MEASURING DISEASE PROGRESSION IN FRIEDREICH'S ATAXIA

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Thesis submitted for the degree of
Doctor of Medicine

June 2023

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Abstract

Friedreich's Ataxia (FRDA) is the commonest autosomal recessive ataxia worldwide, affecting 1 in 40,000. Biologically, there is a deficit in an essential mitochondrial protein, frataxin, caused by a GAA trinucleotide repeat expansion mutations in the frataxin (*FXN)* gene. The size of the GAA repeat expansion determines phenotypic features, including age at onset and individual variation in the progression of FRDA. The condition is not solely characterized by neurodegeneration, but the differential vulnerability of various organs such as heart, pancreas, skeletal muscles to frataxin expression makes it a rather complex and multisystem disease.

The neuropathology initially targets the proprioceptive system (dorsal root ganglia, posterior columns, and spinocerebellar tracts of the spinal cord), followed by atrophy of the cerebellar dentate nuclei and efferent fibres, while later in the disease, the corticospinal tracts degenerate. This combination of sensory neuronopathy and progressive pyramidal weakness is responsible for the typical combination of signs and symptoms specific to FRDA. Unsurprisingly, advanced FRDA patients present with sensory, cerebellar, and pyramidal features that are difficult to disentangle clinically.

The clinical rating scales (the Friedreich Ataxia Rating Scale [FARS] and the Scale for the Assessment and Rating of Ataxia [SARA]) widely used as outcome measures in clinical trials are much more accurate in the assessment of already established motor deficits in ambulatory FRDA patients. However, the sensitivity of these scales to detect small improvements (or stabilizations) over short assessment intervals is low, and this diminishes significantly in non-ambulant patients. Rating scales are therefore not ideally suited as outcome measures in therapeutic trials for what is a rare disease.

In this project, I explored whether electrophysiological measures of corticospinal tract integrity and sensory neuronopathy could be used to quantify disease progression in FRDA. Specifically, I studied 15-30 Hz intermuscular coherence, also known as beta band intermuscular coherence (BIMC), a relatively new marker of upper motor neuron (UMN) function, and motor evoked potential (MEP) central motor conduction time (CMCT), an established marker of UMN function using transcranial magnetic stimulation (TMS), in healthy volunteers and patients with FRDA. I correlated the electrophysiological abnormalities with their GAA repeat size, serial measurements of FARS scores as well as blood Frataxin levels. A few patients had their skin fibroblasts cultured and analysed for quantitative proteomics, to look at alteration in neuroprotective pathway proteins.

I concluded that BIMC is lost very early in the condition and thus not a good method of monitoring progression. Instead, BIMC might have an application in screening for the onset of subclinical disease in asymptomatic individuals with a genetic diagnosis of FRDA, identifying when to initiate disease modifying therapy in such patients. MEP CMCTs showed significant changes with time in patients and thus appear to be a promising method of quantifying disease progression in clinical trials.

As a multi-system disease, the combination of a range of surrogate measures is likely to be significantly more sensitive at detecting disease progression than any measure in isolation. Future studies should aim to extend the preliminary results presented here to develop statistical methods of combining such multi-modal data that will allow quantification of subclinical progression of disease, sufficiently robust to be incorporated as primary outcome measures in clinical trials.

‘ The noblest pleasure is the joy of understanding ’ ~Leonardo Da Vinci.

To my patients and their families

who are waiting, in hope that we would understand enough someday.

Acknowledgements

The first and foremost gratitude must be reserved for one's academic supervisor.

Dr Mark Baker gave me a great opportunity to learn, and to play a part in accelerating research on this rare neurodegenerative condition of Friedreich's Ataxia (FRDA). With his immense knowledge and focus, he has steered me through this non-linear path of completing a doctorate. His dedication to the project has served like an extant anchor, amidst the changes unleashed by the covid pandemic and my family relocations after completing my clinical neurophysiology training. The education I received during this period, in the electrodiagnostic field as well as overcoming challenges of conducting a research study, would go a long way in defining my clinical abilities.

I also owe it to Dr Ming Lai, my clinical training supervisor, to approve of the Out of Programme Research (OOPR) initiative to undertake this study during my higher specialty training. Dr Stephan Jaiser was the famous nerdy medic colleague and a torchbearer for the intricate Matlab scripts for coherence analysis, for which I cannot thank him enough. He was a predecessor, who unreservedly gave the benefit of his experience from methods used in his doctorate study.

Mrs Gail Charlton, the former manager of the department of clinical neurophysiology, RVI, Newcastle, was very thoughtful in offering clinical space for running the research clinics. I simply cannot miss sharing this page with the four neurophysiology secretaries: Elaine Hodgson, Barbara Bell, Lorna Turner and Deb Weedy, who like the invisible pillars of the department, incessantly helped out with patient visits, reports and simply smiling through their day-to-day busyness.

The Mitochondrial lab and the biobank teams at the Institute of Genetics Medicine (IGM) were welcoming and eased me into the new environment. My special thanks to Raf, Rafiqul Hussain, from genomics core facility, who gave his precious time to teach me buffy coat extraction for the Frataxin samples, acquainted me with the laboratory protocols, and processed the initial samples very timely and patiently for me until I was ready. Dr Angela Pyle as the lab manager was always so approachable. In the Biobank, Dr Mojgan Reza, Dan Cox, and other members of the group, kindly guided me with protocols and storage of biopsy samples of skin fibroblasts of my patients, another important thread of my experiments.

Profs Patrick Chinnery and Rita Horvath were the Chief Investigators of the project at different times. I am indebted to them for offering access to the regional ataxia database to initiate the recruitment and that is where I met Mrs Gail Egon, the very helpful Newcastle Ataxia Service nurse. It was extremely generous of Prof Rita Horvath to organise the FRDA repeat length analysis at the Neurogenetics lab in London and lead the proteomics aspect of the study. I would also like to thank Drs Rhys Thomas and Anna Basu for serving on my internal progress review panel and providing valuable feedback and encouragement throughout this period.

We were fortunate to have Dr David Lynch from The Children's Hospital of Philadelphia (CHOP), Philadelphia, one of the eminent researchers in the field, as a collaborator. I am obliged to the scientists from his lab, Yina Dong and Amy Rattelle, who readily joined forces in running assays on Frataxin levels and ensuring their safe transfer.

The Henry Wellcome Building (HWB) at the Institute of Neuroscience (now known as Newcastle University Translational and Clinical Research Institute – NUTCRI) in Newcastle University, was a space that was calm and abuzz in equal measures, nudging

me to grow and evolve with my research, sheltered away from the relentless activity of the hospital. Mrs Beckie Hedley from PGR team and Dr Ann Fitchett, the events co-ordinator, helped with numerous queries of being a new student. I also sincerely appreciate the input of Mrs Kay Garrick, Finance and Research Assistant, giving me insight into accounts whilst I settled the patients' travel and food expenditure claims, created purchase orders for equipment, or shipped samples on dry ice to Philadelphia, USA. It was a steep learning curve!

Indubitably, both sets of parents, family, and friends (both old and new, from Newcastle to Plymouth) have been waiting keenly for me to reach this milestone, through shared struggles and accolades. They deserve all the admiration for being there, sometimes quietly and boisterously at others.

None of this would have materialised without the Friedreich's ataxia patients. The faces of happy children despite their grave condition and their families, who travelled far and wide from the country to greet me in my clinics in Newcastle, regularly for over two and a half years instilled faith in my work. As always, one learns so much from people than textbooks or labs. I earnestly hope I can contribute something tangible towards understanding and management of this condition, even it is like adding a pebble to the huge mountain of research.

And may I conclude by saying, 'force to you' to the charities who supported my study on this rare condition - Ataxia UK, FARA and GoFAR. They strive to find innovative ways to involve patients and caregivers in the ground breaking research and empowering them as well as scientists, through support services, daring fundraisers, the International Ataxia Research Conferences, and a very readable 'Ataxia' magazine.

Publications and Presentations

➢ Abstracts

- *Dhawan V*, *Williams T, Lai H M, Jaiser S R, Baker M R.* Electrodiagnostic findings in facial onset sensorimotor neuropathy (FOSMN)- A review of 3 cases. Presented at **British Society of Clinical Neurophysiology**, May 2019 Spring Scientific Meeting, Liverpool. Published in the Journal of Clinical Neurophysiology.

- *Dhawan V*, *Lai H M, Sweeney K, Baker D, Baker M R*. Reviving a simple and objective tool to test responsiveness during absence seizures. Presented at **British Society of Clinical Neurophysiology**, Oct 2018 Autumn Scientific Meeting, London. Published in the Journal of Clinical Neurophysiology.

- *Dhawan V, Jaiser S R, Chinnery P F, Baker M R.* Monitoring progression of disease in Friedreich's Ataxia: a multi-modal electrophysiological approach. **Poster presentation at 2nd International Ataxia Research Conference (IARC)**, Pisa, Italy, 27th- 30th Sep 2017.

- *Dhawan V, Jaiser S R, Chinnery P F, Baker S N, Baker M R.* Monitoring progression of disease in Friedreich's Ataxia: A multimodal electrophysiological approach. Presented at **Liv Reunion Annual SENFC** (Sociedad Espanola De Neurofisiologia Clinica) meeting with BSCN (British Society for Clinical Neurophysiology), Malaga, Spain, 5th- 7th Oct 2016.

➢ Papers

- *Dhawan V, Jaiser S R, Baker M R.* Motor Evoked Potentials: A Practical Approach for Neurologists. Unpublished. To be submitted to Clinical Neurophysiology Practice Journal.

- *Dhawan V, Baker M R.* A multimodal monitoring study of Friedreich's Ataxia. Unpublished. Work in progress.

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LIST OF ABBREVIATIONS

AF	Atrial fibrillation
AH	Abductor Hallucis
ANOVA	Analysis of variance
APB	Abductor pollicis brevis
BIMC	Beta band intermuscular coherence
CI	Contraindications
CMAPs	Compound muscle action potential
CMC	Corticomuscular coherence
CMCT	Central motor conduction time
CNS	Central nervous system
CSP	Cortical silent period
CST	Corticospinal tract
DC	Direct current
DMSO	Dimethyl Sulfoxide
DOB	Date of Birth
DPBS	Dulbecco's Phosphate-Buffered Saline
DRGopathy	Dorsal Root Ganglionopathy
ECoG	Electrocorticography
EDB	Extensor digitorum brevis
EDC	Extensor digitorum communis
EMG	Electromyogram
FARS	Friedreich's Ataxia Rating Scale
FCS	Fetal calf serum

FDI	First dorsal interosseous
FDS	Flexor digitorum superficialis
GAA	Guanine-Adenine-Adenine
ICARS	The International Cooperative Ataxia Rating Scale
ICF	Intracortical facilitation
IMC	Intermuscular coherence
IntraMC	Intramuscular coherence
ISI	Interstimulus interval
LFP	Local field potential
LOFA	Late Onset Friedreich's Ataxia
LL	Lower limbs
LVEF	Left Ventricular Ejection Fraction
LVH	Left Ventricular Hypertrophy
M1	Primary motor cortex
MtDNA	Mitochondrial DNA
MEG	Magnetoencephalogram
MEP	Motor evoked potential
MG	Medial gastrocnemius
MNCV	Motor Nerve Conduction Velocity
MVC	Maximum voluntary contraction
NCS	Nerve conduction study
NR	Not Reproducible
PBMC	Peripheral blood mononuclear cells
PMCT	Peripheral motor conduction time
RMT	Resting motor threshold

ROC	Receiver-operating characteristic
SAN	Sensory Axonal Neuropathy
SARA	Scale for the assessment and rating of ataxia
SD	Standard deviation
SEPs	Somatosensory Evoked Potentials
SICI	Short interval intracortical inhibition
SNAP	Sensory nerve action potential
SNCV	Sensory nerve conduction velocity
TMS	Transcranial magnetic stimulation
TA	Tibialis anterior
tDCS	Transcranial direct current stimulation
UL	Upper Limb
UMN	Upper motor neuron
YOD	Year of Diagnosis

1 INTRODUCTION

KEY WORDS: Friedreich's ataxia, FRDA, biomarker, BIMC, TMS, MEPs, FARS

1.1 EPIDEMIOLOGY

Friedreich's ataxia (FRDA) is a rare but progressively lethal neurodegenerative and multisystem disease. The condition usually presents in childhood or adolescence causing increasing disability over years and shortening life expectancy.

Nikolaus Friedreich (1825-1882) was the German neurologist and pathologist who described his eponymous ataxia in a series of papers during the 1860s and 1870s. He recorded nine members of three families who developed ataxia, dysarthria, sensory loss, muscle weakness, scoliosis, foot deformity and cardiac symptoms at puberty (Friedreich N., 1863, 1876).

FRDA is an autosomal recessive hereditary ataxia with a very high carrier rate (between 1/60 and 1/90), caused by unstable GAA trinucleotide repeat expansion within the first intron of *FXN* gene (Bidichandani & Delatycki, 1993). Males and females are equally affected. It is the most common inherited ataxia, with prevalence showing large regional differences; estimated at between 1 in 20,000 in south-west Europe and 1 in 250,000 in the north and east European population. Available published literature suggests that prevalences range from

1 in 21,000 in northern Spain to 1 in 750,000 in Finland or 1 in 330,000 in Russians (Vankan, 2013).

1.2 CLINICAL FEATURES: CHARACTERISTICS OF AN HEREDITARY ATAXIA

The progressive neurological syndrome typically manifests as a dorsal root ganglionopathy (DRGopathy), with degeneration and atrophy of the dorsal columns (DC), spinocerebellar tracts (SCT), cerebellum, dentate nuclei and corticospinal tracts (CST).

Affected individuals show signs of gait ataxia, dysmetria of arms and legs, dysarthria, dysphagia, atrophy and weakness of the distal extremities, absence of muscle stretch reflexes, loss of joint and vibratory senses, and superimposed stocking-and-glove type sensory neuropathy (Koeppen, 2011a). Neurogenic urinary, bowel and sexual disturbances have been recognised in FRDA, and are multi-factorial due to interruption of spinal pathways (Panicker et al., 2017). Outside of the nervous system, *FXN* mutations cause cardiomyopathy, which is a frequent cause of premature death. Patients also commonly have glucose intolerance or diabetes, visual and auditory loss, and skeletal changes such as kyphoscoliosis and *pes cavus* (Marmolino et al., 2011).

In a seminal paper by Professor Anita Harding in 1981, the clinical features of 115 patients from 90 families with Friedreich's ataxia were described. The genetics and intrafamilial clustering of clinical features were examined. The study found that dysarthria, signs of pyramidal tract dysfunction in lower limbs

and loss of joint position and vibration sense were not necessarily present during the first five years of symptoms, but eventually developed in all cases. Scoliosis and ECG evidence of cardiomyopathy were found in over two-thirds of the patients studied whilst pes cavus, distal amyotrophy, optic atrophy, nystagmus and deafness were all less frequent. The disorder was progressive in all cases. The mean age of losing the ability to walk was 25 years; 95 per cent were chair-bound by the age of 44 years. Patient with diabetes appeared to be associated with a higher incidence of optic atrophy and deafness (A. E. Harding, 1981) **(Table 1)**.

The patient with FRDA, whilst mentally agile can be severely restricted by the resulting and unremitting accumulation of physical disability (Gibilisco, 2013). The age and intensity of the onset depend upon the GAA trinucleotides repeat expansions. Long expansions lead to an early onset, clinically severe illness, and death in young adult life, whereas patients with short expansions have a later onset and milder signs, some are not even diagnosed during their life time (Koeppen, 2011b). Symptoms typically present at 10-15 years of age but can be earlier or later, with patients being typically wheelchair dependent within 10 -15 years of disease onset. The life expectancy is about 40-50 years, but those with less severe forms of disease continue to live into their sixties. If the symptoms appear after the age of 25, it is called late-onset Friedreich's ataxia (LOFA), and the rate of progression is slower in people with LOFA.

Given that such clinical variability is unusual for a recessive disorder, several authors suggested that FRDA was caused by mutations in several different genes, until in 1988, the gene was mapped to chromosome 9. The meticulous work of several research groups allowed the chromosome region to be narrowed to 150 kb at 9q13 (Montermini et al., 1995; Rodius et al., 1994; Sirugo et al., 1993).

Primary (essential for diagnosis)
Autosomal recessive inheritance
Age of onset before 25 years
Progressive limb and gait ataxia
Absent tendon reflexes in the legs
Extensor plantar response
Axonal sensory neuropathy
Dysarthria (if after five years from onset)
Secondary (additional present in over 66%)
Scoliosis
Pyramidal weakness in lower limbs
Absent reflexes in upper limbs
Distal loss of position and vibration sense
Abnormal ECG (cardiomyopathy)
Additional (less than 50%)
Nystagmus
Optic atrophy
Deafness
Distal amyotrophy (weakness and wasting)
Pes cavus
Diabetes

Table 1. Diagnostic criteria for typical FRDA according to A.E.Harding.

Adapted from (Santos et al., 2010)

1.3 COGNITION IN FRIEDREICH'S ATAXIA

There is limited documentation regarding cognitive function in FRDA. The condition has been traditionally held not to affect cognition and overshadowed by sensory and motor deficits due to effects on spinal cord, peripheral sensory nerves and cerebellum. Recent systematic neuropsychological and neuroimaging studies have revealed marked slowing of information processing speed (not related to motor slowing) and wide-spread visuoconstructive and visuospatial abnormalities, as well as problems with verbal fluency, sustained volitional attention, working memory as well as concrete thinking (Corben et al., 2006; Klopper et al., 2011). The defects observed have been attributed to disruption of cerebello-prefrontal connections as well as parieto-temporal dysfunction and /or direct cortical and cerebellar pathology evident in people with FRDA. These findings point out the importance of health care personnel expecting these patients to function normally in the cognitive domain. Despite these findings, there have been individuals with FRDA known to successfully pursue academic careers.

One of the rare studies highlighted affective difficulty in most FRDA patients in their cohort, ranging from severe depression to normal grief (Flood & Perlman, 1987). The presence of comorbid depression in neurodegenerative disorders is a well-recognized fact. Hitherto, little attention has been paid to impact of depression or health-related quality of life in this progressive and disabling disease. Depressive symptomatology is the most relevant variable for

predicting quality of life. It has been found to affect aspects not only related to motor disability but also present in non-motor dimensions (Nieto et al., 2018; Pérez-Flores et al., 2020).

1.4 GENETICS AND PATHOPHYSIOLOGY: UNSTABLE AND EXPANDING

The X25 gene (now named *FXN* gene according to the HUGO Gene Nomenclature Committee) and the mutations responsible for FRDA were successfully identified in 1996 through an international collaborative effort(Campuzano et al., 1996). The *FXN* gene has been mapped on chromosome 9q13 . The most frequent mutation, in about 95% of the cases, is the expansion of a GAA trinucleotide repeat located within the first intron of the gene. Rarely, there is a point mutation or large deletion in one *FXN* allele and GAA expansion in the other (Palau, 2001). GAA triplet repeat expansions or point mutations in the *FXN* gene suppress its transcription via an epigenetic mechanism, which ultimately causes a deficiency in the levels of functional frataxin protein (Cossée et al., 1997a; Grabczyk & Usdin, 2000).

Frataxin is an 18 kDa soluble protein with 210 amino acids and is expressed in mitochondria. Although its specific functions remain elusive, the general consensus is that the protein assists in controlling cellular iron homeostasis by directly binding iron. It is clearly important in normal mitochondrial function; reduced levels of frataxin result in lower ATP production, and impaired iron utilization, leading to mitochondrial iron accumulation and the accumulation of

multiple mitochondrial DNA deletions. In order to unravel frataxin function further, Campuzano *et al*. detected that the protein is located in mitochondria, associated with the mitochondrial membranes and crests. Using monoclonal antibodies in various human and mouse tissues against different regions of frataxin protein, they also demonstrated that the mitochondrial targeting sequence is encoded by the first 20 amino acids. They proposed that given the shared clinical features between FRDA, vitamin E deficiency and some mitochondriopathies, a reduction in frataxin resulted in oxidative damage (Campuzano et al., 1997). The link between frataxin deficiency and considerable nuclear and mtDNA damage was first demonstrated in yeast lacking Yfh1 (the yeast homolog of *FXN*) (Karthikeyan et al., 2003). Human peripheral blood cells isolated from FRDA patients also exhibited increased nuclear and mtDNA damage. Haugen *et al.* observed that many of the key pathways led by transcription profiling were downregulated, and patients with prolonged frataxin deficiency suffered a systemic survival response to chronic genotoxic stress and consequent DNA damage detectable in blood. (Haugen et al., 2010).

To establish the extent of the mutation burden across the mitochondrial genome in FRDA cells, Bhalla *et al.* looked at a panel of FRDA and control fibroblasts using qPCR and next-generation MiSeq sequencing, respectively. Comprehensive RNA sequencing gene expression analyses were conducted to assess the status of DNA repair and metabolism genes in FRDA cells. The group concluded that downregulation of *FXN* expression in FRDA cells elevated

mtDNA damage, increased mutation load of the mitochondrial genome, and diminished DNA repair capacity (Bhalla et al., 2016).

The further characterisation of mitochondrial dysfunction concentrated on ROS (reactive oxygen species) production, which results in a range of metabolic disturbances including oxidative stress, deficits of iron-sulphur (Fe-S) clusters and defects in haem synthesis (Punga & Bühler, 2010; Santoro et al., 2000). The Fe-Sulphur clusters synthesised in mitochondria are utilized by many enzymes of oxidative phosphorylation and Kreb's cycle, fatty acid breakdown, glutathione synthesis, all of which cascade into altered metabolism in many systems in FRDA (Clark et al., 2018; K. Li, 2019).

PHENOTYPIC VARIABILITY RELATED TO GENETIC EXPANSION

Certain tissues such as sensory neurons and cardiomyocytes exhibit an increase in cellular defects that correlate with the clinical signs. These affected cells are known to have a high mitochondrial content and high metabolic rate; hence these could be more sensitive to frataxin deficiency. Moreover, this tissue specificity partially correlates with the distribution of the transcription of the frataxin gene. Frataxin mRNA is predominantly expressed in DRG and heart, mild in cerebellum, pancreas, liver, and skeletal muscle, and low in other tissues, such as cortex (Koutnikova et al., 1997). Lodi *et al.* pointed out that the disease phenotype was so prominent in the nervous system and the heart , but not clinically apparent in the skeletal muscles in patients with FRDA. One explanation suggested by them was that FRDA patients could not exercise to

the point at which a skeletal muscle defect would be revealed (Lodi et al., 1999). In a similar attempt, Esposito *et al.* demonstrated that skeletal muscle could increase antioxidant defences to a greater level than cardiac muscle, thus rendering the latter more susceptible to oxidant damage. Thirdly, skeletal muscle derive a significant amount of energy from glycolysis, whereas cardiac myocytes derive most of their ATP from the oxidation of free fatty acids. Mitochondrial defects would preferentially be seen in tissues that are most reliant on respiratory oxidation (Esposito et al., 1999). However, the *FXN* expression pattern is broader than expected from the pathology of the disease and therefore, this cannot be the only explanation.

It is increasingly being recognised that part of the tissue-specificity as well as the progressive nature of the disease is likely a direct consequence of the genetic mechanism of the disease. Systematic investigations of mutations in patients and their families have revealed that GAA repeat expansions are highly dynamic, exhibiting both intergenerational and somatic instability. For example, recent observations indicate that GAA repeat mutations increase in length in ageing somatic tissues, particularly in expansion-susceptible tissues such as dorsal root ganglia and cerebellum, and this phenomenon is not seen in other tissues (De Biase et al., 2007; Monrós et al., 1997). The direct involvement of the GAA expansion as the cause of FRDA is demonstrated by the very significant inverse correlation between the size of the smaller of the two expansions and the age of onset, the severity of the disease and the risk of occurrence of systemic features such as cardiomyopathy, scoliosis diabetes and

earlier loss of tendon reflexes (A Dürr et al., 1996; Filla et al., 1996; Montermini et al., 1997). The mechanism of tissue-specific pathology in FRDA seems more relevant with discovery of two novel frataxin isoforms (II and III), specifically expressed in affected cerebellum and heart tissues, respectively (Xia et al., 2012).

There are several other neuromuscular disorders caused by unstable repeats, such as fragile X syndrome (FRAXA), myotonic dystrophy, Huntington disease, and spinocerebellar ataxias. However, FRDA is the only condition known to be caused by a GAA trinucleotide repeat expansion. FRDA reveals a high degree of similarity with other diseases caused by dynamic mutations, particularly with FRAXA, in terms of the molecular mechanism of repeat expansion, intergenerational transmission, and cellular effects of the mutation (Pellegrini & Scorrano, 2007).

1.5 PHARMACEUTICAL TRIALS AND UPCOMING THERAPIES FOR FRDA

Since the identification of the link between FRDA and FXN gene, there has been tremendous progress in understanding the molecular basis and pathology of FRDA. Development of several animal and cellular models of the disease and better diagnostic approaches have also allowed identification of the temporal course of the pathophysiological changes, spurring widespread clinical trials towards establishing clinically effective treatments that delay onset and/or slow the progression of the disease (Martelli et al., 2012). Drug discovery has

been challenging and none of the preclinically promising agents investigated have yet been able to unequivocally demonstrate clinically meaningful symptomatic or disease-modifying capability. However, in this fast-paced and ever-evolving environment, there are excellent possibilities of achieving success in current and future therapeutic interventions for FRDA.

The most prominent factor causing downstream effects is reduced synthesis of frataxin which results in impaired oxidative phosphorylation, mitochondrial biogenesis, and abnormal iron trafficking in the cell (Santos et al., 2010). Hence, one of the most pursued strategies is to increase the intracellular content of frataxin, thus preventing the cascade of protein deficiency that leads to the clinical syndrome. Multiple steps of the pathogenetic cascade are being targeted with pharmacological agents that span across antioxidants (Coenzyme Q10, idebenone, resveratrol (Marmolino et al., 2010)), iron chelators (deferiprone, erythropoietin), interferon gamma (IFNγ), thiamine, enhancers of mitochondrial function and epigenetic modifiers such as nuclear factor erythroid-derived 2-related factor 2 (NRF2) activators and histone deacetylase (HDAC) inhibitors (Aranca et al., 2016; Pallardó et al., 2021).

A class of histone deacetylase (HDAC) inhibitors were shown to reverse *FXN* silencing in primary lymphocytes from individuals with FRDA (Chutake et al., 2016; Herman et al., 2006). Since deficient transcriptional initiation is the major cause of the deficiency of *FXN* transcript (Kumari et al., 2011; Silva et al., 2015; Turchi et al., 2020), reactivation of the epigenetically

silenced *FXN* promoter is likely to be a particularly efficient strategy for restoring *FXN* transcript levels to therapeutically beneficial levels (Soragni et al., 2014). Previous studies have also demonstrated that Nrf2 nuclear factor erythroid 2–related factor 2 (Nrf2) activation is suppressed in FRDA-derived cells, contributing to oxidative stress, mitochondrial dysfunction, and reduced ATP production in FRDA (La Rosa et al., 2021; Shan et al., 2013). Augmentation of Nrf2 activation using a variety of compounds reverses endogenous antioxidant defence mechanisms in animal and cellular models of FRDA. Omaveloxolone is one such drug that has been tested in phase 2 trials and found to be of potential benefit to FRDA patients (Lynch et al., 2020; Lynch & Johnson, 2021). Novel ways of 'unsilencing' *FXN* gene transcription using antisense oligonucleotides are also being explored (Shen et al., 2020).

The next wave of drug development is aiming for agents that restore frataxin protein levels in the mitochondrion e.g., transactivator of transcription-frataxin [TAT-frataxin] (Vyas et al., 2012), or replace or edit the mutated gene e.g., AAV-based gene therapy. Friedreich's ataxia mouse models have been treated with viral vectors encoding for either frataxin or neurotrophins, producing positive results, with hurdles such as immunotoxicity and pheno-toxicity to be overcome (Ocana-Santero et al., 2021).

The much-anticipated prospect of gene therapy offers the exciting possibility of a one-time therapy in FRDA, by advancing the use of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) genome editing technology.

CRISPR/Cas9-mediated gene editing of *FXN* is being investigated as a viable option to restore endogenous frataxin expression. There is also a potential of *ex vivo* gene editing in hematopoietic stem and progenitor cells (HSPC) as a potential option for autologous transplantation therapy (Rocca et al., 2020; Sivakumar & Cherqui, 2022).

2 BACKGROUND AND RATIONALE

2.1 ADDRESSING COMPLEXITIES OF A RARE DISEASE

FRDA is a recessive disease that usually affects families with no known history of the disease. Initiation of neuronal death precedes the appearance of the first symptoms; thus, damage would already have occurred before diagnosis is confirmed. It clinically constitutes one of the inherited ataxias, which are a large group of rare, degenerative and metabolic disorders of enormous clinical and genetic heterogeneity. These factors lead to obvious challenges in designing trials in a rare disease: patient numbers are low; phenotypic expression often variable; and disease time-course lengthy and trajectory not very-well mapped. For these reasons it is very difficult to identify therapeutic subgroups and to power clinical trials according to clinical endpoints.

There is an unmet need to diagnose FRDA earlier, not only to allow patients and their families the opportunity to plan their future lives before they become too unwell, but also to allow new therapies currently being developed that slow or stop the progression of disease to be initiated earlier in the disease course, to the benefit of patients. As expected for a rare disease, the development of novel therapeutics is fraught with difficulties. A major barrier to drug development in rare diseases is the delivery of clinical trials, not only because of the logistics of bringing together trial participants that are geographically dispersed but because of the small numbers they run the risk of being

underpowered, having either insufficient sample sizes or being too short in duration (Lynch & Kichula, 2016). Analysis of outcomes from previous studies tracking disease progression suggest that interventional trials in Friedreich's ataxia require a minimum duration of 2 years with more than 180 participants to be adequately powered. The options then are to either conduct international multi-centre studies, which are very difficult and limit trials to one agent per lifetime! Or use statistically more sensitive measures of disease progression allowing trials to be completed over 12 months with fewer than 100 participants.

There is also evidence that heterozygous carriers of the expansion express approximately 50% of normal frataxin levels but are not symptomatic, suggesting that therapeutic approaches that increase frataxin may be effective even if frataxin is raised only to carrier levels (Lynch et al., 2018; Plasterer et al., 2013). However, even the treatments that correct frataxin levels either in cell-based models or in a patient, are not without side-effects, suggesting a growing need for standardised biological markers of disease progression and to assess patient response to therapeutic interventions.

2.2 NATURAL HISTORY STUDIES IN FRDA

Selection of meaningful and relevant endpoints becomes more fundamental to clinical trial design of a rare disease and requires an understanding of the natural history of the disease and of the normal trajectory without intervention. To address some of these issues, large natural history studies on FRDA have

been established, such as European Friedreich's Ataxia Consortium for Translational Studies (EFACTS) and the Friedreich Ataxia Clinical Outcome Measure Study (FACOMS), which have provided data on the natural history of FRDA for up to 12 years. FACOMS has its main study site in the US, with evaluations conducted at 15 international centres (Patel et al., 2016a). The EFACTS study start date was May 2010, and it is currently running across 11 sites in seven countries in Europe, without a set end date (Reetz et al., 2015, 2016). The systematically collected clinical research data and biological specimens are being integrated for detecting onset and change over the natural course of FRDA, some of which may also be used as potential outcome measures in future clinical trials. These studies have validated measures of neurological change, quality of life and disability and also act as international disease registries enabling facilitation of multi-centre trials. This is also a great effort that has improved patient enrolment globally in FRDA related research (Thomas-Black et al., 2022).

2.3 CHALLENGES FACING CLINICAL TRIALS IN FRDA

As discussed previously, the clinical hallmark of disease is ataxia and is due to a combination of cerebellar and spinocerebellar degeneration, sensory neuropathy, and vestibular nerve involvement. Since no gold standard yet exists to examine disease progression in FRDA, several clinical assessment questionnaires to rate ataxia have been trialled as outcome measures in clinical

trials. The three such validated scales commonly used in quantifying dysfunction in FRDA are:

(i) the International Cooperative Ataxia Rating Scale (ICARS),

(ii) the Friedreich's Ataxia Rating Scale (FARS) and

(iii) Scale for Assessment of Rating of Ataxia (SARA)

ICARS and SARA were developed as tools for standardising scores of impairment in inherited/cerebellar ataxias with a greater weight given to gait and stance, although ICARS also covers oculomotor deficits (Cano et al., 2005; Salcı et al., 2017). ICARS has been found to be time consuming and is difficult to perform for frequent assessments in clinics. FARS was originally designed to capture the whole spectrum of neurologic features of FRDA and also evaluates functional staging for ataxia and activities of daily living. The FARS scale is also complemented by simple quantifiable functional tests with designated performance measures such as the 9-Hole Peg Test, Timed 25-Foot Walk, and PATA Rate Task (PRT) for assessment of dysarthria (Patel et al., 2016a).

To give an indication of numbers, there are about a total of 500-600 patients with the condition in the UK, of which only 25-30 reside in the North-East of England.

Within a larger study of 155 FRDA patients, FARS was found to correlate significantly with activities of daily living, disease duration, and functional disability. Thus, verifying that the FARS and its additional performance measures meet strict standards for construct validity in measuring the

progressive nature of FRDA (Lynch et al., 2006). Both ICARS and FARS show measurable changes over a 12-month period (Delatycki, 2009; Fahey et al., 2007; Schulz et al., 2009). In this study comparing FARS with other scales proposed as outcome measures for FRDA, FARS had the greatest effect size and required fewer patients for an equivalently powered clinical trial, and therefore ideally suited to smaller interventional trials (Fahey et al., 2007). Apart from ICARS, the other two scales examined were Modified Barthel Index (MBI) and Functional Independence Measure (FIM), which are commonly used to measure disability in patients undergoing rehabilitation and are not specific to any diagnosis.

Fahey and colleagues were also able to estimate how large therapeutic trials would need to be if using these clinical scales based on the effect sizes they observed in their study **(Fig 1)**. For instance, if we were to use the Modified Barthel Index (MBI), we would not be able to even consider running a trial in the UK; but it would be possible with using ICARS or FARS (at least 60 patients per treatment arm). However, even trials of this size would struggle with recruitment and may be inadequately powered to detect small treatment effects (Friedman et al., 2010; Patel et al., 2016b).

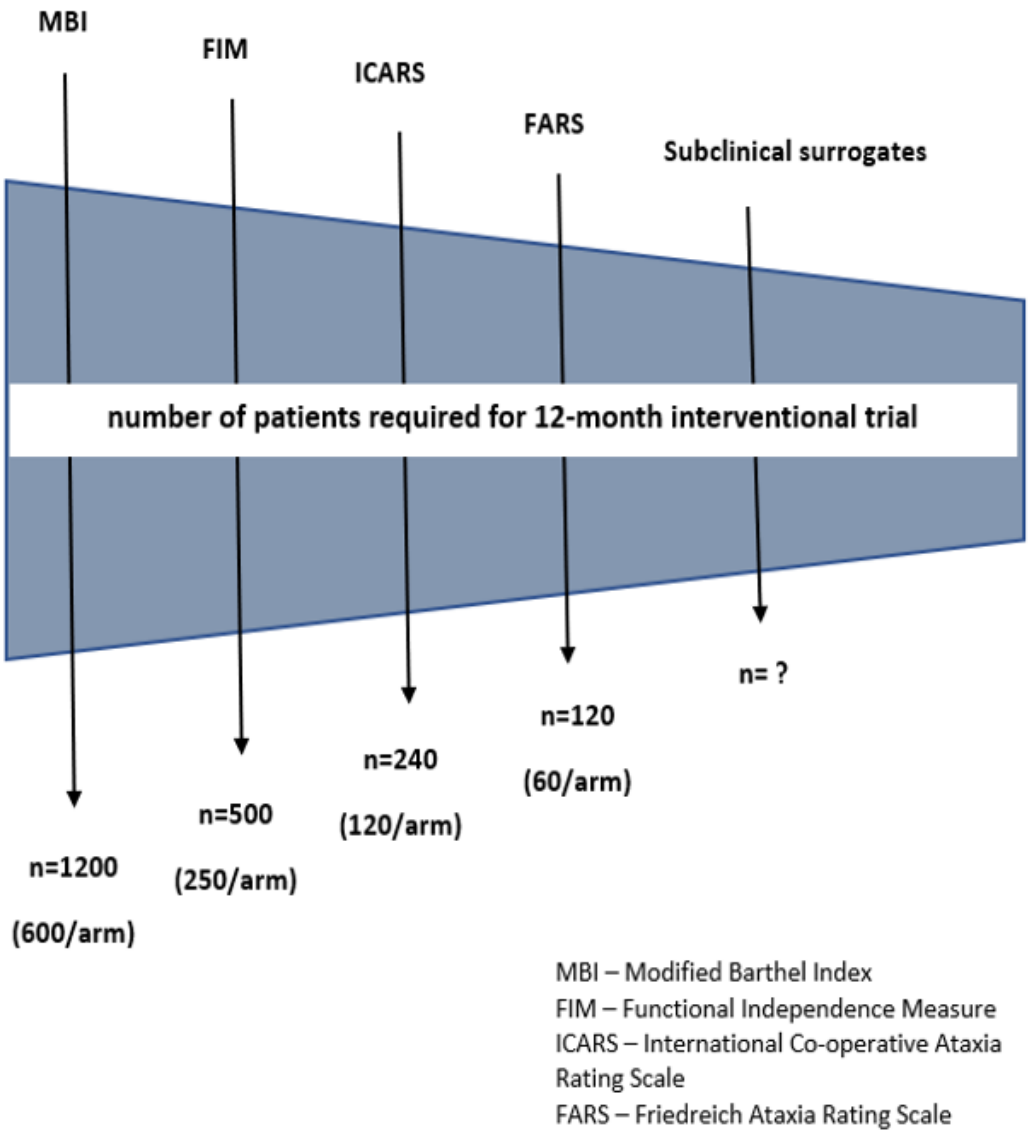


Figure 1. The challenge of therapeutic trials in FRDA.

Estimate of sample sizes (the number of participants needed for a clinical trial) based on effect sizes calculation in a publication by Fahey et al. (2007) JNNP 78:411-413.

2.4 SEARCH FOR AN ADEQUATE BIOMARKER

Through this study, I have tried to stress that one of the major problems with assessing treatment or monitoring regimens for FRDA is the difficulty in observing benefit owing to the slow progression of the disease and the lack of surrogate end points. For example, if a treatment slowed the progression but did not reverse any clinical manifestations, this benefit could take many months or even years to detect. If a treatment is shown to benefit people with FRDA, and in those who can be identified presymptomatically with full neurological function, the intervention may even be able to prevent the appearance of symptoms. Besides, until we have good evidence that correcting the molecular deficit has a measurable clinical effect, it will be difficult to justify the side-effects and costs of these studies.

Although ataxia is by far the most common presenting symptom in FRDA, there is also progressive atrophy of the corticospinal tracts leading to limb weakness, which becomes prominent in advanced disease (Kemp et al., 2016; Koeppen & Mazurkiewicz, 2013). Corticospinal tract degeneration amplifies the pre-existing and disabling effects of incoordination. For example, loss of upper limb dexterity, which is a major contributor to disability for patients, continues to worsen even after patients lose the ability to walk (A Dürr et al., 1996; Filla et al., 1990). Despite this, there is only very sparse reporting in the literature of methods for quantifying pyramidal tract dysfunction in FRDA. One obvious electrophysiological approach to is to measure motor cortical evoked

potentials (MEPs) using transcranial magnetic stimulation (TMS). The few published reports of MEPs in ataxia have been from heterogenous groups of patients with autosomal-dominant and idiopathic cerebellar ataxia, rather than genetically defined subtypes of degenerative ataxia including FRDA (Mondelli et al., 1995; Schwenkreis et al., 2002). Electrophysiological abnormalities, including delayed central motor conduction time (CMCT) and reduced MEP amplitudes, have been found to correlate with the duration and severity of disease in FRDA. Essentially, CMCT has been proposed to be a better index of disease progression than peripheral nerve examination. However, there are few if any serial studies of MEPs in FRDA, evaluated with objective clinical scores and detailed genetic or molecular analyses (Cruz-Martinez & Palau, 1997a; Gilles, Nicolas, et al., 2016; Martínez & Anciones, 1992a).

I have specifically focussed on correlating serially recorded MEPs in upper limbs, with clinical parameters over a period of 2.5 years, to examine its suitability as an outcome measure for future research.

3 OBJECTIVES

3.1 PRIMARY OBJECTIVES

The main objective of this project is to explore whether by combining multi-modal tests incorporating clinical assessments (Friedreich Ataxia Rating Scale (FARS)), electrodiagnostic testing (nerve conduction studies, beta band intermuscular coherence, motor cortical transcranial magnetic stimulation) as well as functional cellular/molecular assays (whole blood Frataxin levels, trinucleotide repeat length, and functional cellular assays of skin fibroblasts) into a screening algorithm, it might be possible to track the progression of disease in Friedreich's ataxia (FRDA) more accurately and efficiently over shorter time scales than are typically used in trials.

3.2 SECONDARY OBJECTIVES

1. To compare the performance of electrodiagnostic markers of disease against that of emerging functional *in vitro* cellular and molecular assays of disease activity in FRDA.

2. To assess what happens to electrophysiological and molecular/cellular measures with increasing duration of disease in a prospective study of FRDA patients.

4 STUDY PARAMETERS

4.1 IMAGING

In other slightly less rare neurological diseases (e.g. multiple sclerosis) surrogates of disease activity (e.g. T2 white matter lesions on MRI in MS) have been used to measure subclinical disease progression, thus overcoming some of the drawbacks with the use of clinical rating scales as outcome measures.

Certain measurable parameters on brain MRI (e.g., cross-sectional area of the superior cerebellar peduncle) are correlated with disease progression in FRDA (Akhlaghi et al., 2011). Studies have also shown significant spinal cord atrophy on MRI, with one study providing evidence for an association between cross-sectional spinal cord area and clinical disability documented by FARS score(Chevis et al., 2013; Wessell et al., 1989). By comparing 3T MRI scans in FRDA patients and age-matched controls it has been proposed that paediatric patients have significant degenerative changes in spinal cord, inferior cerebellar peduncle, and red nucleus, whilst adult patients showed more widespread white matter loss (Rezende et al., 2019).

In the original outline, we proposed that the patients would already have had recent baseline MRI brain imaging, permitting measurements of various neuroimaging markers and comparison with subsequent scans during and after the study period. However, appropriate MRI sequences were not available at

baseline and because we did not have funding to repeat MRI scans during the study, structural MRI was not included in this study.

4.2 MOLECULAR - FRATAXIN, SKIN FIBROBLAST ASSAYS and GAA REPEAT

Insights into the genetic, biochemical, and neuroanatomical basis of FRDA have necessitated that we define molecular hallmarks of disease pathogenesis and understand their relationship to disease status. The testing of peripheral blood cells and a variety of somatic cell types has introduced a powerful tool for *in vitro* disease modelling; for testing enzyme activity, metabolism of cellular substrates and/or looking at gene expression with chronic neurological disease.

In vitro cultures of somatic cells such as skin fibroblasts have contributed invaluably to our understanding of biochemical and cell biological processes. Skin-derived fibroblasts can also be reprogrammed to produce induced pluripotent stem cells (iPSCs). iPSCs represent an important platform in cell culture studies because they can propagate indefinitely and give rise to any cell type in the body, including CNS cell types. Microarrays and proteomic techniques provide a striking improvement in power and throughput over conventional techniques such as reverse transcription-polymerase chain reaction (RT-PCR), and Northern or Western blotting, which allow the simultaneous assessment of only single or small groups of genes and proteins.

4.2.1 FRATAXIN

Measurement of frataxin protein and mRNA levels in tissue - particularly buccal swabs and peripheral blood mononuclear cells is now reasonably established and may provide a means of predicting the rate of progression of neurological dysfunction and may also potentially have the necessary characteristics of a biomarker of therapeutic response in FRDA (Plasterer et al., 2013; Saccà et al., 2011). However, currently there is little data on how these molecular/cellular assays perform longitudinally and how they compare with other metrics.

In a study involving 521 FRDA patients, there was no evidence for change in buccal or blood frataxin levels over time with repeated measures analysis, although linear regression analysis of cross-sectional data predicted a small increase over decades (Lazaropoulos et al., 2015). In research studies, Frataxin levels from patient fibroblasts, lymphocytes, or muscle have historically been measured by Western blotting (analytical technique for separating proteins based on molecular weight using polyacrylamide gel electrophoresis (PAGE)). This is not an efficient or practical method for rapid detection in large scale clinical studies. Lately, the use of lateral flow immunoassays has made more rapid, non-invasive and accurate measurement of frataxin protein levels in the picogram range possible. Moreover, it has been shown that lateral flow testing can distinguish lymphoblastoid cells from FRDA carriers, patients and controls (Willis et al., 2008). The lateral flow test (LFT) applies the same principles of affinity chromatography used in enzyme-linked immunosorbent assays (ELISAs); typically a nitrocellulose membrane acts as a capillary bed along which

test solution flows, interacting first with coloured nanoparticles (or labels) conjugated to antibodies against the target antigen (i.e., frataxin protein) before being concentrated by antibodies anchored to the test strip. LFTs have of course now become almost synonymous with COVID-19 (SARS-CoV-2).

The immunoassay has been extended in Dr David Lynch's lab at The Children's Hospital of Philadelphia (CHOP) to measure frataxin directly from buccal cells and whole blood samples. Work from the Lynch lab has demonstrated significant differences in the mean quantity of frataxin protein extracted from buccal cells between controls, carriers, and patients (100, 50.2, and 20.9% of control, respectively) and in protein extracted from whole blood (100, 75.3, and 32.2%, respectively), although there was some overlap between the groups (Deutsch et al., 2010).

4.2.2 SKIN FIBROBLAST ASSAYS

There is also increasing recognition in hereditary degenerative conditions including FRDA that *ex vivo*/*in vitro* functional cellular assays of skin fibroblasts, which allow the direct assessment and measurement of mitochondrial function in patients, could not only be used for screening potential therapeutic compounds but also be used to measure disease activity (Richardson et al., 2013). Reduction of Frataxin levels in cells and tissues is linked to iron overload and progressive mitochondrial dysfunction, impairment of energy metabolism and accumulation of intracellular oxidative damage. Skin fibroblasts represent a model of primary human cells, with defined mutations and the cumulative

cellular damage of the patients. These cells can be easily isolated from 2 mm punch skin biopsies, a procedure, which does not need stitches and has practically very few complications. FRDA fibroblast cell lines both in human and mouse models, have been successfully created for the purpose of drug discovery or disease mechanism studies, and are maintained as primary fibroblast repositories for future (Y. Li et al., 2016).

4.2.3 GAA TRIPLET REPEAT EXPANSIONS

As has been determined, there is an inverse correlation between the length of the smaller of the two alleles of *FXN* gene and the age at onset. The size of the smaller GAA expansion is also strongly correlated with the rate of disease progression. The number of repeats on the smaller allele account for approximately 50 percent of the variability in age at onset, as compared with less than 20 percent for the allele with the larger expansion (A Dürr et al., 1996).

Unaffected individuals have usually <36 GAA repeats, whereas FRDA patients present with GAA expansions ranging from 70 to >1000, commonly occurring pathogenic repeats lying between 600-1200 (Bidichandani et al., 1998; De Biase et al., 2007). The wide range of GAA repeats reflects the instability of the expansion during transmission, which is characteristic of all mutations with large trinucleotide-repeat expansions (Ashley & Warren, 1995). Thus accurate determination of the number of GAA repeats is important in diseases attributable to complex expanded alleles with multiple repeat interruptions as in FRDA, permitting a more incisive genotype-phenotype correlation.

Another common and critical feature related to the condition, hypertrophic cardiomyopathy, was identified in almost all patients with classic Friedreich's ataxia studied by echocardiography (Morvan et al., 1992). Later, in a study on 187 FRDA patients , Dürr *et al.* showed that the frequency of cardiomyopathy increased with the size of the GAA expansion, as did the occurrence of the extensor plantar response and skeletal deformities. The correlation of *pes cavus* and scoliosis with GAA-expansion size might suggest an early onset of peripheral neuropathy. The association of cardiomyopathy with large expansions is certainly important for prognosis, since cardiomyopathy related complications are a frequent cause of death in FRDA (A Dürr et al., 1996). They also concluded that in Europe and North America, most cases were found to be sporadic and occurred in non-consanguineous families, where autosomal recessive inheritance could not be demonstrated.

Since FRDA is a progressive disease, a full clinical presentation is observed only several years after onset, a fact that makes an earlier diagnosis difficult. Unfortunately, delayed diagnosis impairs genetic counselling for future pregnancies. Direct molecular diagnosis through determination of the size of the GAA expansion should become an essential tool in clinical practice, helping to determine the pathogenicity of the mutation, thus differentiating FRDA from other recessive or sporadic cerebellar ataxias and allowing prognostication, extended family screening and pre-conception genetic counselling (Hoffman-Zacharska et al., 2016; Muthuswamy et al., 2013).

4.3 ELECTROPHYSIOLOGY

In my supervisor's lab (The Movement Laboratory), it was previously shown that subclinical DRGopathy, DC (dorsal column) and CST (corticospinal tract) degeneration could be detected using standard electrodiagnostic tests, namely nerve conduction studies (NCS), somatosensory evoked potentials (SEPs) and motor cortical evoked potentials (MEPs) in patients with mitochondrial disease (M. R. Baker et al., 2011). However, these tests can be uncomfortable and time-consuming, and it would be unpleasant and impractical to base a 3-6 monthly disease monitoring program around such investigations. If another layer of pre-screening were introduced, these investigations could be used much more effectively. Ideally, such a screening test should be painless and easy to deliver, preferably by any healthcare professional with minimal training, and show significant detectable changes with disease progression within an individual.

4.3.1 BETA BAND INTERMUSCULAR COHERENCE (BIMC)

An electrodiagnostic test that measures beta-band intermuscular coherence (BIMC) could meet the exact requirements of a screening test that is painless, easy to deliver by any healthcare professional with minimal training and shows significant detectable changes with disease progression within an individual.

BIMC is a measure of the correlation between pairs of EMG signals within the same limb in the 15–30Hz (beta) frequency range, during weak to moderate sustained voluntary muscle contraction **(Figures 2, 3 & 4)**.

Beta band oscillations have been recorded from the motor cortex in both animals (S. N. Baker et al., 1999; Donoghue et al., 1998; Murthy & Fetz, 1996) and human subjects (Halliday et al., 1998a; Salmelin & Hari, 1994a).

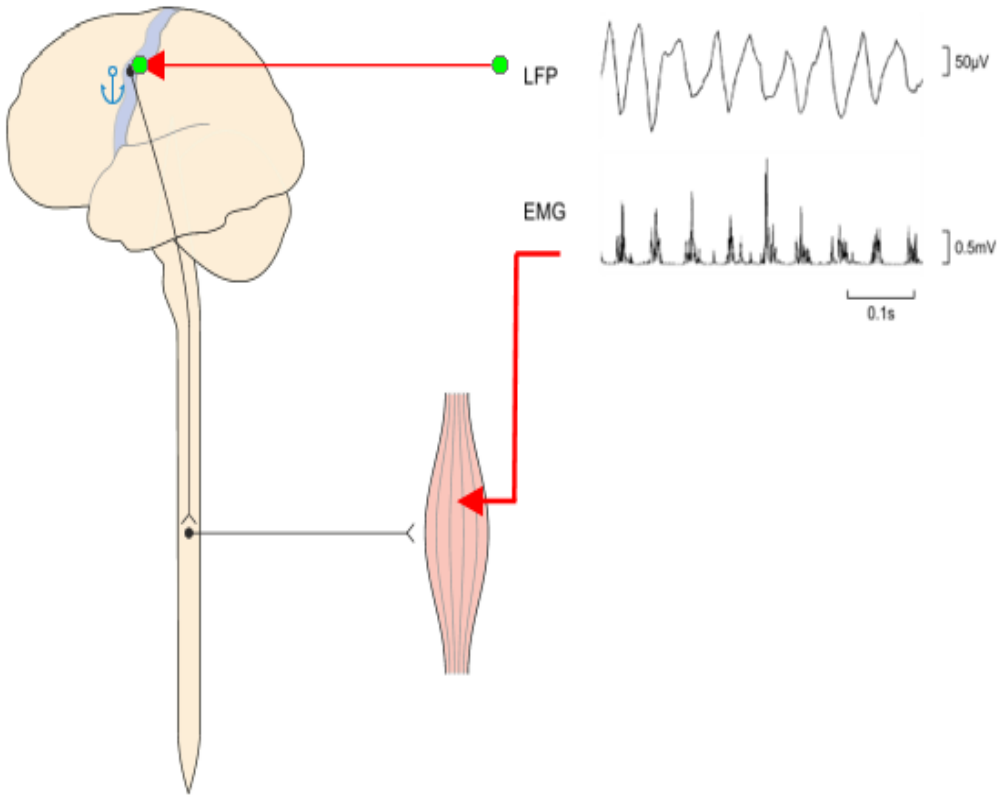


Figure 2. Coherence between oscillations in cortical slow wave activity (local field potentials) and EMG activity.

These oscillations appear to be synchronised throughout large parts of the motor system. Simultaneous EMG recording from a hand muscle involved in the task shows bursts of activity which coincide with the troughs of the cortical oscillation. EMG has been rectified so any negative voltages have been flipped above baseline. [Baker et al. (1997) J Physiol 501:225-41].

During sustained contractions, these oscillations are coherent between sensorimotor cortex and contralateral muscles (corticomuscular coherence, CMC) (Conway et al., 1995; Kilner et al., 2000; Ohara et al., 2001) between co-contracting muscles within the same limb (intermuscular coherence, IMC) (S. N. Baker et al., 1997) and between single motor units in one muscle (intramuscular coherence, IntraMC) (Farmer, Bremner, et al., 1993). Whilst measured between different pairs of signals, CMC, IMC and IntraMC are thought to partly reflect a shared cortical drive.

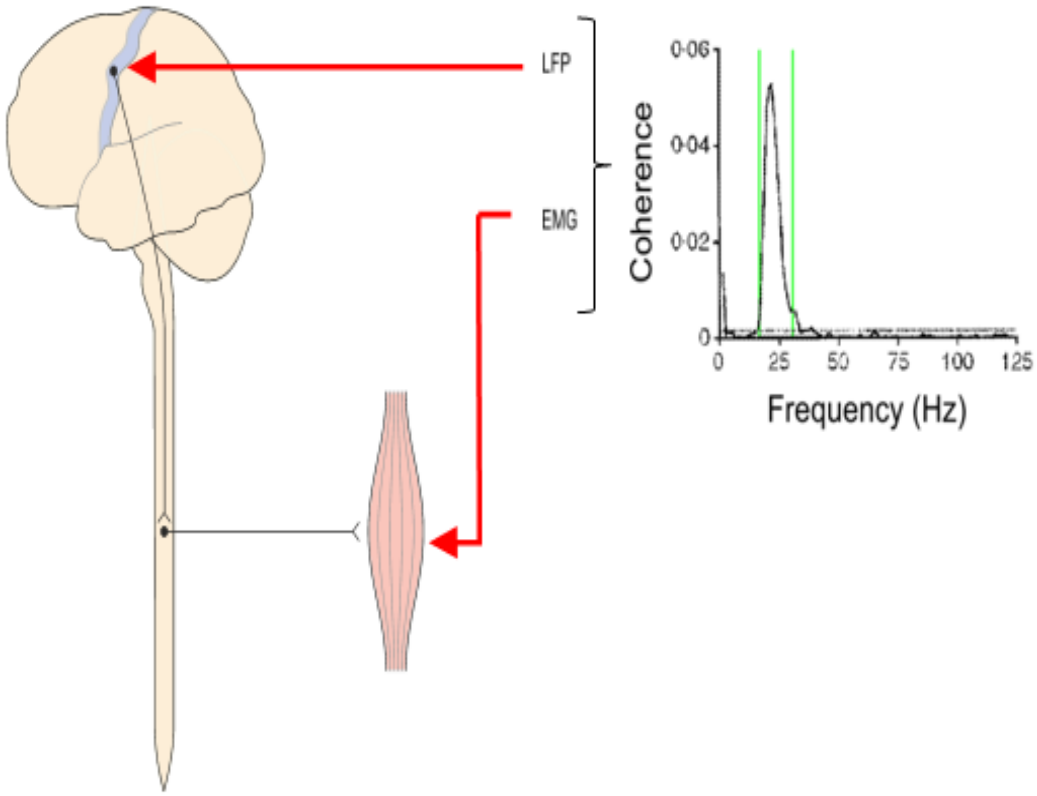


Figure 3. Calculating coherence between two signals.

In order to measure the extent to which signals are correlated in the frequency domain, coherence is calculated. This is a measure of the proportion of the signal within a particular frequency band where there is a constant phase relationship between the two sources and as a correlation coefficient is bounded between 0 and 1. The figure shows the resultant cortico-muscular coherence spectrum. There is significant coherence in the frequency band between 15 and 30Hz, indicated using green lines. This frequency band is called the beta-band. [Baker et al. (1997) J Physiol 501:225-41].

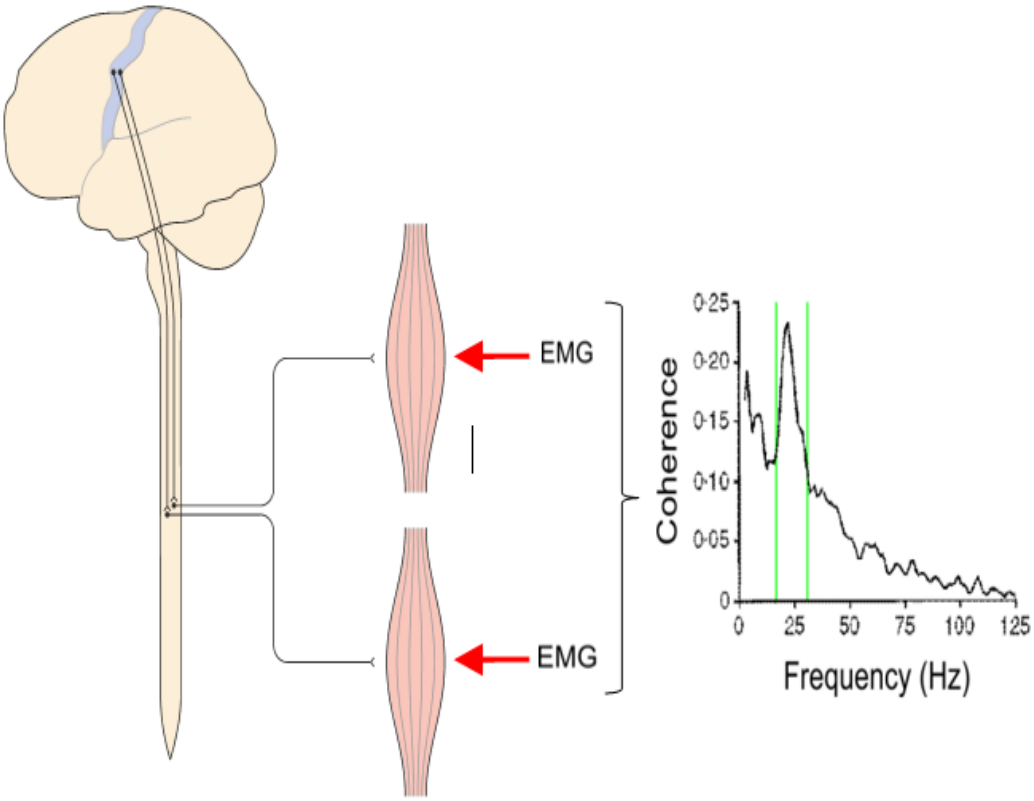


Figure 4. Intermuscular coherence is significant in the beta band.

When multiple muscles are involved as in EMG recorded from hand muscles during a steady state contraction, one can analyse coherence between such muscles. This is intermuscular coherence, and indeed this is significant in the beta-band.[Baker et al. (1997) J Physiol 501:225-41].

In Newcastle, as part of on-going research funded by both the Wellcome Trust and National Institute for Health Research (NIHR), the Baker laboratory has been investigating BIMC as a potential diagnostic test of corticospinal tract disease in various patient groups (Fisher et al., 2012) (Fig 5). In the studies, they have used only surface EMG, primarily because it is painless, and therefore easily applied in children, but also because it is easy to implement. In healthy people there is significant BIMC between pairs of muscles. However, when the corticospinal tract is lesioned, as in spinal cord injuries, BIMC disappears (Farmer, Swash, et al., 1993; Nishimura et al., 2009).

The lab has published preliminary results showing that BIMC can distinguish clearly between patients with Primary Lateral Sclerosis (PLS), a sporadic condition characterized by corticospinal tract degeneration, and age-matched controls. These results also provided preliminary evidence that BIMC changes with disease progression (K. M. Fisher et al., 2012). More recently it has been demonstrated that BIMC can separate patients with autosomal dominant hereditary spastic paraplegia (HSP), a genetically determined disease characterized by CST degeneration, from age-matched controls and patients with PLS. This is important because the process of CST degeneration seen in HSP is similar to that described in FRDA. In HSP, BIMC was usually decreased in the lower limbs and near normal in the upper limbs. [Work published in PhD thesis of Dr S R Jaiser (https://theses.ncl.ac.uk/jspui/handle/10443/2397)].

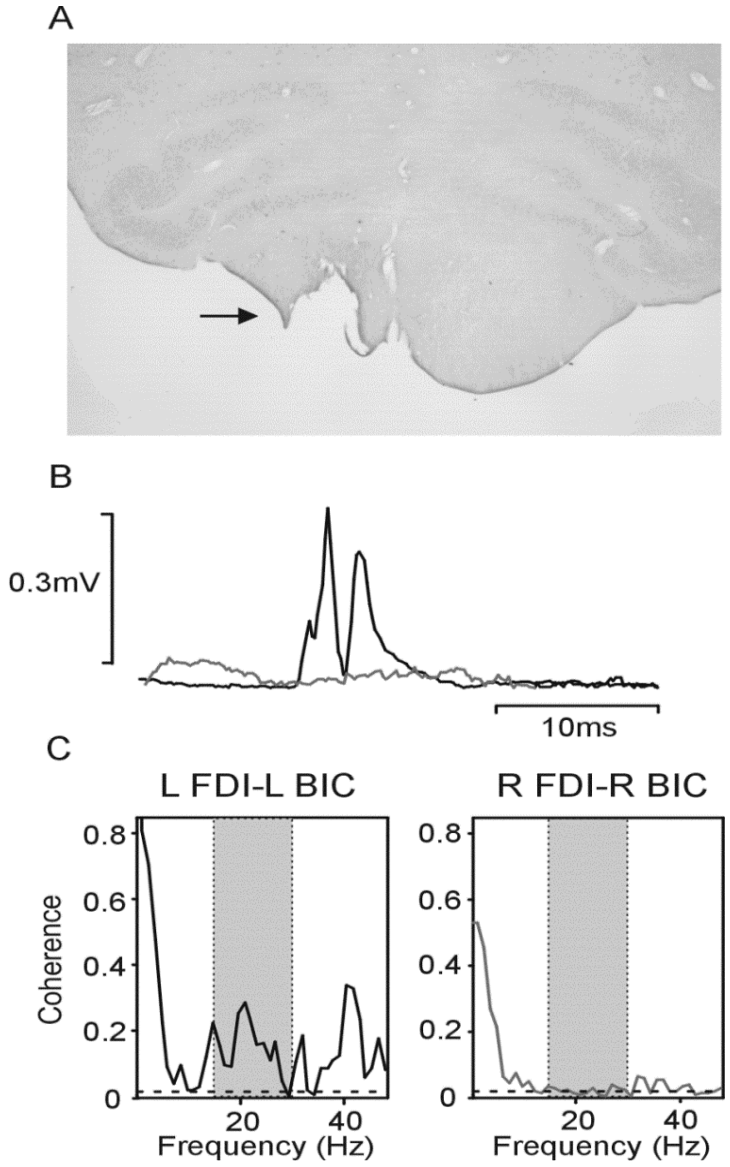


Figure 5. Lesioning pyramid in macaque abolishes BIMC.

(A) Transverse section through the pyramids at the level of the medulla showing the extent of the pyramidal tract lesion in monkey M.

(B) Motor-evoked potentials obtained from left (black) and right (grey) first dorsal interosseous muscles 3 months post lesion to the left pyramidal tract.

(C) Intermuscular coherence plots calculated from EMG data collected during a steady hold task. Dashed line indicates significance level.

BIC = biceps brachii; FDI = first dorsal interosseous; L = left; R = right. Adapted from Fisher et al. (2012) Brain 135(9):2849

RATIONALE OF USING BIMC IN FRDA

Interestingly, EMG-EMG coherence in the beta-band is not simply determined by the coupling between motor cortex and spinal motoneurons provided by CST axons, but also by muscle afferents and their connections back to sensorimotor cortex via the dorsal columns, leading to the current concept of an underlying efferent-afferent feedback loop (Witham et al., 2011). In humans, this model is supported by evidence that beta-band coherence is markedly reduced by deafferentation, whether permanently through severe sensory neuropathy/ neuronopathy/ dorsal root ganglionopathy (Kilner et al., 2004), or reversibly through anaesthesia of the digital nerves (R. J. Fisher et al., 2002) **(Fig 6)**; the latter study concluding that cutaneous input enhances oscillatory synchrony between pairs of hand muscles. The absence of BIMC could thus indicate CST disease, DC disease or DRGopathy – a mixed sensitivity, which fortuitously matches well the profile of affected systems in FRDA.

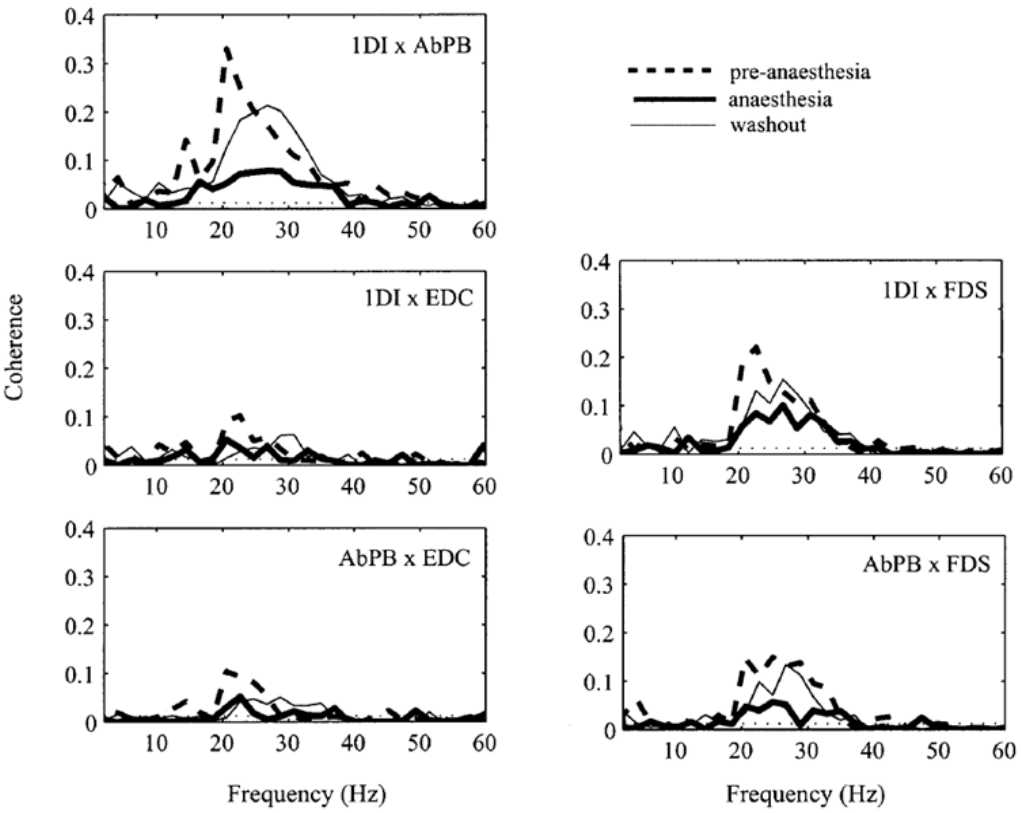


Figure 6. Digital nerve anaesthesia decreases EMG - EMG coherence in a human precision grip task.

Sensory afferent pathways are clearly important, because not only are beta oscillations recorded from muscle afferents, but anaesthetising afferents also reduces BIMC. Adapted from [Fisher et al. (2002) Exp Brain Res 145:207]

In the clinic, although simultaneous multi-channel EMG recording from more than 2 muscles can prove cumbersome, optimisation of methods should help to minimise the variability of results. Since BIMC is non-invasive and can be measured using commonly available EMG equipment, wider application should prove straightforward. Whilst FRDA is the prototypic multi-system ataxic disorder, there are others, and our approach should be applicable to many more such progressive hereditary neurodegenerative disorders.

4.3.2 CORTICAL MOTOR EVOKED POTENTIALS WITH TMS

Transcranial magnetic stimulation (TMS) of the cerebral cortex has proven its credibility as a neurophysiological tool for assessing the integrity of fast conducting corticomotor pathways in a non-invasive way. By quantifying damage to the pyramidal tract system, TMS is used in monitoring disease progression or treatment effect and thus contributes to prognosis (Rossini et al., 1994a; Rothwell et al., 1999; Volz et al., 2015).

TMS makes use of a strong, time-varying magnetic field that traverses bone with little attenuation. Such a field induces a current in conductive tissue in accordance with Faraday's Law (Barker et al., 1985; Barker AT, 2002) and can therefore be used to deliver stimulating currents to many areas of the nervous system where bony structures make direct electrical stimulation difficult, including the brain and the spinal nerve roots (Lemon, 2002). When applied to M1 or the peripheral motor pathways, suprathreshold magnetic stimulation leads to a detectable muscle contraction in a contralateral limb. This response is known as a motor evoked potential (MEP), recordable by electromyography from the surface of the corresponding muscles (Barker et al., 1985). In general, single-pulse TMS (including paired-pulse TMS) is used to explore brain functioning, whereas repetitive TMS (rTMS) is used to induce changes in brain activity that can last beyond the stimulation period.

Initially developed as a tool to diagnose and monitor neurological disorders with upper motor neuron involvement, it is now widely applied experimentally

to advance our fundamental understanding of pathways and neural circuits mediating human behaviour. By painlessly inducing currents to excite cortex, TMS can perturb physiological processes in the brain. Modulation of sensory perception, suppression or facilitation of cognitive capacity and behavioural performance can be obtained by varying the parameters of TMS (A. C. N. Chen et al., 2004). In the UK NHS, TMS is available in some hospitals, although it is usually restricted to research institutes given its expense and rather specialist nature. The technique has been used clinically in assisting in the diagnosis of neurological disorders affecting the CST such as MS, MND and spinal cord diseases (R. Chen et al., 2008; Eisen & Shtybel, 1990).

TMS IN CHILD NEUROLOGY

Although there are fewer published guidelines on TMS in paediatric populations, its practice in children continues to grow (Allen et al., 2017; Garvey & Mall, 2008; Nezu et al., 1997). In pioneering studies started in the 1980s in Newcastle, TMS was employed to study cortical reorganisation in children following congenital or postnatal central nervous system injury, for example, hemiplegic cerebral palsy and stroke (Eyre et al., 1991, 2001). These studies concluded that the functional maturation of the corticospinal tract is completed by the age of 13 years. Whereas the threshold stimulus intensity for exciting peripheral nerves decreases up to the age of 5 years before plateauing, there is a drop in the threshold for cortical activation progressively until the age of 16 years. Also, because axon diameters in both motor and somatosensory

central pathways increase in proportion to height leading to increase in conduction velocity, central conduction time remains constant during growth.

TMS can not only provide an index of maturation of the cortex and corticospinal pathways in children, but it also offers a significant diagnostic potential by detecting patterns of CNS reorganization, as well as guiding synaptic plasticity as a therapeutic strategy respectively, following brain damage (Basu et al., 2010; Smorenburg et al., 2017). Basu at al eloquently highlighted in a case study using TMS, EEG and EMG that cortical plasticity extended beyond reshaping of primary sensory cortical fields in reorganization after sensorimotor cortical injury, where there was transformation of occipital cortex into motor cortex after stroke in early development.

Staudt *et al.* looked at sensorimotor reorganization in patients of congenital hemiparesis using TMS and functional magnetic resonance imaging. It clearly demonstrated that the contralesional hemisphere developed fast-conducting ipsilateral corticospinal pathways to the paretic hand, determined by the maturational stage of the CNS at the time of the insult, as to whether the lesion occurred in the prenatal and perinatal period. Patients with late-third-trimester lesions often did not develop any useful hand function, despite the existence of such ipsilateral corticospinal tracts. The location and extent of the lesion also determined the degree of compensatory changes in the ipsilateral hemisphere and hence the outcome (Staudt et al., 2002, 2004).

TMS has been shown to produce persistent changes in motor output maps in healthy participants, as well as augment motor function during stoke recovery in adults (Rothwell, 2011). The dual stimulation paradigm of directing TMS to motor cortex combined with peripheral nerve stimulation has been shown to directly modulate neuroplasticity in adults (McKay et al., 2002; Stefan et al., 2000). Since the developing paediatric brain is thought to be significantly more plastic than the adult brain, various TMS approaches could help maximise functional outcomes by inducing neuroplasticity changes that may be longer lasting and persist into adulthood (Frye et al., 2008).

TMS IN CHILDHOOD DYSTONIA

Dystonia is a complex movement disorder characterized by involuntary muscle contractions and painful muscle spasms, affecting all ages. Children can present with both genetic (primary) and secondary dystonia (arising from various aetiologies including extreme preterm birth, perinatal hypoxia, or neurometabolic conditions). TMS has been used extensively in adults with dystonia to evaluate the excitability of various cortical areas and has challenged previously held views on the pathophysiology of dystonia. Without appropriate animal models, its pathophysiological mechanisms remained speculative before the TMS era, assumed either to be a pure motor consequence of dysfunction of the basal ganglia loop or a wider network involving multiple brain regions including the cerebellum and the cerebral motor, premotor and sensorimotor cortices (Raike et al., 2005). Loss of inhibition in dystonia has also

been shown through other electrophysiological approaches using the blink reflex and somatosensory evoked potentials (Lozeron et al., 2016).

Findings from TMS studies have shown a loss of inhibition at various levels within motor circuitry in dystonia and also distinguished between primary and secondary types, based on abnormally enhanced cortical plasticity in primary as opposed to normal motor cortex plasticity in secondary dystonia (Kojovic et al., 2013). Another study pointed out a critical difference of abnormally high plasticity (paired associative stimulation through TMS) in the organic dystonia group, versus normal plasticity in psychogenic patients and healthy controls (Quartarone et al., 2009).

Deep Brain Stimulation of the globus pallidus internus (GPi DBS) is of proven benefit in medically refractory dystonia of childhood, but outcomes differ significantly, especially with the heterogeneous population of acquired dystonias. In a cohort of paediatric patients with dystonia, a study using both CMCTs and SEPs provided objective evidence of disruption of sensory connections rather than corticospinal tract damage, as confirmed by the normal TMS MEP latencies in most of the patients, indicating corticospinal tract integrity. The combination of above two neurophysiological parameters was not only more sensitive compared to cranial MRI, but it also substantiated a significant association with DBS outcome – poorer if both CMCTs and SEPs were abnormal and even more so with abnormal SEPs (McClelland et al., 2011). This has contributed to a change in clinical practice by improving selection of

patients for DBS surgery and managing patient and family expectations regarding the potential benefit (McClelland et al., 2018).

TMS STUDIES IN FRDA

For the FRDA patients, we only assessed cortically evoked MEPs, which has been a scarce and mainly experimental investigation so far in this condition (Gilles, Nicolas, et al., 2016; Lanzillo et al., 1994; Nardone et al., 2014).

A single longitudinal study by (Cruz-Martinez & Palau, 1997a; Martínez & Anciones, 1992b) showed electrophysiologic (sensory and motor NCS, EMGs and CMCTs) and clinical work up on a group of 20 patient with FRDA, 13 of which went on to have 2 follow up studies over the next 9 – 12 years. CMCTs were examined only at the time of second and third examinations, using both methods – subtracting MEP latency from cervical latency by stimulation of the cervical roots, as well as the F wave method. They were tested the first time at an age of 7 – 34 years (mean 17.2 ± 6.1). The disease duration ranged from 6 to 20 years (mean 13 ± 4.3). They found that NCS and CMCTs were abnormal in all patients at baseline. Whereas SNAPs and CMAPs, and conduction velocities remained unchanged for several years, there was significant worsening of CMCT threshold, latency and MEP amplitudes at successive testing.

They proposed to have CMCT as the third electrophysiological diagnostic criterion in FA, after reduced SNAP amplitude and absence of H reflex.

I performed a similar electrophysiologic and clinical follow-up, including BIMC and molecular work (Frataxin) over a much shorter time span of 6 monthly intervals. In comparison, my participants had a wider age range (4 – 52 years) and disease duration 2 – 36 years). The aim of my study was to capture changes in a more sensitive and precise manner to find a reliable biomarker to aid with therapy rather than diagnosis. MEPs were the mainstay of my study as well and I hope it can add to the continuity of research in this under investigated condition.

We recorded MEPs in upper and lower limbs using different TMS coils, which optimally target hand or leg representations of motor cortex (circular or figure of 8 *vs* double-cone coil) by single-pulse TMS to the cortex. We measured central motor conduction time (CMCT), that is the delay between activating brain cells and the volley arriving at the level of the spinal cord **(Fig 7)**. CMCT has emerged as the most reliable parameter, which estimates the conduction time from M1 to spinal motor neurons(R. Chen et al., 2008; Groppa et al., 2012; Kumar et al., 1999).

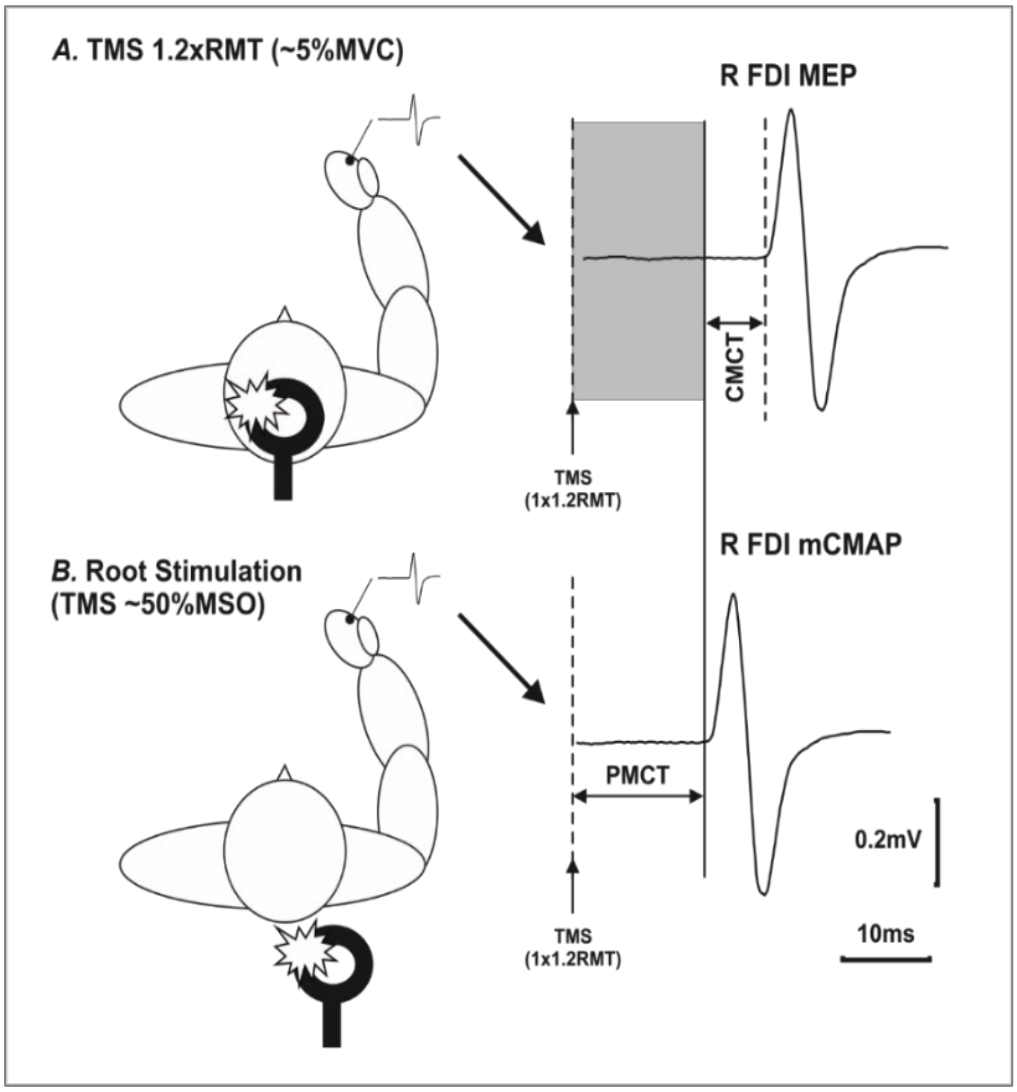


Figure 7. Measuring CMCTs.

Suprathreshold magnetic stimulation of M1 (A) and cervical roots (B) leads to MEPs recordable from the surface of the corresponding muscles.

4.3.3 NERVE CONDUCTION STUDIES

Nerve conduction studies (NCS) involve the application of depolarising electrical pulses to the skin over a peripheral nerve producing: (1) a propagated nerve action potential (NAP) recorded at a distant point over the same nerve, and (2) a compound muscle action potential (CMAP) arising from the activation of muscle fibres in a target muscle supplied by the nerve. In both cases these may be recorded with surface or needle electrodes. NCS test for focal or continuous abnormalities along the length of mixed, motor, or sensory nerves. The findings from various nerves often complement one another and yield different types of information based on distinct patterns of abnormalities for example, the maximum conduction velocity, the shape and size of the CMAP, evidence of temporal dispersion or conduction block, etc.; parameters which depend on the underlying pathophysiology.

Motor responses typically are in the range of several millivolts (mV), as opposed to sensory and mixed nerve responses, which are in the microvolt (μV) range. Thus, motor responses are less affected by electrical noise and other technical factors. In standard practice, surface adhesive electrodes are used for recording, placed over the muscle of interest in a belly–tendon montage. For motor conduction studies, the gain usually is set at 2 to 5 mV per division (Mallik & Weir, 2005). Age-matched ‘‘normal’’ values for NCS parameters are either derived from studies of groups of neurologically normal subjects within the respective labs or culled from the literature.

Previous studies in FRDA have shown absent or severely slowed sensory conduction, with normal or mild delays in motor conduction, even in very mild cases. Polyneuropathy is an early feature of FRDA, which does not appear to correlate with the clinical severity of the condition. Thus whilst NCS can facilitate early diagnosis, they are not suitable for evaluating disease progression (Bouchard et al., 1979; Mondelli et al., 1992; Peyronnard et al., 1976).

4.3.4 SOMATOSENSORY EVOKED POTENTIALS (SEPs)

SEPs are a type of evoked potential, conventionally elicited by electrical stimulation of either mixed or cutaneous nerves. Although small responses can be generated by stimulation of afferent peripheral nerve fibres by tactile, pain, temperature, or other stimuli, these are technically difficult to record. SEPs examine the functional integrity of the peripheral and central nervous system, therefore, a lesion affecting any part of somatosensory pathway may alter the recorded SEP. The abnormalities are not disease specific but can localise the anatomical site of lesions in the central sensory pathway (dorsal roots, spinal cord, brainstem, subcortical and cortical regions) (Yeh et al., 2006).

SEPs are useful in identifying abnormalities causing soft signs or vague symptoms, including the subclinical dorsal column disease encountered in FRDA patients. In addition, SEPs can indicate the type of involvement, for example, impaired conduction caused by axonal loss may result in reduced amplitude, marginally delayed or absent responses, whereas demyelination may result in very prolonged latency responses or absent waveforms.

''Short latency'' SEPs are used for the purpose of clinical interpretation (refer to portion of the waveform normally occurring within 25 ms after stimulation of upper limb nerves, 40 ms after stimulation of the peroneal nerve or 50 ms after stimulation of the tibial nerve). Peripheral nerves are stimulated transcutaneously with electrodes placed on the skin of the selected nerve (median nerve at the wrist, superficial peroneal nerve at the knee or posterior

tibial nerve at the ankle). SEPs are of much smaller amplitude, in the range of microvolts (μV), compared to the compound muscle action potentials (CMAPs) obtained by motor nerve stimulation or voluntary EMG; a large number of SEPs (500-2000) must therefore be averaged to mitigate the effects of scalp muscle artefact and yield a potential that can be measured reliably.

For upper extremity SEPs, the potentials are recorded from the ipsilateral supraclavicular fossa (known as Erb's points or EP), cervical spine at the level of C7, and the scalp over frontal (F3, F4 in accordance with 10 - 20 International System) and parietal areas (CP3, CP4). The components are labelled according to their mean peak latencies and are seen as the early negative polarity components (N9, N13, N14) and the first negative potential (N20) recorded from the scalp overlying the somatosensory cortex contralateral to the stimulated limb. The peak and interpeak latencies are measured. For example, EP to N20 approximates the conduction time between the brachial plexus and the primary sensory cortex (central sensory conduction time - calculated by subtracting the latency of the volley recorded at Erb's point from the N20 latency). For lower limbs, responses evaluate spinal roots at the level of L5 and S1, followed by dorsal column (N21) and cortical potentials (P40).

Many FRDA patients have abnormal SEPs, demonstrating delayed central conduction or absent short latency scalp responses (Jones et al., 1983). These findings suggest a dying back neuropathy with primary axonal degeneration related to the 1st order sensory neuron (Beltinger et al., 1987). With early

appearance of severe axonal neuropathy and/or dorsal root ganglionopathy (DRG), marked attenuation of SEPs is a consistent finding, making it a time-consuming test with no significant role in monitoring disease progression. I therefore omitted SEP testing in patients whose nerve conduction studies had already confirmed a severe sensory neuronopathy or DRGopathy, a feature typically established early in the disease.

4.4 CLINICAL ASSESSMENT: FARS

We chose FARS over ICARS and SARA for our clinical questionnaire assessments. These are all validated and widely accepted scales, which have the sensitivity to elucidate change over time in FRDA patients, and each has its own benefits and drawbacks.

So far, FARS has been shown to be the most responsive instrument based on effect size, and power calculations that prove that fewer participants are required to demonstrate the same effect of an intervention (Fahey et al., 2007; Friedman et al., 2010). The relationship of FARS scale with other rating scales, patient-reported outcomes, disease duration and GAA repeat length are well established. Both SARA and FARS have been successfully used in 2 large natural history studies, in Europe, the European Friedreich's Ataxia Consortium for Translational Studies (EFACTS) (Reetz et al., 2015, 2016), and the United States (Patel et al., 2016a), respectively.

The FARS questionnaire incorporates three subscales: a functional staging for ataxia, a score for activities of daily living (ADL) and a comprehensive neurological examination. The characterisation of neurological deficits (bulbar, upper limb, lower limb, peripheral nerve, and upright stability/gait functions) precisely matches with the profile of sensory and motor systems involved in FRDA. The scale is supplemented by timed measures of performance, to add more quantitative input. It includes an 8 m walk at maximum speed (8MW), the 9-hole peg test (9HPT) for hand dexterity and PATA rate (the rate of repeating

the syllables “PATA” as quickly as possible within a 10 second interval, to measure the severity of dysarthria).

The total scores range from 0 to 159; a higher score indicating a greater level of disability. The scoring for the three domains is as follows:

1. Functional disease staging assessing overall mobility is scored from 0 to 6 (5 being non-ambulatory, confined to but can navigate a wheelchair)
2. Activities of Daily Living scale, scored 0 to 36.
3. Neurological assessment, including the upright stability, scored from 0 to 117.

All the 3 domains (excluding upright stability section of neurological examination), are graded in units of 0.5, if strongly felt by the examiner that the status/task falls between two scores. This allows more incisive grading but at the same time, weakens inter-rater reliability in my opinion.

4.4.1 LIMITATIONS OF THE SCALE

There are certain other inadequacies of a questionnaire being used a clinical assessment tool which also warrant mentioning, particularly when addressing a multisystem disease.

CONFOUNDERS IN SCORING UPPER LIMB DEXTERITY

As has been recognised, the neuropathology of FRDA changes over time. There is an early established pathology of dorsal columns and DRG, followed by cerebellar impairment and degeneration of pyramidal tracts later in the disease. Advanced FRDA patients present with sensory, cerebellar, and pyramidal features that are difficult to disentangle clinically.

For example, the slowing of finger tapping (FT) rate without loss of FT regularity/misses or decrease in amplitude makes pyramidal tract dysfunction the main contributor, rather than cerebellar involvement. Although the scale provides guidance of adding an additional score of 1 to the rating if time of > 6s, the scoring of 'misses' in tapping index fingertip to thumb crease is not adequate as I found out that most of my severely affected FRDA patients were slow but not uncoordinated in performing the task, again highlighting the overlapping pathology of corticospinal tract involvement with upper limb proprioceptive loss. This fact has been nicely demonstrated in the study (Naeije et al., 2021) where they selected an automated method of FT analysis in FRDA to disentangle cerebellar (prominent FT rate variability), extrapyramidal (FT progressive amplitude reduction without slowing of tapping rate), and pyramidal (progressive decrease of FT rate and amplitude) contribution to upper limb loss of dexterity.

CEILING EFFECTS OF GAIT TESTING IN NON-AMBULANT PATIENTS

Gait and incoordination of balance are the most preliminary clinical identifiers for ataxia. Gait ataxia is related to reduced limits of stability and an enlarged postural sway which eventually contributes to reduced functional ambulation and possible falls (Morton & Bastian, 2007). Patients with ataxia exhibit an increased stride width, decreased stride length, increased variability in foot placement and a decreased overall gait velocity.

The upright stability subscale, which determines quantification of progression in the early phase of the disease addresses all of this through its 6 stance-related items (sitting posture, stance-feet apart, stance-feet together, tandem stance, stance on dominant foot and tandem walk). These are scored almost exclusively with extreme values (0 or the maximum, 4). This might indicate that in the context of an otherwise slowly progressing condition, these abilities are lost rapidly. The initial 3 stance items are easier and show preferable inter-item correlations. In contrast, the increasing difficulty of the remaining stance items results in very few patients in a FRDA cohort being able to perform them. In non-ambulatory patients, these items become noisier and contribute little to scoring disease progression. However, these items may provide useful information to the overall FARS score in clinical studies targeting clinically mildly affected individuals with FRDA or those who are very early in the disease process. These results also indicate that all 6 stance items could be useful in classifying patients according to function and in longitudinal analysis of those

individuals with the mildest of FRDA symptoms. The remaining item in the subscore, gait , has the best overall correlation with the total FARS score and in itself directly defines individual ambulatory ability.

Overall, although ceiling effects in upright stability tend less well to the non-ambulatory fraction of the cohort, it seems to be an optimal tool for detecting deterioration in motor function over time in ambulatory FRDA patients, with the introduction of some modifications (Rummey et al., 2019).

It is noteworthy that the assessment of stance and gait (8m/25 ft walk at a normal pace) can be somewhat subjective because it is susceptible to variations resulting from change in type of footwear, use of ankle and foot splints or orthoses (AFOs), and even physiotherapy.

CEILING EFFECTS OF PROPRIOCEPTIVE LOSS

The signs of proprioceptive loss, such as loss of deep tendon reflexes, vibration and joint position sense are usually established early and already score the maximum without showing any change over serial assessments. A study using magnetoencephalography demonstrated that proprioceptive impairment in FRDA is mostly genetically determined and barely progressive after symptom onset (Marty et al., 2019).

FLOOR EFFECTS OF AMYOTROPHY

Given the above fact, the items placed within the peripheral nervous system sub-score itself have weak correlations within the subscale. Especially, muscle strength and atrophy, items which have a slower decline (and thus lower scoring) are somewhat arbitrarily combined with deep tendon reflexes, which, being a cardinal sign of FRDA, are usually diminished or absent on first presentation, therefore receiving a maximum score. Similarly facial atrophy and tongue atrophy items demonstrate a 'floor effect' and correlate weakly with other items in the bulbar subscore. The remaining items in the bulbar subscore, questions on cough and speech, show considerable intercorrelation (Rummey et al., 2019).

CONSTRUCTION OF A MODIFIED FARS (mFARS) SCALE

In the above analysis led by Rummey and colleagues, classical psychometric correlation analysis (item-own vs item-other subscale correlations) confirmed the validity and structure of the FARS neurological examination, but specifically endorsed the modifications leading to the mFARS. **mFARS** has been devised to eliminate "weak" items that correlated poorly and had limited functional significance. The peripheral nervous system (PNS) subscore and two bulbar components of facial and tongue atrophy have been justifiably excluded, demonstrating that the items reflecting functional ability match the most psychometrically robust in the mFARS. In addition, this shows that progression

captured by the FARS examination does not match the deterioration of sensory pathways in FRDA and that sensory loss is perhaps less relevant for the patient.

An addition of 'eyes open or closed' has been added to testing 'stance' items, likely to capture the early features of sensory ataxia before cerebellar ataxia sets in. The authors concluded their study by confirming the validity of mFARS and that it offered an excellent test - retest reliability using data from recent data from several large clinical trials in FRDA (Rummey, Zesiewicz, et al., 2020).

APPLICABILITY OF FARS SCALE IN CHILDREN

There is no specific limitation addressed in literature about applicability of the scale to children. FRDA mainly presents between 5-15 years of age, and patients are somewhat older by the time medical advice is sought or they are enrolled into clinical trials. The two major clinical trials, FACOMS and EFACTS did not have very young children as participants. The FACOMS study had 812 subjects and a mean assessment age of 30.1 ± 15.3 years (mean baseline age was 26.1 years). For EFACTS study, FRDA cohort was stratified by age of onset: early disease onset (≤14 years), intermediate (15-24 years), and late onset (≥25 years), but no age range of the participants was stated. The youngest subject in my study was 4 years old, who was genetically tested for FRDA for having a previously diagnosed older sibling (who was 9 years old and also a participant in my study). The activities of daily living as well as peripheral nerve subscale and upper/ lower limb co-ordination items were not reliably measured in the

youngest participant. Despite completing the FARS scale in all three visits, it was hence decided not to include the results in the final analysis.

COMPLETION OF A COMPACT QUESTIONNAIRE

Though I conducted my study with the original FARS scale, it could take around 30-50 minutes to complete, depending upon the ability and fatigability of the patient. Some would find it impossible to perform 9-hole Peg test within the required time, being exhausted by the time they reached this last item on the scale. With the removal of PNS subscore, the test is not just more compact now, but also stronger in its construct with changes that are to be expected in the natural progression.

Furthermore, the argument remains that some substrates and pathways may change sub-clinically before clinical presentation and are too subtle to be assessed clinically. We make a case that these changes can be captured by electrophysiological tests and help identify and predict progression, allowing investigators to combine or select the best measure for the clinical approach being tested.

We examined the average rates of change with FRDA every 6 months for 2 years and compared the performance of clinical evaluation with electrophysiological (mainly CMCT and BIMC) as well as with molecular assays (Frataxin levels) and GAA repeat expansions, to produce a comprehensive and robust profile of various dysfunctions in this multi-system condition.

5 STUDY DESIGN

5.1 STUDY PROTOCOL, FRDA COHORT AND TIMELINE

The study was set up to assess the sensitivity and specificity of the novel biomarkers (electrophysiological tests - MEPs and BIMC) and investigate their relationship with known biomarkers, including already validated rating scales (FARS scale in our case) and gene repeat sizes.

This was the largest study of its kind to date in the North East of England and has thus established the basis for a future multi-centre longitudinal trial of these tests as markers of progression (or surrogate outcome measure) for therapeutic trials.

These measures were tested in a prospective longitudinal study design in a group of patients with a genetic diagnosis of Friedreich's Ataxia. The participants took part in four study visits over the course of a maximum timeframe of 30 months, with assessments at baseline, 6, 12 and 18 months (and 24 months was proposed if additional funding was made available).

We aimed to perform all assessments for each participant within the same day to avoid unnecessary travel to the centre. However, since the participants hailed from all over the UK (Isle of Man, Southampton), some of them needed air travel as well as overnight accommodation prior to the study day. For the electrophysiological procedures, approximately 4 hours were required on the

first visit and usually up to 3 hours on the consecutive ones (as sensory evoked potentials were deemed to add no further value after baseline). After 1 hour of lunch break, further 2 hours were set aside for the blood tests, clinical questionnaire, including the timed activity test such as PEG test, PATA rate and gait assessments.

5.1.1 FRDA COHORT

The enrolment in the study was stopped when we surpassed the target and recruited 30 patients with an age range of 4 - 52 years (mean = 29 yrs, median = 31 yrs) and disease duration of 2 - 36 years (mean =15.9 yrs). In the study group, there were 7 children (younger than 18 year of age), with an age range of 4 - 18 years (mean = 12 yrs, median = 13 yrs). There were three participants aged 19, hence a third of the group comprised children and young adults.

Sadly, not all of them would continue with the study. Of the 30 patients that were recruited and attended baseline visit, only 17 attended 2nd and 3rd visit, and less than half, 14 patients made it to visit 4. The attrition was on various accounts as shown next in the section of Timeline. The patients recruited earlier to the study were mostly non-ambulant and in advanced diseased state. The results became apparent during the first visit that most of them had a very diminished BIMC, the main marker for the study. Hence the inclusion criteria was revised to include patients who could mobilise at least with support, and I enlisted the help of 'Ataxia UK' to spread the message to FRDA community country wide.

GAA repeat sizing and skin biopsy was done on all except one (n=29) due to issues of consent for a minor. Most patients (n=29) had FARS and blood tests for Frataxin tested at visit one. BIMC was conducted on all patients (n=30) on visit 1. Contra-indications to magnetic stimulation and disease progression leading to absent MEPs meant loss of baseline data (n=23). However, with successive visits, there was loss of data at multiple points for many reasons, such as lack of BIMC led to exclusion of 10 patients after visit 1. The most likely reasons some patients withdrew was ill health and in-patient stay at hospital, making them unable to continue, or practicalities of long distance travelling and overnight stay with family or carer.

Age-matched control (n=5) subjects were recruited from members of the Institute of Neuroscience and their friends and families (aged 5 to 42 years) for MEP measurements. The individuals were tested twice at 6 monthly intervals. Three control specimens were sent for serum Frataxin levels and repeated after 6 months. For adequate analysis of BIMC parameters, controls (n=47) were taken from the existing database of Baker lab (aged 22 to 56 years).

All studies performed in this thesis took place in the department of Clinical Neurophysiology, Royal Victoria Infirmary, Newcastle upon Tyne. We used the services of mitochondrial laboratory and blood bank at the IGM (Institute of Genetic Medicine), Centre for Life in Newcastle.

5.1.2 TIMELINE

The key elements for each stage of the project are highlighted in **Fig 8 -** Study design and flow of participants. To complete the data collection over 4 time points in a span of 2.5 years was a reasonable and achievable timeline. However, evaluating multiple parameters in people with a progressive condition, including children and adolescents, who had sometimes travelled from far off, affected the deliverables.

There was a degree of participant fatigue noticeable near the end of an experiment and if a task was overly repetitive or taking them long to complete. Other factors for me such as changing laboratories for molecular work, learning a new protocol of processing samples for frataxin half way through the study, shipping biological samples overseas packed with the right amount of dry ice, finding suitable disabled access accommodation in Newcastle for patients and families, etc., added to stretch the resources and time. **Fig 9** elucidates subjects included in the final analysis, separately for each parameter, including the reasons for exclusion.

Figure 8. Study design and flow of participants.

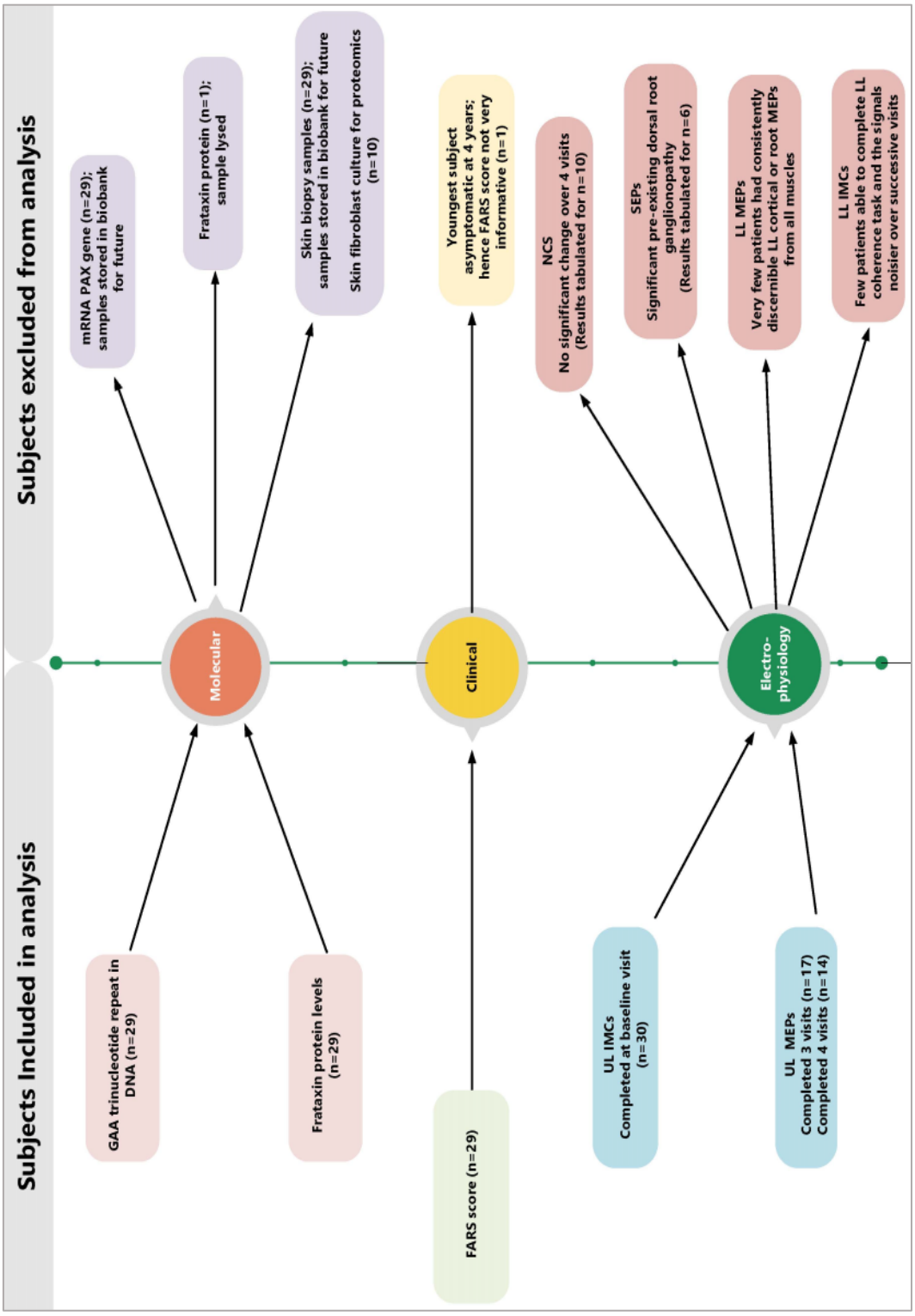


Figure 9. Subjects included in final analysis along with reasons for exclusion.

5.2 ELIGIBILITY CRITERIA

INCLUSION CRITERIA

a) Patients with a molecular genetic diagnosis of FRDA.

   As we started performing the baseline study on the first few patients, it became obvious that BIMC was absent in them due to advanced disease, which was one of the main parameters to be measured in the study. Hence, we refined the inclusion criteria.

b) FRDA patients with disease duration less than 8 years and still independently ambulant or using linked arms for mobility.

EXCLUSION CRITERIA

a) Patients with pacemaker or other contraindications to TMS (presence of metallic foreign body, previous intracranial surgery or trauma, history of epilepsy {relative contraindication}).

b) Patients with other known neurological conditions.

c) Inability to provide informed consent.

Although contraindications to TMS was an exclusion criteria, two patients with implanted medical devices, a cardiac pacemaker and a defibrillator respectively, were still included. This was very early on in the recruitment and FRDA being a rare condition, I did not want to lose patients on whom BIMC studies could be performed. One patient missed to inform about their cochlear implant until the day of the visit. Therefore these three patients were still on the study, but TMS was omitted, with rest of the electrophysiology and molecular tests undertaken as per protocol.

5.3 CONSENT

Written consent was obtained at the beginning of the baseline visit without any difficulty. The patients were provided with the Participant Information Sheet to consider in advance of the visit, and they would then have a full discussion with myself [and the Principal Investigator (PI) if need be] by phone or email to have any questions/concerns addressed. I explained about the nature and objectives of the study and possible risks, burdens and benefits involved, at the outset. The results were not expected to alter the participants' medical management in the near future.

I allowed sufficient time in understanding and addressing potential patient concerns. There were no concerns regarding any of the adult participant's capacity to consent. Most parents were apprehensive about subjecting their children to unpleasant electrophysiological tests, and more so to skin biopsy, as that was a definite invasive procedure. I allayed their doubts by explaining that I was an experienced member of team who would be performing all the tests. The blood samples and electrophysiology are low-risk procedures and generally well tolerated. I also attached photographs of the skin biopsy procedure and assured that I would let a topical local anaesthetic take effect before the biopsy, which would involve 3-4 mm of superficial skin from inner side of arm and leave a small scar. Children had the study explained verbally where necessary at the start of first visit.

Participating subjects all gave written informed consent at the baseline visit, and a copy of the signed consent form was handed to the patient or parent/guardian of the patient in case of participants under 16 years of age. The original signed copy was filed in the Investigator Site File. All parents gave consent, except for the youngest participant who was 4 years old and still asymptomatic despite having a confirmed genetic diagnosis of FRDA. The invasive tests - skin biopsy as well as bloods for testing GAA expansion were omitted, and blood sample for frataxin was taken only for the first study visit in this case.

If a participant withdrew consent at any point during study, they were withdrawn from the study. Identifiable data or tissue already collected with consent was retained and used in the study. No further data or tissue was collected, or any other research procedures carried out on or in relation to the participant.

5.4 RECRUITMENT

I was appointed as a Clinical Research Associate to the institute of neuroscience (ION), Newcastle University before starting the study. This initiative, Out of Programme Training (OOPR), is offered by the deanery to specialty trainees in association with their lead employer trust. Hence, I was able to conduct this research by taking time out of my training in Clinical Neurophysiology at the RVI, Newcastle upon Tyne Hospitals NHS foundation Trust.

The study participants were identified by:

- Database searches at the regional Neuro-Genetics/Ataxia clinic in Newcastle (Chinnery/Horvath).
- Placement of adverts via the UK Ataxia Charity, www.ataxia.org.uk, on their website and in their magazine "the Ataxia".

Understanding and addressing potential patient and investigator concerns is paramount when developing a recruitment strategy. Once the participants were identified, they themselves or parents/guardians had the opportunity to discuss the project and have any queries answered. Since there was no screening visit as that aspect was dealt online or over the phone, gathering and organizing information to the last detail was vital for me to enhance patient preparedness. I had to explain thoroughly about specifics of the appointments, including the various tests, travel, lunch breaks, etc., to reinforce the rigorous requirements of the study. If they expressed their willingness to participate, I sent them an invite letter by post. The GP was contacted and provided with the

information sheet, requesting them to refer the patient to the study. A letter was also sent to the patient's neurologist to keep them informed and receive any additional clinical information that they might consider relevant. I obtained medical history including disease onset and duration, clinical symptoms, and severity of the condition as a part of assessment for the eligibility criteria. Any medications the participant was taking or had recently taken were confirmed with the GP.

Recruitment to the study was not as easy as anticipated, in part because of trial fatigue in the local patient cohort. Despite this we managed to hit and surpass our predefined recruitment target, with the assistance of Ataxia UK. In total, 30 adult and paediatric patients with a genetically confirmed diagnosis of FRDA (age 4 - 52 years; disease duration 2 - 36 years) were prospectively recruited from a database held by the regional and national Neuro-Genetics/Ataxia clinic. The patients were recruited locally from North-East to all over the UK; and arranging their travel and accommodation with the flexibility required around their commitments and mine, was an intense and rewarding learning curve. As our patient cohort were mainly school going children or young employed adults, I would make a note of the participant's personal schedule for the next visit - they preferred periods of school holidays /half-term breaks or sufficient notice to be away from work.

It was heart-warming to finally meet them on the day spending a buzzing morning and afternoon and then have a chance to see them at subsequent 6

monthly visits with their family members. Since there was more time to interact with the patients than in a clinical setting, it was an incredible opportunity to understand a disease pathology thoroughly from its origin in textbooks, pursuing it through to recent research literature and relating it to the lives of real people. For example, I had to work out how to book for the right kind of wheelchair accessible hotels in ample time; and realised the difficulties of travelling with a disability even in the UK. For their monetary reimbursement, I liaised with the university FMS accounts team and financial hub in processing travel and subsistence expense claims in accordance with the policies, mastering aspects of bookkeeping and accounting in this environment.

5.5 FUNDING AND ETHICS

FUNDING

The main funding for the study was split between Ataxia UK (https://www.ataxia.org.uk/), FARA (Friedreich's Ataxia Research Alliance, https://www.curefa.org/) and GoFAR (www.fagofar.org) charities. A later part of funding was also supported by JRESC (Joint Research Executive Scientific Committee, which oversees the NHS/Academic Partnership between the Trust and the Faculty of Medicine of the University of Newcastle upon Tyne) and NIHR.

ETHICS

This study was a part of the bigger project carried out at the Institute of Genetic Medicine (IGM), Centre for Life – 'Genotype and Phenotype in Inherited Neurodegenerative disease'. Ethical approval was obtained for the original project from Health Research Authority, NRES Committee Yorkshire & The Humber - Leeds Bradford (Sep 2014). An addendum was sent it for the updated information sheets and consent forms and additional tests of nerve and muscle electrophysiology and skin biopsy sample , which they endorsed as a minor amendment (Feb 2015).

The Newcastle-upon-Tyne Hospitals NHS Trust acted as a research sponsor of the study, ensuring that NHS indemnity scheme would apply for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm

to participants arising from the management of the research. Where appropriate, samples were analysed outside of UK.

We relayed preliminary results of the data as project reports to the Ataxia UK, the main funder. We intend to disseminate the results of the completed study through published literature in peer reviewed scientific journals and presentation at conferences, as well as share those with the participants and relevant patient groups.

I understand that the time lag between the end of patient participation in this study and the availability of the results might have been longer than expected, but I would ensure that the patients have access to summary findings, as they were informed when the study was coming to a close.

5.6 MATERIAL/SAMPLE STORAGE

Care was taken to ensure that clinical and electrophysiological/molecular data information was held in a secure, central database at Newcastle University. The data was pseudoanonymised, in a way that participant identities would only be recoverable by the designated members of the research team. Physical files have been stored in a locked filing cabinet and office. Access was restricted to researchers directly involved with the study.

The tissue samples were processed and stored in accordance with all applicable legal and regulatory requirements, including the Human Tissue Act 2004 and any amendments thereafter. Blood and skin biopsy samples were collected from patients in accordance with the patient consent form and patient information sheet. It also included all the patient information and documentation supplied in relation to them. The samples have been stored securely in linked anonymised form at the Newcastle MRC Centre Biobank for Neuromuscular diseases, IGM. This meant that data provided to researchers was non-identifiable, but the clinical care team would be able to restore the link if necessary.

DNA was extracted from all the samples for sizing of alleles and sent to Neurogenetics laboratory at UCL. A portion of blood was used to determine levels of frataxin, and the cryopreserved samples were sent to a lab in USA. Blood samples were also extracted for RNA PAX gene to examine expression

profiles and measure other proteins by microarray analysis in the future. These have been stored at – 80 °C in the mitochondrial lab at IGM.

For some patients, we needed to study additional tissues or human cell lines (such as cultured skin fibroblasts) for mitochondrial dynamics/proteomics, in collaboration with Leibniz Institute for Analytical Sciences – ISAS –e.V., Dortmund, Germany (preliminary test performed). Appropriate patient consents were obtained at the start, and these tissues could be requested from the Biobank of the Newcastle MRC Centre (ethical approval is already in place for this). The samples are from a rare condition, and likely to be useful for further research and hence storage is essential for several years.

6 METHODS

As described in the study design and participant flow scheme (Figure 8, Chapter 5), of the 30 subjects recruited, not all could participate in all the parameters involved. For various logistical issues mentioned, the number of participants dropped at the follow-up assessments. After the first study visit, the tests were repeated thrice in total, once at each of the following study visits with the number of patients retained (n=17, n=17, n=14) respectively.

Electrophysiology: At the baseline visit, all the 30 subjects took part in the BIMC task for upper limbs, whereas 23 subjects had TMS MEP tests performed on upper limbs (3 patients had contraindications due to implanted metallic devices, 1 declined the test due to concerns of side effects and in other 3, no MEPs were detectable although attempted in upper limbs). Please refer to Chapter 5.2, Eligibility criteria, for the reasons to have subjects with metallic devices entered into the study.

At baseline, lower limb MEPs and BIMC could only be obtained in the small subset of patients with very mild disease (n=16 for LL MEPs, n=14 for LL BIMC). In this group, where repeated testing was feasible at subsequent visits, this was completed. In the other patients, we decided that it was unreasonable to subject them to TMS at high stimulator intensities in an attempt to record a lower limb MEP at follow-up visits (For e.g., only one or two muscles yielding MEP response from the three being tested). Given the number of lower limb

MEPs completed was very small, I took the decision for statistical reasons not to include these data in the final analysis for thesis. For similar reasons, lower limb BIMC data was omitted from final analysis (please see under section 6.1.4, 'Limitations of coherence task' for details).

NCS studies were performed on upper limbs of all subjects to delineate the extent and distribution of peripheral nerve disease as a baseline for reference. NCS were performed in lower limbs and repeated at future visits where severe axonal neuropathy had not set in, and clear-cut responses were still recordable at least in the upper limbs. SEPs were conducted only on patients who showed preserved SNAPs in nerve conduction studies. Only about a third of the group showed recordable and reproducible signals for SEPs.

FARS scale : The youngest participant was 4 years old and sibling of another study participant. He was clinically asymptomatic except for a hint of falls according to parents. FARS questionnaire was administered but its relevance seemed limited as he was still dependent for most activities of daily living such as dressing up and eating, etc. Accurate examination of upper and lower limb coordination and peripheral nervous system was also impractical. Therefore, baseline clinical data was acquired on 29 patients.

Bloods : Blood test for GAA alleles was performed at the first study visit. All patients except one (29 in total) had their DNA samples tested for GAA trinucleotide repeat expansions, with long-range flanking PCR and agarose gel electrophoresis at the Neurogenetics laboratory at the National Hospital for

Neurology and Neurosurgery, London. A part of my study looked at correlating the expansion size with age at onset and disease severity, as well as comparing expansion size with electrophysiological and clinical parameters.

For the 4 year old subject, the parents did not give consent for the invasive procedure of skin biopsy and testing of gene repeat sizing but allowed blood sample to be taken for Frataxin levels for visit one only. Therefore, bloods for Frataxin were taken from 30 patients, but one sample lysed, leading to 29 samples being processed at baseline. Serum frataxin levels were tested by isolating a buffy coat sample via centrifugation, which was then cryopreserved. The resultant frozen pellets were shipped off to CHOP and analysed using lateral flow immunoassays. Blood samples for RNA PAX gene (n=29) were also extracted at baseline visit. For RNA PAX gene, whole blood was collected in a tube with a special reagent intended for immediate stabilization of intracellular RNA. The samples were frozen and stored for future isolation, to investigate gene expression and for molecular diagnostic testing.

Skin biopsy: Skin biopsy (punch biopsy of 2-3 cm) was taken from the forearm of each participant (n=29, barring the above-mentioned minor participant) at their baseline visit and stored at the Newcastle Biobank for fibroblast culture. Although it was not the main focus of this study, 10 patients had their fibroblasts cultured and mass spectroscopy was carried out to look at alterations in cellular transport proteins in mild and severely affected patients.

6.1 BETA-BAND INTERMUSCULAR COHERENCE(BIMC)

6.1.1 BACKGROUND

As introduced previously, cortical oscillations occur in a normal brain as a steady state process across a broad range of frequencies. Neural activity in motor cortex related to movement oscillates at frequencies around 20 Hz. Although their origin and role are still unclear, these oscillations are associated with similar rhythms in contracting muscles on the opposite sides of the body (corticomuscular coherence) (S. N. Baker et al., 1997; Halliday et al., 1998b; Ohara et al., 2001). It is thought to reflect a connection between the cortex and muscles during continuous muscle activity.

It is now known that the oscillations travel not only from cortex to muscle as expected for a motor command, but also back from muscle to cortex, reflecting sensory input. In all instances, they are task-dependent, being prominent during delay or hold phases and diminished during movement (S. N. Baker et al., 1997; Sanes & Donoghue, 1993).

6.1.2 COHERENCE

Coupling of oscillations between the motor cortex and contralateral muscles can be quantified by coherence analysis. This is what coherence simply is: a linear measure of correlation between two signals at a given frequency. It ranges from 0 for no linear relationship to 1 for a perfect linear relationship,

and as such is equivalent to a *correlation coefficient r^2, except that it is not a single number but rather interpreted as a function of frequency*.

Beta-band coherence between M1 and contralateral limb muscles (corticomuscular coherence, CMC) has been extensively investigated and recorded in monkeys and humans using EEG, ECoG and MEG (Kilner et al., 1999; Salenius et al., 1997; Salmelin & Hari, 1994b). ECoG is invasive, MEG is only available in selected centres and EEG is suboptimal due to its low focality, modest signal-to-noise ratio and the low-pass characteristics of the skull and scalp. By contrast, beta-band coherence can be reliably found between pairs of muscles coactivated in a task or even between pairs of motor units in the same muscle and such beta-band intermuscular or intramuscular coherence partly mirrors the same drive as beta-band CMC.

Previous experiments at Newcastle lab have shown that coherence was particularly marked during steady grip of a compliant, spring-like load (precision grip task), with reported changes in oscillatory synchronization in the 15–30 Hz bandwidth between human motor cortex and hand muscles. It was also demonstrated that coherence varied according to the time course of the task and the level of compliance of the gripped object (Omlor et al., 2011; Riddle & Baker, 2006).

Although the effects of de-afferentation on BIMC are well established, there has been no reported study in literature looking at IMC in FRDA and whether beta-band intermuscular coherence could be a suitable measure of

degeneration in both CST and afferent pathways as is encountered in FRDA. Importantly, IMC is non-invasive as described below and can be performed using routinely available EMG equipment.

6.1.3 RECORDING BIMC

Subjects were seated in a comfortable chair with their arm resting semi-pronated on a cushion. All assessments were carried out on the dominant side. The subjects were kept alert and focussed to continue the task over extended period of time. Surface EMG was recorded from first dorsal interosseous (FDI), flexor digitorum superficialis (FDS) and extensor digitorum communis (EDC) in the upper limb, and extensor digitorum brevis (EDB), tibialis anterior (TA) and medial gastrocnemius (MG) in the lower limb. Adhesive electrodes (Bio-Logic M0476; Natus Medical, Mundelein, IL) were placed in a belly tendon montage over the intrinsic muscles of the hand or foot. For the long muscles of the forearm or calf, the electrodes were placed 4 cm apart, one third along the muscle from its proximal origin. Signals were amplified, band-pass filtered (30Hz-2kHz; Digitimer D360, Digitimer, Welwyn Garden City, UK) and digitised at 5kHz (Micro1401, Cambridge Electronic Devices, Cambridge, UK).

In the upper limb, subjects were asked to perform a repetitive precision grip task. A length of compliant plastic tubing (length 19cm, Portex translucent PVC tubing 800/010/455/800; Smith Medical, Ashford, UK) was attached to the index finger and thumb with Micropore tape (3M Health Care, Neuss, Germany), and subjects were asked to oppose both ends of the tubing when

prompted by visual and auditory cues. At the start of the grey boxes on the screen, the subject received an auditory cue (beep sound) to contract. After 4s, they were given a second cue to relax, and following a pause of 2s the task repeated, and the subject completed 100 such trials per limb **(Fig 10)**. This auxotonic task – so-called because force increases with displacement in a spring-like fashion – required a minimum force of 1N (K. M. Fisher et al., 2012) and was similar to a precision grip task used in previous studies at the motor lab, without measuring digit displacement. In the lower limb, no such device was used, and subjects were asked to dorsiflex ankle and toes in the air while resting the heel on the ground **(Fig 11)**. This is a potential limitation (discussed in the limitations section) but did not affect the ability to measure BIMC in LL muscles as we have shown in previous published studies (Jaiser et al, 2016).

Visual feedback of raw EMG traces was provided to facilitate consistent task performance, particularly the timing of muscle contraction relative to the cues, but not to monitor the level of EMG. Data acquisition and the control of audio and visual feedback were performed using custom scripts written in Spike2 software (Cambridge Electronic Design, UK).

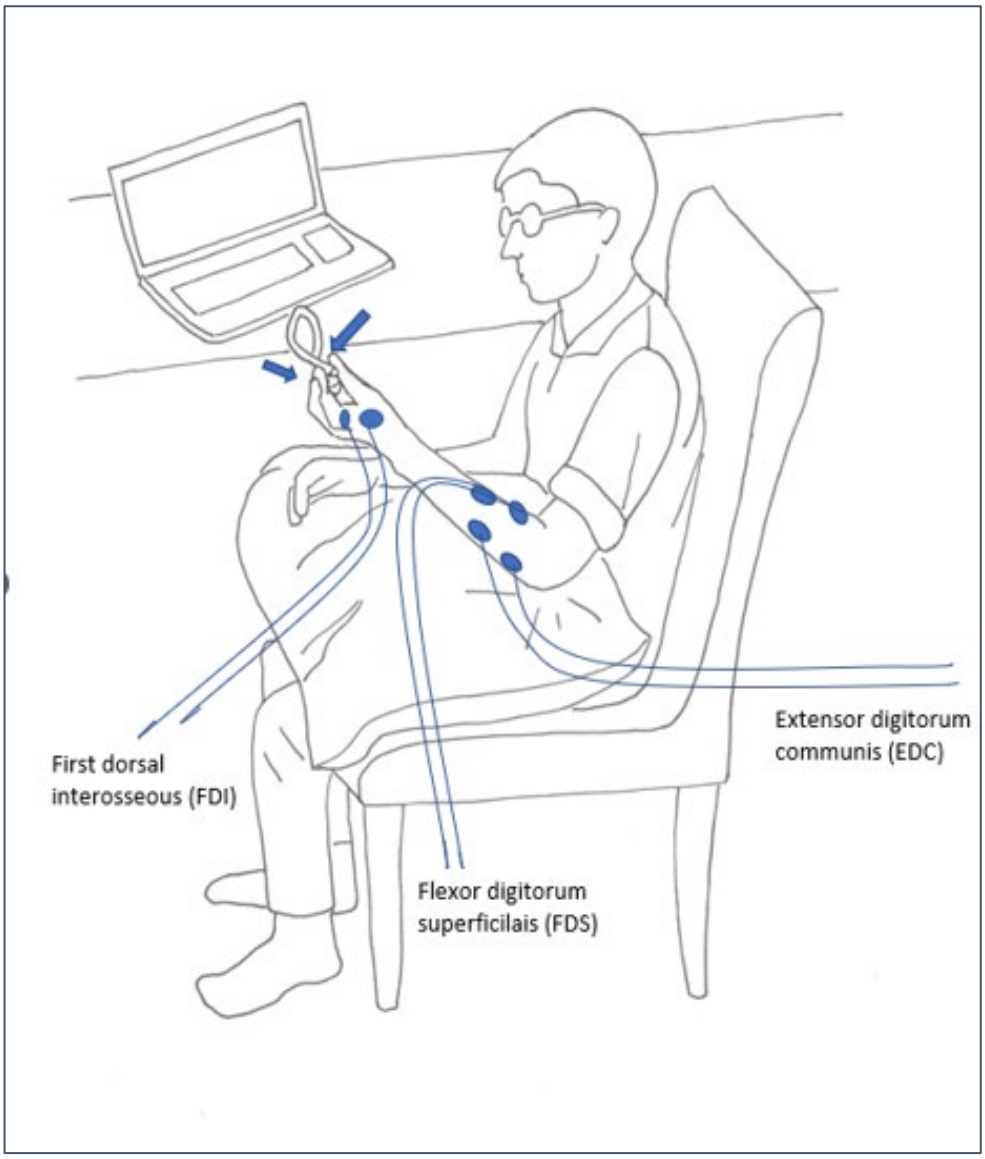


Figure 10. Upper limb BIMC recording – Precision grip against plastic tube.

Coherence Task: This involves squeezing a low compliance spring between the first finger and thumb for 4 seconds, relaxing the grip for 2 seconds before squeezing again. This procedure is repeated at least a hundred times. The EMG signals acquired are then rectified and processed (only stationary segments of EMG during the squeeze phase are used). Coherence is then calculated between the processed first dorsal interosseous (FDI) and extensor digitorum communis (EDC) and flexor digitorum superficialis (FDS) EMG signals.

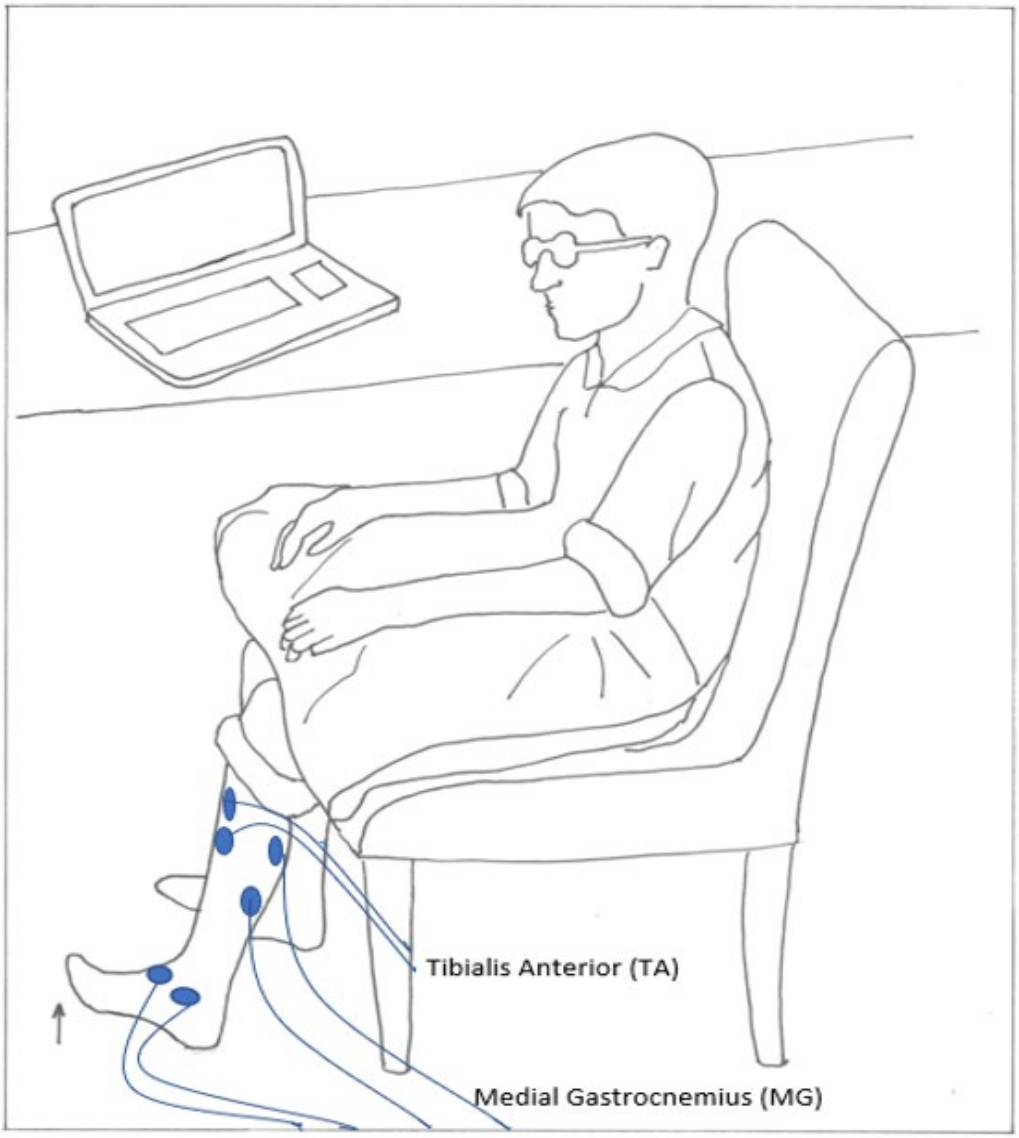


Figure 11. Lower limb BIMC recording - Unrestrained dorsiflexion.

6.1.4 LIMITATIONS OF COHERENCE TASK

Previous studies employed tasks which can be readily standardised but require prolonged activity or a high level of dexterity and thus are not suitable for use in patients with neurological deficits (M. R. Baker & Baker, 2003; S. N. Baker et al., 1997; Evans & Baker, 2003a; Riddle & Baker, 2005a, 2005b, 2006). Our task involved weak, phasic contractions and a minimum of instrumentation. Inherent disadvantages were a greater freedom of movement and less precisely defined targets, making it more difficult to ensure consistent performance. It would be helpful to carry out an experiment where normal subjects perform different tasks in the same session so that resulting coherence estimates can be compared (unpublished studies in our research group have addressed this and shown that there is no difference between beta-band IMC measured using different tasks in the same session).

For coherence analysis, EMG data should be stationary, hence the application of an instrumented approach to control/constrain the coherence task in previous studies. However, this is difficult in practice. The largest study published on coherence in healthy subject of all ages (also from the Baker research group) showed that it was possible to record reliable and comparable beta-band IMC results without instrumentation, which is why I chose not to use instrumentation to control the level of contraction and thus the level of EMG in my study (Jaiser et al., 2016). The main concern was to ensure that only very weak muscle contractions were used; strong contractions reduce beta-band

oscillations within the motor system and instead gamma (Piper) frequency oscillations dominate (Brown et al., 1998). It is however important to note that even if the patients performed the same task as the controls, they may not necessarily have used the same level of contraction. If the patients had weakness, they might have had to use a relatively higher proportion of their MVC to perform the same task.

There is also the phenomenon of moment-to-moment variation of coherence seen during sustained contractions in normal subjects within the same session and within different sessions (Mongold et al., 2022). Within a session, factors such as neuromuscular fatigue may play a role (Forman et al., 2022). Between sessions, the potential causes are that firstly, it not possible to achieve an exactly identical electrode montage at a different visit. In a previous study, electrode position significantly influenced coherence between two intrinsic hand muscles (Keenan et al., 2011). Secondly, coherence amplitude and directionality can change substantially within an individual over a timeframe of one or two years, even with a highly instrumented task, because the balance of afferent and efferent contributions to CMC changes with time (Witham et al., 2011).

Except for one patient who was quite disabled, all the patients recruited to the study could physically perform the IMC task in the upper limbs. The patient who could not perform the finger squeeze task with the tube was provided with a squeeze ball. For the lower limbs, except for the mildly affected, the completion

of task was not straightforward for most patients. The presence of FRDA-induced foot deformities, amyotrophy, proprioceptive loss, spasticity etc. made it impossible for most patients to effectively carry out repetitive toe or ankle movements. For many patients in this situation, especially those have been non-ambulant for a few years, lower limb IMC was not attempted at successive visits if they could not complete in the preceding attempt.

Very young subjects found it difficult to maintain concentration for a steady contraction for a 100 repetitions, there was an intermittent drop-off in EMG activity, more in lower limbs but also noted in upper limbs. A study showed decreased beta band CMC following fatigability in adults with cerebral palsy and neurologically intact adults (Forman et al., 2022).Their findings indicated that compensatory mechanisms to fatigability were present in both groups, and that fatigability affected the corticospinal drive in the same way.

Although IMC recordings were consistently taken early on in the clinic day at each visit, events leading up to the assessments, such as lack of sleep, traveling, fatigue etc. would affect their participation in the task. Therefore, to avoid inconsistencies in data as well as noisy signals due to fluctuations in task performance, we analysed only upper limb IMCs.

RECTIFICATION OF EMGs

It should be acknowledged that there is some debate in the literature regarding the importance of pre-processing EMG data by rectification before calculating beta-band CMC/IMC. (Blouin et al., 2014; Boonstra & Breakspear, 2012; Farina et al., 2013; Keenan et al., 2005; McClelland et al., 2012; McClelland et al., 2014; Myers et al., 2003; Neto & Christou, 2010; Yao et al., 2007).

However, it is important to remember that the compound EMG signal recorded with surface electrodes from skin overlying muscle reflects the sum of the signals from individual motor units, as filtered by the tissues between the motor units and the recording electrodes. The signal from each individual motor unit can be thought of as a timing (delta Dirac) function, which stipulates when the discharges are occurring (generally low frequencies), convolved with the shape of the motor unit action potential, which stipulates what each individual discharge looks like (generally high frequencies, especially when the motor unit is near the recording electrodes so that the intervening tissues confer relatively little low-pass filtering and the action potential looks “sharp”).

The calculation of coherence requires an approximation of the delta Dirac function, so that the timing of discharges between two sources (in our case, two muscles, but other pairs such as cortex-muscle for corticomuscular coherence) can be correlated; the muscle fibre action potential shape is not relevant. Unfortunately, without full decomposition of the compound EMG signal, which is complicated and not usually possible for single channel

recordings (but possible with high-density surface EMG), we cannot really calculate the underlying timing (delta Dirac) functions. Full-wave rectification (or root mean square) of the compound signal is a step which emphasises the timing information over the action potential shape and was the approach I used for the analysis in my thesis.

Whilst this was the rationale for rectifying EMG signals, I acknowledge that there are some potential limitations to this approach. For example, although rectification can enhance the detection of CMC/IMC in some circumstances (particularly in the beta frequency range), the extent to which it does so is variable and is influenced by amplitude cancellation of the EMG signals. This could theoretically cause issues with comparing levels of coherence across studies/conditions where amplitude cancellation may differ (Farina et al., 2014) and might therefore have influenced the results in this study.

6.2 CORTICAL MOTOR EVOKED POTENTIALS

6.2.1 INTRODUCTION

The clinical utility of TMS MEPs includes but is not limited to the investigation of patients with multiple sclerosis, suspected motor neurone disease, myelopathy, cerebral infarction, movement disorders, cerebellar disorders such as Friedreich's ataxia, and peripheral nerve lesions. Other potential applications of TMS MEPs include quantifying damage to the pyramidal tract system and monitoring disease progression or treatment effects, and thus potentially contributing to prognosis. The focus of my experiments was MEPs evoked by single-pulse transcranial magnetic stimulation, the relevant technical and physiological principles, and their correlation with the progression of clinical disease.

6.2.2 BRIEF HISTORY OF TMS

The principles of electromagnetism that underlie TMS were well recognised more than a century ago before its introduction by Barker and colleagues in 1985 (Barker et al., 1985) at the Royal Hallamshire Hospital and University of Sheffield. The reason TMS could not be developed any sooner was due primarily to lack of necessary high-powered electronics, even though the scientific principle of its basis, i.e. electromagnetic induction was discovered by Faraday in 1831. Faraday's law states that a time-varying current in a primary

circuit (coil) will induce an electric field and thereby a current flow in the secondary circuit (brain).

Electrical stimulation is very effective when applied to superficial peripheral nerves using surface electrodes, but it is necessary to use high voltage and hence painful stimuli to penetrate the scalp and skull in order to achieve cortical stimulation (Pascual-Leone, 2002). The revolutionary discovery of transcranial electrical stimulation (TES) by Merton and Morton in 1980 paved the way for its use as a clinical electrodiagnostic method for activating the corticospinal tract but has largely been replaced by TMS since its advent.

TMS has the advantage of being painless and offers a high safety profile in an awake patient. TMS stimulators are now in use world-wide, largely for diagnostic and basic research purposes, but rapidly gaining importance in a variety of therapeutic applications. During cortical stimulation, a large pulse of electric current is discharged through a coil on the scalp inducing a transient magnetic field, which penetrates the skull and induces secondary eddy currents in the underlying brain tissue. TES has found its niche in intra-operative monitoring of the functional integrity of the corticospinal tracts, in comatose patients and a research tool in clinical neurophysiology.

6.2.3 BASIC PRINCIPLES OF TMS

Magnetic stimulation of primary motor cortex generates a volley of responses in corticospinal axons (recorded with epidural surface electrodes over the dorsolateral cord) termed D (D for direct) and I waves (I for indirect) (Terao &

Ugawa, 2002). As the action potentials descend to the spinal cord via the pyramidal tract, the earliest wave recorded, the D-wave, reflects direct activation of corticospinal axons. At higher intensities, the stimulus recruits synaptic inputs to the same corticospinal neurones, causing them to discharge at later intervals. This produces I-waves resulting from activation of mono and polysynaptic inputs to corticospinal neurons. These I-waves are separated by intervals of ~ 1.5 ms and are named as I1, I2 and so forth. There is summation of these multiple descending D- and /or I-wave volleys at the anterior horn cells in the spinal cord, which are then depolarised and generate action potentials when threshold is exceeded.

Different coil orientations preferentially stimulate distinct neuronal pathways evoking different outputs, D or I waves (Volz et al., 2015). Epidural recordings of D-waves are used in combination with SEPs and muscle MEPs for intraoperative monitoring of spinal surgery, as D-waves are not attenuated by use of GA or muscle relaxants.

6.2.4 TMS COILS

The stimulating coil is the only part of a magnetic nerve stimulator that comes in contact with the patient. The electrical field in the tissue is oriented perpendicular to the magnetic field in the stimulation coil. The two most commonly used magnetic coils are a circular coil and figure of eight coil (also named double or butterfly coil).

The orientation of the magnetic field over the brain is important, since large differences can occur with different coil orientations (Rösler et al., 1989). When viewed from above in commonly used monophasic stimulators, clockwise current orientation (Magstim coil B uppermost) within a circular coil centred on the vertex leads to preferential stimulation of the right hemisphere and turning the coil over (Magstim coil side A uppermost) to a counterclockwise current orientation reverses the direction of the induced current and stimulates the left hemisphere. Figure-of-eight coils are designed for more focal stimulation whereas a circular coil effectively stimulates a larger cortical volume. In the newer stimulators capable of rTMS (biphasic and polyphasic ones), stimulation is less dependent on coil orientation and a large circular coil at the vertex activates both hemispheres simultaneously. However, there is no significant difference in the MEP latency using different stimulator types, though other MEP parameters may vary (Rossini et al., 1994b; Rothwell et al., 1999).

6.2.5 SAFETY CONSIDERATIONS OF TMS

Because of the exposure to magnetic fields, contraindications to TMS are similar to those for magnetic resonance imaging (MRI) . A safety check list should be used to screen the patients with implanted metal devices such as cardiac pacemakers or defibrillators, cochlear implants, intrathecal drug delivery pumps, vagus nerve stimulators, history of intracranial surgery and seizures. However, TMS studies on patients with deep brain stimulators in globus pallidus and thalamus would suggest that this is relatively safe provided the coil is not placed in the vicinity of such devices (Kumar et al., 1999; Rossi et

al., 2009). Despite the above concerns, single-pulse TMS is considered extremely safe because of rapid attenuation of the magnetic field with increasing distance from the discharging coil. However, the risk of triggering epileptiform activity is much greater with rTMS even in normal subjects (Wassermann, 1998). Intracranial aneurysmal clips are an absolute contra-indication. Watches and magnetic-sensitive devices e.g., credit cards should be placed away from the area before beginning the examination.

6.2.6 TMS PROTOCOLS AND PAIRED PULSE MEASURES

There are three different kinds of TMS protocols in common use:

Single-pulse TMS (sTMS) provides a single monophasic stimulus to the cortex and results in a detectable muscle contraction in the contralateral limb. We used sTMS in the current project **(Figures 12 & 13)**. The diagnostic sensitivity of the technique is enhanced by quantitative measurements such as onset latency, the size of the MEP (amplitude), cortical silent period (cSP), motor threshold and, in the clinical context, studying multiple muscles above and below a suspected spinal lesion.

Paired pulse TMS (ppTMS) is a further useful tool to investigate multiple excitatory and inhibitory interactions resulting in corticospinal output in the motor cortex (Chen *et al.*, 2008). Paradigms involve stimulating M1 with two pulses, a conditioning stimulus and a test stimulus separated by a specified interstimulus interval (ISI), ranging from 1 to 500 milliseconds. The resulting conditioned motor response is compared to the test motor response elicited by

single-pulse TMS alone, the ratio of which provides a measure of cortical inhibitory-excitatory balance (R. Chen et al., 2008). By varying the stimulation intensities and the ISI, several new TMS biomarkers have emerged, including short-interval intracortical inhibition (SICI), long-interval intracortical inhibition (LICI) and intracortical facilitation (ICF). Short interstimulus intervals result in intracortical inhibition, which is presumably mediated by GABAergic interneurons ($GABA_A$), and longer interstimulus intervals result in intracortical facilitation, which is presumably mediated primarily by glutamate (Ziemann et al., 1996, 1998). A wide range of neurological disorders have been associated with anomalies of intracortical inhibition and facilitation (Berardelli *et al.*, 2008; Chen *et al.*, 2008).

Repetitive TMS (rTMS) uses a train of pulses separated by a regular intertrain interval (ITI) for a specific period of time. The duration of the intertrain interval determines the stimulation frequency, 1 Hz being the most widely used protocol. In most subjects rTMS has an inhibitory effect on the cortex if the time between the pulses is 1 second or more (i.e., 1 Hz or low-frequency repetitive TMS), and has an excitatory effect on the cortex if the frequency of stimulation is faster than 10 Hz and applied in bursts of 2 to 10 seconds with pauses of 20 to 30 seconds in between (i.e., high-frequency repetitive TMS). Importantly, the effects of rTMS outlast the stimulation period (Pascual-Leone, 2002; Romero et al., 2002). The neural plasticity induced by r-TMS has raised considerable interest because of its therapeutic potential, for example in epilepsy, migraine, stroke, chronic pain and depression.

Figure 12. TMS of cortex with a circular coil over the vertex.

Figure 13. TMS of C-spine roots.

In single pulse TMS, CMCT is measured by using a simple method – the circular coil is positioned over the vertex of the scalp to obtain a MEP. Then the coil is positioned over the cervical spine to obtain a peripheral motor conduction time (PMCT) , which is then subtracted from MEP latency to calculate CMCT.

6.2.7 PATHWAY OF MEP GENERATION

MEP is a transcranially induced motor response, observed as a contralateral muscle twitch readily recorded with surface electrodes. A MEP is caused by highly synchronized action potentials in fast-conducting, large diameter axons originating from corticospinal neurons making direct monosynaptic connection with spinal motoneurons **(Fig. 14)**. It is evoked only when the volley of impulses produced in the corticospinal tract is strong enough to bring the spinal motor neurons to their firing threshold, triggering an action potential, which is propagated along the peripheral motor axons. The descending excitatory drive is influenced by external (e.g., intensity of the TMS pulse) and many internal factors (e.g., the maturation and integrity of corticospinal connectivity, activation of other muscles, underlying cortical and spinal oscillatory activity, alertness, etc.).

Facilitation of the MEP, a measure of corticospinal (cortical and spinal) excitability, manifests as shortening of MEP latency, decrease in motor threshold and increase in the area or amplitude of the MEP. Manipulations such as voluntary contraction of the target muscle facilitate the amplitude and shorten the latency of MEP. In intrinsic hand muscles, most of the effect of facilitation occurs with the first 10% of maximal voluntary background contraction of the target muscle (Hess et al., 1986; Thompson et al., 1991), which can be monitored accurately by a force transducer or surface EMG.

A variety of other facilitation manoeuvres have been tried such as conditioning TMS (paired pulse stimuli), tendon, cutaneous nerve or muscle afferent input or even thinking about a movement (Claus et al., 1988; Kiers al., 1997), but background activation has been deemed to be the most effective.

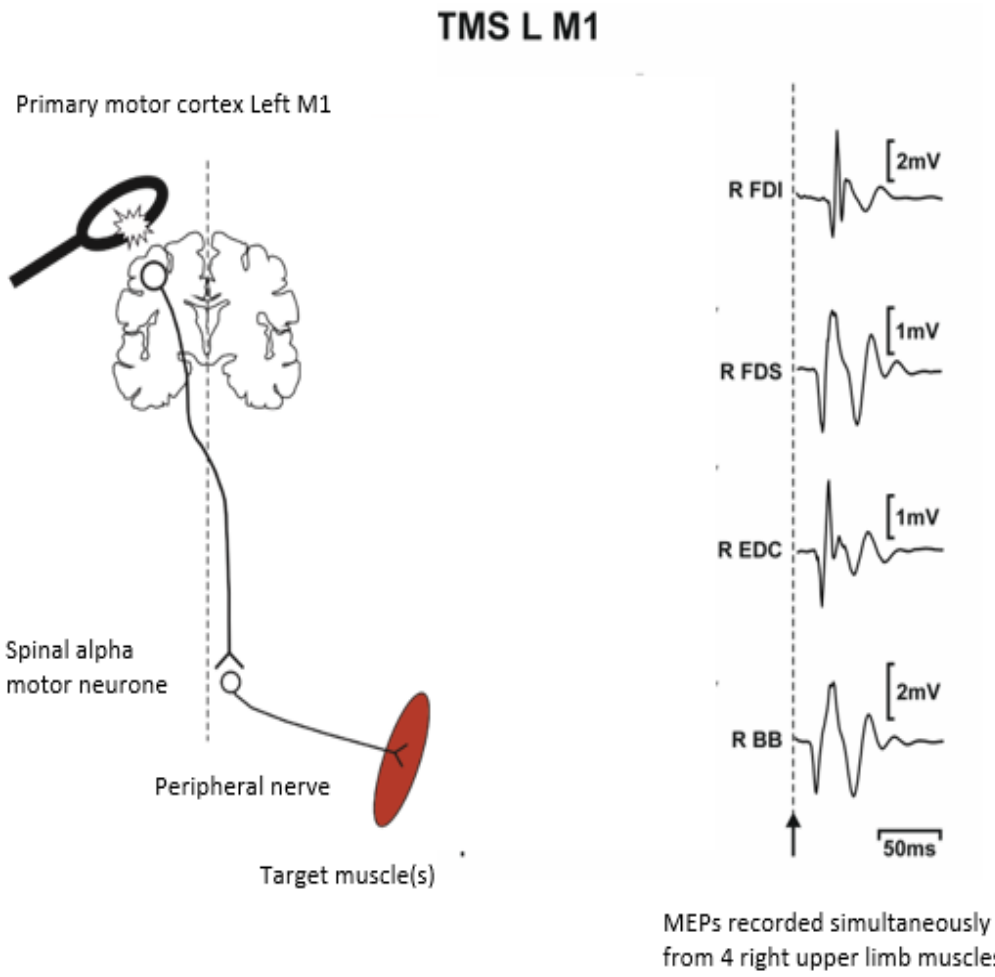


Figure 14. Pathway of MEP generation.

6.2.8 MEP MEASUREMENTS IN CLINICAL NEURODIAGNOSIS

MEP recordings have an inherent variability, even with constant stimulation and recording techniques. Of the different parameters measured with TMS, the latency of the MEP has proven to be the most reliable and useful. In addition, MEP or motor threshold and the cSP may be useful as measures of cortical excitability.

Motor threshold is defined as the stimulus intensity at which the response probability is 50% and is usually measured in muscles at rest (resting motor threshold, RMT). RMT is thought to reflect neuronal membrane excitability because it is increased by drugs that block voltage gated sodium channels (Ziemann et al; 1996b; Chen *et al.*, 1997a), and is also inversely related to the strength of the corticospinal projection, being lower in intrinsic hand muscles than in the proximal upper limb, lower limb or axial muscles. A range of alternative methods for threshold estimation have been proposed but owing to the extent of intrasubject and intersubject variability, the clinical utility of RMT has been called into question (Wassermann, 2002).

Cortical silent period (cSP) refers to the brief suppression of voluntary contraction in a contralateral target muscle following a single cortical stimulus during sustained contraction. It is seen as a period of post-stimulus EMG silence on the MEP trace. The silent period includes cortical and spinal mechanisms and appears to be supported in part by mechanisms independent of those

generating the motor evoked potential that rely predominantly on GABA-B neurotransmitter activity (Chen *et al.*, 2008).

As a marker of disease, cSP has several weaknesses. Interindividual variability of cSP is high (Orth & Rothwell, 2004), measurements of cSP are only comparable between subjects if acquired at a stimulus intensity defined relative to threshold, and cSP might no longer be reliable once threshold is affected by a disease process (Attarian et al., 2005). In addition, cSP has been shown to be abnormal in a wide range of neurological conditions including non-motor conditions (Chen *et al.*, 2008), implying poor specificity and limiting its diagnostic use in conditions involving the motor system.

Threshold tracking TMS (TTTMS) methodologies have recently been adopted by applying ppTMS paradigms, whereby the stimulus intensity for a fixed MEP amplitude (0.2 mV ± 20%) is tracked by a test stimulus. TTTMS constitutes a refinement of SICI and has been reported to discriminate reliably between groups of normal subjects, patients with MND and patients with MND mimic disorders even in the presence of a comparable degree of LMN dysfunction, thus holding great promise as an ALS biomarker (Vucic et al., 2013, 2018; Vucic & Kiernan, 2017).

6.2.9 WHAT IS CMCT AND HOW IS IT CALCULATED?

MEP latency has both a central and peripheral component. Clinically, we measure central motor conduction time (CMCT), which is an estimate of conduction time of corticospinal fibres between motor cortex and spinal motor neurons. It is calculated by subtracting the spinal motor neuron to muscle latency known as peripheral motor conduction time (PMCT) from the cortex to muscle latency [CMCT= MEP latency – PMCT].

PMCT is measured by two main methods, either using the F wave method (PMCT-F) or by magnetic stimulation of the spinal roots (PMCT-M).

F-waves are elicited by conventional electrical stimulation of a peripheral nerve, taking the shortest latency of 20 recordings. PMCT-F is calculated as:

$$\text{PMCT-F} = \frac{(F + M - 1)}{2}$$

where F is the minimal F-wave latency, M the latency of the compound muscle action potential or M-wave (**Fig 15 C,D**) and 1 (in millisecond) is the estimated turnaround time for antidromic activation of the spinal motor neurons (Rossini et al., 1994b; (K. R. Mills & Murray, 1986). If the point of stimulation is moved along the length of the nerve, there are equal but opposite changes in the latencies of M-waves and F-waves; the sum of their latencies remains constant (Kimura J, 2001). I recorded F-waves on motor nerve conduction studies of all the subjects at baseline; NCS were not repeated for each subject thereafter.

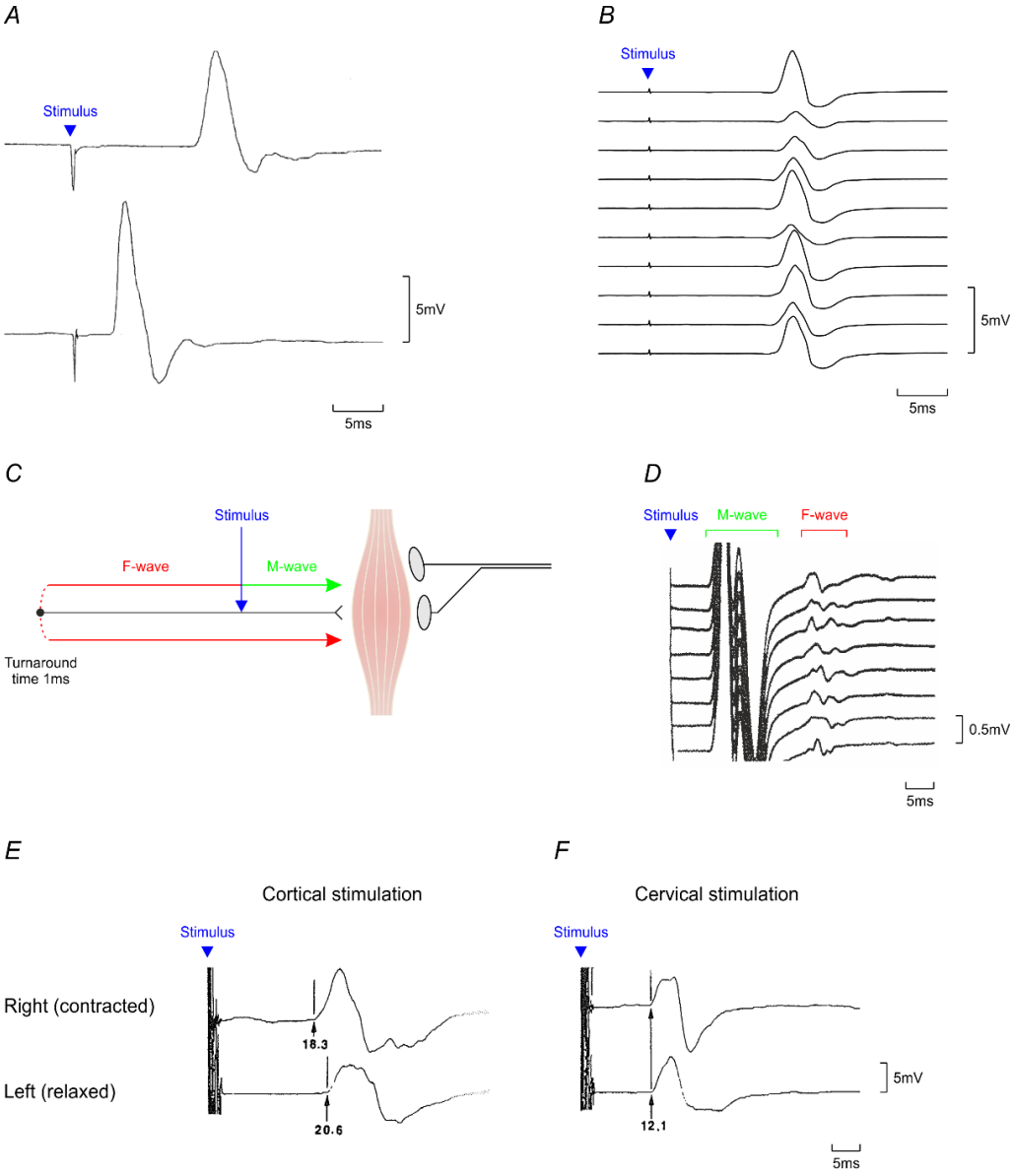


Figure 15. Magnetic and electrical stimulation.

MEPs recorded from abductor digiti minimi (ADM) after magnetic stimulation to the contralateral M1 (A, top) and to the ulnar nerve at the elbow (A, bottom; adapted from Barker *et al.*, 1985).

Variability of MEPs recorded from first dorsal interosseous (FDI) after magnetic stimulation to the contralateral M1 under constant experimental conditions (B).
Generation of M-wave and F-wave after electrical stimulation of a peripheral motor nerve (C).
Consecutive recordings from the thenar muscles showing M-waves and F-waves after ulnar nerve stimulation at the elbow (D; adapted from Kimura, 2001).
MEPs evoked by magnetic stimulation over the cervical spine and over the contralateral M1, during voluntary contraction and at rest.
Voluntary contraction decreases the latency of cortical but not spinal MEPs (E, F; adapted from Kimura, 2001). Figure cited from PhD thesis of Dr S R Jaiser (https://theses.ncl.ac.uk/jspui/handle/10443/2397).

(See Chapter 6, Methods – Electrophysiology). Another major limitation of the F-wave method is that it is only applicable for nerves with recordable F waves i.e. most appropriate for distal muscles in hand and foot and cannot be used for proximal muscles which I tested. I also noted that the younger participants found electrical stimuli on hands more uncomfortable than magnetic stimulation over cervical spine.

PMCT-M is calculated by magnetic stimulation of the lower motor neurons at the level of the exit foramina directly, by placing the coil over cervical and lumbar spine (Chokroverty et al., 1993; Ugawa et al., 1989). This method does not include the conduction time from the anterior horn cell to the intervertebral foramen (intraspinal part), falsely increasing the CMCT as it includes the proximal segment of the spinal nerve root. However, most laboratories use the magnetic stimulation method as it is better tolerated and avoids the use of a further (and more uncomfortable) stimulation modality.

CMCT is the most important MEP parameter in clinical practice and normative values for a large number of muscles in various conditions have been published. There is consensus that CMCT to lower limb muscles is strongly correlated with height but not significantly to upper limb muscles (Furby et al., 1992; Rossini et al., 1987; Toleikis et al., 1991). This may be because of the shorter proximal root segments in the cervical spine, or because height relates less strongly to the length of the CST to the upper limb (Chu, 1989; Claus, 1990).

6.2.10 RECORDING MEPs

Every effort was made to maintain subjects at a constant level of alertness, and all assessments were carried out on the dominant side. Subjects were seated in a comfortable chair with their arm resting on a cushion. Surface EMG was recorded from first dorsal interosseous (FDI), flexor digitorum superficialis (FDS) and extensor digitorum communis (EDC) in the upper limb, and extensor digitorum brevis (EDB), tibialis anterior (TA) and medial gastrocnemius (MG) in the lower limb. Adhesive electrodes (Bio-Logic M0476; Natus Medical, Mundelein, IL) were placed in a belly-tendon montage over the intrinsic muscles of the hand or foot; for the long muscles of the forearm or calf, the electrodes were placed 4cm apart, one third along the muscle from its proximal origin. Signals were amplified, band-pass filtered (30Hz-2kHz; Digitimer D360, Digitimer, Welwyn Garden City, UK) and digitised at 5kHz (Micro1401, Cambridge Electronic Devices, Cambridge, UK).

6.2.11 STIMULATION

Magnetic stimulation was delivered using a Magstim 200 stimulator (Magstim Company, Whitland, UK) at a frequency of 0.2Hz. For upper limb cortical motor evoked potentials (MEPs), a circular coil (13cm outer diameter) was held over the vertex, with its orientation optimised for stimulation of the dominant hemisphere (A side up: left hemisphere, B side up: right hemisphere) **(Fig 12)**. For lower limb cortical MEPs, a double cone coil was used in an analogous manner (posterior coil current: left hemisphere, anterior coil current: right

hemisphere). Stimulation intensity was set at 20% of maximum stimulator output above the resting motor threshold as defined by the Rossini-Rothwell method (Rossini *et al.*, 1994).

Cortical MEPs are facilitated, and their onset latencies minimised by a weak background contraction of the target muscle, with no requirement for strictly controlling the force of the contraction (Chen *et al.*, 2008; Kimura, 2001). Ten MEPs were recorded in the upper limb during opposition of index finger and thumb, and in the lower limb during either dorsiflexion (EDB, TA) or plantarflexion (MG) of ankle and toes. Upper and lower limb root MEPs were recorded at rest with the circular coil centred over the spinous processes of C7 and L1 **(Fig 13)**. The range of stimulation intensities used was 35–80% and 40–100% for cortical MEPs of the upper and lower limbs respectively, and 40–80% and 40–90% for corresponding root MEPs. The wide range of stimulation intensities reflects the range of resting motor thresholds encountered in this population. Spinal root MEPs were usually easily evoked at rest with a circular coil at 50% MSO. However, some patients could not tolerate stimulation at this strength. In this situation, stimulation started at 20% MSO and was increased until the maximum tolerated intensity was identified. Usually spinal magnetic root MEPs can be elicited with a single stimulus. However, when TMS at strengths less than 50% are used, or when there is background EMG activity (e.g. patients with spasticity or dystonia) averaging of 5-10 magnetic root MEPs is required.

6.3 NERVE CONDUCTION STUDIES (NCS)

All the patients were investigated with nerve conduction studies in upper and lower limbs (either left or right), using a standard clinical protocol working with single use adhesive electrodes (Ambu Neuroline 710) on Dantec EMG equipment. These involved stimulation of peripheral nerves to evaluate the function of motor and sensory nerve fibres, repeated at next visit if SNAPs were elicited on the prior visit, even from one upper limb nerve. Peak to peak amplitudes as well as conduction velocities were compared with the normative data for assessment.

6.4 SOMATOSENSORY EVOKED POTENTIALS (SEPS)

SEPs were performed using a standard clinical data acquisition system (Sierra Summit**;** Cadwell, Kennewick, USA) according to the published guidelines, American Clinical Neurophysiology Society, 2006 ("Guideline 9D: Guidelines on Short-Latency Somatosensory Evoked Potentials," 2006) as also used in our routine Neurophysiology clinics.

SEPs were recorded with surface disc electrodes (9 mm diameter) positioned at Erb's point, C7 vertebral spinous process, on the scalp- 2 cm posterior to the C3 (for right upper limb SEPs) and C4 (for left upper limb SEPs) locations. Each cervical electrode was referenced to Fz (10–20 system).

Lower limb SEPs were recorded using electrodes positioned 2 cm anterior to Cz and 2 cm posterior to C, each referenced to Oz. Median nerve was stimulated

at the wrist for upper limb study and the posterior tibial nerve at the ankle for lower limbs. Recordings were made from all 4 limbs. The stimulus current was set at a level above motor threshold such that there was a reliable and visible movement of the thumb or toes.

Cortical traces from 500 stimuli were averaged (after stimulation of each median and tibial nerve in turn). SEP peaks were labelled according to standard nomenclature for upper ((N9, N11, N13, P16, N20 and P25) and lower limbs (N0, P1, N1, P2, N2). Latencies and amplitudes were documented and interpreted relative to the normative data from the lab. SEPs were considered abnormal where latencies were significantly prolonged compared to the control population mean. The N20 was therefore abnormal if the latency was above 22.1 ms (mean + 3SD) from the median nerve at the wrist, and the P1 latency was abnormal if it was greater than 46.7 ms (posterior tibial nerve at ankle; mean + 3SD).

6.5 FRIEDREICH ATAXIA RATING SCALE (FARS)

We used FARS questionnaire at the baseline visit and to quantify disease progression every 6 months. As stated before, FARS questionnaire includes characterisation of neurological deficits, a functional staging and activities of daily living (ADL) assessment. FARSm version had not been devised by the time of my study, so original FARS scale was used, with peripheral nervous system and muscle weakness items included.

I performed a brief neurological examination of all patients at the first visit and documented any important FRDA related features such as level of mobility, muscle weakness, speech disturbance, scoliosis etc. in the clinical research folder. Their clinical records were accessed via their General Practitioners or neurologists, for cardiac involvement, presence of diabetes, ophthalmological abnormalities, whether ataxia was the main presenting symptom and for peripheral neuropathy.

Total FARS score (excluding 9-HPT and PATA rate) was selected as the main variable and correlation analysis carried out against age of onset, duration of disease, GAA repeat expansion size, as well as MEPs and BIMC.

Few patients were selected for further analysis – proteomic profiling of skin fibroblasts as well as to study comparisons between severity of clinical findings in relation to the number of GAA trinucleotide repeat expansions. Based on the spectrum of clinical dysfunction in addition to the results of

electrophysiological investigations performed at the baseline, a subgroup of 10 patients were identified from the cohort. These were categorised into 2 sets: one comprising 5 clinically most severely affected patients and the other one the 5 least affected **(Table 49, Appendix Section B)**. The average disease duration was recorded to be 26 years for the clinically worse ones versus 9.6 years for the mildly affected participants. Unsurprisingly, the former set of patients were all confined to wheelchair with marked truncal ataxia, whilst those in the latter were still ambulant, either independently or using linked arms for support.

The preliminary results from proteomics using mass spectrometry-based techniques on these 10 patients has shown some promising findings **(Table 50**, **Appendix Section B)**.

6.6 GAA TRINUCLEOTIDE REPEAT EXPANSIONS

As mentioned in the study parameters section, various studies have concluded that the size of the GAA repeat length in each allele is important in predicting the age of onset and some features of FRDA. Normal alleles contain between 6 and 34 uninterrupted GAA repeats whereas expanded alleles contain 67-1700 repeats. Thus, FRDA is so far unique among trinucleotide repeat disorders in that it is autosomal recessive, the repeat is intronic, and it is the only disease known to be the result of expansion of a GAA trinucleotide repeat.

The smaller of the two alleles is more important in this regard. The smaller allele size inversely correlates with the amount of residual frataxin present in lymphoblasts, providing a biological explanation for the observed genotype-phenotype correlation with this allele (Campuzano et el; 1997). As for other trinucleotide repeat disorders, repeat sizes cannot be used accurately to predict prognosis in a person but mean allele length is also known to be significantly higher in patients with diabetes and in those with cardiomyopathy.

All the 29 FRDA patients had their DNA samples taken and tested for GAA trinucleotide repeat expansions with long-range flanking PCR on agarose gel electrophoresis, at the Neurogenetics lab at the National Hospital for Neurology & Neurosurgery, London. The gene was not sequenced usually to look for other mutations, only if there was a heterozygous expansion found but it was not so in our study.

Approximately 2% of the mutations described in FRDA patients are point mutations (Cossée et al., 1997b; V. et al., 1996). All patients carrying point mutations are compound heterozygotes with an expanded GAA repeat on the other allele.

6.7 FRATAXIN LEVELS

The levels of serum frataxin protein have been looked at in various studies to develop laboratory-based assays that reflect the benefit of a particular treatment. We used a rapid, non-invasive lateral-flow immunoassay (Luminex xMAP-based immunoassay) by collaborating with researchers at The Children's Hospital of Philadelphia (CHOP), USA. This technique is shown to accurately measure picogram levels of frataxin protein and to distinguish lymphoblastoid cells from FRDA carriers, patients, and controls (Deutsch et al., 2010).

The initial sample processing involved extracting a buffy coat specimen from the blood sample. It has been confirmed in previous studies that peripheral blood mononuclear cells (PBMCs) and buccal swabs have frataxin levels equivalent to those of whole blood. PBMCs are easier to isolate. Buffy coat is that fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets (creamy layer between plasma and column of packed red blood cells) following density gradient centrifugation of the blood. It is named after its "buff-like" colour. Cells in this layer are denser than plasma components but less dense than the red blood cells at the bottom of the tube. I followed the 'Plasma and PBMC Harvesting standard operating procedures (SOP)' at the Newcastle MRC Centre Biobank, IGM.

For the first 16 months of the study, buffy coat processing was kindly carried out by an experienced lab technician, Rafiqul Hussain, from genomics core facility at the Institute of Genetic Medicine in Newcastle upon Tyne. I was

trained by him and finally signed off as competent to process the samples independently thereafter. Prior to that, I had to undergo BioCOSHH risk assessment as well as lab safety induction certifying that I was competent to safely perform unsupervised all aspects of the work, including use of microbiological safety cabinet (MSC-2) and emergency procedures if need be. As per protocol, copies of all risk assessments and standard operating procedures were kept in the laboratory and reviewed regularly and immediately if there were any changes to the risks or the nature of the work. For the rest of the study duration of 18 months, I continued to extract and store the PBMC samples.

6.7.1 METHOD OF BUFFY COAT PREPARATION

The steps detailing the method have been provided in Appendix, Section A (SOP for PBMC Separation by Ficoll Gradients). The following principle is applied for the procedure - anticoagulant-treated blood is layered on the Ficoll-Paque PLUS (Ficoll-Paque Plus: Fisher, 45-001-750) solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. Dulbecco's Phosphate-Buffered Saline (DPBS, Dulbecco's PBS w/o Ca2+/Mg2+: Invitrogen, 14190-250) is a balanced salt solution primarily used to maintain cells in a physiological pH range and providing them with salt ions for osmoregulation. In order to maintain the vitality of cell lines when they have to be preserved for long-term storage, they are frozen down. The best method for cryopreserving cultured

cells is storing them in liquid nitrogen in complete medium in the presence of a cryoprotective agent such as dimethyl sulfoxide (DMSO). The commonly used freezing media comprises of 10% DMSO with 90% fetal calf serum(FCS).

All the steps were performed in a dedicated tissue culture hood using aseptic technique, following safe laboratory procedures for handling bio-hazardous material. Freezing media was prepared freshly and stored chilled at 4° C. The blood sample was diluted with DPBS and mixed with Ficoll-Paque reagent, by gently overlaying the Ficoll with blood. The blood sample was then centrifuged for a total of 30 minutes at the RCF of 400 g (Centrifuge settings: 400 x g for 30 minutes with slow acceleration and no brake (to stop gradually ~ 30 mins). (Eppendorf Centrifuge 5810, **Fig. 16 A**). Most of the top layer (plasma) was removed leaving a small amount to help distinguish PBMC (buffy coat) layer using a 10 ml pipette **(Fig. 16 B)**. Following this, three cycles of washes were performed on the sample using DPBS, at Centrifuge settings of 100 x g for 10 minutes - brake on and high acceleration (max ~ 9). Finally, the DPBS was aspirated off taking care not to disturb the pellet at the bottom of the tube **(Fig. 16 C)**. The distilled pellet was mixed with freezing media and transferred to a cryovial, to be stored at -20 °C for short term, and then transferred to -80 °C for long term storage **(Fig. 16 D)**.

Modification in Protocol

The lab scientist processing Frataxin samples at CHOP suggested to modify the protocol after measuring Frataxin levels in initial samples. This was in order to

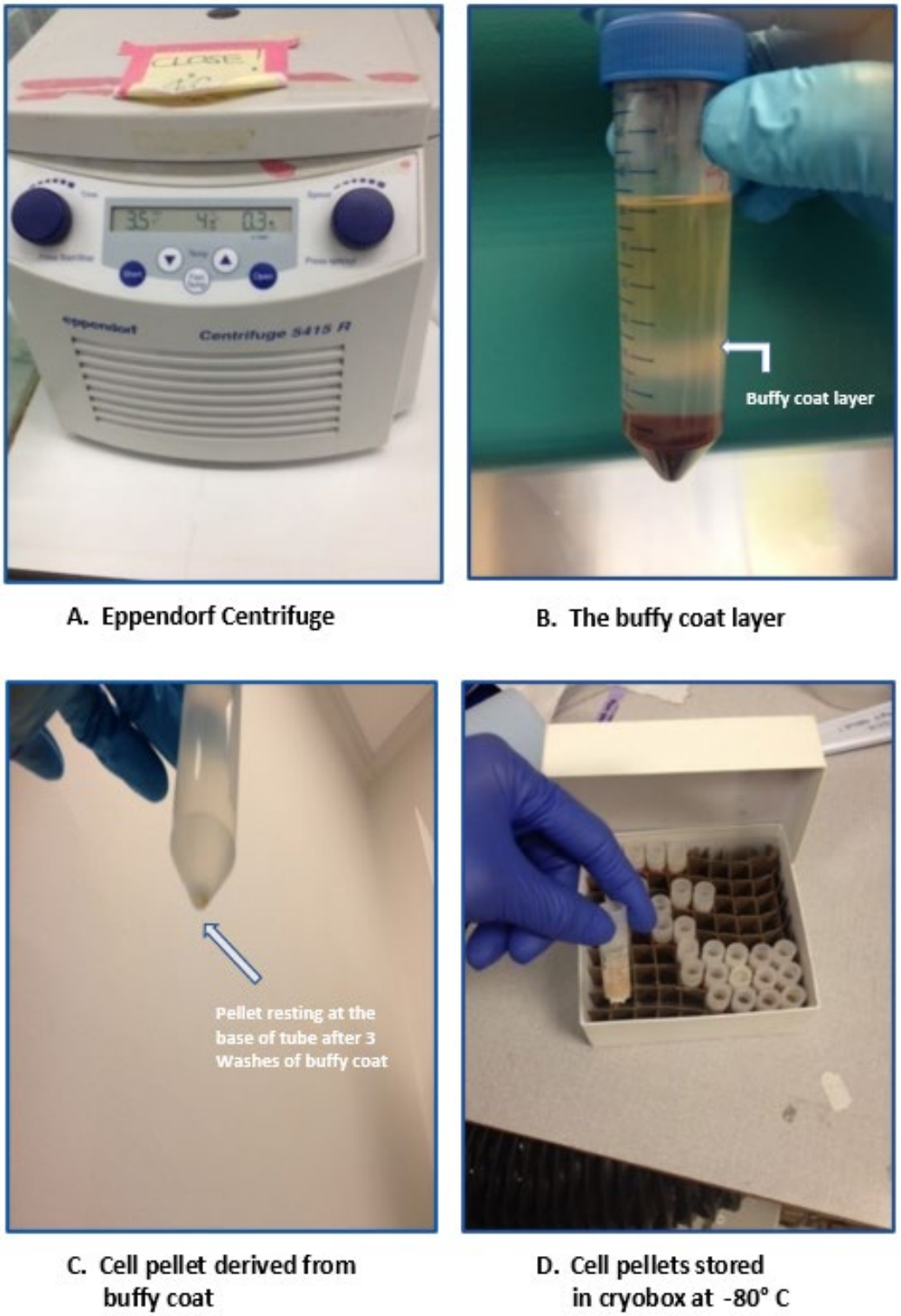
lyse the cell membranes to release Frataxin and make the process easier, as well the fact that extraction buffers improve the stability of protein molecules as they are subjected to various forces during lysis and extraction. It also facilitates the isolation of target proteins from other non-soluble cell components.

Instead of freezing the sample in freezing medium, (after step 24 in the SOPs) in the original protocol, the procedure involved resuspending 10-20 million PBMCs in 100 ul extraction buffer (Pierce RIPA buffer 100 ml) after discarding the supernatant from the pellet. The cells were dispersed by vigorous pipetting, (otherwise cells wouldn't be lysed if they clump together). The cells needed to be counted using a haemocytometer. Following this, the cells and extraction buffer were spun and mixed at maximum speed (13,000rpm) at 4° C, followed by 45 min to 1 hour shaking on orbital shaker in a cold room, and the supernatant stored at - 80 °C. The precipitates such as cell walls obtained after cell lysis were to be discarded. The sample left would be about 900 ul approximately.

I believe that amending the technique mid-way between visit 2 and 3 was not ideal. There was a negative impact on the results (for the samples sent thereafter), due to overtime added to processing samples at end of the day, difficulties in finding the right equipment timely at the lab, as well as change in the sample storage procedures.

For each patient visit, the samples were collected, processed and stored at - 80° C at the mitochondrial lab, and shipped to CHOP at repeated intervals. Great care was taken in ensuring the samples were delivered safely over adequate amount of dry ice to last and maintain a tight temperature control during the shipment (FedEx).

In total, I collected 6 control whole blood samples for frataxin. These were collected in 2 batches of 3 samples each at a 6 month interval. The 3rd control was not available for the second batch and hence a new 6th control was tested. Frataxin protein levels from both controls and FRDA patients were measured with lateral flow immunoassay in nanograms of frataxin per millilitre of blood.



A. Eppendorf Centrifuge

B. The buffy coat layer

C. Cell pellet derived from buffy coat

D. Cell pellets stored in cryobox at -80° C

Figure 16. Extraction of buffy coat - peripheral blood mononuclear cells.

6.8 STATISTICAL ANALYSIS

I have used descriptive statistics, where possible, to summarise the phenotypic characters of the FRDA study group, including their geographical distribution within the UK, to highlight the target population. The datasets of various parameters have been defined with both measures of central tendency and measures of variability.

The SPSS statistical package for ANOVA of repeated measure was applied to assess differences between measurements recorded over time but given the small final sample, there were limitations to these tests. When paired samples were compared, Student's t-test was applied, where data was normally distributed. Where multiple comparisons were used, data was Bonferroni corrected.

I also relied on univariate regression and correlation analyses (Spearman's correlation) to investigate the relationship between electrophysiological tests (MEPs and BIMC) as dependent variables, with progression in clinical features (FARS score) and gene repeat sizes as independent variables, over time. *P* values less than 0.05 were considered significant.

7 RESULTS

7.1 DEMOGRAPHICS OF THE STUDY GROUP

The initial efforts at recruitment targeted the local population of the North-East and participants came from Newcastle-upon-Tyne, Gateshead, Durham, Hartlepool, Penrith, Carlisle and non-urban areas of Cumbria. For the next round of recruitment, with the assistance of Ataxia UK, we recruited patients from as far as the Isle of Man, Dundee, Sheffield, Manchester, Newport (Wales), Sidmouth, Exeter, Stourport on Severn and Southampton, thus representing a wider target population within the UK. All patients were of white Caucasian ethnicity, except one, who was South-East Asian.

Demographics	**n=29**	**Range**	**Median**	**Mean**	**SD**
Males	11				
Females	18				
Right handed patients	25				
Left handed patients	4				
Homozygous for GAA expansion	29				
Age (yrs)		9 - 52	31	29	12
Age of onset (yrs)		4 - 40	11	13	7.29
Disease duration (yrs)		2 - 36	13	16.6	11.1
Children (<18 yrs)	6	9 - 18	13.5	13.7	

Table 2. Demographics of the study group

7.2 FRDA PHENOTYPE

None of the participants had a history of other neurological disorders, except one patient who had previously had a history of epilepsy but was no longer on anti-convulsant drugs. Over half (60 %) were wheelchair dependent and 40% were either independently mobile or using linked arms or furniture support to move about. Over one third of patients (36%) had significant kyphoscoliosis, of which 6 patients (20%) had corrective surgery performed. Two of the patients underwent scoliosis surgery between visits 3 and 4, whereas another patient had a prolonged in-patient stay due to discectomy leading to post-op soft tissue infection during the study.

Five patients (~ 16%) had severe limb and facial weakness, with marked truncal ataxia. A similar number had cardiomyopathy (recorded as severe in two), whilst three had a history of diabetes. Three patients (two with cardiomyopathy) had metallic devices in-situ:, a cardiac pacemaker; a defibrillator; and a cochlear implant **(Table 3)**.

Most patients had sensory neuropathy in the lower limbs and gait ataxia. Speech and swallowing difficulties, issues with dexterity, muscle spasms, cold lower limbs and fatigue were other common features. One patient unfortunately died during the study as a consequence of severe complications of FRDA. He was however excluded after visit 1 due to very advanced state.

Additional clinical features of FRDA	Number of patients affected	
Mobilized independently / with support/rollator frame	12	
Wheelchair users	18	
Kyphoscoliosis	11	
Had corrective surgery for kyphoscoliosis	6	2 had surgery during the study, 1 patient was on a waiting list
Optic neuropathy	4	1 patient was visually impaired
Cardiomyopathy	5	Severe in 2; one of those patients had paroxysmal AF
Diabetes	3	2 had insulin pumps, 1 had diabetes detected during pregnancy
Marked restrictive lung defect	1	On nocturnal ventilatory support
Suprapubic catheter for overactive bladder	1	
Cardiac defibrillator	1	
Cardiac pacemaker	1	
Cochlear implant	1	

Table 3. FRDA phenotypes in the study cohort

7.3 HEALTHY CONTROLS

Serum Frataxin

For Frataxin levels, 6 blood samples were collected from controls in total, who comprised 4 males with an age range of 29 to 53 years. Three blood tests were taken twice, 9 months apart. For the repeat blood tests, the first 2 controls were the same as on first sampling, but the 3rd control was different **(Table 4)**. The normal range for the levels of frataxin measured in healthy controls was provided by the laboratory at CHOP who tested the samples collected as part of our study. The only reason I sent samples from healthy controls was to check that the extraction method I used was giving sensible results and checking repeatability; it was not for generating a control dataset.

May 2016	Frataxin(pg/25ul)
ctrl1	250.96
ctrl2	77
ctrl3	118
Patient 1	49
Patient 2	476
Patient 3	52

Feb 2017 Frataxin sample (pg/25ul)	
Ctrl 1	78.92
Ctrl 2	56.62
Ctrl 3*	60.12

*Control 3 is different in second set of sample, but 1st and 2nd controls are same.

Table 2. Serum Frataxin levels displayed for controls and selected patients, analysed in one batch. Repeat samples for controls 9 months apart.

It has been documented that patients have frataxin levels in peripheral tissues that range from 2 to 30 % of control levels (Deutsch et al., 2010; Nachbauer et al., 2011; Saccà et al., 2013). I did not find this to be the case in my study. The data

below showed variable levels from patients and controls which were sent to CHOP for testing in the same batch. For the second sampling 9 months apart, there was an unexpected reduction in levels of control 1. Besides, the levels between controls and patients tend to overlap.

TMS MEPs

I assessed 5 healthy controls for MEPs, and assessments were repeated once after 12 months. The controls were age-matched (age range 5 - 42 years, 3 male, 2 female) but were a much smaller sample **(Table 5)**. Again, the objective here was not to establish a normal range but to confirm that using my technique there was no significant change in CMCT over a 6 month interval in a cohort of healthy controls. For BIMC, I used controls from the existing Baker lab database (n=47, age range = 22- 56 years) **(Section 7.9).**

TMS- MEP Controls	**Avg CMCT latency** 0 month (ms)	**Avg CMCT latency** 12 month
1	5.7	5.8
2	5.6	5.7
3	5.6	5.6
4	6.9	6.5
5	6.5	6.8

Table 3. MEP controls tested 12 months apart.

The CMCT latencies were averaged across all 3 muscles for individual controls for the 2 time points. The range of latency was between 4.1 to 7.5 ms (normal is 6.7 ± 1.2 ms) in upper limb muscles.* The mean of % change in averaged CMCTs was compared to the mean change in healthy controls (Section 7.7) and found to be significantly different.

**Normal values from Eisen & Shtybel (1990).* Please note that these normal values were derived from 150 control subjects 14 to 85 years.

7.4 FARS

All the 29 participants included had at least 1 visit with a complete neurological, FARS disability staging and ADL items available. The single baseline FARS ratings were analysed as a function of disease duration, age of onset and GAA repeat lengths. Further analysis including 77 data points (29+17+14+14) for 4 visits, revealed that FARS scores ranged from 11 to 137 (0 – 159), with a mean score of 67.6, median score of 60.5 and standard deviation of 33.2. The majority of these individuals were in the early-onset group, i.e., first symptoms of FRDA occurred before age 15 years (n=21, 72.4%), representing the genetically most severely affected individuals as evident by the greater GAA1 (mean 771.90; SD 216.76) and GAA2 (mean 944.28; SD 240.28) repeat sizes in this group, as shown previously (Metz et al., 2013). The mean age of symptom onset was 9.6 years (SD 3.0), and disease duration was 17.4 years (SD 12.3) in this group.

To pass the assumption of homogeneity for ANOVA testing for pairwise comparisons, data were selected from 14 patients for each visit; these were the patients who made it to the fourth and final data set. The estimated marginal means adjusts for any covariates, and the profile plot showed that the means were increasing across time **(Figure 17, Table 6)**. The difference in mean was maximum between baseline and study visit 4; raised by a count of 10 scores. For within the 6 monthly change, maximum change in mean scores was between visits 1 and 2 (addition of ~ 4 ratings) and tapered down to ~ 3

between visits 2 and 3, and ~2 between the penultimate and 4th visit.

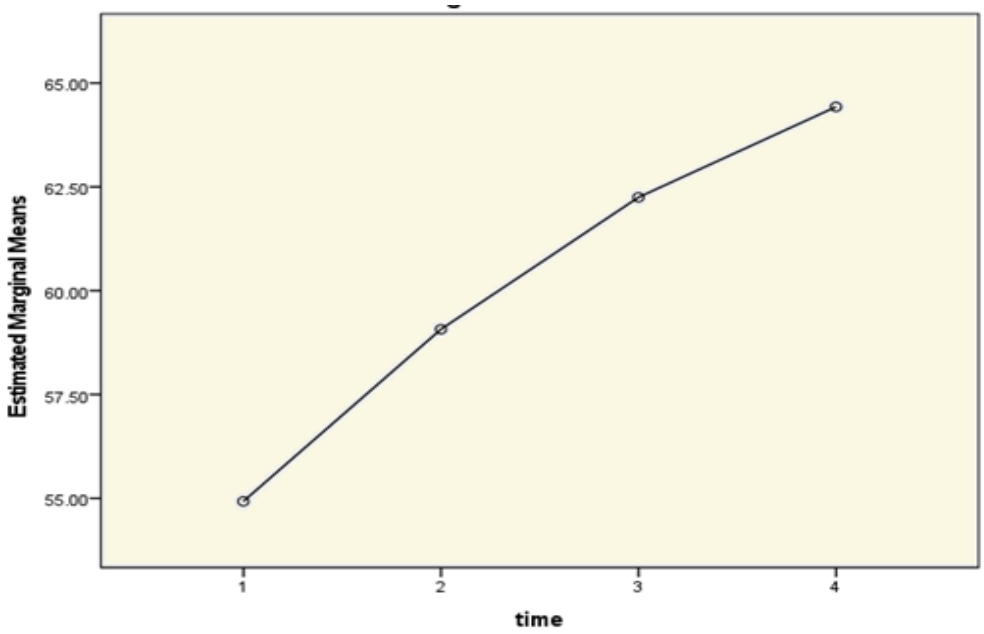


***Figure 17. Plot of estimated marginal means of FARS* - 4 datasets analysed for n=14 patients.**

The consistent increase in mean scores at 6 monthly intervals shows that FARS scale captured the features of increasing disability over the baseline.

Descriptive Statistics	Mean	Std Error	Std. Deviation	N
Baseline	54.9	8.6	32.3	14
Six mths	59.1	8.6	32.0	14
Twelve mths	62.3	8.5	31.9	14
Eighteen mths	64.4	8.7	32.5	14

Table 6. Summary of estimated marginal means of FARS score with time.

The mean ratings increased by a value of ~4 within first 6 months (54.9 to 59.1) and by ~10 in 18 months (i.e. baseline to visit 4 (54.9 to 64.4)).

The change depicted over 6 months was significant between visit 1 and next 3 respective visits and over 12 months between visits 2 and 4. But the test failed to report significant change between visits 2 and 3, as well as between 3 and 4 (Fig 18 followed by summary in Table 7).

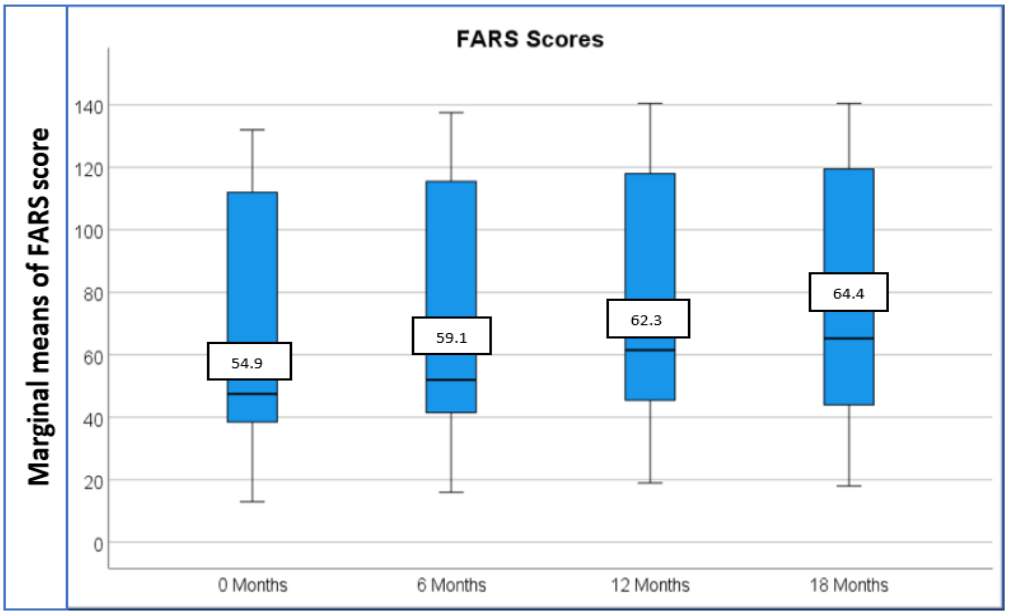


Figure 18. Comparison of mean FARS scores over 4 visits.

Significant change determined between mean scores of visit 1 and 2 (p= 0.001), 1 and 3 (p=0.007), 1 and 4 (0.003) and 2 and 4 (p=0.012). No significant change between study visits 2 and 3 (p =0.95), and 3 and 4 (p=1.0).

Measure FARS Pairwise comparisons

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
1	2	-4.143*	.808	**.001**	-6.652	-1.634
	3	-7.321*	1.771	**.007**	-12.824	-1.819
	4	-9.500*	2.038	**.003**	-15.832	-3.168
2	1	4.143*	.808	**.001**	1.634	6.652
	3	-3.179	1.147	.095	-6.741	.384
	4	-5.357*	1.389	**.012**	-9.673	-1.041
3	1	7.321*	1.771	**.007**	1.819	12.824
	2	3.179	1.147	.095	-.384	6.741
	4	-2.179	1.562	1.000	-7.030	2.673
4	1	9.500*	2.038	**.003**	3.168	15.832
	2	5.357*	1.389	**.012**	1.041	9.673
	3	2.179	1.562	1.000	-2.673	7.030

Based on estimated marginal means

* The mean difference is significant at the .05 level. b. Adjustment for multiple comparisons: Bonferroni.

Table 7. Summary of pairwise comparisons - significant change in 12 months but not within consecutive 6 months except in the first 6 months of study.

Factors effecting FARS ratings

There was an unexpected turn in outcomes between visits 2, 3 and 4. At the first study visit, I identified three patients between the ages of 14 and 33 (who were mobile independently or with minor support) undergoing physiotherapy either in the community or at home. Between the 2nd and 3rd visits and the 3rd and 4th visits, they showed an improvement in upright mobility assessments in the domains of stance feet apart/ feet together /stance on dominant foot. For example, moving from one category (being able to stand for more than 30 sec but less than 45 sec) to the next category (>45 sec to <1min) would drop the score by 1 point. The three trials were timed and averaged as instructed and showed a consistent gain in stability. This led to either no change in their final scores or even a negative value, between visits 2 and 3. The results were testimony to the beneficial effects of rigorous exercise in FRDA and it would have been unethical to question this practice for the purpose of research. Also, it was not always possible for the patients to have the same footwear/ankle and foot orthoses on for the serial measurements, mainly due to change in season and might have introduced slight variation in their mobility scores.

Another 2 patients, both aged 13, were ambulant with support inside the house and needed a wheelchair whilst outside. The condition progressed in both, who then became wheelchair dependent by visit 3 and required scoliosis correction surgery. There was a significant spike in scores for both at visit 3 and following surgery, at visit 4; and these alterations in scores also impacted the analysis.

To try and increase the power, the same test was run on 17 patients from the first 3 study visits, with very similar results (**Fig 19**; **summary tables 8** and **9**).

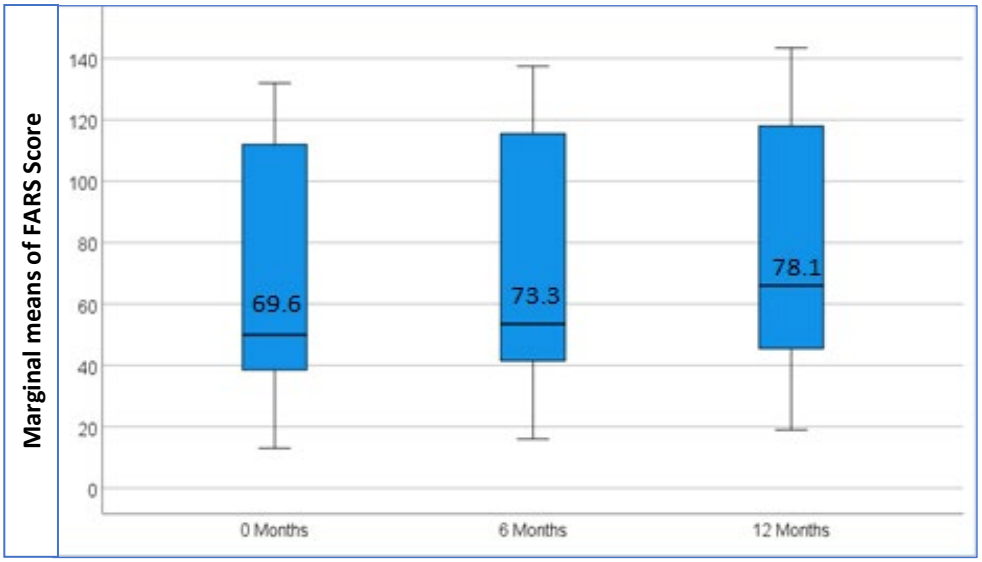


Figure 19. Change in marginal mean of FARS score over first 3 visits.

The change in mean scores was significant between visit 1 and 2 (p< 0.001) and visit 1 and 3 (p=0.005), but not so between study visits 2 and 3 (p=0.65).

General Linear Model- Descriptive Statistics

	Mean	Std. Deviation	N
0 Months	69.65	41.498	17
6 Months	73.26	41.636	17
12 Months	78.18	41.815	17

Measure: FARS Score over three visits (0,6,12 months)

			95% Confidence Interval	
Visits	Mean	Std. Error	Lower Bound	Upper Bound
0	69.647	10.065	48.311	90.983
1	73.265	10.098	51.857	94.672
2	78.176	10.142	56.677	99.676

Table 8. Summary of estimated marginal means with time (3 visits, n=17).

The mean ratings increased by a value of ~ 3.7 within the first 6 months (69.6 to 73.3), by ~ 5 counts between visit 2 and 3 and by ~ 8.5 counts over 18 mths.

(I) Visits	(J) Visits	Mean Difference (I-J)	Std. Error	Sig. [a]	95% Confidence Interval for Difference [a] Lower Bound	Upper Bound
1	2	-3.61*	0.69	**<.001**	-5.47	-1.76
	3	-8.52*	2.27	**.005**	-14.61	-2.44
2	1	3.61*	0.69	**<.001**	1.76	5.47
	3	-4.91	1.92	.065	-10.06	.24
3	1	8.52*	2.27	**.005**	2.44	14.61
	2	4.91	1.92	.065	-.242	10.06

Based on estimated marginal means. a. Adjustment for multiple comparisons: Bonferroni.
*. The mean difference is significant at the .05 level

Table 4 Summary of pairwise comparisons of visits (n=17).

Analogous results to the tests performed with n=14. The changes were significant between visits 1 and 2 and 1 and 3, but not between visits 2 and 3.

FARS scores were assessed against modifiers such as disease duration and age of onset **(Figures 20 & 21)**. The respective chapters show comparisons made with CMCT scores, mean coherence values, GAA repeat length and Frataxin levels. As predicted, scores indicating more severe disease correlated highly significantly with disease duration (p=0.0008) and inversely so with age of onset (p=0.04), when examined in cross section. Hence a younger age of start of symptoms would spell a higher burden of disease. The limited size of the cohort did not allow a detailed analysis, but the effect was very clear just by stratifying patients in 2 groups based on age at onset. The maximal sensitivity of the scale to progression was found in ambulatory patients with onset before age of 15 years, all of whom have less than 20 years of disease duration.

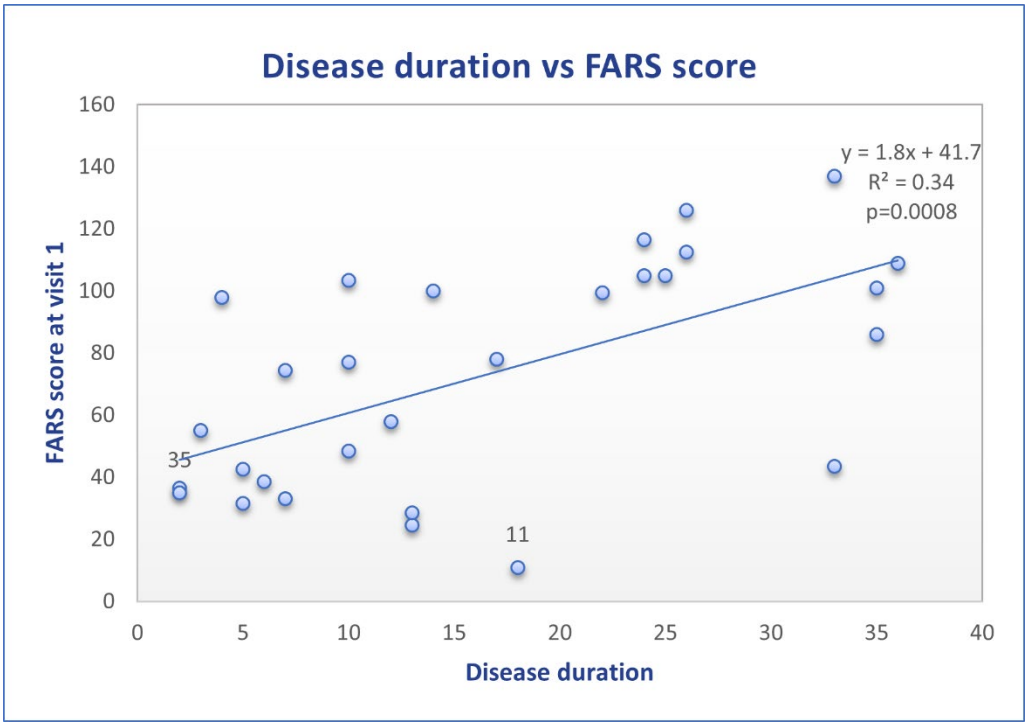


Figure 20. Effect of disease duration on baseline FARS score (R=0.6; p=0.0008).

SUMMARY OUTPUT **FARS vs disease duration**

Regression Statistics	
Multiple R	0.59
R Square	0.35
Adjusted R Square	0.32
Standard Error	29.53
Observations	29

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	12558.75	12558.75	14.40	0.00
Residual	27	23540.28	871.86		
Total	28	36099.03			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	41.75	9.87	4.22	0.0002	21.50	62.01
Disease duration years	1.89	0.49	3.79	**0.0008**	0.87	2.92

Table 10. Summary of effect of disease duration on FARS score.

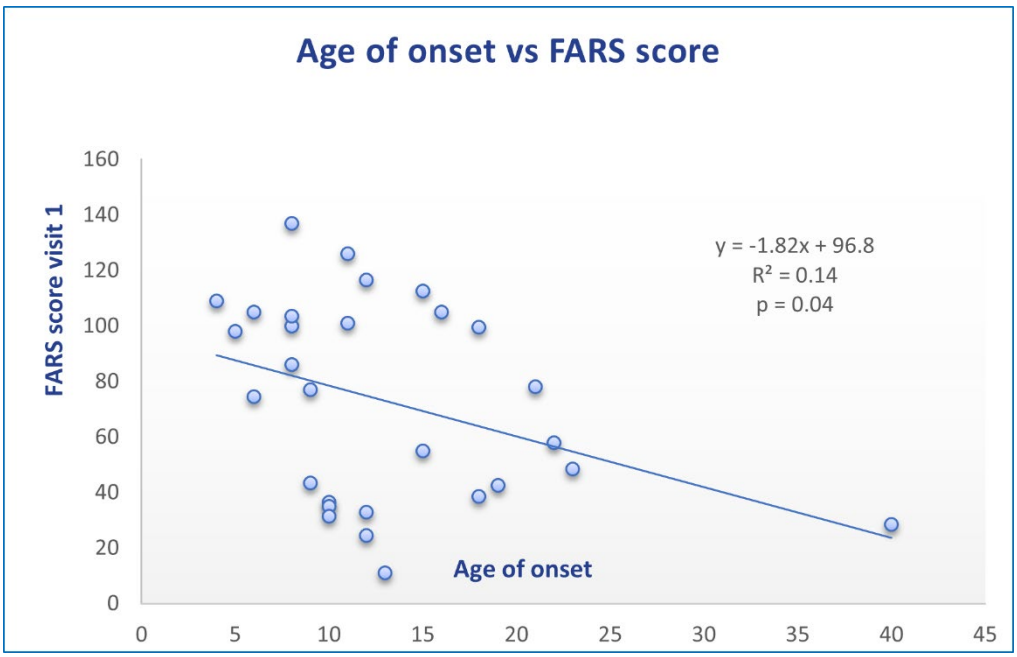


Figure 21. FARS score at baseline with Age of Onset (R=-0.37; p= 0.04).

All the patients had the onset of symptoms before 25 years of age, except for the one who started experiencing symptoms at 40 years of age; classified as LOFA and had a milder disease and lower FARS score.

Age of onset seems to be an overarching predictor of the progression rate, as it reflects the combination of all factors that modify disease severity, such as GAA repeat length and duration of disease. My results showed congruence with several studies that have looked at cross sectional and longitudinal changes, modelling on both age of onset and genetic severity as independent predictors of disease progression. Earlier disease onset is associated with larger numbers of GAA repeats and separating the effects of age and genetic severity is difficult. Progression has been shown to be fastest in younger subjects irrespective of disease duration (Hohenfeld et al., 2022; Patel et al., 2016; Reetz et al., 2015).Loss of ambulation is a critical milestone in the disease course, and early onset FRDA

patients (<15y of age) typically become fully wheelchair dependent after about 11.5y after the onset of first symptoms (Rummey et al., 2020).

As multivariate regression analysis was not performed in my study, it was not possible to separate the confounding effects of subject age, sex and GAA repeat length on the FARS. To add to the overall perspective, the young, still ambulatory participants were undergoing a loss of series of specific functions in a more timely, condensed manner (as quantified by upright stability tasks) whereas the older group showed less substantial change over almost 2 years of assessment.

SUMMARY:

FARS has so far been the most sensitive scale to measure clinical decline in FRDA. In my study, FARS was able to capture the complex features of FRDA in various domains, responsive to the clinical deterioration at each participant visit.

Whilst FARS scores showed significant correlation with disease duration and the age of onset, it was also relatively insensitive to changes over 6 months. This is likely due to the influence of ceiling effects in the disease biology with time and the relative importance of identification of each system affected in the disease phenotype. A significant change in the score was detected by FARS over 12 months, which is in concordance with other studies (Fahey et al., 2007; Friedman et al., 2010; Patel et al., 2016c).

7.5 GAA REPEAT EXPANSIONS

All the patients were found to be homozygous for expansions, with about half the patients having alleles of differing sizes (15 out of 29). The expansion size

Serial No	Age	Disease duration (onset to 2015)	Age of onset (yrs)	Allele 1 (repeats)	Allele 2 (repeats)
1	37	17	21	500	1100
2	33	12	22	500	950
3	39	36	4	1100	1100
4	36	26	11	850	950
5	40	22	18	850	850
6	40	35	8	550	650
7	18	3	15	750	1100
8	14	2	10	680	900
9	33	10	23	850	850
10	43	25	16	800	800
11	21	14	8	900	900
12	36	24	12	1000	1000
13	31	18	13	350	1000
14	42	33	9	900	900
15	19	10	8	850	850
16	45	35	11	750	850
17	30	24	6	1000	1000
18	19	10	9	850	900
19	43	33	8	900	900
20	41	26	15	500	1500
21	25	6	18	500	1000
22	13	2	10	750	750
23	25	13	12	350	350
24	19	7	12	550	680
25	52	13	40	120	120
26	13	7	6	850	1100
27	24	5	19	750	750
28	9	4	5	1100	1350
29	15	5	10	680	1100

Table 11. GAA trinucleotide expansion allele sizes for 29 patients

ranged from 120-1500 repeats. It is noteworthy that the sizes are approximate since they were derived from agarose gel electrophoresis **(Tables 11 and 12)**.

Repeat Range	Estimated Sizing Error (+/-)
100 – 350 rpts	50 rpts
350 – 500 rpts	71 rpts
500 – 850 rpts	118 rpts
850 – 152)00 rpts	235 rpts

Table 12. Estimated margin of error of the sizing for different repeat ranges

For the 29 patients tested, the mean size of the GAA allele 1 (GAA1 or smaller allele) repeats in the *FXN* gene was 726 repeats (n= 29, R= 120 - 1100, SD = 234) and for allele 2 (GAA2 or larger allele) was 905 (n=29, R= 120 - 1500, SD = 260). The patient with the smallest number of repeats (had 120 repeat sizes for both allele 1 and 2) was a late onset Friedreich's Ataxia (LOFA) participant in our study, and there was no overlap with the normal range.

Age of onset with GAA 1	correlation coefficient (R)	p value
All patients (n = 29)	-0.65	0.0001
5 most affected patients	-0.8	
5 mildly affected patients	-0.9	
Age of onset with GAA 2		
All patients (n = 29)	-0.46	0.006
5 most affected patients	0.51	
5 mildly affected patients	-0.36	
Disease duration with GAA 1	0.2	0.32
Disease duration with GAA 2	0.03	

Table 5. Association of gene repeats with age of onset /disease duration.

Similar to the published data (Reetz et al., 2015), we found that the age of disease onset was strongly inversely correlated with the number of GAA repeats on the smaller repeat allele in the *FXN* gene with a coefficient of correlation, **R = -0.8** for the severely affected category patients; and **-0.9** for the milder category patients **(Table 13)**. Please refer to section 6.5 and Appendix B for the clinical stratification of the two subgroups. When analysed for the whole cohort in our study, an R value = -0.65 was determined with GAA1, showing a moderate association, but a very significant p value (p=0.0001) **(Fig 22)**.

There was a weaker inverse correlation with the larger allele size with age of onset (R= -0.46) but again highly significant (p=0.006) **(Fig 23)**. The data in these 2 subsets of patients demonstrate that the size of the GAA repeat expansion correlates with the onset and severity of disease/ FARS scores **(Fig 24)**.

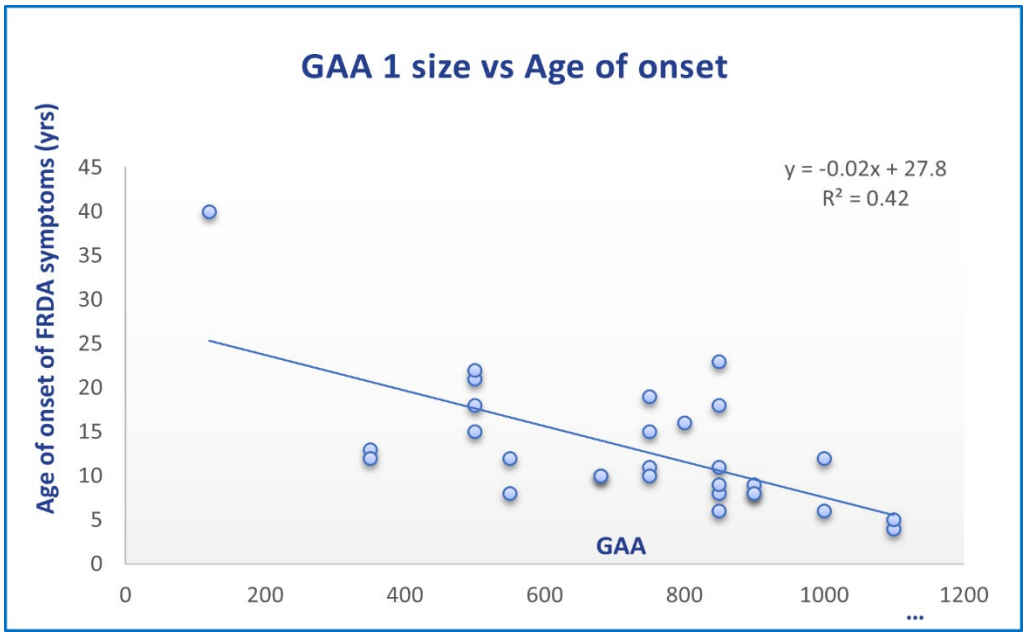


Figure 22. GAA Allele 1 vs Age of onset of symptoms (R=-0.65, p=0.0001)

SUMMARY OUTPUT							GAA 1 size vs Age of onset
Regression Statistics							
Multiple R	0.65						
R Square	0.42						
Adjusted R Square	0.40						
Standard Error	5.64						
Observations	29						
ANOVA							
	df	*SS*	*MS*	*F*	*Significance F*		
Regression	1	629.33	629.33	19.75	0.0001		
Residual	27	860.53	31.87				
Total	28	1489.86					
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	
Intercept	27.75	3.46	8.00	0.00	20.64	34.87	
GAA 1	-0.02	0.004	-4.44	**0.0001**	-0.02	-0.01	

Table 6. Summary output of GAA 1 size vs Age of onset of symptoms.

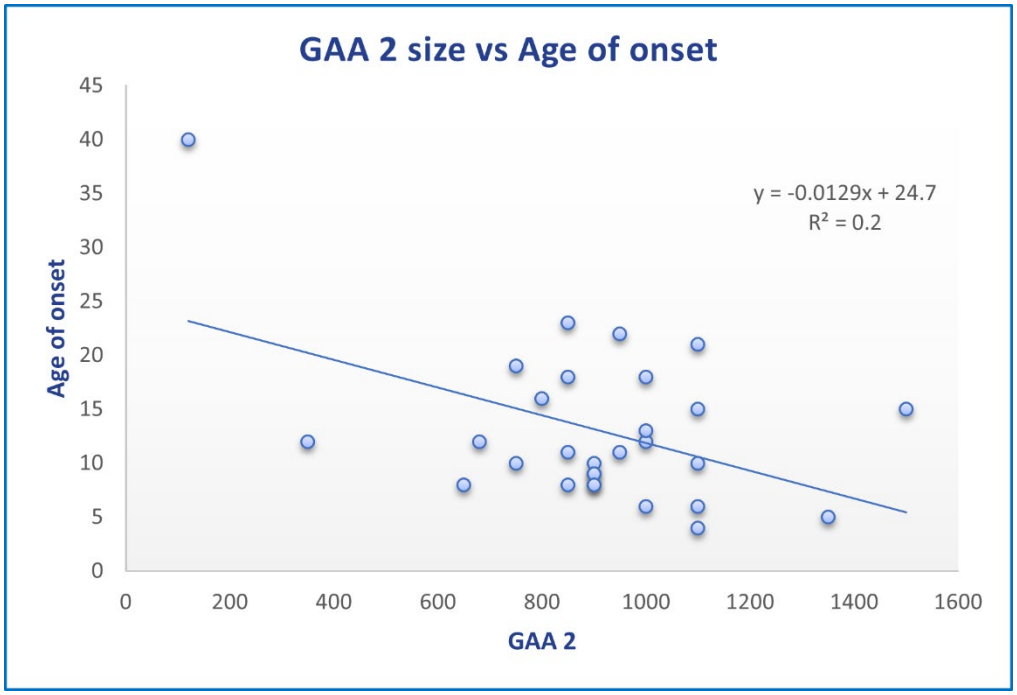


Figure 23. GAA Allele 2 size vs age of onset of symptoms (R= -0.45, p=0.006).

SUMMARY OUTPUT							**GAA 2 size with Age of onset**
Regression Statistics							
Multiple R	0.50						
R Square	0.25						
Adjusted R Square	0.23						
Standard Error	230.51						
Observations	28						
ANOVA							
	df		*SS*	*MS*	*F*	*Significance F*	
Regression	1		472683.95	472683.95	8.90	0.006	
Residual	26		1381526.77	53135.64			
Total	27		1854210.71				
	Coefficients		*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1131.10		89.41	12.65	0.000	947.31	1314.89
21	-18.21		6.11	-2.98	**0.006**	-30.77	-5.66

Table 7. Summary output of GAA 2 size with Age of onset.

Statistically, the correlation coefficient R measures the strength of the relationship between two variables, while R-squared (R^2) measures the amount of variation in the data that is explained by the model. Even when R^2 is low in the regression output tables, low P values still indicate a real relationship between the significant predictor of size of expansions (both smaller and larger allele) and the response variables such as clinical severity and age of onset of symptoms **(Tables 14, 15 and 16)**. No significant correlation was found with either allele size with duration of disease in the study subjects **(Fig 25,table 17)**.

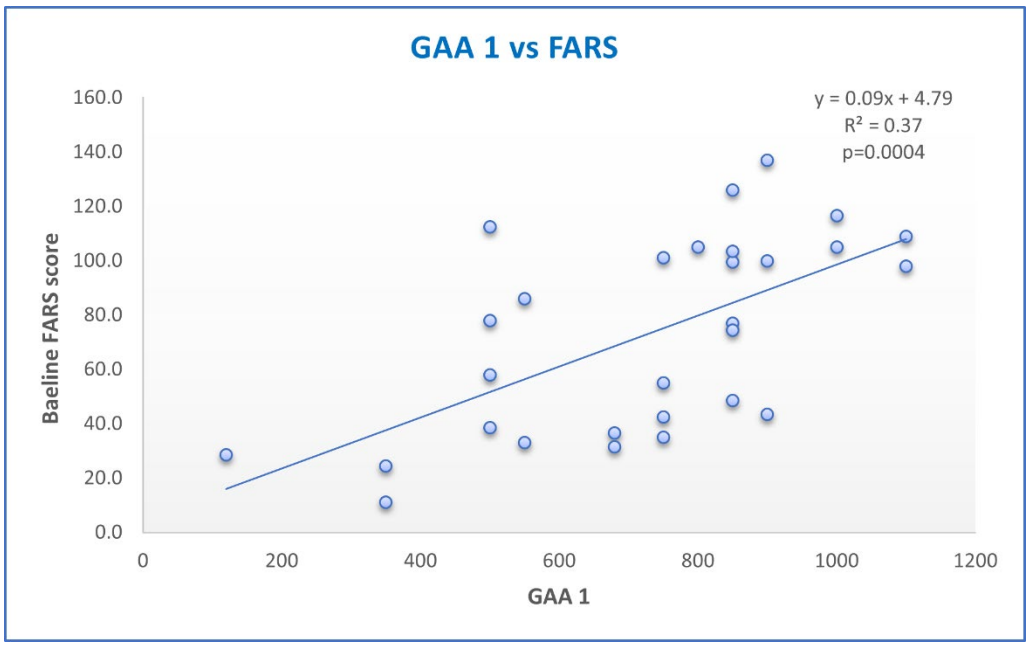


Figure 24. Effect of GAA allele 1 on clinical scores (p=0.0004).

SUMMARY OUTPUT						**GAA 1 Vs FARS score**
Regression Statistics						
Multiple R	0.61					
R Square	0.37					
Adjusted R Square	0.35					
Standard Error	28.91					
Observations	29					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	13536.10	13536.10	16.20	0.0004	
Residual	27	22562.93	835.66			
Total	28	36099.03				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	4.79	17.75	0.27	0.78	-31.64	41.22
Allele 1 (rpts)	0.09	0.02	4.02	**0.0004**	0.04	0.14

Table 8. Summary output of GAA 1 size with FARS score.

For a given dataset, higher variability around the regression line produces a lower R^2 value. As shown previously with the standard deviation in the

demographics section, the data from the FRDA cohort are immensely dispersed in relation to the mean.

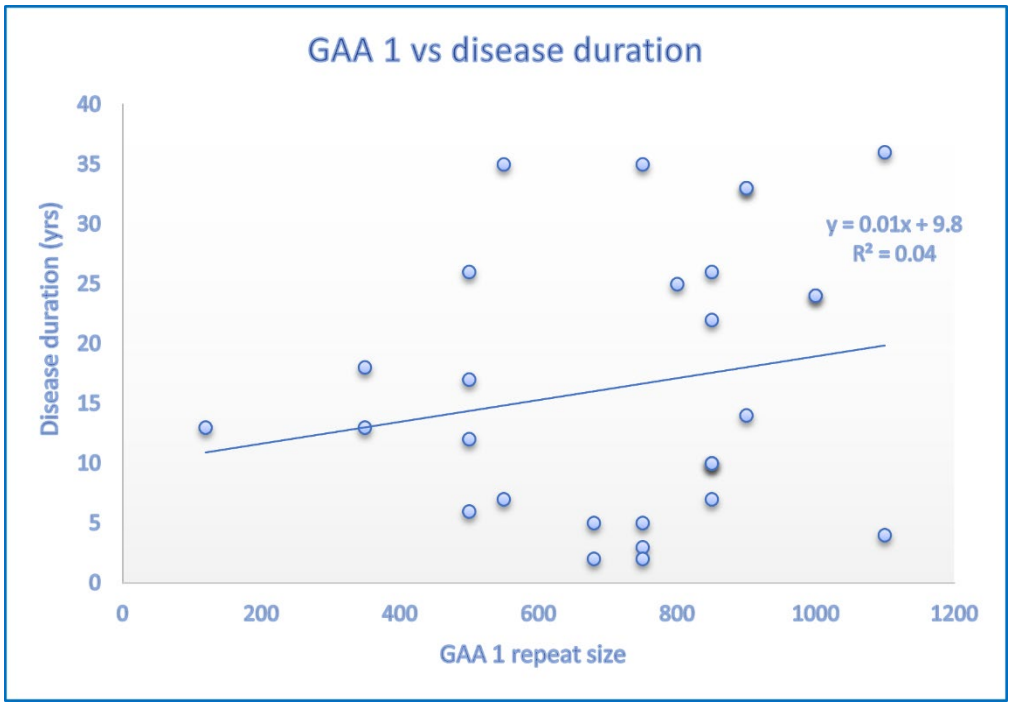


Figure 25. Correlation between GAA 1 and disease duration (R=0.2, p=0.32).

GAA 1 size with disease duration

SUMMARY OUTPUT	
Regression Statistics	
Multiple R	0.19
R Square	0.04
Adjusted R Square	0.00
Standard Error	11.17
Observations	29

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	127.91	127.91	1.02	0.32
Residual	27	3371.27	124.86		
Total	28	3499.17			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	9.83	6.86	1.43	0.16	-4.26	23.91
Allele 1 (rpts)	0.01	0.01	1.01	0.32	-0.01	0.03

Table 9. Summary Output of GAA 1 allele with disease duration.

SUMMARY:

My analysis suggested that even these relatively noisy data have a significant trend with the gene repeats. In particular, the length of the smaller allele was more predictive of disease progression and severity than the length of the larger allele.

Combined with other observations, there was an early deterioration seen for a mean of >900 repeats, and the mean allele length was significantly higher in patients with scoliosis and in those with cardiomyopathy, which is associated with higher disease burden. The phenotype relationship with gene expansion size confirmed by these results has been long established in previous studies, and GAA1 can be used as an important biological variable modulating progression, as well as practical criteria for stratification of clinical trials.

7.6 SERUM FRATAXIN

Frataxin levels were measured in blood and analysed in relation to disease features (FARS). Historically, there was no evidence of change in frataxin levels over time with repeated measures analysis, although linear regression analysis showed a small increase over decades.

As described in the methods, Frataxin levels were measured by my collaborators in the Lynch laboratory at the Children's Hospital of Philadelphia (CHOP). During the study, I learned from my collaborators at CHOP that the lateral flow assay can occasionally yield skewed results, which appear as outliers in the data. These effects can usually be mitigated (or diluted out) by testing more samples. My collaborators also suggested the introduction of an extraction buffer for cell lysis after buffy coat extraction, and a modified method of cryofreezing samples between the second and third visit. Having followed their advice, sample levels varied dramatically for some patients over visits 3 and 4, and results did not change even after back-up samples were re-analysed. For example (some samples did not reveal any cells, and results were documented as 0, to a level documented as high as 1046.57 pg/25 µl (the main outlier in visit 2). Hence, the predictions with GAA repeat sizing, clinical scores and age of onset were run with the data obtained at the baseline visit.

I have summarised information of the characteristics and distribution of values for Frataxin levels assessed over 4 visits (number of datapoints =65; **Table 18**) .

Mean	250.35
Standard Error	29.77
Median	165.91
Mode	N/A
Standard Deviation	240.00
Sample Variance	57599.35
Range	
Minimum	0.78
Maximum	1047.57
Count	65
Confidence Level (95.0%)	59.47

Table 10. A glance at the central tendency and the degree of dispersion of values in Frataxin dataset (4 visits for n=65).

Since the sample sizes were unequal between the four visits, Levene's test for homogeneity of variance was run to check if there was a significant difference between the variances over the four data points. Here, the p-value for the Levene's test was greater than 0.05 (0.6) as desired, indicating that variances were not significantly different from each other (i.e., the homogeneity assumption of the variance was met).

Test of Homogeneity of Variances					
Values	Levene Statistic	df1	df2	Sig.	
	0.61	3	62	0.61	
ANOVA					
Values	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	121.17	3	40.39	1.37	0.26
Within Groups	1827.54	62	29.47		
Total	1948.72	65			

Table 11. Testing for equal variances between samples at 4 visits.

(p>0.05 .i.e. variances are not significantly different from each other).

However, when I proceeded to ANOVA, it showed a much larger variability between groups relative to the variability within groups, meaning the data points were more dissimilar and extreme values would be more likely **(Fig 26, Table 19).**

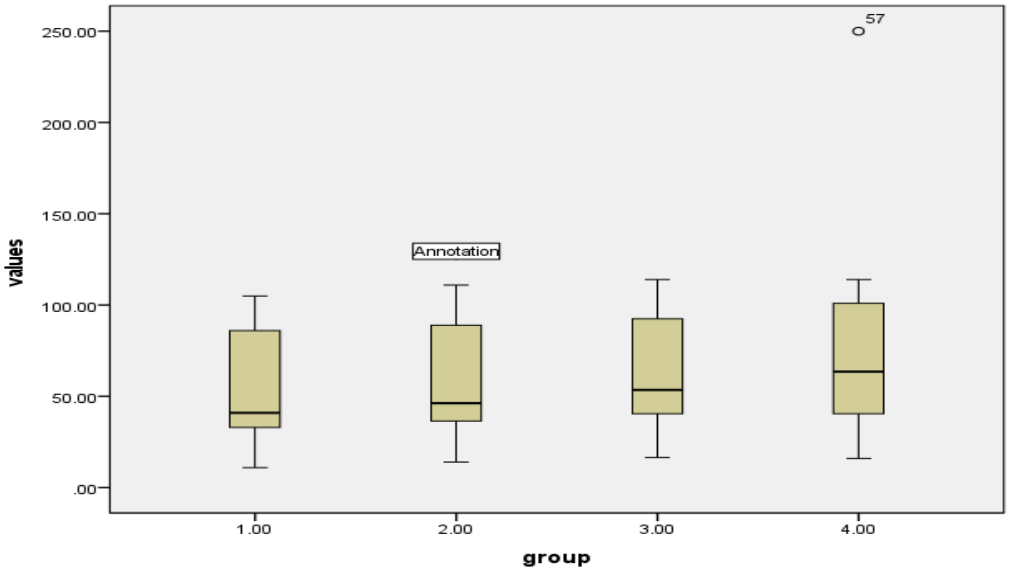


Figure 26. Testing homogeneity of variance for 4 data sets.

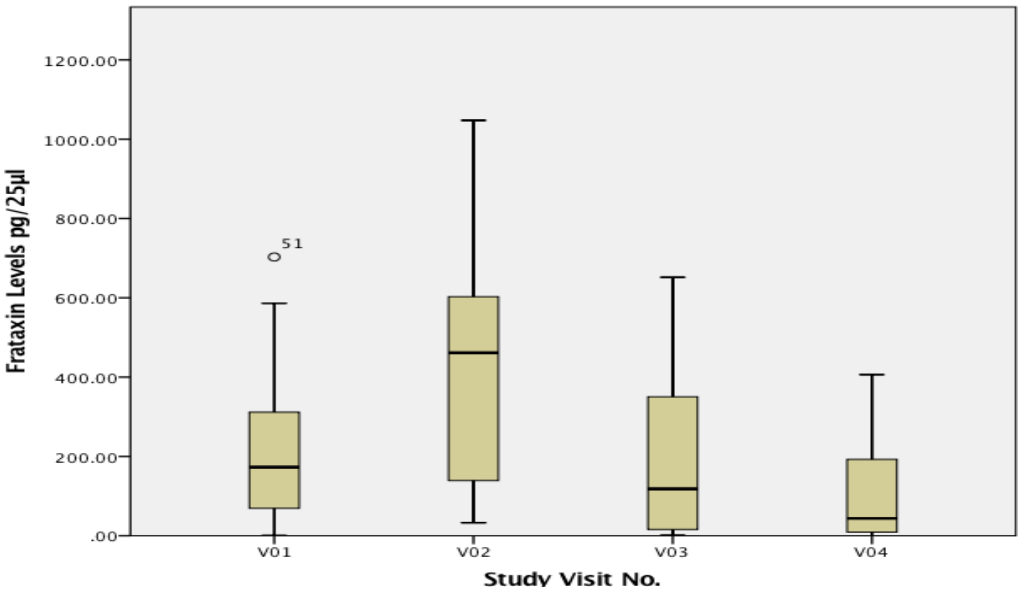


Figure 27. Change in absolute serum Frataxin levels over 4 visits.

No obvious trend with 4 visits; outlier in 2nd visit sample caused skewing.

A box-plot was created to visualise the range of data. As stated before, the results from visit 2 were skewed by an outlier, and there was no overall trend with consecutive visits **(Fig 27)**. Further analysis showed that GAA 1 correlated negatively with serum frataxin levels with a high p-value of 0.002 **(Fig 28)** and frataxin levels could predict clinical status seen with FARS score (R= -0.3, p= 0.04) **(Fig 29)**.

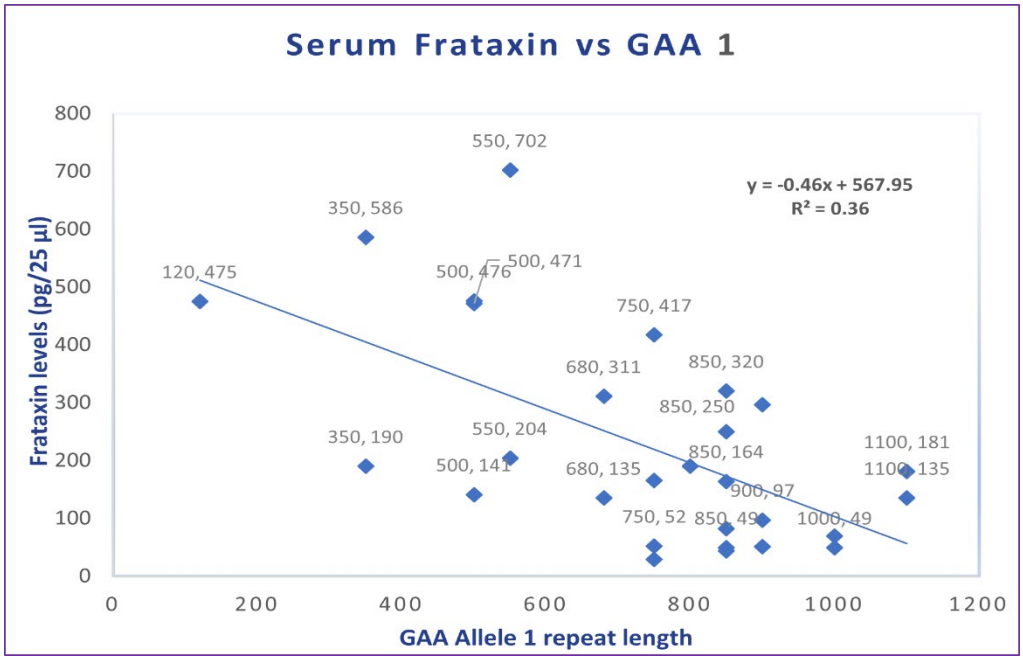


Figure 28. Effect of GAA allele 1 size with Serum Frataxin levels (R = -0.6; p = 0.002).

Regression Statistics ***Frataxin levels vs GAA Allele 1***	
R	-0.58
R Square	0.34
Adjusted R Square	0.31
Standard Error	148.21
Observations	25
p-value	**0.0021**

Table 12. Output Summary of Regression analysis of Frataxin levels with GAA Allele 1.

In the literature, linear regression analysis of FARS scores did not predict frataxin levels very well (Lazaropoulos et al., 2015). Note that multivariate testing to account for age, disease duration and GAA repeat length as independent covariates was not performed for this analysis.

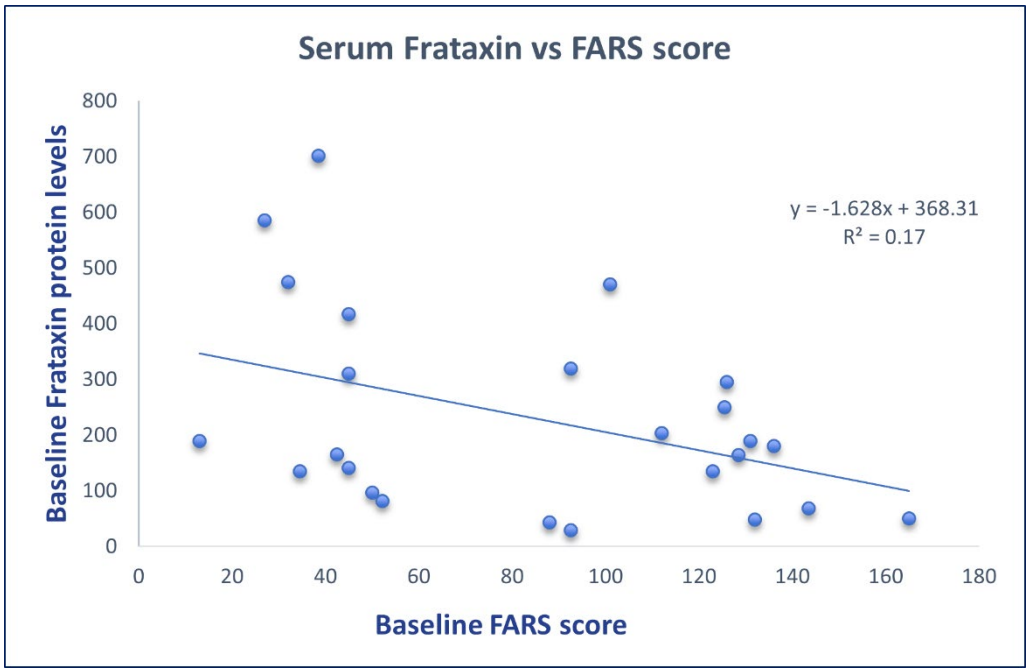


Figure 29. Correlation of clinical severity (FARS) with Serum Frataxin (R = -0.3; p = 0.04).

Regression Statistics Frataxin vs FARS visit 1	
R	-0.41
R Square	0.17
Adjusted R Square	0.13
Standard Error	166.41
Observations	25
p-value	**0.04**

Table 13. Summary Output of Frataxin with baseline FARS score.

Whilst age of onset showed a mild positive correlation (p=0.09) and duration of disease a mild inverse one, the results were not significant on univariate regression testing **(Figures 30 & 31)**. However, with a larger sample size and less noisy data, it is possible that earlier age of onset of symptoms could be correlated with reduced Frataxin levels, but levels do not decline consistently with time.

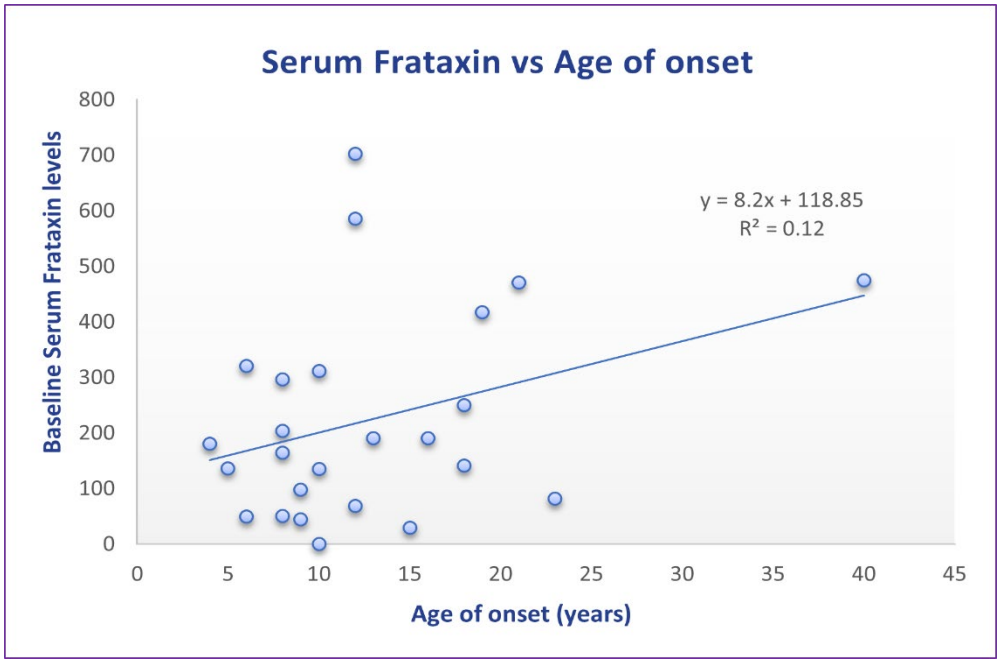


Figure 30. Effect of age of onset on Frataxin levels (R = 0.34, p = 0.09).

Regression Statistics	
Frataxin vs Age of onset	
Multiple R	0.34
R Square	0.12
Adjusted R Square	0.08
Standard Error	177.12
p-value	0.096
Observations	25

Table 14. Summary Output of Frataxin vs Age of Onset.

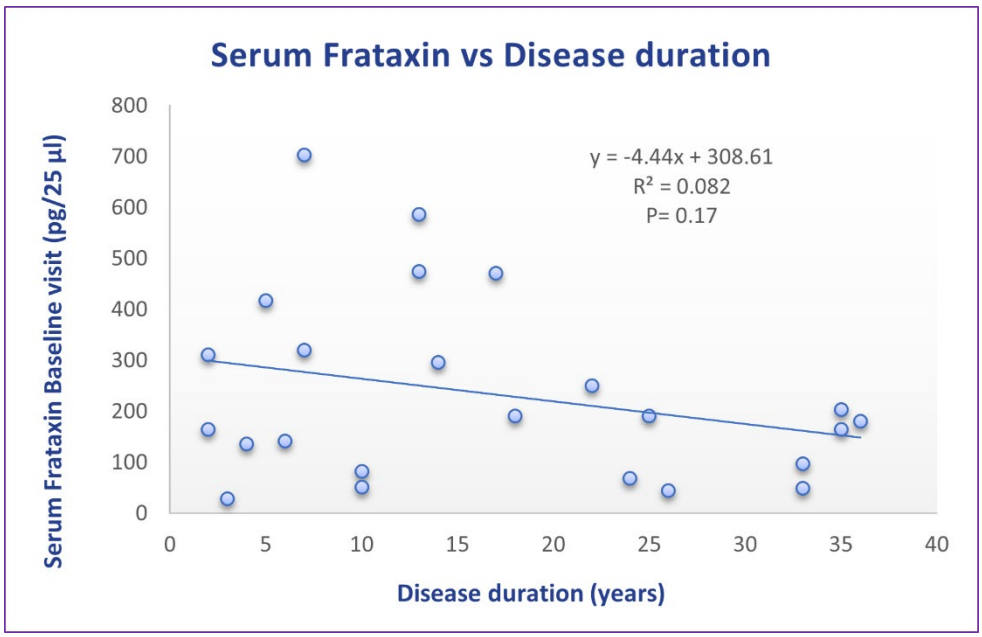


Figure 31. Effect of disease duration on Frataxin levels (R = -0.3, p = 0.17).

SUMMARY:

It has been shown that frataxin levels appear not to change over short periods of time, and do not show a significant relationship between frataxin levels and disease features or progression (Deutsch et al., 2010).

My data show that peripheral frataxin levels reflect disease features in FRDA but cannot accurately predict clinical status. This is consistent with previously published research. However, in these studies there has been an emphasis on interpreting Frataxin levels in the context of specific mutations. For example, compound heterozygous patients for a GAA expansion and a point mutation in *FXN* generally have lower levels of frataxin than those who are homozygous for two GAA repeat expansions (Lazaropoulos et al., 2015).

7.7 SEPs and NCS

Sensory evoked potentials were performed only on patients who had retained but reduced SNAPs on nerve conduction studies. SEP responses were not easily identifiable and appeared delayed and dispersed in most patients, as shown by the findings reproduced in **table 23**. This was the case even with ambulant or relatively mildly affected patients. The lower limbs were affected more and much earlier compared to the upper limbs, a known discrepancy in FRDA.

S No	Upper limbs (ULs) SEPs	Lower limbs (LLs) SEPs	Conclusions
1	Poorly formed.	Poorly formed.	Significant disease affecting the central somatosensory pathways from ULs and LLs.
2	Low amplitudes, central conduction times borderline prolonged.	Absent.	Disease affecting somatosensory pathways from both the ULs and LLs.
3	Latencies delayed, not reproducible.	Latencies delayed, not reproducible.	Disease affecting somatosensory pathways from both the ULs and LLs.
4	Reasonably formed but difficult to reproduce. N20 and P1 latencies were within normal limits.	Reduced in amplitude but reproducible.	Whilst the non-reproducibility of SEPs may raise the possibility of disease affecting central somatosensory pathways, the findings on peripheral NCS would suggest that these findings are more likely to be contributable to the presence of a severe sensory axonal neuropathy, particularly given the normal SEP latencies.
5	Well formed; latencies were within normal limits.	Poorly formed/absent.	Evidence of disease affecting the central somatosensory pathways from the lower limbs only.
6	Poorly formed and of low amplitude.	Not attempted due to marked DRG on NCS.	Evidence of disease affecting somatosensory pathways from both the UL and LLs, with the known discrepancy in FRDA, of LLs being affected more and much earlier compared to the ULs.

Table 15. Results of sensory evoked potential studies in 6 patients.

Peripheral Nerve	Range of Conduction velocity (CV)
Median sensory	45 - 65 m/s
Median motor	49 - 64 m/s
Ulnar sensory	48 - 74 m/s
Ulnar motor	>49 m/s
Peroneal motor	>44 m/s
Tibial motor	>41 m/s
Sural sensory	46 - 64 m/s

Table 16. Accepted conduction velocities in peripheral nerves.

Normal CVs for any given nerve are generally in the range of 50 –60 m/s. [normative values quoted above (Kimura, 2004; Peyronnard et al., 1976)*].*

I performed motor nerve conduction on median, ulnar and tibial nerves and sensory conduction on median, ulnar, superficial peroneal and sural nerves on the dominant side. The main findings from a set of 10 patients are tabulated , repeated 6 monthly on average, over a timescale of 3 or 4 visits **(Table 25).**

There were either absent or reduced amplitude SNAPs, with only borderline slow sensory velocities (~40-45 m/s), consistent with severe sensory neuropathy/neuronopathy or DRGopathy. For median Digit I, the sensory amplitude measured peak to peak from was small, around 3uV or less (normal mean 12 uV ± 3) with low SNCVs ranging between 41 and 57 m/sec (Normal mean: 56.5 m/sec ±5.1). In most patients, no response was obtained at the ankle on stimulating the sural nerve. Please note that patient 10 in the table is a LOFA, with relatively preserved SNAPs (~5 uV - normal mean amplitude 10uV ±4) and conduction velocity (~40 m/sec - normal mean 48.9 ±4.4). MNCV along the median nerve always was above 40 m/s. Amplitude of the M responses was normal, except in very advanced cases. Lower limbs were affected earlier and showed worsened electrophysiology findings as expected.

S. No	Visit no	Motor NCS	Latency (ms)	Peak to peak Amp (uV)	Vel m/s	F wave (ms)	Sensory NCS	Latency (ms)	Peak to peak Amp (uV)	Vel m/s
1	1	R Med-APB (W)	4.5	8300		32.7	Med Dig I	Absent		
		E-W	9.7	7800	44.2					
		R Tib-AH (A)	6.4	5,100		68.8	Sural	Absent		
		PF-Ankle	20.6	5,500	26.8					
	2	R Med-APB (W)	3.8	6,900		28.8	Med Dig I	Absent		
		E-W	8.8	6,500	50					
		R Tib-AH (A)	5.2	7,400		69	Sural	Absent		
		PF-Ankle	19.2	5,800	29.3					
	3	R Med-APB (W)	3.8	5900		32	Med Dig I	Absent		
		E-W	8.6	5600	57.3					
		R Tib-AH (A)	5	5400		71.9	Sural	Absent		
		PF-Ankle	19.2	4700	29.7					
	4	R Med-APB (W)	3.6	6300		32.3	Med Dig I	Absent		
		E-W	8.2	5600	48.9					
		R Tib-AH (A)	6.3	5500		72.9	Sural	Absent		
		PF-Ankle	19.2	4200	30.9					
2	1	R Med-APB (W)	3.4	16800		30.2	Med Dig I	2	3.1	57.5
		E-W	7.6	16200	54.8					
		R Tib-AH (A)	8	2200		67.2	Sural	3.2	1.6	31.3
		PF-Ankle	20.6	1900	35.7					
	2	R Med-APB (W)	3.2	15900		28.9	Med Dig I	2	4.7	55
		E-W	7.5	14900	54.7					
		R Tib-AH (A)	6.2	2200		67.5	Sural	3.4	1.1	22.1
		PF-Ankle	18.7	2000	36.4					
	3	R Med-APB (W)	3	17000		29.3	Med Dig I	1.9	4.2	57.3
		E-W	7.6	16300	54.3					
		R Tib-AH (A)	7.6	1900		68.8	Sural	3.1	3.2	32.3
		PF-Ankle	20.5	1700	34.1					
	4	R Med-APB (W)	4.2	13400		28.8	Med Dig I	2.4	3.1	50
		E-W	8.5	12900	51.9					
		R Tib-AH (A)	7.5	2500		67.5	Sural	absent		
		PF-Ankle	19.8	2000	32.5					
3	1	R Med-APB (W)	4.2	13400		26.2	Med Dig I	2.8	0.7	47.1
		E-W	8.4	10200	52.4					
		R Tib-AH (A)	5.8	13100		52.4	Sural	3.4	0.7	30.9
		PF-Ankle	16.5	8000	34.1					
	2	R Med-APB (W)	4.2	13200		25.8	Med Dig I	2.7	0.9	41.5
		E-W	8	15400	55					
		R Tib-AH (A)	5.2	14700		52.4	Sural	3.4	3	33.8
		PF-Ankle	14.8	14300	37					
	3	R Med-APB (W)	3.4	11800			Med Dig I	2.3	1.3	54.3
		E-W	6.5	13200	67.7	26.7				
		R Tib-AH (A)	6	14500			Sural	3.8	1.2	27.6
		PF-Ankle	16.1	8600	36.1	51				
	4	R Med-APB (W)	3.3	12000		28.3	Med Dig I	2.3	0.5	42
		E-W	7.7	12800	51.8					
		R Tib-AH (A)	6.7	14300		51	Sural	3	1.5	30
		PF-Ankle	15.9	12800	39.7					
4	1	R Med-APB (W)	3.4	11000		26.5	Med Dig I	2.4	1	56.2
		E-W	7.3	18000	55.1					
		R Tib-AH (A)	3.7	7800		51.8	Sural	1.92	3	65.1
		PF-Ankle	12.4	7200	47.7					
	2	R Med-APB (W)	3.8	10500		27.9	Med Dig I	2.3	1.6	47
		E-W	7.9	9300	57.3					
		R Tib-AH (A)	3	17600		53	Sural	Absent		
		PF-Ankle	12.3	14200	45.2					
	3	R Med-APB (W)	3.2	11400		28.3	Med Dig I	2	1.9	48
		E-W	7.8	9400	52					
		R Tib-AH (A)	3.5	15500		53.7	Sural	Absent		
		PF-Ankle	13.6	11900	40.6					
	4	R Med-APB (W)	3.2	10500		28.5	Med Dig I	2	2.7	57
		E-W	8.1	10800	53.1					
		R Tib-AH (A)	3.4	21700		54.7	Sural	Absent		
		PF-Ankle	14.1	13000	39.3					

S. No	Visit no	Motor NCS	Latency (ms)	Peak to peak Amp (uV)	Vel m/s	F wave (ms)	Sensory NCS	Latency (ms)	Peak to peak Amp (uV)	Vel m/s
5	1	R Med-APB (W)	4.9	10800		34.1	Med Dig I	Absent		
		E-W	9.9	6400	53.7					
		R Tib-AH (A)	Absent				Sural	Absent		
		PF-Ankle								
	2	R Med-APB (W)	4.3	9300		32.3	Med Dig I	Absent		
		E-W	8.8	7600	50					
		R Tib-AH (A)	Absent				Sural	Absent		
		PF-Ankle								
	3	R Med-APB (W)	4.4	6900		33.8	Med Dig I	Absent		
		E-W	9	6600	52.2					
		R Tib-AH (A)	Absent				Sural	Absent		
		PF-Ankle								
	4	R Med-APB (W)	5.5	10700			Med Dig I	Absent		
		E-W	9.9	8200	54.5	33.6				
		R Tib-AH (A)	Absent				Sural	Absent		
		PF-Ankle								
6	1	R Med-APB (W)	3.7	15000		25.8	Med Dig I	Absent		
		E-W	8.4	15100	47.9					
		R Tib-AH (A)	5.8	9500		59.4	Sural	Absent		
		PF-Ankle	17.3	4900	30					
	2	R Med-APB (W)	3.9	17000		25	Med Dig I	Absent		
		E-W	9.1	17200	43.3					
		R Tib-AH (A)	5.6	100			Sural	Absent		
		PF-Ankle	NR	NR	NR					
	3	R Med-APB (W)	3.5	13000		27.5	Med Dig I	Absent		
		E-W	8.8	12100	41.5					
		R Tib-AH (A)	7.2	3300		NR	Sural	Absent		
		PF-Ankle	18.9	600	29.9					
	4	R Med-APB (W)	3	15900		22.3	Med Dig I	Absent		
		E-W	7.8	14500	49					
		R Tib-AH (A)	7.1	4500		NR	Sural	Absent		
		PF-Ankle	18.6	1600						
7	1	R Med-APB (W)	3.2	14500		24.8	Med Dig I	2.2	0.4	47.7
		E-W	6.8	13500	55					
		R Tib-AH (A)	4.2	9800		54	Sural	Absent		
		PF-Ankle	1.92	100	NR					
	2	R Med-APB (W)	3	13000		26.3	Med Dig I	2.2	0.6	44.5
		E-W	7.5	12800	51.1					
		R Tib-AH (A)	5.1	8100		55.3	Sural	Absent		
		PF-Ankle	14.8	100	NR					
	3	R Med-APB (W)	2.8	15300		26.8	Med Dig I	Absent		
		E-W	7.5	13700	53.2					
		R Tib-AH (A)	4.2	6800		54.5	Sural			
		PF-Ankle								
	4	R Med-APB (W)	3.7	19800		27.8	Med Dig I	Absent		
		E-W	8.1	12700	55.7					
		R Tib-AH (A)	7.1	8800		NR	Sural	Absent		
		PF-Ankle	19	7200	31.5					

Table 17. Motor and sensory findings in nerve conduction studies in 10 subjects (cont.).

Absent or very low amplitude sensory responses, with only mildly slow SNCVs ~ 40 m/s. CMAPS affected only in advanced patients (LLs > ULs). The findings did not evolve over 18 -24 months.

S. No	Visit no	Motor NCS	Latency (ms)	Peak to peak Amp (uV)	Vel m/s	F wave (ms)	Sensory NCS	Latency (ms)	Peak to peak Amp (uV)	Vel m/s
8	1	R Med-APB (W)	3.1	19800		23.6	Med Dig I	2.5	1.7	56
		E-W	6.4	14700	63					
		R Tib-AH (A)	5.8	17600		50.8	Sural	3	3	38.3
		PF-Ankle	14.3	16600	40.6					
	2	R Med-APB (W)	2.7	17700		24.3	Med Dig I	1.8	3.5	55.9
		E-W	6.2	18500	65.1					
		R Tib-AH (A)	5.5	27600		52.1	Sural	Absent		
		PF-Ankle	15	25300	37.6					
	3	R Med-APB (W)	2.6	21300		24	Med Dig I	1.75	3.5	57.1
		E-W	6	20000	61.8					
		R Tib-AH (A)	5.9	14400		52.4	Sural	Absent		
		PF-Ankle	14.9	12900	41.1					
9	1	R Med-APB (W)	3.4	17700		30	Med Dig I	Absent		
		E-W	8.1	15500	54.3					
		R Tib-AH (A)	3.5	10300		61	Sural	Absent		
		PF-Ankle	16.5	8300	35.4					
	2	R Med-APB (W)	2.8	18600		30.4	Med Dig I	Absent		
		E-W	7.8	15300	50					
		R Tib-AH (A)	3.2	9800		63.1	Sural	Absent		
		PF-Ankle	15.9	1900	37.4					
	3	R Med-APB (W)	3.5	16400		30.9	Med Dig I	Absent		
		E-W	8.1	15000	51.1					
		R Tib-AH (A)	3.9	6600		57.2	Sural	Absent		
		PF-Ankle	18.4	4200	32.4					
10	1	R Med-APB (W)	3.4	19200		23.3	Med Dig I	1.9	9.4	53.2
*		E-W	6.7	20300	59.1					
		R Tib-AH (A)	3.2	12400		45.5	Sural	2.3	13	34.8
		PF-Ankle	12	15300	41.5					
	2	R Med-APB (W)	3.2	16800		25.7	Med Dig I	2.1	12	57.1
		E-W	6.9	15000	58.1					
		R Tib-AH (A)	4.5	19600		55.3	Sural	3	4.4	36.7
		PF-Ankle								
	3	R Med-APB (W)	3.1	24300		21.5	Medi Dig I	1.9	8.5	64
		E-W	6.5	25100	64.7					
		R Tib-AH (A)	3.8	21600		50.8	Sural	2.9	4.8	43.1
		PF-Ankle	12.4	17100	40.7					
	4	R Med-APB (W)	3.3	21100		21.9	Med Dig I	2	9.8	52.5
		E-W	7.2	20000	56.4					
		R Tib-AH (A)	5.7	15100		50.9	Sural	3.1	6.4	37.1
		PF-Ankle	13.8	12800	45.7					

** Patient 10 with well-preserved SNAPs and CMAPs was a LOFA (late onset Friedreich's ataxia), independently mobile and mildly symptomatic.*
W - wrist, E - elbow, A - Ankle, PF - popliteal fossa

SUMMARY:

Both NCS and SEPs measure large proprioceptive neurons, which are frequently absent in FRDA patients at the onset of symptoms.

At the time of subsequent explorations, when the clinical condition had significantly deteriorated in many patients, motor and sensory nerve conduction velocities and amplitude of SNAPs were nearly the same to those found at the first examination without major differences. Noticeably, the sensory responses, especially in the lower limbs, were already absent to start with in many patients. Therefore, my longitudinal evaluation of NCS over 4 visits suggested that the measure might be used to facilitate early diagnosis where genetic testing is delayed. However, neither measure showed significant worsening over time and therefore cannot identify changes associated with disease progression.

7.8 MEPs

Analysis was performed in Matlab (Mathworks, Natick, MA) using custom scripts. The shortest onset latency for each set of ten MEPs was assigned interactively. In the presence of a background contraction, the earliest deflection of the MEP with the shortest latency was often ambiguous on superimposed raw traces because of background EMG activity but could be easily identified by averaging 20 individual MEPs (onsets were clearer with rectified MEPs but this additional processing step was not used in this analysis). Hence, such averages were used for assigning latencies throughout **(Fig 32)**.

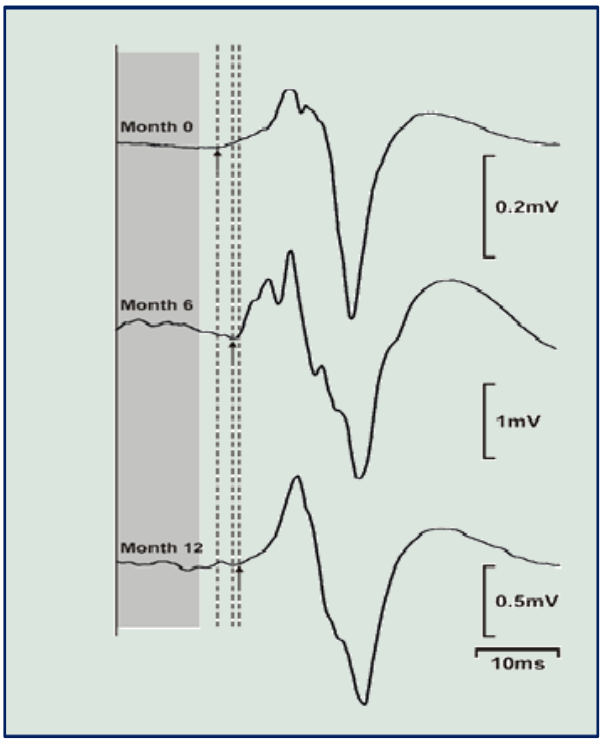


Figure 32. Examples of averaged FDI MEPs from one FRDA patient.

FDI MEPs recorded from a FRDA patient at baseline, 6 months, and 12 months showing increasing onset latency over time. PMCTs have been subtracted for clarity and grey box shows the upper limit of normal for UL CMCT (based on Eisen & Shtybel, 1990). To be noted that CMCTs are prolonged at baseline.

Muscle	CMCT latency in FRDA	Range of CMCT latency in FRDA (ms)	Normal CMCT latency	Range of PMCT in FRDA	Normal PMCT latency	Range of MEP latency in FRDA	Mean normal MEP latency
	Mean ± SD (ms)	ms	Mean ± SD (ms)	ms	Mean ± SD (ms)	ms	Mean ± SD (ms)
FDI	13.1 ± 4.2	5.2 -23.6	7.2 ± 1.4	10.7 - 18.6	15.2 ± 1.6	18.2 - 41.0	20.9 ± 0.8
EDC	12.7 ± 4.9	7.4 -23.2	6.7 ± 1.7	4.9 - 10.1	9.3 ± 1.2	15 - 30	-
FDS	13.7 ± 4.5	7.2 -23.2	7.7 ± 2.0	4.7 - 11.7	8.7 ± 1.2	14.2 - 30.7	-

Table 18. Mean CMCT latency in FRDA cohort in comparison with normative data.

Table 26 compares a collection of CMCT and PMCT latencies from individual FRDA patients (44 data points in 17 patients) to the normative published figures. The figures confirm markedly delayed CMCTs for all the three muscles tested (e.g., mean FDI CMCT latency was **13.1 ± 4.2,** cf. normal **7.2 ± 1.4,** with a range varying from 5.2 to 23.6 ms). MEP amplitude was not formally quantified but were qualitatively diminished and dispersed/desynchronized. In patients with more severe disease, sometimes, resting motor threshold could not easily be determined because only small and unreliable responses could be obtained at the maximum tolerated stimulator output. These observations are consistent with the published literature, which has shown that CST damage in FRDA has both demyelinating and axonal features, leading to preferential loss of fast-conducting fibres in the CST early on in the disease, which will desynchronise the descending volley, increasing the time required for temporal summation at

the LMN and reduce the size of the MEP (Lanzillo et al., 1994; Martínez & Anciones, 1992a).

The first analysis applied randomized blocks ANOVA (one-way ANOVA) that requires 'stacking' the time points, where the muscle latencies were grouped into individual sets known as blocks (n=14). The aim was to minimize the variance among units within blocks relative to the variance among blocks, by controlling for the effects of factors that may confound the results **(Fig 33)**.

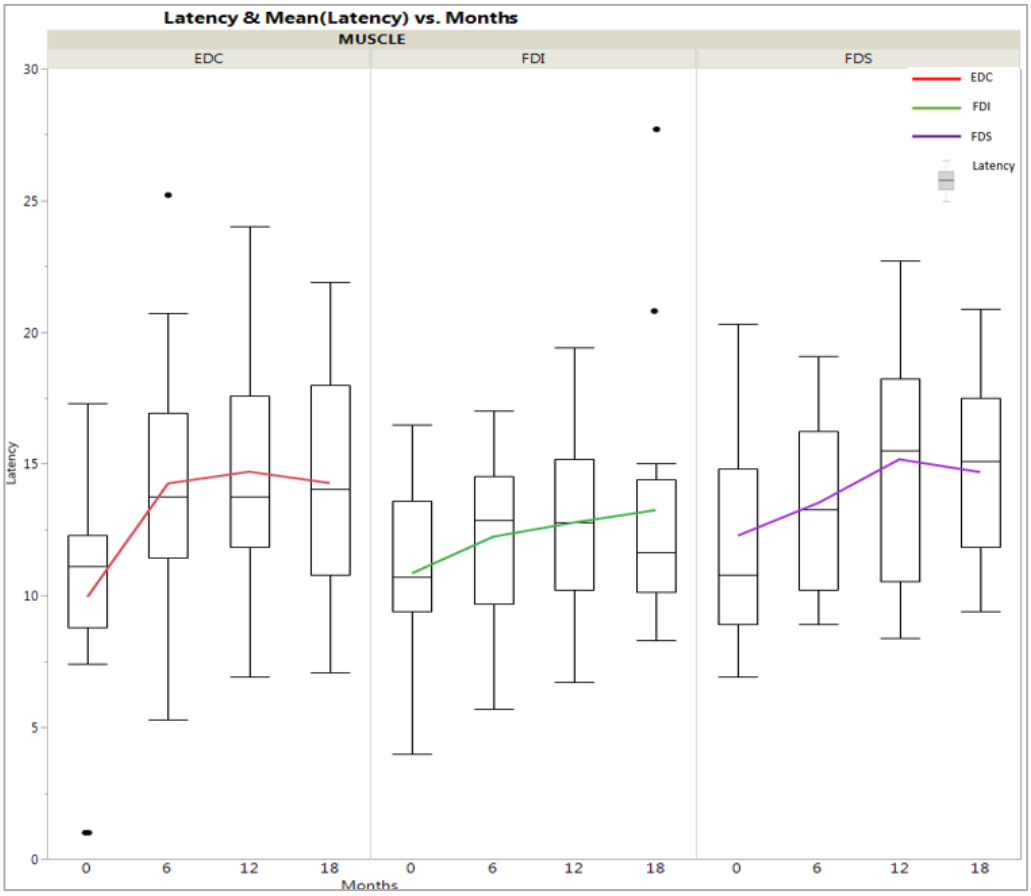
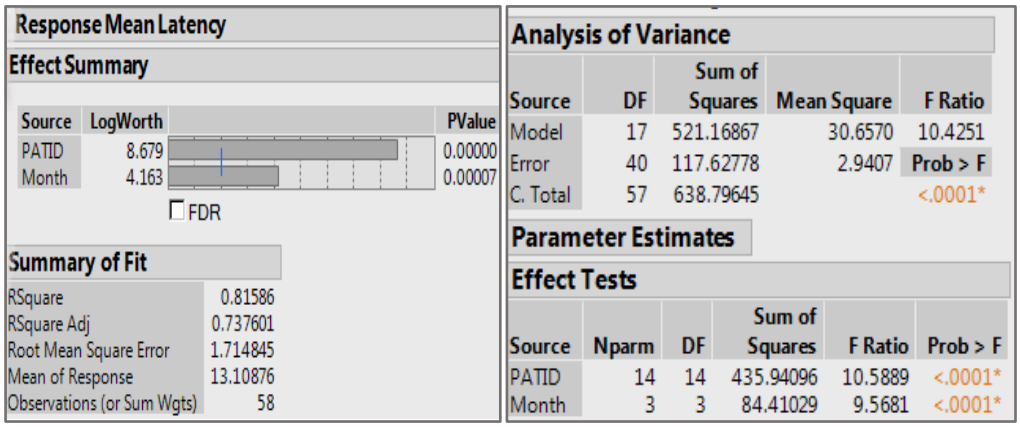


Figure 33. Change in mean absolute CMCT latency of 3 muscles over 4 visits.

The tests for each muscle group separately (EDC, FDS, FDI) were either statistically significant or on the cusp of significance. However, by averaging across muscle groups the changes over time for every 6 months were very highly significant (p<0.0001).



Response Mean Latency

Effect Summary

Source	LogWorth	PValue
PATID	8.679	0.00000
Month	4.163	0.00007

FDR

Summary of Fit

RSquare	0.81586
RSquare Adj	0.737601
Root Mean Square Error	1.714845
Mean of Response	13.10876
Observations (or Sum Wgts)	58

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	17	521.16867	30.6570	10.4251
Error	40	117.62778	2.9407	Prob > F
C. Total	57	638.79645		<.0001*

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
PATID	14	14	435.94096	10.5889	<.0001*
Month	3	3	84.41029	9.5681	<.0001*

Table 19. One-way analysis of variance summary output looking at the increase in mean latencies of 3 muscle groups over 4 visits (R=0.8, p <0.0001).

The figure also shows that that distal muscles are affected early and then plateau but that proximal muscles (with shorter CST axons – here, FDS and EDC) are affected later and thus continue to show the trend. This makes for an argument in favour of recording MEPs from forearm muscles as well as hand, unlike most studies recording from a single hand muscle.

For a more detailed analysis, MEP CMCTs were normalised to baseline, and an increase from baseline on subsequent visits expressed as a percentage change for each muscle for each 6-monthly assessment. Normalisation of data is essential where the pathology of the condition results in very large differences in absolute CMCT between patients. The results of this analysis for upper limb MEP data (insufficient lower limb MEP data could be collected because of the extent of disease in most participants) comparing two groups, patients (n=14; 42 data points) and controls (n=6; 6 data points), are summarised in **Figure 34**.

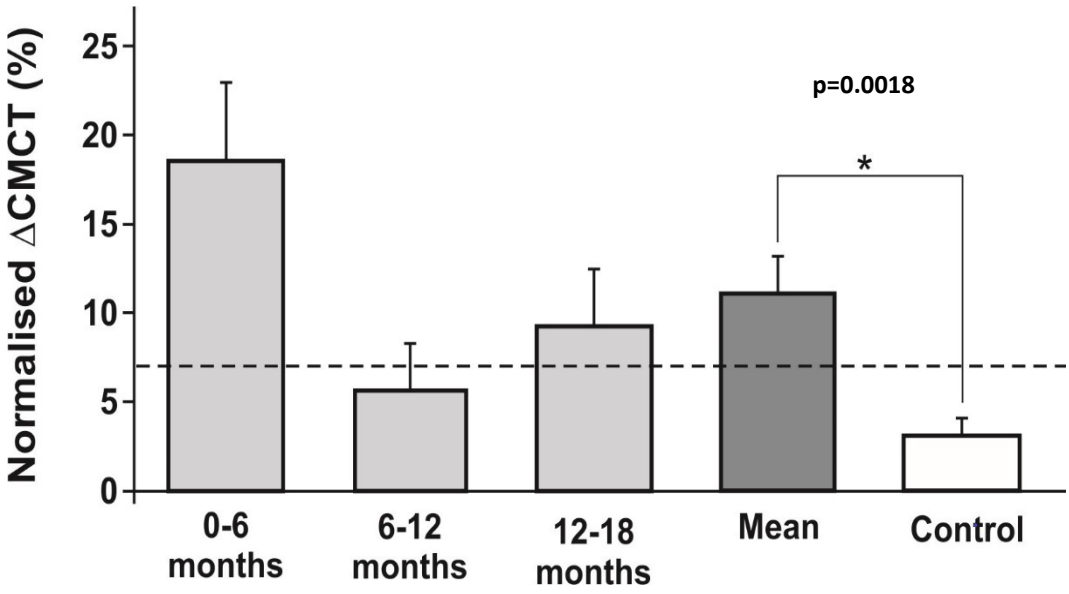


Figure 34. Percentage (%) change in normalised central motor conduction time (ΔCMCT) over 4 visits (baseline, 6 months, 12 months and 18 months) vs controls (baseline and 6 months).

CMCTs were normalised to baseline and averaged (across muscles and patients). Error bars plot one standard deviation of the mean. For comparison, the dashed horizontal line has been plotted to indicate three standard deviations from the mean ΔCMCT over 6 months for the control group (white bar). Note that normalised CMCT increased by 5-20% every 6 months but that none of these changes were significant (allowing for multiple comparisons). Only by averaging all data across the 18-month data collection period (dark grey bar) was there a significant increase in mean ΔCMCT of approximately 10% over 6 months (unpaired t-test; p<0.005).

This analysis confirmed that on average, MEP CMCT increased with time (5-20% at each 6-month follow-up). Whilst the first 6-month change in CMCT was significantly different from the second (p=0.04), as with other data points, accounting for multiple comparisons, none of these changes was significant. All data were averaged across muscles and patients for each 6-month timepoint (averaging across muscles increases the sensitivity of MEP CMCT measurements; (Mills & Nithi, 1998)) and this showed that when all 6-monthly

CMCT changes (n=42) were combined, there was a significant difference between this and the control data set (unpaired t test; $p<0.005$), indicating that MEP CMCTs may be able to monitor disease progression in a small sample of patients (n=14) over an 18 month period of monitoring.

Next, I conducted a linear regression to look at correlation between change in normalised CMCT scores and change in FARS score (n = 42 i.e. 14 patients and 3 datasets) (**Fig 35, table 28)**. The plot implies that there is no strong correlation between % change in CMCT and FARS change when assessed at 6-month intervals, most likely due to FARS not being sensitive to changes over 6 months.

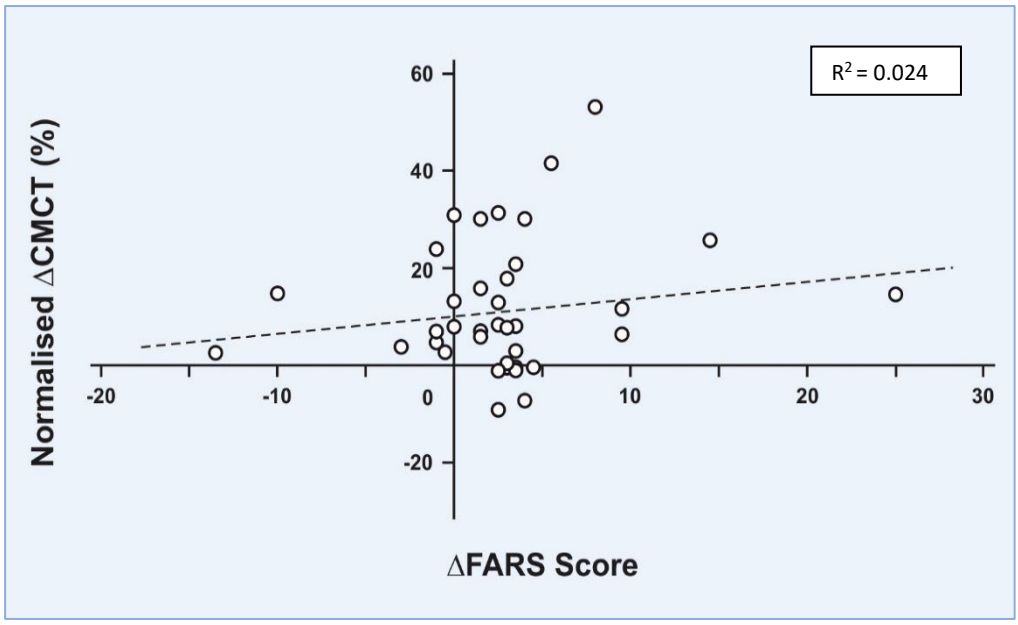


Figure 35. Correlation between % change in CMCT with FARS expressed with R^2.

The dashed line plotted is least squares regression line. It determines the line of best fit for the data between the two variables, providing a visual demonstration of the relationship between the data points. The two sets of data show an appropriate trend in the linear direction.

SUMMARY OUTPUT	**Δ FARS score with Δ CMCT scores (normalised) over 4 visits**
Multiple R	0.35
Standard error of Coefficient	0.39
R Square	0.024
Observations	42
Standard error for Y estimate	14.34
F statistic	0.79
Degrees of freedom	36
Regression sum of squares	163.1
Residual sum of squares	7397.65

Table 20. No significant correlation between % Δ FARS score with Δ CMCT scores (normalised).

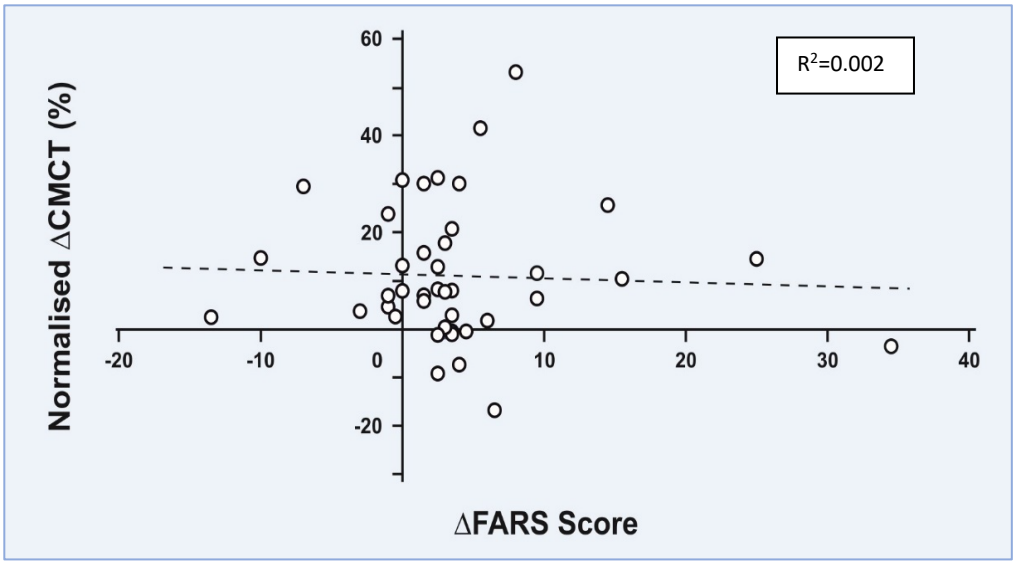


Figure 36. Scatterplot showing the further drop in correlation between % change in CMCT with FARS; after including clinical data from patients who improved with physiotherapy and those with scoliosis correction surgery.

(n=51, with 17 patients x 3 datasets)

There was also no strong correlation between CMCT and FARS over the timescale of my study. This could be due to various accounts - the confounders

which found their way into clinical scoring (see section on FARS results), the FARS scale not being as sensitive as CMCT, and the small subject numbers.

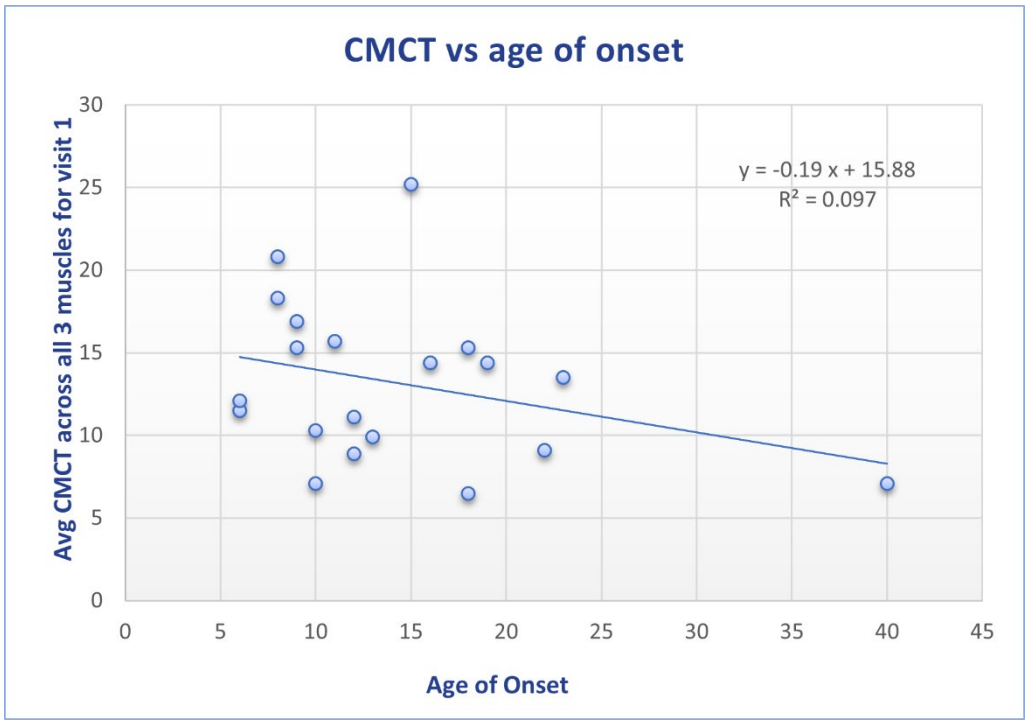


Figure 37. Effect of Age of onset on CMCT (R = -0.31; p = 0.18).

As we hypothesised, a progressive disorder where there is spasticity, MEPs should be abnormal and continue to deteriorate in parallel with clinical worsening. There is no precedent set in research of sequential testing of motor evoked potentials in FRDA or its association with other variables. Baseline CMCTs were averaged across all three muscles and correlational studies were performed with disease duration, GAA 1 and age of onset. Unsurprisingly, CMCT correlated only with disease duration (p=0.03) and not with age of onset or GAA triplet size expansion **(Figures 37, 38 and 39)**.

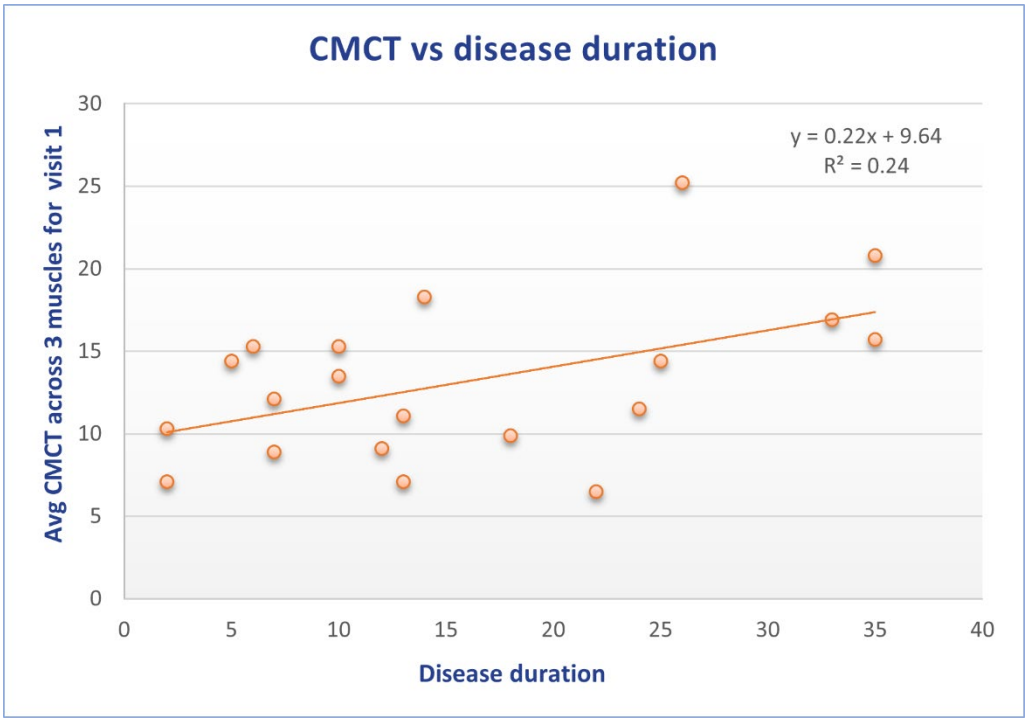


Figure 38. Effect of disease duration on CMCT (R=0.5; p=0.03).

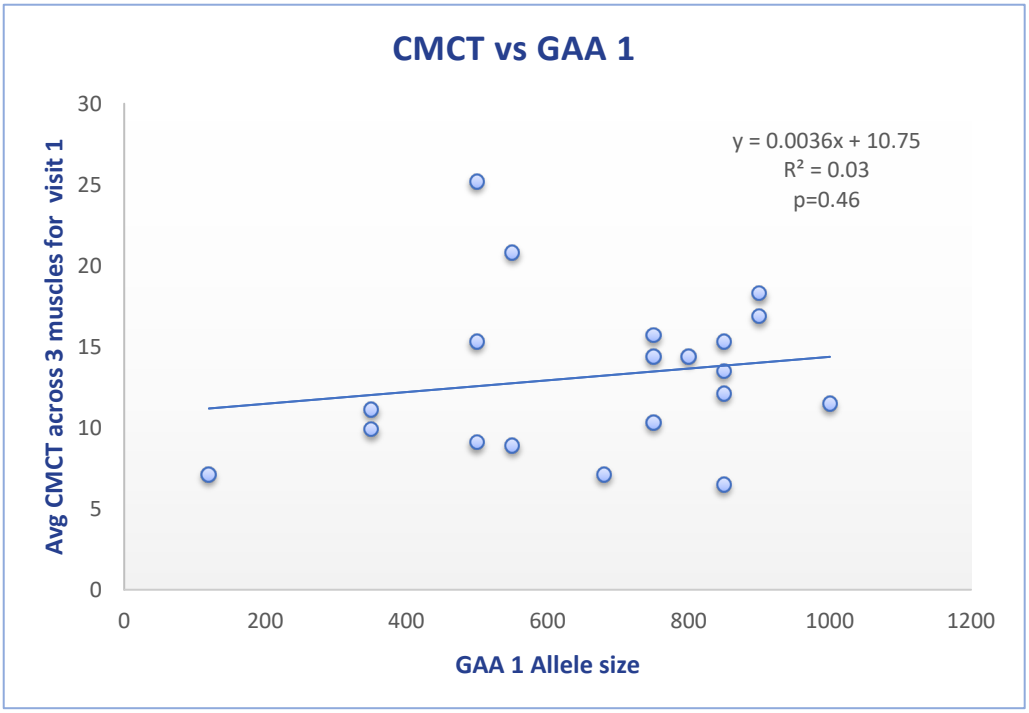


Figure 39. Examining the correlation between GAA 1 allele size and CMCT.

My results are consistent with progressive pyramidal and cerebellar pathways involvement, evidenced by progressive changes in MEPs and FARS respectively over time, as the cause of clinical worsening in FRDA, a pathology which is detected after several years of disease (Cruz-Martinez & Palau, 1997b; I. H. Harding et al., 2020; Naeije et al., 2016, 2021). By contrast, anomalies in DRG and posterior columns of the spinal cord that underlie afferent ataxia are already pronounced early in the course of FRDA and may be, at least in part, developmental. Clinical decline between successive examinations that I conducted, was not compellingly relevant to peripheral nerve abnormalities, which form a main subcomponent of FARS questionnaire. Also, factors other than size of GAA repeat expansion must be influencing phenotypic variability of FRDA.

One potential limitation of the method was that I used average onset rather than earliest onset of latencies , which is different from the study we based our reference values on (Eisen & Shtybel, 1990). However, the MEPs were so significantly delayed in this patient group that I do not think this would have had a major impact on the interpretation of the data; the difference in onset latency for the 2 methods would be of the order of 1-2ms and central motor conduction times in this patient group were an order of magnitude longer. I also elected to pool all the data available by averaging across muscles. Previous publications, for e.g., (Osei-Lah & Mills, 2004)have shown that increasing the number of muscles used to measure CMCT increases the sensitivity of the test.

SUMMARY:

FRDA patients in this study had abnormal MEPs, with prolonged CMCTs (and decreased amplitudes), which worsened over time. These findings mirror the neuropathology, which has shown a reduction in the size of the medullary pyramid, a paucity of myelinated fibres and marked atrophy of the corticospinal tract (Koeppen et al., 2017; Selvadurai et al., 2016), and would lead to desynchronized MEP responses. MEP findings in FRDA thus resemble the upper motor neuron lesion of amyotrophic lateral sclerosis and suggest that a similar "dying back neuropathy" is at work.

I did not quite have enough sampling points (42 with n = 14) to detect a significant change in CMCT over 6 months at successive visits (between second to third and third to fourth visits). However, my data suggest that with that, a sample size of n = 20 – 40 might be sufficient to demonstrate a meaningful change in MEPs over a short interval of 6 months. The results also highlight the temporal course of neuropathological changes in FRDA and variable degree of involvement of proprioceptive, cerebellar, pyramidal, and extrapyramidal systems and the need for multi-evaluation outcome measures. CMCT is the only TMS-based marker which aims to assess corticospinal tract function in isolation, and with relatively well defined neural substrates, making it a sensitive defining and monitoring criteria for FRDA.

7.9 BIMC

Coherence was estimated from EMG recordings performed during a precision grip task (upper limb) or a foot rocking task (lower limb). Data analysis was performed in Matlab (Mathworks, Natick, MA) using custom scripts.

Raw data were visually inspected and the first 100 adequately performed trials examined further. Analysis focussed on the early hold phase of the contraction where beta-band oscillations are known to be maximal **(Figures 40, 41 & 42)**. The wide anatomical spacing between the paired muscles minimised the risk of volume conduction causing inflated coherence values (Grosse et al., 2002).

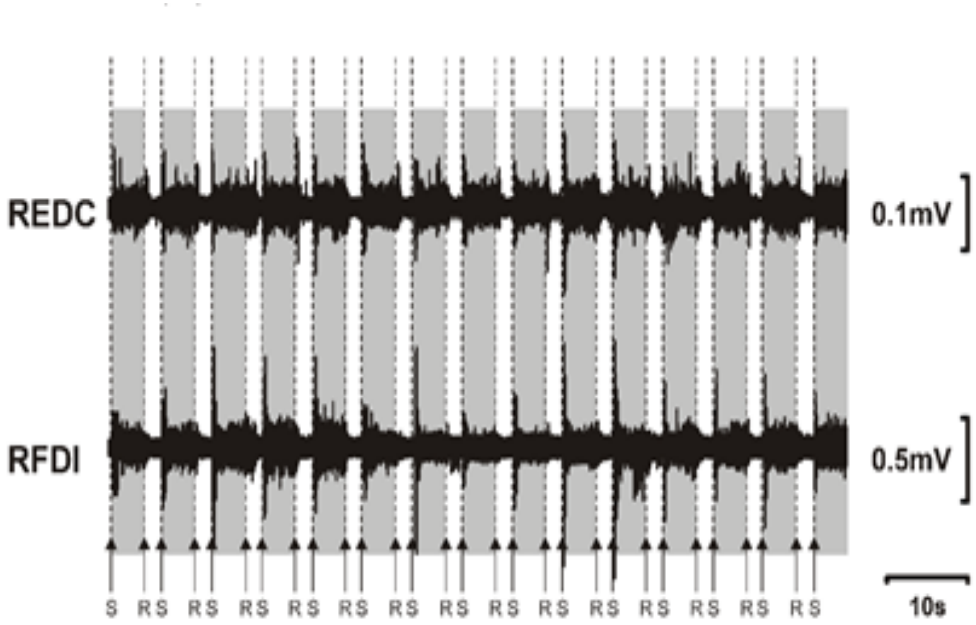


Figure 40. Raw EMG data from a patient performing the BIMC task.

EMG from each mucle, here EDC and FDI, modulated with the task cues, 'squeeze' (S) and 'relax' (R), producing 4s of contraction alternating with 2s of relaxation, and at least 100 repetitions of the 6s task.

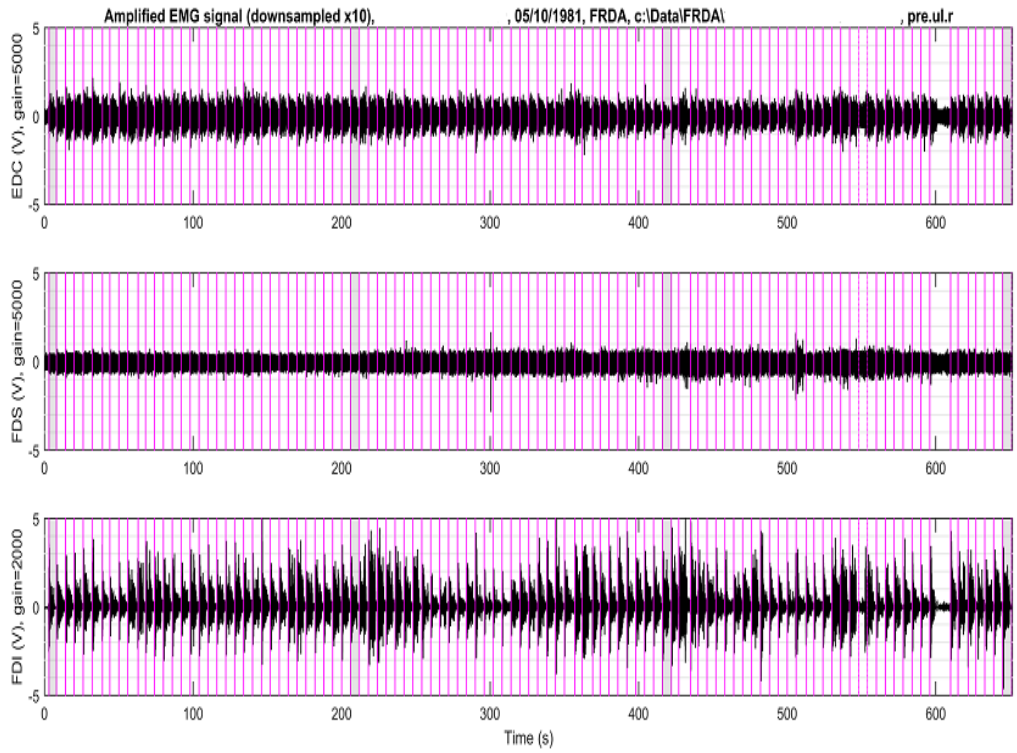


Figure 41. An example of a complete EMG dataset acquired from one patient who completed the upper limb BIMC task. Data are plotted in Matlab.

FDI EMG signal is usually of higher amplitude and shows better modulation than EDC and FDS. Data per limb were collected over 10 minutes (6 s x100 trials), visually inspected and any noisy signals were removed from analysis.

For the upper limb, coherence spectra were calculated between muscle pairs EDC and FDI, as well as between FDS and FDI; for the lower limb, they were computed between MG and EDB, and between TA and EDB. In each subject, coherence was averaged across the 15-30Hz window. The lower limb data has not been included for analysis in this study as any peak in coherence band was much less distinct or absent in lower limbs.

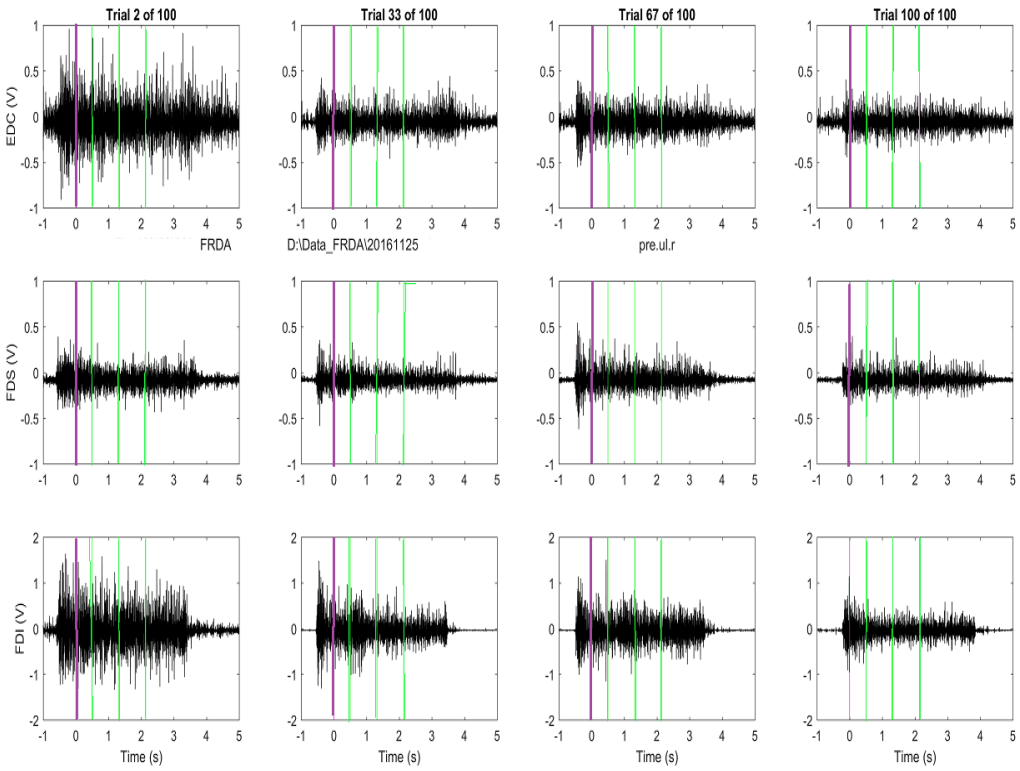


Figure 42. Capturing EMG on the early hold phase of the contraction.

The cued contraction phase of the task is represented by the grey boxes. The matlab automated windows during the hold phase are indicated by the three vertical green lines. These mark the 2 second epoch when EMG is stable or stationary and is the window of analysis. The vertical pink line marks the start of the task (time zero) cued by a high tone. Many subjects showed a drop-off in EMG activity, so the last ~ 1.5s of the 4s active phase did not enter the analysis.

The significance level for averaged coherence was determined using the method described by Benignus in 1969 and later by Evans and Baker in 2003 (Benignus, 1969; Evans & Baker, 2003b). Raw BIMC results are shown in Table 29, where average intermuscular coherence across the beta band is represented by a single number for comparison with the 5% significance level **(Table 29, Figures 45 and 46)**. These data were used in the ROC analysis (see below).

Subject no Study Visit 1	Mean coh EDC-FDI	Mean coh FDS-FDI	Significance level (5%)
1	0.029	**0.096**	0.015
2	0.044	**0.017**	0.014
3	0.007	0.007	0.015
4	0.008	0.007	0.019
5	0.009	0.009	0.017
6	0.006	0.009	0.015
7	0.012	0.005	0.016
8	0.018	**0.031**	0.015
9	0.007	0.007	0.015
10	0.004	0.006	0.014
11	0.005	0.006	0.017
12	0.005	0.008	0.015
13	0.008	**0.017**	0.014
14	0.015	0.006	0.015
15	0.007	0.009	0.015
16	0.007	0.004	0.015
17	0.012	0.008	0.015
18	0.017	0.008	0.016
19	0.018	0.008	0.015
20	0.005	0.006	0.015
21	0.005	0.004	0.016
22	0.007	0.005	0.016
23	0.031	**0.081**	0.015
24	0.048	**0.062**	0.015
25	0.008	0.007	0.015
26	0.009	**0.568**	0.016
27	0.041	**0.049**	0.015
28	0.006	0.006	0.016
29	0.024	0.020	0.032
30	0.007	0.008	0.015

Table 21. Baseline visit data of mean coherence of upper limb muscle pairs.

The significance level for averaged coherence for particular muscle pairs was determined using published methods. Where average BIMC is greater than the significance level the result is shown in bold (8 out of 30 patients). These data were used for the ROC analysis (see below), which revealed a good separation between patients and healthy controls for BIMC.

For comparison, I used a repository of healthy control coherence data collected previously in the Baker lab. Coherence was significantly lower in FRDA as demonstrated in Figure 43 (and Table 29); rarely some more able subjects had a coherence of more than 0.1.

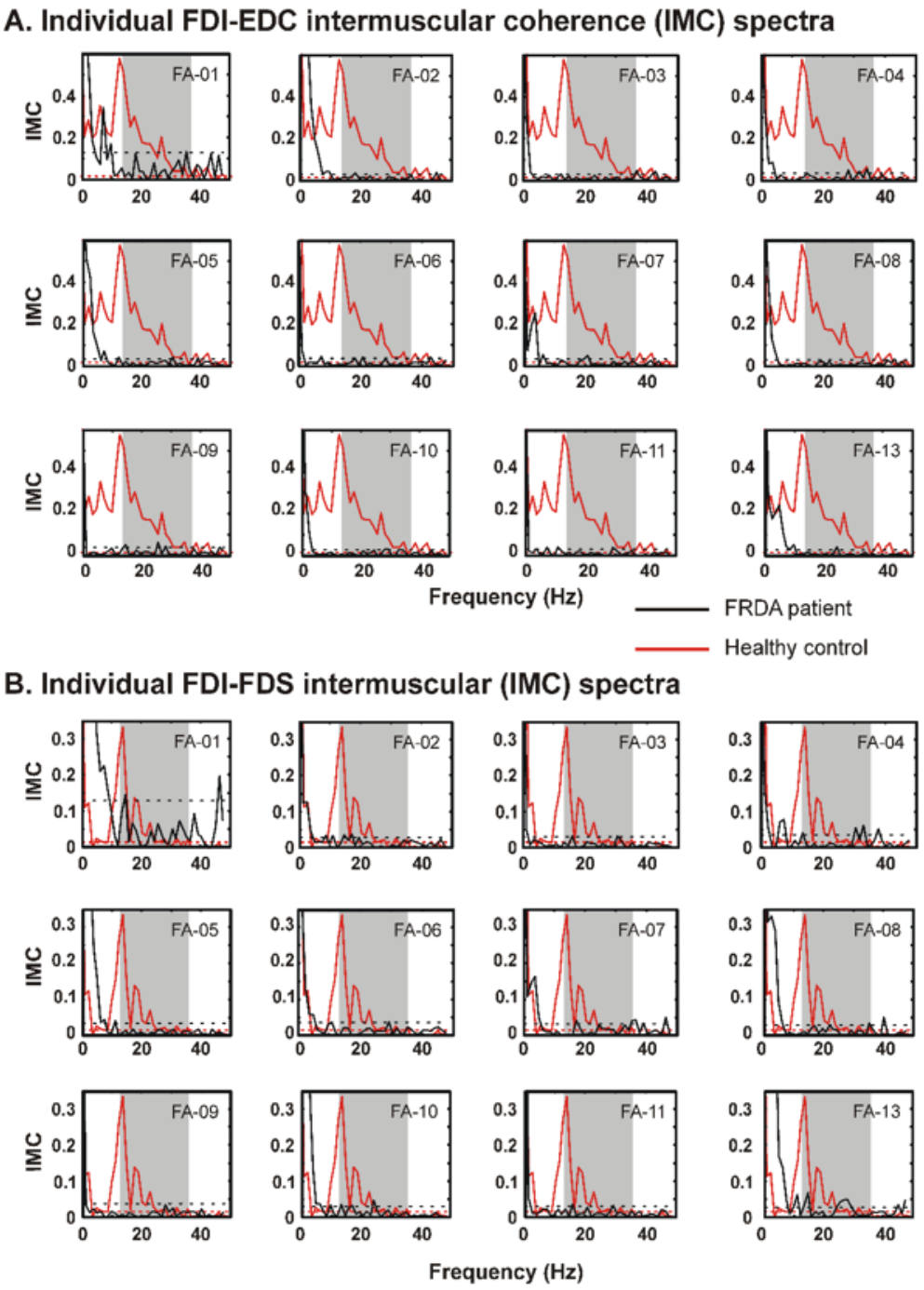


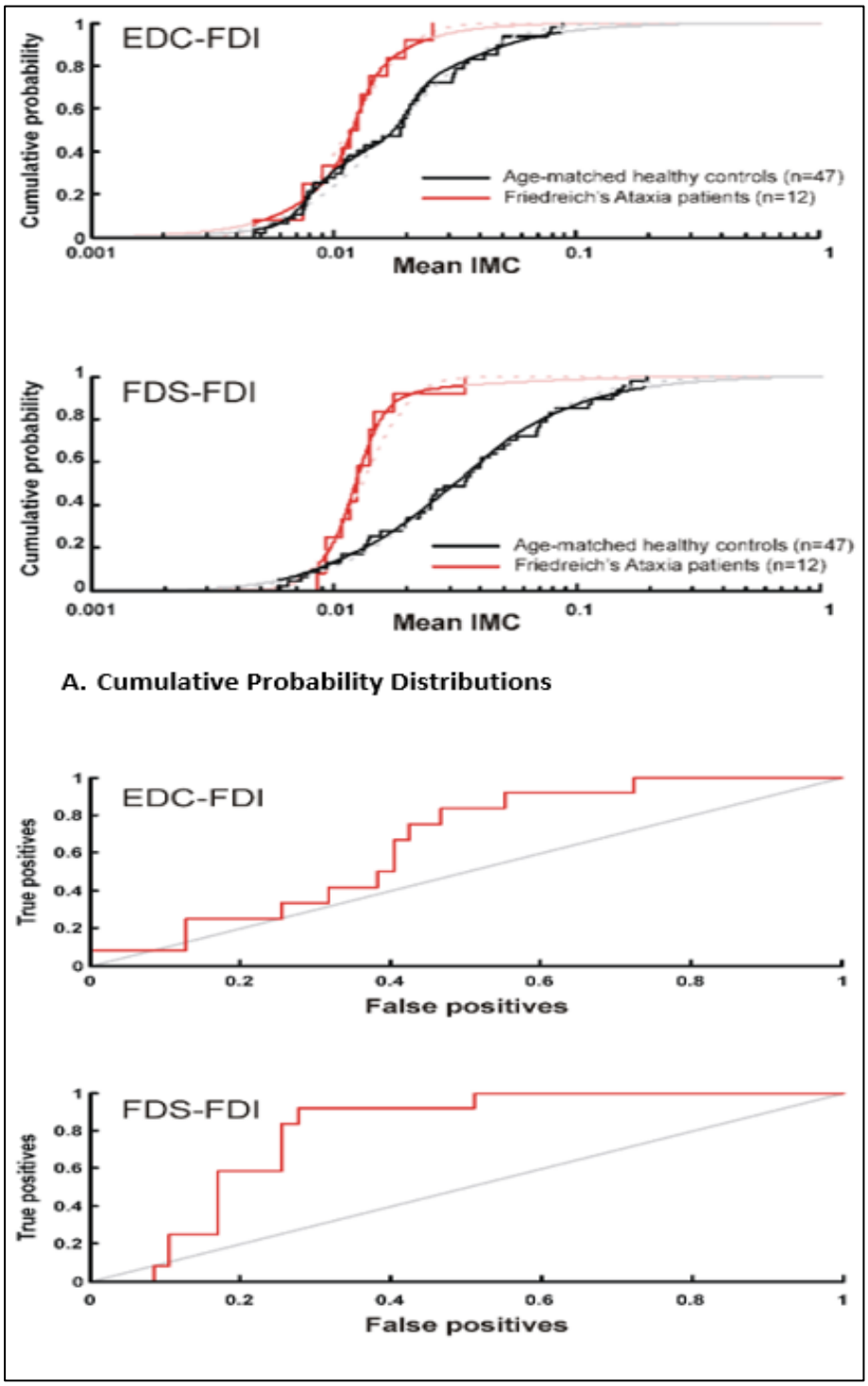
Figure 43. Comparison of control EMG-EMG (intermuscular) coherence spectra with FRDA patients for A. FDI-EDC pair and B. FDI-FDS pair.

The horizontal dashed line indicates the 5% significance level for average coherence. The grey box delineates the boundaries of the beta band (13-35 Hz). In a normal subject (red), coherence spectra shows a clear peak in the 15-30Hz band for each muscle pair. In FRDA subjects (black), average 15-30Hz coherence was decreased in both the muscle pairs, mostly below the significance level.

Figure 43 (A and B) show individual coherence spectra from 12 FRDA patients compared with a single control subject for the purpose of illustration (red is control and black are FRDA patients).

Figure 44 summarises the performance of BIMC as a diagnostic test in FRDA patients. Mean BIMC cumulative probability distribution plots for patients (n=12) and age-matched controls (n=47) are shown in **Fig 44 A** for each muscle pair and show clear separation between controls and patients (note that data have been plotted on a logarithmic scale). By taking the area under the curve of the cumulative probability distributions, it is possible to construct receiver operating characteristic (ROC) curves for each muscle pair **(Fig 44 B)**.

The closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test and hence its diagnostic ability. The ROC curves reveal a good separation between patients and healthy controls for coherence calculated from particular muscle pairs, with better performance for the FDS-FDI pair.



B. Receiver Operating Characteristic Curve for BIMC for both muscle pairs.

Figure 44. BIMC was significantly reduced in FRDA patients compared to controls (A). Coherence was mostly lower than normal, reflected in ROC curves lying above the diagonal (B).

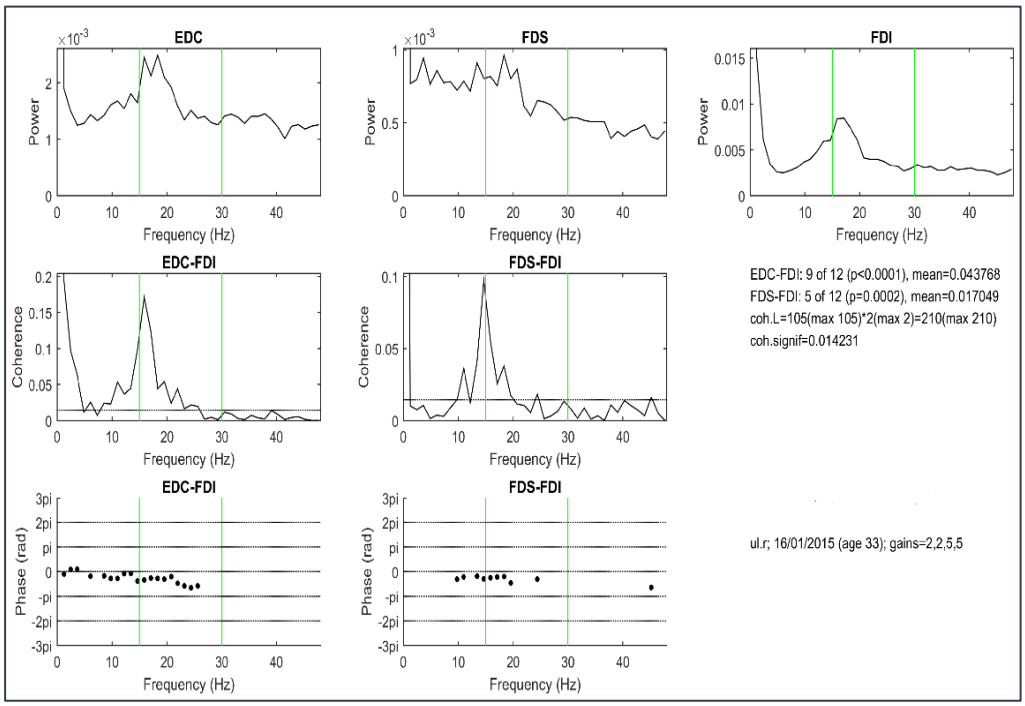


Figure 45. Example spectrograms of normalised EMG power and mean coherence calculated for the muscle pairs EDC-FDI and FDS-FDI from a patient with significant beta-band coherence.

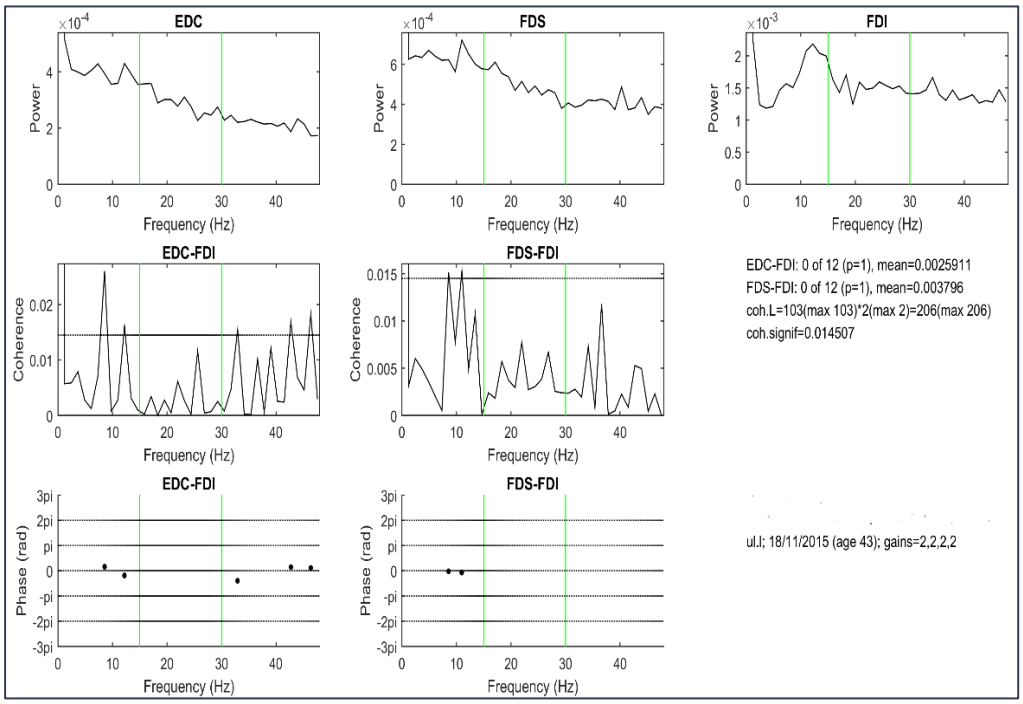


Figure 46. Coherence and power spectra from a patient with advanced disease (avg. coh =0.02) to illustrate the difference compared to the functionally independent patient in fig 45.

The subject with significant coherence in figure 45 was able to mobilize with support, had a total FARS score of 58, with GAA1 repeat size = 500 and GAA2 = 950. Whereas the subject in the latter figure was confined to wheelchair, with a total FARS score of 105, GAA1 size = 800, GAA2 size = 800. The overall distribution was shifted to lower coherence values in patients and could not easily discriminate between the clinical status of the patients.

One of the main hypotheses of my study was that intermuscular coherence would get worse with time and thus, higher clinical score. So far there is very limited literature and only corticokinematic coherence has been looked at in FRDA patients with cortical magnetoencephalography with finger movements, in a single study, as a potential marker of proprioceptive pathways degeneration (Gilles, Brice, et al., 2016).

I looked at the association of mean BIMC for both muscle pairs with FARS score **(Figures 47 and 48)**. It showed an inverse trend as expected. It might show a significant correlation at the population level but not at the individual level. This confirms that FARS scores cannot predict BIMC, except possibly in the very early stages of the disease.

There is data collected for BIMC from the successive three visits, as with other parameters. It would be important to examine this longitudinal data to assess other temporal disease aspects, as well as to demonstrate reproducibility. To keep the study's statistical power, I analyzed and compared only the first visit data from 29 subjects for the purpose of this thesis.

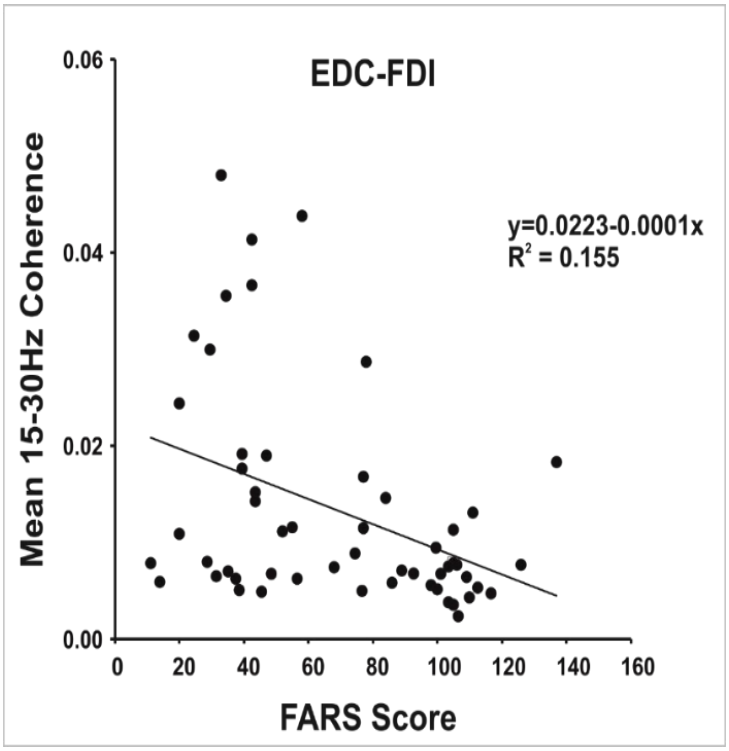


Figure 47. Mean upper limb coherence (EDC-FDI) vs change in FARS score over 24 months.

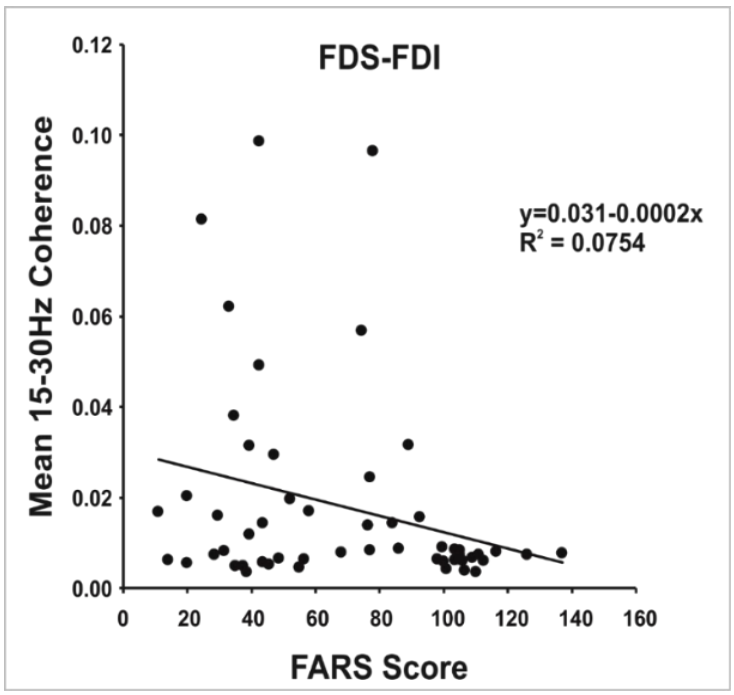


Figure 48. Mean FDS-FDI coherence vs change in FARS score over 24 months.

The charts show an inverse trend but no significant correlation.

SUMMARY:

In patients with FRDA, BIMC was significantly reduced compared to controls. Moreover, only patients with mild/early disease had significant BIMC. There was a negative association between mean coherence in the beta-band in upper limb muscle pairs and FARS score but no significant correlation for either muscle pair.

BIMC did not stand out as a robust test for discriminating CST dysfunction in this patient group. This highlights the role of sensory information in BIMC, and the need to establish optimal recording/processing methods for calculating BIMC in experimental context. Although it was not statistically deduced, patients with higher FARS score had advanced proprioceptive loss in most subjects in the study cohort (as determined by findings on NCS consistent with sensory neuronopathy), and the same patients had extremely diminished averaged coherence for both muscle pairs. It would be intriguing to see if the relationship between clinical scores, sensory studies and BIMC continued as the condition progressed.

So far, BIMC seems more ideally suited to regular screening of asymptomatic/recently diagnosed patients with a genetic diagnosis of FRDA to determine the onset of subclinical disease.

8 GENERAL DISCUSSION

This is the largest study in FRDA population (both paediatric and adults) to date in the North-East of England and an ambitious attempt to open up new modes of investigation. The work presented in this thesis aimed to further the development of electrophysiological biomarkers. We investigated a novel biomarker, beta-band intermuscular coherence (BIMC), together with central motor conduction time (CMCT), a marker based on transcranial magnetic stimulation (TMS) whose neural substrates are relatively well understood. This work confirms the importance of non-invasive methods to be used along with comparison of clinical signs or scores, but also provides a number of cautionary tales for those using these techniques.

8.1 DISCUSSION OF RESULTS

The small sample size for final computations was certainly a shortcoming related to the present research. The results showed high variance in terms of SD and range, for MEP latencies and FARS, and extremely high for Frataxin levels. Given the scarcity of patients, they were not selected randomly from the general population. The cohort comprised a large range in terms of age, length of illness, rate of disability progression and personally significant events in participants, which were out of bounds for the study. These factors and the intrinsic variability of the condition dramatically reduced the statistical power

and therefore, the data cannot easily be generalized to all Friedreich's ataxia patients.

TMS MEPs:

The overarching finding from the analysis is that MEPs could provide a reliable method of detecting a significant subclinical treatment effect in short duration (6-12 month) in small sample (n=20 - 40) trials, and also act as a TMS-based marker of monitoring disease progression in FRDA.

The literature yields very little information and interpretation regarding the presence and evolution of TMS-based measures in FRDA. Studies conducted in hereditary ataxias have been somewhat variable, probably partly attributable to differences in study populations, TMS methods and gold standards for determining normal ranges. Despite TMS-based measures being explored in Friedreich's ataxia as early as the 1990s (Cruz-Martinez & Palau, 1997b; Martínez & Anciones, 1992a), they have not entered routine clinical use. Nonetheless, they have proven to be the best available electrophysiological biomarker of disease progression in FRDA in my study.

The conventional anatomical selective measures such as NCS and SEPs do not work well in monitoring evolution of disease as these do no worsen significantly once established.

CMCT AS A MONITORING TOOL

Prolongation of CMCT has been documented in various disorders such as demyelinating diseases (multiple sclerosis, MS), amyotrophic lateral sclerosis, structural lesions in the corticospinal tract such as stroke and compressive myelopathy, and neurodegenerative disorders including multiple system atrophy and progressive supranuclear palsy. It has been shown to mainly have a diagnostic value in these conditions, when used together with other clinical and electrophysiological measures(Udupa & Chen, 2013).

Only rare reports have shown its ability as a prognostic marker in some of conditions, such as myelopathy and MS. Findings from a study conducted on progressive MS suggested that prolonged CMCT was related to spinal cord lesion load and changes in the CMCT occurred only when spinal cord lesions appeared. Clinical change may therefore occur without either the development of new lesions on MRI scans or an increase in CMCT, possibly through progressive axonal degeneration (Kidd et al., 1998).

A singular study on cerebrotendinous xanthomatosis (CXT) showed that CMCT provided a useful tool for monitoring the effects of replacement therapy with chenodeoxycholic acid (CDCA) for spasticity and pyramidal dysfunction (Mignarri et al., 2011). One group evaluated cervical spondylotic myelopathy (CSM) patients both clinically and with TMS MEPs, pre and post-surgery, suggesting that early surgical intervention could produce a beneficial effect on spinal cord functionality that can be detected by MEPs (Capone et al., 2013).

BIMC:

BIMC has been shown to be a robust measure of CST function in other conditions presenting with progressive CST dysfunction, such as motor neurone disease (MND) and primary lateral sclerosis (PLS). It is important to note however that sensory afferents are likely to play an important role in both 15-30 Hz corticomuscular coherence and intermuscular coherence (Section 4.3.1). Therefore, any diagnostic inference regarding CST function using coherence can only be made in the presence of normal somatosensory evoked potentials and sensory nerve action potentials. I suppose this was one of the main reasons that BIMC did not turn out in the manner expected in this study.

Recent studies have found reduced intramuscular coherence in patients with incomplete spinal cord injury, reflecting impaired supraspinal control (Zipser-Mohammadzada et al., 2022), and one has looked at IMC in spinocerebellar ataxias (SCA), as a marker of differentiating information between the SCA subtypes (Issa et al., 2024). IMC was significantly lower in SCA3 patients than in controls, consistent with the mixed cerebellar, corticospinal, and peripheral nerve dysfunction found with SCA3, but not significantly altered in SCA6 patients, in whom pathological changes are primarily cerebellar with minimal damage to the corticospinal system or peripheral nerves. This also verifies that low IMC represents a lack of integrity in the corticospinal tract or proprioceptive loss or both systems.

The next step therefore would be to carry out a prospective study in a group of patients with very early disease (or indeed those with the gene mutation who are asymptomatic). This would enable us to test the sensitivity and specificity of coherence as a diagnostic test (composite screening for both sensory neuropathy/neuronopathy and CST disease) and to determine at what disease stage changes in coherence become apparent.

Within a few years, the neural substrates of BIMC generation will have been clarified and methods of measuring BIMC will have seen further optimisation, thus nurturing the hope that it could ultimately become a widely used biomarker of pyramidal and may be also for sensory neuropathy /ganglionopathy function.

FARS:

Despite FARS performing better than most of the other parameters tested the sensitivity in capturing changes over a period of less than 12 months is only moderate. This probably reflects the sensitivity of the scale, and not necessarily that only a modest amount of true change is seen in 12 months, as shown by steep clinical decline in certain patients and also by change in MEPs.

Additional analyses of longitudinal changes will be necessary to fully ascertain the utility of FARS scoring, especially in older and non-ambulant patients, and how it can be adequately powered to detect small treatment effects.

It also needs to be emphasised that the complete FARS requires more time to complete, and much of the extra information is collected using other instruments in larger clinical trials. In contrast, the mFARS is quicker to administer and the omission of the peripheral nervous system subscore and 2 items of the bulbar subscore in the mFARS strengthens the overall construct compared with the complete FARS. The subcomponents of the mFARS might prove valuable when investigating temporal differences in loss of function in separate domains e.g., upper and lower limb function, or when focusing on later stage, exclusively non-ambulatory patients.

Serum Frataxin:

Serum frataxin levels showed an association with clinical severity but did not predict progression to a great degree, as shown previously. The data was noisy and did not show a good degree of separation from healthy controls. There are a variety of potentially artefactual reasons why erratic results in frataxin were seen over the visits apart from the sample processing alterations in my study. First, frataxin measurements were being made from samples of unaffected tissue (whole blood); frataxin deficiency in affected tissues may be much greater (De Biase et al., 2007). It has also been hypothesized that the level of frataxin drops over time in affected tissues (but not unaffected) because of progressive expansion of GAA repeats in affected tissues (De Biase et al., 2007).

GAA repeat length:

GAA repeat expansions are thought of as dynamic entities, exhibiting both intergenerational and somatic instability of repeat lengths. Although dissecting the effects of age and genetic severity is difficult, GAA repeat length is a major determinant of progression rate in FRDA.

Both routine management of FRDA patients and clinical trials will benefit from using GAA as a function of progression to different clinical milestones, such as the use of assistive devices, the probability of developing other associated medical conditions such as cardiomyopathy and scoliosis, as well as starting point of treatments.

8.2 INSIGHTS GAINED IN CONTEXT OF EXPERIMENTAL DESIGN

The first lesson learned is that careful selection of specific subgroups of patients, e.g., early- versus late-stage, is very important in a disease like FRDA, depending on the trial's chosen outcome measure. Retrospectively, it is easy to see that the initial set of patients recruited was not optimal for the technique of BIMC due to advanced stage of their illness. These dropouts due to absent or very diminished BIMC early on in the study, led to loss of data at subsequent time points, with less than 50% of the subjects completing the final visit, drastically influencing the statistical power of the study. I enrolled more participants than the minimum required sample size (a target of 20 patients suggested by Ataxia UK) to compensate for expected withdrawals, but the study design where BIMC was the primary outcome measure meant that we had no choice but to exclude those and continue with a reduced sample size.

The second key message through this thesis is the importance of understanding the basic physiology underpinning the methods being utilised and how the diagnostic or prognostic biomarker serves the disease in question.

BIMC appears to be a robust measure of CST function, but that cannot be discerned with certainty unless sensory afferents are intact. Hence for the subset of patients selected, it could not perform the role of gatekeeper for the more involved electrodiagnostic tests, as proposed in the study outline. This is one of the very few studies of coherence in patients of FRDA; which has clearly demonstrated that BIMC is better used as a measure of subclinical illness in

early stage FRDA patients being recently referred from an ataxia clinic, but not suited to be a monitoring tool for FRDA.

On a similar note, given the ceiling effects and subjectivity in FARS scoring, an outcome measure that accurately and objectively quantifies changes in gait and stance might be utilised in addition to FARS in future trials. Several studies have validated the use of accelerometers in assessing physical activity levels (Das et al., 2022; Hickey et al., 2016). Such devices allow home study (reducing the cost and burden associated with travel to study appointments), might reduce inter-rater variability and, if used daily, might provide a much more responsive dataset in the early stages of ataxia.

Thirdly, the potential for confounding should be considered in the design and implementation of the study. There were factors introduced beyond our control, that we failed to eliminate and eventually affected the outcome of the study. Usually, patients who seek additional interventions outside of the study protocol are more likely to be excluded from the study due to violation of the study protocol. We were not in a position to do so, neither ethically, nor was effect of exercising or any other intervention built in our study protocol. As highlighted in results, a few patients who were ambulant with support such as linked arms, were undergoing physiotherapy regimes before or around the start of trial, leading to improvement in FARS score (overall reduction of the score) at successive visits; effects were visible by visit 2 and 3. The reduction in upright mobility scores by being able to stand for longer, to further endpoints

(e.g., from 30sec to 45sec) likely by facilitating proprioception, skewed the results to give a negative change and hence weakened correlation with passage of time. Exercise intervention is now being thought to bring about fundamental changes in physiology by promoting the expression of an iron regulator to bypass the defective gene in maintaining normal mitochondrial function. Studies have emerged showing improvement in functional independence measure in FRDA patients post rehabilitation, which was sustained post discharge (Milne et al., 2012, 2018; Paparella et al., 2023). Intensive coordinating training has been shown to enhance motor performance in cerebellar ataxia (Ilg et al., 2009) and also in other forms of spinocerebellar ataxias. Two patients had spinal scoliosis correction surgery which caused a drastic increase in FARS score, as they were entirely confined to wheelchairs by the next visit. Again, this was the right decision for the patients in their respective disease histories.

8.3 PATIENT-CENTRIC TRIALS

In the process of conducting my research, I have come across a palpable sense of energy and purpose within the leading FRDA scientists, expressing ever new insights into molecular, biochemical, and clinical work, in both human and animal models. We now know that several secondary processes are substantially crosslinked to create a true metabolic disease from Frataxin deficiency. Isolating these pathways, targeting them with therapeutic strategies, and evaluating them in human in clinical trials, would logically move us toward a successful therapy. The presence of a huge number of pharmaceutical and biotechnology companies in the field underscores the importance of the public-private partnership that has grown in the past years. Some of these companies are eager to help take newer discoveries through drug development and into subsequent clinical trials, bolstered by an equally motivated group of patients and carers.

As researchers, we cannot hope to design truly patient focused trials without listening to patients as much as possible. In order to maximise recruitment and retention of participants in rare neurodegenerative diseases, it is paramount to understand their unmet needs, their opinions on acceptability of investigations, time commitments, routes of drug administration, fear of side effects, concerns about stopping current medications etc. Travel burden, both in terms of distance and cost, was a clear deterrent in my group of subjects where mobility was often severely affected. Again, cost reimbursement for travel and

subsistence as we offered, or availability of a travel assistance coordinator would likely improve recruitment. Additionally, consideration of use of wearable devices for home data collection will reduce travel burden for trial participants.

Ethical policies seek to protect the patient from risk, but few have ascertained the attitude to such risk, of patients with inexorable or terminal conditions, for which no mitigating or curative therapies exist. The digital environment post covid has opened up immense opportunities of patient engagement. By employing virtual approaches and cutting need to travel, they have much greater flexibility in being able to do research at a time of their choosing. Even a patient who cannot get out of the house or it takes them two, three hours to just prepare themselves to leave home, can extend their perspective to the regulatory agencies and ethics committees, upon whom rests the eventual decision as to whether a trial can proceed.

Since I examined patients clinically as part of this study, there were exchanges about their concerns and what they would most like to see the treatments address. Symptoms such as vision impairment, hearing loss, swallowing difficulties, issues with dexterity, and the lesser quantified urinary disturbances, bowel and sexual dysfunction are more prominent in late-stage disease and generally overlooked. Also, absence of distal sensation and foot deformities due to repetitive pressure and contractures, which are very common in FRDA, frequently leads to ulceration. These patients would truly

benefit from comprehensive and timely assessments by physical therapists, podiatrists, speech pathologists in a well organised multi-disciplinary setting. Appropriate biomarkers would propel the fast-evolving future clinical trials in FRDA, which may focus on specific subgroups, potentially targeting with specific therapeutic interventions, or more diverse cohorts with broader therapeutic interventions.

9 FAMILIES LIVING WITH FRIEDREICH'S ATAXIA

The news that a child has a genetic, lifelong illness is always devastating for parents, but the diagnosis of a condition like FRDA is particularly overwhelming. Because FRDA is usually diagnosed in children before their teen years, explaining the condition and helping them understand what to expect in the future becomes one of the first challenges a family faces. The disease progresses and affects many different parts of the body, causing disabilities such as scoliosis, diabetes, hearing and speech problems, and heart conditions. This means that a family will need to coordinate healthcare with many different specialists, from orthopedic surgeons to speech therapists. Also, because the disease is rare, the families are unlikely to know someone else who has it.

During the clinical consultations and history taking, I found out that because of its rarity, even physicians may need to be educated towards establishing a diagnosis of FRDA and the current standard of care. For example, one of the 13-year-old participants who underwent correction of spinal scoliosis surgery, termed as 'idiopathic' was diagnosed with FRDA only when the parents approached the surgeon that the child was bumping into things and falling over more after the surgery. The surgeon became suspicious and prompted a Neurology referral, which led to the diagnosis, about 5 years after the onset of symptoms.

This work gave me first hand eye-opening experiences of the highs and lows of being a full-time carer, and of those facing day to day disabilities and uncertainty. The patients, especially those in their teens, despite their need for autonomy and independence and their efforts to be as independent as possible, are constantly dependent on their carers or companions. They risk living in a state of denial and becoming isolated. Nevertheless, most patients and relatives I met with were a galvanized bunch, who always seemed keen to explore opportunities to participate in clinical trials as well as bring awareness to the disease. Many people in the cohort were surprisingly productively employed (one even was a wheelchair-bound football coach), and students were keen on pursuing their studies with help from educational authorities. I would get a common query about accessing physiotherapy in the community, especially for more recently diagnosed, but specialist paediatric physiotherapy services are even rarer I found. Younger women had concerns about effects of pregnancy on their condition, and their ability to raise a family successfully with advancing symptoms. The more able ones seemed keen to have an active social and outdoor life (one followed rock climbing as a hobby) but were sceptical of the perception of public due to unawareness (e.g., being taken as an alcoholic due to gait and speech disturbance).

This proactive spirit has led to creation of an almost world-wide FRDA community with strong networks, including patient organizations, local support groups and national fundraisers, which is also one of the reasons things have moved so fast with the trials. There has also been a tangible positive shift, with

industry and funders engaging patients in the process to define the motivations for and barriers to trial participation, as well as their condition-specific trial preferences.

Ataxia UK and FARA charities have many resources available to help meet the practical challenges of living with FRDA. They assist with some of the issues including accessibility in the workplace and school, travel, coping with everyday activities, employment and education opportunities, navigating medical insurance systems, and getting updates on available assistive technologies.

I must make a mention of 'The Ataxian' - a documentary which captured what was a grueling 9-day non-stop journey for anyone - much more for someone with an energy-depriving affliction. The hero here is Kyle Bryant who was diagnosed with Friedreich's ataxia aged 17. As Kyle lost his ability to play his favourite sports and even walk, he began biking long distances in a specially outfitted "trike." When he was finally relegated to a wheel chair, Kyle decided that enough is enough. He enlisted the help of three friends, Sean, who also has FRDA, John and Mike, and they embarked on the "toughest bike race in the world," the Race Across America (RAAM). The outcome inspired a movement known as rideATAXIA , a nation-wide program of bike rides in the US, that welcomes people of all abilities to ride, and to raise funds for their mission to treat and cure FRDA through research.

London city bridges challenge 2016 -Ataxia UK

10 APPENDIX

A. SOP for PBMC Separation by Ficoll Gradients:

Reagents:

Ficoll-Pacque Plus: Fisher, 45-001-750 (density providing reagent)

DPBS w/o Ca2+/Mg2+: Invitrogen, 14190-250

(Dulbecco's Phosphate-Buffered Saline (DPBS) is a balanced salt solution containing potassium chloride, monobasic potassium phosphate, sodium chloride, and dibasic sodium phosphate).

Freezing media:

10% DMSO (Dimethyl Sulfoxide)

90% FCS (Fetal Calf Serum)

Procedure for donor blood in tube:

*** Perform all steps in tissue culture hood using aseptic technique.**

*** Pre-warm some PBS in an incubator (37 degrees).**

*** Prepare fresh freezing media and store chilled (4 degrees).**

1. Add 40 ml of DPBS in three separate 50 ml falcon tubes.

2. Leave them in an incubator or water bath for 15-20 minutes to prewarm (37 degrees).

3. Put another 15 ml of DPBS in 4th Falcon tube.

4. Label 3 empty Falcon tubes with a patient's name.

5. Pour 15 ml of Ficoll-Pacque plus in one patient labelled Falcon tube.

After Prep is done:

6. Spread paper towel in the hood and place tube rack on it.

7. Transfer blood out of 2 test tubes into the 2nd patient labelled falcon tube - rinse out blood tubes with PBS to maximise recovery.

   Make the total volume up to 20 mls (For e.g., if it is 12.5 ml of blood, add extra 7.5 mls from the 4th Falcon tube.

8. Gently invert 5 times to mix the blood with PBS (solution becomes frothy).
9. Now place together the 20 ml blood sample and falcon tube containing 15 mls of Ficoll pacque reagent.
10. Using a 10-ml pipette (attached to pipette controller), set the gun to slowest speed 'G' (gravity), so the sample trickles out and layers gently.
11. Hold the pipette at 45-degree angle at the mouth of Falcon tube. Slowly and gently overlay the Ficoll with max 20ml diluted blood.

    Be careful not to disturb the Ficoll layer when transporting the tubes.

12. Centrifuge: 400 x g for 30 minutes with slow acceleration and **no brake** (to stop gradually ~30 mins). (Eppendorf Centrifuge 5810)
13. Gently take the centrifuged sample out. Place it with one empty patient labelled falcon and one labelled for waste on the rack.
14. Remove most of the top layer (plasma) leaving a small amount to help distinguish PBMC (buffy coat) layer using another 10 ml pipette
15. With a Pasteur pipette, take the plasma and buffy coat layer and transfer it into patient labelled falcon. Take care to remove the entire layer from both the edge and centre of the tube.
16. To this tube, add DPBS from incubator to the 40 ml mark. Invert and mix softly. (This is the first wash).

17. Centrifuge: 100 x g for 10 minutes - brake on and high acceleration (max ~ 9) from this point.
18. Aspirate PBS off (pellet is very loose and at the bottom of the tube, leave some supernatant (~ 5 ml) mark to avoid cell loss), taking care not to disturb the pellet.
19. Resuspend in more of the warm PBS to reach the 40 ml mark. Invert the tube and forcefully mix the pellet. This is the second wash.
20. Back in Centrifuge: 100 x g for 10 minutes
21. Repeat steps 18-20. This is the third wash. After this spin, the supernatant should be clear. Perform more washes if not.
22. Take the sample out of the centrifuge. Prepare a cryovial with 1.5 ml of freezing media. Label with patient's name, date, visit number and my name.
24. Remove all the excess DPBS, leaving behind approximately 0.5 ml in the tube (taking care not to disrupt the pellet)
23. Add a few drops (0.5 ml) of freezing media to the tube with a Pasteur pipette. Pipette up and down a few times to resuspend/dislodge the pellet.
24. Transfer all contents of the falcon tube to the cryovial (avoiding bubbles).
25. Place cryovial in -20 °C for short term and then transfer to -80 °C for long term storage.

B. Skin Fibroblast Proteomic Profiling

Below are the clinical characteristics of 10 patients, who had their cultured skin fibroblasts looked at for proteomics analysis. FRDA fibroblasts from mild and severe cases were compared with controls to identify and alteration in proteins.

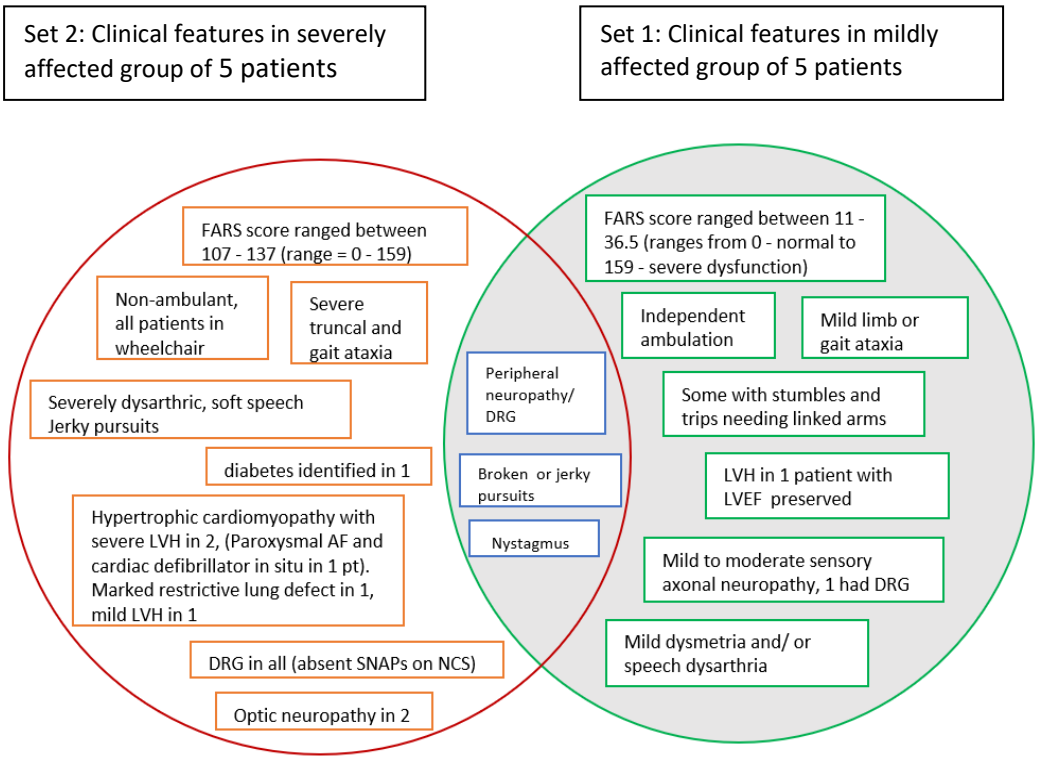


Figure 49. Clinical characteristics and FARS scores of 2 subsets of FRDA patients.

The proteomics part of the study was in collaboration with Leibniz Institute for Analytical Sciences – ISAS –e.V., Dortmund, Germany. Initially, in-vitro studies were performed on the SH-SY5Y cells (human neuroblastoma cell line) depleted for Frataxin-expression and 43 proteins were identified with altered abundances. Next, this data was intersected with the proteomic profiling data obtained from the Frataxin-patient-derived fibroblasts. Five proteins were identified which might represent (according to their fold of regulation) modifying factors determining disease severity **(Table 30).** Unfortunately, the p-Anova in most of the cases (fibroblast data) was not statistically significant, thus requiring additional immunoblot or immunofluorescence studies to confirm the proteomic findings. Confirmational studies need to be performed.

Proteins		Frataxin-depleted SH-SY5Y cells			Fibroblasts mild patients			Fibroblasts severe patients		
Acc #	Name	Fold of regulation	p-Anova	Peptides	Fold of regulation	p-Anova	peptides	Fold of regulation	p-Anova	peptides
P60660	Myosin light polypeptide 6 (MYL6)	0.56	0.00	10	0.71	0.03	8	0.56	0.12	8
Q07021	Complement component 1 Q subcomponent-binding protein, mitochondrial (C1QBP)	0.58	0.00	9	0.86	0.66	9	0.63	0.25	10
P02765	Alpha-2-HS-glycoprotein (FETUA)	1.81	0.00	4	1.56	0.26	2	0.79	0.89	2
P23142	Fibulin-1 (FBLN1)	1.91	0.00	2	0.32	0.01	6	0.23	0.01	1
P17096	High mobility group protein HMG-I/HMG-Y (HMGA1)	2.33	0.00	2	1.15	0.31	2	1.51	0.19	2

Table 22. Preliminary data – alterations in proteins identified on Frataxin depleted SH-SYSY cells and FRDA fibroblast of severe and mild patients.

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Spin

Mama let's spin!
Furniture, canes, rollator frame
turn them all in.

The scourge of my skates and skis,
backpacking and cycling -
in limbo of my metamorphosis.

Aah! Two pairs of dainty wheels
A lightweight and portable mean machine
No more colluding for fleshy limbs.

Cannot reduce speed now,
I am so ready- let gravity win
Cleansing is now an improbable thing.

Watch me outmanoeuvre
the template of this species
with my chrome footprints.

Promise there won't be more changes,
Your heartache and pride
comes with a lifetime guarantee.

Mama let's spin,
me on my wheels,
and you in your comfy heels….

This 14-year-old boy transitioned to wheelchair whilst being my study subject. Notwithstanding his mother's anguish, he was jubilant with the new found independence and speed. The glorious fact that he could pull her along faster or even do some stunts if she held on to the handlebars of the wheelchair, set the scene for this verse. A salute to the spirit of the young boy.