Natural history study of childhood-onset mitochondrial disorders by using outcome measure assessments

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Submitted to Newcastle University for the degree of Doctor of Philiosophy in the Faculty of Medical Sciences

Author's declaration

This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy. I have performed this research at the Wellcome Centre for Mitochondrial Research from 2018 to 2021 under the supervision of Professor Robert McFarland and Professor Grainne Gorman.

I certify that the material in this thesis has not been previously submitted by me for a qualification in this or any other university.

Acknowlegements

First, I thank the Wellcome Centre for Mitochondrial Research for the funding to pursue this clinical research. Before that, I was an NIHR-funded Academic Clinical Fellow in paediatric neurology. My research journey begins in a Newcastle University electrophysiological laboratory that studies the in vitro model of mitochondrial epilepsy in rodent hippocampal brain slices. Preparing these experimental slices from live mice was personally challenging for me. I was, and still am, so grateful that Professor Doug Turnbull, Professor Grainne Gorman and Professor Bobby McFarland came along and offered me a once-in-a-lifetime opportunity to undertake clinical research at the prestigious Wellcome Centre.

I would like to express my deepest gratitude to both of my esteemed supervisors, Bobby and Grainne, for their unwavering support and invaluable advice during my PhD study. At the start of my clinical research, I recall how much I enjoy the road trip with Bobby to attend a family weekend for mitochondrial patients organised by the Lily Foundation. Seeing how passionate he was, and still very much is, in helping those children and families living with the mitochondrial disease has truly inspired me. I also draw my inspiration from Grainne. Her steely determination and relentless pursuit to find a cure for mitochondrial disorder motivates me in the last four years and for many years to come. I am proud of my time working with these two excellent mentors.

I am also eternally grateful to my predecessor of the clinical research associate post, Dr Ng Yi-Shiau. Words cannot express how much he has helped me, as a colleague and as a friend. Over the years, Yi has raised countless constructive discussion points over coffee or lunch breaks. I must also thank Dr Jane Newman who sat next to me for having to put up with my random research ideas and for her expertise when we were on home visits.

This PhD study would not have been possible without the support of many people at the Wellcome Centre for Mitochondrial Research and at the NHS Highly Specialised Service for Rare Mitochondrial Disorders. I am indebted to Alex Bright, Catherine Feeney, Jan Gebbie, Jane Brown, Doug Jerry, Alasdair Blain, Renae Stefanetti, Laura Brown, Clare Massarella and Naomi Thomas. I will never forget the kindness shown

by them throughout my research, especially through the tough times during the COVID-19 pandemic. I would also like to thank Professor Rob Taylor for the opportunities to push my research to the next level. To my collaborators, research participants and their families, you have my deepest thanks.

Finally, I owe my heartiest gratitude to my family. I am forever thankful for my wife's unconditional support, understanding and love throughout the entire research process. For my two children, you remind me of my own passion for paediatrics and what I can do better every day. My parents also deserve my special thanks for the many lessons to accomplish great things in life.

Dedication

To my wife, Jean, and our children, Joshua and Ava

To my parents

Abstract

This thesis aims to study the natural history of two childhood-onset mitochondrial disorders, Leigh syndrome and *RRM2B*-related disease. Current studies have not precisely explored the longitudinal aspects of these disorders to outline their trajectories and the utility of outcome measures to objectively characterise their changes over time. Therefore, the natural history of these two disorders needs a better definition. Using the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) in a large cohort of children with Leigh syndrome caused by various genotypes, I discovered that severe disease burden (high NPMDS scores) and rapid disease progression (rate of NPMDS change per annum > 3) were associated with greater rates of mortality. I also identified several factors such as pathogenic variants in the *SURF1* gene and certain brain imaging changes, that adversely influence their outcomes. In a separate cohort of patients with autosomal recessive pathogenic variants in their *RRM2B* gene, I have also objectively determined their disease burden and progression. I have utilised a range of disease rating scales, performance outcome measures (spirometry, nine-hole peg test and muscle dynamometry) and functional tests (six-minute walk, sit-to-stand and water swallow tests) to provide new natural history data of this rare disorder. These patients performed significantly worse than their predicted values. Of note, their lung forced vital capacity (FVC) deteriorated about 5% per annum. To understand the individual impact of the *RRM2B*-related disorder, I have also explored the use of several patient-reported outcome measures and I found the poor quality of life faced by these patients. All the discoveries in this thesis will serve as robust data for the development of interventional clinical trials in these two conditions where a control arm might not feasible, for the prognostication of disease course in clinical practice, and more importantly, for the provisions of better care for these patients in the future.

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Abbreviations

6MWT	6 minute walk test
ABAT	4-aminobutyrate aminotransferase
AD	Autosomal dominant
ADANE	Autosomal dominant acute necrotizing encephalopathy
ADL	Activities of daily living
ADP	Adenosine diphosphate
AGK	Acylglycerol kinase gene
AIFM	Apoptosis-inducing factor in mitochondrial
ANE	Acute necrotizing encephalopathy
ANT	Adenine nucleotide translocator
AR	Autosomal recessive
ATP	Adenosine triphosphate
ATS	American Thoracic Society
BCS1L	BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone
BMI	Body-mass index
bp	Base pair
BRBGD	Biotin-responsive basal ganglia disease
BTD	Biotinidase
CARU	Clinical ageing research unit
CK	Creatinine kinase
CNS	Central nervous system
CoQ	Coenzyme Q10
COQ9	coenzyme Q9 homolog
COSMIN	Consensus-based standards for the selection of health measurement instruments
COX	Cytochrome c oxidase
CPEO	Chronic progressive external ophthalmoplegia
CRF	Clinical research facility

CSF	Cerebrospinal fluid
CYTB	Cytochrome b
DARS2	Mitochondrial aspartyl-tRNA synthetase
DCA	Dichloroacetate
dCMP	deoxycytidine monophosphate
DFIS	Daily Fatigue Impact Scale
DGUOK	Deoxyguanosine kinase
DHI	Dysphagia Handicap Index
DLD	Dihydrolipoamide dehydrogenase
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
dTMP	deoxythymidine monophosphate
EARS2	Mitochondrial glutamyl-tRNA synthetase
ECG	Electrocardiogram
ECHS1	Enoyl-CoA hydratase, short chain 1
EEG	Electroencephalogram
EMG	Electromyogram
EPC	Epilepsia partialis continua
ETF	Electron transfer flavoprotein
ETFDH	Electron transfer flavoprotein dehydrogenase
ETHE1	Ethylmalonic encephalopathy 1 protein
$FADH_2$	Reduced flavin adenine dinucleotide
FARS2	Mitochondrial phenylalanyl-tRNA synthetase 2
FBXL4	F-box and leucine rich repeat protein 4
FDA	U.S. Food and Drug Administration
Fe-S	Iron-sulphur
FET	Force evaluating and testing
FEV1	Forced expiratory volume in the first second
FIS	Fatigue impact scale
FLAIR	Fluid attenuation inversion recovery
FVC	Forced vital capacity

GCP	Good clinical practice
GDPR	General data protection regulations
GFM1	Mitochondrial translation elongation factor G1
GFM2	Mitochondrial translation elongation factor G2
GTPBP1	GTP-binding protein 1
HSP	Heaving strand promoter
IARS2	Mitochondrial isoleucine-tRNA synthetase
IPO	Intestinal pseudo-obstruction
IQR	Inter-quartile range
IVF	in vitro fertilisation
KSS	Kearns-Sayre syndrome
L	Light strand
LHON	Leber hereditary optic neuropathy
LIAS	Lipoic acid synthetase
LIPT2	Lipoyl(octanoyl) transferase 2
LRPPRC	Leucine-rich pentatricopeptide repeat cassette
LS	Leigh syndrome
LSP	Light strand promoter
MDS	Mitochondrial depletion syndrome
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MEP	Maximal expiratory pressure
MERRF	Myoclonic epilepsy with ragged red fibres
MGME1	Mitochondrial genome maintenance exonuclease-1
MIDD	Maternally inherited diabetes and deafness
MILS	Maternally inherited Leigh syndrome
MIP	Maximal inspiratory pressure
MitoCohort	MRC Mitochondrial Disease Patient Cohort
MNGIE	Mitochondrial neurogastrointestinal encephalopathy
MRC	Medical research council
MRPS34	Mitochondrial ribosomal protein S34
MTATP	Mitochondrially-encoded ATP synthase membrane subunit

MTCO1	Mitochondrially-encoded cytochrome c oxidase I
mtDNA	Mitochondrial DNA
MTFMT	Mitochondrial methionyl-tRNA formyltransferase
MTND	Mitochondrially-encoded NADH:ubiquinone oxidoreductase core subunit
MTO1	Mitochondrial tRNA translation optimization 1
MTTK	Mitochondrially encoded tRNA lysine
MTTL	Mitochondrially encoded tRNA leusine
MTTV	Mitochondrially encoded tRNA valine
MTTW	Mitochondrially encoded tRNA tryptophan
NAD+	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia and retinitis pigmentosa
NARS2	Mitochondrial asparaginyl-tRNA synthetase 2
NCR	non-coding region
NCS	Nerve conduction studies
nDNA	Nuclear DNA
NDUFAF	NADH:Ubiquinone oxidoreductase complex assembly factor
NDUF	NADH:Ubiquinone oxidoreductase core subunit
NGS	Next generation sequencing
NINDS	National Institutes of Health Quality of Life in Neurological Disorders
NMDAS	Newcastle mitochondrial disease adult scale
NMQ	Newcastle Mitochondrial Quality of life measure
NPMDS	Newcastle paediatric mitochondrial disease scale
OPA1	Mitochondrial dynamin like GTPase
OXPHOS	Oxidative phosphorylation system
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PDHc	Pyruvate dehydrogenase complex
PDSS2	Decaprenyl-diphosphate synthase subunit 2
PEG	Percutaneous enteral gastrostomy

PGC1-α	Proliferator-activated receptor gamma coactivator
PGD	Preimplantation genetic diagnosis
PGM	Personal genome machine
PKAN	Pantothenate kinase-associated neurodegeneration
PNPT1	Polyribonucleotide nucleotidyltransferase 1
POLG	Polymerase gamma
PPAR-α	Peroxisome proliferator-activated receptor alpha
PROM	Patient reported outcome measure
PROSPER2B	Prospective observational study of patients with mitochondrial depletion syndrome RRM2B
PS	Pearson syndrome
QOL	Quality of Life
RANBP2	RAN Binding Protein 2
RMND1	Required for Meiotic Nuclear Division protein 1
RNASEH	Ribonuclease H1
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SADS	Sudden adult death syndrome
SANDO	Sensory ataxia neuropathy dysarthria and ophthalmoplegia
SDH	Succinate dehydrogenase
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
SLE	Stroke-like episodes
SUCLA2	Beta subunit of succinyl-CoA ligase
SUCLG1	Alpha subunit of succinyl-CoA ligase
SURF1	Surfeit locus protein 1
TACO1	Translational activator of cytochrome c oxidase 1
TALEN	Transcription activator-like effector nucleases
TCA	Tricarboxylic acid cycle
TFAM	Transcription factor A
TK2	Thymidine kinase 2
TPK1	Thiamine pyrophosphokinase 1
TPP	Thiamine pyrophosphate

TRMU	Mitochondrial tRNA-specific 2-thiouridylase
tRNA	Transfer ribonucleic acid
TSFM	Mitochondrial translation elongation factor
TTC19	Tetratricopeptide repeat protein 19
TWNK	Twinkle mtDNA helicase
TYMP	Thymidine phosphorylase
UQCRQ	Ubiquinol-cytochrome c oxidoreductase
VUS	Variant of unknown significance
WE	Wernicke's encephalopathy
WES	Whole exome sequencing
WGS	Whole genome sequencing

Chapter 1: Introduction

"Whether, in this infant, an unknown toxin or virus was responsible for these lesions, or whether the case was primarily a Wernicke's encephalopathy in an infant, is difficult to decide. The clinical history and course do not help greatly in reaching a decision but are slightly more in favour of a deficiency disorder than an infective or toxic process. Owing to our scant knowledge of early infantile disease processes, it will be an advantage to keep an open mind on the aetiological problem."

Denis Archibald Leigh (1951)

Such was the conclusion of Dr Denis Archibald Leigh (1912-1998) in 1951. Unbeknownst to this brilliant British neuropsychiatrist at that time, he had arguably reported one of earliest conditions related to mitochondrial dysfunction. While the mitochondria had been described earlier as organelles inside cells by Richard Altmann (1894), the link to these conditions was not made until much later. Nonetheless, Leigh's name soon became eponymous with this condition that he described. At that time, he used the term 'subacute necrotising encephalomyelopathy' to encapsulate the features of this condition.

1.1 A brief history of Leigh syndrome

Subacute necrotising encephalomyelopathy, or later known as “Leigh syndrome”, was first described in a 7-month-old male infant who presented to King’s College Hospital, London on 22 April 1947 (Leigh, 1951). This infant had a normal birth and satisfactory development until 6 weeks of age, but from that point developed a range of neurological features including somnolence, blindness, deafness, and spasticity of the limbs. He died within three days of admission from a central nervous system disorder. Subsequent post-mortem autopsy revealed bilateral focal symmetrical subacute necrotic lesions extending from thalami to the pons, inferior olives and posterior columns of the spinal cord. Leigh’s original drawings of these areas are shown in Figure 1. These areas demonstrated severe neuronal damage. Histological findings showed areas of intense vascular, microglial and histiocytic proliferation.

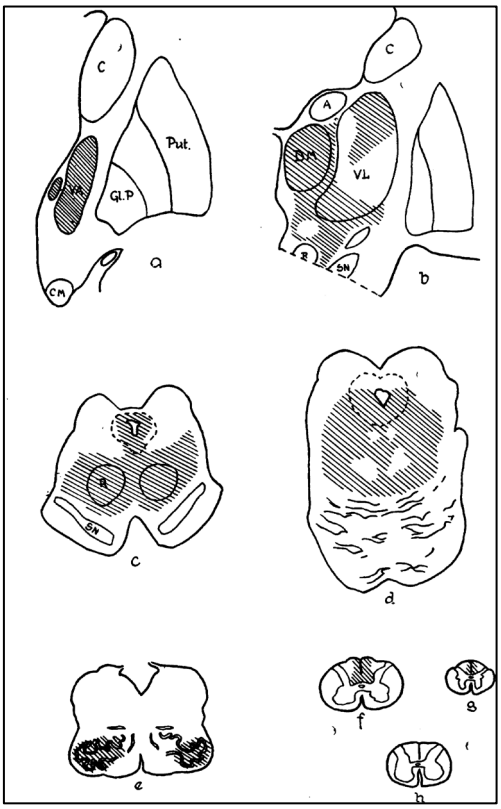

Figure 1-1: This hand-drawn picture by Denis Archibald Leigh showed the location of the lesions (hatched areas) at various cross-sectional levels of the thalamus, brain stem and spinal cord. Reproduced from (Leigh, 1951).

These features that Denis Leigh described become the hallmarks of this condition. At that time, he observed some similarities with Wernicke's encephalopathy, an acute neuropsychiatric disorder resulting from thiamine (Vitamin B1) deficiency. For a while, a biochemical defect in thiamine metabolism has been postulated to be the underlying mechanism in subacute necrotizing encephalomyelopathy (Cooper et al., 1969). Cooper et. al (1969) noticed that the brain tissue from a patient with subacute necrotising encephalomyelopathy contained essentially no thiamine triphosphate, although thiamine and its other phosphate esters were present. Thus, they argued that there was a relationship between this disease and thiamine triphosphate (Cooper et al., 1969).

A defect in energy metabolism was first linked to Leigh syndrome in by Hommes et al. (1968). High serum lactate and pyruvate were noted in a one-year-old boy with clinical features of Leigh encephalomyelopathy and a family history of deaths in three siblings. One of his siblings had a brain autopsy that showed lesions consistent with Leigh syndrome. This boy's liver biopsy, demonstrated an almost complete absence of the enzyme pyruvate carboxylase, which converts pyruvic acid to oxaloacetic acid in the process of gluconeogenesis. Hommes et al. (1968) concluded that Leigh syndrome in this family may be a consequence of a lack of pyruvate carboxylase.

Although Hommes' description had provided the first association of Leigh syndrome with defective energy metabolism, it was not until 1977 that Leigh syndrome was first related to mitochondrial respiratory chain dysfunction. A 6-year-old girl, with autopsy-confirmed subacute necrotising encephalomyelopathy, had previously been shown to have raised blood lactate:pyruvate and beta-hydroxybutyrate:acetoacetate ratios, suggesting a defect in mitochondrial oxidation (Willems et al., 1977). A deficiency in an enzyme found within the mitochondria, the cytochrome c oxidase, was subsequently demonstrated in post-mortem skeletal muscle tissue (Willems et al., 1977).

1.2 Mitochondria

Mitochondria facilitate a variety of vital cellular functions within all nucleated human cells. One primary function of mitochondria is the conversion of energy from food sources into adenosine triphosphate (ATP), the "currency of intracellular energy". A year after Leigh's case report, Palade et. al (1952) showed the very first high-resolution electron micrographs of the mitochondria, replacing the previous Janus Green stains. This technique visualized the second membrane inside these intracellular organelles (Figure 1-2). Not only does this second membrane divide the space in each mitochondrion into the matrix and inter-membrane space, but it also houses the oxidative phosphorylation system (OXPHOS).

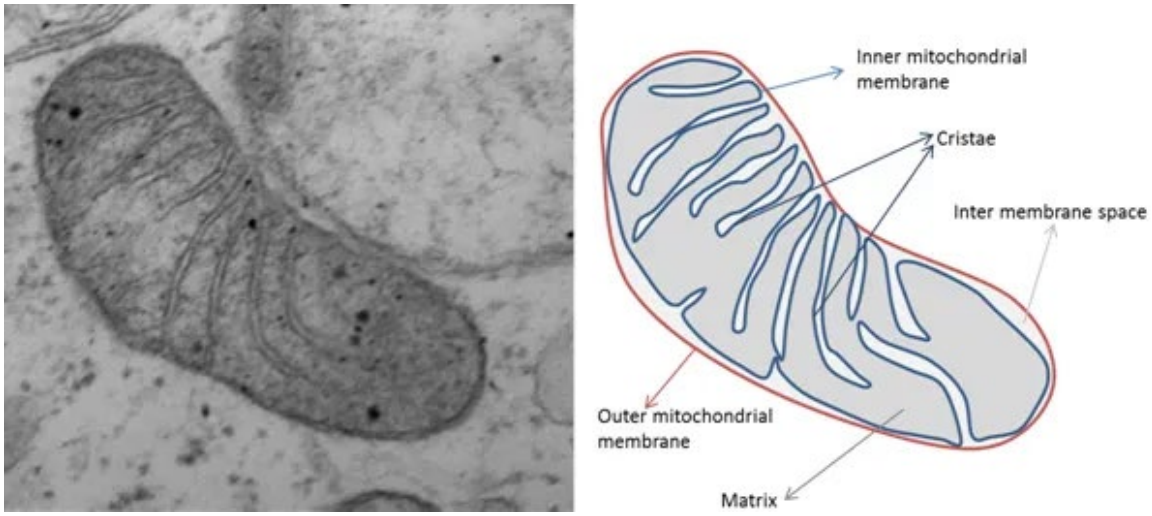

Figure 1-2: An electron microscopy view (left) and a schematic drawing (right) of the cristae, matrix, inner (blue line) and outer membrane (red line) of a single mitochondrion. Reproduced with permission from the patient and public engagement team at Wellcome Centre Mitochondrial Research (2021)

1.2.1 Oxidative phosphorylation system (OXPHOS)

The OXPHOS carries out one of the most important functions of the mitochondria which are to convert energy from glucose and fatty acid into adenosine triphosphate (ATP). Glucose is metabolized to pyruvate through glycolysis during aerobic respiration. Pyruvate then enters the mitochondria and is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Acetyl-CoA participates in a series of reactions known as the tricarboxylic acid cycle (the Kreb cycle) to form carbon dioxide, reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH2) (DiMauro and Schon, 2003). Acetyl-CoA can also be made available for the TCA cycle from fatty acids. Fatty acids undergo beta-oxidation to produce acetyl-CoA and FADH2. The amassed NADH and FADH2 are both oxidized by OXPHOS to derive protons. These protons can then be pumped across the inner membrane to create an electrochemical gradient. This gradient allows the final step of OXPHOS to phosphorylate adenosine diphosphate (ADP) and produce ATP (Schon et al., 2012).

The OXPHOS is made up of five protein complexes (Figure 1-3). The first four complexes are collectively known as the mitochondrial respiratory chain. The first of these complexes is the ubiquinone oxidoreductase, also known as Complex I. It is also the largest complex in the mitochondrial respiratory chain which consists of 45 subunits. 14 of the core subunits are highly conserved across species whilst 31 of them are accessory subunits that had developed through evolution (Sazanov, 2015, Zhu et al., 2016). The NADH, which was donated from the aforementioned glycolysis, is first oxidized by transferring two electrons to the flavin mononucleotide and the iron-sulfur clusters of Complex I. This process also results in four protons passing from the mitochondrial matrix to the intermembrane space. The iron-sulfur carriers then transfer the electrons to Complex III via the coenzyme Q10, also known as ubiquinone. Ubiquinone also shuttles electrons to Complex III from Complex II.

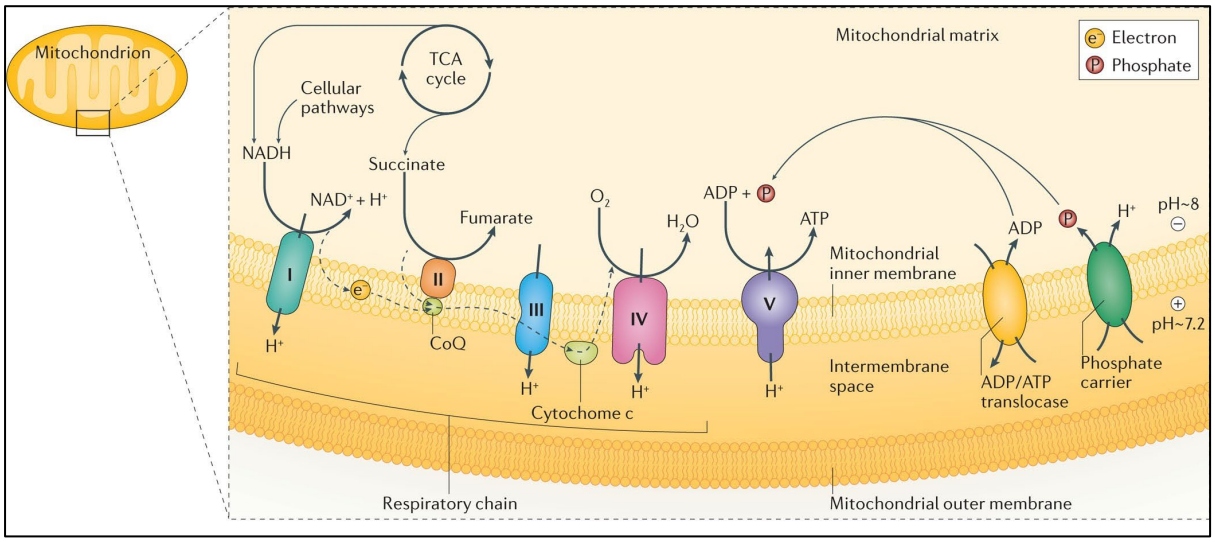

Figure 1-3: Schematic diagram that illustrates the mitochondrial respiratory chain (Complex I to IV) and ATP synthase (Complex V) on the mitochondrial inner membrane. The electron transport between complexes begins at Complex I (left green structure) where there is an entry point for electrons released by NADH from the TCA cycle. CoQ (coenzyme Q10 or ubiquinone) mediates the transfer of electrons. During the transfer along the chain, protons move from the matrix across the inner membrane into the inner membrane space via the complexes. At the end of the chain, Complex IV uses cytochrome c to reduce oxygen molecules which acts as the ultimate electron acceptor. Each NADH removes 10 protons from the matrix. The resulting electrochemical gradient drives the ATP synthase (Complex V) to produce adenine triphosphate (ATP) from ADP and free phosphate. Reproduced from (Gorman et al., 2016)

In contrast to Complex I, Complex II, also known as succinate dehydrogenase, is the smallest complex in the OXPHOS. It acts as a second entry point to the respiratory chain by accepting an electron from succinate. The oxidization of succinate to fumarate in the TCA cycle results in two electrons, which are transferred to ubiquinone via the FADH2. Unlike Complex I, no protons are transported across the membrane by Complex II. The electrons from Complex I and Complex II are shuttled by ubiquinone, also knowns as coenzyme Q10, a small, lipid-soluble molecule consisting of a benzoquinone ring with ten isoprene units (Alcázar-Fabra et al., 2016). When electrons are received by ubiquinone, it is reduced to ubiquinol which is subsequently used by the next complex in the OXPHOS, Complex III or cytochrome c oxidoreductase, to reduce cytochrome c. The process to oxidize ubiquinol back to ubiquinone via Q-cycle mechanism by Complex III also pumps four protons across the inner membrane for each cycle (Trumpower, 1990, Xia et al., 2013).

The cytochrome c, a protein bound to the outer face of the inner membrane, then carries the electrons to Complex IV, also known as the cytochrome c oxidase. Complex IV has 13 different subunits. Subunits I, II and III of Complex IV are core subunits, encoded by mitochondrial DNA while the remaining 10 accessory subunits are nuclear-encoded (Sharma and Wikström, 2016). Complex IV oxidises cytochrome c and transfers the electrons to the bound dioxygen species, the final electron carrier in aerobic respiration, to form molecules of water. The electron transfer also results in four protons moving across the intermembrane space. Being the final recipient of the electrons in the OXPHOS, the rate of Complex IV is determined by the ATP feedback inhibition based on ATP/ADP ratio (Kadenbach and Hüttemann, 2015). As the electron passes through the mitochondrial respiratory chain, protons are pumped into the intermembrane space to create an electrochemical gradient to make ATP from ADP and phosphate.

Complex V, also known as ATP synthase, utilizes this gradient to make ATP. ATP synthase contains two functional units, the F0 and F1, which act as a rotational system (Von Ballmoos et al., 2009). The hydrophobic F0 rotates as it protonates and deprotonates repeatedly when protons flow down the electrochemical gradient from the intermembrane space to the mitochondrial matrix. This alternating ionization of F0 alters the hydrophilic F1 subunits. These changes lead to the phosphorylation of one ATP from ADP and free phosphate for every four protons that flows across the gradient (Okuno et al., 2011).

Apart from the OXPHOS functions, the mitochondria have other crucial functions in cells. They include iron-sulphur biosynthesis, calcium handling, intrinsic cellular apoptosis and many others. The iron-sulphur clusters, primarily synthesized in the mitochondria, participate in electron transport as cofactors (Rouault, 2015). Loss of iron from cells induces a rapid reduction in mitochondrial OXPHOS capacity which is fully reversible with the reintroduction of iron (Rensvold et al., 2016). Mitochondria are also important regulators of cellular calcium ions by sequestering and releasing them (Giorgi 2018 nature). The intrinsic pathways of cellular apoptosis are also regulated by mitochondria. This pathway is initiated by stimuli such as DNA damage, metabolic stress and endoplasmic reticular stress (Taylor et al., 2008).

1.2.2 Mitochondrial genetics

A widely accepted hypothesis on the origins of these double membrane-bound intracellular organelles suggests that mitochondria are the result of an endosymbiotic event between a primitive proteobacterium and the common cellular ancestor of eukaryotes some 1.5 billion years ago, developing one of the most enduring symbiotic relationships in biology with proto-eukaryotes (Sagan, 1967). The less parsimonous alternative theory posits the autogenous 'splitting' of mitochondria from the eukaryotic cell DNA (Cavalier-Smith, 2006, Martin et al., 2015). Regardless of which theory, the result is the persistence of its own mitochondrial deoxyribonucleic acid (Martin and Müller, 2007). The mitochondrial genome is a compact 16,569 base pair, a double-stranded, circular molecule present in the matrix of all mitochondria (Figure 1-4) (Anderson et al., 1981, Robin and Wong, 1988, Larsson and Clayton, 1995).

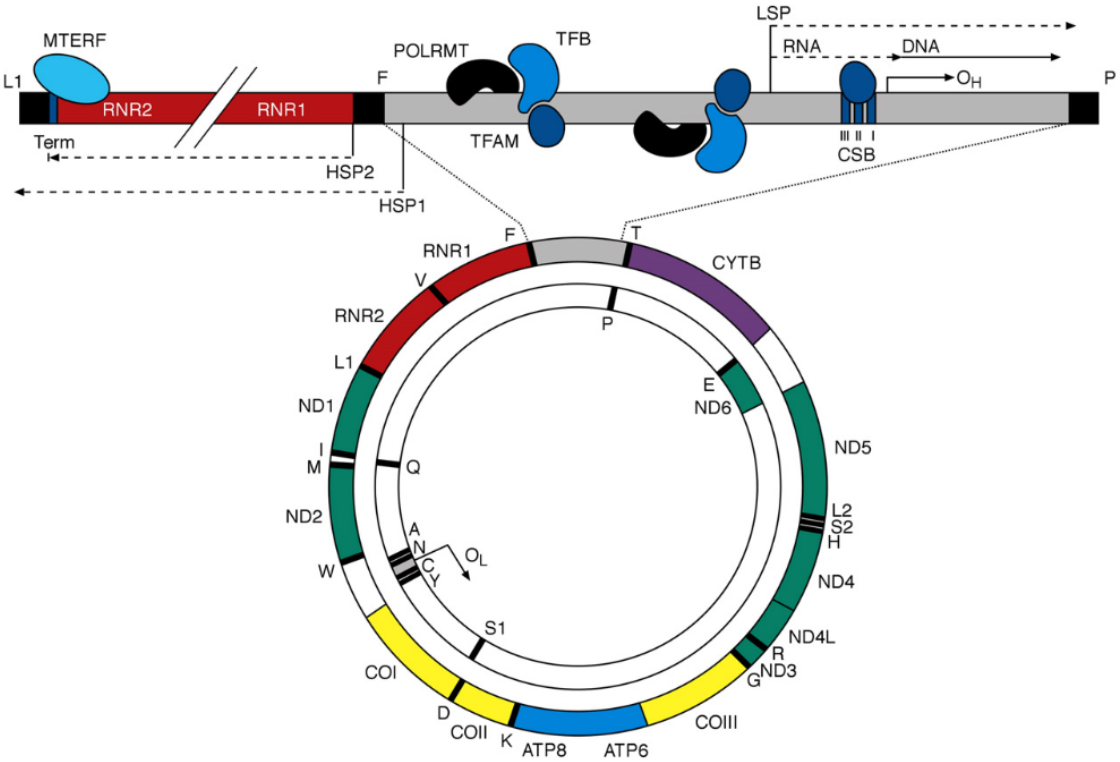

Figure 1-4: Schematic diagram of the human mitochondrial genome (mtDNA) which shows the circular, double-stranded genome with an enhanced view of the D-loop and transcription termination regions in a linear format. The heavy strand is depicted as the outer circle whilst the light strand is drawn in the inner circle. The human mtDNA encodes the 2 mt-rRNAs (red) RNR1 (12S rRNA) and RNR2 (16S rRNA), 22 mt-tRNAs (black bars), and 13 essential subunits of complex I (green), CYTB, a subunit of complex III (purple), catalytic subunits of complex IV (yellow), and MT-ATP6 and MT-ATP8 subunits of complex V (blue). Reproduced from (Tuppen et al., 2010a).

In humans, this semi-autonomous genome encodes 13 structural subunits of the electron transport chain, 2 ribosomal RNA molecules and 22 transfer RNA (tRNA) molecules (Anderson et al., 1981, Taanman, 1999). The two strands of mtDNA differ in their guanine content. They are termed as the heavy strand (H strand) and light strand (L strand). The H strand is particularly guanine rich and encodes most mitochondrial transcripts, whilst the L strand generates the MT-ND6 transcript and eight of the tRNAs. The amount of non-coding DNA within mtDNA is very low in comparison to the nuclear genome, with no intronic regions and just one main non-coding region (NCR) within which the displacement loop (D-loop) is found. The NCR contains both heavy and light strand promoters and other regulatory elements involved in the control of mtDNA replication and transcription (Walberg and Clayton, 1981).

During the transcription process, a polycistronic mRNA transcript is produced from each DNA strand. Most of the mt-rRNA and protein-coding genes on each polycistron are flanked by mt-tRNA genes. The unique folded structures of mt-tRNAs are targeted for endonucleolytic cleavage to allow the release of individual transcripts (Ojala et al., 1981, Taanman, 1999). Many of the mt-tRNAs undergo further maturation and modification crucial for their correct folding and structural stabilisation. Therefore, any point mutations in mt-tRNA genes can cause a loss of mt-tRNA stability that give rise to defective translation and a combined respiratory chain deficiency.

1.2.3 Mutations in the mitochondrial genome

Since the first reports of mitochondrial DNA association with human diseases in 1988 by Holt et al. (1988) and Wallace et al. (1988), pathogenic point variants in the mitochondrial tRNA have been responsible for the majority of mitochondrial diseases despite accounting for only 5-10% of the entire mitochondrial genome (Larsson and Clayton, 1995, Florentz et al., 2003). Several mechanisms have been proposed to explain this over-representation of mt-tRNA point mutations in the aetiology of mitochondrial disease. These include defective processing of the mRNA transcript by RNases P and Z, impaired post-transcriptional mt-tRNA modification, defective interaction of the mt-tRNA with both mitochondria elongation factor Tu and the mitoribosome (Levinger et al., 2004).

Mitochondrial DNA is present in multiple copies in every single cell. In humans, the number of mitochondrial genomes per cell can vary from 100s of copies in sperm to 100,000s of copies in an oocyte. However, this number is dynamic and responsive to cell-specific mechanisms of copy number control (Satoh and Kuroiwa, 1991, Clay Montier et al., 2009). Having a greater replication rate than that of the nuclear DNA, the mitochondrial DNA may accumulate mutations by clonal expansion (Pesole et al., 1999, Florentz et al., 2003, Durham et al., 2006). Mutations in mt-DNA can cause the coexistence of more than one mitochondrial DNA genotype within a single cell, a concept termed heteroplasmy.

The level of heteroplasmy, defined by the proportion of mutant mtDNA, differs between cells within the same tissue, between organs in the same individual and between individuals within the same family. The percentage of mutant mtDNA can vary from 1% to 99%. The level of heteroplasmy can change, either increasing or decreasing, through uneven partitioning of DNA during cell division, a process known as vegetative segregation (Birky et al., 1978). If a mutation is pathogenic, the cell can usually tolerate a mutation load up to a specific biochemical threshold before a respiratory defect will manifest and can be detected through laboratory investigations (Durham et al., 2006). However, a ‘negative selection’ is thought to occur against cells with a high mutant

load with a detrimental effect on respiratory chain function causing the impairment of further proliferation.

In addition, heteroplasmy levels may change rapidly within and between generations during development in a phenomenon known as a mitochondrial genetic bottleneck, first described in Holstein cows transmitting heteroplasmic mtDNA variants (Olivo et al., 1983) and later supported by studies in mice which revealed a significant reduction in mtDNA within the germline (Cree et al., 2008, Wai et al., 2008). This theory argues that only a small proportion of the total number of mtDNA is inherited by the offspring from the mother. The bottleneck effect could result in mature oocytes containing different levels of heteroplasmy and this has been demonstrated in human pedigrees (Wilson et al., 2016). Therefore, the varying severity of clinical phenotypes among family members with a point mutation in mtDNA may be explained by this difference in the levels of transmitted variants (McFarland et al., 2002). The precise mechanism of this mitochondrial genome bottleneck is yet to be defined because studying this phenomenon in humans remains difficult (Zhang et al., 2018).

The aforementioned levels of heteroplasmy can also add further challenges. For instance, carriers of the m.3243A>G variant may be genuinely asymptomatic, particularly if heteroplasmy levels across tissues are uniformly low. Although the phenotypic heterogeneity of this form of mitochondrial disease can at least be partly explained by the levels of heteroplasmy, another concern from epidemiological and clinical perspectives is that medical problems occurring in isolation may not be recognised for their mitochondrial aetiology and the diagnosis will be made only when a 'syndromic' combination of organ involvement occurs. Therefore, the clinical presentation of mitochondrial disease is highly variable and can occur at almost any stage of life, often with the involvement of either single or multiple organs (McFarland et al., 2010); thus posing significant diagnostic challenge for clinicians.

1.2.4 Mutations in the nuclear genome

After the initial breakneck speed of discovery of pathogenic variants in the mtDNA during the 1990s and early 2000s, the field's interest gradually wanes because novel variants became less attainable (DiMauro and Andreu, 2000, DiMauro and Garone, 2010). Instead, the nuclear genome gains more attention and interest, given that it encodes 99% of mitochondrial proteins and most of the respiratory chain subunits (Calvo et al., 2016). Matthews and colleagues reported the first nuclear gene mutation in the X chromosome associated with a disorder that affect mitochondrial function in 1993. They have shown that a mutation in the pyruvate dehydrogenase complex E1 alpha subunit gene, later known as the *PDHA1* gene, had led to a deficiency in pyruvate dehydrogenase complex activity causing Leigh syndrome(Matthews et al., 1993). The first biallelic autosomal mutation in the nuclear genome that affects the mitochondrial function was described by Bourgeron and colleagues in 1995. Two sisters with Leigh syndrome had homozygous mutations in the succinate dehydrogenase gene which encodes the subunit of Complex II (Bourgeron et al., 1995). From then onwards, nuclear gene defects that caused deficiencies in the complexes of the mitochondrial respiratory chain are discovered (Papadopoulou et al., 1999, Ogilvie et al., 2005, Janssen et al., 2006).

To date, there are over 300 pathogenic variants genes associated with mitochondrial disease and new variants are continually being discovered (Thompson et al., 2020). Next-generation sequencing (NGS) over the past decide, has accelerated the discovery of over 150 genes since its implementation. The OXPHOS function is dependent on a wide range of proteins, mostly imported into the mitochondria. The majority of proteins encoded by these nuclear genes have crucial functions in the OXPHOS, either directly or indirectly (Figure 1-5). Defects in the subunits in the complexes, cofactors or assembly factors, for instance, could have direct consequences on the OXPHOS. An example of an indirect effect of nuclear genes is on the expression of mtDNA. Nuclear genes encode for proteins that maintain, transcribe and translate mtDNA. These proteins also need to be imported correctly into the mitochondrial. Therefore, any defects in these processes can adversely affect the OXPHOS system.

mtDNA pathogenic variants: 36/37 genes					
OXPHOS subunits	*(CI) MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6; (CIII) MT-CYB; (CIV) MT-CO1, MT-CO2, MT-CO3; (CV) MT-ATP6, MT-ATP8*				
Ribosomal RNA	*MT-RNR1*				
Transfer RNA	*MT-TA, MT-TC, MT-TD, MT-TE, MT-TF, MT-TG, MT-TH, MT-TI, MT-TK, MT-TL1, MT-TL2, MT-TM, MT-TN, MT-TP, MT-TQ, MT-TR, MT-TS1, MT-TS2, MT-TT, MT-TV, MT-TW, MT-TY*				
Nuclear pathogenic variants: 295 genes					
	Complex I	**Complex II**	**Complex III**	**Complex IV**	**Complex V**
OXPHOS subunits	*NDUFA1, NDUFA2, NDUFA6, NDUFA9, NDUFA10, NDUFA11, NDUFA12, NDUFA13 NDUFB3, NDUFB8, NDUFB9, NDUFB10, NDUFB11 NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8 NDUFV1, NDUFV2*	*SDHA, SDHB, SDHD*	*CYC1, UQCRB, UQCRQ, UQCRC2*	*COX4I1, COX4I2, COX5A, COX6A1, COX6B1, COX7B, COX8A, NDUFA4*	*ATP5F1A, ATP5F1D, ATP5F1E*
OXPHOS assembly factors	*ACAD9, FOXRED1, NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5, NDUFAF6, NDUFAF8, NUBPL TIMMDC1, TMEM126B*	*SDHAF1*	*BCS1L, LYRM7, TTC19, UQCC2, UQCC3*	*CEP89, COX14, COX20, COA3, COA5, COA7, PET100, PET117, SURF1*	*ATPAF2, TMEM70*
Protein import & processing	*AFG3L2, AIFM1, CLPB, CLPP, DNAJC19, GFER, HSPD1, HTRA2, LONP1, MIPEP, PITRM1, PMPCA, PMPCB, SACS, SPG7, TIMM22, TIMM50, TIMM8A, XPNPEP3, YME1L*				
mtDNA replication & maintenance	*ABAT, DGUOK, DNA2, MGME1, MPV17, POLG, POLG2, RNASEH1, RRM2B, SAMHD1, SLC25A4, SSBP1, SUCLA2, SUCLG1, TFAM, TK2, TOP3A, TWNK, TYMP*				
RNA maturation/ modification	*ELAC2, ERAL1, FASTKD2, GTPBP3, HSD17B10, LRPPRC, MRM2, MTFMT, MTO1, MTPAP, NSUN3, PNPT1, PUS1, TRIT1, TRMT10C, TRMT5, TRMU, TRNT1*				
Mitochondrial aminoacyl tRNA synthetases	*AARS2, CARS2, DARS2, EARS2, FARS2, GARS, GATB, GATC, HARS2, IARS2, KARS, LARS2, MARS2, NARS2, PARS2, QRSL1, RARS2, SARS2, TARS2, VARS2, WARS2, YARS2*				
Mitoribosome	*MRPS2, MRPS7, MRPS14, MRPS16, MRPS22, MRPS23, MRPS28, MRPS34, MRPL3, MRPL12, MRPL44, PTCD3*				
Translation	*C12orf65, GFM1, GFM2, RMND1, TACO1, TSFM, TUFM*				
Membrane dynamics & composition	*AGK, CHKB, DNM1L, GDAP1, MFF, MFN2, MSTO1, NME3, OPA1, OXA1L, PGA2G6, PNPLA4, PNPLA8, QIL1, SERAC1, SLC25A46, STAT2, TAZ, TRAK1, VPS13C*				
β-oxidation	*ACADM, ACADS, ACADSB, ACADVL, ACAT1, CPT1A, CPT2, ETFA, ETFB, ETFDH, HADH, HADHA, HADHB, HMGCL, HMGCS2, OXCT1, SLC22A5, SLC25A20*				
TCA cycle	*ACO2, ALDH18A1, CA5A, DLAT, FH, HAAO, IDH3A, IDH3B, KYNU, MDH2, MPC1, PC, PDHA1, PDHB, PDHX, PDK3, PDP1, PPA2, SLC25A12, SLC25A13, SLC25A3*				
Cofactors	*ABCB7, BOLA3, C19ORF12, COA6, COASY, COQ2, COQ4, COQ5, COQ6, COQ7, COQ8A, COQ8B, COQ9, COX10, COX15, CYCS, DLD, FDX1L, FDXR, FLAD1, FXN, GLRX5, HCCS, IBA57, ISCA1, ISCA2, ISCU, LIAS, LIPT1, LIPT2, LYRM4, MECR, MICU1, MICU2, NADK2, NAXE, NFS1, NFU1, PANK2, PDSS1, PDSS2, SCO1, SCO2, SFXN4, SLC19A2, SLC19A3, SLC25A19, SLC25A24, SLC25A26, SLC25A32, SLC25A42, SLC39A8, TPK1*				
Other	*APOPT1, ATAD3A, C1QBP, CHCHD10, D2HGDH, ECHS1, ETHE1, FBXL4, HIBCH, IARS, L2HGDH, OPA3, RTN4IP1, SLC25A1, TMEM65, TXN2*				

Figure 1-5: A list of genes that are currently known to be associated with mitochondrial diseases ordered according to their functions. Reproduced from (Thompson et al., 2020)

1.3 Prevalence of mitochondrial disease

Mitochondrial disease is more prevalent than was originally thought and has now been recognised as among the commonest adult forms of inherited metabolic neurological disorder, with pathogenic mutations in both nuclear and mitochondrial genomes in the United Kingdom (Gorman et al., 2015b). The high-throughput gene-based diagnostic technology available in the last decade, along with detailed phenotyping by highly specialised clinical services and longitudinal natural history studies have accelerated the exponential rise in the identification of novel pathogenic variants causing mitochondrial disease. The most detailed prevalence study in mitochondrial disease determines the prevalence rate of adult mitochondrial disease as 9.6 cases per 100,000 individuals caused by mutations in mtDNA and 12.5 per 100,000 (1 in 8000) when including pathogenic mutations in nuclear genomes (Gorman et al., 2015b). The conservative estimates of a combined prevalence of mutations from both genomes were 23 per 100,000 (1 in 4300) (Gorman et al., 2015b).

1.3.1 Prevalence of childhood mitochondrial disease

Estimating the prevalence of mitochondrial disease in children is even more challenging than in adults. This is partly due to the conspicuous absence of classic syndromic findings, limited useful laboratory tests and lack of clinical consensus on abnormal results. As our understanding of paediatric mitochondrial diseases expands with the advancement in diagnostic technologies and clinical acumen, it becomes apparent that most causative pathogenic variants in children lie in the nuclear genome (Gorman et al., 2015b, Schon et al., 2021). Thus, the true prevalence is likely to be underestimated. Nonetheless, the current best estimate on the prevalence of childhood-onset mitochondrial disorders has been predicted to range from 5 to 15 cases per 10,000 (Uusimaa et al., 2000, Darin et al., 2001, Skladal et al., 2003, Castro-Gago et al., 2006, Ryan et al., 2006, Diogo et al., 2009, Verity et al., 2010, Yamazaki et al., 2014). The substantial variation in prevalence rates, may not only be attributable to study design but also due to the presence of genetic founder mutations. For instance, due to the spread of single ancient European founders in *the POLG* gene, pathogenic variants in this gene are now recognised as one of the most

common causes of childhood-onset recessive mitochondrial disorders in Europe, the USA, New Zealand and Australia (Hakonen et al., 2007). Increased prevalence rates in discrete populations with high consanguinity may also impact prevalence rates as exemplified in Australian Lebanese and Irish travelling communities (Skladal et al., 2003, Ryan et al., 2006) of higher rates of autosomal recessive childhood-onset mitochondrial diseases.

1.3.2 Prevalence of Leigh syndrome

In children, the most common syndromic presentation of mitochondrial disease is Leigh syndrome. This early-onset neurodegenerative disorder is caused by more than 80 pathogenic gene mutations, encoded in two genomes (mitochondrial and nuclear) (Lake et al., 2016, Rahman et al., 2017). Birth prevalence of Leigh syndrome was estimated to be 1:40,000 (Rahman et al., 1996). The estimated prevalence in western Swedish pre-school children was 1:34,000 (Darin et al., 2001), but significant founder effects are influencing the prevalence of Leigh syndrome in some populations. For example, a form of Leigh syndrome, due to a specific homozygous mutation in the *LRPPRC* gene, occurs in the French-Canadian population living in the Saguenay-Lac-Saint-Jean region, where the incidence reaches 1 in 2000 live births (Morin et al., 1993). Until recently, mutations in *LRPPRC* were not described outside of this small insular population and this form of Leigh syndrome was thought to be unique to the region, but subsequent descriptions of Leigh syndrome due to mutations in *LRPPRC* have now been reported more widely in the population (Oláhová et al., 2015). Another example is that of a Leigh-like syndrome, more specifically encephalomyopathy and methylmalonic acidaemia, caused by mutations in *SUCLA2*, where the incidence in the Faroe Islands, a community of approximately 49000 people, approaches 1 in 1700 (Ostergaard et al., 2007).

1.4 Leigh syndrome

Since Dr. Leigh's original report, Leigh syndrome has moved on from a postmortem neuro-histopathological diagnosis to one that can be made during life. There is clinical heterogeneity between patients with respect to the age of onset, duration of illness and symptomatology, but despite this variation, Leigh syndrome is typically characterised by stepwise developmental regression or developmental delay, specific neuroradiological features and abnormal mitochondrial energy metabolism. Stringent diagnostic criteria were first proposed in 1996 to define Leigh syndrome (Rahman et al., 1996), followed by two revisions in 2014 (Baertling et al., 2014) and 2016 (Lake et al., 2016). Three main criteria are generally agreed as follows:

1. Stepwise clinical deterioration: Progressive neurological disease characterised by stepwise decompensation during intercurrent illness. Motor and intellectual developmental delay or regression are often followed by a period of stabilization or recovery before another decline. Seizures and peripheral neuropathy are often present.
2. Bilateral neuro-radiological features: Signs and symptoms of brain stem and/or basal ganglia disease (dystonia spasticity, movement disorder, ataxia and nystagmus). These correspond with bilateral symmetric hyperintense signal changes in the brain stem and/or basal ganglia on T2-weighted magnetic resonance imaging (MRI) of the brain. Proton magnetic resonance spectroscopy may pick up areas of raised lactate levels.
3. Abnormal mitochondrial energy metabolism: Elevation of blood and/or CSF lactate levels. Increased lactate is more consistent in CSF than blood. Plasma amino acid might show raised alanine level. These abnormalities result from defective oxidative phosphorylation (OXPHOS) or pyruvate dehydrogenase complex (PDHc) activities.

'Leigh-like syndrome' terminology is frequently used if patients have clinical features strongly suggestive of Leigh syndrome, but do not satisfy all of these three stringent criteria. This "Leigh-like" designation is often a result of atypical (or normal) neuro-imaging, normal blood and/or CSF lactate levels or unusual neuropathology (Lake et al., 2016). The recent advent of whole genome sequencing lead to more genetic diagnoses being made at an earlier age. Hence, there are patients who carry the pathogenic variants but do not fully express the typical Leigh syndrome phenotypes at the time of diagnoses.

1.4.1 Clinical manifestations

The onset of Leigh syndrome is typically between 3 and 12 months (Rahman et al., 1996) although symptoms can occur in the neonatal period or first become recognized in adult life (McKelvie et al., 2012). The median age of onset is 7 months, with more than 80% of patients presenting before two years old (Naess et al., 2009, Sofou et al., 2014). Late presentation in late adult and slower progression can be seen in some patients, with associated long-term survival. The underlying genetic defect appears to predict life expectancy and extra neurological features (Wedatilake et al., 2013). A multicentre study on Leigh syndrome in Europe revealed that the median time from disease onset to death was 1.8 years with a median age at death of 2.4 years (Sofou et al., 2014).

Initial clinical features during the early infantile period may be non-specific, with feeding difficulties (dysphagia), persistent vomiting and failure to thrive as frequent presentations, often difficult at times to distinguish from other causes such as gastroesophageal reflux. The onset of Leigh syndrome is often triggered by metabolic stressors, such as minor respiratory or gastrointestinal viral infections, which are accompanied, or quickly followed, by a loss of acquired developmental skills (Baertling et al., 2014). Central nervous system features such as encephalopathy, hypotonia, dystonia, spasticity and seizures predominate early in the course of the disease, though as the condition progresses the involvement of the neuromuscular system is also increasingly obvious, with peripheral neuropathy, ptosis and muscle wasting evident. The combined central nervous and neuromuscular system involvement has a

profound impact on motor function and children with Leigh Syndrome can exhibit a wide range of abnormal motor findings (Ruhoy and Saneto, 2014).

A prominent feature of Leigh syndrome is impaired control of eye movement, with nystagmus, strabismus, ptosis and ophthalmoplegia being common clinical findings (Ruhoy and Saneto, 2014). Visual impairment may also be present depending on the precise genetic aetiology of the Leigh syndrome and the associated central nervous system involvement (Åkebrand et al., 2016). Epileptic seizures are common, with both generalized and focal convulsions witnessed in many patients. Basal ganglia and brainstem lesions cause an extrapyramidal movement disorder, respiratory difficulties (apnoea/hypopnoea), bulbar palsy and abnormal thermo-regulation (Sofou et al., 2014). Sensorineural hearing impairment occurs in approximately 20% of patients with Leigh Syndrome (Sofou et al., 2014). Extraneurological manifestations are not uncommon with dilated or hypertrophic cardiomyopathy, hepatic failure, renal tubulopathy and diffuse glomerulocystic kidney disease previously reported (Wang et al., 2008, Naess et al., 2009, Hadzsiev et al., 2010, Van Hove et al., 2010, Duff et al., 2015).

Clinical indicators for poor survival for Leigh syndrome as reported in a multicentre European study, were a history of epileptic seizures, failure to thrive, brainstem lesions and intensive care admissions (Sofou et al., 2014). Age of onset before six months also signifies a poor prognosis though that is true more generally for mitochondrial diseases with the exception of reversible infantile-onset respiratory chain deficiency (Zeharia et al., 2009, Uusimaa et al., 2011). Elevated CSF lactate, reported in most clinical laboratories as above 2 mmol/L, has been linked to early onset of disease, hypotonia, frequent exacerbations and brainstem lesions, though no correlation had been found between high CSF lactate and survival outcome (Sofou et al., 2014). The presence of cardiomyopathy in children with any form of mitochondrial disease usually denotes an unfavourable survival outcome (Holmgren et al., 2003) and cardiac assessment should therefore form part of the clinical assessments of children with Leigh syndrome.

1.4.2 Clinical investigation

A high index of clinical suspicion is key in assessing infants or children who present with stepwise regression of their developmental milestones. In order to establish the clinical diagnosis of Leigh syndrome, a series of assessments and investigations should be considered as follows:

1. Detailed developmental assessment, especially serial assessments
2. Neuro-imaging (MRI and MRS of brainstem / basal ganglia)
3. Electrophysiology studies (EEG if epileptic seizures and nerve conduction studies if peripheral neuropathy suspected/evident)
4. Ophthalmic examination to assess ocular abnormalities
5. Cardiac assessment including echocardiography and ECG for cardiomyopathy
6. Metabolic biochemical studies including serum and CSF lactate and pyruvate levels; urinary organic acids; pH studies
7. Respiratory chain enzyme studies
8. Referral to experts in genetics/mitochondrial service for the consideration of genetics studies (gene panels, exome or whole genome sequencing)

1.4.3 Differential diagnosis

Several disorders share similarities to Leigh syndrome especially bilateral brainstem and/or basal ganglia involvement. Post-infectious encephalopathy secondary to viruses can present with bilateral symmetrical lesions in the brainstem and thalami (Suwa et al., 1999). Another infection-related condition that mimics Leigh syndrome is acute necrotising encephalopathy (ANE), a disorder where previously healthy young children develop a rapidly progressive encephalopathy within days of the onset of a viral infection (Mizuguchi et al., 1995). In the acute phase, cerebral MR imaging typically shows symmetric lesions affecting the basal ganglia (Mizuguchi et al., 1995). Although this condition is sporadic, familial (incompletely penetrant) autosomal dominant ANE (ADANE), due to mutations in the *RANBP2* gene, has been reported(Neilson et al., 2003). Other metabolic disorders, especially methylmalonic aciduria (Zwickler et al., 2012) and propionic acidemia (North et al., 1995) or pantothenate kinase-associated neurodegeneration (PKAN) (Egan et al., 2005), can lead to symmetric basal ganglia involvement. Analysis of blood and urine samples could distinguish these from Leigh syndrome. Another mimic of Leigh syndrome is Wernicke's encephalopathy (WE) secondary to dietary thiamine deficiency which was considered by Denis Archibald Leigh in his original report. Typical neuroradiological features of WE include mamillary body degeneration, bilateral basal ganglia and putaminal MR changes (Zuccoli et al., 2010). This underdiagnosed condition is responsive to thiamine. Thiamine can also be used, in combination with biotin, to treat another Leigh syndrome mimic, biotin-responsive basal ganglia disease (BRBGD). This disorder is caused by mutations in the *SLC19A3* gene, which encodes a thiamine transporter, and can be difficult to distinguish from mitochondrial disease without genetic testing. Brain imaging usually shows symmetric bilateral lesions in the caudate nucleus, putamen and brainstem (Tabarki et al., 2013). This thiamine transporter-2 deficiency can be treated at an early stage with thiamine and high-dose biotin with significant clinical improvement (Fassone et al., 2013).

1.4.4 Biochemical basis

Defective energy metabolism within mitochondria is believed to underline the biochemical basis of Leigh syndrome (DiMauro and Schon, 2003). Biochemical analysis of respiratory chain enzyme activity in a patient with Leigh syndrome may reveal the decreased activity of any of these complexes, but this is not always in isolation and more than one complex may be deficient. Abnormal respiratory chain enzyme activity deficiencies of Complex I or IV are most frequent, though multiple respiratory chain deficiencies also occur with mt-tRNA mutations and defects of mtDNA maintenance or synthesis (e.g. *SUCLA2*, *RRM2B*). Histological and histochemical analysis of the muscle biopsy remains one of the most important diagnostic screens for detecting mitochondrial abnormalities (Old and Johnson, 1989). Skeletal muscle tissue is often affected and is fairly accessible. About 10-20% of patients with normal respiratory chain enzyme activity in skeletal muscle may have defects in the liver or cardiac muscle (Thorburn et al., 2004). A 'hypocapnic hypothesis' has been proposed that questions energy deprivation as the mechanism causing the typical features of Leigh syndrome (Pronicka, 2017). The hypocapnic hypothesis assumes that, for OXPHOS deficient brain cells, the primary harmful factor in triggering Leigh syndrome is a decrease in pCO_2 (hypocapnia) associated with an increase in pH (alkalization) occurring during hyperventilation and not energy deprivation. Evidence supporting this hypothesis remains rather circumstantial.

1.4.5 Genetic basis

Our understanding of the genetic aetiology of Leigh Syndrome has improved enormously over the last 20 years with more than 80 genes now identified in affected patients (Lake et al., 2016). Nevertheless, a substantial proportion of Leigh syndrome patients remain without a genetic diagnosis indicating that there are aspects of mitochondrial metabolism that have not yet been elucidated but are important in maintaining normal mitochondrial function. The genetic basis of Leigh syndrome can be divided into two broad categories – nuclear DNA and mitochondrial DNA associated with Leigh syndrome. These genes and their possible functions are summarized in a schematic diagram (Figure 1-6).

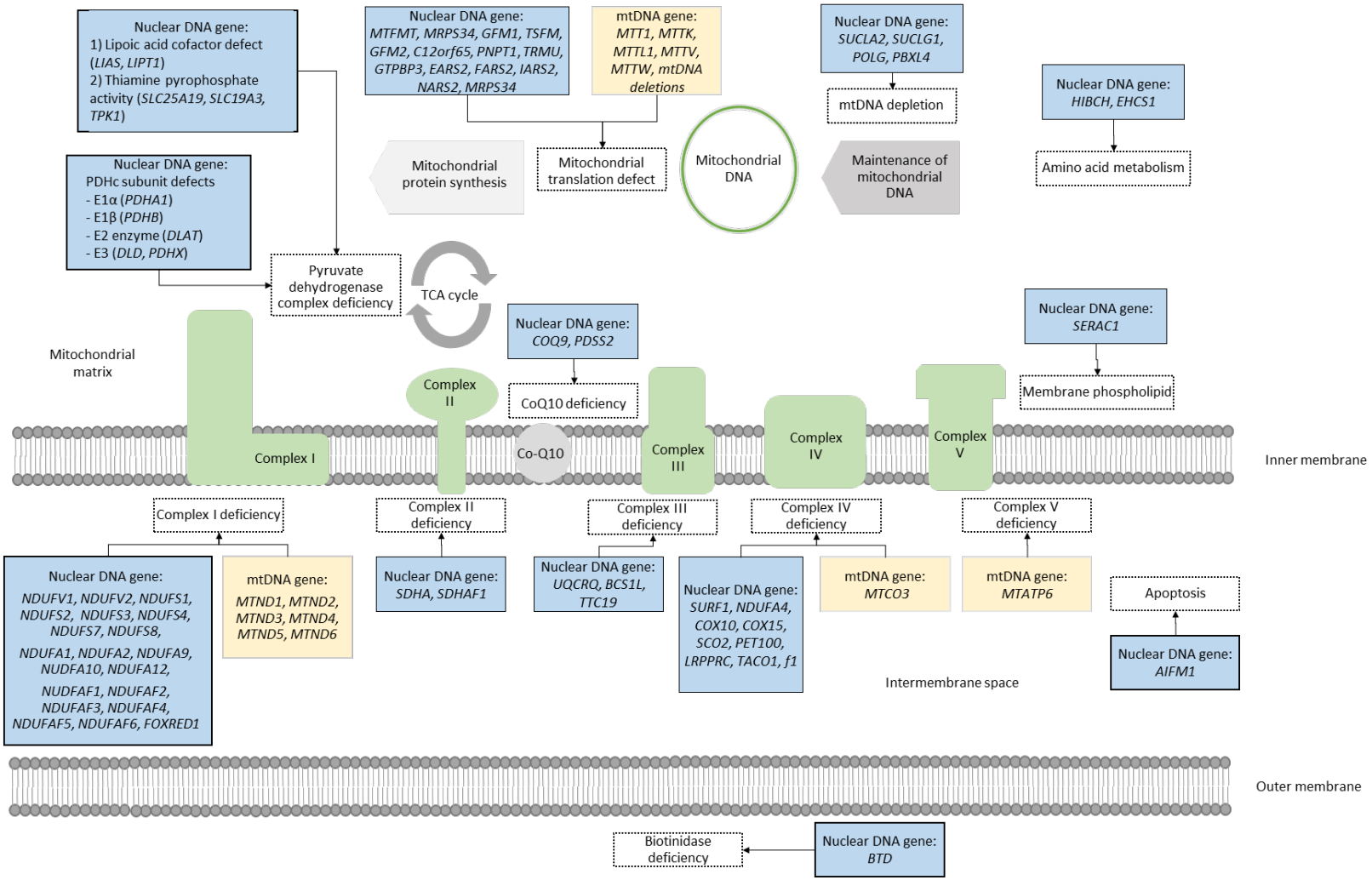

Figure 1-6: Schematic diagram shows the nuclear genes (blue boxes) and mitochondrial genes (yellow boxes) that are currently known to be associated with Leigh or Leigh-like syndrome. These genes are drawn according to their primary function in the mitochondria. Reproduced with permission from (Lim and McFarland, 2019)

1.4.6 Nuclear DNA associated Leigh syndrome

1.4.6.1 Complex I deficiency

The most common biochemical defect in Leigh syndrome is complex I deficiency and mutations affecting the *NDUFS4* (NADH dehydrogenase) subunit, although this may reflect some degree of reporting bias (Fassone and Rahman, 2012). Almost all patients with *NDUFS4* mutations have Leigh syndrome with a characteristic initial presentation at 8 months old, a rapidly progressive course with death occurring by 30 months (Assouline et al., 2012, Fassone and Rahman, 2012). Neuroradiological features of these infants showed bilateral symmetrical basal ganglia lesions and approximately one-third of cases had hypertrophic cardiomyopathy (Assouline et al., 2012). Several other nuclear genetic mutations associated with Leigh syndrome or Leigh-like syndrome affect complex I activity (Tuppen et al., 2010b, Lake et al., 2016). Leigh syndrome mediated by *NDUFS4*, *NDUFV1* and *NDUFS1* seems to have severe forms of the disease (Lake et al., 2016). *NDUFV1* and *NDUFS1* are both core subunits of Complex I (Mimaki et al., 2012) and complete loss of these proteins are probably incompatible with life. Children with these mutations also have a lower median age of death compared to other forms of Leigh syndrome (Sofou et al., 2014, Lake et al., 2016).

1.4.6.2 Complex IV deficiency

Patients with complex IV deficiency constitute approximately 15% of Leigh syndrome (Rahman et al., 1996, Sofou et al., 2014). The most common cause of complex IV deficient Leigh syndrome is mutations in *SURF1*, which encodes a complex IV assembly factor (Wedatilake et al., 2013). *SURF1* patients tend to survive longer than those with typical Leigh syndrome due to other genetic aetiologies, with a median age of death at 5.4 years (Wedatilake et al., 2013). Genetic variants in a different gene, *ETHE1,* impair sulfide detoxification; sulfide is a powerful inhibitor of Complex IV and its accumulation causes a functional cytochrome oxidase deficiency and leads to an encephalopathy related to Leigh syndrome (Tiranti et al., 2009). This fatal condition has been shown to be 'treatable' by reducing sulfide accumulation with metronidazole and N-acetylcysteine (Viscomi et al., 2010). As noted above, inherited homozygous

LRPPRC mutations are a cause of Leigh syndrome with severely low Complex IV activities in brain and liver tissue within the Saguenay-Lac-Saint-Jean region of Quebec (Merante et al., 1993). Leigh syndrome associated with this population has typical mild facial dysmorphism and liver dysfunction (Debray et al., 2011). Although the gene product of LRPPRC has a role in the stability and translation of the mRNA for mitochondrially-encoded Complex IV subunits (Xu et al., 2004), *LRPPRC* is increasingly recognised to have a broader effect on mitochondrial energy processes including ATP synthase (Mourier et al., 2014).

1.4.6.3 Complex II, III and ubiquinone deficiencies

Nuclear gene mutations affecting complex II, complex III and Ubiquinone deficiencies are rare causes of Leigh syndrome. Reports of Leigh syndrome with these biochemical deficiencies include mutations in *SDHA* and *SDHAF1* (complex II), *UQCRQ*, *BCS1L*, and *TTC19* (complex III) and *COQ9* and *PDSS2* (Ubiquinone). From a clinical perspective, early identification of ubiquinone (CoQ10) deficient Leigh syndrome could facilitate initiation of replacement therapy with large doses of CoQ10, with a good survival outcome into adulthood having been reported (Van Maldergem et al., 2002, Rahman et al., 2012). *PDSS2* mutations are known to be associated with seizures and nephrotic syndrome (López et al., 2006). Mutation in *SDHA* (complex II) was one of the earliest reported nuclear mutations described in Leigh syndrome (Bourgeron et al., 1995). Early-onset disease was associated with a rapid demise, but some patients might experience a milder course with survival into late childhood and preservation of cognitive abilities (Pagnamenta et al., 2006, Ma et al., 2013). Mutation in *TTC19* which encodes for a complex III assembly factor causes a form of Leigh Syndrome with characteristic neuroimaging changes that include lesions in the putamen and caudate nuclei, cerebellar atrophy, and the unusual finding of hypertrophic olivary nuclei degeneration (Koch et al., 2015).

1.4.6.4 Mitochondrial DNA depletion

Mitochondrial DNA depletion affects the availability of mitochondrial DNA in the synthesis of key components for complex I, III, IV and V. One of the most common cause of mitochondrial-DNA-depleted Leigh syndrome is a mutation in *SUCLA2* which encodes subunits of succinyl-CoA synthetase within the citric acid cycle (Elpeleg et

al., 2005). Accumulation of succinyl-CoA due to failure in conversion to succinate leads to methylmalonic aciduria. *SUCLA2* mutations cause hypotonia, muscle atrophy, hearing loss and growth retardation (Ostergaard et al., 2007). Manifestations of *SUCLG2* are similar but primarily predominated by recurrent hepatic failure (Valayannopoulos et al., 2010). These two mutations, *SUCLA2* and *SUCLG2*, are thought to impact the interaction of succinyl-CoA synthetase and nucleoside diphosphate kinase which is responsible for mitochondrial nucleotide supply (Ostergaard et al., 2007). Another gene mutation that results in mitochondrial DNA depletion is *FBXL4*. Patients with this mutation present with Leigh-like syndrome along with facial dysmorphism, gastrointestinal dysmotility and renal tubular acidosis (Shamseldin et al., 2012, Huemer et al., 2015).

1.4.6.5 Mitochondrial translation defects caused by nuclear genes

A relatively recently characterised form of Leigh syndrome is that caused by mutations in genes encoding the mitochondrial tRNA modifying enzymes (*MTFMT*, *MTO1*, *TRMU* and *GTPBP3*). These enzymes are responsible for the post-transcriptional modification of mitochondrial tRNAs essential for successful mitochondrial DNA translation. Consequently, Leigh syndrome resulting from such mutations typically has combined mitochondrial respiratory chain deficiencies. Other genes involved in the mtDNA translation machinery that typically lead to a combined mitochondrial respiratory chain deficiency include *MRPS34* (Lake et al., 2017), *GFM1* (Valente et al., 2007), *TSFM* (Ahola et al., 2014), *GFM2* (Glasgow et al., 2017), *C12orf65* (Antonicka et al., 2010) and *PNPT1* (Vedrenne et al., 2012), while those encoding mitochondrial tRNA synthetases (*EARS2* (Martinelli et al., 2012), *FARS2* (Shamseldin et al., 2012), *IARS2* (Schwartzentruber et al., 2014) and *NARS2* (Simon et al., 2015)) often have a more variable impact on the mitochondrial respiratory chain activities (Boczonadi and Horvath, 2014). In 2011 mitochondrial methionyl-tRNA formyltransferase (*MTFMT*) mutations were discovered in two unrelated children with Leigh syndrome, microcephaly and a combined oxidative phosphorylation pathway deficiency (Tucker et al., 2011). Almost all subsequently identified cases are compound heterozygotes carrying one copy of the c.626C>T mutation identified in the original cases. Since its discovery, mutations in *MTFMT* have become one of the most

common nuclear genetic causes of Leigh syndrome in the European population, with a carrier frequency of approximately 1 in 1000 (Haack et al., 2014a).

1.4.6.6 Pyruvate dehydrogenase complex (PDHc) deficiency

The pyruvate dehydrogenase complex is a multi-enzyme platform located on the inner mitochondrial membrane that catalyses the conversion of pyruvate to acetyl CoA and carbon dioxide. The acetyl CoA generated enters the tricarboxylic acid cycle and thus PDHc is a crucial step in the energy transduction process. PDHc-deficient Leigh syndrome presents with many of the typical features of the condition but dysgenesis of the corpus callosum, epilepsy and elevated serum lactate:pyruvate ratio of more than 20 may be diagnostic clues to the genetic aetiology (Patel et al., 2012). Autosomal recessive PDHc deficiencies can be subdivided into several main categories: 1) Lipoic acid synthesis defects (*LIAS and LIPT1*) (Soreze et al., 2013, Baker et al., 2014); 2) PDHc subunit E3 defects (*DLD* and *PDHX*) (Schiff et al., 2006, Quinonez et al., 2013) and 3) absence of thiamine pyrophosphate (TPP) activity (*PDHA1*, *SLC25A19, SLC19A3* and *TPK1*) (Mayr et al., 2011). Of particular note are mutations in *PDHA1* an X-linked gene encoding the E1 alpha subunit of the complex and with unfavourable X-inactivation frequently results in symptomatic females. The gene also has a high *de novo* mutation rate and *PDHA1* mutations are thought to be the leading cause of PDHc-deficient Leigh syndrome(DeBrosse et al., 2012, Patel et al., 2012).

1.4.7 Mitochondrial DNA associated Leigh syndrome

Mitochondrial DNA is inherited maternally and in multicopy and Is considerably more prone to mutation than nuclear DNA yet lacks many of the repair and protective mechanisms present for nuclear DNA. While not completely determining disease expression, the proportion of mutated mtDNA present (the level of heteroplasmy) is nevertheless a key factor in the clinical severity of disease, with tissue-specific thresholds for each individual and each mutation. This concept of a heteroplasmy threshold determining the expression of a biochemical abnormality (and therefore clinical disease) is important in explaining why some family members with sub-threshold heteroplasmy levels remain asymptomatic. Numerous mitochondrial genes have been associated with Leigh syndrome including six that encode for complex I subunits (*MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND5* and *MT-ND6*), one for complex IV subunits (*MTCO3*) and one for complex V subunits (*MT-ATP6*). Among all these, *MT-ND3*, *MT-ND5* and *MT-ATP6* are the leading causes of mitochondrial DNA associated Leigh syndrome. *MT-ATP6* (m.8993T>G or m.8993T>C) is believed to be the cause of about 1 in 10 cases of Leigh syndrome (Rahman et al., 1996, McFarland et al., 2004, Nesbitt et al., 2012, Ma et al., 2013, Ng et al., 2019). Leigh syndrome usually manifests at mtDNA heteroplasmy level above 90%, though several different mutations in the *MT-ND5* gene have been linked with Leigh syndrome at a mutant load below 50% (Kirby et al., 2003). Another group of mtDNA mutations that are associated with Leigh syndrome are those that caused translational defects (*MT-T1, MT-TK, MT-TL1, MT-TV and MT-TW)*. Although m.3243A>G in *MT-TL1* are typically associated with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) or maternally-inherited diabetes and deafness (MIDD) phenotypes and m.8344A>G in *MT-TK* are associated with MERRF; Leigh syndrome has been observed in carriers of these two common mitochondrial tRNA translation defects (Hammans et al., 1993).

1.4.8 Diagnostic strategy

After taking a comprehensive medical history with family pedigree and bedside examination, a series of investigations should be undertaken to define the neuroradiological and biochemical characteristics. Some patients may have a constellation of findings that is suggestive of a specific mutation, which, if identified, might avoid the need for expensive and invasive muscle or skin biopsies for measurement of defective oxidative phosphorylation (OXPHOS) or pyruvate dehydrogenase complex (PDHc) activities. Multi-gene panel testing has become a popular means of investigating a wide range of clinical disorders including mitochondrial disease, but panels quickly become obsolete with new gene discoveries and in several laboratories whole exome or whole genome sequencing is now being employed in the diagnostic algorithm (Alston et al., 2017, Thompson et al., 2020). These powerful next-generation sequencing techniques can detect both nuclear and mitochondrial DNA defects (Calvo et al., 2012, Carroll et al., 2014).

1.4.9 Management

1.4.9.1 Specific treatments

There are possibly six Leigh or Leigh-like syndromes that could be treated with varying degrees of response expected depending on the underlying genetic causes. These conditions should be sought at an early stage in the diagnostic process to avoid delay in instigating disease-modifying treatment. All of these conditions have been discussed in other sections of this chapter and are summarised in Table 1-2. Where there is a high clinical index of suspicion of a particular form of Leigh syndrome it would be reasonable to commence a trial of the relevant supplements while waiting genetic confirmation.

Gene	*Biochemical defect*	*Distinguishing features*	*Treatment (response)*
SLC19A3	Thiamine transporter 2 deficiency	Episodic encephalopathy, paraparesis, dystonia, bilateral necrosis of caudate/putamen	Biotin 5-10mg/kg/ day and thiamine 300-900mg daily (fairly good)(Haack et al., 2014b)
BTD	Biotinidase deficiency	Deafness, optic atrophy, seizures, ataxia, organic aciduria	Biotin 5-10mg daily (fairly good)(Jay et al., 2015)
PDSS2	Coenzyme Q10 deficiency	Refractory seizures, nephrotic syndrome	Coenzyme Q10 10-30mg/kg/day (variable(Hargreaves, 2014, Jay et al., 2015)
ETHE1	Ethylmalonic encephalopathy	Developmental regression, pyramidal & extrapyramidal signs, acrocyanosis, ethylmalonic aciduria	Metronidazole and N-acetylcystine (variable) (Viscomi et al., 2010) (variable); liver transplant (single patient) (Dionisi-Vici et al., 2016)
PDHA1	Pyruvate dehydrogenase complex deficiency	Seizures, dystonia, microcephaly, cerebral atrophy, dysgenesis of corpus callosum, low serum lactate:pyruvate ratio	Thiamine 30-40mg/kg/day (variable) (van Dongen et al., 2015)
TPK1	Thiamine pyrophosphokinase deficiency	Episodic encephalopathy, dystonia, spasticity	Thiamine 20mg/kg/day (variable)(Banka et al., 2014)

Table 1-1: Table listing the six Leigh or Leigh-like syndromes that had specific treatment with varying degrees of success

1.4.9.2 General supportive care

There are currently no specific curative treatments for other causes of Leigh syndrome. However, management of symptoms (e.g spasticity and dystonia) is important, as is prevention, recognition and prompt treatment of exacerbating factors such as fever, dehydration and poor nutrition. Epileptic seizures should be managed by a paediatric neurologist with appropriate use of anticonvulsant medication, ketogenic diet and on occasion epilepsy surgery (vagal nerve stimulator). It is probably advisable to avoid sodium valproate due to its inhibitory effect on mitochondrial

function, though its adverse effects in this respect have not been ubiquitously demonstrated in mitochondrial disease and it has proved an effective anticonvulsant in some patients (Melegh and Trombitas, 1997, Anderson et al., 2002). Dystonia is a common feature in Leigh syndrome with baclofen, benzhexol, tetrabenazine and gabapentin often the symptomatic treatments of choice. Cardiomyopathy can be one of the manifestations of Leigh syndrome (Ruiter et al., 2007) and anti-congestive cardiac therapy should be led by a specialist cardiologist. General health surveillance at regular intervals (6-12 months) to monitor progression is generally recommended (Ruhoy and Saneto, 2014). During acute acidotic crises, sodium bicarbonate or sodium citrate may be considered with close monitoring. Dichloroacetate (DCA) reduces blood lactate by activating the pyruvate dehydrogenase complex. DCA is well tolerated by young children with congenital lactic acidosis (Stacpoole et al., 2008). However, correcting lactic acidosis using DCA has been complicated by peripheral nerve toxicity (Kaufmann et al., 2006). Therefore, the focus of management should be on optimising general supportive care rather than correcting lactate values which might not have clear benefits (Kaufmann et al., 2006, Stacpoole et al., 2008).

1.4.9.3 Family counselling

An extensive, multi-generational family pedigree should be documented during history-taking. Most of the nuclear DNA gene mutations of Leigh syndrome will be inherited in an autosomal recessive manner though some, such as *PDHA1*, *NDUFA1* and *AIFM1* showed X-linked inheritance. Mitochondrial DNA mutations, on the other hand, are often maternally-inherited though some occur *de novo* in the patient. Patients and family members of those with known or suspected to have mitochondrial DNA pathogenic variants should have up-to-date genetic counselling at specialized centres to explore all potential outcomes ranging from prenatal diagnosis to reproductive options (Gorman et al., 2018, Russell et al., 2020). Suspecting that our understanding of genotype, phenotype and potential treatment options of Leigh syndrome will improve in future, DNA banking should be considered for affected individuals. Following the identification of a pathogenic variant in an affected member, there are several options available to prevent the transmission of mitochondrial disease including prenatal diagnosis, preimplantation genetic diagnosis and mitochondrial donation (Gorman et al., 2018).

1.5 Mitochondrial DNA depletion syndrome

Apart from Leigh syndrome, another important 'syndromic' group of childhood-onset mitochondrial disorders result from mitochondrial DNA (mtDNA) depletion. This group of disorders affects the synthesis and replication of mtDNA. In 1991, Moraes and colleagues first noticed a depletion in the expression of mtDNA in muscle and liver tissues of three infants. These infants who died from fatal encephalomyopathy and liver failure had marked COX-deficient fibres and low mtDNA copy numbers in the affected tissues as compared to controls (Moraes et al., 1991). Several major mitochondrial disease phenotypes have mtDNA depletion, namely, the hepatocerebral, encephalomyopathy, pure myopathic and neurogastrointestinal syndromes. mtDNA depletion syndrome has been associated with several nuclear genes – *TK2, SUCLA2, SUCLG, DGUOK, POLG, RRM2B, TWNK,* and *TYMP* (Rötig and Poulton, 2009, Spinazzola et al., 2009, Smits et al., 2010). These nuclear genes encode for proteins that ensure the homeostasis of the mitochondrial deoxyribonucleotide triphosphates (dNTP) pool (Figure 1-7).

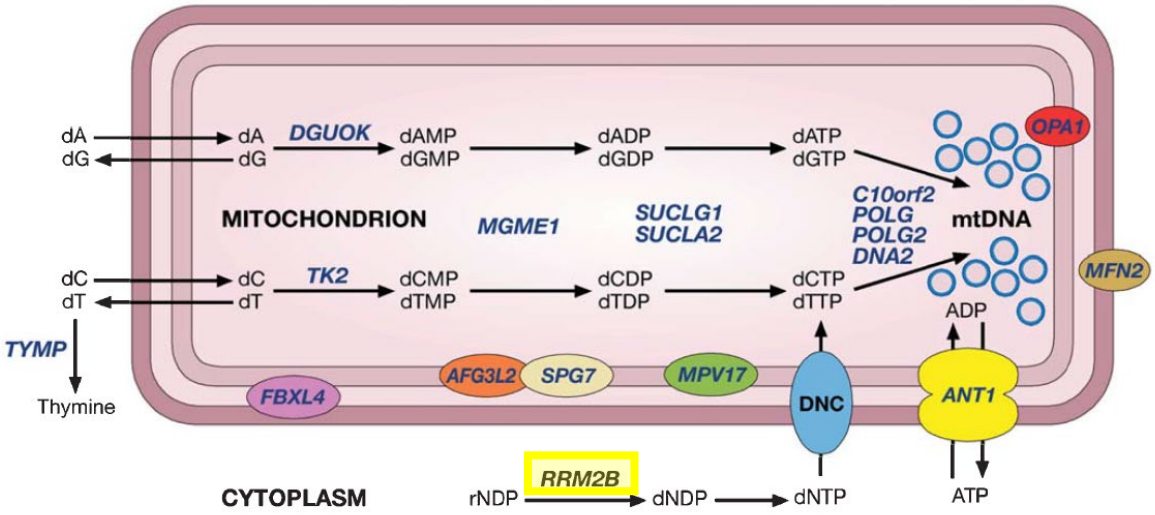

Figure 1-7: Schematic figure of mitochondrial nucleotide metabolism for mtDNA synthesis, replication and repair. Several genes (italicised) involved in these processes to maintain the mtDNA have been associated with depletion and deletion syndromes. RRM2B gene is highlighted in yellow. Reproduced from (Sommerville et al., 2014)

Two major pathways regulate dNTP supply to mitochondria – the salvage and the *de novo* synthesis pathways. The unique mitochondrial dNTP salvage pathway constantly converts former deoxynucleosides, already within the mitochondrial matrix as a result of DNA turnover, into dNTPs (Aaron and Minczuk, 2018). The mitochondrial thymidine kinase 2 is a key driver of the pyrimidine nucleotide salvage pathway as it carries out the initial phosphorylation of pyrimidine precursors (Johansson and Karlsson, 1997). The latter pathway operates in the cytosol where the key regulatory enzymes involved in dNTP synthesis are ribonucleotide reductase (RNR) and thymidylate synthase. RNR is responsible for the reduction of ribonucleotide phosphates to dNTP that maintains the correct amount of mtDNA (Pontarin et al., 2008, Penque et al., 2018). Pathogenic mutations in the *RRM2B* gene encoding the p53-inducible small subunit (p53R2) of the cytosolic dNTP salvage enzyme RNR, have been implicated in mtDNA depletion phenotypes.

1.6 Molecular pathogenesis of *RRM2B* gene

The *RRM2B* is a nuclear gene that encodes the p53-inducible small subunit (p53R2) of the ribonucleotide reductase (RNR) complex). RNR, also known as ribonucleoside diphosphate reductase, catalyses the formation of deoxyribonucleotide triphosphates (dNTP) by removing the 2'-hydroxyl group of the ribose ring from ribonucleotide phosphates (Elledge et al., 1992, Sneeden and Loeb, 2004). This catalysis by RNR is strictly conserved in living cells to regulate the constant DNA to cell mass ratio during division or repair (Tanaka et al., 2000, Torrents et al., 2002, Herrick and Sclavi, 2007). The RNR in humans is a heterodimeric tetramer consisting of large alpha subunits and small beta subunits (Smith et al., 2009). The alpha subunit, the ribonucleotide reductase M1 polypeptide, is encoded by the *RRM1* gene. The smaller beta subunits have two isoforms, the ribonucleotide reductase M2 and ribonucleotide reductase M2 B, which are encoded by *RRM2* and *RRM2B* genes respectively (Tanaka et al., 2000, Smith et al., 2009).The transcription of ribonucleotide reductase M2 B is tightly regulated by the tumour suppressor gene p53 which is activated during nuclear DNA damage (Nakano et al., 2000, Tanaka et al., 2000). The p53R2 has another important role in providing the dNTP needed for mtDNA replication via the salvage pathway (Tanaka et al., 2000) and remains at constant levels in post-mitotic cells (Chabes et al., 2003). The abnormal function of p53R2 disrupts the mtDNA maintenance, accumulation of multiple deletions, and depletion of copy numbers. Kimura and colleagues demonstrated that *RRM2B*-null mice lead to mtDNA depletion. These mice also demonstrated growth retardation, renal failure and early mortality (Kimura et al., 2003). In 2007, Bourdon and colleagues discovered that nonsense, missense and splice-site mutations and in-frame deletions in the *RRM2B* gene leads to the inactivation of the cytosolic p53R2 in humans. A complete lack of mtDNA was found in differentiated skeletal muscles of these patients and led to death shortly after birth (Bourdon et al., 2007). This work was also replicated in several other groups around that time (Bornstein et al., 2008, Acham-Roschitz et al., 2009, Kollberg et al., 2009). This discovery has affirmed the role of p53R2 in the stability and maintenance of human mtDNA.

1.7 Pathogenic variants in the *RRM2B* gene

To date, several pathogenic variants in the *RRM2B* gene have been identified. Many of the reported *RRM2B* mutations in humans are located within a highly conserved region and are also thought to interrupt intramolecular interactions (Pitceathly et al., 2012, Penque et al., 2018). These are summarised in Table 1-3 and illustrated in Figure 1-8. Pathogenic variants in the *RRM2B* gene have been associated with both autosomal recessively (AR) and autosomal dominantly (AD) inherited mitochondrial disease. The severity of the associated histochemical defect in the muscle is more pronounced with autosomal-recessive *RRM2B* mutations than autosomal-dominant mutations (Pitceathly et al., 2012). Most individuals diagnosed with an autosomal dominant *RRM2B* disorders have an affected parent but some individuals may have the disorder as the result of a *de novo* pathogenic dominant variant. Meanwhile, the parents of an affected patient with autosomal recessive *RRM2B* disorders are presumed to be carriers of one *RRM2B* pathogenic variant based on family history. Heterozygotes (carriers) for autosomal recessive *RRM2B* pathogenic variant associated with recessive phenotypes are asymptomatic and are not expected to develop manifestations of the condition.

Nucleotide change	Amino acid change	Inheritance	Phenotype	Reference
c.850C >T	p.Gln284*	AR	Encephalomyopathic	Bourdon (2007)
c.580G >A c.IVS3-2A >G splice site variant	p.Glu194Lys	AR		
c.190T > C c.581A >G	p.Trp64Arg p.Glu194Gly	AR		
c.253_255delGAG c.707G >T	p.Glu85del p.Cys236Phe	AR		
c.671T > G	p.Ile224Ser	AR	Encephalomyopathic	Bornstein (2008)
c.846G > C c.920delA	p.Met282Ile p.Asn307fs	AR		
c.949T > G c.584delG	p.Leu317Val p.Gly195fs	AR		
c.122G > C	p.Arg41Pro	AR	Encephalomyopathic	Spinazzola (2009)
c.328C > T IVS3–2A > C splice site variant	p.Arg110Cys	AR		
c.686G > T	p.Gly229Val	AR	Encephalomyopathic	Kollberg (2009)

c.386T > C	p.Phe123Ser	AR	Encephalomyopathic	Accham Roschitz (2009)
c.329G>A c.362G>A	p.Arg110His p.Arg121His	AR	MNGIE-like syndrome	Shaibani (2009)
c.979C>T	p.Arg327*	AD	arPEO	Tyynismaa (2009)
c.431C>T c.632G>A c.606T>A c.817G>A c.950delT c.952G>T c.965dupA c.122G>A c.583G>A	p.Thr144Ile p.Arg211Lys p.Phe202Leu p.Gly273Ser p.Leu317* p.Glu318* p.Asn322fs p.Arg41Gln p.Gly195Arg	AR AR AD AD AD AD AD	arPEO adPEO	Fratter (2011)
c.122G>A c.391G>A c.253_255delGAG	p.Arg41Gln p.Glu131Lys p.Glu85del	AR AD	Kearns-Sayre-like syndrome adPEO	Pitceathly (2011)
c.341G>A	p.Pro33Ser	AR	arPEO	Takata (2011)
c.48G>A splice site variant c.121C>T c.208G>A c.583G>A c.632G>A c.671T>G c.965dup c.979C>T c.1046C>G c.431C>T c.817G>A c.556A>G c.653C>T	p.Glu16Glu p.Arg41Trp p.Asp70Asn p.Gly195Arg p.Arg211Lys p.Ile224Ser p.Asn322fs p.Arg327* p.Ala349Gly p.Thr144Ile p.Gly273Ser p.Ar186Gly p.Thr218Ile	AD AD AD AD AD AD AD AD AD AR AR	adPEO arPEO	Pitceathly (2012)
c.707 G>A	p.Cys236Tyr	AR	Encephalomyopathic	Stojanovic (2013)
c.328C>T c.846G>C	p.Arg110Cys p.Met282Ile	AR	Kearns-Sayre-like syndrome	Willichowski (2013)
c.414_415delCA c.686G>T	p.Tyr138* p.Gly229Val	AR AR	Encephalomyopathic	Pronicka et al (2016)
c.662A > G	p.Asn221Ser	AR	Encephalomyopathic	Penque (2019)
c.414_415delCA c.(321+1_3221)_(684 + 1_685–1)del	p.Tyr138* p.?	AR	Encephalomyopathic	Iwanicka-Pronicka (2019)

c.686G > T	p.Gly229Val	AR		
c.313G>A c.599G>A	p.Glu105Lys p.Gly200Glu	AR	Encephalomyopathic	Keshavan (2020)
c.181G>C	p.Ala61Pro	AR		
c.165G>A c.(321+1_322-1)_(684+1_685-1)del	p.Met55Ile	AR		
c.846G>C	p.Ala61Pro p.Met282Ile	AR		
c.635_636insAAG	p.Gly212_Leu213insSer	AR		
c.121C>T c.671T>G	p.Arg41Trp p.Ile224Ser	AR		
c.400C>G	p.His134Asp	AR		
c.48G>A c.59C>G	p.Ser20*	AR		

Table 1-2: A list of pathogenic variants in RRM2B (NM_001172477.1) gene (Lim et al., 2021).

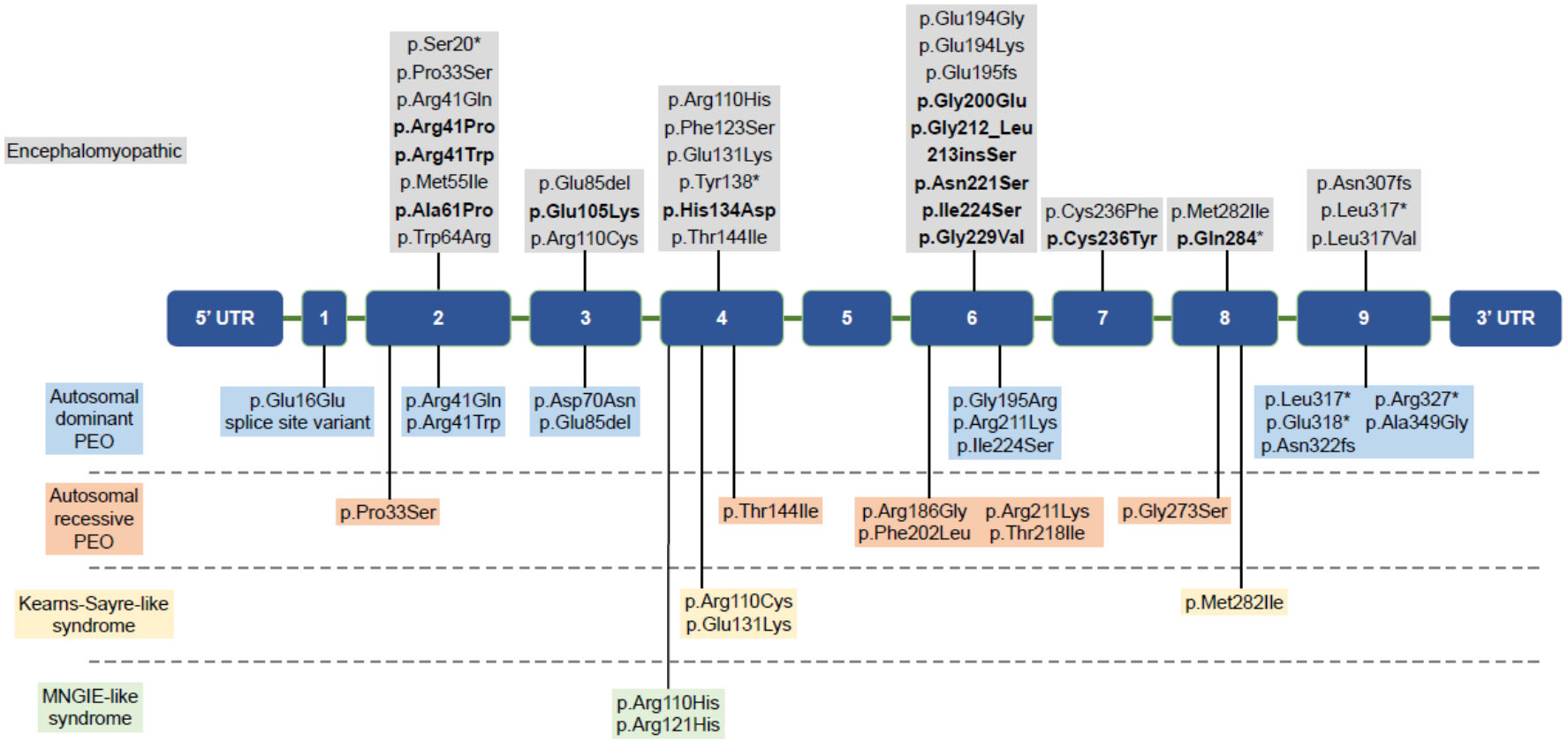

Figure 1-8: Schematic representation of the RRM2B gene structure (NM_001172477.1) illustrating reported pathogenic variants. Coding exons are numbered 1 to 9. RRM2B variants associated with encephalomyopathic phenotype are highlighted in grey, autosomal dominant PEO are highlighted in light blue, autosomal recessive PEO are highlighted in pink, Kearns-Sayre-like syndrome are highlighted in light yellow and MNGIE-like syndrome are highlighted in light green. The variants in bold associated with an encephalomyopathic phenotype have been reported as homozygous changes. Some variants are reported across different phenotypes. Reproduced from (Lim et al., 2021)

1.8 *RRM2B*-related mitochondrial disorder

RRM2B-related mitochondrial disease in general manifests with several clinical phenotypes. First, the mitochondrial DNA depletion syndrome that results in an encephalomyopathic phenotype in infants. This is the most severe phenotype which usually manifests shortly after birth as hypotonia, poor feeding and faltering growth necessitating hospitalisation. There is a likelihood of multisystem involvement including sensorineural hearing loss, renal tubulopathy, and respiratory failure (Keshavan et al., 2020). Second, *RRM2B*-related autosomal dominant progressive external ophthalmoplegia (adPEO) typically has an adult-onset (Pitceathly et al., 2012). Other manifestations include ptosis, bulbar dysfunction, fatigue, and muscle weakness. Third, autosomal recessive progressive external ophthalmoplegia (arPEO) typically has an earlier onset than the adPEO phenotype in childhood (Takata et al., 2011). Finally, mitochondrial neurogastrointestinal encephalopathy (MNGIE)-like phenotype manifests with progressive ptosis, ophthalmoplegia, gastrointestinal dysmotility, cachexia, and peripheral neuropathy (Shaibani et al., 2009). These four phenotypes are summarised in Table 1-4. To date, 78 individuals from 52 families with molecularly confirmed *RRM2B*-related mitochondrial disorders have been reported (Lim et al., 2021) but the true prevalence of this rare condition is currently unknown.

Phenotype	**Mode of inheritance**	**Onset**	**Comments**	**Muscle biopsy findings**
Encephalomyopathic	Autosomal recessive	Infancy	Severe multisystem disease often fatal in early life	mtDNA depletion
Progressive external ophthalmoplegia (recessive)	Autosomal recessive	Early childhood or adulthood	Ptosis, ophthalmoplegia, multisystem involvement and Kearns-Sayre syndrome-like	Multiple mtDNA deletions
Mitochondrial neurogastrointestinal encephalopathy (MNGIE)-like	Autosomal recessive	Childhood or adulthood	Gastrointestinal dysmotility, ophthalmoplegia, ptosis, peripheral neuropathy and leukoencephalopathy	mtDNA depletion
Progressive external ophthalmoplegia (dominant)	Autosomal dominant	Adulthood	Ptosis, ophthalmoplegia, milder disease course and often tissue-specific	Multiple mtDNA deletions

Table 1-3: A summary of the phenotypic spectrum, mode of inheritance, disease onset and muscle biopsy findings associated with pathogenic variants in the RRM2B gene. Reproduced from (Lim et al., 2021)

1.8.1 Clinical manifestations

1.8.1.1 Encephalomyopathic phenotype in RRM2B-related mitochondrial disorders

In the literature, there are currently 31 children who have been reported with this severe form of *RRM2B*-related disorders (Bourdon et al., 2007, Bornstein et al., 2008, Acham-Roschitz et al., 2009, Kollberg et al., 2009, Shaibani et al., 2009, Keshavan et al., 2020). These children typically present in the first few months of life with those affected, dying in early childhood. Myopathy manifesting as muscle hypotonia and weakness, often associated with respiratory insufficiency or failure is almost universally present. The majority of them also has central nervous system findings such as seizures, encephalopathy, developmental delay or regression. Other less frequently reported CNS manifestations include cerebral atrophy and generalized central hypomyelination. Sensorineural hearing loss occurs in over a third of these children. Other common features include proximal renal tubulopathy with nephrocalcinosis and gastrointestinal dysmotility. Given the multisystem involvement, most infants with the encephalomyopathic phenotype have faltering growth and have a poor prognosis. As an autosomal recessively inherited condition, there maybe a family history in affected siblings but the absence of this in a family does not preclude the diagnosis. The encephalomyopathic phenotype of *RRM2B*-related mitochondrial disorder should be suspected when a combination of the aforementioned features is present. Table 1-5 summarises the reported features of this infantile encephalomyopathic phenotype.

Feature		Number of children out of 31 (%)	Comments
Neuromuscular		31 (100%)	Truncal hypotonia is almost universally present; gross motor delay, feeding difficulties, poor head control, generalized weakness, areflexia, ptosis, PEO, uncoordinated swallow, exercise intolerance
Nervous system	**Central**	1-15 (3-48%)	Encephalopathy: gross motor delay; seizures; focal and/or generalized upper motor neuron signs; microcephaly; neurological regression; dystonia MRI shows central hypomyelination; cerebral atrophy;
	Peripheral	2 (6%)	Demyelinating peripheral neuropathy
Respiratory		18(58%)	Respiratory distress, respiratory failure. Artificial ventilation may prolong the expected lifespan on a case-by-case basis
Renal		17 (55%)	Tubulopathy (proximal), nephrocalcinosis, aminoaciduria, glycosuria; lactic acidemia; hypocalcaemia
Faltering growth (previously knowns as failure to thrive)		16(52%)	Likely due to multi-system involvement
Hearing loss		11 (35%)	Sensorineural hearing loss may only be identified on formal assessment
Gastrointestinal		10(32%)	Recurrent vomiting, feed intolerance, chronic diarrhoea, cachexia
Eye		4(13%)	Ophthalmoplegia; pigmentary retinopathy (rod-cone dystrophy); cataracts; megalocornea; blindness; nystagmus
Cardiovascular		4(13%)	Left ventricular hypertrophy, cardiomyopathy, ventricular septal defect

Table 1-4: A summary of the clinical features associated with the encephalomyopathic phenotype of RRM2B disorders. Reproduced from (Keshavan et al., 2020, Lim et al., 2021)

1.8.1.2 Progressive external ophthalmoplegia (PEO) phenotype of RRM2B-related mitochondrial disorder

PEO is characterised by a slowly progressive loss of function of the muscles that move the eye and retract the eyelid. In *RRM2B*-related disorders, this condition can be divided into two categories based on mode of inheritance.

a) Autosomal dominant PEO

The adPEO phenotype is associated with a mild phenotype including proximal muscle weakness, bulbar dysfunction, and fatigue (Table 1-6) (Shaibani et al., 2009, Fratter et al., 2011, Pitceathly et al., 2012, Sommerville et al., 2014). The mean age of disease onset is around 46 years. Tyynismaa and colleagues reported the first individuals with *RRM2B*-related adPEO from two large, unrelated families (Tyynismaa et al., 2005). Apart from PEO and ptosis, other commonly reported findings include myopathy, exercise intolerance, cerebellar ataxia, sensorineural hearing loss, cognitive dysfunction, mood disturbance, bulbar dysfunction and low body mass index (Pitceathly et al., 2012, Sommerville et al., 2014). These features may have variable ages of onset and severity. They are commonly associated with the finding of multiple mtDNA deletions in skeletal muscle biopsy (Fratter et al., 2011, Pitceathly et al., 2012).

Feature		**Number affected out of 42 (%)**
Ophthalmologic	Chronic progressive external ophthalmoplegia	42 (100%)
	Ptosis	40 (95%)
Neuromuscular	Myopathy	37 (88%)
	Exercise intolerance	33 (79%)
Central nervous system	Cerebellar ataxia	35 (83%)
	Sensorineural hearing loss	26 (62%)
	Cognitive dysfunction	24 (57%)
	Bulbar dysfunction	9 (21%)
Mood disturbance		20 (48%)
Low body mass index		9 (21%)
Gastrointestinal dysmotility		6 (14%)

Table 1-5: A summary of the clinical features associated with the autosomal dominant PEO phenotype of RRM2B gene mutations. Reproduced from (Lim et al., 2021).

b) Autosomal recessive PEO

Autosomal recessive PEO (arPEO) as a result of biallelic pathogenic variants in the *RRM2B* gene can present similarly to its dominant phenotype but at a younger age. The mean age of disease onset for arPEO was around seven years old. In this group of patients, the predominantly myopathic phenotype of PEO, ptosis, proximal muscle weakness, and bulbar dysfunction was more severe than the multisystem disorder observed in individuals with a heterozygous *RRM2B* pathogenic variant(Pitceathly et al., 2012). The first patient who harboured the homozygous missense pathogenic variant in *RRM2B* had an insidious onset of PEO from the age of 16 years(Takata et al., 2011). Apart from ophthalmoplegia, two patients who had biallelic variants presented with Kearns-Sayre-like syndrome, single, large-scale deletion of mtDNA disorder. including pigmentary retinopathy, hearing loss, renal tubulopathy and increased CSF protein(Pitceathly et al., 2012). Therefore, arPEO should be suspected in individuals with a Kearns-Sayre-like syndrome when inheritance appears to follow a Mendelian pattern or examination of muscle tissue reveals evidence of multiple mtDNA deletions.

1.8.1.3 Mitochondrial neurogastrointesinal encephalopathy (MNGIE)-like phenotype of RRM2B-related mitochondrial disorder

Shaibani and colleagues reported a 42-year-old woman with *RRM2B* biallelic missense pathogenic variants and mtDNA depletion in clinically relevant tissues. This lady had a 12-year history of progressive ptosis, ophthalmoplegia, gastrointestinal dysmotility, cachexia, peripheral neuropathy, and T2-hyperintense MRI changes in basal banglia (Shaibani et al., 2009). The combination of her symptoms would be compatible with a MNGIE-like syndrome. MNGIE is characterized by progressive gastrointestinal dysmotility, low body-mass index, ptosis, ophthalmoplegia, leukoencephalopathy and demyelinating peripheral neuropathy and symmetric and distal weakness more prominently affecting the lower extremities (Hirano, 2016). MNGIE is a result of biallelic pathogenic variants in the TYMP gene. To distinguish them, the *RRM2B*-related MNGIE-like phenotype is likely to have normal plasma thymidine concentration (<3 μmol/L), normal plasma deoxyuridine concentration (<5 μmol/L), normal thymidine phosphorylase enzyme activity in leukocytes normal (>10% of the control mean), and a negative molecular test for TYMP gene.

1.8.2 Diagnostic strategy

RRM2B-related mitochondrial disorders should be suspected in individuals who present with the clinical features discussed in the previous sections. To confirm a clinical suspicion, the advent of molecular diagnosis has gradually diminished the diagnostic value of invasive muscle biopsy in affected individuals who present with the recognized clinical phenotypes. However, muscle biopsy, if obtained for other reasons, or in cases with atypical features, might demonstrate cytochrome c oxidase (COX)-deficient fibres, subsarcolemmal mitochondrial accumulation (classic "ragged-red" fibres) on histochemistry, mtDNA deletions or depletions on specific tests. The current diagnosis of *RRM2B*-related mitochondrial disorders is established in a proband with biallelic *RRM2B* pathogenic variants or a heterozygous *RRM2B* pathogenic variant identified via molecular genetic testing.

Single-gene testing using sequence analysis of *RRM2B* is now rarely undertaken to establish a genetic diagnosis of this mitochondrial disease. Overlapping phenotypes with common clinical features demand a much broader approach employing, for example, the use of a mitochondrial multigene panel analysis or exome sequencing. A multigene panel that includes *RRM2B* and other genes of interest is most likely to identify the genetic cause of the condition.

1.8.3 Differential diagnoses

Several other gene mutations that affect mtDNA maintenance may mimic *RRM2B*-related mitochondrial disorders. To date, there are pathogenic variants in over twenty nuclear genes known to be associated with mtDNA maintenance (Sommerville et al., 2014, El-Hattab et al., 2017). Table 1-7 summarized the nuclear genes that maintain mtDNA and could potentially present similarly to those with *RRM2B*- related disorders. Multiple genes could be associated with the same clinical features, also known as locus heterogeneity. The significant locus heterogeneity in genes that cause mtDNA maintenance defects renders single-gene testing unreliable in establishing a molecular diagnosis.

Function	Nuclear-encoded mtDNA maintenance genes	Encephalomyopathy	MNGIE-like	Myopathy	Ophthalmoplegia
Mitochondrial DNA synthesis	*POLG*	X	X	X	X
	POLG2			X	
	TWNK				X
	RNASEH1	X			
	MGME1			X	
	DNA2			X	
Mitochondrial nucleotide salvage pathway	*TK2*			X	X
	DGUOK			X	
	SUCLA2	X			
	SUCLG1	X			
	ABAT	X			
Cytosolic nucleotide metabolism	*TYMP*		X		
	RRM2B	**X**	**X**	**X**	**X**
Mitochondrial nucleotide import	*SLC25A4*			X	X
	AGK			X	
Mitochondrial fusion	*OPA1*	X			
	FBXL4	X			

Table 1-6: A summary of nuclear-encoded mtDNA maintenance genes that can mimic phenotypes of RRM2B-related disorders. These genes are arranged according to their function in the mitochondria. "X" denotes the reported phenotypes associated with each gene. The phenotypes associated with RRM2B gene mutations (highlighted in yellow) appears to overlap with other genes. Reproduced from (Lim et al., 2021).

1.8.4 Management

There is currently no definitive cure for *RRM2B*-related mitochondrial disorders. The current management of this disorder focuses on symptomatic management, surveillance for potential complications and supportive long-term care. Depending on the phenotypes, the delivery of patient care is typically implemented by a multidisciplinary team. The infants and young children who present in the early years with encephalomyopathic form of *RRM2B*-related mitochondrial disorder are likely to be hospitalised at the onset of the disease. They might require high-level inpatient care including neonatal or paediatric intensive care for prolonged periods. Paediatric neurologists and their team members might be involved in managing neuromuscular weakness and seizures if present. Respiratory support might be considered by specialist respiratory clinicians in severe cases of myopathy affecting muscles of respiration. Infants who develop renal tubulopathy might also need input from the paediatric nephrology teams. In contrast, individuals who had milder PEO phenotypes are unlikely to need such extensive management. Some might only require cosmetic surgical intervention for severe ptosis or physiotherapy input for myopathy later in life.

Nonetheless, there might be hope for a cure in the future in disorders related to mtDNA maintenance. Thymidine kinase 2 (TK2) is another critical enzyme in the mitochondrial pyrimidine salvage pathway to maintain mtDNA. Deficiency in TK2, caused by mutations in the *TK2* gene, can lead to myopathy with mtDNA depletion. Lopez-Gomez and colleagues used deoxycytidine monophosphate (dCMP) and deoxythymidine monophosphate (dTMP) to treat Tk2-deficient mice in 2017. They found that this molecule-bypass therapy prolonged the lifespan of these mice and restored mtDNA copy number along with respiratory chain enzyme activities (Lopez-Gomez et al., 2017). The success of this therapy led to the use of deoxynucleoside monophosphates and deoxynucleoside in 16 TK2-deficient patients under a compassionate use program in 2019 with some promising indications of efficacy (Domínguez-González et al., 2019). More recently in 2021, Lopez-Gomez and colleagues generated an adeno-associated virus that delivered human TK2 cDNA to Tk2-deficient mice. They found that a combination of this gene therapy and their previous deoxynucleoside administration had resulted in better efficacy than

pharmacological treatment alone (Lopez-Gomez et al., 2021). At present, there is no *RRM2B* molecular bypass therapy or gene therapy that has been published.

With the exception of adPEO, other phenotypes related to *RRM2B* gene mutations are inherited in an autosomal recessive manner. Molecular genetic testing is recommended for the parents of a proband to confirm that both parents are heterozygous for an *RRM2B* pathogenic variant and to allow reliable recurrence risk assessment. Some individuals diagnosed with an *RRM2B*-related disorder have the disorder as the result of a de novo pathogenic variant. The proportion of individuals with an *RRM2B*-related disorder resulting from a de novo pathogenic variant is unknown. Once the *RRM2B* pathogenic variant(s) have been identified in an affected family member, prenatal and preimplantation genetic testing for *RRM2B* mutations could be explored. In some challenging circumstances, clinicians and/or families should consider seeking opinions from specialist centres with expertise in prenatal testing. Differences in perspective may exist among medical professionals and within families regarding the use of prenatal testing. While most specialist centres would consider the use of prenatal testing to be a personal decision, a discussion of these issues may be helpful.

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1.9 The natural history of mitochondrial disorders

From the preceding sections in this Chapter, it is evident that both Leigh syndrome and mtDNA maintenance defects secondary to *RRM2B* gene share a common challenge – there is currently no effective curative or definitive disease-modifying treatment for either condition. The mainstay of current treatment for both relatively rare conditions remains within the realm of supportive care and health surveillance. Looking to the future, several promising experimental treatments are emerging for mitochondrial diseases (Tinker et al., 2021). Some of these might potentially be useful for these two conditions, as highlighted in their respective management sections. However, the rarity of these two conditions limits the number of potential candidates to be enrolled in early phase clinical trials. Therefore, it is important to establish baseline natural history of these conditions for comparison in potential adaptive design trials of novel treatments in the future. Adaptive design allows clinical trials more flexibility by analysing interim results at certain timepoints to modify the subsequent trial course in accordance with prespecified rules without compromising the its inherent validity (Pallmann et al., 2018). This design is appealing for small trials which struggle to demonstrate a large enough effect to reject the null hypothesis. The flexibility of adaptive design means that fewer patients might be required to ensure the same high chance of getting the right answer and a definitive conclusion could be reached earlier.

The natural history of a disease is defined as the course of a disease that encompasses an individual's clinical trajectory from its pathological onset until its outcome, whether that be complete recovery, plateau or death, in an untreated state or following the standard of care (Porta, 2014). Apart from being a comparison arm in new treatments, defining the natural history of diseases could also establish its genotype-phenotype-prognosis correlations (Keshavan and Rahman, 2018). Also known as longitudinal observational studies, natural history study of disease is designed to evaluate different factors, genetic or environmental, and uncover meaningful correlations. The longitudinal aspect of the study tracks the course of the disease over time. In rare diseases, natural history information might not readily be available, but they are "the pillars of epidemiological research" on rare conditions in the development of treatments (Pariser and Gahl, 2014). Data from natural history

study of diseases can also support best practice guidelines for patient care and underline future research priorities.

Historically, clinical drug trial design in mitochondrial disease has been hampered by its relative rarity and genotype-phenotype heterogeneity; and the lack of natural history data, validated biomarkers and outcome measures that correlate with disease progression (Pitceathly et al., 2021). Apart from being rare, there are some inherent challenges in understanding the natural history of mitochondrial disorders. For some mitochondrial disorders, for instance, mtDNA point mutations, disease burden may vary depending on levels of heteroplasmy or mutation load in the affected tissues (Grady et al., 2018). Furthermore, individuals with identical pathogenic variants in the *POLG* gene, responsible for mtDNA maintenance, and may present with variable clinical phenotypes (Rajakulendran et al., 2016). Given the complexity of diagnosing mitochondrial disorders, it is unsurprising that the diagnostic odyssey for patients is often protracted (Grier et al., 2018). The delay in diagnosis might have resulted in advanced disease progression and have narrowed the therapeutic window of opportunity for a putative disease-modifying treatment to achieve its intended benefit (Pitceathly et al., 2021). Natural history studies in mitochondrial disease could potentially identify factors that augment disease severity and progression.

To address the deficit in our understanding of the natural history of mitochondrial disorders, national and international cohorts of mitochondrial patients have been established over the past two decades. The largest living cohort of mitochondrial patients in the UK is the Wellcome Centre for Mitochondrial Research (WCMR) Patient Cohort. The primary aim of this cohort is to understand the natural history of mitochondrial diseases in adults and children. This cohort, since its inception in 2009, has produced several seminal publications in the natural history of mitochondrial diseases, mostly in adult patients (Nesbitt et al., 2013, Grady et al., 2014, Ng et al., 2015a, Ng et al., 2015b, Oláhová et al., 2015, Whittaker et al., 2015, Anagnostou et al., 2016, Martikainen et al., 2016, Ng et al., 2016a, Ng et al., 2016b, Sommerville et al., 2017, Hayhurst et al., 2018, Pickett et al., 2018, Boal et al., 2019, Feeney et al., 2019, Ng et al., 2019). As the understanding of mitochondrial disorders expands over the years from this 'trial-ready' cohort, there is still an unmet need to study the natural history of paediatric mitochondrial disease prospectively.

1.10 Outcome measures in paediatric mitochondrial disease

As mitochondrial research progresses into the era of clinical trials of treatment in the future, there is a need for outcome measures to determine the efficacy of the therapy under investigation. A Cochrane review in 2013 of clinical trials in mitochondrial disorders unearthed the suboptimal standard and quality of studies performed previously (Pfeffer et al., 2013). Of the many flaws highlighted, the most glaring is the lack of good outcome measures. The ‘ideal’ set of outcome measures for clinical trials should be “robust, sensitive, specific, validated for mitochondrial disease and clinically meaningful” (Pitceathly et al., 2021). In reality, the development of 'ideal' outcome measures in mitochondrial disease trials is not straightforward because of the wide phenotypic spectrum, the genetic heterogeneity, the relapsing-remitting disease course and the variable disease progression over several years. A mitochondrial disease clinical trial team must understand these factors, in addition to selecting the outcome measures that could detect a relevant and significant difference between the treatment and the placebo groups. Apart from clinical outcome measures, the regulatory agencies for clinical trials such as the US Food and Drug Administration (FDA) also recommended patient-reported outcome measures that take into consideration "how a patient feels, functions and survives" (FDA, 2006). With these in mind, the mitochondrial research community have come together to standardise the clinical and functional outcome measures for clinical trials. In 2017, an international workshop in Rome, Italy have set out the outcome measures for those with mitochondrial myopathy (Mancuso et al., 2017). A year later, another Delphi-based international workshop proposed outcome measures specifically for children with mitochondrial disorders. They proposed several outcome measures for a natural history study in children with mitochondrial myopathy and with mitochondrial encephalopathy (Koene et al., 2018). The studies in the subsequent Chapters of this thesis have applied these outcome measures and observed how they change over time. The merits and demerits of these outcome measures are discussed in Chapter 2 (Methods) and the relevant methodology sections of individual Chapters.

1.11 Aims and scope

The approach of this thesis takes inspiration from the 'keep an open mind' quote by Denis Archibald Leigh, and not preconceived hypotheses, to carry out natural history studies based on outcome measures for two mitochondrial disorders – Leigh syndrome and *RRM2B*-related diseases. In the Leigh syndrome study, the main aims include the following: (1) to explore the characteristics of children with Leigh syndrome, (2) to determine the disease burden and disease progression of these children using a validated clinical rating scale and (3) to investigate potential factors that influence disease burden and progression. In the *RRM2B*-related mitochondrial disorder study, the main aims are: (1) to assess the disease severity in rare autosomal recessive *RRM2B*-related mitochondrial disease using clinician-reported outcome measures longitudinally; (2) to measure respiratory muscle function, exercise tolerance, limb function, snd swallowing function using a set of performance and functional outcome measurements longitudinally; (3) to investigate patient-reported outcome measures in terms of quality of life, fatigue and swallowing; (4) to explore the relationship of these performance outcome measures, functional tests and patient-reported outcome measures with the clinician-reported outcome measures.

Chapter 2: Methods

2.1 Introduction

This chapter highlights the general methodology principles used in the clinical studies of this thesis. Specific methods are also outlined in the individual Chapters 3, 4, 5 and 6. The Wellcome Centre for Mitochondrial Research Patient Cohort has facilitated the identification of participants of rare mitochondrial disorders in these clinical studies. After gaining informed consent, participants took part in various studies depending on their eligibility criteria. This chapter also outlined outcome measures of this thesis including clinical rating scales, functional assessment, specific bedside tests and patient-reported questionnaires. The studies in this thesis utilised some of these specific outcome measures and the subsequent chapters would indicate which outcome measures are used. This chapter also illustrated the general statistical analysis. All studies in this thesis complied with ethical requirements for clinical research and managed data in accordance with standard regulatory frameworks.

2.2 Settings

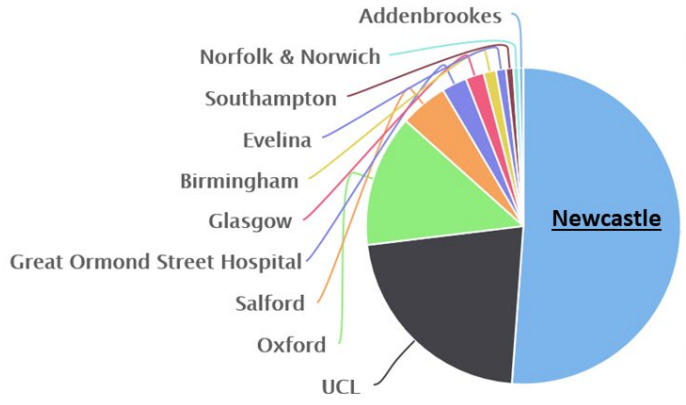
All clinical research activities took place within the Clinical Research Facility (CRF), Clinical Ageing Research Unit (CARU) and inpatient wards of Newcastle upon Tyne NHS Hospitals Foundation NHS Trust as well as the facilities of Wellcome Centre for Mitochondrial Research, Newcastle University, unless stated otherwise. Certain assessments took place at participants' own homes or a location convenient to them. The clinical assessments were primarily dependent on the abilities of participants.

2.3 Identification of participants

Participants from all the studies in this thesis have been identified from the Wellcome Centre for Mitochondrial Research (WCMR) Patient Cohort.

2.3.1 Wellcome Centre for Mitochondrial Research (WCMR) Cohort

Previously known as the UK Medical Research Council (MRC) Mitochondrial Disease Cohort, this cohort (hereafter referred to as MitoCohort) is the largest of its kind and has more than 1800 registered patients of the NHS Highly Specialised Service for Rare Mitochondrial Disorders who have consented to receive information about clinical trials of which they might be eligible. The idea to recruit mitochondrial patients into a cohort was conceptualised in a funding submission to UK MRC in July 2008. The criteria to participate in this cohort are all living adults or children who have clinical, biochemical and/or genetic diagnoses of mitochondrial disease. Asymptomatic carriers of pathogenic variants, which caused mitochondrial disease, are also eligible. The host research site in Newcastle recruited its first patients in March 2009, followed by research sites in London and Oxford who recruited their initial patients in 2010 and 2011 respectively. The success of this cohort has led to UK MRC renewing the funding in 2014. Being the lead among the three centres, the site in Newcastle remains the largest recruiter in this cohort over the years. To date, the research team at Newcastle have recruited 953 patients (Figure 2-1).



Institution	Secondary Institution	Number of Patients
Newcastle		953
UCL		408
Oxford		251
Newcastle	Salford	91
Great Ormond Street Hospital		48
Newcastle	Glasgow	35
Newcastle	Birmingham	24
Newcastle	Evelina	19
Newcastle	Southampton	14
Newcastle	Norfolk & Norwich	11
Newcastle	Addenbrookes	8
Total	1862	

Figure 2-1. Pie chart illustrated the distribution of mitochondrial patients recruited to the MitoCohort. Table showed the number of patients from individual research sites. The Newcastle site also collected data from several other centres.

The MitoCohort captures clinical information and details into a secure database server within Newcastle University. These include medical history, bedside examination findings, laboratory blood tests, ECG, echocardiography results, neuroimaging, neurophysiology studies, relevant radiology findings and molecular genetic testing (Figure 2-2). These details, which are collected from patients at each clinical encounter, are systematically uploaded into the database server. These secure servers in Newcastle University, which have two different interfaces, undergo backup copies daily to minimise the risk of data loss or data theft. Although the primary aim of the MitoCohort is to understand the natural history of mitochondrial disease, it also serves as an avenue for recruiting patients who had been thoroughly characterised into clinical trials. An overseeing committee, which acts as gatekeepers, reviews any study that intends to use data from the cohort. After the approval from this committee, the database was interrogated for patients who fulfilled the eligibility criteria of the relevant studies in this thesis.

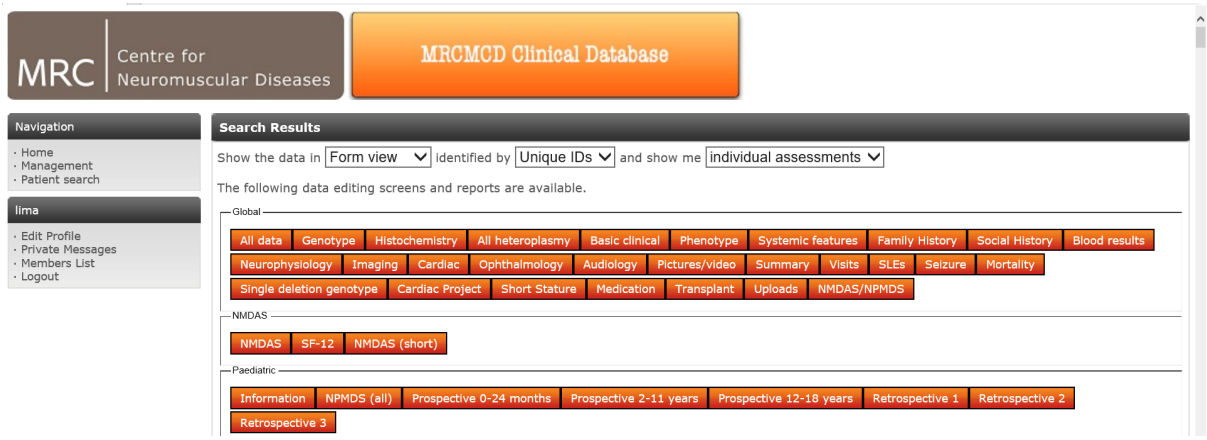


Figure 2-2: Snapshot view of the UK MitoCohort database showed the range of clinical data held in this secure database. Global data screen grouped the available clinical data into different categories.

2.4 Informed consent

The use of patient information from the Mitocohort for studies in this thesis complied with the requirements set by a research ethics committee (REC Ref: 13/NE/0326). Before enrolment, the research team sent invitation letters, along with a copy of the relevant patient information sheets and consent forms to eligible participants after identifying them from the Mitocohort. Children below the age of consent received age-appropriate sheets and assent forms. All consents for the studies are obtained following local ethics policies. All patients are allowed as much time as they wish to consider their decision.

2.5 Study design

The studies in this thesis differed in their designs to certain extents because they set out to answer their unique research questions. The first study aimed to explore the disease burden and progression of children with Leigh syndrome using the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS). This study is a non-interventional longitudinal study that enrolled all children with confirmed diagnoses of Leigh syndrome who had completed two NPMDS assessments at the NHS Highly Specialised Service for Rare Mitochondrial Disorders. This study, which will underpin the setup of clinical trials in the future, collected retrospective and prospective NPMDS data up to March 2020. After the confirmation of consent, this study gathered initial data from the medical notes. At the baseline visit, participants underwent the medical review alongside the NPMDS assessments. These participants had similar NPMDS assessments at their follow-up visits to the research facilities.

The next studies in this thesis are part of a larger project known as a prospective observational study of patients with mitochondrial depletion syndrome, *RRM2B* gene mutations (PROSPER-2B). It was designed as a prospective observational study to evaluate the clinical features of patients with autosomal recessive *RRM2B*-related mitochondrial patients and to develop outcome measures for future studies. The two studies in this thesis focussed on the clinician-reported and patient-reported outcome measures used to delineate the natural history of patients with this condition. The data collection period spanned from 17 June 2019 to 14 June 2021. After consenting to the

studies, participants in this study attended two mandatory visits (baseline and end-of-study visits) and two optional interim visits during the 24-month study period. Of the two optional interim visits, participants had a choice of attending one or both interim visits. During each visit, participants undertook several outcome measures that are outlined in the subsequent methodology sections of this chapter.

2.6 Eligibility criteria

The medical history of potential participants would be reviewed prior to study enrolment to verify that they satisfy the inclusion criteria. These activities as well as eligibility confirmation can be performed before their baseline visits. The eligibility criteria for each study are as follows:

2.6.1 Leigh syndrome cohort

NPMDS assessments for paediatric Leigh syndrome study enrolled participants who fulfilled the following criteria – 1) a confirmed diagnosis of Leigh Syndrome based on diagnostic criteria first proposed in 1996 (Rahman et al., 1996), followed by two revisions in 2014 (Baertling et al., 2014) and 2016 (Lake et al., 2016); 2) aged less than 18 years at the time of recruitment; 3) able, in the opinion of the recruiting investigator to attend routine outpatients' clinical appointments and to complete the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS). Participants were not eligible if any of the following applies – 1) unable to attend the study centre for study visits; 2) unwell and being cared in neonatal or paediatric intensive care and/or unable to complete clinical assessments; 3) other co-existing comorbidities unrelated to mitochondrial disease that would impact on the validity of disease rating scales; 4) participating in any interventional study or clinical trial; 5) any other severe medical or psychiatric co-morbidity, which would make the subject inappropriate for this study.

2.6.2 *RRM2B* cohort

Studies that assessed outcome measures for mitochondrial patients with autosomal recessive *RRM2B* disease enrolled participants who fulfilled the following criteria – 1) a confirmed molecular diagnosis of autosomal recessive *RRM2B*-related mitochondrial disease; 2) able and willing to undertake the essential study

assessments; 3) cognitive capacity to provide informed consent, where appropriate. Participants were not eligible if any of the following applies – 1) participating in any interventional study or clinical trial; 2) other co-existing comorbidities unrelated to mitochondrial disease that is not controlled and would impact the validity of disease rating scales.

2.7 Outcome measures

2.7.1 Demographics and clinical details

Each study extracted clinical data of basic demographics which include current age, age of diagnosis and key clinical features as outlined in their clinic letters. The medical notes also provided information about past medical history, laboratory tests, neuroradiology scans, electrophysiology scans and all relevant investigations. Our study also reviewed the record of concomitant medications and the pertinent physical examination findings. The NHS Highly Specialised Service UKAS-accredited diagnostic laboratory in Newcastle upon Tyne Hospitals Foundation Trust provided the molecular genetic diagnoses for all the study participants. At this NHS clinical laboratory, analysis of the complete mitochondrial genome sequence was undertaken using Ion Torrent PGM (Zierz et al., 2019) or Sanger sequencing (Taylor et al., 2003), as previously reported. Where no genetic defect was identified in mtDNA, nuclear genetic variants were identified using either candidate gene approaches (Sanger sequencing) or next-generation sequencing including both targeted panel and/or whole-exome sequencing (Taylor et al., 2014, Alston et al., 2016). The diagnostic testing strategies for mitochondrial clinical service have been published in the literature(Alston et al., 2017).

2.7.2 Newcastle Paediatric Mitochondrial Disease Scale (NPMDS)

Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) was designed to assess the natural history of mitochondrial disease throughout childhood and to provide a quantifiable measure of the functional disability encountered. It is also a concise, pragmatic tool suitable for use in a clinic setting, yet able to provide a comprehensive assessment. The scale has sections focussing on the current function (Section I), the system-specific involvement (Section II) and the current clinical assessment (Section

III). Each item, with the exception of developmental progress, has four possible responses – normal (0), mild (1), moderate (2), and severe (3) impairment. The total score from all three sections which reflects disease burden can be categorised into mild (0-14), moderate (15-25) and severe (>25) (Phoenix et al., 2006). NPMDS is recommended as an important outcome measure in developing future clinical studies (Koene et al., 2018).

2.7.3 Newcastle Mitochondrial Disease Adult Scale (NMDAS)

Participants who are over 18 years old are assessed using the Newcastle Mitochondrial Disease Adult Scale (NMDAS). It is a semi-quantitative clinical rating scale designed specifically for all forms of adult mitochondrial disease. NMDAS was developed in 2006 to provide a validated and reproducible rating of disease progression (Schaefer et al., 2006). The multi-system involvement of mitochondrial disease necessitated a scale that encompasses many parameters including neurological, renal, diabetes, cardiac and gastrointestinal involvement. Like the NPMDS, NMDAS has three sections – the current function (Section I), the system-specific involvement (Section II) and the current clinical assessment (Section III). In contrast to NPMDS, NMDAS has a possible score of 0-5 for each item within the sections. The higher the score for each item, the more severe the disease burden. This rating scale for adult mitochondrial disease has been used to demonstrate disease burden and progression in several genotypes in the UK (Grady et al., 2014, Pickett et al., 2018).

2.7.4 Assessment of muscle strength

There are many different methods to assess muscle strength and this thesis utilised the hand-held dynamometer testing. The hand-held dynamometer used for measuring muscle force in Newtons, the S.I. unit, was the MicroFET® 2 dynamometer by Hoggan Scientific. The dynamometer is calibrated according to the user manual. Calibration was performed before each test. The chosen muscle group of participants underwent maximal repetitions with consistent verbal encouragement from assessors. Both left and right sides were measured with dedicated 1-minute rest in between each test.

2.7.5 Functional capacity

Participants underwent four different tests for their functional capacity. First, the six-minute walk test (6MWT) measures the distance covered by participants in six minutes. This test was initially based on the American Thoracic Society (ATS) Statement: Guidelines for the Six-Minute Walk test (2002b) and it has since been updated in 2014 by both ATS and the European Respiratory Society(Holland et al., 2014). Participants were asked to walk at a rate suitable to them and can stop or slow down if they feel like doing so. They can resume walking afterwards. The next two tests were the 10-metre walk test at self-selected pace (SSP) and fast-pace (FP). The 10-metre walk test was first introduced in 1987 (Wade et al., 1987) and gradually became a common clinical measure used in rehabilitation with good reliability (Tyson and Connell, 2009). Participants were asked to walk a 14-metre track at their own pace (SSP) or as quickly as possible (FP) with only the middle 10-metre section being timed. Finally, the participants took part in the 30-second sit to stand tests. It is a reliable valid indicator of lower body strength (Jones et al., 1999). Participants were asked to stand from sitting on a standard height chair repeatedly within a 30-second period. The total number of completed repetitions were recorded. All these functional capacity testing took place in predefined circuits in Clinical Ageing Research Unit (CARU), Newcastle.

2.7.6 Pulmonary function tests

Another anatomical system affected by the mitochondrial disease is the muscle of respiration. Participants underwent several pulmonary function tests. First, spirometry is a physiological assessment of the maximal volume of air that the participant can inspire and expire with maximal effort, as measured in volume or flow as a function of time. These are indicated by volume delivered during expiration as forcefully as possible from full inspiration (FVC) and the expiratory volume in the first second (FEV1). The spirometry testing procedure adhered to the latest standardised update in 2019 from the American Thoracic Society and European Respiratory Society (Graham et al., 2019). Next, the sniff nasal inspiratory pressure is a validated, non-invasive test that measures nasal pressure in an occluded nostril during maximal sniff undertaken by the contralateral nostril (Heritier et al., 1994). Other pulmonary measurements included maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP) (2002c, Evans and Whitelaw, 2009). To perform MIP, participants were asked to breathe forcefully against an occluded mouthpiece. Conversely, participants breathed out forcefully against the mouthpiece to perform MEP. The results from the participants were compared against published ranges from healthy subjects (Karvonen et al., 1994, Sclauser Pessoa et al., 2014).

2.7.7 Swallowing assessment

To assess for swallowing dysfunction, participants underwent a 100-ml water swallow test. This bedside test is a sensitive indicator for identifying patients at risk of swallowing dysfunction (Nathadwarawala et al., 1992, Wu et al., 2004, Brodsky et al., 2016). Participants, seated upright, drank 100ml of water from a cup as quickly as possible. A stopwatch with the readability of 1ms measured the swallowing time from the start of lips touching the cup to the completion of the last swallow. Participants who demonstrate signs of choking had to stop drinking immediately regardless of how much water was left in the cup. Swallowing speed (ml/s) can be calculated from the amount of water consumed divided by the time taken from the stopwatch.

2.7.8 Patient-reported outcome measures

Participants, and carers where appropriate, completed questionnaires about their health and wellbeing. Quality of life questionnaires included the NeuroQoL and Newcastle Mitochondrial QoL (NMQoL) questionnaire. NeuroQoL, a reliable and valid self-reported QoL questionnaire, contained 13 brief measures that evaluate physical, mental and social effects in neurological conditions (Cella et al., 2012). Meanwhile, the NMQoL is a valuable assessment tool and consists of 63 items within 16 unidimensional domains, that are specific to mitochondrial disease (Elson et al., 2013). Apart from these two QoL questionnaires, participants also completed the following questionnaire – 1) Dysphagia handicap index (DHI) consists of 25 items divided into emotional, physical, functional and one global subcategory; 2) SWAL-QoL questionnaire is a tool that assesses 10 quality of life concepts which address the desire for eating, dysphagia symptom frequency, mental health, social concerns related to swallowing problems, food selection, fear related to eating, and the burden of dysphagia; (3) Fatigue Impact Scale encompasses 40 items of which, each item is scored from 0 (no problem) to 4 (extreme problem), providing a continuous scale of 0–160.

2.8 Statistical analysis

Statistical analysis in this thesis utilised SPSS version 25 (released in 2017, SPSS Statistics for Windows by IBM Corp. in Armonk, NY). All studies tabulated the collected data using descriptive statistics. Unless stated otherwise, statistical analysis in this thesis reported continuous data using mean and standard deviation or 95% confidence interval, skewed data using median and range, and categorical data using percentages and frequencies. Sensitivity analysis for missing data was investigated. Other outcome data were analysed as appropriate using a mixed-effects model for continuous data and a generalised estimating equation for binary, categorical or count data with appropriate distribution and link function. All tests were two-sided and deemed significant at the 0.05 level. Annotation * indicated significance $p<0.05$, ** indicated significance and $p<0.01$ *** indicated significance $p<0.001$. P values (adjusted or unadjusted) were reported for reasons well documented in the literature and, particularly when testing a priori hypotheses with variables that are not all independent (Perneger, 1998).

Unless stated otherwise, studies used t-tests for continuous variables and chi-square tests for categorical variables. To determine whether baseline outcome measures differed at repeated assessments, studies used the Wilcoxon signed-rank tests and the Mann-Whitney U tests for these matched non-parametric ordinal variables. The phi correlation coefficient (phi) was employed to measure the strength of association between two dichotomous non-parametric variables whilst Kendall's tau-b (τb) correlation coefficient was used for ordinal variables. Continuous variables which fulfilled the assessment for normality were analysed for associations using the Spearman or Pearson's correlation. Factors that might have affected change in variables were analysed using logistic regression. The survival analysis used Kaplan-Meier curves with log-rank test (Mantel Cox) to describe probability of poor outcomes in subgroups of participants. In essence, statistical analysis used tests that were deemed appropriate for the different studies in this thesis. Any specific statistical tests, not outlined here, would be detailed in relevant chapters.

2.9 Ethical and regulatory compliance

All studies in this thesis complied with the Declaration of Helsinki and were conducted in accordance to Good Clinical Practice (GCP) set by the International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. The UK Mitochondrial Disease Patient Cohort: A Natural History Study and Patient Registry obtained a favourable opinion from an independent research ethics committee, NRES Committee North East – Newcastle & North Tyneside 2 (REC Reference Number: 13/NE/0326). All investigators conducted studies in accordance with this ethical opinion.

2.10 Data management

Source data for all studies that included annotations in the participant medical record, standard clinical assessment tools, study-specific assessment worksheets and participant completed questionnaires were stored securely at research sites. Electronic data were held on secure servers within Newcastle University and Newcastle upon Tyne Hospitals NHS Foundation Trust. Participants can only be identified via their unique study ID number rather than by name. Within the study database, participants had unique study ID numbers and no other personal identifiable information. All studies complied with the General Data Protection Regulations (GDPR), 2018 (Laybats and Davies, 2018). Fully anonymised sets of raw data may be made available for third party research purposes with the appropriate data transfer procedures.

Chapter 3: Characterisation of children with Leigh syndrome and their disease burden using the Newcastle Paediatric Mitochondrial Disease Scale

3.1 Introduction

Leigh syndrome is a genetically heterogeneous neurodegenerative disorder, typically characterised by developmental regression, symmetrical brainstem and/or basal ganglia involvement and associated abnormal mitochondrial energy metabolism. Since this eponymous condition was first reported in 1951 by Dr Denis Archibald Leigh (Leigh, 1951), it has become increasingly recognised as one of the commonest syndromic presentations of paediatric mitochondrial disease. Previous studies have characterised the spectrum of clinical phenotype of this devastating syndrome in European mitochondrial centres (Baertling et al., 2014, Sofou et al., 2014, Lake et al., 2016) but they have not systematically measured the disease burden experienced by these children.

Therefore, this chapter set out to characterise children with Leigh syndrome in the UK MitoCohort and to use the validated and established Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) (Phoenix et al., 2006) in determining their disease burden. The primary hypothesis is that children with Leigh syndrome have moderate or severe disease burden by scoring highly on NPMDS assessments. This chapter also compared the NPMDS scores of children with Leigh syndrome with those with other forms of mitochondrial disease. The hypothesis is that children with Leigh syndrome have more disease burden, as indicated by higher NPMDS scores, than children with other forms of mitochondrial disease.

3.2 Specific aims

1. To explore the characteristics of children with Leigh syndrome, focussing on the following clinical investigations – lactate levels, respiratory chain enzyme activities, neuroimaging changes and genotypic spectrum.
2. To determine the disease burden of these children using NPMDS
3. To assess the severity of each phenotype and items scored within the NPMDS.
4. To compare the NPMDS of these children with Leigh syndrome with children with non-Leigh syndrome mitochondrial disease.

3.3 Methods

3.3.1 Study design

This cross-sectional study enrolled all children who had completed NPMDS assessments at research sites with expert clinicians. These children were part of the Mitochondrial Disease Patient Cohort (MitoCohort) UK: A Natural History Study and Patient Registry. (REC: 13/NE/0326). All parents of participants consented to this study which obtained a favourable opinion from an independent research ethics committee, NRES Committee North East – Newcastle & North Tyneside 2 (REC Reference Number: 13/NE/0326). The NPMDS data were collected from the children who attended outpatient clinics in two UK hospitals (Newcastle upon Tyne and Birmingham) between March 2009 and March 2020. Clinicians at the Newcastle study site also reviewed children referred from Northern Ireland, Wales and Scotland. At the time of enrolment, these children were alive and were able to attend clinics at the study sites for assessments. These assessments were performed at their routine clinical outpatient appointments.

3.3.2 Participants

All patients (under 18 years) recruited to the study fulfilled the diagnostic criteria for Leigh syndrome as outlined in the eligibility criteria in the general methods chapter.

3.3.3 Comparison group

All patients (under 18 years) with mitochondrial disease in the UK MitoCohort and not diagnosed with Leigh syndrome.

3.3.4 Outcome measures

3.3.4.1 NPMDS

I used the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) as the clinical outcome measure to determine disease burden objectively. The total score from all three sections, which reflects disease burden can be categorised into mild (0-14), moderate (15-25) and severe (>25)(Phoenix et al., 2006). I scored these children independently during their NPMDS assessments.

3.3.4.2 Medical notes review

I systematically reviewed the medical notes to obtain more details about the diagnostic process and clinical course of these children with Leigh syndrome.

3.3.4.3 Other variables

I collected other variables, including basic demographics, age of disease onset, lactate levels, results of respiratory chain enzyme activities in muscle and genotypes.

3.3.4.4 Neuroimaging

I reviewed their whole brain MRI imaging based on the diagnostic criteria for Leigh syndrome to include symmetrical lesions in the brainstem or basal ganglia structures, namely medulla, pons, midbrain, caudate, putamen, globus pallidus and thalamus. Other areas of interest were also analysed including, subcortical white matter, cerebral cortex, cerebellum and corpus callosum. The neuroimaging investigation was performed at the time of diagnosis of Leigh syndrome.

3.3.5 Statistical analysis

I used SPSS v.25 for statistical analyses. All the statistical methods have also been detailed in Chapter 2. Statistical tests were two-sided with significance set at $p<0.05$. T-tests were used for continuous variables and Chi-square tests for categorical variables. To determine whether baseline NPMDS scores (ordinal variable) differed between the two independent groups (Leigh syndrome vs. comparison groups), we used the Mann-Whitney U test. Correlation coefficients were calculated with the appropriate measure. Cramer V measured the strength of association between two nominal variables, such as the neuroimaging findings. Meanwhile, Kendall's tau-b for ordinal variables, such as the NPMDS scores or the individual item scores.

3.4 Results

3.4.1 Age

3.4.1.1 Age of assessment

Seventy-two children with Leigh syndrome from 68 different pedigrees fulfilled the inclusion criteria to take part in the study. 28 of these children (39%) have consanguineous parents. The mean age of 34 boys and 38 girls who completed the NPMDS assessment was 5.4 years (SD 4.2, 95% CI 4.4-6.4) (Figure 3-1). The girls (6.9 years SD 4.7 years 95%CI 5.32-8.39) were older than the boys (3.8 years SD 3.0, 95%CI 2.72-4.83) at the time of assessment.

3.4.1.2 Age of disease onset

The median age at disease onset was 9 months (IQR 4.25-19) (Figure 3-1). The age of disease onset did not differ significantly between the girls and the boys, t(70)=0.967, p=0.337)

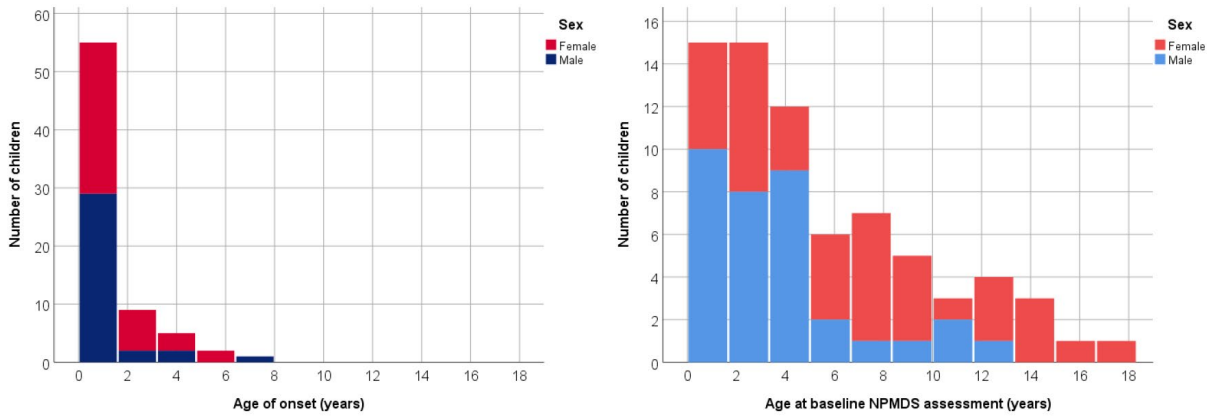


Figure 3-1: Histograms showed the age of onset (years) and age at baseline NPMDS assessment (years) for children with Leigh syndrome.

3.4.2 Supplements

Although vitamins had not been established as disease-modifying treatments, some of these children had various supplements including ubiquinone (36%), riboflavin (17%), thiamine (11%), biotin (7%), folinic acid (5%), L-carnitine (4%) and over-the-counter multivitamin drops (1%). Of the six children with evidence of pyruvate dehydrogenase deficiencies, two had ketogenic diets for epilepsy control, two had declined this treatment, and two had no clinical epilepsy to be considered eligible. None of these treatments influenced the disease course, except for one patient with biotinidase deficiency.

3.4.3 Lactate levels in blood and CSF

Serum lactate levels were available for 50 children; 34 of them (68%) had higher levels than the standardised laboratory reference ranges (>2.2 mmol/L) (Figure 3-2). Cerebrospinal fluid (CSF) lactate levels were measured in 36 children; 23 of them (64%) had high levels (>1.8 mmol/L). Children who had abnormal CSF lactate levels did not differ significantly in disease burden (Z=-0.923, p=0.365).

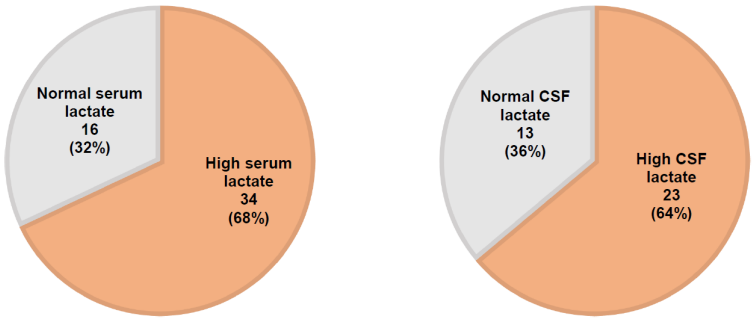


Figure 3-2: Pie charts illustrated the proportion of children with Leigh syndrome who had high serum and high CSF lactate levels respectively. Note that not all the lactate measurements were available for analysis.

3.4.4 Respiratory chain enzyme activities

Thirty-seven children had undergone skeletal muscle biopsy analysis at one of two national mitochondrial diagnostic laboratories in Newcastle and London. Of these, abnormal mitochondrial respiratory chain enzyme activities were reported in 18 cases (49%) (Figure 3-3). The most common respiratory chain defect was an isolated complex I deficiency (n=7), followed by an isolated complex IV deficiency (n=6), followed by defects involving multiple OXPHOS components (n=5). The finding of abnormal mitochondrial respiratory chain enzyme activities also did not differ significantly in disease burden (Z=-0.38, p=0.713)

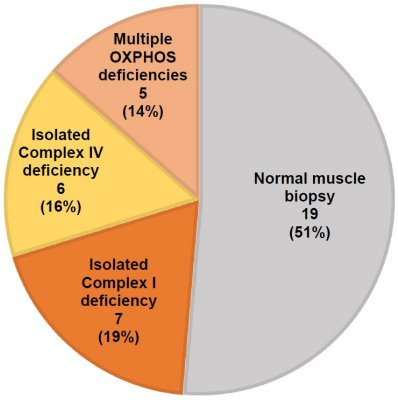


Figure 3-3: Pie chart showed the distribution of muscle biopsy findings from analysis of respiratory chain deficiencies.

3.4.5 Neuroimaging (MRI) findings

The baseline neuroimaging for 63 children was available for analysis. Nine cranial MRI scans had to be excluded because of suboptimal quality for comparison and analysis. These cranial MRI scans were performed at the time of initial investigation at a mean age of 3.2 years (SD 3.8 95%CI 2.0-4.4). Figure 3-4 showed representative neuro-images from this study. Of those suitable for analysis, the most frequent finding was symmetrical putaminal signal abnormality (n=36, 57.1%), followed by symmetrical changes in the globus pallidus (n=26, 41.3%) and caudate (n =25, 39.7%).

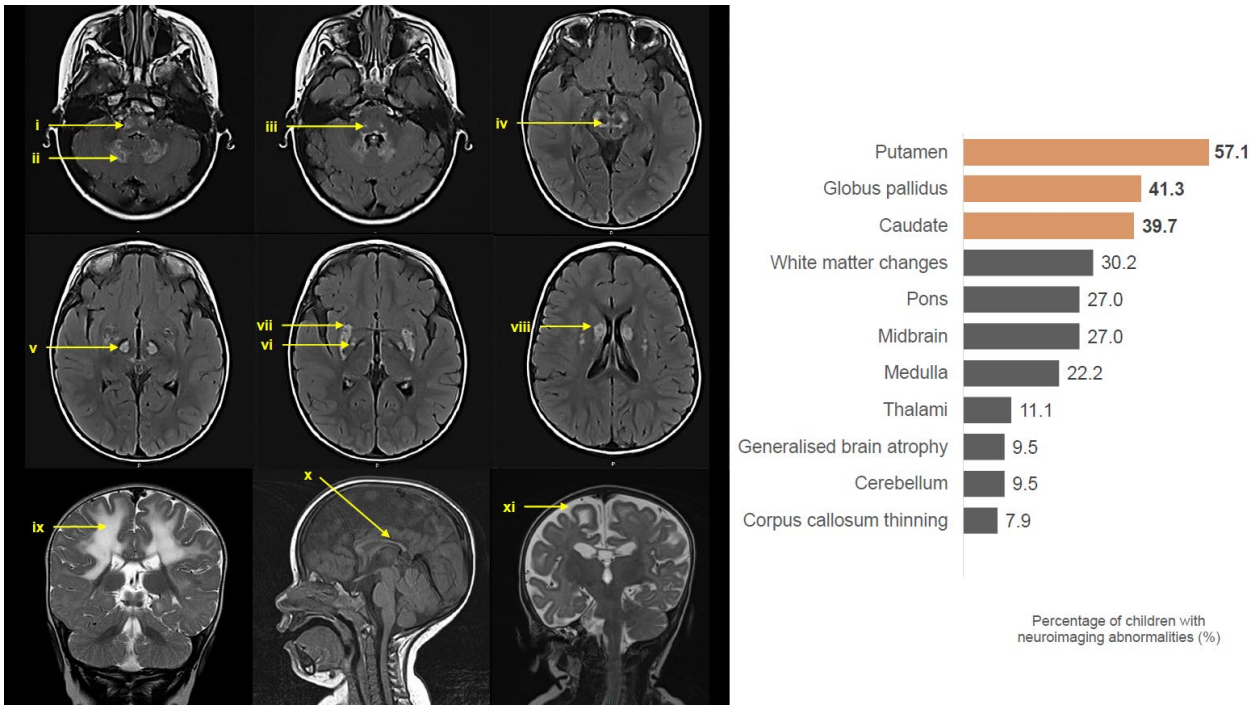


Figure 3-4: A representative neuroimaging changes in these children with Leigh syndrome (i) – medulla, (ii) – cerebellar nuclei, (iii) – pons, (iv) – midbrain, (v) – thalamic, (vi) – globus pallidus, (vii) – putamen, (viii) – caudate, (ix) – white matter involvement, (x) – thinning of corpus callosum and (xi) generalised brain atrophy. The bar chart showed the percentages of neuroimaging abnormalities reported in these 63 scans that had been analysed.

The basal ganglia structures, especially the dorsal striatum (caudate nuclei and putamen), are often observed to be involved together on the MRI. Figure 3-5 summarised the strength of association between findings in each area of the brain into a correlation matrix. Both caudate nuclei and putamen demonstrated a large magnitude (Saneto et al., 2013) of correlation effect, Cramer's V, at 0.57 ($\chi2(1)=20.56$, $p<0.001$). Another large magnitude of correlation includes globus pallidus and putamen with Cramer's V of 0.47 ($\chi2(1)=13.64$, $p<0.001$). Other structures MRI abnormalities that had been observed together include pons and medulla at Cramer's V=0.45 ($\chi2(1)=12.71$, $p=0.001$; midbrain and pons at Cramer's V=0.44 ($\chi2(1)=11.98$, $p=0.001$) and; caudate and cerebellum at Cramer's V=0.29 ($\chi2(1)=5.28$, $p=0.022$.

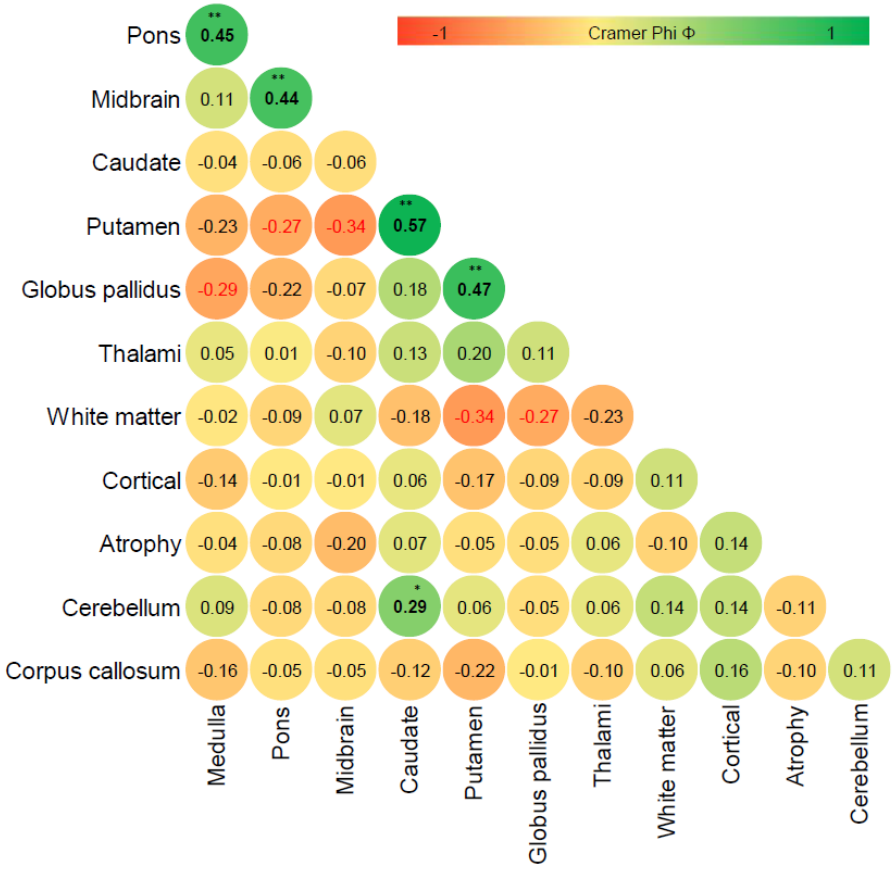


Figure 3-5: Correlation matrix showed the relationships between different MRI neuroimaging findings.

3.4.6 Genotypic spectrum

Fifty-eight (80.5%) children in this cohort have a confirmed genetic diagnosis. Table 3-1 summarised the genotypic spectrum of these children with Leigh syndrome. Leigh syndrome secondary to pathogenic variants in the mitochondrial genome (GenBank accession: NC_012920.1) accounted for just under a quarter of the cohort (n=16, 22.2%, n=16). Pathogenic variants in the *MT-ATP6* gene (m.8993T>G p.Leu156Arg, m.8993T>C p.Leu156Pro and m.9176T>C p.Leu217Pro) were the most common (n=9) mtDNA gene defect in this cohort. Pathogenic variants inherited autosomal recessively accounted for the majority of genetic diagnoses (n=44, 61.1%) with defects in *SURF1* (NM_003172.4) (n=7) being the most common, followed by *NDUFV1* (NM_007103.4) (n=5) and *ECHS1* (NM_004092.4) (n=5). Fourteen children had clinical and biochemical features consistent with a diagnosis of Leigh syndrome but remained genetically undetermined after whole exome sequencing up to the end of the study in March 2020.

Genome	Function	Specific gene	Number of children
Mitochondrial genome	Complex I subunit	*MT-ND1*	1
		MT-ND4	3
		MT-ND5	2
		MT-ND6	1
	Complex V subunit	*MT-ATP6*	9
Nuclear genome	Complex I subunit	*NDUFV1*	5
		NDUFS1	2
		NDUFA9	1
		NDUFA13	1
	Complex I assembly	*NDUFAF6*	2
		NDUFAF8	1
	Complex IV assembly	*SURF1*	7
	Mitochondrial depletion	*SUCLA2*	4
		MPV17	1
	Mitochondrial translation	*MTFMT*	2
		TACO1	2
		c12orf65	1
	Mitochondrial fatty acid beta-oxidation	*ECHS1*	5
	Pyruvate dehydrogenase deficiency	*PDHA1*	2
		PDHX	2
		DLD	1
		Undetermined	1
	Aminoacyl-tRNA synthetase	*NARS2*	2
		DARS2	1
	Others	*BTD*	1
		SLCA19A3	1
Others (presumed nuclear)	Complex I deficiency	-	6
	Complex II & III deficiency	-	1
	Undetermined	-	4
		TOTAL	72

Table 3-1: A summary of the genotypes of children with Leigh syndrome in this study.

3.4.7 NPMDS for children with Leigh syndrome

3.4.7.1 Section I: Current Function

These children had scored highly on several items in the NPMDS (Figure 3-6). Within Section 1: Current Function, the most notable item was their mobility, with only five (7%) of 72 children who had not reported any age-appropriate difficulties. Those who had difficulties with mobility were distributed in three categories, approximately a third each. Twenty-five (35%) children had difficulties walking up the stairs or inclines; twenty (28%) children required support, in the forms of a stick, frame or callipers; and twenty-two (31%) children were entirely dependent on wheelchair or carer to mobilise. The extent of functional impairment because of Leigh syndrome was also evident in other items in the current function, with less than a third of them able to feed (n=20, 28%), self-care (n=21, 29%) and communicate (n=23, 32%) at age-appropriate levels. Twenty-six (36%) children had choked, vomited on feeds, resulting in the reduced intake or had swallowing difficulties, requiring dietary adaptations in the preceding four weeks before NPMDS assessment. Ten children (14%) had supplementary enteral feeding or recurrent aspiration pneumonia. In the most severe category for feeding difficulties, sixteen children (22%) received exclusive enteral fed via gastrostomy or NG tube. The ability to care for personal hygiene, dressing and utensil use were also affected, with twenty-one (29%) children needing help in some of these age-appropriate tasks, twelve (17%) children needing help in all these tasks and eighteen (25%) children entirely reliant on parents or carers without any contribution to self-care. For communication ability, twenty (28%) children had mild impairments, twenty-one (29%) children had moderate impairments and eight (11%) children had severe impairments. The education achievement of these children with Leigh syndrome showed that a third of them struggled to remain in mainstream school or nursery, 24% attended special school or nursery and 4% had not attended school or nursery in the preceding four weeks primarily due to illness. In this section, two-thirds (66%) and 85% of these children also had normal vision and hearing ability, respectively. Five children (7%) had been registered blind and two children (3%) had hearing difficulties despite having hearing aids.

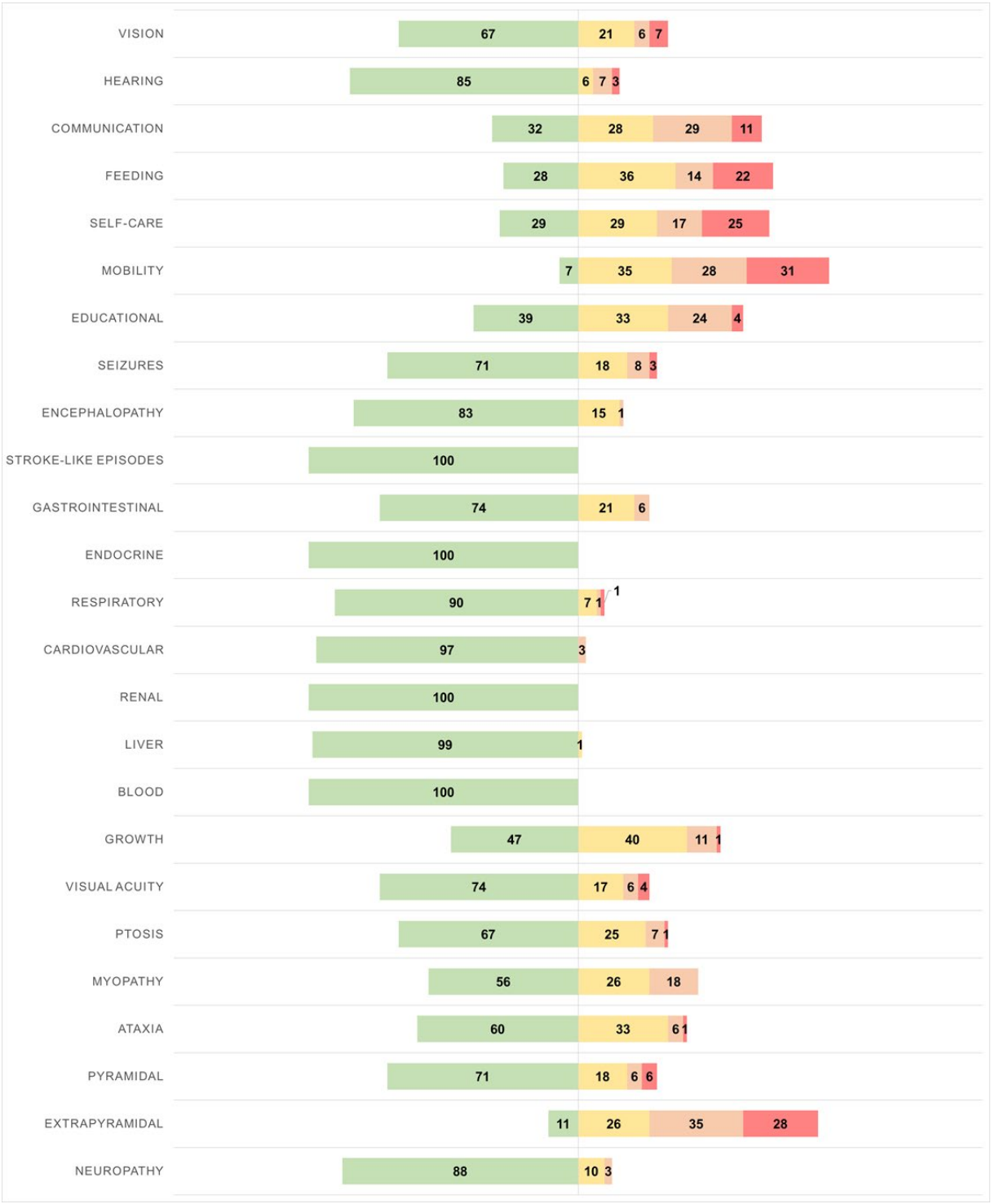


Figure 3-6: Bar chart that showed the scores for each item in the NPMDS for children with Leigh syndrome. Green – Normal, Yellow – Mild, Orange – Moderate, Red – Severe.

3.4.7.2 Section 2: System specific involvement

Among the items in this section, only three items have demonstrated some degree of system involvement. First, about a third of children with Leigh syndrome have reported epileptic seizures. Thirteen children (18%) had myoclonic or absence seizures or less than one generalised tonic-clonic seizure each month in the preceding 12-month period. Six children (8%) reported more than five tonic-clonic seizures per month or more than twenty absence / myoclonic seizures per month during the same period. Only two children (3%) had hospital admissions with prolonged seizures or status epilepticus.

Second, gastrointestinal problems also featured in nearly a third of these children. Fifteen children (21%) reported mild constipation or unexplained vomiting/diarrhoea less than 1 per week. Four children (6%) had moderate constipation that had some relief with laxative treatment. Third, some children had reported episodes of encephalopathy in the preceding 12 months before the assessments; eleven children (15%) had a single episode of personality change, excessive sleepiness, confusion or disorientation. Only one child had more than two episodes per year.

Apart from these three systems, there has been limited involvement from the other systems. None of these children had scored any degrees of disease burden in renal, endocrine or haematological aspects. No stroke-like episodes had been reported in any child. In the respiratory system, five children (7%) had abnormal respiration patterns but not requiring hospitalisation, one child had required hospitalisation but not ventilation, and one child had required artificial ventilation in the preceding 12 months before their NPMDS assessments. Almost all children (97%) had normal cardiovascular function except for two children (3%) who had abnormal echocardiograms. None of them had decompensated cardiomyopathy or required a pacing device. One child had mildly elevated liver function tests but no features of hepatic failure.

3.4.7.3 Section 3: Current clinical assessment

Extrapyramidal signs were the most common examination findings identified (n=64, 89%). A third of these children (n=20, 28%) in this cohort had severe extrapyramidal disorders, characterised by dystonia and/or choreoathetoid movement, which resulted in wheelchair dependency. Another third (n=25, 35%) had moderate generalised dystonia or bilateral extrapyramidal signs and the rest (n=19, 26%) had mild dystonia, focal dystonia or unilateral extrapyramidal signs. On the other hand, pyramidal features were not as common, with thirteen children (18%) who had mild signs, four children (6%) who had moderate signs and four children (6%) who were dependent on wheelchairs due to pyramidal features.

Myopathy or muscle weakness was frequently observed in clinical assessment. Nineteen (26%) children had mild symmetrical weakness in the hip and/or shoulder girdle, whilst thirteen children (18%) had a moderate symmetrical proximal weakness that limits mobility. Another neurological feature that these children exhibited was cerebellar ataxia. A third of them (33%) had ataxic gait or mild upper limb dysmetria. Four (6%) children had required assistance with their ataxic gait, and one child had been wheelchair-dependent because of their cerebellar ataxia. Peripheral neuropathy was not a common finding:. seven children (10%) had areflexia only on examination and two children (3%) had sensory ataxia or distal weakness from peripheral neuropathy.

At bedside ophthalmological examination, twelve (17%) children had visual acuity worse than 6/12 but better than 6/18 on Snellen's chart or no fixation on small objects, four children (6%) had acuity worse than 6/18 but better than 6/60 or impaired fixation on large brightly coloured objects and three children (4%) had no responses to light or unable to count fingers. A quarter of children (n=18, 25%) had gaze-evoked nystagmus, unilateral ptosis or impaired eye movement at extremities. Five children (7%) had bilateral ptosis not obscuring pupils or restricted eye movement, and one child had bilateral ptosis and could only have a flicker eye movement.
Almost all of these children (97%) had some degree of developmental delay. Twenty-eight children (39%) had developmental regression in the preceding twelve months before their NPMDS assessments. The other common clinical feature reported by

these children and their families was growth. Less than half (47%) of these children had normal growth trajectories. Twenty-nine children (40%) had weight or height or both less than 2nd centile. Eight children (11%) had weight or height or both that had fallen and crossed one centile lines on the UK WHO growth reference charts (Wright et al., 2002). One child crossed more than two centile lines. The median weight of this group of children adjusted to their respective ages was on the 9th centile, and the median head circumference was on the 2nd centile.

3.4.7.4 Total NPMDS score

The median total NPMDS score for this group of children with Leigh syndrome was 18 (IQR 12.25-24). Nearly a quarter of these children (n=17, 24%) had total NPDMS scores above 25, indicating severe disease burden(Phoenix et al., 2006). Twenty-eight children (39%) had moderate disease burden (total NPMDS score 15-25) and 27 children (37%) had mild disease burden (total NPMDS score 0-14).

3.4.8 Inter-item relationships

The clinical manifestation of these children with Leigh syndrome can affect several interrelated items within the NPMDS and these items can contribute differently to the total scores. Some of these items are correlated with each other (Figure 3-7). The functional status of the child, feeding, self-care, communication, and mobility were all strongly correlated (Kendall's tau-b (τb) correlation coefficients 0.47 – 0.66, all $p<0.001$). The educational attainment of these children was also correlated with the ability to self-care ($\tau b \approx 0.68$, $p<0.001$) and mobilise ($\tau b \approx 0.45$, $p<0.001$) appropriately for their respective ages. The scores in these five items (communication, feeding, self-care, mobility and education) within the functional status of the children strongly correlated with the total NPMDS scores ($\tau b \approx 0.61 – 0.71$, $p<0.001$). In the clinical assessment section, extrapyramidal signs correlated with mobility at baseline assessment ($\tau b \approx 0.46$, $p<0.001$). The correlation between extrapyramidal signs and the total NPMDS scores was also significant ($\tau b \approx 0.45$, $p<0.001$).

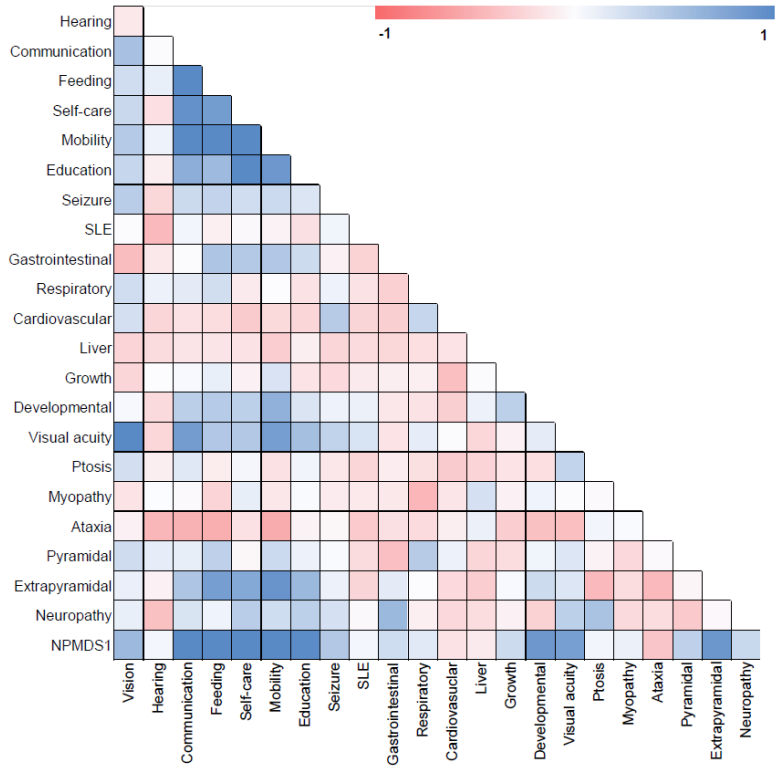


Figure 3-7: Correlation matrix of each item in the NPMDS with each other. Total NPMDS (bottom row) scores have shown strong correlations with several items.

3.4.9 Comparison with other children with mitochondrial disease

Children with Leigh syndrome is reasonably distinct from those other children who had not been diagnosed with Leigh syndrome. During the same study period, a group of children (under aged 18 years) with proven clinical, biochemical and/or genetic mitochondrial disease had been identified as comparisons, hereafter referred to as the 'comparison group' or the 'non-Leigh syndrome group'. These children were not assigned as"controls" because they had not been matched for age, sex or genotypes. Matching controls in rare disease research is challenging due to the lack of large sample size and is not feasible within the study period. The data from these children in the comparison group had been prospectively collected into the UK MitoCohort from their routine outpatient appointments. The eligibility criteria for this comparison group also include the completion of NPMDS assessments during their respective NHS appointments. The aim is to compare the disease burden between these two groups and demonstrate that Leigh syndrome is a discrete subpopulation in a group of children with mitochondrial disease.

3.4.9.1 Age of NPMDS assessment

Fifty-seven children with mitochondrial disorders in the UK MitoCohort who had no features of Leigh syndrome completed their first NPMDS assessments at the clinic during the study period. During these initial NPMDS assessments, their ages (mean = 11.1 years, SD 4.5, 95% CI 9.9-12.3) were significantly older than those with Leigh syndrome $t(127)=7.388, p<0.001$. These children without Leigh syndrome in the comparison group undertook the NPMDS assessments at 11.1 years (SD 4.54, 95%CI 9.92-12.33).

3.4.9.2 Genotypes

Fifty-five of these children in the comparison group have a confirmed genetic diagnosis. Table 3-2 summarised the genotypic spectrum of these children in the comparison group. Mitochondrial disease to pathogenic variants in the mitochondrial genome (GenBank accession: NC_012920.1) accounted for nearly two-thirds of the comparison group (n=37, 65%). Pathogenic variants in the *MT-TL1* gene (m.3243A>G) were the most common (n=20, 35%). Bi-allelic inherited variants that affect mitochondrial DNA maintenance accounted for the majority of nuclear genetic diagnoses (n=16), with defects in *POLG* (NM_002693) (n=7) being the most common. Two children had clinical and biochemical features consistent with a diagnosis of mitochondrial disease but remained genetically undetermined after whole exome sequencing up to the end of the study in March 2020.

Genome	Function	Specific gene	Number of children
Mitochondrial genome	Mitochondrial tRNA	*MT-TL1*	20
		MT-TK	3
		MT-TS2	1
	Complex I subunit	*MT-ND1*	2
		MT-ND3	1
		MT-ND4	2
		MT-ND6	1
	Complex V subunit	*MT-ATP6*	7
Nuclear genome	Mitochondrial DNA maintenance genes	*POLG*	7
		RRM2B	3
		OPA1	3
		TK2	1
		AGK	1
		RMND1	1
	Aminoacyl-tRNA synthetase	*FARS2*	1
Others	Single large-scale deletion of mtDNA	-	1
	Complex I deficiency	-	1
	Undetermined	-	1
		TOTAL	57

Table 3-2: A summary of the genotypes of children with other forms of mitochondrial disease in the comparison group.

3.4.10 NPMDS for the comparison group

3.4.10.1 Section I: Current Function

Children in the comparison group scored differently from those with Leigh syndrome. Within Section 1: Current Function, the most notable item was their mobility (Figure 3-8). Nearly a third (31%) of children with Leigh syndrome used wheelchairs (scoring severe) compared to only 7% of those in the comparison group (U=789, p<0.001). The ability to self-care, including personal hygiene, dressing, utensil use, in the Leigh syndrome group was significantly worse than those in the comparison group (U=1204, p<0.001). A quarter of those with Leigh syndrome needed one-to-one assistance compared to only 2% in the comparison group. Leigh syndrome had also significantly affected the feeding function of children with 22% needing exclusive enteral feeding compared to 5% in the comparison group (U=1108, p<0.001). Another daily function that had been significantly impacted by having Leigh syndrome was the ability to communicate. Less than a third (32%) of children with Leigh syndrome had normal levels of communication as opposed to 78% in the comparison group (U=955, p<0.001).

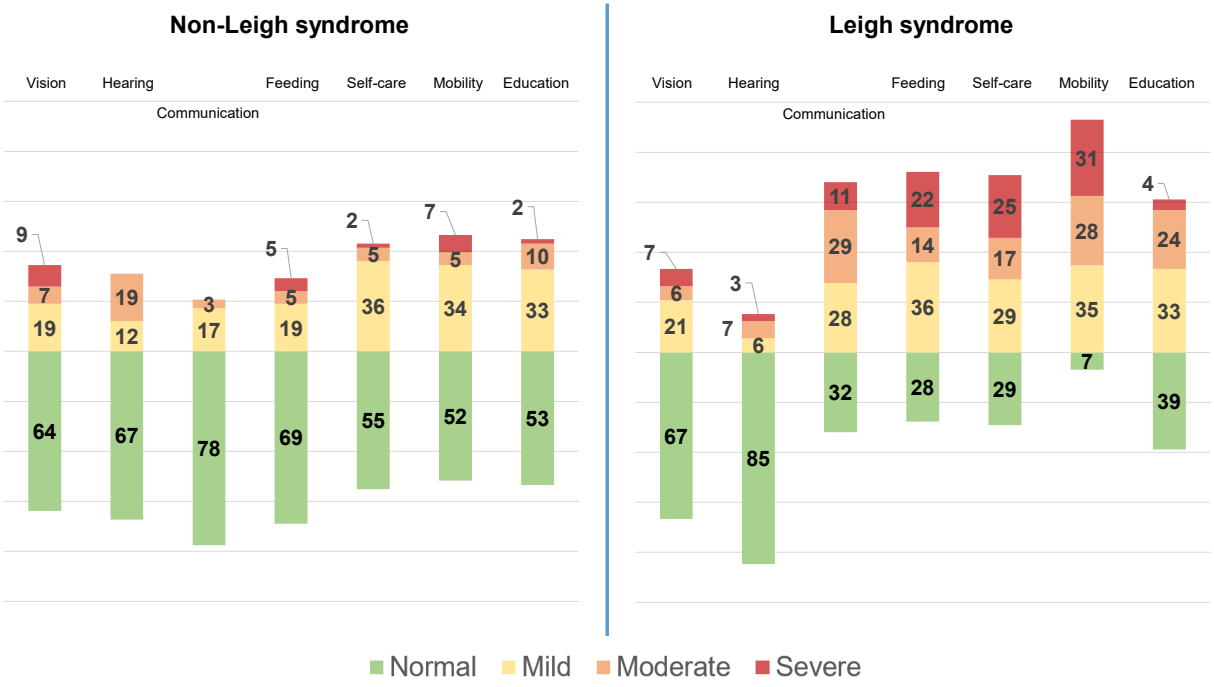


Figure 3-8: Bar chart comparing the Section I (Function) of the NPMDS between non-Leigh syndrome children (comparisons) and Leigh syndrome children.

3.4.10.2 Section II: System specific involvement

The system-specific involvements of both groups have been summarised in Figure 3-9. Children in the comparison group had significantly gastrointestinal involvement than those with Leigh syndrome (U=1657, p=0.025). In the comparison group, 5% of children had severe constipation with no relief from laxative treatment or surgical intervention for dysmotility. None of the Leigh children had been rated as having 'severe' gastrointestinal problems. A higher percentage of children with the non-Leigh syndrome (16%) had 'moderate' gastrointestinal involvement than those with Leigh syndrome (6%). Conversely, a significantly higher proportion of children with Leigh syndrome had some degrees of epileptic seizures than the comparison group (U=1613, p=0.003). None of the children in the comparison group had 'moderate' or 'severe' epileptic seizures whilst, 8% of children with Leigh syndrome had 'moderate' epileptic seizures, and 3% had 'severe' seizures in the preceding 12 months before the NPMDS assessment.

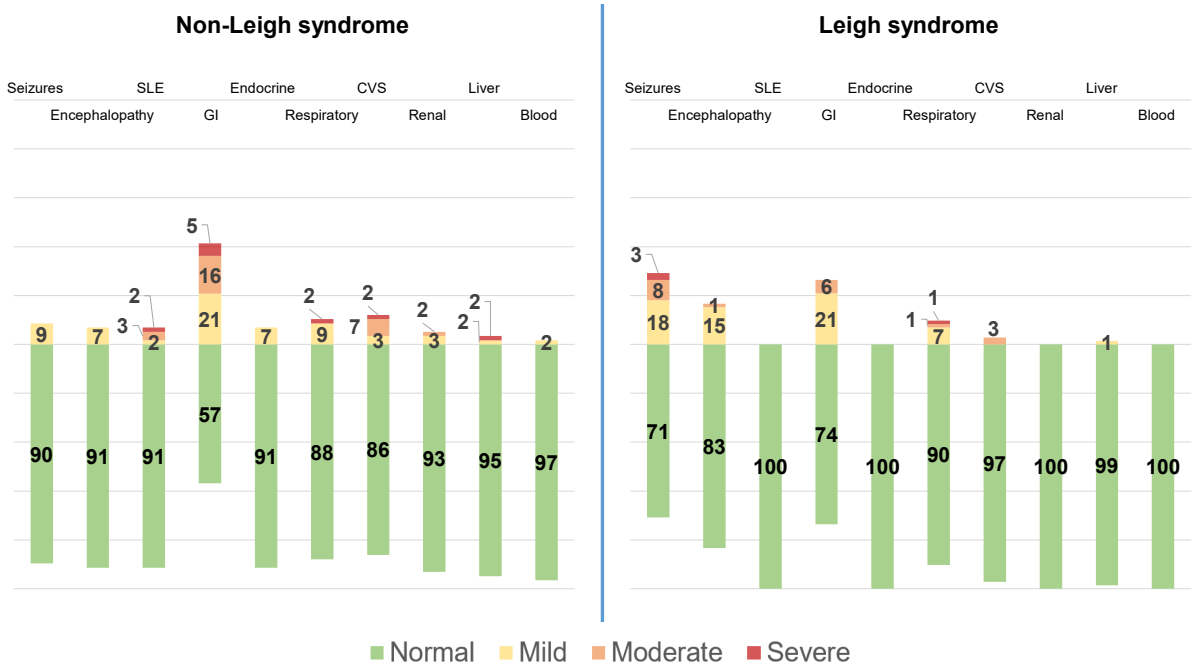


Figure 3-9: Bar chart comparing the Section II (System specific involvement) of the NPMDS between non-Leigh syndrome children (comparisons) and Leigh syndrome children.

3.4.10.3 Section III: Current clinical assessment

During the NPMDS assessment, a significant proportion of children with Leigh syndrome had extrapyramidal features than those in the comparison group (U=228, p<0.001) (Figure 3-10). Whilst nearly almost none of those in the comparison group had any extrapyramidal features, 28% of children with Leigh syndrome had to be in wheelchairs because of their extrapyramidal difficulties. Similarly, a significant proportion of Leigh syndrome children had some degrees of pyramidal features (U=1517, p<0.001). There were no significant differences in other areas of examination findings between the two groups, including growth, visual impairment, ptosis, myopathy, ataxia or neuropathy.

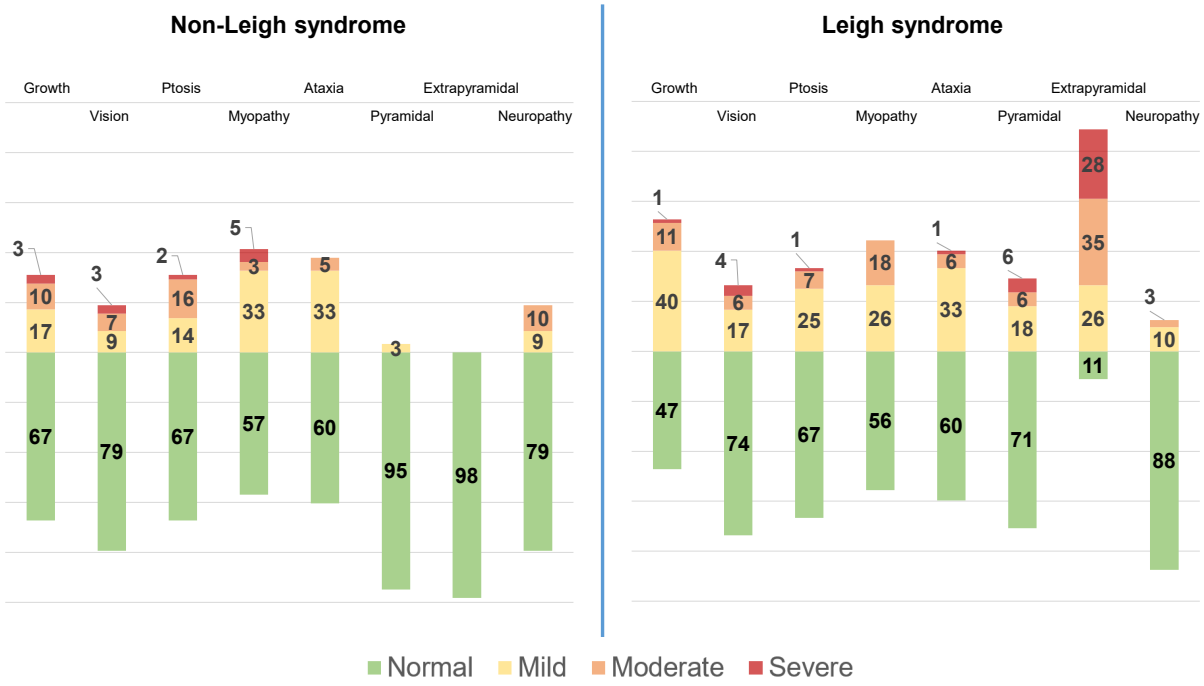


Figure 3-10: Bar chart comparing the Section III (Current clinical assessment) of the NPMDS between non-Leigh syndrome children (comparisons) and Leigh syndrome children.

3.4.10.4 Total NPMDS scores

The median total NPMDS score for the comparison group was 8 (IQR 4-12), which signified a mild disease burden. In contrast, the children with Leigh syndrome had a median score of 18 (IQR 12.25-24), demonstrating moderate disease burden. None of the children in the comparison group syndrome had a total score above 25 which indicated severe disease burden (Figure 3-11), while a quarter of children with Leigh syndrome had severe disease burden. In the comparison group, twelve children (21%) had moderate disease burden (total NPMDS score 15-24) and 45 children (79%) had mild disease burden (total NPMDS score <15). The total NPMDS scores in the Leigh syndrome group was statistically significantly higher than those children in the comparison group (U=629, p<0.001).

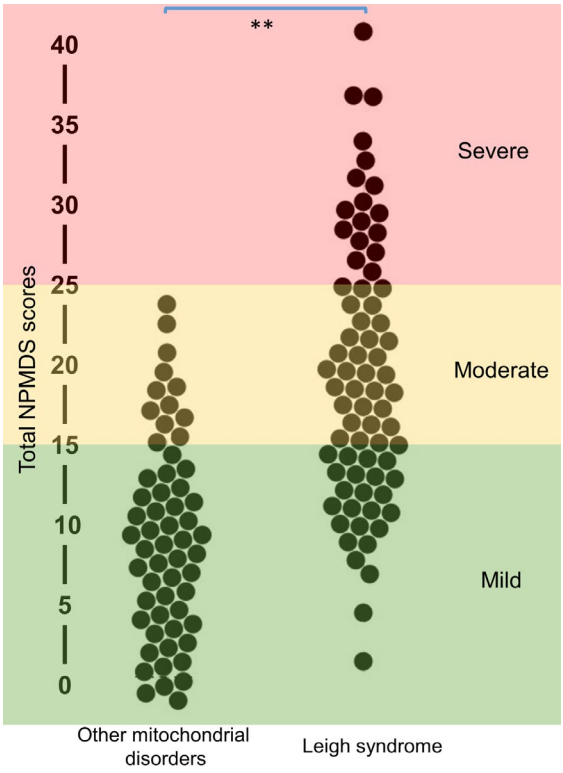


Figure 3-11: Beeswarm plots of children with other forms of mitochondrial disorders (comparison group) and children with Leigh syndrome. The difference between these two groups was statistically significant (p<0.01). None of the children with other forms of mitochondrial disorders (comparison group) had severe disease burden (total NPMDS score >25).

3.5 Discussion

This cross-sectional study has uncovered several significant findings to define the clinical characteristics of children with Leigh syndrome in the UK. These children with Leigh syndrome, as a group, had moderate disease burden when scored using a validated disease scale, the NPMDS. More significantly, a quarter of them had severe disease burden (NPMDS score >25). This observation is unsurprising given the nature of this neurodegenerative disease which results in the most devastating impairment in children (Rahman et al., 1996, Ruhoy and Saneto, 2014). However, no previous studies in the literature had attempted to quantify the disease burden of Leigh syndrome. Having an understanding of how much these children were burdened by their disease not only compels clinicians to prioritise their management but also provides insights into specific areas within NPMDS that contributed to their overall burden. Deterioration of their function, especially communication, feeding, self-care, mobility and education, correlated strongly with their total disease burden. Therefore, this observation had implications on clinical practice to focus on and to consider treatments that could augment these aspects.

Leigh syndrome, also known as subacute necrotising encephalomyelopathy, primarily affects the central nervous system, particularly the basal ganglia structures (Sofou et al., 2014, Alves et al., 2020). These affected structures in the basal ganglia, namely caudate, putamen and globus pallidus, have resulted in extrapyramidal features reported in these children (Martikainen et al., 2016). Exploring the items in the NPMDS scoring demonstrated a large proportion of these children in wheelchairs as a result of their extrapyramidal symptoms and signs. The severity of their extrapyramidal problems had not only affected other functions, including communication, feeding, self-care, and education attainment but also contributed significantly to the overall disease burden. Being primarily a central nervous disease within mitochondrial disorders, a significant proportion of these children with Leigh syndrome also exhibited pyramidal features and epileptic seizures compared to children who had other forms of mitochondrial disease.

NPMDS was designed to assess the natural history of mitochondrial disease throughout childhood; to provide a quantifiable measure of the functional disability encountered and the impact this has on patients and their families and, to be a concise, pragmatic tool suitable for use in a clinic setting, yet able to provide a comprehensive assessment. Phoenix et al. (2006) validated this multidimensional scale and demonstrated its reproducibility. Koene et al. (2018) had recommended NPMDS as an outcome measure for natural history study in children. Therefore, NPMDS had been selected as an outcome measure for this study in children with Leigh syndrome. In this study, the NPMDS assessment tool was applied by those trained in its administration. I had completed these assessments after being trained by my supervisor. Several subsections can afford detailed scrutiny of subtle phenotypic variations arising from the diverse genotypic aetiology of Leigh syndrome. Section I (Function) incorporated patient-reported changes, whilst Section III (Current clinical assessment) is based on the clinically reported outcomes. Not only has this scale shown the severity of each phenotype in detail, but it has also uncovered the proportion of children who had mild, moderate and severe disease burden.

Children with Leigh syndrome tend to have a significantly higher disease burden than other forms of mitochondrial disease. Whilst this study had not utilised matched controls for structured comparisons, the distinctive difference between these two groups is evident. In contrast to their counterparts in the comparison group, children with Leigh syndrome had a significantly more functional disability at appropriate age levels. Furthermore, none of the children in the comparison group had been rated to have, total NPMDS score above 25, which indicates 'severe' disease burden. Although the severity of Leigh syndrome is recognised in the literature (Zhang et al., 2007, Baertling et al., 2014, Sofou et al., 2014, Lake et al., 2016), none had made comparisons of their disease burden with other non-Leigh syndrome children with mitochondrial disease. The indirect comparison in this study had provided new insights into how different children with Leigh syndrome, in terms of disease burden, daily function and clinical features.

Children with Leigh syndrome had generally fared worse in NPMDS items that had statistically significant differences from their counterparts, apart from the gastrointestinal involvement. Those children in the comparison group had a larger proportion of children who had some degrees of gastrointestinal problems than those in the Leigh syndrome group. This difference could be mainly due to the multi-system manifestations of other mitochondrial disorders. In particular, the pathogenic variant m.3243A>G, which accounts for more than a third of the comparison group, had been known to be associated with gut-related problems. Previous reports had linked this pathogenic variant in the *MT-TL1* gene to constipation, chronic gut dysmotility and pseudo-obstruction (Nesbitt and McFarland, 2011, Ng et al., 2016a, Ng et al., 2016b). Although Leigh syndrome is not associated with any particular gut involvement, these children with Leigh syndrome might have secondary gut effects resulting from poor mobility because of their central nervous system involvement or extrapyramidal features (Del Giudice et al., 1999, Sullivan, 2008).

The median age of onset of children with Leigh syndrome in this study was comparable to previously published studies. In a recent sizeable paediatric cohort study in the European countries, Sofou *et al.* (2014) reported median age of onset of 7 months, whilst the earlier seminal paper by Rahman *et al.* (1996) in Leigh syndrome had median onset of 7.5 months. The median age of onset in this study was marginally older at 9 months. These nominal variations in age of onset could be related to the difference in the population sample, the genotypic heterogeneity of the healthcare settings. Despite commonly having a paediatric onset, Leigh syndrome can also be present in adulthood (Nagashima et al., 1999, Goldenberg et al., 2003, Huntsman et al., 2005, McKelvie et al., 2012, Wesolowska et al., 2015, Ng et al., 2019).

Most patients in this study have striatal changes (putamen and caudate) within the basal ganglia and this is similarly reported in the literature (Arii and Tanabe, 2000, Bonfante et al., 2016, Alves et al., 2020). In more recent publications, Bonfante *et al.* (2016) reported basal ganglia changes in 13 of 17 children (76%) and brainstem changes (midbrain and medulla oblongata) in 9 of 17 children (53%) in a small cohort. Meanwhile, Alves et al. (2020) found striatal lesions in 42-53% of the 53 neuroimages they had analysed. Although the number of subjects and proportion of abnormal basal

ganglia changes in my study is similar to these published case series, direct comparisons are not feasible because of variations in the interpretation and reporting of radiological findings. In this cohort of children, I have also demonstrated that some neuro-radiological lesions are detected together more often than might be expected by chance. In particular, the close association of putamen and caudate nuclei lesions is not surprising given the anatomic and functional integration of these two structures (Kumral et al., 1999, Siddiqui and Riley, 2014).

It is also not possible to postulate from the findings of this study how the caudate nuclei lesions disrupt the neural network to other cerebral structures in Leigh syndrome, but this link is worth exploring in future histopathological or animal studies. In a *Ndufs4* knockout mouse model of Leigh syndrome and complex I deficiency, the selective inactivation of striatal neurons led to progressive motor impairment (Chen et al., 2017). In human studies, caudate lesions are often associated with neurobehavioral changes (Mendez et al., 1989, Levy and Dubois, 2005). These changes are usually noted with progressive cognitive decline in adults (Grahn et al., 2008), but this phenomenon in children remains unclear. Alternatively, the presence of caudate lesions could partly result from the advanced stage of the disease process in Leigh syndrome. The caudate lesions in this cohort of children with Leigh syndrome are not seen in isolation but in conjunction with other accompanying symmetrical lesions in the basal ganglia or brain stem. Thence, these lesions might have appeared at later stages as the disease progresses.

One key advantage of this study is the longstanding recruitment that has been delivered by national centres of excellence in the diagnosis and clinical management of patients with mitochondrial disease, where specialist diagnostic laboratories have provided a genetic diagnosis in over 80% of these children compared to much lower yields of genetically-confirmed cases in other cohorts(Ma et al., 2013, Sofou et al., 2014, Lee et al., 2016, Ogawa et al., 2020). This difference in diagnostic yield could also be explained by the varied diagnostic strategies adopted by these expert mitochondrial groups. The diagnostic approach at the NHS Highly Specialised Service for Rare Mitochondrial Disorders and the Wellcome Centre for Rare Mitochondrial Disorders has been published elsewhere (Taylor et al., 2014, Alston et al., 2017).

Furthermore, the gene pool in the UK population, on which this study is based, is likely to be different from those published cohorts in North European or East Asian countries. Having excellent recruitment into the UK MitoCohort had also enabled the ascertainment of the common genotypes that caused Leigh syndrome in this cohort. Although there have been more than 80 genes associated with Leigh syndrome, the most frequently reported genes associated with Leigh syndrome include pathogenic variants in the *SURF1*, *MT-ATP6*, variants affecting Complex I subunits and variants leading to pyruvate dehydrogenase deficiencies (Lake et al., 2016, Rahman et al., 2017). Therefore, this cohort is representative of the genotypic spectrum of Leigh syndrome commonly seen in clinical practice.

In this study, about a third of children with Leigh syndrome who had central nervous system involvement had normal CSF and blood lactate. Lactate levels had been proposed to be an essential biochemical marker to distinguish the diagnosis of mitochondrial disorders (Magner et al., 2011, Yamada et al., 2012, Ruhoy and Saneto, 2014). Given that Leigh syndrome is primarily a central nervous system disease, lactate levels in the CSF is more reliable than the levels in the blood (Yamada et al., 2012). Whilst lactate levels might be elevated for some specific forms of mitochondrial disease, this end-product of glycolysis is not a good discriminator. Lactate level is not specific to mitochondrial dysfunction because an elevation can result from several conditions including shock, sepsis, cardiac arrest, trauma, seizure, ischemia, diabetic ketoacidosis, malignancy, liver dysfunction, toxins, and medications (Andersen et al., 2013). On the other hand, lactic acidaemia during the metabolic crisis could potentially be underrecognised. Poor sampling technique, excessive tourniquet use, or delayed handling post-sampling can also lead to erroneous lactate results. Thus, the interpretation of lactate levels had to consider these factors. Furthermore, the timing of the sampling is another confounding factor. Lactate levels might vary depending on the clinical state of the patients. More crucially, the findings of abnormal CSF lactate levels in this study also did not correlate with the disease burden of Leigh syndrome, suggesting that lactate level measurement might not be as reliable as previously thought, and there is no clinical indication for testing in children for disease management or prognosis.

Alterations of enzyme activities in one or several respiratory chain complexes are frequently associated with mitochondrial disease. Respiratory chain complex activity can be reduced as a result of compromised assembly factor activities or decreased synthesis of other factors in the respiratory chain functions in the mitochondria (Fassone and Rahman, 2012, Koene et al., 2012). To determine these changes, enzymology analysis of skeletal muscle biopsy samples is the most frequently used technique. The skeletal muscle enzymology has historically been the 'gold standard in the diagnostic work-up of mitochondrial disease because this post-mitotic tissue is densely packed with mitochondria, metabolically highly active and demonstrates histopathological correlates of disease (Taylor et al., 2004, Greaves et al., 2010). However, the procedure to obtain skeletal muscle involved the need for general anaesthetics along with a skilled paediatric surgical team, and this might be perceived as a risk or logistical challenge by clinicians looking after these children with Leigh syndrome. In this study, only about half of the children had muscle biopsy procedures, reflecting the low uptake of this diagnostic procedure. In addition, the decreasing costs and increasing quality of next-generation genetic tests, such as whole-exome sequencing or panels, has promoted these tests to the forefront as the first-tier diagnostic tools for neuromuscular diseases instead of skeletal muscle analysis. Even if the children had their muscle biopsy analysed, the yield of respiratory chain analysis is low in this study. About half of the children with Leigh syndrome had normal findings in respiratory chain analysis of their muscles. In many instances, muscle enzymology is not diagnostic of Leigh syndrome (Ruhoy and Saneto, 2014). Also, having abnormal findings in muscle respiratory chain analysis did not correlate significantly with disease burden in this cohort of children with Leigh syndrome. Therefore, the role of muscle biopsy in children with Leigh syndrome is gradually shifting towards proving pathogenic variants instead of an initial diagnostic investigation.

There are several limitations to this study. First, the small sample size might have resulted in relatively low statistical power. However, the number of children with Leigh syndrome, which is a relatively rare paediatric mitochondrial disorder, recruited into this study is considerable when compared to other more extensive international studies (Ma et al., 2013, Sofou et al., 2014, Lee et al., 2016, Ogawa et al., 2020). Second, the NPMDS has some shortcomings despite its usefulness. NPMDS might

have suffered from ceiling effect in children who had severe extrapyramidal features or mobility problems. The ceiling effect described how subjects who are at or near the possible upper limit cannot be estimated above a certain level or that their variance is difficult to determine (Everitt and Skrondal, 2002, Cramer and Howitt, 2004). For instance, a Leigh syndrome patient with extensive basal ganglia lesion in a wheelchair might develop pain, discomfort or pathological fractures secondary to their severe dystonia, but these could not be scored more than 3 points in the 'extrapyramidal' section. Third, there is an absence of perfect controls to make valid comparisons. Children with Leigh syndrome presented to clinic or medical attention at a younger age than their comparisons because of their age of onset. Although there were cases of adult-onset Leigh syndrome, there is a preponderance to pre-school children. The comparisons used in this study had limited data points such as neuroimaging, lactate levels or muscle respiratory chain deficiencies for analysis. There were no clinical indications or ethical approval to subject children with other forms of mitochondrial diseases to invasive and extensive investigations. Furthermore, the genotypic spectrums of the two groups differ. Thus, it is not possible to compare if any of these factors influence the disease burden between these two groups. Nonetheless, the distinction between these two groups regarding disease burden is stark, highlighting that children with Leigh syndrome have more clinical needs than their counterparts. Lastly, this study only provided a cross-sectional view of the children with Leigh syndrome. As a neurodegenerative disease, a cross-sectional study is not able to capture the disease progression or the natural evolution of Leigh syndrome.

This study into children with Leigh syndrome using the NPMDS has not only provided a certain level of granularity in the severity of each clinical phenotype but also highlighted their disease burden. I have also established the characteristics of these children, including their age of onset, lactate levels, respiratory chain enzyme activities, neuroimaging changes and genotypic spectrum. Despite the lack of direct controls, Leigh syndrome remains a distinct group with a significantly higher disease burden than children with other forms of mitochondrial disease. This cross-sectional work, which could not delineate the disease progression, has also highlighted a need for longitudinal data in Leigh syndrome to understand its natural history further.

Chapter 4: A longitudinal study of children with Leigh syndrome to assess their disease progression and factors influencing their disease trajectory

4.1 Introduction

As discussed in Chapter 3, there is a need to explore the disease progression of Leigh syndrome using the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS). In the current literature, there have also been no cohort studies that longitudinally assess disease progression in Leigh syndrome using any validated disease rating scale. Recent publications on children with Leigh syndrome in European mitochondrial centres (Baertling et al., 2014, Ruhoy and Saneto, 2014, Sofou et al., 2014, Lake et al., 2016) have not provided quantitative information about the disease progression over time. Despite being regarded as a paediatric neurodegenerative condition, the exact amount of disease progression of Leigh syndrome in children over time remains unclear.

The cohort of children with Leigh syndrome in Chapter 3 has also offered an opportunity to answer this research question. Given that these children are being followed up in the NHS Highly Specialised Service for Rare Mitochondrial Disorders in Newcastle, NPMDS assessments can be repeated in their subsequent visits to clinics. Their follow-up NPMDS assessments can then be compared with their baseline assessments to determine how much disease burden they had accumulated over the follow-up period. Furthermore, the NPMDS, which has been designed to monitor the rate of disease progression in children (Phoenix et al., 2006), is the most practical and time-efficient method compared to the other disease rating scale (Koene et al., 2016). This chapter set out to assess children with Leigh syndrome longitudinally using the NPMDS. The NPMDS also can delineate the exact disease burden accumulated within specific clinical features. After establishing the disease progression, this study also aims to establish the clinical variables that can potentially influence the disease trajectory.

4.2 Specific aims

5. To determine the rate of disease progression in children with Leigh syndrome using the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS)
6. To investigate potential factors that influenced disease burden and progression as well as mortality.

4.3 Methods

4.3.1 Study design

This longitudinal study followed up a cohort of children with Leigh syndrome who had completed baseline NPMDS assessments at research sites with expert clinicians and repeated these assessments at their subsequent outpatient clinic appointments. These children were part of the Mitochondrial Disease Patient Cohort (MitoCohort) UK: A Natural History Study and Patient Registry. (REC: 13/NE/0326). All parents of participants consented to this study which has a favourable opinion from an independent research ethics committee, NRES Committee North East – Newcastle & North Tyneside 2 (REC Reference Number: 13/NE/0326). The NPMDS data were collected from the children who attended outpatient clinics from March 2009 to March 2020. At the time of follow-up assessment, these children were alive and were physically able to attend clinics at the study sites. After completing their follow-up data collection, I compared their baseline and follow-up assessments individually to determine the rate of change in their NPMDS scores.

4.3.2 Participants

All patients (under 18 years) recruited to the study fulfilled the diagnostic criteria for Leigh syndrome as outlined in the eligibility criteria in Chapter 2.

4.3.3 Outcome measures

4.3.3.1 NPMDS at follow-up visits

I have repeated the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) as the clinical outcome measure to determine objectively disease burden at follow-up visits. The total score from all three sections which reflects disease burden can be categorised into mild (0-14), moderate (15-25) and severe (>25). At their follow-up assessments, I scored these children independently and I was blinded to their previous (baseline) NPMDS scores to reduce bias.

4.3.3.2 Variables

I have used the following variables, from Chapter 3, in my analysis for possible influences on the disease progression, namely age of disease onset, growth centiles, genotypes and neuroimaging changes. In addition to these clinical variables, I have also followed up with these patients up to March 2020 for any mortality related to their underlying mitochondrial disease. The disease burden (total NPMDS scores) and rate of disease progression (rate of change in NPMDS scores) have also been evaluated as potential factors in the mortality of children with Leigh syndrome.

4.3.4 Statistical analysis

I used SPSS v.25 for statistical analyses. All the statistical methods have also been detailed in Chapter 2. Statistical tests were two-sided with significance set at $p<0.05$. T-tests were used for continuous variables and Chi-square tests for categorical variables. To determine whether baseline NPMDS scores (ordinal variable) differed significantly between the two time points of this same group of children, I used the Wilcoxon signed-rank test (Wilcoxon, 1947). Correlation coefficients were calculated with the appropriate measure. Meanwhile, Kendall's tau-b are used for ordinal variables, such as the NPMDS scores or the individual item scores. To predict the probability of death based on one or more independent variables, I used binomial logistic regression. Of all the methods of calculating the variation in this regression, I used Nagelkerke R^2 value, which is a modification of Cox & Snell R^2 and the value of Nagelkerke R^2, a pseudo-R^2, will have lower values than in multiple regression (Nagelkerke, 1991). I have also used the Kaplan-Meier method (Kaplan & Meier, 1958), a nonparametric method used to estimate the probability of survival of these children past the given time during the study period. I compared the survival distributions of two or more groups of factors for equality with the log-rank test. The log-rank test interrogates the null hypothesis that there is no difference in the overall survival distributions between the groups by calculating a $\chi 2$-statistic (Mantel, 1966).

4.4 Results

Seventy-two children with Leigh syndrome from 68 different pedigrees fulfilled the inclusion criteria to take part in this longitudinal study.

4.4.1 Change in NPMDS

4.4.1.1 Section I: Current Function

There were trends of significant disease progression in several items of the NPMDS when these children had been reassessed at follow-up appointments. In Section I (Current Function), the proportion of children who were wheelchair-dependent or fully reliant on their carer for mobility had increased from 30.6% at baseline to 56.9% at follow-up (Figure 4-1). A Wilcoxon signed-rank test also showed that children with Leigh syndrome had a statistically significant decline in their mobility over the follow-up period (Z=-4.7, p<0.001). Indeed, the median rating for the mobility item which was 2 (‘Moderate’ – requiring support to walk) at baseline had risen to a median score of 3 (‘Severe’ – fully wheelchair or carer dependent) at follow-up assessments. This significant change in the severity of physical function was also evident in the other items in Section I (Figure 4-1). The dependence on wheelchairs or carers for mobility was reflected in the loss of ability to self-care (including personal hygiene, dressing or utensil use) at age-appropriate levels. Although nearly a third of the children had normal ability for self-care at age-appropriate levels, this fell to 18% at follow-up. Furthermore, 43.1% of these children were fully reliant on parents with no contribution to self-care or one-to-one assistance with self-care at baseline which had increased significantly from a quarter of children (25.0%) (Z=-3.9, p<0.001). Despite the proportion of children who had normal feeding ability remained relatively unchanged throughout the study period, those children who had difficulties at baseline assessments deteriorated significantly when assessed at follow-up (Z=-3.9, p<0.001) (Figure 4-1). The proportion of children who had to be exclusively fed via gastrostomy or nasogastric tube had doubled from 22.2% to 45.8%. Similarly, the ability to communicate effectively at an age-appropriate level with parents and strangers worsened significantly (Z=-4.4, p<0.001). The percentage of children whose speech was not understood by family or friends and who required communication aids doubled from 11.1% to 26.4%.

From their baseline to follow-up assessments, children attending mainstream nursery or school with comparable academic achievement to peers dropped from 38.9% to 22.2% (Figure 4-1). There was a significant shift towards attending special school or not attending school due to their illnesses (Z=-4.9, p<0.001). The percentage of children not attending school or nursery settings because of their illness soared to 18.1% from 4.2%, whilst those attending special school or nursery increased to more than a third (34.9%) from less than a quarter (23.6%). The percentages of vision and hearing impairments in these children were less marked compared to other aspects of functional activities in this Section I (Figure 4-1). Nearly two-thirds of have normal visual function without any concerns from parents or carers at baseline and retain this normal function at follow-up. Nonetheless, those who had visual impairments progressed significantly (Z=-3.0, p=0.003). 6.9% of these children with Leigh syndrome had registered been blind or unable to recognise the face at baseline and this percentage had doubled to 13.9% at follow-up assessments. Whilst the majority of these children with Leigh syndrome (78-85%) had a normal hearing function, there had also been a progression in their hearing impairment (Z=-2.6, p=0.008). 8.3% of the children in this cohort had poor hearing function even with aids.

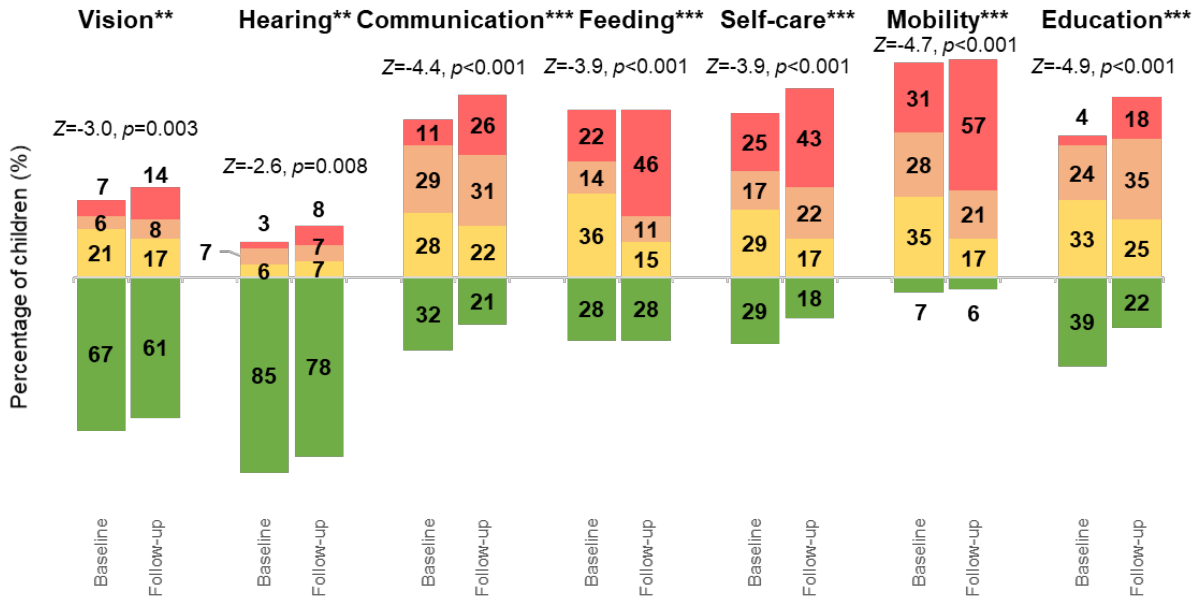


*Figure 4-1: Percentages of children with Leigh syndrome were rated as normal (green), mild (yellow), moderate (amber) and severe (red) in selected items of Section I (Current Function) within the NPMDS. Baseline and follow-up NPMDS scores were arranged alongside each other for comparisons. * indicated significance <0.05, ** indicated significance <0.01 *** indicated significance <0.001.*

4.4.1.2 Section II: System Specific Involvement

Epileptic seizures and gastrointestinal systems were the two items in the System Specific Involvement that changed significantly from baseline to follow-up NPMDS assessments (Figure 4-2). The percentage of Leigh syndrome children with epileptic seizures had increased from 29.2% at baseline assessments to 37.5% at follow-up assessments. A Wilcoxon signed-rank test showed that children with Leigh syndrome had a statistically significant change in the severity of their epileptic seizures (Z=-3.2, p=0.002). The percentage of children who had at least one episode of prolonged seizures or status epilepticus in the preceding nearly doubled from 2.8% to 5.6%.

There was also significant deterioration in gut-related symptoms (Z=-3.0, p=0.002) (Figure 4-1). A quarter (25.8%) of these patients had some forms of gut dysmotility symptom (NPMDS ≥ 1) at baseline assessment but at follow-up NPMDS ratings, more than a third (36.2%) had gut-related issues with 4.2% having severe constipation with no relief from the laxative treatment. On the other hand, there was no significant change in respiratory, cardiovascular, hepatic, endocrine, renal or haematological systems over time.

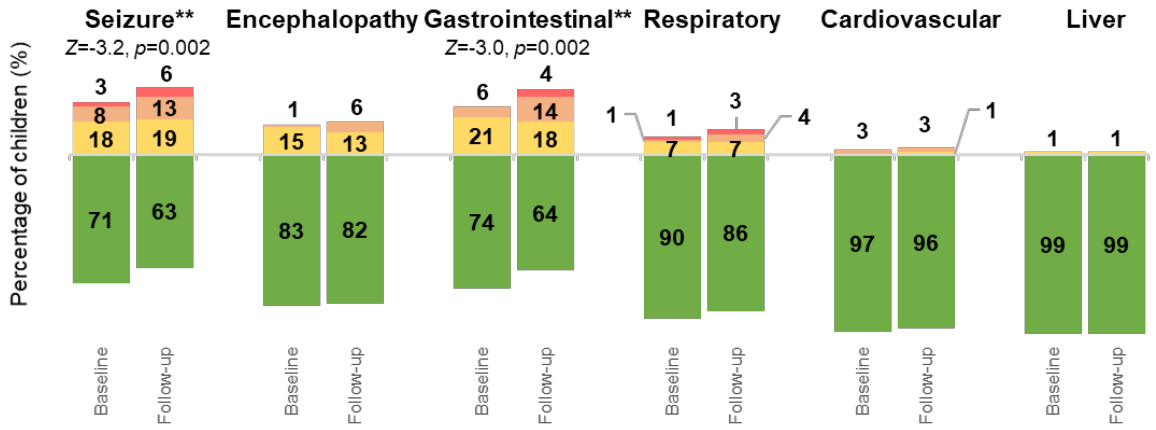


*Figure 4-2: Percentages of children with Leigh syndrome were rated as normal (green), mild (yellow), moderate (amber) and severe (red) in selected items of Section II (System specific involvement) within the NPMDS. Baseline and follow-up NPMDS scores were arranged alongside each other for comparisons. * indicated significance <0.05, ** indicated significance <0.01 *** indicated significance <0.001.*

4.4.1.3 Section III: Current Clinical Assessment

Of all the assessment items in Section III, the most striking change is the extrapyramidal findings (Figure 4-3). The percentages of children with severe extrapyramidal features characterised by dystonia and dyskinesia had risen significantly at follow-up (Z=-4.0, p<0.001). Children with Leigh syndrome needing wheelchairs because of severe extrapyramidal difficulties increased from 27.8% at baseline to 52.8%. At follow-up, almost all patients had developed certain degrees of extrapyramidal features (94.4%).

Likewise, the percentage of children with muscle weakness or myopathy had increased from baseline (Z=-3.7, p<0.001) (Figure 4-3). None of these children had been wheelchair-bound due to myopathy at baseline but 9.7% of them had subsequently needed wheelchairs at follow-up because of myopathy. The number of children who had moderate symmetrical proximal weakness that limited mobility had also increased marginally, from 18.1% to 22.2%. Other neurological examination findings had also changed considerably. Cerebellar ataxia also increased from baseline (Z=-2.7, p=0.003) with 19.4% of children needing assistance with gait abnormality or severe limb dysmetria at follow-up. Another neurological sign that worsened significantly were pyramidal signs (Z=-2.6, p=0.017). The only neurological feature that did not change significantly was the severity of neuropathy features (Z=-1.4, p=0.177).

Their visual acuity of children with Leigh syndrome had significantly become more severe than from their initial assessments (Z=-3.5, p<0.001) (Figure 4-3). 9.7% of these children had acuity worse than 6/60 or no response to light or unable to count fingers at follow-up assessments, compared to 4.2% at baseline. The ophthalmoplegia or eyelid ptosis had also progressed significantly (Z=-3.9, p<0.001). More children (9.7%) had bilateral ptosis obscuring pupils and with >50% restriction to eye movements at follow-up appointments than at baseline (1.4%). Although the developmental scores had not differed significantly between the two assessments (Z=-0.012, p=0.991), almost all of these children have some degree of developmental delay, with nearly a third (29.2%) having severe developmental regression in the four months preceding their follow-up assessments.

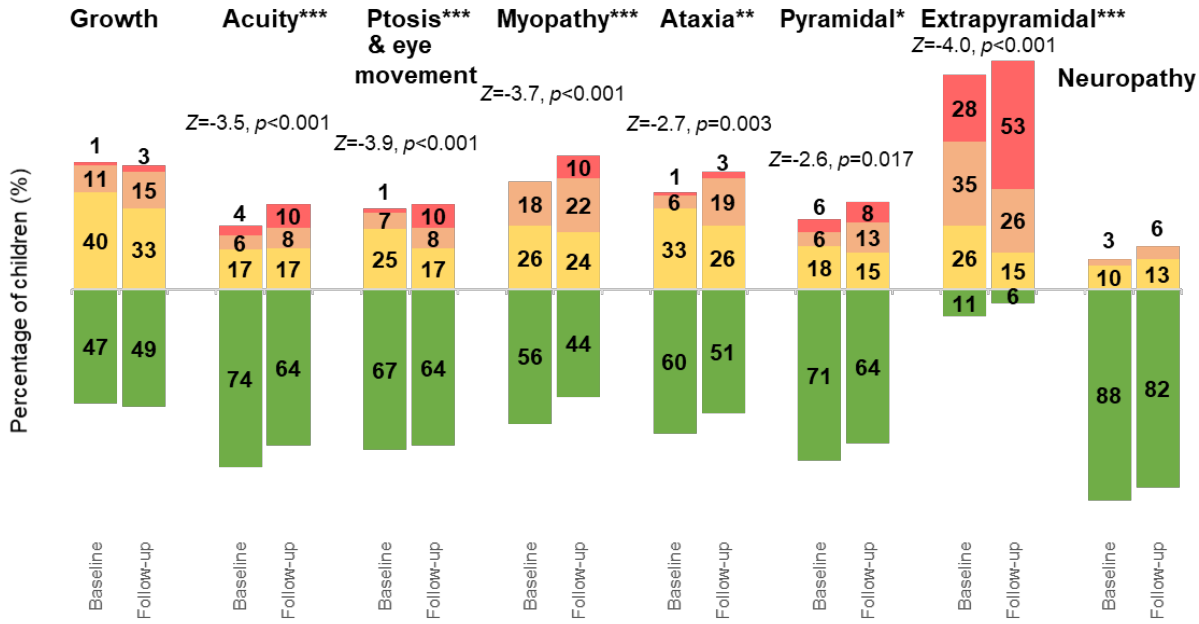


*Figure 4-3: Percentages of children with Leigh syndrome were rated as normal (green), mild (yellow), moderate (amber) and severe (red) in selected items of Section III (Current clinical assessment) within the NPMDS. Baseline and follow-up NPMDS scores were arranged alongside each other for comparisons. * indicated significance <0.05, ** indicated significance <0.01 *** indicated significance <0.001.*

4.4.1.4 Change in total NPMDS score

The median NPMDS scores at baseline and follow-up assessments were 18 (IQR 12-24) and 24 (IQR 17-31) respectively (Figure 4-4A). A Wilcoxon signed-rank test confirmed that this change in NPMDS scores was significant (Z=-6.9, p<0.001). The general upward trend of total NPMDS scores for these children over time (Figure 4-4B). Almost all of the children experienced an increase in the NPMDS score from their respective baseline assessments. The only exception is one child who has a diagnosis of biotinidase deficiency (bi-allelic pathogenic *BTD* gene variants) and improved clinically with the administration of high-dose biotin. This improvement was evident by the fall in the total NPMDS score from 14 to 2. Only 16 children (22.2%) had a high disease burden with NPMDS score above 25 at their respective baselines, but the number of children with the high disease burden nearly doubled to 30 (41.7%) at their respective follow-up appointments.

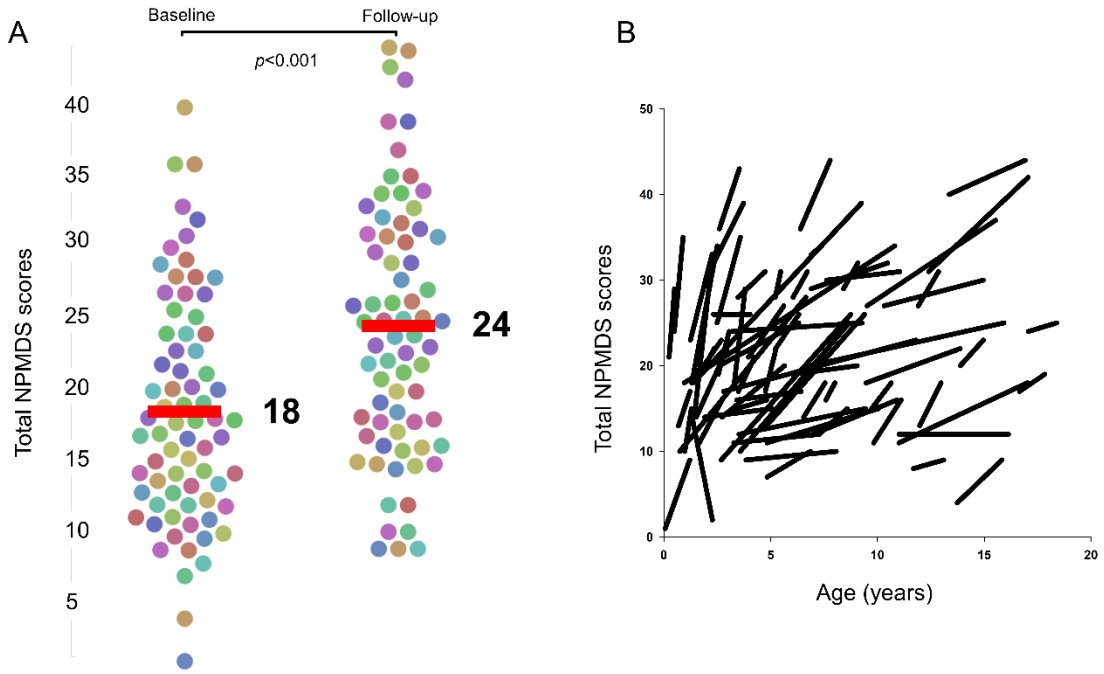


Figure 4-4: The change of total NPMDS scores for all participants. (A) A beeswarm plot of the total NPMDS scores for each individual patient in this study at baseline (left swarm) and at follow-up (right swarm). Red lines indicate the median scores. The median score rose from 18 at baseline to 24 at follow-up NPMDS assessments. The difference between these two assessments was significant, p<0.001. (B) A vector graph showing these changes according to their ages. Each individual line showed the change in NPMDS scores from baseline to follow-up assessments for every patient in this study.

4.4.2 Inter-item relationships

Akin to the correlation matrix in Chapter 3, some of the items scored at follow-up NPMDS assessments have been shown to be correlated with each other (Figure 4-5). The correlation matrix at follow-up is almost identical to the analysis at baseline, suggesting that there had been no significant changes in the relationships between the items scored in NPDMS. At follow-up, the functional status of the children, such as feeding, self-care, communication and mobility were still well correlated (Kendall's tau-b (τ_b) correlation coefficients 0.53 – 0.67, all p<0.001). The educational attainment of these children remains also significantly correlated with the ability to self-care ($\tau_b \approx$ 0.52, p<0.001) and communicate ($\tau_b \approx 0.51$, p<0.001) appropriately for their respective ages. The scores in these five items (communication, feeding, self-care, mobility and education) within the functional status of the children at follow-up assessment were still strongly correlated with the total NPMDS scores ($\tau_b \approx 0.53 – 0.68$, p<0.001). In the clinical assessment section, extrapyramidal signs showed a slightly better correlation with mobility at follow-up assessments ($\tau_b \approx 0.50$, p<0.001) than at baseline ($\tau_b \approx 0.46$, p<0.001).

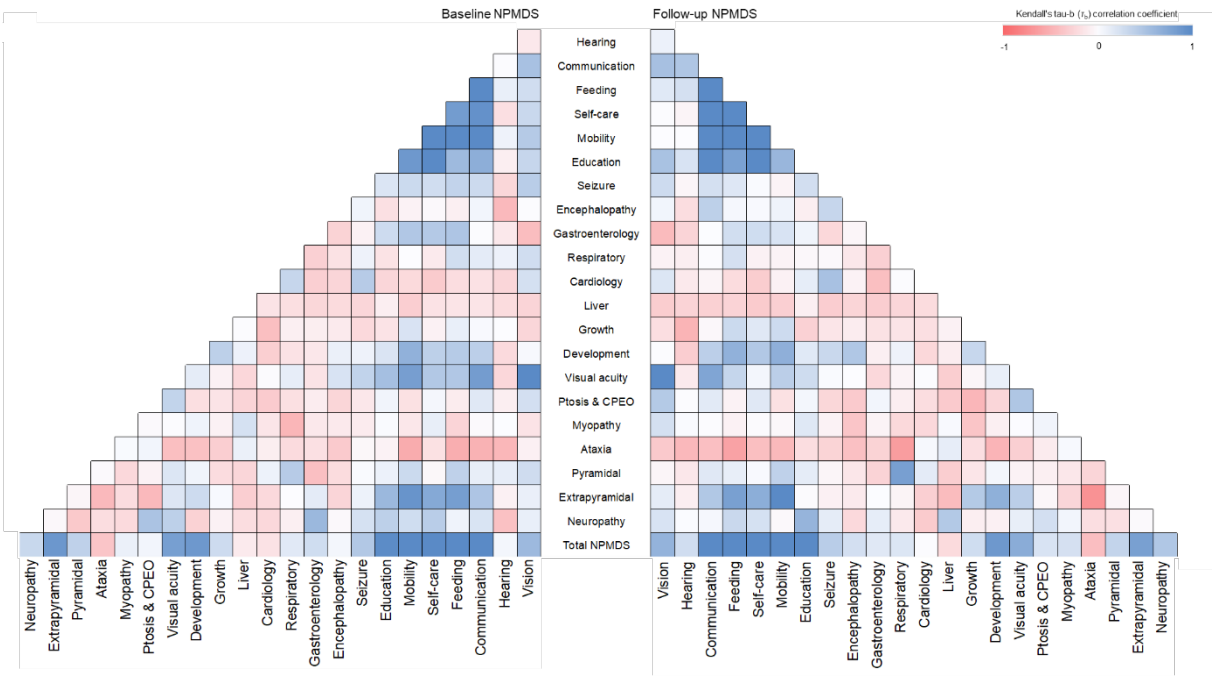


Figure 4-5: The inter-item relationships of the NPMDS items in this cohort of children with Leigh syndrome. Any positive Kendall's tau-b (τb) correlation coefficients were shown in blue. The correlation matrix at baseline (left) and at follow-up assessments (right) were similar.

4.4.3 Rate of change in NPMDS scores

The children in this cohort gained on average 4.5 NPMDS scores annually (SD 6.5, 95%CI 3.0-6.1). This increment of NPMDS scores per annum differed among genotypes (Figure 4-6 from A to F). One particular group of genotypes stood out distinctively. Children who harbour pathogenic *SURF1* variants (n=7), impairing the assembly of complex IV, had the highest rise in NPMDS scores per annum of 11.5 (SD 7.7, 95%CI 4.3-18.5) (Figure 4-6C). Compared to the other genotypes, the annual NPMDS scores increment in patients with pathogenic *SURF1* variants was significantly higher, $t(70)=3.1$, $p=0.002$.

The second fastest gain of NPMDS scores were patients harbouring pathogenic *MT-ATP6* gene variants in the mitochondrial genome (m.8639T>G, m.8993T>G, m.8993T>C, m.9035T>C, m.9176T>C) encoding a structural subunit of Complex V with 5.9 points per annum (SD 8.1 95%CI 0-12.1) (Figure 4-6D), but this rate of change was not significantly different from other genotypes in this cohort. Other pathogenic mtDNA variants in the *MT-ND1, MT-ND4*, and *MT-ND5* genes had a slower change in annual NPMDS scores (mean 1.4, SD 1.4, 95%CI 0.1-2.7) (Figure 4-6B). There were no associations or relationships between the rate of change and the heteroplasmy levels of all these mtDNA variants.

Pathogenic variants in *PDHA1, PDHX*, and *DLD* genes causing pyruvate dehydrogenase deficiency had the third-highest rate of change of NPMDS scores at 4.7 per annum (SD 4.2 95%CI 0.3-9.2) (Figure 4-6E). This is followed by pathogenic variants in nuclear genes (*NDUFV1, NDUFS1, NDUFA9, NDUFA13, NDUFAF6,* and *NDUFAF8*) affecting Complex I assembly proteins and structural subunits (mean 2.9, SD 4.5 95%CI 0.1-5.8) (Figure 4-6A), other nuclear genotypes (mean 2.6, SD 5.0, 95%CI 0.3-5.0) (Figure 4-6F)

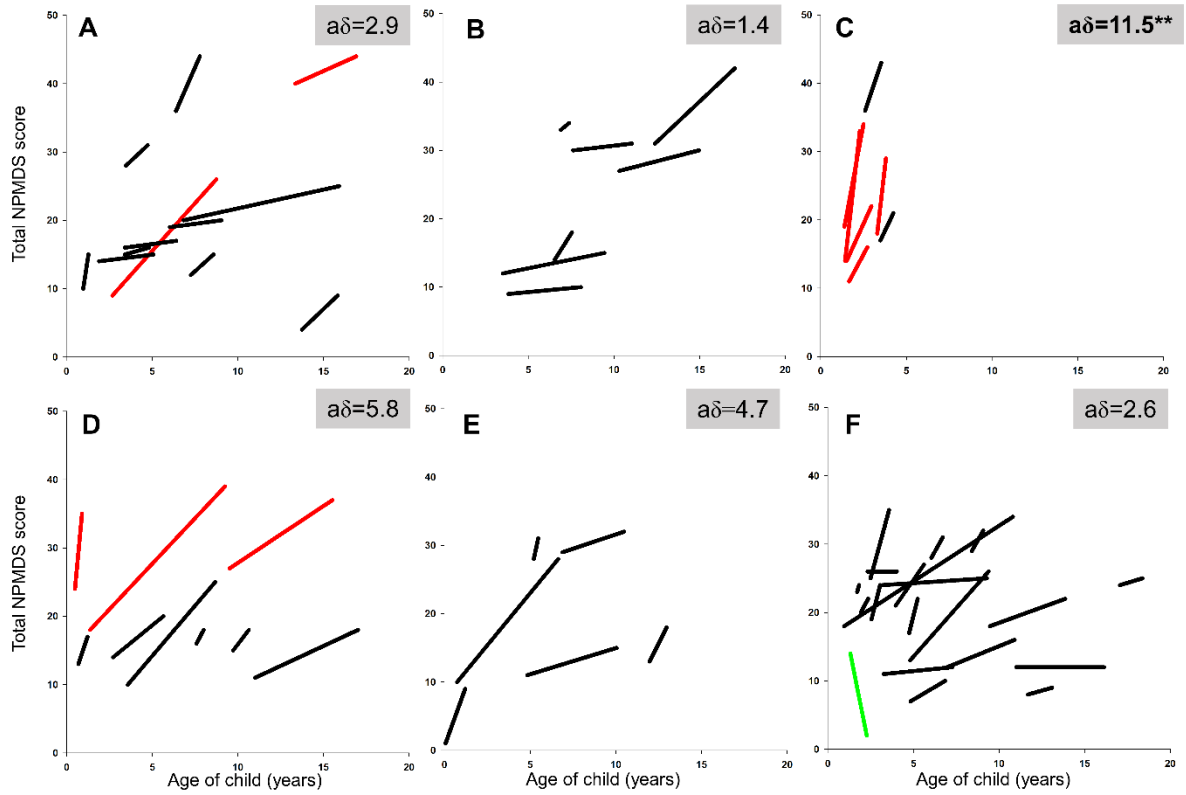


Figure 4-6: The vector plots for NPMDS scores at baseline and at follow-up in this study based on their genotypes. The red vector lines indicate patients who had died since the end of the study. Only one child improved during the follow-up (green line). aδ is the change of NPMDS scores per annum. Vector plots categorised into several groups according to genotypes – (A) Complex I assembly factor and structural protein variants (NDUFV1, NDUFS1, NDUFA9, NDUFA13, NDUFAF6, NDUFAF8), (B) mtDNA-encoded Complex I subunits variants (MT-ND1, MT-ND4, MT-ND5, MT-ND6), (C) Pathogenic SURF1 gene variants affecting complex IV assembly, (D) MT-ATP6 gene variants, (E) Pathogenic variants affecting pyruvate dehydrogenase complex (PDHA1, PDHX, DLD) and, (F) Other pathogenic nuclear gene variants. Children without a known genetic diagnosis are not shown here.

4.4.4 Survival

After their follow-up NPMDS assessments, twelve children (16.6%) died and the mean age of death was 7.3 years (SD 6.0, 95% CI 3.5-11.1). The mean interval between their last follow-up NPMDS and their deaths was 8.7 months (SD 8.7 95% CI 3.1-14.3). The genotypes of these twelve children were *SURF1* (n=5), *MTATP6* (n=3), *NDUFAF6* (n=1), *NDUFS1* (n=1) and genetically undetermined (n=2). The red vector lines in Figure 4-6, marked those children with a confirmed molecular genetic diagnosis who had died. Among all the domains within the follow-up NPMDS, the deceased group had scored significantly higher in the following items – Feeding function (Z=-2.5p=0.013), Respiratory involvement (Z=-4.9, p<0.001) and Pyramidal assessment (Z=-2.4, p=0.015).

4.4.5 Possible influence on outcomes

4.4.5.1 Early age of onset on mortality

The median age of onset for children who died was 11 months (IQR 2-17) and the median age of onset for those who survived was 9 months (IQR 5.5-19.5). Comparing these two medians independently, the difference was not statistically significant (p=0.545). Twenty children (27.7%) had their onset of disease at 6 months or less in this cohort. The log-rank test of differences in the survival of children with disease onset at 6 months or less and those with later disease onset of more than 6 months was not statistically significant ($\chi2(1) = 3.3$, p=0.07) (Figure 4-7).

4.4.5.2 Early age of onset on severe disease burden

Early disease onset (under 6 months) has a significantly higher NPMDS score at follow-up assessments (U=339.5, p=0.022), signifying greater disease burden. The log-rank test of differences in the probability of having severe disease burden (total NPMDS > 25) of children with disease onset under 6 months and those with later disease onset was statistically significant ($\chi2(1) = 13.5$, p<0.001) (Figure 4-7).

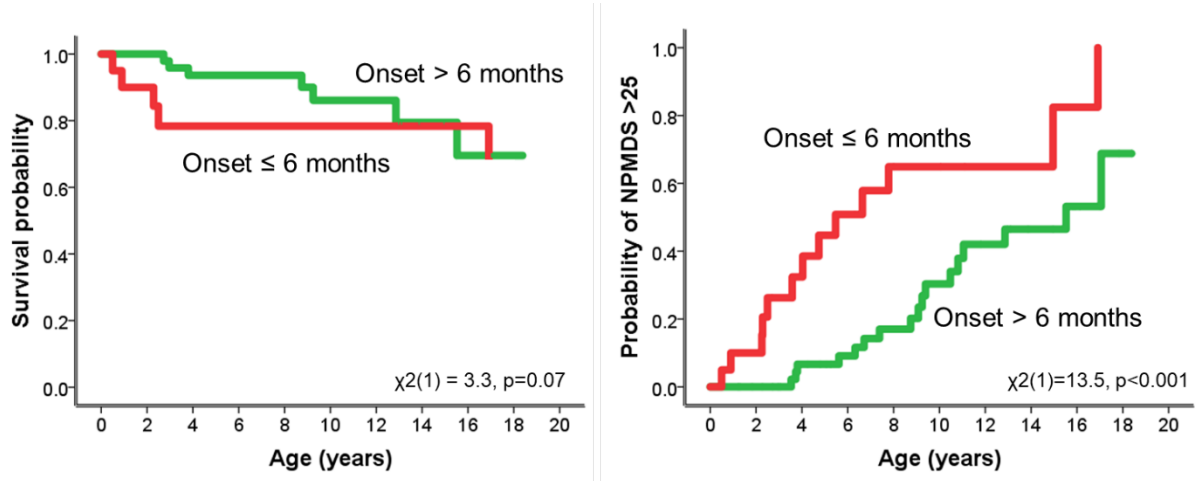


Figure 4-7: The Kaplan-Meier (K-M) curves on survival probability from mortality and from severe disease burden (NPMDS score >25). K-M curve on the left compared the survival probability of children who had the age of disease onset 6 months or below with children who had the age of disease onset after 6 months. K-M curve (one minus survival) on the right compared probability of having severe disease burden (NPMDS score > 25) between these two groups.

4.4.5.3 Faltering growth on mortality

More than half of these children with Leigh syndrome (n=37, 51.4%) weighted the 2nd centile or had fallen across two centile lines on the UK WHO growth charts. These children fulfil the criteria of faltering growth, previously known as failure to thrive (Gonzalez-Viana et al., 2017). Failure to thrive had been proposed as a predictor of poorer survival in children with Leigh syndrome (Sofou et al., 2014). In this study, those children with faltering growth also had poorer survival than those who were thriving. The log-rank test of differences in the survival of children between these two groups was statistically significant ($\chi2(1) = 5.3$, $p=0.021$) (Figure 4-8).

4.4.5.4 Faltering growth on disease burden

Not only would these children with faltering growth have a higher likelihood of dying, but they were also more likely to develop severe disease burden at follow-up assessments. At their follow-up assessments, twenty-one children with faltering weight and fourteen children without faltering weight had severe disease burden (NPMDS score <25). The log-rank test of differences between these two groups was statistically significant ($\chi2(1) = 13.2$, $p<0.001$) (Figure 4-8).

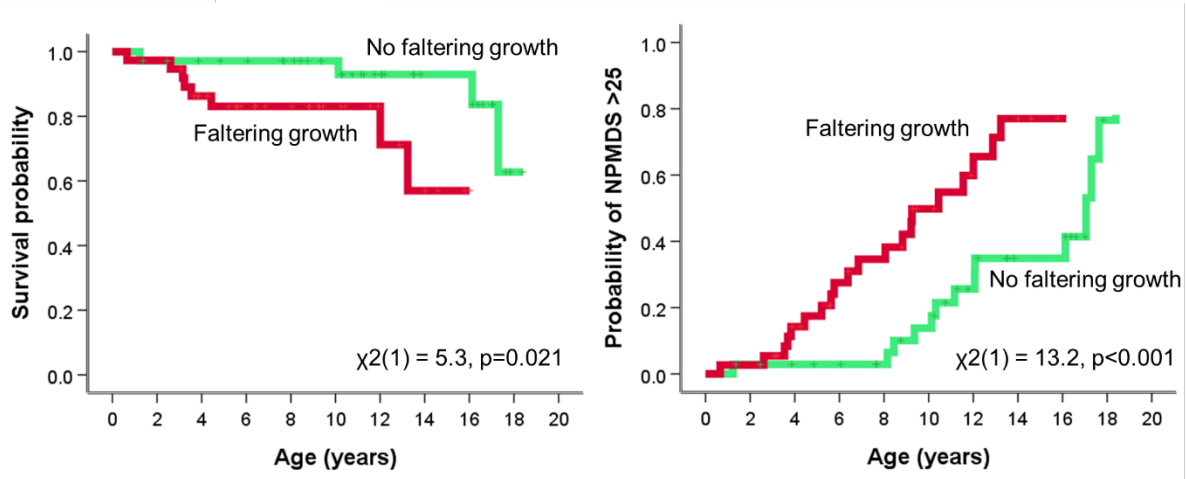


Figure 4-8: The Kaplan-Meier curves on survival probability from mortality and from severe disease burden (NPMDS score >25). K-M curve on the left compared the survival probability of children who had faltering growth with those who had no faltering growth. K-M curve (one minus survival) on the right compared probability of having severe disease burden (NPMDS score > 25) between these two groups.

4.4.5.5 SURF1 on mortality

Of the twelve children who had died after their last follow-up assessments, five of them harboured pathogenic variants *SURF1.* In comparison to pathogenic gene variants in nuclear or mitochondrial DNA, children with Leigh syndrome as a result of pathogenic recessive variants in the *SURF1* gene were significantly associated with poorer survival ($\chi2(2) = 41.8$, $p<0.001$) (Figure 4-9). Indeed, children with Leigh syndrome secondary to *SURF1* gene variants had a 3.1-fold increased risk of death (RR 3.1, 95%CI 1.0-10.1).

4.4.5.6 SURF1 on disease burden

The difference in probability of developing severe disease burden (NPMDS > 25) was also statistically significant ($\chi2(2) = 25.7$, $p<0.001$). Once again, children with Leigh syndrome due to recessive *SURF1* pathogenic variants had fared worse than those with pathogenic variants in the nuclear or mitochondrial genome (Figure 4-9). The pathogenic *SURF1* variants identified were the pathogenic c.312-321del10insAT, c.792_793delAG, c.574_575insCTGCp. (Arg192fs) and c.488T>G variants.

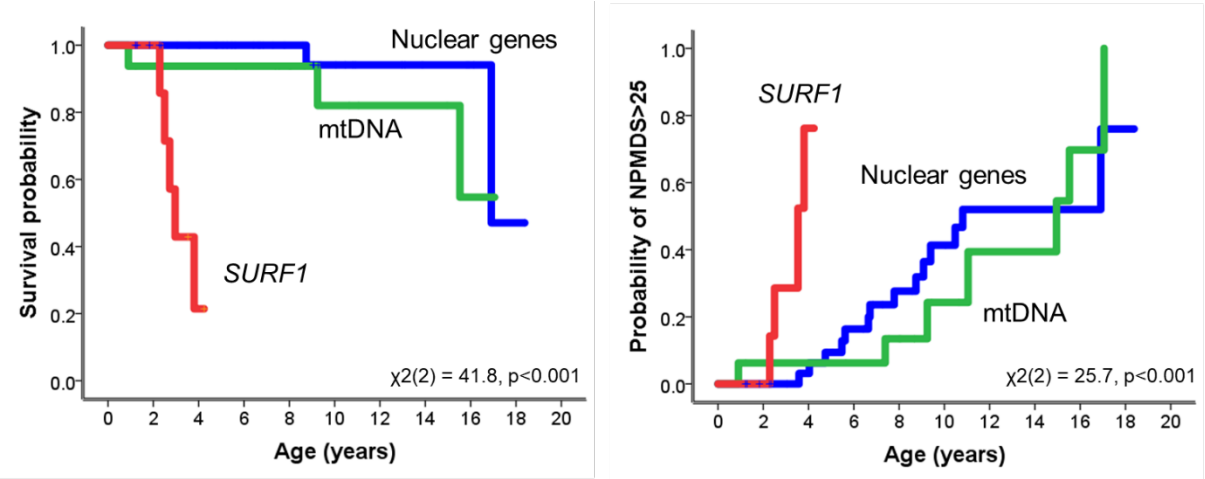


Figure 4-9: The Kaplan-Meier (K-M) curves on survival probability from mortality and severe disease burden (NPMDS score >25). K-M curve on the left compared the survival probability of children who had different genotypes (SURF1, mtDNA variants and nuclear gene variants). K-M curve (one minus survival) on the right compared probability of having severe disease burden (NPMDS score > 25) among these three groups.

4.4.5.7 Neuroimaging changes

Bilateral caudate nuclei signal abnormality is positively correlated with higher follow-up NPMDS score (rpb=0.27, p=0.033), incremental change when assessed per annum (rpb=0.34, p=0.007) and mortality (rpb=0.36, p=0.004) (Figure 4-10). Bilateral cerebellar signal changes were identified in 30% of those who died compared with 5.7% of those who were still alive ($\chi(1)=5.8$, p=0.046). Generalised brain atrophy at initial neuroimaging is associated with higher NPMDS scores at follow-up assessments ($\chi(27)=47.5$, p=0.009).

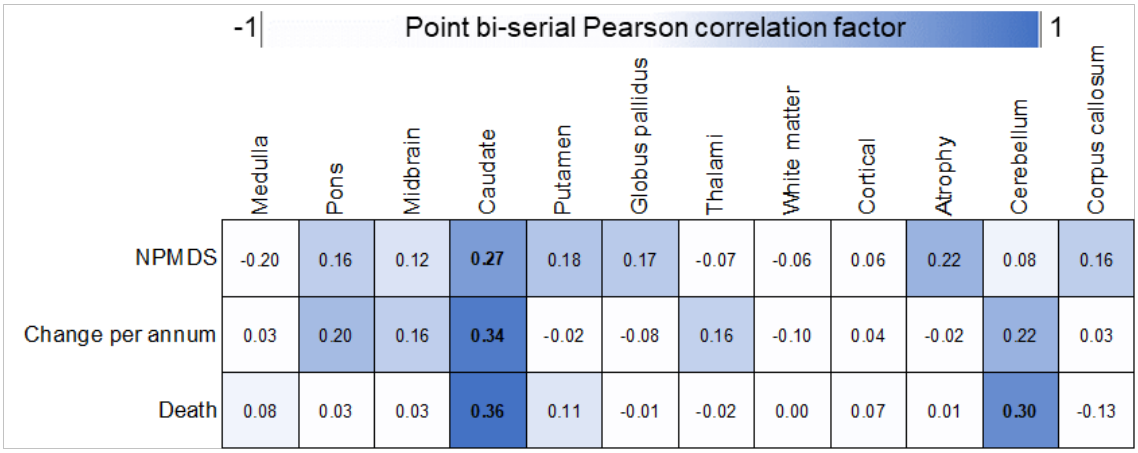


Figure 4-10: The point biserial Pearson correlation factor for each abnormal neuroimaging change against the disease burden (follow-up NPMDS scores), disease progression (NPMDS score change per annum) and death. Blue indicates a positive correlation.

Bilateral caudate nuclei and cerebellar signal abnormalities had also been associated with poorer survival, as demonstrated by log-rank tests of $\chi2(1) = 7.11$, p=0.008 and $\chi2(1) = 4.16$, p=0.041, respectively (Figure 4-11). Whilst individual changes in the medulla, pons and midbrain had not affected mortality, these brainstem lesions, as a group, had demonstrated with higher mortality $\chi2(1) = 4.82$, p=0.028 (Figure 4-11).

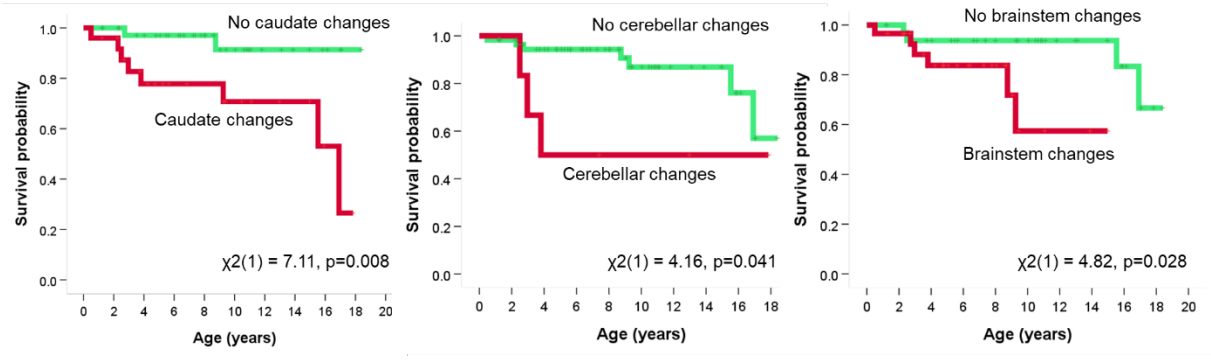


Figure 4-11: The Kaplan-Meier curves on survival probability for children with different abnormalities in the brain neuroimaging.

4.4.5.8 Other variables

Other proposed predictors of mortality in children with Leigh syndrome include epileptic seizures and elevated lactate levels (Sofou et al., 2014). However, these two factors did not affect mortality in this study. Having epileptic seizures or history of epilepsy had not shown a statistical difference in survival $\chi2(1) = 1.61$, p=0.205. The presence of abnormal lactate levels in CSF or serum or both had not been associated with poorer survival $\chi2(1) = 0.04$, p=0.834.

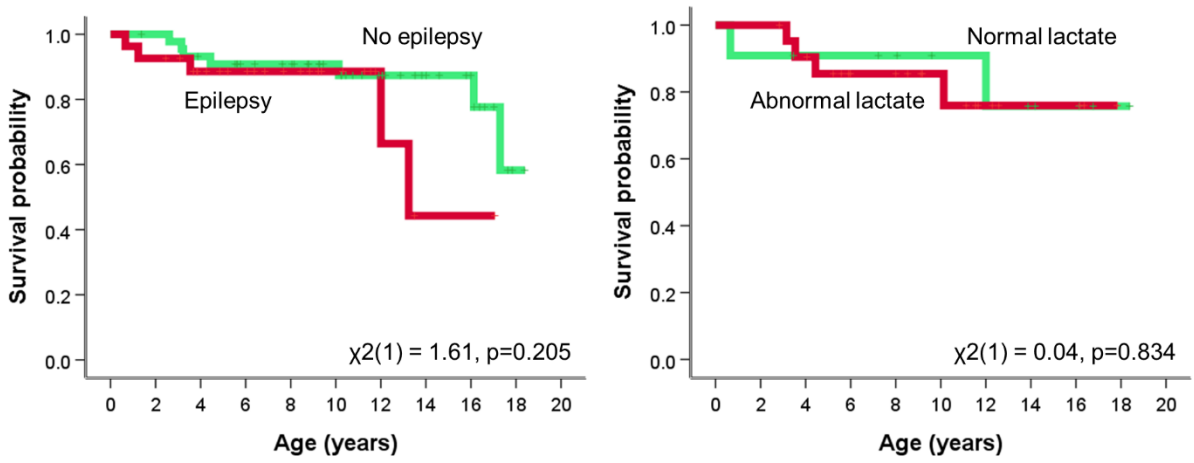


Figure 4-12: The Kaplan-Meier (K-M) curves on survival probability of these children. K-M curve on the left compared the survival probability of children who had epilepsy. K-M curve on the right compared the survival probability of children who had raised lactate levels.

4.4.5.9 Disease burden

Severe disease burden, as indicated by total NPMDS > 25, had been linked with poorer survival. Children who died had a higher follow-up NPMDS median score of 32 (IQR 26.75-36.5) than those who survived, where the median score was 22.5 (IQR 16-29.5) (Z=-3.0, $p<0.01$). However, there was no difference in the baseline NPMDS scores for the surviving and the deceased groups with medians of 17 (IQR 12-24) and 18.5 (IQR 14-26.25) respectively (Z=-0.59, $p=0.562$). None of the children who had NPMDS scores 0-14 (mild disease burden) at follow-up had died. Of those who scored between 15 and 25 at follow-up NPMDS assessments (moderate disease burden), only two of them had died. Ten children who scored above 25 at follow-up NPMDS assessments (severe disease burden) had died. A log-rank test determined that there were statistically significant differences in the survival distributions of these three groups. Children with severe disease burden (total NPMDS score > 25) have higher mortality than those with lower burden ($\chi2(2) = 11.4$, $p=0.003$) (Figure 4-13).

4.4.5.10 Disease progression

Another indicator of poor survival is the change of NPMDS per annum. Children who died exhibited significantly faster disease progression before their deaths as evidenced by the higher acquisition of annual NPMDS scores per annum (11.5, SD 11.0 95%CI 4.5-18.6) than those who remained alive (3.2, SD 4.0, 95%CI 2.1-4.2), ($t(70)=4.6$, $p<0.001$). In particular, those children whose NPMDS scores rose by more than 3 points per annum have significantly worse survival in the log-rank test of differences ($\chi2(1) = 11.3$, $p=0.001$) (Figure 4-13).

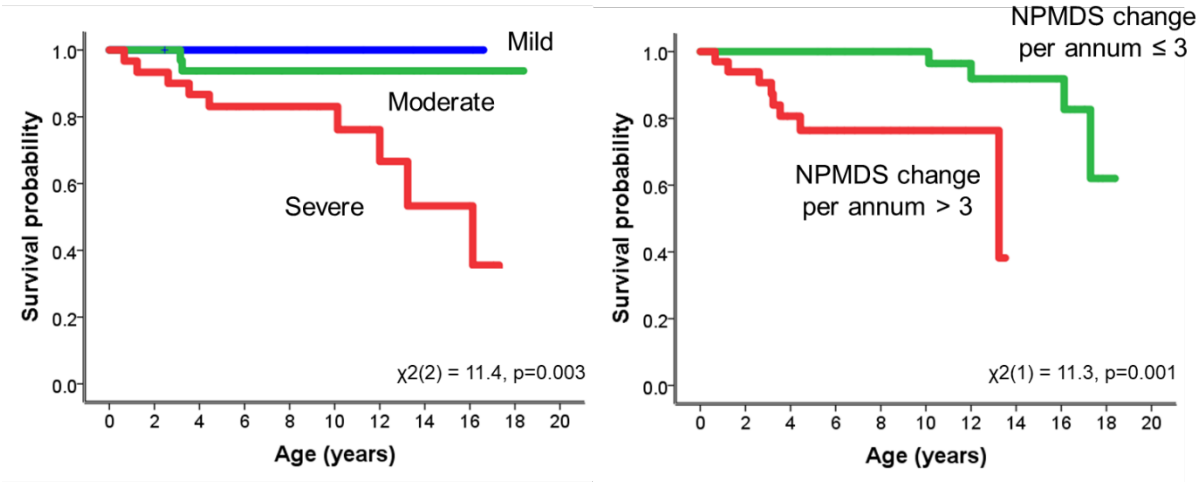


Figure 4-13: The Kaplan-Meier (K-M) curves on survival probability. K-M curve on the left compared the survival probability of children who had mild (NPMDS score 0-14), moderate (NPMDS 15-25) and severe (NPMDS >25) disease burden. K-M curve on the right compared the survival probability of children who had different disease progression indicated by NPMDS change per annum.

4.4.7 Logistic regression on disease burden and progression

Having severe disease burden at follow-up assessments and higher accumulation of NPMDS score per annum were still significantly associated with mortality even after controlling for other independent variables of sex and age of onset ($p<0.01$). Binomial logistic regression was undertaken to determine the effects of the NPMDS scores at follow-up and the score increment per annum on the likelihood of dying. The model explained 48.7% (Nagelkerke R2) of the variance in mortality and correctly classified 83.3% of cases, suggesting that disease burden and progression were both likely to be predictive of the increased probability of demise.

4.5 Discussion

This longitudinal study has provided new natural history data to define the clinical evolution of Leigh syndrome. This group of children have been followed up within MitoCohort (UK), the largest cohort of patients with mitochondrial disease in the UK. The major advantage of this longitudinal study in Leigh syndrome over previously published data from cross-sectional observational studies is the systematic chronological recording of a validated mitochondrial disease-specific rating scale that quantified clinical changes in individual patients over time. Repeated use of the NPMDS at multiple outpatient clinic attendances not only provides quantitative data on the disease severity of Leigh syndrome at each visit, but also allows disease trajectories to be plotted for individual patients.

This study has revealed that children with Leigh syndrome have considerable disease progression. The current function section (Section 1) of the NPMDS highlighted the deterioration in the severity of all items, particularly mobility, education, communication and self-feeding skills. Each of these functional activities is interrelated and contribute significantly to the overall disease burden. Most notably, more than half of these children progressed to full wheelchair or carer dependence for their mobility at follow-up assessments. The loss of mobility is an established risk for increased mortality in the general paediatric population (Nissen et al., 2018). Being wheelchair-bound might signify a milestone of Leigh syndrome disease progression and herald potential development of other associated problems. The effect of this significant change in mobility was evident in the other items of Section I as illustrated in the correlation matrix. Other impairments of physical function, such as feeding skills, communication and self-caring abilities, have also long been recognised as poor prognostic factors in disabled children (Hutton et al., 1994, Ashwal, 2005, Cohen et al., 2008). However, none of these functional difficulties in Section I have any direct links with death in this study. Causes of death in children with functional impairments could be multi-factorial (Sidebotham et al., 2014, Rousseau et al., 2015). Deteriorating physical function is unlikely to be the singular factor leading to deaths in children with Leigh syndrome, but its potential as a surrogate factor is yet to be established.

Of the clinical phenotypes rated in the NPMDS, the progression of extrapyramidal features was the most prominent. The prominence of these extrapyramidal features is unsurprising because one of the hallmarks of Leigh syndrome is the neuro-radiological features that involve the basal ganglia. Despite the recognition of this hallmark, no published studies have explored the precise progression of this movement disorder. Martikainen et al. (2016) had subjectively reported the slowly progressive and stepwise nature of extrapyramidal movement disorder in eleven paediatric patients. This study has, on the other hand, objectively demonstrated the significant change in extrapyramidal signs of a larger cohort of children with Leigh syndrome and established the rate of progression over time.

Almost all children with Leigh syndrome in this cohort had demonstrated significant disease progression over the study period. Follow-up assessments showed more than twice the number of children who had severe disease burden than at baseline assessments. Although this study valued the rate of progression of 4.5 NPMDS scores per annum on average for these children, individual children in this cohort progressed differently. These variable progression rates reflect the heterogeneity of Leigh syndrome which has more than 80 pathogenic genes, both in nuclear and mitochondrial genomes, identified in affected patients (Lake et al., 2016). Each of these pathogenic gene variants is likely to exert different disease mechanisms and effects on the mitochondrial respiratory chains; even a different T to C, instead of T to G transition at position 8993 within the *MT-ATP* gene (mitochondrial genome) can lead to more severe phenotypes and disease progression rates (Debray et al., 2007, Sofou et al., 2018, Ng et al., 2019). This study had further illustrated the breadth of disease progression rates across several common genotypes of Leigh syndrome.

This study has also expanded the current understanding of disease burden and progression of the commonest syndromic presentation of paediatric mitochondrial disease. Disease burden and progression in certain adult mitochondrial diseases such as single large scale deletion of mitochondrial DNA and pathogenic variant m.3243A>G have been thoroughly described (Grady et al., 2014, Grady et al., 2018, Pickett et al., 2018), whereas in paediatric mitochondrial diseases, like Leigh syndrome, the clinical course remain under-researched. Despite mitochondrial

disease in early childhood is often rapidly progressive in most cases (McFarland et al., 2010), little is known about how much and how quickly it progresses. Not only has this study answered these previously unresolved questions in paediatric Leigh syndrome, but it has also established that those who accumulate more than 3 NPMDS scores per annum or carry severe disease burden as indicated by NPMDS scores more than 25 are more likely to have worse survival. Knowing that the high disease burden and rapid disease progression as poor prognostication factors is crucial. From a clinical perspective, this study has illustrated that the use of this objective scale in clinics for patients with Leigh syndrome is of value for monitoring, prognostication, and advance care planning. Clinicians could potentially gain a better idea of prognosis by applying the NPMDS serially and provide informed counselling or palliative care at the end stages of this devastating neurodegenerative disease process. Given the growing landscape for therapeutic intervention in mitochondrial diseases, this study will also be of substantial value to the design of future trials as a historical metric of standard clinical care for those where a control arm, might not be feasible.

Twelve children (16.6%) died in this cohort, contrary to the higher mortality of Leigh syndrome reported by previous cross-sectional studies (Rahman et al., 1996, Naess et al., 2009, Sofou et al., 2014, Ogawa et al., 2020). This higher survival rate could be attributed to the design of this study that focussed on children who have likely already survived acute crises in their early years. Although this design may have been biased towards better survival, this cohort represented the Leigh syndrome population would be more likely enrolled on trials of treatment. Those with significant disease burden and deterioration to death might not fulfil eligibility criteria or the narrow window of opportunity for early phase trials. Other known barriers for participation in clinical trials for mitochondrial diseases include those who are on multiple concomitant medications and those who could potentially have rapid disease progression (Zolkipli-Cunningham et al., 2018). Early case reports of genetic conditions are often descriptions of strikingly fatal, severe or novel disease manifestations, but almost inevitably these underestimate the breadth of phenotypes and the survival associated with any given genetic defect. This is true for Leigh syndrome and the less severe phenotypes in this cohort contrast with those previously published and probably contribute to this survivor bias.

Apart from disease burden and progression discussed previously, there are also other potential predictors of poorer outcomes in these children. Several predictors had been suggested in the literature (Baertling et al., 2014, Ruhoy and Saneto, 2014, Sofou et al., 2014, Lake et al., 2016, Ogawa et al., 2020). While this study could not find a significant association with mortality in some of them, including the presence of epilepsy or abnormally high lactate levels, it has substantiated the others, as discussed below. In addition, this study has also uncovered new potential factors that might influence the disease trajectory of Leigh syndrome.

Disease onset before 6 months of age has been suggested as a poor prognostic factor in some studies of children with Leigh syndrome (Sofou et al., 2014, Ogawa et al., 2020). In this study, early disease onset is not a significant predictor of mortality but instead is associated with a higher probability of developing severe disease burden (NPMDS scores >25). However, the relatively small number, especially of rare pathogenic variants, means that it is not possible to draw valid comparisons with other studies. Furthermore, the median age of onsets and the interquartile ranges from each study vary, which could partly be explained by the genotypic heterogeneity of Leigh syndrome.

More importantly, this study demonstrated the link between faltering growth with both mortality and disease burden in children with Leigh syndrome. Previously known as failure to thrive, faltering growth is characterised by a slower rate of weight gain than expected for age, sex, and current weight in childhood (Gonzalez-Viana et al., 2017). NICE guidelines proposed that thresholds for concern for faltering growth in children include a fall across more weight centile spaces or when current weight is below 2nd centile for age (National Guideline, 2017). Although faltering growth is often multifactorial, it has consistently been associated with poor outcomes in general paediatric practice (Rudolf and Logan, 2005, de Souza Menezes et al., 2012, Valla et al., 2018). Failure to thrive in Leigh syndrome has also been proposed by Sofou et al. (2014) as a possible predictor of mortality and this study has substantiated their observations. More than half of this cohort of children with Leigh syndrome have fulfilled the criteria for faltering growth. These children who had lower than expected growth were significantly more at risk of high disease burden and mortality.

Suboptimal growth is well-recognised in mitochondrial disease. Boal et al. (2019) estimated that 1 in 3 and 1 in 10 of these adults with mitochondrial disease had the height of >1SD and >2SD, respectively, below the population mean after adjusting for multiple comparisons. There are several explanations on how mitochondrial dysfunction impacts growth. Mitochondrial dysfunction can adversely affect fetal and placental growth (Wakefield et al., 2011). At birth, infants with mitochondrial disorders are significantly lighter than their healthy comparison groups (von Kleist-Retzow et al., 2003, Tavares et al., 2013, Feeney et al., 2019). Furthermore, neuromuscular manifestations of mitochondrial dysfunction affect skeletal integrity and longitudinal bone growth. Mice with limbs that had been paralysed limb had significantly shorter lengths, suggesting that muscle movements stimulate chondrocytes in the growth plates (Killion et al., 2017). Finally, growth during early childhood is also influenced by the interaction of many factors that include nutrition and the co-existence of chronic disease. In children with Leigh syndrome, poor growth is also influenced by the lack of mobility or feeding difficulties secondary to their extensive neurological manifestations.

Another interesting finding from this study is the association of caudate involvement with disease burden, progression and mortality in these children with Leigh syndrome. The majority of patients in this study have striatal changes (putamen and caudate) within the basal ganglia and this is similarly reported in the literature.(Stenton et al., Arii and Tanabe, 2000, Alves et al., 2020) I have also demonstrated in this cohort of children that some neuro-radiological lesions are detected together more often than might be expected by chance. In particular, the close association of putamen and caudate nuclei lesions is not surprising given the anatomic and functional integration of these two structures (Kumral et al., 1999, Siddiqui and Riley, 2014). In a *Ndufs4* knockout mouse model of Leigh syndrome and complex I deficiency, the selective inactivation of striatal neurons led to progressive motor impairment (Chen et al., 2017).

In human studies, caudate lesions are often associated with neurobehavioral changes (Mendez et al., 1989, Levy and Dubois, 2005). These changes are usually noted with progressive cognitive decline (Grahn et al., 2008) but this phenomenon in children remains unclear. It is not possible to postulate from the findings of this study how the

caudate nuclei lesions disrupt the neural network to other cerebral structures in Leigh syndrome but this link is worth exploring in future histopathological studies. Alternatively, the presence of caudate lesions could partly be a result of an advanced stage of the disease process in Leigh syndrome. The caudate lesions in this cohort of children with Leigh syndrome are not seen in isolation but in conjunction with other accompanying symmetrical lesions in the basal ganglia or brain stem.

The significant association of brainstem lesions with mortality in Leigh syndrome shown in this study has also supported a similar observation by Sofou et al. (2014). The brainstem, the caudal portion of the brain that connects the diencephalon to the spinal cord and the cerebellum, has three main parts, namely the medulla, pons and midbrain (Hurley et al., 2010). Dysfunction in these parts can lead to impairment of consciousness, cardiorespiratory failure, and increased mortality (Sharshar et al., 2003, Sharshar et al., 2011, Mazeraud et al., 2016). In the mouse model of Leigh syndrome, Quintana et al. (2012) reported bilateral lesions in the dorsal brainstem, one of the principal sites of neuronal gliosis, of the most severely affected mice. These mice developed progressively worsening of breathing difficulties before their premature death but their life span could be prolonged by restoring the selective inactivation of *Ndfus4* in the brainstem (Quintana et al., 2012). Despite this observation in the mouse model, coupled with clinical observations from this study and Sofou et al. (2014), there are no post-mortem histopathological studies in children with Leigh syndrome of these lesions to determine the exact mechanisms or disease process.

Children with Leigh syndrome due to the pathogenic variants in *SURF1* in this cohort have poorer outcomes when followed up longitudinally. The *SURF1* gene encodes for an assembly factor of mitochondrial cytochrome *c* oxidase (COX) (Zhu et al., 1998). Children who harbour pathogenic variants in the *SURF1* gene have rapid disease progression and high mortality as compared to other genotypes. However, this poor prognosis is in contrast to the published cross-sectional study on SURF1 deficiency (Wedatilake et al., 2013). Whilst the median age of death of participants with pathogenic *SURF1* variants is lower, I cannot draw any consequential parallels because the previously published cohort have different inclusion criteria and included patients who survived into adulthood. In this smaller group of *SURF1*-related Leigh

syndrome patients, the pathogenic, homozygous c.792_793del variant was found in two unrelated patients who were deceased. The other two deceased unrelated children with pathogenic *SURF1* variants were heterozygous for a common c.312_321del10insAT (p.Pro104_Leu105ins*) variant with another pathogenic variant on the other allele.

My intention to conduct a longitudinal study of Leigh syndrome in a way that is suitable in paediatric outpatient settings has led to some limitations. First, there is the use of only two time points. For some children with Leigh syndrome, this approach did not capture a stepwise deterioration, fluctuating course of disease burden in patients encountering metabolic crisis or impacts of pharmacological treatments for common symptoms such as dystonia and constipation, and erroneously abbreviated their disease course to a linear decline. The interval between the time points was also not standardised. Second, there is a lack of matched controls to compare the disease trajectory of Leigh syndrome. There is a lack of longitudinal data on a sufficient number of children with other forms of mitochondrial disease to draw any comparisons. As discussed as a limitation in Chapter 3, I have also observed a ceiling effect from the ordinal scoring of certain items in the NPMDS which can substantially underestimate the progression of Leigh syndrome when these children lose ambulation. Having outcome measures that incorporate upper limb functions in future studies, instead of NPMDS only, could address this and reduce bias. Finally, the variations in disease progression across genotypes have also highlighted the importance of focussing on one genotype in longitudinal research to avoid misinterpretation of findings from a sample that only shares similar phenotypes.

In summary, this study have established objective information on the disease progression of Leigh syndrome caused by different genotypes. Furthermore, I have identified several predictors of disease trajectory in this devastating neurodegenerative condition. To strengthen these findings further and to explore the natural history of Leigh syndrome in more detail, there is a need for large-scale international collaborative studies that recruit more patients, especially those with rarer genotypes, to explore a range of sensitive outcome measures alongside NPMDS in a longitudinal assessment of these patients.

Chapter 5: Exploring clinician-rated outcome measure, performance outcome measures and functional tests in autosomal recessive *RRM2B*-related disorders

5.1 Introduction

Chapter 3 and 4 have utilised the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) as a clinician-reported outcome measure in cohorts of children with Leigh syndrome and other forms of mitochondrial disease. Another version of this clinician-reported outcome measure for adult mitochondrial patients exists in the form of the Newcastle Mitochondrial Disease Adult Scale (NMDAS) (Schaefer et al., 2006). Akin to the NPMDS, the NMDAS has been widely used in several studies to understand disease burden and progression (Grady et al., 2014, Ng et al., 2016b, Pickett et al., 2018, Rocha et al., 2018). Beyond these two clinician-reported outcome measures, there are other proposed measures to study the natural history of mitochondrial disease and the impact of treatment on the disease trajectory. As a plethora of emerging pharmacological and non-pharmacological therapies are being developed to treat mitochondrial disease, an international consortium on Clinical Trial Readiness in Mitochondrial Myopathies had recommended a consensus set of outcome measures (Mancuso et al., 2017). This Chapter explores the use of these proposed outcome measures, which include clinician-reported outcome measures, performance outcome measures and functional tests, in a group of rare mitochondrial disorders caused by autosomal recessive, pathogenic variants in the *RRM2B* gene.

Pathogenic variants in the *RRM2B* gene can lead to mitochondrial DNA maintenance defects, and they are an important cause of adult-onset and childhood-onset mitochondrial disorders. To date, 78 individuals from 52 families with molecularly confirmed pathogenic variants *RRM2B* have been reported (Pitceathly et al., 2012, Lim et al., 2021). There are several phenotypes of *RRM2B*-related mitochondrial disease. Chapter 1 (Introduction) has detailed the clinical characteristics, diagnosis, molecular genetics, and management of this *RRM2B*-related condition. This Chapter discusses the late-onset myopathic phenotype caused by biallelic *RRM2B* pathogenic variants. Unlike the devastating encephalomyopathy phenotype in infancy (Keshavan et al., 2020), this group of patients experienced a later disease onset and survived to adulthood; thus, allowing recruitment into clinical studies and investigation of outcome measures.

The most pragmatic and feasible outcome measures were chosen from the consensus set by Mancuso et al. (2017) to reflect some of the inherent phenotypes in patients with *RRM2B*-related disorders. Respiratory muscle weakness is commonly reported in these patients who had myopathies (Bourdon et al., 2007, Bornstein et al., 2008, Kollberg et al., 2009, Keshavan et al., 2020). The primary mechanism for respiratory muscle weakness in most myopathies is driven by an insufficient performance of the diaphragm, intercostal muscles and/or accessory muscles (Pfeffer et al., 2015). Symptoms can begin insidiously and present alongside tiredness or fatigue. Some patients might complain of shortness of breath, sleepiness, or morning headache (Bourke and Gibson, 2002, Laghi and Tobin, 2003). Spirometry testing of myopathic patients characteristically reveals lower than predicted forced vital capacity (FVC) and normal ratio with the forced expiratory volume in 1 second (FEV1/FVC) (Ambrosino et al., 2009). Other validated non-invasive measurements of respiratory muscle strength include the sniff nasal inspiratory pressure (SNIP), maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP) (Wilson et al., 1984, Heritier et al., 1994, Uldry and Fitting, 1995, Schoser et al., 2017, Laveneziana et al., 2019). Despite being widely used in neuromuscular diseases, these measurements had not been extensively investigated in mitochondrial patients, especially those with pathogenic variants in the *RRM2B* gene.

In addition to respiratory weakness, swallowing difficulties secondary to bulbar dysfunction in patients with pathogenic variants in the *RRM2B* gene had also been highlighted by previous publications (Fratter et al., 2011, Pitceathly et al., 2012). The reported clinical problem related to oropharyngeal muscle weakness is comparable to patients with other forms of myopathies who have a spectrum of swallowing difficulties, ranging from slow eating, difficulty in chewing to recurrent aspirations (Argov and de Visser, 2021). Coupled with the coexisting gastrointestinal problems in mitochondrial diseases, swallowing difficulties can also precipitate the risk of malnutrition and low body mass index (de Laat et al., 2015). Prior to becoming clinically symptomatic, impaired swallowing might be detected by numerous bedside methods. Nevertheless, the evidence for these methods had been considered to be only at a ‘fair’ level in a meta-analysis by Audag et al. (2019), further perpetuating the lack of consensus in bedside testing or screening for swallowing difficulties. For primary mitochondrial

myopathies, Mancuso et al. (2017) reached an international consensus on using the timed water swallow as one of the functional tests to assess swallowing. Thus, this Chapter examined the utility of this bedside water swallow test in patients with autosomal recessive *RRM2B* variants.

The international workshop recommended other functional tests were the 6-minute walk test (6MWT) and sit-to-stand tests (Mancuso et al., 2017). The 6MWT was initially developed by the American Thoracic Society (2002a) as a submaximal exercise test to assess aerobic capacity and endurance. It has since become a widely used outcome measure in neuromuscular clinical research because of its sensitivity to fatigue-related changes (Montes et al., 2010, Tveter et al., 2014, Dunaway Young et al., 2016). Meanwhile, the sit-to-stand test was developed to provide a reliable indicator for leg strength in active older adults (Jones et al., 1999). Newman et al. (2015) had evaluated these timed walk and sit-to-stand tests in a group of patients with m.3243A>G-related mitochondrial disease. However, no study in the literature had applied these tests in *RRM2B*-related mitochondrial disease. Given the mtDNA depletion or multiple deletions in the muscle of these patients with pathogenic *RRM2B* variants, myopathy was a prominent manifestation. (Pitceathly et al., 2012, Lim et al., 2021). Thus, these functional tests might identify the deficits of muscle strength and endurance in the lower limbs.

Apart from the tests mentioned earlier for lower limb function, Mancuso et al. (2017) also proposed a performance outcome measure for the upper extremities, namely the nine-hole peg test. The nine-hole peg test was originally created in 1971 as a measure of hand and finger dexterity. It has since been considered a standard research metric in various neurological disorders (Kellor et al., 1971, Mathiowetz et al., 1985, Earhart et al., 2011, Feys et al., 2017, Cutellè et al., 2018). Upper limb dexterity is key in carrying out activities of daily living. Coincidentally, the NMDAS has items in Section 1 (Current Function) that rate activity of daily living such as handwriting, utensil use, dressing and personal hygiene. Therefore, the nine-hole peg test not only quantifies the upper limb performance of these myopathic patients with autosomal recessive *RRM2B* mitochondrial disorders but also could be correlated with their respective scores in Section 1 of the NMDAS.

These tests that interrogate the function of upper and lower extremities could be impacted by the proximal myopathy experienced by these patients with autosomal recessive, pathogenic variants in the *RRM2B* gene. Therefore, quantitative dynamometry for the muscle strength, which is also part of the recommended measures by Mancuso and colleagues, is the other performance outcome measure for these patients in this Chapter. The quantitative dynamometry testing of muscle groups is a sensitive tool to measure strength in specific muscle groups (Drouin et al., 2004, Stark et al., 2011, Barden et al., 2012). Although the dynamometry measurements are highly dependent on the operator, a moderate level of reliability was observed using the handheld dynamometer, coupled with standardised protocol and instructions for patients (Buckinx et al., 2017). Measuring each muscle group in the upper and lower limbs could potentially discern the degree and the patterns of muscle weakness in patients with *RRM2B* related mitochondrial diseases.

In essence, all these outcome measures by Mancuso et al. (2017) have been selected to capture the key clinical characteristics of this autosomal recessive *RRM2B*-related disorder, in particular, the respiratory muscle weakness, exercise tolerance, limb function, swallowing function. Whether these proposed outcome measures agreed in a workshop are feasible in practice remains to be seen. Recently, Montano and colleagues have applied some of these outcome measures in an Italian cohort of patients with primary mitochondrial myopathy (Montano et al., 2020). However, to date, no published studies have applied these outcome measures in those who harboured the biallelic pathogenic variants in the *RRM2B* gene. It is also of interest to know how different the results of these outcome measures are from normative values and how they correlate with each other in this rare disorder. Another unexplored territory for mitochondrial research is obtaining longitudinal data for this genotype through the natural history study in the absence of any intervention. By understanding how these outcome measures evolve over time, only then can clinicians and researchers appreciate the clinically meaningful change if a novel intervention was to be developed and introduced in the future. Molecular bypass therapy in the form of deoxythymidine and deoxycytidine has demonstrated clinical efficacy in autosomal recessive *TK2* variants (Garone et al., 2014, Lopez-Gomez et al., 2017, Domínguez-González et al., 2019), which cause depletion and multiple deletions of mtDNA, similar

to those caused by autosomal recessive *RRM2B* variants. The therapies had been administered to sixteen participants on compassionate use in that intervention study without a control arm. Without a robust natural history study, it remains unclear if the reported improvements in disease severity or function were robust. With the potential translation of molecular bypass therapy from those used in *TK2*-deficient patients on the horizon, it is more imperative than ever to understand the natural history of autosomal recessive *RRM2B* variants.

5.2 Specific aims

1. To assess the disease severity in rare autosomal recessive *RRM2B*-related mitochondrial disorder using the clinician-reported outcome measure, Newcastle Mitochondrial Disease Scale (NMDAS)
2. To measure the respiratory muscle function, exercise tolerance, limb function, swallowing function in this group of disorders the using a set of outcome measurements, which includes the following:
    a. Performance outcome measures
        i. Spirometry
        ii. Sniff nasal inspiratory pressure
        iii. Nine-hole peg test
        iv. Quantitative muscle dynamometry
    b. Functional tests
        i. Six-minute walk test
        ii. Sit-to-stand test
        iii. Timed water swallow test
3. To explore the correlation of these performance outcome measures and functional tests with the clinician-reported outcome measure.
4. To assess these clinician-reported, performance outcome measures and functional tests longitudinally.

5.3 5.3 Methods

5.3.1 Study design

This cross-sectional study assessed a group of participants who had autosomal recessive *RRM2B* variants at the research sites or at their home environment. These participants were part of the Prospective Observational Study of Patients with Mitochondrial Depletion Syndrome *RRM2B* (PROSPER2B) (REC: 18/WM/0354). All participants consented to this study, which has a favourable opinion from the independent research ethics committee, NRES Committee West Midland Solihull. To recruit participants with this rare condition, this study has permission to screen patients in the largest UK database for mitochondrial disorders, the Mitochondrial Disease Patient Cohort (MitoCohort) UK: A Natural History Study and Patient Registry. (REC: 13/NE/0326). The PROSPER2B study was sponsored by the Newcastle upon Tyne Hospitals NHS Foundation Trust and was funded by the patient charity, Lily Foundation, UK and the Australian Mitochondrial Disease Foundation (AMDF).

Before recruitment, I screened the UK MitoCohort for potential participants who fulfilled the eligibility criteria. Participants in the UK MitoCohort had consented to be contacted for research studies. Therefore, all eligible participants had been given the opportunity to take part in this study. In addition to sending the eligible patients the study information sheets, I also discussed the aims of the study and the methodology of data collection with them. All participants understood the requirements of this study, but they are free to withdraw from the study without affecting their care and to decline the outcome measures that they feel are not appropriate.

After these participants had consented to participate, I carried out the study assessments at the Clinical Ageing Research Unit (CARU) and at the Campus of Ageing and Vitality (CAV) in Newcastle. Participants who were unable to travel to these research sites had the opportunity to be assessed at their home environment. The research team and I have travelled to the home of some of these patients with the relevant study equipment. There are only a certain number of assessments that can be performed in-home settings because of space limitations. Participants who attended the research site in Newcastle were able to undertake the six-minute walk test that required predefined distances in a gait laboratory.

5.3.2 Study window

I collected the research data in this Chapter from 17 June 2019. The baseline assessments for all participants were completed by 16 December 2019. All participants had their follow-up and end-of-study assessments completed by 14 June 2021.

5.3.3 Participants

All patients recruited to the study fulfilled the diagnostic criteria for autosomal recessive *RRM2B*-related mitochondrial disease. The inclusion and exclusion criteria have been outlined in Chapter 2 (General Methods).

5.3.4 Characteristics and medical background

I reviewed the medical notes of the participants. During their study assessments, I revisited their background medical history and their ongoing medical symptoms. The basic demographics, including sex, weight, height and BMI were noted. I also confirmed the pathogenicity of the variants in their individual genetic results.

5.3.5 Genetic findings

The NHS Highly Specialised Service UKAS-accredited diagnostic laboratory in Newcastle upon Tyne Hospitals Foundation Trust provided the molecular genetic diagnoses for all the study participants. The *RRM2B* nuclear genetic variants were identified using either candidate gene approaches (Sanger sequencing) or next-generation sequencing, including both targeted panel and/or whole-exome sequencing (Taylor et al., 2014, Alston et al., 2016). The diagnostic testing strategies for mitochondrial clinical service have been published (Alston et al., 2017).

5.3.6 Specific outcome measures

These outcome measures have been detailed in Chapter 2 (General Methods). I have highlighted the outcome measures used in this Chapter and summarised them as follows:

1. Clinician-reported outcome measures
    a. Newcastle Mitochondrial Disease Scale (NMDAS)

    NMDAS has been designed to assess patients with mitochondrial disease clinically (Schaefer et al., 2006). It is a scale to evaluate the multisystem involvement, determine disease burden, and understand the natural history of adult mitochondrial disease. The scale has three sections, namely Current Function, System Specific Involvement and Current Clinical Assessment (Table 5-1). Each section contains questions that pertain to specific clinical problems and each question of the scale can be scored from a 0 to 5 in the order of increasing severity.

Current function	System-specific involvement	Current clinical assessment
• Vision • Hearing • Speech • Swallowing • Handwriting • Cutting food and handling utensils • Dressing • Hygiene • Exercise tolerance • Gait stability	• Psychiatry • Migraine • Seizures • Stroke-like episodes • Encephalopathic episodes • Gastrointestinal symptoms • Diabetes mellitus • Respiratory muscle weakness • Cardiovascular system	• Visual acuity • Ptosis • Progressive external ophthalmoplegia • Dysphonia / Dysarthria • Myopathy • Cerebellar ataxia • Neuropathy • Pyramidal involvement • Extrapyramidal involvement • Cognition

Table 5-1: Table shows all the individual items within the three sections in the NMDAS

2. Performance outcome measure

    a. Spirometry

    The device used for this study is the portable MicroLab® 3500 Spirometer with SPC Software which is compliant with the American Thoracic Society and European Respiratory Society standards. Output of interest from this spirometer was the Forced Vital Capacity (FVC) and Forced Expiratory Volume in one second (FEV_1), expressed in litres (L). All participants are given three attempts but only the best results are used. The predicted FVC and FEV_1 percentage were calculated from other variables including age, sex and height, based on the ERS official statement and reference ranges (Quanjer et al., 2012).

    b. Sniff nasal inspiratory pressure

    The MicroMedical® MicroRPM (Respiratory Pressure Meter) is a handheld device that provides measurements for sniff nasal inspiratory pressure (SNIP) via the adapted nasal probes. It can also measure the maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP) at the mouth using the flanged rubber mouthpiece via the inspiratory or the expiratory pressure valves, respectively. Like the spirometry, participants are also given three attempts to obtain the maximal pressures. Output values for SNIP, MIP and MEP are expressed in cm H_2O. The maximal SNIP values for healthy adults can be predicted from the following equation (Uldry and Fitting, 1995):

    SNIP (cm H_2O) for male = 126.8 – 0.424 x Age in years

    SNIP (cm H_2O) for female = 94.9 – 0.22 x Age in years

    The maximal MIP and MEP values can also be predicted from the following equation proposed by Wilson (1984), as follows:

    MIP (cm H_2O) for male = 142 - (1-03 x Age in years)

    MIP (cm H_2O) for female = -43 + (0-71 x Height in cm)

    MEP (cm H_2O) for male = 180 - (0-91 x Age in years)

    MEP (cm H_2O) for female = 3.5 + (0.55 x Height in cm)

c. Nine-hole peg test

The nine-hole peg test is a hand function test that focuses on fine manual dexterity, and it is frequently used in clinical neuroscience research. The current commercially available nine-hole peg test consists of a plastic console with a shallow round dish on one end and nine peg holes on the other side (Grice et al., 2003). Along with a brief demonstration, the participants were given standardised instructions and followed the established protocol (Mathiowetz et al., 1985, Grice et al., 2003). A stopwatch was used to time this test from the moment the first peg was picked until the last peg had been returned to the dish. In the current version of the console, Grice et al. (2003) produced the average time taken for this test and its respective standard deviations of healthy participants based on their age groups.

d. Quantitative dynamometry for muscle strength

Handheld dynamometry is an objective way to quantify muscle force production compared to bedside manual muscle testing (Jackson et al., 2017). In this study, the device used for measuring muscle force in Newtons, the S.I. unit, was the MicroFET® 2 dynamometer by Hoggan Scientific LLC. This device measured the force generated by handgrip, elbow flexion, shoulder abduction, knee extension and hip flexion bilaterally. The normative values and ranges for these forces, in Newtons, are available for comparison depending on gender weight, age and dominant sides (Andrews et al., 1996, Steiber, 2016, Benfica et al., 2018).

3. Functional tests
    a. Six-minute walk test

    This is a sub-maximal exercise test developed to assess aerobic capacity and endurance. Participants who attended the research site in Newcastle walked a predefined circuit for six minutes, but participants who were assessed at home had not taken part in this test. The distance (metres) completed by the participants in this timed test is used as the outcome measure. Enright et al. (1998) established reference equations for predicting the total distance walked during six minutes (6MWT distance) for healthy adults as follows:

    Predicted 6MWT distance for male = (7.57 × height in cm) − (5.02 × age in years) − (1.76 × weight in kg) − 309 metres
    (subtract 153 metres for the Lower Limit of Normal)

    Predicted 6MWT distance for female = (2.11 × height in cm) − (5.78 × age in years) - (2.29 × weight in kg)+ 667 metres
    (subtract 139 metres for the Lower Limit of Normal)

    Walking speeds

    The walking speeds of these participants can be measured over 10-metres distances. Participants were asked to walk these distances at their self-selected pace and at their fastest pace. The reference values for these two walking speeds for adults are available in the literature (Bohannon, 2009, Bohannon and Andrews, 2011).

    b. Sit-to-stand test

    This 30-second sit-to-stand, also known as the chair stand test, was designed to test strength in lower limbs and endurance in adults (Rikli 1999). Participants were asked to stand from, and sit back down on, standard seats repeatedly within 30 seconds whilst crossing their arms and holding against their chest. An average number of complete stands

for those below 65 years ranged from 14 to 19 in men and 12 to 17 in women (Jones et al., 1999, Rikli and Jones, 1999).

c. Timed water swallow test

This 100ml water swallowing test is a sensitive indicator in identifying patients at risk of swallowing dysfunction when compared to videofluoroscopic examination of swallowing (Wu 2004). Participants were asked to drink 100ml of water as quickly as possible whilst being timed by a stopwatch. Their swallowing speeds (10ml/s) were determined by the amount of water drunk divided by the time elapsed. Swallowing speed of less than 10ml/s is an index of abnormal swallowing, and the swallowing speed of adults with no swallowing difficulties can be predicted from the following equation (Nathadwarawala et al., 1992):

Swallowing speed for male (m/s) = (-0.548 x Age in years) + 57.55
Swallowing speed for male (m/s) = (- 0.324 x Age in years) + 37.84

5.3.7 Statistical analysis

I used SPSS v.25 for statistical analyses. All the statistical methods have also been detailed in Chapter 2. Statistical tests were two-sided with significance set at $p<0.05$. For individual participants, their actual and predicted values were compared in pairs using the dependent t-tests or paired-samples t-test. I calculated the Spearman rank-order correlation coefficient to determine the relationships between the outcome measures (Spearman, 2010). Kendall's Tau-b is the alternative to Spearman's correlation in cases where a monotonic relationship between two outcome measures do not exist on scatterplots. To determine whether baseline NMDAS scores (ordinal variable) differed significantly between the two-time points of this same group of participants, I used the Wilcoxon signed-rank test (Wilcoxon, 1947). Paired T-tests were used to interrogate the differences of other outcome measures (continuous variable), namely spirometry percentages, sniff nasal inspiratory pressure, time taken to complete nine-hole peg test, quantitative muscle dynamometry, distance covered in six-minute walk test and time taken to complete water swallow test.

5.4 Results

I identified 13 patients who had autosomal recessive *RRM2B*-related mitochondrial disorders from the UK MitoCohort (Figure 5-1). One patient was deceased. Of the remaining twelve patients, two of them declined to take part in this study. Five of the participants who consented to take part were unable to travel to the research site in Newcastle but agreed for home visits. Thus, these participants were assessed at their homes. These participants who were assessed at home could not undertake functional tests that require gait laboratory facilities.

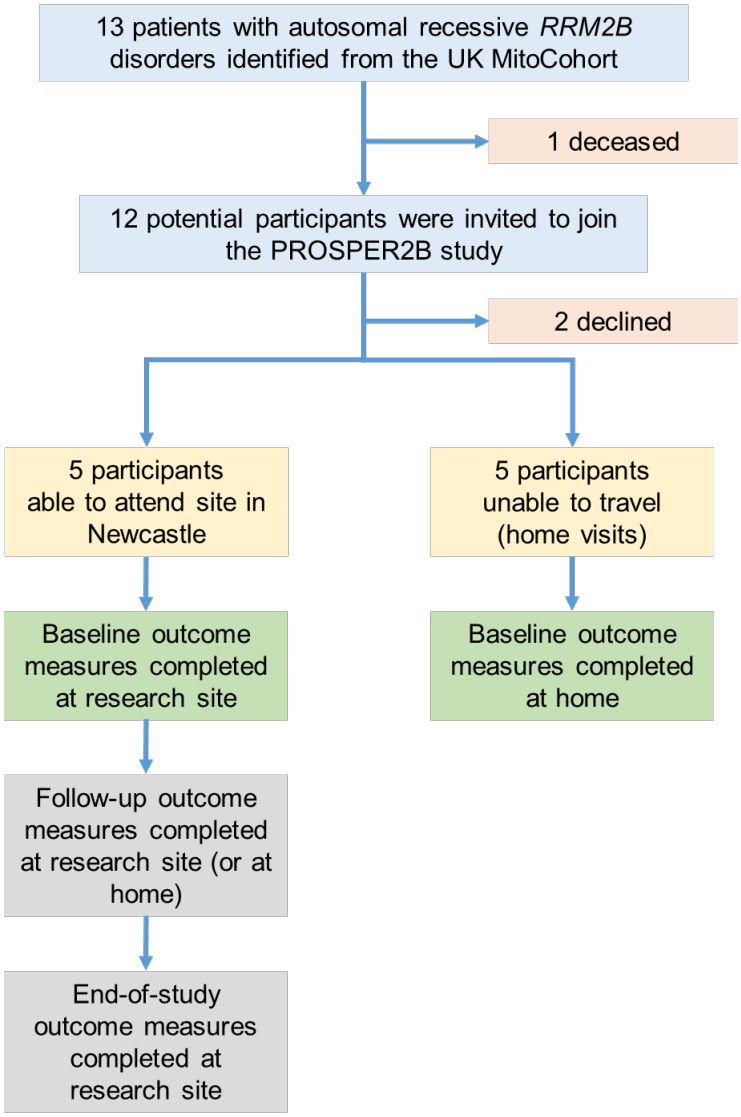


Figure 5-1: Flow chart shows the number of patients who were screened from the UK MitoCohort, invited to join the study, consented, completed the assessments, and were included in the analysis. Ten participants completed the baseline assessments. Five of them had further follow-up and end-of-study assessments.

5.4.1 Characteristics

Ten participants, four males and six females, from six family pedigrees, took part in the study at the mean age of 33 years (SD 13.1; 95% CI 23.6-42.4) (Table 5-2). The age of the oldest and the youngest participant was 51.9 years and 17.2 years, respectively. The mean age of disease onset was 11.8 years (SD 9.2; 95% CI 5.2-18.4). Half (P1, P2, P3, P4 and P5) had pre-pubertal age of disease onset (range 0.9 years to 8.5 years). On the other hand, one participant, P7, first presented with severe sensorineural hearing loss in infancy. Four remaining participants (P6, P8, P9 and P10) had disease onset during adolescence (range 15.6 years to 17.0 years). The average age at which these participants had confirmed genetic diagnosis of *RRM2B*-related mitochondrial disease was 27.4 years (SD 13.5; 95% CI 17.7-37.2)

5.4.2 Clinical features

From their medical records, the commonly documented clinical features are summarised in Table 5-1. Eyelid ptosis and chronic progressive external ophthalmoplegia were universally present in all participants. All participants had complained of sensorineural hearing loss except for P8. The hearing loss for five of them (P1, P2, P4, P5 and P9) had subsequently progressed to the stage that they required cochlear implants. The first symptom experienced by five participants (P1, P3, P4, P5 and P6) was hearing loss. Two participants (P9 and P10) had ptosis as their first symptoms in adolescence. Whilst P2 had faltering growth in childhood, P7 had a low body-mass index (BMI) in adolescence, and P8 had symptoms of myopathy in the third decade as their first presenting complaints, respectively. Most of the participants described some degrees of proximal myopathy or muscle weakness in their clinical courses except for one of the participants (P3). However, P3 had complained of fatigue or excessive tiredness, similarly to the patients who had symptoms of myopathy. These symptoms had reportedly impacted their activities of daily living. Another common symptom that these participants (P1, P2, P4, P5, P6, P7, P8 and P9) had raised in their previous clinical appointments was dysphagia. Six of the participants (P1, P2, P3, P5, P7 and P10) had low BMI (BMI <18.5) at their clinical appointments. All participants in this study had previous sleep studies and seven of them have non-invasive ventilatory support at night.

	P1	P2*	P3*	P4	P5	P6	P7^	P8^	P9^	P10^
Sex	F	F	M	F	F	M	F	M	M	F
Pathogenic variants	c.362G>A p.(Arg121His) and c.1037C>T p.(Thr346Ile) novel	c.817G>A p.(Gly273Ser) and c.181G>A p.(Ala61Thr)	c.817G>A p.(Gly273Ser) and c.181G>A p.(Ala61Thr)	c.686G>T p.(Gly229Val) and c.482C>T p.(Thr161Ile)	c.404C>G p.(Ser135*) and c.998G>A p.(Arg333His)	c.431C>T, p.(Thr144Ile) and c.946T>G, p.(Ser316Ala)	c.122G>A, p.(Arg41Gln) and c.817G>A, p.(Gly273Ser)	c.122G>A, p.(Arg41Gln) and c.817G>A, p.(Gly273Ser)	c.122G>A, p.(Arg41Gln) and c.817G>A, p.(Gly273Ser)	c.122G>A, p.(Arg41Gln) and c.817G>A, p.(Gly273Ser)
Age of onset (years)	2.7	2.3	8.5	7.4	0.9	17.0	16.7	30.4	16.7	15.6
Age of diagnosis (years)	14.6	15.3	11.5	31.7	11.7	47.0	30.8	46.5	31.8	34.0
Current age (years)	17.2	23.7	18.0	35.9	18.4	51.9	37.7	51.4	36.6	39.6
Weight (kg)	27	46	58	67	40.1	64	35	63	63	44
Height (cm)	1.54	1.71	1.79	1.66	1.52	1.78	1.58	1.65	1.66	1.62
BMI (kg/m2)	11.4	15.7	18.1	24.3	17.4	20.2	14.0	23.1	22.8	16.8
Ptosis	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Ophthalmoplegia	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Hearing loss	✓	✓	✓	✓	✓	✓	✓		✓	✓
Cochlear implant	✓	✓		✓	✓				✓	
Myopathy	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Fatigue	✓	✓	✓	✓	✓		✓	✓	✓	✓
Dysphagia	✓	✓		✓	✓	✓	✓	✓	✓	
Low BMI	✓	✓	✓		✓		✓			✓
Additional features		TPN Hyperthyroidism		Sleep apnoea				Asthma	Learning difficulties	Hypothyroidism

Table 5-2: A summary of the characteristics for all participants in this study, including the pathogenic variants in the RRM2B gene, age of disease onset, age of molecular genetic diagnosis, age at the time of enrolment to study, weight, height and the common clinical features documented by clinicians in their medical notes. F=female ; M=male ; TPN=total parenteral nutrition

5.4.3 Pathogenic variants

All participants harboured autosomal recessive pathogenic variants in the *RRM2B* genes (NM_001172477.1), and their variants in their respective exons are summarised in Figure 5-2.

Of six pedigrees in this group of participants, P2 and P3 were siblings with non-consanguineous parents. P7, P8, P9 and P10 shared the same pedigree. P2 and P3 had two pathogenic variants, the c.817G>A p.(Gly273Ser) and the c.181G>A p.(Ala61Thr). These had been associated with recessive CPEO presentations in early adulthood (Fratter et al., 2011). Sequential COX/SDH histochemistry evidenced their significant mitochondrial dysfunction with 34% COX-deficient and 5% ragged-red fibres. The long-range PCR of their muscle DNA sample demonstrated multiple mtDNA rearrangements. The other family pedigree of P7, P8, P9 and P10 also had the same variants. They harboured two heterozygous pathogenic variants in each of them, the pathogenic c.817G>A p.(Gly273Ser) and c.122G>A p.(Arg41Gln) that had also previously been reported (Pitceathly et al., 2012).

The other participants in this cohort also have extensive laboratory work and published literature to substantiate the pathogenicity of their respective variants. P1 harboured two heterozygous *RRM2B* variants, the pathogenic c.362G>A p.(Arg121His) variant and the c.1037C>T p.(Thr346Ile) variant. The variant had been described in a case of autosomal recessive MNGIE-like presentation with mtDNA depletion (Shaibani et al., 2009). The muscle biopsy findings of P1 revealed 25% COX-deficient fibres and ragged-red subsarcolemmal accumulations. The long-range PCR of her muscle also showed multiple mtDNA rearrangements. P4 also had a significantly large number of COX-deficient fibres on Southern blotting multiple mtDNA rearrangements on long-range PCR. She was heterozygous for the pathogenic c.686G>T p.(Gly229Val) variant and likely pathogenic c.482C>T p.(Thr161Ile) variant that had previously been reported (Kollberg et al., 2009, Pronicka et al., 2016).

Although P5 had no mtDNA rearrangement on long-range PCR, she had evidence of marked mtDNA depletion in her skeletal muscle along with evidence of multiple respiratory chain deficiency involving complexes I, III and IV. Her ratio of *MTND1* (mtDNA) to 18S rDNA (nuclear) using the real-time PCR assay was 5.02 (normal control range 9.00-40.00). The sequencing of her *RRM2B* gene uncovered compound heterozygous variants c.404C>G p.(Ser135*) and c.998G>A p.(Arg333His) that had been classified as pathogenic and likely pathogenic according to the ACGS guidelines (Ellard et al., 2020). The *RRM2B* gene sequencing of P6, who had clinical features and affected siblings, showed that he was heterozygous for the c.431C>T p.(Thr144Ile) and c.946T>G p.(Ser316Ala) pathogenic variants. The pathogenicity of c.431C>T p.(Thr144Ile) had also previously been reported (Fratter et al., 2011). The c.946T>G p.(Ser316Ala) variant was classed as a likely pathogenic variants given that his affected siblings shared similar clinical symptoms consistent with *RRM2B*-related disease in the presence of both variants. The two participants who did not join this study were the siblings of P6.

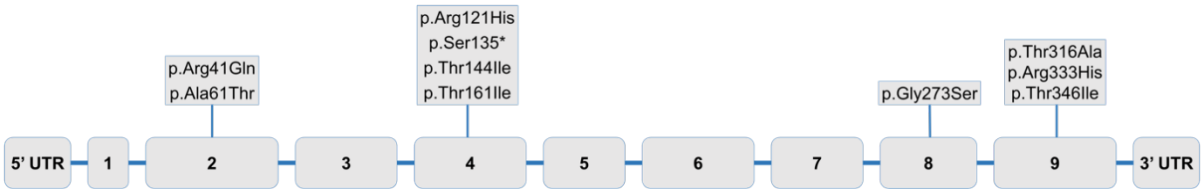


Figure 5-2: Schematic representation of the RRM2B gene structure (NM_001172477.1) illustrating the pathogenic variants found in this cohort of participants. Coding exons are numbered 1 to 9.

5.4.4 Newcastle Mitochondrial Disease Adult Scale (NMDAS)

All ten participants completed the NMDAS assessments. The mean total NMDAS scores for all these participants was 53.9 (SD 12.5; 95%CI 45.0-62.8). The results of these scores for individual items of the NMDAS are outlined in Figure 5-3.

Section I – Current Function

In the first section of NMDAS (Current Function), all the participants had some degree of exercise intolerance. Four participants (P2, P4, P7 and P8) had the most severe degree of which they could only walk less than 25 metres on the flat and were unable to do the stairs independently in the 4 weeks preceding the assessment. All functions related to activities of daily living, such as handling utensils, dressing, hygiene and gait, had been affected to varying degrees. The other notable function in this section that had considerably been affected was hearing. Corresponding to the findings of their medical notes, five participants had the most severe score which denotes having cochlear implants. Some of these participants also reported various levels of difficulties with their speech and swallowing function. Of note, P10 had required a communication aid because of speech problems and P4 had required gastrostomy enteral feeding because of swallowing difficulties.

Section II – System Specific Involvement

The second section of NMDAS interrogates system-specific involvement in the preceding twelve months before the assessment. Of all the items in this section, respiratory muscle weakness reported in all participants was the most prominent (Figure 5-3). Seven of the participants who had forced vital capacity (FVC) less than 45% scored the worst level of respiratory muscle weakness. The other two noticeable system involvements were the gastrointestinal system and psychiatric health. Eight of the ten participants had some bowel symptoms, ranging from mild constipation to severe dysmotility requiring surgical procedures. Meanwhile, seven participants had complained of mild to severe mental health symptoms. In contrast to these three affected systems, the other features related to central nervous systems, such as seizures, stroke-like episodes, migraine, encephalopathy, were mostly absent in these patients with *RRM2B*-related mitochondrial disorders.

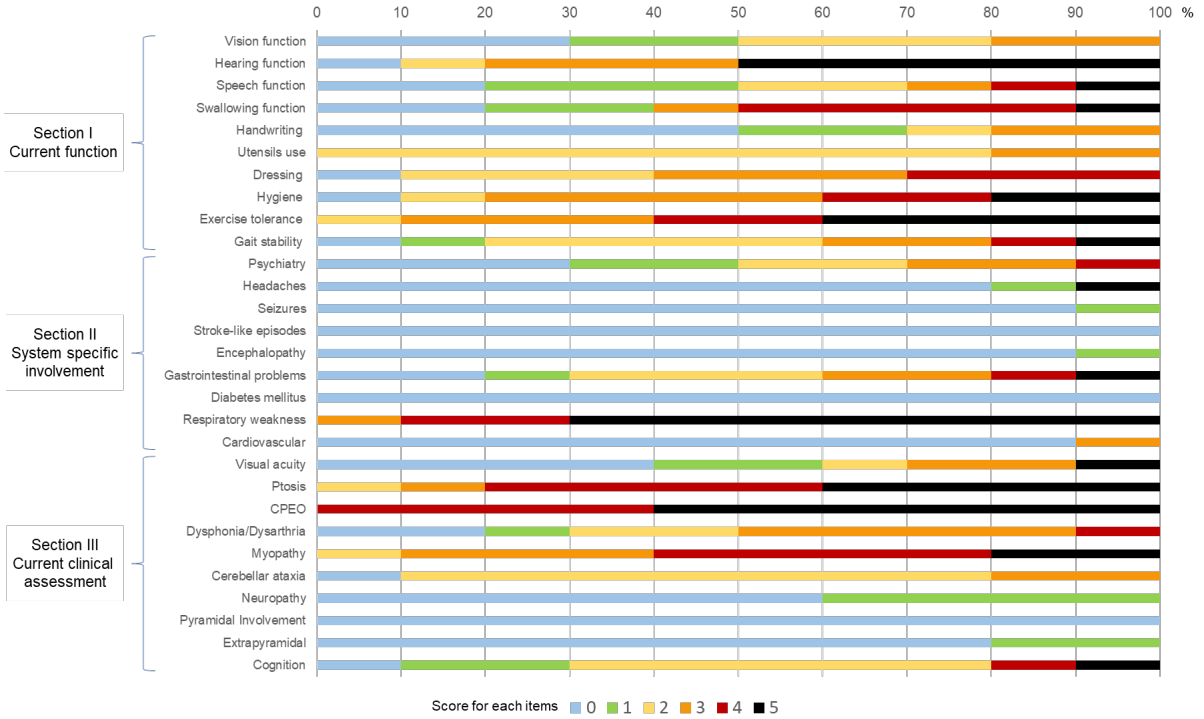


Figure 5-3: Bar chart shows the percentages of participants' scores in each item within the three sections (Current Function, System-specific Involvement, Current Clinical Assessment) of the Newcastle Mitochondrial Disease Adult Scale (NMDAS)

Section III – Current Clinical Assessment

The clinical assessment section rates the status of each medical examination findings at the time of NMDAS scoring. As highlighted in their medical background, all participants had chronic progressive external ophthalmoplegia (CPEO) and eyelid ptosis. Six of the participants (P1, P4, P7, P8, P9 and P10) had complete CPEO, typified by minimal adduction or only a flicker of eye movement on the worst side. The remaining four participants had abduction of the worst eye below 30% of normal at bedside examination. The worst degree of bilateral eyelid ptosis, which obscured more than two-thirds of pupils, was found in four participants (P4, P5, P6, P10), and this was followed by bilateral ptosis obscuring more than a third of pupils in the other four participants (P2, P3, P7 and P9). P1 and P8 had unilateral ptosis and bilateral ptosis, obscuring less than a third of the pupils.

On examination, all participants had signs of proximal myopathy. Two participants (P2 and P4) had scored with the worst ratings with wheelchair dependence primarily due to proximal muscle weakness. Four participants (P6, P8, P9 and P10) had waddling gait and were unable to rise from 90-degree squats unaided. Three participants (P1, P3 and P7) had a moderate proximal weakness (MRC scale 4-/5) with some difficulty rising from 90-degree squats. Although P5 had not specifically complained of proximal muscle weakness as a problem in the medical notes, this participant had clear weakness in the hip and shoulder (MRC scale 4/5) on examination.

Other NMDAS examination findings of note include signs of reduced visual acuity, dysphonia or dysarthria, ataxia, and cognitive difficulties. These examination findings had been noted in these participants to varying severity, but they were not as pronounced as the aforementioned myopathy, ptosis and CPEO in these participants. In keeping with the lack of central nervous system involvement in Section II, there were relatively few signs of pyramidal, extrapyramidal or neuropathy.

5.4.5 Correlation of NMDAS

A Spearman's rank-order correlation was run to determine the relationships among items in the NMDAS, with the resulting coefficients illustrated in a matrix (Figure 5-4). Some of the items in the NMDAS which assessed similar aspects of a particular clinical feature correlated strongly and positively. There was a strong and positive correlation between the reported speech difficulties in Section I (Current Function) and the clinical findings of dysarthria or dysphonia in Section III (Clinical Assessment), which was statistically significant (r=0.88, p=0.001). Likewise, the reported gait instability in Section I and the findings of cerebellar ataxia also shown a strong and statistically significant correlation (r=0.78, p=0.008). Apart from these variables in the matrix, there were several other strong correlations, but they did not reach statistical significance.

These correlation coefficients in the matrix, which measure the strength of relationships, also indicated the effect size. Several items in the NMDAS had robust effects on the total NMDAS scores. Of those coefficients that had strong and positive correlations with total NMDAS scores, four of them had shown to be statistically significant. Changes in disease severity for exercise intolerance and gait instability in Section I significantly affected the total NMDAS scores (r=0.80-0.92, p=0.006-<0.001). In Section III, visual acuity and cerebellar ataxia correlated significantly with NMDAS scores (r=0.77-0.80, p=0.01-0.005). No items in Section II (System Specific Involvement) had any significant magnitude of effect on NMDAS scores.

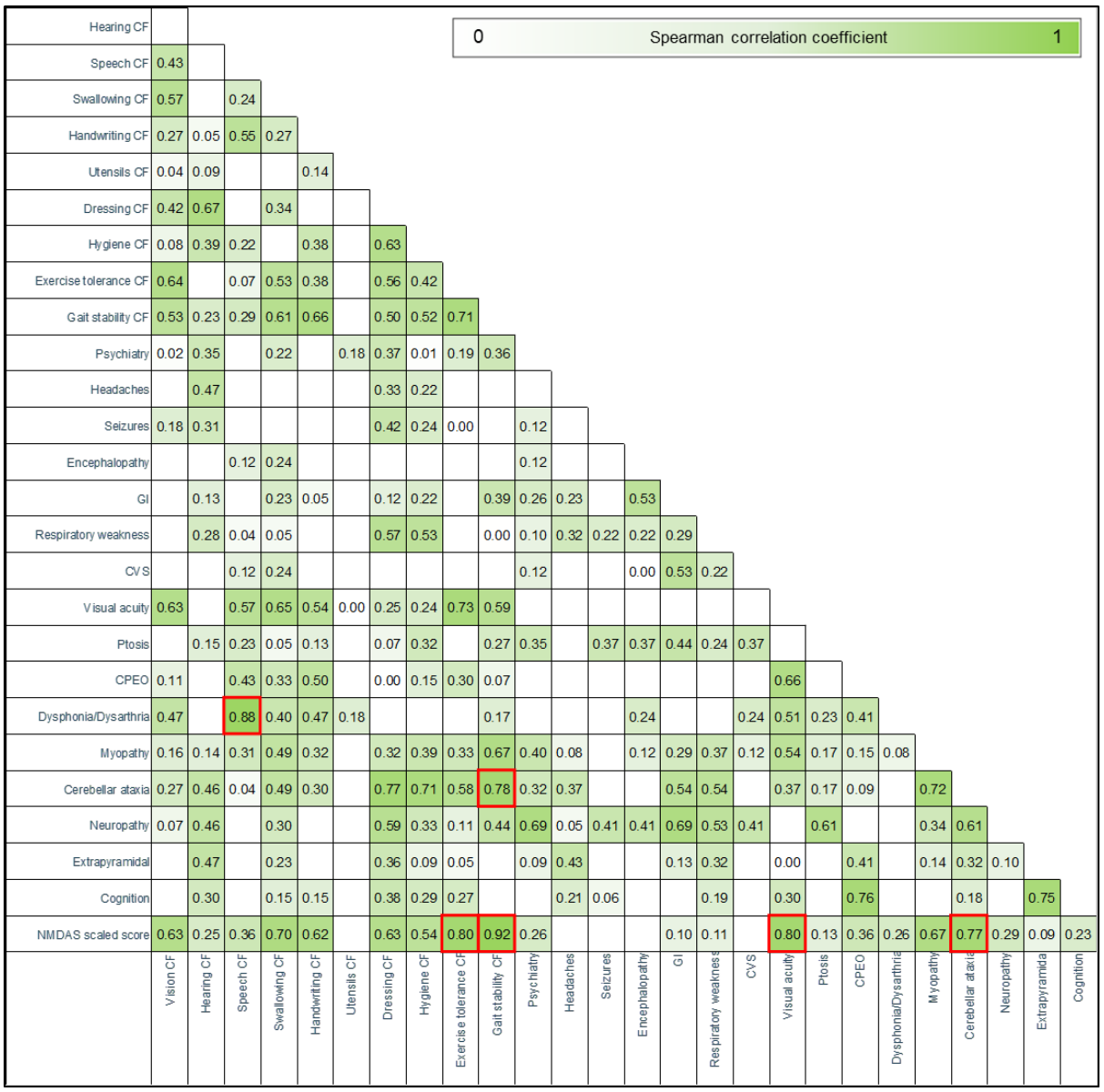


Figure 5-4: Correlation matrix shows item-item relationships in the NMDAS using Spearman's rank-order correlation coefficients. Those statistically significant coefficients have red borders. The NMDAS total scaled score is also shown.

5.4.6 A longitudinal study of the NMDAS

Of the ten participants, five have interim follow-up visits at home and have returned to the research site for end-of-study visits. The total NMDAS scores for these five participants recorded during these visits are summarised in Figure 5-5. These scores generally fluctuate but, three of the five participants (P2, P4 and P5) had higher NMDAS scores at the end of the study than their baseline scores. However, the average NMDAS score for this group of 52.6 (SD 17.8; 95%CI 30.5-74.8) at baseline visits did not differ significantly from 54.6 (SD 27.0; 95%CI 21.1-88.2) at end-of-study visits (Wilcoxon signed-rank Z = 0.41 p=0.69). There was no statistical difference for each item within NMDAS too. As a group, the rate of change in NMDAS was about 1 point per annum (SD 6.1; 95%CI -6.6-8.5). The cumulative NMDAS points scored in each domain for these ten participants at each visit are outlined in Figure 5-6. Of all the items in the NMDAS, respiratory weakness, hearing function, chronic progressive external ophthalmoplegia, ptosis, and exercise tolerance scores accumulated the most scores during the study period. These five items have consistently scored higher than other items in the NMDAS.

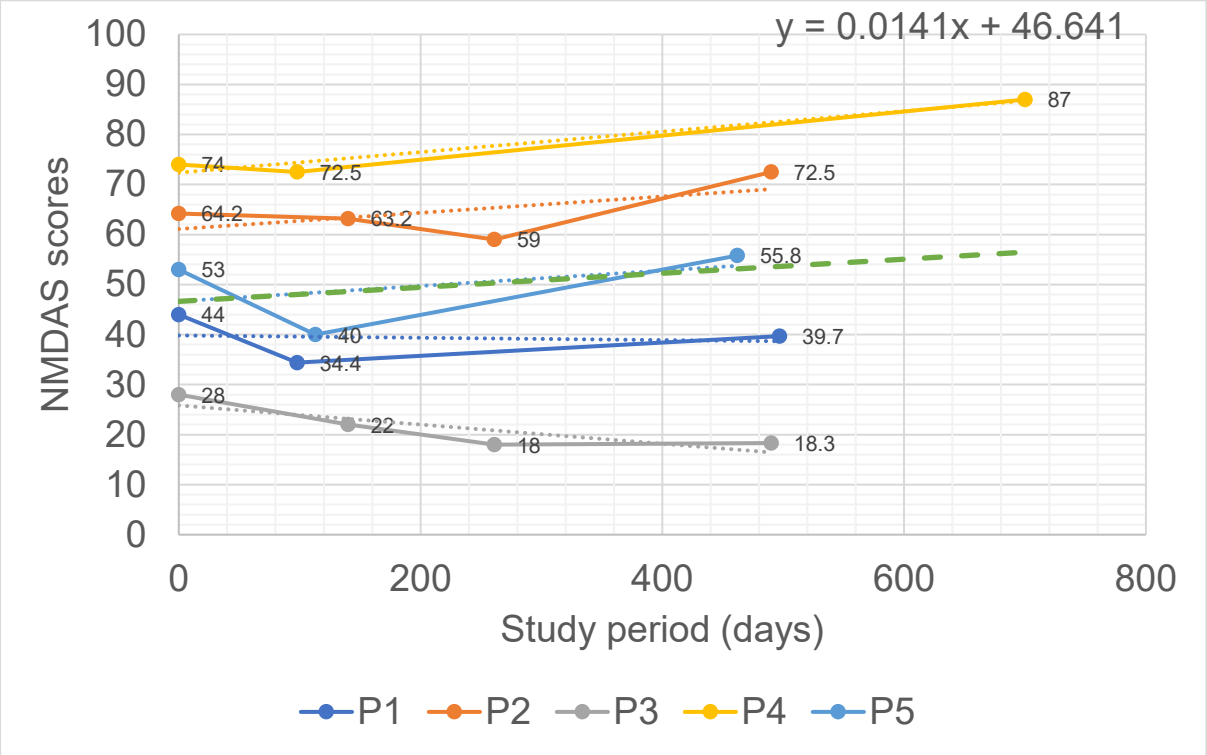


Figure 5-5: Line chart shows the total NMDAS scores for five individual participants (P1 to P5) during the study period with trendline (dashed green)

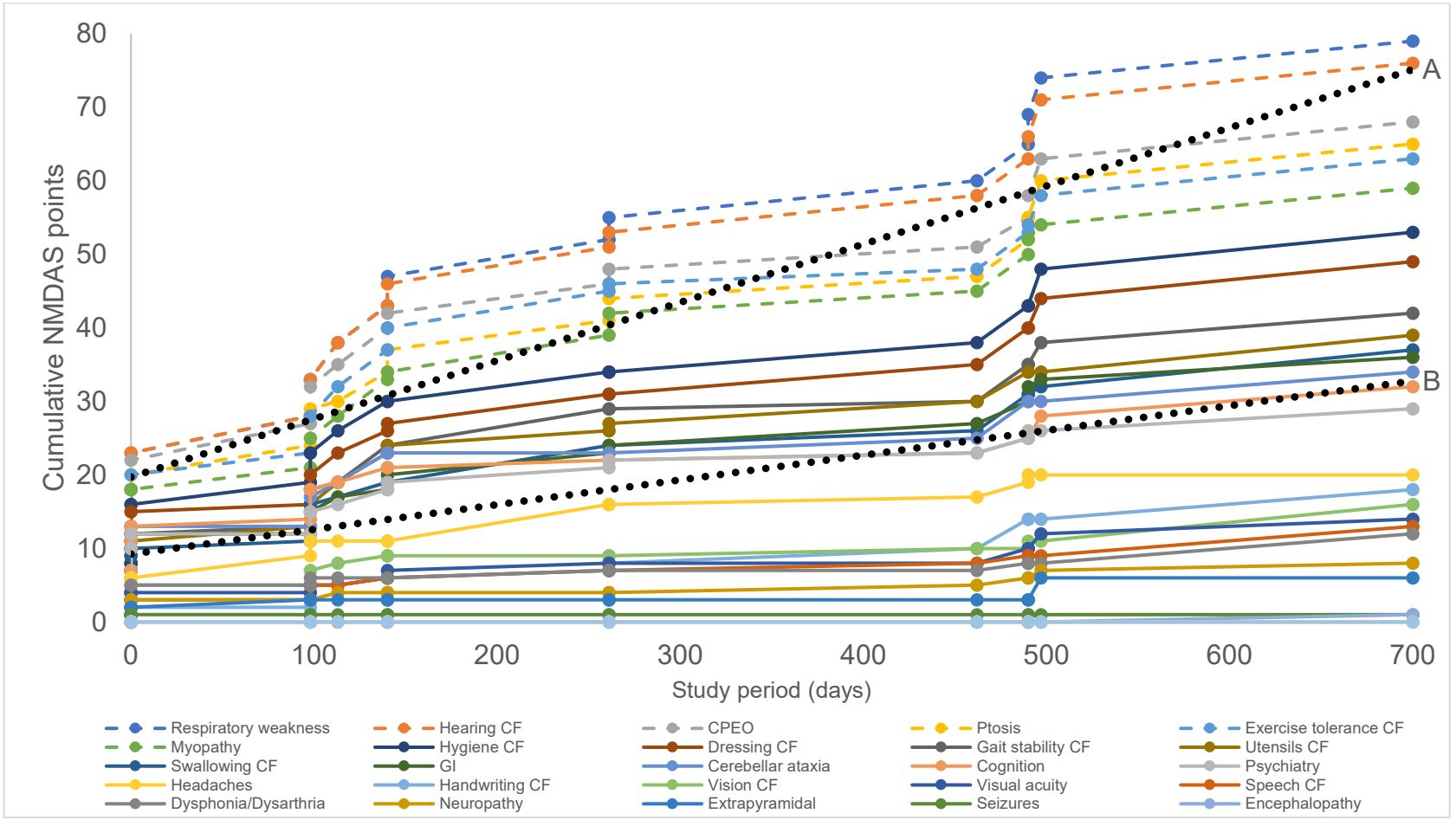


Figure 5-6: The cumulative scores for individual items in the NMDAS for five participants during the study period. Respiratory weakness, hearing function, chronic progressive external ophthalmoplegia (CPEO), ptosis, exercise tolerance and myopathy items in the NMDAS (dashed lines) appeared to have higher rates of scoring than other items (full lines) at each study visit time points. There was a clear distinction in how much disease burden that these participants experience in these particular items over the study period. The trendlines for these two groups of items has been shown in black dashed lines (A) and (B).

5.4.7 Spirometry (FVC and FEV_1)

The spirometry results from this group of participants are outlined in Table 5-3. After three attempts, the best forced vital capacity (FVC) and best forced expiratory volume in one second (FEV_1) results were selected for analysis for each participant. One participant, P6, could not perform the third attempt because of tiredness. All the participants' techniques and procedures for spirometry satisfied the quality criteria set by standard guidelines (Miller et al., 2005, Graham et al., 2019).

The mean of their best FVC was 1.47 litres (SD 0.69, 95% CI 0.89-2.04). On average, the predicted percentage FVC for these participants was only 38.1% (SD 10.5, 95% CI 29.3-46.9), which was significantly lower than the healthy population (Quanjer et al., 2012). Whilst the mean of their FEV_1 was 1.35 litres (SD 0.53, 95% CI 0.91-1.79). Although the predicted percentage FEV_1 of 41.5% (SD 9.1% 95%CI 33.8-49.1) placed these participants in the 'Severe' grade (35-49% of predicted FEV_1) on lung disease severity scale (Pellegrino et al., 2005), they were reduced in proportion to their respective FVC.

From the age, height and sex of these participants, their individual predicted FVC and FEV_1 values were calculated from published reference equations (Quanjer et al., 2012). The distribution of their actual and predicted values were compared in Figure 5-7 and Figure 5-8. The paired-samples t-test was also determined their actual FVC values differed significantly from their respective predicted values, $t(9) = -13.8$, $p<0.001$. Likewise, their actual FEV_1 values also diverged significantly from their predicted values, $t(9) = -13.3$, $p<0.001$.

All participants achieved FEV1/FVC ratio above the lower limit of normal 0.70 (Pellegrino 2005). The FEV_1/FVC ratio for them ranged from 0.82 to 0.99. Taken together with the lower than predicted values in FVC and FEV_1, these spirometry results suggested patterns of restrictive lung disease, most likely secondary to their underlying neuromuscular disorders.

Participant ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
FVC trial 1 (litres)	0.87	1.04	2.84	1.20	1.07	1.67	1.40	1.39	2.37	0.71
FVC trial 2 (litres)	0.85	1.05	2.92	1.38	1.21	1.60	1.48	1.51	2.39	0.83
FVC trial 3 (litres)	0.84	1.06	3.00	1.45	1.18	-	1.57	1.49	2.42	0.89
Best FVC (litres)	0.87	1.06	3.00	1.45	1.21	1.67	1.57	1.51	2.42	0.89
% of predicted FVC	29%	34%	52%	40%	36%	34%	55%	35%	52%	25%
FEV_1 trial 1 (litres)	0.84	1.03	2.46	1.14	0.57	1.66	1.37	1.39	2.37	0.68
FEV_1 trial 2 (litres)	0.83	1.04	2.42	1.06	1.17	1.63	1.41	1.49	2.09	0.82
FEV_1 trial 3 (litres)	0.81	1.05	2.42	1.33	0.92	-	1.43	1.48	2.39	0.87
Best FEV_1 (litres)	0.84	1.05	2.46	1.33	1.17	1.66	1.43	1.49	2.39	0.87
% of predicted FEV_1	32%	39%	49%	42%	40%	43%	58%	44%	64%	29%
FEV_1/FVC ratio	0.97	0.99	0.82	0.92	0.97	0.99	0.91	0.99	0.99	0.98

Table 5-3: Table shows the forced vital capacity (FVC) results, forced expiratory volume in 1 second (FEV_1), the percentages of predicted FVC and the percentage of predicted FEV_1 for all ten participants.

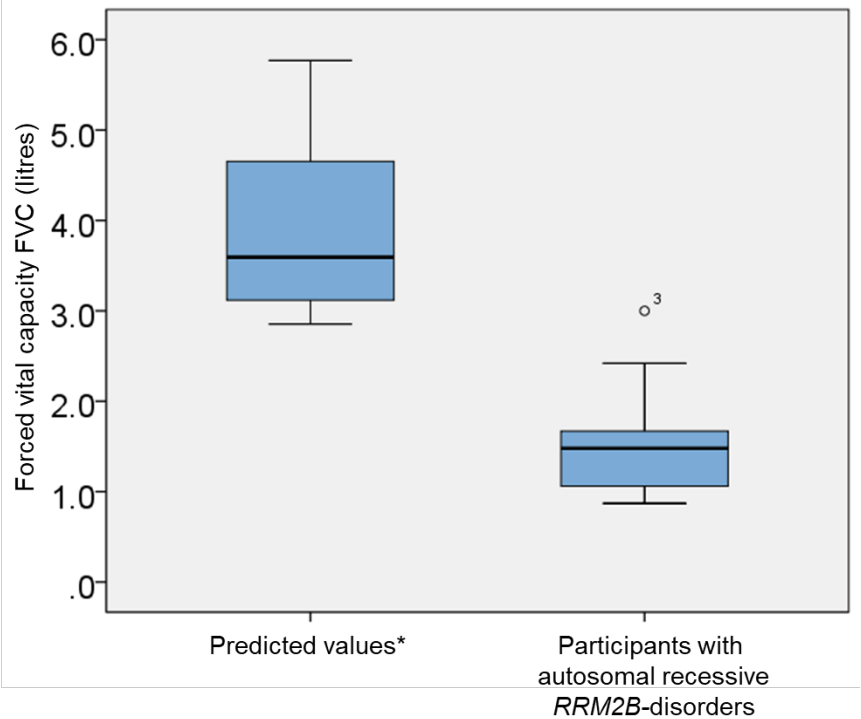


Figure 5-7: Boxplot shows the difference between the predicted FVC values (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

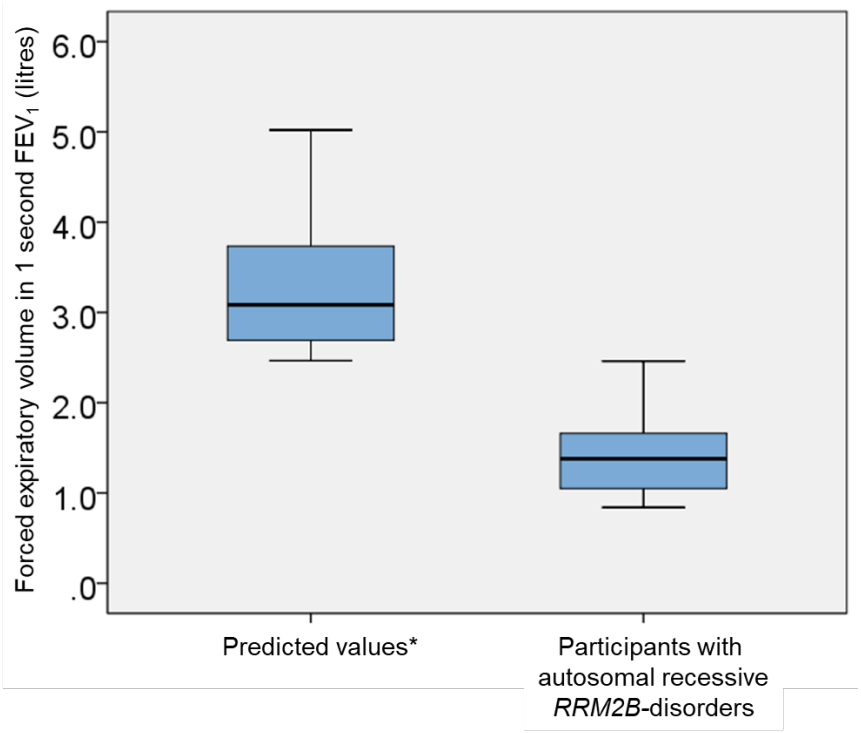


Figure 5-8: Boxplot shows the difference between the predicted FEV_1 values (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

5.4.8 Sniff nasal inspiratory pressure (SNIP)

The SNIP results, along with the maximal inspiratory and expiratory pressures (MIP and MEP), for the participants are summarised in Table 5-4. One participant, P9, could not perform the SNIP, MIP and MEP tests because of moderate learning difficulties. One other participant, P8, could not complete the MIP test because of tiredness, and the performance did not satisfy the requirements of the test. All remaining participants collaborated satisfactorily with the coordination and volitional contraction required for these manoeuvres (Uldry and Fitting, 1995). Of all the participants, P2 and P10 had non-invasive ventilation at night.

Of the nine participants who completed the SNIP, their mean value was 19.1 cmH2O (SD 22, 95%CI 0.7-37.5) and their mean maximal predicted value was 94.3 cmH2O (SD 11.7, 95%CI 84.5-104.0) (Figure 5-9). These two values differed significantly for individual participants as determined by paired-samples t-test, $t(8)=-16.0$, $p<0.001$. Essentially, these participants had only achieved 18.7% (SD 17.6, 95%CI 4.0-33.4) of their predicted maximal SNIP values.

The mean maximal inspiratory pressure (MIP) from the mouth for the eight participants who completed the tests was 19.5 cmH2O (SD 19.3, 95%CI 3.3-35.7). These values were also significantly lower than their predicted values of 79.4 cmH2O (SD 19.1 95%CI 63.4-95.4), ($t(7)=17.2$, $p<0.001$) (Figure 5-10). Similar to the nasal measurements, these participants only managed 22.4% (SD 15.9, 95%CI 9.1-35.6) of their predicted MIP from their inspiratory manoeuvres through the mouthpiece.

The maximal expiratory pressure (MEP) for the nine participants, on average, was 29.1 cmH2O (SD 19.4, 95%CI 12.9-45.4). Again, these values were significantly lower when compared to their predicted values of 109.0cmH2O (SD 32.2 95%CI 82.0-135.9) in pairs, $t(8)=9.9$, $p<0.001$ (Figure 5-11). They only reached 25.8% (SD 13.6 95%CI 14.5-37.2) of their predicted MEP values.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Sex	F	F	M	F	F	M	F	M	M	F
Age (years)	17.2	23.8	18.1	35.9	18.4	53.6	39.3	53.0	38.2	41.2
Maximal inspiratory pressure (MIP), (cm H2O)	7	10	64	21	24	17	10	N/A	N/A	3
Predicted MIP, (cm H2O)	66	78	123	75	64	87	69	N/A	N/A	72
% of predicted MIP	11%	13%	52%	28%	37%	20%	14%	N/A	N/A	4%
Maximal expiratory pressure (MEP), (cm H2O)	18	12	67	36	38	38	13	23	N/A	11
Predicted MEP, (cm H2O)	88	98	158	95	87	163	90	144	N/A	93
% of predicted MEP	20%	12%	42%	38%	44%	23%	14%	16%	N/A	12%
Sniff nasal inspiratory pressure (SNIP), (cm H2O)	15	8	73	16	14	10	9	8	N/A	8
Predicted SNIP, (cm H2O)	91	90	119	87	91	104	86	105	N/A	86
% predicted SNIP	16%	9%	61%	18%	15%	10%	10%	8%	N/A	9%

Table 5-4: Table summarises the results of the maximal inspiratory pressure (MIP), maximal expiratory pressure and sniff nasal inspiratory pressure (SNIP) for these ten participants.

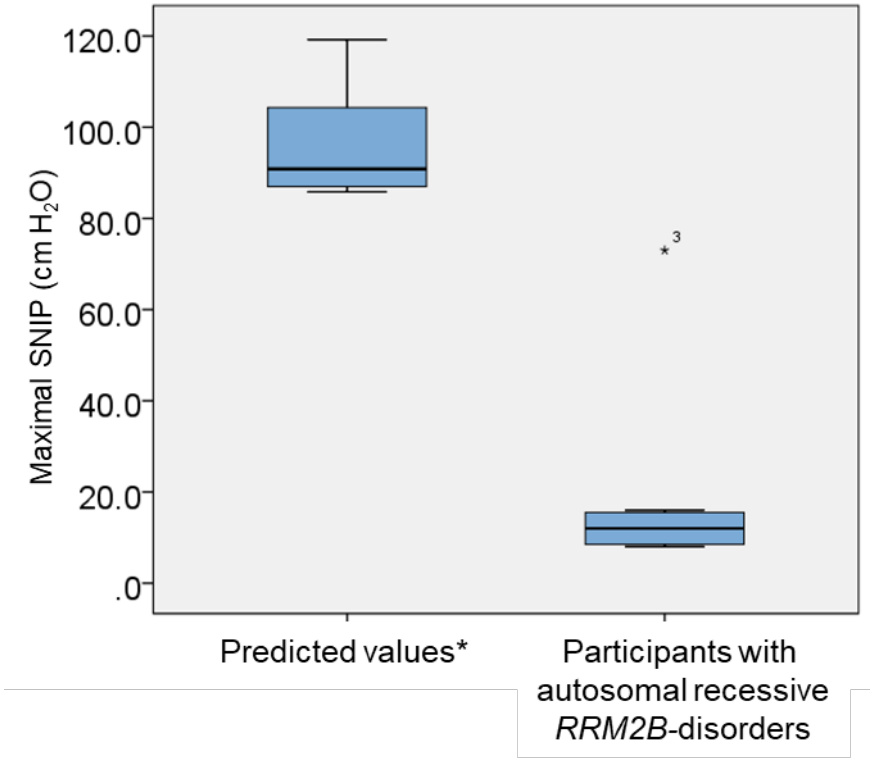


Figure 5-9: Boxplot shows the difference between the predicted sniff inspiratory pressure (SNIP) values (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

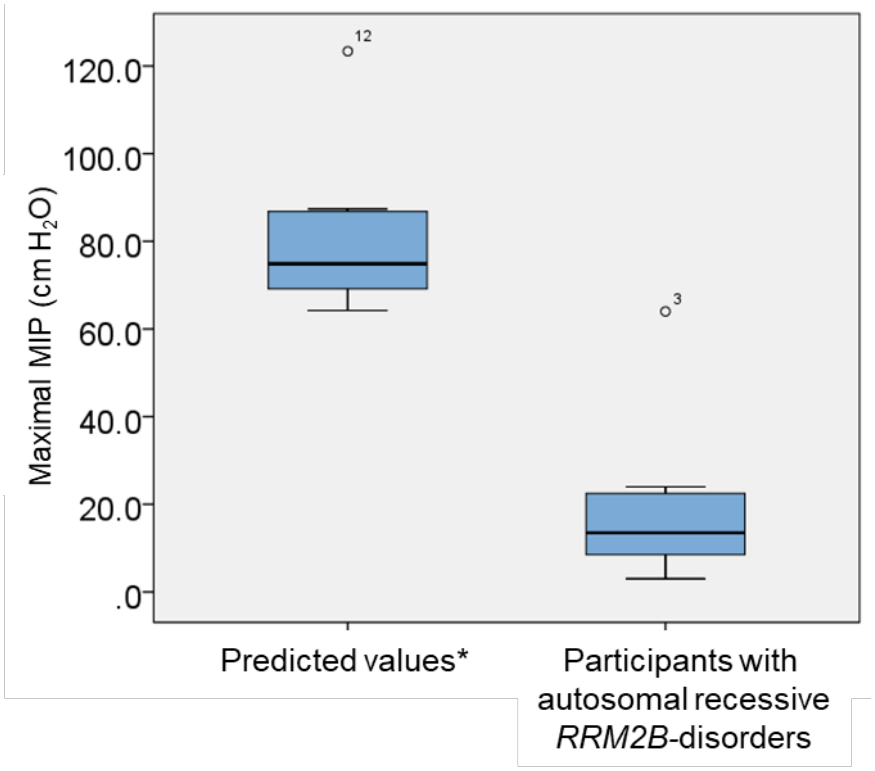


Figure 5-10: Boxplot shows the difference between the predicted maximal inspiratory pressure (MIP) values (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

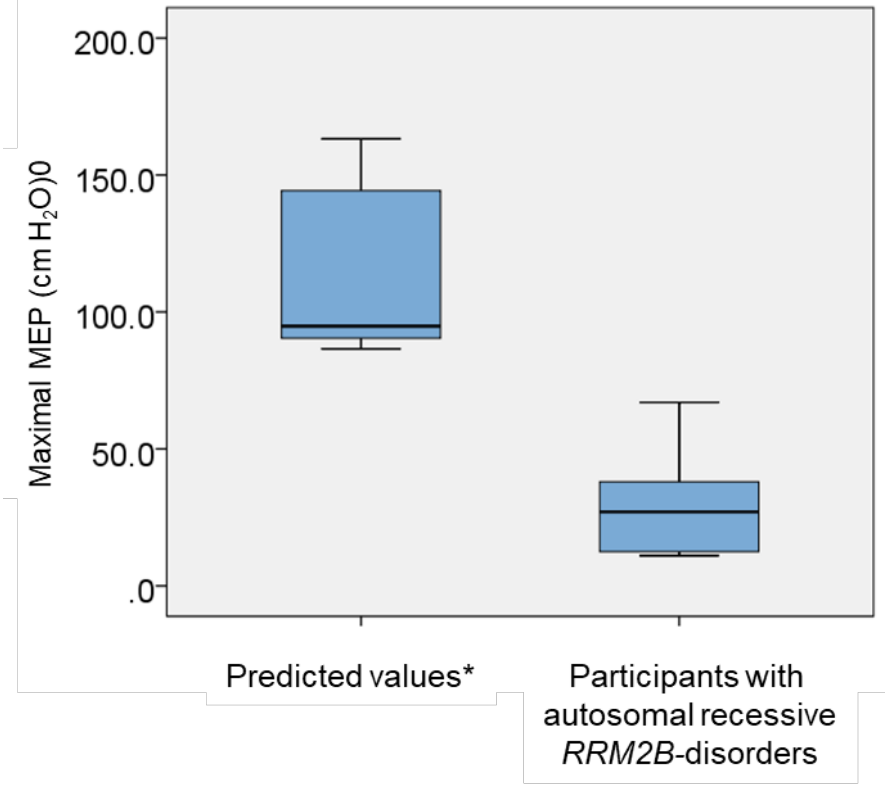


Figure 5-11: Boxplot shows the difference between the predicted maximal expiratory pressure (MEP) values (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

A Spearman's rank-order correlation was run to determine the relationships between these lung function values and related items in the NMDAS (Table 5-5). The NMDAS items that could affect lung function tests were respiratory weakness exercise tolerance, myopathy, speech difficulties, swallowing and dysphonia/dysarthria. Other non-related items in NMDAS were also explored but none had demonstrated significant correlations. Of these related items, there was a strong and negative correlation between the severity of respiratory weakness and the percentage of predicted FVC ($r=-0.80$, $p<0.001$) (Figure 5-12). This correlation was expected because the respiratory weakness item in the NMDAS had been scored according to the spirometry results. Other related items in the NMDAS and the total NMDAS scores have not demonstrated any significant correlations with FVC and FEV_1.

Unlike the spirometry, the SNIP, MIP and MEP values were not assessed as part of NMDAS scoring. Of these, the percentage of predicted SNIP had a strong and significant effect size on speech function in the NMDAS ($r=-0.74$, $p=0.02$) (Figure 5-13). Although SNIP had negative correlations with other related items, the effect size was weak or very weak ($r=-0.16$ to -0.42) with no statistical significance. Both percentages of predicted MIP and MEP has not demonstrated statistically significant correlations with these related items in the NMDAS. Of note, the percentage of predicted MEP had moderate and negative correlations ($r=-0.58$) with speech difficulties but the effect size did not reach statistical significance.

NMDAS items	% of predicted FVC	% of predicted FEV1	% of predicted MIP	% of predicted MEP	% of predicted SNIP
Respiratory weakness	-.803**	-.764*	-0.38	-0.1	-0.41
Exercise tolerance CF	0.41	0.22	0.05	-0.21	-0.25
Myopathy	-0.23	-0.12	-0.31	-0.44	-0.4
Speech CF	-0.08	0.17	-0.41	-0.58	-.744*
Swallowing CF	0.38	0.47	0.11	0.03	-0.16
Dysphonia/ Dysarthria	0.27	0.53	-0.05	-0.24	-0.42
Total NMDAS scaled score	0.26	0.18	-0.14	-0.33	-0.38

*Table 5-5: Table shows Spearman's rank-order correlation coefficients between items scored in the NDMAS and the respiratory-related outcome measures (FVC, FEV1, MIP, MEP and SNIP). * denotes p<0.05, ** denotes p<0.01*

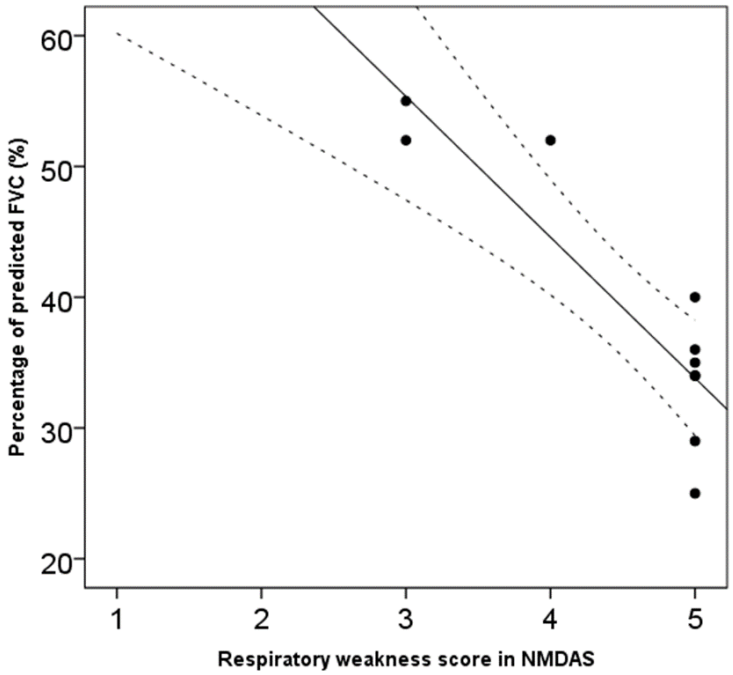


Figure 5-12: Scatter plot shows the relationship between the percentage of predicted FVC and the respiratory weakness score in the NMDAS for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

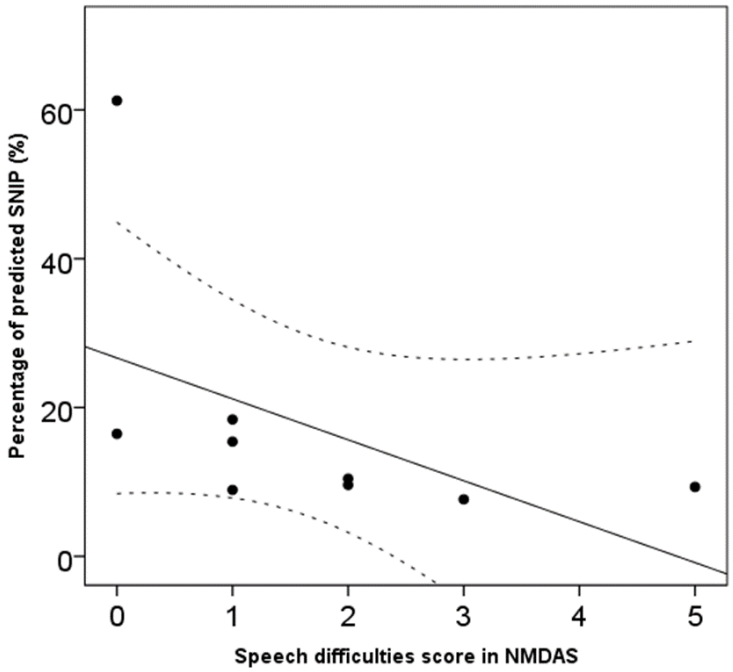


Figure 5-13: Scatter plot shows the relationship between the percentage of predicted SNIP and the speech difficulties score in the NMDAS for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

5.4.9 A longitudinal study of spirometry

Of the ten participants, five had interim follow-up visits at home and returned to the research site for end-of-study visits. The percentages of predicted FVC and FEV_1 for these five participants recorded during these visits are summarised in Figure 5-14 and Figure 5-15 respectively. The percentages of predicted FVC for all five participants had fallen significantly from 38.2% (SD 8.7, 95%CI 27.4-50) at baseline to 30.4% (SD 10.0, 95%CI 17.9-42.9) at the end of study ($t(4)=3.0$, $p=0.039$). The rate of reduction in percentage of predicted FVC was 5.2% per annum (SD 3.2, 95%CI 1.2-9.1). Meanwhile, the average percentage of FEV_1 for this group of participants did not differ significantly at baseline ($\mu=40.4$, SD 6.1, 95%CI 32.8-48.0) and at end-of-study visits ($\mu=31.2$, SD 14.0, 95%CI 13.8-48.6) ($t(4)=1.9$, $p=0.123$). The percentage of predicted FEV_1 decreased by 6.6% per annum (SD 8.2, 95%CI -3.6-17.0).

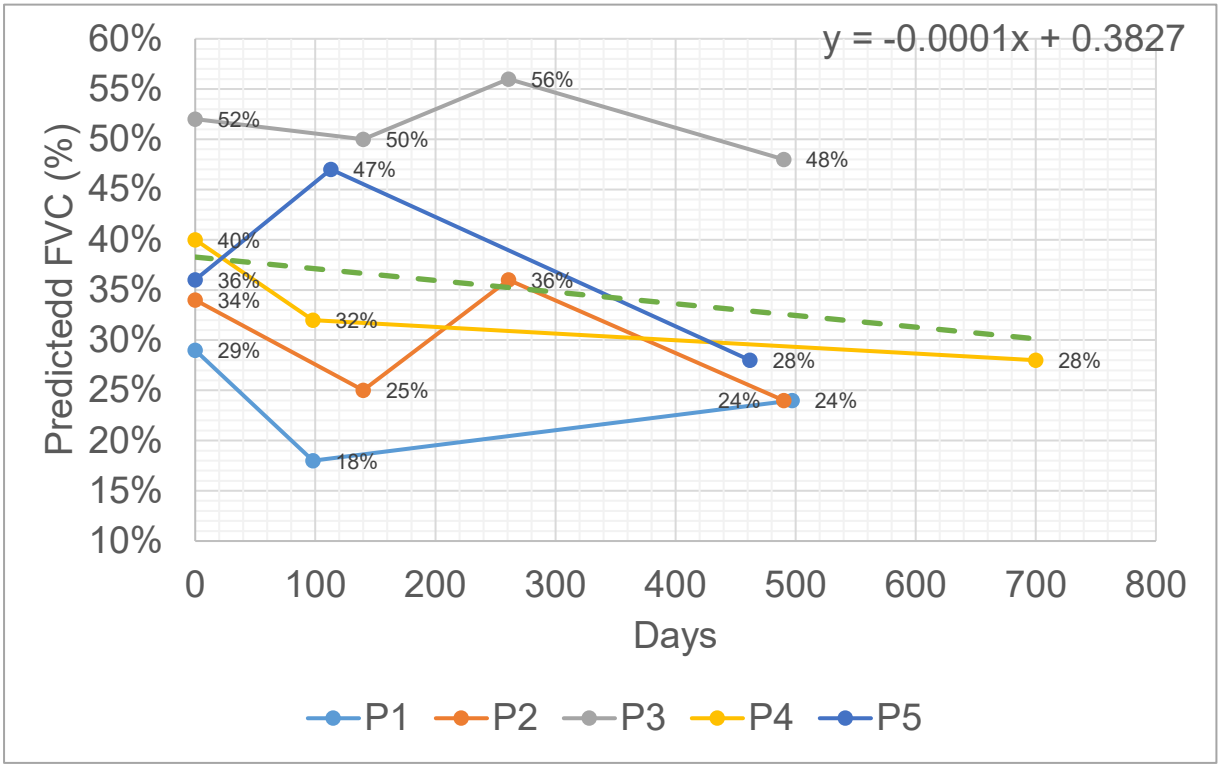


Figure 5-14: Line chart shows the percentages of predicted forced vital capacity (FVC) for five participants (P1 to P5) during the study period, including trendline (dashed green).

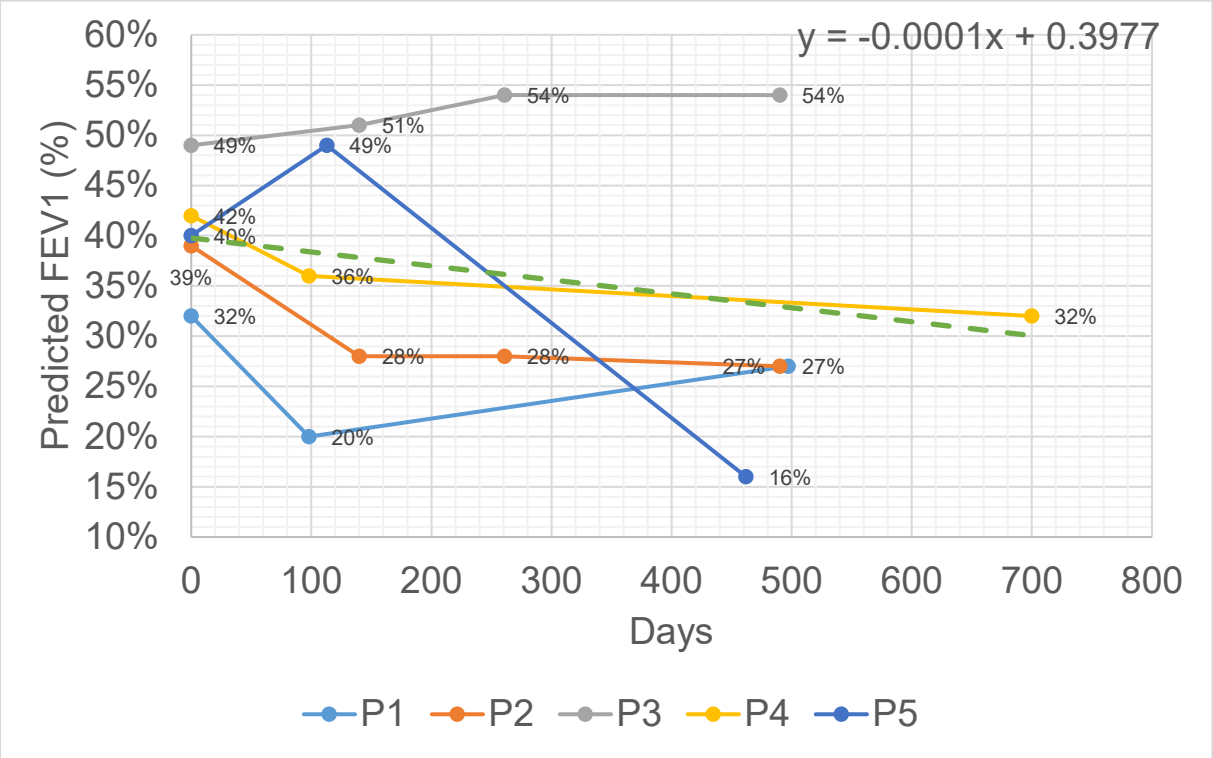


Figure 5-15: Line chart shows the percentages of the predicted forced expiratory volume in 1 second (FEV1) for five participants during the study period, including trendline (dashed green).

5.4.10 A longitudinal study on SNIP, MIP and MEP

Five participants have interim follow-up visits at home and have returned to the research site for end-of-study visits. The percentages of predicted SNIP, MIP and MEP and SNIP for these five participants recorded during these visits are summarised in Figure 5-16, Figure 5-17 and Figure 5-18, respectively. The percentage of predicted SNIP had generally remained unchanged during the study period (t(4)=-1.3, p=0.26). Although the average percentage of predicted MIP for this group of participants decreased by 4.1% per annum (SD 13.9, 95%CI -13.2-21), it did not differ significantly at baseline (μ=28.1%, SD 17.3, 95%CI 6.7-49.6) and at end-of-study visits (μ=21.7%, SD 23.9, 95%CI -8.0-51.4) (t(4)=0.74, p=0.5). The average percentage of predicted MEP for this group of participants also did not differ significantly at baseline (μ=31.4%, SD 14.2, 95%CI 13.8-49.0) and at end-of-study visits (μ=26.9%, SD 11.1, 95%CI 13.1-40.7) (t(4)=0.80, p=0.47). The percentage of predicted MEP decreased by 3.5% (SD 10, 95%CI -8.8-15.9).

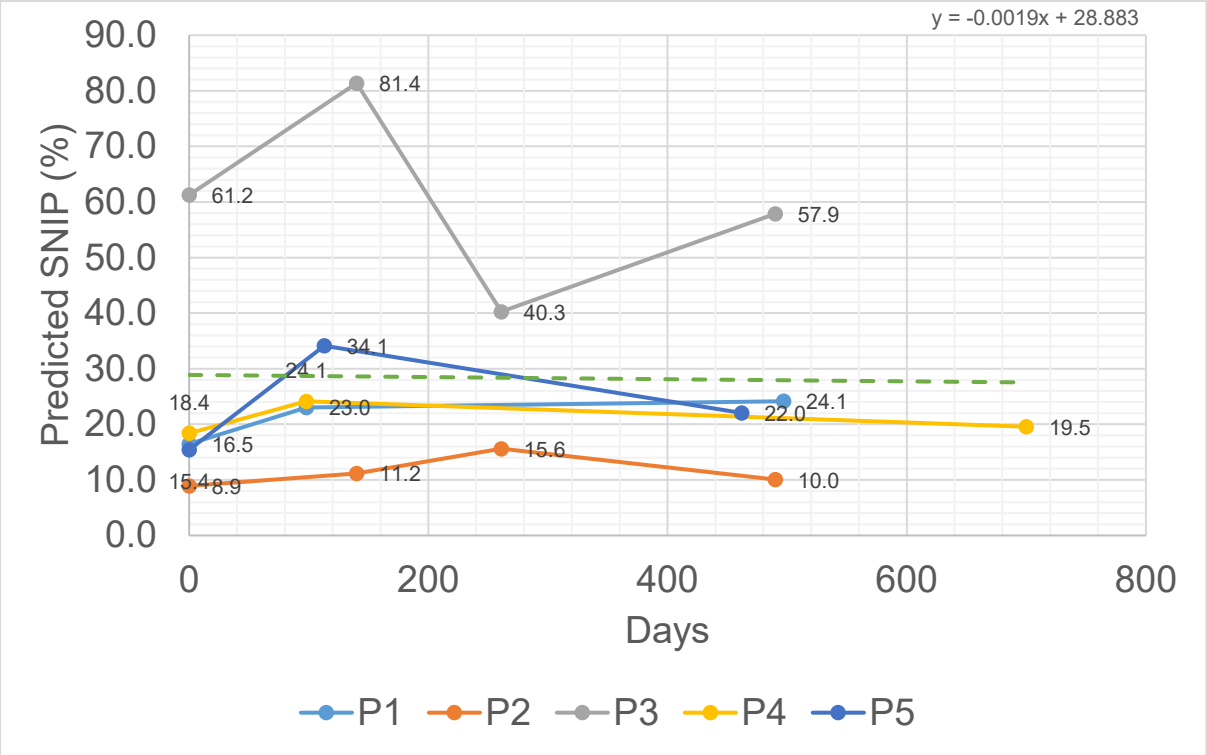


Figure 5-16: Line chart shows the percentages of predicted sniff nasal inspiratory pressure (SNIP) for five participants during the study period, including trendline (dashed green).

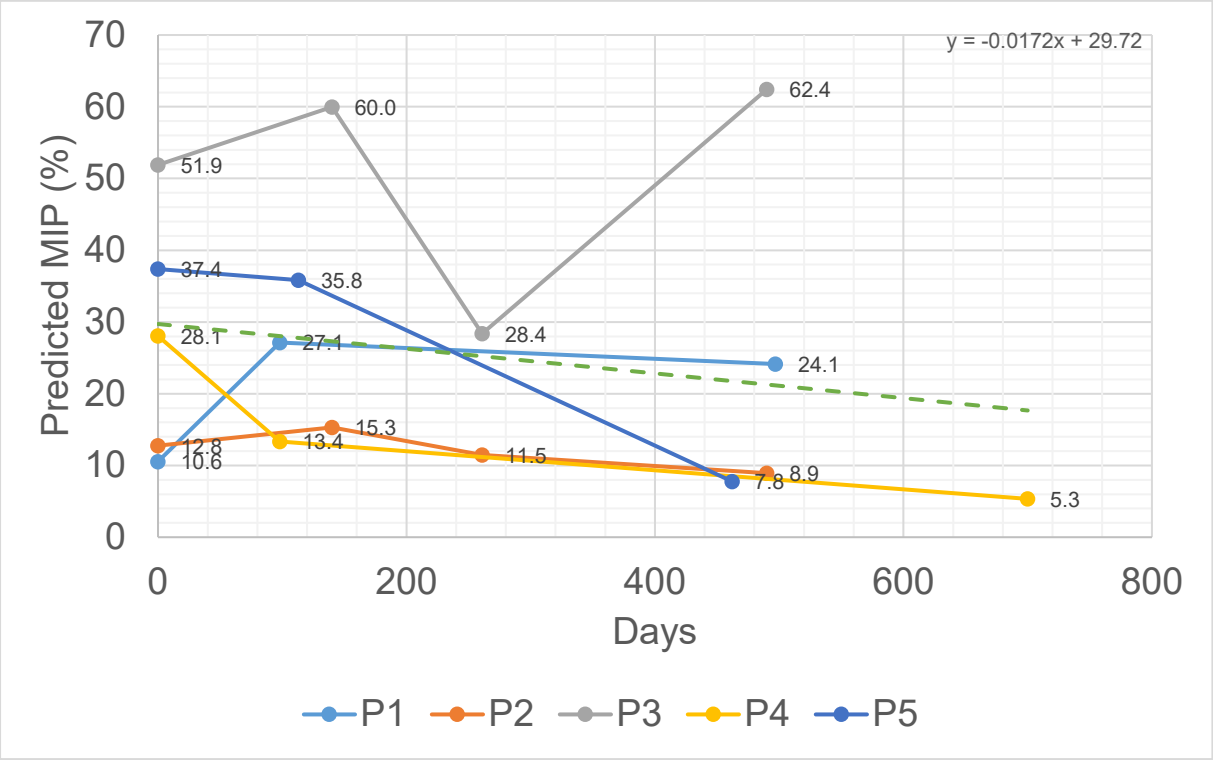


Figure 5-17: Line chart shows the percentages of predicted maximal inspiratory pressure (MIP) for five participants during the study period, including trendline (dashed green).

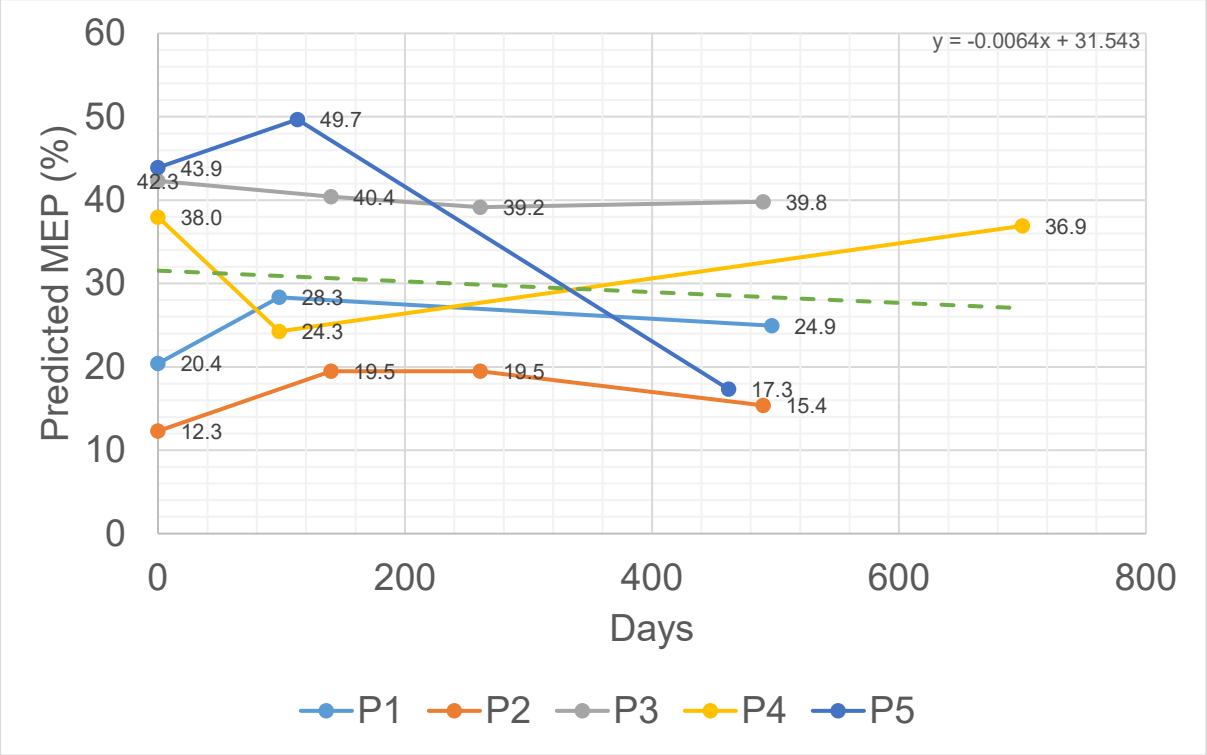


Figure 5-18: Line chart shows the percentages of predicted maximal expiratory pressure (MEP) for five participants during the study period, including trendline (dashed green).

5.4.11 Nine hole peg test

The results of the nine-hole peg test (NHPT) for all the participants are tabulated in Table 5-6. All participants had two trials of the NHPT and only the better times, recorded in seconds, were used for analysis. One participant, P9, could not complete a second trial of the dominant hand satisfactorily and had refused to undertake a repeat trial.

On average, the time taken by these participants to complete the NHPT using the dominant and non-dominant hands were 25.4 seconds (SD 2.5, 95%CI 23.3-27.4) and 28.8 seconds (SD 3.9, 95%CI 25.6-32.1), respectively. Their best times were compared with the normative values expected for each participant (Figure 5-19 and Figure 5-20). In this group of participants, their NHPT results for both dominant and non-dominant hands were significantly slower than the expected values $t(9)=3.2$, $p=0.01$ and $t(9)=4.2$, $p=0.002$, respectively.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Hand dominance	Left	Right	Right	Right	Right	Right	Right	Right	Right	Right
Dominant Trial 1 (seconds)	26.85	35.05	25.05	36.47	23.84	22.25	28.90	41.03	69.07	25.50
Dominant trial 2 (seconds)	24.42	25.35	30.04	28.69	25.87	21.31	37.13	36.72	n/a	25.35
Best dominant (seconds)	24.42	25.35	25.05	28.69	23.84	21.31	28.90	41.03	69.07	25.35
Non-dominant trial 1 (seconds)	30.32	52.26	35.06	36.12	31.02	26.29	31.10	55.90	78.12	31.84
Non-dominant trial 2 (seconds)	29.08	37.87	25.13	29.15	26.94	26.67	28.28	44.03	64.51	27.88
Best non-dominant (seconds)	29.08	37.87	25.13	29.15	26.94	26.29	28.28	44.03	64.51	27.88

Table 5-6: Table shows the time taken to complete the nine-hole peg test in these ten participants.

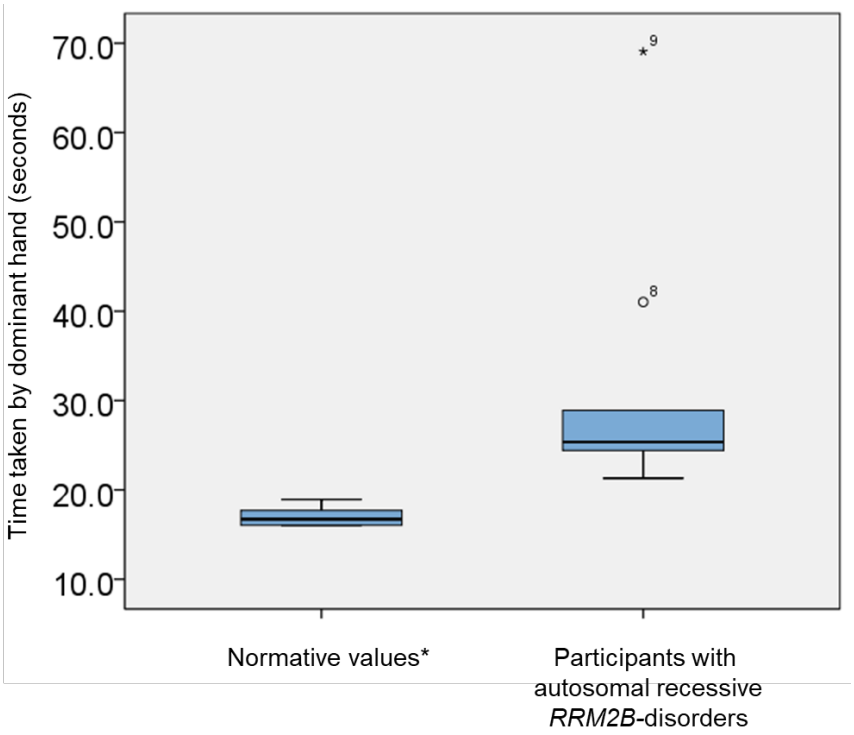


Figure 5-19: Boxplot shows the difference between the normative values for the time taken to complete the nine-hole peg test with the dominant hand (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

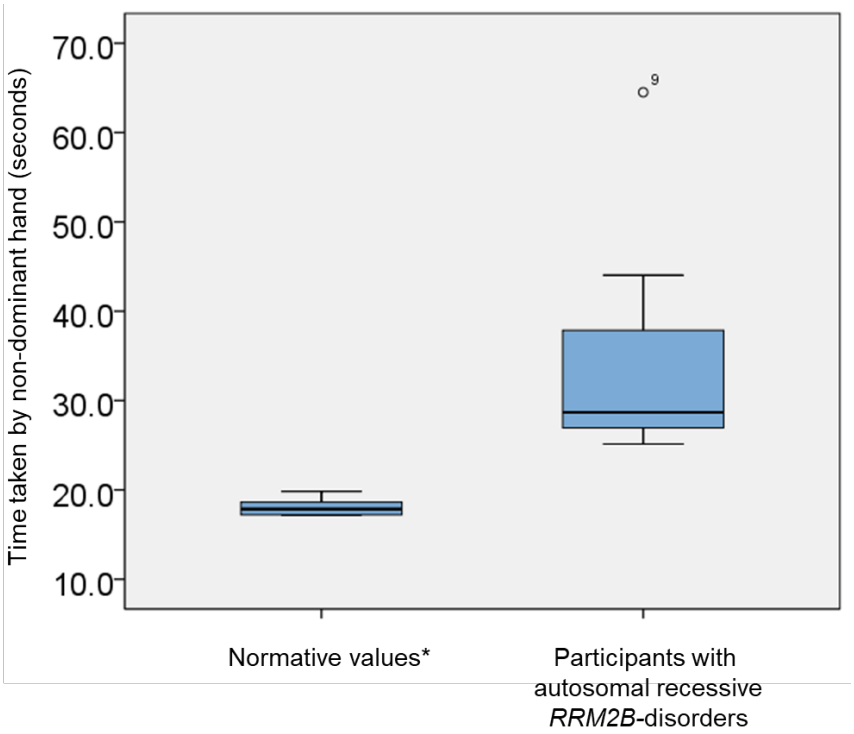


Figure 5-20: Boxplot shows the difference between the normative values for the time taken to complete the nine-hole peg test with a non-dominant hand (right) and those obtained by these ten participants with autosomal recessive RRM2B-disorders (left).

A Spearman's rank-order correlation was run to determine the relationships between these NHPT and questions in the NMDAS. The positive correlation and their respective statistical significance are summarised in Table 5-7. Of the items in the NMDAS that had a positive correlation with the NHPT, only exercise tolerance (r=0.68-0.71, p=0.02-0.03) (Figure 5-21) and visual acuity (r=0.81-0.95, p<0.001) (Figure 5-22) had reached statistical significance. Of note, NHPT also had a significant effect size on the total NMDAS scores (r=0.70-0.76, p=0.01-0.03) (Figure 5-23).

NMDAS items	Best dominant	Best non-dominant
Vision CF	0.60	0.66
Handwriting CF	0.58	0.34
Dressing CF	0.09	0.55
Exercise tolerance CF	0.711*	0.680*
Gait stability CF	0.47	0.49
Visual acuity	0.947*	0.812*
CLEO	0.68	0.57
Myopathy	0.37	0.53
Cerebellar ataxia	0.18	0.54
Cognition	0.29	0.40
Total NPMDS scaled scores	0.693*	0.758*

Table 5-7: Table shows Spearman's rank-order correlation coefficients between the results of the nine-hole peg tests and the items in the NMDAS

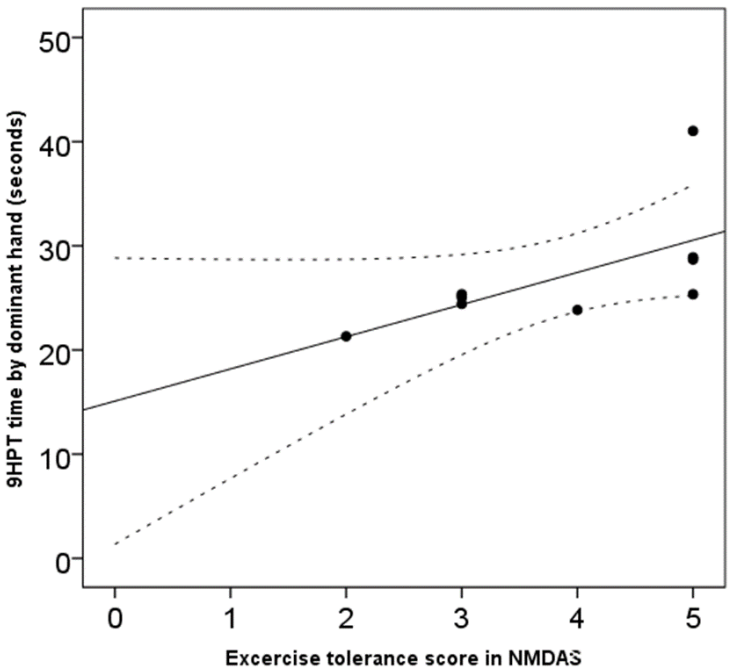


Figure 5-21: Scatter plot shows the relationship between the time taken to complete the nine-hole peg test and the exercise tolerance score in the NMDAS for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

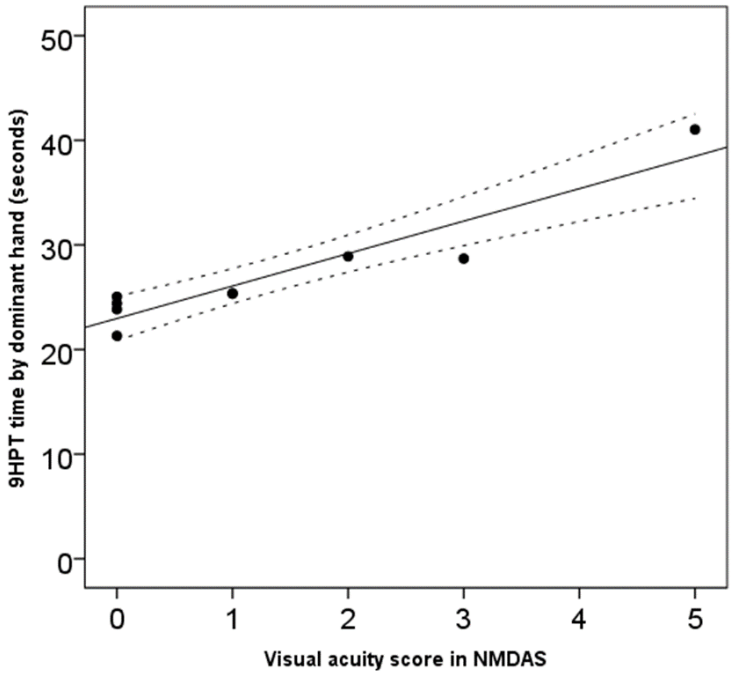


Figure 5-22: Scatter plot shows the relationship between the time taken to complete the nine-hole peg test and the visual acuity score in the NMDAS for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

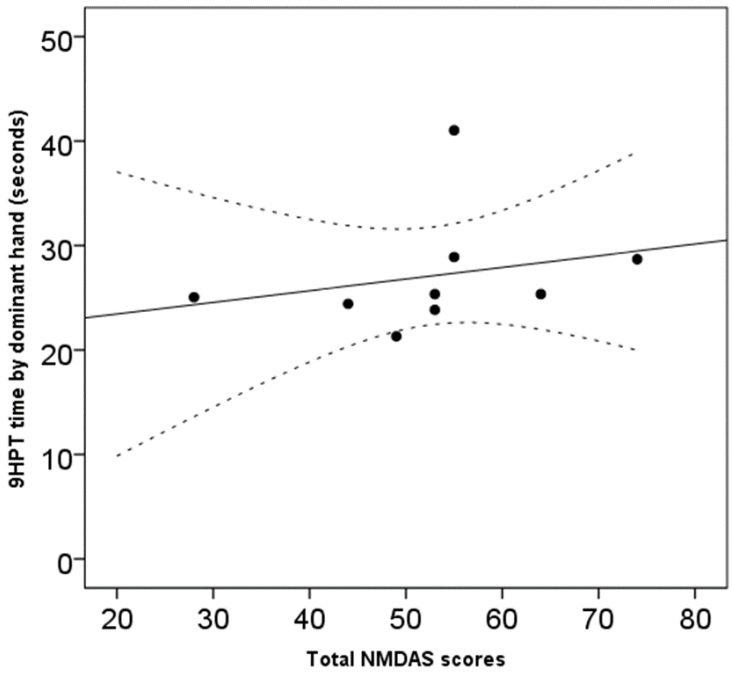


Figure 5-23: Scatter plot shows the relationship between the time taken to complete the nine-hole peg test and the total NMDAS scores for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

5.4.12 A longitudinal study on nine-hole peg test (NHPT)

Five participants have interim follow-up visits at home and have returned to the research site for end-of-study visits. The time taken by the participants to complete the NHPT using their dominant and non-dominant hands during these visits are summarised in Figure 5-24 and Figure 5-25. These participants, using their dominant hands, required longer time in these tests at end-of-study visits (μ=35.5 seconds, SD 11.9, 95%CI 20.8-50.2) than at baseline visits (μ=30.8 seconds, SD 4.7, 95%CI 24.9-36.8), but these were not statistically significant (t(4)=0.904, p=0.417). Similarly, they also performed slower with their non-dominant hands at end-of-study visits (μ=38.6 seconds, SD 12.9, 95%CI 22.6-54.7) than at baseline visits (μ=36.9 seconds, SD 8.9, 95%CI 25.9-48.0) but not significantly slower (t(4)=0.484, p=0.654).

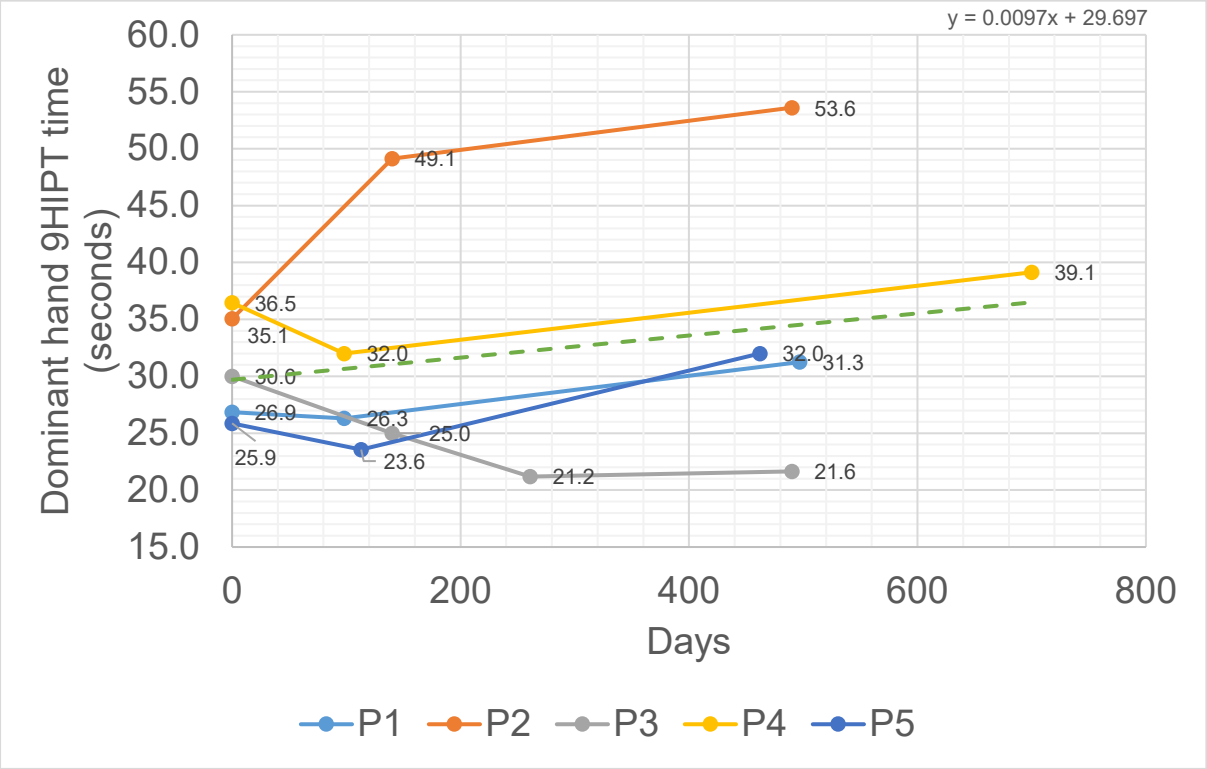


Figure 5-24: Line chart shows the time taken to complete the nine-hole peg test using their dominant hands for five participants during the study period, including trendline (dashed green).

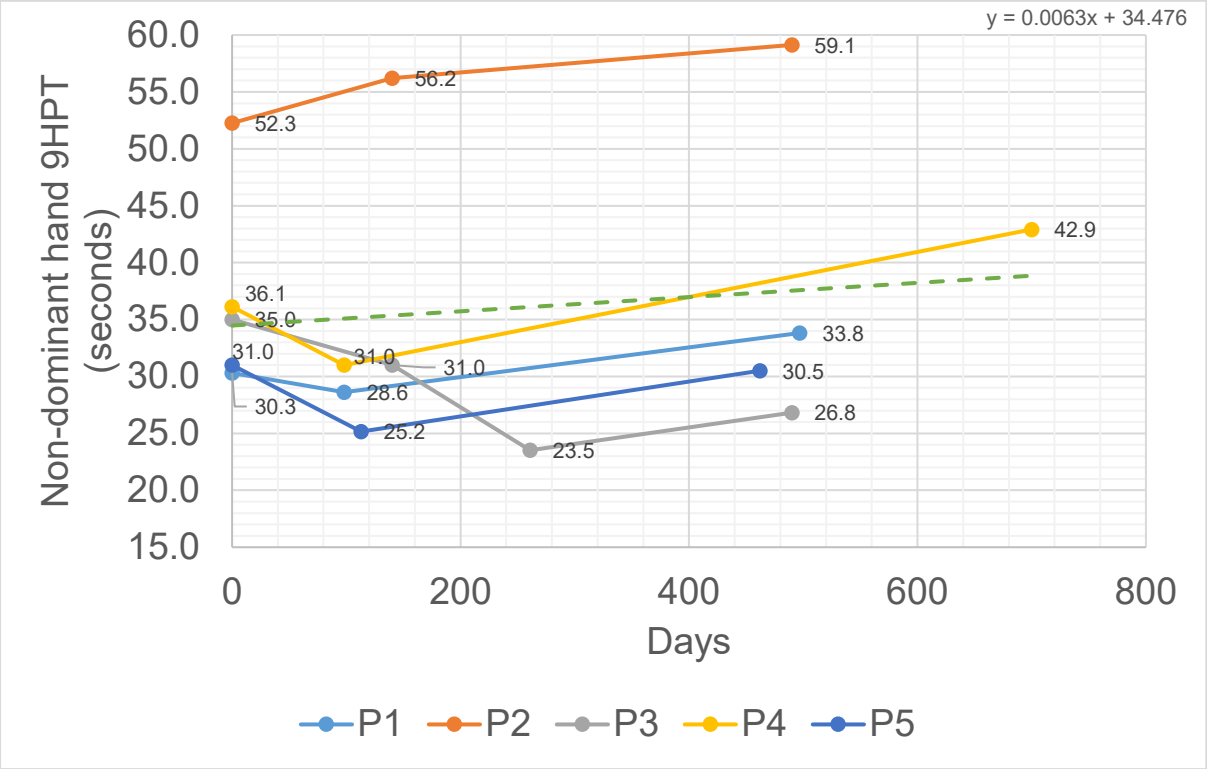


Figure 5-25: Line chart shows the time taken to complete the nine-hole peg test using their non-dominant hands for five participants during the study period , including trendline (dashed green).

5.4.13 Quantitative dynamometry for muscle strength

The forces generated by these participants in handheld dynamometry testing are summarised in Figure 5-26. Of all the participants, only P1 was left-handed. Their mean grip strength for right and left hands was 144 Newtons (SD 65.4 95%CI 97.7-192.2) and 125 Newtons (SD 42.7 95%CI 95.2-156.3). Moving proximally, their elbow flexion produced 48 Newtons (SD 18.5, 95%CI 34.7-61.3) on the right and 50 Newtons (SD 17.0, 95%CI 37.9-62.2). The weakest force generated was in their shoulder abduction movements. These participants, on average, only managed 36 Newtons (SD 15.4, 95%CI 25.3-47.4) on the right shoulder and 34 Newtons (SD 15.6, 95%CI 22.4-44.7) on the left shoulder. In the lower limbs, the mean forces measured in their knee flexion were 95 Newtons (SD 47.2, 95%CI 61.5-129.1) on the right side and 109 Newtons (SD 63.1, 95%CI 64.6-154.9). Their hip flexions generated, on average, 39 Newtons (SD 39, 95%CI 11.2-67.1) on the right side and 42 Newtons (SD 44.0, 95%CI 11.4-74.3) on the left side. All these results were lower than the forces generated by a healthy population of 50-59 years old of 244-417 Newtons for handgrip, 160-292 Newtons for elbow flexion, 124-238 Newtons for shoulder abduction, 294-447 Newtons for knee extension and 128-205 Newtons for hip flexion (Andrews et al., 1996).

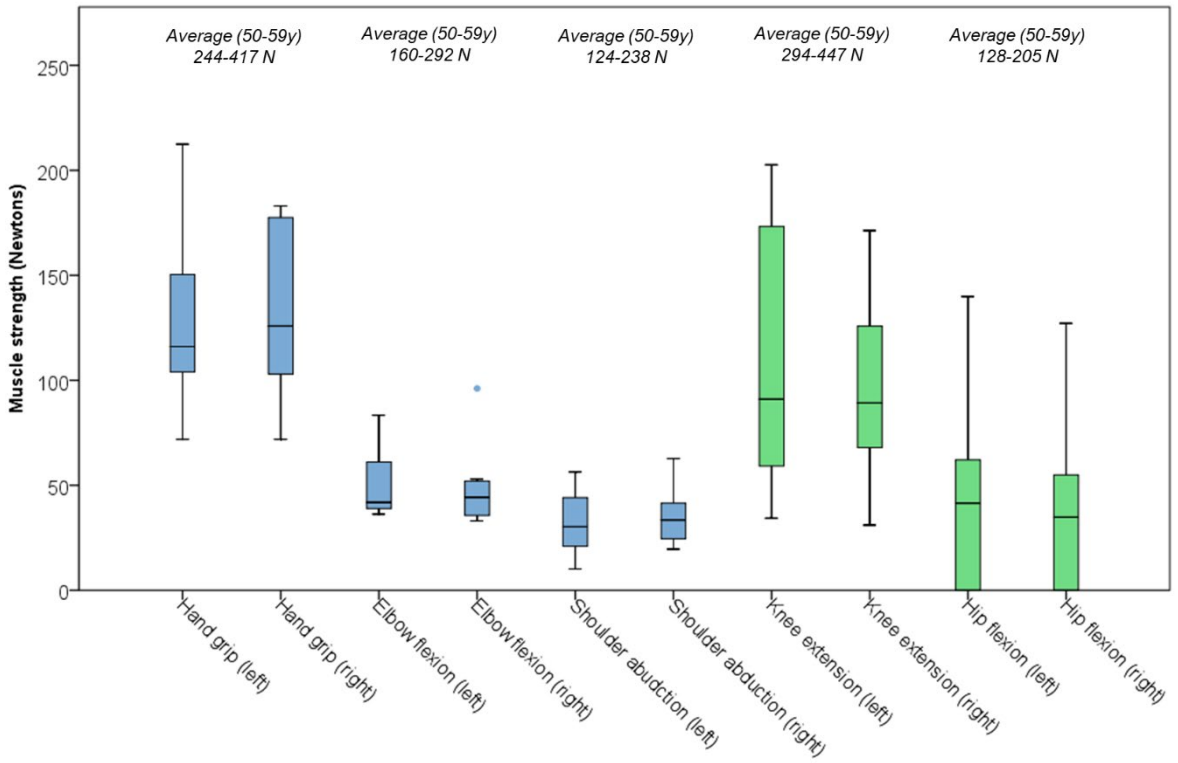


Figure 5-26: Boxplot shows the distribution of muscle strength in different movements performed by the ten participants with autosomal recessive RRM2B-disorders. Blue coloured box plot indicates upper limb movement and green coloured box plot indicates lower limb movement. Average values achieved by a healthy population of 50-59 years olds for each movement are shown above the respective boxplots.

5.4.14 A longitudinal study of quantitative dynamometry

Five participants returned to the research site for end interim follow-up visits at home and have returned to the research site for end-of-study visits. The muscle strength of these participants generally decreased at the end of the study period, but these changes were not statistically significant. The mean difference of strength from baseline and the results of paired t-tests for each of the muscle groups are outlined in Table 5-8. Despite achieving lower muscle strength at the end of study, the general trends in muscle strength for their grip (Figure 5-27), elbow flexion (Figure 5-28), shoulder abduction (Figure 5-29), knee extension (Figure 5-30) and hip flexion (Figure 5-31) appear to fluctuate during the study window.

	Mean difference (Newtons)	Standard deviation	Lower 95% CI	Upper 95% CI	Paired t-test values	p-values
Grip strength right	19.5	16.3	-0.7	39.8	2.68	0.06
Grip strength left	12.3	28.7	-23.3	47.9	0.96	0.39
Elbow flexion right	8.8	10.0	-3.6	21.3	1.97	0.12
Elbow flexion left	8.1	8.3	-2.1	18.4	2.20	0.09
Shoulder abduction right	5.5	8.7	-5.3	16.2	1.41	0.23
Shoulder abduction left	8.7	13.7	-8.4	25.7	1.41	0.23
Knee extension right	15.8	27.0	-17.8	49.3	1.30	0.26
Knee extension left	15.5	21.8	-11.7	42.7	1.59	0.19
Hip flexion right	8.0	35.2	-35.7	51.8	0.51	0.64
Hip flexion left	11.6	32.2	-28.4	51.5	0.80	0.47

Table 5-8: Tables shows the difference of muscle strength (Newtons) at the baseline and at the end-of-study assessment for five participants.

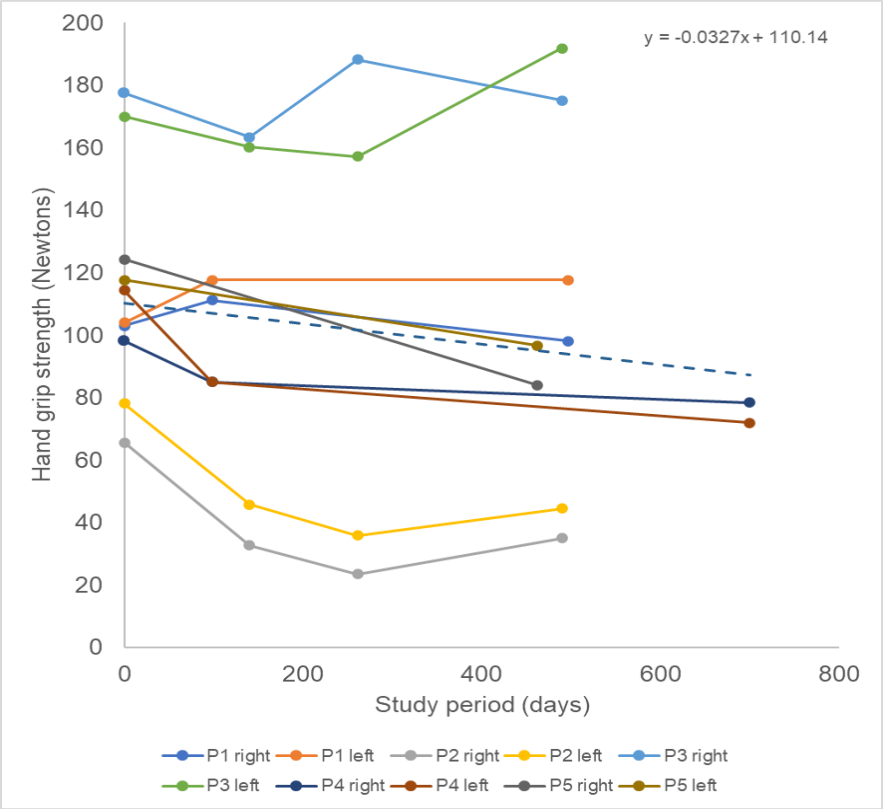


Figure 5-27: Line chart shows the grip strength for five participants during the study period.

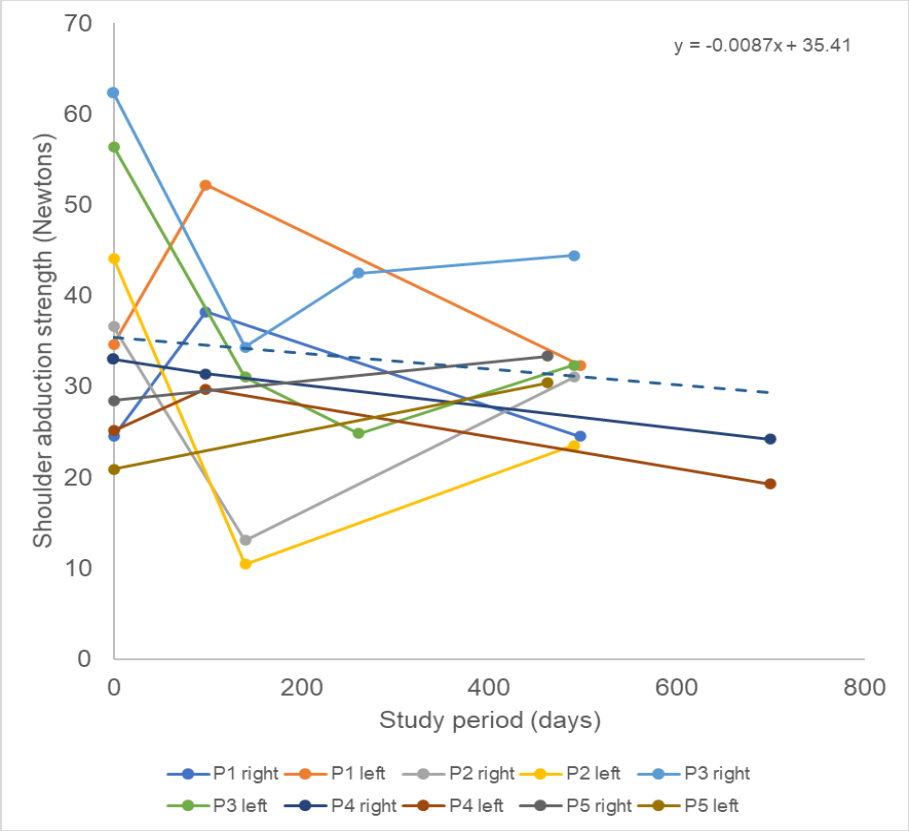


Figure 5-28: Line chart shows the shoulder abduction strength for five participants during the study period.

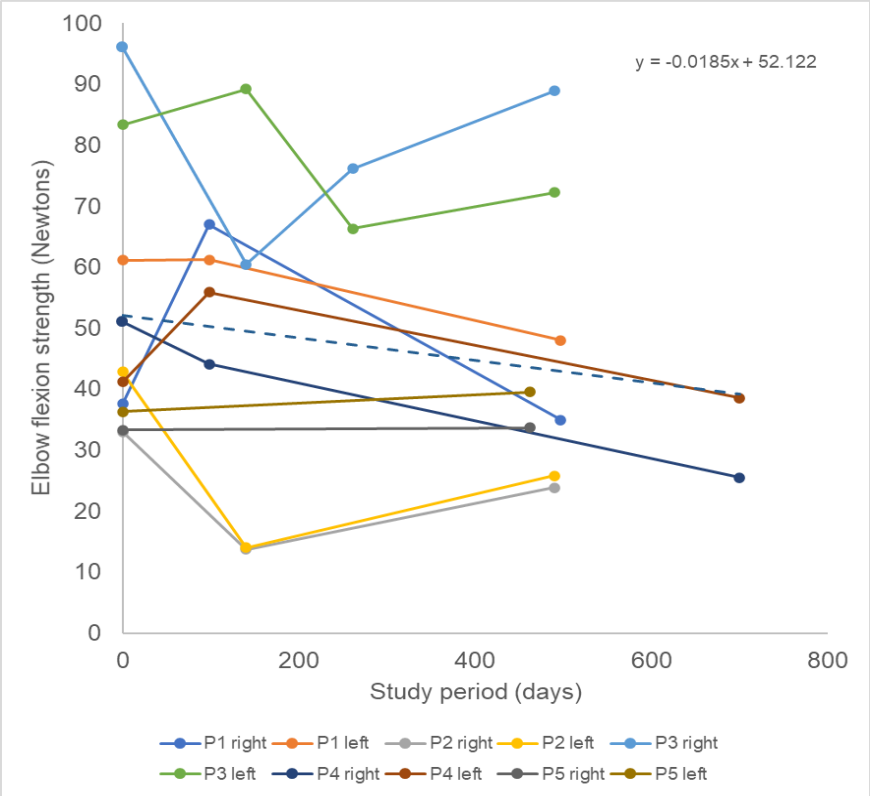


Figure 5-29: Line chart shows the elbow flexion strength for five participants during the study period.

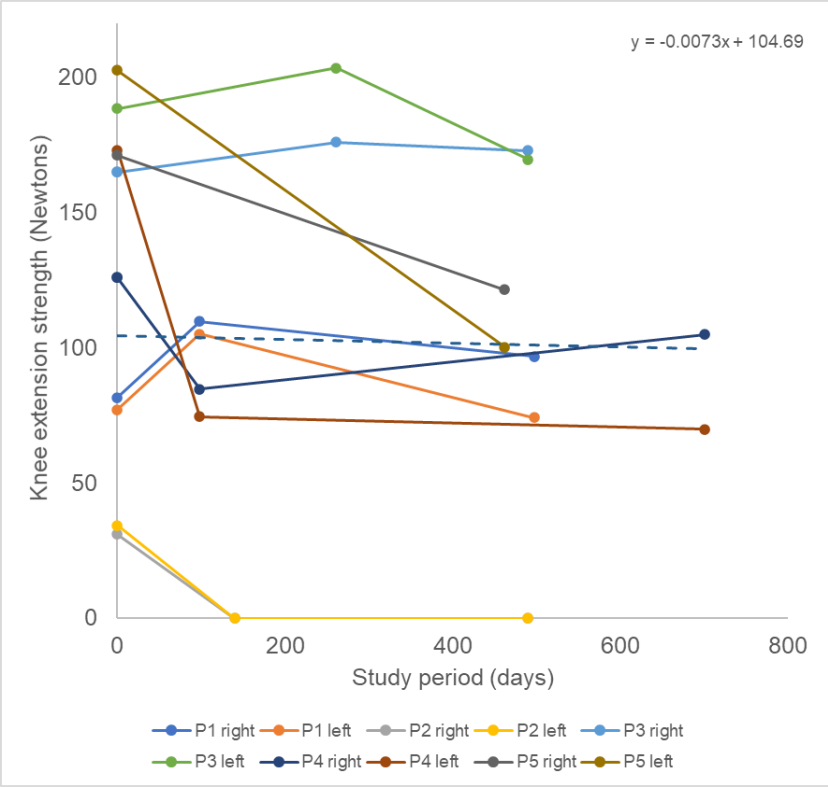


Figure 5-30: Line chart shows the knee extension strength for five participants during the study period.

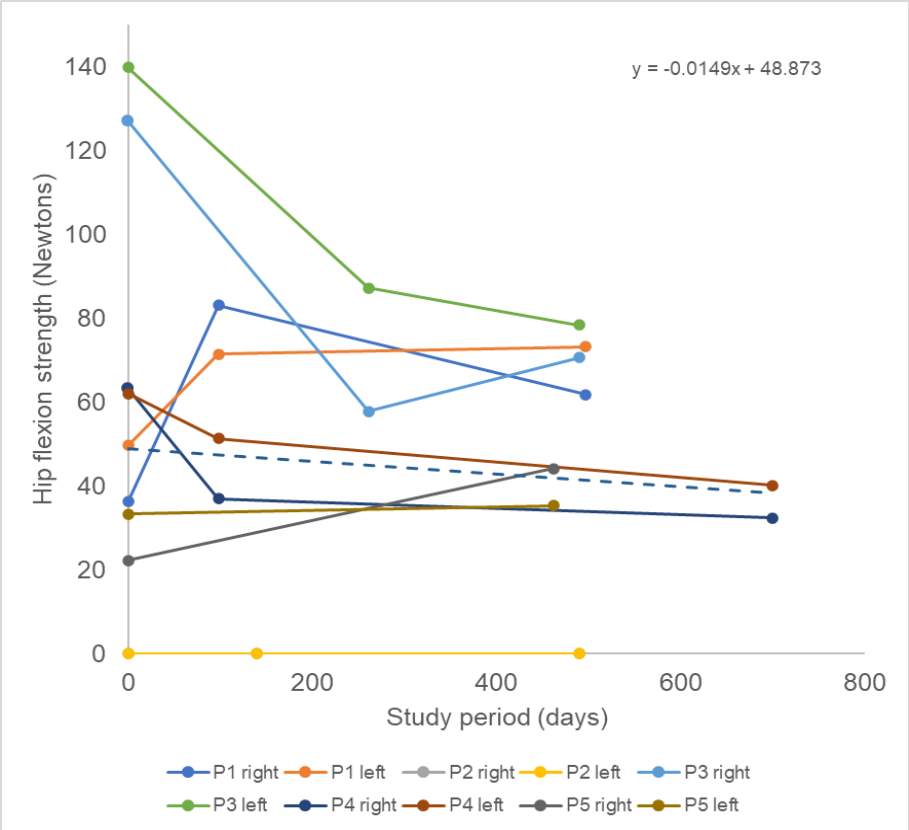


Figure 5-31: Line chart shows the hip flexion strength for five participants during the study period.

5.4.15 Six-minute walk test

The six-minute walk test (6MWT) was not undertaken by five of the participants (P6, P7, P8, P9 and P10) who had home visits. There was no appropriate space in their home environments to undertake these tests safely. Of those who attended the research site, only four participants (P1, P3, P4 and P5) completed the 6MWT. Being a wheelchair user, P2 did not participate in this assessment. In 6 minutes, P1, P3, P4 and P5 walked 280m, 569m, 125m and 405m, respectively. The average distance covered by these four participants was 318m (SD 151 95%CI 78-561).

None of the participants reached their individual predicted distances or their lower limit of normal distances (Figure 5-32). The difference between the predicted distances and the actual distances were statistically significant, t(3)=-10.1, p=0.002). On average, the percentage of distance covered was 39.8% (SD 16.7, 95%CI 13.2-66.3). Although Spearman's rank-order correlation determined a strong and negative relationship between the 6MWT and the NMDAS (Figure 5-33), the statistical significance was not reached, likely due to the small number (n=4) in this study.

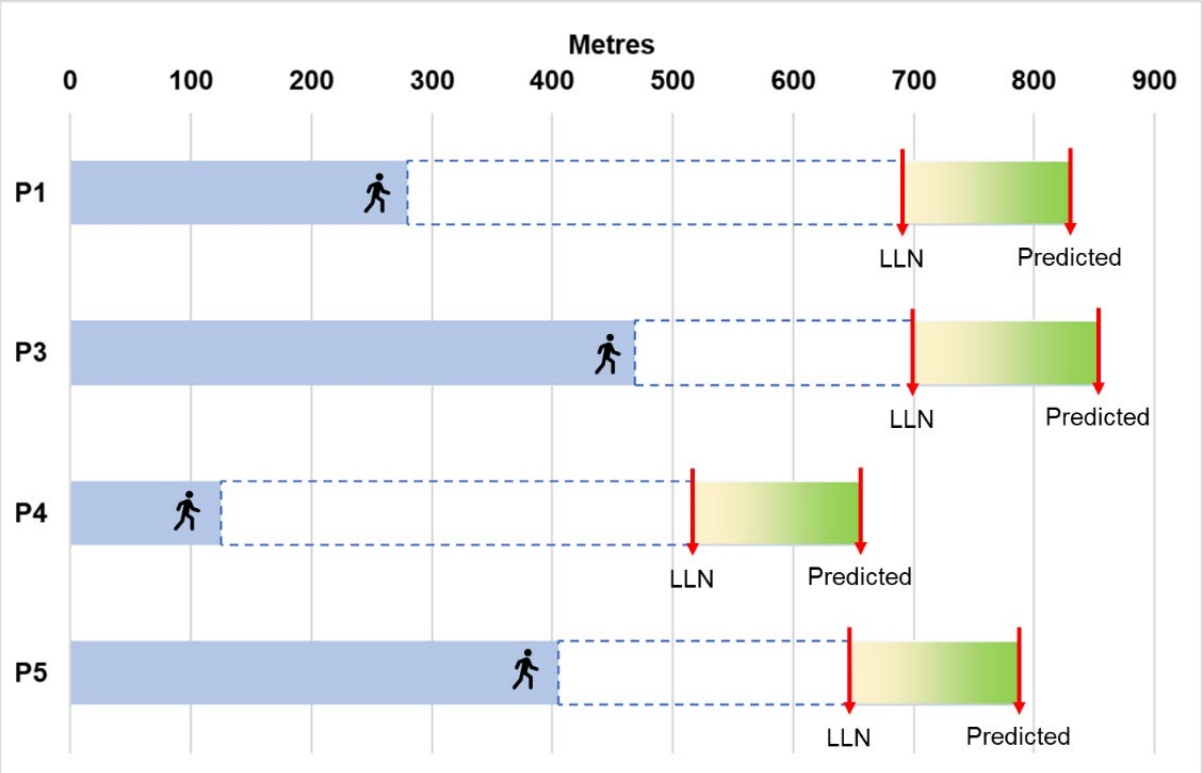


Figure 5-32: Figure shows the distance (in metres) completed by four participants in the six-minute walk tests. None of them reached their lower limit of normal (LLN) or their predicted distances.

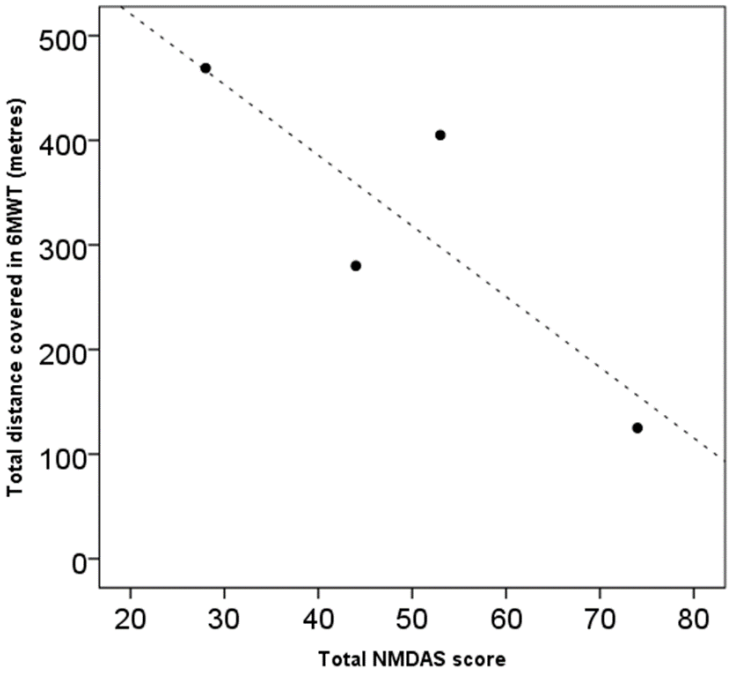


Figure 5-33: Scatter plot shows the relationship between the distance covered in the 6-minute walk test and the total NMDAS scores for the four participants in this study. A linear regression line (dashed line) is also shown.

5.4.16 A longitudinal study on the 6-minute walk test

The four participants who completed the 6MWT at baseline assessments returned to the research sites for further assessments during the study period. Their results are summarised in Figure 5-34. At the end of the study period, three of them (P3, P4 and P5) had not been able to walk further than the distance they managed at their respective baseline assessments. As a group, the average distance for these four participants was 345 metres (SD 188.3, 95%CI 45.1-644.4) at baseline and 312 metres (SD 219.4, 95%CI -37.4-660.8) at end-of-study assessments. The difference was not statistically significant (t(4)=0.85, p=0.44). These four participants lost on average 18 metres per annum in the 6 MWT (SD 47.8, 95%CI -41.6-77.2).

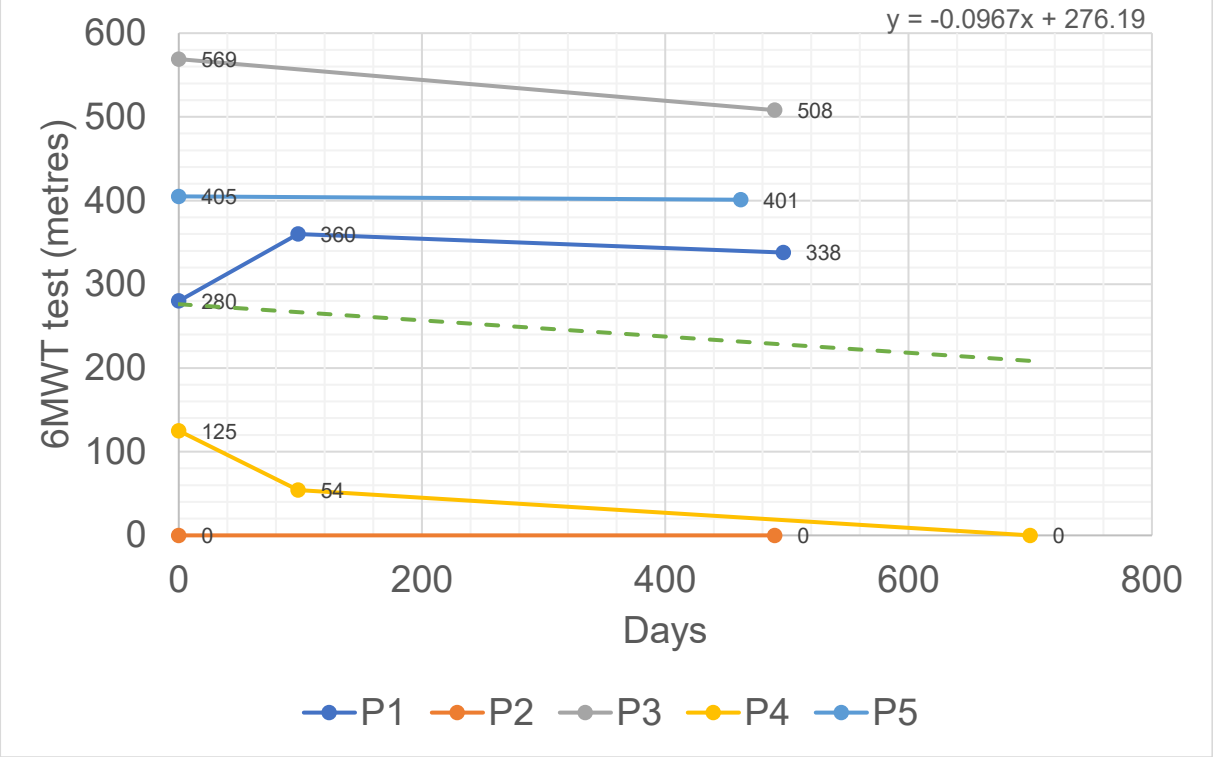


Figure 5-34: Line chart shows the distance achieved in the 6-minute walk test (6MWT) for five participants during the study period, including trendline (dashed green).

5.4.17 Walking speeds

Four of the participants who had taken part in the 6MWT also walked 10-metre distances at self-selected speeds and at their fastest walking speeds (Table 5-9). In a 10-metre distance, the average self-selected speed of walking of 0.9 m/s (SD 0.4 95%CI 0.3-1.5) and the fast-paced speed of walking of 1.7m/s (SD 0.8, 95%CI 0.4-3.0) for these participants was lower than predicted but they were not statistically significant. These participants increased their speed significantly between self-selected speeds and fast-paced speeds, $t(3)=-3.6$, $p=0.036$.

There was a negative, strong, and statistically significant relationship between walking speed and NMDAS scores. As the disease burden (total NMDAS score) increases, both the self-selected pace ($r=-0.96$, $p=0.37$) (Figure 5-35) and fast walking speed ($r=-0.97$, $p=0.28$) (Figure 5-36) fall. Of all the items in the NMDAS, the exercise tolerance score correlated strongly and significantly with the self-selected walking speed ($r=-0.97$, $p=0.027$) (Figure 5-37) and fast-paced walking speed ($r=-0.99$, $p=0.007$) (Figure 5-38). No other items in the NMDAS have demonstrated significant correlations.

	P1	P3	P4	P5
Self-selected speed (m/s)	1.1	1.3	0.4	0.7
Fast-paced speed (m/s)	2.2	2.5	0.6	1.5
Predicted self-selected speed* (m/s)	1.4	1.4	1.4	1.4
Predicted fast-paced speed* (m/s)	2.5	2.5	2.3	2.5

*Table 5-9: Table shows the speed of walking at both self-selected pace and fast face for the five participants. * predicted speeds are based on published reference values (Bohannon and Andrews, 2011).*

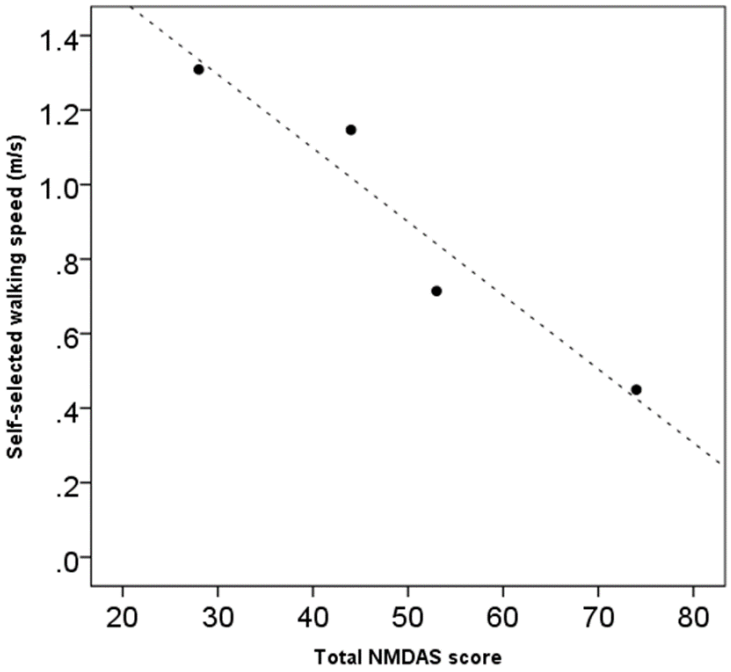


Figure 5-35: Scatter plot shows the relationship between the self-selected walking speed and the total NMDAS scores for the four participants in this study. A linear regression line (dashed line) is also shown.

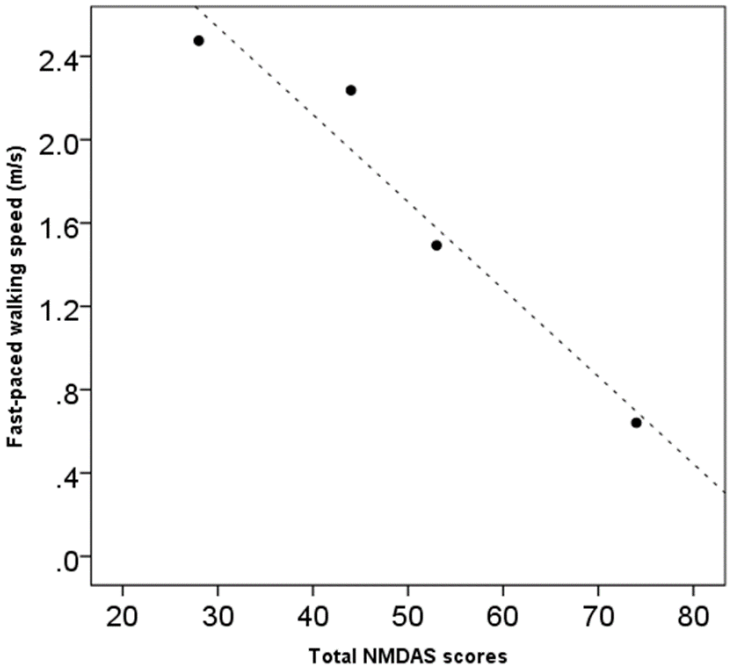


Figure 5-36: Scatter plot shows the relationship between the fast-paced walking speed and the total NMDAS scores for the four participants in this study. A linear regression line (dashed line) is also shown.

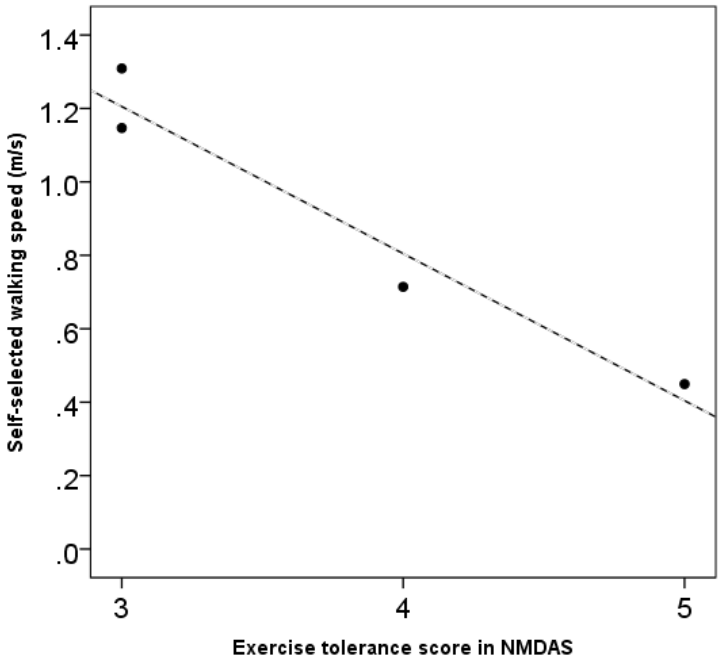


Figure 5-37: Scatter plot shows the relationship between the distance covered in the self-selected walking speed and the exercise tolerance score in the NMDAS for the four participants in this study. A linear regression line (dashed line) is also shown.

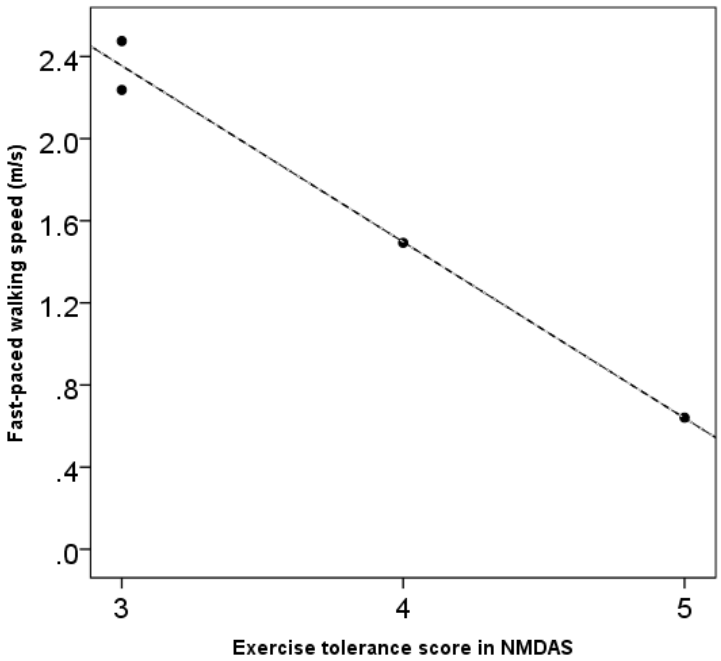


Figure 5-38: Scatter plot shows the relationship between the distance covered in the self-selected walking speed and the exercise tolerance score in the NMDAS for the four participants in this study. A linear regression line (dashed line) is also shown.

5.4.18 A longitudinal study on walking tests

The four participants who had their walking speeds measured at baseline assessments returned to the research sites for further assessments during the study period. Their walking speeds at their self-selected pace and their fastest pace are summarised in Figure 5-39 and Figure 5-40, respectively. As a group, the average walking speed at a self-selected pace for these four participants was 0.94 m/s (SD 0.44, 95%CI 0.24-1.64) at baseline and 0.91 m/s metres (SD 0.48, 95%CI 0.14-1.67) at end-of-study assessments. Although their average self-paced walking speed declined by 0.02m/s per annum (SD 0.115, 95%CI -0.158-0.127), there was no statistical difference between the baseline and end-of-study assessments (t(4)=0.449, p=0.677). Similarly, there was no statistical difference in the fast-paced walking test (t(4)=0.793, p=0.472) despite a fall of 0.10 m/s per annum (SD 0.318, 95%CI -0.493-0.297). Their average fast-paced walking speed was 1.71 m/s (SD 0.82, 95%CI 0.40-3.03) and 1.52 m/s (SD 1.09, 95%CI -0.20-3.25) at baseline and end-of-study assessments, respectively.

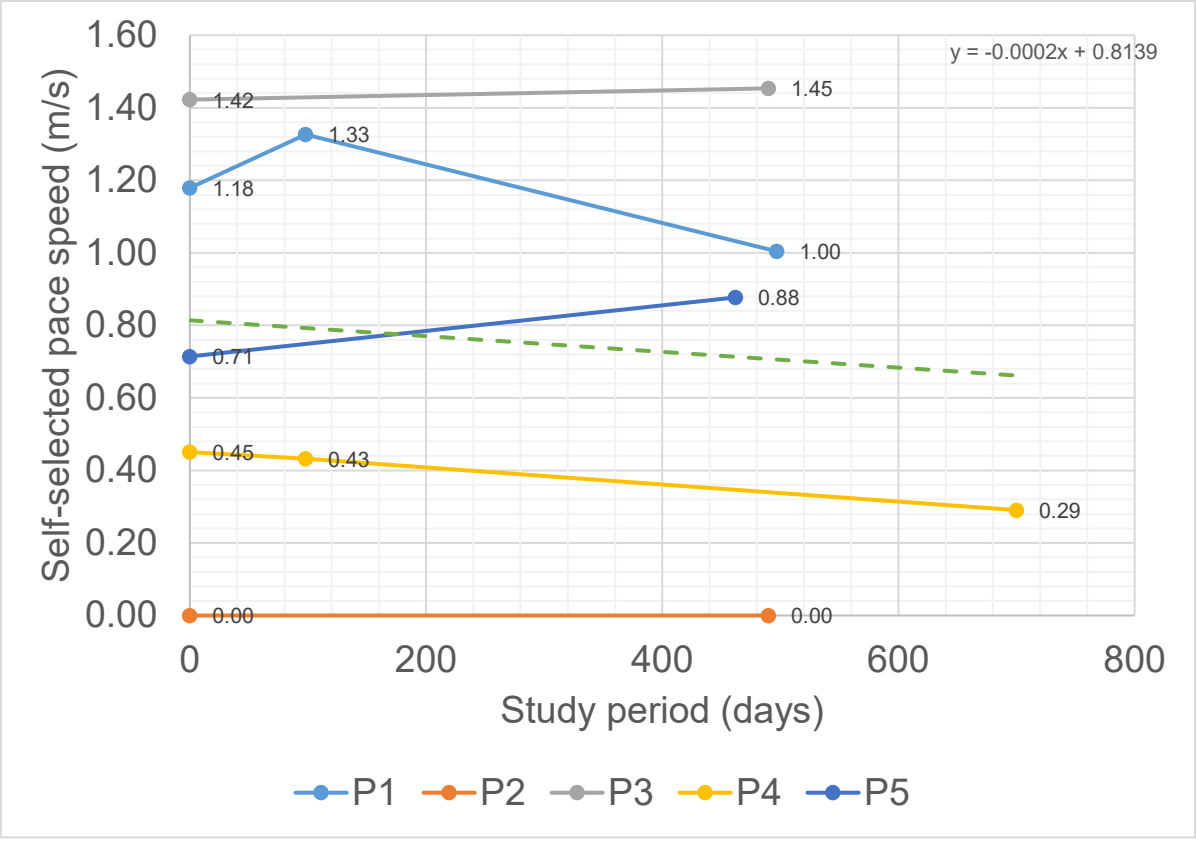


Figure 5-39: Line chart shows the self-selected walking speed for five participants during the study period, including trendline (dashed green).

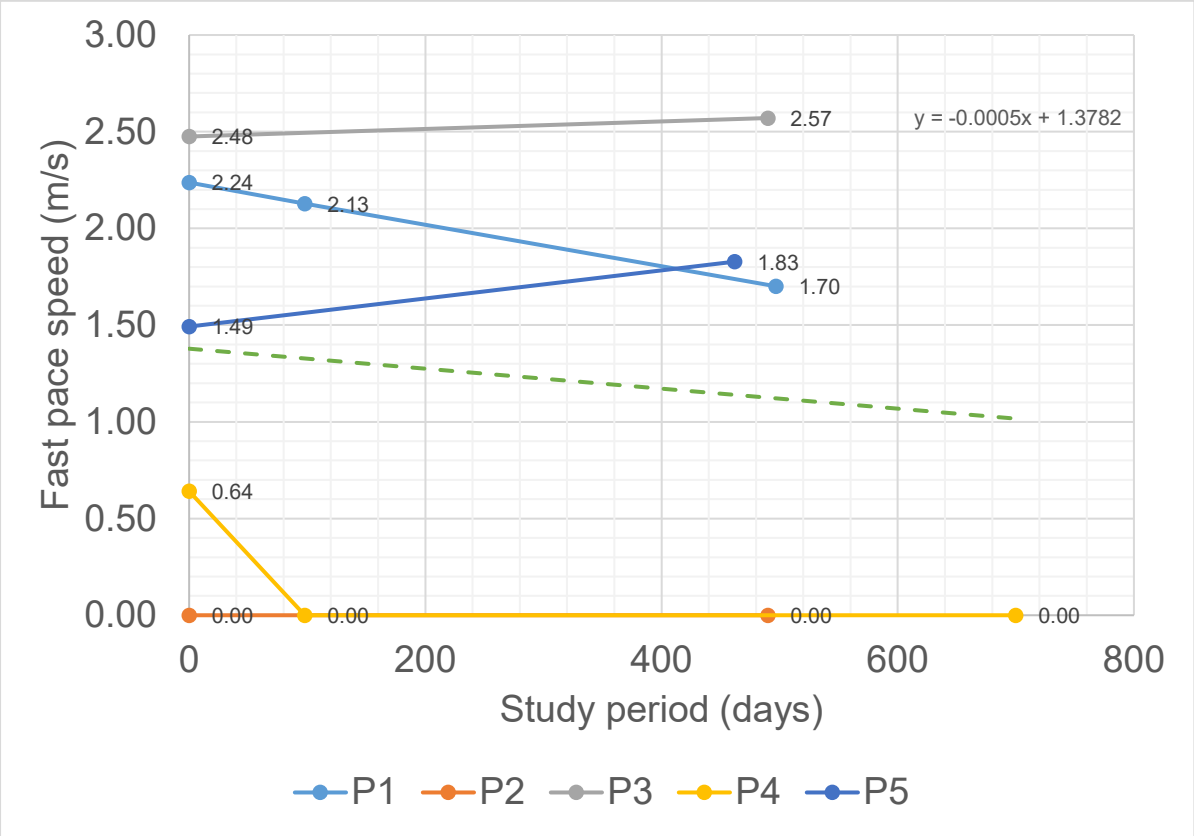


Figure 5-40: Line chart shows the fast-paced walking speed for five participants during the study period, including trendline (dashed green).

5.4.19 30-second sit to stand

Of the ten participants at research sites and at home, only four (P1, P3, P5, P6) were able to attempt the 30-second sit to stand tests, along with the average number of complete stands for healthy older populations (Jones et al., 1999) (Table 5-10). The remaining six participants reported that they had been limited by their ability to stand unaided repeatedly and it was deemed unsafe to proceed. P1 and P5, both females, performed four and nine complete stands, respectively but these were below the lower limit of an average number of twelve stands. Likewise, P3 and P6, both males, who completed ten and six stands respectively, were also lower than the lower limit of an average of fourteen stands. The 30-second sit to stand test was not repeated in subsequent interim or end-of-study visits because of suboptimal performance and safety concerns.

Participant	Number of complete stands in 30 seconds	The average number of complete stands for 60-64 years old (based on sex)*	The average number of complete stands for 90-94 years old (based on sex)*
P1	4	12 – 17 (female)	4 – 11 (female)
P3	10	14 – 19 (male)	7 – 12 (male)
P5	9	12 – 17 (female)	4 – 11 (female)
P6	6	14 – 19 (male)	7 – 12 (male)

Table 5-10: Table summarises the number of complete stands in 30 seconds for four participants who managed to do this test safely. The remaining participants had not undertaken this outcome measure. These four participants performed worse than the average 60–64-year-olds and on par with 90-94 year-olds based on published reference values (Jones et al., 1999).*

5.4.20 100ml water swallow test

All participants completed the timed water swallow test except for P10, who had stopped the test after drinking 25ml because of coughing episodes and declined further repeat testing. The results of the timed swallow test of 100ml of water of the nine participants are summarised in Table 5-11. The mean time taken to consume 100mls of water was 24.8 seconds (SD 17.0, 95%CI 11.7-37.9) and the mean time per swallow was 4.1 seconds (SD 2.8, 95%CI 2.0-6.3). For each swallow, the average volume was 19.0mls (SD 7.9, 95%CI 12.9-25.0).

The mean swallowing speed for these participants was 6.9 ml per second (SD 5.8, 95%CI 2.4-11.4). These results were significantly slower than their individual predicted swallowing speed at a mean speed of 31.8 ml per second (SD 6.9, 95%CI 26.6-37.1), $t(8)=-15.7$, $p<0.001$ (Figure 5-41). Their swallowing speed made up 20.3% (SD 14.3, 95%CI 9.4-31.3) of the predicted speed for their respective ages.

ID	P1	P2	P3	P4	P5	P6	P7	P8	P9
Amount taken (ml)	100	100	100	100	100	100	100	100	100
Number of swallows	7	4	4	14	6	6	8	3	5
Time taken (seconds)	23.2	27.2	5.5	34.0	6.9	60.0	34.4	11.0	20.7
Volume per swallow (ml per swallow)	14.3	25.0	25.0	7.1	16.7	16.7	12.5	33.3	20.0
Average time per swallow (seconds)	3.3	6.8	1.4	2.4	1.1	10.0	4.3	3.7	4.1
Swallow capacity (ml/s)	4.3	3.7	18.1	2.9	14.6	1.7	2.9	9.1	4.8
Predicted swallowing speed * (ml/s)	32.3	30.1	47.6	26.2	31.9	28.2	25.1	28.5	36.6
Percentage of predicted swallow speed (%)	13.4	12.2	38.1	11.2	45.8	5.9	11.6	31.8	13.2

Table 5-11: Table summarises the results of the 100-ml water swallow test for nine participants. P10 had not completed the assessment safely. The predicted speed of swallowing is based on a reference equation by Nathadwarawala et al. (1992)*

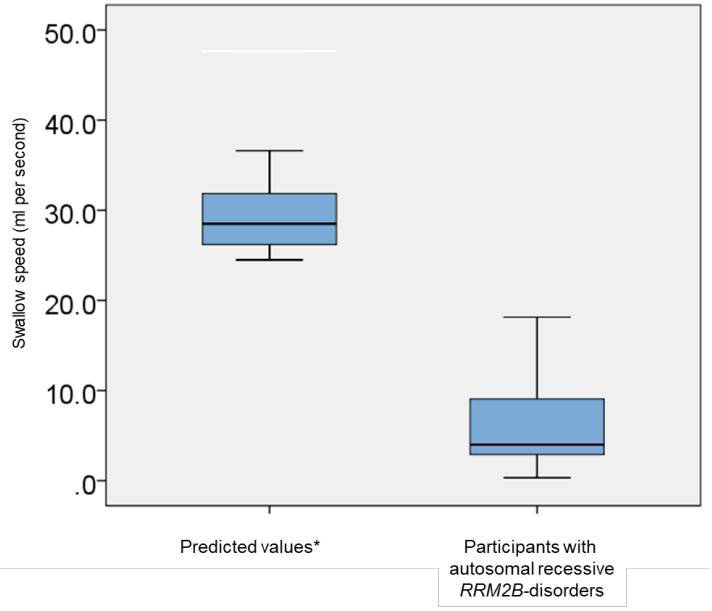


Figure 5-41: Boxplot shows the difference between the predicted values for water swallow speed (right) and those obtained by the nine participants with autosomal recessive RRM2B-disorders (left).

A Spearman's rank-order correlation was run to determine the relationships between their swallowing speeds and related items in the NMDAS in Table 5-12. There was a strong and negative correlation for both swallowing speeds and their percentages with gastrointestinal involvement (r=-0.71 to -0.79, p=0.012-0.03) (Figure 5-42). Although swallowing speed had a strong negative correlation with the NMDAS swallowing function, only the percentage of predicted swallowing speed was statistically significant (r=-0.73, p=0.027) (Figure 5-43).

NMDAS items	Swallow speed (ml/s)	Percentage of predicted swallow speed (%)
Speech CF	-0.248	-0.291
Swallowing CF	-0.638	-0.726*
Exercise tolerance CF	-0.105	-0.105
Gait stability CF	-0.443	-0.528
Gastrointestinal	-0.785*	-0.709*
Dysphonia/Dysarthria	-0.188	-0.248
Myopathy	-0.442	-0.624
Cerebellar ataxia	-0.505	-0.505
Total NMDAS scaled scores	-0.328	-0.429

Table Table 5-12: summarises the Spearman's rank-order correlation coefficients between the swallow speed and the items in the clinician-rated NDMAS.

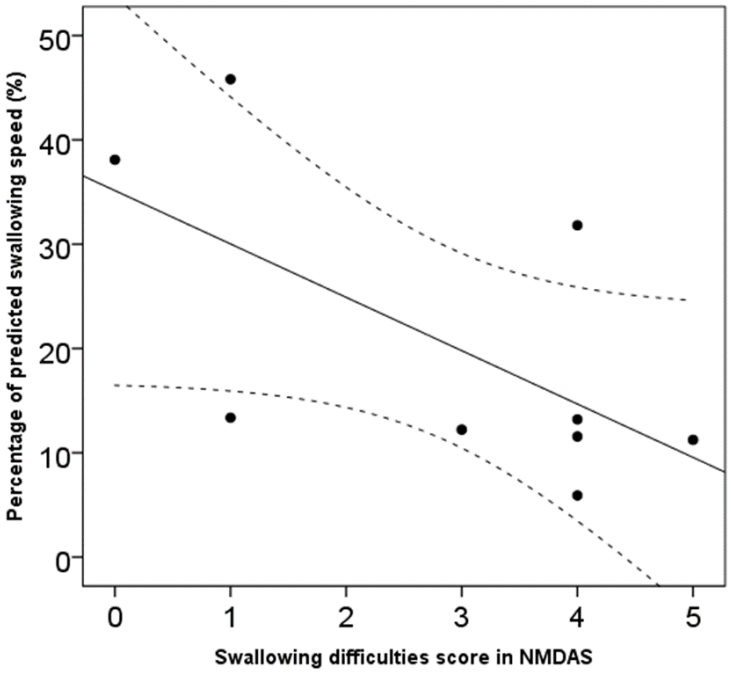


Figure 5-42: Scatter plot shows the relationship between the percentage of predicted swallowing speed and the swallowing difficulty score in the NMDAS for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

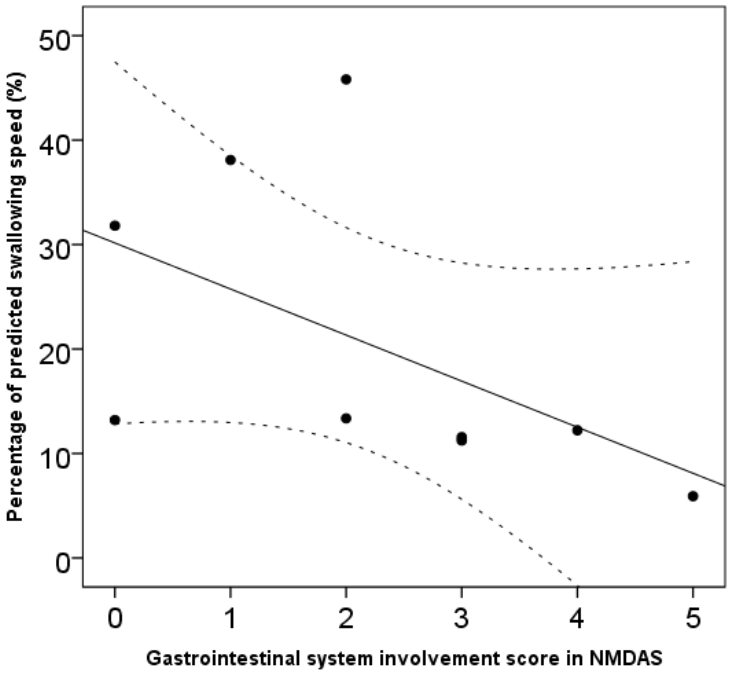


Figure 5-43: Scatter plot shows the relationship between the percentage of predicted swallowing speed and the gastrointestinal system involvement score in the NMDAS for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

5.4.21 A longitudinal study on 100ml water swallow test

Five participants have interim follow-up visits at home and have returned to the research site for end-of-study visits. Their swallowing speed in the 100ml water swallow test (ml/s) for these five participants recorded during these visits is summarised in Figure 5-44. At the end of the study period, the swallow speed for four participants (P1, P2, P4 and P5) had decreased from those obtained at their respective baseline assessments. As a group, the average swallowing speed for these participants was 8.7 ml/s (SD 7.1, 95%CI -2.6-20.0) at the baseline and 5.3 ml/s (SD 7.4, 95%CI -6.4-17.0) at the end of the study. Whilst these participants might have lost swallowing speed by 2.6 ml/s per annum (SD 4.1, 95%CI -2.4-7.6), the difference was not statistically significant (t(4)=1.492, p=0.210).

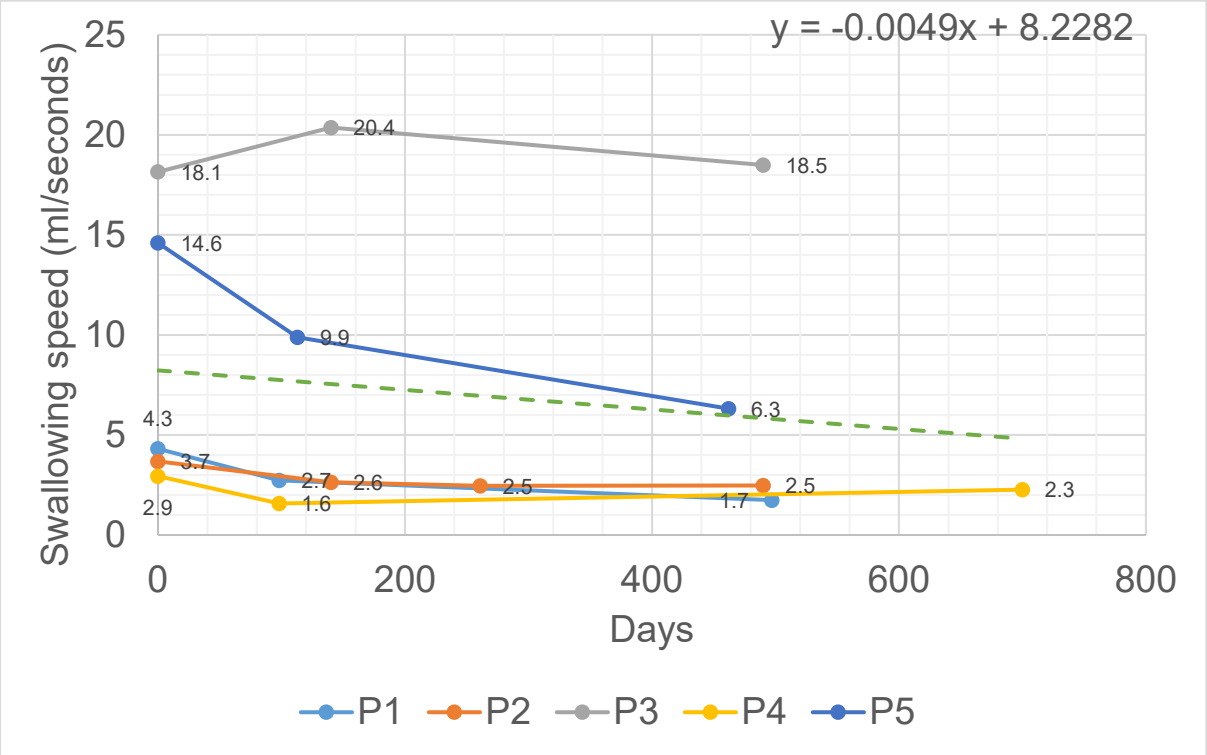


Figure 5-44: Line chart shows the swallowing speed in the 100ml water swallow test for five participants during the study period, including trendline (dashed green).

5.5 Discussion

Several key findings emerge from this study of outcome measures in participants with autosomal recessive *RRM2B* gene variants. First, respiratory weakness, hearing problems, chronic external ophthalmoplegia (CPEO), ptosis and exercise intolerance in the NMDAS featured prominently in this group of patients. These features had also been graded on the extreme end of the spectrum throughout the study period. CPEO and ptosis appear to be universal attributes of this condition, in line with the findings from previous clinical cross-sectional studies on patients who harboured the pathogenic variants in the *RRM2B* gene (Tyynismaa et al., 2005, Shaibani et al., 2009, Fratter et al., 2011, Pitceathly et al., 2012). Pitceathly and colleagues (2012) had included autosomal dominant variants of the *RRM2B* gene in their cohort of 31 patients. There were only four participants in that study with autosomal recessive variants. They shared similar characteristics with this cohort of ten participants. The disease severity for those four participants was not rated using the NMDAS. Therefore, the NMDAS data in this study have contributed a more precise definition of the disease severity for this group of rare mitochondrial disorders. Furthermore, the follow-up results throughout the study have also provided new insights into the longitudinal change of the disease severity.

There was a delay in achieving confirmatory genetic diagnoses for these patients. While the average age of disease onset was during their childhood at about 11 years, the average age at which these participants had confirmed genetic diagnosis of *RRM2B*-related mitochondrial disease was in their adulthood, at about 27 years. This delay could be due to several factors. The disease onset at an early age might be non-specific for instance hearing loss or tiredness which could be due to other more common conditions. Furthermore, there might be a lack of pattern recognition of this rare condition among clinicians. Mitochondrial disorders are notoriously difficult to diagnose reliably due to their clinical heterogeneity. The diagnostic odyssey of mitochondrial patients might take many years and average consultations with eight clinicians before arriving at their final diagnoses (Grier et al., 2018). In addition to that, they might have to undergo protracted investigations including blood tests, muscle

biopsies, neuroimaging (MRI) specialist metabolic tests and genetic tests. Since pathogenic variants of *RRM2B* are associated with 'mitochondrial DNA depletion' on initial tests, some of those in this cohort of participants had been given this label as their diagnoses before their variants were confirmed at much later rounds of genetic investigations.

One interesting observation of the phenotype of this cohort of participants was the low body-mass index (BMI), only ranging from 11 to 24 kg/m2. Low BMI is invariably reported in mitochondrial disorders in the literature. In a French cohort, the mean body mass index (BMI) was 20.2 with a range of 13.5 to 27.1 kg/m2 (Guillausseau et al., 2001). Whittaker and colleagues reported a mean BMI of 22.3 kg/m2, in m.3243A > G variant carriers (Whittaker et al., 2007), whereas in a Japanese study the patients had a mean BMI of 20.4 kg/m2 (Suzuki et al., 2003). In cohorts of patients harbouring the pathogenic variants in *RRM2B* gene, low BMI had been reported in numerous cases (Pitceathly et al., 2012). The cause of low BMI could be multifactorial. At a histological level, mitochondrial myopathy patients demonstrated a higher ratio of type II to I fibers and a tendency towards the lower cross-sectional area, indicating potential skeletal muscle atrophy (Gehrig et al., 2016). Having less muscle bulk translates to lower weight. This skeletal muscle atrophy is likely to be driven by dysfunctional mitochondria which trigger catabolic signalling pathways that feed-forward to the nucleus to promote the activation of muscle breakdown(Romanello and Sandri, 2016). Apart from the inherent muscle atrophy, there are also other external factors. Patients with mitochondrial diseases were known to have reduced oral intake due to dysphagia, gastroparesis, and intestinal pseudo-obstruction (Read et al., 2012, de Laat et al., 2015, Ng et al., 2016a). In essence, the low BMI in this cohort was consistent with similar observations made by other studies in mitochondrial disorders.

The clinical constellation of CPEO, ptosis, myopathy, hearing loss, bulbar dysfunction and fatigue experienced by these participants with autosomal recessive *RRM2B* variants appear to overlap with those with autosomal dominant inheritance (Tyynismaa et al., 2005, Shaibani et al., 2009, Fratter et al., 2011, Pitceathly et al., 2012) and it is generally accepted that autosomal recessive *RRM2B* patients have more severe phenotypes with multisystem involvement (Pitceathly et al., 2012, Sommerville et al.,

2014). The severity of recognised phenotypes and the multi-systemic involvement in these participants had been captured in the NMDAS, which showed considerable disease burden at a mean total score of 53.8. However, the lack of publications on disease ratings for those with autosomal dominant *RRM2B* disease limits any direct comparisons to test this hypothesis. It was also beyond the scope of this study to include an autosomal dominant comparison study arm. On the other hand, these participants in this study are clearly distinct from another form of autosomal recessive *RRM2B* disorder, the infantile-onset mitochondrial DNA depletion syndrome. This syndrome due to biallelic pathogenic *RR2MB* variants presents with encephalomyopathy features in the very first months of life and rapidly deteriorated with respiratory failure leading to assisted ventilation (Bourdon et al., 2007, Bornstein et al., 2008, Kollberg et al., 2009, Keshavan et al., 2020). In contrast, participants in this study did not deteriorate significantly during the study period, with an increment of approximately 1 NMDAS point per annum.

The respiratory muscle strength in this group of participants was significantly weaker than their predicted values in all the performance outcome measures that quantified their FVC, FEV_1, SNIP, MIP and MEP. This data confirms the respiratory muscle weakness previously described in mitochondrial disease (Kim et al., 1991, Cros et al., 1992, Amornvit et al., 2014) and in those who harboured the pathogenic *RRM2B* gene variants (Fratter et al., 2011, Pitceathly et al., 2012, Keshavan et al., 2020). However, there is still limited published results on quantitative respiratory muscle profile for mitochondrial patients, particularly pathogenic *RRM2B variants*, to make meaningful comparisons with this study. The FVC and FEV1 have previously been reported as normal in heterogeneous cohorts of mitochondrial patients (Flaherty et al., 2001, Montano et al., 2020). Thus, this study has highlighted the distinct nature of respiratory weakness in this genotype among other mitochondrial diseases. Of the ten participants, seven of them had been using non-invasive ventilation therapy at night. The use of NIV is one of the hallmarks of severe respiratory weakness. Future studies should consider analysing the use of nocturnal NIV in more depth. It would be helpful to know how the use of nocturnal NIV affect the retention of CO2 and the quality of sleep in sequential sleep studies. Sleep studies could be conducted before and after the NIV to measure effect size of this intervention.Another interesting correlation is the

significant relationship between SNIP and clinician-rated speech difficulties. SNIP has been widely studied in other neurological conditions which affect bulbar function (Chaudri et al., 2000, Murray et al., 2019). Given the strong correlation shown in this group of participants, SNIP could potentially be a good outcome measure of their bulbar function. This study also goes beyond the current published literature on mitochondrial disease to demonstrate the degree of weakness in various respiratory muscle tests and the longitudinal changes during the study period. Of all the parameters that measured respiratory muscle strength, the FVC for five participants reduced significantly at the end of the study, falling by about 5% per annum. Such dramatic decline in FVC in this group of patients is comparable to the 5.9% yearly loss of FVC in myopathic patients with Duchenne muscular dystrophy who had not been treated with glucocorticoids (McDonald et al., 2018). It is difficult to explain the lack of significant deterioration in SNIP, MIP and MEP for the *RRM2B* patients in this study, but this could be due to the 'floor effect' of these tests; these myopathic participants might be so near the lower limit of these scales to test their respiratory weakness that any variance is no longer discernible.

Similarly, the quantitative muscle dynamometry, which showed low levels of strength in skeletal muscle groups, had not changed significantly during the study. In all the participants with autosomal recessive *RRM2B* gene variants in this study, their muscle strength in shoulder abduction, elbow flexion, knee extension and hip flexion movements were substantially lower than those expected for the age and sex. Muscle weakness as a result of mitochondrial myopathy is a well-recognised phenomenon (DiMauro et al., 1985, Gorman et al., 2016, Vincent et al., 2016). The skeletal muscle fibres rely on their large number of mitochondria, known for their role in ATP synthesis, to fuel the energy-demanding process of muscle contraction (Chance and Williams, 1956, Vincent et al., 2016). Those participants who had pathogenic variants in the *RRM2B* gene failed to encode p53-controlled ribonucleotide reductase to maintain mtDNA (Bourdon et al., 2007, Bornstein et al., 2008), are likely short of mitochondria for sustained muscle contraction. The findings from these myopathic participants also agree with reduced contractile properties in thigh and calf muscle found in patients with other forms of mitochondrial myopathies (Poulsen et al., 2019). In these participants with autosomal recessive *RRM2B* variants, their proximal muscles in the

hips and shoulder appear to be affected to a greater degree than their distal muscles groups. Proximal muscle weakness is recognised in most mitochondrial myopathies (Chinnery and Turnbull, 1997, Ahmed et al., 2018) and also those associated explicitly with pathogenic variants of the *RRM2B* gene (Pitceathly et al., 2012, Keshavan et al., 2020). Some argued that muscle contraction in mitochondrial myopathy could be affected by peripheral neuropathy or central nervous system involvement (Mancuso et al., 2012b, Lax et al., 2017). The likelihood of neurogenic involvement in this group of participants is probably minimal due to the absence of such evidence from their clinical assessments. However, this study did not have electrophysiology studies to exclude this possibility.

Their poor muscle strength in the lower limbs is also translated to significantly reduced performance in the functional tests in this study. All four of the participants who can take part in the 6-minute walk tests (6MWT) could not reach the lower limit of normal distance expected of them at baseline or at end-of-study assessments. The 6MWT is a widely used outcome measure in neuromuscular disease research (Takeuchi et al., 2008, Mcdonald et al., 2013). A group of Italian mitochondrial patients of different genotypes walked significantly shorter distances (366-429 metres ± 44-57) than their predicted distance in the 6MWT (Montano et al., 2020). In the UK, mitochondrial patients with m.3243A>G point mutations also performed worse than controls in the 6MWT (486 metres ± 107 vs 513 metres ± 52) but the difference was not statistically significant (Newman et al., 2015). For comparisons, the participants in this study who, on average, could only reach 318 metres ± 151, and these were significantly lower than their predicted distances. The poorer performance in this group with autosomal recessive *RRM2B* variants could partly be due to their higher disease burden (NMDAS score) compared to the m.3243A>G group and the Italian cohort (53.9±12.9 vs 16±8 and 18.5±10.5). However, there might be other unexplored factors that affect 6MWT because, similar to the m.3243A>G and the Italian cohorts (Newman et al., 2015, Montano et al., 2020), there was no significant correlation between their 6MWT distances and their respective NMDAS scores. The inability of participant to complete or achieve their lower limit of normal in the 6MWT limits the usefulness of this test in future trials. Furthermore, those participants who had mobility or significant health problems (e.g. P5-P10 in this study) might not travel to the research site to perform

this arduous test. The walking speed over the 10-metre distance had demonstrated strong and significant correlations with their NMDAS scores. This correlation between walking speed and NMDAS score was also observed in the m.3243A>G cohort (Newman et al., 2015). In addition to the NMDAS score, the walking speed of this group of participants with *RRM2B* variants also correlated strongly with their exercise intolerance severity rating, although the strength of this correlation could be affected by the small number of participants in this study. In essence, the 6MWT and walking speeds results have shed further light on their suitability and relevance as functional tests in mitochondrial disease research.

In contrast, the 30-second sit-to-stand test (STS) is unlikely to be suitable as an outcome measure for clinical research in myopathic patients with autosomal recessive *RRM2B* gene variants. All ten participants in this study were offered the opportunity to participate in this functional test at home or research sites, but only four could manage to complete it. Not only did these four participants poorly, but STS was also deemed unsafe because of the risk of falls during the assessments. The STS, recommended by the international workshop (Mancuso et al., 2017), was initially included in this study based on its extensive use as a measure of lower limb strength (Jones et al., 1999, McCarthy et al., 2004, Bohannon, 2009) and its potential advantage as a remote functional test at home settings instead of repeated visits to research sites. The results of STS from the four participants were worse or, at best, on par with healthy 90 to 94-year-old adults (Jones et al., 1999, Rikli and Jones, 2012). STS use in other groups of mitochondrial diseases has demonstrated a difference from control groups (Newman et al., 2015, Montano et al., 2020). However, those mitochondrial disorders differ from this group of participants in terms of disease burden and ambulation. Some of the severely myopathic participants in this study are wheelchair dependent, and STS is possibly able to discriminate between ambulant and non-ambulant patients in a binary fashion. Compared to timed functional tests, the ordinal variable obtained from those who participate in the STS test also limits the sensitivity to changes. Furthermore, some researchers critically questioned the use of STS and have demonstrated its over-dependence on other factors such a balance and psychological status (Lord et al., 2002, Netz et al., 2004). Taken together with the findings from this study, the STS

is not a feasible or sustainable outcome measure for lower limb strength in this group of patients with autosomal recessive *RRM2B* gene variants.

Upper limb dexterity in these participants has been shown to be significantly reduced in the nine-hole peg tests. In this cohort, the time taken to complete the nine-hole peg test correlated significantly with exercise intolerance, visual acuity and total NMDAS score. However, there was no direct correlation with other activities of daily living in the NMDAS, raising doubts on the initial notion that upper limb dexterity of these participants affects handwriting, dressing, utensil use and personal hygiene. However, the effect size on these domains in the NMDAS might be diminished due to the small number of patients assessed in this study. Despite taking a longer time to complete the tests at the end of the study than at baseline, the difference at these two time points of assessment was not statistically significant. Furthermore, there was an improvement in the performance of this test at the interim visits for some participants. This learning effect might have affected the results when they were asked to do the tests in the longitudinal arm of the study. The magnitude of the learning effect probably differs between participants. This learning effect for the peg tests had also been observed seen in other conditions and appeared to become less pronounced over time (Haverkate et al., 2016, Granström et al., 2019). To address this learning effect, future trials could consider giving a few opportunities for participants to practice the test before recording their best efforts. There is currently no published research on the use of the nine-hole peg test as an outcome measure in mitochondrial disease research for comparison despite being recommended by the international workshop for mitochondrial research (Mancuso et al., 2017). Thus, to my knowledge, this present study is the first to generate data on nine-hole peg tests values for myopathic patients with mitochondrial dysfunction along with its longitudinal changes.

Another outcome measure that had generated new data for mitochondrial research is the results from 100ml water swallow tests. Coupled with the scores from clinician-rated NMDAS, these findings have substantiated the reports of bulbar dysfunction in *RRM2B* deficiency by previous cross-sectional studies (Fratter et al., 2011, Pitceathly et al., 2012), but there is currently no published data on this genotype for comparison. The swallowing speed of 6.9ml/s achieved by the participants in this study was

significantly lower than their predicted values. Montano and colleagues (2020) have demonstrated that the swallowing speed for 103 Italian patients with other forms of mitochondrial disease of 8.7ml/s was slower than normal values, but the difference was not statistically significant. They also found that the swallowing speed of these patients correlated significantly with their NMDAS scores (Montano et al., 2020).

Contrary to the Italian cohort, this study could not find a correlation with the total NMDAS score. Instead, the swallowing speed of these participants correlated significantly with the clinician's rating scale for swallowing difficulties and gastrointestinal problems. The multi-systemic involvement of autosomal recessive *RRM2B* variants, which contributed independently to the total NMDAS may be the reason why there was no direct correlation in this cohort. This study demonstrated that the direct attribution of swallowing speed with swallowing and gastrointestinal is a more plausible proposition than the loose correlation with disease burden in general.
Several weaknesses in this study should be considered. First, the low number of participants, especially in the longitudinal part of the study, hinders the generalisation of the results to other forms of mitochondrial disease. The low numbers have also led to a large variance in some outcome measures and weaken their effect sizes. To enhance recruitment, this study had screened for patients who harboured the autosomal recessive *RRM2B* gene in the MitoCohort database, which is possibly the largest mitochondrial disease patient cohort in the UK. Thus, it is unlikely that a larger living cohort of autosomal recessive *RRM2B* patients exists in the UK. Second, there is also a likelihood of survival bias because some patients with aggressive disease courses might have been excluded. However, it is notable that this study aims not to determine the prevalence of autosomal recessive *RRM2B* gene variants. Of those who had been screened from the cohort, three of them did not enrol into the study and their data is neither available nor being consented for analysis in this study. In a study with small numbers like this, outliers in any of these three non-participants could potentially distort the research data. To mitigate this, the consented data in the MitoCohort was reviewed, and limited analysis suggests that these non-participants share similar characteristics with the study participants. This study was also hampered by five participants (P6 to P10) who could not complete the follow-up and end-of-study visits at the research sites for the longitudinal arm of the project. They had declined to travel

for health reasons. Of the ten participants, five participants (P1 to P5) were younger and had earlier disease onset compared to the other five participants (P6 to P10). Despite these differences, there was no difference in their clinical phenotypes at baseline visits. Without the data from a longitudinal study on these five patients (P6 to P10), the exact disease progression and changes in outcome measures could not be determined or compared between these the younger and the older groups within this cohort. Another limitation in this study is a lack of reliable outcome measures that could objectively quantify the severity of ophthalmoplegia or ptosis features in this disorder. The current outcome measures for these features are vaguely subjective but automated software algorithms are currently being developed and validated (Bodnar et al., 2016, Choi et al., 2016, Thomas et al., 2020). This study also suffers from limitations associated with the inter-rater reliability of the outcome measures, and the results of the outcome measures might be influenced by the assessor. The accuracy of quantitative dynamometry testing, for instance, depending on the force generated against the tester (Wikholm and Bohannon, 1991, Rodriguez-Perea et al., 2021). To reduce this bias, these participants are given the same instructions and are tested by the same assessor at each visit, where possible. Lastly, not all of the outcome measures proposed by Mancuso et al. (2017) are feasible in the research environment where this study was carried out. Some of the performance outcome measures or biomarkers require specialised equipment and highly skilled technicians. Whether there is a likelihood that these untested outcome measures might be more reliable and responsive than those selected for this study remains unknown.

Despite some of these shortcomings, this study has several important implications for clinical practice and future research. From a clinical perspective, these participants with autosomal recessive *RRM2B* variants have demonstrated they are likely to perform poorly in clinical assessments that involve muscle strength and continue to achieve suboptimal results for at least a year. Of all the outcome measures in this study, some of the outcome measures are probably not suitable for clinical trials such as the STS. This study has provided new datasets on the outcome measures for this condition which could be subsequently taken forward to a future interventional trial. Based on the findings, the suitable outcome measures which could be taken forward to future trials include NMDAS, lung spirometry in particular FVC and FEV1,

quantitative muscle dynamometry, nine-hole peg test and timed water swallow test.While previous research has focussed on basic descriptions of autosomal recessive *RRM2B* in cross-sectional studies, this study has provided new, detailed, and quantitative data on the natural history of this rare condition. The data produced from this study could be utilised as a historical metric for future intervention trials for this rare condition where a placebo arm is possibly not ethical. In this group of participants, future research that focussed on other unexplored outcome measures and biomarkers is also warranted. Moving forward, future natural history studies should consider developing and comparing the findings from this group of participants with other forms of mitochondrial disease who share similar phenotypes such as single large-scale deletion of the mtDNA or mitochondrial dysfunction secondary to mtDNA maintenance defects. Having shown the extensive burden in these participants who harboured biallelic pathogenic variants in the *RRM2B* gene in this Chapter, the next research question of interest is to investigate their thoughts or feelings about their conditions.

Chapter 6: Investigating patient-reported outcome measures in autosomal recessive *RRM2B*

6.1 Introduction

Chapter 5 has explored the clinician-rated outcome measure, performance outcome measures and functional tests in autosomal recessive *RRM2B*-related disorders. However, questions regarding how these participants feel about their own symptoms and quality of life remains. Understanding what matters to them is equally important as those outcome measures studied in Chapter 5. Increasingly, patients are placed at the centre of healthcare research to ensure that research is robust and generates value for money (Devlin et al., 2010, Nelson et al., 2015, Squitieri et al., 2017). Furthermore, healthcare policymakers, regulatory bodies and pharmaceutical companies can gain unique insight from patients themselves in developing treatment (Boyce et al., 2014, Vodicka et al., 2015, Crossnohere et al., 2021).

Patient-reported outcome measures (PROM) can be defined as standardised and validated questionnaires that are completed directly with regards to their own health status, perceived level of impairment, and quality of life patients without the interpretation of their responses by clinicians (Weldring and Smith, 2013, Kingsley and Patel, 2017, Higgins et al., 2019). Conventional outcomes that measured disease burden and physical functions might demonstrate only the physiological effect of disease but not provide a holistic view by patients on the multifaceted disease process. For instance, patients may perform poorly in individual clinical assessments and functional tests, while PROM may reveal that these patients were affected by other factors such as personal preferences, quality of life or mental health issues. Thus, patients' experience of their condition is an integral part of clinical research and can complement the conventional outcome measures.

In mitochondrial disease, the International Workshop led by Mancuso and colleagues (2016) had also considered the importance of patient-reported questionnaires in the clinical trial readiness. Since then, patient-reported outcomes on quality of life, fatigue, and mental health problems have been studied in a group of mitochondrial patients with m.3243A>G point mutation (Verhaak et al., 2016). Patient-reported outcome measures have also recently shaped the design of pharmacological trials in some forms of mitochondrial disease (Janssen et al., 2019, Newman et al., 2020, Tinker et

al., 2021). To date, no previous research study has investigated the patient-reported outcome measures in patients with autosomal recessive *RRM2B* variants.
This Chapter explores several PROMs in a cohort of participants with autosomal recessive *RRM2B* gene variants, namely Neuro-QOL(Cella et al., 2012, Gershon et al., 2012), Newcastle Mitochondrial disease Quality of life scale (Elson et al., 2013), Fatigue Impact Scale (Fisk et al., 1994b), Daily Fatigue Impact Scale (Fisk and Doble, 2002), SWAL-QOL (McHorney et al., 2000, McHorney et al., 2002), and Dysphagia Handicap Index (Silbergleit et al., 2012). These questionnaires have been developed and validated by their respective publications. The methodology section of this Chapter outlines the properties of these questionnaires along with their scoring systems.

The Neuro-QOL, a comprehensive PROM developed under the initiative of the National Institutes of Health Quality of Life in Neurological Disorders (NINDS) in 2012, is aimed at neurological disorders. It has been shown to be a valid measure in some neurological disorders such as multiple sclerosis (Elbers et al., 2012, Miller et al., 2016), epilepsy (Victorson et al., 2014), Huntington's disease (Carlozzi et al., 2020) and Parkinson's disease (Nowinski et al., 2016, Mills et al., 2020) with good psychometric properties. However, the validation of Neuro-QOL in mitochondrial populations with neuromuscular disorders has been limited. In 2013, health-related quality of life scale specific to mitochondrial disease was developed and validated by Elson and co-workers in Newcastle (Elson et al., 2013). It has recently been employed in a treatment trial for adults with primary mitochondrial myopathy (Karaa et al., 2020). These two PROMS, one generic and the other mitochondrial-disease-specific, have the potential to comprehensively address a multitude of issues pertaining to the quality of life.

Two PROMs related to fatigue is this Chapter are the Fatigue Impact Scale and the Daily Fatigue Impact Scale. Fatigue is one of the key manifestations of myopathic patients with mitochondrial DNA maintenance secondary to pathogenic variants in the *RRM2B* gene (Pitceathly et al., 2012, Lim et al., 2021). Fatigue Impact Scale (FIS), a widely used tool in fatigue research, has been employed in 132 mitochondrial patients by Gorman and colleagues (Gorman et al., 2015a). Their study showed that nearly

two-thirds of these patients had reported excessive symptomatic fatigue (FIS > 40), whilst nearly a third reported functionally limiting fatigue symptoms (FIS > 80) (Gorman et al., 2015a). Daily Fatigue Impact Scale (DFIS), an adapted version of FIS by the original authors for daily administration, had not been employed in mitochondrial patients before. Instead of the lengthy 40-question FIS, DFIS comprises only eight items after undergoing Rasch analysis (Fisk and Doble, 2002). Despite not being used in mitochondrial disease specifically, DFIS has shown good psychometric properties in other neurological conditions, such as Parkinson's disease and multiple sclerosis (Martinez-Martin et al., 2006, Benito-León et al., 2007, Elbers et al., 2012, Serrano-Dueñas et al., 2018). The brief DFIS, which retains good psychometric properties, is potentially helpful for daily fatigue assessment in these patients with mitochondrial disease.

Swallowing problems or dysphagia are common in mitochondrial disease patients, with nearly half of the patients reporting more difficulties than control (Read et al., 2012). In this study, about a third of participants had multiple mtDNA deletions, but it was unclear how many of them harbour biallelic pathogenic variants of the *RRM2B* gene. Swallowing problems have been speculated to cause malnutrition and low BMI for some patients with mitochondrial disease (de Laat et al., 2015, Hedermann et al., 2017, Boal et al., 2019). Given the low BMI identified in the previous Chapter, there is a clear need to explore the impact of dysphagia on these patients with autosomal recessive *RRM2B* gene variants. To this end, the SWAL-QOL and Dysphagia Handicap Index were utilised. The SWAL-QOL has previously shown that poorer swallowing related quality of life for 14 mitochondrial patients who carry the *POLG* gene variants (Vogel et al., 2017), but it has not been studied in *RRM2B*-related conditions. Meanwhile, the DHI, which was developed to be more concise and easier to complete than SWAL-QOL (Silbergleit et al., 2012), has never been utilised in mitochondrial patients to date.

The results from PROMs may correlate with each other or with other conventional outcome measures studied in Chapter 5. Calculating the correlation coefficient is one of the best ways to assess the construct validity, particularly the convergent validity of PROMs. The Consensus-based Standards for the selection of health Measurement

Instruments (COSMIN) sets out several methods to assess validity and so determine the quality of the PROM (Mokkink et al., 2010, Terwee et al., 2012). Apart from construct validity, another key analysis is the internal consistency of these PROMs. Internal consistency, calculated by Cronbach α statistic, determines the homogeneity of items to measure the same latent variable reliably.

6.2 Specific aims

5. To investigate patient-reported outcome measures in terms of quality of life, fatigue and swallowing in rare autosomal recessive *RRM2B*-related disorders, using the following questionnaires:
    a. Neuro-QOL
    b. Newcastle Mitochondrial Quality of life measure (NMQ)
    c. Fatigue impact scale
    d. Daily fatigue impact scale
    e. Swallowing quality of life questionnaire (SWAL-QOL)
    f. Dysphagia handicap index
6. To explore the relationships between these patient-reported outcome measures and other outcome measures.
7. To ascertain the internal consistency of these questionnaires.

6.3 Methods

6.3.1 Study design

A group of participants who had autosomal recessive *RRM2B* variants answered a set of questionnaires at the research sites or at their home environment. Like Chapter 5, these participants were part of the Prospective Observational Study of Patients with Mitochondrial Depletion Syndrome *RRM2B* (PROSPER2B) (REC: 18/WM/0354). All participants consented to this study. I collected the questionnaire data in this Chapter between 17 June 2019 and 16 December 2019. All participants understood the questionnaires used in this study, but they can decline to answer that they felt the questions were inappropriate or applicable to them.

6.3.2 Participants

All patients recruited to the study fulfilled the diagnostic criteria for autosomal recessive *RRM2B*-related mitochondrial disease. The inclusion and exclusion criteria had been outlined in Chapter 2 (General Methods). These participants had also completed the clinician-rated outcome measure, performance outcome measures and functional tests in the PROSPER2B study as presented in Chapter 5.

6.3.3 Administration of questionnaires

All questionnaires used as patient-reported outcome measures in this Chapter have been printed on A4 papers with a clear presentation of the texts and headings to make the questions easy to follow. The original texts from all six questionnaires had not been altered in any way in accordance with copyright requirements. The question format for these questionnaires was primarily the Likert scale. Standardised and clear instructions had been given to participants on how to complete these questionnaires. The language used in these questionnaires were English. It was acceptable to define a term but not to define a concept in cases where there was a subjective interpretation of the quality of life. All participants had the optimal time needed to provide complete data set on the day of their assessments.

6.3.4 General quality of life questionnaires

6.3.4.1 Neuro-QOL

The Neuro-QOL is a set of self-reported that assessed health-related quality of life in adults with neurological problems. The version which is used in this study contained 12 domains to evaluate symptoms, concerns and pertinent issues. I adhered to the method of administering and interpreting Neuro-QOL as published in the literature (Cella et al., 2012, Gershon et al., 2012, Disorders and Stroke, 2015). Each response option can be assigned a value, ranging from, for instance, 1 = Never for the lowest possible score to 5 = Always for the highest possible score per question. The scores from each question summed up the total raw score for each scale. The raw scores from each scale in the Neuro-QOL could be converted to standardised T-scores with a mean of 50 and a standard deviation of 10 (Disorders and Stroke, 2015). The reference population for the meaning of these t-scores was a clinical reference population for specific scales, namely the Stigma, Fatigue, Emotional and Behavioural Dyscontrol, and Sleep Disturbance measures. All other adult measures used a general population reference sample. A higher or lower Neuro-QOL T-score represents more or less of the concept being measured, depending on the questions in each domain. High scores suggest worse self-reported health in domains such as Anxiety, Depression, Fatigue, Emotional and Behavioural Dyscontrol, Sleep Disturbance, and Stigma. On the contrary, low scores in some domains, namely Upper Extremity Function, Lower Extremity Function, Cognitive Function, Positive Affect and Well-Being, Ability to Participate in Social Roles and Activities, Satisfaction with Social Roles and Activities, indicate undesirable self-reported health.

6.3.4.2 Newcastle Mitochondrial Quality of life measure (NMQ)

The NMQ is a set of mitochondrial disease-specific, health-related quality of life measures. It has 63 items within 16 unidimensional domains, namely, Mobility, Activities of daily living, Fatigue, Vision, Communication, Cognition, Food and digestion, Pain, Muscle stiffness, Migraine, Emotional wellbeing, Stigma, Family role, Personal relationships, Social role / Support and Diabetes.

Each response to the questions can be scored as follows: Never = 5; rarely = 4; sometimes = 3; often = 2; and always = 1. Then, the raw score, a sum of all items in the domain, for each NMQ domain can be transformed to a 0–100 score by the following formula: (Total domain score - the number of items)/(maximum possible - minimum possible score per domain) × 100 (Elson et al., 2013). Unlike the Neuro-QOL, the NMQ has no published values for the reference population. Without a t-score generated from item response analysis in its initial conceptualisation and validation, it can be assumed that each question carries equal weight. The higher the percentage NMQ domain score, the greater the perceived quality of life and health status (Elson et al., 2013).

6.3.5 Fatigue specific questionnaires

6.3.5.1 Fatigue impact scale (FIS)

FIS is a self-reported questionnaire that asks participants to rate the extent to which fatigue has caused problems for them during the last four weeks in relation to exemplar statements (Fisk et al., 1994b). In this study, the published guide on administering and interpreting FIS had been adhered to (Fisk et al., 1994a, Fisk et al., 1994b).

There are 40 questions in FIS which can take up to 5 minutes for a non-fatigued person to complete but might take longer for a fatigued participant. The responses to all questions in FIS are set as follows – No Problem (0), Small Problem (1), Moderate Problem (2), Big Problem (3), Extreme Problem (4). All these questions in FIS can be summed up to 160, the maximum score.

FIS consists of three subscales that represent how fatigue impacts Cognitive (10 questions), Physical (10 questions) and Psychosocial function (20 questions) (Fisk et al., 1994b, Frith and Newton, 2010). Cognitive subscale interrogates participants about their concentration, memory, thinking and organisation of thoughts. Physical subscale reflects on motivation, effort, stamina and coordination. Psychosocial subscale depicts the isolation, emotions, workload resulting from fatigue (Frith and Newton, 2010).

6.3.5.2 Daily Fatigue Impact Scale (DFIS)

In contrast to FIS, Daily Fatigue Impact Scale (DFIS) asks participants how much of problem fatigue has been for them on the day when the questionnaire was completed. The authors of the original FIS used Rasch analyses to reduce the 40 items in FIS to eight for DFIS and adapted the DFIS for daily use (Fisk and Doble, 2002). Despite this reduction to the minimum number of items, Fisk et al. (2002) still demonstrated DFIS as a valid measure of the subjective daily experience of fatigue on top of its practical use and ease of scoring. Similar to FIS, the responses to each question in DFIS can be scored ordinally on a 0 (No Problem) to 4 (Extreme Problem) Likert scale. The total scores for DFIS can range from 0 to 32. Both FIS and DFIS had not published normative values or t-scores on reference populations.

6.3.6 Swallow-specific questionnaire

6.3.6.1 Swallowing quality of life questionnaire (SWAL-QOL)

SWAL-QOL is a detailed 44-item questionnaire that measured quality of life for patients with oropharyngeal dysphagia (McHorney et al., 2000). These items are distributed across ten domains: Burden, Eating Desire, Eating Duration, Food Selection, Social, Mental Health, Fear, Communication, Fatigue, and Sleep domains. Each item in the SWAL-QOL is rated on a Likert scale from 0 to 4, corresponding to the severity level from worst to best. The sums of scores from all items within a domain can be expressed in the maximum possible score percentages. Then, a total SWAL-QOL, expressed in 0-to-100 metric, can be obtained by dividing ten from the sum of all domains. The higher the value of SWAL-QOL, total or domains, indicate lower impairment. Thus, the 'normal' value of SWAL-QOL is a maximum score of 100.

6.3.6.2 Dysphagia Handicap Index (DHI)

DHI is a 25-item questionnaire that measures the handicapping effect of dysphagia on emotional, functional and physical aspects of the participant's lives (Silbergleit et al., 2012). This DHI has three ordinal values in its Likert scale for all 25 items, 'Never', 'Sometimes' and 'Always'. The scoring system for DHI dictates that 'Never' equals zero, 'Sometimes' equals a 2 and 'Always' equals a 4 (Silbergleit et al., 2012).

All subscales of DHI, Emotional, Functional and Physical, can be combined to provide an overall score that reflects the participant's perception of their dysphagia. The maximum possible total DHI score is 100. A recent meta-analysis of published DHI scores on subjects who had no history of dysphagia or neurological disease as well as no history of head or neck malignancy has generated a normative value of 2.49 (95%CI 0.51-4.48) with an age range of 20-86 years (Sobol et al., 2021). At the end of the DHI 25 questions, a self-reported severity scale from 1 to 7. These values are grouped into four severity categories, as follows – Normal =1; Mild = 2 or 3; Moderate = 4 or 5; and Severe = 6 or 7.

6.3.7 Statistical analysis

I used SPSS v.25 for statistical analyses. All the statistical methods have also been detailed in Chapter 2. I have used descriptive statistics to describe the patient-reported outcomes on quality of life for each questionnaire. All related outcome measures from Chapter 5 have been used in the correlational analysis. I also calculated the Spearman rank-order correlation (Spearman, 2010) to determine the relationships among these outcome measures. Convergent validity with Spearman coefficients lower than 0.4 was considered weak, between 0.40 and .69 moderate, between 0.7 and 0.89 strong and above 0.9 very strong correlations (Strauss and Smith, 2009, Schober et al., 2018). The significance level is set at $p<0.05$. I also calculated Cronbach's alpha, the commonest measure of internal consistency or "reliability", for these questionnaires. A value of 0.7 or higher was considered satisfactory (Bland and Altman, 1997, Tavakol and Dennick, 2011).

6.4 Results

6.4.1 Participants

In this study, all ten participants, six females and four males, who had autosomal recessive *RRM2B*-related mitochondrial disorders, completed their questionnaires (Figure 6-1). Five of these participants who consented to take part were unable to travel to the research site in Newcastle but agreed for home visits. Thus, they answered these questions in their respective home environments. The clinical phenotypes of these participants have been detailed in Chapter 5.

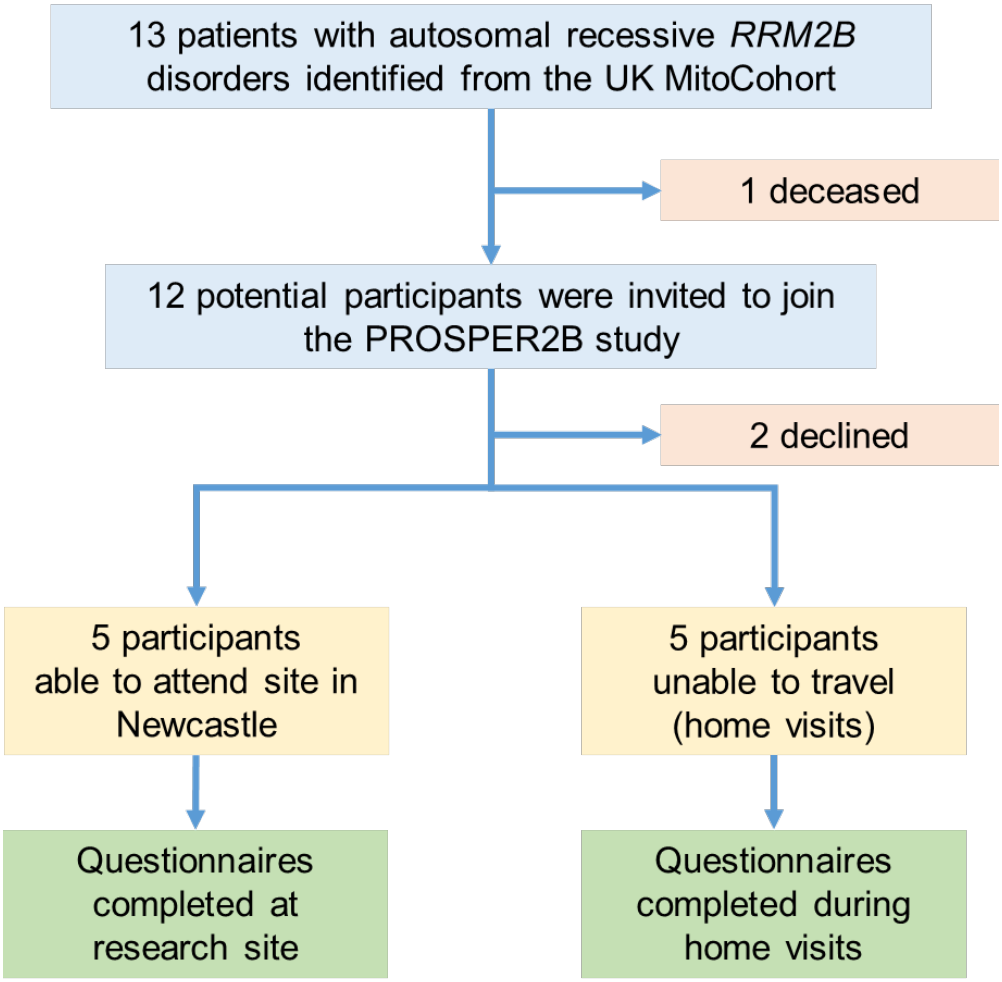


Figure 6-1: Flow chart shows the number of patients who were screened from the UK MitoCohort, invited to join the study, consented, completed the questionnaires, and were included in the analysis.

6.4.2 Neuro-QOL

The responses of these participants regarding the function of their upper and lower extremities in the Neuro-QOL are summarised in Figure 6-2 and Figure 6-3, respectively. In the upper extremity, all participants had reported at least some difficulty in picking up coins from a table. Half of them were unable to do this task. Other functions that these participants had 'much difficulty' or 'unable to do' include writing with a pen or pencil, making a phone call or brushing their teeth. The lower limb function appeared to be more challenging, with all participants reporting at least 'some difficulty' getting in and out of a car, pushing a heavy door, getting up from the floor independently and having a walk for at least 15 minutes. Most notably, eight of these participants were 'unable' to get up from lying on their backs without help, and the remaining two could do this with 'much difficulty'.

Figure 6-4 shows the cognitive function responses from these participants. Four of the ten participants complained that they 'very often' must read something several times to understand it and had trouble concentrating. All participants reported that their thinking was slow to some degree. However, about a third of them could read and follow complex instructions and manage their time to do their daily activities. Four of the ten participants had no problems planning and keeping appointments that were not part of their weekly routine.

The answers from these participants about their positive effect and wellbeing domain are shown in Figure 6-5. Half of them 'always' or 'often' felt that their life was worth living and that they had a sense of wellbeing. Six of the ten participants 'always' or 'often' considered that their lives were satisfying and had meanings. Conversely, some of the participants reported that they had 'sometimes' (n=5), 'rarely' (n=2) or 'never' (n=1) felt many areas of their lives were interesting to them. No participants have reported that they were 'always' cheerful or 'always' had a sense of balance in their lives.

The ability to participate in social roles and activities has been outlined in Figure 6-6. The majority of participants had 'never' or 'rarely' been able to participate in leisure activities, do all the regular activities with their friends, and keep their social commitments. Half of the ten participants also described that they 'rarely' or 'never' keep with their family responsibilities or do all their regular family activities.

In addition to their abilities, their satisfaction with their respective social roles and activities are also noted in Figure 6-7. More than half of them conveyed that they were 'quite a bit' or 'very much' disappointed by their ability to socialise with families. They were also 'quite a bit' or 'very much' bothered by their limitations in regular activities with friends. Eight of ten of them were also either 'a little bit' or 'not at all' satisfied with their ability to do household chores or tasks. Most of them (n=7) were either 'a little bit' or 'not at all' satisfied with their abilities to do things for fun outside their homes.

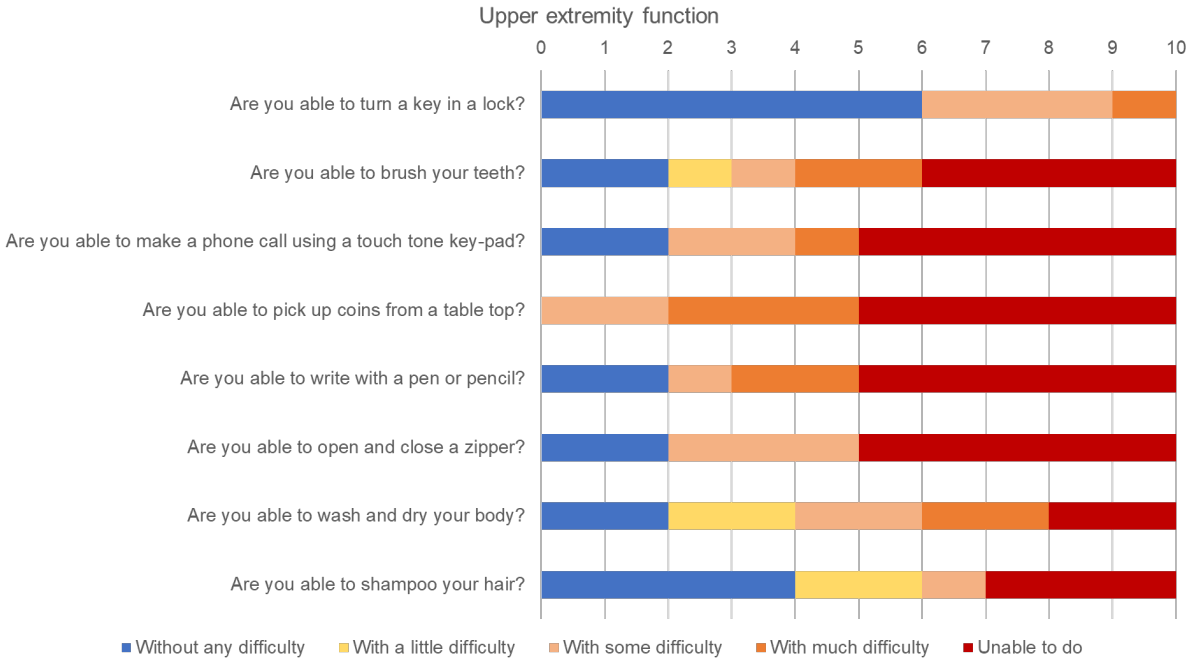


Figure 6-2: Bar graph shows the responses of ten participants regarding the function of their upper extremities in the Neuro-QOL.

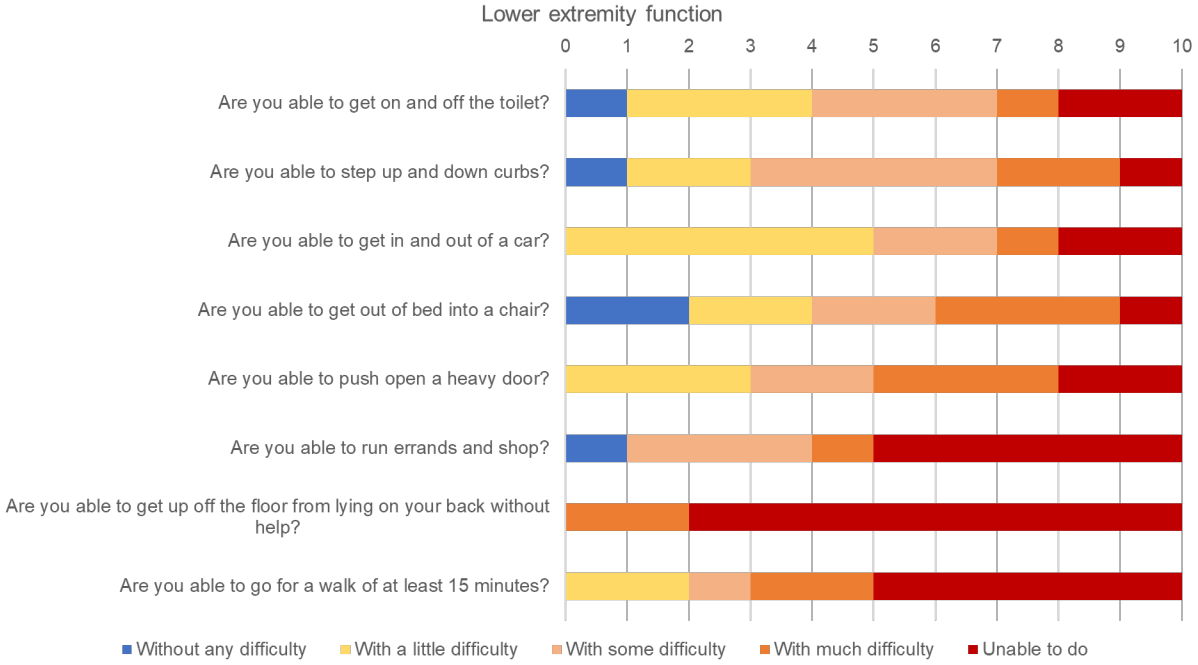


Figure 6-3: Bar graph shows the responses of ten participants regarding the function of their lower extremities in the Neuro-QOL.

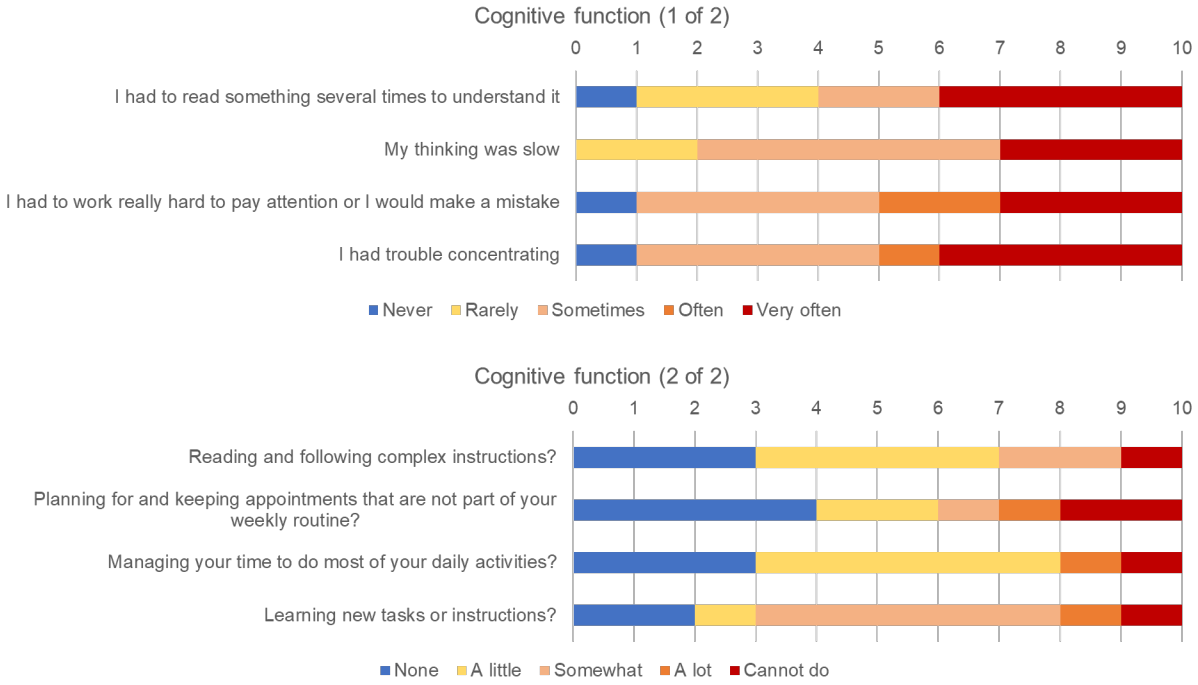


Figure 6-4: Bar graph shows the responses of ten participants regarding their cognitive functions in the Neuro-QOL.

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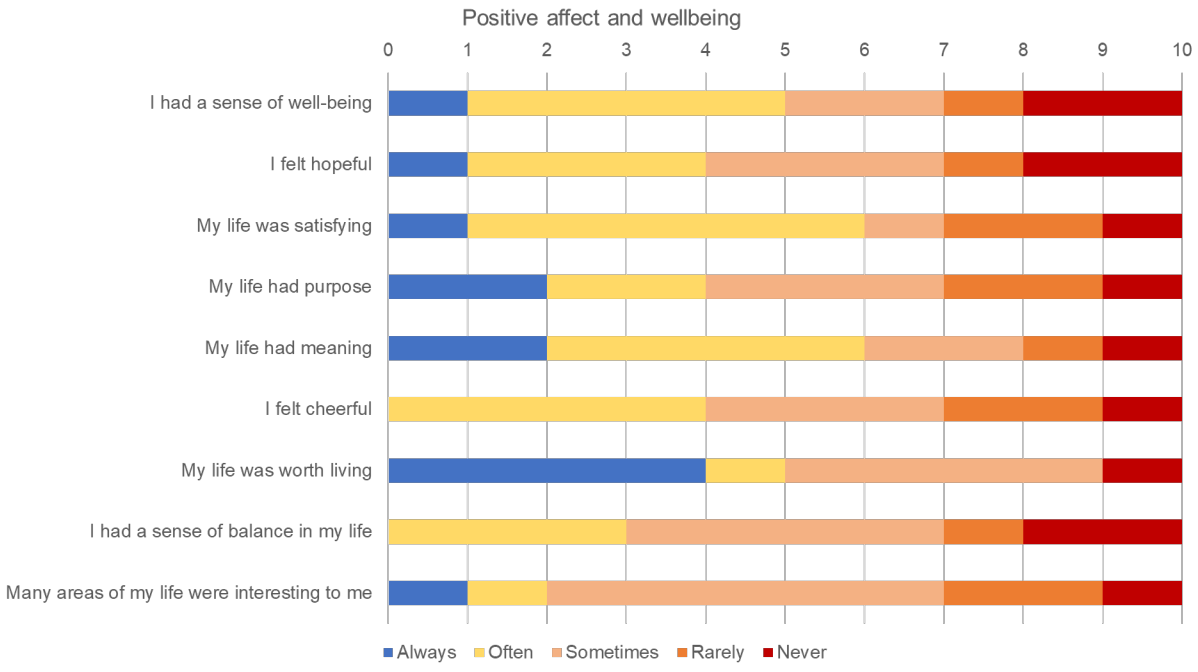

Figure 6-5: Bar graph shows the responses of ten participants regarding their positive affect and wellbeing in the Neuro-QOL.

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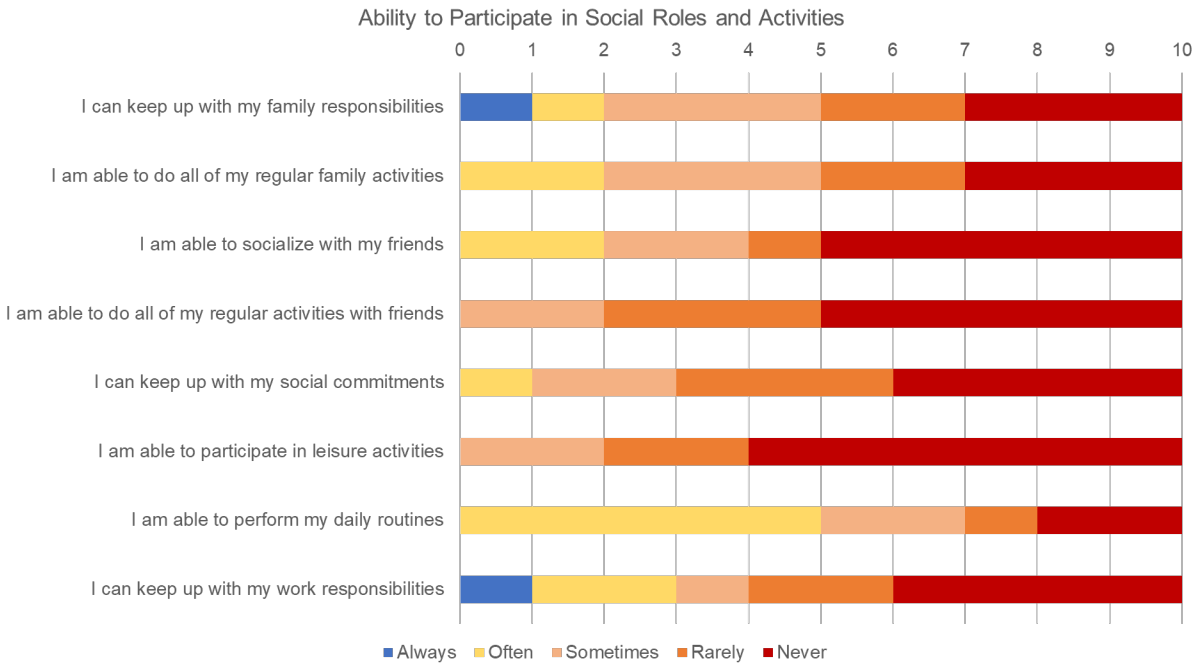

Figure 6-6: Bar graph shows the responses of ten participants regarding their abilities to participate in social roles and activities in the Neuro-QOL.

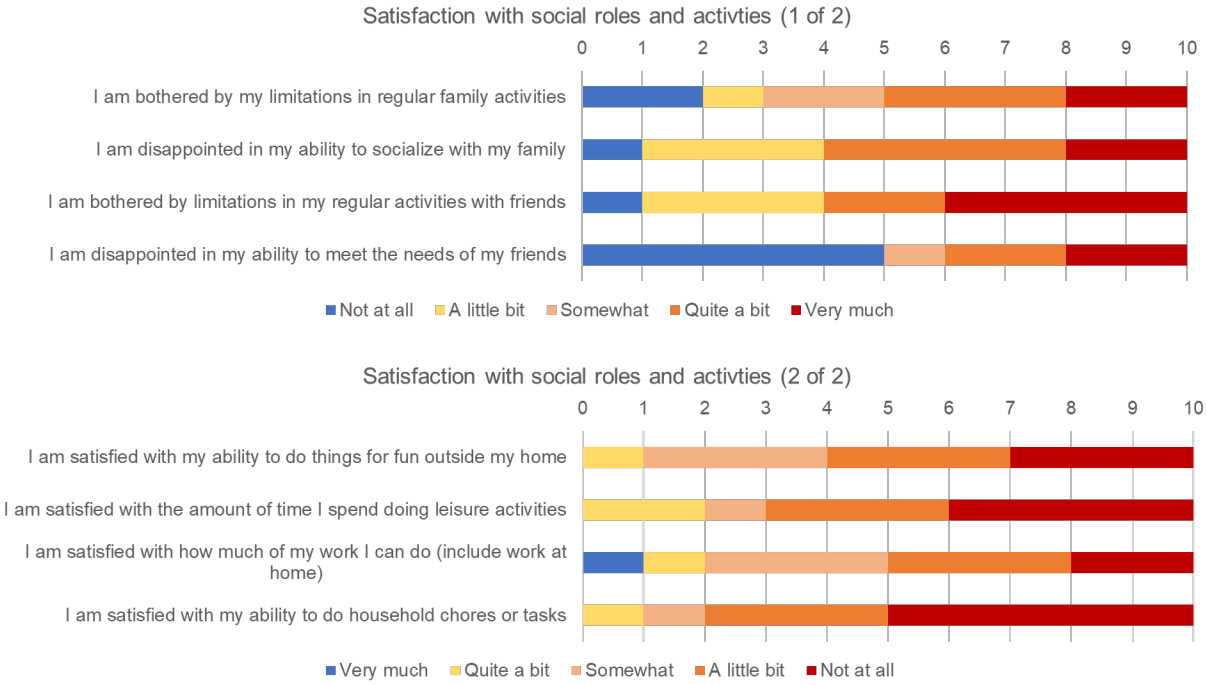

Figure 6-7: Bar graph shows the responses of ten participants regarding their levels of satisfaction with social roles and activities in the Neuro-QOL.

These participants also shared their anxieties in the Neuro-QOL (Figure 6-8). Many situations had made more than half of them ‘often’ or ‘always’ worry. Four of the ten participants felt that their worries ‘often’ or ‘always’ overwhelmed them. Eight participants ‘sometimes’ felt tense or nervous. Although three participants had never experienced nervousness when their normal routines were disturbed, the other three in this group ‘often’ or ‘always’ felt otherwise. Depression in this group of participants appeared to be less pronounced than their anxieties (Figure 6-9). The majority of them had ‘never’ or ‘rarely’ felt they had no reason for living or their lives were empty. Half of them ‘never’ or ‘rarely’ felt nothing could cheer them up, worthless or nothing was interesting. Only two participants, who were on antidepressant medications, had reported ‘often’ or ‘always’ in these questions about their depression.

Similarly, their responses in the emotional and behavioural dyscontrol domains in Figure 6-10 showed they coped relatively well in these areas. Most of these patients had ‘never’ or ‘rarely’ had trouble controlling their tempers, behaviours, or conflicts with others. Six of the ten participants also had ‘never’ or ‘rarely’ said, did things without thinking or became irritable around other people. Half of them were ‘never’ or ‘rarely’ bothered by little things. Fatigue seemed to be the most markedly affected domain in the Neuro-QOL for these participants (Figure 6-11). No participants had reported ‘never’ in any questions within this domain. More than half of these participants had ‘often’ or ‘always’ felt tired, fatigued and that they had no energy. Some participants experienced sleep disturbances (Figure 6-12). More than half of them was ‘often’ or ‘always’ sleepy during the daytime. Nine participants ‘sometimes’ or ‘always’ had to force themselves to get up in the morning.

The responses to the stigma these participants faced because of their illnesses are shown in Figure 6-13. Seven participants ‘sometimes’, ‘often’ or ‘always’ felt left out of things due to their conditions. More than half of them had, at least ‘sometimes’, felt embarrassed because of their physical limitations. However, nine of these participants had stated that people were ‘never’ or ‘rarely’ unkind to them or seemed uncomfortable with them because of their conditions. They also 'never' or 'rarely' found some people acted as though it was their fault that they had the illnesses.

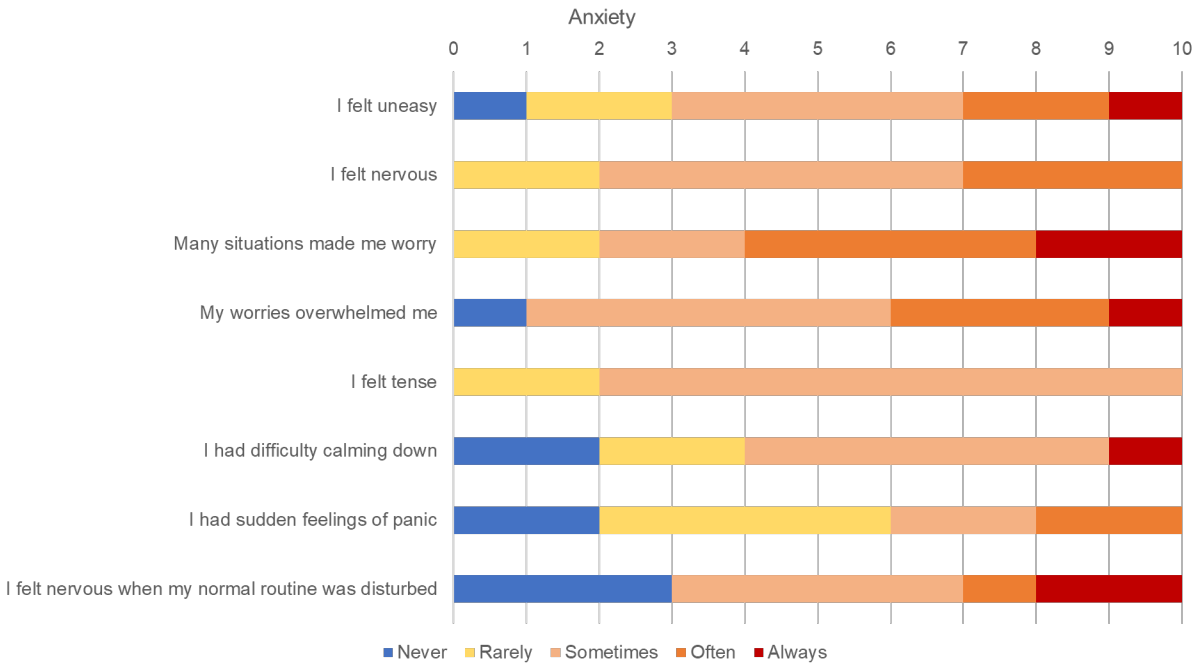

Figure 6-8: Bar graph shows the responses of ten participants regarding their levels of anxiety in the Neuro-QOL.

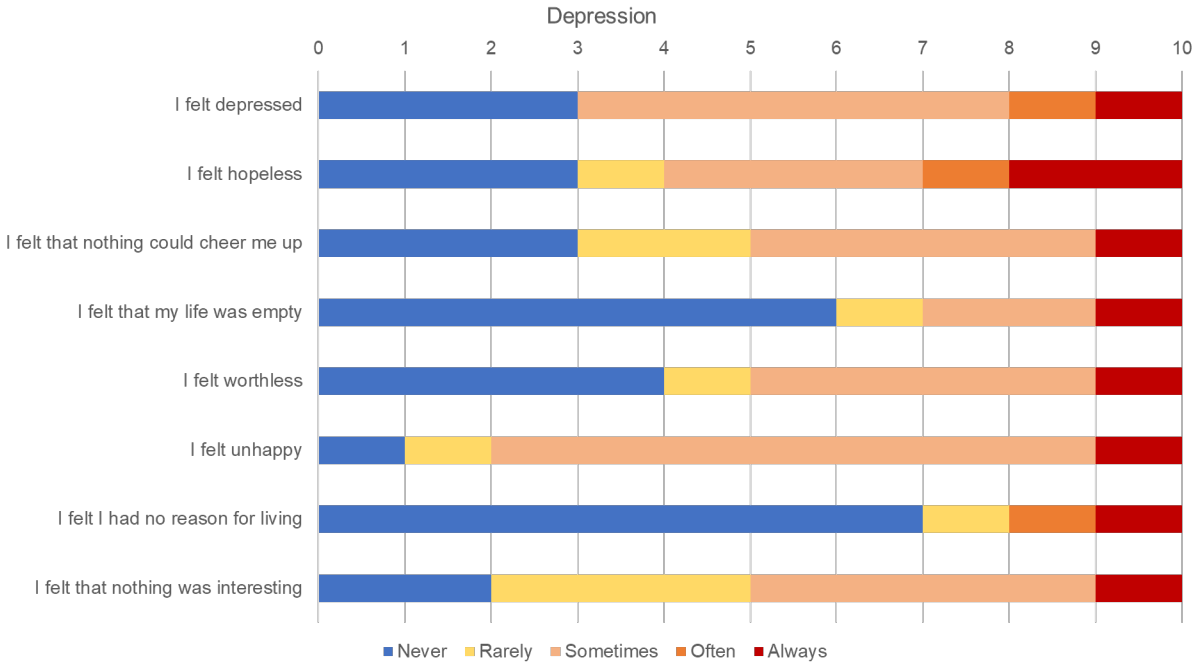

Figure 6-9: Bar graph shows the responses of ten participants regarding their levels of depression in the Neuro-QOL.

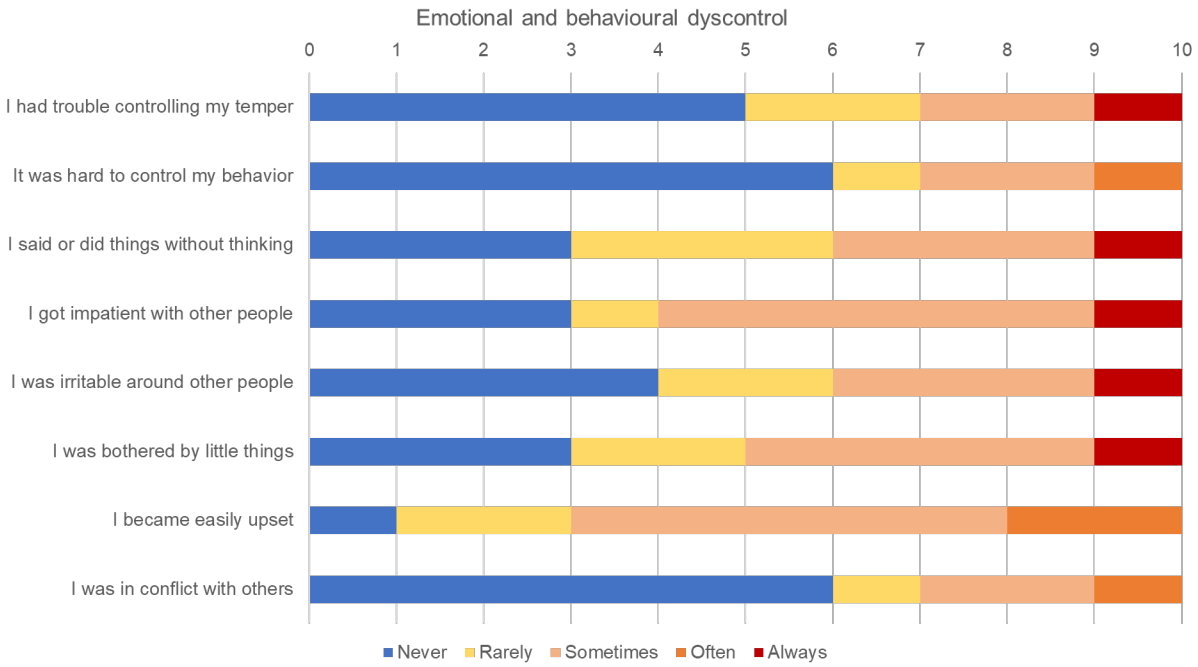

Figure 6-10: Bar graph shows the responses of ten participants regarding the emotional and behavioural dyscontrol in the Neuro-QOL.

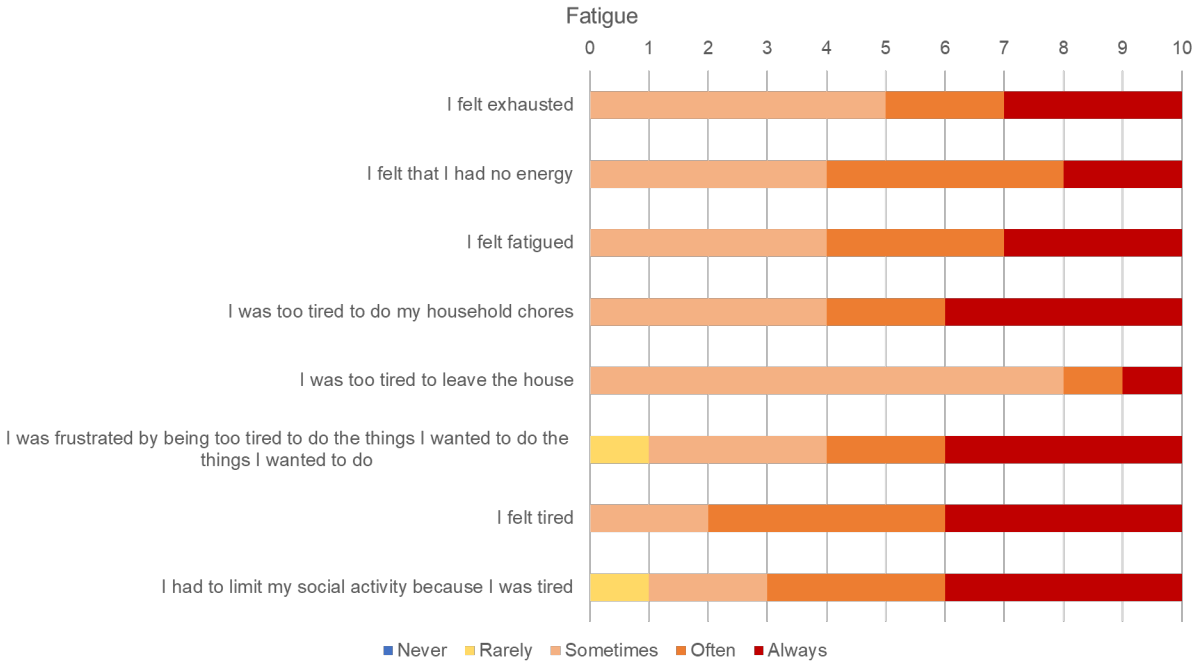

Figure 6-11: Bar graph shows the responses of ten participants regarding their levels of fatigue in the Neuro-QOL.

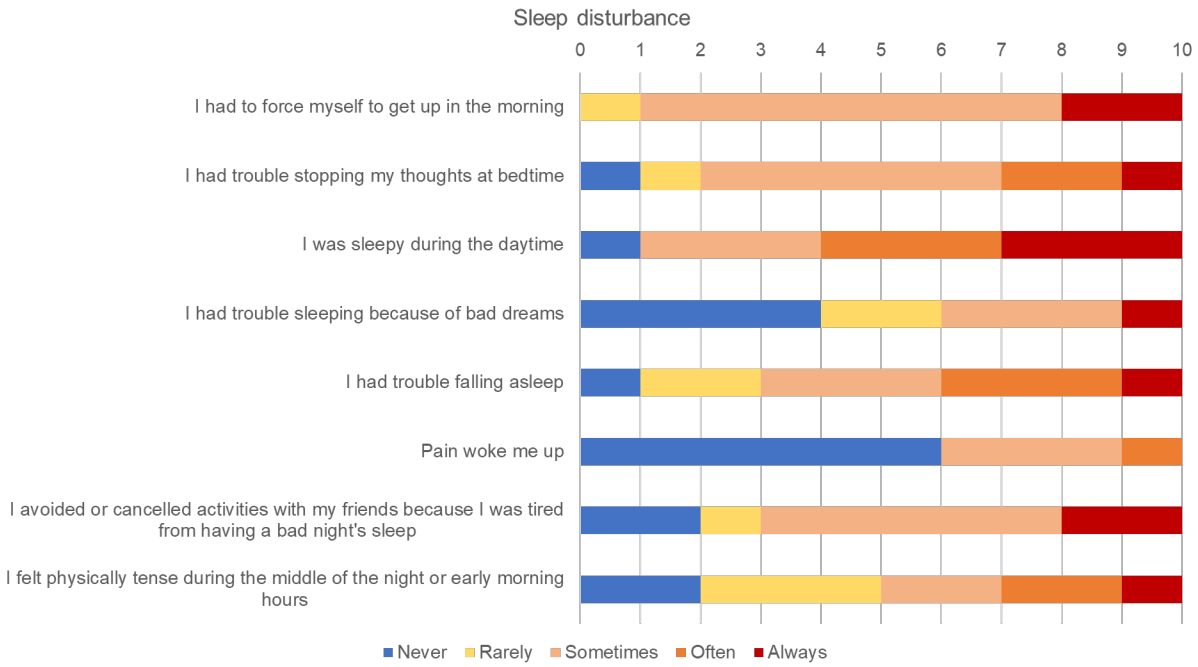

Figure 6-12: Bar graph shows the responses of ten participants regarding their experiences with sleep disturbance in the Neuro-QOL.

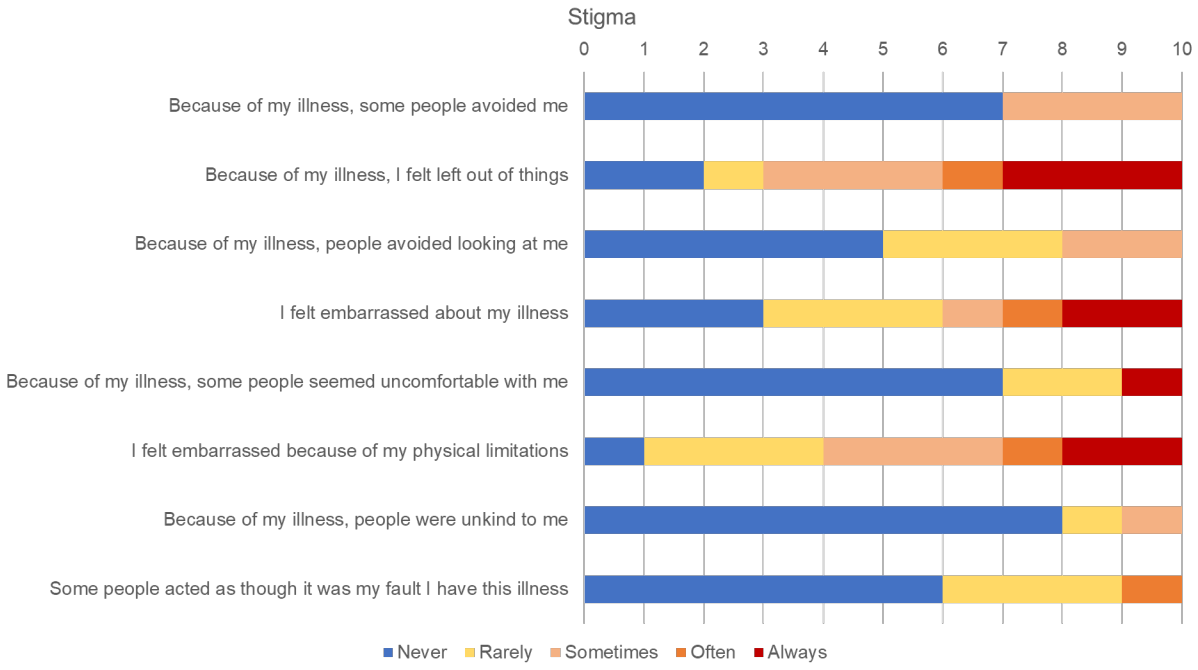

Figure 6-13: Bar graph shows the responses of ten participants regarding their feelings of stigma in the Neuro-QOL.

6.4.2.1 T-scores for Neuro-QOL domains

The domains which have low Neuro-QOL T-scores to indicate undesirable self-reported health are shown in Figure 6-14. In this group of domains, the mean t-scores were 32.3 (SD 10.6, 95%CI 24.7-39.9) for Upper Extremity Function and 31.1 (SD 5.3, 95%CI 27.3-34.9) for Lower Extremity Function. These limb function t-scores were more than one standard deviation (SD) lower than the reference population (less than 40). Their Ability to Participate in Social Roles and Activities which had a mean t-score of 35.1 (SD 6.8, 95%CI 30.2-39.9), was also 1 SD lower. Likewise, their Cognitive Function domain had a mean t-score of 38.8 (SD 10.5, 95%CI 31.3-46.3). The mean t-scores for other domains were also lower than reference population but remained within the 1 SD. Meanwhile, Positive Affect and Well-Being domain scored 47.5 (SD 8.3, 95%CI 41.5-53.4) and Satisfaction with Social Roles and Activities scored 40.6 (SD 3.1, 95%CI 38.4-42.8).

Figure 6-15 displays the domains which have high Neuro-QOL t-scores to indicate undesirable self-reported health. Of these, Fatigue scored, on average, the highest at 59.4 (SD 6.9, 95%CI 54.4-64.2) followed by Sleep Disturbance at 59.0 (SD 5.9, 95%CI 54.8-63.3) and Anxiety 58.0 (SD 3.8, 95%CI 55.4-60.7). Although the mean t-scores for these three domains were higher than the reference population of 50, they were lower than the 60-mark, which was 1 SD. Other domains in this group were Stigma with a mean t-score of 54.8 (SD 3.7, 95%CI 52.2-57.5), Depression with a mean t-score of 53.0 (SD 8.7, 95%CI 46.6-59.2) and Emotional and Behavioural Dyscontrol with a mean t-score of 50.7 (SD 11.5, 95%CI 42.5-58.9).

6.4.2.2 Correlation

A Spearman's rank-order correlation was run to determine the relationships between their t-scores of these domains with related clinician-rated outcome measure (NMDAS), performance outcome measures and functional tests outlined in Chapter 3. No significant correlation coefficient could be determined.

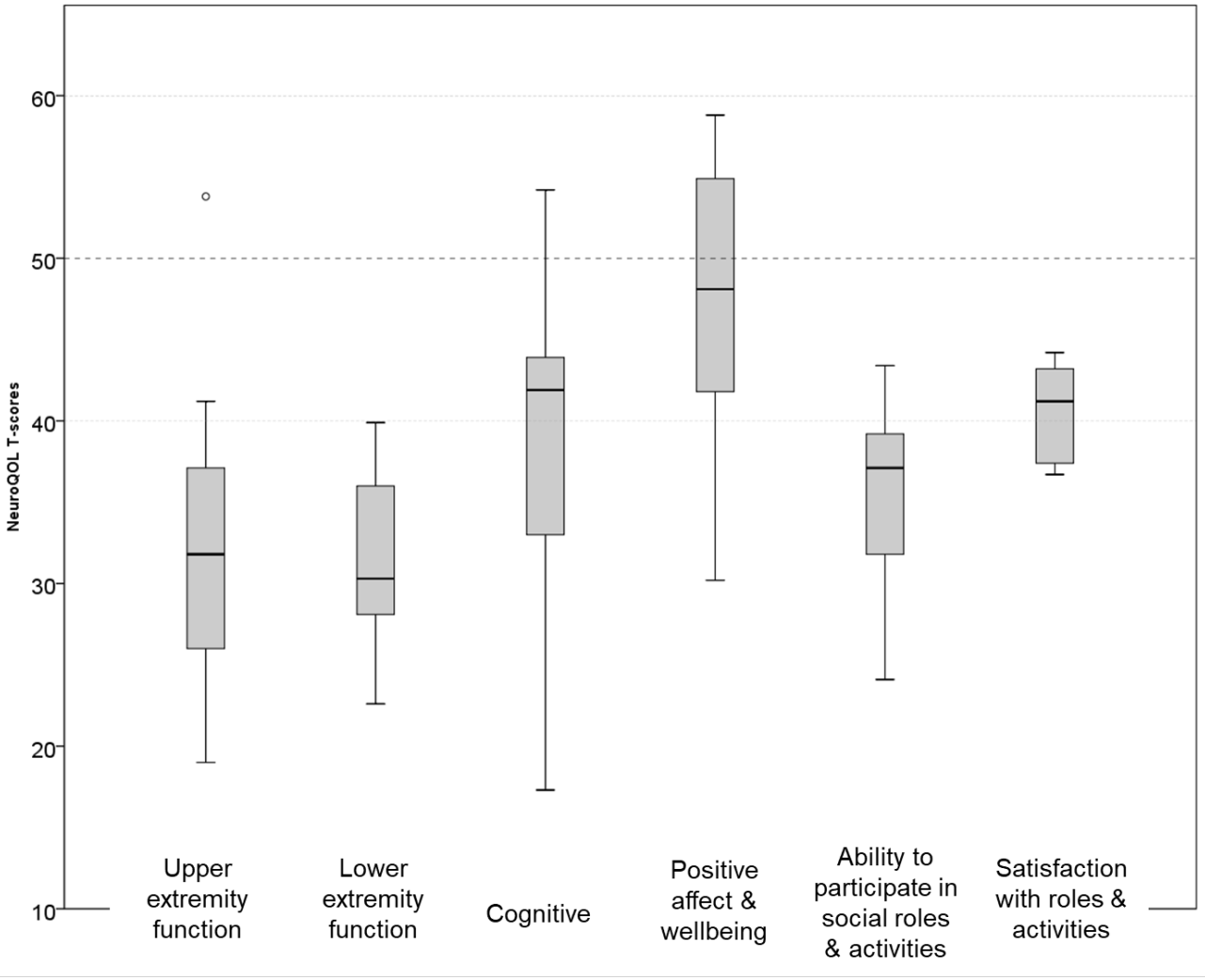

Figure 6-14: Box and whisker plots show the distribution of Neuro-QOL t-scores for six domains. Low scores in these six domains indicate undesirable self-reported health. Standardised T-scores with a mean of 50 is shown (dashed line) along with a standard deviation of 10 higher or lower than the reference population are also displayed (grey dotted lines).

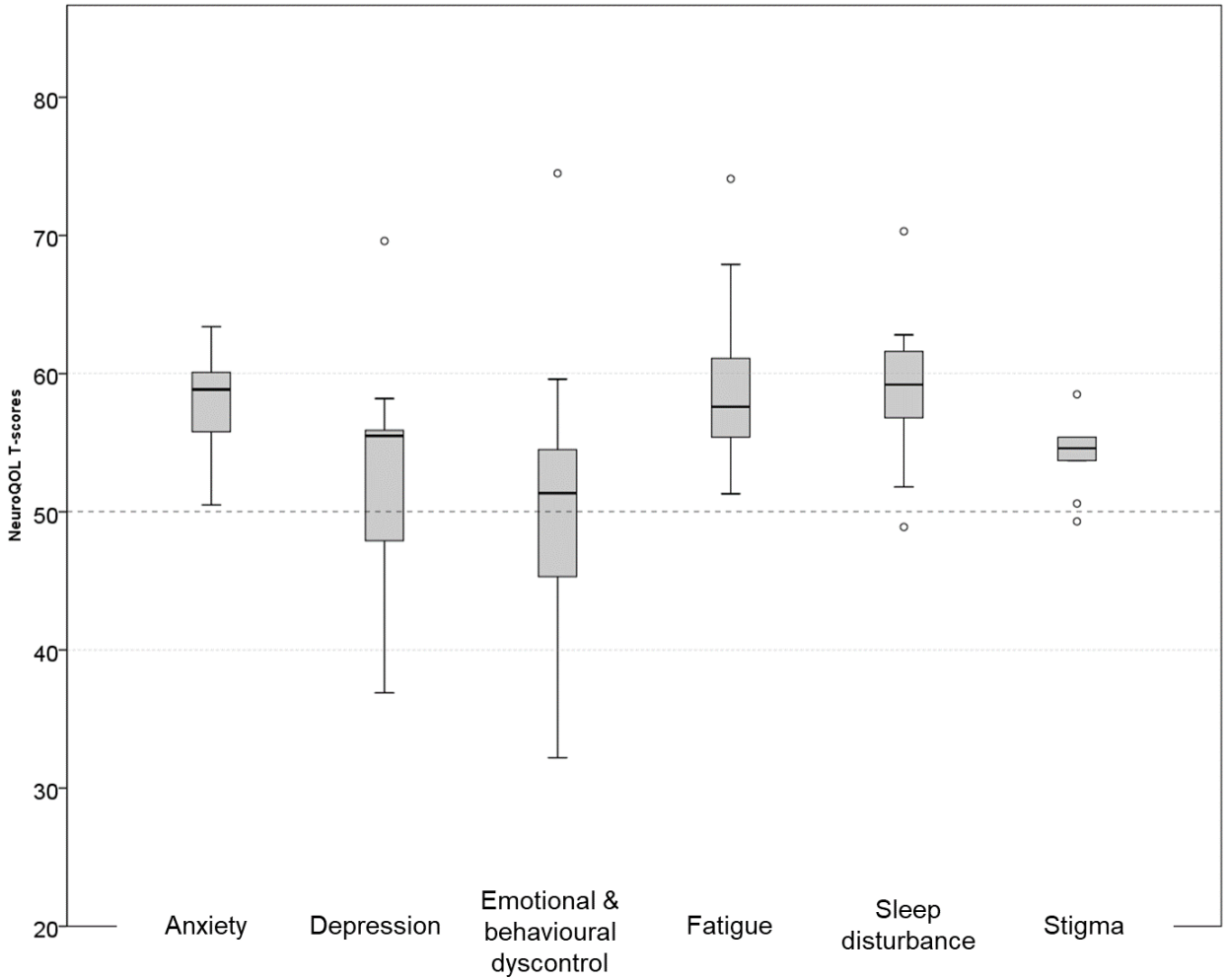

Figure 6-15: Box and whisker plots show the distribution of Neuro-QOL t-scores for six domains. High scores in these six domains indicate undesirable self-reported health. Standardised T-scores with a mean of 50 is shown (dashed line), along with a standard deviation of 10 higher or lower than the reference population are also displayed (grey dotted lines).

6.4.3 Newcastle Mitochondrial Quality of life measure (NMQ)

The responses of these participants to NMQ questions are summarised in Figure 6-16 and Figure 6-17. The domains in NMQ and their respective question numbers (Qn) are as follows: Mobility (Q1-Q5), Activities of daily living (Q6-Q9), Fatigue (Q10-Q13), Vision (Q14 and Q15), Communication (Q16-Q21), Cognition (Q22), Food and digestion (Q23-Q28), Pain (Q29-Q32), Muscle stiffness (Q33-Q36), Migraine (Q37-Q39), Emotional wellbeing (Q40-Q42), Stigma (Q43-Q45), Family role (Q46-Q49), Personal relationships (Q50-Q55) and Social role / Support (Q56-Q58).

No participants in this cohort had reported any features of diabetes mellitus. Thus, the 16th domain (Q59-Q63) of the NMQ with regards to diabetes mellitus was not answered. In those 15 domains that were asked of these participants, several questions related to employment, work or sexual relationships were answered as they were not applicable to some of these participants. Even if taking this into account, the response rate for all questions in NMQ for these participants was still high at 97.1%.
All participants had responded that they had ‘sometimes’ (n=4), ‘often’ (n=2) or ‘always’ (n=4) had difficulty getting around outside their home environments. Inside their home environments, nine of them ‘sometimes’ had encountered difficulty getting around. More than half (n=6) of them ‘always’ worried about falling over in public and had difficulty carrying bags of shopping. Four of the ten participants had not reported any difficulties in any activities of daily living in this NMQ. Five of the remaining participants had ‘often’ or ‘always’ encountered difficulty in washing themselves or doing buttons or shoelaces.

These participants also rated that they ‘sometimes’ (n=1), ‘often’ (n=7) or ‘always’ (n=2) felt tired in their Fatigue domain. Seven of them had were not able to take up employment or miss work because of their poor energy levels. In the Vision domain, half of the ten participants had complained that they ‘often’ or ‘always’ had difficulty reading small print. However, the majority of them (n=6), 'never' or 'rarely' had difficulty completing domestic activities because of poor vision.

Consistent with their hearing impairment phenotype, all these participants with autosomal recessive *RRM2B* gene variants had some degrees of difficulty hearing what other people were saying. Six of them ‘always’ had this problem, and they ‘always’ had to have people repeat themselves to them. Eight of these participants ‘sometimes’ or ‘always’ had difficulty communicating with people on the phone. In the Cognition domain of NMQ, four participants ‘never’ or ‘rarely’ found it difficult to make decisions or felt confused in their thinking abilities.

Six of the ten participants had either ‘sometimes’ or ‘always’ had lost their appetites, felt discomfort and felt unable to eat out because of their digestive problems. Although the type or source of pain was not specified in this NMQ questionnaire, half of all the participants could not take up employment or miss work because of unspecified pain, which could be related to muscle stiffness. In the subsequent “Muscle Stiffness” domain, six participants reported that they could not take up employment because of this problem. In contrast, most of these participants had not been affected to such levels by migraines or headaches.

In the Emotional Wellbeing domain, six of the participants ‘sometimes’ or ‘always’ felt weepy and tearful. They also ‘sometimes’ or ‘often’ felt frustrated, angry or bitter. As a result of their illness, half of these participants ‘always’ felt unable to talk to others. Eight of them ‘often’ or ‘always’ felt dependent on their family members but, most of them ‘never’ (n=4) or ‘rarely’ (n=2) felt they were a burden to their families. They ‘never’ or ‘rarely’ felt a lack of support from their families or friends. Beyond their families and friends, seven of these participants ‘often’ or ‘always’ struggled to make close personal relationships. Six participants 'always' felt people did not understand their condition and their condition had interfered with their social lives.

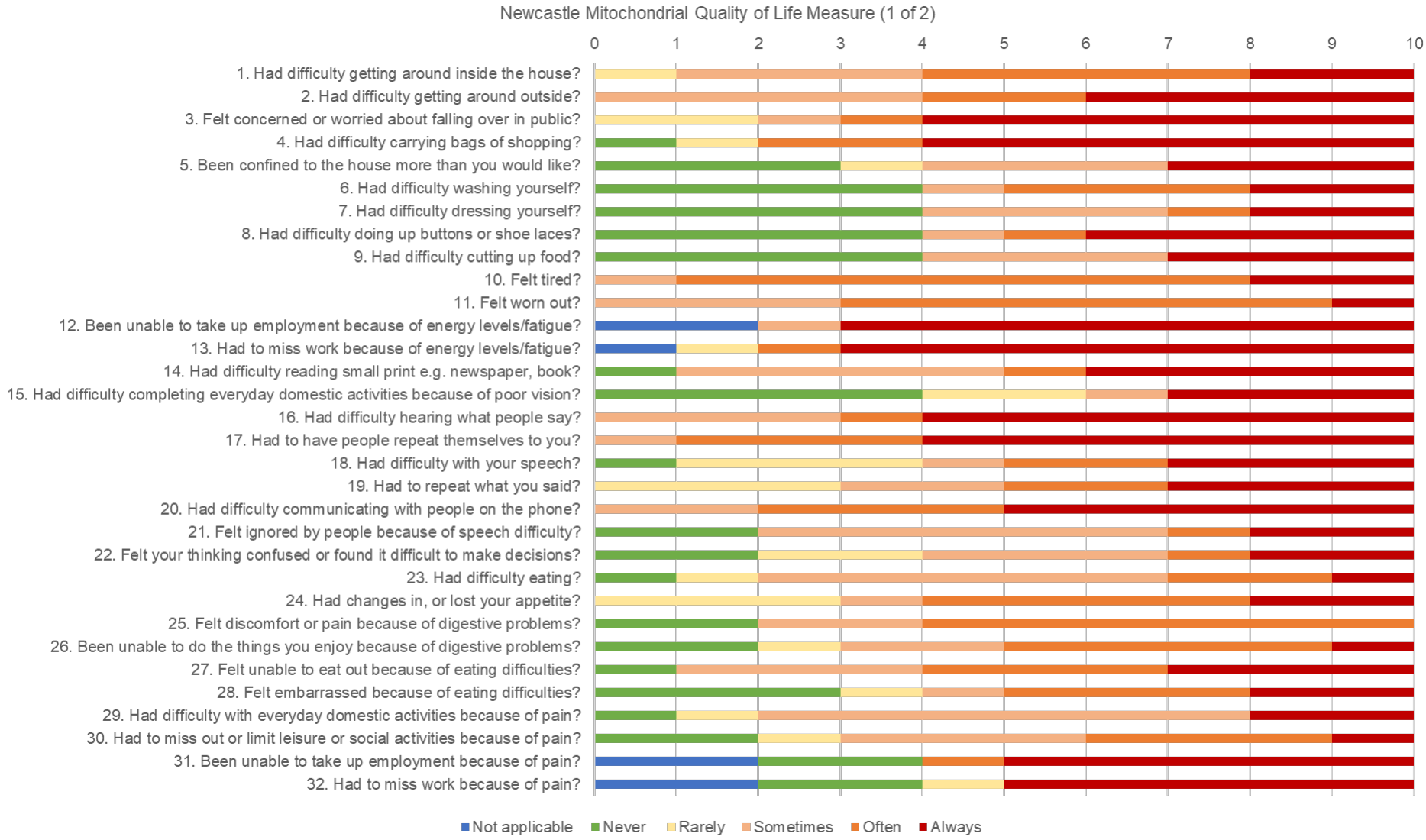

Figure 6-16: Bar graph shows the responses of ten participants in the NMQ (part 1 of 2).

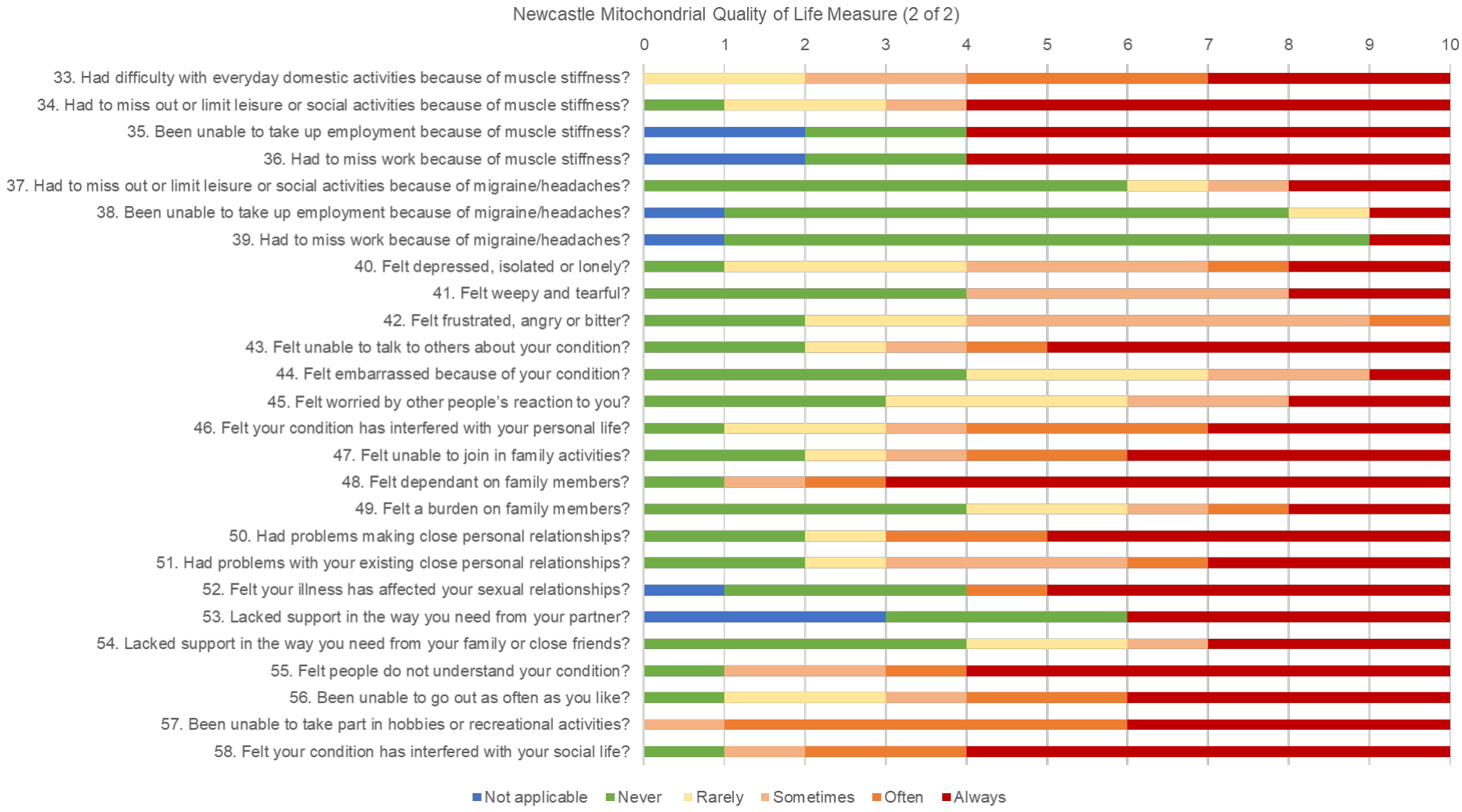

Figure 6-17: Bar graph shows the responses of ten participants in the NMQ (part 2 of 2).

6.4.3.1 NMQ domains scores

The mean scores for each domain in the NMQ are tabulated in Table 6-1, and the distributions of these scores are illustrated in Figure 6-18. Of all the domains, Fatigue appeared to have scored the lowest with a mean of 19.0 (SD 12.4, 95%CI 10.1-27.8). This is followed by Social Role or Support 24.2 (SD 23.4, 95%CI 7.4-40.9), Muscle Stiffness 28.1 (SD 31.9, 95%CI 5.3-28.1), Communication 29.2 (SD 21.1, 95%CI 14.1-44.2) and Mobility 31.0 (SD 21.6, 95%CI 15.6-46.4). On the contrary, Migraine or Headaches domain had scored highly at 76.7 (SD 41.0, 95%CI 47.4-106.0), suggesting that this domain had relatively less undesirable self-reported outcomes.

NMQ domain	Mean scores	Standard deviation, 95% confidence intervals
Fatigue	19.0	(SD 12.4, 95%CI 10.1-27.8)
Social role/support	24.2	(SD 23.4, 95%CI 7.4-40.9)
Muscle stiffness	28.1	(SD 31.9, 95%CI 5.3-50.9)
Communication	29.2	(SD 21.1, 95%CI 14.1-44.2)
Mobility	31.0	(SD 21.6, 95%CI 15.6-46.4)
Family role	38.8	(SD 28.8, 95%CI 18.1-59.4)
Pain	41.2	(SD 33.6, 95%CI 17.2-65.3)
Food and digestion	43.3	(SD 23.9, 95%CI 26.2-60.4)
Personal relationships	44.2	(SD 36.1, 95%CI 18.3-70.0)
Vision	46.3	(SD 35.9, 95%CI 20/6-71.9)
Cognition	52.5	(SD 36.2, 95%CI 26.6-78.4)
Activities of daily living	53.1	(SD 39.9, 95%CI 24.6-81.6)
Stigma	56.7	(SD 23.2, 95%CI 40.1-73.2)
Emotional wellbeing	57.5	(SD 25.9, 95%CI 39.0-76.0)
Migraine	76.7	(SD 41.0, 95%CI 47.4-106.0)

Table 6-1: Table summarises the mean scores for individual domains within the NMQ, ranked from lowest to the highest. Low scores in the NMQ indicate undesirable self-report health outcomes.

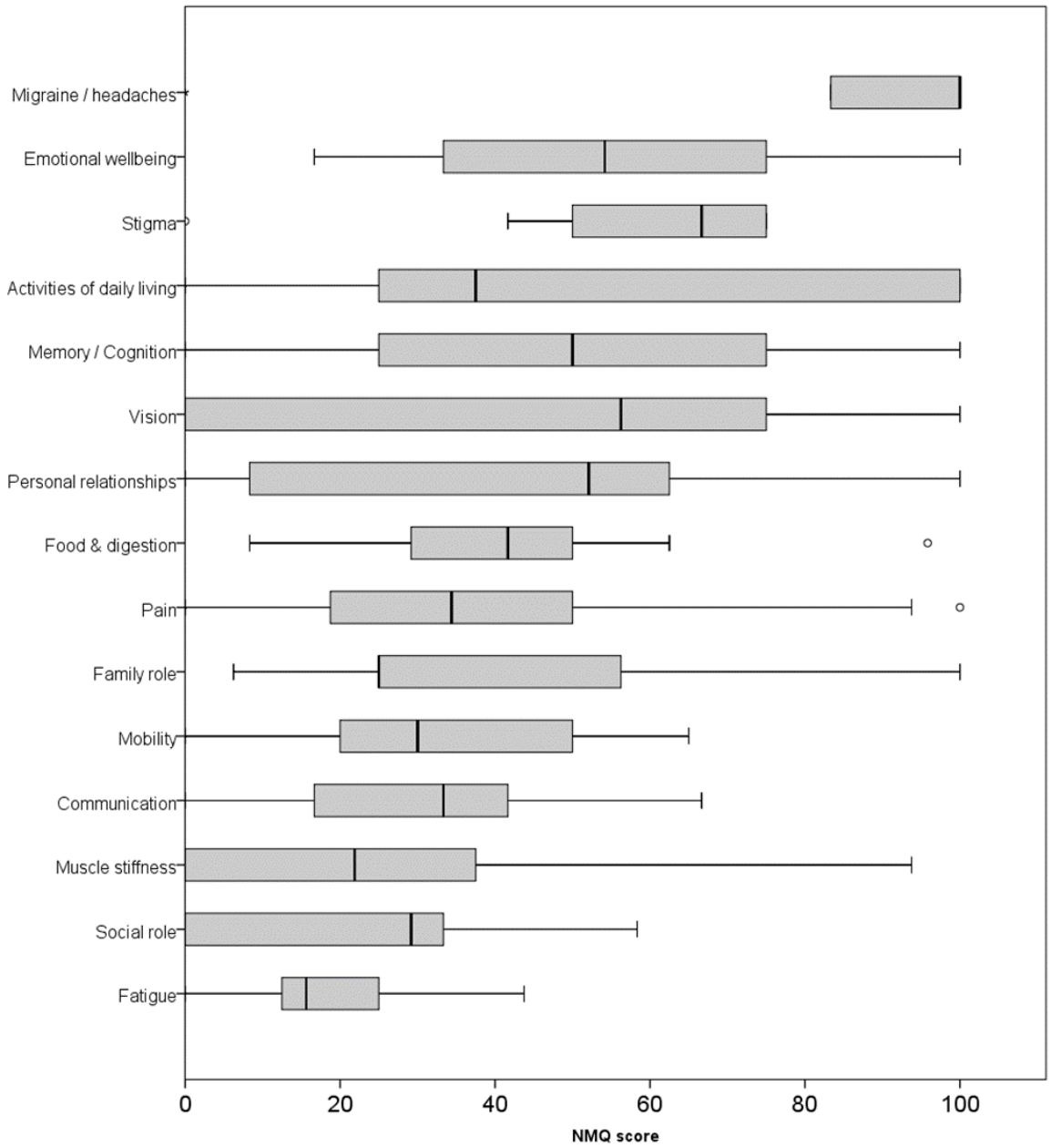

Figure 6-18: Box and whisker plot shows the distribution of NMQ scores for all domains. Low scores indicate undesirable self-reported health.

6.4.3.2 Correlation

A Spearman's rank-order correlation was run to determine the relationships between NMQ domains with related clinician-rated outcome measure (NMDAS), performance outcome measures and functional tests outlined in Chapter 3. Of all the domains in NMQ, the Activities of Daily Living subscale score correlated negatively with the total NMDAS scores (r=0.67, p=0.031) (Figure 6-19). No other significant correlations had been identified.

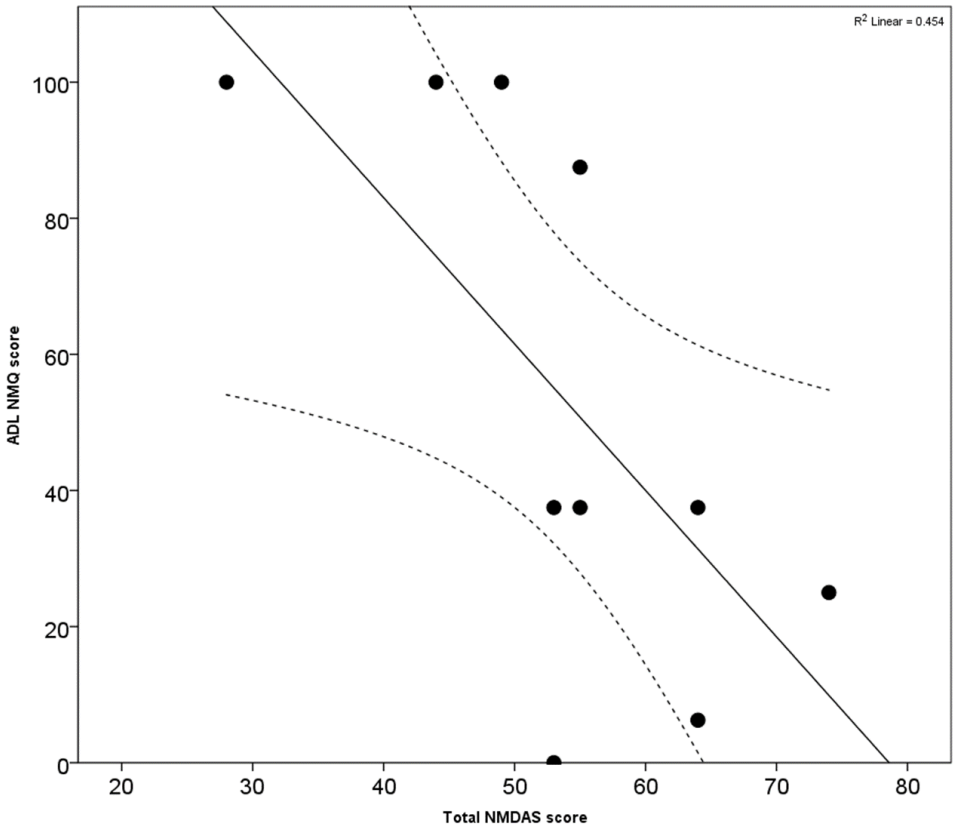

Figure 6-19: Scatter plot shows the relationship between Activities of Daily Living (ADL) score in the NMQ and the total NMDAS score for the ten participants in this study. Linear regression line (unbroken line) with its confidence intervals (dashed lines) are also shown.

6.4.4 Relationship between Neuro-QOL and NMQ

Spearman's rank-order correlations interrogated the relationships between Neuro-QOL and NMQ domains (Figure 6-20). The NMQ has a unidirectional scoring, of which the high scores indicate unfavourable outcomes while the Neuro-QOL has bi-directional scoring, depending on the domains as described in the Methods section. Hence, the significant Spearman coefficients in the matrix could be negative or positive.

In this group of participants, the Vision domain in the NMQ correlated significantly with several Neuro-QOL domains, notably Positive Affect and Wellbeing ($r=0.88$, $p<0.01$) and Depression ($r=0.85$, $p<0.01$). Positive Affect and Wellbeing in the Neuro-QOL also correlated significantly with Personal Relationships ($r=0.95$, $p<0.01$) and Cognition ($r=0.85$, $p<0.01$) domains in the NMQ. Other significant and strong correlations include Personal Relationships in NMQ with Depression in Neuro-QOL ($r=0.92$, $p<0.01$), Food and Digestion in NMQ with Satisfaction with Social Roles in Neuro-QOL ($r=0.92$, $p<0.01$), Pain in NMQ with Sleep Disturbances in Neuro-QOL ($r=0.88$, $p<0.01$).

Both Neuro-QOL and NMQ questionnaires shared some very similar domains, namely Fatigue and Stigma. However, the correlation between Neuro-QOL Fatigue and NMQ Fatigue domains were weak and not statistically significant ($r=-0.32$, $p=0.37$). Stigma domains in both Neuro-QOL and NMQ questionnaires also did not correlate strongly or significantly ($r=-0.38$, $p=0.28$). Even though the Neuro-QOL lower extremity function t-score and the Mobility item in NMQ enquired about similar quality of life measures in these participants, these two did not demonstrate strong or significant correlations ($r=0.42$, $p=0.23$).

	Anxiety tscore NeuroQOL	Depression tscore NeuroQOL	Fatigue tscore NeuroQOL	Emotion and behaviour dyscontrol tscore NeuroQOL	Sleep disturbance tscore NeuroQOL	Stigma tscore NeuroQOL	Upper extremity tscore NeuroQOL	Lower extremity tscore NeuroQOL	Cognitive tscore NeuroQOL	Positive affect & wellbeing tscore NeuroQOL	Ability to participate in social roles tscore NeuroQOL	Satisfaction with social roles tscore NeuroQOL
Mobility NMQ score	-0.06	-.667*	-.716*	-0.27	-0.61	0.04	0.44	0.42	.644*	.656*	0.35	0.48
ADL NMQ score	-0.44	-0.43	-0.24	-.679*	-0.11	-0.26	.717*	0.58	.731*	0.48	0.48	0.19
Fatigue NMQ score	-0.01	-0.55	-0.32	-0.24	-0.58	-0.09	0.33	0.43	0.55	.634*	0.25	.807**
Vision NMQ score	-0.31	-.850**	-0.42	-.703*	-.696*	-0.06	.786**	.661*	.857**	.882**	.752*	0.50
Communication NMQ score	-0.11	-.718*	0.02	-0.44	-.729*	-0.04	0.33	0.30	0.47	.812**	0.42	0.59
Memory cognition NMQ score	-0.19	-.746*	-0.23	-0.53	-.748*	-0.06	.637*	0.59	.763*	.854**	0.46	.684*
Food and digestion NMQ score	-0.29	-0.56	0.01	-0.42	-0.62	-0.27	0.17	0.53	0.34	.645*	0.30	.920**
Pain NMQ score	-0.26	-.763*	-0.26	-0.51	-.881**	0.33	.643*	0.55	0.61	.810**	0.62	0.60
Muscle stiffness NMQ score	0.03	-0.38	-0.29	-0.23	-0.53	0.16	0.54	0.33	0.53	0.48	0.33	0.57
Migraine headaches NMQ score	0.14	-0.31	-0.43	-0.01	-0.54	0.28	0.40	0.36	0.28	0.22	0.42	-0.04
Emotional wellbeing NMQ score	-0.58	-.743*	-0.48	-.675*	-0.47	-.688*	0.41	.875**	.680*	.674*	0.29	0.24
Stigma NMQ score	-0.51	-0.26	0.16	-0.39	-0.09	-0.38	-0.09	0.47	0.02	0.23	0.18	0.43
Family role NMQ score	-.677*	-.671*	-0.24	-.759*	-0.33	-0.41	0.40	.700*	0.54	.635*	0.48	0.38
Personal relationships NMQ score	-0.25	-.923**	-0.52	-0.60	-.795**	-0.02	.659*	0.52	.826**	.945**	0.55	0.38
Social role NMQ score	-0.06	-.650*	-0.16	-0.36	-0.62	0.03	0.34	0.36	0.49	.719*	0.48	.731*

*Figure 6-20: Correlation matrix shows item-item relationships between Neuro-QOL and NMQ using Spearman's rank-order correlation coefficients. Statistically significant coefficients with ** indicate p<0.05 and ** indicate p<0.01.*

6.4.5 Fatigue impact scale (FIS)

The mean score for FIS in this group of participants were 99.6 (SD 33.5, 95%CI 75.6–123.6) out of the maximal 160. The breakdown of all responses from these participants for the Fatigue Impact Scale (FIS) is illustrated in Figure 6-21. In the cognitive section of FIS, six of the ten participants reported that they had ‘big problem’ or ‘extreme problem’ in that they found themselves more forgetful and had slowed down their thinking in the last four weeks. Half of all the participants also had ‘big problem’ or ‘extreme problem’ in paying attention for long periods of time, making decisions, and finishing tasks that require thinking.

Their scores in the Physical section of the FIS reflected their poor physical ability. Nine of these ten participants found ‘big problem’ or ‘extreme problem in that their muscles felt weaker than they should, they had to limit their physical activities, and they required more frequent or longer periods of rest in the last four weeks. Seven of them complained that they had trouble maintaining physical effort for long periods and were less able to complete tasks that required physical effort.

These physical limitations had some impact on their psychosocial wellbeing. In this section, most of the participants (n=7) had a 'big problem' or 'extreme problem’ in their abilities to travel outside their homes in the last month. More than half (n=6) had rated ‘big problem’ or ‘extreme problem’ in relying on others for help, less motivated to engage in social activities, having few social contacts outside their home, difficulty in dealing with anything new, less able to deal with emotional issues, difficulty in participating in family activities and unable to provide emotional support to their families. All participants had found minor difficulties seem like major difficulties as a ‘moderate’ to ‘extreme problem’.

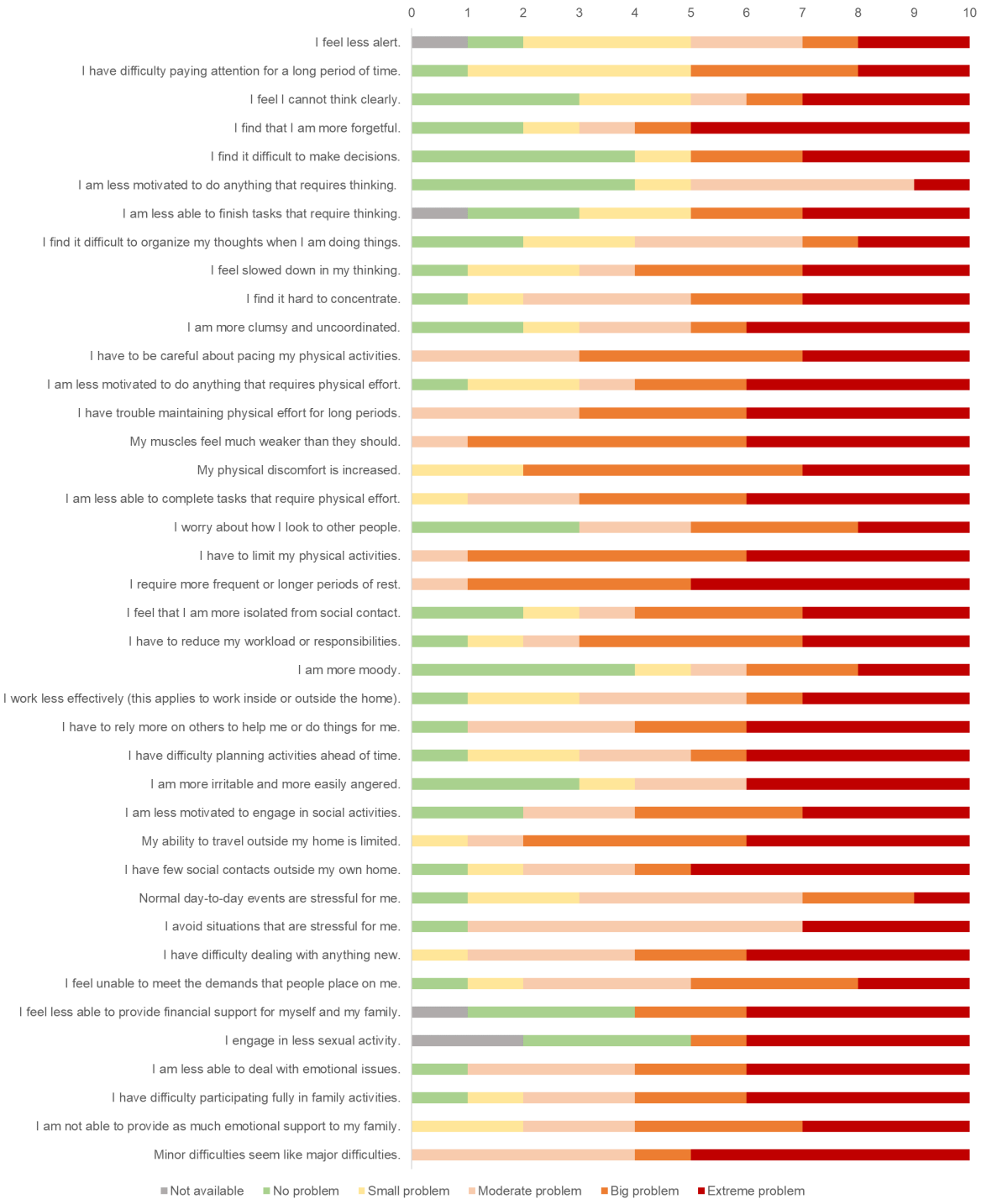

Figure 6-21: Bar graph shows the responses of ten participants in the Fatigue Impact Scale (FIS).

6.4.5.1 Correlations

A Spearman's rank-order correlation was run to determine the relationships between scores FIS scores with other related measures in Chapter 5. Of all the outcome measures, FIS correlated significantly with several. First, the FIS score demonstrated a strong and significant correlation with the total NMDAS score, a measure of disease burden (r=0.71, p=0.02) (Figure 6-22). There was also a moderate and significant correlation with one of the items in the NMDAS, the severity of exercise tolerance (r=0.67, p=0.03) (Figure 6-23). Lastly, FIS scores had a moderate and significant correlation with the nine-hole PEG test (r=0.66, p=0.04) (Figure 6-24). Notably, there were no significant correlations with myopathy item in the NMDAS (r=0.39, p=0.27) or 6-minute walk test (r=0)

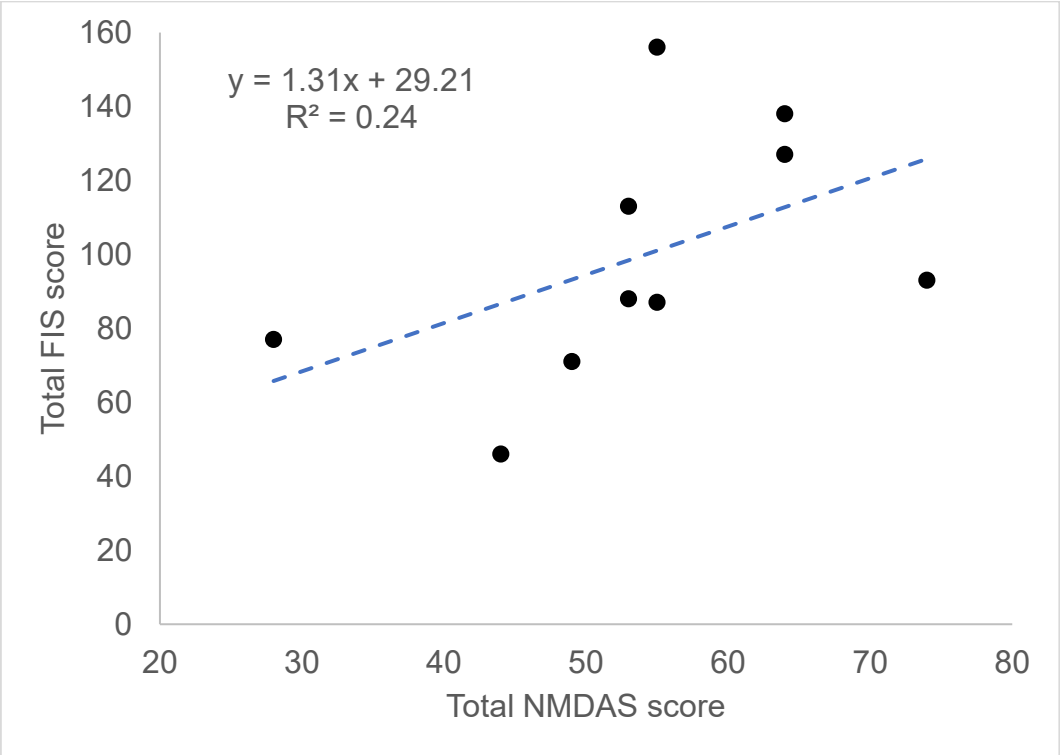

Figure 6-22: Scatter plot shows the relationship between total FIS scores and the total NMDAS score for the ten participants in this study. A linear regression line (dashed line) is also shown.

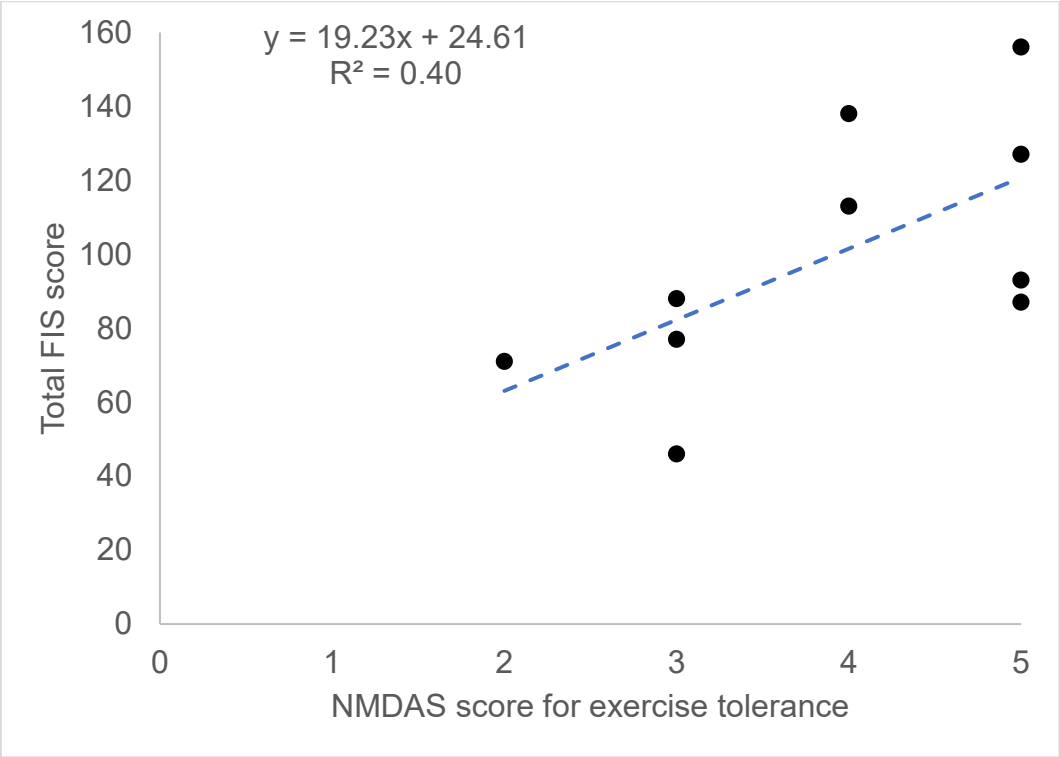

Figure 6-23: Scatter plot shows the relationship between total FIS scores and the NMDAS score for exercise tolerance for the ten participants in this study. A linear regression line (dashed line) is also shown.

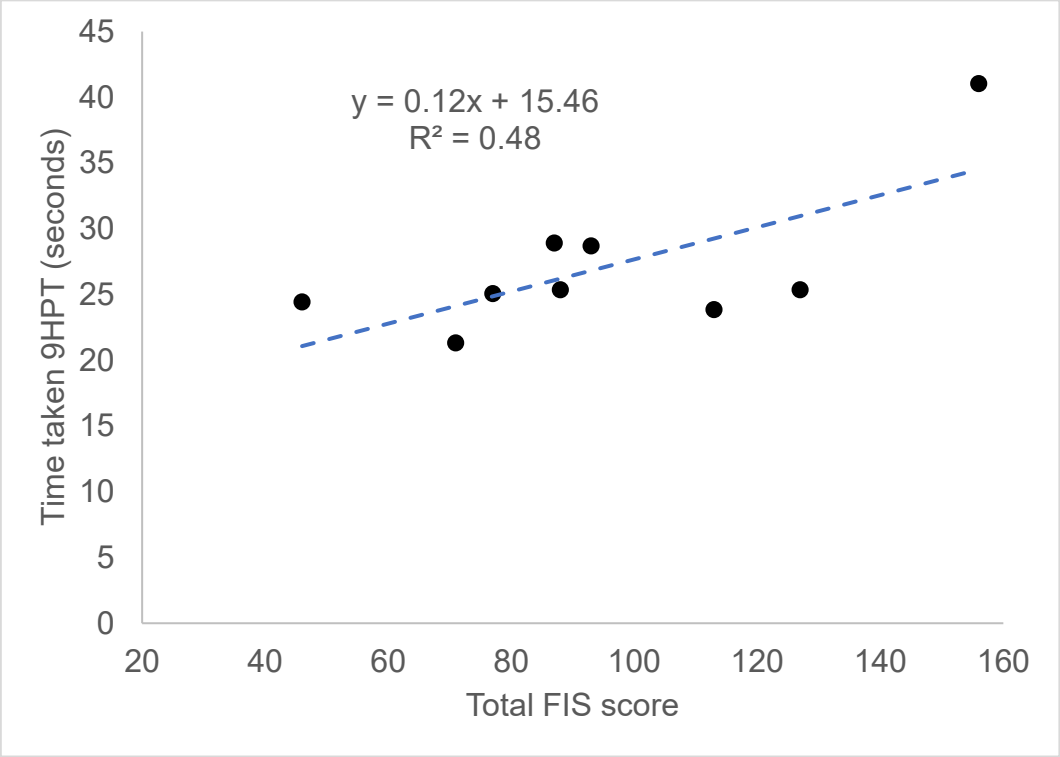

Figure 6-24: Scatter plot shows the relationship between the time taken to complete the nine-hole peg test and the total FIS scores for the ten participants in this study. A linear regression line (dashed line) is also shown.

6.4.6 Daily Fatigue Impact Scale (DFIS)

The mean score for the Daily Fatigue Impact Scale (DFIS) for this group of participants was 19.7 (SD 7.4, 95%CI 14.4-25.0), and the breakdown of their responses to the eight questions in DFIS are summarised in Figure 6-25. On the day of assessment, nine of the ten participants had recognised that they had 'big' or 'extreme' problems in their fatigue, resulting in limitations to their physical activities. Eight had 'big' or 'extreme' problems in maintaining physical effort for a long period of time. Seven had felt less motivated to do anything physical and had to reduce their workload. Two participants had not found that slowing down or feeling less alert to be of any problems, but two others felt these were ‘extreme problems’ to them.

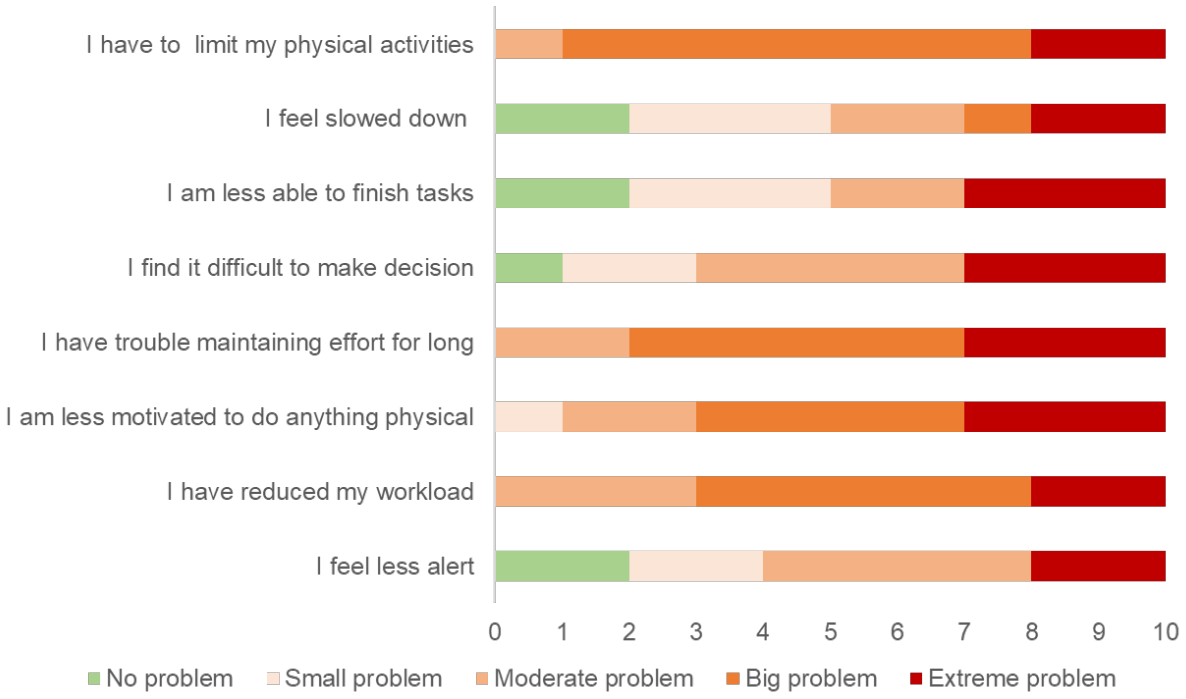

Figure 6-25: Bar graph shows the responses of ten participants in the Daily Fatigue Impact Scale (DFIS).

6.4.6.1 Correlation

A Spearman's rank-order correlation was also run to determine the relationships between scores DFIS scores with other related measures in Chapter 5. Akin to the longer version FIS, the DFIS score also correlated strongly and significantly with the time taken to complete the nine-hole peg test (r=0.70, p=0.03) (Figure 6-26). However, their similarities did not extend to other outcome measures. There were no correlations with total NMDAS scores (r=0.36, p=0.31) (Figure 6-27) or, exercise tolerance (r=0.33, p=0.36) (Figure 6-28). Despite these differences, FIS and DFIS still demonstrated moderate and significant correlation with each other (p=0.69, p<0.026) (Figure 6-29).

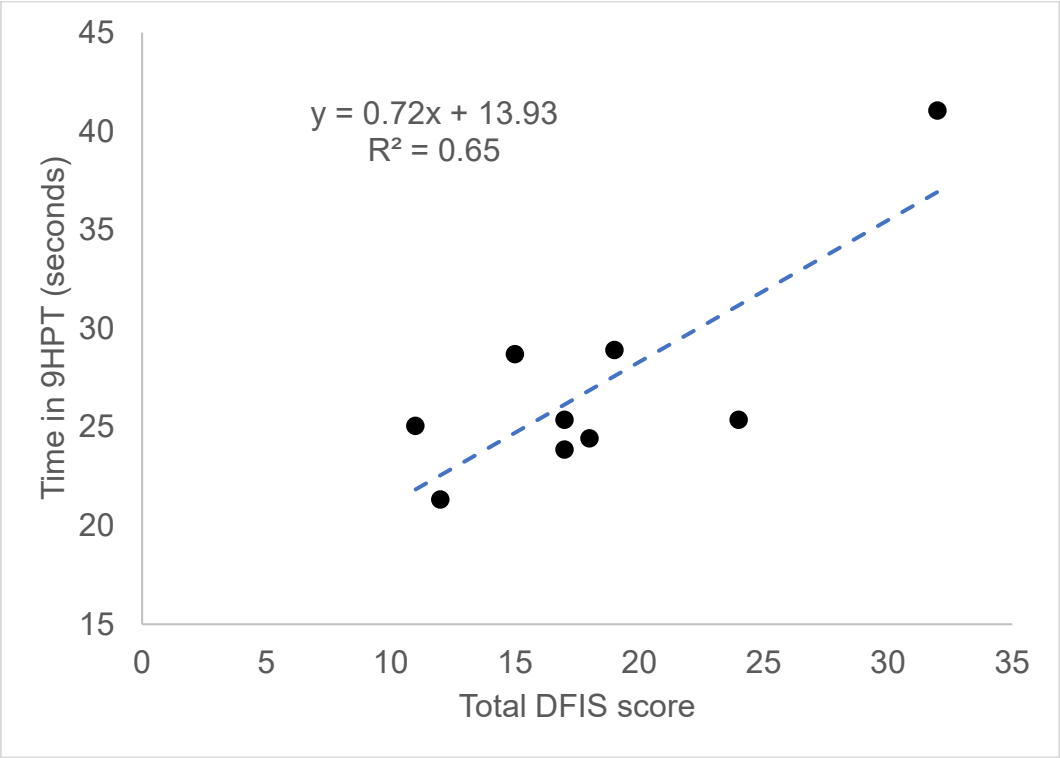

Figure 6-26: Scatter plot shows the relationship between the time taken to complete the nine-hole peg test and the total DFIS scores for the ten participants in this study. A linear regression line (dashed line) is also shown.

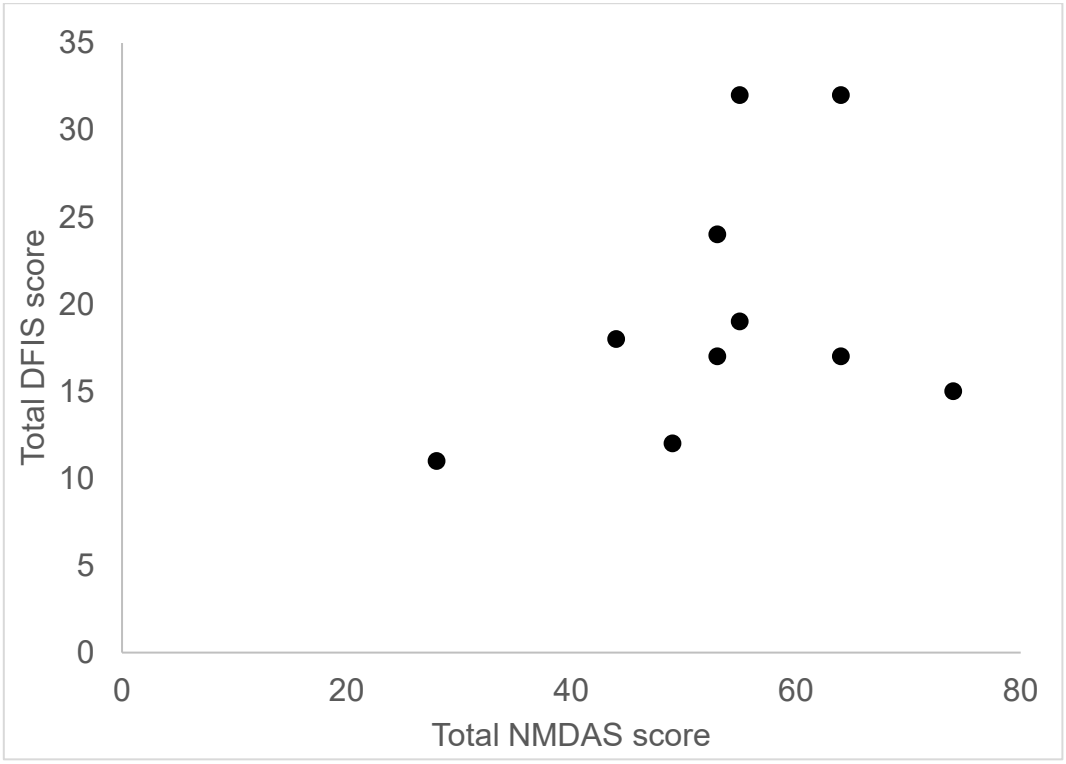

Figure 6-27: Scatter plot shows the relationship between the total DFIS scores and total NMDAS scores for the ten participants in this study.

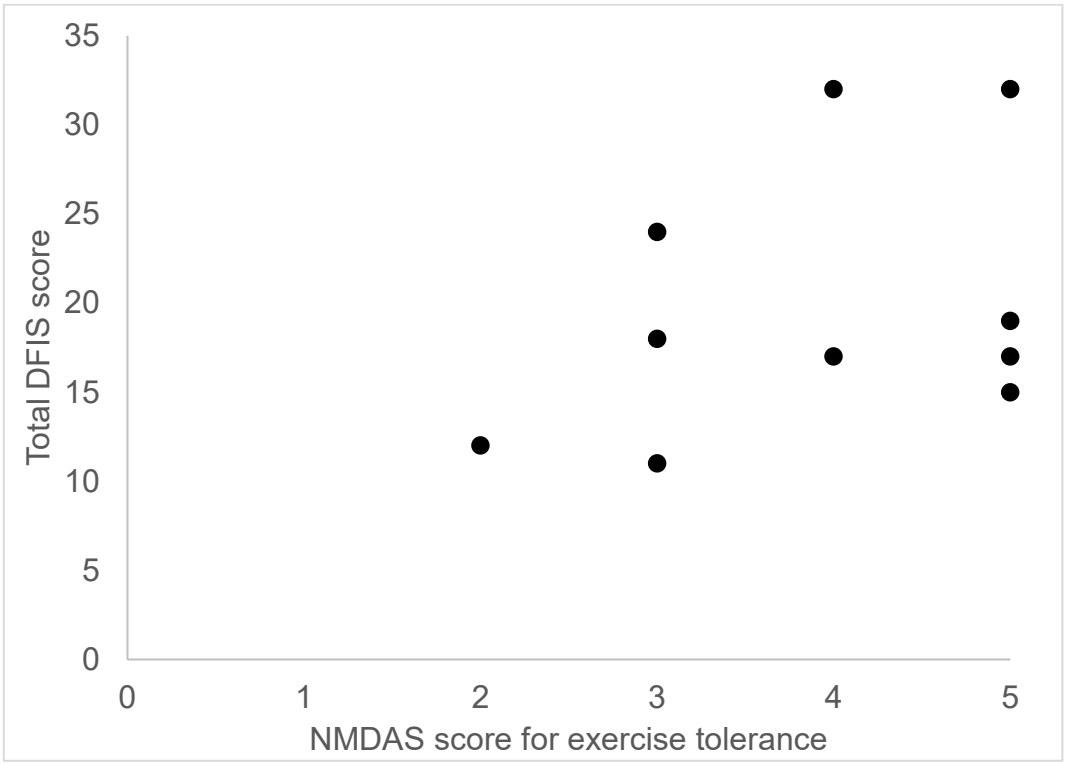

Figure 6-28: Scatter plot shows the relationship between the total DFIS scores and the NMDAS score for exercise tolerance for the ten participants in this study.

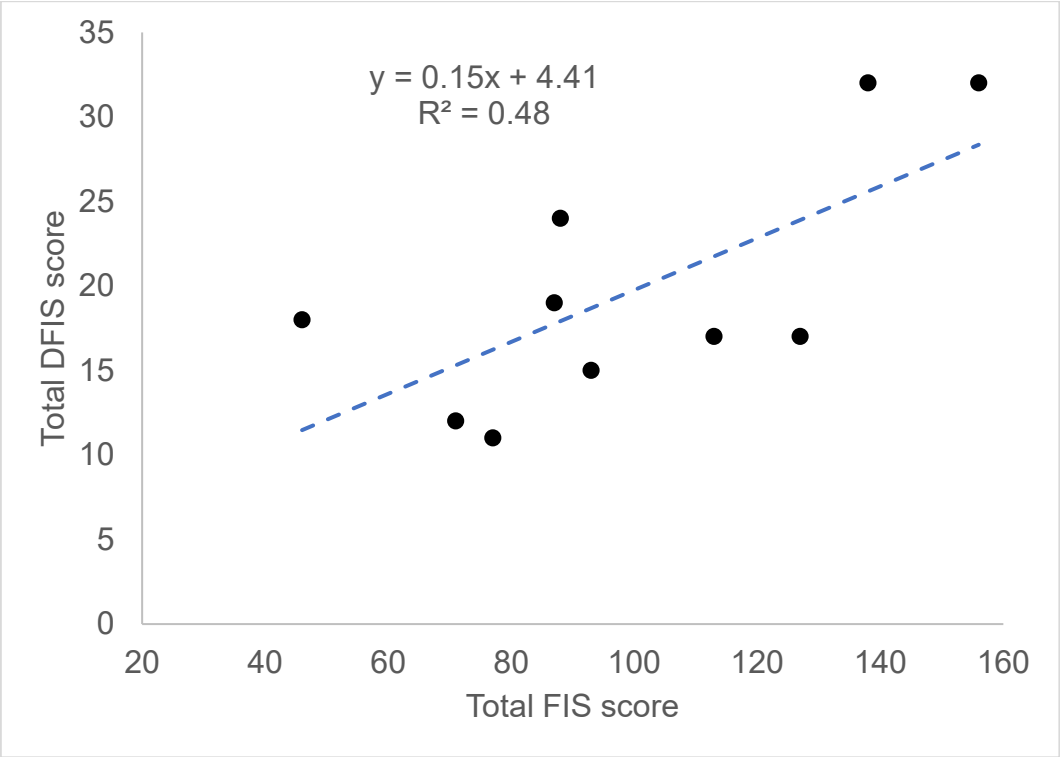

Figure 6-29: Scatter plot shows the relationship between the total DFIS scores and the FIS for the ten participants in this study. A linear regression line (dashed line) is also shown.

6.4.7 Swallowing Quality of Life questionnaire (SWAL-QOL)

The responses from these participants to the Swallowing Quality of Life questionnaire (SWAL-QOL) are summarised from Figure 6-30 to Figure 6-34. SWAL-QOL have ten quality-of-life subscales and a symptoms frequency scale.

In the Burden subscale, half of all the participants did not find that swallowing problem a major distraction in their lives. With regards to this, two other participants felt that this statement was ‘a little true’ and another participant rated this as ‘somewhat true’. Compared to the Burden subscale, the subscale that probed their Eating Desire were rated poorer. Half of these ten participants did not enjoy eating anymore, and four of them rarely felt hungry. Their Eating Duration had also reportedly increased, with eight participants feeling that they took longer to eat than other people.

Six participants ‘strongly agreed’ or ‘agreed’ that it was difficult to find food that they liked and had problems figuring what they can eat. In the Social subscale, six participants also stated that they did not go out to eat because of their swallowing problems. Half of all participants felt that their swallowing problem made it hard for them to have social lives and social gathering had not been enjoyable because of their swallowing problems. In the Mental Health subscale, six participants found their swallowing problems frustrated them and four of them felt discouraged by their swallowing problems. In the Fear subscale, six participants responded, ‘always true’ or ‘often true’ to the statement ‘I never know when I am going to choke’.

Three of the participants noticed that people had a hard time understanding them, and it had been difficult for them to speak clearly 'all of the time'. The majority of these participants also rated that they felt tired (n=9) and weak (n=7), either ‘all of the time’ or ‘most of the time’. Four participants complained of trouble sleeping, and five participants had trouble staying asleep, ‘all of the time’ or ‘most of the time’.

In the symptom frequency scale of SWAL-QOL, six participants ‘almost always’ or ‘often’ have to clear their throat. Five participants complained that they ‘often’ had thick and excessive saliva or phlegm. Three participants ‘often’ felt that food sticking in their throats and another three ‘sometimes’ felt this problem too.

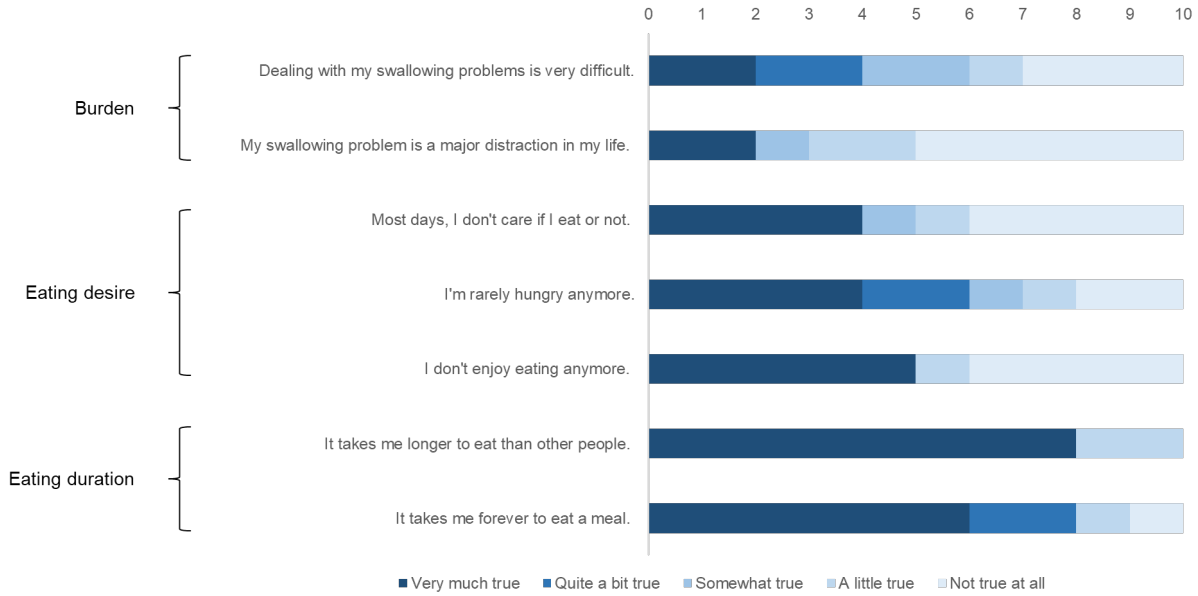

Figure 6-30: Bar graph shows the responses by ten participants regarding burden, eating desire and eating duration in the SWAL-QOL

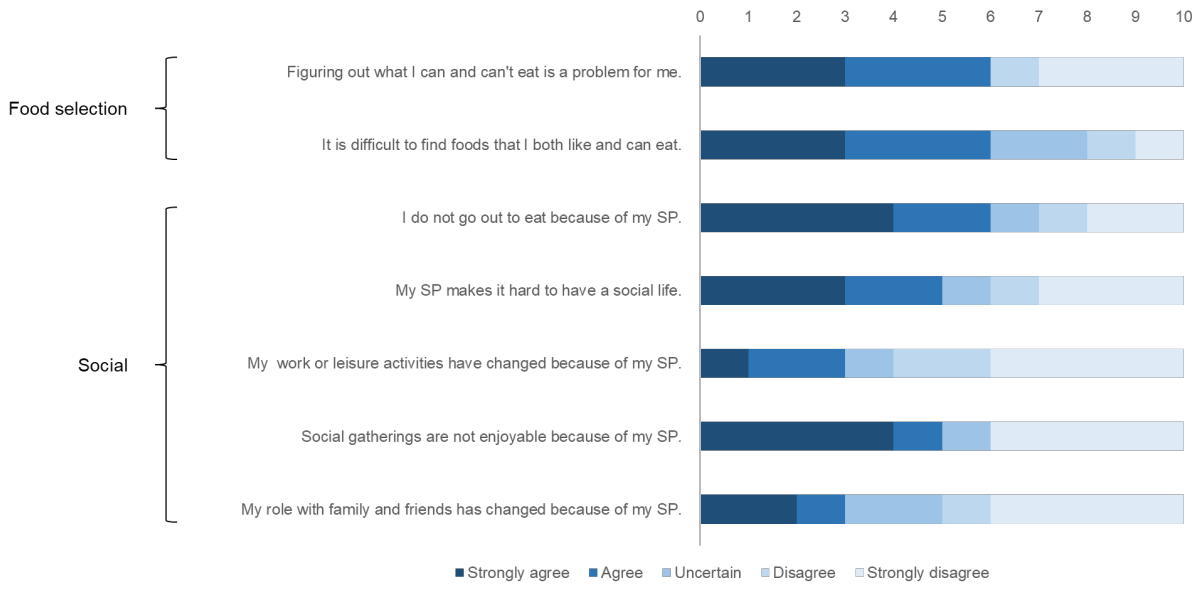

Figure 6-31: Bar graph shows the responses by ten participants regarding food selection and social issues in the SWAL-QOL

Figure 6-32: Bar graph shows the responses by ten participants regarding mental health problems and feelings of fear in the SWAL-QOL

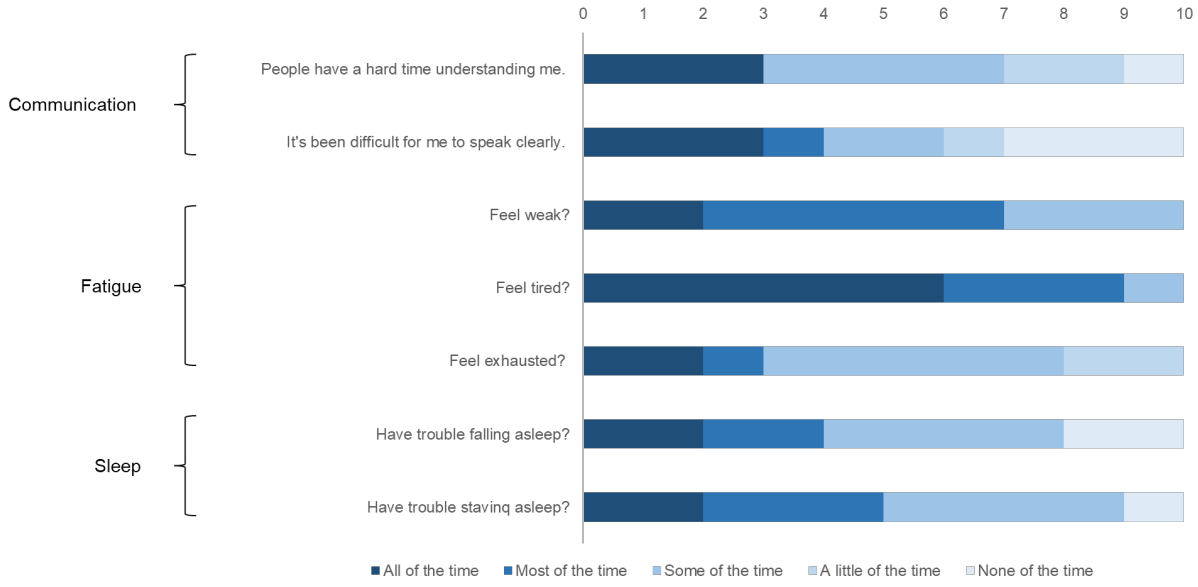

Figure 6-33: Bar graph shows the responses by ten participants regarding communications, fatigue and sleep problems in the SWAL-QOL

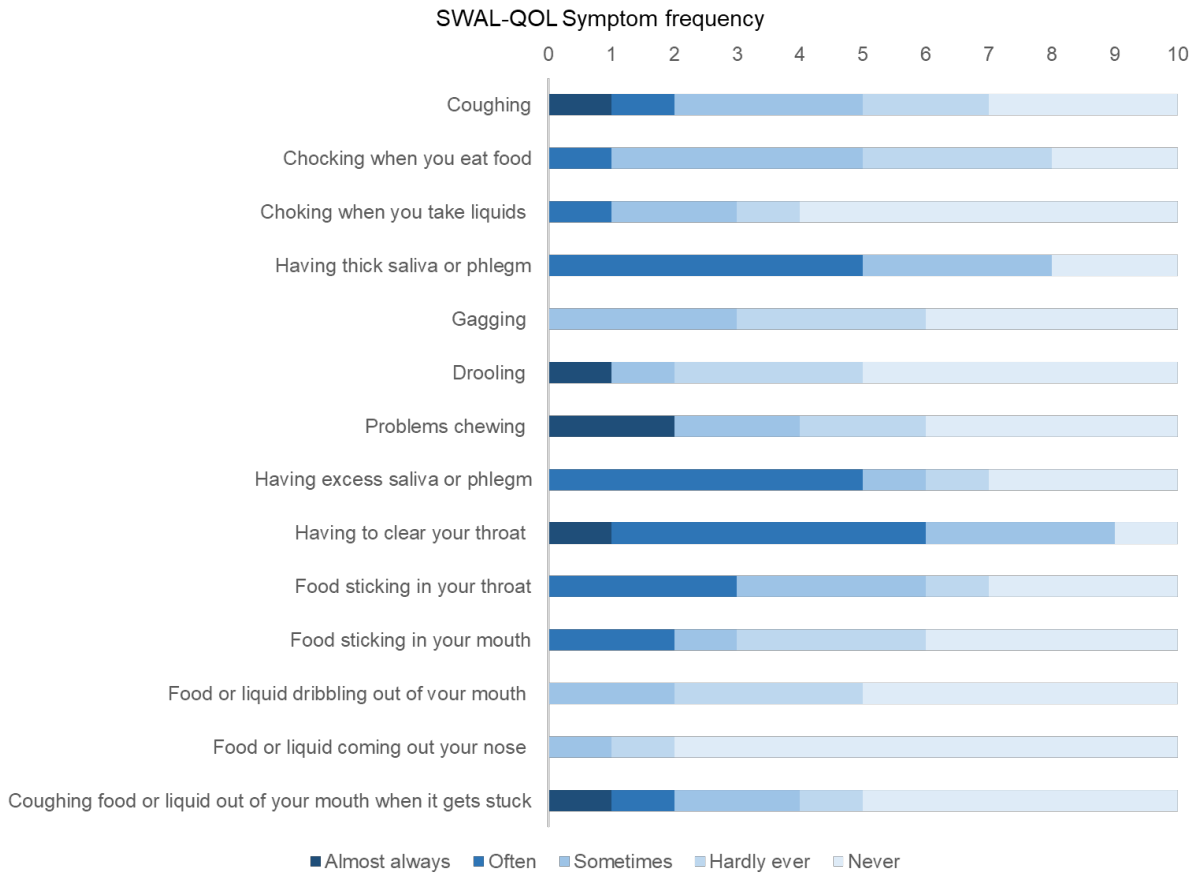

Figure 6-34: Bar graph shows the responses of ten participants regarding the frequency of their swallowing symptoms in the SWAL-QOL.

The scoring in each quality-of-life subscale of the SWAL-QOL is expressed as percentages of the maximum possible domain score (Figure 6-35). In other words, lower percentages indicate a less favourable quality of life measures. For these participants, the mean total SWAL-QOL percentages stood at 55% (SD 20.7, 95%CI 39.9-69.6). Of the ten subscales, four subscales scored less than the total SWAL-QOL percentages. Eating Duration at 35% (SD 26.7, 95%CI 15.9-54.2) fared the worst, closely followed by Fatigue at 42% (SD 12.2, 95%CI 33.3-50.7). The other two subscales were Food Selection and Sleep Disturbance, which scored 52% (SD 28.6, 95%CI 31.5-72.5) and 53% (SD 22.1, 95%CI 37.2-68.8) respectively.

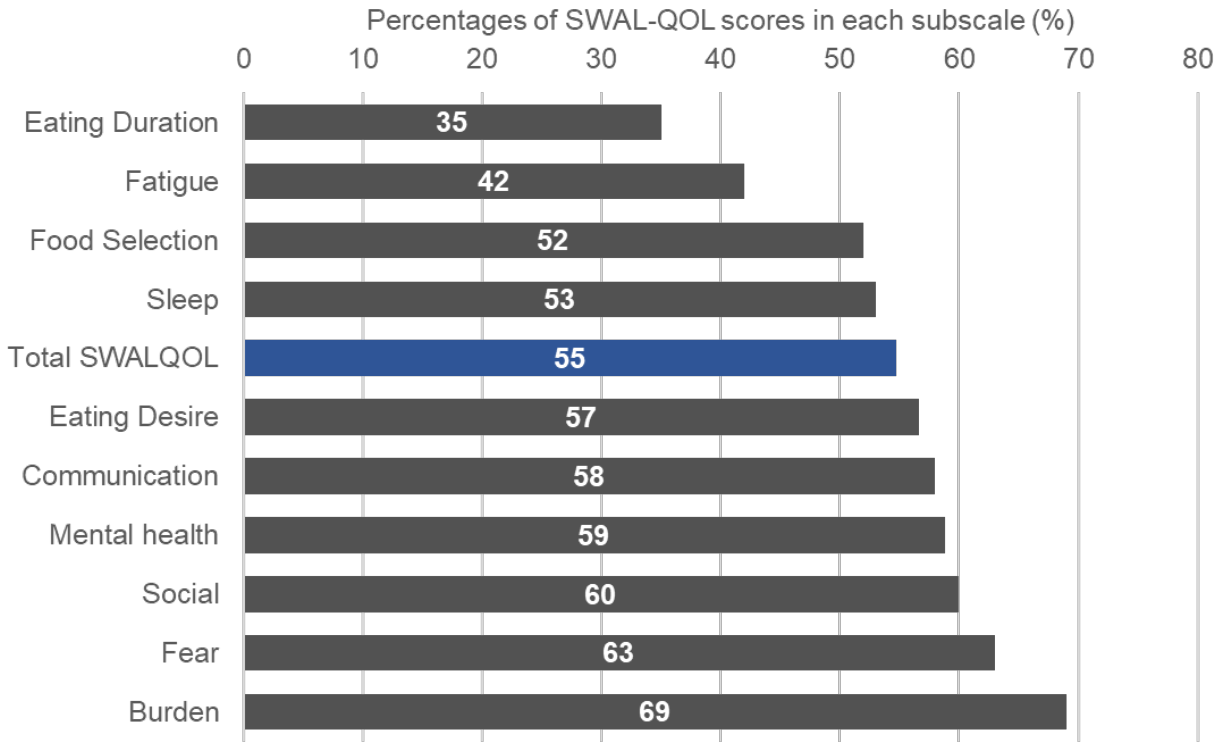

Figure 6-35: Bar chart summarises the mean percentages for individual sections within the SWAL-QOL, ranked from lowest to the highest. Low scores in the SWAL-QOL indicate undesirable self-reported swallowing-related health outcomes.

6.4.7.1 Correlations

The self-reported symptom frequency scores within the SWAL-QOL correlated with the other ten subscales about the quality of life (r=0.82, p=0.004) (Figure 6-36). A Spearman's rank-order correlation was run to determine the relationships between SWAL-QOL with other outcome measures in Chapter 5. SWAL-QOL scores have shown a negative, strong, and significant correlation with swallowing function ratings in the NMDAS (r=0.78, p=0.014) (Figure 6-37). However, SWAL-QOL did not show any significant association with the swallowing speed from the 100ml water swallow tests (r=0.53, p=0.139) (Figure 6-38).

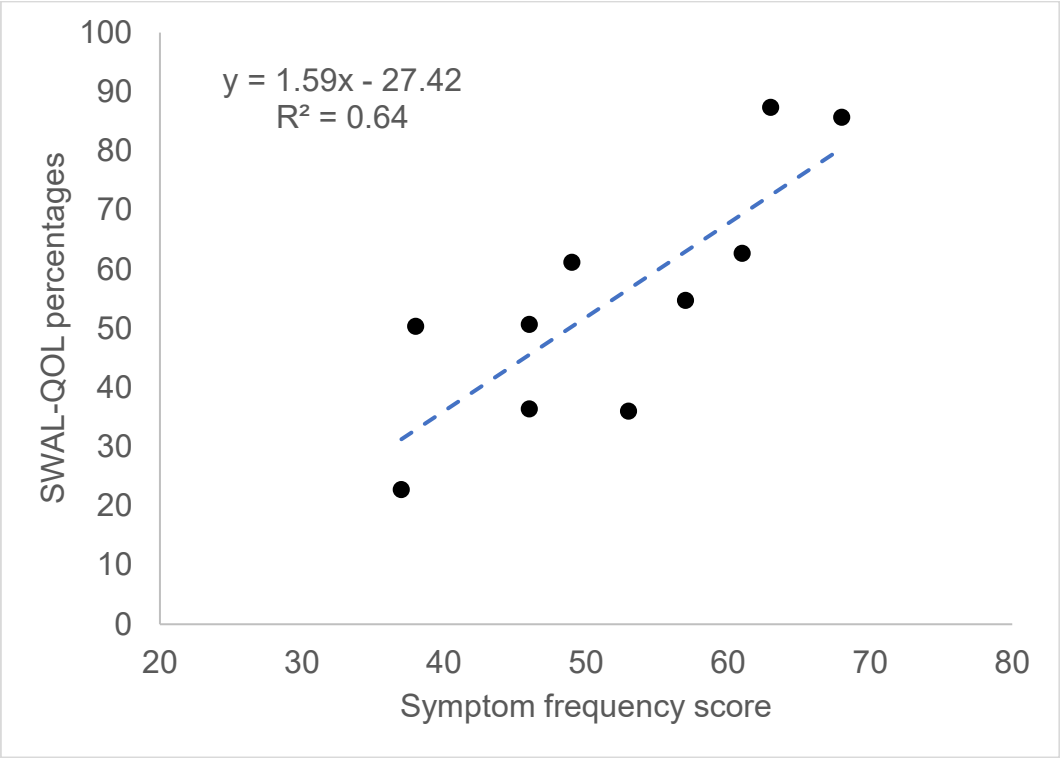

Figure 6-36: Scatter plot shows the relationship between the total SWAL-QOL percentages and the self-reported symptom frequency score for the ten participants in this study. A linear regression line (dashed line) is also shown.

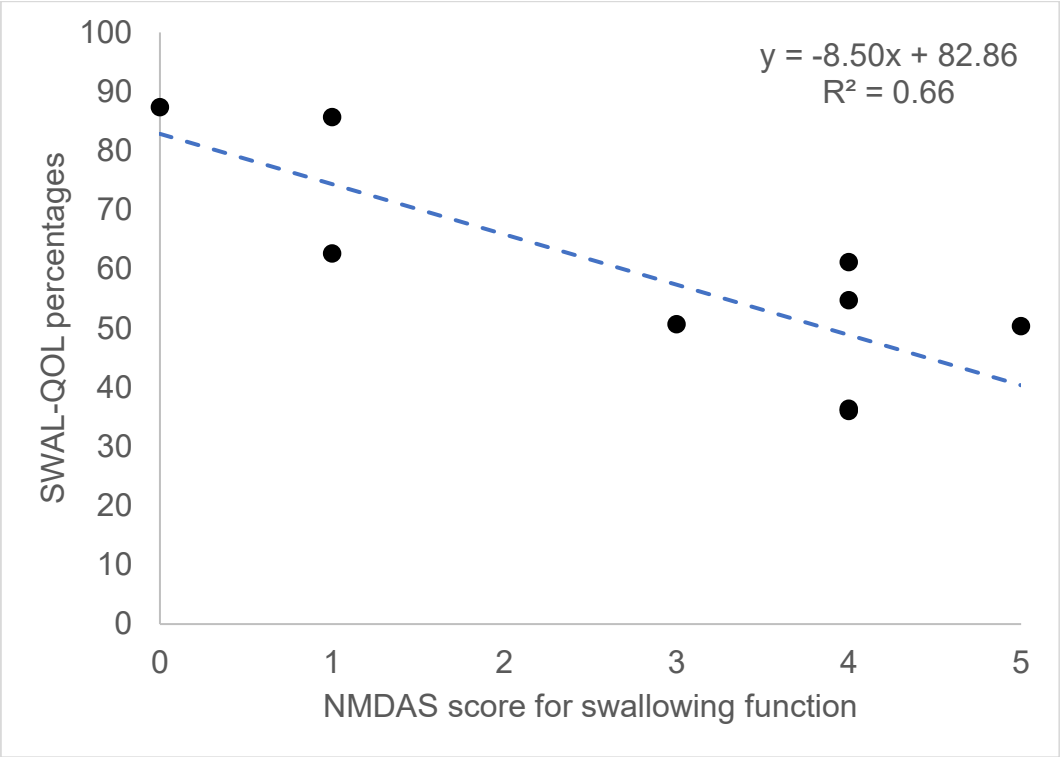

Figure 6-37: Scatter plot shows the relationship between the total SWAL-QOL percentages and the swallowing function score in the NMDAS for the ten participants in this study. A linear regression line (dashed line) is also shown.

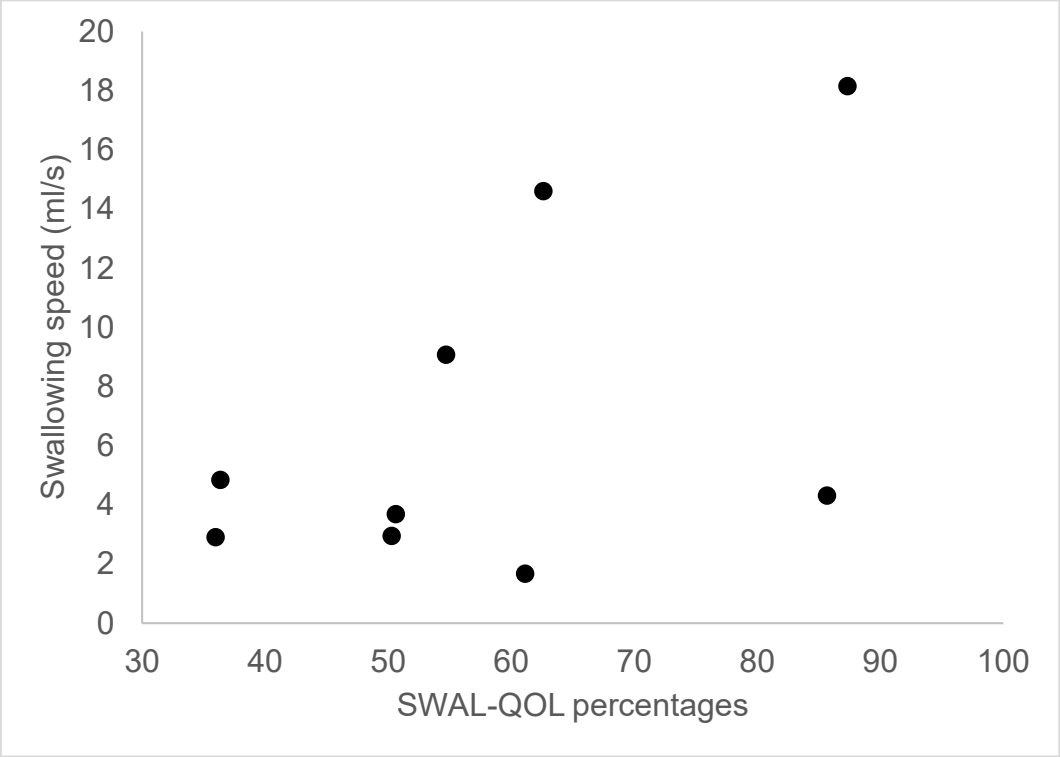

Figure 6-38: Scatter plot shows the relationship between the swallowing speed in the 100ml-water swallow tests and total SWAL-QOL percentages for the ten participants in this study.

6.4.8 Dysphagia Handicap Index (DHI)

Dysphagia Handicap Index (DHI) questionnaires have three subsections, namely Physical, Functional and Emotional. Figure 6-39 outlines the answers from these ten participants to the DHI questionnaires. In the Physical subsection of DHI, half of all participants expressed that they 'always' need to drink fluid to wash food down, and the other half 'sometimes' had to do this. Eight of these participants noted that they ‘often’ (n=6) or ‘always’ (n=2) had to swallow again before food would go down and choke when they took their medications.

Their dysphagia had also impacted their function. In this section, nine participants felt that they ‘always’ (n=8) or ‘sometimes’ (n=1) took longer them longer to eat a meal than it used to. Eight of them ate smaller meals more often due to their swallowing problems. Six of them 'always' avoid some foods because of their swallowing problems, and five participants ‘always’ changed the ways they swallow to make it easier to eat.

In the Emotional subsection, five out of the ten participants claimed that they ‘always’ felt embarrassed to eat in public and did not enjoy eating as much as they are used to. On the other hand, seven participants reported that they never felt handicapped or became angry at themselves because of their swallowing problems.

In this group of participants, the mean scores for Physical, Functional and Emotional subsections were 14 (SD, 95%CI 9.9-18.1), 19 (SD 12.4, 95%CI 9.9-27.7) and 11 (SD 6.7, 95%CI 5.8-15.4). The total score for these three subsections was 43.4 (SD 23.0, 95%CI 27.0-59.9). These mean scores were higher than the results compared to 323 controls in a meta-analysis of DHI normative value, which was 2.49 with a confidence interval of 0.51-4.48 (Sobol et al., 2021).

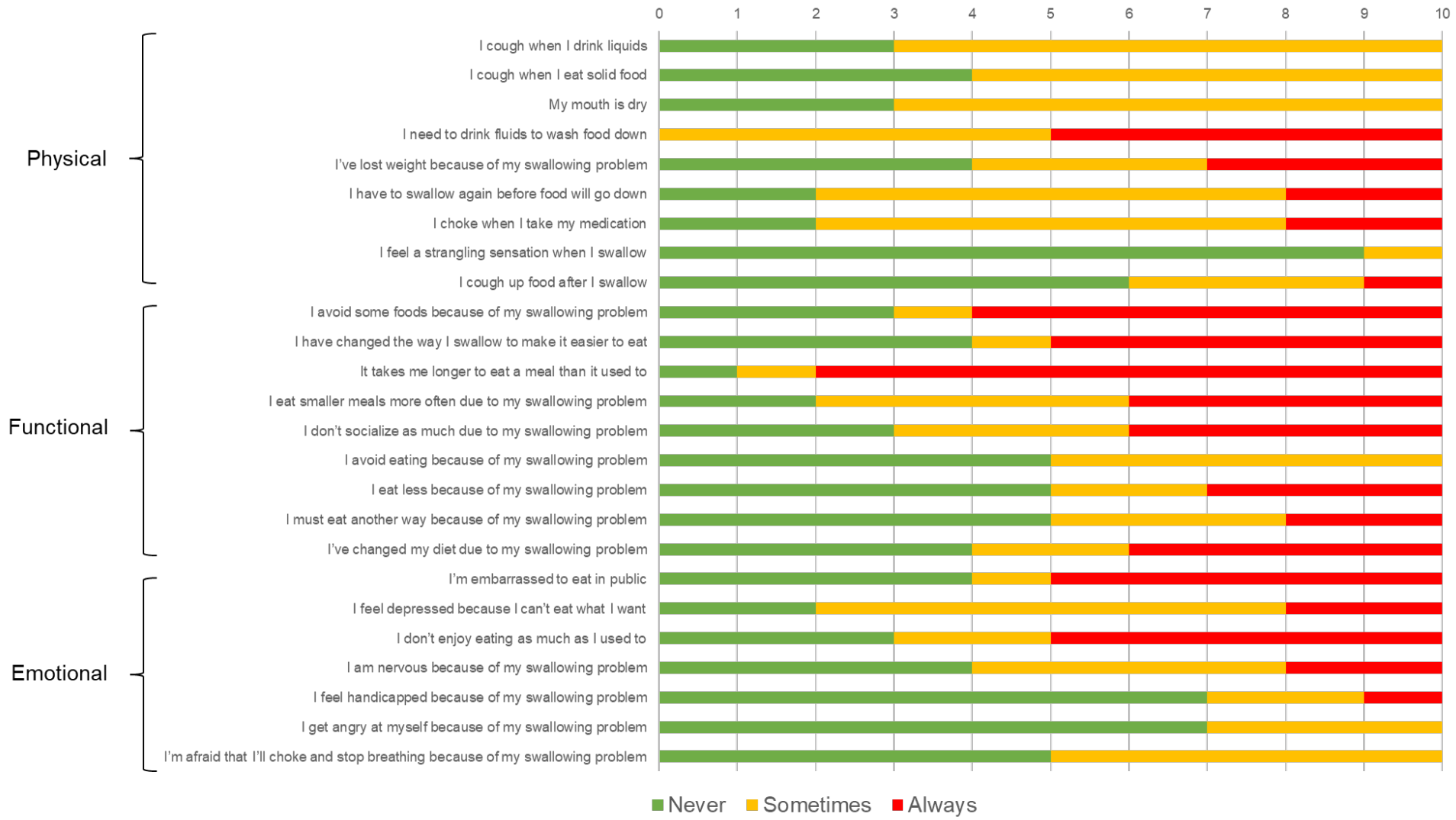

Figure 6-39: Bar graph shows the responses of ten participants in the Dysphagia Handicap Index (DHI).

6.4.8.1 Correlations

The total DHI scores for all three subsections correlated strongly and significantly with self-reported severity rating within the DHI (r=0.94, p<0.001) (Figure 6-40). A Spearman's rank-order correlation was run to determine the relationships between SWAL-QOL with other outcome measures in Chapter 5. Similar to the SWAL-QOL, the DHI has shown a very strong and significant correlation with the swallowing function ratings in the NMDAS (r=0.82, p=0.007) (Figure 6-41). There was also no correlation with swallow speed from the 100-ml water swallow tests (r=-0.42, p=0.27) (Figure 6-42). The DHI and SWAL-QOL correlated strongly with each other SWAL-QOL (r=-0.79, p=0.007) (Figure 6-43).

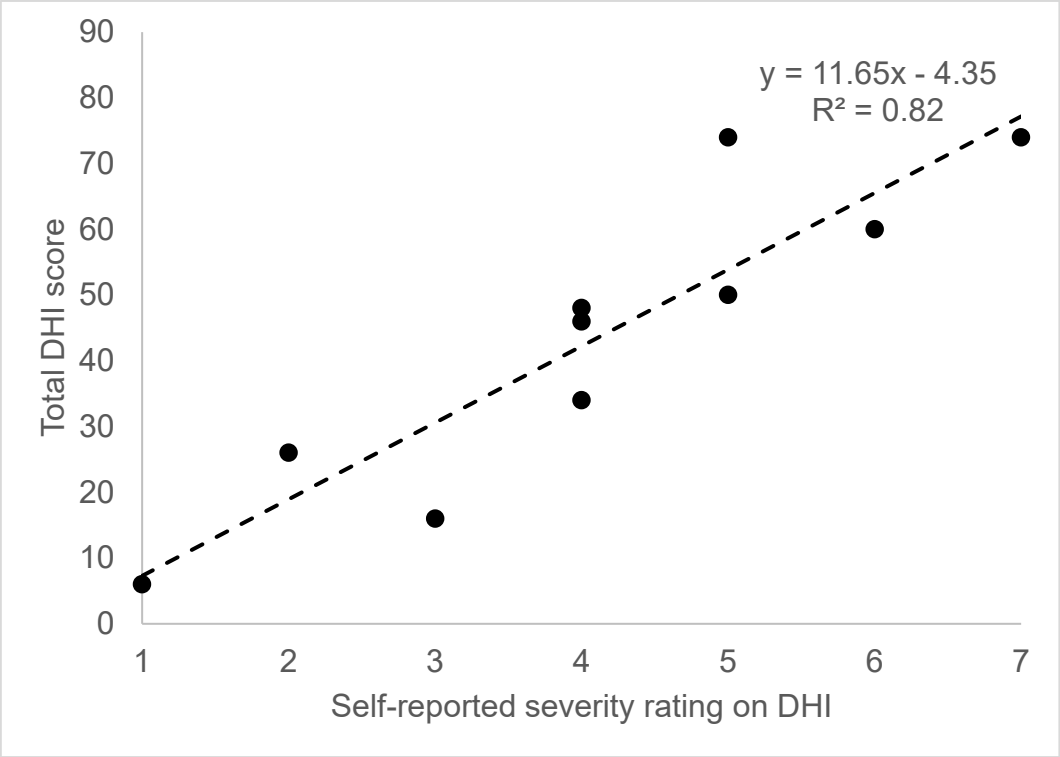

Figure 6-40: Scatter plot shows the relationship between the total DHI score and the self-reported severity rating in the DHI for the ten participants in this study. A linear regression line (dashed line) is also shown.

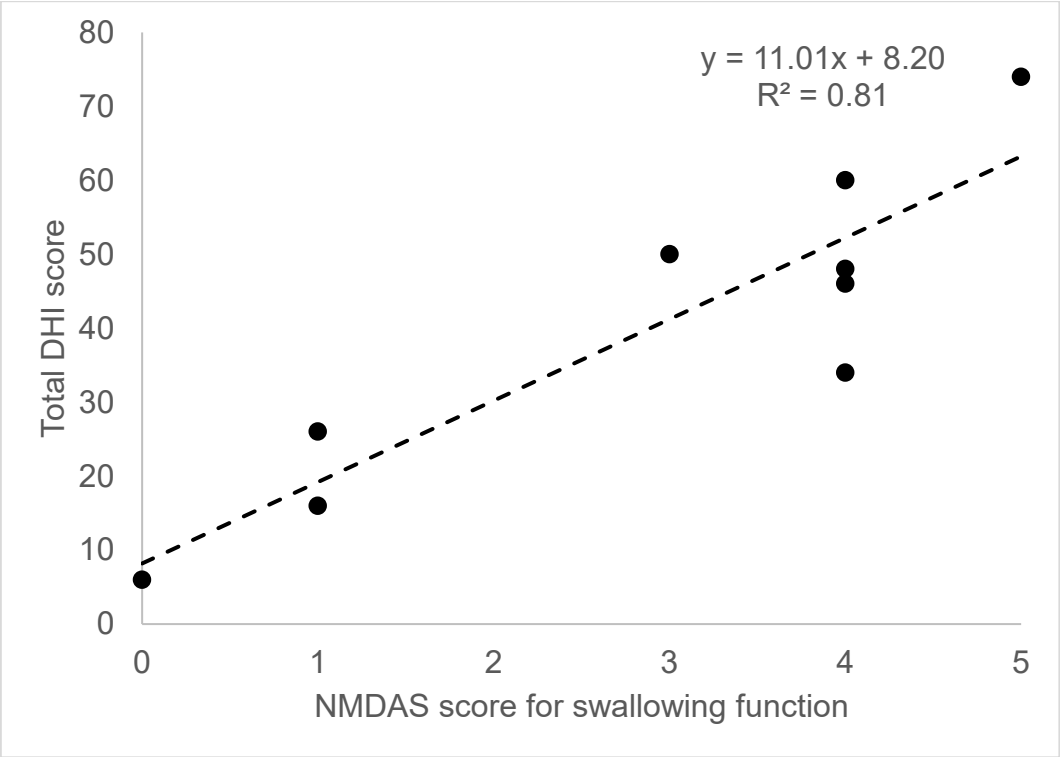

Figure 6-41: Scatter plot shows the relationship between the total DHI scores and the swallowing function score in the NMDAS for the ten participants in this study. A linear regression line (dashed line) is also shown.

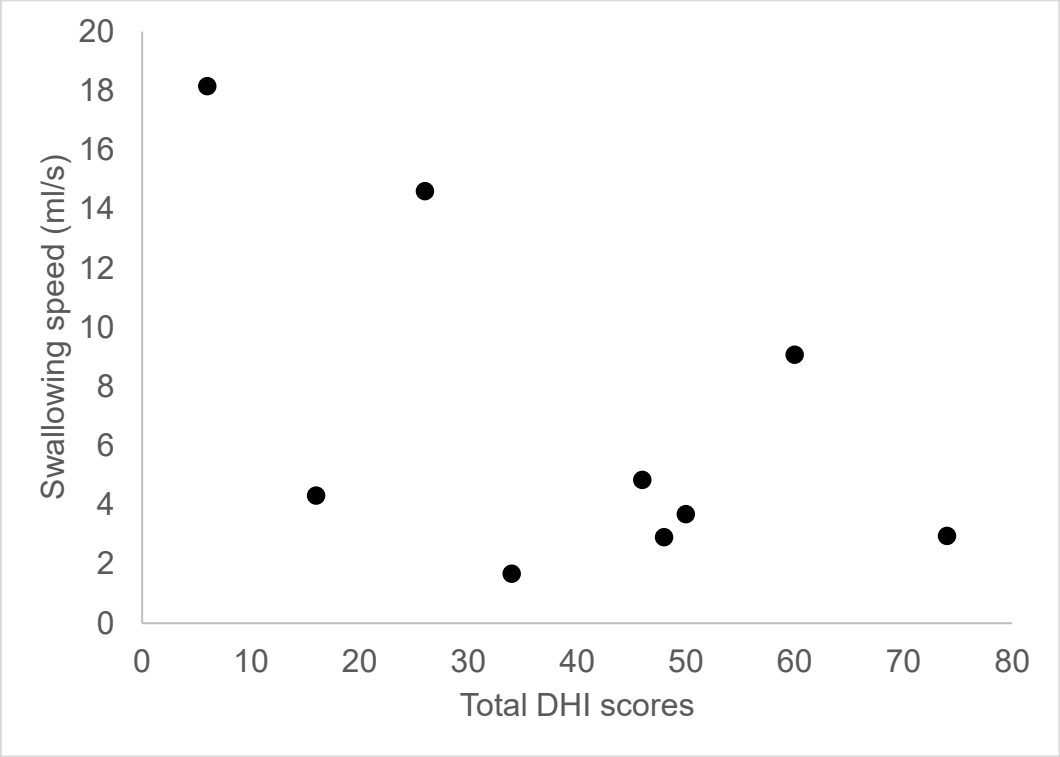

Figure 6-42: Scatter plot shows the relationship between the swallowing speed in the 100-ml water swallow tests and the total DHI scores for the ten participants in this study.

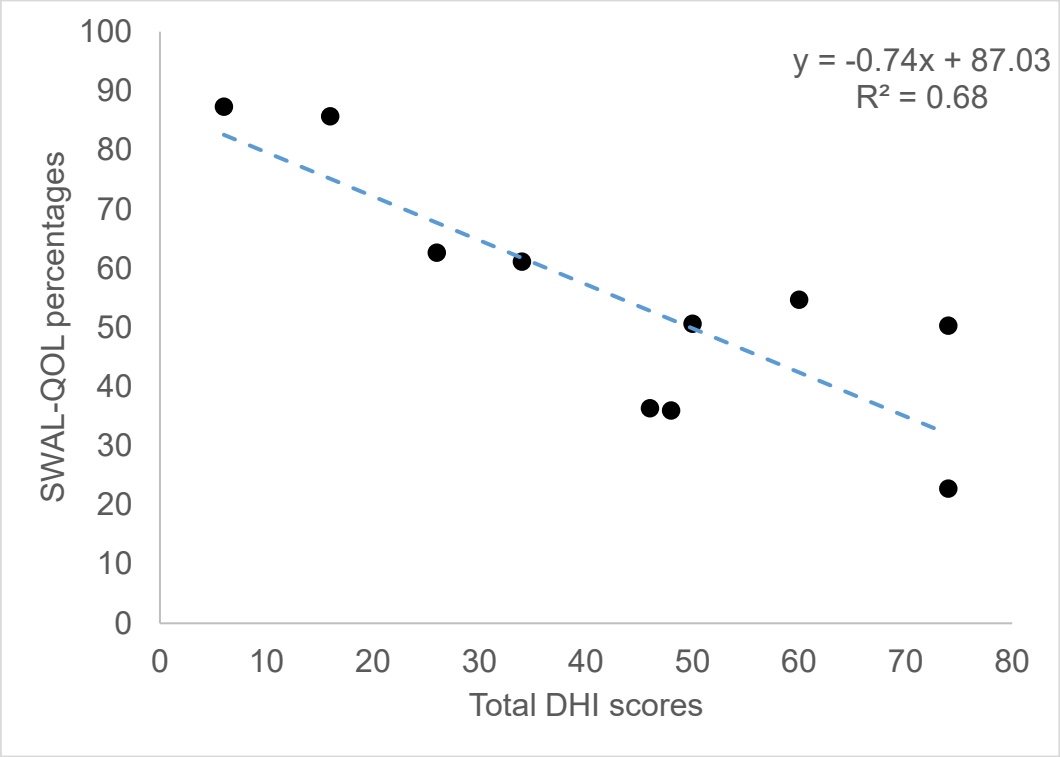

Figure 6-43: Scatter plot shows the relationship between the total SWAL-QOL percentages and the total DHI scores for the ten participants in this study. Linear regression line (dashed line) is also shown.

6.4.9 Internal consistencies

An internal consistency analysis was carried on these patient-reported outcome measures. The Cronbach's alpha values for all six questionnaires answered by this cohort of participants are summarised in Table 6-2. All six scales had demonstrated a high level of internal consistency. Both Neuro-QOL (α = 0.865) and NMQ (α = 0.951) were employed to measure the quality-of-life construct in these participants. The shortened version DFIS (α = 0.914) had a slightly lower Cronbach's alpha than FIS (α = 0.914) but both had achieved acceptable reliability in measuring the fatigue construct. Meanwhile, SWAL-QOL (α = 0.966) and DHI (α = 0.935) had also shown a satisfactory level of internal consistency in the measurement of the swallowing construct.

Questionnaires	Cronbach's alpha
Quality of life questionnaires	
Neuro-QOL	0.865
Newcastle Mitochondrial QoL	0.951
Fatigue-specific questionnaires	
Fatigue Impact Scale	0.950
Daily Fatigue Impact Scale	0.914
Swallowing-specific questionnaires	
SWAL-QOL	0.966
Dysphagia Handicap Index	0.935

Table 6-2: Table summarises the Cronbach's alpha values for the six questionnaires used in this study.

6.5 Discussion

From the results of this Chapter, several key findings are evident. First, Neuro-QOL has shown that these participants have poorer quality of life and has highlighted some domains that they struggled with as compared to the reference population, namely upper extremity function, lower extremity function, ability to participate in social roles and fatigue. The NMQ, a mitochondrial disease-specific PROM, also demonstrated a similar reduction in quality of life across other areas of their health, like the Neuro-QOL. This finding, pertaining to quality-of-life, ties in well with previous studies that reached similar conclusions using different PROMs on another group of mitochondrial patients. Kim and colleagues in 2010 observed that mothers of children with mitochondrial disease perceived poorer health-related quality of life and higher caregiver burden (Kim et al., 2010). Meanwhile, a cohort of adult Italian patients with mitochondrial myopathies secondary to mtDNA single deletion or multiple deletions registered low SF-36 quality of life scores (Orsucci et al., 2012). More recently, impaired quality of life has also been reported in patients with m.3243A>G point mutation (Verhaak et al., 2016) and with *POLG* gene mutations (Hikmat et al., 2020). Even though all these studies used different PROMs, together with results from this Chapter further substantiate the prevailing argument that mitochondrial disease has a significant impact on quality of life. Regardless of which PROMs has been utilised, a consensus is needed on this reduction in quality of life to be translated into a tangible value for the healthcare economic evaluation. By putting this together, only can the mitochondrial clinical research community determine the quality-adjusted life years in a cost-utility analysis and persuade public or private bodies for future investments into clinical care and research.

Second, fatigue featured more prominently than other domains in the NMQ and Neuro-QOL questionnaires in this group of participants with autosomal recessive *RRM2B* gene variants. Patients and clinicians may define fatigue differently, but it is generally described as lack of energy, tiredness, and low tolerance towards physical activity. In mitochondrial disease, the inadequate conversion to ATP from oxidative phosphorylation, which provides energy for cellular function, has been regarded as a putative mechanism for fatigue (Mancuso et al., 2012a, Nunnari and Suomalainen,

2012, Filler et al., 2014). In this cohort, fatigue in both Neuro-QOL and NMQ were perceived along with high levels of anxiety and sleep problems. Others have shown that mental health and sleep problems are covariates in perceived fatigue in other groups of mitochondrial disease (Gorman et al., 2015a, Parikh et al., 2019). Although there are some significant correlations of fatigue with some other items in the Neuro-QOL and NMQ questionnaire, this study did not uncover other correlations with the conventional outcome measures described in Chapter 5. The lack of correlations in this small sample size makes it difficult to speculate on the construct validity of these two scales.

In contrast, the Fatigue Impact Scale (FIS), which specifically enquires about participants' fatigue-related health, has demonstrated satisfactory convergent validity with other outcome measures in Chapter 5. The FIS score rated by participants increases as the clinician-reported disease burden and exercise intolerance rises. These findings are in accordance with findings reported by others that pointed out mitochondrial disease severity is associated with fatigue (Mancuso et al., 2012a, Gorman et al., 2015a, Parikh et al., 2019). Gorman et al. (2015) also administered the FIS questionnaire in a group of mitochondrial patients and a third of them had scored more than 80. This cohort of *RRM2B* participants scored nearly 100 on average, putting them at the worse end of the perceived fatigue spectrum among other mitochondrial patients. These participants who scored highly on FIS also performed slowly at nine-hole peg tests. Daily Fatigue Impact Scale (DFIS), the abbreviated version of FIS, has also shown a strong correlation with the nine-hole peg test but not the clinician-rated outcome measures. Under certain circumstances, the convergent validity DFIS can be misconstrued as less worthy than its predecessor. However, the DFIS, despite its brevity, still retain strong correlations with FIS scores in this study. Both scales that measure perceived fatigue should be considered in their own merit because they are designed to capture participants' perceptions at different lengths of time. FIS might be also affected by the other factors. This study could have explicitly evaluated the impact of sleep disturbances from respiratory weakness on patient-reported fatigue scores in more depth. Children and adults with neuromuscular disorders are known to have a high frequency of sleep-disordered breathing (Labanowski et al., 1996). Sleep disordered breathing might lead to significant

morbidity and increased mortality (Arens and Muzumdar, 2010). These patients often complained of fatigue, tiredness, sleepiness and lack of energy (Chotinaiwattarakul et al., 2009). In this cohort of participants, their predisposing factors to sleep-disordered breathing include a reduced activity of respiratory muscles and poor lung mechanics due to their underlying neuromuscular weakness. Without non-invasive ventilatory (NIV) support, sleep-disordered breathing at night could impact their fatigue levels the next day. Quality of life questionnaires are often sensitive to sleep disorders and their treatment with NIV, probably in part because they include fatigue-related constructs (Siccoli et al., 2008). NIV is consistently effective in improving the quality of life and physiological parameters in the affected patients (Tsolaki et al., 2011). Therefore, the FIS score in this study is likely to be negatively affected by sleep dysfunction secondary to respiratory muscle weakness and positively affected by the use of NIV. Future studies should explore these relationships in more detail.

Another key finding in this study is related to patient-reported swallowing problems. These SWAL-QOL and Dysphagia Handicap Index (DHI) questionnaires have shown poor swallowing related quality of life in these participants with autosomal recessive *RRM2B* gene variants. A similar pattern of results of SWAL-QOL was obtained by Vogel and colleagues (2017) in 14 German mitochondrial patients who harboured a different gene mutation, the *POLG* variants. They also found mediocre rates of fatigue scores affecting their swallowing (55% in *POLG* patients vs 42 % *RRM2B* patients). Participants with pathogenic variants in their *RRM2B* genes in this cohort have also reported longer eating duration but not by those in *POLG* gene mutations in the German study (Vogel et al., 2017). In this Chapter, the SWAL-QOL and Dysphagia Handicap Index (DHI) scores completed by participants correlate strongly with other swallowing-related, clinician-rated outcome measure in the NMDAS in the previous Chapter. One shortcoming of these two scales is that they have not demonstrated similar convergent properties as the NMDAS with functional tests such as the swallowing speed of the 100ml water timed test. Nonetheless, both SWAL-QOL and DHI demonstrated reasonable convergent validity with each other. To date, this is also the first time DHI, a more concise tool, has been studied in mitochondrial patients. The general findings attributable to swallowing difficulties in this group of *RRM2B* participants from SWAL-QOL and DHI have shown very little difference. Thus, at this

stage of understanding and limited use in a small number of participants, DHI is as good as the SWAL-QOL. It is well justified to examine the validity of DHI in a larger group of mitochondrial patients in the future.

Patient-reported outcome measures (PROMs) have huge potential to be leveraged into decision-making by clinicians and researchers because they measure what matters most to people (Calvert et al., 2019). People care about the impact of illness on their wellbeing and the most rational way is to ask them directly. However, challenges exist in collecting and incorporating the data, valuing the data, making sense of the data and using the data to make changes to patient care (Boyce et al., 2014). The six questionnaires in this Chapter also face these well-documented complexities. In the pursuit to collect data for these questionnaires, participants had been asked to complete multiple sets, often containing overlapping items, which can be perplexing and vulnerable to heuristic responses. For instance, questions about 'feeling tired' were asked in different formats in Neuro-QOL, NMQ and SWALQOL. These repeated questioning of the same construct might be considered unnecessarily burdensome, but they could also be examined for correlations to support their individual construct validity. In order for PROMs to be interpreted meaningfully for stakeholders, they may have to be integrated into 'big data' models (Calvert et al., 2015). Therefore, the results from this small cohort of rare *RRM2B* gene variants could play a small contributory part for future trials with a larger number of participants to understand PROMs in mitochondrial disease.

There are several limitations to this study. First, there is a risk of response biases in these self-reported PROMS because of deficits in memory, motivation, communication or knowledge. These well-researched response biases refer to answers by respondents that might have been distorted by mental shortcuts that they rely on (Podsakoff et al., 2003, Bradburn et al., 2004). On one end, some participants might choose socially desirable responses, a social desirability effect, to conform with social norms and present themselves in a good light, leading to underreporting of the impact on quality of life. On the other, some might overreport their difficulties because they are reflecting the subtle cues within these questionnaires to erroneously confirm the purpose of this study. These biases are inherent in all subjective PROMs and

eliminating them is not possible. However, these response biases were reduced in this study by checking the answers to the individual questionnaires. There have been no apparent tendencies for naysaying or yea-saying effect where participants respond negatively or positively to all items regardless of their content. Neither has there been any participants who chose categories towards the ends of a response scale (extreme reporting bias) or categories towards the middle of the scale (no-opinion reporting bias). Another limitation of this study is the generalisability of the results. Due to the small number of participants and the rare autosomal recessive *RR2MB* genotypes, this study suffers low potential to apply its findings in other forms of mitochondrial diseases. Nevertheless, it still provides robust data on this specific group of participants and offers modest indications of the feasibility of these questionnaires for future studies. Lastly, this study has not explored the quality-of-life indicators for some of the common features of *RRM2B* disorders such as ptosis and ophthalmoplegia. This is partly explained by the dearth of validated PROMs in the current literature for these features.

Notwithstanding these limitations, all these six questionnaires have exceeded the 0.7 thresholds of internal consistency in measuring their latent constructs. The Cronbach's alpha for all PROMs in this Chapter has, in fact, been higher than their original validation studies. The high levels of alpha are likely due to the homogeneity of this group of predefined participants in their disease manifestations. Along with the construct and convergent validity this study explores, the psychometric properties for some of these PROMs have made them a real potential for use in future mitochondrial disease clinical trials. Future studies should consider how these PROMs perform longitudinally (test-retest reliability) and how PROMs differ between assessors (inter-rate reliability). Future studies are also needed to establish how PROMs can be integrated into other clinical, laboratory, biomarker, and genetic data.

Chapter 7: General discussion, conclusion and future work

7.1 General discussion

Over the past few decades, our understanding of childhood-onset mitochondrial disease has expanded dramatically since the very first description by Dr Denis Archibald Leigh. This thesis has sought to broaden the mitochondrial field by adding novel perspectives and insights to, as Dr Leigh puts it, to ‘our scant knowledge’. Several important findings have been uncovered. Before the studies reported in this thesis, no studies had attempted to quantify the disease burden and progression of children with the most severe form of mitochondrial disorders, Leigh syndrome. In Chapter 3, I explored the indepth characteristics of children with Leigh syndrome and assessed their disease burden. Children with Leigh syndrome, as a group, demonstrated moderate disease burden when scored using the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) ratings and, a quarter of them had a severe burden of disease (NPMDS score >25). This finding is consistent with other previous studies or reports that described the severe impairment caused by this neurodegenerative syndrome in a more subjective manner (Rahman et al., 1996, Baertling et al., 2014, Ruhoy and Saneto, 2014, Sofou et al., 2014, Lake et al., 2016, Sofou et al., 2018). This work expanded the definition of disease severity by highlighting specific aspects of their function, especially communication, feeding, self-care, mobility, and education, which correlated strongly with their total disease burden. The meticulous quantification of disease burden has key implications not only in directing future reseach, but also on current clinical practice. Our findings will hopefully help focus clinical pratice on identifying and manageing these burdensome symptoms faced by these children, and also potentially inform healthcare commissioning bodies who should prioritise the needs of these children in future service provisions.

More importantly, Chapter 4 in this thesis explored disease burden longitudinally and confirmed that children with Leigh syndrome have considerable disease progression. My systematic chronological recording of NPMDS scores quantified their clinical course over time. The repeated use of the NPMDS at multiple time points not only provides quantitative data on the disease severity of Leigh syndrome at each time point but also allows disease trajectories to be plotted for individual patients. Almost all children with Leigh syndrome significantly progressed during the follow-up window.

More than half of them were wheelchair-bound at the end of the study. Being wheelchair-bound, an established risk for increased mortality in the general paediatric population (Nissen et al., 2018) might signify a milestone of Leigh syndrome disease progression and herald potential development of other associated problems. The significant deterioration in mobility in these children has been shown to impact other impairments of physical functioning, such as feeding skills, communication and self-caring abilities, that have been shown to be poor prognostic factors in disabled children (Hutton et al., 1994, Ashwal, 2005, Cohen et al., 2008). On average, these children with Leigh syndrome accumulated approximately 4.5 NPMDS points per year with individual children in this cohort demonstrating variable progression rates, ranging from 1.4 to 11.5 NPMDS points per year.

The variable progression rates are consistent with the demonstrable clinical heterogeneity of Leigh syndrome that has been reported in association with more than 80 pathogenic variants, involving both the nuclear and mitochondrial genomes (Lake et al., 2016). One particular genotype emerged from the study. Children with Leigh syndrome due to the pathogenic variants in *SURF1* in this cohort exhibited poorer outcomes. The *SURF1* gene encodes for an assembly factor of mitochondrial cytochrome *c* oxidase (COX) (Zhu et al., 1998). These children were characterised by a rapid disease trajectory and high mortality as compared to other genotypes. Apart from their genotype, I have also examined other factors that could influence the mortality and disease trajectory of these children. First, I have demonstrated that those children with Leigh syndrome who died in this cohort, had a greater disease burden (‘severe’ category) and faster disease progression (rate of NPMDS change > 3). In short, those who start 'badly' and deteriorate 'quickly', have higher mortality. Next, there was a significant association of caudate and brainstem involvement with disease burden, progression and mortality in these children with Leigh syndrome, which supported a similar observation by Sofou et al. (2014). However, there is currently no post-mortem histopathological studies in children with Leigh syndrome of these lesions to support our findings and to determine the exact mechanisms that may lead to poor outcomes. Finally, I have also evaluated other predictors of mortality including elevated lactate levels and epileptic seizures that had been suggested in the literature

(Sofou et al., 2014, Lake et al., 2016, Ogawa et al., 2020) but I could not find a significant association with mortality.

Beyond the genetically heterogenous Leigh syndrome cohort, this thesis also explored new territories in another form of childhood-onset mitochondrial disease caused by pathogenic variants within a single gene, the *RRM2B* gene. Chapter 5 and 6 in this thesis featured the largest cohort of the rare autosomal recessive *RRM2B*-related mitochondrial disorders in the UK. Similar to the Leigh syndrome cohort, I interrogated the disease burden and progression of this disorder. Instead of using single clinician-rated outcome measures, the study in the autosomal recessive *RRM2B* cohort had utilised a range of outcome measures, including functional tests, performance measures, and patient-reported outcome measures. The basic clinical characteristics of this cohort corresponded with findings from previous clinical cross-sectional studies in patients who harboured childhood-onset, autosomal recessive *RRM2B* variants (Tyynismaa et al., 2005, Shaibani et al., 2009, Fratter et al., 2011, Pitceathly et al., 2012). I have demonstrated the considerable disease burden in this cohort with a mean total Newcastle Mitochondrial Disease Adult Scale (NMDAS) score of 53.8 but, unlike the Leigh syndrome cohort, the participants did not deteriorate significantly during the study period, with an increment of only 1 NMDAS point per annum. Prior to this study, the quantitative aspects of their disease burden and progression had not previously been known or established.

Chapter 5 has also provided new, detailed, and quantitative data on the natural history of this rare autosomal recessive *RRM2B*-related disorder. I found that the respiratory muscle strength in this group of participants was significantly weaker than their predicted values in all of the performance outcome measures (FVC, FEV_1, SNIP, MIP and MEP) and substantiated the subjective descriptions of respiratory weakness in pathogenic *RRM2B* gene variants (Fratter et al., 2011, Pitceathly et al., 2012, Keshavan et al., 2020). Of all the parameters that measured respiratory muscle strength, the forced vital capacity (FVC) of their lungs fell significantly by about 5% per annum, which is comparable to the 5.9% yearly loss of FVC in myopathic patients with Duchenne muscular dystrophy who had not been treated with glucocorticoids (McDonald et al., 2018). The myopathic feature of these participants was quantified

using muscle dynamometry and I noticed their proximal muscles in the hips and shoulder appear to be affected to a greater degree than their distal muscles groups. Their poor muscle strength is also apparent in significantly reduced performance in functional tests. None of the participants in this *RRM2B* cohort reached their predicted distances in the six-minute walk test. I also found that their walking speeds significantly correlated negatively with their disease burden and exercise intolerance. These participants also performed poorly in other validated tests, including the 9-hole peg test, 100ml water swallow test and sit-to-stand test. Their performance, on average, had also deteriorated over time during the study window of two years.

In addition to these tests, I also investigated how these participants who harboured autosomal recessive *RRM2B* mutations feel and think about their conditions by using validated patient-reported outcome measures. They include the Neuro-QOL, Newcastle Mitochondrial Quality of life measure (NMQ), Fatigue impact scale (FIS), Daily fatigue impact scale, Swallowing quality of life questionnaire (SWAL-QOL) and Dysphagia handicap index. Both Neuro-QOL and NMQ demonstrated a similar reduction in quality of life across several aspects of their health. Fatigue was a major issue for them, and this is also evident in the questionnaires that specifically interrogated it, the FIS. Gorman et al. (2015) had previously administered the FIS questionnaire in a heterogenous group of mitochondrial patients and a third of them had scored more than 80, indicative of severe symptomatic fatigue experienced in the past one month. This cohort of participants harbouring rare autosomal recessive variants in the *RRM2B* gene, scored nearly 100 on average, putting them at the worse end of the perceived fatigue spectrum among mitochondrial patients. These patient-reported outcome measures (PROMs) have potential to be leveraged into meaningful decision-making by clinicians and researchers because they measure what matters most to patients (Calvert et al., 2019). People care about the impact of illness on their wellbeing and the most rational way is to ask them directly. The participants who with the *RRM2B* gene variants have unequivocally felt that impact on their quality of life.

The findings from the natural history studies of these two cohorts, Leigh syndrome and autosomal recessive *RRM2B* variants, using validated outcome measures have unearthed several differences between both groups of patients. The Leigh syndrome

cohort, which accrued 4.5 NPMDS points per year, appeared to exhibit a much more aggressive disease progression than the autosomal recessive *RRM2B*-related disorders that only accumulated in average 1 NMDAS point per year. The difference might partly be explained by the use of different rating scales. Other more plausible possibilities are that these two cohorts were captured at different time points in their respective clinical disease trajectories. *RRM2B*-related mitochondrial disorder cohort, which was older than those in the Leigh syndrome cohort might have undergone early disease progression that then plateaued. Leigh syndrome also appears to have a higher rate of mortality with nearly one in seven children dying during the study window. The lack of mortality in the young people and adults with *RRM2B*-related mitochondrial disorder could be due to survivor bias because it remains unknown how many of them had succumbed to the disease prior to reaching adulthood. Whilst both Leigh syndrome and *RRM2B*-related disorders are considered mitochondrial diseases, they differ in many ways discussed previously but not least, the nature and severity of organs affected by the disease process. Leigh syndrome, also known as subacute necrotising encephalomyelopathy, primarily affects the central nervous system, particularly the brainstem and basal ganglia structures (Sofou et al., 2014, Alves et al., 2020). On the other hand, the autosomal recessive *RRM2B*-related disorders predominantly affect skeletal muscles.

Notwithstanding their aforementioned differences, both cohorts shared a common theme in this thesis. These two rare disorders demonstrated a significant disease burden. The data in this thesis, which measured and quantified their respective disease burdens, have important implications for the health economics of rare disorders especially mitochondrial disorders. The findings of the high disease burden in this thesis have substantiated the notion that rare diseases expend a disproportionate volume of healthcare related resources in relation to their incidence in the population. Healthcare costs per patient with rare diseases were recently estimated to be approximately three-five hold higher than age-matched controls and were comparable to other high-cost diseases, like cancer and heart failure (Tisdale et al., 2021). This will have significant implications for healthcare planning and provision and costings of future therapeutic treatments. To compare disease burden between different diseases, a common set of metrics that captures various aspects of the

disease is required in a form of health-adjusted life years (Whitehead and Ali, 2010). One of the commonly used metrics is the disability-adjusted life year (DALY), which takes into account the overall disease burden and relies on disability weight (Global Burden of Disease Study, 2017). Disability weight is determined by how a disease affects a patient. Therefore, the data on disease burden and quality of life from this thesis could potentially support the calculation of DALY and contribute to future healthcare budget planning for these two rare disorders.

Apart from new discoveries and potential impact on health economics, the longitudinal collection of data in this thesis and its interpretation could also be invaluable to future clinical trials of rare diseases. Future research projects in these two conditions might choose to use the data in this thesis as historical control data to fully or partially substitute conventional controls. The rapid deterioration of children with Leigh syndrome, in particular high rate of mortality, is a concern in designing an ethical and feasible control arm to any trial. Although the 'gold-standard' interventional trial design for Leigh syndrome is a classical randomised, controlled trial (RCT), this might be confounded by the small number of living participants. Furthermore, the rapid and irreversible progression of Leigh syndrome might compel parents to prefer an active intervention instead of just placebo for their children. Several alternative trial designs such as crossover studies or open-label extension might address this, but the adaptive trial design with historical control is most suitable. An adaptive trial design, which analysed its interim results at certain timepoints, can reduce the number of sample size, shorten the trial and eliminate ineffective treatment earlier without compromising the validity. The historical data in this thesis can also increase the feasibility and efficiency of studies into these rare diseases, which might face recruitment difficulties, due to respective small sample size. By 'borrowing' historical control data, future studies could improve the power of the current control arms, reduce Type 1 error in trials, and provide more accurate results in the development of treatments (Viele et al., 2014, Jiao et al., 2019). The natural history data from this thesis which tracks the longitudinal disease course in the absence of treatment are highly recommended to provide endpoints prior to the initiation of interventional trials (Ghadessi et al., 2020). Therefore, natural history data of these participants who received standard care

without specific treatment, may have important roles to play in the design, delivery, and interpretation of future clinical trials.

Several outcome measures were studied and analysed in this thesis. The Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) and the Newcastle Mitochondrial Disease Adult Scale (NMDAS) were designed to quantify improvement or deterioration over a period of time (Phoenix et al., 2006, Schaefer et al., 2006). These scales scored the 'global' ratings of patients at two or more time points to determine their clinical trajectories. These 'global' rating scales are popular in neuroscience research (Rosen et al., 1984, Disease, 2003, Roxburgh et al., 2005, Schmitz-Hübsch et al., 2006). The popularity of these scales as an outcome measure is driven by their ease of administration, interpretability in busy clinical settings and more importantly they are freely available without specialist skills or training (Kamper et al., 2009). However, disease rating scales have a major pitfall that can be encapsulated by the theory of implicit change (Ross, 1989). This theory suggests that individuals could not accurately remember their previous state. They retrospectively applied their ideas of change over time based on their own views or interpretations of their current state. As a result, disease rating scales are liable to under or overscore when measurements from two or more time points are compared. To overcome this, several other quantitative performance outcome measures and functional tests can be utilised to complement the clinician-rated disease scales in this thesis. These outcome measures differentiate what individuals 'think' they can do and what they 'actually can do'. I have discovered several correlations between the rating scales and these performance-based outcome measures. These correlations have provided insights into how clinicians should interpret the commonly used rating scales in their future practice. The concurrent use of patient-reported outcome measures about their quality of life has also offered new perspectives on how their diseases affect them beyond the clinicians' interpretation from disease rating scales. Therefore, a future interventional trial in mitochondrial disorders must have a range of outcome measures to capture any meaningful changes. The choice of outcome measure is likely to be dependent on the specific mode of action of the drug or intervention. Of the outcome measures that had been studied, I propose that a combination of clinician-rated outcome measures (NPMDS or NMDAS depending on the age), patient-reported outcome measures

(Newcastle Mitochondrial Quality of Life Questionnaire (NMQ) and daily fatigue impact scale (DFIS)) and specific performance tests (muscle dynamometry and lung function tests) should be included.

These outcome measures have their own inherent merits and demerits. Clinician-rated disease rating scales have been used in many medical conditions. They were initially trialled on a specific group of patients in the development phase to confirm that they measure what they were designed to measure and that their construct can detect a change in what they measure over a pre-defined period of time (Sinha et al., 2008). The two clinician-rated disease rating scales, the NMDAS and NMPDS, used in this study had been constructed to determine the disease burden of mitochondrial disorders in adults and children respectively (Phoenix et al., 2006, Schaefer et al., 2006). The comprehensive approach of these two rating scales addressed the heterogeneity of clinical features, genetic mutations and biochemical deficiencies associated with mitochondrial disease. They covered many of the clinical features that are either not apparent at diagnosis or are found in only a minority of patients with that particular genetic mutation, for instance, stroke-like episodes. Another advantage of these two scales was that they were concise, efficient and clinic-friendly tools. The clinician-rated measure also differs from other outcome measures such as performance tests or self-reported questionnaires. It is primarily completed by a health care professional who uses clinical judgement and reports on patient behaviours or signs that are observed. A clinician can make accurate assessments of observations that reflect patient feeling, function, or predict survival. For example, in the NMDAS and NPMDS, a clinician could examine the degree of ophthalmoparesis whereas patients do not typically do so themselves. However, the main drawback of clinician-rated outcome measure is that it is only the perspective of the clinicians on what patients can actually do rather than what they perceive they can do. The latter is best assessed using patient-reported outcome measures (PROM). The use of PROM in clinical trials has gained popularity over time. Consecutive reviews of ClinicalTrials.gov in 2007–2013 evidenced that the use of PROM had doubled over that period (Vodicka et al., 2015). One of the key strengths of PROM is to provide unique information on the impact of a medical condition from the patients' perspective. PROM data could enrich the understanding of the patients' experience with information that could not be

gained from biomedical outcomes alone, as certain aspects of health are relatively hidden from clinicians, such as pain or fatigue. Furthermore, some outcomes such as the degree of bothersome symptoms could be subjective and best collected through patient self-reporting (Au et al., 2010). However, PROM has several weaknesses including poor design, missing self-reported data from questionnaires and reporting biases. To address these issues, PROM should be developed in accordance with the SPIRIT-PRO guidance (Calvert et al., 2018). A plan to facilitate the handling of unavoidable missing data should be employed, particularly in trials involving children with aggressive diseases such as Leigh syndrome who might not survive to complete all scheduled follow-up assessments. PROM findings should also be published according to CONSORT-PRO and ISOQOL PRO reporting guidelines (Brundage et al., 2013, Calvert et al., 2013) in a timely fashion. Both clinician-rated and patient-reported outcome measures often suffer from subjectivity to individual interpretations and the ordinal nature of the scoring systems.

On the other hand, functional or performance tests objectively assess the function of the patients based on the tasks that they could complete and then quantify them using predefined standards. In Chapter 5 of this thesis, examples of these tests include spirometry, a nine-hole peg test, quantitative muscle dynamometry, a six-minute walk test and timed water swallow test. Using these functional tests as outcome measures is common in clinical research. Since they are less reliant on the subjective judgment of researchers than other types of outcome measurements, they may be particularly attractive for use in multicenter trials for standardising assessment, as they can reduce measurement error and variability when collecting data (Richardson et al., 2019). The use of performance and functional outcome measures also overcomes the limitations of self-reported out PROM in which participants might not accurately recall their own functional abilities. In this thesis, not all the PROM correlated with the functional tests. For instance, their ability to walk a certain distance in a 6-minute walk test and self-reported perception of physical activity differs, suggesting their own perceptions appeared to be at odds with their true ability to perform a particular task. However, functional tests also have their own inherent limitations such as establishing the validity of these in tests in patients living with mitochondrial disease. There are currently no functional tests had been designed specifically as an outcome measure

for mitochondrial disorders. Therefore, the level of evidence needed to support its validity comes into question.

Some of the outcome measures might have suffered from floor or ceiling effects in extreme cases. The floor or ceiling effect described how subjects who are at or near the possible upper and lower limit cannot be estimated beyond a certain level or that their variance is difficult to determine (Everitt and Skrondal, 2002, Cramer and Howitt, 2004). If floor or ceiling effects are present, extreme items are likely missing at the lower or upper end of the scale, indicating limited content validity. As a consequence, patients with the lowest or highest possible score cannot be distinguished from each other, thus reliability is reduced. Furthermore, the responsiveness is limited because minor changes cannot be measured in these patients (Terwee et al., 2007). A large floor or ceiling effect could also imply limited instrument range, measurement inaccuracy, and response bias, all of which indicate inadequate questionnaire performance (Bernstein et al., 2019). In this study, several outcome measures might have encountered this effect. For instance, the NPMDS in the Leigh syndrome study. A Leigh syndrome patient with extensive basal ganglia lesion in a wheelchair might develop pain, discomfort or pathological fractures secondary to their severe dystonia, but these could not be scored more than the maximum 3 points in the 'extrapyramidal' section. Meanwhile, the *RRM2B* patients seem to have hit the ‘floor’ effect in some respiratory weakness tests such as SNIP, MIP and MEP. Their respiratory weakness was so severe that it might be so near the lower limit of these scales and any variance is no longer discernible. As for the patient-reported outcome measures in Chapter 6, if a huge number of participants rated the lowest or the highest possible score on a particular questionnaire, then that suggests that all of those patients have the same quality of life. Hence, a number of self-reported questionnaires cannot reliably differentiate among those at these extreme ends of the spectrum. It is important to recognise and appreciate the presence of these floor or ceiling effects in natural history studies or small pilot studies. Future interventional larger studies that use these scales or instruments need to make adjustments or calibrations to mitigate these floor or ceiling effects.

There is currently no specific cure for these rare mitochondrial conditions. Thence, it is challenging to predict the effect size on these conditions without knowing the exact mechanism of a hypothetical drug. Nonetheless, a future clinical trial for rare mitochondrial disorder might look like this. The most fundamental approach is to study the safety and efficacy of the drug versus control or placebo. Taking advantage of what had been discovered in this study, the primary endpoint could be a change in one of the aforementioned outcome measures. The safety outcome could be defined as any serious adverse event including mortality. The primary analysis must include intention-to-treat and all randomised participants. The study should also adopt the concept of the Prospective Randomised Open, Blinded End-point approach (PROBE) (Hansson et al., 1992) with a group of clinicians who would measure the outcome measures while blinded to both treatment and time. Participants are to be randomised initially to the intervention or control arm. Given the small numbers, the study might need to be run at several sites, nationally and internationally. Outcome measures are to be collected at baseline and regular intervals, depending on the pharmacokinetics of the drug. There is a need for adaptive interim analyses at every 5-10 participants to monitor safety, futility, and efficacy. The trial may terminate early if the intervention is superior or futile compared to the control. With the adaptive trial design, there is also the advantage of continual reassessment of the randomisation process using either covariates or early observable outcomes. For instance, the adaptive design could include ‘play the winner’ in which more participants are allocated to the more effective treatment. Clinical researchers must also consider the probability that would make them randomise their next patient due to efficacy or due to futility. Taking into consideration the chronicity of these mitochondrial disorders, cross-over design might be factored in at later stages of the trial course, but this might be limited by the carryover effects or the non-linear effect of the hypothetical drug. There is also currently limited evidence that adaptive trial or cross-over designs had been successfully completed in any mitochondrial disorders research to date. More importantly, expert clinical researchers should be consulted throughout the design and delivery of any potential trial. This proposed study must also be handled with caution since there is still no known drug for mitochondrial disorders in the development to guide the trial design.

7.2 Conclusion

The purpose of this thesis was to understand the natural history of childhood-onset mitochondrial disorders using validated outcome measures. I have objectively quantified disease burden and progression in children with Leigh syndrome, the most common syndromic presentation, and the most severe forms of mitochondrial disorder. Using validated paediatric mitochondrial disease scale, I charted the disease trajectories of Leigh syndrome caused by various genotypes. I have also identified several factors that might influence their disease trajectories and mortality. I also discovered that severe disease burden and rapid disease progression in these children with Leigh syndrome are associated with greater rates of mortality. In the largest UK cohort of patients with rare autosomal recessive mutations in the *RRM2B* gene, I have also objectively measured their disease burden and progression. I have utilised several outcome measures – clinician-rated outcome measures, performance outcome measures, functional tests – to provide new insights into the natural history of this rare *RRM2B*-related mitochondrial disorder. To understand the impact of the disorder on their lives, I have also explored the use of several patient-reported outcome measures and discovered the poor quality of life faced by these patients. All the new discoveries in this thesis will serve as robust data for the development of interventional clinical trials in these conditions where a control arm is not feasible; for the research directions of natural history studies in other rare mitochondrial disorders; for the prognostication of disease course in clinical practice, and more importantly, for the provisions of better care for these patients in the future.

7.3 Future work

7.3.1 A prospective study in children with Leigh syndrome

Despite having determined the disease burden and progression of children with Leigh syndrome using NPMDS, several unresolved research issues remain. First, the disease progression for Leigh syndrome might be more variable than what I had captured in the two time points. Therefore, I had planned to add interim follow-up assessments between the two time points to chart the disease trajectory more accurately. Ideally, the more assessments in future study designs, the more granular one might define disease trajectories. However, there is a need to balance excessive visits to research sites against the risks of these children travelling with debilitating conditions for non-clinical purposes. Next, NPMDS might not be adequately sensitive to define minor changes, especially at the extreme end of the scales. Hence, several outcome measures suggested by the international workshop (Koene et al., 2018) should be utilised to detect clinical changes over a predefined study period.

The Leigh syndrome cohort children were unable to answer patient-reported, quality-of-life questionnaires but their parents or carers could. Little is known about the burden of looking after children with severe mitochondrial disease especially those with Leigh syndrome. Studies have adapted the original version of the caregiver burden scale (Zarit et al., 1980) to learn about the impact of caregiving on parents or carers of children with chronic diseases (Raina et al., 2004, Ekim and Ocakci, 2016, Javalkar et al., 2017, Fitzgerald et al., 2018). To address all these unresolved research issues, I submitted my pilot data during my PhD studies, and I had successfully secured further funding from the Leigh Syndrome International Consortium to undertake a new prospective study. The design of this new study, also known as ‘Leigh syndrome – Investigating Outcomes and Natural history (LION)’, was based on lessons that I had realised in this thesis. After gaining the necessary approval, the LION study, my potential postdoctoral project, had started recruiting participants and would be collecting data for next few years.

7.3.2 Remote application of disease rating scales

The NPMDS had conventionally been administered by trained clinicians in face-to-face appointments. This reliance on face-to-face assessments limited a wider reach for patients who could not travel to clinical or research sites. To travel to these clinics, there is a considerable travel time, financial and personal costs to patients that were often unaccounted for. Therefore, the next logical evolutionary step of the NPMDS, which was designed in 2006 by Phoenix and colleagues at our research centre, is to adapt it for remote use. Remote use of NPMDS, without the need for children and their families to be physically present in clinics, could become part of the long-term health surveillance in between their scheduled clinics. The move towards this remote use of NPMDS was also accelerated by the COVID-19 global pandemic in 2020, an unprecedented health crisis, during my research studies in this thesis. The ensuing national lockdown to curb the spread of the COVID-19 virus, to protect lives and to save the NHS came into force. This unique challenge to the delivery of clinical care for patients meant that all face-to-face or in-person appointments were moved to virtual platforms and brought forth the era of telemedicine (Bashshur et al., 2020, Hollander and Carr, 2020, Portnoy et al., 2020).

To support the delivery of telemedicine for children with mitochondrial disease, there is a demand for a new electronic rating scale to replace NPMDS and it should also allow parents or carers to rate the disease severity without the interpretation from a clinician. I supervised a medical student in her Maters by Research (MRes) project to develop an online patient-reported disease rating scale known as the 'Mitochondrial disease In Children – a parent/carer Reported Outcome measure' (MICRO). We carried out pilot and validation studies to determine the preliminary validation of MICRO. MICRO was able to designate disease severity rating as accurately as the original NPMDS. The parents or carers who were interviewed about the use of MICRO, found it an acceptable and practical tool to monitor their children's health at home. There are plans to undertake further validation in a larger group of children with mitochondrial diseases. I foresee the potential of MICRO as the next-generation disease rating scale in paediatric mitochondrial disorders.

7.3.3 Natural history studies in rare mitochondrial disorders

These natural history studies of rare conditions using outcome measures have given me and my colleagues the knowledge and experience to undertake similar research in other rare mitochondrial genotypes which might have not been extensively explored previously. Leigh syndrome, caused by over 80 known genes (Lake et al., 2016), might be the commonest syndromic presentation of paediatric mitochondrial disorders but disorders caused by individual genes are rare. As I have learnt in these studies, different genotypes have different disease burdens and progression rates. Moving forward, next-generation gene sequencing including whole exome and whole genomes, are revolutionising the molecular genetic diagnoses of mitochondrial disorders (Taylor et al., 2014, Schon et al., 2021). Having a confirmed molecular genetic diagnosis is a key step in the application of precision medicine in managing the complexity of mitochondrial disorders (Boggan et al., 2019). At present, the natural history of mitochondrial disorders caused by these pathogenic variants in very rare genes remains relatively understudied. I envisage that the methodology applied in this thesis is vital in determining the natural histories of these rare diseases. The data obtained from natural history studies of these rare genotypes could be used in future clinical trials. Furthermore, the relative success of molecular bypass therapy in autosomal recessive *TK2* mutations (Garone et al., 2014, Lopez-Gomez et al., 2017, Domínguez-González et al., 2019) have led to pharmaceutical interests to translate the technology in other rarer mtDNA maintenance genes. The use of molecular bypass technology in the *RRM2B* cohort could be explored as a future research project. Apart from that, outcome measures in mitochondrial disorders could be complemented by novel biomarkers. Serum biomarkers in mitochondrial disorders are still being established (Davis et al., 2016, Steele et al., 2017, Lehtonen et al., 2021). Thus, there is a potential to explore biomarkers alongside natural history study and outcome measures in future research. Exploring the research in rare genetic diagnoses of mitochondrial diseases also meant that we need to work collaboratively with other specialist centres around the world to enrich the sample size and interpretability of the results. No matter which direction future research in very rare mitochondrial disorders take, the experience from this thesis has given me a great platform to begin my career as an independent clinical researcher.

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