



**CLINICAL, MOLECULAR AND HISTOPATHOLOGICAL ANALYSIS OF
MYOTONIA CONGENITA IN A MALAYSIAN FAMILY WITH A RARE
c.1667T>A (p.Ile556Asn) VARIANT IN *CLCN1***

By

NURUL HUDA BINTI MUSA

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

January 2025

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Special dedication to
MC patients and their family members,
normal control individuals and
all rare disease patients

To my beloved family:
Abah, Mama, Husband, Khadijah,
Damia, Danish, Syaffiyah, Wafirah,
Liana, Fairuz, Azliza, Akif, Aidil, Aryan

SCMG Team:
Dr Thila, Aissvarya, Haliza



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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January 2025

Chairman : Associate Professor Thilakavathy a/p Karuppiah, PhD
Faculty : Medicine and Health Sciences

Myotonia congenita (MC) is a rare neuromuscular disease resulting from genetic variants in the *CLCN1* gene, which encodes skeletal muscle chloride channels. It has a prevalence of approximately 1 in 100,000 individuals worldwide. It is characterised by muscle stiffness which causes by delayed muscle relaxation during contraction. This study was aimed to determine the clinical, molecular aspects and histopathological changes of MC. The study reports a 49-year-old female proband who presented with muscle stiffness started in early childhood. Clinical diagnosis was established using electrophysiological and blood analysis, whole exome sequencing (WES), *in silico* and Sanger sequencing, molecular gene expression, and epigenetic studies. The functional effects of the variant were characterised through protein expression and histopathological analysis. The proband was diagnosed with MC after myotonic discharge on electromyography, clinical examination with transient muscle weakness, warm-up phenomenon, and muscular hypertrophy. No additional family member had MC except her brother who had similar symptoms. Serum creatine kinase

levels were elevated in both proband and her brother with 447 U/L and 228 U/L respectively. Furthermore, WES revealed a previously reported pathogenic *CLCN1* heterozygous variant NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn), in the proband, her brother, and observed in all their asymptomatic children, showing a dominant with an incomplete penetrance inheritance. Characterisation of this variant using multiple *in silico* prediction tools showed a damaging and deleterious effect on the CLC-1 protein, and with low allele frequency, it indicates that the variant is a rare and uncommon. Thus, the variant was classified as pathogenic according to ACMG guidelines. Additionally, most allele counts were found in South Asian ancestry, highlighting the need to monitor asymptomatic carriers in the Southeast Asian population. The multiplex ligation-dependent probe amplification analysis revealed neither exon deletion nor duplication found in *CLCN1*, which rules out the recessive form of MC. Meanwhile, epigenetics analysis through DNA methylation demonstrated a hypermethylated CpG site in the proband located in the *CLCN1* enhancer region. This hypermethylation is implicated in the reduction of gene expression and subsequently, protein expression, which suggests that epigenetics might play a role in the genotype-phenotype correlation. Histopathological analysis showed an increase in the internal nuclei by 33%, indicating hindrance of muscle contraction. Additionally, the diameter of muscle fibres varied, displaying hypertrophic muscle fibres and the occurrence of type 2 fast-twitch fibres predominance, reflecting a hypertrophic condition and rapid relaxation of muscle stiffness in the patient, respectively. Ultrastructural analysis using transmission electron microscopy showed a normal Z-line with an irregular arrangement of fibres showing narrowing and splitting of myofibrils and reduction of mitochondria. Thus, these histopathological findings correlate with the mild phenotype in the MC patient. In conclusion, this study describes

the first case of MC in Malaysia caused by a known pathogenic *CLCN1* variant. Collectively, the c.1667T>A (p.Ile556Asn) variant is a rare variant that displayed incomplete penetrance causing clinical, molecular, and mild histopathological changes. The findings of this study have elucidated the c.1667T>A (p.Ile556Asn) variant in MC and provide an understanding of its impact will lead to personalised medicine thereby improving diagnosis, genetic counselling, and treatment.

Keywords: Autosomal dominant, Case report, Chloride channel, *CLCN1*, Myotonia congenita, Thomsen disease

SDG: GOAL 3: Good health and well-being, GOAL 4: Quality education

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**ANALISIS KLINIKAL, MOLEKULAR DAN HISTOPATOLOGI PESAKIT
MIOTONIA KONGENITA DALAM SEBUAH KELUARGA DI MALAYSIA
DENGAN VARIAN JARANG JUMPA c.1667T>A (p.Ile556Asn) PADA *CLCN1***

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Miotonia kongenita (MC) adalah penyakit neuromuskular jarang berlaku yang disebabkan oleh varian genetik dalam gen *CLCN1* yang mengkodekan saluran klorida pada otot rangka. Ia dicirikan oleh kekakuan otot yang berlaku disebabkan oleh lambatan pengenduran otot selepas pengecutan. Kajian ini bertujuan untuk menentukan aspek klinikal, molekul, dan perubahan histopatologi bagi pesakit MC. Kajian melaporkan satu kes klinikal melibatkan seorang wanita (proban) berusia 49 tahun yang mengalami kekakuan otot sejak di usia awal kanak-kanak. Diagnosis klinikal dijalankan menggunakan analisis elektrofisiologi dan darah, penjujukan seluruh exom (WES), *in silico*, dan penjujukan Sanger, ekspresi gen molekul, serta kajian epigenetik. Kesan fungsian varian dicirikan melalui ekspresi protein dan analisis histopatologi. Proban telah didiagnos MC selepas pelepasan mionik dikesan pada elektromiografi, dan hasil pemeriksaan klinikal menunjukkan kelemahan otot sementara, fenomena 'warm-up', dan otot hipertrofi. Abangnya juga mempunyai simptom yang sama; namun, tiada ahli keluarga tambahan yang menunjukkan

simptom MC. Tahap serum kreatina kinase meningkat pada proban dan abangnya dengan masing-masing mencatatkan bacaan 447 U/L dan 228 U/L. Selain itu, WES melaporkan varian heterozigot patogen NM_000083.3(*CLCN1*) c.1667T>A (p.Ile556Asn), pada gen *CLCN1*, terdapat pada proban, abangnya, dan juga terdapat pada semua anak mereka yang tanpa gejala menandakan pewarisan ketembusan yang tidak lengkap. Pencirian varian ini menggunakan pelbagai perkakas ramalan *in silico* menunjukkan kesan yang merosak dan merbahaya pada ClC-1 protein, dan kekerapan alel yang rendah, menunjukkan bahawa varian ini adalah varian yang jarang dijumpai. Oleh itu, varian ini diklasifikasikan sebagai patogen mengikut garis panduan ACMG. Selain itu, kebanyakan kiraan alel ditemui dari keturunan Asia Selatan, mengisyaratkan keperluan untuk memantau pembawa tanpa gejala dalam populasi Asia Tenggara. Analisis ‘multiplex ligation-dependent probe amplification’ (*MLPA*) mendedahkan tiada deletan atau penggandaan exon yang ditemui pada *CLCN1*, yang menolak pewarisan MC secara resesif. Sementara itu, analisis epigen melalui metilasi DNA menunjukkan kesan hipermetilasi di tapak CpG pada proban yang terletak di kawasan penggalak *CLCN1*. Hipermetilasi ini dikaitkan dengan pengurangan ekspresi gen dan ekspresi protein, yang mencadangkan bahawa epigen mungkin memainkan peranan dalam korelasi genotip-fenotip. Analisis histopatologi menunjukkan peningkatan nukleus di dalam gentian otot sebanyak 33%, menandakan halangan dalam kontraksi otot. Selain itu, diameter gentian otot juga berbeza, menandakan keadaan otot hipertrofik dan dominasi sel otot fiber sentak cepat jenis 2 menandakan pengecutan cepat kekakuan otot pada pesakit. Analisis ultrastruktur menggunakan mikroskop elektron transmisi menunjukkan garis Z yang normal dengan susunan gentian otot yang tidak teratur menunjukkan penyempitan dan pemisahan miofibril serta pengurangan mitokondria. Oleh itu, penemuan histopatologi ini berselari dengan

fenotip ringan pada pesakit MC. Kesimpulannya, kajian ini menerangkan kes pertama MC di Malaysia yang disebabkan oleh varian *CLCN1* patogen yang telah dilaporkan. Secara kolektif, varian c.1667T>A (p.Ile556Asn) adalah varian jarang jumpa yang menunjukkan ketusukan tidak lengkap dan menyebabkan perubahan klinikal, molekul, dan histopatologi. Penemuan kajian ini telah menjelaskan varian c.1667T>A (p.Ile556Asn) dalam MC dan memberikan pemahaman tentang kesannya yang akan membawa kepada kepersisan perubatan untuk menambahbaik diagnosis, kaunseling genetik, dan rawatan pesakit.

Kata kunci: Autosom dominan, *CLCN1*, Laporan kes, Miotonia kongenita, Penyakit Thomsen, Saluran klorida

SDG: MATLAMAT 3: Kesihatan Yang Baik dan Sejahtera, MATLAMAT 4: Pendidikan berkualiti

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LIST OF ABBREVIATIONS

%	Percentage
.fsa	File extension for sequence analysis
1D-PAGE	One-dimensional polyacrylamide gel electrophoresis
AAV	Adeno-associated virus
ACMG	American College of Medical Genetics
ADM	Abductor digiti minimi
Asn	Asparagine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BA	Benign stand-alone
BMC	Becker myotonia congenita
bp	Base pair
BP	Benign supporting
BS	Benign strong
BSA	Bovine serum albumin
C1	Child 1 (PB's daughter)
C2	Child 2 (Proband's son)
C3	Child 3 (Proband's daughter)
CADD	Combined annotation-dependent depletion
CAMP	Compound muscle action potential
CASP2	Caspase 2
CBS	Cystathionine β -synthase
cDNA	Complementary DNA

CGIs	CpG islands
Chr	Chromosome
CK	Creatine kinase
Cl ⁻	Chloride ion
CLC-1	Chloride channel protein 1 gene
<i>CLCN1</i>	Chloride voltage-gated channel gene
CMAP	Compound muscle action potential
COX	Cytochrome c oxidase
Cp	Crossing point
CpG	Cytosine-phosphate-Guanine
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated protein 9
Ct	Threshold cycle
dbSNP	Single nucleotide polymorphism database
DD	DNA denaturation
DM1	Myotonic dystrophy type 1
<i>DMPK</i>	Dystrophia myotonica protein kinase
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EMG	Electromyography
ER	Endoplasmic reticulum
ExAC	Exome aggregation consortium
FMRS	Fragment MLPA reaction score

FRSS	Fragment run separation score
FSLP	Final-normalisation Signal Sloping Probes
FT4	Thyroxine
g/L	Grams per litre
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase
GATK	Genome analysis toolkit
GERP++	Genomic evolutionary rate profiling
gnomAD	Genome aggregation database
GRCh	Genome reference consortium human
h	Hour
H&E	Hematoxylin and Eosin
HCl	Hydrochloric acid
HET	Heterozygous
HGMD	Human gene mutation database
HGVS	Human Genome Variation Society
HOM	Homozygous
HSAAS	Hospital Sultan Abdul Aziz Shah
Hz	Hertz
IBS	Institute of Bioscience
IHC	Immunohistochemistry
Ile	Isoleucine
INDEL	Insertion/deletion variant
INFORMM	Institute for Research in Molecular Medicine
JKEUPM	Ethic Committee for Research Involving Human Subjects

kb	Kilobase
<i>KCNJ2</i>	Potassium voltage-gated channel subfamily j member 2
L	Litre
LOVD	Leiden Open Variation Database
m/s	Meters per second
MAF	Minor Allele Frequency
MC	Myotonia congenita
MGT	Modified Gomori trichrome
MH	Malignant hyperthermia
min	Minute
miRNAs	MicroRNAs
mL	Millilitre
MLPA	Multiplex Ligation-dependent Probe Amplification
mM	Millimolar
mmol/L	Millimoles per Litre
mRNA	Messenger Ribonucleic Acid
ms	Millisecond
MutPred	Mutation prediction
mV	Millivolt
NADH	Nicotinamide Adenine Dinucleotide
NCBI	National Center for Biotechnology Information
NCS	Nerve conduction studies
NDM	Nondystrophic myotonia
ng/μL	Nanogram/microlitre

NGS	Next-generation sequencing
NTC	No template control
°C	Degree Celsius
OMIM	Online Mendelian Inheritance in Man
PB	Proband's brother
PBW	Proband's sister-in-law
PCR	Polymerase chain reaction
PM	Pathogenic moderate
PMC	Paramyotonia congenita
PolyPhen-2	Polymorphism Phenotyping v2
PP	Pathogenic supporting
PROVEAN	Protein variation effect analyzer
PS	Pathogenic strong
PSLP	Preliminary signal sloping probes
PVDF	Polyvinylidene fluoride
PVS	Pathogenic very strong
QC	Quality control
qRT-PCR	Quantitative real-time polymerase chain reaction
REVEL	Rare Exome Variant Ensemble Learner
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNS	Repetitive nerve stimulation
RPQ	Reference probe quality

RS	Rank score
RSQ	Reference sample quality
s	Seconds
scAAV	Self-complementary AAV
SCM	Sodium-channel myotonia
SCN4A	Sodium voltage-gated channel alpha subunit 4
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscope
SET	Short Exercise test
SIFT	Sorting intolerant from tolerant
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
ssAAV	Single-stranded AAV
TAE	Tris acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris Buffered Saline with Tween 20
TEM	Transmission electron microscope
TF	Transcription factors
T _m	Melting temperature
TMC	Thomsen myotonia congenita
TSH	Thyroid-stimulating hormone
U/L	Units per litre

UPM	Universiti Putra Malaysia
UTR	Untranslated region
V	Volt
VUS	Variant of uncertain significance
w/v	Weight per volume
WES	Whole exome sequencing
WGS	Whole genome sequencing
x g	Times gravity
xlsx	Microsoft excel open XML spreadsheet
$\Delta\Delta G$	Gibbs free energy of unfolding
μL	Microlitre
μM	Micromolar
μm	Micrometer



CHAPTER 1

INTRODUCTION

1.1 Background

Myotonia congenita (MC) is a rare neuromuscular disease primarily affecting the skeletal muscle. It is characterised by dysfunction of chloride channels in the plasma membrane, with myotonia as the main symptom. Myotonia is defined by the delayed relaxation of muscle fibres following voluntary contractions or mechanical stimulation. This condition is distinguished by the spontaneous repetitive electrical activity of the skeletal muscle membrane, commonly observed as a ‘myotonic run’ on electromyography (EMG) (Nojszewska et al., 2019).

MC is caused by a variant in the skeletal muscle chloride ion channel gene, *CLCN1*; and inherited in a dominant or recessive manner. Over 318 different variants have been identified in MC, including deletions, nonsense, and missense variants (Duno and Vissing, 2021). Traditionally, the disease has been divided into Thomsen’s myotonia congenita (TMC), which is autosomal dominant (OMIM #160800), and Becker’s myotonia congenita (Becker’s Disease, BMC), which is autosomal recessive (OMIM #255700). BMC is prevalent in many countries, particularly in Western countries while Sasaki et al. (2020) showed that TMC is more prevalent in Japan. The global prevalence of MC, including both BMC and TMC is estimated to be approximately 1 in 100,000 individuals (Emery, 1991). This remains the only reported prevalence estimate, with no recent data published to date. Similarly, specific data on the

prevalence in Asia, including Malaysia is limited and likely underreported due to the lack of widespread genetic testing. In TMC, the age of onset is early compared to BMC

which is usually later in life but the myotonia condition is more severe in BMC with more common transient weakness. Myotonia severity can range from mild to moderate, and patients can experience normal life without the progression of the symptoms. Even though some phenotype findings are more common in the BMC than in the TMC, considerable phenotype overlapping exists and complicates the genotype-phenotype correlation.

The traditional Mendelian pattern of inheritance does not always fully explain the patterns observed in MC. Instead, terms such as incomplete penetrance with variable expressivity offer a more accurate description of the modes of transmission within certain families affected by MC. This phenotypic variability refers to a wide range of clinical manifestations and severity of the symptoms observed among individuals affected even though family members carry the same variant.

The specific molecular processes underlying the diverse clinical symptoms observed in MC are not well understood due to limited available genotype/phenotype data (Meyer et al., 2020). Numerous theories have been suggested to explain the clinical variation including differential in allelic expression, the influence of trans-acting genetic modifiers, the impact of epigenetic, hormonal, or environmental factors, as well as reduced penetrance (Cannon, 2015; Suetterlin et al., 2014). These proposed mechanisms, either individually or in combination, may elucidate why identical

variants lead to varying degrees of channel malfunction among different patients (Kubisch, 1998), thereby influencing the manifestation of the MC phenotype.

Several investigations are required to diagnose MC including physical examination, electrophysiological evaluation using EMG, electrophysiology test which is a short exercise test (SET), blood tests to check serum creatine kinase (CK) levels, and genetic analysis. Advances in next-generation sequencing (NGS) technologies have revolutionised the field of genetic testing and diagnosis and positively impacting the identification of disease-causing variants in MC. NGS techniques, including whole genome sequencing (WGS), whole-exome sequencing (WES), and targeted gene panel sequencing, enable simultaneous screening of multiple genes, thereby offering a comprehensive approach to variant identification. These methods have substantially accelerated the discovery of novel variants and improved the understanding of genotype-phenotype correlations in MC.

In Malaysia very limited data available on the number of reported cases. To date, only three cases of MC have been reported in Malaysia from 2008: one case from a hospital in the northern region (Looi et al., 2008), where no genetic analysis was conducted, and two cases involving siblings included in this study. Similar to international practices, MC is typically diagnosed through clinical evaluation and electromyography (EMG), with some cases confirmed via genetic testing. For management, patients are often prescribed medications such as mexiletine to alleviate the symptoms. This study therefore aims to determine the clinical, molecular and histopathological changes underlying MC in a Malaysian family.

1.2 Problem Statement

The prevalence of MC varies significantly across different geographical regions (Matthews et al., 2010), affecting approximately 1 in 100,000 of a population globally (Emery, 1991) and the prevalence is higher in certain regions as in Northern Scandinavia where it occurs 9 in 100,000 population (Sun et al., 2001). This is the most updated report on the prevalence of MC highlighting the regional difference with no other recent studies available. Patients commonly experience muscle stiffness as the main symptom, which is rarely life-threatening, however, affected individuals often encounter challenges in carrying out daily activities leading to lifetime disability. In addition, there is evidence that some MC patients may have an altered response to anaesthetic drugs (Looi et al., 2008). There was a case of an undiagnosed MC patient in Malaysia who developed a life-threatening masseter spasm following anaesthetic administration that caused potentially lethal complications (Looi et al., 2008). Thus, careful preoperative history questions on the muscular symptoms and family history are vital.

A few medications have been used to treat MC such as mexiletine, a class 1B antiarrhythmic compound that reduces muscle stiffness but may cause adverse side effects, such as gastrointestinal discomfort, tremors, dizziness, nausea in some patients (Modoni et al., 2020; Suetterlin et al., 2015) while others do not respond to the medication. Moreover, MC exhibits significant genetic heterogeneity and presents with overlapping clinical features, with a limited understanding on genotype-phenotype correlations (Meyer et al., 2020). Despite progress has been made in

understanding MC, the wide range of genetic and phenotypic variability still presents challenges in diagnosis, treatment, genetic counselling, and research planning.

Clinical variability has been observed among individuals with MC, where a wide range of symptoms can be seen with the same variant. Individuals can exhibit variations in the severity of muscle stiffness, age of onset, progression of the disease, and specific symptoms experienced. In some cases, incomplete inheritance or variants with dual inheritance further complicates the genotype-phenotype correlation, making it challenging to predict clinical outcomes based solely on genetic findings. Thus, comprehensive analyses such as gene, protein, allelic expression, epigenetic, and histopathological studies are required to better understand the disease mechanism and its clinical manifestations.

1.3 Hypothesis

It is hypothesised that MC patients presenting with myotonic features may carry a variant in the *CLCN1* gene. MC patient will exhibit an increase in serum creatine kinase (CK), presence with myotonic discharge, muscular hypertrophy, and short exercise test (SET) characterised by the Fournier pattern II. The variable phenotypes observed in MC patients carrying the variant in the *CLCN1* are due to differential changes in gene or protein expression and methylation between patients. It is also hypothesised that the muscle tissue of MC patient will exhibit histopathological changes.

1.4 Objectives

The overarching aim of this project is to study the clinical, molecular and histopathological changes underlying MC in a Malaysian family with the variant in *CLCN1*. The specific objectives are:

1. To determine the clinical presentation of a family with MC using clinical examination, laboratory investigations, and neurophysiology studies.
2. To investigate the genetic profile and inheritance patterns of the variant in MC patient.
3. To determine the molecular characteristics of the variant through gene expression and epigenetics using skeletal muscle tissue.
4. To determine the chloride channel (ClC-1) protein expression in the MC patient and provide a comprehensive examination of the histological features.

1.5 Significance of the Study

This study is the first molecular study on the variant screening of MC among patients in Malaysia. The findings of this study will contribute novel insight into the molecular knowledge of MC among patients in Malaysia and act as a cornerstone to develop better treatment and genetic counselling for the patients. This study also may lead the way to precision and personalised medicine based on genetic assessment.

CHAPTER 2

LITERATURE REVIEW

2.1 Myotonia Congenita

Myotonia congenita (MC) is a genetic neuromuscular channelopathy that affects the skeletal muscle (Lossin and George, 2008). It is a rare genetic disorder caused by *CLCN1* variants affecting the chloride ion channel (ClC-1) and characterised by muscle stiffness (myotonia) and the inability of the muscle to relax after voluntary contraction or mechanical stimulation. This is due to the electrical instability of the muscle membrane itself, leading to repetitive action potentials with a single stimulus known as “myotonic runs”.

MC is one of the first proven human channelopathies and the most common inherited muscle channelopathy (Horga, 2013). It was first described by Julius Thomsen, a Danish physician who was himself affected by the disease. Thomsen described the wide range of symptoms among affected family members. Myotonia was severe in his younger brother and sister, but it was barely noticeable in Thomsen's mother and older brother. He also described myotonia's gradual disappearance by repetitive motions, known as the "warm-up" effect. As more myotonia families were discovered, it became evident that the phenotypic differed greatly from one family to the other. Later in 1977, Peter Emil Becker demonstrated the existence of a recessive MC variant with muscular manifestations that is intermediate between Thomsen's relatives and patients with dystrophic myotonia (Becker, 1977). Hence MC was divided into a recessive

disorder (Becker's disease, BMC) and a dominant disorder (Thomsen's disease, TMC).

2.1.1 Prevalence

The prevalence of myotonia congenita (MC) varies significantly across geographical regions (Matthews et al., 2010), affecting approximately 1 in 100,000 individuals worldwide (Emery, 1991). It is notably more prevalent in specific areas, such as Northern Scandinavia, where the occurrence rises to 9 in 100,000 (Sun et al., 2001). This remains the only reported prevalence estimate, with no recent data published to date. BMC is more frequent with a frequency of 0.6 per 100,000 in the general population, compared to 0.3 frequencies for Thomsen disease (<http://raredisease.org>). Although BMC is more frequent, TMC in Germany is estimated at around twice (1 in 23,000) that of the recessive type (1 in 50,000) (Becker, 1977). However, no specific data on the prevalence of MC in Asia, including Malaysia, has been reported.

2.1.2 Types of Myotonia Congenita

The hallmark of MC is myotonia, that is muscle stiffness particularly after a period of rest or after a sudden movement. Another common clinical sign in both forms of MC is muscular hypertrophy where the patient develops an abnormal increase in muscle size that gives an athletic appearance (Mankodi, 2008). It is further divided based on their mode of inheritance; it can be inherited either as an autosomal dominant (Thomsen's myotonia congenita, TMC) or autosomal recessive (Becker's myotonia congenita, BMC) trait. BMC is clinically more severe and there are few other clinical

features that differentiate between these two types of MC (**Table 2.1**). In general, TMC is characterised by early onset usually at birth or very early in infancy, and the severity ranges from mild to moderate myotonia with no progression of the symptoms. Hypertrophy is more prevalent in adulthood but rare in childhood and the child may have abnormally developed muscle in the limbs, as well as delayed relaxing of the eyelids after forceful closure during sneezing or crying (Jurkat-Rott et al., 2002).

Table 2.1: Clinical features of myotonia congenita

Features	Myotonia congenita	
	Thomsen's (TMC)	Becker's (BMC)
Age of onset	Infancy	Early childhood
Inheritance	Autosomal dominant	Autosomal recessive
Myotonia	Mild to moderate	Moderate to severe
Distribution of myotonia	Generalised; face, arms>legs	Generalised; legs>face, arms
Transient weakness	No	Yes
Duration of weakness	None	Variable
Progressive weakness	No	Some patients
Provocative factors	Cold, stress, pregnancy	Cold, stress, pregnancy
Alleviating factors	Exercise	Exercise
Myotonia	Present	Present

(Chrestian et al., 2006)

On the other hand, BMC, reported by later onset (four to 12 years), range of myotonia from moderate to severe, moderate muscle hypertrophy, and myotonia with transient weakness and even permanent weakness (Lossin and George, 2008). It has been also reported that the age of onset could be earlier in BMC, however, a study by Fialho et al. (2007) observed no difference in the age of onset.

Other differences between the two forms are BMC affects primarily the lower limb and progresses to the arms, neck, and facial muscles, while for the TMC form, affected individuals develop muscle rigidity and hypertrophy. Mild cold exacerbation and significant muscle pain were equally common features in TMC and BMC cases (Fialho et al., 2007). Even though some phenotype findings are more common in the BMC rather than TMC, there is considerable phenotypic overlap which can complicate genotype-phenotype correlations.

2.1.3 Clinical Phenotypes

2.1.3.1 Myotonia

MC forms a spectrum of disorders with significant overlap in clinical phenotypes that can cause complex and difficulty in diagnosis. The two forms of MC share common symptoms but also exhibit distinct differences in their clinical presentation. Myotonia refers to the delayed relaxation of muscles after voluntary contraction, leading to muscle stiffness, because the muscles remain hyperexcitable and continue to contract even after movement has stopped. To estimate the severity of myotonia, Becker developed a semiquantitative measure of general impairment based on the patient's history (Becker, 1977). In cases of severe myotonia, the symptoms can be immediately apparent, posing significant challenges in daily activities and being difficult to conceal from others. In contrast, mild myotonia only slightly impedes the affected individual, often causing no significant discomfort due to the muscle abnormality. Myotonia that is localised to a specific area of the body, such as the legs, is also classified as a mild.

Myotonia is the most common symptom and reported in most of the patients (Trivedi et al., 2013). It is clinically variable and ranges from myotonic discharge that is only detectable on EMG to severe disabling muscle stiffness at an early age. Almost every skeletal muscle in the body may exhibit stiffness, and it is frequently found in leg and handgrip myotonia, in up to three-quarters of the patients (Trivedi et al., 2013). Stiffness is more prominent in the extremities, especially legs and hands and usually relieved after repeated contractions of the muscle called the “warm-up” phenomenon (Heatwole et al., 2013; Trivedi et al., 2013). Characteristic symptoms may include difficulties in the relaxing hand grip, starting to walk or run, arising from a chair, climbing stairs, opening eyes completely after forcibly closing them, and arising from bed at night (Crews et al., 1976). In addition to the extremities, myotonia in MC can also affect the tongue muscles, causing anomalies that lead to speech difficulties and complicate activities such as chewing and swallowing (Duno and Vissing, 2021).

2.1.3.2 Transient weakness

Transient weakness was present on clinical testing in approximately 70% (9 of 13) of BMC patients with two mutated alleles (Deymeer et al., 1998) whereas it has not been reported as a clinical symptom in patients with TMC. Meanwhile, approximately one-third of BMC patients having a weakness phenotype (Olave-Rodriguez et al., 2021), one-third have transient weakness but no atrophy, and one-third having relatively mild features that are indistinguishable from TMC (Colding-Jørgensen et al., 2003). Thus, pronounced difference was observed between the two forms of MC.

2.1.3.3 Warm-up phenomenon

In MC patients, muscle stiffness will lessen with exercise which known as the warm-up phenomenon, where myotonia is reduced or abolished with repeated muscle contraction. The effect is short-lived and will wear off after 5 min of rest. One potential mechanism for the warm-up effect is the increased activity of the muscle sodium-potassium ATPase pump induced by exercise, which facilitates the clearance of extracellular potassium from transverse tubules (T-tubules). However, a recent study by Novak et al., 2015 showed that the slow inactivation of sodium channels as a key contributor to the warm-up phenomenon. Although MC is not fatal, severely affected patients may be exposed to potentially harmful situations due to their inability to move away quickly. The absence of progressive weakness and systemic symptoms distinguishes these disorders from dystrophic myotonia.

2.1.4 Diagnosis of MC

Several investigations are required for the diagnosis of MC. These include physical examination, electrophysiology studies, laboratory analysis, and molecular genetic analysis which can include either WES or WGS.

2.1.4.1 Physical Examination

Physical examination using the myotonia bedside test is used to confirm clinical myotonia (Stunnenberg et al., 2020). The first test is percussion myotonia in the thenar muscles. A tap applied with a reflex hammer on the thenar eminence will indicate

myotonia is present if there is delayed muscle relaxation following 5 to 10 s of maximal muscle contraction (Trip et al., 2009). The second test is the handgrip test in which the patient is asked to make a forceful fist for several seconds and then asked to open the hand as fast as possible (Matthews et al., 2010). MC patients will show an abnormally delayed ability to fully release the handgrip and should check for the presence of a warm-up phenomenon where there is a decrease of myotonia after repetitive contractions (Heatwole et al., 2013). The third test is the transient paresis test in the biceps (Trip et al., 2009). Further examination is to observe the presence of muscle hypertrophy; an athletic-like appearance with above-average muscle mass that occurs in the upper extremities, lower extremities, and facial muscle (Stunnenberg et al., 2020).

2.1.4.2 Clinical electrophysiology

Specialised techniques in neurophysiology have helped to precisely diagnose muscle channelopathies. Electrophysiological evaluation using EMG follows a standardised protocol for assessment of skeletal muscle (Nojszewska et al., 2019). Four muscles are usually examined (extensor digitorum muscle, dorsal interosseous I muscle, vastus lateralis muscle, and tibialis anterior muscle) (**Figure 2.1**) in which the compound muscle action potential (CMAP) will be recorded following supramaximal electrical stimulation of the ulnar nerves at the wrist.

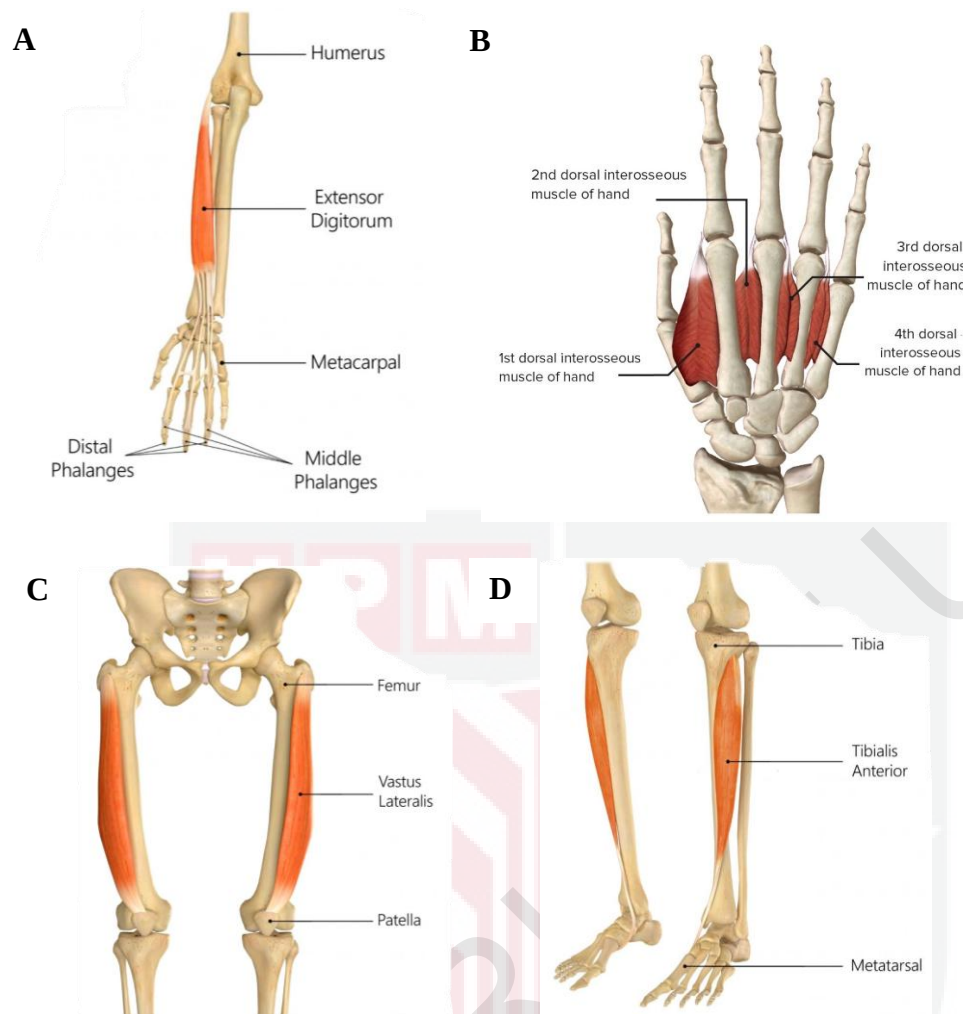


Figure 2.1: Structure of the four muscles examined during EMG. (A) extensor digitorum muscle, (B) dorsal interossei I muscle, (C) vastus lateralis muscle, and (D) tibialis anterior muscle.

Recordings are repeated before and after the voluntary contraction of the stimulated muscle. On EMG, myotonia appears as prolonged, spontaneous trains of muscle unit action potentials that display varying amplitude and frequency. The sound produced is often described as the sound of a dive bomber or a revving engine (Fournier, 2004). In response to short (10 – 20 s) or long (3 – 5 min) exercise tests, different types of muscle channelopathies will give different magnitudes of CMAP readings (Streib, 1982). The Short Exercise Test (SET), which is an electrophysiological based test, monitors the CMAP amplitude following three short bursts of exercise (Streib, 1987). The CMAP

amplitude is an indirect measure of sarcolemmal excitability and indicates the ability to depolarise and repolarise. This test allows identification of the Fournier patterns I – III, which can help to distinguish between *SCN4A* or *CLCN1* gene channel variants.

Fournier patterns are characterised by changes in the CMAP amplitude of the abductor digiti minimi muscle after a series of three exercises in the muscle at room temperature and after cooling. In Pattern I, there is a progressive decrease in CMAP amplitude after each exercise that does not recover after a period of rest. Pattern II shows a decrease in CMAP amplitude immediately following exercise that recovers after a period of rest; and pattern III by shows no significant decrement, indicating a normal response. Cold temperatures may aggravate or reveal patterns I and II. Fournier patterns I and III are typically associated with sodium voltage-gated channel alpha subunit 4 gene (*SCN4A*), while Fournier pattern II is commonly associated with variants in the *CLCN1* (Fournier et al., 2004). After exercise, CMAP amplitude decreases immediately at room temperature which rapidly recovers following subsequent trials of exercise (reduces with repetition), confirming transient weakness. This pattern of transient weakness is most observed in BMC and can also be seen in the TMC.

2.1.4.3 Laboratory analysis

Additionally, determining serum levels of creatine kinase (CK) is a key tool for diagnosis. An elevated CK level can indicate muscle damage caused by myotonia. CK levels of MC patients typically range from normal (normal <200 U/L) to moderate increase (more than 1.5 times the upper limit of the normal value) (Duno and Vissing, 2021; Moghadam et al., 2016).

2.1.4.4 Genetic analysis

Genetic analysis is one of the established analyses for MC disease to identify variant in *CLCN1* gene (Brugnoni et al., 2021; Ferradini et al., 2017). Molecular genetic testing is most likely to identify the genetic cause through comprehensive screening using WGS or WES, and recent studies on MC have increased utilisation of WES to uncover variants (Elaraby et al., 2024; He et al., 2024). WES targets the protein-coding regions of the genome, where over 85% disease-causing variants are found, offering a focused and cost-effective approach (Majewski et al., 2011; Botstein and Risch, 2003). On the other hand, WGS analyses the entire genome, including non-coding regions, for a more comprehensive assessment.

2.1.4.5 Anaesthetic complication

Management of MC patients should include the clinicians being aware of the potential anaesthetic complications, in which use of the depolarising muscle relaxant suxamethonium should be avoided (Radkowski et al., 2023; Hassan, 2021; Thiel, 1967). MC is reported to be linked to the increased risk of episodes that are similar to malignant hyperthermia (MH) (Haberer et al., 1989; Heiman-Patterson et al., 1988). This is based on case reports prior to the development of genetic testing. In one report, a 5-year-old boy with MC phenotype experienced a hyperthermia-like episode after receiving a non-triggering anaesthetic and passed away in spite of pre-treatment with dantrolene. This child also experienced other complications, including a highly elevated creatine kinase (CK) level (Haberer et al., 1989). Another 12-year-old girl who had been diagnosed as moderately severe MC, was administered a depolarising

anaesthetic, and later passed away due to a classic MH reaction (Saidman and Eger, 1964). Initial reports suggested MH complication in MC, but current research indicates that the association was likely due to overlapping symptoms. Both conditions can cause muscle rigidity and hypertonia. It has been clarified that MC does not carry the same risk factors as MH, but it does lead to muscle stiffness and myotonia (Bandschapp and Iaizzo, 2013; Farbu et al., 2003). In MC patients, this can result in problems like jaw muscle stiffness, which can affect breathing and lower oxygen levels. This complication can still occur especially in undiagnosed asymptomatic individuals. This was previously reported when an undiagnosed MC patient in Malaysia developed a life-threatening masseter spasm following the induction of anaesthesia that caused potentially lethal complications (Looi et al., 2008). Thus, for the clinical management of MC, it is important that a careful preoperative history question on the muscular symptoms and family history in patients with a positive MC diagnosis is performed.

2.2 Molecular Pathophysiology

2.2.1 Ion Channels

Ion channels are transmembrane pores that allow ions to enter and exit cells by allowing them to pass through the membrane. They can be distinguished based on which principal ions, such as sodium, chloride, sodium, and potassium ions, can be passed through the channel. The main voltage-gated ion channels in skeletal muscle are the chloride, potassium, sodium, and calcium channels (Jurkat-Rott and Lehmann-Horn, 2004). The voltage-gated potassium, sodium, and calcium channels are

members of the same gene superfamily, and share high sequence similarity (Hille, 2002), while chloride channels differ from the other channels as they form homodimers with two separate pores (**Figure 2.2**). Ion channels can exist in three different conformations; 1) “open” where ion can pass through the pore, 2) “closed” (resting state) where the ion is unable to pass through the pore and 3) “inactivated” (refractory period) where the ion channel is temporarily unable to open (Duran et al., 2010).



Figure 2.2: Diagrammatic representation of (A) chloride channel (CLC-1) and (B) sodium channel (Nav1.4). (A) The chloride channel consists of two identical subunits each with its own ion-conducting pore while (B) the sodium channel comprises a single ion-conducting pore formed by the interaction between four homologous domains. (Source: Matthews et al., 2010).

2.2.2 Chloride Channel (CLC-1 Channel)

The human chloride channel (CLC-1) is a voltage-dependent channel, that opens upon membrane depolarisation and important to stabilise skeletal muscle resting membrane potential (Jentsch et al., 2002). It is a transmembrane protein consists of 988 amino

acids (~120 kDa), and almost exclusively expressed in skeletal muscle although low levels can be found in the heart, kidney, and smooth muscle (Steinmeyer et al., 1991). This channel is a homodimer and contains two protopores (two identical pores) lined in opposite directions with a ‘double-barrelled’ opening of functional channels (**Figure 2.3**) (Jentsch and Pusch, 2018; Chen, 2005). The gating behaviour of ClC-1 is complex. The opening and closing of two pores in the channels are controlled by two distinct gating mechanisms (**Figure 2.4**). The common gate (“slow gate”) controls the opening and closing of the two pores simultaneously which causes the deactivation of both pores, while the “fast gate” controls the opening and closing of each individual pore and operates independently from the partner fast gate (Duffield et al., 2003; Dutzler et al., 2002). The activation of the ClC-1 ion-conducting pathway therefore requires the opening of both the common-gate and the fast-gate.

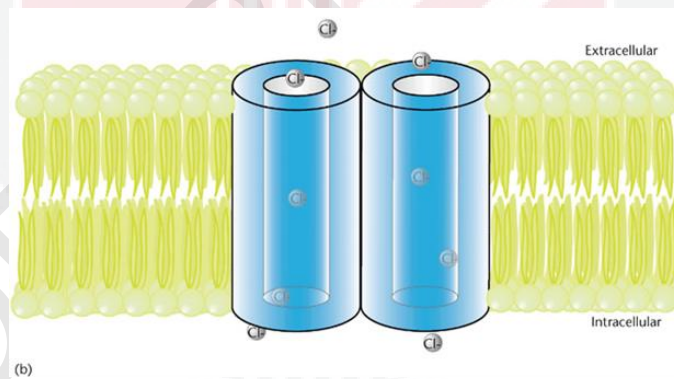


Figure 2.3: Structure of ClC-1 chloride channel. (Source: Pusch, 2002)

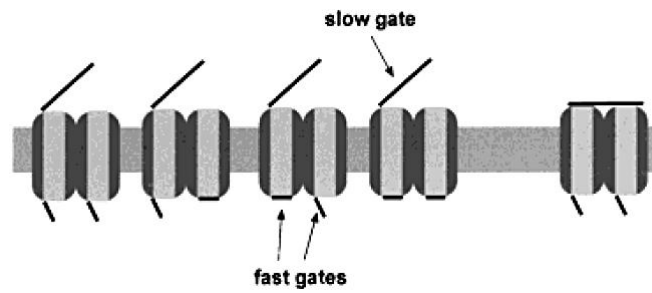


Figure 2.4: Gating mechanism of ClC-1. (Source: Pusch, 2002)

The ClC-1 membrane consists of an amino (N)-terminal transmembrane portion and carboxyl (C)-terminal cytoplasmic portion. The transmembrane portion is composed of 18 α -helices (helices A – R) and the cytoplasmic portion consists of cystathionine β -synthase (CBS) domains (CBS1 and CBS2), which fold into an ATP-binding site (**Figure 2.5**). Most of the transmembrane helices are notably tilted and not perpendicular to the membrane (Tseng et al., 2011). Among all the 18 helices, helices H, I, P, and Q are located on the dimer interface which is the subunit between the two pores while residue at helices D, F, N, and R form the ion-conducting pore known as the protopore (Duffield et al., 2003).

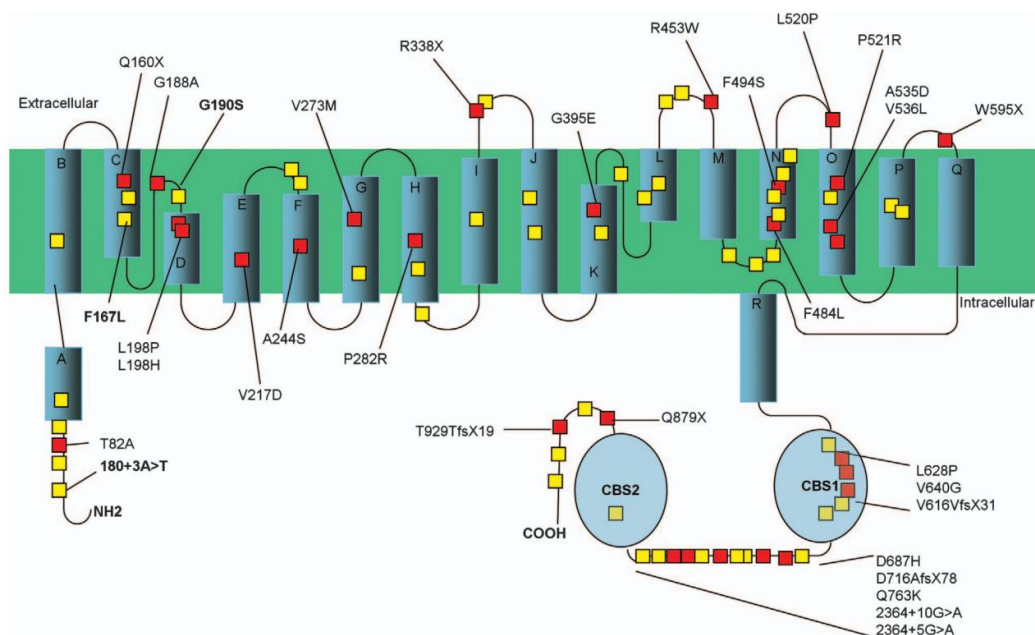


Figure 2.5: CIC-1 membrane topology shows the localisation of the variants on the protein. The protein consists of 18 α -helices (helices A – R) and the cytoplasmic portion consists of cystathionine β -synthase (CBS) domains (CBS1 and CBS2) (Source: Brugnoli et al., 2013)

2.2.3 Pathophysiology

Cl^- is the most abundant anion in most organisms (Luscher et al., 2020). In skeletal muscle, chloride conductance is unusually high. It accounts for up to 85% of the resting membrane conductance while the remaining 15% is attributed by other ion channels primarily potassium channel (Pedersen et al., 2016). During muscle contraction, action potentials propagated along the sarcolemma and into the T-tubules, causing the efflux of potassium and membrane depolarisation which lead to contraction. Chloride channels play a crucial role in counteracting this depolarising effect by ensuring high chloride conductance, which balances the accumulation of potassium in the T-tubules (**Figure 2.6A**). The combined activity of potassium and chloride channels repolarise the sarcolemma and stabilise the resting potential in normal muscle.

However, variants in the *CLCN1* produce defective ClC-1 protein that causes a reduction in chloride conductance in myotonic muscle (Altamura et al., 2020). This eventually leads to the accumulation of potassium in the T-tubule lumen causing depolarisation of the surface membrane (**Figure 2.6B**). The depolarisation increases the excitability of the muscle membrane, triggering repetitive action potential and lead to delayed relaxation, a characteristic feature of myotonia observed in MC patients (Barchi, 1975). Barchi (1975) has shown that decreasing chloride permeability to 20% is sufficient to cause myotonia.

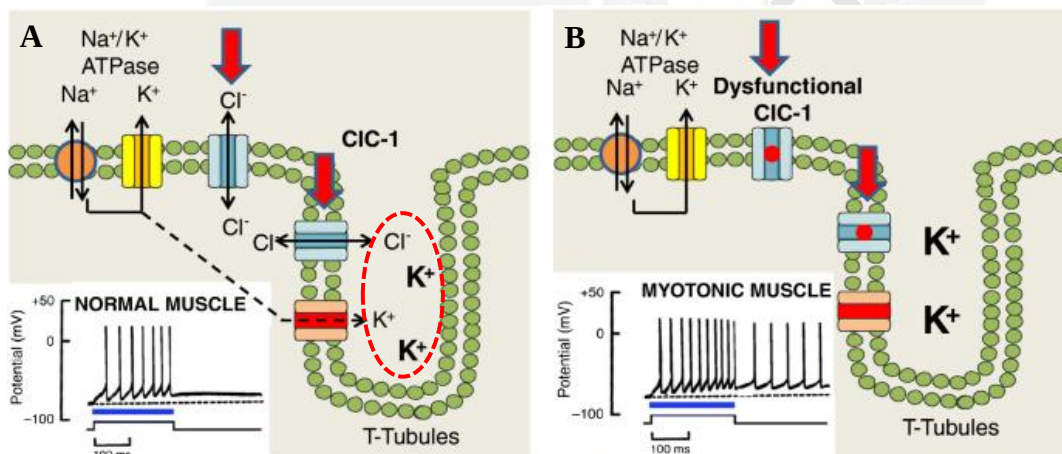


Figure 2.6: Pathophysiology of ClC-1 chloride channel. Diagram showing the contribution of chloride channel to skeletal muscle excitability and action potential in (A) normal and (B) myotonic muscle. The dysfunctional chloride channel in myotonic muscle caused buildup of potassium which propagated to the surface membrane and triggered spontaneous action potential and hyperexcitability of muscle fibre. (Source: Altamura et al., 2020)

Variants in voltage-gated ion channels can affect channel function such as gating, voltage-sensing, permeation, assembly, and trafficking (Enkvetchakul, 2010). In the ClC-1 chloride channel, variants lead to increased skeletal muscle membrane excitability, resulting in delayed muscle relaxation, a hallmark of myotonia in MC patients (Imbrici et al., 2015a; Jentsch et al., 2002). For BMC, variants impair the

gating and conductance of both subunits, resulting to a loss of chloride conductance. However, if a mutated subunit is paired with a wild-type, the channel's function may not be impaired enough to cause the disease. The chloride conductance must be reduced by more than 50% to produce myotonia, which is why individuals with a single recessive allele remain asymptomatic (Furman, 1978).

In contrast, TMC is caused by a variant that affects the "common gate" of both subunits within the heterodimer, particularly at the dimer interface (Lossin, 2008; Duffield, 2003; Saviane et al., 1999). These variants cause a dominant-negative effect, where the mutant subunit interferes with the wild-type subunit and requires more positive voltages to open both subunits, explaining the dominant inheritance (Jeng et al., 2020). Additionally, some variants exhibit both dominant and recessive inheritance patterns, likely due to their impact on both fast and slow gating mechanisms (Weinberger et al., 2012). Thus, the inheritance pattern of MC depends on the specific variant's effect on CLC-1 gating mechanism, whether it impairs both subunits in recessive forms (BMC) or interferes with common gating in dominant forms (TMC).

2.3 Variant and Complex Genotype-Phenotype Correlation

The *CLCN1* (OMIM# 118425) gene that causes MC is located on 7q35 and is expressed extensively in the sarcolemma (George et al., 1993; Koch et al., 1992). To date, over 318 *CLCN1* variants have been identified including missense, nonsense, indels and splice variants (Duno and Vissing, 2021; Morales and Pusch, 2020; Liu, 2015; Brugnoli, 2013). The variants are located throughout the entire gene sequence, including the N-terminal, transmembrane, and C-terminal domains of CLC-1 protein

(Fialho et al., 2007). Nonsense, splice-site, and frameshift variants resulting in the truncation of the channel protein typically exhibit a recessive inheritance, unless they occur near the C-terminus in which they may exhibit dominant inheritance (Richardson et al., 2014). Missense variants can be associated with either recessive or dominant inheritance, while variants with exclusive dominant inheritance are less common (Tang and Chen, 2011).

The types of variants that cause MC whether it is dominant, or recessive are significant as they can cause phenotypic variability. Most of the variants are found in recessive pedigrees, whereas approximately 27 variants exclusively result in TMC presumably through a dominant-negative effect (Fialho et al., 2007). Plassart-Schiess et al. (1998) have noted that classic Mendelian patterns do not always account for the clinical forms of MC and propose that instead of classifying the condition based on dominant or recessive inheritance, incomplete penetrance with variable expressivity better describes the complex mode of transmission in some families.

Few variants (p.A313T, p.R317Q, p.I556N, p.L198V, p.A218T) have been shown to exhibit incomplete penetrance. p.A313T was found in two unrelated families where in one family, the proband carried the variant while the mother who also carried the variant, was normal (Plassart-Schiess, 1998). p.R317Q variant that was initially found in a dominant pedigree was later described in one heterozygote member with MC while two others were asymptomatic. Another variant, p.I556N, categorised as a dominant variant with incomplete penetrance, affected two female heterozygotes from Indian descent with mild symptoms and late onset, while a male carrier was unaffected (Plassart-Schiess, 1998). Functional analysis for this p.I556N variant revealed a slight

difference from the wild-type parallel with the mild or normal phenotype (Kubisch et al., 1998). Few other variants (p.L198V, p.L283F, and p.F307S) were also found in MC patients with unaffected parents.

Despite being asymptomatic, myotonic bursts sometimes can be present in an asymptomatic heterozygous individual for certain variants including variants that can be both dominant and recessive alleles (Colding-Jørgensen et al., 2003). Four asymptomatic carriers in recessive pedigrees described by Becker (1977) had a brief myotonic discharge (100 – 500 ms duration) when tested with EMG. Abnormal EMG was found in one asymptomatic individual with the p.A531V variant and two asymptomatic individuals with the p.R894X variant. However, other individuals with these variants showed normal EMG results. For non-functional variants such as p.Q74X, fs433X, fs503X (Mailander et al., 1996), and p.Q658X (de Diego et al., 1999), approximately half of the heterozygous carriers exhibited abnormal EMG, while the remaining carriers had normal EMG results. This variability in EMG findings may be attributed to different inheritance patterns.

One of the unique phenomena of MC is that the same pathogenic variant can behave as both dominant and recessive alleles. The p.G230E has been reported in eight autosomal dominant pedigrees in which one of the 20 individuals heterozygous for the variant were asymptomatic, while in another additional family, the variant was found in the recessive pedigree (Brugnoni et al., 1999; Koty et al., 1996). The p.R894X variant has been reported in four dominant pedigrees as well as more than 10 recessive pedigrees which suggests the variant has clearly a dual inheritance pattern (Marinakos et al., 2024; Colding-Jørgensen et al., 2003).

2.3.1 Factors Explaining Phenotypic Variability

The molecular mechanisms underlying the phenotypic variability seen in MC is not well understood with a lack of genotype-phenotype correlation data (Meyer et al., 2020). Further studies are required to gain a deeper understanding of the genotype-phenotype relationships. Differential allelic expression, trans-acting genetic modifiers, epigenetics, and hormonal factors are some of the explanations suggested to explain the phenotypic variability observed in MC (Cannon, 2015, Suetterlin et al., 2014). These suggested mechanisms (alone or in combination) may help the design of functional studies to investigate why the same variant results in varying degrees of channel dysfunction in different patients.

2.3.2 Differential in Allelic Expression

Differential in allelic expression would also provide the explanation why some variants in *CLCN1* gene can behave as both dominant and recessive (Duno et al., 2004). In a diploid organism, the two alleles are expected to be expressed with a 1:1 ratio, however some alleles can show significant differences from the expected ratio resulting in a high frequency of allele differential expression (Lo et al., 2003). A proposed hypothesis is that the difference in the expression of an alternative allele within the gene containing causal heterozygous variants may affect how phenotypic traits appear in individuals with the same genotype. This proposed mechanism is primarily relevant to dominantly inherited conditions in which haploinsufficiency is the underlying cause, such as Lynch syndrome (Hesson et al., 2015) and hypertrophic cardiomyopathy (Glazier et al., 2019). In these conditions, allelic imbalance could lead

to either increased expression of the wild-type allele, thereby compensating for haploinsufficiency and reducing penetrance, or decreased expression of the wild-type allele, exacerbating haploinsufficiency and resulting in higher penetrance (Jordan et al., 2019). Allelic imbalance is reported to affect as many as 88% of human tissue specific genes and is possibly induced by genetic modifiers or random factors (Aguet et al., 2016).

Differential allelic expression has been suggested as a possible explanation for intrafamilial variation in TMC pedigrees (Colding-Jorgensen, 2005; Duno et al., 2004). A study that examined allele-specific gene expression of transcribed single-nucleotide polymorphisms (SNPs) reported 54% (326 from 602) of genes can have a preferential expression of one allele with 28% (170 from 602) of the genes showing one of the alleles expressed four-fold greater than the second allele (Lo et al., 2003). A study by Duno et al. (2004) suggested the influence of differential allelic expression on disease progression, which may explain the symptomatic variability of myotonic members in dominant families. For example, the study demonstrating the p.R894X variant with both dominant and recessive inheritance, reported a severely affected dominant patient, expressing twice the amount of p.R894X mRNA relative to the wild-type allele. This specific variant is widely observed across diverse populations, representing one of the most prevalent variants. Located in the final exon of *CLCN1* gene, this variant results in the truncation of the terminal 94 amino acids from the C-terminus, causing a significant reduction in chloride conductance. This decrease is attributed to a notable decline in the expression of the protein on the cell surface (Meyer et al., 1995).

2.3.3 Epigenetics

Epigenetics is the study of heritable and persistent changes in gene expression that can regulate gene activity but does not directly modify the DNA sequence (Berger et al., 2009). Even though epigenetic modifications remain after several rounds of cell replication, these alterations rarely segregate and can be reversed. To the best of our knowledge, no study has been conducted to determine the epigenetic mechanisms of MC.

There are three main epigenetic mechanisms: changes in DNA methylation, chromatin remodelling, and the expression of regulatory microRNAs (miRNAs). Together with histone changes, DNA methylation, the first layer of a heritable epigenetic marker, appears to be important in inhibiting gene reactivation (Feng et al., 2007). DNA methylation plays a crucial role in regulating tissue-specific gene expression, alternative splicing, prevention of cryptic initiation of transcription from alternative promoters, and X chromosome inactivation. These processes have been demonstrated to influence disease development (Velasco and Francastel, 2019). Research over the last decade has shown that epigenetic modification can play a role in the pathogenesis of the neuromuscular disorders. The variation in clinical phenotype and age of onset reported for myotonic dystrophy type 1 (DM1) patients, has been associated with the DNA methylation of CpG islands in the *DMPK* gene (Cerrato et al., 2020; Bennett et al., 2019; Legare et al., 2019; Buckley et al., 2016).

MicroRNAs (miRNAs), small non-coding RNAs about 22 nucleotides long, can regulate gene expression and counteract specific abnormal changes. Working with

additional proteins, they form the RNA-induced silencing complex (RISC) to degrade, destabilise, or block the translation of mRNA messengers (Kim and Nam, 2006). Mature miRNAs typically bind to the 3' untranslated regions (3'UTRs) of mRNAs, but they can occasionally bind to the 5'UTR or even the amino acid coding sequence. Previous studies manipulating microRNA levels, showed that these molecules play a critical role in regulating protein function in various mammalian processes, including cancer, immune regulation, and cardiac function (Abolhasanzadeh et al., 2024; Khameneh et al., 2024; Zhang et al., 2024) . Notably, certain microRNAs identified for their importance in heart development and function were also found to be highly expressed in skeletal muscles (Chen et al., 2006; Zhao et al., 2005).

A study by Zaharieva et al. (2013) reported microRNAs taken from the blood of Duchenne muscular dystrophy patients showed the expression of specific muscle-enriched microRNAs, changed significantly as the dystrophic disease progressed. Comparable findings were reported in muscles of dystrophic mdx mice, in which expression of a specific muscle-enriched microRNA, miR-206, when compared to normal mice muscles was observed (Roberts et al., 2013). To date, no specific human microRNA variants have been directly associated with a neuromuscular disease. There are, however, some examples in which manipulating certain microRNAs genetically, leads to muscle phenotypes similar to those found in human patients with neuromuscular diseases (Liu et al., 2011).

2.4 DNA Sequencing for Variant Detection

2.4.1 Sanger Sequencing

Sanger sequencing, also known as ‘first-generation sequencing’, is the gold standard for DNA sequencing. It is commonly utilised for variant detection in genetic studies (Grada and Weinbrecht, 2013). When investigating a specific gene or region, researchers design primers that target the desired sequence. By sequencing the amplified DNA fragments, potential variants within the DNA sequence can be identified. To detect variants, the obtained sequence is compared to the reference genome, allowing for the identification of single nucleotide changes, insertions, and deletions (Al-Shuhaib and Hashim, 2023).

2.4.2 Next-Generation Sequencing (NGS)

The field of molecular genetics has made significant progress, especially with the introduction of NGS techniques such as WES and WGS. These approaches are useful tools for finding the causative variants and providing a genetic diagnosis. WGS analyses the entire DNA, while WES focuses on the protein-coding regions of the genome. The sequencing strategy is to begin with WES, since 85% of disease variants are located in the protein-coding regions of genes (Choi et al., 2009). These advances have greatly improved the ability of clinicians to accurately identify specific gene variants.

NGS, introduced over a decade ago, is a significant improvement over traditional sequencing methods and has been widely adopted in clinical genetic testing. It has become a powerful tool for diagnosing and analysing rare diseases (Vinkšiel et al., 2021; Boycott et al., 2013). NGS allows for comprehensive genetic analysis, leading to the detection of disease-causing variants and higher diagnostic success. Its capacity to sequence entire genes in a single read increases the chances of identifying harmful variants, even in cases with varied or atypical clinical features. NGS comprises short-read sequencing and long-read sequencing methods. Short-read sequencing generates DNA fragments of 250 to 800 base pairs (bp), making it cost-effective and suitable for high-throughput tasks like whole genome sequencing and gene panel testing. It efficiently identifies single nucleotide polymorphisms (SNPs) and small variations but may struggle with complex regions and structural variations due to its shorter read length. In contrast, long-read sequencing produces reads that can generate sequences >10 kb, making it ideal for analysing complex genomes, detecting large structural variants, and resolving repetitive sequences (Hu et al., 2021a). NGS facilitates the identification of disease-causing variants by comparing the patient's genomic data with reference sequences or samples from healthy individuals. This comparison allows clinicians to determine whether specific variants are associated with known rare diseases or potentially novel disorders (Cho et al., 2017).

Overall, the utilisation of NGS has significantly advanced the understanding and management of rare genetic disorders (Vinksel et al., 2021). Its capacity to perform thorough genetic analysis, identify disease-causing variants, increase diagnostic yield, provide efficiency and speed, and contribute to the discovery of novel disease genes has revolutionised the field of rare disease diagnosis and molecular analysis.

The NGS involves four steps: nucleic acid isolation, library preparation, cluster generation, sequencing, and data analysis (Hu et al., 2021a). In the case of variant determination, once the sequence has been determined, it will then be processed computationally by aligning the sequence to the reference genome, identifying the variants, and annotating the variant with positional information and various bioinformatics tools.

2.4.3 Variant Prioritisation and Interpretation

One of the crucial steps in NGS data analysis is variant annotation, prioritisation, and interpretation. This process involves the identification and selection of candidate variants from a huge amount of data generated by sequencing technologies. The aim is to filter the single nucleotide polymorphism (SNP) and focus on the candidate variant to be analysed. Ten of thousands of exonic variants in the human genome detected in the sequence mostly are synonymous that do not cause amino acid change, and benign variants that might not have a functional impact (Shen et al., 2013). These variants have to be filtered out leaving only the remaining as potential candidate variants. Then, these candidate variants can be filtered using population frequency, gene function, *in silico* prediction score, and various other bioinformatics tools to come out with only a few candidate variants.

Population frequency, often measured by Minor Allele Frequency (MAF) indicates how common a particular variant occurs in a specific population, and those variants with a low MAF are often prioritised for further investigation. Specifically, a MAF threshold of less than 1% is commonly used to select variants that are likely pathogenic

(Mahlich et al., 2017). This threshold helps to filter out common variants that are unlikely to contribute to disease, allowing researchers to focus on rare variants that may have a significant impact on health.

2.4.4 *In Silico* Prediction Scores

In silico prediction tools are then used to assess the variant potential impact on protein function. Various software have been developed by bioinformaticians to predict the effect and pathogenicity of the variant on amino acid substitutions including SIFT, CADD, PROVEAN, and PolyPhen-2. *In silico* prediction tools play a crucial role in evaluating the potential pathogenicity of the variants, particularly in the rare diseases (Licata et al., 2023). These tools utilise various algorithms to assess the likelihood that a variant will impact protein function or contribute to disease (Ghosh et al., 2017). By analysing sequence conservation, structural changes, and functional predictions, these tools help to prioritise variants for further analysis. Understanding their cut-off scores is essential for distinguishing between benign and potentially pathogenic variants, providing valuable insights into genetic disorders.

SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>) predicts whether an amino acid substitution affects protein function and the rank score range is 0 to 1, whereby a variant that has a rank score > 0.5 is predicted to be deleterious while those with a score of < 0.5 are predicted to be tolerated variants (Kumar et al., 2009). PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) predicts the functional impact of amino acid substitutions and the rank score range is 0 to 1. A score of > 0.5 is considered probably damaging and has a deleterious effect on protein function, while variants with < 0.5

score are possibly damaging and lower the likelihood of having a significant impact on protein function (Adzhubei et al., 2010). PROVEAN (<http://provean.jcvi.org/>) is a software tool which predicts whether an amino acid substitution or indel has an impact on the biological function of a protein, and from a rank score of 0 to 1, the variants with a score of > 0.5 indicate a deleterious effect, and < 0.5 are neutral (Choi and Chan, 2015). MutPred (<http://mutpred.mutdb.org/>) evaluates the pathogenicity of amino acid substitutions by integrating evolutionary, structural, and functional features of proteins and the score ranges from 0 to 1, with > 0.5 indicating more likely to be deleterious and < 0.5 less likely to be deleterious or polymorphism (Li et al., 2009).

Meanwhile, PhyloP measures the conservation of nucleotide sequences across different species by assessing evolutionary rates, whether a particular nucleotide or region is conserved relative to its expected evolutionary rate. The higher the rank score value (> 0.5) indicates greater conservation (Pollard et al., 2010). GERP++_RS score range is -12.3 to 6.17, whereby a variant with > 2 is considered deleterious or also specifically termed as “highly conserved” in this prediction tool and those < 2 is less conserved (Davydov et al., 2010). Multiple algorithm tools such as CADD (<https://cadd.gs.washington.edu/>) provide a Phred-scaled score that combines multiple sources of data including conservation, functional annotations, and allele frequencies to predict the impact of variants on gene function (Rentzsch et al., 2019). CADD_phred score range is 0 to 35, and a variant with a score > 20 is considered deleterious in protein function. Furthermore, REVEL integrates multiple predictors and statistical models to assess pathogenicity of a missense variant whether is likely to be deleterious or benign (Ioannidis et al., 2016). REVEL rank score ranges from 0

to 1, where variants with a score > 0.5 are often considered to be deleterious, and < 0.5 is benign.

Additionally, databases aggregating variant population frequencies are a useful method to explore pathogenicity by assessing the prevalence of the variant in the population. Among the population frequency databases available are 1000genomes, and ExAC (Exome aggregation consortium). These databases can be helpful in filtering out common SNPs as an example of a dominant variant, the variant can be considered as not causative if it is found in more than 1% of the population (Oliver et al., 2015). By combining MAF data with other factors, such as functional predictions and clinical relevance, researchers can enhance their ability to identify potential pathogenic variants effectively.

2.4.5 The American College of Medical Genetics and Genomics (ACMG)

Guidelines

The final step in determining the clinical significance of the variant is using The American College of Medical Genetics and Genomics (ACMG) guidelines. ACMG guidelines provide a standardised framework for the classification and interpretation of the variants (Richard et al., 2015). These guidelines offer a standardised way to interpret genetic data by considering several factors. These include how common a variant is in the population, its predicted impact on protein function, and evidence from functional studies and family inheritance patterns. This approach ensures that variants are evaluated consistently and accurately. By applying these criteria, variants can be classified into categories such as pathogenic, likely pathogenic, benign, or likely

benign. This structured approach is important because it ensures consistency in how variants are interpreted across different laboratories and clinical settings. The ACMG guidelines help identify variants that may be disease-causing and aid in making accurate diagnoses, ultimately guiding treatment options and genetic counselling for the patients.



CHAPTER 3

CLINICAL EXAMINATION OF TWO SIBLINGS WITH MYOTONIA CONGENITA

3.1 Introduction

Myotonia congenita (MC) is an inherited neuromuscular disorder caused by variants on the chloride channel encoded gene, *CLCN1*. Myotonia in MC exhibits considerable clinical variability, ranging from myotonic discharges only detectable on the electromyography (EMG) test (Arzel-Hezode et al., 2010) to the disabling muscle stiffness at an early age. The leg muscles are commonly affected, and about three-quarters of patients exhibit handgrip myotonia (Trivedi et al., 2013). Nearly every skeletal muscle in the body can exhibit muscle stiffness, but symptom tends to improve with exercise, a response known as the warm-up phenomenon (He et al., 2024; Chrestian et al., 2006). The clinical picture depends on whether the disease is inherited as autosomal dominant Thomsen disease (TMC) (OMIM#160800) or autosomal recessive Becker disease (BMC) (OMIM#255700). These two types of MC can be further distinguished by clinical presentation.

In TMC, the age of onset is normally at birth and early infancy (one year) and muscle hypertrophy (body-builder appearance of muscle) which is rare in childhood, are common in adulthood. TMC severity can range from mild to moderate, and patients can experience normal life without progression of the symptoms. On the other hand, the age of onset of BMC is usually later in life, which is during early childhood (3 – 8 years) (Heatwole and Moxley, 2007), but some studies showed much earlier onset in

BMC which is before three years old (Dupre et al., 2009). The predominant features of TMC are a painless, transient and muscle stiffness that primarily affects both the upper extremities and the facial muscles (Davies and Hanna, 1999).

BMC is more common with more severe symptoms and is frequently associated with transient muscle weakness and muscle hypertrophy in contrast to TMC. TMC is less common and usually presents with asymptomatic to moderately severe symptoms with myotonia more commonly seen in the upper limbs (Matthews et al., 2010; Lossin and George, 2008; Becker et al., 1977). BMC can also be differentiated from TMC by the presence of a slowly progressive weakness in some patients (Gilitwala et al., 2023), occasional atrophy of certain muscle groups and transient episodes of proximal weakness that are common in most of the patients (Shapiro and Ruff, 2002). Additionally, nearly half of BMC patients (56%) first experience symptoms in their lower extremities before any involvement in their upper extremities compared with only 12% in TMC (Becker et al., 1977). Even though some phenotypic features are more common in the recessive than in the dominant form such as the presence of transient weakness and prominent myotonia symptoms in the lower limb, considerable phenotype overlap exists and complicates the genotype-phenotype correlation. In a study by Liu et al. (2015), both BMC and TMC patients reported transient weakness and lower limb stiffness at the age of onset.

In addition to the clinical history and physical examination, electrophysiological studies are important to confirm the presence of myotonia by showing myotonic bursts (waxing and waning muscle potentials in frequency and amplitude) with needle EMG (Hehir and Logigian, 2012). More specific tests, such as repetitive nerve stimulation

(RNS), short exercise test (SET), and muscle cooling can help to differentiate between various myotonia phenotypes. However, the sensitivity and specificity of these tests can vary depending on the variant. The present study was conducted to determine the clinical characteristics of a family with MC, i.e., clinical symptoms, laboratory investigations, and electrophysiology study.

3.2 Materials and Methods

3.2.1 Study Ethics

This study involving human participants was reviewed and approved by JKEUPM (Ethic Committee for Research Involving Human Subjects), Universiti Putra Malaysia (JKEUPM-2020-135) (**Appendix A1**). All patients, participants, and their family members provided written informed consent to participate in this study (**Appendix A2 and A3**).

3.2.2 Study Design

This study used a convenient sampling method by recruiting patients referred to Hospital Sultan Abdul Aziz Shah (HSAAS). This clinical study was conducted at the Neurophysiology Laboratory, HSAAS, Selangor, Malaysia, and the genetic and molecular study was conducted at the Medical Genetic Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Selangor, Malaysia.

3.2.3 Sampling

A 49-year-old woman with the MC phenotype referred to HSAAS was recruited for this study. The study was focused on a family with two patients (siblings) suspected of MC. The patients' first-degree relatives including proband's older brother and all their children aged 14 to 17 years were recruited for the study. Additionally, other family members; including the patients' second-degree relatives (maternal and paternal siblings, and maternal grandaunt) were also recruited for physical examination to assess for any symptoms of MC.

3.2.4 Physical Examination

Physical examination was conducted by the neurologist to evaluate the features of MC. A detailed medical history was obtained including the age of onset, progression and the family history of similar symptoms. Myotonia was assessed through delayed handgrip relaxation and repeated eyelid closure. This was followed by the examination of the muscle to assess muscular hypertrophy, particularly on the calves and biceps, muscle strength test and gait analysis.

3.2.5 Clinical Examination

3.2.5.1 Blood Test

The patients, as well as first-degree relatives, were subjected to clinical and physical examination, blood tests, and electrodiagnostic studies. Five millilitres of blood samples were collected in an EDTA blood collection tube (BD Vacutainer, USA) from the study subjects, kept under room temperature and blood test was conducted by the HSAAS laboratory. Blood tests conducted include a full blood count, differential count, hemoglobin, liver function, renal function, serum creatine kinase (CK), serum electrolytes analysis (calcium, phosphate, magnesium) and a thyroid function test.

3.2.5.2 Electrodiagnostic Studies

3.2.5.2.1 Electromyography (EMG)

Needle electromyography (EMG) was utilised to detect the presence of electrical myotonia (Nojszewska et al., 2019), which is caused by membrane hyperexcitability leading to mild depolarisation of the muscle membrane, resulting to spontaneous and self-sustained bursts of muscle fibre action potentials. EMG examination was carried out by the neurologist from the Neurology Department, HSAAS in all subjects using the Natus Nicolet AT2 Amplifier (Natus Neurology, Natus Medical, Pleasanton, USA) (**Figure 3.1**) following a standardised protocol for electrophysiology assessment of skeletal muscle (Fournier et al., 2004). Four muscles were examined (extensor digitorum, dorsal interosseous, vastus lateralis, and tibialis anterior muscle). An

electromyographic examination was performed using standardised EMG protocols offering a reproducible and painless method for electrophysiological assessment of skeletal muscle. Needle EMG can be used to detect the presence of myotonic discharge. These myotonic potentials are characterised by repetitive discharges firing at a rate of 20 – 80 Hz, in which the amplitude and frequency of the potentials wax and wane, producing a characteristic “dive bomber” sound (Miller, 2008).



Figure 3.1: Natus Nicolet AT2 Amplifier (Natus Neurology, USA) equipment used to conduct EMG examination.

3.2.5.2.2 Short Exercise Test (SET)

An electrophysiology test, a short exercise test (SET) was conducted to identify the Fournier pattern I – III. It is the main electrodiagnostic distinction between different forms of myotonic disorder (Fournier et al., 2006). It involves an exercise in combination with the determination of compound muscle action potential (CMAP). The CMAP amplitude is an indirect measure of sarcolemmal excitability, giving an indication of the ability of active fibres to depolarise and repolarise (Streib, 1987). Exercise can act as a trigger to sarcolemmal depolarisation and therefore specialised short exercise testing can be used to help to differentiate between the types of

channelopathy (Fournier et al., 2006, 2004; McManis et al., 1986). The CMAP were recorded before and after periods of exercise at specific time points. SET procedure was conducted by stimulating the ulnar nerve at the wrist (**Figure 3.2**).

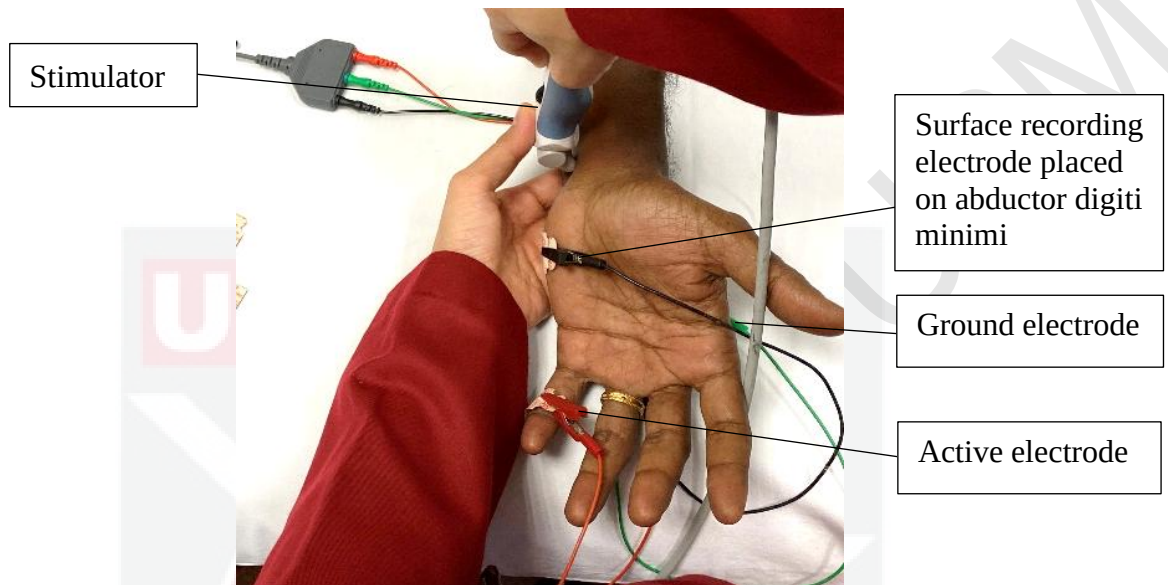


Figure 3.2: Short exercise test (SET) procedure. The ulnar nerve was stimulated at the wrist using stimulator, patient was then asked to contract the abductor digiti minimi (ADM) as strongly as possible and the potential was recorded using surface recording electrode that was placed on ADM. The ground electrode was placed between the active electrode and stimulator.

During the procedure, supramaximal CMAP were recorded at baseline. Patients were then asked to contract the abductor digiti minimi (ADM) as strongly as possible for 10 s, and CMAP were then taken immediately after exercise at 10-s intervals for a total of 60 s. This protocol was repeated three times and repeated with cooling by soaking the hand in icy water for 5 min. Post-exercise CMAP amplitudes were then calculated as percent change from the average pre-exercise baseline measurement.

In normal subjects, the CMAP does not decline when the stimulation applied at the wrist. Any decrement in CMAP of over 10% was considered abnormal on the test, which corresponds to other standardised values reported in the literature (Shapiro and

Preston, 2003). Different variations in the CMAP determined the Fournier patterns that distinguished the different type of non-dystrophic myotonia (NDM). Patients with MC commonly exhibit Fournier pattern II with an initial decrement of post-exercise CMAP, which was improved by 60 s and less pronounced in subsequent trials (Fournier et al., 2004). Pattern I is typical in paramyotonia congenita (PMC) patients, in whom decrements are maximal after the third trial, and Pattern III, which does not show any abnormal decrement, is typical for sodium-channel myotonia (SCM) patients (Tan et al., 2011; Kuntzer et al., 2000). This test shows distinct patterns depending on the channel defect and therefore can be helpful in guiding diagnosis and genetic testing. The amplitude of CMAP, expressed as a percentage of its value before the trials was plotted against the time elapsed after the first exercise trials.

3.2.5.2.3 Nerve conduction studies (NCS)

Nerve conduction studies (NCS) were performed on upper and lower limbs. It was performed unilaterally with surface stimulation on the nerves recording from muscles. Pairs of electrodes were used, one to initiate the impulse and the other to record the response further along the path of the nerve. The amplitude, latency and velocity were recorded (Nojszewska et al., 2018).

3.3 Results

3.3.1 Clinical Phenotypes

3.3.1.1 Proband

The proband is a 49-year-old woman, who was referred to HSAAS for further follow-up of her MC diagnosis. She is of Sri Lankan Tamil ethnicity and is the second born of two siblings from a non-consanguineous marriage. She disclosed episodes of muscle stiffness (myotonia) and cramps beginning in early childhood at five years old, but these symptoms did not limit her daily activities. She had frequent falls especially when she tried to walk after a period of rest. She was clinically diagnosed with MC at the age of 24, after a positive myotonic discharge finding on EMG. The proband did not undergo genetic analysis at the initial diagnosis. She defaulted clinical follow-up and did not notice any further worsening of her symptoms.

Her pregnancy and history of giving birth (at the age of 34- and 35-years old) were unremarkable. She gave birth by caesarean section via epidural anaesthesia for both pregnancies and has never been given general anaesthesia. There was no extra-muscular manifestation, e.g., cataracts, endocrine dysfunction, or any previous cardiac problem which excludes the possibility of myotonic dystrophy. During referral, she complained of difficulty initiating muscle contraction and mild transient weakness after rest, which improved after exercise (warm-up phenomenon) (**Figure 3.3**). The myotonia affected all skeletal muscles, including the bulbar muscles, where she also

has difficulty swallowing, and myotonia caused cramps and pain in the sternal muscle during sudden sneezing.

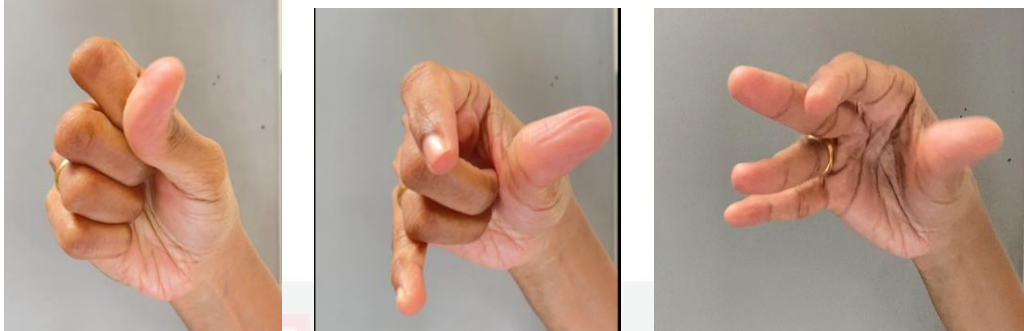


Figure 3.3: Proband has difficulty releasing a finger after a hand grip. After grip hand that trigger the initial contraction, it took 8 – 10 s for the proband to fully release the handgrip.

3.3.1.2 Family members

3.3.1.2.1 Patient 2

Proband's older brother or patient 2 (hereafter referred to as PB) is a 51-year-old man who had the similar symptoms. He first noticed muscle stiffness at the age of 13 with hand and leg cramps but remained undiagnosed until this study was conducted. The patient described his symptoms as mild myotonia compared to his sister, had no limitations in daily activities, and could still play sports. He had mild difficulty climbing up the stairs, releasing hand grips on objects, and experiencing eye-closure myotonia.

3.3.1.2.2 Children

First-degree relatives included in the study are the proband's and PB's children. PB has one 17-year-old daughter (referred to as C1), while the proband has a 15-year-old son (C2) and a 14-year-old daughter (C3). The children of both the proband and PB did not have any clinical manifestations and were clinically classified as normal children.

3.3.2 Physical Examination

Upon physical examination, the proband was reported to develop muscle hypertrophy (an abnormal muscle enlargement) on the right and left biceps (**Figure 3.4A and B**) and right and left calves (**Figure 3.4C and D**). Meanwhile, PB exhibited mild muscle hypertrophy in the bilateral biceps and calves (photographs not shown). The strength in both the upper and lower limbs, for both proximal and distal regions was normal, with no myotonia when testing reflexes as it does not involve the nerve and spinal reflex pathway. Gait and sensory examination were normal for both siblings. Physical examination of the other family members, including the three normal children, showed no signs of hypertrophy. The spouses of the proband and PB were not available for clinical examination; however, based on the observations and interviews, there are no signs of myotonia in these individuals, indicating that they do not exhibit any physical symptoms.

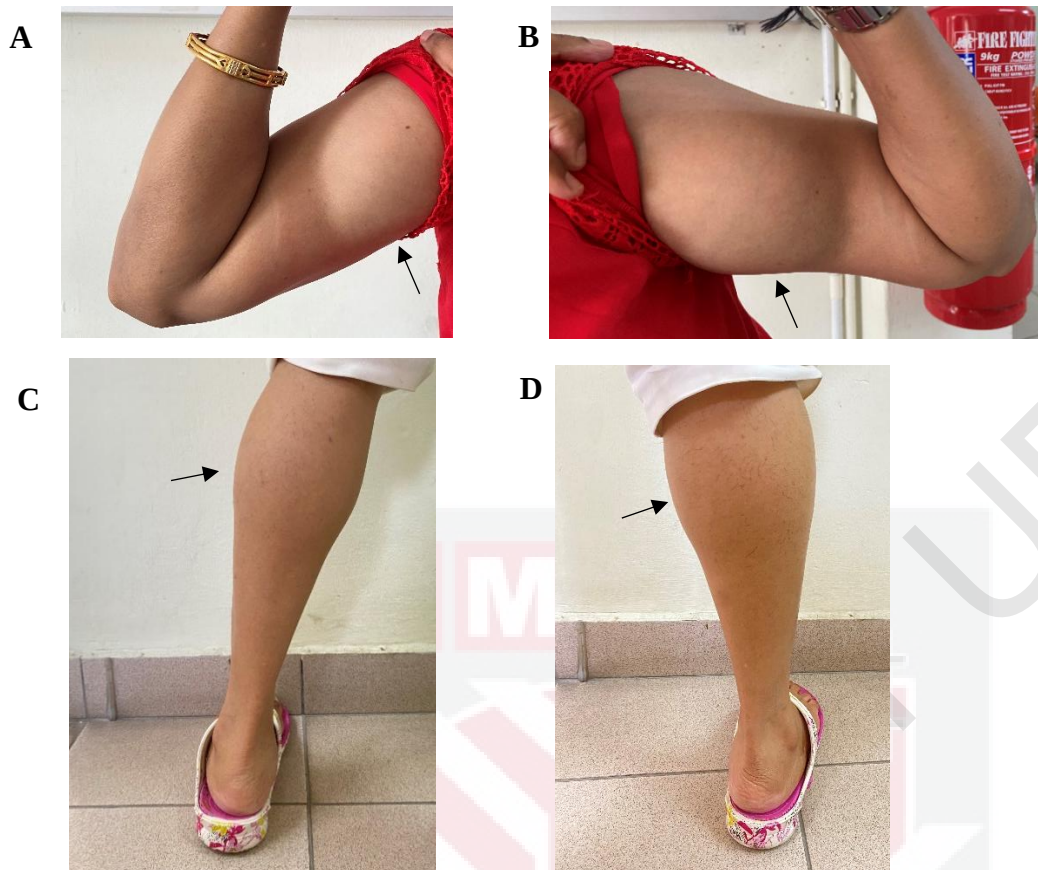


Figure 3.4: Proband's muscle hypertrophy on right (A) and left (B) bilateral biceps and right (C) and left calves (D). The arrow indicates enlargement of the muscle (muscle hypertrophy).

3.3.3 Blood Test Marked an Increased in Serum Creatine Kinase (CK) Value

Blood samples were collected from the proband, PB and the children and were analysed for a full blood count, liver function, renal function, serum CK, and a thyroid function tests (**Table 3.1**). Blood profiles which included full blood count, differential count, hemoglobin, renal and liver function, were normal for both proband and PB. A thyroid function test conducted on both patients also showed a normal level of thyroid-stimulating hormone (TSH) and thyroxine (FT4). In contrast, both the proband and PB had an elevated serum CK level of 447 U/L and 228 U/L, respectively (normal range < 201 U/L) (Nardin et al., 2009) (**Figure 3.5**). A blood test on all normal children showed normal CK values ranging between 72 – 122 U/L.

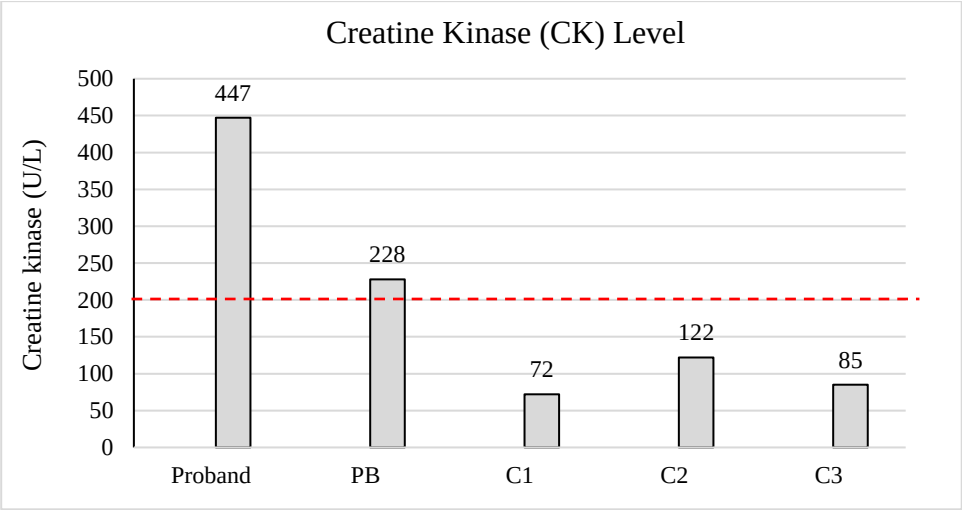


Figure 3.5: MC patients show an increased in CK value. Proband shows an elevated creatine kinase (CK) level with 447 U/L and a slight increase in PB with 228 U/L, while the children (C1: Child 1, C2: Child 2, C3: Child 3) reported a normal CK value. The red line indicates the cut-off value for the normal range CK which is less than 201 U/L (Nardin et al., 2009).

Table 3.1: Blood test analysis. Blood test including calcium, CK, ESR, FBC, differential count, hemoglobin, liver function test, phosphate, renal function, and thyroid function test.

Test	Parameters	Proband	PB	C1	C2	C3	Reference Range
Calcium	Serum calcium (mmol/L)	2.31	2.34	2.33	2.5	2.34	2.10 - 2.55
	Adjusted calcium (mmol/L)	2.25	2.26	2.21	2.34	2.24	2.10 - 2.55
	Albumin (g/L)	43	44	46*	48*	45	32 - 45
CK	Creatine kinase (U/L)	447*	228*	72	122	85	<170
ESR	Erythrocyte sedimentation rate (mm/h)	N/A	2	6	4	13	<18
Full blood count (FBC)	White blood cell (WBC) ($10^3/\mu\text{L}$)	7.3	6.7	5.3	7.3	7.1	4.1 - 11.4
	Red blood cell (RBC) ($10^6/\mu\text{L}$)	4.65	4.84	4.39	5.15	4.61	3.9 - 5.2
	Hemoglobin (HB) (g/dl)	13.2	14.4	10.6*	13.6	10.8*	11.6 - 15.1
	Hematocrit (HCT) (%)	40.6	42.9	33*	41	34*	35.1 - 44.9
	Mean corpuscular volume (MCV) (fL)	87.3	88.6	75.2	79.6*	73.6*	80.6 - 95.5
	Mean corpuscular hemoglobin (MCH) (pg)	28.4	29.7	24.2*	26.3*	23.4*	26.9 - 32.3
	Mean corpuscular hemoglobin concentration (MCHC) (g/dL)	32.5	33.5	32.2	33.1	31.8*	31.9 - 35.3
	Red cell distribution width (RDW) (%)	13.4	12.9	16*	13.3	15.4*	12 - 14.8
	Red cell distribution width Std dev (fL)	40.7	40.3	42.4	37.6*	39.8	39–46
	Platelet ($10^3/\mu\text{L}$)	314	249	266	355*	330	171 - 399
	Mean platelet volume (MPV) (fL)	7.5	8.2	8.3	8.4	7.7	7.5 - 11.2
Differential count	Neutrophils (NE) (%)	66.5	43.8	45.8	41.4	55.6	40.0 - 80.0
	Lymphocyte (LY) (%)	22.5	32.9	35.7	47.3*	31.8	20 - 40
	Monocyte (MO) (%)	7.2	9.8	9.5	7	9.6	2 - 10
	Eosinophil (EO) (%)	3.4	12.4*	8*	3.5	2.4	1.0 - 6.0
	Basophil (BA) (%)	0.7	1.1	1	0.8	0.6	0.0 - 2.0

	Neutrophils (NE) # (10*3/ μ L)	4.9	2.9*	2.4	3.0*	3.9	3.9 - 7.1
	Lymphocyte (LY) # (10*3/ μ L)	1.6*	2.2	1.9	3.5	2.2	1.8 - 4.8
	Monocyte (MO) # (10*3/ μ L)	0.5	0.7	0.5	0.5	0.7	0.4 - 1.1
	Eosinophil (EO) # (10*3/ μ L)	0.3	0.8	0.4	0.3	0.2	0.0 - 0.8
	Basophil (BA) # (10*3/ μ L)	0.1	0.1	0.1	0.1	0	0.0 - 0.1
Hemoglobin A1c	HBA1C (NGSP) (%)	5.3	5.3	5.4	5.7	5.7	< 5.7
	HBA1C (IFCC) (mmol/mmol)	34	34	35	39	39	< 39
Liver function Test	Albumin (g/L)	N/A	44	46*	48*	45	32 - 45
	Total proteins (g/L)	N/A	72	75	80	78	60 - 80
	Bilirubin total (umol/ L)	N/A	7	5	4	3	<15
	Alanine aminotransferase (ALT) (U/L)	N/A	10	18	13	9	<34
	Alkaline phosphatase (ALP) (U/L)	N/A	69	64	333*	185	57 - 254
	Globulin (g/L)	N/A	28	29	32	34	20 - 39
	A/G ratio	N/A	1.57	1.59	1.5	1.32	1.0 – 2.5
	Serum Magnesium (mmol/L)	0.84	0.86	0.77	0.8	0.84	0.70 - 0.91
Phosphate	Serum phosphorus (mmol/L)	1.14	1.22	1.26	1.55	1.60*	0.90 - 1.55
Renal profile	Sodium (mmol/L)	140	136	139	142	143	136 - 145
	Potassium (mmol/L)	4.7	4.6	4.1	4.3	4.9	3.4 - 5.1
	Chloride (mmol/L)	103	104	103	104	106	98 - 107
	Urea (mmol/L)	2.2	3.7	3.6	2.7*	2.9	2.76 - 8.07
	Creatinine (μ mol/L)	73	106	49	67	50	50 – 106
Thyroid function test	Thyroid stimulating hormone (TSH) (mIU/L)	2.25	1.72	0.77	3.3	3.82	0.51 - 4.3
	Free thyroxine (FT4) (pmol/L)	12.5	14	13.9	15.1	13.9	12.6 - 21

Note : Asterisk (*) indicates values that fall outside the reference range, and bold value indicate an elevated CK value. N/A: Data not available

3.3.4 Electrodiagnostic Testing

Recently, specialised clinical electrophysiology protocols have aided precise diagnosis in muscle channelopathies. The electrodiagnostic testing in this study included EMG and a short exercise test (SET) for all subjects.

3.3.4.1 Electromyography (EMG) Showed Myotonic Discharges

EMG on the first dorsal interosseous, biceps, and tibialis anterior of the proband during rest demonstrated myotonic discharges with dive bomber sounds. This is an electrophysiological hallmark of MC, and her brother exhibited a similar myotonic discharge (**Figure 3.6A**). Meanwhile, normal children exhibited electrical silence which is the absence of electrical potential at rest (**Figure 3.6B**).

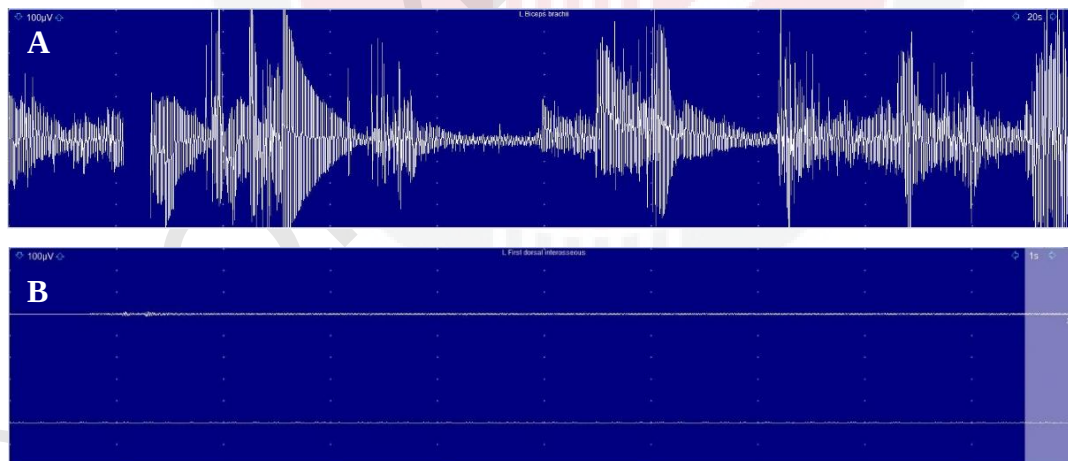


Figure 3.6: Electromyography (EMG) readings in a patient with MC and the patient's normal children. (A) A representative EMG of the MC patient showed myotonic discharges during rest while (B) a representative from normal children showed an absence of electrical potential readings.

3.3.4.2 Short Exercise Test (SET) Revealed Fournier II Pattern

A short exercise test (SET) was performed on all subjects. During the test, patients were required to contract the ADM as strongly as possible for 10 s, and serial CMAP were taken immediately after exercise and relaxation at 10-s intervals for 60 s (data shown in **Appendix A4**). During this test, both proband and her brother (PB) showed an initial post-exercise CMAP decrement with reductions of -21% (**Figure 3.7A**) and -19% (**Figure 3.7B**), respectively, followed by improvement after 60 s. Additionally, both graphs show the decrement after exercise recovers quickly and diminishes after three successive repetitions, reflecting the transient weakness observed clinically. This reduction in CMAP revealed a Fournier pattern II suggesting MC. A CMAP amplitude reduction of more than 10% for the SET is considered abnormal (Fournier et al., 2004). The SET was also performed after cooling, which showed similar findings at room temperature. Repeated SET performed on all three children at room temperature showed no significant change in CMAP amplitude (**Figure 3.7C and Appendix A5**). Results obtained from symptomatic family members (MC patients) were pooled together, while the three normal children were pooled in another graph plot (**Figure 3.8**).

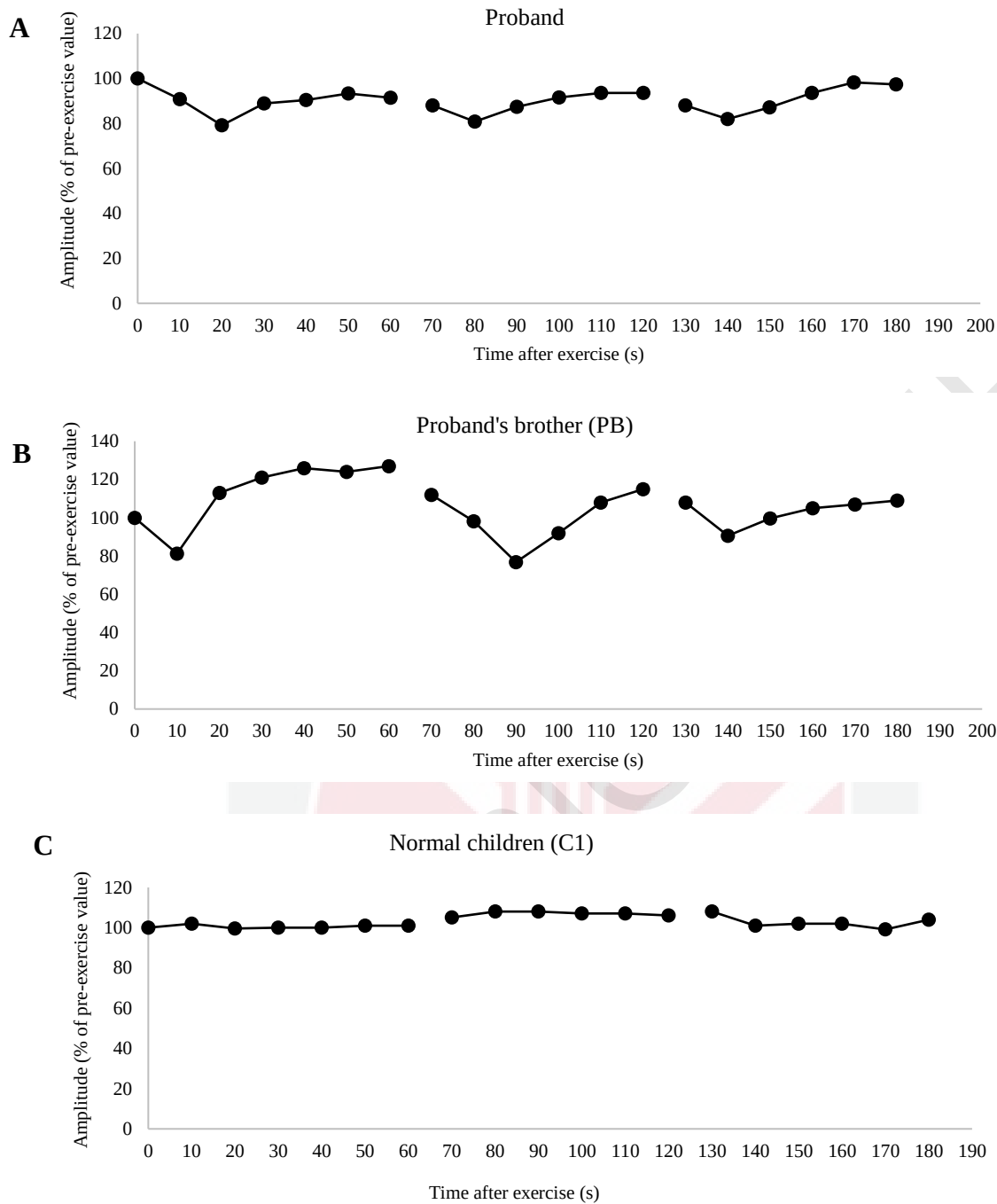


Figure 3.7: Repeated short exercise test in MC patients and a representative from a normal child. (A) Proband and (B) PB show a reduction in CMAP amplitude following three successive short exercises at room temperature while (C) normal children (C1) show no reduction in CMAP post-SET, which was similar to the other two children (Appendix A4). The amplitude of the CMAP, expressed as a percentage of its value before the trials, is plotted against the time elapsed after the first exercise trials.

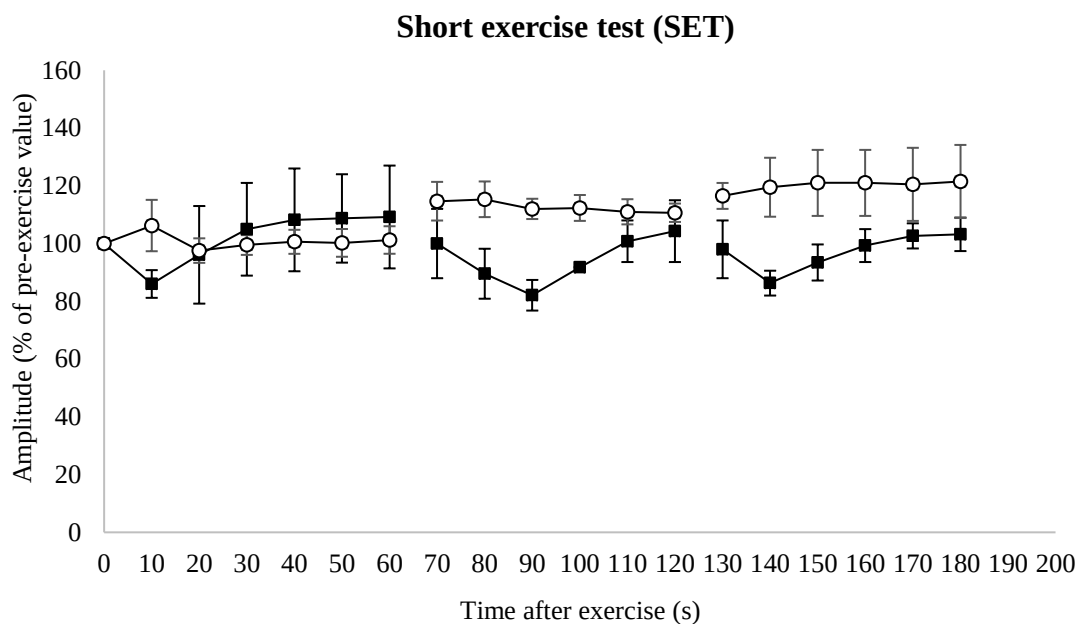


Figure 3.8: Comparison of the repeated short exercise test (SET) in MC patients and normal children. Changes in CMAP amplitude on left ulnar nerve following three successive short exercises at room temperature in two MC patients (■) and 3 normal children (○). SET shows an initial decrement in CMAP, which subsequently improves (Fournier Pattern II) in MC patients. The amplitude of the CMAP, expressed as a percentage of its value before the trials, is plotted against the time elapsed after the first exercise trials. Symbols and vertical bars represent mean $\pm$ SEM.

3.3.4.3 Nerve Conduction Studies (NCS) Showed Normal Amplitudes, Velocity, and Latency

Nerve conduction studies (NCS) use electrical impulses to assess nerve damage and can be used to study sensory, motor, and mixed nerves. NCS revealed normal amplitudes, velocity, and latency in the upper and lower limbs for both the Proband and her brother (PB), in which most of the values fall within the normal range. The amplitude parameter showed a normal value of higher than 4 mV for the median nerve and 6 mV for the ulnar nerve, reflecting the normal number of conducting fibres and reduced axonal loss (**Table 3.2**) (Huynh and Kiernan, 2011). Conduction velocities recorded were higher than 50 m/s demonstrating the integrity of the myelin sheath

which is important for impulse conduction and this velocity is reduced in demyelinating sheath.

Table 3.2: Nerve conduction study recorded on latency, amplitude, and velocity in Proband (top) and PB (bottom)

Proband				
Nerve / Sites		Latency (ms)	Amplitude (mV)	Velocity (m/s)
Motor NCS				
Median nerve	Wrist	2.92 (<4 ms)	17.7 (>4 mV)	-
	Elbow	6.2 (<4 ms)	10.2 (>4 mV)	52 (>50 m/s)
Ulnar nerve	Wrist	2.08 (<3 ms)	10.2 (>6 mV)	-
	Below elbow	2.45 (<3 ms)	8.4 (>6 mV)	61.5 (>50 m/s)
	Above elbow	2.19 (<3 ms)	8.4 (>6 mV)	-
Sensory NCS				
Median nerve	Digit II	2.92 (<3.5 ms)	73.4 (<80 μ V)	-
	Mid palm	1.82 (<2.3 ms)	75.4 (<80 μ V)	56.9 (>50 m/s)
Ulnar nerve	Digit V	2.81 (<3.5 ms)	33.7 (<60 μ V)	-
	Mid palm	1.67 (<2.3 ms)	66.2 (<60 μ V)	66.8 (>50 m/s)
PB				
Nerve / Sites		Latency (ms)	Amplitude (mV or μ V)	Velocity (m/s)
Motor NCS				
Median nerve	Wrist	3.06 (<4 ms)	7.0 (>4 mV)	-
	Elbow	7.19 (<4 ms)	5.6 (>4 mV)	56 (>50 m/s)
Ulnar nerve	Wrist	2.50 (<3 ms)	8.8 (>6 mV)	-
	Below elbow	6.56 (<3 ms)	9.5 (>6 mV)	62 (>50 m/s)
	Above elbow	7.98 (<3 ms)	7.9 (>6 mV)	49 (>44.8 m/s)
Sensory NCS				
Median nerve	Digit II	2.33 (<3.5 ms)	50.2 (<80 μ V)	56 (>50 m/s)
	Mid palm	1.21 (<2.3 ms)	57.1 (<80 μ V)	66 (>50 m/s)
Ulnar nerve	Digit V	2.27 (<3.5 ms)	32.2 (<60 μ V)	48 (>50 m/s)
	Mid palm	1.08 (<2.3 ms)	29.8 (<60 μ V)	74 (>50 m/s)

Note: the values in bracket indicate the normal range.

3.3.5 Other Family Members

MC symptoms were not reported in the children of both proband and PB aged between 14 and 17 years old, and other family members including maternal and paternal

siblings, and maternal grandaunt when physically assessed by the neurologist. Additionally, the family history assessment conducted through interviews revealed that both deceased parents did not exhibit any MC symptoms. EMG tests for the children did not reveal myotonic discharge (**Figure 3.6B**). Normal CK levels were also recorded (< 201 U/L). The children's SET results did not show a reduction in CMAP post-exercise (**Figure 3.7C**). The clinical manifestations of the proband, PB, and the normal children are summarised in **Table 3.3**.

Table 3.3: Summary of clinical examination of Proband, PB, and normal children

Clinical examination	Proband	PB	C1	C2	C3
Sex	Female	Male	Female	Male	Female
Age at examination	49	51	17	15	14
Age at onset	5	13	N/A	N/A	N/A
Clinical myotonia:					
Myotonia	+	+	-	-	-
Muscular hypertrophy	+	+	-	-	-
Transient weakness	+	+	-	-	-
Warm up phenomenon	+	+	-	-	-
Serum creatine kinase (U/L)	447*	228*	72	122	85
Myotonic discharge	+	+	-	-	-
CMAP decrement	21%	19%	No	No	No
Fournier pattern	II	II	III	III	III
Nerve conduction studies	Normal	Normal	Normal	Normal	Normal

Note: + (present), - (absence). Asterisk (*) indicates values that fall outside the reference range, and bold value indicate an elevated CK value which is the clinical feature of MC (normal < 201 U/L). Fournier pattern II characterised by a drop in CMAP amplitude that improves after 60 seconds, while Fournier pattern III represents by no abnormal decrement in CMAP (Refer **Appendix A6** for Fournier pattern).

3.4 Discussion

MC is characterised by muscle stiffness present from childhood and affects all striated muscle groups, including the extrinsic eye muscles, facial muscles, and tongue. The stiffness experienced can be alleviated through repeated muscle contractions, known as the "warm-up" phenomenon, and is typically accompanied by muscle hypertrophy (Duno and Vissing, 2021). This has been observed in most of MC cases (Miao et al, 2018; Lakraj et al., 2013), including the MC patients in the present study.

In this study, clinical examination was conducted to confirm MC in a single family. Despite the absence of symptoms in previous generations in family members, two siblings are affected by the neuromuscular disorder. There is a phenotypic heterogeneity between the siblings, including differences in age of onset, severity of myotonia, and CK levels in which the proband had earlier onset, more severe symptoms, and higher CK value than her older brother (PB). This finding is similar to a study reported by Portaro et al. (2015), which presented two affected siblings without any family history. The study demonstrated both similarities and variations in the sibling's phenotypes. The disease onset in both was 10 years old however, the CK value was slightly increased in the sister (400 U/L) while her brother had elevated values reaching 1200 U/L. The brother seems to be more affected, as indicated by the higher CK levels, and more pronounced muscle hypertrophy. Although there are reports indicating distinct differences in the clinical presentation characteristics (e.g. age of onset, muscle weakness, presence of hypertrophy, and the distribution of myotonia) of TMC and BMC, some studies show no significant differences between TMC and BMC (Hu et al., 2021b).

In addition to the physical examinations, serum CK levels can indicate a muscle disorder. This study reports the CK levels of the proband was double that of her brother (PB), whose CK value was slightly above the normal upper limit (200 U/L) which possibly reflects the severe symptoms in the proband compared to her brother. Serum CK base levels in the general population typically range from 35 to 175 U/L (Gagliano et al., 2009). The CK level was defined as normal if the CK level was less than the upper normal limit; mildly elevated if the CK level was less than 10 x the upper normal limit (200 U/L); and markedly elevated if the CK level was higher than 10 x the upper normal limit (Jackson and Barohn, 2013). Recommended acceptable upper limits of normal CK levels have suggested an increase of 1.5 times the present limits (Kyriakides et al., 2010). The values can vary in patients with MC. Referring to the sibling in the study by Portaro et al. (2015), the two siblings affected by MC exhibited different CK levels, with the brother's CK value being three times higher than his younger sister. On the other hand, a study by Sparber et al. (2020) reported a 6-year-old boy with calf stiffness, muscle hypertrophy, and myotonic runs in biceps brachii and vastus lateralis but the CK level was normal. Gao et al. (2010) also reported normal CK values in two girls with MC, aged 6.5 and 8. Thus, as demonstrated by Vereb et al. (2021), CK values can vary among patients, where only 60% of the patients had an elevated CK level.

CK levels are significantly influenced by muscle mass and muscle damage. A greater amount of muscle mass and heightened vulnerability of muscles result in an increased release of CK from myocytes into the serum, consequently leading to elevated CK levels (Totsuka et al., 2002). Other parameters in blood profiles such as the electrolyte and liver function show a normal level in most MC patients (Lyons et al., 2010).

Thyroid function appeared to be normal in MC patients and it should be checked because hypothyroidism can cause clinical and electrical myotonia (Passeri et al., 2014).

To enhance the precision of MC diagnosis, needle EMG, a specialised clinical electrophysiology protocol was used. Electrodiagnostic tests have become a valuable tool for diagnosing and managing neuromuscular disorders. Utilising EMG, myotonia was diagnosed by observing prolonged, spontaneous trains of muscle unit action potentials that fluctuate in amplitude and frequency, resembling the sound of a dive bomber or a revving engine, as described by Fournier et al. (2004). These patterns of action potentials were observed for both the patients presenting with MC in this study.

Furthermore, variations in the CMAP following different stimuli readily detected electrical myotonia, allowing for indirect measurement of sarcolemmal excitability. The CMAP size displays variations during SET (10 – 20 s) (McManis et al., 1986; Streib, 1982), and the decrement in SET varies among different MC patients. For instance, a 14-year-old boy with TMC exhibited an 11% decrease in CMAP amplitude during SET (Thomas et al., 2008), whereas a 56-year-old woman with BMC showed a 62% decrease (Chrestian, 2006). Previous studies suggest that this pattern of CMAP decrement can offer insights into the specific type of MC, where TMC typically shows a variable reduction and BMC is often associated with a larger decrease in CMAP amplitude (Chrestian et al., 2006). MC patients in this study may suggest TMC as characterised by a CMAP decrement of less than 30% (Streib, 1987). However, whether decrement studies can be used to distinguish between TMC and BMC remains

unresolved, and it may depend on the specific variant associated with the phenotype (Colding-Jorgensen, 2003).

Following SET analysis, three distinct electrophysiological patterns; Patterns I, II, and III were identified for each non-dystrophic myotonia group. These unique patterns have a crucial role in distinguishing MC subtypes. Patients with chloride channel variants typically exhibit Pattern II, characterised by an immediate CMAP decrement ($> 10\%$) after exercise at room temperature, followed by quick recovery and diminishing with repetition, a pattern reflective of the transient weakness observed clinically. Specifically for MC, the Fournier pattern II can be observed in BMC, while TMC is reported to show the Fournier pattern II or III (Matthews et al., 2010). MC patients in this study showed Fournier pattern II, suggesting the diagnosis of MC condition. NCS use electrical impulses to assess nerve damage and results in previous studies have reported normal values (Hu et al., 2021), which is consistent with the result shown in this study. This sophisticated approach to electrophysiological assessment has significantly advanced our understanding and diagnosis of MC.

From the MC characteristics stated, both patients in this study displayed the classic triad of MC characteristics; myotonia, warm-up phenomenon, and muscular hypertrophy. Upon comparing the proband and her brother (PB), milder symptoms were observed in the proband's brother, manifested by a lower reduction in SET, milder hypertrophy, lower CK value and a later onset. The age of onset, disease progression, and symptoms may vary within the same family and among different families even the individuals carrying identical CLC-1 variants (Morales and Pusch, 2020; Colding-Jørgensen, 2005; Duno et al., 2004). This discrepancy in symptom

severity emphasises the variability within MC clinical presentations. Although certain phenotypes may suggest one type of MC, the overlap of additional phenotypic features makes it challenging to accurately determine whether it is TMC or BMC. Therefore, further genetic analysis is essential to confirm the type of MC. Meanwhile, for adolescent children that appear normal, continual monitoring is necessary as some conditions can develop after the age of 20 (Portaro et al., 2015; Plassart-Schiess et al., 1998).

3.5 Conclusion

In conclusion, this study provides a comprehensive analysis of the clinical phenotype of MC within a single Malaysian family, representing the first such investigation in Malaysia. The two siblings presenting with muscle stiffness confirmed the MC clinical diagnosis. The variability in symptom severity observed between the two patients in this family underscores the complexity of MC presentations as reflected by the range of myotonic symptoms, CK value, and age of onset. This study contributes valuable insights to the clinical characterisation and diagnostic complexity of MC, offering a foundation for future research and clinical management strategies in this rare neuromuscular disorder. Further investigation through genetic analysis holds promise for elucidating the underlying genetic mechanisms contributing to MC manifestations, which will be the cornerstone for improved diagnostic accuracy and targeted therapeutic interventions.

CHAPTER 4

GENETIC ANALYSIS AND INHERITANCE OF THE c.1667T>A (p.Ile556Asn) VARIANT IN A PATIENT WITH MYOTONIA CONGENITA

4.1 Introduction

Myotonia congenita is caused by variants in the *CLCN1* gene, encoded for chloride channel (ClC-1) which include missense, nonsense, and frameshift variants, as well as deletions and insertions. To date, more than 318 pathogenic variants (Duno and Vissing, 2021) across the 23 exons or within introns of the *CLCN1* gene have been reported in different populations (**Figure 4.1**) (Palma Milla et al., 2019; Portaro et al., 2018; Liu et al., 2015; Ulzi et al., 2012).

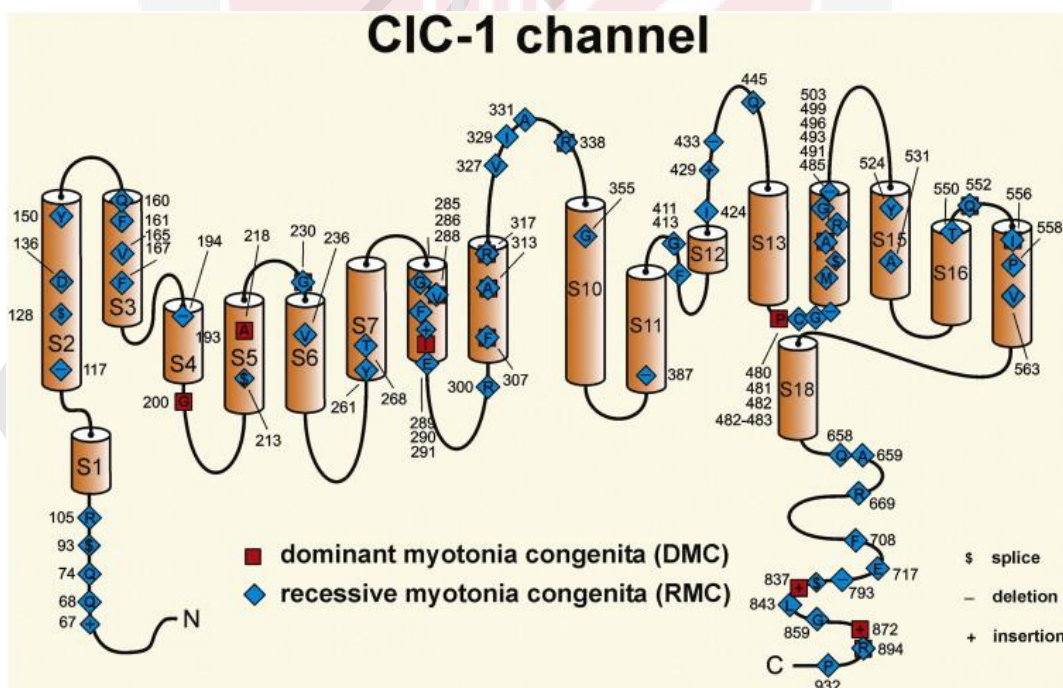


Figure 4.1: Membrane topology of the chloride channel, CLC-1. The model shows the skeletal muscle chloride channel monomer, CLC-1 with 18 helices (S1 – S18 represent helices from A – R, respectively) and the variants that span the entire length of the gene (Source: Jurkat-Rott et al., 2002).

The intensive search for variants that followed its discovery showed that the recessive form is more common, and the dominant form is rare with only 27 variants related to TMC as well as variants reported with an undefined inheritance pattern (Morales and Pusch, 2020). The same *CLCN1* variants can also be inherited as either a recessive or dominant allele in pedigrees, suggesting that dominance of an allele may contribute to MC phenotypic variability (Portaro et al., 2018; Kubisch et al., 1998). Furthermore, *CLCN1* variants can lead to incomplete or reduced penetrance (Plassart-Schiess et al., 1998). The complexity and variability in the manifestation of *CLCN1* gene defects are further complicate by the observation that some families harbour three *CLCN1* variants (Sun et al., 2001) and atypical cases, where myotonia only appears during pregnancies (Morton, 2020) and asymptomatic cases of dominant MC (Passeri et al., 2014).

A concerning issue for asymptomatic cases of MC is that the patients may have an altered response to anaesthetic drugs. There are cases of undiagnosed MC patients in Malaysia who developed life-threatening masseter spasms following induction of anaesthesia that caused potentially lethal complications (Looi et al., 2008). With this life-threatening complication, it is critical for MC patients to be diagnosed and seek treatment early in life. Consequently, understanding the genetic basis of MC is crucial for accurate diagnosis, genetic counselling, and the potential development of targeted therapies.

The diagnosis of MC now includes advanced methods including next-generation sequencing (NGS), which allows for more precise and comprehensive identification of genetic variants. Before the advancement of NGS, routine molecular diagnosis relied on a gene-by-gene approach, which focused on specific genes; as in the case of

myotonia, where *CLCN1* and *SCN4A* were screened (Maggi et al., 2017; Kato et al., 2016). Variants in the *SCN4A* gene encoding the skeletal muscle sodium channel cause sodium channel myotonia, hyperkalemic periodic paralysis and paramyotonia congenita. This gene-by-gene approach was costly, time-consuming, and challenging to identify the causative variant. NGS provides an effective and rapid strategy for detecting variants in humans by enabling all coding exons of the genome to be sequenced in a single test (Rizzo and Buck, 2012). Nowadays, many studies use a whole-exome sequencing (WES) approach, which was cost-effective and allows detailed analyses of the protein-coding exome to detect common and rare variants (Lohmann and Klein, 2014). NGS is now routinely used in a diagnostic setting as testing and can be designed to perform either targeted or genome-wide capture of human genes to rapidly diagnose a disease (Rabbani et al., 2014). Being currently in its infancy in Malaysia, this technology is rapidly developing and growing as the choice of molecular testing in Malaysia's healthcare setting.

To the best of our knowledge, no genetic MC studies have been reported in Malaysia, and unavailable data on the number of MC cases in Malaysia's hospital as most of the patient remain undiagnosed and the prevalence in this population is still unknown. Only one clinical case of an MC patient has been reported at a hospital in the northern region, but no genetic study has been conducted. The present study was conducted to investigate the genetic profile and inheritance patterns of the variant found in MC patients.

4.2 Materials and Methods

4.2.1 Sample Collection

Informed consent was obtained from the study participants, i.e., (1) WES; proband only, (2) Sanger sequencing; proband, her first-degree relatives; proband's brother (PB), sister-in-law (PBW), children and niece (C1 – C3), and her second-degree relatives (maternal and paternal siblings, maternal grandaunt), and (3) Multiplex ligation-dependent probe amplification (MLPA) included proband, her first-degree relatives (PB, PBW, C1 – C3) and normal control individuals. Six millilitres of blood samples were collected in an EDTA blood collection tube (BD Vacutainer, USA) from the study subjects (Proband and her first-and second-degree relatives) using a 5 mL syringe (Terumo, Japan) and 21 G x 1½” needle (Terumo, Japan).

4.2.2 DNA Extraction

Genomic DNA was extracted from the whole blood (2 mL) using the QIAamp® DNA Blood Midi Kit (Qiagen, Germany) according to the “Purification of DNA from Whole Blood” protocol recommended by the manufacturer. In a 15 mL centrifuge tube, 200 µL of QIAGEN protease and 2 mL blood were mixed briefly and 2.4 mL buffer AL was added into the mixture followed by vigorous shaking. The mixture was then incubated in a water bath (Mettler, Germany) at 70 °C for 10 min and 2 mL of 99.8% absolute ethanol (Systerm, Malaysia) was added to the mixture and mixed vigorously using a vortex mixer (FinePCR, Finevortex, South Korea). The mixture was then transferred into the spin column and centrifuged at 1,000 x g for 3 min

(Eppendorf MiniSpin®, Germany), and the filtrate was discarded. Two millilitres of Buffer AW1 were added into the column and centrifuged at 2,800 x g for 1 min. The filtrate was discarded and 2 mL Buffer AW2 was added and centrifuged at 2,800 x g for 15 min. DNA samples were then eluted with 300 µL of Buffer AE. The purity (A260/A280 and A260/A230) and concentration (in ng/µL) of the extracted DNA were measured using a UV spectrophotometer (PCRmax Lambda, USA). All DNA samples had an A260/A280 ratio of 1.8 and an A260/A230 ratio of 2.0 – 2.5 were used for downstream analysis.

4.2.3 Whole Exome Sequencing (WES) and Bioinformatics

Genomic DNA (3 µg) from proband were sent for WES. WES was outsourced to Macrogen Inc., (Seoul, South Korea). The captured DNA was sequenced as paired-end reads (150 bp) with 60 x read depth on a NovaSeq 6000 sequencer (Illumina, United States). Library preparation and exome capture was performed using the SureSelect V5-post kit (Agilent Tech. Ltd., United States). The sequencing reads were aligned to the human reference genome (GRCh38/hg38) assembly using the mapping program BWA (Burrows-Wheeler Aligner). Quality control (QC) of the sequence read alignments in WES was performed using standard metrics, including mapping quality, percentage of reads aligned to the reference genome, mean read depth, coverage uniformity, and percentage of target regions covered at ≥ 20 x. These metrics ensured the accuracy and reliability of the sequencing data for downstream variant analysis. The sequence variant calling and variant filtering were carried out using the genome analysis toolkit GATK software, and sequence variants were annotated to dbSNP and SNPs from the 1000 Genome Project using the SnpEff program.

The variant call annotations were supplied from Macrogen in an Excel file (.xlsx) format. The Excel file contained thousands of variants including SNVs (single nucleotide variants) and indels (insertions and deletions). The identified candidate variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richard et al., 2005).

4.2.3.1 Variant Analysis (Filtering)

WES from the proband was queried for variants in *CLCN1* (the MC gene) and the myotonia-related gene (*SCN4A*). Gene coding variants identified were prioritised based on the following filtering strategies: (1) only coding variants were included (as WES covers over 95% exonic regions with 85% focusing on Mendelian-causal variants), while non-coding variants as intronic and untranslated region (UTR) were not prioritised because they are not targeted by WES, (2) only variants with minor allele frequency (MAF) < 0.1% (Bamshad et al., 2011) (documented in dbSNP and the 1000 Genomes databases) were included as the variants are expected to be very rare while the variants with MAF > 0.1% were excluded because at this prevalence, the disease would likely be observable in the population and (3) variants that had been classified as benign in the ClinVar database (National Center for Biotechnology Information, 2019) were excluded. ClinVar is a comprehensive database for information on the clinical significance of the variants. The web-based platform incorporates data from research publications, clinical laboratories, and expert curation to classify variants as pathogenic, likely pathogenic, benign, or uncertain significance. Additional tools were used to prioritise candidate variants, including *in silico* analysis

with computational methods to predict the potential impact of variants on protein function.

4.2.3.2 Bioinformatic Tools for Variant Analysis

4.2.3.2.1 *In Silico* Prediction Tools

After the filtering process, the remaining variants were analysed further to assess their potential pathogenicity using *in silico* tools. The tools used to predict the impact on the protein function included SIFT (Sorting Intolerant From Tolerant), PolyPhen-2 (Polymorphism Phenotyping), PROVEAN (Protein Variation Effect Analyzer), and MutPred. For conservation analysis, the PhyloP, and GERP++ (Genome Evolutionary Rate Profiling) tools were used. Multiple algorithm tools were also used which included CADD (Combined Annotation Dependent Depletion), ClinPred and REVEL (Rare Exome Variant Ensemble Learner). All of the *in silico* prediction tools provided scores based on the tool's algorithms that provided a normal to pathogenic range for interpretation of the variant on the impact of protein function and conservation constraints (**Table 4.1**).

Table 4.1: *In silico* tools used to assess their potential pathogenicity and their cut-off score for rare diseases.

<i>In silico</i> tools	Functions	Score for rare disease	References
SIFT (RS)	Predicts whether an amino acid substitution affects protein function.	> 0.5 (Deleterious) < 0.5 (Tolerated)	Kumar et al., 2009
Poly-Phen2 (RS)	Predicts the impact of an amino acid substitution on the structure and function of a protein	> 0.5 (Damaging) < 0.5 (Benign)	Adzhubei et al., 2010
PROVEAN (RS)	Predicts whether an amino acid substitution or indel has an impact on the biological function of a protein	> 0.5 (Deleterious) < 0.5 (Neutral)	Choi and Chan, 2015
MutPred (RS)	Predicts impact of amino acid substitutions and small indels on protein function, stability, and disease association	> 0.5 (Deleterious) < 0.5 (Polymorphism)	Li et al., 2009
ClinPred (RS)	Predict the pathogenicity of genetic variants, particularly in clinical relevance and use features derived from sequence, evolutionary and functional data	> 0.5 (Damaging) < 0.4 (Benign)	Alirezaie et al., 2018
PhyloP (Vertebrate) (RS)	Measures the conservation of nucleotide sequences across species.	> 0.5 (More conserved) < 0.5 (Less conserved)	Pollard et al., 2010
GERP++	Assesses the evolutionary conservation of nucleotide sequences by identifying constrained elements.	≥ 2 (Highly conserved) < 2 (Less conserved)	Davydov et al., 2010
CADD Phred	Phred-scaled score that combines multiple sources of data to predict the impact of variants.	≥ 20 (Deleterious)	Rentzsch et al., 2019
REVEL (RS)	Integrates several predictors and statistical models to assess the pathogenicity of missense variants.	> 0.5 (Deleterious) < 0.4 (Benign)	Ioannidis et al., 2016

Note: RS is a rank score, which is the ratio of the rank of the score over the total number of tool scores.

4.2.3.2.2 Protein Stability and Dynamics

A textual file describing the three-dimensional structure of ClC-1 (Uniprot F8VZY0) was obtained from AlphaFold (<https://alphafold.ebi.ac.uk/entry/P35523>, last accessed on 5 August 2024) and uploaded to DynaMut2 (<http://biosig.unimelb.edu.au/dynamut2>, last accessed on 5 August 2024) along with the location and identity of amino acid substitution. DynaMut2 is a tool used for analysing the effects of the variant on protein stability and dynamics. The predicted impact of a variant on protein stability was expressed by the difference in the Gibbs free energy of unfolding ($\Delta\Delta G$), measured in kcal/mol (<https://biosig.lab.uq.edu.au/dynamut2/help>, last accessed on 5 August 2024). This value is defined as the difference between the unfolding free energy between the mutated protein and its wild-type form. A negative $\Delta\Delta G$ indicates a destabilising effect on the protein, while a positive $\Delta\Delta G$ indicates a stabilising effect (Benevenuta et al., 2023). The prediction is accompanied by the interactive viewer of wild-type and mutated residue which enables the analysis of interatomic interactions.

4.2.3.2.3 Evolutionary Conservation

The evolutionary conservation of the variant site was estimated by aligning the protein sequences of ClC-1 from nine animals across diverse species using HomoloGene (<https://www.ncbi.nlm.nih.gov/homologene/>, last access 30 November 2022). HomoloGene provides a list of orthologs detected in nine completely sequenced genomes: *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Macaca mulatta* (rhesus monkey), *Canis lupus* (grey wolf), *Bos taurus* (domestic cattle), *Mus musculus*

(house mouse), *Rattus norvegicus* (Norway rat), *Gallus gallus* (red junglefowl), *Danio rerio* (zebrafish). These species were ranked based on their evolutionary distance from humans. In the aligned sequences, the position of the amino acids affected by the variant was identified, and the same amino acid was checked to see if it was present across all or most species. The same amino acid across all species indicated a highly conserved region and conservation at a specific amino acid position indicates that this region is crucial for the protein's function or structure.

4.2.4 ACMG Classification

The selected variants were then determined for their pathogenicity according to the ACMG variant classification guidelines (Richard et al., 2015). Pathogenicity should be determined by aggregating the entire body of evidence and arriving at a single conclusion. Each pathogenic criterion is weighted as very strong (PVS1), strong (PS1–4); moderate (PM1–6), or supporting (PP1–5), and each benign criterion is weighted as stand-alone (BA1), strong (BS1–4), or supporting (BP1–6). A summary of the ACMG criteria supporting pathogenicity is provided in **Table 4.2**. The classification of benign variants is attached in **Appendix B1**. The criteria for the identified variant; c.1667T>A (p.Ile556Asn) was selected based on the observed evidence, and these criteria were then combined according to the scoring rules in **Table 4.3** (Richard et al., 2015).

Table 4.2: ACMG variant classification criteria for pathogenic variant. The criteria were based on the ACMG guidelines (Richard et al., 2015)

ACMG Criteria	Definition
Very strong PVS1	Null variant in a gene where loss-of-function is a known mechanism of disease
Strong	PS1 Same amino acid change as a previously established pathogenic variant but a different nucleotide change
	PS2 De novo variant, with parental relationships confirmed, in a patient with the disease and no family history
	PS3 Well-established functional studies supportive of damaging effects on gene or gene product
	PS4 Prevalence of the variant in affected individuals is significantly increased compared with prevalence in controls
Moderate	PM1 Located in a mutational hotspot and/or critical and well-established functional domain
	PM2 Absent from controls
	PM3 For recessive disorders – detected in trans with a pathogenic variant
	PM4 Protein length changes resulting from inframe deletions/insertions in a nonrepetitive region and stop-loss variants
	PM5 Novel missense change at an amino acid position where a different missense change has been reported as pathogenic
	PM6 Assumed de novo, but without confirmation of paternity and maternity
Supporting	PP1 Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease
	PP2 Missense variant in a gene with a low rate of benign missense variation where missense variants are a common mechanism of disease
	PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product
	PP4 Patient's phenotype/family history is highly specific for a disease with a single genetic aetiology
	PP5 Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation

Table 4.3: ACMG rules for combining criteria to classify sequence variants
(Richard et al., 2015)

Classification	Criteria
Pathogenic	(i) 1 Very strong (PVS1) AND a) ≥ 1 Strong (PS1–PS4) OR b) ≥ 2 Moderate (PM1–PM6) OR c) 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) OR d) ≥ 2 Supporting (PP1–PP5) (ii) ≥ 2 Strong (PS1–PS4) OR (iii) 1 Strong (PS1–PS4) AND a) ≥ 3 Moderate (PM1–PM6) OR b) 2 Moderate (PM1–PM6) AND ≥ 2 Supporting (PP1–PP5) OR c) 1 Moderate (PM1–PM6) AND ≥ 4 supporting (PP1–PP5)
Likely pathogenic	(i) 1 Very strong (PVS1) AND 1 moderate (PM1–PM6) OR (ii) 1 Strong (PS1–PS4) AND 1–2 moderate (PM1–PM6) OR (iii) 1 Strong (PS1–PS4) AND ≥ 2 supporting (PP1–PP5) OR (iv) ≥ 3 Moderate (PM1–PM6) OR (v) 2 Moderate (PM1–PM6) AND ≥ 2 supporting (PP1–PP5) OR (vi) 1 Moderate (PM1–PM6) AND ≥ 4 supporting (PP1–PP5)
Benign	(i) 1 Stand-alone (BA1) OR (ii) ≥ 2 Strong (BS1–BS4)
Likely benign	(i) 1 Strong (BS1–BS4) and 1 supporting (BP1–BP7) OR (ii) ≥ 2 Supporting (BP1–BP7)
Uncertain significance	(i) Other criteria shown above are not met OR (ii) the criteria for benign and pathogenic are contradictory

4.2.5 Sanger Sequencing

Sanger sequencing analysis was then carried out to validate the candidate variant identified via WES. Although the read depth of WES is > 50 , Sanger sequencing was still conducted to truly confirm its presence in the proband and to ascertain if the variant is also present in all participated family members, which include the first-degree relatives (PB, PBW, C1 – C3) and second-degree relatives (maternal and paternal siblings and maternal grandaunt). The variant was then subjected to a segregation analysis in the family.

4.2.5.1 Primer Design

The position of the variant identified by WES in the *CLCN1* gene was located in the NCBI database (NCBI Reference Sequence: NG_009815.2) (<https://www.ncbi.nlm.nih.gov/nuccore/1731765503>, last accessed on 7 August 2024) to be used as an input to design forward and reverse primers using Primer3 (<https://primer3.ut.ee/>, last accessed on 13 June 2023), an online primer design tool to identify potential primers. The sequence of both the forward and reverse primer was selected so the 3' end of each primer sequence was at least 40 base pairs away from the target variant.

The designed primers were selected based on the following criteria: (1) primer length between 18 – 22 bases long, (2) melting temperature approximately at 56 – 62 °C, (3) the sequence starts with cytosine or guanine (C or G), and (4) GC content (percentage of guanine and cytosine bases) between 50 – 60%. Potential forward and reverse

primer sequences were then checked using NCBI Blast (National Center for Biotechnology Information Basic Local Alignment Search Tool) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed on 13 June 2023) to cross-check their specificity to the particular gene and would not anneal 100% to any other sequence in the human genome. PCR primers were purchased from Integrated DNA Technologies, USA. **Table 4.4** shows the primers designed to amplify the sequence of interest in the *CLCN1* gene.

Table 4.4: c.1667T>A (p.Ile556Asn) primers detail

Variant	Primer	Primer Sequence	Length	GC %	Tm (°C)	Amplicon size
I556N	Forward	5'-TTATTCCCATCCCATCCCCA-3'	20	50	57.8	232 bp
	Reverse	5'-TAGAGAGAGGGCTGCAGGCT-3'	20	60	62.2	

Note: Tm: melting temperature, bp: base pair

4.2.5.2 Polymerase Chain Reaction (PCR)

The sequence of interest was amplified using optimised PCR conditions with a MultiGene™ Mini Personal Thermal Cycler (Labnet International, USA). Optimisation of the PCR using a thermal gradient was performed to determine the optimal temperature for specific and efficient primer binding. In this study, the thermal gradient was conducted by varying the annealing temperature range between 50 – 58 °C.

The DNA samples were subjected to PCR reaction. For a 50 µL PCR reaction, genomic DNA was amplified in PCR tubes containing 25 µL of 2x MyTaq™ Mix (Bioline,

United Kingdom), 0.4 μM of each I556N forward and reverse primers, and 50 ng of genomic DNA and nuclease-free water (Vivantis Inc., USA) was added to bring the total volume to 50 μL (**Table 4.5**). A non-template control (NTC) which consists of all the components without a DNA template was included as a technical control to ascertain no DNA contamination in the reagents and water.

Table 4.5: PCR component

Component	Initial concentration	Final concentration	Volume (μL)
MyTaq master mix	2x	1x	25
Forward primer	10 μM	0.4 μM	2
Reverse primer	10 μM	0.4 μM	2
DNA sample	25 ng/ μL	100 ng	2
Water (PCR grade)	-	-	19
Total volume (μL)			50 μL

Thermal cycling was initiated with the following condition: initial denaturation at 95 $^{\circ}\text{C}$ for 3 min, then 30 cycles of 95 $^{\circ}\text{C}$ (15 s), 58 $^{\circ}\text{C}$ (15 s) and 72 $^{\circ}\text{C}$ (10 s), and finally cooled the PCR product at 4 $^{\circ}\text{C}$ (**Table 4.6**).

Table 4.6: Thermal cycling profile

Steps	Temperature	Duration of Cycles	Cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	15 s	30
Annealing	58 °C	15 s	
Extension	72 °C	10 s	
Cooling	4 °C		

4.2.5.3 Gel Electrophoresis

The amplified PCR products were then visualised and analysed by 1.5% (w/v) agarose gel electrophoresis using an MP-250N gel electrophoresis system (Major Science, Taiwan). Gels were made with 0.9 g agarose powder (Vivantis Technologies, Malaysia) mixed with 60 mL 1x Tris-Acetate-EDTA (TAE) buffer (First Base, Singapore) in a conical flask and heated using a microwave oven (Elba, Italy). Molten agarose was allowed to cool at around 65 °C, and 3 µL of ViSafe Red gel stain (Vivantis Inc., USA) was added to the gel, poured into gel trays and allowed to solidify. Five microlitres of each sample were loaded into the gel. A 100 bp DNA ladder (Vivantis Technologies, Malaysia) was used as a reference marker for product size. Electrodes were attached to the electrophoresis chamber and PCR samples were electrophoresed at 60 V for 120 min. PCR products on the gel were visualised using gel documentation (G: BOX iChemi XR, SynGene Inc., USA) to confirm the amplification of the sequence of interest at 232 bp.

4.2.5.4 PCR Purification and Sequencing

PCR products were purified using MEGAquick-spin Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea) according to the manufacturer's protocol. Briefly, 225 µL (5 volumes) of BNL Buffer was added to 45 µL PCR product in a microcentrifuge tube and mixed well before being transferred to the column in the collection tube. The tubes were then centrifuged at 11,000 x g for 30 s and the flow through was discarded. Next, 750 µL Washing Buffer was added to the column and centrifugation was repeated once. Forty microlitre of Elution Buffer was added to the column and centrifuged at full speed for 1 min to elute the purified PCR product.

The purified PCR products were then sent for Sanger sequencing service (Apical Scientific, Malaysia). Sequencing traces were then viewed and analysed using Seq Scanner Software 2 (Version 2. Applied Biosystems, USA). The presence of the variant was identified by aligning the sequencing results with the genomic DNA sequence by utilising the NCBI BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed on 13 June 2023). The resulting sequences were then compared with the *CLCN1* NCBI reference sequence. The literature and databases such as Leiden Open Variation Database (LOVD, <https://databases.lovd.nl/shared/variants/0000812999#00005230>, last accessed on 4 August 2024) and the Human Gene Variant Database (HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>, last accessed on 4 Aug 2024) were used to find out whether the detected sequence variation have been reported previously.

4.2.6 Pedigree Construction

Pedigree was constructed to trace the inheritance pattern of the variant within the family. Detailed family history was collected through structured interviews with affected individuals and their relatives. Information on the health status, age, and any symptoms of the family members was documented. Using this data, a multi-generation pedigree chart was created following guidelines from Bennet et al. (2008). Males were represented by squares and females by circles, while affected individuals were shaded, unaffected individuals were left unshaded and family members carrying the variant without displaying symptoms are indicated by a dot within their respective square or circle. Horizontal lines were used to connect parents, and vertical lines indicated their offspring. The pedigree was analysed for patterns consistent with Mendelian inheritance. This detailed pedigree analysis was crucial in understanding the genetic transmission of the trait and in identifying potential carriers of the variant.

4.2.7 MLPA (Multiplex Ligation-Dependent Probe Amplification)

MLPA was performed using a commercially available kit (SALSA MLPA kit P350-A1 *CLCN1-KCNJ2*), following the manufacturer's protocol (MRC-Holland, Netherlands). The kit contains probes for all 23 exons of *CLCN1*, four probes for *KCNJ2* (potassium inwardly rectifying channel, associated with Andersen-Tawil Syndrome), reference probes and *CASP2* as a upstream probe. MLPA was performed as directed by the manufacturer's guidelines and the *CLCN1* result were analysed.

4.2.7.1 Selecting Reference Sample

Reference samples are DNA samples obtained from healthy individuals and a total of six reference samples were used in this experiment. The first MLPA experiment was run for the reference samples as an internal validation to estimate the reproducibility of each probe within each MLPA run. From the analysis, the standard deviation was examined for all probes individually to ensure there are no large differences in reproducibility for certain probes. The standard deviation needs to be ≤ 0.10 for all probes in the experiment.

4.2.7.2 MLPA Technique

Briefly, 80 ng of DNA was mixed with Tris EDTA buffer (pH 8.0) in a microcentrifuge tube to a total of 5 μL to prevent depurination during the initial denaturation step. The tubes were heated to 98 °C for 5 min for denaturation and cooled to 25 °C (**Table 4.7**). Then, 3 μL of hybridisation master mix was added to each reaction. The hybridisation master mix was prepared by adding 1.5 μL MLPA buffer and 1.5 μL probe mix per reaction. For the hybridisation reaction, the DNA mix was heated to 95 °C for 1 min and kept at 60 °C for 18 h. Next, 32 μL of Ligase-65 master mix was added to each DNA mix for ligation reaction. For each reaction, 25 μL of sterile ultrapure water was mixed with 3 μL of ligase buffer A, 3 μL of ligase buffer B and 1 μL Ligase-65 enzyme. The ligase and DNA mix was then heated to 54 °C for 15 min and then 98 °C for 5 min and finally cooled to 20 °C.

Table 4.7: Thermal cycler program for MLPA reaction

Steps	Temperature (°C)	Duration	Cycle
DNA denaturation	98	5 min	1
Hybridisation reaction	95	1 min	1
	60	18 h	
Ligation reaction	54	15 min	1
	98	5 min	
PCR reaction	95	30 s	30
	60	30 s	
	72	60 s	
	72	5 min	1
Cooling	12		

Consequently, a 10 µL polymerase master mix was added to each MLPA reaction for PCR. This polymerase master mix was prepared by mixing 7.5 µL sterilised ultrapure water with 2 µL SALSA PCR master mix, and 0.5 µL SALSA polymerase. The PCR reaction was then run as the following steps for 30 cycles: 30 s at 95 °C, 30 s at 60 °C, 60 s at 72 °C and final elongation for 5 min at 72 °C followed by cooling at 12 °C.

The fragment analysis of the PCR products by capillary electrophoresis was outsourced to Apical Scientific (Malaysia). Amplified fragments were analysed using an ABI 3730xl capillary sequencer (Applied Biosystems, USA), which used POP-7 polymer, and the Gene Scan™ 500 ROX was used as the labelled size standard. These parameters information was used for data analysis in Coffalyser.Net (www.mrcholland.com).

4.2.7.3 MLPA Data Analysis

Fragment analysis results (.fsa files) were quantitatively analysed using Coffalyser.Net software following the manufacturer’s protocol and the parameters were set as in **Table 4.8**.

Table 4.8: Parameter setup on Coffalyser.Net

Items	Parameters
Capillary electrophoresis instrument	ABI 3730xl capillary sequencer
Labelled size standard	GeneScan 500 ROX
Polymers	POP-7 polymer
Dye	FAM
Filter Set	Dye Set D

Fragment analysis results (.fsa files) for all the samples were uploaded in the software and the sample type was labelled as sample, reference and no DNA sample. The data analysis was then started, and the manual bin set was checked to ensure that all probes fall inside the bin. For every sample, the FRSS (Fragment Run Separation Score) and FMRS (Fragment MLPA Reaction Score) scores were checked to ensure that all samples showed four green bars for both scores and a green icon in the DNA and DD (DNA denaturation) columns. These scores include quality assessment of the sample DNA, MLPA reaction, capillary separation and normalisation steps, and green bar and icon represent high-quality scores, and the results were accepted for the next analysis.

When all samples showed green bars and green icons, comparative analysis was started. The PSLP (Preliminary Signal Sloping Probes), FSLP (Final-normalisation Signal Sloping Probes), RSQ (Reference Sample Quality) and RPQ (Reference Probe

Quality) scores for every sample were checked to ensure all samples had four green bars for all scores and the sample results were analysed. Coffalyser.Net automatically performs control fragment checks to ensure minimal quality requirements are met. MLPA probe mixes also contain internal control fragments that have to be analysed before the final result calculation. These include the Benchmark fragment, Q-fragments (DNA quantity and ligation indicators), D-fragments (DNA denaturation indicators), and gender indicator fragments (for chromosomes X and Y). The probe mix contains nine quality control fragments that give signal when there are problems that affect the MLPA results (**Table 4.9**).

Table 4.9: Control fragments of SALSA MLPA probe mixes

Name	Length (nucleotide)	Interpretation
Benchmark fragment	92	Benchmark to compare other quality control fragments.
Quantity fragments (Q-fragments)	64, 70, 76, 82	High when DNA amount is too low, or ligation failed. Median of Q fragment signals >33% of the 92-nucleotide benchmark fragment → DNA quantity insufficient or ligation failed.
Denaturation fragments (D-fragments)	88, 96	Low in case of poor sample DNA denaturation. Signal <50% of the 92-nucleotide benchmark fragment → DNA denaturation insufficient.
X & Y fragments	100, 105	Control for sample mix-up

4.2.7.4 MLPA Data Interpretation

The final relative quantification was performed on samples and reference DNA samples that were included in the same MLPA experiment (both PCR amplification and capillary fragment analysis) and were tested with the same probe mix lot in order to exclude any possible inter-run variation.

Relative probe signals from each sample were compared to the reference samples (unrelated individuals without MC) to determine the relative copy number of the target sequences in a sample. In a normal individual, the relative signal for each probe is expected to be 1.0, reflecting the presence of two copies of the target sequence in the genome. Results were analysed using Coffalyser.Net software (MRC Holland, Netherlands), and a peak ratio range > 1.25 suggests duplications, while those < 0.75 suggest deletions.

4.3 Results

4.3.1 Whole Exome Sequencing (WES) Analysis

4.3.1.1 Variant Filtering

WES analysis reported the total reads of 35,948,082 reads and 60.6 mean depth of coverage. At least 88% of target RefSeq coding sequences were covered by greater than 20 reads. **Figure 4.2** shows a filtering strategy for variants identified within *CLCN1* and *SCN4A* genes. WES identified 94,360 variants with 88.2% single nucleotide variants (SNVs) and 11.8% insertion deletions (indels) variants. From these, the variants were filtered only to myotonia-related genes which resulted in a total of 16 variants in *CLCN1* gene and five in the *SCN4A* gene (**Figure 4.2**).

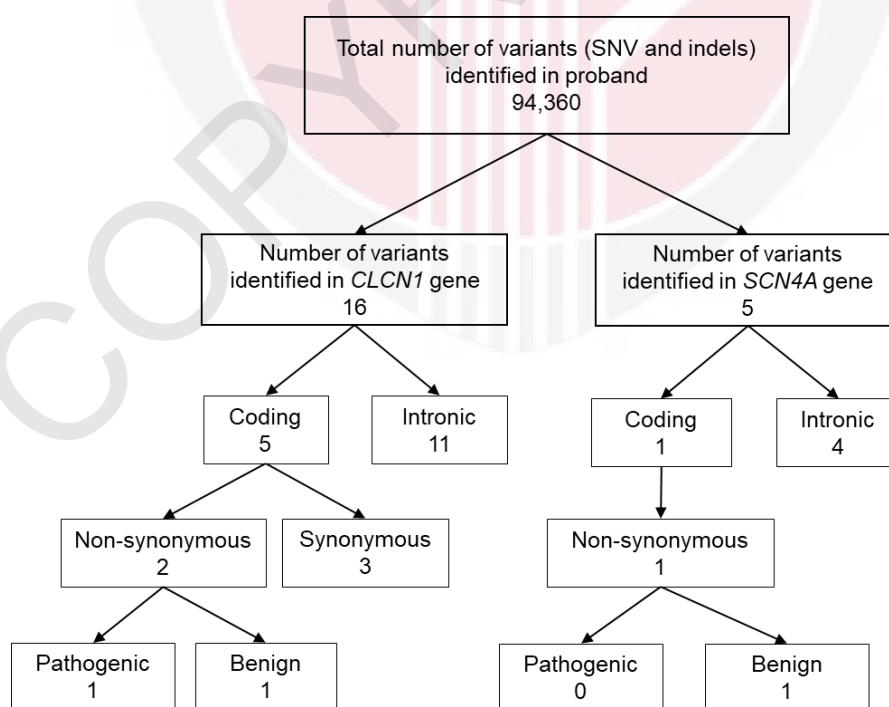


Figure 4.2: Filtering strategy for variants identified within *CLCN1* and *SCN4A*

Out of the 16 *CLCN1* variants, five coding variants included two missense (non-synonymous) and three synonymous variants, while the remaining 11 were intronic variants. Intronic variants located in non-coding sequences of the gene, and synonymous variants which do not change the amino acid sequence were not prioritised as it has fewer functional sequences than coding variants. For the two missense *CLCN1* variants, one was identified as pathogenic based on ClinVar classification while the other was benign. Five variants were found in *SCN4A*, i.e., one benign missense variant and four intronic variants which were excluded due to high frequency and prevalence in the normal population. The details of the variants are listed in **Table 4.10**.

Table 4.10: Variants found in *CLCN1* gene and a myotonia-related gene, *SCN4A* via WES analysis.

Gene	HGVS.c	HGVS.p	Zygosity	Effect	dbSNP ID	Chr	Pos	Exon	Ref allele	Alt allele	Total read	Alt read	ClinVar
<i>CLCN1</i>	c.9A>G	p.Gln3Gln	HET	Synonymous variant	.	Chr7	143,316,221	1/23	A	G	109	50	.
<i>CLCN1</i>	c.261C>T	p.Thr87Thr	HOM	Synonymous variant	rs6962852	Chr7	143,319,835	2/23	C	T	33	33	Benign
<i>CLCN1</i>	c.301+27T>A	.	HET	Intron variant	rs6948526	Chr7	143,319,902	2/22	T	A	17	11	.
<i>CLCN1</i>	c.352G>T	p.Gly118Trp	HOM	Missense variant	rs10282312	Chr7	143,320,714	3/23	G	T	28	28	.
<i>CLCN1</i>	c.433+72C>G	.	HOM	Intron variant	rs7800971	Chr7	143,320,867	3/22	C	G	10	10	.
<i>CLCN1</i>	c.774+146T>C	.	HET	Intron variant	rs2272257	Chr7	143,323,532	6/22	T	C	68	25	.
<i>CLCN1</i>	c.774+170G>A	.	HET	Intron variant	rs2272256	Chr7	143,323,556	6/22	G	A	89	40	.
<i>CLCN1</i>	c.774+301T>C	.	HOM	Intron variant	rs2272255	Chr7	143,323,687	6/22	T	C	174	174	.
<i>CLCN1</i>	c.774+465G>A	.	HET	Intron variant	rs41276051	Chr7	143,323,851	6/22	G	A	68	38	.
<i>CLCN1</i>	c.775-397G>A	.	HET	Intron variant	rs12667732	Chr7	143,324,017	6/22	G	A	27	18	.
<i>CLCN1</i>	c.1065-108G>A	.	HET	Intron variant	rs2242493	Chr7	143,331,443	9/22	G	A	16	7	.
<i>CLCN1</i>	c.1167-141G>T	.	HET	Intron variant	rs2367941	Chr7	143,332,278	10/22	G	T	17	9	.
<i>CLCN1</i>	c.1167-113T>C	.	HET	Intron variant	rs117748860	Chr7	143,332,306	10/22	T	C	18	11	.

CLCN1	n.1341+359_1341+361delTTC	.	HET	Intron variant	.	Chr7	143,332,856	11/21	CTCT	C	64	29	.
CLCN1	c.1667T>A	p.Ile556Asn	HET	Missense variant	rs80356697	Chr7	143,342,013	15/23	T	A	81	42	Pathogenic
CLCN1	c.2154C>T	p.Asp718Asp	HET	Synonymous variant	rs2272251	Chr7	143,345,744	17/23	C	T	42	12	Benign
SCN4A	c.3720+60dupC	.	HOM	Intron variant	rs58028318	Chr17	63,945,299	19/23	A	AG	33	33	.
SCN4A	c.1570A>G	p.Ser524Gly	HOM	Missense variant	rs6504191	Chr17	63,963,708	10/24	T	C	28	28	Benign
SCN4A	c.1453-132C>A	.	HOM	Intron variant	rs2331408	Chr17	63,963,957	9/23	G	T	5	5	.
SCN4A	c.1243-41A>G	.	HOM	Intron variant	rs2877373	Chr17	63,964,718	8/23	T	C	3	3	Benign
SCN4A	c.1036+89dupT	.	HOM	Intron variant	rs11418006	Chr17	63,967,933	6/23	C	CA	19	16	.
SCN4A	c.703+55T>C	.	HOM	Intron variant	rs2302236	Chr17	63,971,107	5/23	A	G	37	37	.
SCN4A	c.483-25T>C	.	HOM	Intron variant	rs9892013	Chr17	63,971,875	3/23	A	G	40	40	Benign

Note: Bold is the c.1667T>A (p.Ile556Asn), a missense variant that is pathogenic according to ClinVar classification. Chr: chromosome, HGVS.c: Human Genome Variation Society's coding DNA level, HGVS.p: Human Genome Variation Society's protein level, dbSNP: Database of Single Nucleotide Polymorphisms, ClinVar: ClinVar clinical significance, HOM: homozygous, HET: heterozygous.

4.3.1.2 Variant Analysis Found a Variant in *CLCN1*

Filtering strategy resulted in one candidate variant found on proband that was located specifically in *CLCN1* which is the gene that caused MC. It is a missense variant NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) reported as pathogenic by ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/RCV001224306.2/>, last accessed on 7 August 2024) (**Table 4.11**). The variant caused the substitution of T to A at coding position 1667, which is located at exon 15. This change resulted in substitution of isoleucine for asparagine at the amino acid residue 556 (p.Ile556Asn). The read call was heterozygous with 81 total reads and 42 alternate reads.

Table 4.11: Details of the NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) variant

Items	Details
Gene	<i>CLCN1</i>
HGVS.c	c.1667T>A
HGVS.p	p.Ile556Asn
Zygosity	Heterozygous
Effect	Missense variant
dbSNP ID	rs80356697
Chromosome	Chromosome 7
Position	143,342,013
Exon	15/23
Reference allele	T
Alternate allele	A
Alternate read	42
Total read	81
ClinVar ClinSig	Pathogenic
gnomAD allele frequency	0.000007434

Note: HGVS.c: Human Genome Variation Society's coding DNA level, HGVS.p: Human Genome Variation Society's protein level, dbSNP: Database of Single Nucleotide Polymorphisms, ClinVarSig: ClinVar clinical significance.

The allele frequency of rs80356697 in the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/variant/7-143342013-T-A?dataset=gnomad_r4, last accessed on 5 Aug 2024) database is 0.000007434 indicating that the variant is present in approximately 7 in every 1,000,000 alleles. gnomAD showed that there is a 12 allele counts which means 12 times this variant was found among all the individuals whose genomes were sequenced and included in the gnomAD dataset (**Figure 4.3**). Moreover, all 11 allele counts were found in South Asian. The allele count refers to the number of times a specific allele (a variant of a gene) appears in a population or sample. This low allele count indicates a rare variant, and it is uncommon in the general population. This variant was selected to be further characterised and subjected to test of segregation.

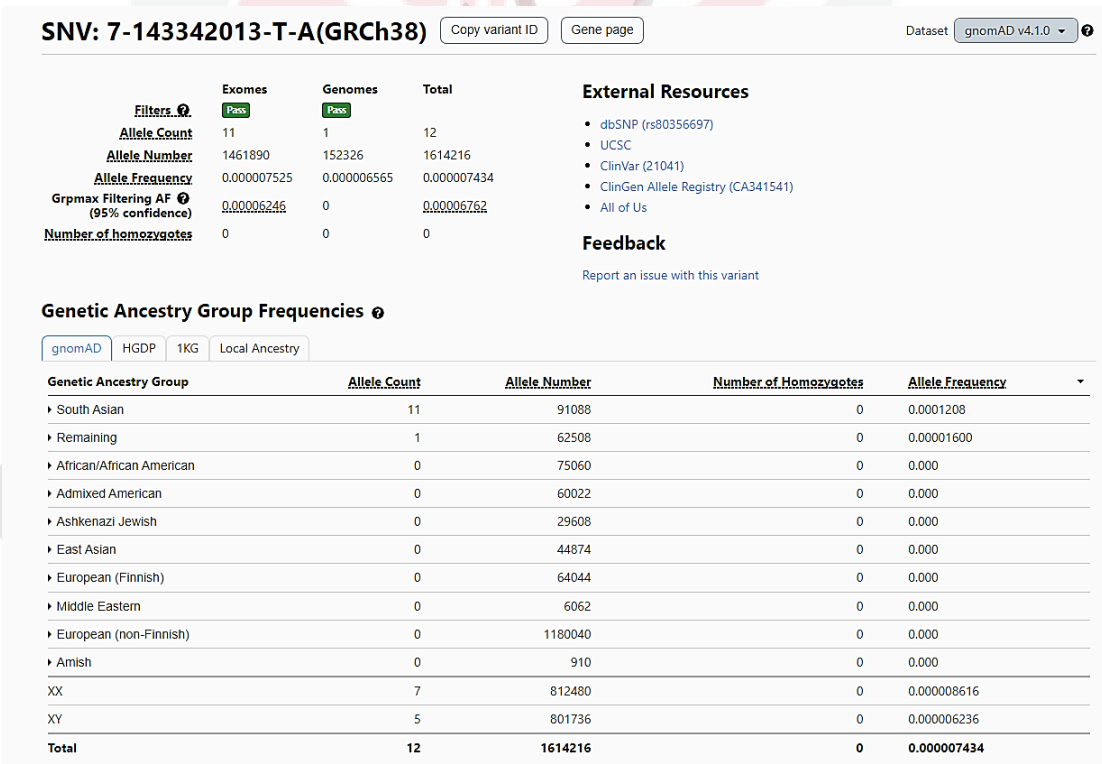


Figure 4.3: Genetic ancestry group frequency shows 12 alleles count for c.1667T>A (p.Ile556Asn) variant in the gnomAD database. (https://gnomad.broadinstitute.org/variant/7-143342013-T-A?dataset=gnomad_r4, last accessed on 5 Aug 2024)

4.3.1.3 Bioinformatic Tools for Variant Analysis

4.3.1.3.1 *In Silico* Prediction Tools Predicted the Variant to Be Damaging

Table 4.12 shows the *in silico* prediction score for the identified c.1667T>A (p.Ile556Asn). This variant has a phred scaled score of 29.7 by CADD and a REVEL ranked score of 0.97773 which was predicted to be deleterious and damaging, respectively. Several other *in silico* tools also predicted the variant to be damaging and deleterious with a ranked score of > 0.5, predicted by SIFT (0.9121), PolyPhen-2 (0.9058), MutPred (0.9158), and PROVEAN (0.8964). Meanwhile ClinPred, a tool that uses ClinVar as a training set reported a score of 0.969031, indicating that the variant is predicted to be pathogenic. The score of 0.8815 by PhyloP and 6.04 by GERP++ indicates that the region is highly conserved.

Table 4.12: Summary of the *in silico* prediction score of the c.1667T>A (p.Ile556Asn) variant

<i>In silico</i> tools	<i>In silico</i> prediction scores	Range	Cut-off value
CADD Phred	29.7*	0 to 35	≥ 20.0
REVEL (RS)	0.97773*	0 - 1	> 0.5
SIFT (RS)	0.91219*	0 - 1	> 0.5
PolyPhen-2 (RS)	0.90584*	0 - 1	> 0.5
MutPred (RS)	0.91581*	0 - 1	> 0.5
PROVEAN (RS)	0.89648*	0 - 1	> 0.5
Clinpred (RS)	0.969031*	0 - 1	> 0.5
PhyloP (vertebrate) (RS)	0.8815*	0 - 1	> 0.5
GERP++	6.04*	-12 to 6.17	> 2

Note: RS: rank score. Asterisk (*) indicates values that exceed the cut-off value for pathogenic variant.

4.3.1.3.2 DynaMut Predicted Destabilising Effect of the Variant

Protein stability using DynaMut in a wild-type protein showed an isoleucine amino acid (hydrophobic) at position 556 interacted with phenylalanine (hydrophobic) at position 547 (**Figure 4.4A**) creating 12 hydrophobic interactions due to both being from the same hydrophobic group. It also creates three hydrogen, three Van der Waals and eight polar bonds with the phenylalanine. On the other hand, changes from T to A at the 1667 coding sequence position result in the substitution of the amino acid isoleucine to asparagine (hydrophilic) (**Figure 4.4B**). This change caused the loss of nine hydrophobic interactions due to the polar nature of asparagine. Besides, it also increased one hydrogen bond and Van der Waals interaction (**Table 4.13**). In summary, the c.1667T>A (p.Ile556Asn) variant is predicted to reduce CLC-1 protein stability ($\Delta\Delta G$) by 2.49 kcal/mol (**Figure 4.4C**), suggesting a destabilising effect of the CLC-1 protein due to decreased hydrophobic interaction.

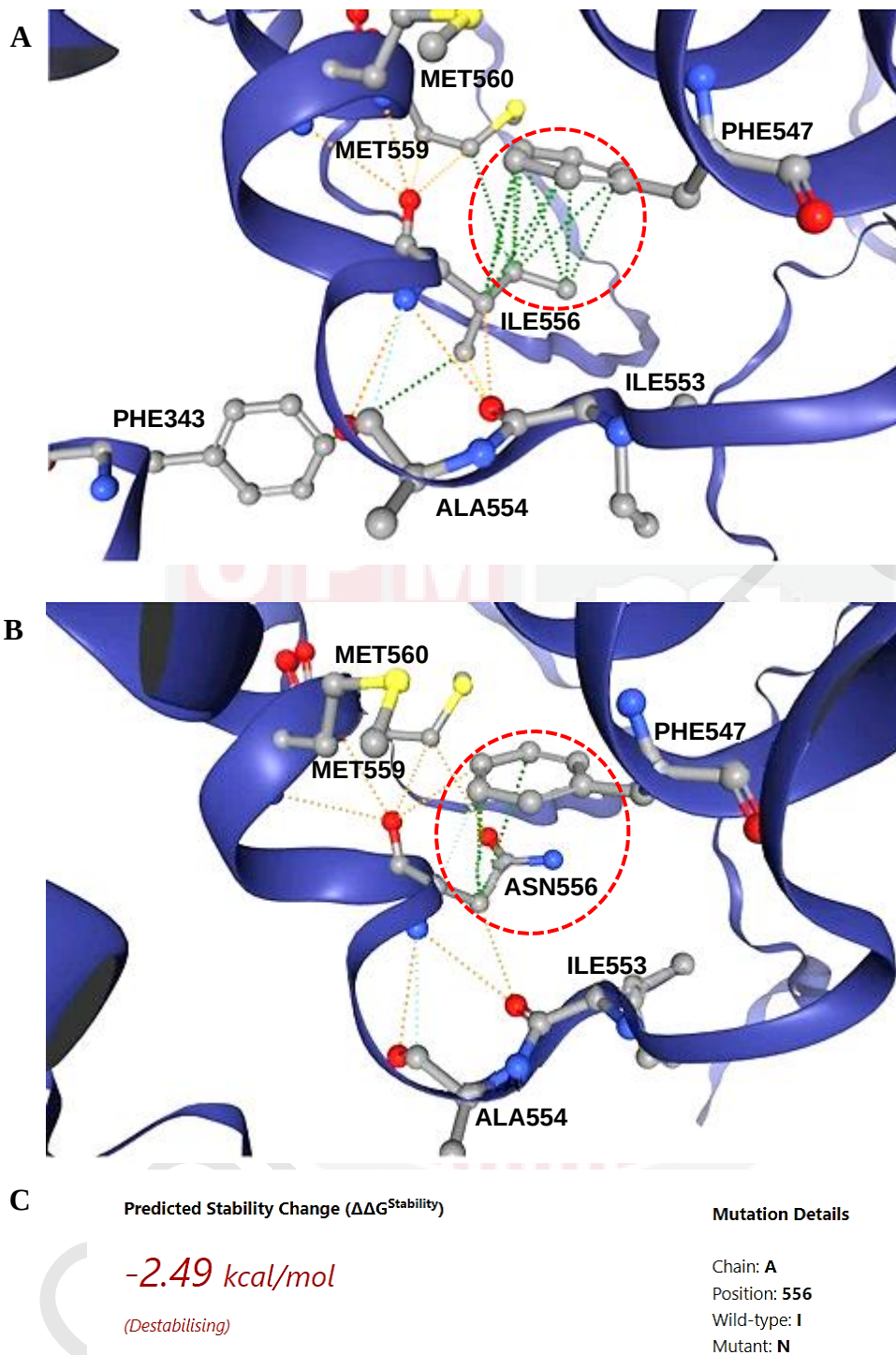


Figure 4.4: Protein stability changes predicted by DynaMut2 for c.1667T>A (p.Ile556Asn) variant. (A) A wild-type protein shows an interaction between isoleucine at position 556 and phenylalanine at position 547 (red dashed circle). It shows a higher hydrophobic interaction between isoleucine (hydrophobic) and phenylalanine (hydrophobic). (B) A mutant protein with the changes of asparagine at position 556. Due to the hydrophilic property of asparagine, it reduces the hydrophobic interaction with phenylalanine that destabilises the CIC-1 protein. (C) Predicted stability change by -2.49 kcal/mol indicating the destabilising effect. (https://biosig.lab.uq.edu.au/dynamut2/results_prediction/173773761553)

Table 4.13: Summary of the changes in the interaction between wild-type and mutant ClC-1 protein predicted by DynaMut2.

Interaction	Wild-type Protein	Mutant Protein	Changes in interaction
Hydrophobic	12	3	- 9
Hydrogen bond	3	4	+1
Van der Waals	3	4	+ 1
Clash	2	1	- 1
Polar	8	8	No change

4.3.1.3.3 Evolutionary Conservation Revealed a Highly Conserved Region

Protein conservation analysis by an alignment of ClC-1 protein sequences revealed that the variant localised at the highly conserved region. This is shown by the unchanged isoleucine position across diverse species including mammals, birds, and fish indicates the critical role of this residue extends beyond mammals (**Figure 4.5**).

Organism				
H.sapiens	520	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 569
P.troglodytes	520	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 569
M.mulatta	268	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 317
C.lupus	520	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 569
B.taurus	520	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 569
M.musculus	520	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 569
R.norvegicus	520	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQIAH	I	LPMMVAVILANMV 569
G.gallus	540	LPGGYAVIGAAALTGAVSHTVSTAVICFELTGQISH	I	LPMMVAVILANMV 589
D.rerio	512	LPGGYAVIGAAAMTGAVTHTVSTAVICFELTGQISH	I	LPMMVAVILANMV 561
D.rerio	464	IPGGYAVIGAAALTGAVTHTVSTAVICFELTGQISH	I	LPMMVAVILANMV 513

Figure 4.5: Multi-species conservative analysis of ClC-1 protein shows that the variant localised at highly conserved I556 (pointed with the red box). H.sapiens: *Homo sapiens* (human), P.troglodytes: *Pan troglodytes* (chimpanzee), M.mulatta: *Macaca mulatta* (rhesus monkey), C.lupus: *Canis lupus* (grey wolf), B.taurus: *Bos taurus* (domestic cattle), M.musculus: *Mus musculus* (house mouse), R.norvegicus: *Rattus norvegicus* (Norway rat), G.gallus: *Gallus gallus* (red junglefowl), D.rerio: *Danio rerio* (zebrafish).

4.3.2 ACMG Classified the Variant as Pathogenic

The c.1667T>A (p.Ile556Asn) variant was then determined their pathogenicity based on the criteria following the method in the ACMG guidelines (Richard et al., 2015) as summarised in **Table 4.14**. Based on the evidence the variant met the following criteria: PS4, PM1, PP2, PM2, PM5, and PP3. These criteria were then combined according to the scoring rules: 1 strong (PS), 3 moderates (PM), and 2 supporting (PP) criteria, which classified the variant as a pathogenic variant.

Table 4.14: ACMG suggested classification for c.1667T>A (p.Ile556Asn)

Category	DESCRIPTION	Evidence
PS4	For dominant rare disorders, appeared in affected cases while extremely rare in the population (PM2 met), or the prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls	It is a dominant rare disorder, PM2 met
PM1	Non-truncating non-synonymous variant is located in a variant hot spot and/or critical and well-established functional domain	p.Ile556Asn located at the Q helices which is at the dimer interface that is the functional domain
PM2	Extremely low frequency in gnomAD population databases	gnomAD database reported a low allele frequency (0.000007434)
PM5	Different amino acid change as a known pathogenic variant	The same codon with a different amino acid change was found in c.1666A>T (p.Ile556Phe)
PP2	Missense variant in a gene with low rate of benign missense variants and for which missense variant is a common mechanism of a disease	Missense variant is a common mechanism (Appendix B2)
PP3	For a missense or a splicing region variant, computational prediction tools unanimously support a deleterious effect on the gene	All nine <i>in silico</i> tools predicted the variant to be damaging and deleterious
Met criteria: 1 strong (PS), 3 moderate (PM), and 2 supporting (PP)		
Suggested classification: Pathogenic		

4.3.3 Sanger Sequencing Validates the Presence of the Variant

Sanger sequencing was used to validate the candidate variant identified through WES analysis. PCR optimisation for primers was first conducted to ensure specificity and reproducible amplification of the target DNA sequence. The temperature at 58 °C was identified as the best annealing temperature (**Appendix B3**); the temperature that

produced specific amplification of the target PCR product without non-specific bands and was then used for subsequent PCR reactions. PCR was then conducted using all subject samples, electrophoresed at 1.5% agarose gel (**Figure 4.6** and **Appendix B4**) and subjected to Sanger sequencing.

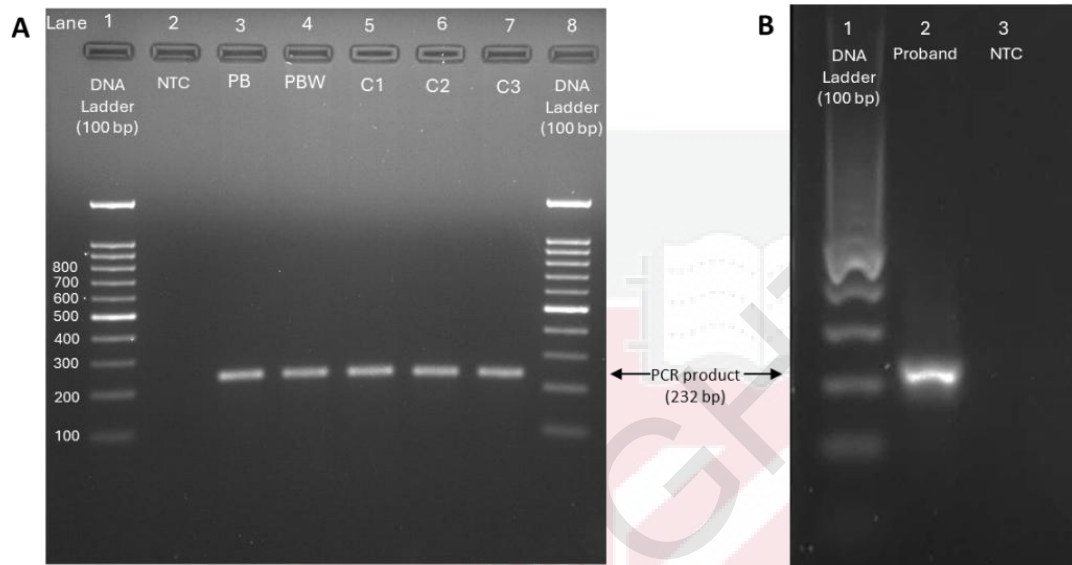


Figure 4.6: Gel electrophoresis of PCR reaction. Gel electrophoresis was performed on 1.5% agarose gel using a 100 bp DNA ladder as a molecular weight marker. (A) Lane 1: 100 bp DNA ladder, Lane 2: non-template control (NTC), Lane 3: proband's brother (PB), Lane 4: PB's wife (PBW), Lane 5: PB's daughter (C1), Lane 6: proband's son (C2), Lane 7: Proband's daughter (C3), Lane 8: 100 bp DNA ladder. (B) Lane 1: 100 bp DNA ladder, Lane 2: proband, and Lane 3: non-template control (NTC). Lane 3 – 7 (A) and Lane 2 (B) represent the amplification of 232 bp sequence of interest contained c.1667T>A (p.Ile556Asn) variant.

The pathogenic variant c.1667T>A (p.Ile556Asn) identified through the proband's whole exome sequencing (WES) data analysis was confirmed in the proband and other family members using Sanger sequencing. Analysis results revealed the presence of this variant in proband, PB, and all three children aged between 14 to 17 years old, all in a heterozygous state (**Figure 4.7**). The variant could not be confirmed in proband's parents as both had passed away. Sanger sequencing was also conducted on maternal

and paternal siblings and maternal grandaunt to look for the presence of the variant in the previous generation and the results showed none of them had the variant (**Appendix B5**).



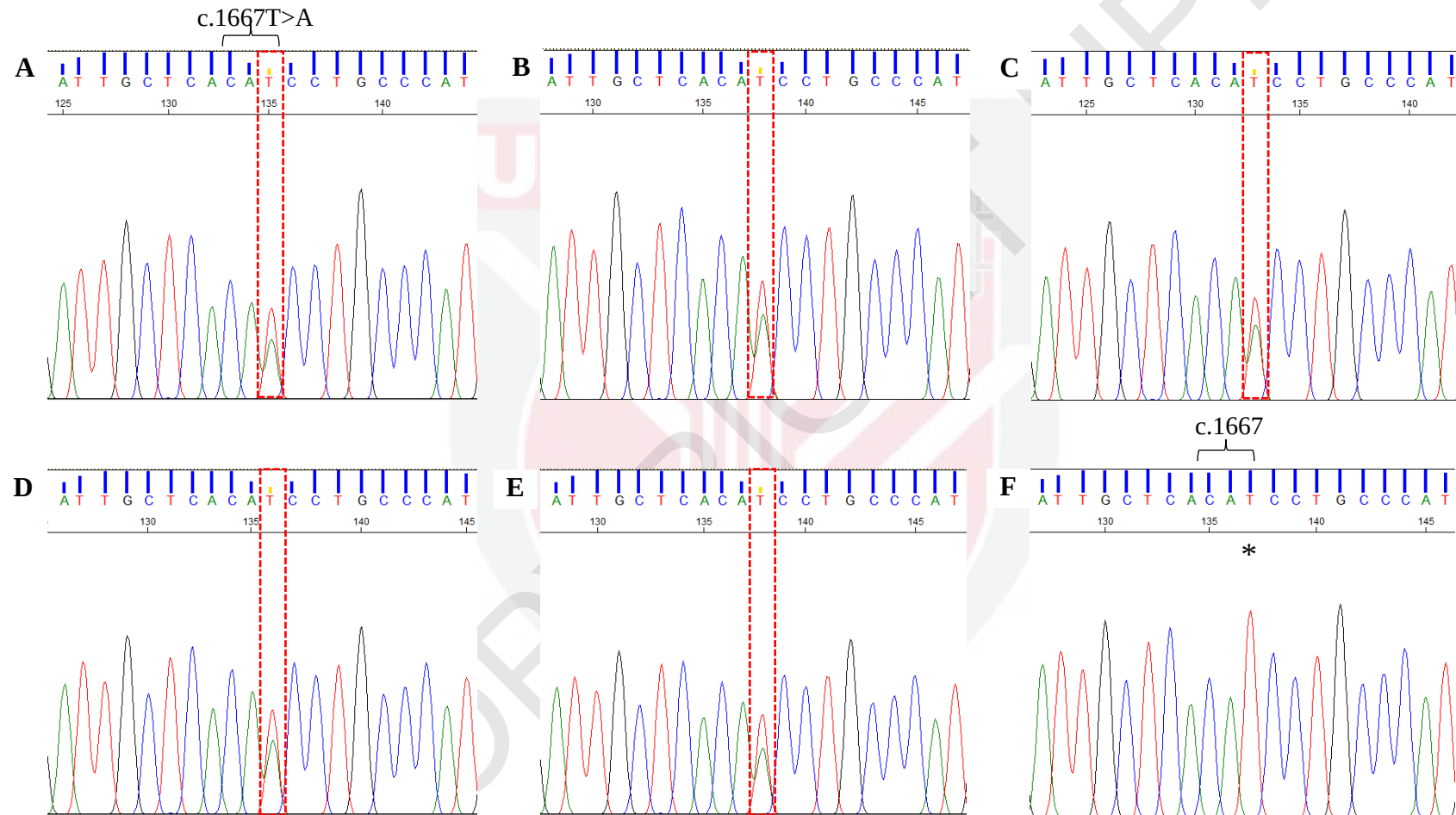


Figure 4.7: Sanger sequencing chromatogram. Forward sequence traces show a missense heterozygous variant for NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) in exon 15 of *CLCN1*. The variant present in (A) Proband, (B) Proband's brother (PB), (C) PB's daughter (C1), (D) proband's son (C2), and (E) proband's daughter (C3). (F) PB's wife (PBW) has a homozygous wild-type genotype (T/T, homozygous). The highlighted box denotes the heterozygous substitution of T to A. Asterisk (*) shows the wild-type allele.

4.3.4 Pedigree Analysis Showed an Incomplete Penetrance Inheritance

After completing the Sanger analysis, a family pedigree was constructed following Bennet et al., (2008). Family pedigrees represent family relationships and the transmission of genetic traits across generations, which is essential for identifying patterns of inheritance. Five-generational pedigree was constructed for this family consisting of proband, first-degree relatives (PB, PBW, C1 – C3) and second-degree relatives (aunt, uncle and maternal grandaunt) (**Figure 4.8**). First-tier pedigree consists of great-grandparents who are both deceased, the second tier consists of a maternal grandaunt (II:5) and the third tier consists of paternal (III:3, III:4, III:8) and maternal siblings (III:18 and III:19) all without the presence of the c.1667T>A (p.Ile556Asn) variant. The fourth tier consists of a proband (IV:3) and her brother (IV:2) who both have the heterozygous c.1667T>A (p.Ile556Asn) variant, while the brother's wife (IV:1) has a normal genotype (wild type). Followed in the fifth tier are their children (V:1, V2, and V:3) who all have the missense c.1667T>A (p.Ile556Asn) variant in heterozygous. Since all the children do not present with any symptoms, they are denoted as an asymptomatic carrier. This finding revealed the incomplete penetrance of this dominant c.1667T>A (p.Ile556Asn) variant, where some individuals carrying the variant showed symptoms of the disease while others did not.

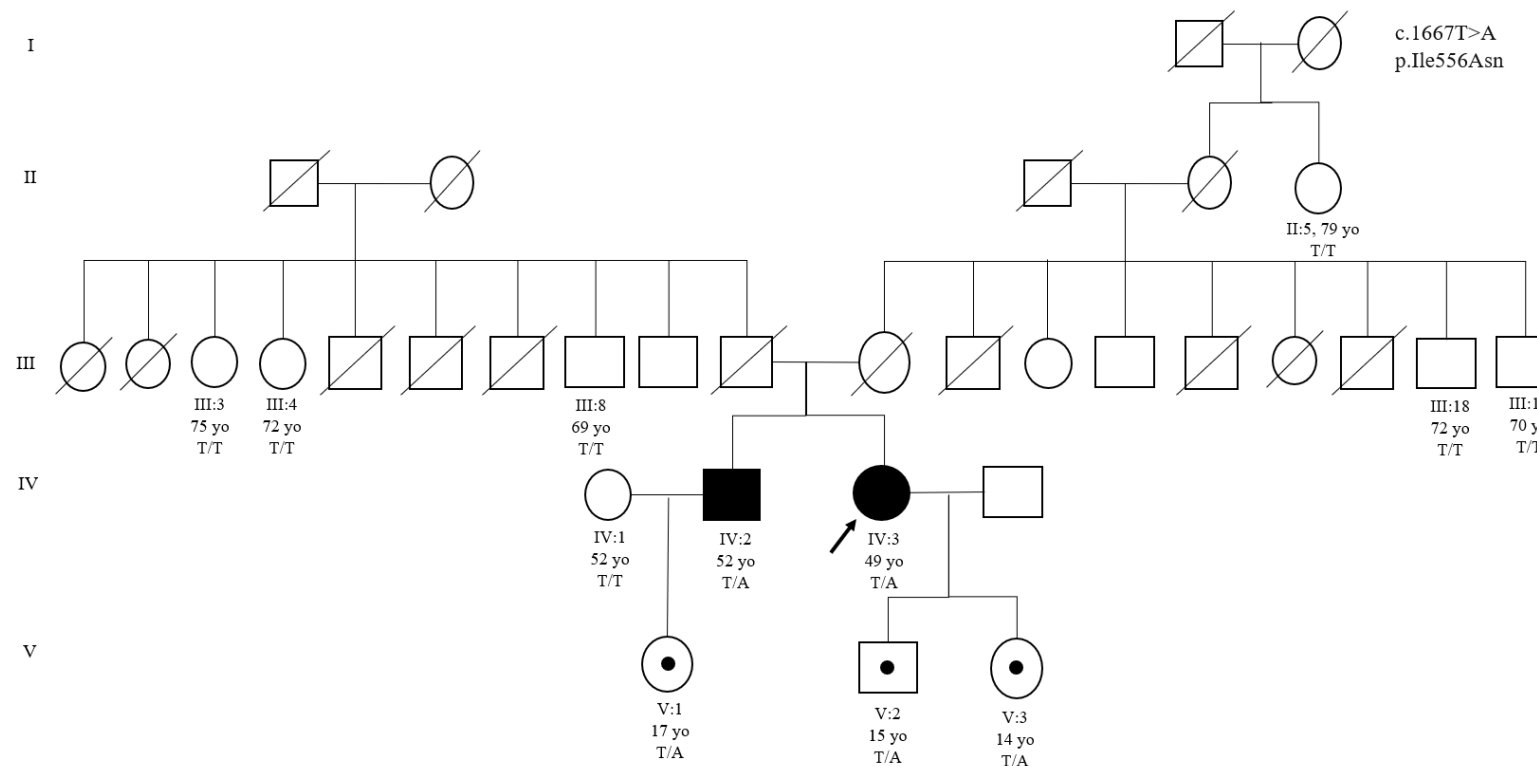


Figure 4.8: The pedigree of a Malaysian family of Sri Lankan Tamil ethnicity with MC shows the segregation of *CLCN1* c.1667T>A (p.Ile556Asn) pathogenic variant. The variant is present in Proband (IV:3), PB (IV:2), and their asymptomatic children; C1 (V:1), C2 (V:2), C3 (V:3). The variant was absent in PBW (IV:1), all paternal (III:3, III:4, and III:8) and maternal (III:18 and III:19) siblings, and maternal great aunt of the proband (II:5). The arrow indicates the proband who underwent WES. Individuals in each generation of the pedigree are numbered consecutively from left to right. Circles indicate females; squares indicate males; a horizontal line connecting a male and a female symbol indicates mating (marriage line). Solid symbols indicate the affected individual with MC, open symbols indicate family members show normal phenotype, and black dots indicate asymptomatic carriers.

4.3.5 MLPA Analysis Excludes a Copy Number Variation (CNV) On the Alternate Allele

A previous study reported that exon deletions or duplications in *CLCN1* are present in 6% of MC cases (Rayan et al., 2012). Therefore, MLPA analysis of *CLCN1* was carried out to investigate the possibility of exon deletions or duplications in *CLCN1* that can't be detected through WES analysis. The pedigree analysis showed that the variant follows a dominant inheritance pattern with incomplete penetrance, and due to the proband's phenotype resembling a recessive inheritance, MLPA analysis was conducted to determine the possibility of the coexistence of an exon deletion and duplication on the second allele.

Internal validation for the reference samples showed the standard deviation ≤ 0.10 for all probes in the experiment, indicating no large differences in reproducibility for the probes (**Appendix B6**). Out of six reference samples, three samples with the lowest standard deviation were selected to be included in each MLPA experiment together with non-template control (NTC). Before conducting the comparative analysis, the FRSS, FMRS, DNA, and DD scores were confirmed to display four green bars and icons, indicating high-quality scores, and these results were acceptable for further analysis (**Appendix B7**). Subsequently, the PSLP, FSLP, RSQ, and RPK exhibited green bars and icons for all samples, meeting the minimum quality criteria (**Appendix B7**). Internal control fragments, including benchmark, Q-fragments, D-fragments, and gender indicators, were assessed, with all samples passing the requirement (**Appendix B8**).

Results of the MLPA analysis showed that the average probe ratio was falls within the 0.75 – 1.25 range demonstrating the absence of exon deletions and duplications of the *CLCN1* gene in the proband (**Figure 4.9**), her brother, and all their children (**Appendix B9**). **Figure 4.10** shows the probe ratio of the proband was similar to the reference samples (normal control) for all 23 exons.

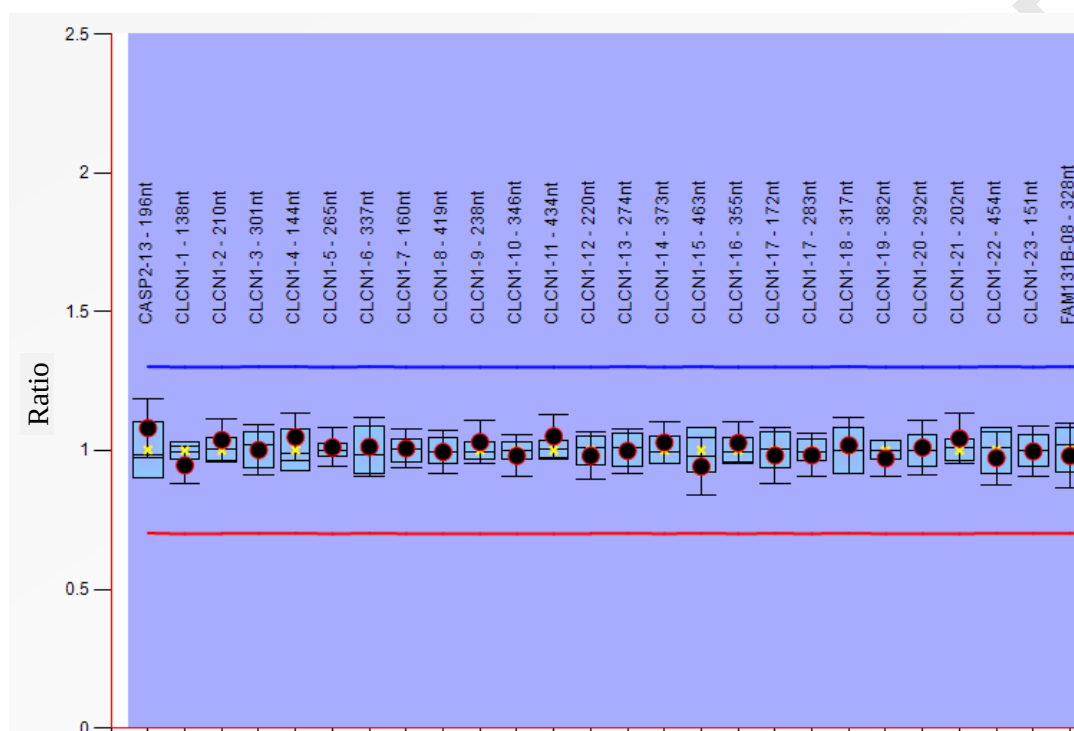


Figure 4.9: A ratio chart of MLPA result of the MC patient using SALSA MLPA kit P350-A1 *CLCN1-KCNJ2* region probe mix. The black spots represent MLPA probes, the upper blue line indicates a peak ratio of 1.3 and any probes above this line represent a duplication, the lower red line indicates a peak ratio of 0.75 and any probes below this line represent a deletion, and the probes between the two lines are considered as normal two copies. The chart shows no deletion or duplication as all the spots are between both cut-off lines in the ratio chart. The analysis also contains a *CASP2* gene as the upstream probe.

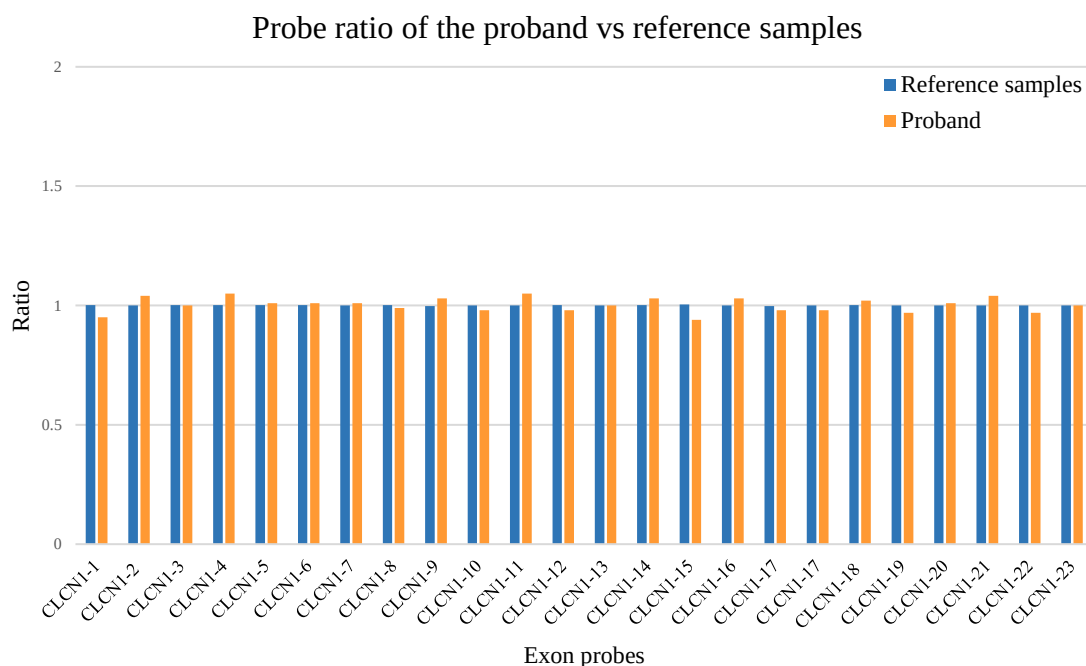


Figure 4.10: Figure shows the probe ratio of the proband (orange bars) vs reference samples (blue bars) for the 23 exons.

4.4 Discussion

Whole Exome Sequencing (WES) is a powerful and widely used method for identifying causal variants in inherited disorders. It is a technique that sequences all protein-coding regions of the genome, which constitute about 1 – 2% of the genome and contain approximately 85% of known disease-related variants (Bamshad et al., 2011; Botstein and Risch 2003). As WES can identify hundreds of thousands of variants in an individual, the main challenge is implementing filtering strategies to identify candidate pathogenic variants and distinguish them from benign variants. WES analysis revealed one heterozygous variant in *CLCN1* gene, NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn), which was classified as pathogenic by ClinVar in the proband. *SCN4A*, a myotonia-related genes were also used in the filtering strategies, but no candidate variant was found.

In silico tools were used to help prioritise and interpret which variants that are more likely to be related to disease. Further characterisation of this variant on its effect on the protein showed a damaging and deleterious effect by multiple *in silico* prediction tools used (SIFT, PolyPhen-2, MutPred, PROVEAN, ClinPred), and with a low allele frequency (0.000007434) indicates a rare variant that is very uncommon to be found in general populations. Moreover, most of the alleles were found in the South Asian ancestry (Plassart-Schiess et al., 1998). Conservation scores by PhyloP and GERP++ and evolutionary conservation alignment by Homologene showed that this variant localised at a highly conserved amino acid residue across diverse species. This suggests that the variant is important for the structure and function of the protein, as any variation or changes in the conserved residue may disrupt the structure or function of the protein potentially leading to disease (Lesk et al., 2008).

Variant interpretation using the ACMG standards for curation includes predictions from *in silico* algorithms as one of the eight recommended criteria for evidence. It states that ‘If all of the *in silico* programs tested agree on the prediction, then this evidence can be counted as supporting. If *in silico* predictions disagree, then this evidence should not be used in classifying a variant’ (Richard et al., 2015). It also encourages the use of multiple algorithms and meta-predictors like REVEL and CADD that combine multiple individual predictors to generate a score (Ghosh et al., 2017). Generally, all of the evidence and analyses classified this c.1667T>A (p.Ile556Asn) variant as pathogenic following ACMG classification.

Segregation analysis was then conducted, and the result showed the proband’s elder brother (PB) who presented with similar MC symptoms also carries the heterozygous

c.1667T>A (p.Ile556Asn) variant. However, the proband's deceased parents and all other family members (maternal and paternal siblings, children of the proband and her affected brother) did not present any MC phenotypic characteristics. Genetic analysis revealed that all three children from both patients carried the same c.1667T>A (p.Ile556Asn) heterozygous variant as their parent, despite the absence of any physical and clinical symptoms. Therefore, they were classified as asymptomatic carriers. This variant found in the heterozygous state in proband is a dominant variant as the presence of a single copy of the altered allele is sufficient to express the phenotype and cause the disease. While this study identified the c.1667T>A (p.Ile556Asn) variant as dominant with incomplete penetrance, previous research has shown that this variant can exhibit both dominant and recessive inheritance patterns, with incomplete dominance and reduced penetrance (Kubisch et al., 1998; Plassart-Schiess et al., 1998).

The incomplete penetrance of the variant and the proband's phenotype resembling a recessive clinical form led to the decision to conduct MLPA analysis. Moreover, Rayan et al. (2012) have identified exon deletions or duplications in *CLCN1* in 6% of patients with MC. This is a commonly used method to identify the possibility of the coexistence of an exonic deletion and duplication associated with the c.1667T>A (p.Ile556Asn) variant. The analysis revealed no deletion or duplication in the exonic regions of *CLCN1* in the proband. A study by Brugnoli et al., (2022) reported a recessive MC patient with a compound heterozygous variant; p.G482R and a novel deletion of the last three exons (exon 21, 22 and 23) of *CLCN1*. A missense p.G482R variant was identified in this patient and his asymptomatic mother, while the heterozygous deletion was also found in his asymptomatic father. Several cases of exon deletions in the *CLCN1* gene have been reported, affecting various regions of the

chloride channel (Brugnoni et al., 2022; Morrow et al., 2013, Rayan et al., 2012). All reported cases involve patients with recessive MC suggesting that MLPA should be used to detect the second variant in cases clinically suggestive of recessive MC. The findings of this study revealed that the c.1667T>A (p.Ile556Asn) variant exhibits an incomplete penetrance inheritance in this family.

Extensive literature searches for the c.1667T>A (p.Ile556Asn) missense variant revealed two other case reports for this variant (Arzel-Hezode et al., 2010; Plassart-Schiess et al., 1998). Both studies reported individuals with heterozygous c.1667T>A (p.Ile556Asn), typically had mild myotonic symptoms and myotonia discharges, similar clinical phenotypes were seen in patients in this study. The mild symptoms exhibited by the patient have been clarified through functional analysis. A study on the c.1667T>A (p.Ile556Asn) variant showed severe shifts of the voltage dependence; however, co-expression of the variant with wild-type ClC-1 showed only a slight shift (Kubisch et al., 1998), which explains the milder symptoms in patients with a heterozygous variant.

Nevertheless, a previous study has reported this missense variant exhibiting phenotypic variability (Colding-Jorgensen, 2005). The c.1667T>A (p.Ile556Asn) variant has been reported to show incomplete dominance and reduced penetrance in families (Kubisch et al., 1998; Plassart-Schiess et al., 1998). Plassart-Schiess et al. (1998) initially reported this variant in a family of Indian descent with heterozygous c.1667T>A (p.Ile556Asn) was less affected by MC than the homozygote family members. Two of three heterozygous individuals in the family appeared to have mild myotonia, while another member remained asymptomatic. Similar findings were

reported in the study by Arzel-Hezode et al. (2010), where two French siblings who were homozygous exhibited more severe symptoms compared to their heterozygous mother. Interestingly, their father, who was heterozygous, was also asymptomatic (Arzel-Hezode et al., 2010). The phenomenon of incomplete penetrance in heterozygotes is supported in the present study. Among five individuals who are heterozygous for the c.1667T>A (p.Ile556Asn) variant, two members exhibited myotonia, while the other three members were asymptomatic carriers.

The *CLCN1* gene provides instructions for making a chloride channel called ClC-1 protein. ClC-1 protein is a homodimer with each subunit having its own independent ion pathway (protopore) (Saviane et al., 1999; Ludewig et al., 1996). The crystal structure reveals that each subunit of ClC-1 contains 18 α -helical regions, which reside within the membrane, vary in length and are often oblique, with most not spanning the entire membrane (Dutzler et al., 2002). Autosomal dominant or TMC is described by the dominant-negative effect of mutated ClC-1 channel subunits in which the variant in one subunit can impair the function of the normal subunit, by interfering with the interaction between the two subunits (Grunnet et al., 2003). This hypothesis is particularly applicable to dominant variants located at the dimer interface (junction between two monomers). This has been shown by the cluster of dominant variants at the interface of the channel monomers, particularly concentrated in the H and I helices and some also occurring in the P and Q helices. The helices at the dimer interface of ClC-1 protein (H, I, P, and Q helices) play a crucial role in its functional domain, especially in the common gating mechanism of the chloride channel (Duffield et al., 2003). Moreover, the c.1667T>A (p.Ile556Asn) variant have been showed to be localised at the Q helices which are located at the dimer interface.

Dominant variants such as p.I290M are normally located at one of the monomers residing at the dimer interface or its proximity which causes an effect on the common or slow gate mechanism (Imbrici et al., 2015b). Likewise, the variant found in this study, c.1667T>A (p.Ile556Asn) is localised in the dimer interface with a weak dominant-negative effect (Skalova et al., 2013). This indicates that variants in the helices forming the dimer interface can alter the CLC-1 common gating process by modifying the energy of the open and closed channel states (Duffield et al., 2003) and explains the dominant-negative impact exerted on the unaffected wild-type monomer (Fialho et al., 2007).

4.5 Conclusion

In conclusion, this study has identified a reported rare heterozygous NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) pathogenic variant in *CLCN1* gene, marking the first reported MC study in Malaysia. The variant showed autosomal dominant inheritance with incomplete penetrance of MC in this family. Understanding the genotype-phenotype correlation and modes of transmission for this variant will shed light on the affected family to predict the risk of the disease transmission and the probability of the children developing the disease at the age of over 20 years as found by Plassart-Schiess et al. (1998). This *CLCN1* variant was submitted to the Leiden

Open	Variation	Database
		(https://databases.lovd.nl/shared/variants/0000812999#00005230 , last accessed on 6 August 2024) with individual ID 00384614).

CHAPTER 5

INVESTIGATING THE MOLECULAR IMPACT OF THE c.1667T>A (p.Ile556Asn) VARIANT WITH *CLCN1* GENE EXPRESSION AND EPIGENETICS STUDIES

5.1 Introduction

The phenotypic variability observed with MC may result from genetic modifiers that influence the disease penetrance, age of onset, and severity of the disease (Imbrici et al., 2016). MC can be inherited as autosomal dominant (TMC) or autosomal recessive (BMC). Although dominant variants may show full penetrance, a dominant inheritance pattern with incomplete penetrance has been observed in some pedigrees (Plassart-Schiess et al. 1998), suggesting that other modifying factors may contribute to the variability of myotonia. The variant found in this study, c.1667T>A (p.Ile556Asn) has been reported for both dominant and recessive inheritance with incomplete dominance and reduced penetrance observed (Kubisch et al., 1998; Plassart-Schiess et al., 1998). The molecular mechanisms underlying the clinical heterogeneity seen in myotonic channelopathies are unknown, with limited data on genotype-phenotype correlations (Meyer et al., 2020). Understanding the underlying molecular mechanisms is essential for effective diagnosis, prognosis, genetic counselling, and development of targeted therapies.

Gene expression analysis is a powerful tool for investigating the impact of a variant on a gene's function and how this can contribute to the disease (Wolf et al., 2023). This analysis measures the level of mRNA being produced in patients versus controls and reflects how actively the *CLCN1* gene is being transcribed into RNA, which can

ultimately impact the ClC-1 protein production (Conte et al., 2020; Duno et al., 2004). For this study, comparing the mRNA expression level in MC patients carrying the variant with the normal control individual can help to determine whether the variant leads to increased (upregulate) or decreased (downregulate) *CLCN1* expression (Portaro et al., 2015). Quantitative real-time polymerase chain reaction (qRT-PCR) is an effective technique that measures the amount of specific mRNA which indicates the gene's expression level (Kubista et al., 2006). Gene expression can also help to explain the incomplete penetrance, by analysing how the gene expression levels may vary among individuals with the same variant. Multiple hypotheses have been proposed to explain the clinical variability, including epigenetic, trans-acting genetic modifiers, differential allelic expression, hormonal or environmental factors; and dominance with reduced penetrance (Cannon, 2015, Suetterlin et al., 2014).

Epigenetics is another factor that could affect the phenotypic variability. It is the study of heritable and persistent changes in gene expression that can regulate gene activity but does not directly modify the DNA sequence (Berger et al., 2009). Even though epigenetic modifications remain after several rounds of cell replication, these alterations rarely segregate and can be reversed. DNA methylation as the first layer of a heritable epigenetic mark seems to play a role in preventing gene reactivation together with histone modifications and regulate gene expression (Portela and Esteller, 2010; Feng et al., 2007). It is a widely studied epigenetic modification, that involves the addition of a methyl group to the DNA molecule typically at the fifth carbon of the cytosine within CpG dinucleotides. This usually occurs at CpG sites (sites where cytosine is followed by guanine) and regions containing high levels of CpG dinucleotides (called CpG Island) localised at or near gene promoters (Illingworth et

al., 2010) that can inhibit gene expression (Deaton and Bird, 2011). There is increasing evidence that epigenetic modification in neuromuscular disorders is playing a role in the pathogenesis of the disease (Cerrato et al., 2020; Bennett et al., 2019; Buckley et al., 2016). To the best of our knowledge, no study has been conducted to determine the epigenetic mechanisms of MC.

Molecular characterisation of tissue-specific diseases requires the use of appropriate tissue samples (Kumar et al., 2016). For neuromuscular diseases, muscle tissue is essential for analysing gene expression and DNA methylation. In particular, the effect of variants on the *CLCN1* expression is studied primarily in skeletal muscle, the main site of *CLCN1* transcription and expression. *CLCN1* is a gene related to muscle function, and data from The Human Protein Atlas showed that the *CLCN1* gene is highly expressed in skeletal muscle with relatively low expression in the blood (<https://www.proteinatlas.org/search/CLCN1>, last accessed on 11 August 2024). Additionally, epigenetic modifications showed tissue-specific patterns, making it essential to use muscle tissue for accurate analysis, as other tissues like blood display different DNA methylation patterns (Titcombe et al., 2022). Hence, this study was conducted to determine the molecular impact of the c.1667T>A (p.Ile556Asn) variant through gene expression and epigenetic studies using skeletal muscle tissue.

5.2 Materials and Methods

5.2.1 Sample Collection

Informed consent was obtained from the study participants, and muscle tissue samples were collected for the gene expression and DNA methylation from the proband (hereafter referred to as MC patient) and an unrelated normal control individual. Due to the limited number of individuals who consented to undergo muscle biopsy, this molecular study includes only one MC patient (proband) and one normal control.

5.2.1.1 Muscle Biopsy

Muscle biopsy specimens were collected from the left biceps brachii. The muscle biopsy procedure was performed at the HSAAS operation theatre by an orthopaedic surgeon from the Department of Orthopaedics, HSAAS. During the procedure, the skin was cleaned with surgical skin cleanser (betadine), and sterile draping was placed around the surgical area. A local anaesthetic was then injected avoiding puncturing and infiltration of the underlying muscle tissue. An anaesthetic was injected along the whole incision line to provide adequate anaesthesia which set in within 2 min. The skin was then incised with a scalpel. Beneath the subcutaneous tissue, the muscle is covered by a layer of fascia. When the muscle has been exposed, a group of fibres is separated out bluntly from the belly of the muscle, avoiding the tendinous regions where muscle fibres normally are smaller, with increased connective tissue. Parallel to the length of the muscle fibre, a muscle sample was clamped at both ends and a muscle tissue measuring 4 cm in length and 1 cm in diameter was excised (**Figure 5.1**). After

closing the fascia, the subcutaneous tissue was sutured to close the skin and the biopsy site then be bandaged. The patient was monitored in daycare to observe any signs of infection or bleeding at the surgical site.



Figure 5.1: Muscle tissue was clamped at both ends and a muscle cylinder measuring 4 x 1 cm was excised. (Source: <https://neupsykey.com/acquired-myopathies/>)

5.2.1.2 Muscle Tissue Preparation and Processing

Muscle tissue was taken to the laboratory and cut into several fragments (**Figure 5.2**): (A) both ends of the tissue sections were snap-frozen immediately in dry ice before being stored at -80 °C until further extraction process, (B) 1 x 1 cm tissue was fixed in 4% cold glutaraldehyde and sent to Institute of Bioscience (IBS), UPM for electron microscopy study, and (C) 1 x 0.5 cm fresh muscle tissue was wrapped in aluminium foil, placed in an airtight container and sent to the Pathology Department, Universiti Malaya for histopathological analysis service.

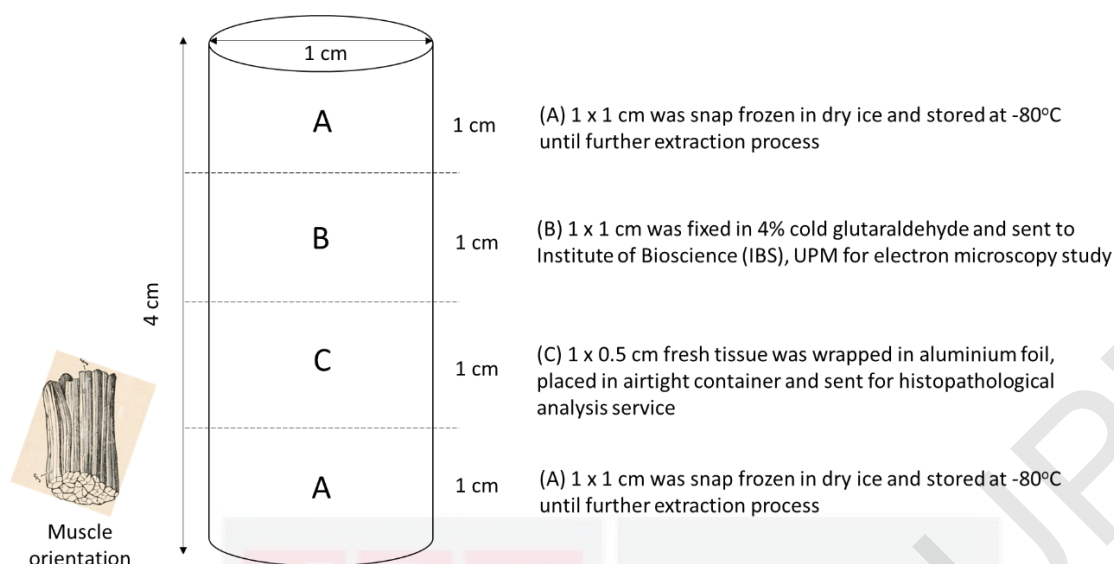


Figure 5.2: Work plan for muscle tissue allocation and subsequent processing.

(A) both ends of the tissue sections were snap-frozen immediately in dry ice before being stored at -80 °C until further extraction process, (B) 1 x 1 cm tissue was fixed in 4% cold glutaraldehyde and sent for electron microscopy study, and (C) 1 x 0.5 cm fresh muscle tissue was wrapped in aluminium foil, placed in an airtight container and sent for histopathological analysis service. The muscle was positioned longitudinally along its length as shown in the image at the lower left (Source of muscle orientation image: <http://surl.li/pqntzr>).

5.2.2 Gene Expression Analysis

5.2.2.1 RNA Extraction

The RNA extraction from muscle tissue sample was conducted using the MasterPure complete DNA and RNA purification kit (Epicenter, USA) following the manufacturer's protocol with some modifications i.e., using a homogeniser pestle as another method for tissue disruption. Briefly, 5 mg of muscle tissue samples were weighed (BT224S weighing balance, Sartorius, Germany) and placed in a microcentrifuge tube containing 300 µL of lysis solution. The muscle tissues were then homogenised using a disposable homogeniser pestle and incubated at 65 °C for 15 min and subsequently placed on ice for 5 min. After tissue disruption, the process

proceeded with protein precipitation and formation of pellet followed by the RNA precipitation and washing steps following the manufacturer's protocol. In the final step, the RNA pellet was resuspended in 20 μ L RNase-free water. The purity (A260/A280 and A260/A230) and concentration (in ng/ μ L) of the extracted RNA were measured using a UV spectrophotometer (PCRmax Lambda, USA). The RNA samples with a purity ratio (A260/A280) of 1.9 - 2.0 were used for downstream analysis. Following RNA extraction, total RNA was treated with DNase I to remove any residual genomic DNA contamination. Using the GRiSP DNase I Set (GRiSP Research Solutions, Portugal), total RNA was incubated with DNase I enzyme (0.5 μ L per μ g of RNA) in a reaction mix containing DNase I buffer (1x), and RNase-free water was added to a total volume of 20 μ L. The reaction was incubated at 37 °C for 30 min. Following incubation, DNase I was inactivated by adding 1 μ L of stop solution and incubating at 65 °C for 10 min.

5.2.2.2 cDNA Synthesis

Reverse transcription of RNA resulting in complementary DNA (cDNA) was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) following Procedure A of the manufacturer's protocol, utilising random hexamer primer in a reaction volume of 20 μ L. Each reaction was set up as presented in **Table 5.1**. The template-primer mix was incubated at 65 °C for 10 min in a PeQSTAR block thermal cycler (VWR, USA) with a heated lid and immediately cooled on ice. Then, the reverse transcription (RT) mix was added to the tube followed by incubation in the thermal block as the following: 10 min at 25 °C, 60 min at 50 °C, and 5 min at 85 °C.

The tubes were immediately cooled on ice. The synthesised cDNA was stored at -20 °C until further analysis.

Table 5.1: Reverse transcription components of transcriptor kit

Step	Component	Volume	Final concentration	Volume
Template-primer mix	Total RNA		1 µg total RNA	Variable
	Random Hexamer Primer		60 µM	2 µL
	Water, PCR Grade		-	to a total volume of 13 µL
RT (reverse transcription) mix	Transcriptor Reverse Transcriptase Reaction Buffer		8 mM MgCl ₂	4 µL
	Protector RNase Inhibitor		20 U	0.5 µL
	Deoxynucleotide Mix		1 mM each	2 µL
	Transcriptor Reverse Transcriptase		10 U	0.5 µL

5.2.2.3 Primer Designing

The primers for *CLCN1* (target gene) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (reference gene) (**Table 5.2**) were designed following the method previously described in Section 4.2.5.1 in Chapter 4 with modifications so that the forward and reverse primers were designed to span exon-exon boundaries. This is important to ensure that they specifically amplify cDNA (complementary DNA) derived from mRNA, rather than amplifying genomic DNA. Hence, the *CLCN1* primers were designed to overlap exon 14 and exon 15 boundaries, and *GAPDH* primers were designed to overlap exon 2 and 3. The melting temperature (T_m) is the temperature at which half of the DNA strands in a sample become denatured. The T_m provided in the table was determined by the manufacturer based on the specific

properties of the primer, such as its length and GC content. It was used to determine the optimal annealing temperature in qRT-PCR, which is typically set 3 – 5°C lower than the T_m to ensure efficient and specific primer binding during amplification.

GAPDH was selected as the reference gene because β -Actin (*ACTB*) initially run in Western blot showed varying expression between a normal control and an MC patient, making it unsuitable for accurate normalisation (see section 6.3.1). The primer pairs for *CLCN1* produced a 262 bp amplicon, while pair for *GAPDH* produced a 66 bp amplicon. Despite the smaller amplicon size, the reaction demonstrated high efficiency with 100% as shown in **Figure 5.4**, which falls within the acceptable range of 90–110%, ensuring reliable amplification.

Table 5.2: List of *CLCN1* and *GAPDH* primers for qRT-PCR

Gene	Primer	Exon	Primer Sequence	Length (bp)	GC %	Tm (°C)	Amplicon size (bp)
<i>CLCN1</i>	Forward	14	5'-GAGCTGCATTTG GAAGGCTG-3'	20	55	59.8	262
	Reverse	15	5'-TAGAGAGAGGGC TGCAGGCT-3'	20	60	62.2	
<i>GAPDH</i>	Forward	2	5'-AGCCACATCGCT CAGACAC-3'	19	58	60.1	66
	Reverse	3	5'-GCCCAATACGAC CAAATCC-3'	19	53	56.3	

5.2.2.4 PCR Efficiency

The PCR efficiency was assayed for the primers before reverse transcription quantitative PCR was conducted. PCR efficiency refers to the effectiveness of the PCR amplifying the target DNA during each cycle. Ideally, PCR efficiency should be close to 100%, meaning that the amount of DNA doubles with each cycle. It typically expressed as a percentage, and if the target DNA doubles in quantity during the exponential phase of PCR amplification, the assay is considered to have 100% efficiency. The amplification efficiency should be between 90% and 110% for reliable and precise quantification. The PCR efficiency was conducted by generating a standard curve for each primer set using undiluted cDNA (from a normal control) as the starting template. To prepare for the serial dilution of the cDNA template, the starting template which was undiluted cDNA synthesised from 1 µg of mRNA was further diluted (1:5) in RNase-free water (**Figure 5.3**).

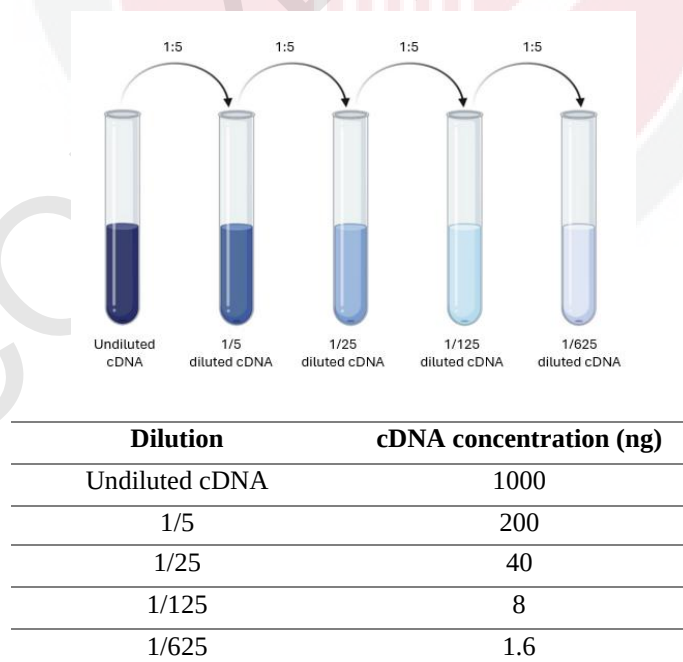


Figure 5.3: Serial dilution of cDNA sample for standard curve and PCR efficiency. For each dilution step, one volume of cDNA (2 µL) from the former tube and four volumes of water (8 µL) was added to the latter tube.

The PCR reactions were carried out in a working volume of 20 μL in triplicate containing 1x LightCycler 480 SYBR Green I Master (Roche, Switzerland), 0.5 μM of forward and reverse primers, nuclease-free water (Vivantis Inc., USA) and 1 μL cDNA template as shown in **Table 5.3**. No template control (NTC) was included for each primer pair to ensure each sample was free from contamination.



Table 5.3: qRT-PCR components

Components	Initial concentration	Final concentration	Volume (μL)
Water, PCR grade	-	-	7
Forward primer	10 μM	0.5 μM	1
Reverse primer	10 μM	0.5 μM	1
SYBR Green	2x	1x	10
cDNA template		1000 – 1.6 ng	1
Total volume (μL)			20 μL

The qRT-PCR was performed on LightCycler® 480 (Roche, Switzerland). Cycling protocols were performed as follows: denaturation at 95 °C for 5 min; 40 cycles denaturation (95 °C, 10 s), optimal annealing temperature (55 °C, 30 s for *CLCN1* and 58 °C, 20 s for *GAPDH*), extension (72 °C, 30 s) followed by melting analysis using a temperature range of 55 °C to 95 °C with 10 acquisitions per every 1 °C increment. The qRT-PCR data were collected and analysed using the Light Cycler release LCS480 1.5.1.62 software.

PCR efficiency was assessed by plotting the $\log_{10}$ of cDNA concentrations over C_p (crossing point) values obtained from the experimental results. The C_p is defined as the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence. This value represents the amount of target gene found in the sample and it is used to calculate experimental results.

The slope of the standard curve was utilised to estimate the PCR amplification efficiency in a real-time PCR reaction. This standard curve is graphically represented as a semi-log regression line plotting the C_p value against the log of the input nucleic

acid. Using the slope of the standard curve, the efficiencies of each gene assay was calculated by the following equation:

$$Efficiency (E) = (10^{\left(-\frac{1}{Slope}\right)} - 1) \times 100$$

A slope of -3.32 on this standard curve signifies a PCR reaction with 100% efficiency. Slopes that are more negative than -3.32 (e.g., -3.9) indicate reactions with efficiencies lower than 100%.

5.2.2.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

All the qRT-PCR experiments were performed in agreement with the MIQE guidelines for qPCR (Bustin et al., 2009). The qRT-PCR experiments were performed using LightCycler® 480 (Roche, Switzerland). Each reaction was performed using SYBR Green (Roche, Switzerland) in triplicate on a single-plex reaction. The setup of the reaction consisted of 100 ng/μL of cDNA, 1x LightCycler 480 SYBR Green I Master (Roche, Switzerland), 0.5 μM of each forward and reverse primer, and nuclease-free water (Vivantis Inc., USA) for a final volume of 20 μL. The qRT-PCR conditions were performed as follows: 95 °C for 5 min, then 40 cycles of 95 °C for 10 s, 55 °C, 30 s for *CLCN1* or 58 °C, 20 s for *GAPDH*, 72 °C for 30 s and continue with melting curve analysis (**Table 5.4**).

Table 5.4: Thermal cycling profile for qRT-PCR

Steps	Temp (°C)	Time	Cycle
Initial denaturation	95	5 min	1x
Denaturation	95	10 s	40x
Annealing	55 (<i>CLCN1</i>)	30 s	
	58 (<i>GAPDH</i>)	20 s	
Elongation	72	30 s	
Melting Curve Analysis	55 - 95 (<i>CLCN1</i>) 58 - 95 (<i>GAPDH</i>)	Continuous	1x

To determine the level of expression, the differences (Δ) between the threshold cycle crossing points (C_p) or known as threshold cycle (C_t) were determined from the amplification curve and the relative gene expression was calculated using the expression by comparing with the normal control ($\Delta\Delta C_t$). The $\Delta\Delta C_t$ (delta delta cycle threshold) method was used to compare gene expression levels between samples. It involves normalising the *CLCN1* expression level by dividing it by the reference (*GAPDH*) gene expression level in the same sample. The fold-change in gene expression was determined by dividing the normalised expression level of the experimental sample by that of the control sample using the following formula:

$$CLCN1 \text{ gene expression relative to } GAPDH = 2^{-\Delta\Delta C_t}$$

$$\Delta C_t = C_{tCLCN1} - C_{tGAPDH}$$

$$\Delta\Delta C_t = \Delta C_t - \text{average } \Delta C_{t\text{calibrator}}. \text{ Calibrator is a median value of normal control.}$$

5.2.3 DNA Methylation

5.2.3.1 DNA Extraction from Muscle Tissue

The DNA extraction from muscle tissue sample was conducted using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol with some modifications i.e., using a homogeniser pestle as a method of disruption. Briefly, 20 mg of muscle tissue samples were weighed (BT224S weighing balance, Sartorius, Germany) and placed in a 1.5 mL microcentrifuge tube containing 180 µL Buffer ATL solution. The muscle tissues were then disrupted using a disposable homogeniser pestle and 20 µL of proteinase K was added and the tissue mixture was incubated at 56 °C for 2 h until the tissue was completely lysed. Subsequently, the process proceeded with lysis, binding and washing steps following the manufacturer's protocol. In the final step, the DNA was eluted with 100 µL Buffer AE. The purity and concentration (in ng/µL) of the extracted DNA were measured using a UV spectrophotometer (PCRmax Lambda, USA). The DNA samples with a purity ratio (A260/A280) of 1.7 – 1.8 were used for downstream analysis.

5.2.3.2 CpG Site Determination

CpG assay was designed using PyroMark Assay Design software. The DNA sequence of interest that includes the specific region targeted for CpG island was obtained by searching for the *CLCN1* gene in the UCSC Genome Browser (<https://genome.ucsc.edu/index.html>, last accessed on 20 Aug 2024). The CpG island was made visible by navigating to the 'Regulation' section and adjusting the settings to

show the CpG islands. The island appeared as a green box, with a number indicating the count of CpG sites within (**Appendix C1**). The green box was then right-clicked, and the option 'Get DNA for CpG' was selected to retrieve the DNA sequence, and the CpG site sequence is shown in **Appendix C2**. This DNA sequence was then used to design the PyroMark primers.

5.2.3.3 Primer Designing

PCR primer and sequencing primer were designed using PyroMark Assay Design 2.0 Software (Qiagen, Germany). DNA sequence retrieved contained 42 CpG sites and six sets of PCR primers were designed targeting different sequences within the CpG site in the *CLCN1* (**Table 5.5**). The analysis was proceeded with four primer sets; Primer 1, 2, 3, and 5 that covered most of the CpG sites (**Appendix C3**). The PyroMark primer sets (Qiagen, Germany) consisted of the forward, reverse and sequencing primer (**Table 5.6**) were used. The reverse primers were synthesised as the biotin-labelled primers allowing for the synthesis of the biotinylated amplicons.

Table 5.5: List of primer sets within the CpG sites in *CLCN1*

Primer set	CpG site covered by the primer	Number of CpG sites covered	Amplicon length (bp)
Primer 1*	Site 4 – 12	9	203
Primer 2*	Site 13 – 19	7	181
Primer 3*	Site 20 - 27	8	181
Primer 4 ^σ	Site 25 – 29	5	267
Primer 5*	Site 27 – 37	11	317
Primer 6 ^σ	Site 38 – 40	3	117

Note: * Indicate primer sets 1, 2, 3, and 5 that were used for the analysis

^σIndicate primer set 4 and 6 were excluded due to the low number of CpG sites

Table 5.6: Details of the primers used for the analysis

Primer sets	Sites	Primers	Primer sequences	Nt	Product size (bp)
Primer 1	Site 4 – 12	Forward	5'-AGGGATTGAGTGAGGTTAATTT-3'	22	203
		Reverse (Biotin-labelled)	5'-CCACCCAACCCAACAAAATCATTAAAT-3'	27	
		Sequencing	5'-AGTTTTTTAGGAAGTTGAGAA-3'	20	
Primer 2	Site 13 – 19	Forward	5'-AGGGAGTGGGTTTTTTTTTAGATT TAATGA-3'	29	181
		Reverse (Biotin-labelled)	5'-AAACCCCTCCCCAACCAA-3'	18	
		Sequencing	5'-GGGTTTTTTTTTAGATTTAATGATT-3'	24	
Primer 3	Site 20 – 27	Forward	5'-TATTTGTGTTTTGAGAGTAGGTTG-3'	29	181
		Reverse (Biotin-labelled)	5'-AAACCCCTCCCCAACCAA-3'	18	
		Sequencing	5'-TATTTGTGTTTTGAGAGTAGGTTG-3'		
Primer 5	Site 27 – 37	Forward	5'-AGGGAGTGGGTTTTTTTTTAGATT TAATGA-3'	29	317
		Reverse (Biotin-labeled)	5'-CTTTTCCCTTTACTCCTAATCTATCC-3'	26	
		Sequencing	5'-GTTGGGGAGGGGTTT-3'	15	

Note: Nt – nucleotide, bp: base pair

5.2.3.4 Bisulfite Treatment and Pyrosequencing

The DNA samples from the normal control and MC patient, which were extracted from muscle tissue, were outsourced to the Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia for bisulfite treatment and pyrosequencing. DNA methylation analysis was conducted using bisulfite pyrosequencing, a highly sensitive and accurate protocol. The outsourced laboratory followed the

manufacturer's protocol. Briefly, 1.5 µg of DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Germany). Treatment with sodium bisulfite distinguished methylated and unmethylated cytosine by selectively converts cytosine to uracil; however, methylated cytosine (5-methylcytosine) was protected from deamination and the CG sequence was preserved. Briefly, the bisulfite reactions were prepared in 200 µL of PCR tubes by adding the following components: 1.5 µg of genomic DNA in a total volume of 20 µL, 85 µL of bisulfite mix, 35 µL of DNA protect buffer, and the total volume was brought to 140 µL using RNase-free water. The mixture was then incubated in a thermal cycler for bisulfite conversion as the following (Table 5.7).

Table 5.7: Bisulfite conversion thermal cycler condition

Steps	Time	Temperature (°C)
Denaturation	5 min	95
Incubation	25 min	60
Denaturation	5 min	95
Incubation	85 min (1 h 25 min)	60
Denaturation	5 min	95
Incubation	175 min (2 h 55 min)	60
Hold	30 min	20

After the conversion completed, bisulfite-converted DNA was purified using spin columns, followed by a desulfonation step with desulfonation buffer to remove residual bisulfite, and the bisulfite-converted DNA was then eluted in 20 µL of elution buffer. Bisulfite-converted DNA was used to amplify the CpG site by using PyroMark PCR Kit (Qiagen, Germany) with the biotinylated primers by the Pyromark CpG

Assay kit (Qiagen, Germany) (**Table 5.6**). In parallel with the experimental samples, control DNA from the EpiTect PCR Control DNA Set (Qiagen, Germany) (fully methylated and unmethylated) was subjected to the same bisulfite conversion and PCR amplification. Briefly, the PCR reactions were prepared in PCR tubes with a total volume of 25 uL by adding the following components: 10 ng bisulfite converted DNA, PyroMark PCR master mix (1x), CoralLoad concentrate (1x), forward and reverse primers (0.2 µM each), and RNase-free water. The PCR mixture was then incubated in a thermal cycler for as the following cycling protocol (**Table 5.8**).

Table 5.8: Cycling protocol for PyroMark PCR master mix

Steps	Time	Temperature (°C)	Cycle
PCR activation	15 min	95	1x
3-steps cycling			
Denaturation	30 s	94	45x
Annealing	30 s	56	
Extension	30 s	72	
Final extension	10 min	72	1x

After PCR amplification was completed, biotinylated PCR products were immobilised on streptavidin-coated Sepharose beads. PCR template strands were purified using the PyroMark® Q96 Vacuum Workstation. It was used to prepare single-stranded DNA for pyrosequencing by isolating and purifying biotinylated PCR products. Initially, the immobilised PCR products were captured using the PyroMark® Q96 Vacuum Workstation. The filter probe tips contain immobilised PCR were washed thoroughly with 70% ethanol, followed by washed with a denaturation solution to separate the DNA strands, leaving single-stranded DNA on the beads. A final wash with washing

buffer was then performed to remove unbound components like primer and unbiotinylated DNA. The immobilised biotinylated single-stranded DNA was released into a sequencing plate that contained sequencing primer, and ready for pyrosequencing analysis.

Pyrosequencing was then performed to analyse CpG site methylation with PyroMark Q96 ID System (Qiagen, Germany) using PyroMark Gold Q96 Reagents (Qiagen, Germany). The sequencing mix was prepared, which included the biotinylated single-stranded DNA, sequencing primers, and the PyroMark Gold Q96 Reagents containing essential enzymes and buffers. This mixture was then loaded into the PyroMark Q96 ID System. The system performed real-time sequencing, detecting nucleotide incorporation into the growing DNA strand and generating sequence data.

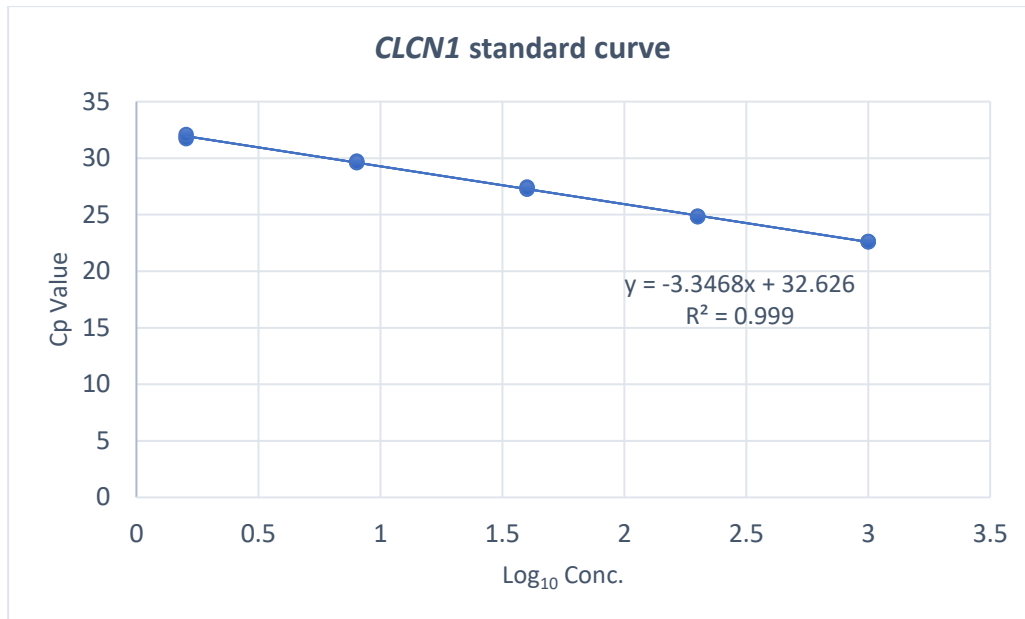
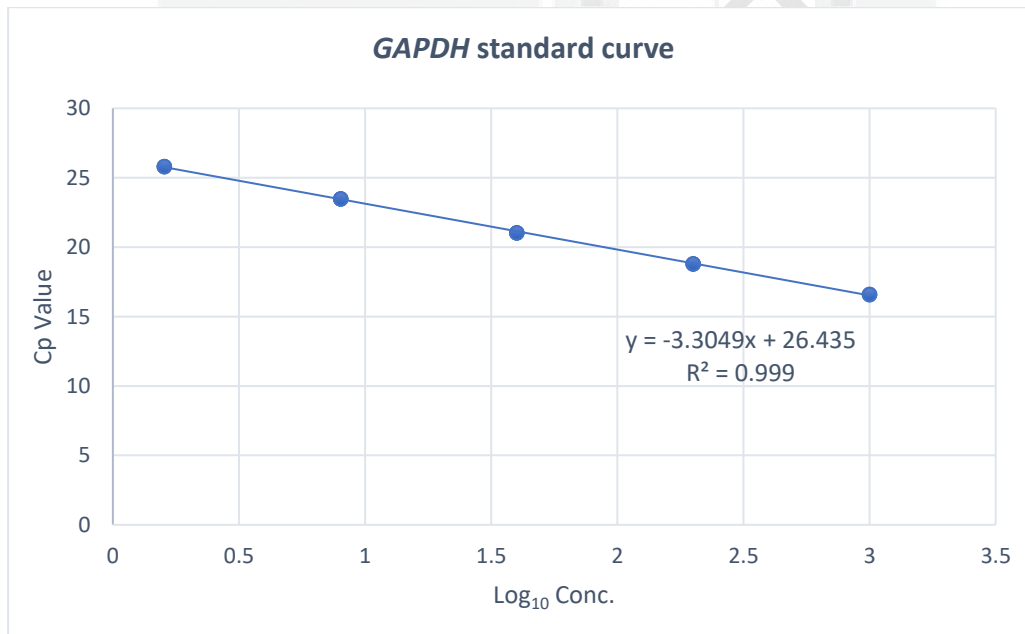
The pyrosequencing reaction was started as the sequencing primer attached to a biotinylated-single-stranded template. When a CpG site was methylated, the “C” in the CpG site was not converted to “U” during bisulfite conversion, thereby detecting of only “C” in that site. Nucleotides were then incorporated in a specific order that complemented the template sequence. As nucleotide was added, pyrophosphate (PPi) molecule was released producing visible light that appears as a peak on a pyrogram. The peak's height indicates the number of identical nucleotides added. The percentage of DNA methylation value for each CpG site was displayed on the pyrogram and calculated by dividing the peak height of cytosine (C) by the combined peak heights of cytosine (C) and thymine (T). Unmethylated and methylated DNA samples from commercial kit (EpiTect PCR Control DNA, Qiagen, Germany) were used as controls to ensure conversion efficiency and accuracy in methylation analysis.

5.3 Results

5.3.1 *CLCN1* Gene Expression Reduced in MC Patient

5.3.1.1 PCR Efficiency

PCR efficiency was determined for each primer pair using serial dilutions of control cDNA to ensure the efficiency of PCR amplification and the specificity of the primers for the *CLCN1* and *GAPDH*. The C_p value amplification curves (**Appendix C4**) were used to create a standard curve. The standard curve generated for the *CLCN1* showed a slope of -3.347 and a y-intercept of 32.626, with an R^2 value of 0.999 (**Figure 5.4A**). This results in a PCR efficiency of 98.971%, with an amplification factor of 1.989, indicating that the amount of DNA is nearly doubled with each cycle. The standard curve for *GAPDH* revealed a slope of -3.305, a y-intercept of 26.435, and an R^2 value of 0.999 (**Figure 5.4B**). The efficiency was calculated as 100.717%, reflected in an amplification factor of 2.007, which suggests slightly more than doubling of the target DNA with each PCR cycle. The calculated PCR efficiencies of the *CLCN1* and *GAPDH* primers were 1.98 (99%) and 2.00 (100%), respectively, at which the values obtained from this experiment fall within the acceptable range for PCR efficiency, between 1.9 (90%) and 2.1 (110%). Thus, both genes showed high amplification efficiency and confirmed the reliability and accuracy of the qRT-PCR assays.

A**B**

Gene	Slope	Y-intercept	R ² value	qRT-PCR efficiency (%)	Amplification factor
<i>CLCN1</i>	-3.347	32.626	0.999	98.971	1.989
<i>GAPDH</i>	-3.305	26.435	0.999	100.717	2.007

Figure 5.4: qRT-PCR of the target gene, *CLCN1* and the reference gene, *GAPDH* show the 5x dilution series from which the standard curves were generated. (A) The *CLCN1* standard curve shows a slope of -3.347 and an R^2 value of 0.999, with a qRT-PCR efficiency of 98.97% indicating a 1.989 amplification factor. **(B)** *GAPDH* standard curve shows a slope of -3.305 and an R^2 value of 0.999, with qRT-PCR efficiency of 100.717% indicating a 2.007 amplification factor. All dilutions were run as technical replicate (n=3) of single extraction of sample. Both PCRs were specific for cDNA and exhibited similar amplification efficiencies as indicated by the slope of the standard curves.

5.3.1.2 Melting Curve Analysis

Melting temperature (T_m) calling analysis was conducted to assess primer specificity. The melting peak analysis for the *CLCN1* revealed that the peaks on the right (blue peaks) correspond to the target product, with a melting temperature of 86 °C. In contrast, the peaks on the left (red peaks) are from the NTC, which showed a lower melting temperature of 74 °C and appeared after 35 cycles, suggesting the presence of primer-dimers. The melting curve analysis for *GAPDH* displayed a single peak at around 82 °C (blue peaks) (Figure 5.5B), indicating a specific amplification of the target product, with NTC showing no peak (green line). The analysis revealed a single melting peak for both primers, indicating their specificity to the targets.

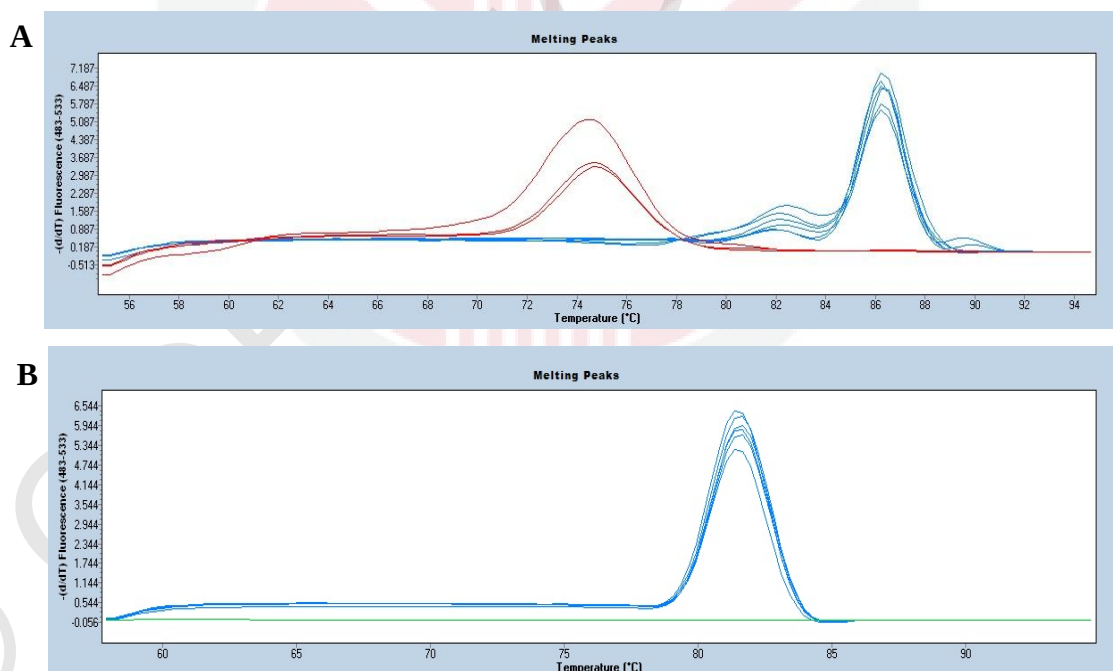


Figure 5.5: Melting curve analysis of *CLCN1* and *GAPDH*. (A) The *CLCN1* graph displays the target product with a peak at 86 °C (blue) and NTC with a lower melting temperature peak at 74 °C (red). The NTC shows a late amplification peak at 74 °C after 35 cycles, suggesting the presence of primer-dimers. (B) The *GAPDH* shows a single peak at 82 °C corresponding to the target product, and no peak is observed for the NTC.

5.3.1.3 *CLCN1* Relative Expression

The *CLCN1* relative gene expression of the MC patient and normal control was quantified by qRT-PCR using cDNA isolated from the biceps brachii muscle. The Cp (crossing point or cycle threshold) values generated from the amplification curve represent the number of PCR cycles required for the fluorescent signal to exceed a threshold level, indicating the presence of a target nucleic acid. **Figure 5.6** shows the amplification curve for the *CLCN1* and *GAPDH*. The normal control tissue had Cp values of 31.74, 31.19, and 31.15, with an average of 31.36 for the *CLCN1* gene, while the MC patient tissue had Cp values of 30.03, 30.1, and 30.47, with an average of 30.2 (**Figure 5.6A**). In comparison for the *GAPDH*, normal control tissue reported an average Cp value of 23.07 based on triplicate values of 23.06, 23.09, and 23.07 (**Figure 5.6B**). Meanwhile, the MC patient tissue showed an average Cp value of 20.99, with triplicate values of 20.98, 20.98, and 21.00.

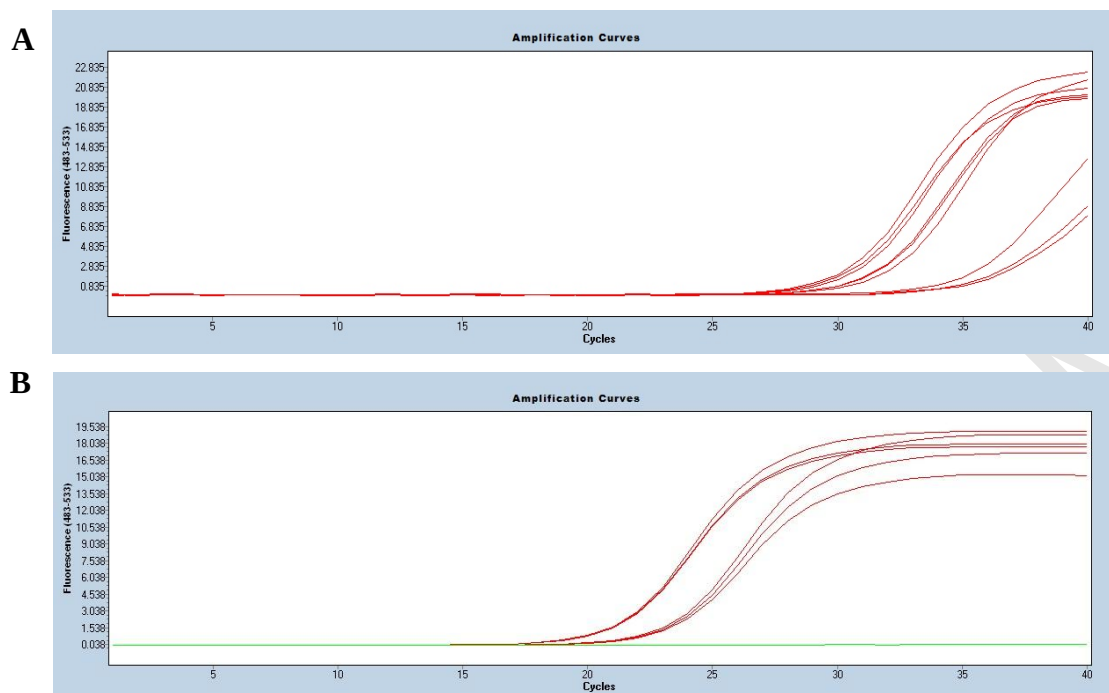


Figure 5.6: The graph illustrates the amplification curve for the *CLCN1* and *GAPDH* gene obtained from the qRT-PCR analysis. The amplification curve shows an increase in fluorescence intensity, indicating the accumulation of the target product over the PCR cycles. The point at which the fluorescence signal surpasses the threshold line represents the C_p (crossing point) value. It reflects the cycle number at which the gene product is first detected above background levels. (A) The amplification curve for *CLCN1* shows normal control tissue had an average C_p value of 31.36 while MC tissue had a C_p average of 30.2. (B) The amplification curve for *GAPDH* shows normal control tissue had average C_p value of 23.07 while MC tissue had a C_p average of 20.99.

The C_p values were used to determine the relative expression level of the *CLCN1* gene.

Table 5.9 shows the analysis of a relative expression level of the *CLCN1* expression between a normal control tissue and MC patient tissue, normalised with *GAPDH*. ΔC_t values (the difference between *CLCN1* and *GAPDH* C_t values) were calculated to normalise *CLCN1* expression, and $\Delta\Delta C_t$ values were determined using the normal control as a calibrator. As a result, the ΔC_t values for normal control and the MC patient were 8.29 and 9.2, respectively. The 8.29 value was then used as a calibrator resulting in a $\Delta\Delta C_t$ of 0.00 in normal control and 0.92 in MC patient. The relative fold change in the expression of *CLCN1* was 0.53 for the MC patient. The trend suggested

a 47% reduction in *CLCN1* expression in the MC patient compared to the normal control (Figure 5.7).

Table 5.9: Relative expression levels of the *CLCN1* gene, normalised to *GAPDH*, in the MC patient carrying the c.1667T>A (p.Ile556Asn) variant as compared to normal control

Sample	<i>CLCN1</i> average Cp	<i>GAPDH</i> average Cp	ΔCt (<i>CLCN1</i> – <i>GAPDH</i>)	$\Delta\Delta Ct$ ($\Delta Ct - 8.29$)	$2^{-\Delta\Delta Ct}$
Normal control	31.36	23.07	8.29	0.00	1.00
MC patient	30.20	20.99	9.21	0.92	0.53

Note: $\Delta Ct = Ct_{CLCN1} - Ct_{GAPDH}$, $\Delta\Delta Ct = \Delta Ct - \text{average } \Delta Ct_{\text{calibrator}}$. Calibrator (median value of normal control).

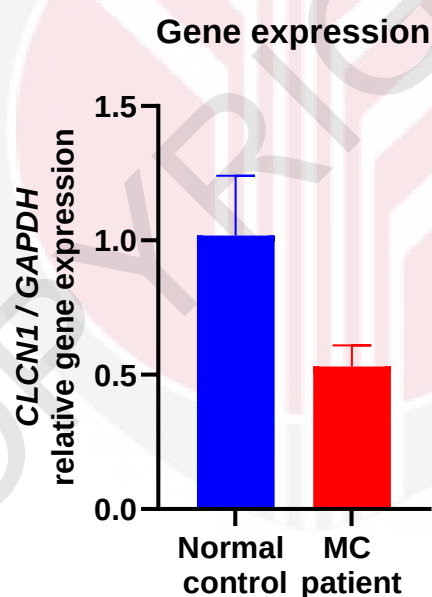


Figure 5.7: Relative quantification of the *CLCN1* gene expression normalised with *GAPDH*. The *CLCN1* gene expression shows a two-fold downregulation trend in the MC patient as compared to normal control. Each bar represents the mean $\pm$ SD of technical replicate (n=3) of single extraction of sample.

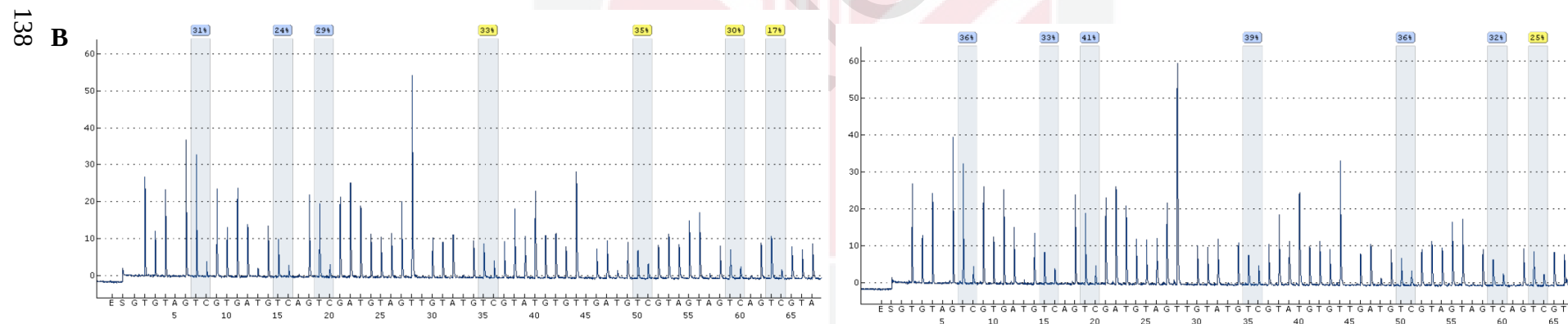
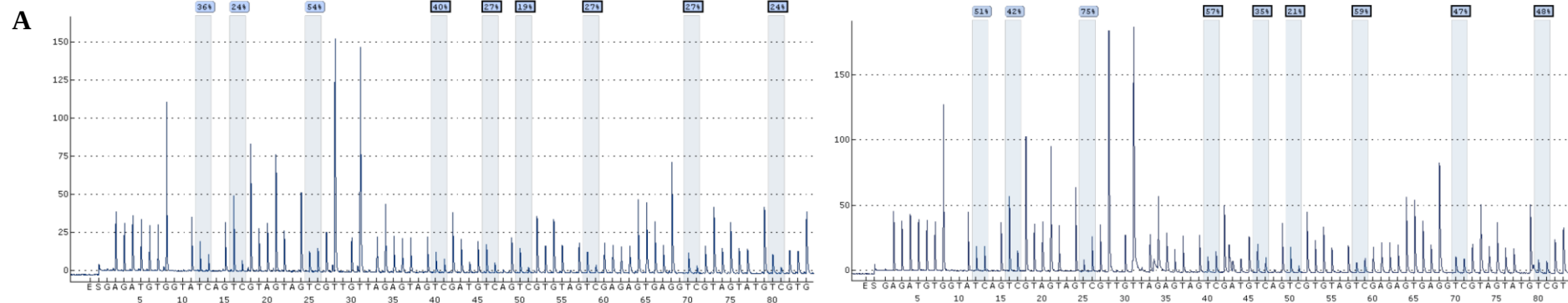
5.3.2 DNA Methylation Increased in MC Patient

Due to the large size of the PCR product, the CpG sites were divided into six regions and four different sets of PyroMark primers were designed to sequence CpG region: (1) sites 4 – 12, (2) sites 13 – 19, (3) sites 20 – 27, and (4) sites 27 – 37. When a CpG site was methylated, the cytosine (C) in the CpG site remains unchanged during bisulfite conversion, resulting in the detection of only cytosine (C) at that site. However, if the CpG site was unmethylated, the cytosine (C) was converted to uracil (U) during bisulfite conversion, which is then detected as thymine (T) during PCR. At the corresponding CpG site, the presence of only cytosine (C) was interpreted as 100% methylation, while only thymine (T) as 0% methylation, and a combination of both C and T indicated partial methylation, ranging from 0% to 100%.

The pyrosequencing pyrograms showed the CpG sites are shaded, and the percentage of the methylation level is shown above the shaded area (**Figure 5.8**). The methylation percentage at each CpG site was calculated by dividing the peak height of cytosine (C) by the combined peak heights of cytosine (C) and thymine (T), as displayed in the computer-generated sequencing chromatogram. The average methylation level for the DNA control set across all CpG sites and methylated control was 2.2% for unmethylated control and 79.9% for methylated control (**Appendix C5**).

Normal control

MC patient



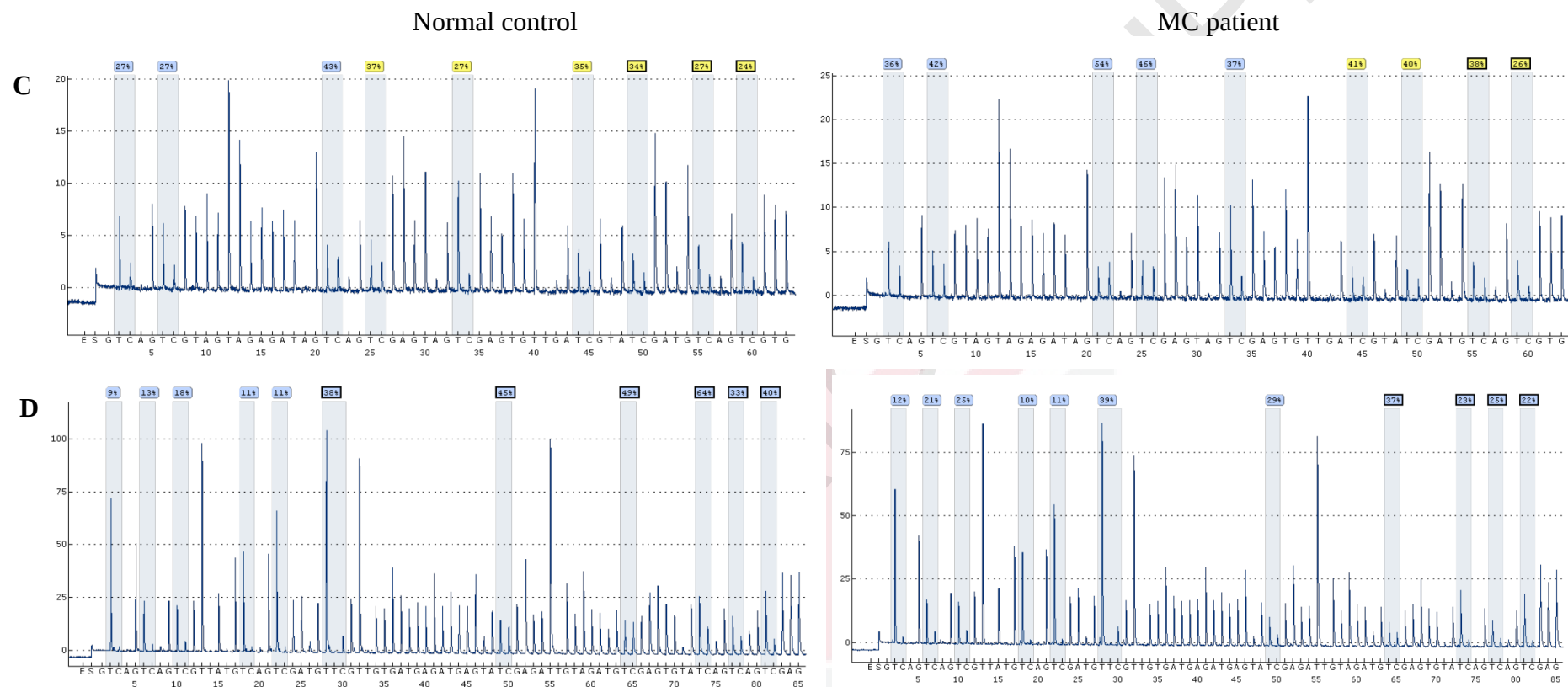


Figure 5.8: DNA methylation effect of the CpG sites 4 – 37 in the *CLCN1* located at the enhancer region in normal control (left panel) and MC patient (right panel). The shaded area represents the CpG sites, and the percentage shown above the shaded area is the methylation level of the CpG site. Pyrosequencing pyrograms show the DNA methylation status for the (A) CpG sites 4 – 12, (B) CpG sites 13 – 19, (C) CpG sites 20 – 27, and (D) 28 – 37. Overall, the normal control shows a range of DNA methylation between 17 – 54% across all 34 CpG sites, while (B) MC patient shows an increase in the DNA methylation (hypermethylation) with 21 – 75%. The blue value indicates that the peak height data meets the minimum quality requirements, while the yellow value indicates that the peak height exceeds the quality threshold. Both values are reliable for the analysis. The methylation level of each CpG site can be found in **Appendix C5**.

In muscle tissue of normal control, the DNA methylation profile showed a DNA methylation level from CpG site 4 – 29 ranging between 13 – 54%. On the other hand, the MC patient revealed a higher DNA methylation (hypermethylation) at CpG sites 4 – 29 with a range of 21 – 75% (**Figure 5.9**). The end region from CpG sites 30 – 37 showed a reversed pattern in which normal control tissue had increased methylation levels as compared to MC tissue. The MC patient showed hypermethylation compared to normal control in the first three-quarter of the CpG region, noticeably, CpG sites 10, 11, and 12 recorded the highest increment of methylation in the MC patient compared to the normal control with the increment of 119, 74 and 100%, respectively. Conversely, the reversed pattern was observed at the quarter end of the CpG region where the normal control reported the hypermethylation as compared to MC patient tissue.

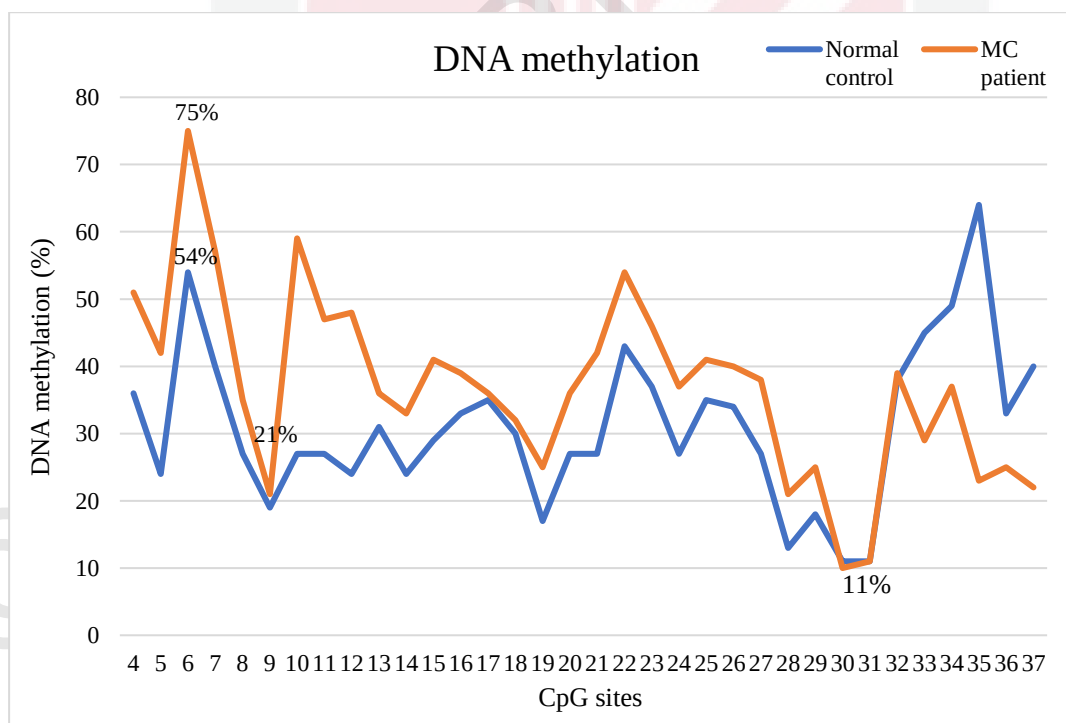


Figure 5.9: DNA methylation profile across 34 CpG sites in *CLCN1*. Methylation analysis of the MC patient shows a trend of increased DNA methylation as compared to normal control, particularly at CpG sites 4 – 29. Conversely, the methylation pattern reversed toward the end quarter of the region at which the normal control was hypermethylated at CpG sites 30 – 37.

Figure 5.10 shows an increased trend of DNA methylation levels in the MC patient carrying the c.1667T>A (p.Ile556Asn) variant. The boxplot shows that the MC patient exhibits higher DNA methylation levels (hypermethylation) from sites 4 – 29, as compared to the normal control, with median values of 37% and 27%, respectively.

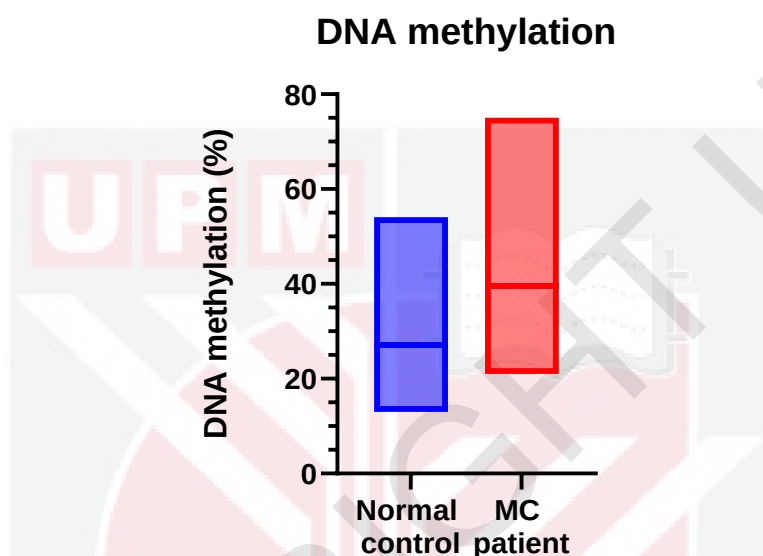


Figure 5.10: The box plot shows a comparison of DNA methylation value in tissue samples of normal control and the MC patient from CpG sites 4 – 29. The boxes plot for minimum and maximum values, and the lines inner the boxes represent the median values. Methylation analysis across 28 CpG sites of a normal control shows a range of methylation levels between 13 – 54%, with a median value of 27%. On the other hand, the MC patient reported a higher DNA methylation with a range of 21 – 75%, and a median value of 37%.

5.4 Discussion

MC has been known for its variable clinical presentation and one hypothesis to explain this variability is the expression of the *CLCN1* gene. In this study, the MC patient with c.1667T>A (p.Ile556Asn) variant showed a mild myotonia, therefore the expression of the *CLCN1* gene was studied. Moreover, to date, gene expression in relation to this variant has never been reported. In this study, the heterozygous MC patient showed a

trend of reduced *CLCN1* gene expression when compared to the normal control. This suggested that altered expression of *CLCN1* in the patient may cause the mild myotonia phenotype. The brother and the asymptomatic children with the heterozygous variant did not consent for muscle biopsy, therefore the gene expression analysis could not be performed, to assess the gene expression trend in the asymptomatic carriers.

In contrast to this finding, previous studies have shown no changes in *CLCN1* gene expression between MC patients and normal individuals (Imbrici et al., 2016; Portaro et al., 2015). A study by Portaro et al. (2015) reported two MC patients. One patient had compound heterozygous alleles with the p.T82A and p.G190S *CLCN1* variant in trans, while another patient had a homozygous p.G270V variant. Neither patient exhibited changes in *CLCN1* expression when compared to normal controls. A similar finding reported by Imbrici et al., (2016) showed the gene expression of *CLCN1* in the MC patient carrying the p.T335N variant, was within the range observed in control individuals.

Several other hypotheses have been proposed to explain the clinical variability among patients with the same variant, including transacting genetic modifiers; epigenetic, hormonal, or environmental factors; and dominance with low penetrance (Cannon, 2015; Suetterlin et al., 2014). To better understand the differential expression of *CLCN1* gene, where the result from this study did recapitulate previous studies, epigenetic studies were conducted.

Epigenetics describes the changes in gene expression that occur when DNA is modified without changing in nucleotide sequence. Epigenetic changes are essential for controlling the accessibility of DNA for transcription (Farsetti et al., 2023; Fedoriw et al., 2012). DNA methylation plays a key role in regulating tissue-specific gene expression, alternative splicing, prevention of cryptic initiation of transcription from alternative promoters, and X chromosome inactivation. These processes have been demonstrated to influence disease development (Velasco and Francastel, 2019). It is typically examined in the context of CG dinucleotide (CpG) where the cytosine base can be modified at the fifth carbon position by the addition of a methyl (CH₃) group. The association between DNA methylation at the CpG site and the inhibition of gene expression was first demonstrated in a study of CpG islands (CGIs), which is the region that has a high frequency of CpG dinucleotides. CGIs typically located in the promoter of the protein-coding gene, and it was found that the CpG island in the promoter region of active genes was shown to be unmethylated when compared to coding regions (Illingworth et al., 2010; Deaton and Bird, 2009). Although CGIs are mainly found in promoter regions, they can also be present in enhancer regions.

Research over the last decade has shown that epigenetic modification can play a role in the pathogenesis of neuromuscular disorders. The variation in clinical phenotype and age of onset reported for myotonic dystrophy type 1 (DM1) patients, has been associated with the DNA methylation of CpG islands in the *DMPK* gene (Cerrato et al., 2020; Bennett et al., 2019; Legare et al., 2019; Buckley et al., 2016). At present, this study is the first to determine the methylation status of the *CLCN1* gene in the MC patient. The findings revealed that out of all CpG regions in the enhancer, three-quarters were hypermethylated in the MC patient as compared to the normal control,

with a reversed pattern observed in the remaining quarter where normal control tissue was hypermethylated. This hypermethylation occurs at the CpG site which is located at the enhancer region of *CLCN1* (**Appendix C1**). Enhancers are regulatory DNA sequences that enhance the transcription of associated genes. It displays variable tissue-specific methylation patterns and can function as alternative promoters for the genes in which they are located (Illingworth et al., 2008). The methylation status of these enhancers can impact their ability to interact with transcription factors and other regulatory proteins, thereby influencing gene expression.

In normal individuals, enhancer regions are usually hypomethylated, which allows transcription factors (TF) to bind and keeps the chromatin open for transcription (Loyfer et al., 2023). In contrast, hypermethylation of enhancers leads to closed chromatin, inhibiting TF binding and reducing gene expression (Baribault et al., 2018). For the MC patient in this study, most of the *CLCN1* gene enhancer region is hypermethylated, suggesting a repressive environment that may decrease *CLCN1* expression. However, the last quarter of this region showed hypomethylation in the MC patient when compared to the normal control. Enhancers often showed variable methylation to regulate gene expression (Baribault et al., 2018). The overall hypermethylation suggests *CLCN1* downregulation, while the hypomethylation in the last quarter of the enhancer may indicate an attempt by the cellular regulatory machinery to counterbalance the overall downregulation state, perhaps to maintain minimal expression levels of *CLCN1* necessary for cellular function (Barral and Déjardin, 2023). Although the last quarter is hypomethylated, the enhancer region in MC patients is still mainly hypermethylated compared to normal controls, likely having a significant impact on gene expression and chromatin structure.

Enhancer regions are *cis*-regulatory sequences that can significantly enhance the transcription of associated genes, often functioning over long distances. These non-promoter CGIs, known as orphan CGIs, are found in intergenic or intragenic regions far from annotated promoters (Kowalczyk et al., 2012). Hypermethylation in enhancer CGIs leads to the loss of enhancer signatures and results in the downregulation of target genes (Bae et al., 2016). Thus, the hypermethylation observed at the CpG site within the enhancer region of the *CLCN1* gene suggests a potential mechanism for transcriptional repression, which correlates with the downregulation of the *CLCN1* gene that was found in this study. This suggests that an increase in DNA methylation in the MC patient may indicate a potential epigenetic mechanism contributing to the pathogenesis of MC.

5.5 Conclusion

This study is the first to examine the effects of variant c.1667T>A (p.Ile556Asn) on *CLCN1* gene expression and methylation status in the MC patient. The findings suggest that hypermethylation at the CpG site located at the enhancer region downregulates the *CLCN1* gene that contributes to the phenotype presentation. Further research is needed to include asymptomatic carriers, which could help elucidate the role of epigenetics in modulating disease expression and contribute to a more comprehensive understanding of the mechanisms underlying incomplete penetrance in MC.

CHAPTER 6

DETERMINING THE FUNCTIONAL EFFECTS OF THE c.1667T>A (p.Ile556Asn) VARIANT VIA PROTEIN EXPRESSION AND HISTOCHEMICAL ANALYSIS

6.1 Introduction

Myotonia congenita (MC) is an inherited muscle disorder caused by loss-of-function variants in the chloride channel 1 gene (*CLCN1*) encoding the skeletal muscle chloride channel (ClC-1). These variants lead to disrupted ion movement in muscle membranes and changes in muscular excitability. The ClC-1 channel is a homodimer comprising two identical subunits with an ion-conducting pathway or protopore.

Functional studies have been performed to characterise the variants to study their impact on the biological function of genes or proteins. This includes examining the variant effects on ClC-1 protein expression (Imbrici et al., 2016; Raheem et al., 2012), ClC-1 protein localisation (Altamura et al., 2020), interaction with other molecules, and impact on physiological processes (Kubisch et al., 1998). Several methods have been used, including an electrophysiological study by Kubisch et al. (1998) using *Xenopus* oocyte expression to analyse the effect of several variants including c.1667T>A (p.Ile556Asn), the same heterozygous variant found in the proband. The study demonstrated that c.1667T>A (p.Ile556Asn) slightly shifts the voltage dependence when co-expressed with wild-type, indicating mild changes when the variant is in the heterozygous state (Kubisch et al., 1998). This finding may explain the mild phenotype observed in the MC patient in the current study.

Furthermore, another functional study conducted by Altamura et al. (2020) studied the molecular defect of the p.G411C variant by combining biochemical and electrophysiological approaches. Protein expression analysis indicated that the p.G411C variant does not affect the total protein expression. Instead, it reduces the proportion of the total proteins present at the surface membrane. Further biochemical analysis demonstrated that most of the mutant channels did not reach the plasma membrane but were trapped inside the cells (Altamura et al., 2020). Another functional study carried out by Imbrici et al. (2016) on *CLCN1* variants also showed there is no significant difference in protein expression between wild-type and mutant alleles. In contrast, others have shown a reduction in the protein expression suggesting this might be due to a defect in trafficking (Lee et al., 2013; Richman et al., 2012). If protein expression is unaffected, further analysis can be conducted to determine whether the protein is located on the membrane or if there are issues with protein trafficking. The findings from these experiments are crucial in understanding the broader implications of the variant on muscle function.

While genetic, molecular, and functional research has been studied extensively, histopathological analysis in affected muscles has received less attention. This is due to the process being time-consuming, and fewer studies not placing emphasis on investigating the structural changes in muscle tissue caused by genetic abnormalities. Despite the gap in this type of investigation, histopathological analyses are important for confirming the diagnosis and to identify secondary changes that might occur due to the defect in the ion channel. These findings may also correlate with the clinical presentation of MC.

Routine staining for MC includes hematoxylin and eosin (H&E), myosin ATPase staining (pH 4.3, 4.6 and 9.4), modified Gomori trichrome (MGT), nicotinamide adenine dinucleotide (NADH), succinate dehydrogenase (SDH), and cytochrome c oxidase (COX). Staining muscle with H&E stains nuclei blue and the cytoplasm light red (Wang et al., 2017). Using H&E staining, evaluation of the general architecture, fibres size, shape, number, and position of the nucleus can be performed (Dubowitz et al., 2013). The transverse section of normal muscles has roughly the same fibre size, each comprising hundreds of myofibrils, and the nuclei occupy the periphery of the fibres. In disease muscle, the muscle fibres size varies and is no longer uniform and the nuclei start to migrate centrally (Walters and Baborie, 2020). Previous H&E studies have shown MC patients have variable muscle fibres size and increased internal nuclei (Portaro et al., 2015) while others report normal morphology (Hu et al., 2021b; Miao et al., 2018).

NADH is an oxidative enzyme staining procedure that reveals intracellular structure and myofibrillar organisation. In diseased muscles as in myopathies, NADH staining can highlight structural changes such as lobulated fibres, and moth-eaten fibres (**Figure 6.1**), which is a result of intermyofibrillary network disruption and mitochondrial abnormalities (Mitchell and Waclawik, 2007).

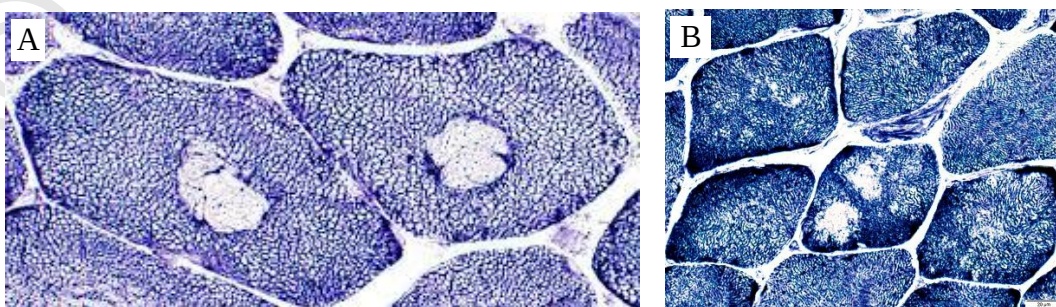


Figure 6.1: Lobulated fibres and moth-eaten appearance observed in NADH staining. (Source: <https://neuromuscular.wustl.edu/pathol/>)

MGT can demonstrate abnormal accumulation of mitochondria that can be seen as ragged red fibres (Preedy and Peters, 2002) in cases of mitochondrial disease and nemaline rods (**Figure 6.2**) that can be found in nemaline rod myopathy, one of the hereditary neuromuscular disorders (Sharma et al., 2007).

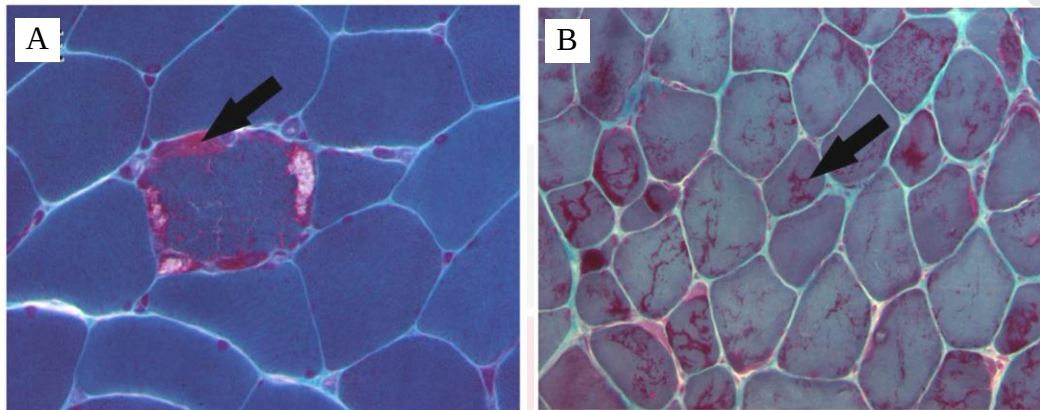


Figure 6.2: Ragged red fibres (left) and nemaline rods (right) observed in a patient with mitochondrial diseases and nemaline rod myopathy, respectively. Note that the arrow is pointing to the ragged red fibres structure (Cotta et al., 2021).

SDH and COX are mitochondrial enzymes used to study mitochondrial pathology. Abnormal structures such as lobulated fibres can be stained with SDH, while fibres lacking COX activity appear white compared to the adjacent fibres' staining pattern in COX, which is characteristic of mitochondrial myopathy. These routine staining experiments (NADH, SDH and COX) have been conducted previously on MC patients and normal oxidative enzyme activity was reported (Hu et al., 2021b; Miao et al., 2018).

Skeletal muscle is made up of muscle fibres of polygonal shape and uniform size. There are two types of muscle fibres: type 1 'slow-twitch' and type 2 'fast-twitch'. These types of muscle fibres can be determined with myosin ATPase staining by

incubating the tissue sections with adenosine triphosphatase (ATPase) at various pH values. ATPase is an enzyme important for adenosine triphosphate (ATP) production, which is responsible for muscle contraction (Cain et al., 1962). In normal muscle, type 1 and type 2 fibres are distributed randomly in a mosaic pattern, while MC patients have been reported to exhibit a predominance of type 2 fibres (Meng et al., 2016, Portaro et al., 2015; Lakraj et al., 2013). Most muscle diseases exhibit some or all of these abnormalities, although a few may appear normal with minimal signs of disease. If standard staining procedures do not indicate any abnormalities, immunohistochemical staining may provide evidence of a specific disease process.

Immunohistochemical staining has been widely used to ascertain the presence or absence of abnormal accumulation or abnormal distribution of certain proteins in neuromuscular disorders. Raheem et al. (2012) conducted an immunohistochemical assay for sarcolemma chloride channel abundance for the patients with myotonia and revealed a total loss of CLC-1 protein in homozygous patients with R894X nonsense variant, and patients with compound heterozygous showed either total or subtotal loss of CLC-1 protein.

The ultrastructural analysis of muscle structure can be visualised using electron microscopy (EM) techniques. The main abnormalities that can be seen in muscular fibres include inclusion bodies (aggregates of proteins or other substances found within cells), vacuolar changes such as the presence of rimmed vacuoles, myofibrillar disorganisation, lack of mitochondria (marked by a decreased number of paired dots observed above the Z line), and Z-line disorientation (**Figure 6.3**) (Fernandez et al., 2005). This analysis serves to identify changes that are not evident with light

microscopy. Thus, this chapter aims to determine the CLC-1 protein expression in the MC patient and provide a comprehensive examination of the histological features.

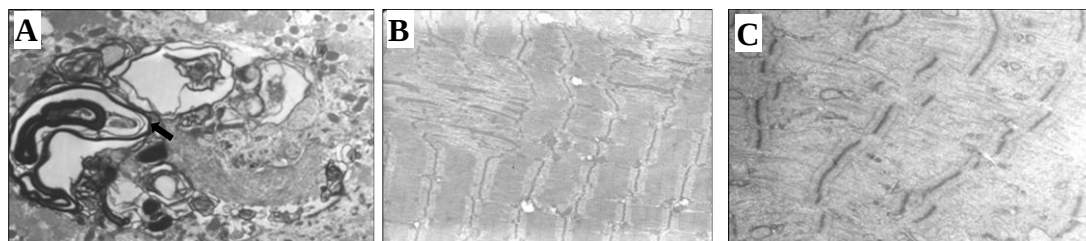


Figure 6.3: Vacuolar changes such as (A) the presence of rimmed vacuoles (arrow), (B) myofibrillar disorganisation and lack of mitochondria, and (C) Z-line disorientation visualised using TEM (Fernandez et al., 2005).

6.2 Materials and Methods

6.2.1 Sample Collection

Informed consent was obtained from the study participants, and muscle tissue samples were collected for the protein expression, histochemical and electron microscopy analysis from the proband (hereafter referred to as MC patient) and an unrelated normal control individual. Due to the limited number of individuals who consented to undergo muscle biopsy, this functional study includes only one MC patient (proband) and one normal control.

6.2.2 Muscle Biopsy

For muscle biopsy procedure refer to Chapter 5, Section 5.2.1.1

6.2.3 CLC-1 Protein Expression

6.2.3.1 Protein Extraction

Fifty milligrams of biceps tissues were weighed and transferred into a 2 mL microcentrifuge tube. The tissues were homogenised using an Ultra-Turrax® Homogenizer (S10N - 5 G disperser, Ika® T10 Basic, China) in an ice-cold buffer containing 200 µL of radioimmunoprecipitation assay (RIPA) cocktail which include 160 µL of RIPA buffer (Pierce™, Thermo Scientific, USA), 20 µL of phosphatase inhibitor (PhosSTOP, Roche, Switzerland) and 20 µL of protease inhibitor (SIGMAFAST™, Sigma-Aldrich, Germany). Homogenates were incubated on ice for 30 min and centrifuged (Eppendorf MiniSpin®, Germany) at 13,000 x g for 30 min at 4 °C. The supernatants were then collected and proceeded with protein quantification.

6.2.3.2 Protein Quantification

Total protein was quantified using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA) following the manufacturer's protocol. Briefly, diluted bovine serum albumin (BSA) standard was prepared in a microcentrifuge tube by diluting 1 mL albumin standard (2 mg/mL) into a set of diluted standards with RIPA buffer as shown in **Table 6.1**.

Table 6.1: Preparation of diluted albumin (BSA) standards

Dilution scheme for standard microplate protocol			
Vial	Volume of RIPA buffer (μL)	Volume and source of BSA (μL)	Final BSA concentration (μg/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

BSA provided in the kit was used as a standard throughout the experiments. Two hundred microlitres of BCA working reagent was prepared for each sample and diluted by mixing 50 parts of BCA Reagent A with 1 part of BCA reagent B (50:1, Reagent A: B). In a 96-well microplate, 1 μL of protein sample and a dilution of standards were mixed with 199 μL of working reagent in triplicate and mixed well. The microplate was then covered with aluminium foil and was incubated in an incubator (Sastec, Malaysia) at 37 °C for 30 min. The absorbance was read at 562 nm using a VersaMax™ Plus Rom v1.23 ELISA plate reader (Molecular Devices®, USA) via SoftMax Pro 5.4 software (Molecular Devices®, USA). The standard curve established using BSA was plotted (**Appendix D1**), and the protein concentration of each sample was then determined from the curve and 20 μg of protein needed for one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was calculated (**Appendix D2**).

6.2.3.3 One-dimensional Polyacrylamide Gel Electrophoresis

The one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was carried out using Mini-PROTEAN® Precast gels (Bio-Rad Laboratories, USA). For each sample, 20 µg of total protein extracts were added to a microcentrifuge tube containing 4x Laemmli Sample Buffer (BioRad, USA) (62.8 mM Tris HCl pH 6.8, 26.3% (w/v) glycerol, 2.1% (w/v) sodium dodecyl sulphate (SDS), 0.01% (w/v) bromophenol blue, 5% β-mercaptoethanol). The protein mixture was vortexed, heated using a heat block (WiseTherm® HB High-performance Heating Block, Germany) at 100 °C for 5 min and kept on ice until loading.

The 4 – 20% Mini PROTEAN® TGX™ Precast Polyacrylamide Protein Gel cast gels were assembled facing each other and secured into the electrophoretic module, Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (BioRad, USA) according to the manufacturer's instruction. The electrophoretic module was then placed into the electrophoretic tank and the tank was filled with 1x SDS running buffer (**Table 6.2**). A total of 5 µL of Precision Plus Protein Dual Color Standards (Bio-Rad, USA) and 20 µL of each protein mixture were loaded into the wells of cast gel. The lid was placed on the electrophoretic tank and the electrical leads were plugged into the power pack. The cast gel was run at 80 V for 10 min and then increased to 120 V for 60 min at room temperature until the bromophenol front dye ran off the gel.

Table 6.2: Solutions used for Western blot

Buffer/solutions	Components
SDS running buffer	100 mL 10 x Tris/Glycine/SDS (BioRad, USA), 900 mL dH ₂ O
10x TBS buffer	24 g Tris base (BioRad, USA), 88 g NaCl (System, Malaysia) in 1 L dH ₂ O, pH 7.6
TBST washing buffer	100 mL 10 x TBS buffer, 900 mL dH ₂ O, 1 mL Tween-20 (Sigma Aldrich, USA)
10x transfer buffer	30.4 g Tris (BioRad, USA); 144.2 g Glycine (BioRad, USA) in 1 L of dH ₂ O
Transfer buffer	700 mL dH ₂ O, 100 mL 10 x Transfer buffer, 200 mL methanol
Blocking solution	50 mL 1 x TBST with 2.5 g blotting grade blocker, non-fat milk (BioRad, USA)
Ponceau S	0.1 % Ponceau-S (Amresco, USA) in 1% Acetic acid, reusable

Note: dH₂O: distilled water, TBS: tris-buffered saline, SDS: sodium dodecyl sulfate, TBST: tris-buffered saline with Tween 20

The electrophoretic module was removed from the electrophoretic tank and the cast gels were dissembled from the electrophoretic module. The gel cassette sandwich was opened gently, and the resolving gel was gently removed for immunoblotting.

6.2.3.4 Immunoblotting

Proteins were transferred from the gel to the polyvinylidene fluoride (PVDF) membrane (BioRad, USA) using electro-blotting in a sandwich assembly at 25 V for 7 min and continued for another 3 min for the second run using Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories GmbH, Germany).

After transfer, the membrane was stained for 5 min in Ponceau S solution (**Table 6.2**) until the protein bands were distinctly visible. The membrane was then washed in

TBST solution (**Table 6.2**) three times for 5 min and then blocked in blocking solution (**Table 6.2**) for 1 h at room temperature on a shaker (Sastec Benchtop Shaker ST-344, Malaysia). The primary antibody (ClC-1 rabbit-polyclonal, 1:1,000) and housekeeping protein (GAPDH mouse-polyclonal, 1:10,000) was added, and the membrane was incubated overnight, at 4 °C. The membrane was then washed three times for 20 min with TBST. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Elabscience, USA, 1:2,000) was added to membrane-targeted ClC-1 and the goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Elabscience, USA, 1:20,000) was added to membrane-targeted GAPDH. The membrane was then incubated in the mixture for 1 h at room temperature, then the membrane was washed three times for 10 min with TBST (Refer to **Appendix D3** for antibodies dilution).

6.2.3.5 Immunodetection

The Enhanced chemiluminescence (ECL) Substrate Detection Kit (Elabscience®, USA) was used to detect a horseradish peroxidase-conjugated secondary antibody. Two mL of ECL working solution were freshly prepared in a 15 mL centrifuge tube by mixing equal parts of ECL Substrate A and ECL Substrate B, (1 mL of ECL substrate A with 1 mL of ECL substrate B). The ECL working solution (500 µL) was added to the membrane (3 x 4 cm), incubated for 5 min in the dark, and visualised using the Syngene G: BOX gel documentation system (Synoptics Ltd, Syngene, G BOX, UK). The expected size bands were determined to be correct by comparing the bands on the membrane with the pre-stained protein marker.

6.2.3.6 Band Intensity and Protein Expression

Densitometric scans of western blots were quantified by using ImageJ Lab software (National Institutes of Health, USA), which allows the chemiluminescence detection of each experimental protein band. The blot image was converted into an 8-bit image and each protein band of interest was selected using the rectangle selection tool. The absolute signal intensity was adjusted by subtracting the local background. The intensity of each protein band was normalised to the intensity of the reference protein, GAPDH as described in the formula below and the mean relative protein expression for the MC patient and normal control of each protein was calculated.

$$\text{Normalise protein expression} = \frac{\text{Intensity of the protein of interest (ClC-1)}}{\text{Intensity of the GAPDH}}$$

6.2.4 Histochemical Analysis

The fresh muscle tissue was immediately sent and outsourced to the Pathology Department, Faculty of Medicine, Universiti Malaya for histopathological analysis. In brief, the muscle tissue was snap frozen and staining procedures using H&E, myosin ATPase staining (pH 4.3, 4.6 and 9.4), MGT, NADH, SDH, and COX were performed. For H&E staining, muscle fibres were examined for their length to identify any abnormalities, specifically fibres exceeding 100 μm or measuring less than 50 μm , and the number of fibres with internal nuclei was counted, and the percentage was calculated by comparing to the total number of muscle fibres using the formula below:

$$\text{Internal nuclei (\%)} = \frac{\text{Number of fibres with internal nuclei}}{\text{Total number of muscle fibres}} \times 100\%$$

Myosin ATPase staining was performed to determine the distribution of type 1 and type 2 fibres. The number of type 1 and 2 fibres was determined by counting the muscle fibres for each type over the total number of muscle fibres. The percentage for each fibres type were calculated as shown in the formula below. The percentages were compared between the MC patient and the normal control.

$$\text{Muscle fibres (\%)} = \frac{\text{Number of fibres for each muscle type}}{\text{Total number of muscle fibres}} \times 100\%$$

Myosin ATPase at different pH values, was used to differentially stain type 1 and type 2 fibres. ATPase pH 4.3 stained type 1 fibres dark and type 2 fibres light whereas ATPase pH 9.4 stained type 1 fibres light and type 2 fibres dark. In addition, ATPase pH 4.6 differentiates further 2 fibre types in which type 2A fibres were stained light, type 2B stains with an intermediate shade, and type 1 fibres dark. MGT, NADH, SDH and COX staining experiments were conducted to determine the mitochondrial pathology, abnormalities, and myofibrillar organisation.

To detect the presence of CLC-1 protein on the sarcolemma, frozen sections (6 µm) of the muscle tissues were slices and mounted on adhesive-coated slide glass (Platinum Pro, Matsunami, Japan) and stained using polyclonal *CLCN1* antibody (1:100) (Antibodies-online, GmbH, Germany). The sections were hydrated by soaking three times in PBS for 5 min and incubated in endogenous blocking solution EnVision FLEX Peroxidase-Blocking Reagent (Dako, Denmark) for 5 min. This was followed by flush washing with phosphate buffer saline (PBS) and subsequent incubation with CLC-1 primary antibody (1:100) for 30 min at room temperature. Incubation with EnVision FLEX/HRP-tagged secondary antibody (Dako, Denmark) (100 µL) was

done for 20 min at room temperature. Diaminobenzidine (EnVision™ FLEX DAB + Chromogen, Dako, Denmark) was used as substrate chromogen before being counterstained with Tissue-Tek hematoxylin solution (Sakura Finetek, Japan). The staining was then analysed using a light microscope (Olympus Microscope Bx40, Japan) and compared to the normal control.

6.2.5 Electron Microscopy

The muscle tissue biopsy was immediately fixed in 4% cold glutaraldehyde (Agar Scientific, UK) and sent to the Institute of Biosciences (IBS), Universiti Putra Malaysia for ultrastructural analysis following the IBS standard in-house protocol. In brief, the muscle tissue was cut into 1 cm³ slices for SEM and 1 mm³ slices for TEM and fixed in cold 4% glutaraldehyde (Agar Scientific, UK) for 12 h at 4 °C. Both SEM and TEM tissues were then washed three times with 0.1M sodium cacodylate buffer (Agar Scientific, UK) for 10 min. Post-fixation was then performed with 1% osmium tetroxide (Agar Scientific, UK) in sodium cacodylate buffer (Agar Scientific, UK) for 2 h at 4 °C and dehydrated in an acetone series: 35%, 50%, 75%, and 95% acetone, each for 10 min, followed by 100% acetone for 15 min, for three times.

The next processing steps was different between SEM and TEM tissue sample. SEM analysis was conducted by placing the tissue specimen into critical dryer for 30 min and was mounted on a metal stub using a sticky carbon disc which increase the conductivity. Silver-containing glue was also applied for more conductivity. Specimens were then coated with gold using Bal-tec SCD 005 sputter coater (Bal-Tec

AG, Switzerland) to prevent charge buildup on the specimen surface and analysed using a scanning electron microscope (SEM) (JSM IT-100, JEOL, Japan).

For TEM analysis, the muscle tissue specimen was then infiltrated with a different ratio of acetone (Systerm, Malaysia) and resin (Agar Scientific, UK). It was first infiltrated in an equal mixture of acetone and resin (1:1) for 1 h, followed by a 1:3 acetone-to-resin mixture for 2 h. After that, the muscle tissue was soaked in 100% resin overnight and then continue for another 2 h. The tissue specimen was then embedded in resin and continue with polymerisation in an oven at 60 °C for 24 h. Semithin sectioning was performed using glass knife and ultramicrotome to cut 1 µm sections and place them onto a glass slide. The tissue was then stained with toluidine blue (Electron Microscopy Sciences, UK), dried and washed. Ultrathin sections (80 nm) were then prepared, stained with uranyl acetate (Ted Pella. Inc, US) and lead nitrate stain (Electron Microscopy Sciences, UK), and analysed using TEM (JEM-2100F, JEOL, Japan).

6.3 Results

6.3.1 CLC-1 Protein Expression Reduced in MC Patient

CLC-1 protein expression was analysed using Western blot by extracting the total CLC-1 protein from the biceps brachii of the MC patient and a normal control individual. GAPDH was used as a housekeeping protein. Due to the limited number of individuals who consented to undergo muscle biopsy, this study includes only one MC patient (proband) and one normal control. As shown in **Figure 6.4A**, the band size of CLC-1

protein can be detected at 109 kDa while a GAPDH band was detected at 36 kDa (the whole blot images can refer **Appendix D4**). GAPDH was selected as the reference protein because β -Actin protein showed varying expression between a normal control and an MC patient, making it unsuitable for accurate normalisation (**Appendix D5**). The intensity of each protein band was normalised to the GAPDH, and the mean relative protein expression for each protein in the MC patient and normal control is shown in **Appendix D6**. The MC patient had lower band intensity compared to the normal control, with total protein levels reduced by nearly 50% (**Figure 6.4B**) compared to normal control which suggests a trend of downregulation of the ClC-1 protein in the MC patient. This suggests that the c.1667T>A (p.Ile556Asn) variant may lead to altered ClC-1 protein expression, which is likely to contribute to myotonia in this patient.

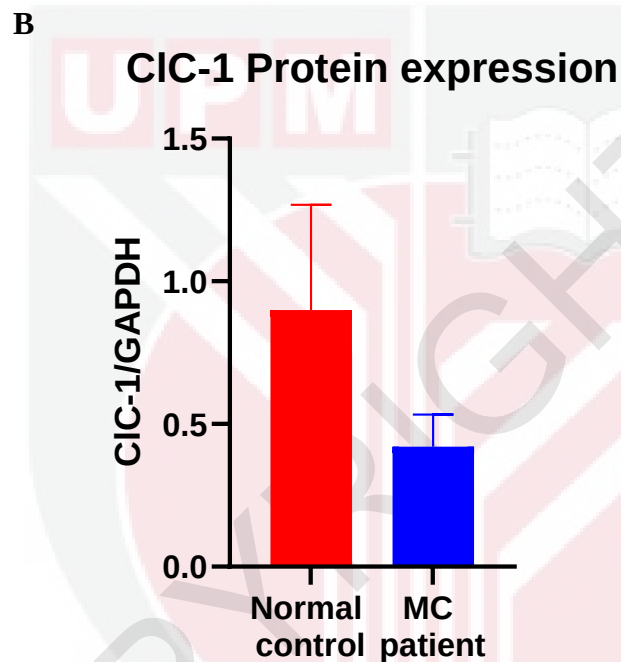
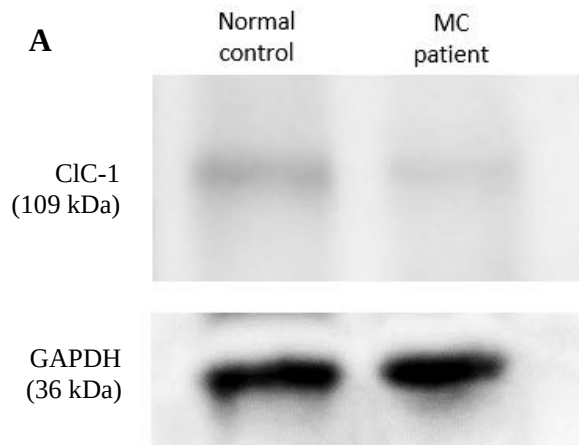


Figure 6.4: Quantification of total protein amount in biceps brachii muscle from the MC patient and one normal control. (A) Western blot analysis of total CLC-1 protein shows the MC tissue has reduced signal intensity compared to the normal control when normalised with GAPDH protein. (B) Quantification of the total protein shows a 50% downregulation trend of CLC-1 protein in the MC tissue as compared to normal wild-type. Each bar represents the mean $\pm$ SD of technical replicates from duplicate experiments, conducted using protein from a single extraction.

6.3.2 Histochemical Analyses Showed Mild Changes in MC Tissue

Histochemical analyses were conducted to evaluate possible morphological, enzymatic, and ultrastructural alterations in the skeletal muscle of the MC patient.

6.3.2.1 Hematoxylin and Eosin (H&E) Staining Showed Increased Internal Nuclei and Variability in Muscle Fibres Size

H&E staining was used to determine the general morphology, including the nuclei position and fibres size. The number of muscle fibres with internal nuclei in the whole tissue section was counted (**Appendix D7**) and the length of muscle fibre was measured. In normal control tissue, most of the muscle fibres contain nuclei located typically along the periphery or perimysium (**Figure 6.5A**), whereas in the MC patient, some muscle fibres exhibited nuclei positioned internally (**Figure 6.5B1**). The H&E staining analysis revealed that 33.8% of the muscle fibres in the MC patient had internal nuclei, compared to only 5.8% in the normal control.

In addition to the altered position of nuclei, the MC tissue exhibited variability in muscle fibres size, with noticeable differences in fibres diameter. Some hypertrophic muscle fibres were observed, measuring over 100 μm , with few fibres in the MC patient being 120 μm in length (**Figure 6.5B2**). Additionally, atrophic fibres were present, with diameters smaller than 50 μm , including fibres as small as 35 μm (**Figure 6.5B3**).

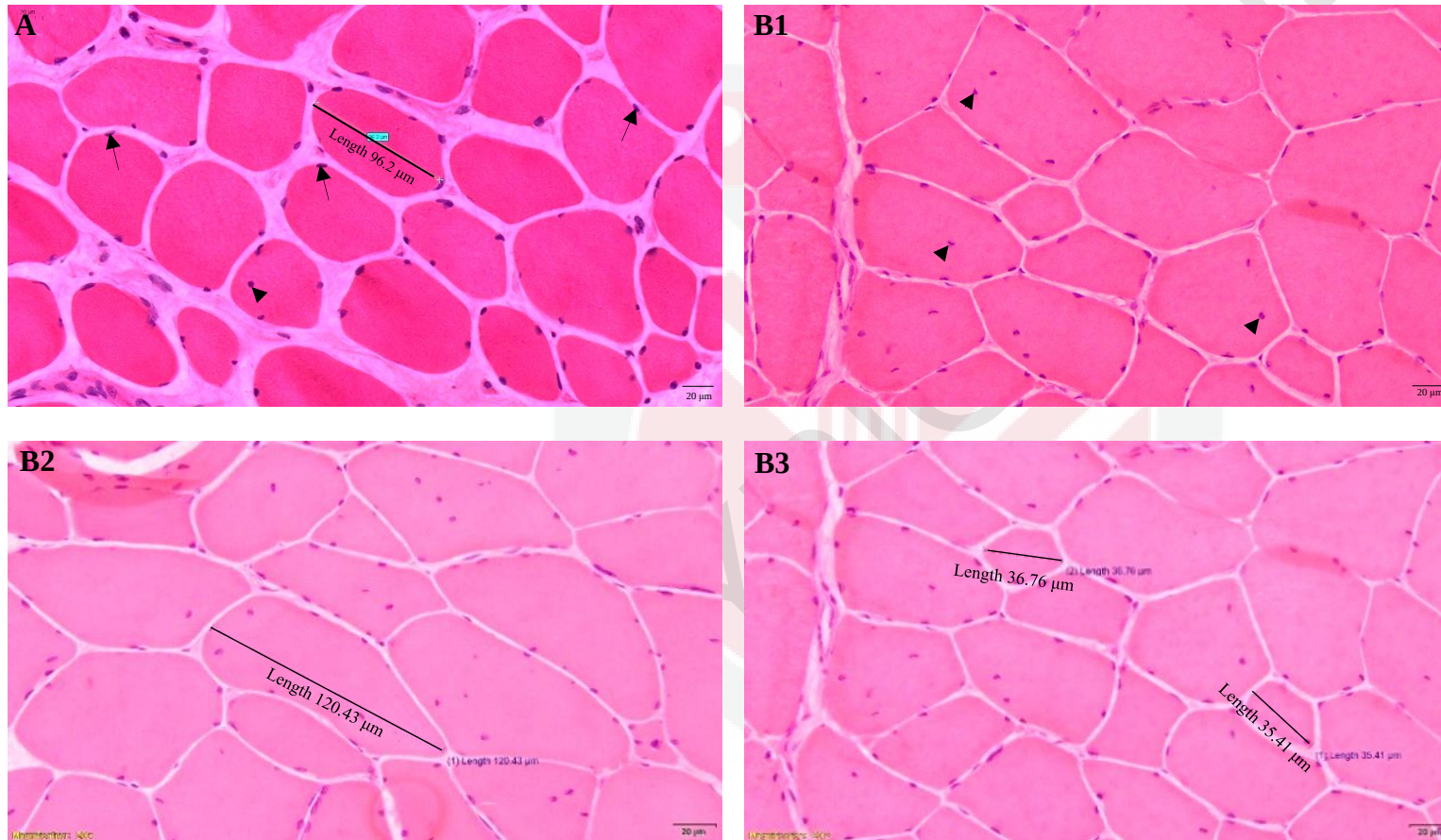


Figure 6.5:
Histopathological findings for left biceps brachii stained with haematoxylin and eosin (H&E) of (A) normal control and (B1 – B3) MC tissue.
 (A) Normal control shows peripheral nuclei (black arrow) with a uniform size of muscle fibres while (B1) muscle tissue shows an increase of internal nuclei (arrowhead), (B2) fibres size variation with 120 μm of hypertrophic muscle fibres (muscle fibres size $\geq 100 \mu\text{m}$), and (B3) 35 μm of atrophic fibres (muscle fibres $< 50 \mu\text{m}$).

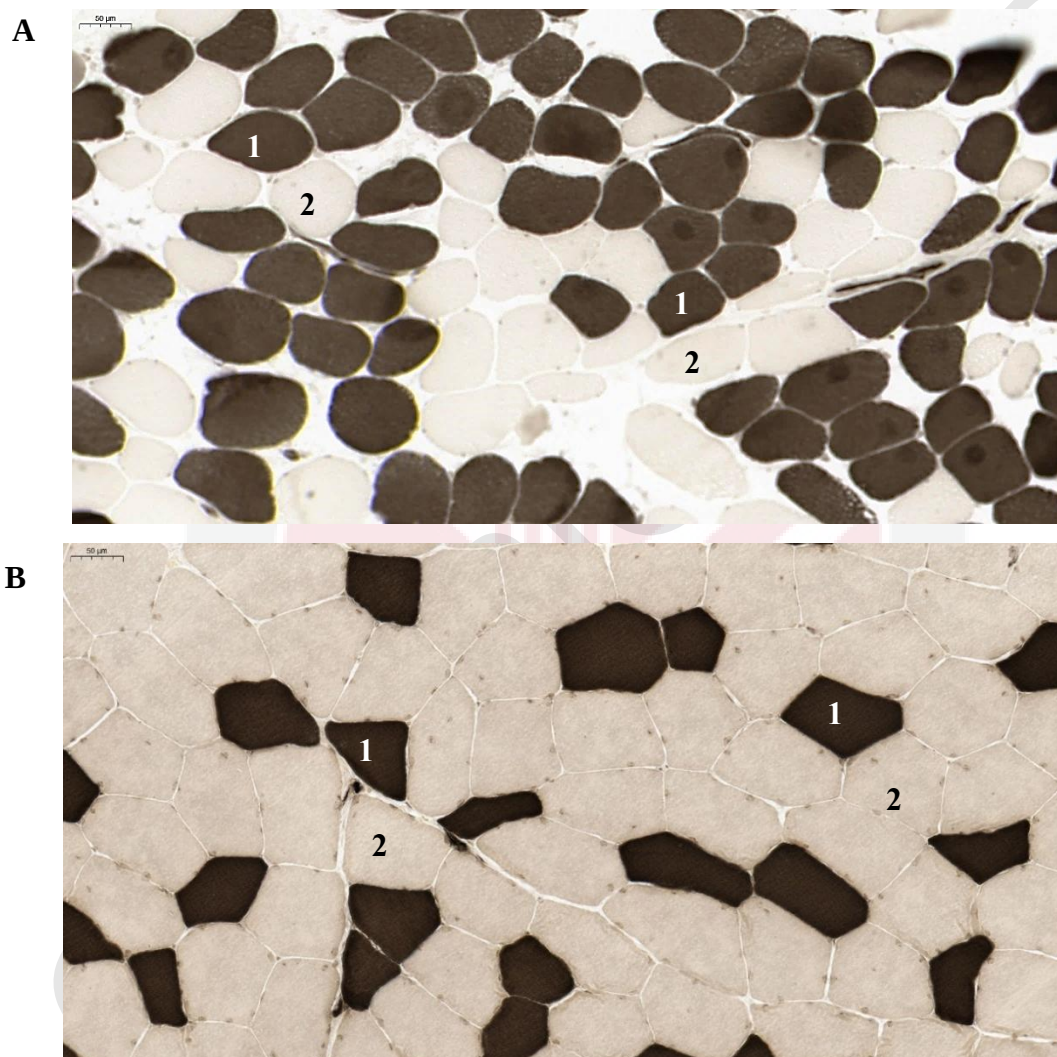
6.3.2.2 Myosin ATPase Revealed Predominance of Type 2 Fibres

Myosin ATPase staining was conducted at different pH levels, differentiated between type 1 and type 2 muscle fibres. The total number of type 1 and type 2 muscle fibres stained at different pH; pH 4.3, 4.6, and 9.4 were counted across all sections (**Appendix D8**).

Myosin ATPase staining for pH 4.3 stained type 1 fibres dark and type 2 fibres light. The normal control tissue had an equal representation of dark and light band patterns with a proportion of 49.9% of type 1 and 50.1% of type 2 fibres (**Figure 6.6A**). In contrast, reduced dark band fibres (type 1, 23.1%) were observed in the MC tissue with an increased proportion of light band fibres (type 2, 76.9%) (**Figure 6.6B**). Meanwhile ATPase pH 4.6 stained type 1 dark with further type 2 fibres differential staining in which type 2A fibres stained light and type 2B fibres stained with an intermediate shade. Normal control tissue showed that most of the muscle fibres were dark bands (type 1, 55.1%) followed by intermediate bands of type 2B (39.8%) and a small number of type 2A with only 5.1% (**Figure 6.7A**). If the number of types 2A and 2B fibres were counted and represented as type 2 collectively, the proportion of type 1 and type 2 is 55.1% and 44.9%, respectively.

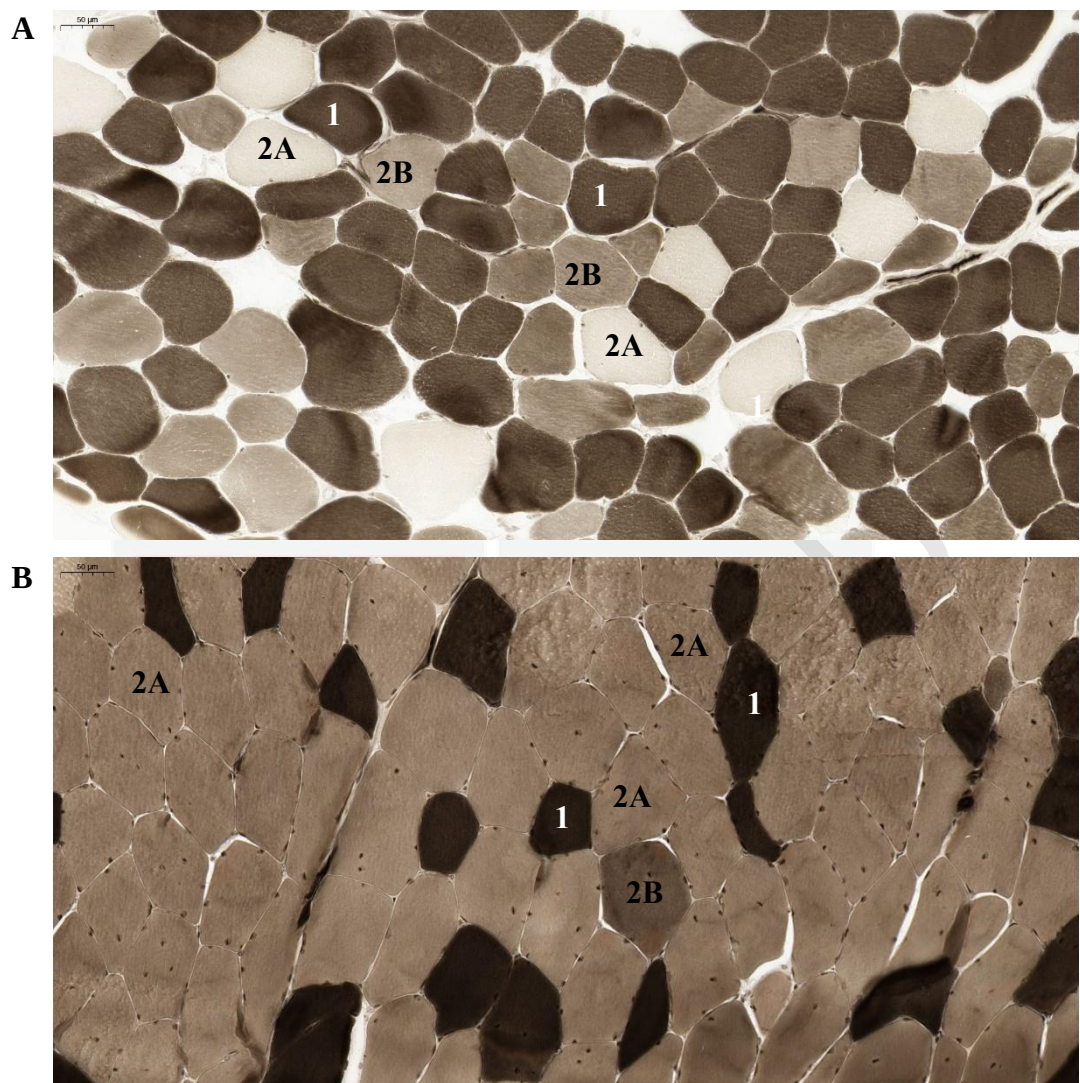
Meanwhile, for the MC tissue, the light band (type 2A) accounted for nearly two-thirds of the fibres (77.1%) followed by the dark band (type 1) with 22.3% and only 0.5% of the intermediate band (type 2B fibres) (**Figure 6.7B**). Additionally, ATPase pH 9.4 stained type 1 fibres light and type 2 fibres dark, and normal control tissue showed nearly equal proportion of light (type 1) and dark fibres (type 2) with 53.4% and

46.6%, respectively (**Figure 6.8A**). In contrast, the MC patient tissue showed an increase in dark bands (type 2) with 78.9% fibres and a reduction in light bands (type 1) with 21.1% (**Figure 6.8B**). Overall, examination of the myosin ATPase staining for all three pH showed a consistent predominance of type 2 fibres with a reduction of type 2B fibres in the MC tissue.



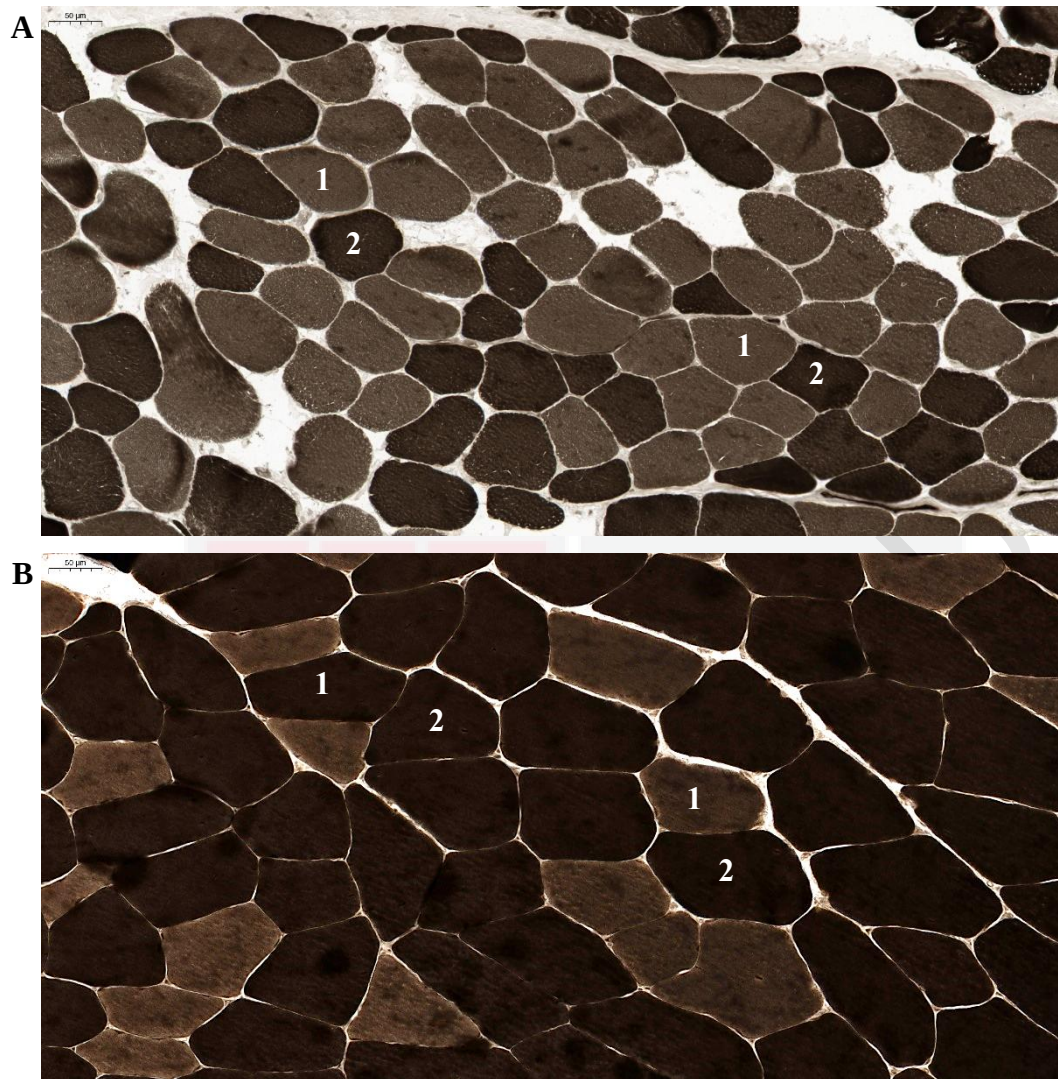
pH 4.3		Muscle fibres distribution (%)	
Type / Sample		Normal control	MC patient
Type 1 (Dark)		49.9	23.1
Type 2 (Light)		50.1	76.9

Figure 6.6: Myosin ATPase staining at pH 4.3. ATPase pH 4.3 stained type 1 fibres dark and type 2 fibres light. (A) Result shows an equal proportion of light and dark bands in normal control tissue while (B) the MC tissue shows an increased proportion of light bands (type 2) with reduced dark bands (type 1).



pH 4.6 Type / Sample	Muscle fibres distribution (%)	
	Normal control	MC patient
Type 1 (Dark)	55.1	22.3
Type 2A (Light)	5.1	77.1
Type 2B (Intermediate)	39.8	0.5

Figure 6.7: Myosin ATPase staining at pH 4.6. ATPase pH 4.6 stained type 1 fibres dark, type 2A fibres light and type 2B fibres with intermediate shade. (A) Normal control tissue shows the highest proportion of dark bands (type 1), followed by intermediate shade (type 2B) and a small number of light bands (2A). (B) MC tissue shows mostly light bands (type 2A), reduced dark bands (type 1) and very few fibres with intermediate shade (type 2B).



pH 9.4	Muscle fibres distribution (%)	
Type / Sample	Normal control	MC patient
Type 1 (Light)	53.4	21.1
Type 2 (Dark)	46.6	78.9

Figure 6.8: Myosin ATPase staining at pH 9.4. ATPase pH 9.4 stained type 1 fibres light and type 2 fibres dark. (A) Normal control shows nearly equal percentage of type 1 and type 2 fibres while (B) results revealed MC tissue shows muscle fibres largely dark bands (type 2) with a reduced portion of light bands (type 1) indicate a predominance of type 2 fibres.

6.3.2.3 Additional Staining Showed Normal Condition

COX, SDH, NADH, and MGT staining experiments were conducted to determine if the mitochondrial and myofibrillar organisation abnormalities were present. Results demonstrated normal enzymatic activity for COX (**Figure 6.9A**), SDH (**Figure 6.9B**), and NADH (**Figure 6.9C**), confirming by the absence of abnormal intracellular structure indicating normal myofibrillar organisation in both MC patient and normal control. Additionally, no special hyperchromatic structures were observed in the myofibrillar cytoplasm indicating a normal MGT staining (**Figure 6.9D**) (refer to **Figures 6.1 and 6.2** for the abnormalities structure stained with NADH and MGT).

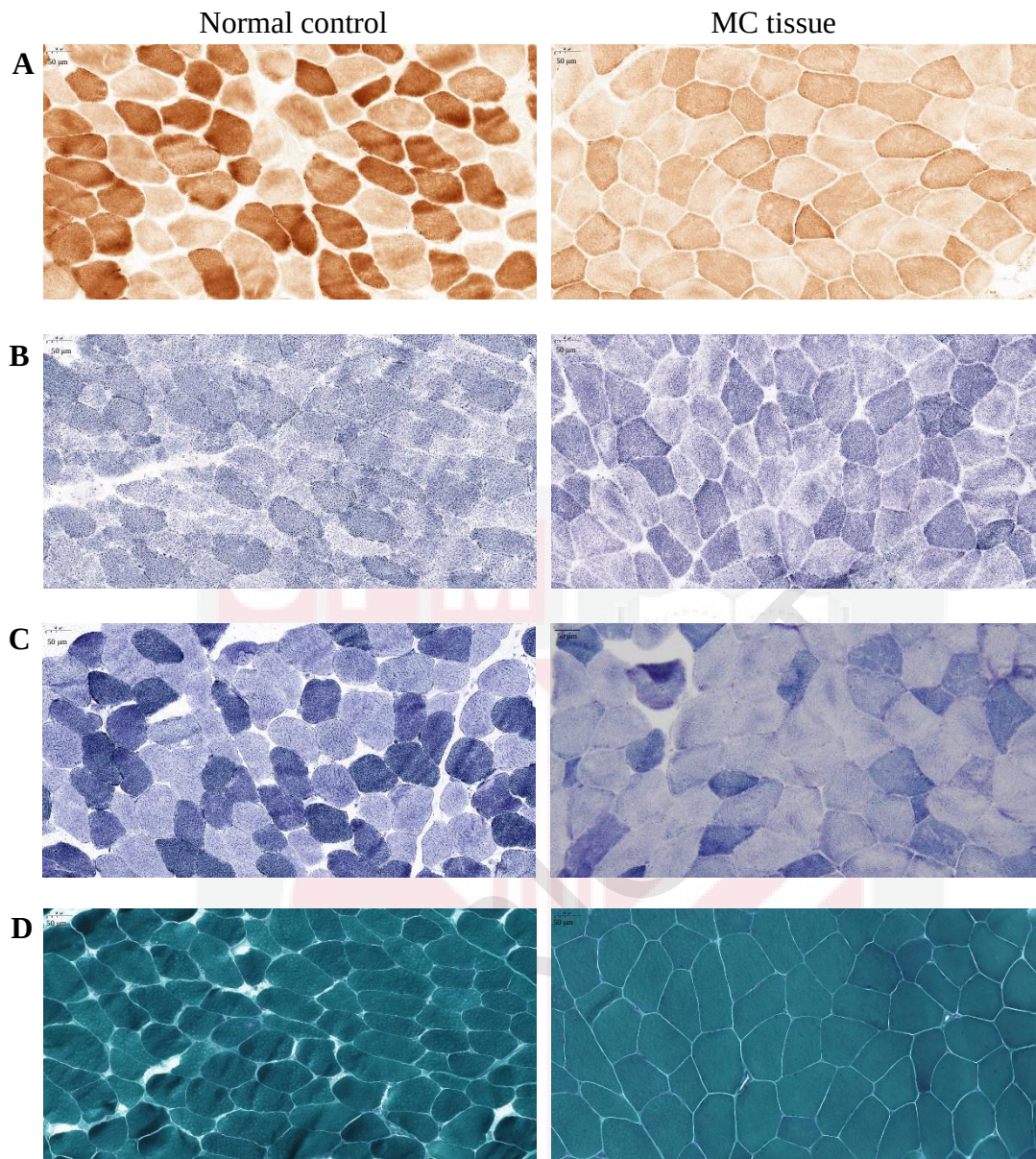


Figure 6.9: Cytochrome C oxidase (COX), Succinate dehydrogenase (SDH), Nicotinamide adenine dinucleotide (NADH), Modified Gomori Trichrome (MGT) staining of normal control (left) and MC tissue (right). No abnormal intracellular structures with normal myofibrillar organisation were observed in both MC patient and normal control for (A) COX (B) SDH, and (C) NADH staining, and (D) no special hyperchromatic structures were observed in myofibrillar cytoplasm with MGT staining.

6.2.3.4 Immunohistochemistry (IHC) Demonstrated Reduced ClC-1 Protein Expression in the Sarcolemma

Immunohistochemistry (IHC) was also conducted to visualise the presence of ClC-1 protein in the sarcolemma membrane. To enhance the staining, peroxidase was utilised, and chromogen was added in which the enzymatic reaction produces a visible, brown-coloured deposit at the site of the protein-antibody complex. Although the unstable cryostat temperature during sectioning procedure caused endomysial space between the normal control tissue fibres, the IHC staining displayed a brown line encircling the muscle fibres indicating the presence of ClC-1 protein in the sarcolemma (**Figure 6.10A**). In contrast, tissue from the MC patient revealed reduced ClC-1 protein expression in the sarcolemma which can be observed by the partial absence of the lining surrounding the muscle fibres (**Figure 6.10B**). The absence of the lining is more pronounced in the MC patient, which aligns with the Western blot analysis showing a downregulation trend of ClC-1 total protein expression (as discussed in section 6.3.1).

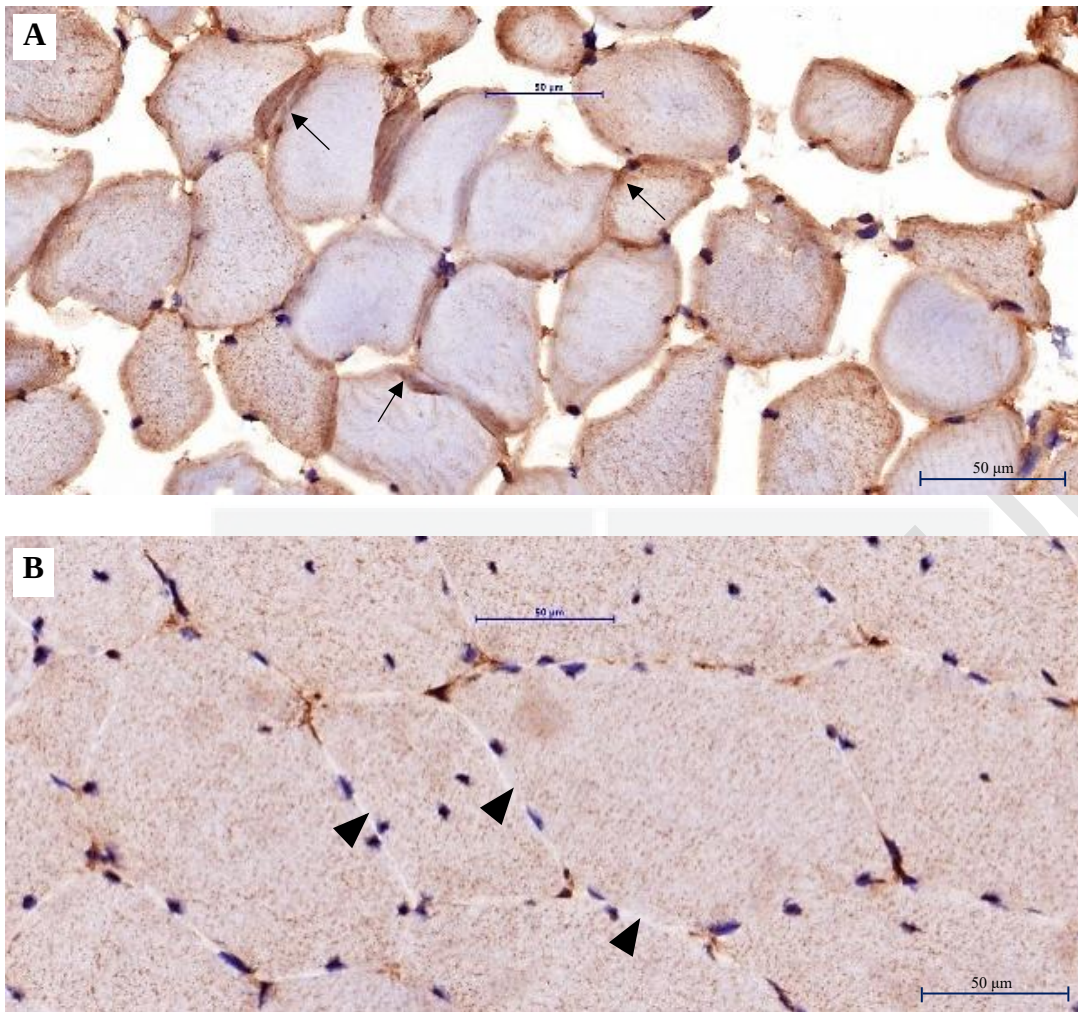


Figure 6.10: Immunohistochemical staining to detect localisation of CIC-1 protein on sarcolemma in normal control and MC tissue. (A) In normal control, CIC-1 protein can be seen as a brown lining (black arrow) surrounding the muscle fibres while (B) in MC tissue there is a partial absence of brown lining (arrowhead) indicating a reduced CIC-1 protein on the sarcolemma. Scale bar = 50 µm.

6.3.3 Electron Microscopy Showed Changes in MC Tissue

Ultrastructural analysis was conducted by examining the biceps muscle under a scanning electron microscope (SEM) and transmission electron microscope (TEM). The SEM micrograph of the muscle tissue depicted a normal striation with a visible A-I banding pattern, alongside a slightly rough surface showed fine wrinkles and well-organised parallel rows of paired openings corresponding to the level of the tubular

system (**Figure 6.11**). Due to the insufficient amount of tissue sample from the normal control, SEM was not able to be conducted for the normal control tissue as re-collection of tissue was not a viable option. However, the structure of tissue from the MC patient appears to be normal according to Sakuragawa et al. (1973) (**Appendix D9**).

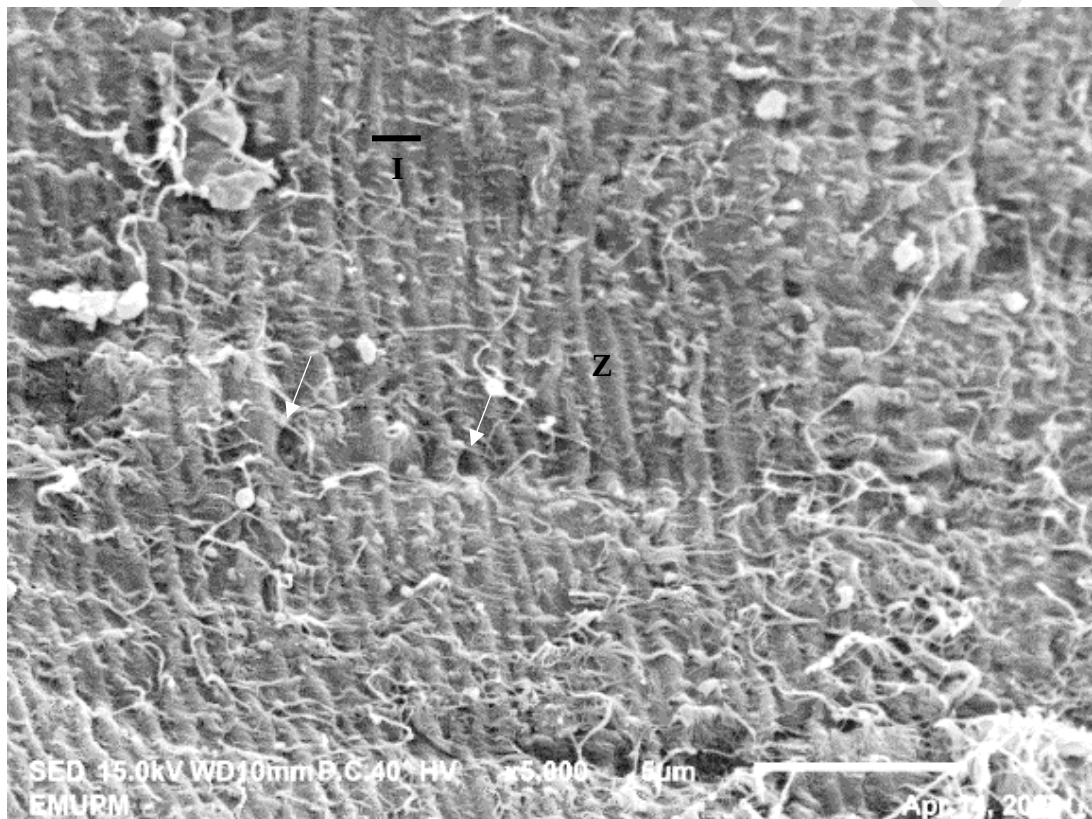


Figure 6.11: Photomicrograph of MC tissue from scanning electron microscope (SEM). The micrograph shows a normal striation with the repetitive A-I banding pattern. I: I band, Z: Z line, white arrow indicates parallel rows of opening corresponding to tubular system. Magnification 5,000 x, scale bar = 5 µm.

The TEM micrograph of normal muscle tissue revealed a longitudinal section of a skeletal muscle fibres showing the repetitive band pattern of the myofibrils, which are the contractile units. Each pale I band is bisected by a dense Z line, with mitochondria typically positioned in pairs on either side of the Z lines (**Figure 6.12**). The darker A

band is divided by a pale region or H zone (open arrow) and transected by the M line (white arrow).

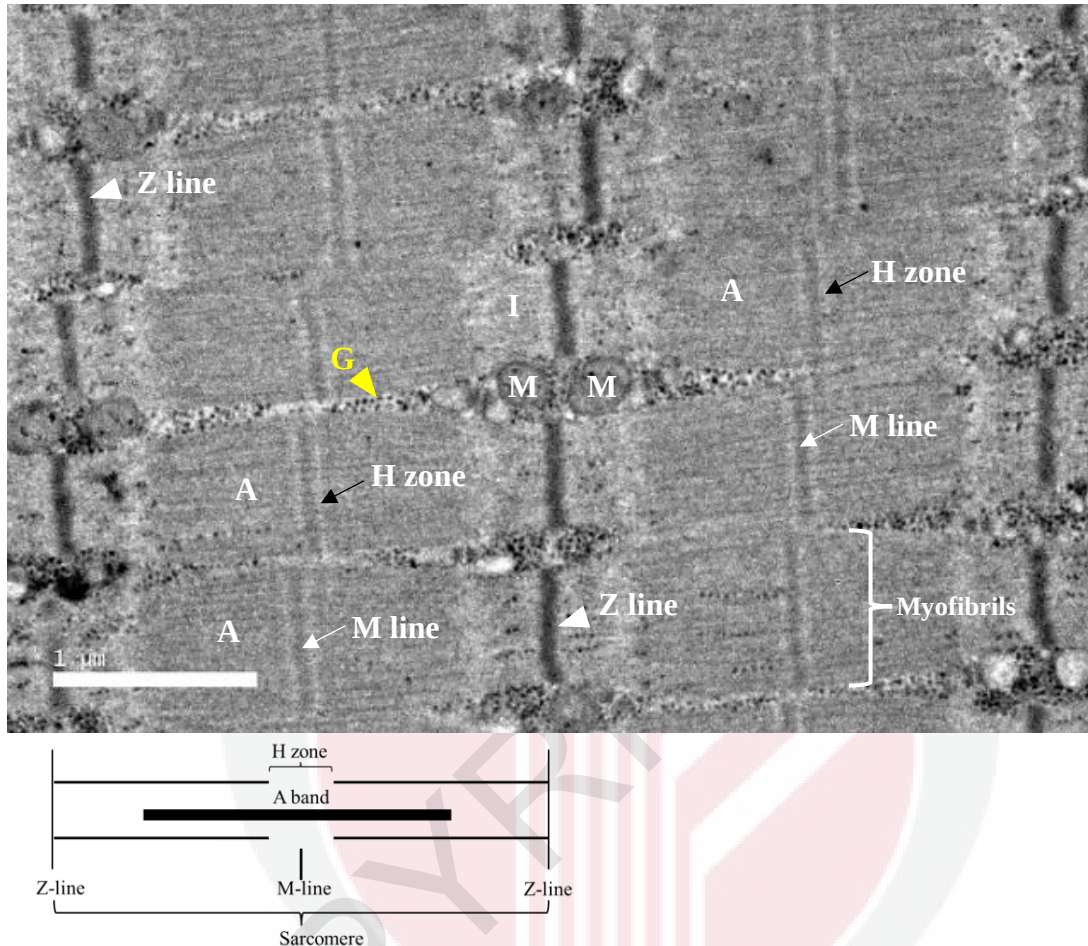


Figure 6.12: Micrograph of a normal biceps brachii muscle under the transmission electron microscope (TEM). The micrograph shows a repetitive banding pattern of myofibrils. The darkly staining Z line (white arrowhead) is composed of largely actin, that bisects the pale I band which consists of mainly fine actin filaments. The A bands are divided by a pale region, or H zone (black arrow), where myosin heads are absent, and this zone is transected by the M line (white arrow). Note the typical disposition of the round-shaped mitochondria (M) in pairs symmetrically disposed in relation to the Z lines. The small dense granules within the sarcoplasm are glycogen (G) particles. Tissue processed with uranyl acetate stains, magnification 5,000 x, scale bar = 1 μm.

In contrast, there was an irregular arrangement of the muscle fibres in the MC tissue showing splitting and narrowing myofibrils, expansion of the intermyofibrillar spaces

in some areas and a reduction of mitochondria at the junction of the A and I band (Figure 6.13). The I region was not visible due to fully contracted muscle fibres. Despite the irregular arrangement, the TEM micrograph showed normal Z lines.

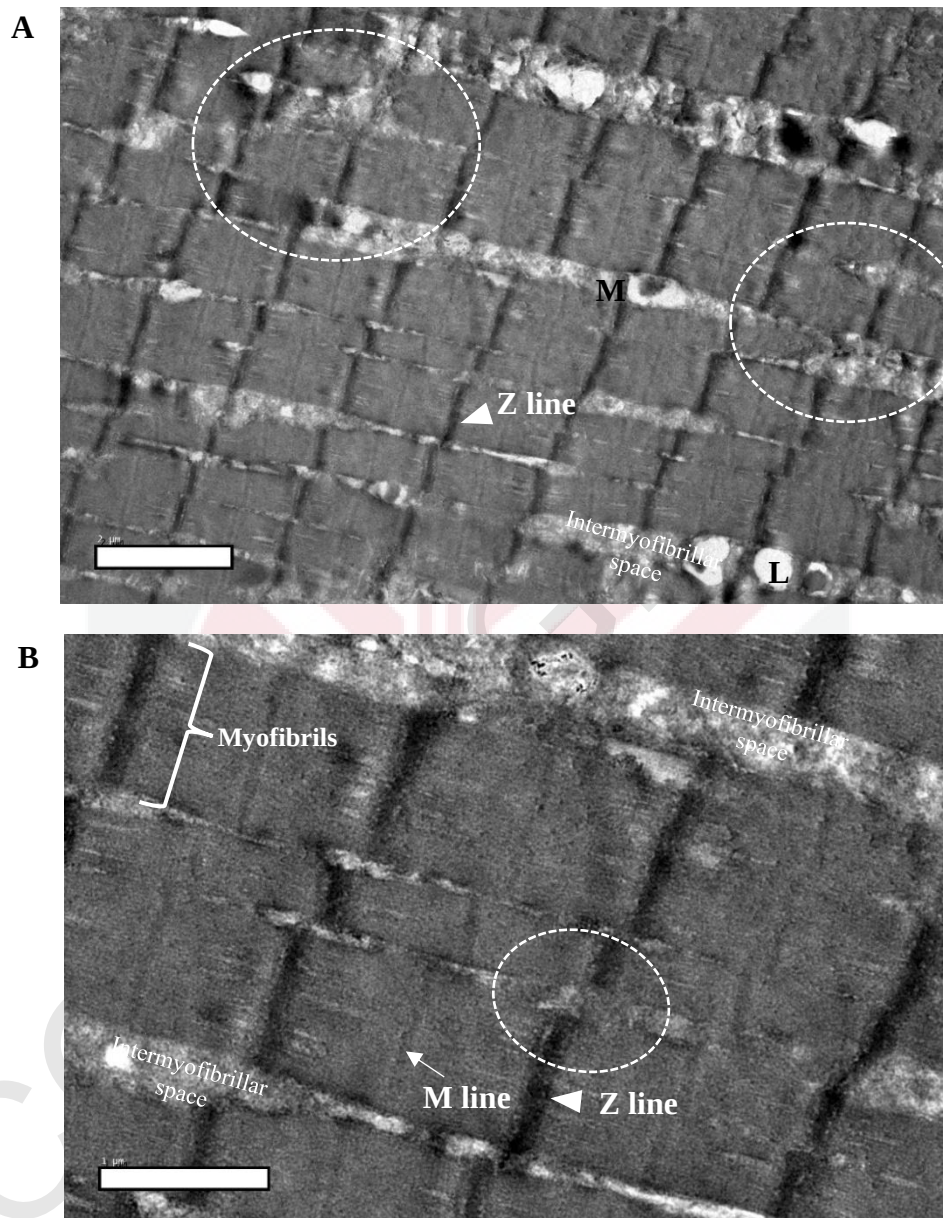


Figure 6.13: Micrograph of transmission electron microscopy (TEM) from biceps brachii muscle from MC patient (proband). The micrograph from the MC patient shows an irregular alignment of the muscle fibres with narrowing and splitting myofibrils (white dashed circle), expansion of intermyofibrillar spaces and a reduction of mitochondria at the junction of the A and I band. The I region was not visible due to fully contracted muscle fibre. A lipid droplet (L) can be seen in the intermyofibrillar space. Despite the irregular arrangement, the TEM micrograph shows normal Z lines (white arrowhead). H zone transected by the M line (white arrow). Tissue processed with uranyl acetate stains, magnification 2,000 x, scale bar = 2 μm (A) 5,000 x, scale bar = 1 μm (B).

6.4 Discussion

In this chapter, the c.1667T>A (p.Ile556Asn) variant was functionally characterised, and the result showed that the variant reduced the total protein expression level in the MC patient compared to the normal control. This finding was not consistent with a different *CLCN1* variant reported previously. Imbrici et al. (2016) conducted a study on both muscle tissue and transfected cells which revealed that the total amount of ClC-1 protein was similar in the control and the MC patient carrying p.T335N. This suggests the p.T335N variant is unlikely to affect the synthesis of the ClC-1 protein and that the gating dysfunction of mutant ClC-1 channels may be the main cause of reduced chloride conductance and consequent myotonia in the patient. In contrast to c.1667T>A (p.Ile556Asn) variant, which is positioned at the dimer interface, this variant is located in the extracellular loop connecting helices I and J. Alterations in the I-J loop could potentially disrupt intermonomeric interactions at the dimer interface, leading to improper channel gating (Imbrici et al., 2016).

Apart from the expression pattern using muscle tissue, a few other studies used transfected HEK293T cells to determine the effect of a variant on protein expression. Altamura et al., (2020) conducted a study using a HEK293T cells transfected with c.1231G>T (p.G411C) variant. No significant difference between the mutant and wild-type cells in the total protein expression was demonstrated, however reduced levels of the protein expressed at the surface membrane were reported. This result is consistent with another report by Richman et al. (2012), which also observed similar total protein expression between wild-type and mutant cells (HEK293T cells transfected with c.697G>A (p.Gly233Ser) variant) but noted a reduction in surface expression. These

findings suggest that the mutant protein has abnormal trafficking to the plasma membrane. It was found that the reduction in surface protein expression was due to the failure of the mutant protein to pass the endoplasmic reticulum (ER) quality control system and implicated defective membrane trafficking. This process is frequently impacted by disease-associated mutant ion channels (Lee et al., 2013).

This current study reported a differential ClC-1 protein expression, and no other studies have been conducted to examine the impact of the c.1667T>A (p.Ile556Asn) variant on protein expression. In addition to previous reports stating that surface membrane protein expression is influenced by membrane trafficking, this finding suggests another possible reason that might affect the level of ClC-1 protein expression. The previous molecular characterisation (Chapter 5) discussed a hypermethylation effect of the CpG site on *CLCN1* occurring in the enhancer region. Hypermethylation of the enhancer CpG island removes the enhancer signature, leading to downregulation of the target gene and consequently, reducing protein expression (Bae et al., 2016). Hypermethylation has been demonstrated in this study, where the MC patient exhibited high levels of DNA methylation (hypermethylation), which was correlated with reduced mRNA expression, eventually leading to reduced protein expression. Therefore, epigenetic modifications, such as hypermethylation of enhancer regions, can downregulate target genes and reduce protein expression, and could potentially explain the phenotypic variability of varying genotype-phenotype correlations. Unfortunately, this could not be confirmed with other family members as the parents did not consent to the biopsy of the children, while the other two being underage for the study.

Following the examination of the variant and its impact on protein expression, the next experimental focus was to conduct a thorough histopathological analysis of the affected tissues. While genetic testing offers valuable insights into the genetic factors involved in a disease, additional functional analysis is needed to understand the pathophysiology and investigate the effects on muscle tissue. Muscle biopsies provide the opportunity for directly assessing muscle structure, function, and pathology which enables clinicians to evaluate histological abnormalities. Histology knowledge of skeletal muscle facilitates understanding of the tissue, cellular, and molecular mechanisms involved in the adaptive response to the disease.

Previous pathological analyses of myotonia patients have shown mild, non-specific abnormalities (Engel and Brooke, 1966) whereas others have reported normal muscle conditions (Samaha et al., 1967). One significant finding of this study is the increased internal nuclei in the MC tissue following H&E staining. Muscle fibres in the MC tissue were reported to have an increase of internal nuclei with 33% which is elevated as compared to less than 5% in normal individuals. Healthy muscle only has 3 – 5% of its fibre-containing nuclei located internally, within the cells, but many diseases lead to an increase in internal nuclei (Exeter and Connell, 2010). This finding is consistent with previous reports for other variants of the *CLCN1* gene that have demonstrated an increase in the internal nuclei in MC patients (Meng et al., 2016; Portaro et al., 2015). Enrich Kuhn et al., (1979) also reported central nuclei in six dominant MC patients and all four recessive MC cases. In contrast, the MC patient was reported with a heterozygous p.T550M variant exhibited normal muscle fibres without internal nuclei (Hu et al., 2021b). Furthermore, central nucleated muscle fibres are indicative of newly regenerated muscle fibres during muscle regeneration (Wang

et al., 2017). Disruption of nuclei positioning results in hindered muscle contraction and occurs in a multitude of muscle disorders (Roman and Gomes, 2018).

Besides the position effect of the nuclei, MC tissue also showed muscle fibres size variability with increased muscle fibres over 100 μm or hypertrophic fibres. This finding was similar to a study by Portaro et al. (2015), which showed variability in muscle fibres size in the MC patient with a compound heterozygous variant (p.R453W and p.G190S variant) as well as a study by Hata et al. (2019) which reported similar significant fibres hypertrophy in half of the muscle fibres in myotonia patients. In contrast, Hu et al. (2021b) observed no changes in fibres size in a patient with a dominant c.1649C>T (p.T550M) variant. The hypertrophic fibres in the MC patient reflect the muscular appearance of the patient shown earlier in **Figure 3.4** (Chapter 3). Typically, the diameter of muscle fibres along with the presence of adipocytes and fibrotic areas are linked to muscle strength (Yue et al., 2016).

Upon staining the muscle fibres with ATPase under three distinct pH conditions, a mosaic pattern of different fibre types was revealed. Another notable finding from this study is the predominance of type 2 fibres. The predominance of muscle fibres type 2 for the biceps brachii muscle can be reported when the number is more than 77% (Cotta and Paim 2016; Dastgir et al. 2016).

The results from this study are similar to other studies which showed patients with the *CLCN1* gene variant commonly reported to have a predominance of type 2 fibres (Meng et al., 2016; Portaro et al., 2015; Lakraj et al., 2013; Ulzi et al., 2012). A study by Hata et al., (2019) reported the pathological findings of myotonia patients caused

by a variant of the sodium channel. *SCN4A* variants also showed a predominance of type 2A fibres with a reduction of type 2B fibres. Among the two muscle fibre types, type 2 fibres are considered fast-twitch and predominantly use glycogen for energy production which is responsible for short-duration, high-intensity activity, and quick bursts of energy. Conversely, type 1 fibres, known as slow-twitch fibres utilise oxidative metabolism and responsible for a long duration and low-intensity activity (Westerblad et al., 2010). Type 2 fibres can be further classified as type 2A which is less easily fatigued, and type 2B muscles which is easily fatigued (Polla et al., 2004). A study by Crews et al., (1976) demonstrated the abnormality in the proportion of muscle fibre types appears to be a universal finding in MC. His study demonstrated an absence of Type 2B fibres in all eight patients, and more than 55% of the fibres were Type 2A in 4 out of 7 MC tissues.

The condition of muscle fibres having difficulty relaxing after contraction, and the consequence of the predominance of type 2 fibres in MC patients can be explained as the neurophysiology adaptation. MC causes the stiffness of muscle and the preference for fast-twitch type 2 muscle fibres can be explained by the need for the muscle to relax rapidly. Fast-twitch fibres are designed for short bursts of intense contractions, and they can quickly return to their resting state compared to slow-twitch type 1 fibres, which are designed for sustained contractions (Schiaffino and Reggiani, 2011). In the context of myotonia, fast-twitch fibres are better suited to handle the impaired relaxation mechanism because they are naturally equipped for quick contractions followed by rapid relaxation.

Regardless of the specific variants in the causative channels, the muscle abnormalities associated with MC are characterised by moderate and nonspecific changes in muscle structure (Fisher et al., 1975). Several MC cases with mutated *CLCN1* (Portaro et al., 2015; Gurgel-Giannetti et al., 2012; Ulzi et al., 2012) usually showed a mild variation in fibres size and type 2 predominancy, along with a reduction or lack of type 2B fibres. Furthermore, all other staining (COX, SDH, NADH and MGT) showed normal enzymatic activities and mitochondrial and myofibrillar organisation. These findings are consistent with other studies that demonstrated normal enzymatic activity in the MC patient with a recessive splice-site variant (Miao et al., 2018) and a patient with a dominant p.T550M variant (Hu et al., 2021b).

Ultrastructural analysis using electron microscopy revealed a normal striation (SEM) and normal Z-line (TEM) in the MC patient despite irregular arrangements of the fibres, narrowing and splitting of myofibrils, and reduction of mitochondria. In diseases such as Duchenne muscular dystrophy which leads to the destruction of surface structures and muscle atrophy, SEM micrograph reported the A-I banding pattern disappears (Sakuragawa et al., 1973). TEM analysis on a recessive MC patient revealed an increased number of enlarged mitochondria, lipid droplets, glycogen granules, and remnants of myofibrils (Kuhn et al., 1979). A study by Samaha et al. (1967) on MC tissue disclosed no abnormalities in the patient, while another study by Hata et al., (2019) showed a variant on *SCN4A* exhibited Z-streaming.

Ultrastructural analyses are essential when the patient is suspected of congenital myopathies, inflammatory myopathies, myofibrillar myopathies, vacuolar myopathies, and diseases of neuromuscular junctions (Goebel et al., 2013). Although

electron microscopy investigation is rarely conducted on MC patients and other myotonic disorders and myotonic dystrophies, this current finding and some others (Hata et al., 2019) suggest that the variant on the *CLCN1* gene did change the structure of the muscle.

6.5 Conclusion

In conclusion, this study revealed a reduction in total protein expression of the MC patient carrying an incomplete penetrance of the heterozygous variant, c.1667T>A (p.Ile556Asn) in the *CLCN1* gene. The incomplete penetrance observed may be attributed to an epigenetic effect, as demonstrated by hypermethylation in the enhancer region that reduced gene expression and ultimately influenced the ClC-1 protein expression. Histopathological analysis demonstrated a mild histopathological change with increased internal nuclei, variability in fibres size, type 2 fibres predominance, narrowing and splitting of myofibrils, and reduction of mitochondria in the muscle of the MC patient that might influence the phenotypes.

CHAPTER 7

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

7.1 Research Summary and Conclusion

This study aimed to determine the clinical, molecular and histopathological analysis of myotonia congenita (MC) in a Malaysian family with a rare NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) variant in *CLCN1*. This study started with a clinical examination to confirm MC condition in a Malaysian family. Two siblings in the family of Sri Lankan ethnicity were affected with MC despite no other family members showed similar symptoms. They both exhibited the classic MC triad with myotonia, warm-up phenomenon, and muscular hypertrophy, with the proband had earlier onset, more severe symptoms, and higher CK values than her older brother, indicating phenotypic heterogeneity. Additionally, the MC patients exhibited a Fournier Pattern II on electrophysiology analysis, supporting the diagnosis of MC, and confirming the myotonia is caused by a *CLCN1* gene defect.

Further genetic analysis via WES identified a rare heterozygous variant in the *CLCN1* gene, c.1667T>A (p.Ile556Asn). The variant predicted to be deleterious and damaging with low allele frequency, and predominantly found in the South Asian population, making screening for this variant is important for South Asian patients. Furthermore, the location of the variant in the Q helices of the ClC-1 protein, which is at the dimer interface, suggests a dominant-negative effect on chloride channel function (Skalova et al., 2013). Segregation analysis confirmed the variant was present in the proband,

her affected brother, as well as the asymptomatic carriers' children, which suggests the incomplete penetrance of the autosomal dominant condition. Meanwhile, the ACMG classification revealed this variant as pathogenic.

Further molecular characterisation of the c.1667T>A (p.Ile556Asn) variant was performed to investigate the impact of the variant on *CLCN1* gene expression and epigenetic factors. Molecular analysis revealed the *CLCN1* gene expression was downregulated in the MC patient compared to a normal control, indicating that the variant may affect *CLCN1* expression. Although previous studies have reported no difference in the expression level of the *CLCN1* gene between MC patients and normal controls, other modifying factors are believed to influence this phenotypic variability (Portaro et al., 2014; Duno et al., 2004). Therefore, further epigenetic studies were performed with a specific focus on DNA methylation, which is a commonly studied mechanism for understanding gene regulation. This study is the first to examine the DNA methylation status of the *CLCN1* gene in an MC patient, and the results identified hypermethylation at the CpG sites, located in the *CLCN1* gene enhancer region. Hypermethylation in this region may suppress gene transcription, aligning with the observed decreased *CLCN1* expression. These findings suggest a potential epigenetic mechanism by which the variant c.1667T>A (p.Ile556Asn) influences the MC phenotype.

Functional analysis of the variant in the *CLCN1* gene showed a decreased in CLC-1 protein expression in the MC patient compared to the normal control. This finding suggested that hypermethylation of the *CLCN1* enhancer region in this patient may affect mRNA expression which has consequently contributed to the decreased of CLC-

1 protein levels observed in the MC patient. Histopathological analysis of MC tissue showed increased internal nuclei, increased muscle fibre size variability, type 2 fibre predominance and ultrastructural abnormalities such as splitting myofibrils and reduction of mitochondria.

Thus, the findings from this study provide valuable insights into MC and its underlying genetic and molecular mechanisms. These insights have the potential to benefit not only MC patients and their families but also clinicians, researchers, and the broader community in various impactful ways. For the MC patient, the findings highlight the potential risks and the importance of continuous monitoring of the asymptomatic children for any later onset, which improve the genetic counselling and disease management. It also raises awareness about the possibility of passing the genetic variant to future generations, helping families make informed decisions. It also serves as a guideline for the clinicians in diagnosing, genetic testing, and molecular evaluation of MC. This includes enhancing the role of genetic counselling in clinical practice. Furthermore, the findings also highlight the need for thorough preoperative history checks to prevent complications.

It can also encourage other researchers to further explore into methylation pattern and other epigenetic factors as contributors to phenotypic variability in MC. Such research could reveal additional mechanisms underlying disease variability and open new target for therapeutic interventions. Eventually it can also increase public awareness, and encourage individuals affected by MC, or those with a family history of the condition, to seek early diagnosis. Gathering more samples will provide statistical and prevalence data, which are critical for public health planning.

In conclusion, this study marks the first documented case of MC in Malaysia, showing that two affected siblings had varying symptoms, thereby highlighting the phenotypic variability within one family. By combining clinical, molecular, and epigenetic data the impact of the NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) variant was further elucidated and highlights the need to monitor asymptomatic carriers in the Southeast Asian population. The findings of this study have helped define the pathogenic role of the gene variant in MC and provide a valuable target for understanding disease mechanisms and therapy development.

7.2 Study Limitations and Recommendations for Future Research

There are several limitations in this study, and future research should focus on addressing these challenges. A key limitation of this study was the inability to recruit more MC patients, as many remain undiagnosed due to their ability to adapt to the condition without seeking medical attention. Future studies should aim to establish a multi-centre approach for MC. MC is a rare disorder, and NM_000083.3(*CLCN1*):c.1667T>A (p.Ile556Asn) variant is even rarer, therefore, multi-centre research studies that involve countries across the region can enhance the research scope. This will expand the study population and allow for a more thorough evaluation of clinical variability.

Another limitation is the inability to perform allelic expression analysis, as this requires muscle samples from normal heterozygous individuals, specifically the asymptomatic children, and consent for the procedure could not be obtained. Thus, the chances of identifying more asymptomatic carriers are higher through a multi-centre

study, making allelic expression pattern analysis more feasible. Future research could then include this analysis to better understand the incomplete penetrance associated with the variant.

Besides that, WES was conducted only on the proband due to financial constraints. In future studies, WES could be performed on the proband's brother and their asymptomatic children, followed by a merged analysis to expand the search for other potential candidate genes or genetic modifiers. Additionally, whole-genome sequencing (WGS) can be included in future research to provide a more comprehensive genetic analysis. WGS offers a thorough examination of the entire genome, including coding and non-coding regions, which can help identify rare variants, structural variations, and regulatory elements that might contribute to incomplete penetrance and complex genotype-phenotype correlations. Incorporating WGS can significantly enhance the understanding of the genetic factors underlying the MC condition.

Currently, there is no treatment for MC. Future research should focus on finding effective treatments for the disease or ways to alleviate the symptoms. Exploring personalised therapeutic approaches for MC could significantly advance treatment options. One of the strategies involves repurposing of drugs that can directly modulate chloride channel activity, particularly those targeting ClC-1 and other channel such as calcium channels, which could reveal their potential to indirectly modulate chloride channel functions. Furthermore, evaluating epigenetic modifying agents such as DNA methyltransferase inhibitors or histone deacetylase inhibitors presents another

potential therapeutic strategy. These can be achieved through bioinformatics and animal model studies.

More advanced therapeutic approaches include gene-editing strategies such as clustered regularly interspaced short-palindromic repeat-associated protein 9 (CRISPR-Cas9) and viral-mediated gene therapy. CRISPR-Cas provides a powerful tool for correcting pathogenic variant at the DNA level. In MC, which is caused by *CLCN1* variants, CRISPR-Cas9 can repair defective sequences, and eventually restoring chloride channel function. Adeno-associated virus (AAV) vectors offer a potential approach for delivering functional *CLCN1* gene copies or regulatory elements to muscle cells. This strategy, used in other neuromuscular disorders, could help restore chloride channel function in MC. These advancements highlight CRISPR's potential to correct genetic defects, offering hope for treating neuromuscular diseases caused by specific mutations.

Collectively, these research directions could enhance the understanding and management of MC and potentially lead to the development of effective treatments and improved patient outcomes.

REFERENCES

- Abolhasanzadeh, N., Sarabandi, S., Dehghan, B., Karamad, V., Avci, C. B., Shademan, B., & Nourazarian, A. (2024). Exploring the intricate relationship between miRNA dysregulation and breast cancer development: insights into the impact of environmental chemicals. *Frontiers in Immunology*, 15, 1333563.
- Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A.S., & Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. *Nature Methods*, 7(4), 248-249.
- Aguet, F., Brown, A. A., Castel, S. E., Davis, J. R., Mohammadi, P., Segre, A. V., A.V., Zappala, Z., Abell, N.S., Fresard, L., Gamazon, E.R., Gelfand, E., & Montgomery, S. B. (2016). Local genetic effects on gene expression across 44 human tissues. *BioRxiv*, 074450.
- Alirezaie, N., Kernohan, K. D., Hartley, T., Majewski, J., & Hocking, T. D. (2018). ClinPred: prediction tool to identify disease-relevant nonsynonymous single-nucleotide variants. *The American Journal of Human Genetics*, 103(4), 474-483.
- Al-Shuhaib, M. B. S., & Hashim, H. O. (2023). Mastering DNA chromatogram analysis in Sanger sequencing for reliable clinical analysis. *Journal of Genetic Engineering and Biotechnology*, 21(1), 115.
- Altamura, C., Desaphy, J. F., Conte, D., De Luca, A., & Imbrici, P. (2020). Skeletal muscle CLC-1 chloride channels in health and diseases. *Pflügers Archiv-European Journal of Physiology*, 472, 961-975.
- Arzel-hézode, M., Sternberg, D., Tabti, N., Vicart, S., Goizet, C., Eymard, B., Fontaine, B., & Fournier, E. (2010). Homozygosity for dominant mutations increases severity of muscle channelopathies. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 41(4), 470-477.
- Bae, M. G., Kim, J. Y., & Choi, J. K. (2016). Frequent hypermethylation of orphan CpG islands with enhancer activity in cancer. *BMC Medical Genomics*, 9, 89-97.
- Bamshad, M. J., Ng, S. B., Bigham, A. W., Tabor, H. K., Emond, M. J., Nickerson, D. A., & Shendure, J. (2011). Exome sequencing as a tool for Mendelian disease gene discovery. *Nature Reviews Genetics*, 12(11), 745-755.
- Bandschapp, O., & Iaizzo, P. A. (2013). Pathophysiologic and anesthetic considerations for patients with myotonia congenita or periodic paralyses. *Pediatric Anesthesia*, 23(9), 824-833.
- Barchi, R. L. (1975). Myotonia: an evaluation of the chloride hypothesis. *Archives of Neurology*, 32(3), 175-180.

- Baribault, C., Ehrlich, K. C., Ponnaluri, V. C., Pradhan, S., Lacey, M., & Ehrlich, M. (2018). Developmentally linked human DNA hypermethylation is associated with down-modulation, repression, and upregulation of transcription. *Epigenetics*, 13(3), 275-289.
- Barral, A., & Déjardin, J. (2023). The chromatin signatures of enhancers and their dynamic regulation. *Nucleus*, 14(1), 2160551.
- Becker PE, Knussmann R, Kuhn E, Passarge MF (1977). Myotonia congenita and syndromes associated with myotonia. *Topics in Human Genetics* 3. Stuttgart: Georg Thieme; 1–178.
- Benevenuta, S., Birolo, G., Sanavia, T., Capriotti, E., & Fariselli, P. (2023). Challenges in predicting stabilizing variations: An exploration. *Frontiers in Molecular Biosciences*, 9, 1075570.
- Bennett, R. L., French, K. S., Resta, R. G., & Doyle, D. L. (2008). Standardized human pedigree nomenclature: update and assessment of the recommendations of the National Society of Genetic Counselors. *Journal of Genetic Counseling*, 17, 424-433.
- Bennett, S. A., Tanaz, R., Cobos, S. N., & Torrente, M. P. (2019). Epigenetics in amyotrophic lateral sclerosis: a role for histone post-translational modifications in neurodegenerative disease. *Translational Research*, 204, 19-30.
- Berger, S. L., Kouzarides, T., Shiekhata, R., & Shilatifard, A. (2009). An operational definition of epigenetics. *Genes & Development*, 23(7), 781-783.
- Botstein, D., & Risch, N. (2003). Discovering genotypes underlying human phenotypes: past successes for Mendelian disease, future approaches for complex disease. *Nature Genetics*, 33(3), 228-237.
- Boycott, K. M., Vanstone, M. R., Bulman, D. E., & MacKenzie, A. E. (2013). Rare-disease genetics in the era of next-generation sequencing: discovery to translation. *Nature Reviews Genetics*, 14(10), 681-691.
- Brugnoni, R., Galantini, S., Confalonieri, P., Balestrini, M. R., Cornelio, F., & Mantegazza, R. (1999). Identification of three novel mutations in the major human skeletal muscle chloride channel gene (*CLCN1*), causing myotonia congenita. *Human Mutation*, 14(5), 447-447.
- Brugnoni, R., Kapetis, D., Imbrici, P., Pessia, M., Canioni, E., Colleoni, L., de Rosbo, N. K., Morandi, L., Cudia, P., Gashemi, N., Bernasconi, P., Desaphy, J. F., Conte, D., & Mantegazza, R. (2013). A large cohort of myotonia congenita probands: novel mutations and a high-frequency mutation region in exons 4 and 5 of the *CLCN1* gene. *Journal of Human Genetics* 58 (9), 581 – 587.
- Brugnoni, R., Maggi, L., Canioni, E., Verde, F., Gallone, A., Ariatti, A., Filosto, M., Petrelli, C., Logullo, F.O., Esposito, M., & Ruggiero, L. (2021). Next-generation sequencing application to investigate skeletal muscle

- channelopathies in a large cohort of Italian patients. *Neuromuscular Disorders*, 31(4), 336-347.
- Brugnoni, R., Modoni, A., Canioni, E., Imbrici, P., Tincheva, S., Todorova, A., Maggi L., & Monaco, M. L. (2022). From clinical to molecular diagnosis: relevance of the MLPA in one case of recessive myotonia congenita – case report. Research Square.
- Buckley, L., Lacey, M., & Ehrlich, M. (2016). Epigenetics of the myotonic dystrophy-associated DMPK gene neighborhood. *Epigenomics*, 8(1), 13-31.
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. and Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. 611 – 622.
- Cain, D. F., Infante, A. A., & Davies, R. E. (1962). Chemistry of muscle contraction: adenosine triphosphate and phosphorylcreatine as energy supplies for single contractions of working muscle. *Nature*, 196(4851), 214-217.
- Cannon, S. C. (2015). Channelopathies of skeletal muscle excitability. *Comprehensive Physiology*, 5(2), 761.
- Cerrato, F., Sparago, A., Ariani, F., Brugnoletti, F., Calzari, L., Coppedè, F., De Luca, A., Gervasini, C., Giardina, E., Gurrieri, F., Lo Nigro, C., Merla, G., Miozzo, M., Russo, S., Sangiorgi, E., Sirchia, S. M., Squeo, G. M., Tabano, S., Tabolacci, E., et al. (2020). DNA methylation in the diagnosis of monogenic diseases. *Genes*, 11(4), 355.
- Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F.L., & Wang, D. Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nature Genetics*, 38(2), 228-233.
- Chen, T. Y. (2005). Structure and function of CLC channels. *Annual Review of Physiology*, 67, 809-839.
- Cho, Y., Lee, C.H., Jeong, E.G., Kim, M.H., Hong, J.H., Ko, Y., Lee, B., Yun, G., Kim, B.J., Jung, J., & Jung, J., (2017). Prevalence of rare genetic variations and their implications in NGS-data interpretation. *Scientific Reports*, 7(1), 9810.
- Choi, M., Scholl, U.I., Ji, W., Liu, T., Tikhonova, I.R., Zumbo, P., Nayir, A., Bakkaloğlu, A., Özen, S., Sanjad, S., & Nelson-Williams, C., (2009). Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences*, 106(45), 19096-19101.
- Choi, Y., & Chan, A. P. (2015). PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics*, 31(16), 2745-2747.

- Chrestian, N., Puymirat, J., Bouchard, J. P., & Dupré, N. (2006). Myotonia congenita—a cause of muscle weakness and stiffness. *Nature Clinical Practice Neurology*, 2(7), 393-399.
- Colding-Jørgensen, E. (2005). Phenotypic variability in myotonia congenita. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 32(1), 19-34.
- Colding-Jørgensen, E., DunØ, M., Schwartz, M., & Vissing, J. (2003). Decrement of compound muscle action potential is related to mutation type in myotonia congenita. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 27(4), 449-455.
- Conte, E., Fonzino, A., Cibelli, A., De Benedictis, V., Imbrici, P., Nicchia, G. P., Pierno, S., & Camerino, G. M. (2020). Changes in expression and cellular localization of rat skeletal muscle ClC-1 chloride channel in relation to age, myofiber phenotype and PKC modulation. *Frontiers in Pharmacology*, 11, 714.
- Cotta, A., & Paim, J. F. (2016). Muscle biopsy reporting. *Archives of Pathology & Laboratory Medicine*, 140(9), 879-879.
- Cotta, A., Carvalho, E., da-Cunha-Júnior, A. L., Valicek, J., Navarro, M. M., Junior, S. B., da Silveira, E.B., Lima, M.I., Cordeiro, B.A., Cauhi, A.F. and Menezes, M.M., & Paim, J. F. (2021). Muscle biopsy essential diagnostic advice for pathologists. *Surgical and Experimental Pathology*, 4, 1-20.
- Crews, J., Kaiser, K. K., & Brooke, M. H. (1976). Muscle pathology of myotonia congenita. *Journal of the Neurological Sciences*, 28(4), 449-457.
- Dastgir, J., Rutkowski, A., Alvarez, R., Cossette, S. A., Yan, K., Hoffmann, R. G., Sewry, C., Hayashi, Y.K., Goebel, H.H., Bonnemann, C., & Lawlor, M. W. (2016). Common data elements for muscle biopsy reporting. *Archives of Pathology & Laboratory Medicine*, 140(1), 51-65.
- Davies, N. P., & Hanna, M. G. (1999). Neurological channelopathies: diagnosis and therapy in the new millennium. *Annals of Medicine*, 31(6), 406-420.
- Davydov, E. V., Goode, D. L., Sirota, M., Cooper, G. M., Sidow, A., & Batzoglou, S. (2010). Identifying a high fraction of the human genome to be under selective constraint using GERP++. *PLoS Computational Biology*, 6(12), e1001025.
- De Diego, C., Gamez, J., Plassart-Schiess, E., Lasa, A., Del Rio, E., Cervera, C., Baiget, M., Gallano, P., & Fontaine, B. (1999). Novel mutations in the muscle chloride channel *CLCN1* gene causing myotonia congenita in Spanish families. *Journal of Neurology*, 246, 825-829.
- Deaton, A. M., & Bird, A. (2011). CpG islands and the regulation of transcription. *Genes & Development*, 25(10), 1010-1022.
- Deymeer, F., Çakirkaya, S., Serdaroğlu, P., Schleithoff, L., Lehmann-Horn, F., Rüdél, R., & Özdemir, C. (1998). Transient weakness and compound muscle action

- potential decrement in myotonia congenita. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 21(10), 1334-1337.
- Dubowitz, V., Sewry, C. A., & Oldfors, A. (2013). *Muscle biopsy: a practical approach: expert consult; online and print*. Elsevier Health Sciences.
- Duffield, M., Rychkov, G., Bretag, A., & Roberts, M. (2003). Involvement of helices at the dimer interface in ClC-1 common gating. *The Journal of General Physiology*, 121(2), 149-161.
- Duno, M., & Vissing, J. (2021). Myotonia congenita (Vol. Seattle (WA): University of Washington, Seattle; 1993-2022.
- Dunø, M., Colding-Jørgensen, E., Grønnet, M., Jespersen, T., Vissing, J., & Schwartz, M. (2004). Difference in allelic expression of the *CLCN1* gene and the possible influence on the myotonia congenita phenotype. *European Journal of Human Genetics*, 12(9), 738-743.
- Dupré, N., Chrestian, N., Bouchard, J. P., Rossignol, E., Brunet, D., Sternberg, D., Brais, B., Mathieu, J., & Puymirat, J. (2009). Clinical, electrophysiologic, and genetic study of non-dystrophic myotonia in French-Canadians. *Neuromuscular Disorders*, 19(5), 330-334.
- Duran, C., Thompson, C. H., Xiao, Q., & Hartzell, H. C. (2010). Chloride channels: often enigmatic, rarely predictable. *Annual Review of Physiology*, 72(1), 95-121.
- Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., & MacKinnon, R. (2002). X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature*, 415(6869), 287-294.
- Elaraby, N. M., Ahmed, H. A., Dawoud, H., Ashaat, N. A., Azmy, A., Galal, E. R., Elhussen, Y., Awady, H.E., Metwally, A.M., & Ashaat, E. A. (2024). Clinical and molecular characterization of myotonia congenita using whole-exome sequencing in Egyptian patients. *Molecular Biology Reports*, 51(1), 766.
- Emery, A. E. (1991). Population frequencies of inherited neuromuscular diseases—a world survey. *Neuromuscular Disorders*, 1(1), 19-29.
- Engel, W. K., & Brooke, M. H. (1966). Histochemistry of the myotonic disorders. In *Progressive Muskeldystrophie Myotonie· Myasthenie: Symposium vom 30. November bis 4. Dezember 1965 anlässlich der 125. Wiederkehr des Geburtstages von Wilhelm Erb* (pp. 203-222). Springer Berlin Heidelberg.
- Enkvetchakul, D. (2010). Genetic disorders of ion channels. *Missouri Medicine*, 107(4), 270.
- Erich Kuhn, S., Fiehn, W., Seiler, D., & Schröder, J. M. (1979). The autosomal recessive (Becker) form of myotonia congenita. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 2(2), 109-117.

- Exeter, D., & Connell, D. A. (2010, June). Skeletal muscle: functional anatomy and pathophysiology. In *Seminars in Musculoskeletal Radiology* (Vol. 14, No. 02, pp. 097-105). © Thieme Medical Publishers.
- Farbu, E., Sjøfteland, E., & Bindoff, L. A. (2003). Anaesthetic complications associated with myotonia congenita: case study and comparison with other myotonic disorders. *Acta Anaesthesiologica Scandinavica*, 47(5), 630-634.
- Farsetti, A., Illi, B., & Gaetano, C. (2023). How epigenetics impacts on human diseases. *European Journal of Internal Medicine*, 114, 15-22.
- Fedoriw, A., Mugford, J., & Magnuson, T. (2012). Genomic imprinting and epigenetic control of development. *Cold Spring Harbor Perspectives in Biology*, 4(7), a008136.
- Feng, W., Shen, L., Wen, S., Rosen, D. G., Jelinek, J., Hu, X., Huan, S., Huang, M., Liu, J., Sahin, A.A. and Hunt, K.K., & Yu, Y. (2007). Correlation between CpG methylation profiles and hormone receptor status in breast cancers. *Breast Cancer Research*, 9, 1-13.
- Fernandez, C., Figarella-Branger, D., Meyronet, D., Cassote, E., Tong, S., & Pellissier, J. F. (2005). Electron microscopy in neuromuscular disorders. *Ultrastructural Pathology*, 29(6), 437-450.
- Ferradini, V., Cassone, M., Nuovo, S., Bagni, I., D'Apice, M. R., Botta, A., Novelli, G., & Sangiulio, F. (2017). Targeted next generation sequencing in patients with myotonia congenita. *Clinica Chimica Acta*, 470, 1-7.
- Fialho, D., Schorge, S., Pucovska, U., Davies, N. P., Labrum, R., Haworth, A., Stanley, E., Sud, R., Wakeling, W., Davis, M.B., Kullmann, D. M., & Hanna, M. G. (2007). Chloride channel myotonia: exon 8 hot-spot for dominant-negative interactions. *Brain*, 130(12), 3265-3274.
- Fisher, E. R., Danowski, T. S., Ahmad, U., Breslau, P., Nolan, S., & Stephan, T. (1975). Electron microscopical study of a family with myotonia congenita. *Archives of Pathology*, 99(11), 607-610.
- Fournier, E., Arzel, M., Sternberg, D., Vicart, S., Laforet, P., Eymard, B., Willer, J.C., Tabti, N., & Fontaine, B. (2004). Electromyography guides toward subgroups of mutations in muscle channelopathies. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, 56(5), 650-661.
- Fournier, E., Viala, K., Gervais, H., Sternberg, D., Arzel-Hézode, M., Laforêt, P., Eymard, B., Tabti, N., Willer, J.C., Vial, C., & Fontaine, B. (2006). Cold extends electromyography distinction between ion channel mutations causing myotonia. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, 60(3), 356-365.
- Furman, R. E., & Barchi, R. L. (1978). The pathophysiology of myotonia produced by aromatic carboxylic acids. *Annals of Neurology: Official Journal of the*

American Neurological Association and the Child Neurology Society, 4(4), 357-365.

Gao, F., Ma, F. C., Yuan, Z. F., Yang, C. W., Li, H. F., Xia, Z. Z., Shui, Q.X. & Jiang, K. W. (2010). Novel chloride channel gene mutations in two unrelated Chinese families with myotonia congenita. *Neurology India*, 58(5), 743-746.

George Jr, A. L., Crackower, M. A., Abdalla, J. A., Hudson, A. J., & Ebers, G. C. (1993). Molecular basis of Thomsen's disease (autosomal dominant myotonia congenita). *Nature Genetics*, 3(4), 305-310.

Ghosh, R., Oak, N., & Plon, S. E. (2017). Evaluation of in silico algorithms for use with ACMG/AMP clinical variant interpretation guidelines. *Genome Biology*, 18, 1-12.

Gilitwala, Z., Satpute, S., & Patil, S. (2023). A Detailed Clinical Approach to Non-dystrophic Myotonia: A Case Report of Two Brothers With Myotonia Congenita. *Cureus*, 15(6).

Glazier, A. A., Thompson, A., & Day, S. M. (2019). Allelic imbalance and haploinsufficiency in MYBPC3-linked hypertrophic cardiomyopathy. *Pflügers Archiv-European Journal of Physiology*, 471, 781-793.

Goebel, H. H., & Stenzel, W. (2013). Practical application of electron microscopy to neuromuscular diseases. *Ultrastructural Pathology*, 37(1), 15-18.

Grada, A., & Weinbrecht, K. (2013). Next-generation sequencing: methodology and application. *Journal of Investigative Dermatology*, 133(8), 1-4.

Grunnet, M., Jespersen, T., Colding-Jørgensen, E., Schwartz, M., Klaerke, D. A., Vissing, J., ... & Dunø, M. (2003). Characterization of two new dominant ClC-1 channel mutations associated with myotonia. *Muscle & Nerve*, 28(6), 722-732.

Gurgel-Giannetti, J., Senkevics, A. S., Zilbersztajn-Gotlieb, D., Yamamoto, L. U., Muniz, V. P., Pavanello, R. C., Oliveira, A.B., Zatz, M., & Vainzof, M. (2012). Thomsen or Becker myotonia? A novel autosomal recessive nonsense mutation in the *CLCN1* gene associated with a mild phenotype. *Muscle & Nerve*, 45(2), 279-283.

Hasan, S. S. (2021). Anesthetic management of a patient with myotonia congenita. *Anaesthesia, Pain & Intensive Care*, 25(6), 816-818.

Hata, T., Nagasaka, T., Koh, K., Tsuchiya, M., Ichinose, Y., Nan, H., Shindo, K. & Takiyama, Y. (2019). Pathological findings in a patient with non-dystrophic myotonia with a mutation of the *SCN4A* gene; a case report. *BMC Neurology*, 19, 1-6.

He, Y., Qiu, Y., Xiong, Y., Shen, Y., Jiang, K., Yi, H., Huang, P., Zhu, Y., Zhu, M., Zhou, M., Hong, D. & Tan, D. (2024). Clinical and genetic characteristics of myotonia congenita in Chinese population. *Channels*, 18(1), 2349823.

- Heatwole, C. R., Moxley, R. T. (2007). The non-dystrophic myotonias. *Neurotherapeutics*, 4, 238 – 251.
- Heatwole, C. R., Statland, J. M., & Logigian, E. L. (2013). The diagnosis and treatment of myotonic disorders. *Muscle & Nerve*, 47(5), 632-648.
- Hehir, M. K., & Logigian, E. L. (2012). Electrodiagnosis of myotonic disorders. *Physical Medicine and Rehabilitation Clinics of North America*, 24(1), 209-220.
- Heiman-Patterson, T., Martino, C., Rosenberg, H., Fletcher, J., & Tahmouh, A. (1988). Malignant hyperthermia in myotonia congenita. *Neurology*, 38(5), 810-810.
- Hesson, L. B., Packham, D., Kwok, C. T., Nunez, A. C., Ng, B., Schmidt, C., Fields, M., Wong, J.W., Sloane, M.A., & Ward, R. L. (2015). Lynch syndrome associated with two MLH1 promoter variants and allelic imbalance of MLH1 expression. *Human Mutation*, 36(6), 622-630.
- Hille, B. (2002). Ion channels and the electrical properties of membranes. In Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (Eds.), *Molecular Biology of the Cell* (4th ed., pp. 293-323). Garland Science.
- Horga, A., Raja Rayan, D. L., Matthews, E., Sud, R., Fialho, D., Durran, S. C., Burge, J. A., Portaro, S., Davis, M. B., Haworth, A., & Hanna, M. G. (2013). Prevalence study of genetically defined skeletal muscle channelopathies in England. *Neurology*, 80 (16), 1472 – 1475.
- Hu, C., Shi, Y., Zhao, L., Zhou, S., & Li, X. (2021b). Myotonia congenita: clinical characteristic and mutation spectrum of *CLCN1* in Chinese patients. *Frontiers in Pediatrics*, 9, 759505.
- Hu, T., Chitnis, N., Monos, D., & Dinh, A. (2021a). Next-generation sequencing technologies: An overview. *Human Immunology*, 82(11), 801-811.
- Huynh, W., & Kiernan, M. C. (2011). Nerve conduction studies. *Australian family Physician*, 40(9), 693-697.
- Illingworth, R., Kerr, A., DeSousa, D., Jørgensen, H., Ellis, P., Stalker, J., Jackson, D., Clee, C., Plumb, R., Rogers, J. and Humphray, S., Cox, T., Langford, C., & Bird, A. (2008). A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS Biology*, 6(1), e22.
- Imbrici, P., Altamura, C., Camerino, G. M., Mangiatordi, G. F., Conte, E., Maggi, L., Brugnioni, R., Musaraj, K., Caloiero, R., Alberga, D., Marsano, R.M., Ricci, G., Siciliano, G., Nicolotti, O., Mora, M., Bernasconi, P., Desaphy, J. F., Mantegazza, R., & Camerino, D. C. (2016). Multidisciplinary study of a new CLC-1 mutation causing myotonia congenita: a paradigm to understand and treat ion channelopathies. *The FASEB Journal*, 30(10), 3285.

- Imbrici, P., Altamura, C., Pessia, M., Mantegazza, R., Desaphy, J. F., & Camerino, D. C. (2015b). ClC-1 chloride channels: state-of-the-art research and future challenges. *Frontiers in Cellular Neuroscience*, 9, 156.
- Imbrici, P., Maggi, L., Mangiatordi, G. F., Dinardo, M. M., Altamura, C., Brugnoli, R., Alberga, D., Pinter, G.L., Ricci, G., Siciliano, G., Micheli, R., Annicchiarico, G., Lattanzi, G., Nicolotti, O., Morandi, L., Bernasconi, P., Desaphy, J. F., Mantegazza, R., & Camerino, D. C. (2015a). ClC-1 mutations in myotonia congenita patients: insights into molecular gating mechanisms and genotype–phenotype correlation. *The Journal of Physiology*, 593(18), 4181–4199.
- Ioannidis, N. M., Rothstein, J. H., Pejaver, V., Middha, S., McDonnell, S. K., Baheti, S., Musolf, A., Li, Q., Holzinger, E., Karyadi, D., Cannon-Albright, L.A., Teerlink, C. C., Stanford, J. L., Isaacs, W. B., Xu, J., Cooney, K. A., Lange, E.M., Schleutker J, Carpten JD, Powell IJ, Cussenot O, Cancel-Tassin G, Giles G. G., MacInnis, R. J., Maier, C., Hsieh, C. L., Wiklund, F., Catalona, W. J., Foulkes, W. D., Mandal, D., Eeles, R. A., Kote-Jarai, Z., Bustamante, C. D., Schaid, D. J., Hastie, T., Ostrander, E. A., Bailey-Wilson, J. E., Radivojac, P., Thibodeau, S. N., Whittemore, A.S., & Sieh, W. (2016). REVEL: an ensemble method for predicting the pathogenicity of rare missense variants. *The American Journal of Human Genetics*, 99(4), 877–885.
- Jackson, C. E., & Barohn, R. J. (2013). A pattern recognition approach to myopathy. *CONTINUUM: Lifelong Learning in Neurology*, 19(6), 1674–1697.
- Jeng, C. J., Fu, S. J., You, C. Y., Peng, Y. J., Hsiao, C. T., Chen, T. Y., & Tang, C. Y. (2020). Defective gating and proteostasis of human ClC-1 chloride channel: molecular pathophysiology of myotonia congenita. *Frontiers in Neurology*, 11, 76.
- Jentsch, T. J., & Pusch, M. (2018). CLC chloride channels and transporters: structure, function, physiology, and disease. *Physiological Reviews*, 98(3), 1493 – 1590.
- Jentsch, T. J., Stein, V., Weinreich, F., & Zdebik, A. A. (2002). Molecular structure and physiological function of chloride channels. *Physiological Reviews*, 82(2), 503–568.
- Jordan, W., Rieder, L. E., & Larschan, E. (2019). Diverse genome topologies characterize dosage compensation across species. *Trends in Genetics*, 35(4), 308–315.
- Jurkat-Rott, K., & Lehmann-Horn, F. (2004). Ion channels and electrical properties of skeletal muscle. *Myology*, 203–231.
- Jurkat-Rott, K., Lerche, H., & Lehmann-Horn, F. (2002). Skeletal muscle channelopathies. *Journal of Neurology*, 249, 1493–1502.
- Kato, H., Kokunai, Y., Dalle, C., Kubota, T., Madokoro, Y., Yuasa, H., Uchida, Y., Ikeda, T., Mochizuki, H., Nicole, S., Fontaine, B., Takahashi, M. P., & Mitake, S. (2016). A case of non-dystrophic myotonia with concomitant mutations in

- the *SCN4A* and *CLCN1* genes. *Journal of the Neurological Sciences*, 369, 254-258.
- Khameneh, S. C., Razi, S., Lashanizadegan, R., Akbari, S., Sayaf, M., Haghani, K., & Bakhtiyari, S. (2024). MicroRNA-mediated metabolic regulation of immune cells in cancer: an updated review. *Frontiers in Immunology*, 15, 1424909.
- Kim, V. N., & Nam, J. W. (2006). Genomics of microRNA. *TRENDS in Genetics*, 22(3), 165-173.
- Koch, M. C., Steinmeyer, K., Lorenz, C., Ricker, K., Wolf, F., Otto, M., Zoll, B., Lehmann-Horn, F., Grzeschik, K.H., & Jentsch, T. J. (1992). The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science*, 257(5071), 797-800.
- Koty, P. P., Pegoraro, E., Hobson, G., Marks, H. G., Turel, A., Flagler, D., Cadaldini, M., Angelini, C., & Hoffman, E. P. (1996). Myotonia and the muscle chloride channel: dominant mutations show variable penetrance and founder effect. *Neurology*, 47(4), 963-968.
- Kowalczyk, M. S., Hughes, J. R., Garrick, D., Lynch, M. D., Sharpe, J. A., Sloane-Stanley, J. A., McGowan, S. J., De Gobbi, M., Hosseini, M., Vernimmen, D., Brown, J. M., Gray NE, Collavin, L., Gibbons, R. J., Flint, J., Taylor, S., Buckle, V. J., Milne, T. A., Wood, W. G., & Higgs, D. R. (2012). Intragenic enhancers act as alternative promoters. *Molecular Cell*, 45(4), 447-458.
- Kubisch, C., Schmidt-Rose, T., Fontaine, B., Bretag, A. H., & Jentsch, T. J. (1998). ClC-1 chloride channel mutations in myotonia congenita: variable penetrance of mutations shifting the voltage dependence. *Human Molecular Genetics* 7, 1753.
- Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular aspects of Medicine*, 27(2-3), 95-125.
- Kumar, P., Henikoff, S., & Ng, P. C. (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature Protocols*, 4(7), 1073-1081.
- Kumar, V., Sanseau, P., Simola, D. F., Hurle, M. R., & Agarwal, P. (2016). Systematic analysis of drug targets confirms expression in disease-relevant tissues. *Scientific Reports*, 6(1), 36205.
- Kuntzer, T., Flocard, F., Vial, C., Kohler, A., Magistris, M. R., Labarre-Vila, A., Gonnaud, P. M., Ochsner, F., Soichot, P., Chan, V., & Monnier, G. (2000). Exercise test in muscle channelopathies and other muscle disorders. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 23(7), 1089-1094.
- Kyriakides, T., Angelini, C., Schaefer, J., Sacconi, S., Siciliano, G., Vilchez, J. J., & Hilton-Jones, D. (2010). EFNS guidelines on the diagnostic approach to pauci-

or asymptomatic hyperCKemia. *European Journal of Neurology*, 17(6), 767-773.

- Lakraj, A. A., Miller, G., Vortmeyer, A. O., Khokhar, B., Nowak, R. J., & DiCapua, D. B. (2013). Novel mutations in the *CLCN1* gene of myotonia congenita: 2 case reports. *The Yale Journal of Biology and Medicine*, 86(1), 101.
- Lee, T. T., Zhang, X. D., Chuang, C. C., Chen, J. J., Chen, Y. A., Chen, S. C., Chen, T.Y., & Tang, C. Y. (2013). Myotonia congenita mutation enhances the degradation of human CLC-1 chloride channels. *PloS One*, 8(2), e55930.
- Légaré, C., Overend, G., Guay, S. P., Monckton, D. G., Mathieu, J., Gagnon, C., & Bouchard, L. (2019). DMPK gene DNA methylation levels are associated with muscular and respiratory profiles in DM1. *Neurology: Genetics*, 5(3), e338.
- Lesk, A. M., Sangar, V., Parkinson, H., & Whisstock, J. C. (2008). Bioinformatics of protein function. In *Structural Proteomics And Its Impact On The Life Sciences* (pp. 79-119). World Scientific.
- Li, B., Krishnan, V. G., Mort, M. E., Xin, F., Kamati, K. K., Cooper, D. N., Mooney, S.D. & Radivojac, P. (2009). Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics*, 25(21), 2744-2750.
- Licata, L., Via, A., Turina, P., Babbi, G., Benevenuta, S., Carta, C., Casadio, R., Cicconardi, A., Facchiano, A., Fariselli, P. and Giordano, D., Isidori, F., Marabotti, A., Martelli, P. L., Pascarella, S., Pinelli, M., Pippucci, T., Russo, R., Savojardo, C., Scafuri, B., Valeriani, L., & Capriotti, E. (2023). Resources and tools for rare disease variant interpretation. *Frontiers in Molecular Biosciences*, 10, 1169109.
- Liu, N., Bezprozvannaya, S., Shelton, J. M., Frisard, M. I., Hulver, M. W., McMillan, R. P., Wu, Y., Voelker, K. A., Grange, R. W., Richardson, J. A., Bassel-Duby, R., & Olson, E. N. (2011). Mice lacking microRNA 133a develop dynamin 2-dependent centronuclear myopathy. *The Journal of Clinical Investigation*, 121(8), 3258-3268.
- Liu, X. L., Huang, X. J., Shen, J. Y., Zhou, H. Y., Luan, X. H., Wang, T., Chen, S. D., Wang, Y., Tang, H. D., & Cao, L. (2015). Myotonia congenita: novel mutations in *CLCN1* gene. *Channels*, 9(5), 292-298.
- Lo, H. S., Wang, Z., Hu, Y., Yang, H. H., Gere, S., Buetow, K. H., & Lee, M. P. (2003). Allelic variation in gene expression is common in the human genome. *Genome Research*, 13(8), 1855-1862.
- Lohmann, K., & Klein, C. (2014). Next generation sequencing and the future of genetic diagnosis. *Neurotherapeutics*, 11(4), 699-707.
- Looi, I., Bakar, A. A., Lim, C. H., Khoo, T. H., & Samuel, P. E. (2008). Anaesthetists' nightmare: masseter spasm after induction in an undiagnosed case of myotonia congenita. *Medical Journal of Malaysia*, 63(5), 423.

- Lossin, C., & George Jr, A. L. (2008). Myotonia congenita. *Advances in Genetics*, 63, 25-55.
- Loyfer, N., Magenheim, J., Peretz, A., Cann, G., Bredno, J., Klochendler, A., Fox-Fisher, I., Shabi-Porat, S., Hecht, M., Pelet, T., Moss, J., Drawshy, Z., Amini, H., Moradi, P., Nagaraju, S., Bauman, D., Shveiky, D., Porat, S., Dior, U. et al. (2023). A DNA methylation atlas of normal human cell types. *Nature*, 613(7943), 355-364.
- Ludewig, U., Pusch, M., & Jentsch, T. J. (1996). Two physically distinct pores in the dimeric CIC-0 chloride channel. *Nature*, 383(6598), 340-343.
- Lüscher, B. P., Vachel, L., Ohana, E., & Muallem, S. (2020). Cl⁻ as a bona fide signaling ion. *American Journal of Physiology-Cell Physiology*, 318(1), C125-C136.
- Lyons, M. J., Duron, R., Molinero, I., Sangiuolo, F., & Holden, K. R. (2010). Novel *CLCN1* mutation in carbamazepine-responsive myotonia congenita. *Pediatric Neurology*, 42(5), 365-368.
- Maggi, L., Ravaglia, S., Farinato, A., Brugnioni, R., Altamura, C., Imbrici, P., Camerino, D.C., Padovani, A., Mantegazza, R., Bernasconi, P., Desaphy, J.F., & Filosto, M. (2017). Coexistence of *CLCN1* and *SCN4A* mutations in one family suffering from myotonia. *Neurogenetics*, 18, 219-225.
- Mahlich, Y., Reeb, J., Hecht, M., Schelling, M., De Beer, T. A. P., Bromberg, Y., & Rost, B. (2017). Common sequence variants affect molecular function more than rare variants? *Scientific Reports*, 7(1), 1608.
- Mailänder, V., Heine, R., Deymeer, F., & Lehmann-Horn, F. (1996). Novel muscle chloride channel mutations and their effects on heterozygous carriers. *American Journal of Human Genetics*, 58(2), 317.
- Majewski, J., Schwartzentruber, J., Lalonde, E., Montpetit, A., & Jabado, N. (2011). What can exome sequencing do for you? *Journal of Medical Genetics*, 48(9), 580-589.
- Mankodi, A. (2008). Myotonic disorders. *Neurology India*, 56(3), 298-304.
- Marinakakis, N. M., Svingou, M., Papadimas, G. K., Papadopoulos, C., Chroni, E., Pons, R., Pavlou, E., Sarmas, I., Kosma, K., Apostolou, P., Sofocleous, C., & Kekou, K. (2024). Myotonia congenita in a Greek cohort: Genotype spectrum and impact of the *CLCN1*: c. 501C> G variant as a genetic modifier. *Muscle & Nerve*.
- Matthews, E., Fialho, D., Tan, S. V., Venance, S. L., Cannon, S. C., Sternberg, D., Fontaine, B., Amato, A.A., Barohn, R.J., Griggs, R.C., Hanna, M.G., & CINCH investigators. (2010). The non-dystrophic myotonias: molecular pathogenesis, diagnosis and treatment. *Brain*, 133(1), 9-22.

- McManis, P. G., Lambert, E. H., & Daube, J. R. (1986). The exercise test in periodic paralysis. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 9(8), 704-710.
- Meng, Y. X., Zhao, Z., Shen, H. R., Bing, Q., & Hu, J. (2016). Identification of novel mutations of the *CLCN1* gene for myotonia congenital in China. *Neurological Research*, 38(1), 40-44.
- Meyer, A. P., Roggenbuck, J., LoRusso, S., Kissel, J., Smith, R. M., Kline, D., Arnold, W. D. (2020). Genotype-Phenotype Correlations and Characterization of Medication Use in Inherited Myotonic Disorders. *Frontiers in Neurology*, 26, 11:593.
- Meyer-Kleine, C., Steinmeyer, K., Ricker, K., Jentsch, T. J., & Koch, M. C. (1995). Spectrum of mutations in the major human skeletal muscle chloride channel gene (*CLCN1*) leading to myotonia. *American Journal of Human Genetics*, 57(6), 1325.
- Miao, J., Wei, X. J., Liu, X. M., Kang, Z. X., Gao, Y. L., & Yu, X. F. (2018). A case report: autosomal recessive Myotonia congenita caused by a novel splice mutation (c. 1401+ 1G> A) in *CLCN1* gene of a Chinese Han patient. *BMC Neurology*, 18, 1-3.
- Miller, T. M. (2008). Differential diagnosis of myotonic disorders. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 37(3), 293-299.
- Mitchell, J. A., & Waclawik, A. J. (2007). Muscle biopsy in diagnosis of neuromuscular disorders: the technical aspects, clinical utility, and recent advances. *Journal of Histotechnology*, 30(4), 257-269.
- Modoni, A., D'Amico, A., Primiano, G., Capozzoli, F., Desaphy, J. F., & Lo Monaco, M. (2020). Long-term safety and usefulness of mexiletine in a large cohort of patients affected by non-dystrophic myotonias. *Frontiers in Neurology*, 11, 300.
- Moghadam-Kia, S., Oddis, C. V., & Aggarwal, R. (2016). Approach to asymptomatic creatine kinase elevation. *Cleveland Clinic Journal of Medicine*, 83(1), 37.
- Morales F, Pusch M. (2020). An Up-to-Date Overview of the Complexity of Genotype-Phenotype Relationships in Myotonic Channelopathies. *Frontier Neurology*, 17, (10) 1404.
- Morrow, J. M., Matthews, E., Rayan, D. L. R., Fischmann, A., Sinclair, C. D., Reilly, M. M., Thornton, J.S., Hanna, M.G. & Yousry, T. A. (2013). Muscle MRI reveals distinct abnormalities in genetically proven non-dystrophic myotonias. *Neuromuscular Disorders*, 23(8), 637-646.
- Morton, A. (2020). Myotonic disorders and pregnancy. *Obstetric Medicine*, 13(1), 14-19.

- Nardin, R. A., Zarrin, A. R., Horowitz, G. L., & Tarulli, A. W. (2009). Effect of newly proposed CK reference limits on neuromuscular diagnosis. *Muscle & Nerve*, 39(4), 494-497.
- Nojszewska, M., Gawel, M., Kierdaszuk, B., Sierdziński, J., Szmidt-Sałkowska, E., Seroka, A., Kamińska, A.M. & Kostera-Pruszczyk, A. (2018). Electromyographic findings in sporadic inclusion body myositis. *Journal of Electromyography and Kinesiology*, 39, 114-119.
- Nojszewska, M., Lusakowska, A., Gawel, M., Sierdzinski, J., Sulek, A., Krysa, W., Elert-Dobkowska, E., Seroka, A., Kaminska, A.M. & Kostera-Pruszczyk, A. (2019). The needle EMG findings in myotonia congenita. *Journal of Electromyography and Kinesiology*, 49, 102362.
- Novak, K. R., Norman, J., Mitchell, J. R., Pinter, M. J., & Rich, M. M. (2015). Sodium channel slow inactivation as a therapeutic target for myotonia congenita. *Annals of neurology*, 77(2), 320-332.
- Olave-Rodriguez, J. A., Bonilla-Escobar, F. J., Candelo, E., & Rodriguez-Rojas, L. X. (2021). First Two Case Reports of Becker's Type Myotonia Congenita in Colombia: Clinical and Genetic Features. *The Application of Clinical Genetics*, 473-479.
- Oliver, G. R., Hart, S. N., & Klee, E. W. (2015). Bioinformatics for clinical next generation sequencing. *Clinical Chemistry*, 61(1), 124-135.
- Palma Milla, C., Prior De Castro, C., Gómez-González, C., Martínez-Montero, P., I. Pascual Pascual, S., & Molano Mateos, J. (2019). Myotonia congenita: mutation spectrum of *CLCN1* in Spanish patients. *Journal of Genetics*, 98, 1-10.
- Passeri, E., Sansone, V. A., Verdelli, C., Mendola, M., & Corbetta, S. (2014). Asymptomatic myotonia congenita unmasked by severe hypothyroidism. *Neuromuscular Disorders*, 24(4), 365-367.
- Pedersen, T. H., Riisager, A., de Paoli, F. V., Chen, T. Y., & Nielsen, O. B. (2016). Role of physiological ClC-1 Cl⁻ ion channel regulation for the excitability and function of working skeletal muscle. *Journal of General Physiology*, 147(4), 291-308.
- Plassart-Schiess, E., Gervais, A., Eymard, B., Lagueny, A., Pouget, J., Warter, J. M. Fardeau, M., Jentsch, T. J., & Fontaine, B., (1998). Novel muscle chloride channel (*CLCN1*) mutations in myotonia congenita with various modes of inheritance including incomplete dominance and penetrance. *Neurology* 50, 1176 – 1179.
- Polla, B., D'Antona, G., Bottinelli, R., & Reggiani, C. (2004). Respiratory muscle fibres: specialisation and plasticity. *Thorax*, 59(9), 808-817.
- Pollard, K. S., Hubisz, M. J., Rosenbloom, K. R., & Siepel, A. (2010). Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Research*, 20(1), 110-121.

- Portaro, S., Altamura, C., Licata, N., Camerino, G. M., Imbrici, P., Musumeci, O., Rodolico, C., Conte Camerino, D., Toscano, A., & Desaphy, J. F. (2015). Clinical, molecular, and functional characterization of *CLCN1* mutations in three families with recessive myotonia congenita. *Neuromolecular Medicine*, 17, 285-296.
- Portaro, S., Cacciola, A., Naro, A., Milardi, D., Morabito, R., Corallo, F., Marino, S., Bramanti, A., Mazzon, E., & Calabrò, R. S. (2018). A case report of recessive myotonia congenita and early onset cognitive impairment: Is it a causal or casual link?. *Medicine*, 97(22), e10785.
- Portela, A., & Esteller, M. (2010). Epigenetic modifications and human disease. *Nature Biotechnology*, 28(10), 1057-1068.
- Preedy, V. R., & Peters, T. J. (Eds.). (2002). *Skeletal muscle: pathology, diagnosis and management of disease*. Cambridge University Press.
- Pusch, M. (2002). Myotonia caused by mutations in the muscle chloride channel gene *CLCN1*. *Human Mutation*, 19(4), 423-434.
- Rabbani, B., Tekin, M., & Mahdieh, N. (2014). The promise of whole-exome sequencing in medical genetics. *Journal of Human Genetics*, 59(1), 5-15.
- Radkowski, P., Suren, L., Podhorodecka, K., Harikumar, S., & Jamrozik, N. (2023). A Review on the Anesthetic Management of Patients with Neuromuscular Diseases. *Anesthesiology and Pain Medicine*, 13(1).
- Raheem, O., Penttilä, S., Suominen, T., Kaakinen, M., Burge, J., Haworth, A., Sud, R., Schorge, S., Haapasalo, H., Sandell, S. and Metsikkö, K., Hanna, M., & Udd, B. (2012). New immunohistochemical method for improved myotonia and chloride channel mutation diagnostics. *Neurology*, 79(22), 2194-2200.
- Raja Rayan, D. L., Haworth, A., Sud, R., Matthews, E., Fialho, D., Burge, J., Portaro, S.I.M.O.N.A., Schorge, S., Tuin, K., Lunt, P. and McEntagart, M., & Hanna, M. G. (2012). A new explanation for recessive myotonia congenita: exon deletions and duplications in *CLCN1*. *Neurology*, 78(24), 1953-1958.
- Rentzsch, P., Witten, D., Cooper, G. M., Shendure, J., & Kircher, M. (2019). CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Research*, 47(D1), D886-D894.
- Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., & Rehm, H. L. (2015). ACMG Laboratory Quality Assurance Committee Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, 17(5), 405-424.
- Richardson, R. C., Tarleton, J. C., Bird, T. D., & Gospe Jr, S. M. (2014). Truncating *CLCN1* mutations in myotonia congenita: variable patterns of inheritance. *Muscle & Nerve*, 49(4), 593-600.

- Richman, D. P., Yu, Y., Lee, T. T., Tseng, P. Y., Yu, W. P., Maselli, R. A., Tang, C.Y., & Chen, T. Y. (2012). Dominantly inherited myotonia congenita resulting from a mutation that increases open probability of the muscle chloride channel CLC-1. *Neuromolecular Medicine*, 14, 328-337.
- Rizzo, J. M., & Buck, M. J. (2012). Key principles and clinical applications of “next-generation” DNA sequencing. *Cancer Prevention Research*, 5(7), 887-900.
- Roberts, T. C., Godfrey, C., McClorey, G., Vader, P., Briggs, D., Gardiner, C., Aoki, Y., Sargent, I., Morgan, J.E., & Wood, M. J. (2013). Extracellular microRNAs are dynamic non-vesicular biomarkers of muscle turnover. *Nucleic Acids Research*, 41(20), 9500-9513.
- Roman, W., & Gomes, E. R. (2018, October). Nuclear positioning in skeletal muscle. In *Seminars in Cell & Developmental Biology* (Vol. 82, pp. 51-56). Academic Press.
- Saidman, L. J., Havard, E. S., & Eger, E. I. (1964). Hyperthermia during anesthesia. *JAMA*, 190(12), 1029-1032.
- Sakuragawa, N., Sato, T., & Tsubaki, T. (1973). Scanning Electron Microscopic Study of Skeletal Muscle: Normal, Dystrophic, and Neurogenic Atrophic Muscle in Mice and Humans. *Archives of Neurology*, 28(4), 247-251.
- Samaha, F. J., Schroeder, J. M., Rebeiz, J., & Adams, R. D. (1967). Studies on myotonia: Biochemical and electron microscopic studies on myotonia congenita and myotonia dystrophica. *Archives of Neurology*, 17(1), 22-33.
- Sasaki, R., Nakaza, M., Furuta, M., Fujino, H., Kubota, T., & Takahashi, M. P. (2020). Mutation spectrum and health status in skeletal muscle channelopathies in Japan. *Neuromuscular Disorders*, 30(7), 546-553.
- Saviane, C., Conti, F., & Pusch, M. (1999). The muscle chloride channel CLC-1 has a double-barreled appearance that is differentially affected in dominant and recessive myotonia. *The Journal of General Physiology*, 113(3), 457-468.
- Schiaffino, S., & Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiological Reviews*, 91(4), 1447-1531.
- Shapiro, B. E., & Preston, D. C. (2003). Repetitive nerve stimulation and exercise testing. *Physical Medicine and Rehabilitation Clinics*, 14(2), 185-206.
- Shapiro, B., & Ruff, R. (2002). Disorders of skeletal muscle membrane excitability: myotonia congenita, paramyotonia congenita, periodic paralysis, and related disorders. *Neuromuscular disorders in clinical practice*. Boston: Butterworth-Heinemann, 987-1020.
- Sharma, M. C., Gulati, S., Atri, S., Seth, R., Kalra, V., Das, T. K., & Sarkar, C. (2007). Nemaline rod myopathy: A rare form of myopathy. *Neurology India*, 55(1), 70-74.

- Shen, H., Li, J., Zhang, J., Xu, C., Jiang, Y., Wu, Z., Zhao, F., Liao, L., Chen, J., Lin, Y. and Tian, Q., & Deng, H. W. (2013). Comprehensive characterization of human genome variation by high coverage whole-genome sequencing of forty four Caucasians. *PloS One*, 8(4), e59494.
- Skálová, D., Zídková, J., Vohánka, S., Mazanec, R., Mušová, Z., Vondráček, P., Mrázová, L., Kraus, J., Réblová, K., & Fajkusová, L. (2013). *CLCN1* mutations in Czech patients with myotonia congenita, *in silico* analysis of novel and known mutations in the human dimeric skeletal muscle chloride channel. *PLoS One*, 8(12), e82549.
- Sparber, P., Sharova, M., Filatova, A., Shchagina, O., Ivanova, E., Dadali, E., & Skoblov, M. (2020). Recessive myotonia congenita caused by a homozygous splice site variant in *CLCN1* gene: a case report. *BMC Medical Genetics*, 21, 1-5.
- Steinmeyer, K., Ortland, C., & Jentsch, T. J. (1991). Primary structure and functional expression of a developmentally regulated skeletal muscle chloride channel. *Nature*, 354(6351), 301-304.
- Streib, E. W. (1987). Differential diagnosis of myotonic syndromes. *Muscle Nerve*, 10, 603-615.
- Streib, E. W., Sun, S. F., & Yarkowsky, T. (1982). Transient paresis in myotonic syndromes: a simplified electrophysiologic approach. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 5(9), 719-723.
- Stunnenberg, B. C., LoRusso, S., Arnold, W. D., Barohn, R. J., Cannon, S. C., Fontaine, B., Griggs, R.C., Hanna, M.G., Matthews, E., Meola, G. and Sansone, V.A., & Statland, J. M. (2020). Guidelines on clinical presentation and management of nondystrophic myotonias. *Muscle & Nerve*, 62(4), 430-444.
- Suetterlin, K., Männikkö, R., & Hanna, M. G. (2014). Muscle channelopathies: recent advances in genetics, pathophysiology and therapy. *Current Opinion in Neurology*, 27(5), 583-590.
- Suetterlin, K. J., Bugiardini, E., Kaski, J. P., Morrow, J. M., Matthews, E., Hanna, M. G., & Fialho, D. (2015). Long-term safety and efficacy of mexiletine for patients with skeletal muscle channelopathies. *JAMA neurology*, 72(12), 1531-1533.
- Sun, C., Tranebjærg, L., Torbergesen, T., Holmgren, G., & Van Ghelue, M. (2001). Spectrum of *CLCN1* mutations in patients with myotonia congenita in Northern Scandinavia. *European Journal of Human Genetics*, 9(12), 903-909.
- Tan, S. V., Matthews, E., Barber, M., Burge, J. A., Rajakulendran, S., Fialho, D., Sud, R., Haworth, A., Koltzenburg, M., & Hanna, M. G. (2011). Refined exercise testing can aid DNA-based diagnosis in muscle channelopathies. *Annals of Neurology*, 69(2), 328-340.

- Tang, C. Y., & Chen, T. Y. (2011). Physiology and pathophysiology of CLC-1: mechanisms of a chloride channel disease, myotonia. *BioMed Research International*, 2011(1), 685328.
- Thiel, R. E. (1967). The myotonic response to suxamethonium. *British Journal of Anaesthesia*, 39(10), 815-821.
- Thomas, J., Tarleton, J., & Baker, S. K. (2008). Recessive *CLCN1* mutation presenting as Thomsen disease. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 38(5), 1515-1517.
- Titcombe, P., Murray, R., Hewitt, M., Antoun, E., Cooper, C., Inskip, H. M., Holbrook, J.D., Godfrey, K.M., Lillycrop, K., Hanson, M. & Barton, S. J. (2022). Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function. *Epigenetics*, 17(6), 653-664.
- Totsuka, M., Nakaji, S., Suzuki, K., Sugawara, K., & Sato, K. (2002). Break point of serum creatine kinase release after endurance exercise. *Journal of Applied Physiology*, 93(4), 1280-1286.
- Trip, J., Drost, G., Ginjaar, H. B., Nieman, F. H. M., Van Der Kooi, A. J., de Visser, M., van Engelen, B. G. M., & Faber, C. G. (2009). Redefining the clinical phenotypes of non-dystrophic myotonic syndromes. *Journal of Neurology, Neurosurgery & Psychiatry*, 80(6), 647-652.
- Trivedi, J. R., Bundy, B., Statland, J., Salajegheh, M., Rayan, D. R., Venance, S. L., Wang, Y., Fialho, D., Matthews, E., Cleland, J. and Gorham, N., & CINCH Consortium. (2013). Non-dystrophic myotonia: prospective study of objective and patient reported outcomes. *Brain*, 136(7), 2189-2200.
- Tseng, P. Y., Yu, W. P., Liu, H. Y., Zhang, X. D., Zou, X., & Chen, T. Y. (2011). Binding of ATP to the CBS domains in the C-terminal region of CLC-1. *Journal of General Physiology*, 137(4), 357-368.
- Ulzi, G., Lecchi, M., Sansone, V., Redaelli, E., Corti, E., Saccomanno, D., Pagliarani, S., Corti, S., Magri, F., Raimondi, M. and D'Angelo, G., & Lucchiari, S. (2012). Myotonia congenita: novel mutations in *CLCN1* gene and functional characterizations in Italian patients. *Journal of the Neurological Sciences*, 318(1-2), 65-71.
- Velasco, G., & Francastel, C. (2019). Genetics meets DNA methylation in rare diseases. *Clinical Genetics*, 95(2), 210-220.
- Vereb, N., Montagnese, F., Gläser, D., & Schoser, B. (2021). Non-dystrophic myotonias: clinical and mutation spectrum of 70 German patients. *Journal of Neurology*, 268, 1708-1720.
- Vinkšiel, M., Writzl, K., Maver, A., & Peterlin, B. (2021). Improving diagnostics of rare genetic diseases with NGS approaches. *Journal of Community Genetics*, 12, 247-256.

- Walters, J., & Baborie, A. (2020). Muscle biopsy: what and why and when?. *Practical Neurology*, 20(5), 385-395.
- Wang, C., Yue, F., & Kuang, S. (2017). Muscle histology characterization using H&E staining and muscle fiber type classification using immunofluorescence staining. *Bio-protocol*, 7(10), e2279-e2279.
- Weinberger, S., Wojciechowski, D., Sternberg, D., Lehmann-Horn, F., Jurkat-Rott, K., Becher, T., Begemann, B., Fahlke, C., & Fischer, M. (2012). Disease-causing mutations C277R and C277Y modify gating of human ClC-1 chloride channels in myotonia congenita. *The Journal of Physiology*, 590(15), 3449-3464.
- Westerblad, H., Bruton, J. D., & Katz, A. (2010). Skeletal muscle: energy metabolism, fiber types, fatigue and adaptability. *Experimental cell research*, 316(18), 3093-3099.
- Wolf, S., Melo, D., Garske, K. M., Pallares, L. F., Lea, A. J., & Ayroles, J. F. (2023). Characterizing the landscape of gene expression variance in humans. *PLoS Genetics*, 19(7), e1010833.
- Yue, F., Bi, P., Wang, C., Li, J., Liu, X., & Kuang, S. (2016). Conditional loss of Pten in myogenic progenitors leads to postnatal skeletal muscle hypertrophy but age-dependent exhaustion of satellite cells. *Cell Reports*, 17(9), 2340-2353.
- Zaharieva, I. T., Calissano, M., Scoto, M., Preston, M., Cirak, S., Feng, L., Collins, J., Kole, R., Guglieri, M., Straub, V. and Bushby, K., & Muntoni, F. (2013). Dystromirs as serum biomarkers for monitoring the disease severity in Duchenne muscular Dystrophy. *PloS One*, 8(11), e80263.
- Zhang, Z., Zou, Z., Zhang, H., & Zhang, D. M. (2024). Regulatory network analysis based on integrated miRNA-TF reveals key genes in heart failure. *Scientific Reports*, 14(1), 13896.
- Zhao, Y., Samal, E., & Srivastava, D. (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*, 436(7048), 214-220.

APPENDICES

Appendix A1. Ethical approval by the Medical Research and Ethics Committee and University Putra Malaysia Research Ethics Board (JKEUPM-2020-135)

Ref. no: UPM/TNCPI/RMC/JKEUPM/1.4.18.2 (JKEUPM)
Date: 5 November 2020

Dear Prof./Dr./Mr./Ms.,

APPLICATION FOR JKEUPM ETHICAL CLEARANCE: APPROVED

With reference to the above, I am pleased to inform you that your application for ethical clearance for the research project entitled 'Clinical and Genetic Analysis of Myotonia Congenita in a Single Family in Malaysia' has been approved.

Please note that the official letter of approval will be issued as soon as possible. However, the ethical clearance is considered effective from the date of this email, and you may now proceed with your research.

Kindly remind the ethical approval is required in the case of amendments/ changes to the study documents/ study sites/ study team.

Researchers should also complete a Study Final Report upon study completion. The form can be obtained from the Ethics Committee for Research Involving Human Subjects (JKEUPM) website (<http://www.tncpi.upm.edu.my/faildokumen>).

If you have any enquiries, please contact Ms. Nurulhasanah Ishak (03-97691605) or Ms. Nor Ellia Abd Ajis (03-97691244).

Note: Please use this reference number for any transaction:- JKEUPM-2020-135

Thank you.

Yours faithfully,

Prof. Dr. Zamberi Sekawi
Chair
Ethics Committee for Research Involving Human Subjects
Universiti Putra Malaysia



PEJABAT TIMBALAN NAIB CANCELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

Reff. : UPM/TNCPI/RMC/1.4.18.2 (JKEUPM)
Date : 24 May 2022

Dr Abdul Hanif Khan Bin Yusof Khan
Department of Neurology
Faculty of Medicine and Health Science
Universiti Putra Malaysia
43400 UPM Serdang, Selangor

Dear Sir/ Madam,

PROJECT TITLE: CLINICAL, MOLECULAR AND HISTOPATHOLOGICAL ANALYSIS OF MYOTONIA CONGENITA IN A SINGLE-FAMILY IN MALAYSIA

JKEUPM REF. NO: JKEUPM-2020-135

PRINCIPAL INVESTIGATOR: DR. ABDUL HANIF KHAN YUSOF KHAN

STUDENT: NURUL HUDA MUSA

Your letter dated 11 April 2022 is referred.

The committee has approved your proposed amendment on 24 May 2022. The documents have been approved for use:

1. Respondent's Information Sheet / Consent- (Malay) -Healthy Control, Version 7 dated 23/05/2022
2. Respondent's Information Sheet / Consent (Malay) -MC Patient, Version 7 dated 23/05/2022
3. Respondent's Information Sheet / Consent- (English) -Healthy Control, Version 7 dated 23/05/2022
4. Respondent's Information Sheet / Consent (English) -MC Patient, Version 7 dated 23/05/2022

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) ☎ 603-9769 1002 📠 603-9769 4134, Pejabat Pentadbiran TNCPI ☎ 603-9769 1608, Pejabat Pengarah Pusat Pengurusan Penyelidikan (RMC) ☎ 603-9769 1601, Pejabat Pengarah Putra Science Park (PSP) ☎ 603-9769 1291
🌐 <http://www.tncpi.upm.edu.my>

5. Proposal (English), Version 6 dated 11/04/2022
6. Curriculum Vitae of:
 - a. Nurul Huda Musa

Thank you.

"WITH KNOWLEDGE WE SERVE"

Yours faithfully,



PROF.DR. ZAMBERI SEKAWI

Chair

Ethics Committee for Research involving Human Subjects
Universiti Putra Malaysia

Appendix A2. An English and Malay version of informed consent give to MC patient.

Version 7 JKEUPM-2020-135, 23rd May 2022



JAWATANKUASA ETIKA UNIVERSITI UNTUK
PENYELIDIKAN MELIBATKAN MANUSIA (JKEUPM)
UNIVERSITI PUTRA MALAYSIA, 43400 UPM SERDANG,
SELANGOR, MALAYSIA

FORM 2.4: RESPONDENT'S INFORMATION SHEET AND INFORMED CONSENT FORM (MC PATIENT)

Please read the following information carefully and do not hesitate to discuss any questions you may have with the researcher.

1. STUDY TITLE: Clinical, molecular and histopathological analysis of Myotonia Congenita in a single family in Malaysia

2. INTRODUCTION:

Myotonia congenita (MC) is a rare inherited genetic disorder attributed to mutations in the *CLCN1* gene. MC characterized by difficulties in muscle relaxation and muscle weakness. The prevalence worldwide is small with more new mutations being found. To date, there is no prevalence data in Asian countries with no family study being reported in Malaysia. Therefore, the objectives of this study are to determine the clinical and genetic characteristics of MC in a family suspected with MC. We will perform the clinical examination by conducting a thorough family history (including family pedigree), physical examination, laboratory testing, and neurophysiology studies, i.e., nerve conduction study (NCS) and electromyography (EMG). Genetic studies will be performed to screen the *CLCN1* gene. If a new mutation is found, the presence of the new mutation will be examined in individuals without MC. Gene expression, protein expression and histopathological studies will be performed and require muscle biopsies procedure conducted on the confirmed MC patients and on the individuals without MC condition to obtain muscle tissue samples.

3. WHAT WILL YOU HAVE TO DO?

If you agree to participate in this study:

- For suspected patient and their family: will undergo a physical examination, neurophysiology testing (NCS and EMG) and have to donate around 10 ml of blood for routine blood investigation and genetic testing and undergo muscle biopsies procedure to obtained muscle tissue samples.
- Physical examination – neurophysiology testing will be done by doctors, nurses and medical assistant involved with this study.
- Muscle biopsies – a minimally invasive minor surgical procedure to obtain small sample of muscle tissue samples.
- We will compensate your travel fee to Hospital Pengajar UPM for this study analysis.

4. WHO SHOULD PARTICIPATE IN THE STUDY?

- You are eligible for this study if you are with a clinical condition of MC or a family member of an individual with a clinical condition of MC. If you have children at age more than 7 years old, he/she should also participate in the study.

5. WHAT WILL BE THE BENEFITS OF THE STUDY:

(a) TO YOU AS THE SUBJECT?

This is a preliminary research study on mutation screening of MC related genes in a family with MC, therefore this study will not benefit the subject directly during the research period. However, if we diagnosed you or one of your family members as possible MC, we will offer a follow up and medication under our Neurology Clinic in Hospital Pengajar UPM.

(b) TO THE INVESTIGATOR?

Identification of clinical profile, genetic mutations and histopathological changes in muscle tissue of MC patients in a single-family in Malaysia.

6. WHAT ARE THE POSSIBLE RISKS?

1. For nerve conduction study (NCS): it involves the application of mild electrical current to assess the integrity of the nerve. Possible risks are very minimal and most commonly cause mild transient discomfort.
2. For electromyography (EMG): it involves placing a small needle and application of small electrical current at the muscle examined. This test is generally safe with possible risk mainly involves bleeding at the site of needle placement, which can be stopped by applying pressure at the area. Rare side effects include infection at the site of needle placement.
3. For blood taking: there is a small risk of hematoma (bruise) at the site of blood drawing; however, this can be avoided by pressing at the place where the needle was injected using cotton until the bleeding stops.
4. For muscle biopsies procedure: Muscle biopsy is a minimally invasive procedure. The associated risks are small that includes pain, redness, swelling or bleeding around the biopsy site. There is small risk of infection to occur at the biopsy site which may present with fever and pain over the biopsy site. However, the reported risk is small and will not cause any harm.
5. You may withdraw from the study at any point of time if you are no longer interested in joining the study. If you are a patient receiving treatment for MC, withdrawing from the study would not affect the plan treatment.

7. WILL THE INFORMATION THAT YOU PROVIDE AND YOUR IDENTITY REMAIN CONFIDENTIAL?

Your identity and your medical records and data relating to this research study will be kept confidential, except as required by law, and the study sponsor. The leftover sample will be disposed of at the end of the research study unless you consent to donate your blood sample, it will be stored for future studies related to MC.

8. WHO SHOULD YOU CONTACT IF YOU HAVE ADDITIONAL QUESTIONS DURING THE COURSE OF THE RESEARCH?

Dr. Abdul Hanif Khan Yusof Khan
Department of Neurology
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor D.E.
Email: ahanifkhan@upm.edu.my

Dr.Thilakavathy Karuppiah
Clinical Genetics Unit,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor D.E.

Please initial here if you have read and understood the contents of this page_____

9. CONSENT

I Identity Card No.....
address.....
..... hereby voluntarily agree to take part in the research stated above.

I have been informed about the nature of the research in terms of methodology, possible adverse effects and complications (as written in the Respondent's Information Sheet). I understand that I have the right to withdraw from this research at any time without giving any reason whatsoever. I also understand that this study is confidential and all information provided with regard to my identity will remain private and confidential.

☐ I agree to donate my blood sample for future study related to MC.**

☐ I agree to donate my muscle sample for future study related to MC.**

☐ I agree that the images/photos/video recordings/voice recordings related to me to be used in any form of publication or presentation (if applicable).**

* delete where necessary

** tick (/) in the box if you are agree

Signature
(Respondent)

Signature
(Witness)

Date:.....

Name:.....

I/C No.:.....

I confirm that I have explained to the respondent the nature and purpose of the above-mentioned research.

Date:

Signature
(Researcher)



**JAWATANKUASA ETIKA UNIVERSITI UNTUK
PENYELIDIKAN MELIBATKAN MANUSIA (JKEUPM)
UNIVERSITI PUTRA MALAYSIA, 43400 UPM SERDANG,
SELANGOR, MALAYSIA**

**BORANG 2.4: PENERANGAN DAN PERSETUJUAN RESPONDEN
(PESAKIT MC)**

Sila baca maklumat berikut dengan teliti. Sekiranya anda mempunyai sebarang pertanyaan, sila kemukakan kepada penyelidik.

1.TAJUK KAJIAN: Analisis klinikal, molekular dan histopatologikal Myotonia Congenita (MC) dalam satu keluarga di Malaysia

2. PENGENALAN

Myotonia congenita (MC) adalah penyakit genetik yang menyebabkan gangguan pergerakan otot disebabkan mutasi dalam gen *CLCN1*. Pesakit MC akan mengalami kesusahan untuk merehatkan otot dan akan mengalami kelemahan otot. Penyakit ini jarang berlaku di dunia dengan 160 mutasi yang dilaporkan dalam gen ini dan lebih banyak mutasi baru yang dijumpai. Sehingga kini, tiada data kekerapan di negara-negara Asia dan tiada kajian keluarga yang dilaporkan di Malaysia. Oleh itu, objektif kajian ini adalah untuk menentukan ciri-ciri klinikal dan genetik MC dalam keluarga yang disyaki dengan MC. Kami akan menjalankan analisis klinikal dengan menjalankan sejarah keluarga yang menyeluruh (termasuk salasilah keluarga), pemeriksaan fizikal, ujian makmal, dan kajian neurofisiologi, iaitu kajian pengaliran saraf (NCS) dan elektromilografi (EMG). Kajian genetik akan dilakukan untuk menyaring gen *CLCN1*. Jika mutasi baru ditemui, kehadiran mutasi gen akan diperiksa dalam individu tanpa MC. Kajian ekspresi gen, ekspresi protein dan kajian histopatologi akan dijalankan dan memerlukan prosedur biopsi otot dijalankan ke atas pesakit MC yang telah disahkan dan individu tanpa penyakit MC untuk mendapatkan sampel tisu otot.

3. APAKAH YANG PERLU ANDA LAKUKAN?

Jika anda bersetuju untuk mengambil bahagian dalam kajian ini:

- Bagi pesakit MC dan keluarga mereka: anda akan menjalani pemeriksaan fizikal, ujian neurofisiologi (NCS dan EMG) dan perlu mendermakan sekitar 10 ml darah untuk penyiasatan darah rutin dan ujian genetik dan menjalani prosedur pembedahan kecil untuk mendapatkan sampel tisu otot.
- Pemeriksaan fizikal - ujian neurofisiologi akan dilakukan oleh doktor, jururawat dan pembantu perubatan yang terlibat dengan kajian ini.
- Biopsi otot - prosedur pembedahan kecil dan minor untuk mendapatkan sedikit sampel tisu otot.
- Kami akan membayar kos perjalanan anda ke Hospital Pengajar UPM bagi analisis kajian ini.

4. SIAPA YANG BOLEH MENYERTAI KAJIAN INI?

- Anda layak untuk kajian ini jika anda mempunyai keadaan klinikal MC atau ahli keluarga seseorang dengan keadaan klinikal MC. Sekiranya anda mempunyai anak berusia lebih dari 7 tahun, dia juga harus turut serta dalam kajian ini.

5. APAKAH FAEDAH MENYERTAI KAJIAN INI?**(a) KEPADA ANDA SEBAGAI PESERTA?**

Ini adalah kajian penyelidikan awal mengenai penyingkapan mutan bagi gen yang berkaitan dengan MC dalam keluarga dengan MC, oleh itu kajian ini tidak akan memberi manfaat secara langsung kepada subjek semasa tempoh penyelidikan. Walau bagaimanapun, jika anda atau salah satu daripada ahli keluarga anda mungkin MC, kami akan menawarkan untuk menerima rawatan di bawah Klinik Neurologi kami di Pengajar Hospital UPM.

(b) KEPADA PENYELIDIK?

Pengenalan profil klinikal, mutasi genetik dan perubahan histologi bagi pesakit MC dalam satu keluarga di Malaysia.

6. ADAKAH IA BERISIKO?

1. Untuk kajian konduksi saraf (NCS): ia melibatkan penggunaan arus elektrik ringan untuk menilai integriti saraf. Risiko mungkin sangat minima dan biasanya menyebabkan ketidakselesaan sementara.
2. Untuk electromyography (EMG): ia melibatkan meletakkan jarum kecil dan penggunaan arus elektrik kecil pada otot yang diperiksa. Ujian ini secara amnya selamat dengan kemungkinan risiko terutamanya melibatkan pendarahan di tempat penempatan jarum, yang boleh dihindarkan dengan menggunakan tekanan di kawasan tersebut. Kesan sampingan yang jarang berlaku termasuk jangkitan di lokasi penempatan jarum.
3. Untuk pengambilan darah: terdapat risiko kecil hematoma (lebam) di tempat lukisan darah; Walau bagaimanapun, ini boleh dielakkan dengan menekan di tempat jarum disuntik menggunakan kapas sehingga pendarahan berhenti.
4. Untuk prosedur biopsi otot bagi mendapatkan sampel tisu otot: biopsi otot adalah prosedur invasive minimum, dan risiko yang berkaitan adalah kecil termasuk kesakitan, kemerahan, bengkak atau pendarahan di sekitar kawasan biopsi. Terdapat risiko kecil jangkitan berlaku di kawasan biopsi yang mungkin disertai dengan demam dan sakit di kawasan biopsi. Namun, risiko adalah kecil dan tidak akan mengundang bahaya.
5. Anda boleh berhenti dari menyertai kajian ini pada bila-bila masa sekiranya anda tidak berminat lagi untuk mengikuti kajian ini. Sekiranya anda seorang pesakit yang mendapat rawatan MC, menarik diri dari kajian tidak akan mempengaruhi pelan rawatan.

7. ADAKAH MAKLUMAT DAN IDENTITI SAYA KEKAL RAHSIA?

Identiti dan rekod perubatan anda dan data yang berkaitan dengan kajian penyelidikan ini akan dirahsiakan, kecuali seperti yang dikehendaki oleh undang-undang, dan penaja kajian. Baki sampel darah akan dilupuskan pada akhir kajian penyelidikan melainkan jika anda bersetuju menderma sampel darah anda, ia akan disimpan untuk kajian masa depan yang berkaitan dengan MC.

8. SIAPA YANG SAYA PERLU DIHUBUNGI SEKIRANYA SAYA MEMPUNYAI SOALAN TAMBAHAN SEMASA MENGIKUTI PENYELIDIKAN INI?

Dr. Abdul Hanif Khan Yusof Khan
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Dr. Thilakavathy Karupiah
Unit Genetik Klinikal,
Fakulti Perubatan Dan Sains Kesihatan,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor D.E.

___ Sila tandatangan di sini sekiranya anda telah membaca dan memahami kandungan halaman ini ___

9. PERSETUJUAN

Saya No Kad Pengenalan
beralamat.....
..... dengan ini bersetuju untuk mengambil bahagian secara sukarela dalam penyelidikan yang tersebut di atas *(kajian klinikal/percubaan ubat-ubatan/rakaman video/kumpulan sasaran/temuduga/ soal selidik).

Saya telah diberi penjelasan secara menyeluruh mengenai penyelidikan ini dari segi metodologi, risiko dan komplikasi (seperti tertulis pada Helaian Penerangan Responden). Saya memahami bahawa saya berhak menarik diri dari penyelidikan ini pada bila-bila masa tanpa memberi sebarang alasan. Saya juga memahami bahawa sebarang maklumat yang berkaitan identiti saya akan dirahsiakan.

☐ Saya bersetuju untuk menderma sampel darah saya untuk kajian masa depan yang berkaitan dengan MC**

☐ Saya bersetuju untuk mendermakan sampel otot saya untuk kajian masa depan yang berkaitan dengan MC**

☐ Saya bersetuju bahawa rakaman gambar / gambar / video / rakaman suara yang berkaitan dengan saya digunakan dalam sebarang bentuk penerbitan atau pembentangan (jika berkenaan)**

* potong mana yang berkenaan

** tanda (/) di dalam kotak yang anda setuju

Tandatangan
(Responden)

Tandatangan
(Saksi)

Tarikh :

Nama :

No. K/P:

Saya mengesahkan bahawa saya telah menerangkan kepada responden ini sifat dan tujuan penyelidikan yang tersebut di atas.

Tarikh

Tandatangan
(Penyelidik)

Appendix A3. An English and Malay version of informed consent give to normal control individual without MC conditions.

Version 7 JKEUPM-2020-135, 23rd May 2022



UPM
UNIVERSITI PUTRA MALAYSIA

**JAWATANKUASA ETIKA UNIVERSITI UNTUK
PENYELIDIKAN MELIBATKAN MANUSIA (JKEUPM)
UNIVERSITI PUTRA MALAYSIA, 43400 UPM SERDANG,
SELANGOR, MALAYSIA**

FORM 2.4: RESPONDENT'S INFORMATION SHEET AND INFORMED CONSENT FORM (INDIVIDUALS WITHOUT MC CONDITION)

Please read the following information carefully and do not hesitate to discuss any questions you may have with the researcher.

1. STUDY TITLE: Clinical, molecular and histopathological analysis of Myotonia Congenita in a single family in Malaysia

2. INTRODUCTION:

Myotonia congenita (MC) is a rare inherited genetic disorder attributed to mutations in the *CLCN1* gene. MC characterized by difficulties in muscle relaxation and muscle weakness. The prevalence worldwide is small with more new mutations being found. To date, there is no prevalence data in Asian countries with no family study being reported in Malaysia. Therefore, the objectives of this study are to determine the clinical and genetic characteristics of MC in a family suspected with MC. We will perform the clinical examination by conducting a thorough family history (including family pedigree), physical examination, laboratory testing, and neurophysiology studies, i.e., nerve conduction study (NCS) and electromyography (EMG). Genetic studies will be performed to screen the *CLCN1* gene. If a new mutation is found, the presence of the new mutation will be examined in individuals without MC. Gene expression, protein expression and histopathological studies will be performed and require muscle biopsies procedure conducted on the confirmed MC patients and on the individuals without MC condition to obtain muscle tissue samples.

3. WHAT WILL YOU HAVE TO DO?

If you agree to participate in this study:

- a) You will undergo muscle biopsies procedure to obtained muscle tissue samples.
- b) You will have to donate 5 ml of blood for genetic testing.
- c) Muscle biopsies – a minimally invasive minor surgical procedure to obtain small sample of muscle tissue samples.
- d) We will compensate your travel fee to Hospital Pengajar UPM for this study analysis.

4. WHO SHOULD PARTICIPATE IN THE STUDY?

1. You are eligible for this study as a control subject if you are an adult individual without MC condition with age more than 18 years old.

5. WHAT WILL BE THE BENEFITS OF THE STUDY:

(a) TO YOU AS THE SUBJECT?

This is a preliminary research study on mutation screening of MC related genes in a family with MC, therefore this study will not benefit the individuals without MC condition during the research period. The subject will be important to compare on molecular profile and histopathological changes.

(b) TO THE INVESTIGATOR?

Identification of clinical profile, genetic mutations and histopathological changes in muscle tissue of individuals without MC to make a comparison with the condition in MC patients.

6. WHAT ARE THE POSSIBLE RISKS?

1. For blood taking: there is a small risk of hematoma (bruise) at the site of blood drawing; however, this can be avoided by pressing at the place where the needle was injected using cotton until the bleeding stops.
2. For muscle biopsies procedure: Muscle biopsy is a minimally invasive procedure. The associated risks are small that includes pain, redness, swelling or bleeding around the biopsy site. There is small risk of infection to occur at the biopsy site which may present with fever and pain over the biopsy site. However, the reported risk is small and will not cause any harm.
3. You may withdraw from the study at any point of time if you are no longer interested in joining the study.

7. WILL THE INFORMATION THAT YOU PROVIDE AND YOUR IDENTITY REMAIN CONFIDENTIAL?

Your identity and your medical records and data relating to this research study will be kept confidential, except as required by law, and the study sponsor. The leftover sample will be disposed of at the end of the research study unless you consent to donate your blood sample, it will be stored for future studies related to MC.

8. WHO SHOULD YOU CONTACT IF YOU HAVE ADDITIONAL QUESTIONS DURING THE COURSE OF THE RESEARCH?

Dr. Abdul Hanif Khan Yusof Khan
Department of Neurology
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor D.E.
Email: ahanifkhan@upm.edu.my

Dr. Thilakavathy Karupiah
Clinical Genetics Unit,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor D.E.

Please initial here if you have read and understood the contents of this page_____

9. CONSENT

I Identity Card No.....
address.....
..... hereby voluntarily agree to take part in the research stated above.

I have been informed about the nature of the research in terms of methodology, possible adverse effects and complications (as written in the Respondent's Information Sheet). I understand that I have the right to withdraw from this research at any time without giving any reason whatsoever. I also understand that this study is confidential, and all information provided with regard to my identity will remain private and confidential.

☐ I agree to donate my blood sample for future study related to MC.**

☐ I agree to donate my muscle sample for future study related to MC.**

☐ I agree that the images/photos/video recordings/voice recordings related to me to be used in any form of publication or presentation (if applicable).**

* delete where necessary

** tick (/) in the box if you are agree

Signature
(Respondent)

Signature
(Witness)

Date:.....

Name:.....

I/C No.:.....

I confirm that I have explained to the respondent the nature and purpose of the above-mentioned research.

Date:

Signature
(Researcher)



**JAWATANKUASA ETIKA UNIVERSITI UNTUK
PENYELIDIKAN MELIBATKAN MANUSIA (JKEUPM)
UNIVERSITI PUTRA MALAYSIA, 43400 UPM SERDANG,
SELANGOR, MALAYSIA**

**BORANG 2.4: PENERANGAN DAN PERSETUJUAN RESPONDEN
(INDIVIDU TANPA PENYAKIT MC)**

Sila baca maklumat berikut dengan teliti. Sekiranya anda mempunyai sebarang pertanyaan, sila kemukakan kepada penyelidik.

1.TAJUK KAJIAN: Analisis klinikal, molekular dan histopatologikal Myotonia Congenita (MC) dalam satu keluarga di Malaysia

2. PENGENALAN

Myotonia congenita (MC) adalah penyakit genetik yang menyebabkan gangguan pergerakan otot disebabkan mutasi dalam gen *CLCN1*. Pesakit MC akan mengalami kesusahan untuk merehatkan otot dan akan mengalami kelemahan otot. Penyakit ini jarang berlaku di dunia dengan 160 mutasi yang dilaporkan dalam gen ini dan lebih banyak mutasi baru yang dijumpai. Sehingga kini, tiada data kekerapan di negara-negara Asia dan tiada kajian keluarga yang dilaporkan di Malaysia. Oleh itu, objektif kajian ini adalah untuk menentukan ciri-ciri klinikal dan genetik MC dalam keluarga yang disyaki dengan MC. Kami akan menjalankan analisis klinikal dengan menjalankan sejarah keluarga yang menyeluruh (termasuk salasilah keluarga), pemeriksaan fizikal, ujian makmal, dan kajian neurofisiologi, iaitu kajian pengaliran saraf (NCS) dan elektromiografi (EMG). Kajian genetik akan dilakukan untuk menyaring gen *CLCN1*. Jika mutasi baru ditemui, kehadiran mutasi gen akan diperiksa dalam individu tanpa MC. Kajian ekspresi gen, ekspresi protein dan kajian histopatologi akan dijalankan dan memerlukan prosedur biopsi otot dijalankan ke atas pesakit MC yang telah disahkan dan individu tanpa penyakit MC untuk mendapatkan sampel tisu otot.

3. APAKAH YANG PERLU ANDA LAKUKAN?

Jika anda bersetuju untuk mengambil bahagian dalam kajian ini:

- Anda akan menjalani prosedur pembedahan kecil untuk mendapatkan sampel tisu otot.
- Anda perlu menderma 5 ml darah untuk ujian genetik.
- Biopsi otot - prosedur pembedahan kecil dan minor untuk mendapatkan sedikit sampel tisu otot.
- Kami akan membayar kos perjalanan anda ke Hospital Pengajar UPM bagi analisis kajian ini.

4. SIAPA YANG BOLEH MENYERTA KAJIAN INI?

- Anda layak untuk kajian ini sebagai subjek kawalan jika anda adalah individu tanpa penyakit MC berumur lebih 18 tahun.

5. APAKAH FAEDAH MENYERTAI KAJIAN INI?

(a) KEPADA ANDA SEBAGAI PESERTA?

Ini adalah kajian penyelidikan awal mengenai penyingkiran mutan bagi gen yang berkaitan dengan MC dalam keluarga dengan MC, oleh itu kajian ini tidak akan memberi manfaat kepada individu tanpa penyakit MC semasa tempoh penyelidikan. Subjek adalah penting untuk membuat perbandingan profil molekular dan perubahan histopatologikal.

(b) KEPADA PENYELIDIK?

Pengenalan profil klinikal, mutasi genetik dan perubahan histologi tisu otot bagi individu tanpa penyakit MC bagi membuat perbandingan dengan keadaan pesakit MC.

6. ADAKAH IA BERISIKO?

1. Untuk pengambilan darah: terdapat risiko kecil hematoma (lebam) di tempat lukisan darah; Walau bagaimanapun, ini boleh dielakkan dengan menekan di tempat jarum disuntik menggunakan kapas sehingga pendarahan berhenti.
2. Untuk prosedur biopsi otot bagi mendapatkan sampel tisu otot: biopsi otot adalah prosedur invasive minimum, dan risiko yang berkaitan adalah kecil termasuk kesakitan, kemerahan, bengkak atau pendarahan di sekitar kawasan biopsi. Terdapat risiko kecil jangkitan berlaku di kawasan biopsi yang mungkin disertai dengan demam dan sakit di kawasan biopsi. Namun, risiko adalah kecil dan tidak akan mengundang bahaya.
3. Anda boleh berhenti dari menyertai kajian ini pada bila-bila masa sekiranya anda tidak lagi berminat untuk mengikuti kajian ini.

7. ADAKAH MAKLUMAT DAN IDENTITI SAYA KEKAL RAHSIA?

Identiti dan rekod perubatan anda dan data yang berkaitan dengan kajian penyelidikan ini akan dirahsiakan, kecuali seperti yang dikehendaki oleh undang-undang, dan penaja kajian. Baki sampel darah akan dilupuskan pada akhir kajian penyelidikan melainkan jika anda bersetuju menderma sampel darah anda, ia akan disimpan untuk kajian masa depan yang berkaitan dengan MC.

8. SIAPA YANG SAYA PERLU DIHUBUNGI SEKIRANYA SAYA MEMPUNYAI SOALAN TAMBAHAN SEMASA MENGIKUTI PENYELIDIKAN INI?

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Dr.Thilakavathy Karupiah
Unit Genetik Klinikal,
Fakulti Perubatan Dan Sains Kesihatan,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor D.E.

____ Sila tandatangan di sini sekiranya anda telah membaca dan memahami kandungan halaman ini ____

9. PERSETUJUAN

Saya No Kad Pengenalan
beralamat.....
..... dengan ini bersetuju untuk mengambil bahagian secara sukarela dalam penyelidikan yang tersebut di atas *(kajian klinikal/percubaan ubat-ubatan/rakaman video/kumpulan sasaran/temuduga/ soal selidik).

Saya telah diberi penjelasan secara menyeluruh mengenai penyelidikan ini dari segi metodologi, risiko dan komplikasi (seperti tertulis pada Helaian Penerangan Responden). Saya memahami bahawa saya berhak menarik diri dari penyelidikan ini pada bila-bila masa tanpa memberi sebarang alasan. Saya juga memahami bahawa sebarang maklumat yang berkaitan identiti saya akan dirahsiakan.

- ☐ Saya bersetuju untuk menderma sampel darah saya untuk kajian masa depan yang berkaitan dengan MC**
- ☐ Saya bersetuju untuk menderma sampel otot saya untuk kajian masa depan yang berkaitan dengan MC**
- ☐ Saya bersetuju bahawa rakaman gambar / gambar / video / rakaman suara yang berkaitan dengan saya digunakan dalam sebarang bentuk penerbitan atau pembentangan (jika berkenaan)**

* potong mana yang berkenaan

** tanda (/) di dalam kotak yang anda setuju

Tandatangan
(Responden)

Tandatangan
(Saksi)

Tarikh :

Nama :

No. K/P:

Saya mengesahkan bahawa saya telah menerangkan kepada responden ini sifat dan tujuan penyelidikan yang tersebut di atas.

Tarikh

Tandatangan
(Penyelidik)

Appendix A4. Short exercise test (SET)

Proband			PB		
Time Interval (s)	Amplitude	Relative Amplitude	Time Interval (s)	Amplitude	Relative Amplitude
	mV	%		mV	%
0	9.4	100	0	8.9	100
10	8.5	90.8	10	7.2	81.2
20	7.4	79.2	20	10	113
30	8.3	88.9	30	10.8	121
40	8.5	90.4	40	11.2	126
50	8.8	93.4	50	11	124
60	8.6	91.4	60	11.3	127
70	8.3	88	70	10	112
80	7.6	80.9	80	8.7	98.2
90	8.2	87.4	90	6.8	76.8
100	8.6	91.6	100	8.2	91.9
110	8.8	93.6	110	9.6	108
120	8.8	93.6	120	10.2	115
130	8.3	88	130	9.6	108
140	7.7	82	140	8	90.6
150	8.2	87.2	150	8.8	99.7
160	8.8	93.6	160	9.3	105
170	9.2	98.3	170	9.5	107
180	9.1	97.4	180	9.7	109

Average (MC Patients)		
Time Interval (s)	Relative amplitude	SEM
	%	
0	100	0.0
10	86	4.8
20	96.1	16.9
30	104.95	16.1
40	108.2	17.8
50	108.7	15.3
60	109.2	17.8
70	100	12.0
80	89.55	8.7
90	82.1	5.3
100	91.75	0.2
110	100.8	7.2
120	104.3	10.7
130	98	10.0
140	86.3	4.3
150	93.45	6.3
160	99.3	5.7
170	102.65	4.4
180	103.2	5.8

Child 1 (C1)

Time Interval (s)	Amplitude	Relative Amplitude
	mV	%
0	7.8	100
10	7.2	92.7
20	7.6	97.4
30	7.6	98.1
40	7.9	102
50	8.1	104
60	8.2	106
70	8.5	109
80	8.2	106
90	8.4	108
100	8.6	110
110	8.5	110
120	8.6	111
130	8.6	111
140	8.3	107
150	8.3	107
160	8.3	107
170	8.2	105
180	8.2	106

Child 2 (C2)

Time Interval (s)	Amplitude	Relative Amplitude
	mV	%
0	6.6	100
10	6.8	103
20	5.9	90.3
30	6.2	94.4
40	6.1	92.8
50	6	90.7
60	6	91.7
70	8.4	128
80	8.3	127
90	7.8	119
100	7.9	121
110	7.8	119
120	7.6	116
130	8	122
140	8.7	132
150	8.8	135
160	8.9	135
170	8.9	136
180	9	137

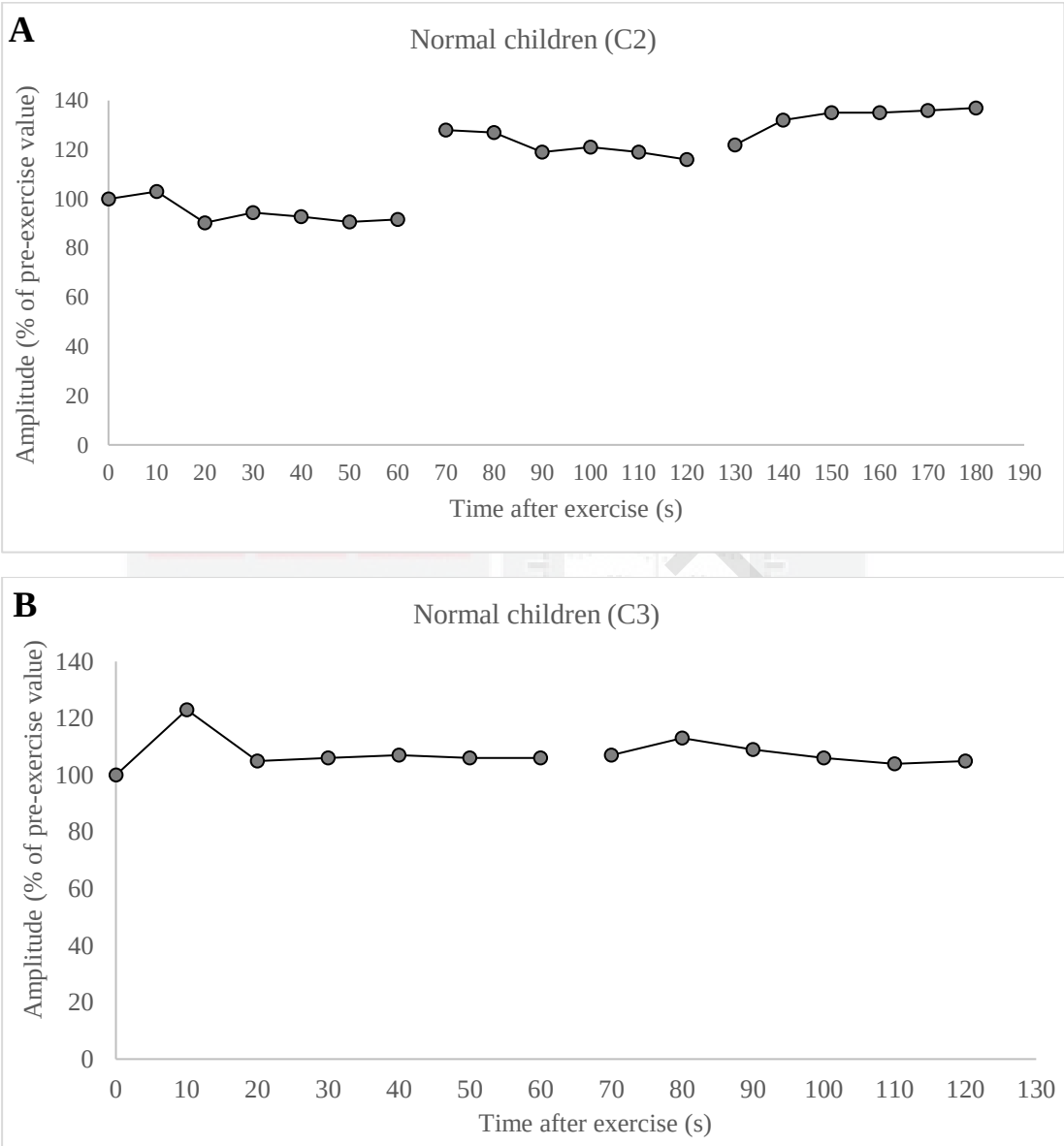
Child 3 (C3)

Time Interval (s)	Amplitude	Relative Amplitude
	mV	%
0	6.6	100
10	8.2	123
20	7	105
30	7	106
40	7.1	107
50	7.1	106
60	7	106
70	7.1	107
80	7.5	113
90	7.2	109
100	7.1	106
110	6.9	104
120	7	105

**Average
(Normal children)**

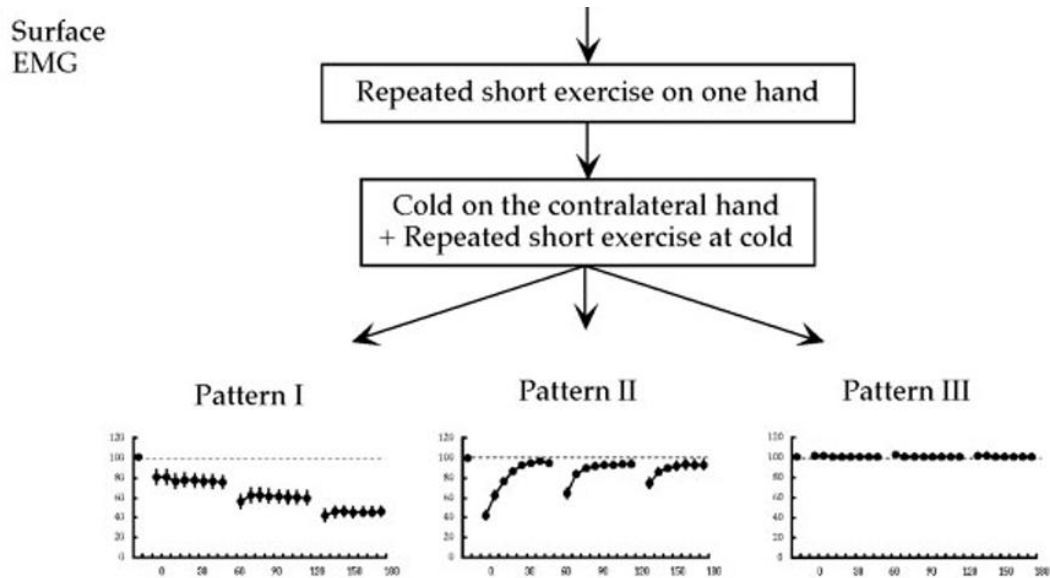
Time Interval (s)	Relative amplitude	SEM
	%	
0	100	0.0
10	106.2	8.9
20	97.6	4.2
30	99.5	3.4
40	100.6	4.2
50	100.2	4.8
60	101.2	4.8
70	114.7	6.7
80	115.3	6.2
90	112.0	3.5
100	112.3	4.5
110	111.0	4.4
120	110.7	3.2
130	116.5	4.5
140	119.5	10.2
150	121.0	11.4
160	121.0	11.4
170	120.5	12.7
180	121.5	12.7

Appendix A5. Repeated short exercise test (ET) normal children.



Repeated short exercise test in normal children. (A) Child 2 and (B) Child 3 showed no reduction in CMAP post SET. The amplitude of the CMAP, expressed as a percentage of its value before the trials, is plotted against the time elapsed after the first exercise trials.

Appendix A6. Fournier Pattern graph. Electromyography (EMG) patterns obtained by repeated short exercise test showing the different patterns that determine the Fournier pattern.

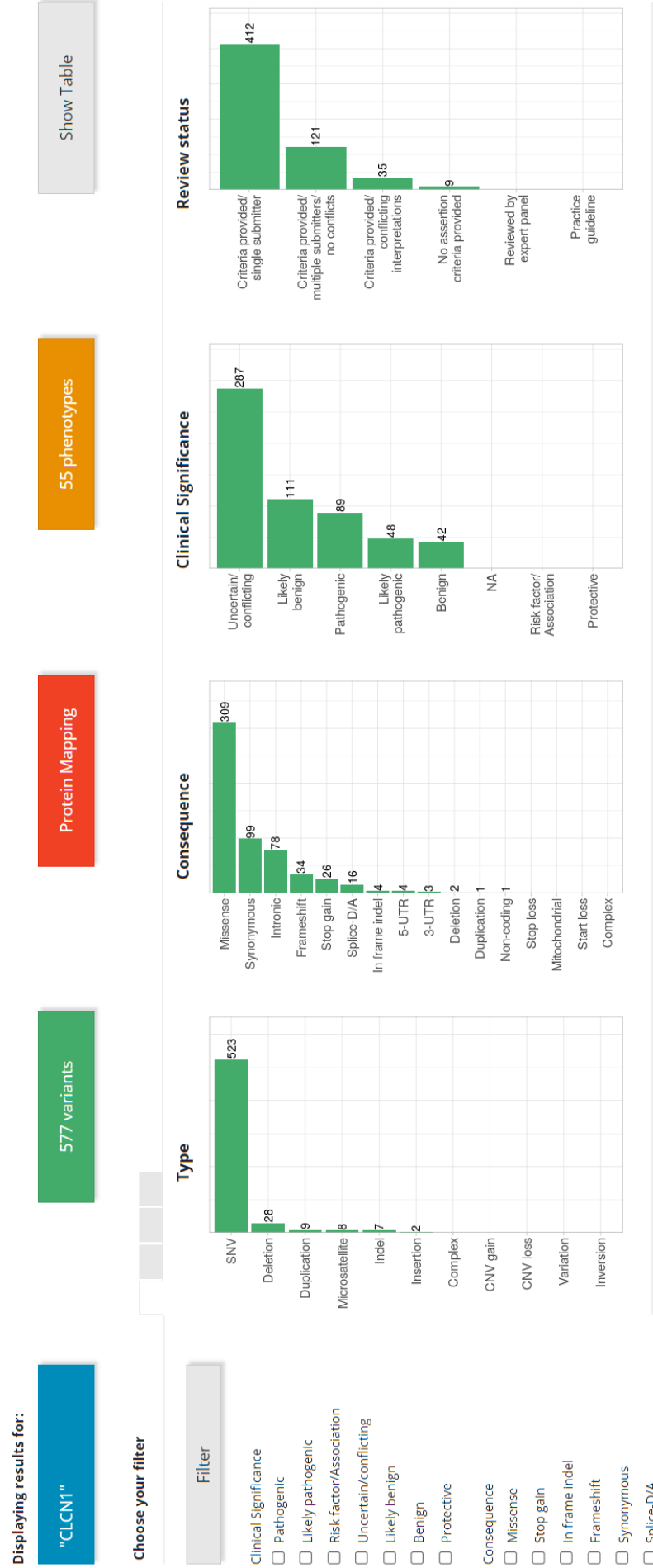


(Source: Fournier et al., 2006)

Appendix B1. ACMG variant classification criteria for benign variant. The criteria were based on the ACMG guidelines (Richard et al., 2015).

ACMG Criteria		Definition
Stand-alone	BA1	Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium
	BS1	Allele frequency is greater than expected for disorder
Strong	BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age
	BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing
	BS4	Lack of segregation in affected members of a family Caveat: The presence of phenocopies for common phenotypes (i.e., cancer, epilepsy) can mimic lack of segregation among affected individuals. Also, families may have more than one pathogenic variant contributing to an autosomal dominant disorder, further confounding an apparent lack of segregation.
	BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease
Supporting	BP2	Observed in <i>trans</i> with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in <i>cis</i> with a pathogenic variant in any inheritance pattern
	BP3	In-frame deletions/insertions in a repetitive region without a known function
	BP4	Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant.
	BP5	Variant found in a case with an alternate molecular basis for disease
	BP6	Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation
	BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved

Appendix B2. Summary on *CLCN1* variants by Simple ClinVar shows missense variant is a common mechanism. (Source: <https://simple-clinvar.broadinstitute.org/>, last accessed 25 September 2024)



577 variants

Protein Mapping

55 phenotypes

Show Table

Type

Consequence

Clinical Significance

Review status

SNV 523

Deletion 28

Duplication 9

Microsatellite 8

Indel 7

Insertion 2

Complex

CNV gain

CNV loss

Variation

Inversion

Missense 309

Synonymous 99

Intronic 78

Frameshift 34

Stop gain 26

Splice-D/A 16

In frame indel 4

5-UTR 4

3-UTR 3

Deletion 2

Duplication 1

Non-coding 1

Stop loss

Mitochondrial

Start loss

Complex

Uncertain/conflicting 287

Likely benign 111

Pathogenic 89

Likely pathogenic 48

Benign 42

NA

Risk factor/Association

Protective

Criteria provided/single submitter 412

Criteria provided/multiple submitters/no conflicts 121

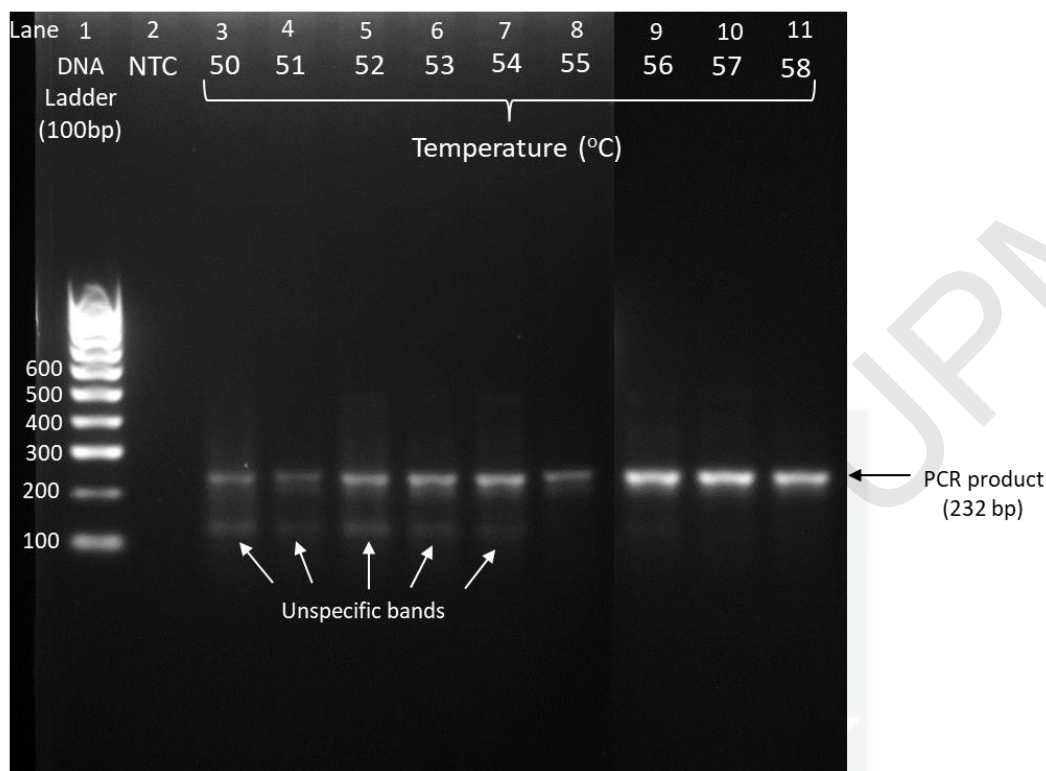
Criteria provided/conflicting interpretations 35

No assertion criteria provided 9

Reviewed by expert panel

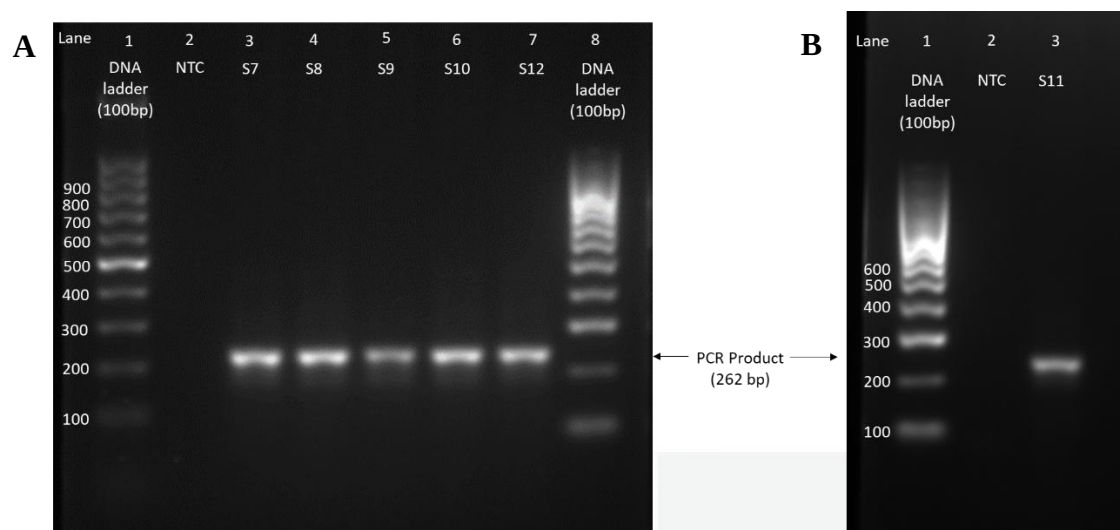
Practice guideline

Appendix B3. The temperature (58 °C) was identified as the best annealing temperature.



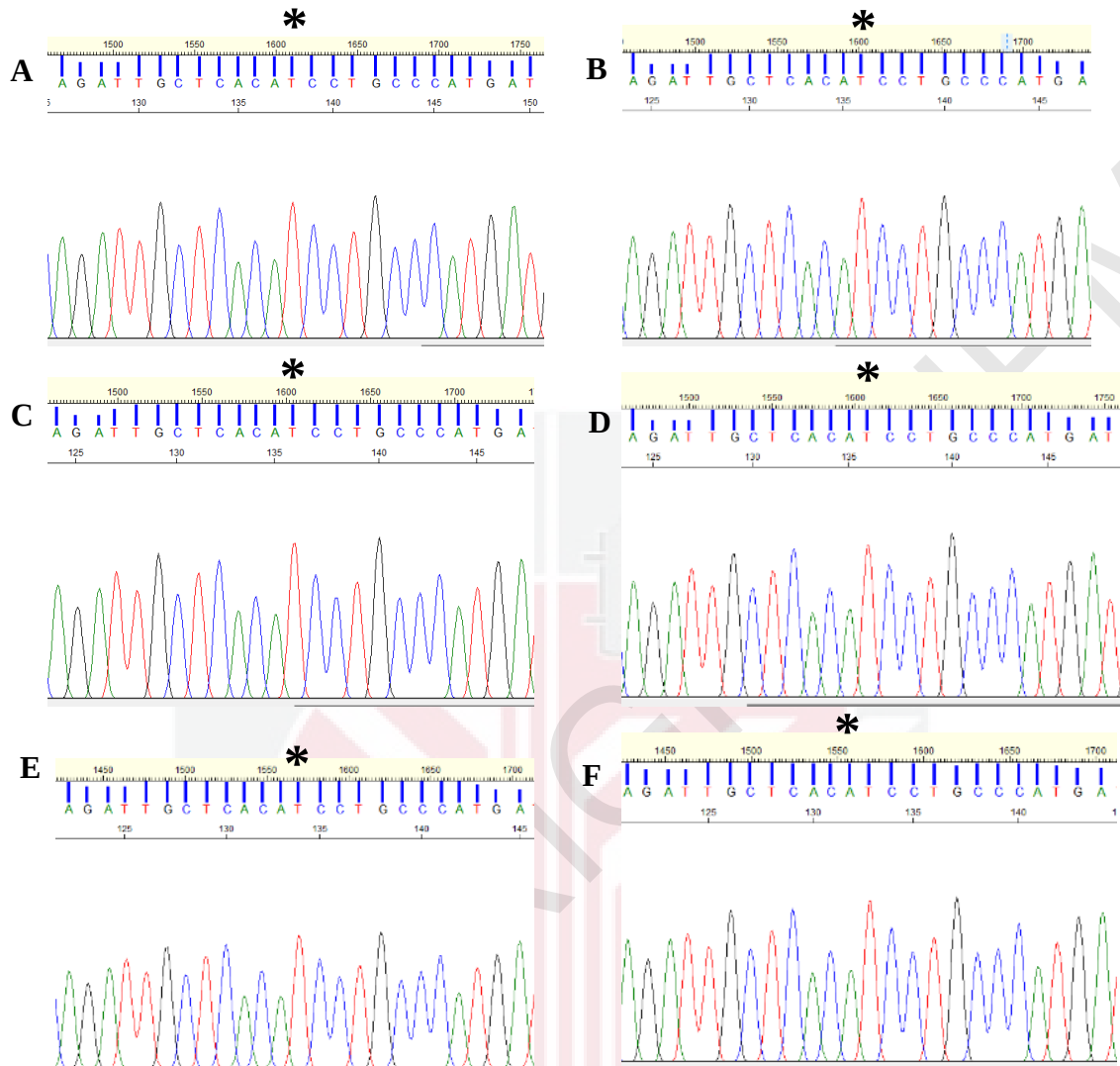
Gel electrophoresis of thermal gradient PCR. Gel electrophoresis was performed on 1.5% agarose gel using a 100 bp DNA ladder as a molecular weight marker, and the PCR products from different annealing temperature (50 – 58 °C) were loaded at lane 3 – 11. Lane 1: 100 bp DNA ladder, lane 2: NTC, lane 3: 50 °C, lane 4: 51 °C, lane 5: 52 °C, lane 6: 53 °C, lane 7: 54 °C, lane 8: 55 °C, lane 9: 56 °C, lane 10: 57 °C and lane 11: 58 °C. Annealing temperature at 58 °C gave the clearest, and specific band at 232 bp while other temperatures showed unspecific bands.

Appendix B4. Gel electrophoresis of PCR reaction from second-degree relatives.



Gel electrophoresis of PCR reaction. Gel electrophoresis was performed on 1.5% agarose gel using a 100 bp DNA ladder as a molecular weight marker. (A) Lane 1: 100 bp DNA ladder, lane 2: no template control (NTC), lane 3: S7 (III:3), lane 4: S8 (III:4), lane 5: S9 (III:8), lane 6: S10 (II:5), lane 7: S12 (III:19), lane 8: 100 bp DNA ladder. (B) Lane 1: 100 bp DNA ladder, lane 2: NTC, and lane 3: S11 (III:18). Lane 3 – 7 (A) and Lane 3 (B) represent the amplification of 232 bp sequence of interest contained c.1667T>A (p.Ile556Asn) variant.

Appendix B5. Sanger sequencing of second-degree relatives



Sanger sequencing chromatogram. Forward sequence traces showing a homozygous wild-type genotype (T/T, homozygous) in Proband's second-degree relatives, indicating an absence of the c.1667T>A (p.Ile556Asn) variant in (A) S7 (III:3), (B) S8 (III:4), (C) S9 (III:8), (D) S10 (II:5), (E) S11 (III:18) and (F) S12 (III:19). Asterisk (*) shows the wild-type allele.

Appendix B6. MLPA internal validation for the reference samples showed the standard deviation ≤ 0.10 for all probes in the experiment.

	Probe target info	AllSamples										
		Intra					Inter					
		Mean	First qu...	Median	Third q...	Min	Maximu...	Stdev	MAD			
07q (n=26)	CASP2-13	0.99	0.93	1	1.06	0.89	1.12	0.08	0.05			
	CLCN1-1	1.19	0.96	1	1.04	0.95	1.04	0.04	0.04			
	CLCN1-2	1.01	0.92	1	1.01	0.9	1.04	0.05	0.04			
	CLCN1-3	0.99	0.99	1	1.02	0.95	1.06	0.04	0.05			
	CLCN1-4	1.58	1.01	1	1.02	0.99	1.04	0.02	0.01			
	CLCN1-5	1.05	0.95	1	1.06	0.92	1.12	0.07	0.04			
	CLCN1-6	1.21	0.98	1	1.02	0.96	1.04	0.03	0.02			
	CLCN1-7	1.16	0.99	1	1.01	0.95	1.03	0.03	0.02			
	CLCN1-8	1.08	0.98	1	1.01	0.97	1.02	0.02	0.01			
	CLCN1-9	1.05	0.98	1	1.02	0.9	1.06	0.06	0.05			
	CLCN1-10	1.17	0.98	1	1.01	0.9	1.03	0.04	0.03			
	CLCN1-11	1.03	0.99	1	1.01	0.94	1.02	0.03	0.02			
	CLCN1-12	1.02	0.99	1	1.04	0.92	1.05	0.05	0.05			
	CLCN1-13	0.95	0.98	1	1.04	0.95	1.07	0.04	0.03			
	CLCN1-14	0.99	1	1	1.01	0.99	1.01	0.01	0.01			
	CLCN1-15	0.89	0.98	1	1.02	0.95	1.02	0.03	0.02			
	CLCN1-16	1.24	0.99	1	1.01	0.96	1.01	0.02	0.01			
	CLCN1-17	1.14	0.99	1	1.01	0.94	1.05	0.03	0.03			
	CLCN1-17	1.1	0.97	1	1.01	0.9	1.01	0.05	0.01			
	CLCN1-18	0.92	0.98	1	1.01	0.97	1.03	0.02	0.02			
	CLCN1-19	1.06	1.01	1	1.01	1	1.03	0.01	0			
	CLCN1-20	1.24	1.03	1	1.09	0.95	1.11	0.06	0.05			
	CLCN1-21	1.09	0.98	1	1.01	0.89	1.04	0.05	0.04			
	CLCN1-22	0.89	0.99	1	1.02	0.94	1.03	0.03	0.03			
	CLCN1-23	1.28	0.98	1	1.01	0.97	1.03	0.02	0.01			
17q (n=5)	FAM131B-08	1.13	1.01	1	1.03	0.97	1.06	0.03	0.02			
	KCNJ2-1	1.09	0.99	1	1.03	0.89	1.06	0.06	0.04			
	KCNJ2-1	1.2	1.01	1	1.04	0.98	1.07	0.03	0.02			
	KCNJ2-2	0.93	0.99	1	1.02	0.94	1.06	0.05	0.06			
	KCNJ2-2	1.12	0.98	1	1.02	0.97	1.03	0.02	0.02			
	KCNJ2-2	1.04	0.99	1	1.01	0.99	1.02	0.01	0.01			
References...	Reference	02-227.843800	0.97	1	1.01	0.88	1.01	0.05	0.01			
	Reference	04-055.298100	1	0.99	1	0.99	1.02	0.01	0.01			
	Reference	05-132.037620	1	0.99	1	0.97	1.01	0.01	0.01			
	Reference	06-168.809400	0.99	0.99	1	0.89	1.07	0.07	0.06			
	Reference	14-076.842490	0.88	0.98	1	0.91	1.02	0.04	0.02			
	Reference	15-046.490040	1.11	1	1	0.99	1.02	0.01	0.01			
	Reference	16-009.939680	0.99	0.98	1	0.95	1.05	0.03	0.02			
	Reference	18-051.279450	1.04	1.03	1	1	1.1	0.04	0			

Appendix B7. Fragment analysis scores were confirmed to displayed four green bars and icons.

Experiment: 2nd run (Adjust bin)

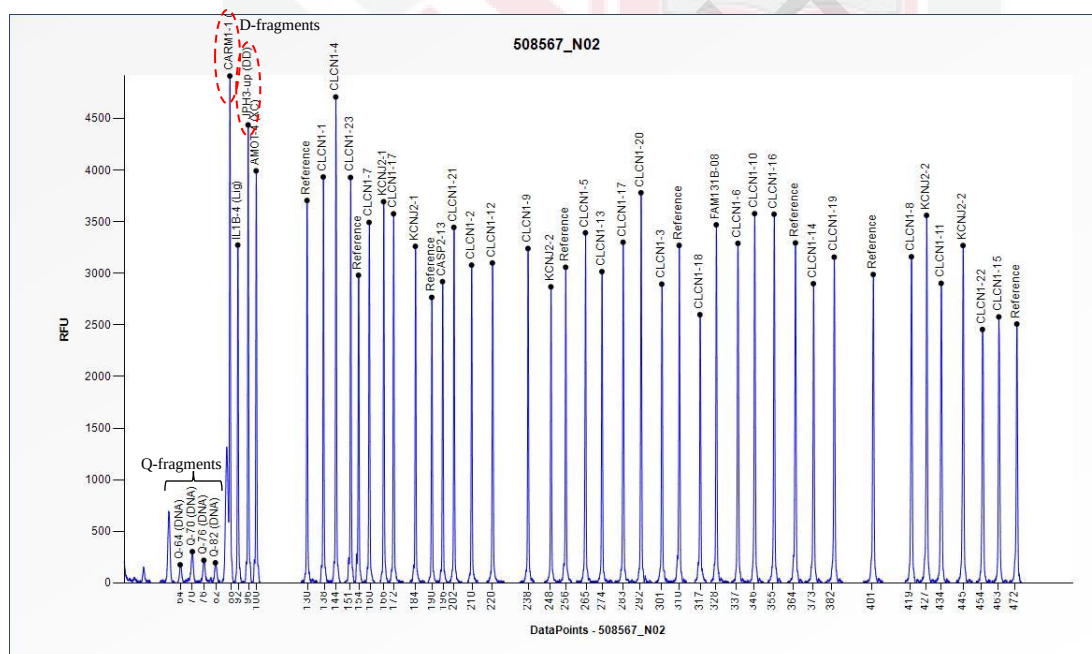
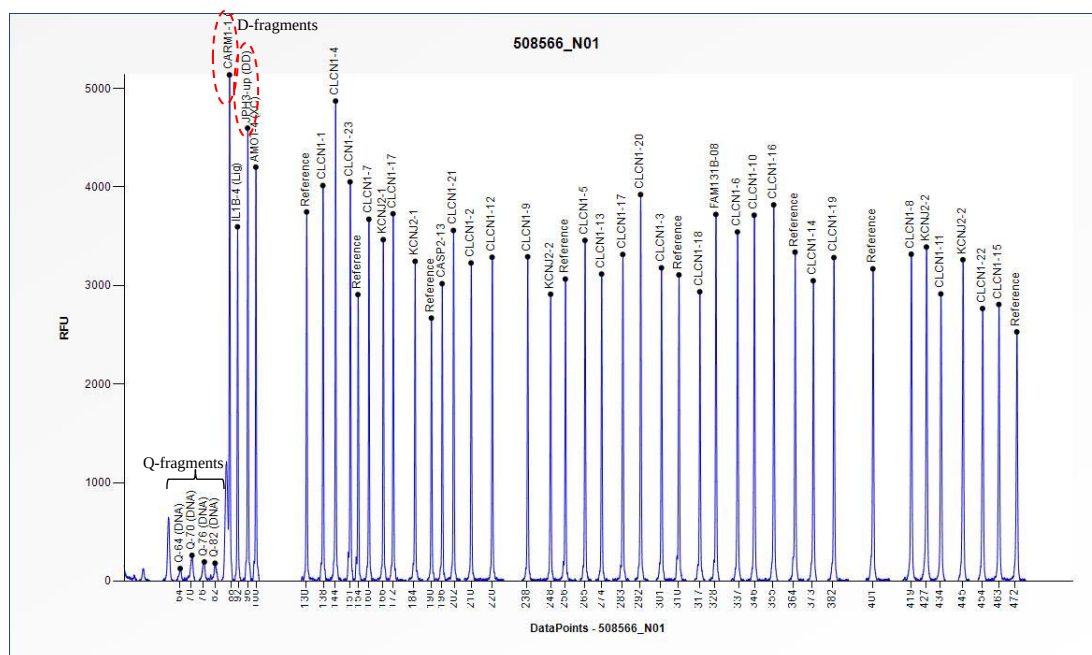
details fragment analysis comparative analysis

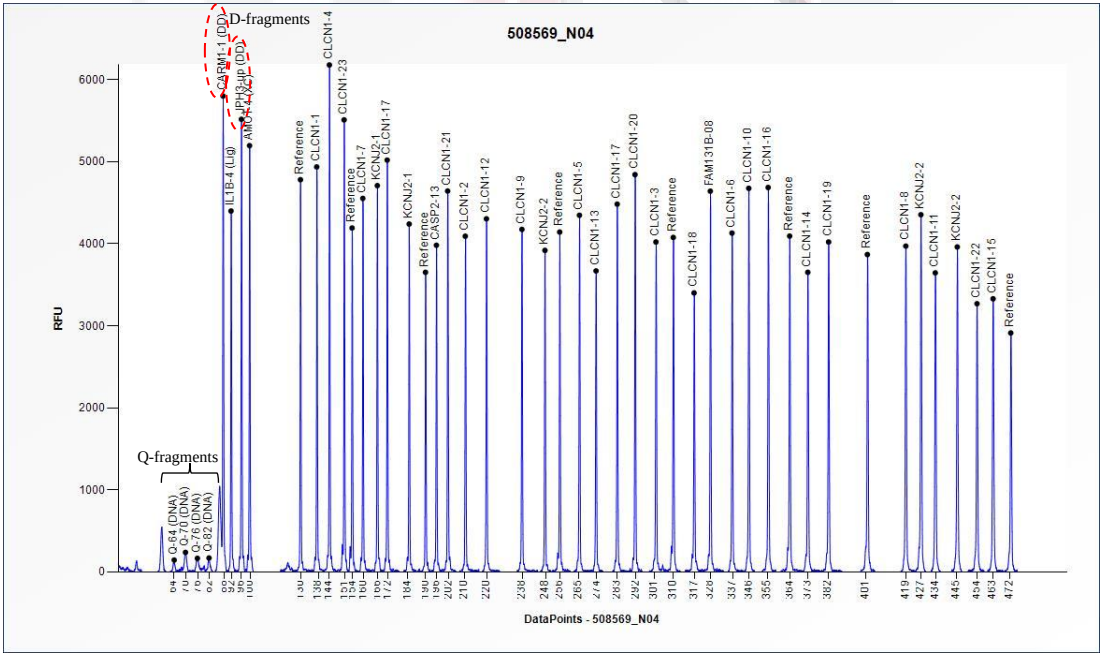
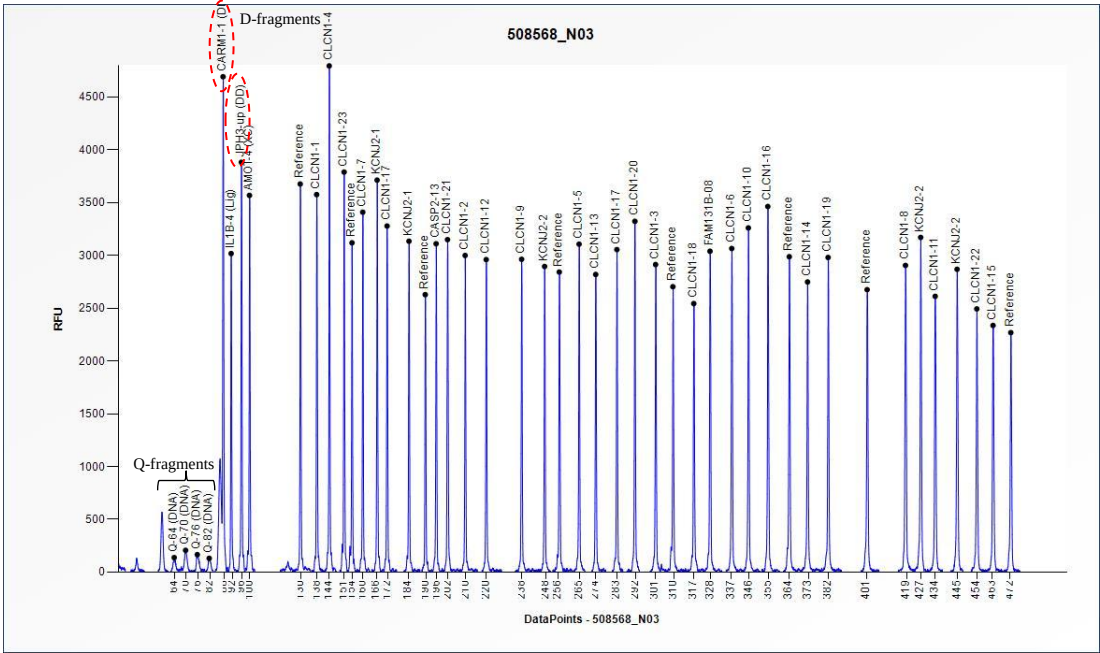
	sample name	sample type	bin smpl	FRSS	FMRS	probes	DNA	DD	X	Y	Pos
1	508566_N01	reference	☐			39/39					A7
2	508567_N02	reference	☐			39/39					B7
3	508568_N03	reference	☐			39/39					C7
4	508569_N04	reference	☐			39/39					D7
5	508570_N06	reference	☐			39/39					E7
6	508571_S01	sample	☐			39/39					F7
7	508572_S03	sample	☐			39/39					G7
8	508573_S04	sample	☐			39/39					H7
9	508574_S05	sample	☐			39/39					A8
10	508575_S06	sample	☐			39/39					B8
11	508576_NTC	no DNA	☐			0/0					C8

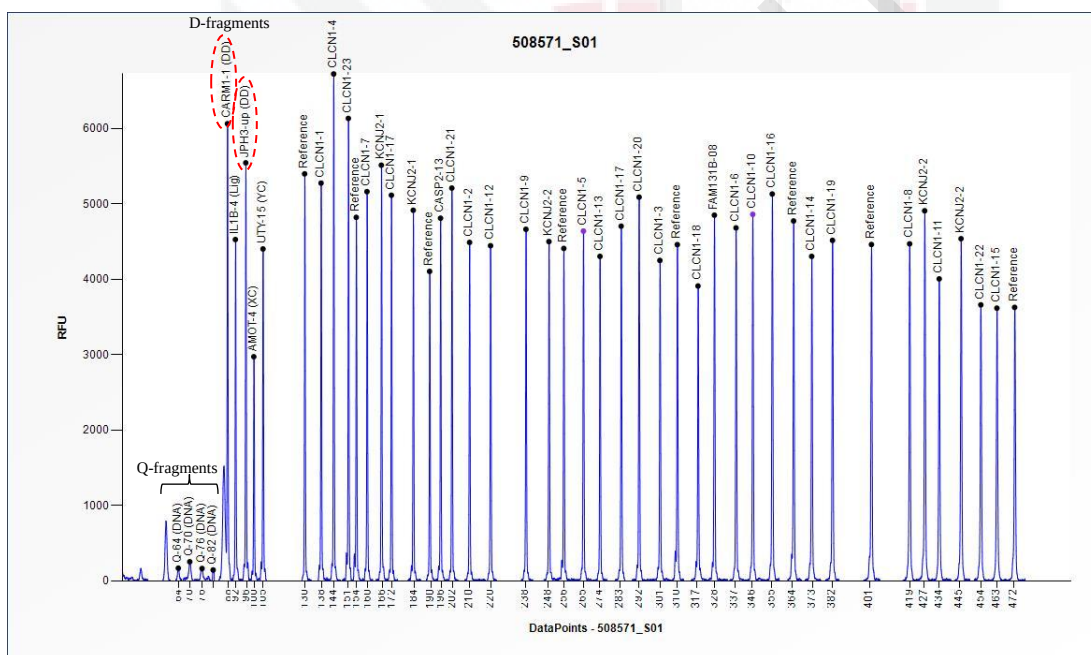
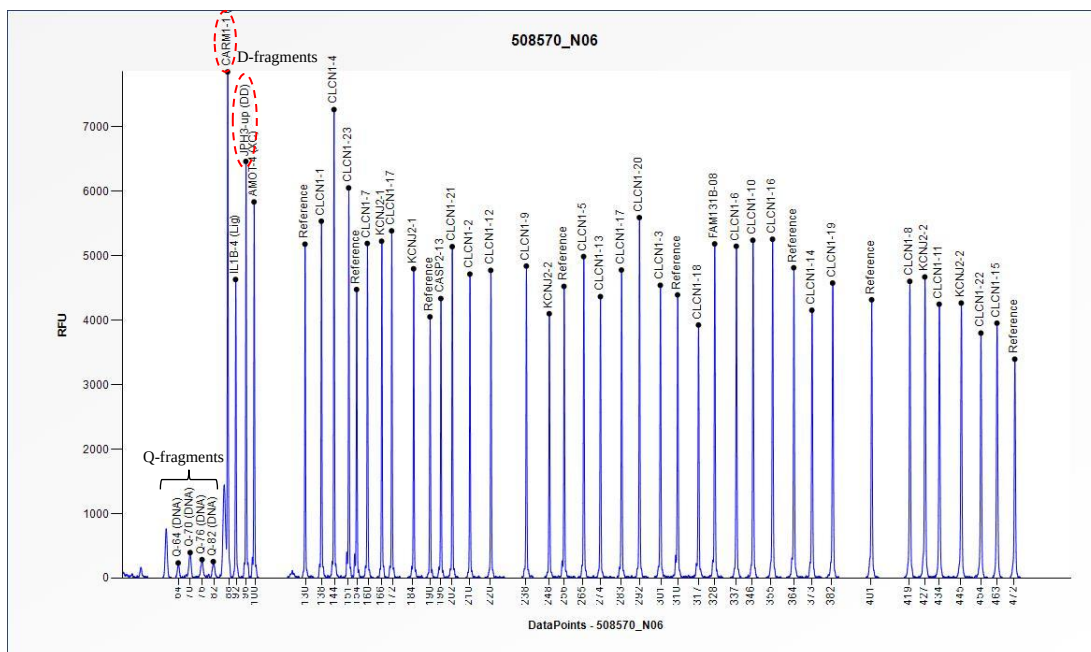
details fragment analysis comparative analysis

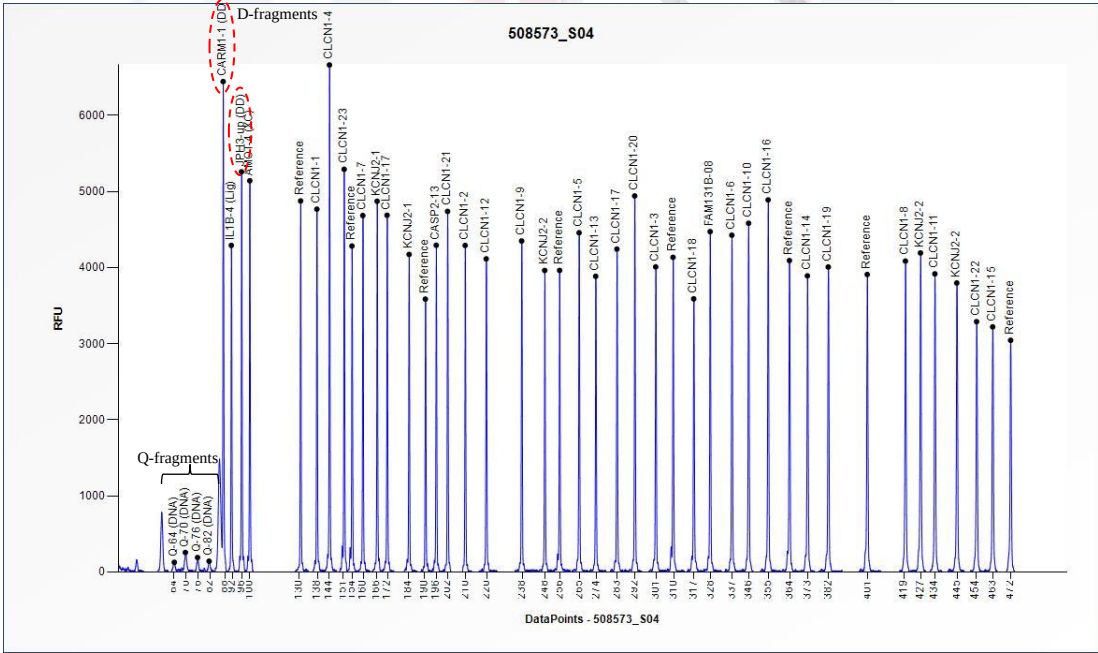
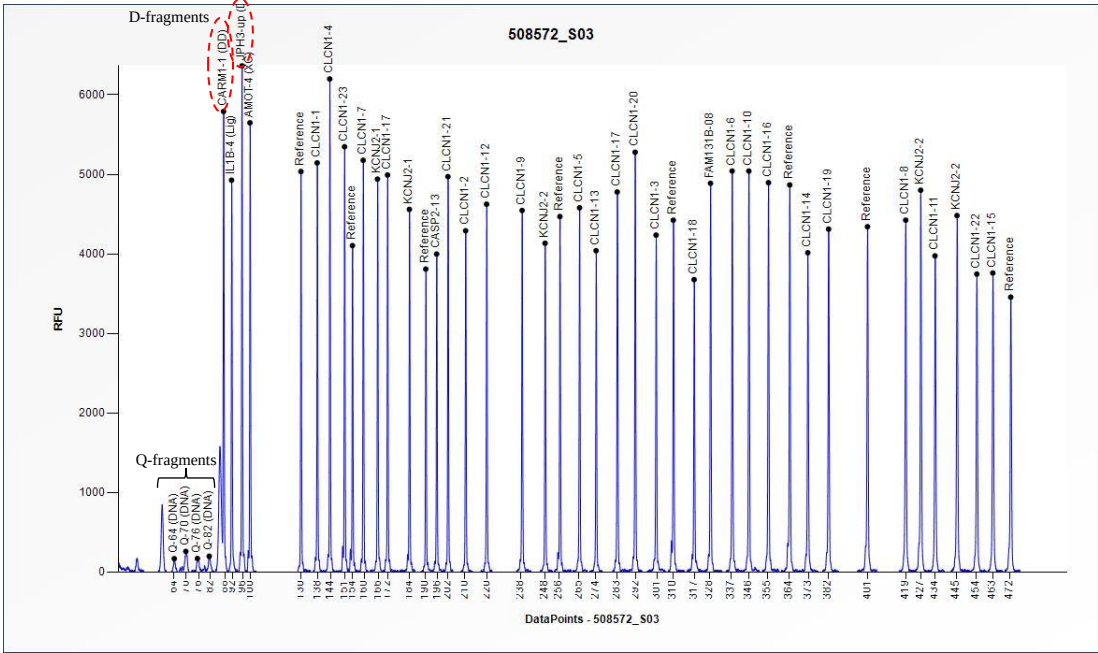
	sample name	sample type	FMRS	X	Y	gender	analy...	CAS	PSLP	FSLP	RSQ	RPQ
1	508566_N01	reference				female	☐					
2	508567_N02	reference				female	☐					
3	508568_N03	reference				female	☐					
4	508569_N04	reference				female	☐					
5	508570_N06	reference				female	☐					
6	508571_S01	sample				male	☐					
7	508572_S03	sample				female	☐					
8	508573_S04	sample				female	☐					
9	508574_S05	sample				female	☐					
10	508575_S06	sample				male	☐					

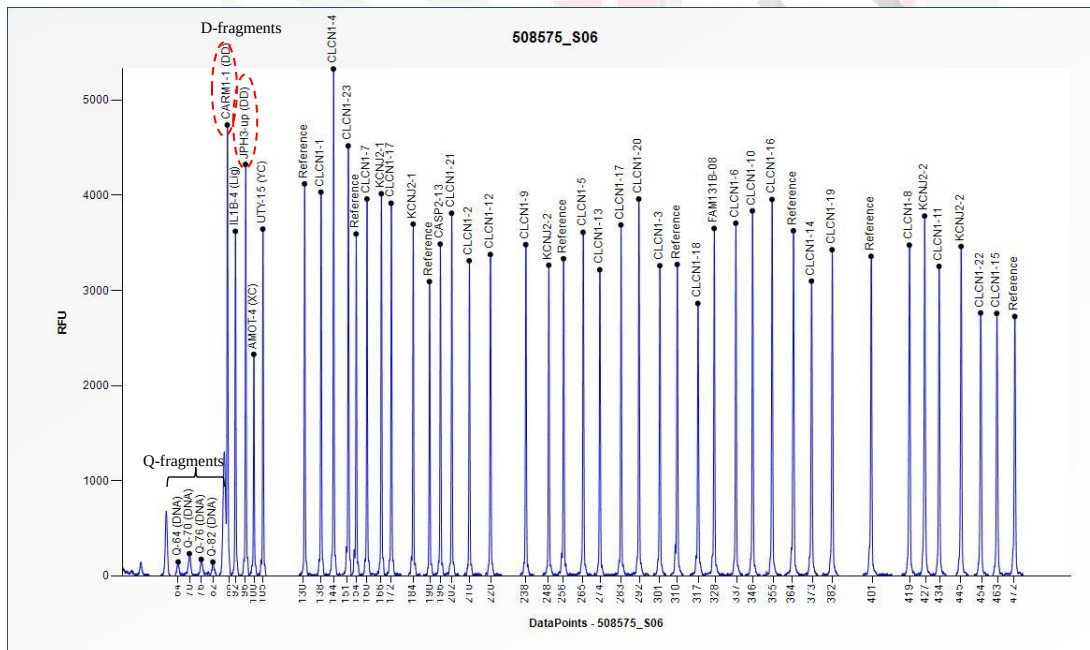
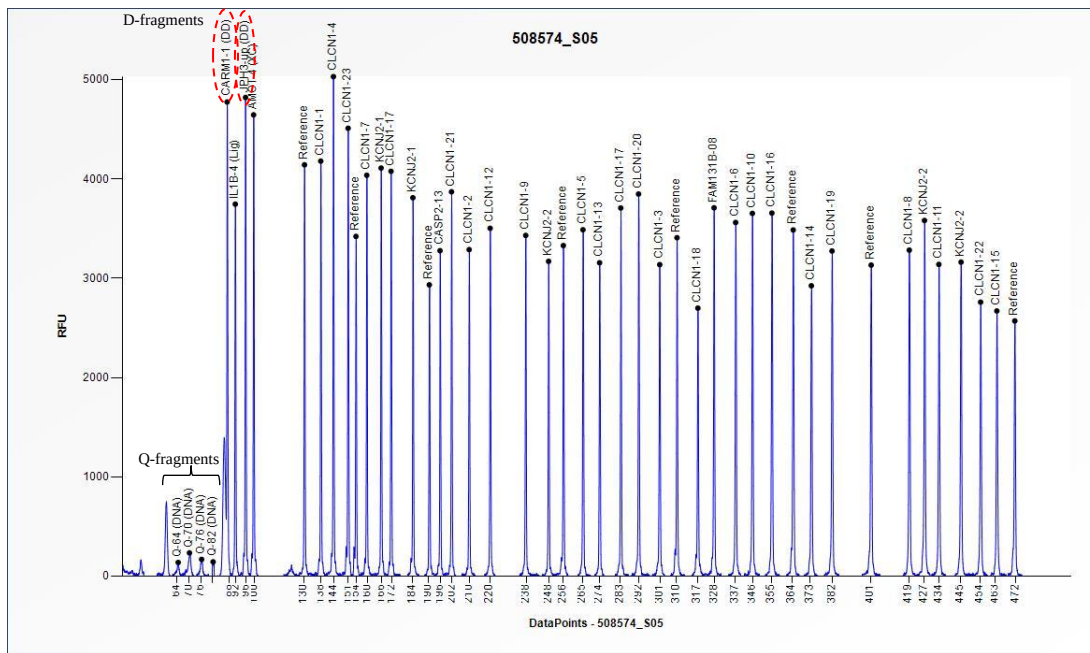
Appendix B8. Internal control fragments.





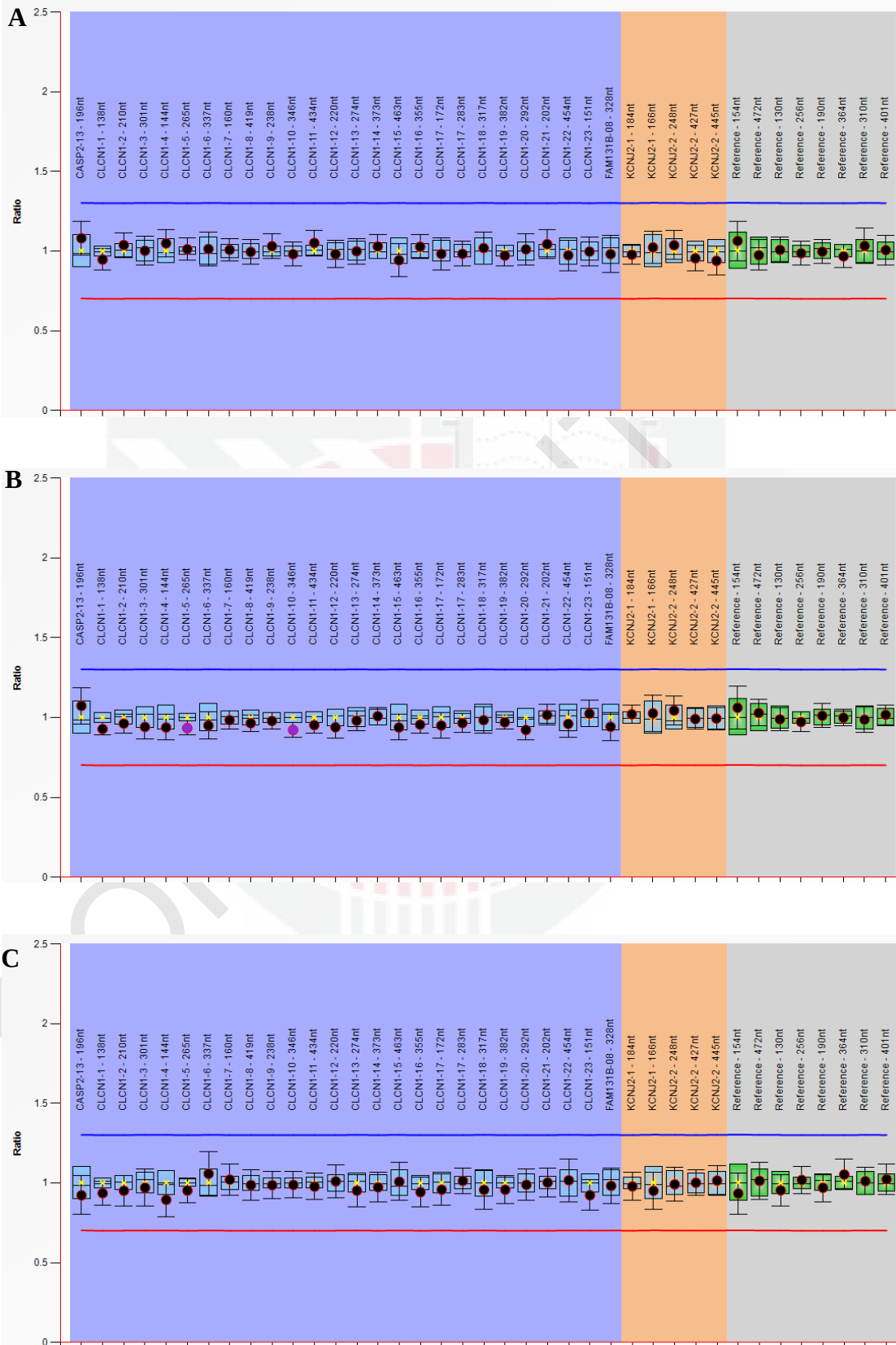


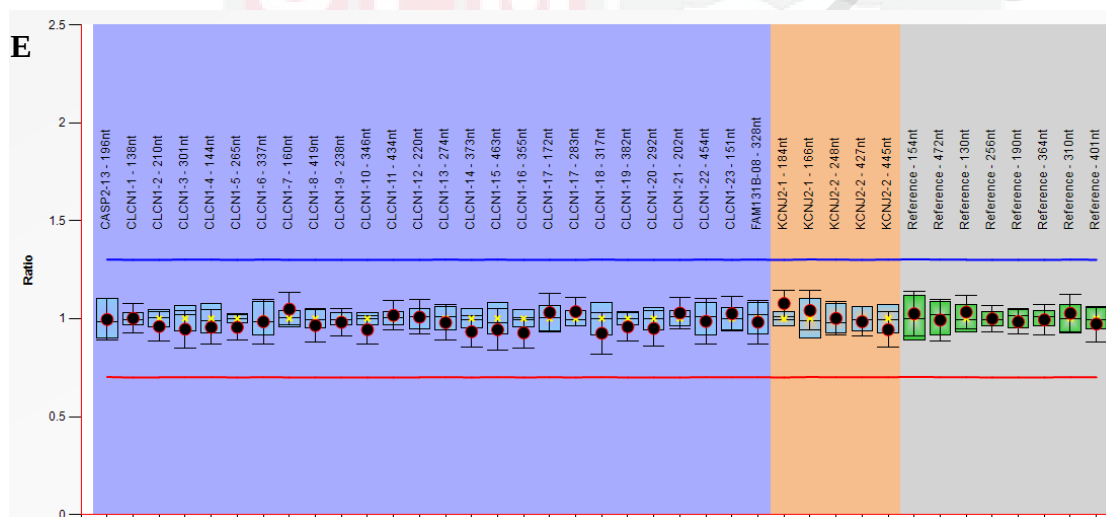
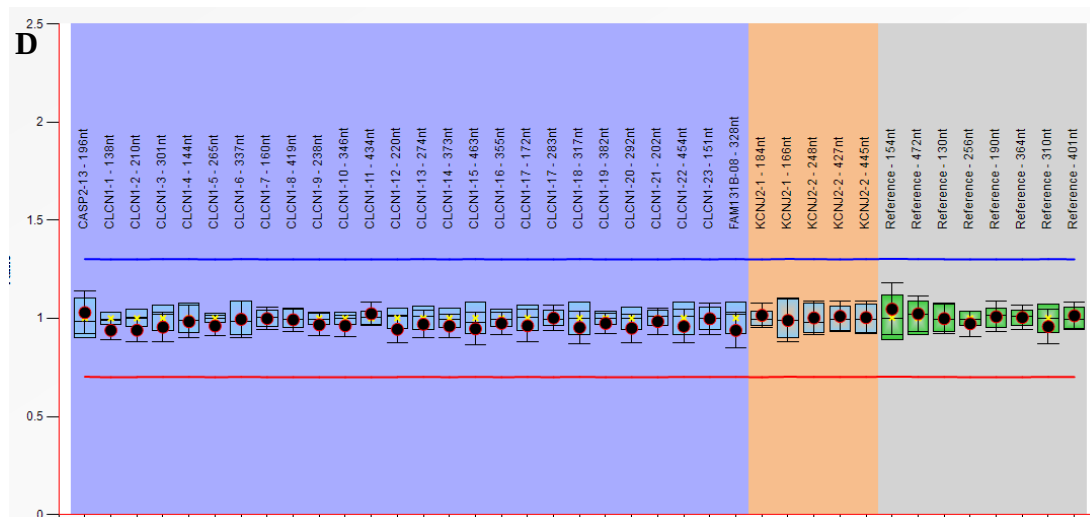




Internal control fragments in four reference samples (N01, N02, N03, N04, N06), Proband's brother (PB, S01), PB's daughter (C1, S03), Proband (S04), Proband's son (C2, S05) and Proband's daughter (C3, S06). The four Q-fragments (64, 70, 76 and 82 nt) show decreased in height indicated in all samples indicate sufficient DNA addition and successful ligation. The two D-fragments (88 and 96 nt) (dashed circle) show increased in height in all samples indicate a complete and sufficient denaturation.

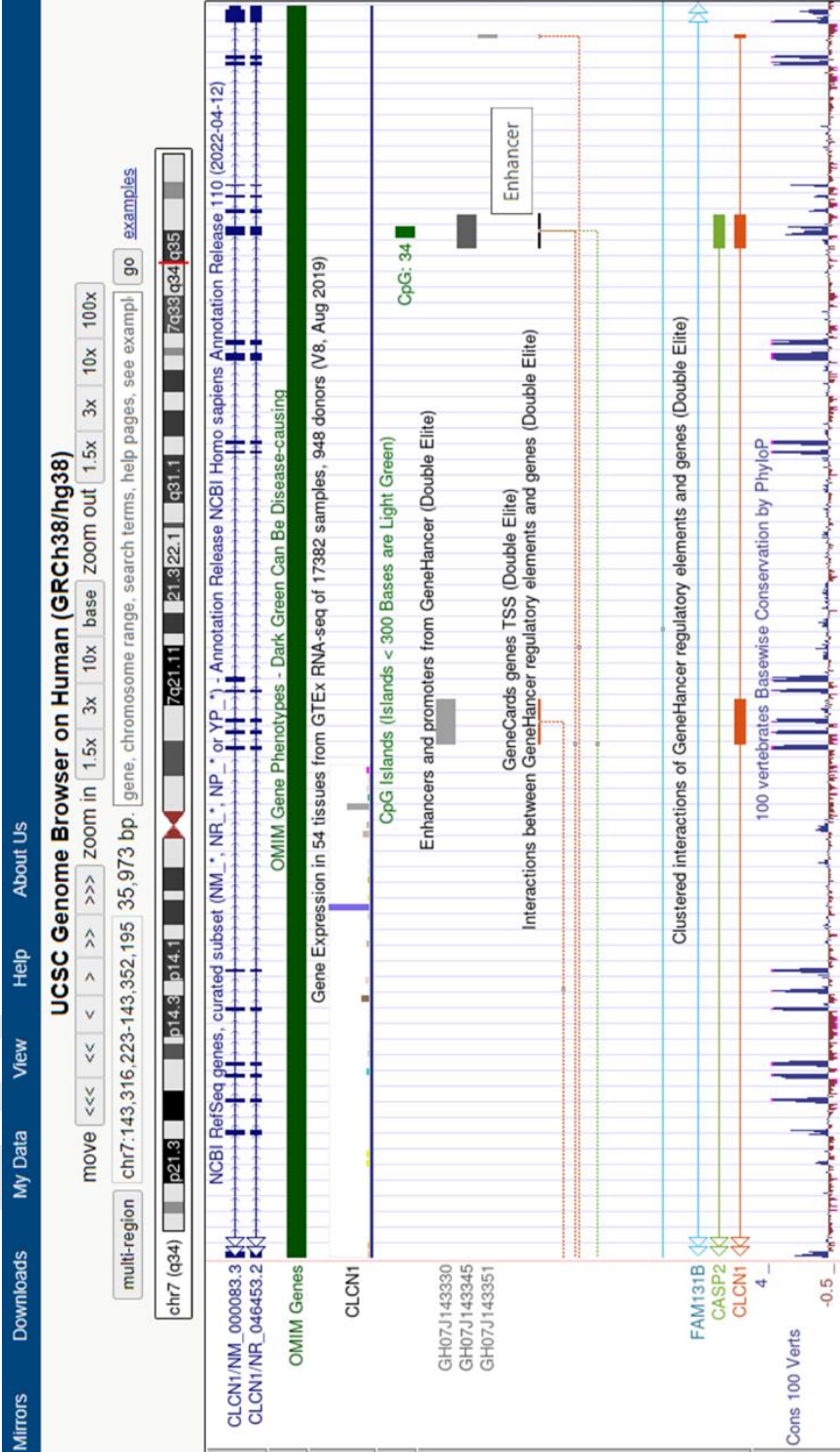
Appendix B9. Ratio charts of MLPA result of the first-degree relatives using SALSA MLPA kit P350-A1 *CLCN1-KCNJ2* region probe mix.





A ratio chart of MLPA result of the patients and family members using SALSA MLPA kit P350-A1 *CLCN1-KCNJ2* region probe mix. (A) Proband, IV:3 (B) Proband's brother (PB), IV:2 (C) PB's daughter (C1), V:1 (D) Proband's son (C2), V:2 and (E) Proband's daughter (C3), V:3. The black spots represent MLPA probes, the upper blue line indicates a peak ratio of 1.3 and any probes above this line represent a duplication, the lower red line indicates a peak ratio of 0.75 and any probes below this line represents a deletion, and the probes between the two lines are considered as normal two copies. The chart is showing no deletion or duplication as all the spots are between both cut-off lines in the ratio chart. The analysis also contains a *CASP2-13* upstream probe and three exon probes for *KCNJ2*, which are other genes analysed by this kit.

Appendix C1. The CpG island appeared as a green box, with a number indicating the count of CpG sites within. The green box was then right clicked, and the option 'Get DNA for CpG' was selected.

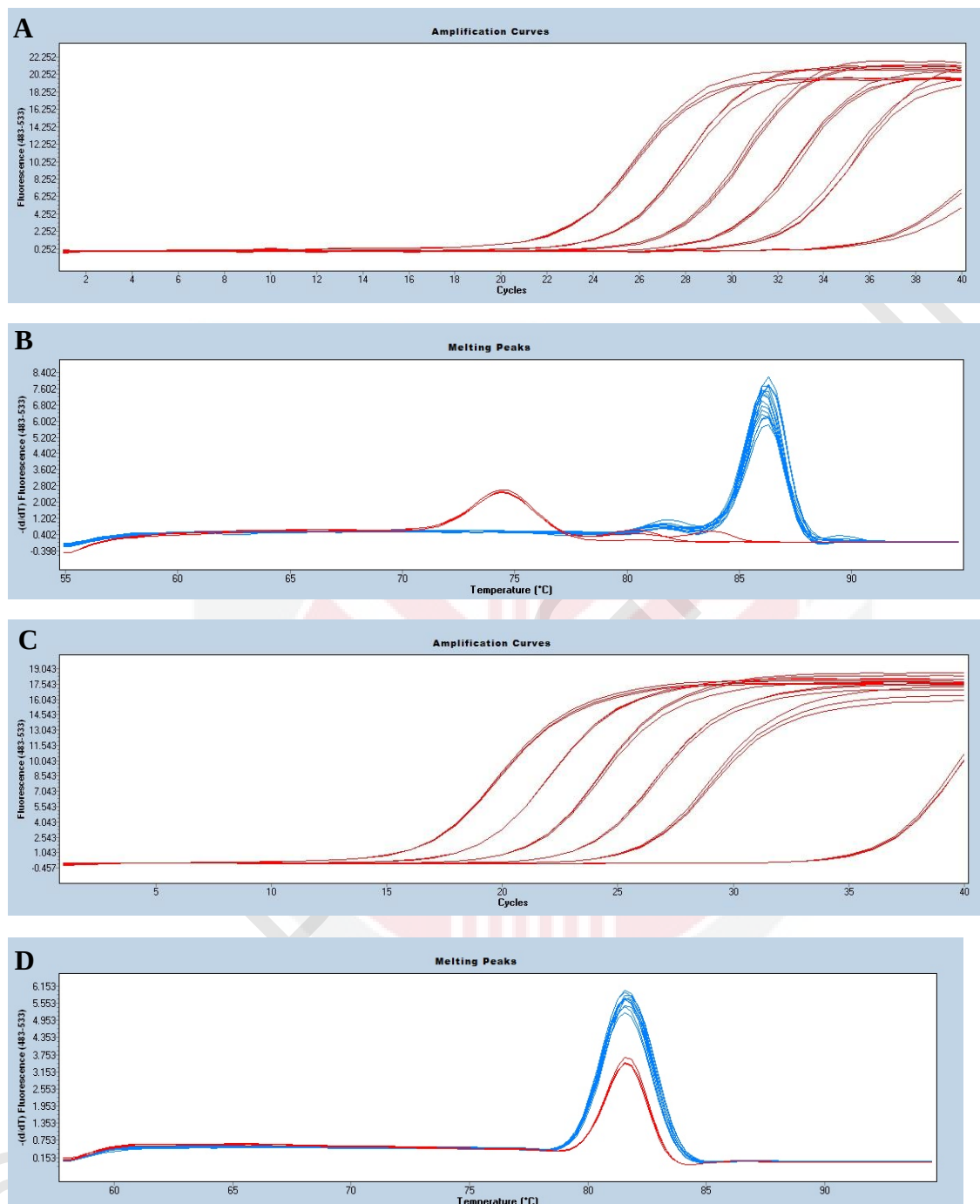


Appendix C2. The CpG site sequences retrieved.

```
>hg38_dna range=chr7:143345116-143346074 5'pad=300 3'pad=300 strand=+ repeatMasking=none
GAGAGATATAATGTTTGATTGCTTATAGACTTCTTTCAAAGCGTGTTCA
CGTCTCAGCTTATTTGAATGGCTTCTCATGTTTCTTTTATTTGGACAGC
ATTGATTAGCCTCCTTAATTATCAGCTCACCTATCTGAAGTCTATATAAG
GCCTCATCAAAATAAGTACAAATTAGCCCAATTAGTGCTCTACCAATTGG
CCCCGTACCTGCTAAATGGATGAAATAAAAGGGATTGAGTGAGGTTAATC
TTGGAGGATTCTTAAATGAGTTTCCAGGAAGCTGAGAAAGATGTGGGGA
CGCCGGGCAGGGTGGCGCTCTCTGTTCTTCTCAGGAGTGCGGAGCGC
GGTGCTGCGAGAGGGCTTGGAGGGGGCGCTCAGGCAGGGCGTGGGTTTCC
CTCAGATTCAATGATCCTGCTGGGCTCGGTGGAGCGGTGCGAACTGCAGG
CCCTCCTGCAGCGCCACCTGTGTCTGAGCGCAGGCTGCGCGCAGCCCAA
GAGATGGCGCGGAAGTTGTGCGAGCTGCCTTACGACGGGAAGGCGCGGCT
GGCTGGGGAGGGGCTCCCCGGCGCGCTCCAGGCCGGCCCGAGTCTTTCG
CCTTTGTGGATGAGGATGAGGACGAAGACCTCTCTGGCAAGAGCGAGGTG
ACCGCGCCGGGAAGGGCTAGGGAGTGGGATAGATCAGGAGCAAAGGGAAA
AGCGTCGTCTGGGCTGGGCTGGGCTGGGACAGGCGTAGTTTCCCT
GAAAGTAGTGGGAAGAGGGAGGGAGAGTCTGGTAAGTGAAGAAAGCCAGA
AATTCTGGATCAGGTACCATAGAGACCAGTGCCTCTGGGTCCAGGGACTA
AAGAAGATAAGGTTGCAGATGATGGTATCCTCGACAATTCAAAGGGATAT
AGGAATTTCTGAAGTGGGGGAAGAATAGAGTTTCGCTTCCAGAACATC
CACCAACTG
```

https://genome.ucsc.edu/cgi-bin/hgc?hgsid=1570922031_6pXWGKArsE9gMjQKvQa1NavTA4g&g=htcGetDna2&table=&i=mixed&l=143345415&r=143345774&getDnaPos=chr7%3A143%2C345%2C416-143%2C345%2C774&db=hg38&hgSeq.cdsExon=1&hgSeq.padding5=300&hgSeq.padding3=300&hgSeq.casing=upper&boolshad.hgSeq.maskRepeats=0&hgSeq.repMasking=lower&boolshad.hgSeq.revComp=0&submit=get+DNA

Appendix C4. The amplification curves and melting peaks from serial dilutions for *CLCN1* (A, B) and *GAPDH* (C, D) to determine the efficiency of PCR amplification.

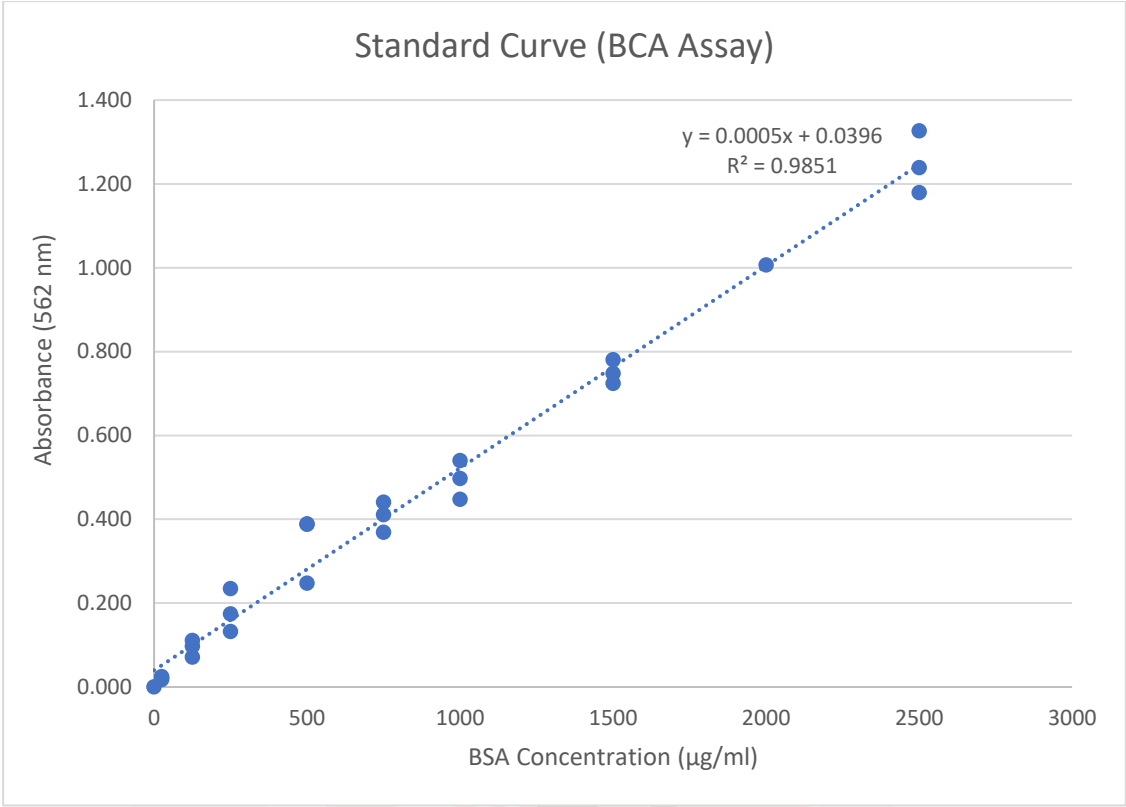


Appendix C5. The methylation level of the 24 CpG sites was analysed in normal control and an MC patient by pyrosequencing assay.

CpG sites	Normal control	MC patient	MC (Methylated control)	UC (Unmethylated control)
4	36	51	81	0
5	24	42	74	5
6	54	75	72	5
7	40	57	58	0
8	27	35	54	0
9	19	21	88	0
10	27	59	86	10
11	27	47	79	0
12	24	48	93	6
13	31	36	75	0
14	24	33	100	0
15	29	41	94	0
16	33	39	83	9
17	35	36	75	10
18	30	32	66	0
19	17	25	38	0
20	27	36	89	0
21	27	42	82	0
22	43	54	83	0
23	37	46	83	0
24	27	37	80	0
25	35	41	76	0
26	34	40	78	0
27	27	38	73	0
28	13	21	85	0
29	18	25	93	0
30	11	10	99	8
31	11	11	77	6
32	38	39	89	7
33	45	29	81	0
34	49	37	85	8
35	64	23	88	0
36	33	25	79	0
37	40	22	81	0
Average	30.6	42.1	77.5	1.9

Appendix D1. The absorbance readings at 562 nm for BSA dilution standards are shown in the top table, and the standard curve established using BSA is shown in the bottom graph.

Well	BSA concentration ($\mu\text{g/mL}$)	Absorbance at 562 nm	
		Before blank subtraction	After blank subtraction
A1	2500	1.339	1.239
B1	2500	1.279	1.179
C1	2500	1.427	1.327
A2	2000	0.989	0.889
B2	2000	1.248	1.148
C2	2000	1.107	1.007
A3	1500	0.848	0.748
B3	1500	0.881	0.781
C3	1500	0.824	0.724
A4	1000	0.640	0.540
B4	1000	0.597	0.497
C4	1000	0.548	0.448
A5	750	0.541	0.441
B5	750	0.511	0.411
C5	750	0.469	0.369
A6	500	0.489	0.389
B6	500	0.348	0.248
C6	500	0.488	0.388
A7	250	0.274	0.174
B7	250	0.335	0.235
C7	250	0.232	0.132
A8	125	0.197	0.097
B8	125	0.171	0.071
C8	125	0.211	0.111
A9	25	0.125	0.025
B9	25	0.122	0.022
C9	25	0.118	0.018
A10	0	0.106	0.000
B10	0	0.098	0.000
C10	0	0.095	0.000
Mean blank =		0.100	



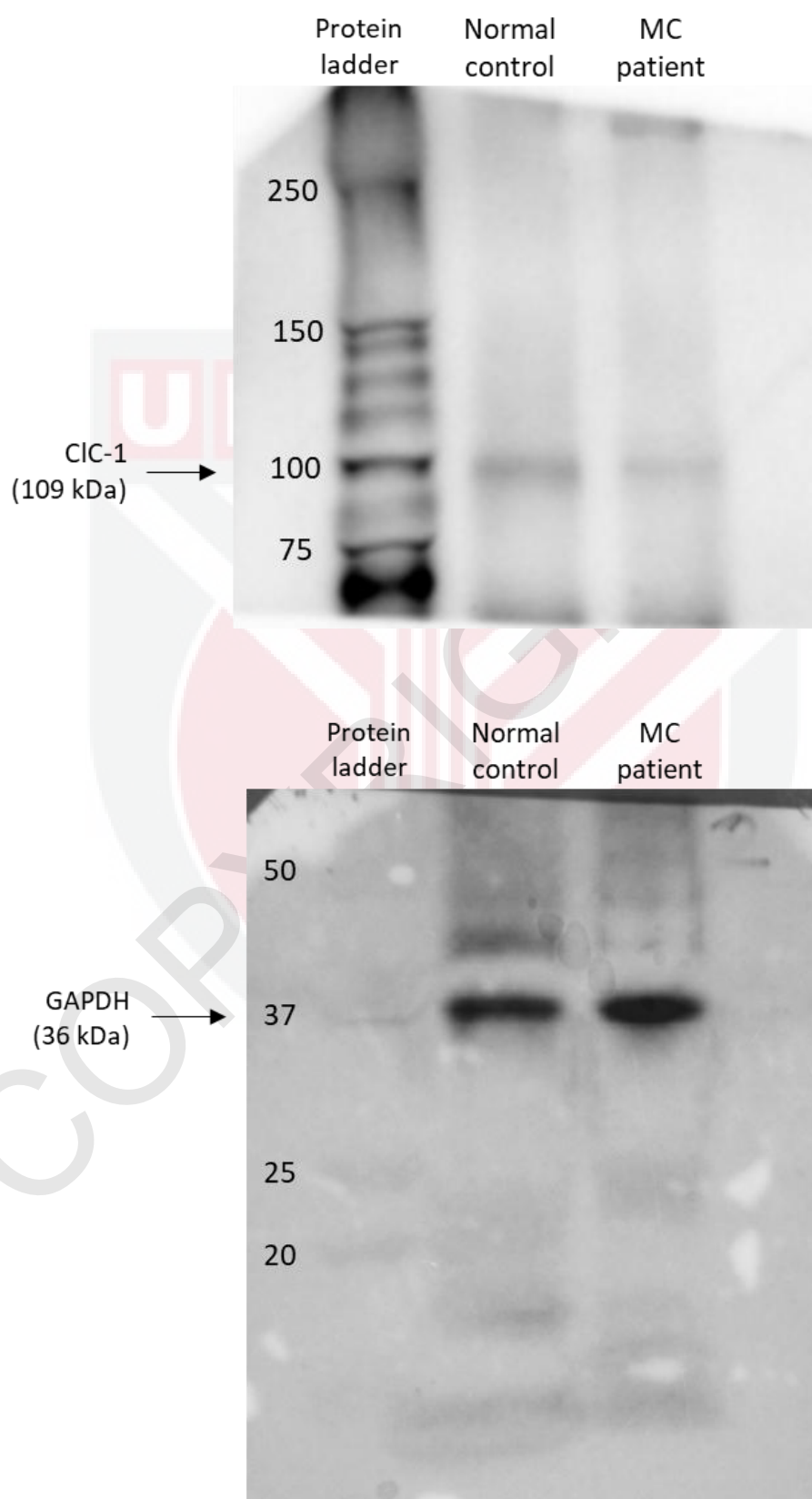
Appendix D2. Protein quantification and preparation for Western blot

Samples	Replicates	Before blank subtraction	After blank subtraction	Conc. (µg/mL) = (abs-c)/m = (abs -0.0396)/0.0005	Normalized conc. (µg/µL)	Mean normalized concentration (µg/µL)	Volume needed for 20 µg (µL)	Loading buffer (µL)	Ripa Buffer (µL)
MC patient	1	0.354	0.254	428.80	4.29	3.721	21.50	12	14.50
	2	0.306	0.206	332.80	3.33				
	3	0.317	0.217	354.80	3.55				
Normal control	1	0.497	0.397	714.80	7.15	6.515	12.28		23.72
	2	0.446	0.346	612.80	6.13				
	3	0.453	0.353	626.80	6.27				

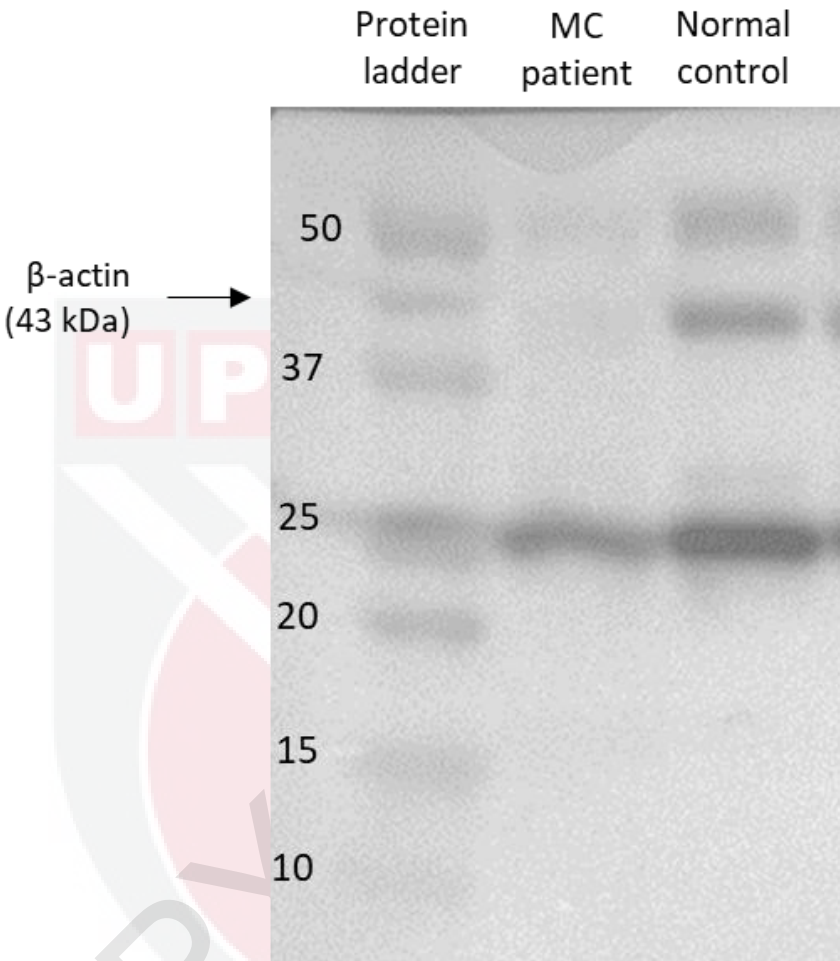
Appendix D3. Antibodies dilution for immunoblotting. The blots were incubated with respective target antibodies in 4 mL of 5% blocking solution following desired volume dilution as the table below.

Antibody (Gene, host)	Primary antibody		Secondary Antibody	
	Dilution	Volume (µL)	Host	Dilution Volume (µL)
CIC-1 (Rabbit)	1:1,000	4.0	Anti-rabbit	1:2,000 2.0
GAPDH (Mouse)	1:10,000	0.4	Anti-mouse	1:20,000 0.2

Appendix D4. Whole blot from western blot analysis of total ClC-1 protein (top blot) shows the MC tissue has reduced signal intensity compared to the normal control when normalised with GAPDH protein (bottom blot).



Appendix D5. Whole blot from western blot analysis of β -actin shows the MC tissue has reduced signal intensity compared to the normal control shows varying expression between normal control and MC patient.



Appendix D6. The Mean Relative Protein Expression for Each Protein in MC Patient and Normal Control.

PROTEIN	CIC-1				GAPDH			
	Normal control		MC patient		Normal control		MC patient	
	1	2	1	2	1	2	1	2
Mean Grey Value (MGV)	189.18	164.63	209.35	181.88	182.63	138.68	184.21	144.93
Inverted Formula	65.82	90.37	45.65	73.12	72.37	116.32	70.79	110.07
BG Mean Grey	222.54	231.16	225.08	214.69	237.10	242.64	239.82	240.86
Inverted BG	32.46	23.85	29.92	40.31	17.9	12.36	15.18	14.14
NET CIC-1 / GAPDH	33.36	66.52	15.73	32.81	54.47	103.96	55.61	95.93

Protein expression		Normalised to normal control	
SAMPLES	Protein Expression	SAMPLES	Protein Expression
			1 2
Normal control	0.61	Normal control	1.00 1.00
MC patient	0.28	MC patient	0.46 0.53

Note: The data represent as technical replicates from duplicate experiments (1 and 2), conducted using protein from a single extraction.

Appendix D7. H&E Internal nuclei.

Samples		MC Patient		Normal control	
Section		Fibres with internal nuclei	Fibres with normal nuclei	Fibres with internal nuclei	Fibres with normal nuclei
1		64	127	40	145
2		32	58	5	260
3		32	117	10	197
4		30	60	4	183
5		38	74	5	64
6		40	153	12	251
7		60	107	11	218
8		47	68	5	112
9		64	105	2	73
10		65	56	3	51
TOTAL		472	925	97	1554
Internal nuclei (%)		33.8%		5.8%	

Appendix D8. Myosin ATPase. The total number of type 1 and type 2 muscle fibres stained at different pH; pH 4.3, 4.6, and 9.4 were counted across all sections in normal control and MC tissue.

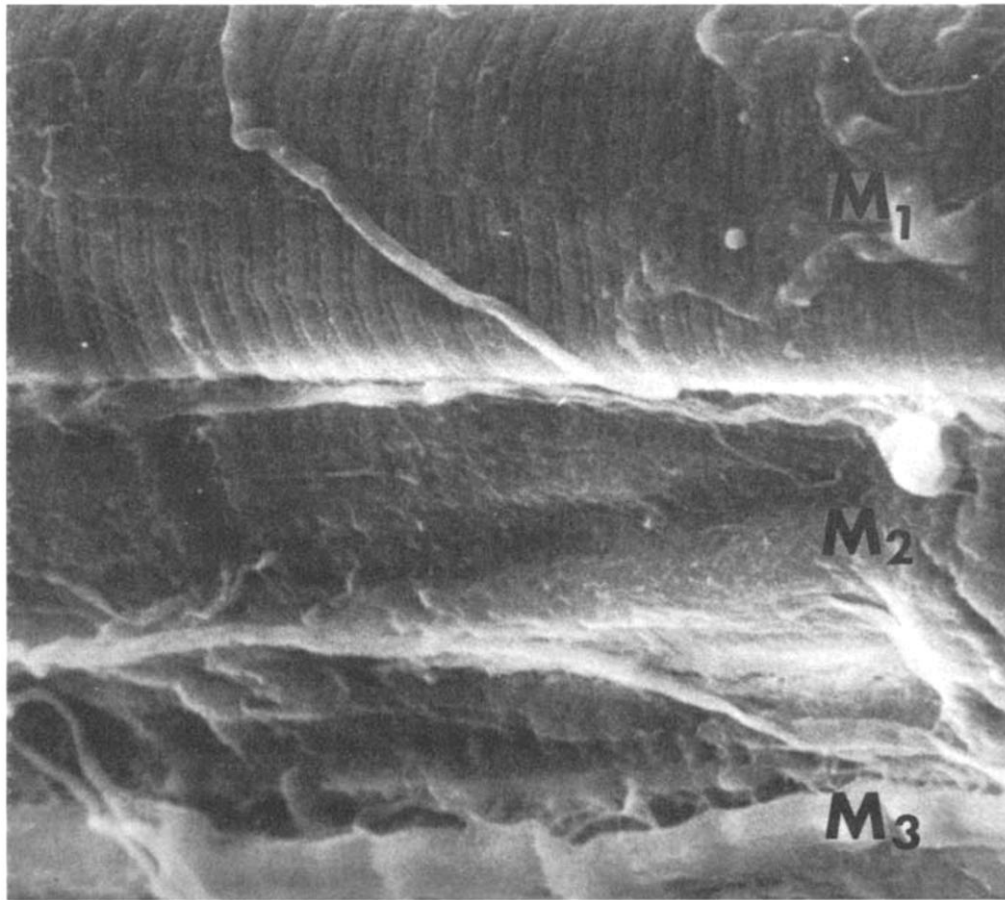
Normal control tissue

ATPase pH	pH 4.3		pH 4.6			pH 9.4	
Section	Type I	Type II	Type I	Type IIA	Type IIB	Type I	Type II
	Dark	Light	Dark	Light	Intermediate	Light	Dark
1	194	205	150	10	102	148	128
2	219	223	134	18	110	159	157
3	173	163	322	28	241	158	133
4	155	152	146	14	91	270	223
TOTAL	741	743	752	70	544	735	641
%	49.9	50.1	55.1	5.1	39.8	53.4	46.6

MC tissue

ATPase pH	pH 4.3		pH 4.6			pH 9.4	
Section	Type I	Type II	Type I	Type IIA	Type IIB	Type I	Type II
	Dark	Light	Dark	Light	Intermediate	Light	Dark
1	127	379	119	374	1	88	320
2	97	357	95	434	5	108	441
3	97	376	133	405	2	119	428
4	95	275	129	431	3	117	427
TOTAL	416	1387	476	1644	11	432	1616
%	23.1	76.9	22.3	77.1	0.5	21.1	78.9

Appendix D9. The micrograph of DMP (Duchenne muscular patient) shows M1, almost normal muscle fibre; M2, atrophic muscle fibre; and M3; complete atrophic muscle fibre (original magnification, 1,600 x) (Sakuragawa et al., 1973).



BIODATA OF STUDENT

Nurul Huda Musa began her journey in academia with her graduation from Universiti Teknologi MARA (UiTM) in 2009, where she earned a First Class Bachelor's Degree in Science (Hons.) Biology. Her academic excellence was immediately recognised, as she was awarded the prestigious Young Lecturer Scheme Scholarship by the Ministry of Higher Education (MoHE). This opportunity allowed her to pursue her master's degree in Neuroscience at UiTM's Faculty of Pharmacy, where she delved deeper into her fascination with research on neuroinflammation in Alzheimer's Disease. In 2014, she graduated and marked the beginning of her career in higher education. Following her Master's, she spent seven fulfilling years as a lecturer at UiTM. Her dedication to teaching and research led to another great opportunity, being awarded with the IPTA Academic Training Scheme from MoHE, enabling her to pursue her PhD in Human Genetics at Universiti Putra Malaysia (UPM). This marked a major turning point in her career.

During her PhD, she was awarded the Wellcome Connecting Science Travel Award Bursary, the Malaysian Neuroscience Society (MNS) Education Award, and the UPM Outbound Programme Travel Award. These recognitions allowed her to travel to Cambridge, UK, for advanced training in Next-Generation Sequencing (NGS) and to Acibadem University, Istanbul, Turkiye, for an intensive three-month genomic analysis course. These experiences not only expanded her technical expertise but also enriched her global perspective, enabling her to bring cutting-edge knowledge back to Malaysia. Her doctoral research focused on the clinical and genetic analysis of Myotonia congenita, a rare neuromuscular disorder. Remarkably, her study was the

first of its kind in Malaysia, shedding light on a condition that had long been under-researched in the region.

She published one article, co-authored another, and participated in several conferences and symposiums, winning two awards for best micrograph at a microscopy conference and a travel award. She was also awarded a full sponsorship to attend a summer course at Universitas Gadjah Mada (UGM) in Jogjakarta, Indonesia. Other than academic contribution, she participated as an organizer in three conferences and symposiums held in the faculty and three community events.

LIST OF PUBLICATIONS

Publication:

Musa, N. H., Thilakavathy, K., Mohamad, N. A., Kennerson, M. L., Inche Mat, L. N., Loh, W. C., Abdul Rashid, A.M., Baharin, J., Ibrahim, A., Wan Sulaiman, W.A. and Hoo, F.K., & Yusof Khan, A. H. K. (2023). Case report: Incomplete penetrance of autosomal dominant myotonia congenita caused by a rare *CLCN1* variant c.1667T> A (p. I556N) in a Malaysian family. *Frontiers in Genetics*, 13, 972007.

Proceedings:

Musa, N. H., Yusof Khan, A. H. K., Kennerson, M. L., Thilakavathy, K. (2022). A Rare *CLCN1* Variant c.1667T>A (p.I556N) Showing an Incomplete Penetrance in a Family with Myotonia Congenita. [Abstract]. In: Genome Informatics, Wellcome Connecting Science, UK, 21 – 23rd September 2022.

Musa, N. H., Yusof Khan, A. H. K., Kennerson, M. L., Thilakavathy, K. (2023). Genetic Characterization of a Myotonia Congenita Patient with a Rare c.1667T>A (p.I556N) *CLCN1* Variant Using MLPA and *In Silico* Analysis. [Abstract]. In: The 15th Malaysia International Genetics Congress (MiGC15), Bangi Resort Hotel, 7 – 9th March 2023.

Musa, N. H., Yusof Khan, A. H. K., Kennerson, M. L., Thilakavathy, K. (2023). Comparative Evaluation of Sample Preparation and RNA Isolation Techniques for Gene Expression Analysis in Neuromuscular Diseases. [Abstract]. In: The 8th International Biotechnology Symposium, 29 – 30th August 2023.

Musa, N. H., Yusof Khan, A. H. K., Kennerson, M. L., Wong, K. T., Hussin, H., Mohamed Ramli, F. A., Thilakavathy, K. (2023). Histopathological and ultrastructural characterization of skeletal muscle in a Malaysian patient with Thomsen congenital myotonia. [Abstract]. In: 31st Scientific Conference of Microscopy Society Malaysia. 18th December 2023.