Treatment of Muscle Weakness and Fatigue in Neurofibromatosis Type 1

Emily Vasiljevski

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Primary Supervisor: A/Prof. Aaron Schindeler

Co-supervisors: Prof. Joshua Burns and Prof. David G Little

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Faculty of Medicine and Health
Declaration of Contributions

The work presented in this thesis was conducted during my full-time candidature to fulfil the requirements for the degree of Doctor of Philosophy through the Faculty of Medicine and Health, University of Sydney. Unless otherwise stated, all experiments were conducted by myself at the Orthopaedic Research and Biotechnology Unit at The Children’s Hospital at Westmead or the Clinical Research Centre at Kids Research. I completed the drafting of published manuscripts included in this thesis and the thesis itself with guidance from my primary supervisor and co-authors. Initial experimental conception was conducted in conjunction with my supervisors.

In Chapter 2, the in situ muscle physiology and associated data analysis was performed by Dr. Peter Houweling from the Murdoch Children’s Research Institute (Mel, VIC), and I assisted with the experiments and data interpretation. The LC-MS and GC-MS lipidomics methods were established and ran on mass spectrometers by Dr. Thusitha Rupasinghe from Metabolomics Australia (Mel, VIC). Lipidomics data analysis and figure production was also completed by Thusitha Rupasinghe. Figures 4 and 5 histology was completed by Miss Tarneet Kaur. I conducted data analysis in conjunction with her and figure production thereafter.

In Chapter 3, thematic analysis of interview transcripts was conducted by Dr. Karen Scott and myself, both individually and independently.

In Chapter 4, biochemical experiments were conducted by The Pathology Department and Biochemical Genetics Department at The Children’s Hospital at Westmead. Hand-held dynamometry strength testing was performed by Ms. Anita Mudge and Ms. Gabrielle Donlevy, both from the Paediatric Gait Analysis Service. The normative data was obtained by Dr. Marnee McKay, Dr. Jennifer Baldwin and Prof. Joshua Burns.
Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purpose anywhere else. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis has been acknowledged.

Emily Vasiljevski

August 2020

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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August 2020
Authorship Attribution Statement


I reviewed the literature, conceptualised and wrote the drafts of the published manuscript.


I conceptualised the study, and carried out the investigation, formal analysis and wrote the drafts of the published manuscript.

In addition, the following manuscripts have been submitted to peer-reviewed journals, and the candidate’s contributions include:

- Chapter 3 of this thesis is the manuscript of “Emily R Vasiljevski, Karen Scott, David Stevenson, and Aaron Schindeler. Parents’ experiences using research into L-carnitine supplementation to treat their child’s muscle weakness and fatigue in neurofibromatosis type 1. PLOS ONE.”

I conceptualised the study, and carried out the investigation, formal analysis and wrote the drafts of the manuscript.

- Chapter 4 of this thesis is the manuscript of “Emily R Vasiljevski, Joshua Burns, Paula Bray, Gabrielle Donlevy, Anita Mudge, Kristi Jones, Matthew A Summers, Andrew

I conceptualised the study, and carried out the investigation, formal analysis and wrote the drafts of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published paper, permission to include the published material has been granted by the corresponding author.

Emily Vasiljevski

August 2020

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

A/Prof. Aaron Schindeler

August 2020
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Abstract

Neurofibromatosis type 1 (NF1), previously termed von Recklinghausen’s disease, is a genetic disorder characterised by the development of tumours on the nerves. NF1 can also affect muscle, with hypotonia, muscle weakness and fatigue able to have a profound impact on paediatric quality of life. This thesis investigates an underlying metabolic myopathy, and the potential of dietary intervention to treat the muscular symptomology in NF1.

In Chapter 2, we utilised the Nf1Prx1−/− mouse model to test a range of dietary interventions, including 1) a medium-chain fatty acid (MCFA)/L-carnitine combination, 2) MCFA/L-carnitine with reduced dietary compliance (5/7 days/week), 3) MCFA alone, 4) L-carnitine alone, 5) a “mitochondrial cocktail” (L-carnitine, riboflavin, CoQ10 and creatine), and 6) a low fat diet. Oil Red O (ORO) analysis revealed a significant reduction of intramyocellular lipid (IMCL) accumulation following every dietary intervention, excluding the low fat diet. The MCFA/L-carnitine combination resulted in a significant reduction of IMCL within four weeks, but this was not sustained upon reversion to a standard diet. Lipidome analysis revealed consistent and separable changes in muscle lipidome profiles, by genotype and dietary treatment. Nf1−/− deficient muscle demonstrated significantly elevated acylcarnitine levels (particular C16 and C18:1) compared to WT. Taken together, these data support the theory of an underlying metabolic problem that can be treated by dietary intervention.

In Chapter 3, we used a qualitative approach to explore the decision-making process of families who chose to self-supplement their child with L-carnitine. Thematic analysis revealed that primary muscular symptoms and psychosocial repercussions, the ineffectiveness of current treatment strategies, and research availability and accessibility were driving factors in their decision. During L-carnitine supplementation, every parent perceived a significant improvement in their child’s physical abilities. Further guidance around the optimum dose for
their child, research accessibility and sharing health findings, and the development of a clinical trial were queried. This study hints at the potential of L-carnitine supplementation in the context of NF1.

In Chapter 4, we conducted the first Phase 2a clinical trial of L-carnitine supplementation for NF1-associated muscle weakness and fatigue. Six children aged between 8-12 years with a confirmed diagnosis of NF1, history of muscle weakness and fatigue, and naïve to L-carnitine supplementation were enrolled in the study. There were no adverse events or side effects reported throughout the study with kidney and liver function tests confirming the safety profile, and compliance rate was high. There was evidence of improved foot strength (plantarflexion and dorsiflexion movements), as well as long jump and six minute walk distances. Plasma acylcarnitines were low, but not within a range clinically linked to carnitine deficiency. Upon three-month follow up, four out of six families elected to continue L-carnitine supplementation. These data support a Phase 3 clinical trial of L-carnitine supplementation in NF1.
Original Publications Included in this Thesis

Over the course of this thesis, several studies have been written into research articles, peer reviewed and/or are in the process of being reviewed and published, and are included in manuscript format.

Chapter 1 (Part 2) – Review publication


Chapter 2 – Experimental publication


Chapter 3 – Experimental publication

- Emily R Vasiljevski, Karen Scott, David Stevenson, Aaron Schindeler. Parents’ experiences using research into L-carnitine supplementation to treat their child’s muscle weakness and fatigue in neurofibromatosis type 1. PLOS ONE (currently under review).

Chapter 4 – Experimental publication

- Emily R Vasiljevski, Joshua Burns, Paula Bray, Gabrielle Donlevy, Anita Mudge, Kristi Jones, Matthew A Summers, Andrew Biggins, Craig F. Munns, Marnee J McKay, Jennifer N Baldwin, David G Little, Aaron Schindeler. L-carnitine supplementation for muscle weakness and fatigue in children with neurofibromatosis

**Other Publications**

Original publications that I was a contributing author during my candidature, include:


**National and International Conference Presentations**


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Chapter 1 Prologue

To orient the reader to the context of this thesis, the first chapter will provide an introduction to Neurofibromatosis Type 1 (NF1), including a brief historical background, the molecular genetics, and clinical presentation with a focus on NF1 muscle (Part 1). It also includes a general outline of skeletal muscle development and function, and highlights the importance of the NF1 gene for normal development. In Part 2 of this chapter, we then extensively review the Lipid Storage Myopathies (LSMs), which feature similar histopathology and muscular symptoms to NF1. We speculate that the current treatments and future directions of LSMs could be used to guide the treatment of NF1 muscle weakness and fatigue. The fundamental hypothesis of this thesis is that NF1 features an underlying metabolic myopathy, and that dietary intervention has the potential to treat the muscular symptomology in NF1.
Chapter 1: Part 1

Introduction
1. Neurofibromatosis type 1

1.1. The Neurofibromatoses, and an introduction to Neurofibromatosis type 1

The Neurofibromatoses (NF) are a group of complex genetic disorders caused by inherited or spontaneous mutations in tumour suppressor genes (1). NF describes a group of three conditions: Neurofibromatosis Type 1 (NF1), Neurofibromatosis Type 2 (NF2) and Schwannomatosis. These are generally global conditions, however in some cases neurofibromas can be restricted to one part of the body (e.g. Segmental NF) (2). NF1 is the most common form of NF, and the most common autosomal dominant disorder. It affects ~1:3000 individuals of both sexes and across ethnic lines (3).

1.2. The history of Neurofibromatosis type 1

NF1, formerly known as von Recklinghausen’s disease (VRD), has a long standing history that can be traced back to Ancient Egyptian times. The Ebers Papyrus is a 110 paged medical scroll from 1536 BC, and is the first report suspected to describe the disorder (4). Other forms of documentation, including sketches by a Cistercian monk have been found from the 13th century (5). These showed NF1 patients as grotesque individuals with sack-like tumours and bulbous nodules (Figure 1). Due to the ‘unnatural’ content of the drawings they were historically catalogued under ‘monsters’ (5). It wasn’t until the late eighteenth century that documentation became more scientific in nature (6). Notably, the British physician Mark Akenside is acknowledged as the first to comment on the heritable nature of NF1 (5). In 1882, von Recklinghausen coined benign tumours that arose from the peripheral nerve sheath as ‘neurofibromas’ (6). It was because of this the condition was named VRD, later to be re-named NF1 at the National Institutes of Health (NIH) Consensus Development Conference in 1987.
“The Elephant Man,” remains part of the NF1 culture zeitgeist, and popularized a 1980 American historical drama film (Figure 2) (5). It described the life of a young man named Joseph Carey, who had various skin and bone deformities and was cruelly presented in circuses as the “Elephant Man.” His initial diagnosis was NF1, and although this proved incorrect in his life it brought widespread exposure of NF1 to the public (5).

Figure 1. An early sketch of someone with NF1 (5).
Figure 2. A real photograph of the Elephant Man (5).

1.3. Diagnostic criteria of Neurofibromatosis type 1

The modern diagnostic criteria for NF1 were published at the National Institutes of Health (NIH) Consensus Development Conference (Table 1) (7). It relies heavily on the pivotal involvement of the skin, nervous system and bone in NF1. Characteristic features of NF1 include skin hyperpigmentation or ‘café-au-lait’ macules, and excessive freckling in the inguinal or axillary regions of the body. Alongside these, neurofibromas and melanocytic hamartomas of the iris, also known as Lisch nodules, become apparent in most individuals by the time adulthood is reached. Less common are optic gliomas and osseous lesions. A first degree relative with NF1 also forms part of the criteria. The presence of two or more of these
conditions are required for a clinical diagnosis (7). The majority of NF1 patients are diagnosed clinically, with genetic testing reserved for ambiguous cases (8).

Limitations exist for these NIH diagnostic criteria in infancy, as a number of the conditions manifest from late childhood. For example, cutaneous neurofibromas develop in only 14% of NF1 children under 10 years of age, and this increases to 94% by the time early adulthood is reached (9). Furthermore, Lisch nodules can only be observed in 22% of patients by the age of 5 years, however this increases to 96% by the time they reach 20 years of age (9). Additionally, approximately 50% of cases represent a de novo origin, and the first degree relative criterion is made redundant in these circumstances. It is estimated that only half of all sporadic cases can be diagnosed by 1 year of age because of this (10). A clinical diagnosis of NF1 can confidently be made using these NIH diagnostic criteria by 8 years old in most children, and by 20 years old in all NF1 patients. The addition of other criteria, including short stature, macrocephaly and T2 hypersensitivity have been suggested to provide a more reliable diagnosis (10), however no changes have been made.
Table 1 NIH diagnostic criteria (7) requires 2 or more criterion to fulfil a NF1 diagnosis.

<table>
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<th>Number</th>
<th>Feature</th>
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<tr>
<td>6 or more</td>
<td>Café-au-lait macules (&gt;5mm diameter pre puberty and &gt;15mm diameter post puberty)</td>
</tr>
<tr>
<td>2 or more</td>
<td>Neurofibromas</td>
</tr>
<tr>
<td>1</td>
<td>Plexiform neurofibroma</td>
</tr>
<tr>
<td>Excess</td>
<td>Inguinal or axillary freckling</td>
</tr>
<tr>
<td>2 or more</td>
<td>Lisch nodules</td>
</tr>
<tr>
<td>1</td>
<td>Optic glioma</td>
</tr>
<tr>
<td>1</td>
<td>Osseous lesion, e.g. sphenoid dysplasia</td>
</tr>
<tr>
<td></td>
<td>First-degree relative, including parent, sibling or offspring, diagnosed with NF1 by the above criteria</td>
</tr>
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1.4. The **NF1** gene encodes the Ras-GTPase neurofibromin

The **NF1** gene is large and spans 350 kb of genomic DNA at chromosome 17q11.2 (11, 12). It encodes neurofibromin, a 327kDa protein that has several alternative isoforms (13). Neurofibromin is constitutively expressed, but has particularly high levels of expression in the nervous system including neurons, Schwann cells and oligodendrocytes. Neurofibromin contains a central, RAS-specific GTPase activating protein (GAP) domain. This interacts directly with RAS to cause a conformational change, and catalyses the conversion of the active GTP-bound RAS to inactive GDP-bound RAS. Thus neurofibromin is a negative regulator of RAS activity.

Mutations in **NF1** result in the constitutive activation of RAS, and its downstream signalling axes, including the canonical RAS-mitogen activated protein kinase (MAPK) signalling pathway, and RAS-phosphatidylinositol-3 kinase (PI3K)-mammalian target of rapamycin (mTOR). Hyper activation of these pathways have been demonstrated in multiple cell types in NF1, including glial cells, Schwann cells, cardiomyocytes, myoblasts, osteoblasts and osteoclasts, and underlie aberrant proliferation, differentiation and metabolism (14-21). Aside from this, RAS proteins are known to interact with more than twenty ‘down-stream’ effectors that stimulate various molecular pathways (Figure 3), and this is likely the reason for significant phenotypic diversity observed in NF1 (22). Pharmacological inhibition of RAS signalling in tumour development have shown some promise in NF1, and are currently being tried in clinical trials (23).

The mutation rate for the **NF1** gene is estimated to be between 1×10^{-4} and 3.1×10^{-5}, which is considerably higher than the average mutation rate of most other inherited disease genes (24). Systemic heterozygosity is most commonly observed in NF1, with every somatic cell featuring one inactivated copy of the **NF1** gene. However somatic mosaicism, including generalised, and
a localised form in one segment or dermatome of the body have been reported in NF1 (25). Mosaicism describes a mixed population of normal cells as well as cells harbouring a \( NF1 \) gene mutation. The calculated prevalence of mosaic NF1 (MNF1) in the general population is 1:36000, but this is likely to be an underestimate. MNF1 often presents with a much milder phenotype than NF1, and is generalised more often than segmental (25). Furthermore, individuals with NF1 who harbour a heterozygous germline mutation may acquire a second, somatic ‘hit’ in accordance to the “two-hit” hypothesis first proposed by geneticist Alfred Knudson in 1971. Data have emerged indicating a second, somatic ‘hit’ (\( NF1^{+/} \)) can promote NF1 tumourigenesis (26-28), with biallelic inactivation of the \( NF1 \) gene recently being identified in up to 74% of plexiform neurofibromas (29, 30). The two-hit hypotheses has been implicated in the development of NF1 bone abnormalities, including tibial pseudoarthroses (31) (32). However, the cell of origin, which harbours a somatic ‘second hit’ and drives pseudoarthrosis development remains unclear. The surrounding soft tissue to the cortical bone at the pseudoarthrosis site has been shown to feature biallelic inactivation, and it is possible that the \( NF1 \) somatic mutation originally occurred in mesenchymal progenitors (33). Further, a somatic ‘second hit’ has been demonstrated in NF1 kidney tissue, which resulted in adrenal hyperplasia (34),
Figure 3: Ras signalling pathway illustration highlighting the diverse downstream signalling pathways of RAS proteins (DBGET, 2019).
1.5. Clinical presentation of Neurofibromatosis type 1

NF1 is a complex disorder that can affect various physiological systems, including the nervous, cardiovascular and musculoskeletal systems.

1.5.1. Tumorigenesis

Individuals with NF1 are predisposed to a range of benign and malignant tumours, which involve neural-crest derived cells, however also require interactions with other cell types, including endothelial cells, smooth muscle cells and mast cells (35, 36). Neurofibromas are the primary tumour manifestation of NF1, and exhibit extensive cellular heterogeneity, including hyperproliferative Schwann cells, perineural cells, fibroblasts and mast cells (30).

There are three major types of neurofibromas: cutaneous, spinal, and plexiform neurofibromas. Cutaneous neurofibromas are small tumours associated with nerves in the skin. They are the most frequently observed neurofibromas in clinic and develop in 99% of individuals with NF1 (37). Majority of patients report pruritus (itch) on a daily or almost daily basis, and this can frequently precede the development of a new cutaneous neurofibroma or be associated with established tumours (38). The pathophysiology of pruritus is not completely understood in NF1. It is speculated that mast cells are involved, and they release histamine that could contribute to the itch (39). Cutaneous neurofibromas may become pedunculated as they grow, and can catch on clothing and cause cosmetic problems (39). Although they very rarely progress to malignancy, they can be surgically removed or laser treated if problematic (40). Alternatively, emollients and antihistamines may provide some benefit, and excessive heat should be avoided (39).

Spinal neurofibromas are slow-growing, benign nerve sheath tumours that develop in the spinal canal of ~40% of NF1 patients (30). They can grow along the dorsal sensory roots causing
significant pain and radicular sensory changes, however this occurs rarely in childhood and affects only 2% of adults with NF1 (41). The majority of cases are asymptomatic and require careful monitoring. Surgical resection is reserved for severe cases (42).

Plexiform neurofibromas are diffuse, grow along the length of a nerve, and often involve major nerve trunks and nerve plexi (30, 43). They grow at an unpredictable rate with periods of rapid growth followed by relative inactivity. Approximately 30-50% of NF1 patients develop a plexiform neurofibroma in their lifetime. They frequently cause significant pain, disfigurement and neurological deficits. Removing a benign plexiform neurofibroma is usually difficult because of their infiltration into surrounding soft tissue and high vascularity (30, 43). A number of agents, including anti-angiogenesis drugs and fibroblast inhibitors have been tested in clinical trials to limit tumour growth, however there is little evidence to support their application (44). Approximately 10-15% of all plexiform neurofibromas undergo malignant transformation and therefore require careful health monitoring (30).

Malignant peripheral nerve sheath tumours (MPNST’s) are highly invasive and aggressive soft tissue sarcomas. Individuals with NF1 have 8-13% risk of developing a MPNST in their lifetime, however, those carrying large genomic deletions have an increased risk of MPNST (45, 46). MPNST’s arise from pre-existing plexiform neurofibromas, as well as de novo. Early diagnosis of MPNSTs is crucial as the response rate to chemotherapy is low, and only complete surgical resection has been shown to be curative. Radiotherapy is used if the MPNST is not amendable to surgical resection (47).

Optic pathway gliomas are a slow-growing brain tumour (astrocytoma) that arise in or around the optic nerve in approximately 15% of NF1 individuals (48). The predominant symptom of an optic pathway glioma is progressive vision loss. Sometimes these tumours cause blindness because the optic nerve is damaged from the increasing pressure on and around it (49). These
tumours can transform to malignancy, however they optimistically have a high cure rate. Management of optic gliomas requires regular ophthalmological monitoring for vision loss and tumour expansion (50). The role of debulking surgery in the management of optic pathway gliomas is still highly controversial, and should be discussed on a case-by-case basis involving a multidisciplinary team of ophthalmologists, oncologists and neurosurgeons. There is a consensus that if the tumour threatens complete vision loss then surgery should proceed (50).

1.5.2. **Learning, attention and behavioural difficulties**

A range of characteristic cognitive deficits are reported in children with NF1. Defective visuospatial processing remains a major trait of the NF1 cognitive phenotype (51-54). Visuospatial processing refers to one’s ability to identify visual and spatial relationships among objects and is mainly tested by The Judgement of Line Orientation, however, can also be assessed using automated paired associate learning tasks and electrophysiological tools. The intellectual ability of NF1 children is slightly lower than their siblings and other healthy children with a lower mean full-scale IQ and lower academic achievement, however are still within a normal range (55, 56). Furthermore, children with NF1 perform poorer in mathematics, reading and writing related tasks, however this has not been shown to be related to IQ achievement discrepancies (52, 57). Impairments in executive function, including planning, behavioural organization, cognitive flexibility, working memory, error detection and correction, are also frequently noted in affected children (56, 58).

Children with NF1 require a detailed developmental assessment before they start school, and yearly assessments there on to monitor progression. A special education needs coordinator should also be involved early on, as well as close liaison between teachers, allied health professionals and educational psychologists to ensure optimal support (39).
Attention deficits, and poorer social skills and reduced social competence have been linked to the NF1 phenotype. Greater than 26% of children with NF1 fall on the autism spectrum (59). Children with NF1 are more likely to internalize and externalize problems, have weaker facial expression recognition, experience more loneliness than their siblings, have greater anxiety symptoms, sleep disturbance and lower participation in skilled activities. Cognitive performance, social interaction and emotional function are frequently linked and interventions that focus on all three domains would be most likely to be of benefit.

Attention-deficit/hyperactivity disorder (ADHD) is present in approximately 40-50% of affected children, and is associated with impaired cognitive function, learning and academic achievement (57, 60). The HMG-CoA reductase inhibitor, lovastatin showed substantial promise, with prepulse inhibition, a neurological phenomenon reported in ADHD, being significantly improved in Nf1+/− mice (61). A significant reduction of Ras-MAPK signalling in the cortex and hippocampus was thought to underlie these benefits (61). Unfortunately, clinical translation was unsuccessful (59). Nonetheless, methylpenidate is a stimulant that has shown to have a beneficial effect on ADHD symptoms (60, 62, 63).

1.5.3. Cardiovascular complications

NF1 can cause a vasculopathy that is usually recognised in childhood or early adulthood (64). Often pregnancies can illuminate a vasculopathy (64). Cardiovascular manifestations may include renal artery or pulmonary stenosis, hypertension, congenital heart disease, and hypertrophic cardiomyopathy (65, 66). Cerebrovascular abnormalities are usually caused by stenosis or occlusions of the internal carotid, middle cerebral or anterior cerebral artery (64). Neurofibromas can also develop in the heart, or compress the heart or major blood vessels, which obstructs blood flow (67). The heterogeneity of cardiovascular abnormalities may be
related to the heterogeneity of NF1. The type and incidence of cardiovascular malformations in NF1 have not been well-defined, however renal artery stenosis has been observed in 1-2% of NF1 patients (39, 64). Further, congenital heart defects has been reported in up to 6.4% of individuals with NF1 (68).

The histopathology of NF1 vascular lesions have previously been described and classified (69, 70). They are based on three factors; vessel diameter, histology and morphology. Early diagnosis of vascular lesions is vital in order to limit its capacity to cause long-term hemodynamic consequence and risk bacterial endocarditis (64). It also reduces the risk of arrhythmias, pulmonary hypertension and right ventricular dysfunction later in life. Further, NF1 vasculopathy is a frequent cause of premature death in individuals with NF1. Therefore, blood pressure must be checked annually. If blood pressure is high, it should be checked three more times within the month to verify the findings. A cardiologic assessment, including M-mode, 2D and colour Doppler scan echocardiogram is a part of obligatory routine care. It is crucial that any patient who presents with a possible heart murmur should be examined by echocardiography for congenital heart disease. Surgical revascularisation can be undertaken to improve blood pressure, and a nephrectomy performed in cases with parenchymal lesions or severe renal artery disease to prevent grafting (64).

1.5.4. Skeletal involvement

The skeletal phenotypes of NF1 can be classified as either generalised or focal manifestations. Generalised skeletal manifestations are usually milder, and include reduced bone mineral density (BMD) or osteopenia, osteoporosis, and short stature, whereas focal skeletal manifestations cause significant morbidity, and include long-bone dysplasia, sphenoid wing dysplasia and short angle scoliosis.
Generalised skeletal manifestations are more frequently observed than focal abnormalities. Decreased BMD has been observed in up to 50% of individuals with NF1 at an early age, which was not gender specific (71-74). However, interpreting this has been challenging, particularly because of modifications to the osteoporosis criteria in children, and the severity of osteoporosis in this paediatric population remains unclear (75). It is speculated that underlying vitamin D and calcium deficiencies could contribute to this phenotype in childhood. Following puberty, growth velocity declines to below normal, and 20-30% of NF1 adults have a height below the 3rd centile (75). The cause of short stature is unknown, nevertheless growth hormone deficiency and scoliosis have been implicated.

Long bone dysplasia is observed in 3-4% of NF1 patients, and is a challenging skeletal abnormality (76). It usually presents in infancy with unilateral anterolateral bowing, and most commonly involves the tibia. The bowed long bone usually sustains fracture leading to a non-union or “pseudarthrosis.” Pseudarthroses frequently require multiple surgeries and are often unsatisfactory or require amputation. Thus, routine bracing to prevent fracture of the dysplastic long bone is the current standard for treatment (77, 78). Continuation of bracing after fracture has also been considered in order to delay surgery until mid-childhood and improve surgical outcome (75). Cranial defects involving the sphenoid wing are observed in approximately 11% of individuals with NF1 (76), and vertebral deformities manifest in 10-33% of children with NF1 (79). Both of these focal abnormalities are frequently associated with neurofibromas, and therapies targeting tumour reduction or stabilisation could improve skeletal outcomes (75). Surgery is reserved for highly progressive cases of spinal curvature (>45° before maturity or 55° after maturity). Unidentified genetic or epigenetic factors may influence the development of focal skeletal manifestations (80).

A number of mouse models have been developed to determine the role of Nf1 in bone cells. Delayed osteoblast differentiation, increased osteoclast numbers and bone resorption, and
delayed injury repair have been demonstrated in Nf1-deficient mice (81, 82). Preclinical mouse models have been used to assess the potential efficacy of selected drugs on bone formation, repair and remodelling. A combination of bisphosphonates and bone morphogenetic protein 2 has been shown to improve net bone production in an in vivo model of heterotopic bone formation (83). Lovastatin treatment has been tried in the Nf1Prx1−/− mouse model and resulted in improved cortical bone injury healing (82). To date, clinical trials assessing therapeutic agents for bone abnormalities are limited.

1.5.5. Muscle manifestations of NF1

Reduced muscle size in NF1 was first demonstrated in 2005, by Stevenson et al who analysed 40 individuals with NF1 using peripheral quantitative computed tomography (pQCT) scanning. They demonstrated a significant reduction in muscle cross sectional area at the 66% tibia site compared to age matched controls (84). Previously, outside of the NF1 context, muscle cross sectional area has been associated with muscle geometry, which is a critical factor for normal bone architecture (84). Comparable reductions in muscle size were observed in a paediatric cohort, which showed children with NF1 had a significant reduction in lean tissue mass using dual-energy X-ray absorptiometry (DEXA).

Reductions in strength were not scientifically confirmed until Souza et al (2009) performed hand grip dynamometry testing of 21 study participants compared to aged, gender, and physical activity matched controls. They showed that maximal force grip strength normalized to forearm muscle size was significantly reduced in individuals with NF1 (85). Later, maximal isometric strength of 15 upper and lower limb muscle groups were evaluated in children with NF1 and age, sex, height and weight-matched controls using hand-held dynamometry (86). Strength deficits of 3-43% were observed in the NF1 cohort, with hip flexion, hip abduction,
plantarflexion and dorsiflexion being the most affected muscle groups (86). Aerobic exercise performance has also been assessed in a small cohort of 17 individuals with NF1, along with age, gender and bodyweight matched controls. They demonstrated that individuals with NF1 have a reduced maximal oxygen uptake (VO₂ max), and reduced maximal systolic blood pressure (87), however this could be due to lower physical activity levels in the NF1 cohort that was not accounted for.

Johnson et al measured the motor proficiency of children with NF1, which describes the activation and sequencing of movement patterns (88). Motor proficiency was examined using the BOT 2. The BOT 2 is a self-administered test measuring fine and gross motor skills, including fine manual control, manual coordination, body coordination, and strength and agility. An overall score for motor proficiency is generated from these tests. Of the NF1 population, 19% scored in the average category, 54% scored in the below average category and 27% scored well below average. Less efficient movement patterns result in a higher energy expenditure and greater fatigue, which would likely effect the amount of physical activity. The greatest difference between NF1 kids and normative data was observed in balance and strength, followed by running speed and agility, then fine motor integration (88).

Approximately 30% of children and adolescents with NF1 report a significantly poorer self-concept for physical and sporting abilities. The only major predictor for physical abilities self-concept was social problems, which highlights the interrelationship between sport and social/emotional welfare.

A conditional Nf1 knockout mouse model, using a MyoD transgene designed to drive Nf1 deletion in skeletal muscle cells (Nf1M_myod⁻/⁻) was developed to better understand the pathobiology and guide potential therapies (89). The Nf1M_myod⁻/⁻ mouse model was the first model to demonstrate the importance of Nf1 as a regulator of muscle development and
metabolism. Whilst sarcomeric protein structure remained normal, large intramyocellular lipid droplets were observed on electron microscopy, and later confirmed by Oil Red O histology (89). These findings proved consistent with human NF1 muscle samples validating the usefulness of the \( Nf1^{MyoD^{-/-}} \) mouse model (90). Liquid Chromatography Mass Spectrometry (LC-MS) analysis of the lipid revealed up to a 20-fold increase in triglyceride and cholesterol ester species containing long-chain fatty acids (90). \( Nf1^{MyoD^{-/-}} \) mice fail to thrive and die within the first week of life (89). Thus, it cannot be used to test potential therapies over an extended period. The \( Nf1^{Prx1^{-/-}} \) mouse model is limb specific, and although it was originally used to study Nf1 skeletal development it can also be useful for Nf1 muscle therapy development (81). They similarly present with intramyocellular lipid accumulation, reduced grip strength in adulthood, as well as other metabolic changes, including increases in succinate dehydrogenase and medium-chain acyl-CoA dehydrogenase activity, and decreased expression of CTP-I, FATP4 and CD36 (89). A modified diet enriched with medium-chain fatty acids and supplemented with L-carnitine shows significant promise in ameliorating lipid deposition and improving grip strength using the \( Nf1^{Prx1^{-/-}} \) mouse model (90). Dietary intervention represents a highly promising therapeutic approach for NF1 muscle symptoms, as elaborated upon in the review paper featured in part 2 of this thesis introduction entitled “\textbf{Lipid storage myopathies: Current treatments and future directions.}”
2. Skeletal muscle development and function

2.1. Early embryonic development and myogenesis

Three distinct germ layers form during early embryonic development: the ectoderm, the mesoderm and the endoderm. Briefly, the ectoderm is the outermost layer of cells that goes on to form the nervous system, epidermis, and various neural crest-derived tissues (91). The endoderm is the innermost layer that develops into the gastrointestinal and respiratory tracts, endocrine glands and organs, and urinary system. The mesoderm is the middle layer between the ectoderm and endoderm, and gives rise to the axial skeleton, cartilage, connective tissue, non-epithelial blood cells, kidneys and muscles (91).

The mesoderm anatomically separates into the paraxial mesoderm, the intermediate mesoderm and the lateral plate mesoderm (92). The paraxial mesoderm progressively segments to form pairs of somites from head to tail (92). Polarity increases, and the somites compartmentalise into two distinct parts; ventral and dorsal. The ventral part undergoes an epithelial to mesenchymal transition to form the sclerotome. The dorsal part remains as an epithelium layer and forms the dermomyotome (93). The four margins of the dermomyotome fold ventrally to form lip-like borders, known as the dermomyotome lips. Muscle progenitor cells delaminate from the dermomyotome lips, and proliferate and differentiate under the dermomyotome to form the myotome. There are a portion of progenitor cells that delaminate from the ventrolateral lip and migrate to the limb bud before proliferation and differentiation (93).

Two distinct waves of differentiation occur during embryonic myogenesis; (i) the first wave is during embryonic myogenesis (e10-12.5 in the mouse), where a scaffold of primary, multinucleated myotubes are formed from a fraction of differentiated and fused embryonic myoblasts (94). This is important for shaping and positioning the orientation of individual muscles. (ii) The second wave is during foetal myogenesis (e14.5-17.5 in the mouse), which
occurs when successive waves of foetal myoblasts proliferate using the surface of primary myotubes. These then fuse among themselves to form secondary myotubes that gradually separate from the primaries (94).

The last phase of myogenesis is characterised by specific muscle formation, and involves the maturation of myotubes into fully functional myofibres (94). Coordinated development of the metabolic machinery of the cell, as well as the ion transport membrane system and contractile elements are necessary for normal muscle formation. The primary muscle fibres tend to become slow muscle fibres, in contrast to secondary muscle fibres that usually acquire features of fast muscle fibres (94). Specific muscles of the body arise from a distinct and highly regulated pattern of development. The deep back muscles, ventrolateral body wall muscles, and medial shoulder girdle muscles originate solely from the myotome (93). Limb, tongue and lateral shoulder girdle muscles are derived from the migrating progenitor cells. Some muscles, including other shoulder girdle muscles, the trapezius and sternocleidomastoid muscle originate from the lateral plate mesoderm (93).

### 2.2. Myogenic regulation

A host of myogenic regulatory factors (MRFs) have been discovered to govern the various stages of myogenesis (95). The delamination of muscle progenitor cells from the dermomyotome is governed by Pax3 transcription factor and c-Met/HGF, whereas c-Met/HGF and Lbx1 transcription factors are required for migration of the progenitor population that delaminate from the ventrolateral lip of the dermomyotome. The extensive proliferation of muscle progenitor cells is controlled by multiple MRFs, including Pax3, c-Met/HGF, Mox2, Myf5 and MyoD. Myf5 and MyoD remain upregulated to determine the cellular fate of muscle progenitor cells, converting them to myoblasts. This is followed by the downregulation of Myf5
and MyoD, and upregulation of desmin and myogenin to cause terminal differentiation, where myoblasts fuse to form multi-nucleated fibres called myotubes. Both Lbx1 and Mox2 are involved in specific muscle formation (95).

The myogenic regulatory factors are spatially and temporally regulated by a fine balance of positive regulators, such as wingless-type MMTV integration site (WNTs) and sonic Hedgehog (SHH) genes, and negative regulators, such as bone morphogenetic proteins (BMPs) and the Notch signalling pathway (92).

2.1. Satellite cells

A progenitor population originating from the central dermomyotome co-expresses transcription factors Pax7 and Pax3 within the skeletal muscles during embryonic development (96). In the final stages of foetal development, the progenitor population produces cells in a satellite position around myofibres, which are marked by the expression of Pax7. In adult muscle, these satellite cells reside between the basal lamina and sarcolemma of muscle fibres. They are mitotically quiescent, however can become ‘activated’ upon exercise (overloading) and acute or chronic muscle injury for skeletal muscle regeneration. Following activation, the satellite cells move outside of the basal lamina, and start to cycle and co-express Pax7 and MyoD (96). Environmental cues, namely Notch and IGF-1 signalling stimulate satellite cell proliferation, and Wnt signalling induces differentiation (97). During muscle regeneration, myotubes form through a three-step mechanism (98). Firstly, cell adhesion proteins of the Ig superfamily reduce the distance between cell membranes to 20-50nm. Secondly, actin-polymerization machinery is activated, and transient actin-based structures are formed. Finally, the lipid bilayer is destabilized, which facilitates fusion between two proximal outer leaflets of the bilayers, followed by fusion between the two distal inner leaflets. Cellular cytoplasm’s then
contact each other leading to the mixture of intracellular components within the syncytium (98). Adult myogenesis during muscle regeneration recapitulates many of the cellular and molecular aspects of developmental myogenesis (Figure 4) (97).

It is likely that there are two subsets of satellites cells. The first subset maintain myogenic markers and are capable of rapid differentiation permitting adult myogenesis. The second lose expression of myogenic markers and leave the cell cycle. These possess stem-cell like properties that allow for prolonged self-renewal, and maintenance of the satellite cell pool (96).

Figure 4: Adult myogenesis recapitulates many of the cellular and molecular aspects of developmental myogenesis (97).
2.2. Other progenitor cell types that are important for muscle regeneration

Aside from satellite cells, there are a number of other cells that reside in skeletal muscle or infiltrate from the general circulation, which contribute to the cellular response to muscle injury. Fibro-adipogenic progenitors (FAPs) are a multi-potent progenitor population that reside in skeletal muscle, and rapidly proliferate upon skeletal muscle damage (99). They match proliferation rates of satellite cells to maintain a transient local environment that favours satellite cell differentiation (100). Further, they secrete a number of molecules, including IL-6, IL-33, WISP1, Follistatin and IL-10, to mediate signalling pathways, and regulate muscle regeneration. FAPs provide a source of extracellular matrix proteins, which are vital for tissue remodelling. There is also evidence of crosstalk between FAPs and infiltrating immune cells, which is necessary for balancing inflammatory processes and regeneration (100). Infiltrating immune cells, including macrophages, eosinophils, neutrophils and regulatory T-cells, secrete cytokines that stimulate proliferation of satellite cells and FAPs, and assist in degradation of injured tissues.

Pericytes are another multipotent progenitor population that are embedded into the capillary basal lamina, and sit closely in relation to satellite cells. They have gained more attention recently because of their newly-discovered myogenic potential (101), and possible therapeutic purposes in degenerative diseases (102). A recent study suggested that pericytes have a role in regulating satellite cell activation, and were also implicated in muscle hypertrophy regulation (103). However, further investigations are necessary to further elucidate the precise role of pericytes in muscle regeneration.
2.3. Defects in muscle regeneration

Defects in muscle satellite cells have been reported in a number of neuromuscular diseases, such as Pompe disease, Duchenne muscular dystrophy and Myasthenia gravis (104). Altered satellite cell activation, proliferation and/or differentiation, as well as changes in total numbers and/or self-renewal all contribute to the progressive degeneration of skeletal muscle in these patients. The skeletal muscle gradually becomes pathologically replaced by fat or fibrotic tissue (104). The presence of centralised nuclei on muscle biopsy are routinely found in muscle wasting diseases, and are a clear indication of a constant state of muscle degeneration/regeneration (105). Additionally, progressive phagocytosis of muscle fibres, increased variability in fibre size and fibre splitting are all reported (105).

Muscle degeneration was suspected in Nf1 limb muscles. Generalised muscle fibrosis, variability in muscle fibre size and substitution of muscle with fat was noted on histological examination using the Nf1Prx1−/− transgenic mouse model (106). However, a characteristic feature of this mouse model is cartilaginous hip fusion, which causes the mice to drag their hind limbs. Therefore, it is difficult to distinguish whether there is muscle degeneration or muscular atrophy/muscle wasting due to disuse of the limb muscles and muscle unloading. To further understand this, the Nf1MyoD−/− model was developed to investigate the importance of Nf1 for the actin cytoskeleton (89). This was also based on previous findings that neurofibromin is essential for regulating actin cytoskeleton dynamics via the Rho-ROCK-LIMK2-Cofilin pathway (107). Curiously, electron microscopy examination of the sarcomeres revealed no abnormalities. Instead, intramyocellular lipid accumulation was discovered, and confirmed shortly thereafter in NF1 human muscle biopsies (89, 108). A number of other Nf1 metabolic findings, including elevated lipids containing long-chain fatty acids and fatty acid transporter alterations, lead to the speculation that Nf1 is a critical regulator of lipid metabolism. This is
described in my review paper, however an underlying question remains regarding whether *NF1* has a fundamental role in myogenesis and muscle differentiation.

### 2.4. *NF1* signalling affects pathways important for myogenesis

The function and relative significance of *NF1* and downstream signalling pathways, including the canonical RAS-MAPK remain ambiguous in the context of myogenesis.

High levels of neurofibromin protein are detectable in the developing skeletal muscle of rats at E16 (109). At E16 skeletal muscle contains fused myotubes, as well as proliferating myoblasts. This suggests that *Nf1* expression could be important later on in the myogenic program. *Nf1* expression has also been examined during *in vitro* muscle differentiation (110). It was shown that *Nf1* expression proceeded nAChR expression, a marker of myoblast differentiation. It is possible that the NF1 gene is not involved in the early stages of differentiation.

Key insights into the role of *Nf1* during myogenesis have come from the *Nf1Prx1* knockout mouse model featuring conditional inactivation of *Nf1* in the limb bud mesenchyme (106). No overt changes were observed in migration and proliferation of progenitor muscle cells, however abnormalities in muscle differentiation were detected. A reduction in myogenic expression was noted at E12.5 and one day later, at E13.5, a defect in muscle formation was noticeable. Further, at E14.5 there was a drastic decrease in Desmin positive cells in the ventral muscle groups. This caused a significant shift of the MyoD/Desmin positive cell ratio towards the MyoD population. Together, these findings indicate a severe disruption of myoblast terminal differentiation. Signalling components known to be downstream of RAS, namely ERK1/2 and MEK were increased in the lysates of Nf1 muscle.
Studies outside the context of NF1 can provide key insight into the role of signalling components downstream of NF1, however for the purpose of this literature review only some findings will be included to introduce the importance of *NFI* for differentiation in the myogenic program.

It has been shown that constitutively activated MEK1 represses the transcriptional activity of MyoD, Myf5 and myogenin (111). Activated MEK1 also compromised the ability of MyoD to activate the myogenin gene, inhibiting myogenic conversion of fibroblasts. Further, in C2C12 myoblast culture myocytes expressing activated MEK1 failed to efficiently form multinucleated myotubes after 5 days in differentiation medium. However, the addition of a MEK1 inhibitor resulted in precocious differentiation. Taken together, these data suggest that MEK1 negatively regulates the switch from proliferation to differentiation by the inhibition of MRF function (111).

It has been observed that endogenous MEK1 is activated in differentiated myoblasts, and localised in their nuclei (112), however it was further examined whether MEK1 associates with MyoD in their nuclei (113). A GST pulldown assay was performed using recombinant His6-tagged constitutively active MEK1 (His6MEKEE) and GST-MyoD proteins. The His6MEKEE co precipitated with GST-MyoD, and not with GST alone, suggesting that activated MEK1 directly interacts with MyoD. Notably, their interaction increased in the presence of ATP. They were further able to demonstrate that MyoD is phosphorylated directly by activated MEK1, and phosphorylation was repressed with MEK inhibition, through *in vitro* kinase assays using His6MEKEE. Thus eliminating the possibility that kinases up or downstream of MEK1 phosphorylated MyoD (113).

There is some discrepancy in the literature regarding RAS-MAPK signalling, and its effect on myogenic differentiation (111, 114, 115). A study by Jo, *et al* 2009, used the Tet-Off expression
system to investigate the effects of active MEK1 overexpression at different stages of myogenesis. Firstly, they showed that MEK1, similar to ERK1/2 increased gradually until day 2, and then decreased in differentiating C2C12 cells. Notably, immunostaining detected MEK1, ERK1/2 and pERK1/2 in the cytoplasm, whereas pMEK1 was almost exclusively in the nucleus, suggesting an important stimulatory role in muscle differentiation. They showed that MEK inhibitors introduced on day 1 in differentiating medium significantly decreased the protein level of myosin heavy chain, the promoter activity of muscle creatine kinase, and the number of fully differentiated myotubes, whereas ERK inhibition did not exert the same effect. They then investigated the differing roles of MEK1 at different times of activation during differentiation. Using a C2C12 cell line that constitutively expressed active nuclear MEK1 under the control of the Tet-response element, they demonstrated that MEK1 activation during early stage differentiation results in the repression of myogenesis, whereas mid-stage activation lead to its stimulation. This is an important finding as inherited or de novo mutations in NF1, and loss of function results in early hyper activation of RAS and downstream signalling pathways. Although this study reasons some discrepancy in the literature, it illustrates the complexity of RAS-MAPK signalling for myogenesis. Further investigation is required to fully elucidate the role of NF1 and RAS-MAPK in myogenesis (112).
3. References


Chapter 1: Part 2

Lipid Storage Myopathies: Current Treatments and Future Directions

Part 2 of this chapter provides an extensive review of literature concerning the treatment of lipid storage myopathies, which can be used to guide treatment of NF1 muscle weakness and fatigue. This has been previously peer reviewed and published, and is included here in manuscript format.

Review

Lipid storage myopathies: Current treatments and future directions

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ABSTRACT

Lipid storage myopathies (LSMs) are a heterogeneous group of genetic disorders that present with abnormal lipid storage in multiple body organs, typically muscle. Patients can clinically present with cardiomyopathy, skeletal muscle weakness, myalgia, and extreme fatigue. An early diagnosis is crucial, as some LSMs can be managed by simple nutraceutical supplementation. For example, high dosage L-carnitine is an effective intervention for patients with Primary Carnitine Deficiency (PCD). This review discusses the clinical features and management practices of PCD as well as Neutral Lipid Storage Disease (NLSD) and Multiple Acyl-CoA Dehydrogenase Deficiency (MADD). We provide a detailed summary of current clinical management strategies, highlighting issues of high-risk contraindicated treatments with case study examples not previously reviewed. Additionally, we outline current preclinical studies providing disease mechanistic insight. Lastly, we propose that a number of other conditions involving lipid metabolic dysfunction that are not classified as LSMs may share common features. These include Neurofibromatosis Type 1 (NF1) and autoimmune myopathies, including Polymyositis (PM), Dermatomyositis (DM), and Inclusion Body Myositis (IBM).

1. Introduction

Lipid storage myopathies (LSMs) are a group of genetic disorders characterized by excessive and pathological lipid accumulation, chiefly within muscle fibers [1]. There are four classic LSMs with a defined genetic cause: Neutral Lipid Storage Disease with Myopathy (NLSD-M), Neutral Lipid Storage Disease with Ichthyosis (NLSD-I), Primary Carnitine Deficiency (PCD) and Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) [2]. These conditions are associated with dysfunction in intracellular triacylglycerol catabolism, mitochondrial fatty acid oxidation, or transport of carnitine, acyl-carnitines, and/or long chain fatty acids [1]. Lipid dysmetabolism can cause a range of clinical features, but most commonly encompass a progressive myopathy with muscle weakness, myalgia, and fatigue [1].

Triacylglycerols are a broad category of non-polar lipid molecules made up of three fatty acids bound to a glycerol backbone. They are the predominant metabolic substrate utilized at rest, during routine activities, and for low to moderate-intensity exercise (30–65% maximal

Abbreviations: ABHD5, α/β-Hydrolase Domain-5; AC, adenylylate cyclase; anti-ACVR2B, mouse monoclonal activin receptor type IIb; ATGL, adipose triglyceride lipase; BEZ, bezafibrate; CACT, carnitine translocase; CD36, cluster of differentiation 36; CGI-58, comparative gene identification-58; CoA, coenzyme A; CoQ10, coenzyme Q10; CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; DM, dermatomyositis; EM, electron microscopy; ETF, electron transfer flavoprotein; ETFDH, electron transfer flavoprotein dehydrogenase; ETF-QO, electron transfer flavoprotein -ubiquinone oxidoreductase; FABP4, fatty acid binding protein-4; FACS, fatty acyl-CoA synthetase; FADH2, flavin adenine dinucleotide; FAT, fatty acid translocase; FATP, fatty acid transport protein; GC-MS/MS, gas chromatography - tandem mass spectrometry; HDL-C, high-density lipoprotein-cholesterol; HSL, hormone sensitive lipase; IBM, inclusion body myositis; IMCL, intramyocellular lipid; TCA, tricarboxylic acid; jvs, juvenile visceral steatosis; LSM, lipid storage myopathy; MADD, multiple acyl-CoA dehydrogenase deficiency; MCAF, medium chain fatty acids; MCT, medium chain triglycerides; MGL, monoglyceride lipase; MS/MS, tandem mass spectrometry; NADH, nicotinamide adenine dinucleotide; NaHB, sodium-D-L-3-hydroxybutyrate; NEFA, non-esterified fatty acid; NF1, neurofibromatosis type 1; PM, polymyositis; PNPLA2, Patatin-like Phospholipase Domain Containing 2; PPAR, peroxisome proliferator-activated receptors; SLC22A5, Solute Carrier -avin adenine dinucleotide; FAT, fatty acid translocase; ETF, electron transfer flavoprotein; ETFDH, electron transfer flavoprotein dehydrogenase; ETF-QO, electron transfer flavoprotein -ubiquinone oxidoreductase; FABP4, fatty acid binding protein-4; FACS, fatty acyl-CoA synthetase; FADH2, flavin adenine dinucleotide; FAT, fatty acid translocase; FATP, fatty acid transport protein; GC-MS/MS, gas chromatography - tandem mass spectrometry; HDL-C, high-density lipoprotein-cholesterol; HSL, hormone sensitive lipase; IBM, inclusion body myositis; IMCL, intramyocellular lipid; TCA, tricarboxylic acid; jvs, juvenile visceral steatosis; LSM, lipid storage myopathy; MADD, multiple acyl-CoA dehydrogenase deficiency; MCAF, medium chain fatty acids; MCT, medium chain triglycerides; MGL, monoglyceride lipase; MS/MS, tandem mass spectrometry; NADH, nicotinamide adenine dinucleotide; NaHB, sodium-D-L-3-hydroxybutyrate; NEFA, non-esterified fatty acid; NF1, neurofibromatosis type 1; PM, polymyositis; PNPLA2, Patatin-like Phospholipase Domain Containing 2; PPAR, peroxisome proliferator-activated receptors; SLC22A5, Solute Carrier -avin adenine dinucleotide; FAT, fatty acid translocase; ETF, electron transfer flavoprotein; ETFDH, electron transfer flavoprotein dehydrogenase; ETF-QO, electron transfer flavoprotein -ubiquinone oxidoreductase; FABP4, fatty acid binding protein-4; FACS, fatty acyl-CoA synthetase; FADH2, flavin adenine dinucleotide; FAT, fatty acid translocase; FATP, fatty acid transport protein; GC-MS/MS, gas chromatography - tandem mass spectrometry; HDL-C, high-density lipoprotein-cholesterol; HSL, hormone sensitive lipase; IBM, inclusion body myositis; IMCL, intramyocellular lipid; TCA, tricarboxylic acid; jvs, juvenile visceral steatosis; LSM, lipid storage myopathy; MADD, multiple acyl-CoA dehydrogenase deficiency; MCAF, medium chain fatty acids; MCT, medium chain triglycerides; MGL, monoglyceride lipase; MS/MS, tandem mass spectrometry; NADH, nicotinamide adenine dinucleotide; NaHB, sodium-D-L-3-hydroxybutyrate; NEFA, non-esterified fatty acid; NF1, neurofibromatosis type 1; PM, polymyositis; PNPLA2, Patatin-like Phospholipase Domain Containing 2; PPAR, peroxisome proliferator-activated receptors; SLC22A5, Solute Carrier

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oxygen consumption). While most body triacylglycerol (> 80%) is derived from sources of dietary fat, endogenous triacylglycerol synthesis occurs primarily in the adipocytes and liver; this is fueled by excess available glucose and/or precursors of hepatic gluconeogenesis [3].

In healthy individuals triacylglycerol is primarily stored in subcutaneous and visceral adipose tissue [4]. While minimal, skeletal muscle also contains triacylglycerol stores that can provide energy for muscle activity [5]. These are primarily stored in adipocytes situated between muscle fibers, but can also be present in small, lipid droplets within muscle fibers termed intramyocellular lipid (IMCL) inclusions [5]. Resting tissue IMCL levels can vary in response to environment and energy demand; indeed while increased muscle IMCL is a hallmark of metabolic disease, in elite endurance trained athletes IMCL stores are dramatically elevated as a response to chronic training (termed fat adaptation) [6].

2. Overview of fatty acid metabolism

In order to contextualize the pathways dysregulated in lipid storage myopathy patients, it is important to define the normal mechanism for the breakdown of fats. In this section we provide a brief overview of the key pathways and enzymatic steps in fatty acid metabolism relevant to lipid storage diseases. For an in-depth and complete review of metabolic biochemistry we recommend reviews by Zechner, et al. [7], Longo, et al. [8], Houten, et al. [9], and Letts and Sazanov [10].

Briefly, triacylglycerol stored in cells are hydrolyzed sequentially into glycerol and fatty acids via lipolytic pathways. Next, the fatty acids are transported across the mitochondrial membrane and then broken down by beta-oxidation to generate acetyl-CoA. Finally, within the mitochondria, acetyl-CoA molecules are used to fuel classical aerobic respiration pathways of the TCA cycle and the electron transport chain. Many of the key regulatory enzymes and factors governing these processes are linked to lipid storage myopathies.

2.1. Intracellular lipolysis

The enzyme adipose triglyceride lipase (ATGL) catalyzes the initial and often rate-limiting step of intracellular lipolysis [11]. This involves the conversion of a triacylglycerol molecule to diacylglycerol, which releases a fatty acid [11]. ATGL is predominately expressed in adipose tissue and to a lesser extent in non-adipose tissue including liver, heart and skeletal muscle [7]. It has been speculated that other neutral lipases may play a role in the initiation of lipolysis in non-adipose tissues [7].

Diacylglycerols are subsequently hydrolyzed by hormone sensitive lipase (HSL), to monoacylglycerols, which are finally hydrolyzed by monoacylglyceride lipase (MGL) to liberate the final fatty acid [7]. HSL can also hydrolyse triacylglycerols and monoacylglycerols, but with lesser specificity [7].

Lipolysis occurs largely in response to energy demand, and is initiated by the release of hormones such as glucagon, epinephrine, and norepinephrine [7, 12]. Adipose tissue, these trigger the activation of adenylate cyclase (AC) and subsequently Protein Kinase A (PKA) [12]. PKA phosphorylates Perilipin-1 causing CGI-58 to dissociate from Perilipin 1 and induce ATGL activity [12] (Fig. 1 A). In non-adipose tissue ATGL and its co-activator CGI-58 are recruited, and directly bind to Perilipin-5 to induce the hydrolysis of triacylglycerol to diacylglycerol [13, 14] (Fig. 1 B).

2.2. Fatty acid transport

After fatty acids are released into circulation, they are transported in the blood bound to serum albumin, a protein with high-affinity fatty acid binding sites [15]. When the fatty acid-albumin complex reaches the target cell, it is thought that the fatty acid then dissociates to begin the cellular uptake process [15, 16]. Several membrane-associated fatty acid binding transport proteins, in particular members of the Fatty Acid Transport Protein (FATP) family, promote the uptake of fatty acids into various cell types [17]. FATP rapidly translocate to the plasma membrane in response to insulin, and facilitate fatty acid transport across cellular membranes [17]. Additionally, Fatty Acid Translocase (FAT/CD36) is another key fatty acid transporter at the plasma membrane, which modulates long-chain fatty acid oxidation during times of rapid energy demand [18].

2.3. Carnitine shuttling of fatty acyl-coenzyme A’s

Once transport proteins take up the non-esterified fatty acids (NEFAs), the NEFAs are coupled with Coenzyme A (CoA) to form short-chain (< C8), medium-chain (C8–12), long-chain (C12-C24), or very long-chain acyl-CoA (> C24), depending on the length of the fatty acid carbon chain [17]. This is catalyzed by a family of intracellular enzymes with different chain-length specificities, known as the Fatty Acyl-CoA Synthetase’s (FACS). This conjugation prevents NEFAs from re-entering the circulation and facilitates their transport across the mitochondrial membrane [17] (Fig. 2).

While short and medium-chain acyl-CoA can passively diffuse across the mitochondrial membrane, long-chain and very long-chain acyl-CoA require active transport via the Carnitine Shuttle system [8]. Carnitine Palmitoyltransferase I (CPT I) is a key enzymatic regulator of carnitine bioactivity and catalyzes the binding of free carnitine and long chain/very long chain acyl-CoAs. Carnitine Translocase (CCT) then shuttles acylcarnitine across the inner mitochondrial membrane. Inside the mitochondrial matrix, the acylcarnitine is separated back into a long chain acyl-CoA and free carnitine by Carnitine Palmitoyltransfase II (CPT II) (Fig. 2) [8].

The action of CPT I requires sufficient endogenous carnitine to be present [19]. Carnitine can be synthesized from lysine and methionine by kidney and liver cells, but this is insufficient and humans require carnitine intake from dietary sources [19]. Free carnitine (unbound to fatty acid CoA molecules) is circulated in the blood and is actively transported into tissues by tissue-specific plasma membrane carnitine transporters [20]. These include Organic Cation/Carnitine Transporter 2 (OCTN2) (Fig. 2) [20].

2.4. Beta-oxidation

Beta-oxidation describes the enzymatic removal of carbon molecules from a fatty acid chain, beginning at the beta-carbon. This is catalyzed by a family of acyl-CoA dehydrogenases, which include short-, medium-, long-, and very long- chain acyl-CoA dehydrogenase [9, 21]. There are four enzymatic steps within a cycle, and each cycle liberates a 2-carbon acetyl-CoA molecule. The acetyl-CoA molecules can subsequently be metabolized via the tricarboxylic acid (TCA) cycle (also known as the Krebs or Citric acid cycle). The reduced intermediates, including Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide (FADH2), feed electrons from the 2-carbon acetyl-CoA to the electron transport chain [9] (Fig. 3).

2.5. The Electron transport chain and oxidative phosphorylation

The mitochondrial electron transport chain is a series of protein complexes that transfer electrons from electron donors, to electron acceptor molecules via a series of biochemical redox reactions [10]. Electrons are transferred to ubiquinone through electron acceptors including Electron Transfer Flavoprotein (ETF) and Electron Transfer Flavoprotein - Ubiquinone Oxidoreductase (ETF-QO) [22]. The respiratory chain and oxidative phosphorylation are not specific to lipid precursors, as acetyl-CoA is also a product of glucose and amino acid metabolism [23] (Fig. 3).
3. Lipid storage myopathies

Individuals with LSMs do not produce or produce inadequate amounts of crucial enzymes in the pathways just described. This results in intracellular lipid accumulation, which is associated with muscle weakness, myalgia and fatigue; these can have a profound impact on an individual’s quality of life.

Today, many lipid storage myopathies are effectively managed, but they are often reliant on an early and accurate diagnosis and can otherwise be lethal. A subset of lipid storage myopathies is still lacking in effective management strategies, and requires further extensive research.

3.1. Neutral lipid storage disease

Neutral lipid storage diseases (NLSD) are rare (prevalence unknown with ~50 cases reported in the literature) autosomal recessive disorders of lipolysis. They are clinically heterogeneous; however classically present with myopathy (NLSD-M, OMIM: #610717) and/or ichthyosis (NLSD-I, OMIM: #275630), which is dry, thickened, scaly skin. NLSD is often diagnosed by the presence of lipid inclusions within leukocytes, which is assessed from a peripheral blood smear. These lipid inclusions are often diagnosed by the presence of lipid inclusions within leukocytes, (NLSD-I, OMIM: #275630), which is dry, thickened, scaly skin. NLSD is of lipolysis. They are clinically heterogeneous; however classically...
metabolism [35, 37, 38]. Additional long-term treatment studies are required to determine the therapeutic potential of Bezafibrate.

Beta-blockers have also been tried in a clinical setting due to a partial rescue of patient myoblasts and fibroblasts reported in the literature [27]. However this was unsuccessful [35, 37]. Enzyme replacement therapy could be a potential treatment option, which has been under explored. Successful enzyme therapy has been observed in Fabry disease and Pompe disease [39], and may be applicable here.

3.1.2. Neutral lipid storage disease with ichthyosis (NLSD-I)

NLSD-I, also known as Chanarin-Dorfman syndrome, is caused by mutations in the Comparative Gene Identification-58 (CGI-58) also annotated as α/β-Hydrolase Domain-5 (ABHDS5) gene [25]. The CGI-58/ABHDS5 protein interacts with factors associated with the surface of lipid droplets, including the perilipins, and co-activates ATGL [40, 41]. Some mutant versions of CGI-58 have been shown to lose the ability to interact with perilipin; they are not recruited to lipid droplets and thus fail to activate ATGL [40, 41].

Patients with NLSD-I show a markedly different clinical phenotype and can be affected earlier in life compared to those with NLSD-M, despite CGI-58 and ATGL functioning in the same biochemical pathway [42]. NLSD-I can feature global developmental delay (including motor function and IQ), hepatomegaly and intestinal disease, but not cardiomyopathy. However the characteristic feature of the condition is ichthyosis, specifically non-bullous congenital ichthyosiform erythroderma (Table 2). The underlying mechanism is believed to be the excessive incorporation of nonpolar lipids into the lamellar membrane. This is followed by the formation of a non-lamellar phase within the stratum corneum interstices (outer skin layer), which impairs barrier function [43]. Ichthyosis is not observed in patients with NLSD-M. This suggests an ATGL-independent function of CGI-58 in the skin, and potentially other organs. Seasonal fluctuation of ichthyosis have also been observed, leading to speculation that temperature elevation may affect ATGL activity or other lipolytic pathways [44, 45]. Both skin and liver biopsies frequently aid in the diagnosis of NLSD-I, as well as genetic sequencing of CGI-58/ABHDS5 (Table 2).

Similar to NLSD-M, there is no curative treatment for NLSD-I. Topical application of urea-containing emollients, and dietary intervention are the mainstays for clinical treatment (Table 2). One of the original case reports for NLSD-I describes treatment with a gluten-free diet, which was reported to ameliorate gastrointestinal symptoms and slightly improve muscle strength [46]. The patient was initially prescribed prednisone (25-30 mg daily/1 year), vitamin D (5000 U daily/1 year), nystatin, and tetracycline however these had no effect [46]. Despite the beneficial response to a gluten-free diet, there have been no other reports involving this type of dietary intervention.
A combination of high carbohydrate, low fat diet supplemented with medium-chain triacylglycerols has shown to be a beneficial therapeutic dietary strategy (Table 2). There are several other recent case reports in the literature that outline other dietary interventions, including low fat [47, 48], LCFA-restricted [49] and protein-restricted carbohydrate-rich diets [50], however have no clinical report to treatment effects, and therefore have been excluded from Table 2. This suggests these dietary interventions may have had little therapeutic benefit.

It is commonly advised throughout the literature that retinoids including acitretin should be avoided in cases where a NLSD-I patient has disturbed liver function. However, there are three recent reports where patients with impaired liver function have been prescribed acitretin [51–53]. In all cases, the individuals showed improvements in liver function, suggesting a review of clinical retinoid usage could be justified. Although retinoids may not be appropriate for all patients with impaired liver function, they could provide a viable treatment option provided they are used with caution and there is careful monitoring.

NLSD-I can be misdiagnosed as Sjogren-Larsson Syndrome, which also presents with ichthyosis [54]. Some clinical studies have shown in these patients the severity of itching is reduced with zileuton treatment [51–53]. This could be beneficial for NLSD-I patients but has yet to be investigated.

A Cgi58−/− knockout mouse model was generated in order to investigate the role of Cgi58 in triacylglycerol catabolism [56]. The study found Cgi58−/− mice failed to thrive and died within 16 h after birth, in contrast to heterozygous Cgi58+/− mice, which were comparable to wildtype littermates. Cgi58−/− mice featured marked triacylglycerol levels in carcasses, hypoglycemia, hepatic steatosis, and lamellar ichthyosis associated with loss of skin permeability barrier function. Glucose injections, increased humidity and Vaseline® application failed to rectify the defects or extend the lifespan [56]. A more recent study in Cgi58−/− mice showed that the lethal skin barrier defect could be rescued by promoting Cgi-58 expression in the suprabasal epidermal layers, a prerequisite for a functional skin permeability barrier [57].

3.2. Primary carnitine deficiency

Primary Carnitine Deficiency (PCD, OMIM: #212140) is an autosomal recessive disorder caused by mutations in the Solute Carrier Family 22 Member 5 (SLC22A5) gene. The SLC22A5 gene encodes for the Organic Cation/Carnitine Transporter 2 (OCTN2) protein. It is responsible for the majority of cellular carnitine uptake, and does so in a sodium-dependent manner. The majority of PCD patient mutations in SLC22A5 have been shown to reduce transporter activity, and transporter activity has been shown to correlate with disease severity [58, 59].

The incidence of PCD varies with a frequency of 1:3000–1:120,000 worldwide. It typically presents from early infancy or childhood, and chiefly manifests with liver, skeletal and cardiac muscle involvement. Patients can experience episodes of hypoketotic hypoglycemia, hyperammonemia, hepatomegaly, and hepatic encephalopathy. Additionally, skeletal muscle weakness, impaired motor function, prominent fatigue and cardiomyopathy are also commonly reported (Table 3). Low levels of free and total carnitine indicated by routine biochemistry, or tandem mass spectrometry (MS/MS) suggests carnitine deficiency (PCD, OMIM: #212140) is an autosomal recessive disorder caused by mutations in the Solute Carrier Family 22 Member 5 (SLC22A5) gene. The SLC22A5 gene encodes for the Organic Cation/Carnitine Transporter 2 (OCTN2) protein. It is responsible for the majority of cellular carnitine uptake, and does so in a sodium-dependent manner. The majority of PCD patient mutations in SLC22A5 have been shown to reduce transporter activity, and transporter activity has been shown to correlate with disease severity [58, 59].

The cardiac and skeletal muscle function of PCD patients is rescued by pharmacological doses of carnitine (Table 3). Carnitine supplementation restores carnitine plasma levels, however muscle tissue

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**Fig. 3. Beta oxidation and the electron transport chain.** Beta oxidation is the catabolic process by which a fatty acyl-CoA molecule is broken down within the mitochondrial matrix to produce 1) an acetyl CoA molecule that feeds into the Krebs cycle and 2) NADH and FADH2 which act as reducing agents and transfer electrons across the Electron Transport Chain (ETC) complexes. The initial rate-limiting step of beta oxidation is catalyzed by a class of enzymes, known as the Acyl-CoA Dehydrogenases. These enzymes transfer electrons to the electron acceptors Electron Transfer Flavoprotein (ETF) and Electron Transfer Flavoprotein-Ubiquinone Oxioreductase (ETF-UO). They then transfer the electrons to complex 1, Ubiquinone, of the electron transport chain (ETC). The ETC involves a series of 5 protein complexes, transferring electrons through a series of redox reactions to the ATPSynthase (complex 5) resulting in the phosphorylation of ADP into ATP.

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<table>
<thead>
<tr>
<th>Lipid storage myopathy</th>
<th>Author/year</th>
<th>Patient</th>
<th>Clinical presentation</th>
<th>Diagnostic testing</th>
<th>Treatment</th>
<th>Clinical outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLSD-M</td>
<td>Muggenthaler, et al., 2016 [37]</td>
<td>22 years of age, Male</td>
<td>- Syncope, shortness of breath, muscle weakness, severe dilated biventricular cardiomyopathy.</td>
<td>- Muscle biopsy: lipid storage - Sanger seq.: homozygous missense mutation c.497A &gt; G in PNPLA2</td>
<td>- Beta-blocker, ACE inhibitor, Eplerenone, warfarin and a defibrillator was implanted</td>
<td>- Initial improvements in shortness of breath - Condition deteriorated and patient required cardiac implantation (follow-up not stated).</td>
</tr>
<tr>
<td>NLSD-M</td>
<td>Pasanisi, et al., 2016 [110]</td>
<td>26 years of age, Male</td>
<td>- Muscle weakness, walking difficulties, left ventricular dilation, ↓ left ventricular systolic function</td>
<td>- Muscle biopsy: lipid storage - Gene analysis: homozygous deletion of seven nucleotides in exon 7 (c.41_47_delGCTGCGG) of PNPLA2</td>
<td>- Enalapril (5 mg bid) and ivabradine (7.5 mg/di)</td>
<td>- Ejection fraction ↑ - Improved exercise capacity (oxygen consumption increased from 48 to 57% of the predicted value) (18 month follow-up) - Salbutamol was not tolerated and dexamethasone was stopped after no clinical improvement (2 month follow-up) - ↓ myalgia and fatigue with fenofibrate treatment (follow-up not stated).</td>
</tr>
<tr>
<td>NLSD-M</td>
<td>Pennisi, et al, 2015 [111]</td>
<td>72 years of age, Female</td>
<td>- Exercise intolerance, myalgia and muscle weakness.</td>
<td>- Peripheral blood smear: Jordans anomaly - Muscle biopsy: mild non-specific myopathic changes - Gene analysis: c.497A &gt; G in exon 5 and c.1442C &gt; T in exon 10 of PNPLA2</td>
<td>- Salbutamol (4 mg, two divided doses), dexamethasone, (25 mg/day), and fenofibrate (160 mg/day)</td>
<td>- Over 10 years cardiac function deteriorated. Patient was discharged and received medication in outpatient settings.</td>
</tr>
<tr>
<td>NLSD-M</td>
<td>Kaneko, et al, 2014 [112]</td>
<td>59 years of age, Male</td>
<td>- Muscle weakness, cardiomegaly with dilation of the left ventricle and decreased ejection fraction.</td>
<td>- Muscle biopsy: lipid storage - Peripheral blood smear: Jordans anomaly - Gene analysis: Homozygous c.576delC PNPLA2 mutation</td>
<td>- Beta-blockers, ACE inhibitors and diuretics - Intensive care and cardiovascular treatment - Oral Carnitine (1 g/daily)</td>
<td>- No significant clinical improvement was observed and treatment ceased (6 month follow-up).</td>
</tr>
<tr>
<td>NLSD-M</td>
<td>Perrin, et al, 2013 [26]</td>
<td>17 years of age, Male</td>
<td>- Fatigue, increased difficulties in running and walking, and myalgia after prolonged exercise.</td>
<td>- Muscle biopsy: lipid storage - Peripheral blood smear: Jordans anomaly - Gene analysis: homozygous mutation c.865C &gt; T in PNPLA2</td>
<td>- Implantable cardioverter defibrillator in patient one and BEZ treatment in both patients (Bezalip, Actavis, 400 mg/daily).</td>
<td>- No improvements in grip strength or cardiac function (7 month follow-up) - Some improvement in leg strength. - The only notable difference was cardiac and muscle fat content.</td>
</tr>
<tr>
<td>NLSD-M</td>
<td>Van De Weijer, et al., 2013 [18]</td>
<td>37 and 39 years of age, Female</td>
<td>- Progressive muscle weakness, diffuse hypokinesia of the left and right ventricles of the heart with ↓ ejection fraction. - Mild myopathy and reduced exercise intolerance (heterozygote carrier).</td>
<td>- Muscle biopsy: lipid storage - Peripheral blood smear: Jordans anomaly - Gene seq. Analysis: deletion causing a frame shift at amino acid position 270 in PNPLA2 of both patients. Additionally patient one has a missense mutation 548C &gt; T.</td>
<td>- Implantable cardioverter defibrillator in patient one and BEZ treatment in both patients (Bezalip, Actavis, 400 mg/daily).</td>
<td>- Some improvement in leg strength. - The only notable difference was cardiac and muscle fat content.</td>
</tr>
</tbody>
</table>

Table notes: We searched for articles in the Medline via OvidSP (1946-Present), PubMed, Scopus and ProQuest Research Library databases using the following strategy, combining terms with OR: “Primary Carnitine Deficiency”, “Multiple Acyl-CoA Dehydrogenase Deficiency”, “Glutaric Acidemia Type II”, “Neutral Lipid Storage disease with Ichthyosis”, “Chanarin-Dorfman Syndrome” and “Neutral Lipid Storage Disease with Myopathy”. To be included in this review studies needed to meet the following criteria: Clinical case reports, controlled and/or uncontrolled clinical trials involving an intervention and follow-up. Reports including unconfirmed diagnoses, or non-intervention observational studies were not included. Only English-written articles published within the last seven years were assessed (2011–2017 inclusive).
<table>
<thead>
<tr>
<th>Author and year</th>
<th>Patient (age and gender)</th>
<th>Clinical presentation</th>
<th>Confirmation diagnostic testing</th>
<th>Treatment</th>
<th>Reported clinical outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLSD-I Verma, et al.</td>
<td>8 years of age, Male</td>
<td>Generalised scaling, erythema, disseminated hypopigmentation, skin thickening and hyperkeratosis</td>
<td>Skin biopsy: Hyperkeratosis</td>
<td>Topical emollients and acitretin (10 mg/day)</td>
<td>Dramatic improvements of the skin condition and liver function tests were observed (2 month follow-up)</td>
</tr>
<tr>
<td>NLSD-I Arora, et al.</td>
<td>Newborn, Female</td>
<td>Congenital ichthyosis, and hepatomegaly</td>
<td>Liver biopsy: Macrocellular hepatosteatosis and Jordans anomaly</td>
<td>Oral acitretin (10 mg biweekly) and a diet with MCT’s and LCFAs.</td>
<td>Improved in clinical condition; 3 month follow-up.</td>
</tr>
<tr>
<td>NLSD-I Kazemi, et al.</td>
<td>29 years of age, Female</td>
<td>Developmentally delayed in all spheres, generalised scaling of the whole body, brown hyperpigmentation and hyperkeratosis</td>
<td>Gene analysis: homozygous nonsense mutation c.297 &gt; C in exon 3 of ABHD5.</td>
<td>Moderate amounts of carb, and MCT’s, and oral acitretin (0.5 mg/kg/day)</td>
<td>Transaminases levels continually fluctuated (follow-up period and 1 year follow-up).</td>
</tr>
<tr>
<td>NLSD-I Gupta, et al.</td>
<td>18 months of age, Male</td>
<td>Progressive upper abdominal distension, hepatomegaly, and generalised ichthyosis and hyperkeratosis</td>
<td>Liver biopsy: Macrocellular steatosis, cirrhosis and steatohepatitis</td>
<td>UDCA, vitamin E and localemollients</td>
<td>Improvement in clinical condition and liver function tests following dietary intervention, UDCA and vitamin E treatment, UDCA and vitamin E initiated at 4 years old.</td>
</tr>
<tr>
<td>NLSD-I Waheed, et al.</td>
<td>67</td>
<td>Generalised scaling and erythema reduced</td>
<td>Liver biopsy: Absent hepatosteatosis.</td>
<td>No treatment</td>
<td>Improvement in clinical condition (observing dietary and discontinuous)</td>
</tr>
<tr>
<td>NLSD-I Missaglia, et al.</td>
<td>1 year of age, Male</td>
<td>Motor and cognitive development normalized (2 year follow-up).</td>
<td>Liver biopsy: No significant findings.</td>
<td>Prednisolone (tapering dose of 0.5 mg/kg)</td>
<td>Liver enzymes have normalized (6 months follow-up).</td>
</tr>
<tr>
<td>NLSD-I Israeli, et al.</td>
<td>8 years of age, Male</td>
<td>Skin lesions were significantly reduced.</td>
<td>Liver biopsy: Normalised and no significant findings.</td>
<td>Prednisolone (tapering dose of 0.5 mg/kg)</td>
<td>Liver enzymes have normalized (6 months follow-up).</td>
</tr>
<tr>
<td>NLSD-I Huigen, et al.</td>
<td>1 year of age, Female</td>
<td>Developmentally delayed and hypotonia.</td>
<td>Liver biopsy: Severe macro and micro-vesicular steatosis and Jordans anomaly.</td>
<td>Prednisolone (tapering dose of 0.5 mg/kg)</td>
<td>Liver enzymes have normalized (6 months follow-up).</td>
</tr>
</tbody>
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**Table 2**

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<thead>
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<th>Lipid storage disease</th>
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<td>Oral acitretin (10 mg biweekly) and a diet with MCT’s and LCFAs.</td>
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<td>NLSD-I</td>
<td>2013 [51]</td>
<td>Progressive upper abdominal distension, hepatomegaly, and generalised ichthyosis and hyperkeratosis</td>
<td>Liver biopsy: Macrocellular steatosis, cirrhosis and steatohepatitis</td>
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<tr>
<td>NLSD-I</td>
<td>2016 [114]</td>
<td>Generalised scaling of the whole body, brown hyperpigmentation and hyperkeratosis</td>
<td>Gene analysis: homozygous nonsense mutation c.297 &gt; C in exon 3 of ABHD5.</td>
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<td>NLSD-I</td>
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Table 3: Treatment-outcome case reports of Primary Carnitine Deficiency (PCD) patients since 2011.

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>PCD</td>
<td>Ravindranath, et al., 2017 [118]</td>
<td>8 months of age, Male</td>
<td>- Recurrent vomiting, lethargy, jaundice, enlarged liver, non-ketotic hyperglycemia and hyperammonemia</td>
<td>MS/MS: ↓ free &amp; total carnitine &amp; medium-chain dicarboxylic aciduria.</td>
<td>- Intra venous dextrose, sodium benzoate, &amp; uncooked cornstarch in water</td>
<td>- No episodes of hypoglycemia ammonia levels &amp; liver function normalized (4 month follow-up).</td>
</tr>
<tr>
<td>PCD</td>
<td>Lahrouchi, et al., 2017 [66]</td>
<td>3 years of age, Female</td>
<td>- Progressive fatigue, shortness of breath, pallor of skin and conjunctivae and left ventricular hypertrophy.</td>
<td>Whole exome seq: heterozygous c.1316dupT in exon 8 of SLC22A5.</td>
<td>- Carnitine (100 mg/kg/day), MCT (30% total energy intake), and ↓ LCFAs.</td>
<td>- ↑ Activities of daily living. Exercise tolerance normalized to age matched controls (8 month follow-up)</td>
</tr>
<tr>
<td>PCD</td>
<td>Gantigara, et al, 2017 [139]</td>
<td>8 years of age, Male</td>
<td>- Altered sensorium, increased frequency of seizures and global developmental delay with hypotonia.</td>
<td>Serum: ↓ free carnitine</td>
<td>- Levocarnitine &amp; levetiracetam</td>
<td>- Patient seizure free (2 months follow-up)</td>
</tr>
<tr>
<td>PCD</td>
<td>Jun, et al, 2016 [77]</td>
<td>3 years of age, Female</td>
<td>- Tonic-clonic seizures and deeply drowsy. Experienced a severe course of hypoglycemic encephalopathy. This was attributed to consumption of pivalic acid-containing antibiotics.</td>
<td>MS/MS: ↓ free carnitine, Sanger Seq.: compound heterozygous mutations C.1354G &gt; A and c.231_234del in SLC22A5.</td>
<td>- Midazolam, phenobarbital and phenytoin, 10% glucose bolus, hydrocortisone (5 mg/kg/day), three divided doses.</td>
<td>- Blood glucose stable (35 day follow-up).</td>
</tr>
<tr>
<td>PCD</td>
<td>Deswal, et al, 2016 [120]</td>
<td>9 months of age, Male</td>
<td>- Jaundice, failure to gain weight, mild motor developmental delay, frequent breathing problems, cardiomagaly and biventricular hypertrophy.</td>
<td>Serum: ↓ free carnitine, Sanger Seq.: compound heterozygous mutations C.1354G &gt; A and c.231_234del in SLC22A5.</td>
<td>- Oral l-carnitine (100 mg/kg/day)</td>
<td>- Patient transferred to rehabilitation medicine for status epilepticus, disabled visual fixation, impaired visual acuity and impaired locomotion and transitional movements.</td>
</tr>
<tr>
<td>PCD</td>
<td>Mahale, et al, 2016 [121]</td>
<td>8 months of age, Female</td>
<td>- Fever, loose stools, excessive irritability, lethargy, hepatomegaly and worsening sensorium.</td>
<td>MS/MS revealed ↓ free carnitine (2.6 mM/l).</td>
<td>- Mechanical ventilator, intravenous fluids, and levocarnitine (100 mg/kg/day, divided doses).</td>
<td>- Gradually improved in sensorium and was extubated (follow-up not stated).</td>
</tr>
<tr>
<td>PCD</td>
<td>Al-shareef, et al, 2015 [122]</td>
<td>35 years of age, Female</td>
<td>- History of schizo-affective disorder, lithium-induced diabetes, insulin and hyperthyroidism. Drowsiness, personality changes, fatigue, generalised weakness, and deteriorating mobility.</td>
<td>Routine biochemistry diagnosis VHS. On 3 week follow-up serum: ↓ free and total carnitine</td>
<td>- Sodium valproate (1000 mg, divided in two doses).</td>
<td>- Within 3 days mental status improved achieving a 10/10 abbreviated mental test score.</td>
</tr>
<tr>
<td>PCD</td>
<td>Mudfa-Albayrak, et al., 2015 [123]</td>
<td>9 years of age, Male</td>
<td>- Dysmorphic appearance, fatigue and hypertrophic cardiomyopathy.</td>
<td>MS/MS revealed ↓ free carnitine, Genetic testing identified a homozygous c.1427T &gt; G mutation in SLC22A5.</td>
<td>- Oral carnitine (200 mg/kg/day)</td>
<td>- Clinical improvement was observed, particularly ↓ fatigue (2 month follow up)</td>
</tr>
<tr>
<td>PCD</td>
<td>Yilmaz, Atay, et al, 2015 [124]</td>
<td>12 years of age, Male</td>
<td>- Prominent fatigue, upper extremity muscle weakness, and decreased deep tendon reflexes. Congestive heart failure, hypoglycemic hypoketotic and encephalopathy secondary to PCD.</td>
<td>Serum: ↓ free carnitine, An SLC22A5 mutation was found (mutation details not stated).</td>
<td>- Mechanical ventilator, intravenous fluids, antibiotics and antiepileptic medication.</td>
<td>- It was reported that heart failure ceased (follow-up not stated).</td>
</tr>
<tr>
<td>PCD</td>
<td>Kumar, et al, 2015 [125]</td>
<td>3 months of age, Male</td>
<td>- Altered sensorium, fever, hypotonia, abnormal body movements, tachypnea, tachycardia and seizures and hepatomegaly.</td>
<td>Serum: Deficiency of free carnitine</td>
<td>- Methylninethionine, sodium bicarbonate, and antiepileptic medications.</td>
<td>- No post-treatment report on muscle function or hypoglycemic hypoketotic encephalopathy</td>
</tr>
<tr>
<td>PCD</td>
<td>Yilmaz, Seker, et al, 2015 [126]</td>
<td>3 years of age, Male</td>
<td>- Upper airway infection, cardiac insufficiencies, including cardiomagaly and left ventricular hypertrophy.</td>
<td>Serum: ↓ free and total carnitine, Next gen Seq.: homozygous c.597_597delG mutation in SLC22A5.</td>
<td>- Normalization of all clinical parameters reported (follow-up not stated).</td>
<td>- This patient has been followed for 14 years. Over time, improvements in cardiac functions were observed, including improvements in left ventricular ejection fraction. No further details given.</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Lipid storage myopathy</th>
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<tr>
<td><strong>PCD</strong></td>
<td>Wang, et al, 2014</td>
<td>5 children (1-14 years of age, 1 Female and 4 Males)</td>
<td>All patients presented with dyspnea, cardiomyopathy and enlargement of the left ventricle.</td>
<td>High-throughput seq.: heterozygous c.338G &gt; A, c.760C &gt; T and c.865C &gt; T SLC22A5 mutations. Serum: mean value 1.37 ± 0.66 μmol/L - Carnitine (200–300 mg/day p.o), plus digoxin, diuretics and vasodilators</td>
<td>- All patients presented with dyspnea, cardiomyopathy and enlargement of the left ventricle. Treatment included carnitine (200–300 mg/day p.o), digoxin, diuretics and vasodilators. Left ventricular function returned to normal and heart size normalized for all patients (4 month to 2 year follow-up).</td>
</tr>
<tr>
<td><strong>PCD</strong></td>
<td>Agnetti, et al, 2013</td>
<td>3.5 years of age, Female</td>
<td>Fatigue and marked pallor, cardiomegaly and severe congestive heart failure.</td>
<td>Serum: ↓carnitine - Muscle biopsy: lipid storage</td>
<td>Oral carnitine (3 g/day to 2 g/day after 6 months)</td>
</tr>
<tr>
<td><strong>PCD</strong></td>
<td>De Biase, et al, 2012</td>
<td>20 years of age, Female</td>
<td>Syncopal episode caused by ventricular tachycardia, and abnormal QT interval.</td>
<td>After newborn screened positive, serum: ↓total and free carnitine - Molecular analysis: c.95A &gt; G and c.136C &gt; G mutations in SLC22A5 - Carnitine (3 g increased to 4.2 g/day).</td>
<td>Metoprolol (50 mg to 75/day). Implantable defibrillator and mexiletine (450 mg/day). - High dose carnitine (3960 mg/day)</td>
</tr>
<tr>
<td><strong>PCD</strong></td>
<td>Mazzini, et al, 2011</td>
<td>22 years of age, Female</td>
<td>Irregular heart rhythm, witnessed ventricular fibrillation arrest requiring resuscitation.</td>
<td>Gene Seq.: A142S and R488H mutations on allele 1 and 1588G &gt; T on allele 2 of SLC22A5. Serum: ↓free carnitine - Endomyocardial biopsy: lipid storage.</td>
<td>Cardioverter-defibrillator implanted. Oral levocarnitine (3 g/day) and MCFA's</td>
</tr>
</tbody>
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Notes: We searched for articles in the Medline via OvidSP (1946-Present), PubMed, Scopus and ProQuest Research Library databases using the following strategy, combining terms with OR: “Primary Carnitine Deficiency”, “Multiple Acyl-CoA Dehydrogenase Deficiency”, “Glutaric Acidemia Type II”, “Neutral Lipid Storage Disease with Ichthyosis”, “Chanarin-Dorfman Syndrome”, and “Neutral Lipid Storage Disease with Myopathy”.

To be included in this review studies needed to meet the following criteria: Clinical case reports, controlled and/or uncontrolled clinical trials involving an intervention and follow-up. Reports including unconfirmed diagnoses, or non-intervention observational studies were not included. Only English-written articles published within the last seven years were assessed (2011-2017 inclusive).
Table 4  
Treatment-outcome case reports of multiple Acyl-CoA dehydrogenase de
cificiency (MADD) patients since 2011.

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<tr>
<td>MADD</td>
<td>Vattemi et al., 2017 [132]</td>
<td>40 years of age, Female</td>
<td>- Diffuse myalgia, muscle stiffness, exercise intolerance and progressive weakness.</td>
<td>- Muscle biopsy: atrophic fibers and lipid storage - Gene screening: no mutations in ETFB, ETFB or ETFDH.</td>
<td>- Riboflavin (100 mg/day slowly lowered to 20 mg/day).</td>
<td>- Muscle weakness had completely resolved (22 years follow-up)</td>
</tr>
<tr>
<td>MADD</td>
<td>Creanza et al., 2017 [133]</td>
<td>18 years of age, Female</td>
<td>- Pregnant</td>
<td>- Ongoing myalgia and weakness with prednisolone (8 year follow up). - Symptom free with riboflavin, CoQ10 and carnitine and L-carnitine (up to 4 g/day follow up)</td>
<td>- Carnitine, 11 with riboflavin (100 mg/day), pyridoxine (300 mg/day), c-carnitine (up to 4 g/day during third trimester) and protein supplements (up to 30 g/day during second trimester) throughout pregnancy - During delivery and postoperative period carnitine (3 g/day) and an isotonic solution (with 10% dextrose) was administered.</td>
<td>- Pregnancy progressed and patient gave birth without complications</td>
</tr>
<tr>
<td>MADD</td>
<td>Pooja et al., 2017 [134]</td>
<td>24 years of age, Male</td>
<td>- Exercise-induced weakness and fatigue</td>
<td>- MS/MS: ↑medium and long chain acyl carnitines</td>
<td>- Riboflavin (100 mg/day), CoQ10 (60 mg/day) and carnitine (500 mg/day)</td>
<td>- Dramatic improvement in exercise tolerance (6 month follow up).</td>
</tr>
<tr>
<td>MADD</td>
<td>Vengalil et al., 2017 [135]</td>
<td>6 adults (17–40 years of age, 1 Female and 5 Males)</td>
<td>- Exertional myalgia, and muscle weakness</td>
<td>- Muscle biopsy: lipid storage - MS/MS: Δacylcarnitines and carnitine - Muscle biopsy: lipid storage</td>
<td>- Initially received a course of oral steroids (for polymyositis) - Riboflavin, CoQ10 and carnitine and restriction of fat intake.</td>
<td>- No definite benefits of oral steroids. - All patients reported alleviation of symptoms: fatigue, muscle weakness, dysphagia, myalgia and dropped head syndrome (follow-up period not stated). - Ongoing myalgia and weakness with prednisolone (8 year follow up). - Symptom free with riboflavin (6 month follow up)</td>
</tr>
<tr>
<td>MADD</td>
<td>Fu et al., 2016 [136]</td>
<td>23 years of age, 68 years of age and 15 years of age, Male</td>
<td>- Muscle weakness and exercise intolerance</td>
<td>- Gene analysis: 3 compound heterozygous variants of ETFDH including c.892C &gt; T, c.453delA and c.449_453delTAACA, c.1227A &gt; C, c.65A &gt; G, c.920C &gt; G and c.1450 T &gt; C in ETFDH</td>
<td>- Prednisolone (60 mg per day) (for polymyositis) - Proton pump inhibitors (for irritable bowel syndrome) - Riboflavin (60-90 mg/day) and CoQ10 (60 mg/day)</td>
<td>- All treated with c-carnitine, 11 with riboflavin, 4 with CoQ10, 1 with MCT's, all on adapted diet and limited fat intake - Followed up for up to 27 years there was a good response, 11/13 continued to display minor symptoms of exercise intolerance</td>
</tr>
<tr>
<td>MADD</td>
<td>Behin et al., 2016 [132]</td>
<td>13 Adults (21–55 years of age, 8 Female and 5 Male)</td>
<td>- Exercise intolerance, myalgia and muscle weakness</td>
<td>- Muscle biopsy: lipid storage - Gene analysis: ETFDH mutations including c.250G &gt; A, c.591G &gt; A, c.622G &gt; C, c.587C &gt; G, c.1732C &gt; T, c.79C &gt; T, c.406-2A &gt; G, c.1366C &gt; T, c.877G &gt; C, c.245C &gt; T and c.769T &gt; C.</td>
<td>- Riboflavin (60 mg/day), carnitine (2g/ day) and CoQ10 (100 mg/day), vitamin B12 (500μg/day)</td>
<td>- Strength returned (two weeks follow-up) - 2/6 patients reported none to mild alleviation of sensory disturbances (18 month follow up).</td>
</tr>
<tr>
<td>MADD</td>
<td>Wang et al., 2016 [137]</td>
<td>6 Adults (33–54 years of age, 1 Female and 5 Male)</td>
<td>- Proximal limb weakness and sensory neuropathy, particularly distal limb numbness</td>
<td>- Muscle biopsy: myofibre size variability and lipid storage - Urine: 12-hydroxyglutaric acid, 2-hydroxyadipic acid and palmitic acid - Muscle biopsy: lipid storage - Metabolites: 2-hydroxyglutaric acid, glyceric acid and 2-hydroxyadipic acid.</td>
<td>- Riboflavin (60 mg/day), carnitine (2g/ day) and CoQ10 (100 mg/day), vitamin B12 (500μg/day)</td>
<td>- Strength returned (two weeks follow-up) - 2/6 patients reported none to mild alleviation of sensory disturbances (18 month follow up).</td>
</tr>
<tr>
<td>MADD</td>
<td>Peng et al., 2015 [138]</td>
<td>46 years of age, Male</td>
<td>- Bent spine, dropped head and numbness</td>
<td>- Muscle biopsy: myopathia and muscle weakness</td>
<td>- Riboflavin (60 mg/day), after 3 weeks dropped down to riboflavin (30 mg/ day)</td>
<td>- Spine and dropped head completely resolved 93 week follow-up - Muscle strength improved and mild</td>
</tr>
</tbody>
</table>

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### Table 4 (continued)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>MADD</strong> Zhuo, et al, 2015 [139]</td>
<td>9 years of age, Female</td>
<td>- Muscle weakness, intermittent vomiting and hepatoplenomegaly</td>
<td>- Serum: &gt; G in exon 7, and c.920C &gt; G in exon 8 of EFTDH. - Muscle biopsy: lipid storage - Gene screening: c.770A &gt; G in exon 10 and c.1450T &gt; C in exon 11 of ETFDH.</td>
<td>Riboflavin (100 mg/day) and l-carnitine (50 mg/kg/day), &lt;1% daily caloric &lt;1% fat diet</td>
</tr>
<tr>
<td><strong>MADD</strong> Wen, et al, 2015 [140]</td>
<td>24 years of age, Male</td>
<td>- General fatigue, weakness, muscle pain, intermittent nausea and vomiting</td>
<td>- Serum: &gt; G in exon 7, and c.920C &gt; G in exon 8 of EFTDH. - Muscle biopsy: myofibre size variability and lipid storage - Gene screening: compound heterozygous mutations c.1211T &gt; C in exon 10 and c.1450T &gt; C in exon 9 of ETFDH.</td>
<td>Prednisone therapy (60 mg/day) (for polymyositis) - l-carnitine (2 g/day) and Riboflavin (30 mg)</td>
</tr>
<tr>
<td><strong>MADD</strong> Ersoy, et al, 2015 [141]</td>
<td>19 years of age, Female</td>
<td>- Progressive muscle weakness, dyspnea and hepatomegaly</td>
<td>- Serum: acyl carnitines and organic acid analysis: &gt; G in exon 9 of ETFDH.</td>
<td>Carnitine (1 g), riboflavin (100 mg) and CoQ10 (100 mg), hemodialysis and physiotherapy</td>
</tr>
<tr>
<td><strong>MADD</strong> Gautschi, et al, 2015 [142]</td>
<td>2.5 years of age, Male</td>
<td>- Progressive muscle weakness, severe metabolic decompensation, hypoglycaemia and diffuse leukodystrophy.</td>
<td>- Serum: acyl carnitines and urinary organic acids suggestive of MADD. - Gene analysis: homozygous mutation c.1106G &gt; C in exon 9 of ETFDH.</td>
<td>A fat and protein restricted diet and carnitine, riboflavin (100 mg/day) and ubiquinone (5-10 mg/kg/day). - Racemic mixture of NaHB (3-4% of daily caloric need)</td>
</tr>
<tr>
<td><strong>MADD</strong> Rosenbohm, et al, 2014 [143]</td>
<td>25 years of age, Female</td>
<td>- Erythematous rash, rhabdomyolysis, and progressive quadriaparesis becoming bedbound</td>
<td>- Muscle biopsy: lipid storage - MS/MS: l-2-OH glutarate, ethylmalonic, 3-OH-propionate - Gene analysis: compound heterozygous missense mutations (890G &gt; T/W297 L and 950C &gt; G/P317R) in ETFDH.</td>
<td>Riboflavin (200 mg/day), CoQ10 (15 mg/kg/day), a diet with &lt;1% fat (10-15%), &lt;1% carb, and medium protein levels (10%)</td>
</tr>
<tr>
<td><strong>MADD</strong> Shioya, et al, 2014 [90]</td>
<td>31 years of age, Male</td>
<td>- Muscle weakness and limb fatigability</td>
<td>- Serum: &gt; G in exon 7, and c.920C &gt; G in exon 8 of EFTDH. - Muscle biopsy: lipid storage - Serum: &gt; G in exon 7, and c.920C &gt; G in exon 8 of EFTDH. - Gene analysis: homozygous c.1544G &gt; T mutation in ETFDH.</td>
<td>l-carnitine, and riboflavin (105 mg/day) or BEZ (8.2 mg/kg/day). - Riboflavin was added to l-carnitine and BEZ treatment.</td>
</tr>
<tr>
<td><strong>MADD</strong> Zhu, et al, 2014 [144]</td>
<td>13 adults (26–57 years of age, 4 Females and 9 Males)</td>
<td>- Muscle weakness, exercise intolerance, myalgia and recurrent vomiting after fatty meals</td>
<td>- Gene analysis: compound heterozygous missense mutations (890G &gt; T/W297 L and 950C &gt; G/P317R) in ETFDH.</td>
<td>l-carnitine (2 g/day), riboflavin (60 mg/day), CoQ10 (90 mg/day). - Less responsive patients (3) were administered a higher dose of riboflavin (120 mg/day) and prednisone (30 mg/day)</td>
</tr>
</tbody>
</table>
| **MADD** Van Rijt, et al., 2014 [84] | Newborn, Female | - Hypoglycaemia, developmental delay, severe tachyarrhythmias and progressive cardiomypathy. | - Gene analysis: homozygosity for a mutation in ETF (specific mutation not stated). | Frequent feedings, restriction of fat, riboflavin, CoQ10. - NaHB was added (increased from (continued on next page)
Recurrent vomiting associated with fatigue resolved by treatment. Gastric enteritis improved with oral rehydration solution. Gave birth to a child by C-section. Remained stable with no hypoglycemia or acidosis during or after delivery.

- Intravenous carnitine (660 mg 3 times daily), riboflavin (100 mg), intravenous 10% dextrose and ondansetron, commercial oral rehydration solution during pregnancy.
- Muscle biopsy: lipid storage
- Urine: abnormal excretion of glutarate, methylmalonic acid and 2-hydroxyglutaric acids
- MS/MS: [acyl carnitines
- Gene analysis: compound heterozygous mutations including c.1522C > A and c.1583,1584insA in ETFDH.
- At diagnosis blood acyl-carnitine and urinary organic acid profiles were consistent with MADD.
- PCR and bidirectional DNA seq.: homozygous c.1601C > T mutation in exon 12 of ETFDH.
- Intravenous carnitine (660 mg 3 times daily), riboflavin (100 mg), intravenous 10% dextrose and ondansetron, commercial oral rehydration solution during pregnancy.
- Intravenous carnitine (During delivery 1000 mg every 8h, after delivery 1320 mg and reduced back to pre-delivery levels).
- Muscle biopsy: lipid storage
- Serum: ↑ free carnitine and acyl carnitine
- Urine: ↑ glutarate and 2-OH-glutarate
- Gene analysis compound heterozygous missense ETFDH mutation c.1211T > C in exon 11 and c.1786G > A in exon 13.
- Muscle weakness and fatigue gradually improved (3 week follow up).
- Muscle weakness and fatigue resolved (6 month follow up).

Table 4 (continued)

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<tr>
<td>MADD Wen, et al, 2013</td>
<td>34 Adults (6–50 years of age, 18 Females, 16 Males)</td>
<td>All adults showed proximal muscle weakness and exercise intolerance</td>
<td>Muscle biopsy: lipid storage</td>
<td>450 mg/kg/day to 900 mg/kg/day and finally 2600 mg/kg/day.</td>
<td>CoQ10 (60 mg/day), and after two weeks riboflavin therapy</td>
<td>Partial recovery of muscle weakness with CoQ10 (2 week follow-up), and on the addition of riboflavin muscle strength almost returned to normal (2 month follow-up).</td>
</tr>
<tr>
<td>MADD Zhao, et al, 2012</td>
<td>55 years of age, Male</td>
<td>Osphagia, muscle weakness, difficulty walking, fatigue, swallowing and speaking anorexia and intermittent nausea</td>
<td>Muscle biopsy: lipid storage</td>
<td>Riboflavin (100 mg/day) and ↓ protein/fat and ↑ carb diet</td>
<td>Symptoms subsided and exercise intolerance improved within a few weeks (6 year follow-up).</td>
<td></td>
</tr>
<tr>
<td>MADD Trakadis, et al, 2012</td>
<td>24 years of age, Female</td>
<td>Pregnant haha this is not a disease. Was she doing a standard preg test or something?</td>
<td>At diagnosis blood acyl-carnitine and urinary organic acid profiles were consistent with MADD.</td>
<td>Intravenous carnitine (660 mg 3 times daily), riboflavin (100 mg), intravenous 10% dextrose and ondansetron, commercial oral rehydration solution during pregnancy.</td>
<td>Recurrent vomiting associated with fatigue resolved by treatment. Gastrenteritis improved with oral rehydration solution. Gave birth to a child by C-section. Remained stable with no hypoglycemia or acidosis during or after delivery.</td>
<td></td>
</tr>
<tr>
<td>MADD Gotelli, et al, 2012</td>
<td>20 years of age, Male</td>
<td>Waddling and stepping gait, reduced reflexes and muscle weakness</td>
<td>Muscle biopsy: lipid storage</td>
<td>Riboavin (100 mg/daily) and ↓ fatty acid, ↓ protein and ↑ carb diet</td>
<td>Able to walk normally with no significant weakness (1 year follow-up).</td>
<td></td>
</tr>
<tr>
<td>MADD Rosa, et al, 2012</td>
<td>7 months of age, Male</td>
<td>Developmental delay and muscle weakness</td>
<td>Muscle biopsy: lipid storage</td>
<td>Carnitine (1 g/day) and after one month additional riboflavin (100 mg/day) and ubiquinone (50 mg/day) treatment</td>
<td>Modest improvement of limb weakness with carnitine (1 month follow-up).</td>
<td></td>
</tr>
<tr>
<td>MADD IZUMI, et al, 2011</td>
<td>56 years of age, Male</td>
<td>Fatigability, muscle weakness, myalgia, weight loss and dysarthria.</td>
<td>Muscle biopsy: lipid storage</td>
<td>Riboavin (100 mg/day), l-carnitine (50 mg/kg/day) and ↓ fat diet</td>
<td>Improved motor function, and effect of therapy on intellectual disability (6 month follow-up).</td>
<td></td>
</tr>
<tr>
<td>MADD Kaminsky, et al, 2011</td>
<td>55 years of age, Female</td>
<td>Severe myalgia, muscle weakness,</td>
<td>Muscle biopsy: lipid storage</td>
<td>Riboavin (30 mg/day), l-carnitine (50 mg/kg/day) and ↓ fat, ↓ protein, ↑ carb diet</td>
<td>Able to walk normally and myalgia resolved (6 month follow-up).</td>
<td></td>
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Notes: We searched for articles in the Medline via OvidSP (1946-Present), PubMed, Scopus and Proquest Research Library databases using the following strategy, combining terms with OR: “Primary Carnitine Deficiency” “Multiple Acyl-CoA Dehydrogenase Deficiency”, “Gluutaric Acidemia Type II”, “Neutral Lipid Storage disease with Ichthyosis”, “Chanarin-Dorfman Syndrome” and “Neutral Lipid Storage Disease with Myopathy”.

To be included in this review studies needed to meet the following criteria: Clinical case reports, controlled and/or uncontrolled clinical trials involving an intervention and follow-up. Reports including unconfirmed diagnoses, or non-intervention observational studies were not included. Only English-written articles published within the last seven years were assessed (2011–2017 inclusive).
carnitine concentration reaches only 5–10% of normal [60]. This is due to the persistent impairment of the OCTN2 carnitine membrane transporter that limits carnitine uptake in muscle to passive diffusion [61]. Nonetheless, the increases in muscle carnitine are sufficient to regain normal physiological function [60, 61]. Additionally, carnitine may act via antioxidant activity [62]. In skeletal muscle, reactive oxygen and nitrogen species are normally synthesized at low levels during force production. However, when reactive species accumulation overtakes tissue antioxidant capacity, then oxidative stress activates pathophysiologic signalling leading to reduced muscle strength capacity and fatigue [62]. L-carnitine has been shown to protect key enzymes of the antioxidant defense system from further peroxidative damage [63].

Multiple long-term follow-up studies have been reported to ascertain the efficacy of lifetime carnitine supplementation [64, 65] showing it’s safe and effective. Notably many undeveloped countries lack newborn genetic screening, which can result in preventable mortalities of young children with PCD [66]. This highlights the importance of both newborn screening and early intervention before irreversible organ damage occurs [67].

Individuals who are heterozygous for SLC22A5 gene are reported to have mildly reduced plasma carnitine levels, and they can progressively develop benign cardiac hypertrophy. It is estimated these asymptomatic PCD individuals may encompass up to 1% of the population. It is unclear whether SLC22A5 heterozygosity leads to an increased risk of cardiomyopathy [68–71]. The addition of sodium pivalate to the drinking water of rodents to induce a PCD phenotype is a well-established pre-clinical model. Pivacic acid and carnitine form a pivaloylcarnitine ester; this is excreted to induce carnitine deficiency [72]. This model has proven useful for studying the cardiac manifestations of carnitine deficiency syndromes, including asymptomatic PCD [72]. In the pivalic acid-treated rat model, β-adrenergic stimulation of the myocardium resulted in a 50% mortality rate. This was rescued by carnitine supplementation [73]. The heterozygous juvenile visceral steatosis (jvs/+ mouse) is an alternative genetic rodent model of asymptomatic PCD [74]. The jvs/+ mice are prone to cardiomyopathy and heart failure when challenged by surgically induced pressure overload on the heart [75]. These models justify further clinical research into the cardiac risks associated with asymptomatic PCD, and whether these individuals may benefit from prophylactic carnitine treatment.

3.2.1. HIGH RISK: Pivacic acid containing antibiotics are contraindicated for PCD patients

The death of five adults with PCD has recently been attributed to the consumption of antibiotics containing pivic acid, a chemical that depletes carnitine by esterification [76]. These cases were reported in the Faroe Islands, an archipelago in the North Atlantic Ocean with the highest reported frequency of PCD worldwide (1:3000). Additionally, a recent case report associated pivic acid containing antibiotics with an extremely severe hospital course for another patient [77]. Pivic acid is commonly used to increase oral bioavailability of antibiotics, including pivampicillin and pivmecillinam. Current studies are estimating the prior mortality associated with pivic acid in PCD patients, as well as determining the risk associated with these drugs in asymptomatic heterozygous individuals. As PCD is not widely recognized as a contraindication for prescription of antibiotics containing pivic acid, this may continue to cause preventable deaths [76]. The following is a list of available pivic acid containing antibiotics that should be avoided in suspected or diagnosed PCD individuals: Cefidorein pivoxil, cefcapene pivoxil, ceferam pivoxil, pivmecillinam and pivampicillin.

3.3. Multiple acyl-coenzyme a dehydrogenase deficiency (MADD)

Multiple Acyl-Coenzyme A Dehydrogenase Deficiency (MADD, OMIM: #231680), also known as Glutaric Acidemia Type II, is an autosomal recessive genetic disorder caused predominately by mutations in the Electron Transfer Flavoprotein (ETFA, ETFB) or Electron Transfer Flavoprotein Dehydrogenase (ETFDH) genes. These genes encode the α and β subunits of Electron Transfer Flavoprotein (ETF) and Electron Transfer Flavoprotein-Ubiquinone Oxidoreductase (ETF-QO) respectively. Disruption of ETF and ETF-QO function impairs the transfer of electrons received from acyl-CoA dehydrogenases, compromising the β-oxidation of fatty acids [78].

MADD has a clinically heterogeneous phenotype, but is classically characterized based on age at presentation. There are two types of neonatal-onset MADD, with (type I) or without (type II) congenital anomalies. Neonatal-onset MADD is severe and often lethal, and can feature hepatomegaly, non-ketotic hypoglycemia, metabolic acidosis, hypotonia, and sometimes cardiomyopathy [79, 80]. Adult-onset MADD predominantly features muscle-related symptoms, including weakness, fatigue, and myalgia (Table 4). Excessive lipid build-up has been observed in muscle biopsies of patients with both neonatal and adult onset types [81, 82], which aids in diagnosis. Elevated urinary organic acids, including glutaric acid, and acyl carnitines are also characteristic diagnostics. Finally, most cases report mutations in the three “classical” MADD genes (ETFA, ETFB and ETFDH), which confirm diagnosis. If a mutation cannot be identified then other genes associated with riboflavin transport, for example SLC25A2, SLC25A2 and SLC25A3, or FAD transport or synthesis, including SLC25A32 and FLAD1 can be investigated [83] (Table 4). MADD is associated with a genotype-phenotype correlation, with ETFA and ETFB mutations linked to early onset disease and ETFDH mutations late onset [80, 84, 85].

Most adult-onset patients are responsive to riboflavin supplementation (riboflavin responsive (RR)—MADD), which ameliorates symptoms. Carnitine, CoQ10, and vitamin B12 are also recommended to enhance clinical outcomes (Table 4). However, neonatal-onset MADD (mutations in ETFA and ETFB) is often lethal and cannot be treated [79]. There has been one treatment-outcome case report within the last 8 years detailing a patient with neonatal-onset MADD (confirmed mutation in the ETFA gene). The patient underwent sodium-D-L-3-hydroxybutyrate (NaHB) therapy, which improved clinical outcome (Table 4). NaHB therapy could be further investigated for treatment of neonatal-onset MADD.

MADD has predominately been modeled in vitro using patient fibroblasts and genetically modified mammalian cell lines. Recently, bezafibrate, a fibrate drug commonly used to lower human serum lipid levels, has been tried [86] following promising findings of its application in other metabolic diseases [87–89]. Fibrate are known to induce FATP and FACS expression in adipose tissue, which promotes cellular NEFA uptake through PPAR activation [36]. In vitro, bezafibrate significantly reduced the levels of palmitoylcarnitine (C16) and octanoylcarnitine (C8), suggesting a possible improvement of β-oxidation capacity [86], which could be translatable. However, there has been one clinical report of bezafibrate treatment within the last 8 years, and it was only found beneficial when supplemented with riboflavin [90].

Aside from in vitro studies, Zebra fish (Danio rerio) have been found to be a good in vivo model of MADD. They feature mitochondrial and metabolic abnormalities, including reduced oxidative phosphorylation, increased glycolytic flux and hyperplastic neural progenitor cells [91, 92]. In an etfa-null Zebrafish model, increased Mechanistic Target of Rapamycin Complex 1 (mTORC1) signalling was treated with rapamycin, and this partially reversed the MADD phenotype [92]. In an etfdh-null Zebrafish model, it was shown that the PPARγ-ERK pathway was up regulated, and a PPARγ agonist partially rescued the model [91]. However, the clinical relevance of these studies is yet to be ascertained.

3.3.1. HIGH RISK: MADD patients are frequently misdiagnosed with polymyositis

MADD patients are often mistakenly diagnosed with polymyositis, and treated with glucocorticoids. Mistreatment with glucocorticoids was compared with riboflavin in a cohort of 45 patients with late-onset...
MADD. 18 were initially administered glucocorticoids (1 mg/kg/day), and the remaining 27 patients took riboflavin (90-120 mg/day) and coenzyme Q10 (60 mg/day) for at least one month. Muscle strength was only improved in the riboflavin group [93]. Moreover, glucocorticoids have the potential to increase lipid levels and lipid accumulation, which would be particularly harmful in these patients. Potential adverse events include metabolic disorder, Cushing syndrome, and gastrointestinal ulcers [94].

3.3.2. HIGH RISK: MADD patients are particularly sensitive to dietary changes

Care also needs to be taken when advising MADD deficiency patients on dietary changes. A 2014 case study reported a 33-year old woman who presented with a history of progressive proximal muscle weakness [78]. The patient was taking citalopram (20 mg/day) and hydrocodone/ibuprofen (7.5 mg/200 mg for pain, however still required hospital admission). Gabapentin (300 mg 3 times daily) and hydrocodone/ibuprofen were further prescribed. Serum creatinine was relatively low, and carnitine supplements were given four times a day (1 mg). Symptoms persisted. A cocktail of carnitine (4 mg/twice daily), coenzyme Q10 (150 mg twice daily) and riboflavin (100 mg twice daily) was then given. However treatment failed to reverse symptoms, and respiratory function declined resulting in death. Notably, prior to death the patient revealed deliberate restriction of carbohydrate intake as a means of weight loss [78], potentially exacerbating metabolic decline.

These studies highlight the shortfalls that can still occur in the clinical care of MADD deficiency, due to either misdiagnosis or neglecting an appropriate clinical interrogation of dietary practices.

4. Metabolic involvement in classically non-metabolic syndromes

In a clinical and genetic analysis study of LSMs only 24% of cases were attributed to mutations in the ‘classic’ LSM genes, including PNPLA2, CGI-58 or ABHD5, SLC22A5, ETF-A, ETF-B and ETF-DH [2]. Thus, we speculate that some LSMs may be associated with genes not previously associated with metabolic disorders, including Neurofibromatosis type 1 and autoimmune myopathies.

4.1. Neurofibromatosis type 1 (NF1)

Neurofibromatosis Type 1 (NF1) is a complex, multi-system autosomal dominant genetic disorder affecting ~1:3000 individuals. Individuals with NF1 present with a spectrum of characteristic clinical and sub-clinical features including a high tumor burden, skin pigment abnormalities, osseous dysplasias, and cognitive abnormalities. While there are many anecdotal reports of reduced muscle strength, fatigability, and decreased motor function in NF1, these have historically been attributed to neuromuscular causes [95]. However, several recent seminal studies have highlighted the prevalence and impact of muscle weakness in well-described paediatric cohorts [96] and revealed a biological mechanism using genetic mouse models [97, 98].

Most notably, murine Nf1 knockout muscle [97] and human NF1 muscle biopsies [99] exhibit substantively elevated levels of intramyocellular lipid. A 2018 study has revealed that this is associated with an increase in long-chain fatty acids in a murine model and that the muscle lipid phenotype could be rescued by a reduced long-chain fatty acid diet and i-carnitine supplementation [99]. These data suggest that NF1 shares many features of a LSM both in terms of muscle function and histology, and may be potentially treated using a targeted metabolic intervention.

4.2. Autoimmune myopathies

Polymyositis (PM), Dermatomyositis (DM), and Inclusion Body Myositis (IBM) are a rare and heterogeneous group of autoimmune myopathies with a combined prevalence of 15–32 cases per 100,000 [100]. These diseases are characterized by muscle weakness and fatigue [101–103]. An abnormal immune response has been implicated by the infiltrations of inflammatory cells in histopathology of muscle biopsies. High levels of serum triacylglycerol, non- high-density lipoprotein-cholesterol (non HDL–C), and very low-density lipoprotein-cholesterol (VLDL-C) are common to PM/DM patients [102, 103]. Intramyocellular lipid droplets have been observed in PM patient muscle tissue [104]. Similarly, accumulations of free (non-esterified) cholesterol are found in the muscle fibers of patients with IBM [101]. Potential mechanisms for muscle dysfunction and fatigue include impaired fatty acid metabolism and reduced energy bioavailability and/or lipotoxicity.

In a clinical setting patients respond poorly to immune therapy [105], despite being the traditional approach to autoimmune myopathies. Treatment for example, the phase IIb/III multi-center international study for Bimagrumab, an anti-ACVR2B monoclonal antibody to improve muscle performance failed to achieve its primary outcome goals [106]. Therefore, a need to develop another targeted therapy for autoimmune myopathies is necessary. Triheptanoin oil is a medium odd-chain fatty acid-enriched oil that has been speculated to improve muscle performance and is currently registered for phase I trial for treatment of IBM [ACTRN1261400082606]. Statins are potent cholesterol-lowering drugs, which may benefit patients with PM/DM/IBM [107]. While they may affect lipid droplet formation, statins have also been associated with muscle pain, weakness and fatigue [108]. It is unknown whether statins have a positive or negative affect on muscle in the context of PM/DM/IBM [107]. There are cases of PM/DM, which have been triggered by prior exposure to statin use, and these were associated with a more aggressive clinical course [109].

5. Conclusion

Lipid Storage Myopathies (LSMs) represent a diverse class of metabolic disorders that result in lipid accumulation and impaired muscle function. The underlying causes of disease have been linked to genes associated with triacylglycerol lipolysis, fatty acid transport, and beta-oxidation. Based on the different and often highly effective treatments available for LSM, early and correct diagnosis is imperative. Conditions such as MADD deficiency are associated with a higher than acceptable rate of misdiagnosis, which can result in delays to treatment and high morbidity. For PCD, understanding the risks associated with pivalic acid-containing antibiotics is critical to reduce mortality in this patient population. Finally, conditions such as NF1 and PM/DM/IBM have not been historically classified as LSMs, but exhibit many features common to LSMs. Thus there may be justification to reevaluate the current classification systems to accommodate these related conditions.

Conflicts of interest

Authors have no conflicts of interest to declare.

Acknowledgement

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References

[6] V.B. Schrauwen-Hinderling, et al., The increase in intramyocellular lipid content is


Key Questions Forming the Focus of this Thesis

The literature review raises several questions in the field of NF1-muscle where a deeper biological understanding could aid in the development of clinical therapeutics.

Specifically this thesis aims to address the following key questions:

1) Utilising a dietary intervention of L-carnitine and medium chain fatty acids showed significant promise in prior studies. However, many mechanistic questions remain that would impact on therapeutic translation:

   a) The timeline of muscle improvements are unclear and do they continue to occur over time?

   b) Are the treatment effect sustained if there is a reversion to standard diet?

   c) Is L-carnitine supplementation sufficient to improve the muscle phenotype?

   d) What are the effects of reduced subject compliance (“cheat days”), additional mitochondrial supplements, and a low fat diet?

2) A number of individuals have contacted our group based on our prior publication after commencing self-medication of their children with L-carnitine. This raised questions not only regarding their experiences with safety and efficacy, but also the decision making process that led them to trial the therapy.

3) Is L-carnitine supplementation a safe and feasible approach to treat NF1-associated muscle weakness and fatigue in children?
Chapter 2 Prologue

In the previous introductory chapter we suggest that NF1 patient treatment could benefit from disease categorisation as a lipid-storage metabolic myopathy, and highlight work by our group that demonstrates the potential of dietary intervention using the limb-specific \( Nf1_{Prx}^{-/} \) mouse model. Notably however, the study was limited to a single time point and did not address the beneficial dietary component or effect of reversion to standard diet.

In Chapter 2 we hypothesise that a reduction in muscle lipid can be observed at an earlier time point, and that a range of dietary treatment strategies, including L-carnitine supplementation alone could benefit Nf1 muscle using \( Nf1_{Prx}^{-/} \) mice, however dietary effects are not sustained following reversion to standard diet. We conduct \textit{in situ} muscle physiology to assess the effect of several different dietary strategies on muscle function, and analyse using other modalities including Oil Red O and lipid mass spectrometry.
Chapter 2

Evaluating modified diets and dietary supplement therapies for reducing muscle lipid accumulation and improving muscle function in neurofibromatosis type 1

Chapter 2 has been previously peer reviewed and published, and is included here in manuscript format.

Evaluating modified diets and dietary supplement therapies for reducing muscle lipid accumulation and improving muscle function in neurofibromatosis type 1 (NF1)

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Abstract

Neurofibromatosis type 1 (NF1) is a genetic disorder that affects a range of tissue systems, however the associated muscle weakness and fatigability can have a profound impact on quality of life. Prior studies using the limb-specific Nf1 knockout mouse (Nf1^Prx1^−/−) revealed an accumulation of intramyocellular lipid (IMCL) that could be rescued by a diet supplemented with L-carnitine and enriched for medium-chain fatty acids (MCFAs). In this study we used the Nf1^Prx1^−/− mouse to model a range of dietary interventions designed to reduce IMCL accumulation, and analyze using other modalities including in situ muscle physiology and lipid mass spectrometry. Histological IMCL accumulation was significantly reduced by a range of treatments including L-carnitine and high MCFAs alone. A low-fat diet did not affect IMCL, but did provide improvements to muscle strength. Supplementation yielded rapid improvements in IMCL within 4 weeks, but were lost once treatment was discontinued. In situ muscle measurements were highly variable in Nf1^Prx1^−/− mice, attributable to the severe phenotype present in this model, with fusion of the hips and an overall small hind limb muscle size. Lipidome analysis enabled segregation of the normal and modified chow diets, and fatty acid data suggested increased muscle lipolysis with the intervention. Acylcarnitines were also affected, suggestive of a mitochondrial fatty acid oxidation disorder. These data support the theory that NF1 is a lipid storage disease that can be treated by dietary intervention, and encourages future human trials.

Introduction

Neurofibromatosis type 1 (NF1) is a complex genetic disease that can have a profound impact on childhood development and adult quality of life. A clinical diagnosis of NF1 relies on...
fulfilling at least two of the seven diagnostic criteria; café au lait macules, skinfold freckling, neurofibromas, Lisch nodules, optic pathway tumors, bone dysplasia, and a family history [1]. Individuals with NF1 are predisposed to neural tumor formation and development of neurological, musculoskeletal and vascular abnormalities that contribute to the morbidity of the disorder. However, the reduced muscle tone, muscle weakness, poor co-ordination, and increased fatigability associated with NF1 are being increasingly appreciated as major burdens of disease [2]. These can lead to significant functional impairment and reduced quality of life in children, particularly when combined with other features of NF1 such as learning and behavioral difficulties [3].

Key insights into the role of NF1 in muscle have come from conditional Nf1-deficient mouse lines. A limb-specific Nf1 knockout mouse (Nf1Prx1−/−) was generated using a Prx1-cre transgene to drive deletion of Nf1 in cells of the mesenchymal lineage in the fore and hind limbs. This mouse strain has reduced muscle weight, muscle weakness, fibrosis and impaired myoblast differentiation in the developing limbs [4]. To more specifically investigate the function of Nf1 in muscle, Sullivan et al. generated a Nf1 knockout mouse specifically deleting the gene in skeletal muscle (Nf1MyoD−/−) [5]. Nf1MyoD−/− pups are born with a reduced body weight, exhibit stunted growth and failure to thrive, and maternal infanticide typically occurs during the first week of life. Electron microscopy analysis of Nf1MyoD−/− muscle revealed excessive accumulations of intramyocellular lipid (IMCL), consistent with a metabolic myopathy [5]. This was confirmed at the light microscopy level by Oil Red O staining. These novel findings led us to speculate that NF1 may have a key role in the regulation of muscle lipid metabolism.

More recently, Summers et al. published a report examining the IMCL found in Nf1MyoD−/− muscle [6]. Lipidomics identified an increase in triglycerides, diglycerides, and cholesterol esters containing long-chain fatty acids (LCFAs). This led to the hypothesis that a deficiency in LCFAs metabolism may underlie the muscle weakness. Consequently, a dietary intervention where Nf1Prx1−/− mice were treated with a diet enriched for medium-chain fatty acids (MCFA) and supplemented with 300mg/kg L-carnitine led to reversal of the muscle lipid phenotype and improved forelimb grip strength. L-carnitine has previously been shown to treat muscle weakness in patients with other metabolic myopathies [7–9].

However, the mouse chow containing 70% octanoic acid as a MCFA source [6] is an approach that is not directly translational to dietary modification in humans. Moreover, it was unknown whether L-carnitine supplementation alone would be sufficient to produce significant reductions in muscle lipid and improvements in strength; an intervention that could be more readily adopted than changes in dietary fat intake. Thus the aim of this study was to use the published preclinical model to guide future clinical trials in individuals with NF1.

In addition to positive control (70% FAs ≤C12:0, 300mg/kg/day L-carnitine) and negative control (standard chow) groups, a number of other treatments were tested. These included a mitochondrial cocktail of L-carnitine, CoQ10, riboflavin (VitB2) and creatine [10]. This combination nutraceutical therapy can target many pathways of cellular energy dysfunction, including mitochondrial energy depletion and oxidative stress, which can be amendable by this approach in several lipid storage myopathies (LSM) [11]. We further included a group modelling limited dietary compliance, being fed normal chow 2 days per week. Finally, we tested mice given a low fat (<2%) diet, to see whether a generic low-fat strategy is effective.

The primary outcome measure for this study was IMCL accumulation, as measured by Oil Red O staining, and detailed in some cases by lipidomics. Our prior report had shown variability with grip strength testing in Nf1Prx1−/− mice. Thus, in situ muscle physiology testing was performed to better functionally assess the effects of dietary interventions on hind limb muscle strength and fatigability.
Study design

The screening of dietary strategies and dietary supplements (Table 1) was performed over 8 weeks, as previously shown to produce a significant decrease in IMCL in group 2 compared to group 1 [6]. Outcome measures included functional and histological assessments (n = 10/group). Tibialis anterior (TA) muscle was measured for maximum specific muscle force (mN/mm²), as well as the rate of muscle fatigue and recovery [12–14]. Hind limb wet muscle weight was also measured and frozen mouse quadriceps muscle, with other muscle groups (soleus, TA, and gastrocnemius) collected for comparison via Oil Red O staining for neutral lipids.

In this subsequent study, High MCFA chow + L-carnitine was tested in a longitudinal study to assess the onset of reduced IMCL as well as the effects of longer-term treatment (Table 2). A third group was also tested, which was reverted to standard chow at week 8 to examine the potential restoration of IMCL with an unrestricted diet. Outcome measures for this study were histological staining (Oil Red O, n = 6 per group per time point) as well as a more detailed lipid analysis by LC-MS/MS and GC-MS/MS mass spectrometry of the week 8 time point (n = 8 per group).

Materials and methods

Mouse strains and husbandry

All animal experiments were approved by the Westmead Hospital Animal Ethics Committee, The Children’s Hospital at Westmead/Children’s Medical Research Institute Animal Ethics Committee (protocol number: K319) or Murdoch Children’s Research Institute Animal Ethics Committee (protocol number: A879), and performed according to their prescribed guidelines. Prx1-Cre transgenic mice [15] and Nf1<sup>flox/flox</sup> mice [16] (sourced from Jackson laboratory USA) were crossed to produce first generation Prx1-Cre<sup>+/−</sup> Nf1<sup>flox/+</sup> mice. They were then backcrossed to the parental Nf1<sup>flox/flox</sup> strain to generate experimental homozygous knockout animals Prx1 Cre<sup>+/−</sup> Nf1<sup>flox/flox</sup>.

<table>
<thead>
<tr>
<th>Group</th>
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<td>2</td>
<td>Nf1&lt;sup&gt;Prx1&lt;/sup&gt;−/−</td>
<td>Standard chow</td>
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<td>3</td>
<td>Nf1&lt;sup&gt;Prx1&lt;/sup&gt;−/−</td>
<td>Modified chow</td>
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<td>4</td>
<td>Nf1&lt;sup&gt;Prx1&lt;/sup&gt;−/−</td>
<td>Modified chow, standard chow (w8-16)</td>
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<td>Weeks 12, 16</td>
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The original registered report protocol can be found at https://osf.io/mjc8u.
Nf1<sub>Prx1</sub>−/− mice were distinctly smaller than their littermates. To ensure their survival and reduce maternal rejection, pups were given daily saline injections of 0.1 mL up until four weeks of age. Samples were collected at three weeks of age for genotyping by quantitative real-time PCR for the Cre and Nf1<sup>flox</sup> alleles (Transnet YX, TN, USA). All Nf1<sub>Prx1</sub>−/− mice used in this study were age matched females.

All experimental animals were monitored twice daily throughout the course of the study. If any mice showed signs of distress or deterioration, or greater than >10% weight loss then 0.1 mL saline injections were administered daily until weight normalized. Humane endpoints were defined as >10% weight loss or significant signs of distress that persisted. Mice were anesthetized by isoflurane inhalation prior to cardiac puncture and euthanasia by cervical dislocation after the completion of studies.

Modified diets and dietary supplement therapies

Female mice were grouped housed 3–5 mice per cage, and fed <i>ad libitum</i> either standard AIN93M rodent chow pellets, or were assigned one of the modified diet chows. Those on the intermittent feeding regimens received 5 days of modified chow followed by 2 days of standard chow. All modified diet formulas were based on AIN93M and were designed to contain equal amounts of digestible energy (15.7 MJ/kg), carbohydrates (65.6–65.8%), protein (13.8–13.9%) and total fats (4%), excluding the low fat chow, which contained 15.0 MJ/Kg of digestible energy, a higher amount of carbohydrates (68.6%), and minimal requirements for total fats (1.8%).

High MCFA chow contains octanoic acid (C8:0, 2.8%) as its predominant lipid source, representing 70% of the total fatty acids content. LCFAs were included at minimal levels for animal health (Palmitic Acid 16:0, 0.07%; Stearic Acid 18:0, 0.03%; Oleic Acid 18:1, 0.17%; Linoleic Acid 18:2 n6, 0.61%; Linolenic Acid 18:3 n3, 0.30%). In contrast, the standard AIN93M, contains 100% of fatty acids as ≥C16:0. Palmitic Acid 16:0, 0.17%; Stearic Acid 18:0, 0.08%; Oleic Acid 18:1, 2.22%; Gadoleic Acid 20:1, 0.04%; Linoleic Acid 18:2 n6, 0.86%; Linolenic Acid 18:3 n3, 0.56%). The low fat chow was based on the AIN93M diet but had reduced fat (<2%).

L-carnitine was added to standard or high MCFA chow, at a concentration of 1.71g/kg, achieving a desired daily dose of 300mg/kg/mouse/day. The mitochondrial cocktail chow consisted of L-carnitine added at 1.71g/kg (300mg/kg/mouse/day), CoQ10 added at 0.114g/kg (20mg/kg/mouse/day), creatine added at 0.057g/kg (10mg/kg/mouse/day) and riboflavin (active vitamin B2) added at 0.0684g/kg (12mg/kg/mouse/day). All additives were based on standard chow consumption rates (Specialty Feeds, WA, Australia).

<i>In situ</i> muscle physiology

After 8 weeks of treatment <i>in situ</i> assessment of the TA was performed using the 1300A Whole Mouse Test System and 701C stimulator (Aurora Scientific). The mice were anaesthetized using isoflurane inhalation and placed on a heated platform (37°C) for the procedure. Briefly, a small incision was made in the distal end of the animal's leg and the skin is retracted halfway up the leg to expose the TA. The distal tendon of the TA was surgically isolated and the knee joint exposed. Surgical silk was used to secure the tendon to the dual-mode lever arm and the foot and knee were secured. The muscle was then stimulated to contract by placing electrodes adjacent to the sciatic nerve. The optimal length (Lo) was determined based on production of maximum twitch force (Pt), resting muscle length was recorded. The TA was then stimulated to contract (5 – 200Hz, with 2 minutes rest between each contraction) to generate a force frequency curve and maximal tetanic force (Po) was achieved at 150Hz. Absolute force (mN),
specific force (mN/mm²), and the rate of muscle fatigue and recovery (%) were determined as previously outlined in Garton, et al. 2018 [17]. An additional control group of standard chow fed C57/BL6 mice (n = 10) were compared to the Nf1Prx1⁻/⁻ test groups. The operator was blinded to treatment in all cases.

**Tissue collection and histological staining**

Mice were euthanized via cervical dislocation and muscles were dissected out and weighed, discarding of overlying fascia and adipose tissue. Muscle tissues were surface coated in Tissue-Tek® O.C.T. Compound (Sakura Finetek USA), placed on a thin piece of tin foil and frozen in liquid nitrogen supercooled isopentane (2-methyl butane) and stored at -80 °C. 8um sections were cut on a Leica CM1950 Clinical Cryostat, and captured on Superfrost™ Plus Microscope Slides (Fisher Scientific, USA) and stored at 4 °C prior to staining.

Oil Red O staining were performed as previously published [6]. Quantification was done using Fiji ImageJ, by quantifying total lipid stained red area as a percentage of total section area.

**Liquid chromatography–mass spectrometry (LC-MS) lipid analysis**

Please see the Supporting information (S1 File) for a complete description of lipidomics materials and methods. Lipids for LC-MS analysis were extracted using a modified Bligh Dyer extraction protocol. Lipids were analyzed using Agilent LC 1290 binary pump coupled with Ascentis Express RP amide (50 × 2.1 mm, 1.8μ), and separated lipid species were detected using Agilent QQQ 6490 mass spectrometer, using multiple reaction monitoring (MRM) as previously published [6].

For GC-MS based fatty acid analysis, dried samples and dried fatty acid calibration mix were derivatised with 5 μL of Meth-Prep™ II (Grace Davison Discovery). The samples were then analyzed on a GC-MS system comprised of a Gerstel 2.5.2 Autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole mass spectrometer (Agilent, Santa Clara, USA) [18, 19].

**Statistical analysis**

For Oil Red O analysis, the average lipid area from four sections from n = 10 (study 1) or n = 6 (study 2) individual mice were compared by ANOVA with Tukey’s post-hoc multiple comparisons test (multiple groups) or two-tailed Student’s t-test (two groups). For muscle physiology measures, similar parametric comparisons were made using n = 10 mice. Experimental results are expressed as mean ± SEM. P-values of <0.05 were considered statistically significant.

**Results**

**Measuring strength, fatigability and muscle recovery in Nf1Prx1⁻/⁻ mice**

A prior study using the Nf1Prx1⁻/⁻ mice used grip strength as the primary functional outcome measure [5], however more detailed assessment was sought using in situ muscle physiology. Following 8 weeks of ad libitum access to the allocated dietary treatments outlined in Table 1, the maximum specific force was measured in all groups of Nf1Prx1⁻/⁻ mice. Notably, the low fat diet regimen yielded a 66% increase in maximal specific force of the TA muscle compared with standard chow (Fig 1D) (n = 10 P < 0.02). The increased maximum specific force was not associated with changes in TA muscle wet weight or body weight (Fig 1A and 1B). Other diet intervention groups did not demonstrate significant changes compared with standard chow (Fig 1C and 1D).
Analysis of TA muscle fatigability measured following 120 contractions and after 1, 3 and 10 min recovery (Fig 2A–2F) showed an extremely high amount of intragroup variability. No significant differences in muscle function in terms of fatigue and recovery could be detected between groups. Nevertheless, the challenges associated with reliably carrying out the protocol
Fig 2. Dietary treatment effects on muscle fatigue and recovery in Nf1Prx1-/ TA muscles were unobservable. Fatigue and recovery response of (A) MCFA enriched diet supplemented with L-carnitine (B) MCFA enriched diet supplemented with L-carnitine treatment modelling cheat days (5/7 days) (C) MCFA enriched diet (D) L-carnitine supplementation alone (E) L-carnitine in combination with riboflavin, CoQ10 and creatine, a common mitochondrial mix and (F) Low fat diet, at final fatigue measure (120th contraction), 1, 3 and 10 min recovery period (arrows) showed no difference. N = 10 in situ TA muscles per group. Data presented as group mean ± SEM. p-values were assessed by non-parametric ANOVA.

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on muscles that were smaller and weaker than WT likely added to the high variance seen in
the data.

Nf1<sub>Prx1</sub>−/− mice were characterized relative to wild type mice to confirm reduced wet muscle
weight and strength. The wet muscle weight (mg) of the TA in Nf1<sub>Prx1</sub>−/− mice was reduced by
81% compared to WT mice (S1A Fig). Additionally, they exhibited an 83% reduction in abso-
lute force (mN) (S1B Fig).

Multiple dietary interventions reduced IMCL in Nf1<sub>Prx1</sub>−/− mice

The major prior finding that prompted this study was that high MCFA diet + L-carnitine
could reduce IMCL accumulation in Nf1<sub>Prx1</sub>−/− mice [6]. Analysis of the two components of
this treatment separately, as well as modeling cheat days (5/7 days) and a “mito mix” all sup-
port the concept of dietary intervention for this condition. Oil Red O staining of sections
taken from the mid-belly of the quadriceps was used to show histological changes in lipid
droplet density. MCFA + L-carnitine, MCFA, L-carnitine and mito mix treatment yielded a
55–69% reduction in IMCL, whereas MCFA + L-carnitine (5/7 days) gave a 40% reduction
(Fig 3) (n = 10, P < 0.001). This can be visualized in representative sections (Fig 3). In contrast,
the low fat diet did not change IMCL via histology (Fig 3).

The response to dietary intervention occurs within 4 weeks and subsides
after cessation of treatment

Longitudinal assessment of Nf1<sub>Prx1</sub>−/− mice fed a high MCFA diet + L-carnitine diet was per-
formed. Histological staining confirmed a reduction of IMCL as early as 4 weeks of treatment
(n = 6, p < 0.03) (Fig 4A). This reduction was consistent with prior findings with dietary inter-
vention at a single time point of 8 weeks [6]. Moreover, the reduction persisted concomitant
with treatment out to the final time point of 16 weeks (n = 6, p < 0.02) (Fig 4B and 4C). For
mice that received MCFA + L-carnitine for 8 weeks and were then reverted to standard chow,
IMCL was found to re-accumulate after a further 4 and 8 weeks (n = 6, p < 0.003) (Fig 5).
These data suggest that dietary changes need to be maintained in order to prevent the build-up
of new IMCL.

Changes in lipidome profile in response to Nf1 deficiency and dietary
intervention

To help elucidate the mechanism underlying the changes in IMCL accumulation, muscle sam-
ples underwent lipid mass spectrometry analysis for a variety of lipid species including triglyc-
erides (TGs), diglycerides (DGs) and free fatty acids. Three test groups: WT mice on standard
chow, Nf1<sub>Prx1</sub>−/− mice on standard chow, and Nf1<sub>Prx1</sub>−/− mice on modified chow (MCFA + L-
carnitine) were examined after 8 weeks of dietary intervention. This utilized homogenized
quadriceps muscle with all associated subcutaneous adipose tissue carefully removed.

Principal Component Analysis (PCA) was used to determine whether the samples clustered
into distinct groups, which indeed was the case (Fig 6A). The first two components captured
>60% of the variance across the dataset (PC1: 40%, PC2: 21.6%). This supports the concept
that not only genotype but also diet leads to consistent and separable changes in muscle lipi-
dome profile. Hierarchical clustering analysis and heat map visualization of the top 50 lipid
species selected based on fold change suggested substantive increases in species of DG, includ-
ing DG 16:0, DG 16:1, DG 18:1, species of TG, including TG16:1, TG 18:1, and species of phos-
phatidylglycerol (PG), including PG 34:1, PG 34:2 and PG 36:2 in modified chow treated
Nf1<sub>Prx1</sub>−/− mice (Fig 6B). Total TG, total DG and total PGs were all significantly elevated in
Fig 3. Dietary modifications in Nf1<sup>−/−</sup> mice rescue IMCL accumulation. Histological analysis using Oil Red O showed up to 69% reduction in IMCL accumulation. Scale bar; 200μm at 10x magnification, n = 10. Data presented as group mean ± SEM. p-values were assessed by one way ANOVA. **p = 0.0083, ***p = 0.0001 and ****p<0.0001.

https://doi.org/10.1371/journal.pone.0237097.g003
Fig 4. *Nf1*<sup>-/-</sup> mice fed MCFA + L-carnitine chow show reduced IMCL accumulation within 4 weeks of treatment. A significant reduction of IMCL accumulation was observed in quadriceps muscle of *Nf1*<sup>-/-</sup> mice fed MCFA + L-carnitine chow compared to standard chow fed *Nf1*<sup>-/-</sup> mice at all time points; (A) 4 weeks (B) 8 weeks and (C) 16 weeks of treatment. (D) IMCL accumulation did not significantly increase over 12 weeks in standard chow fed *Nf1*<sup>-/-</sup> mice. (E) IMCL reduction plateaued after 4 weeks of MCFA + L-carnitine treatment in *Nf1*<sup>-/-</sup> mice. Scale bar; 200um at 10x magnification, n = 6. Data presented as group mean ± SEM. p-values were assessed by one way ANOVA.

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Nf1\(^{-/-}\) mice fed the modified chow (n = 8, P < 0.0001) (Fig 6C). In contrast, total CE, LPC, LPE, PC, PE, PI and PS were unaltered (Fig 6C). Fatty acid analysis using GC-MS confirmed increases in several FA species including palmitoleic acid (C16:1), oleic Acid (C18:1n9c) and eicosapentaenoic acid (C20:5n3) in modified chow-fed Nf1\(^{-/-}\) mice (Table 3).

Strikingly, total acylcarnitines were elevated in Nf1\(^{-/-}\) mice compared to WT mice (n = 8, p < 0.003) (Fig 7). Detailed analysis of individual acylcarnitine species revealed significantly increased levels of acylcarnitine 14:0 (n = 8, p < 0.024), 16:0 (n = 8, p < 0.0001), 16:1 (n = 8, p < 0.0013), 16:0 (n = 8, p < 0.0013), 18:1 (n = 8, p < 0.0001) and 18:2 (n = 8, p < 0.0002). Acylcarnitine 16:1 and 18:1 (n = 8, p < 0.0001) were further increased with modified chow feeding (Fig 7).

The full lipidomics data set is included in the Supporting information (S2 File).

Discussion

Murine studies have previously shown that Nf1 deficiency is associated with accumulation of IMCL high in LCFAs. This study was prompted by our prior finding that a modified diet enriched with MCFAs and supplemented with L-carnitine could decrease muscle IMCL and improve grip strength in Nf1\(^{-/-}\) mice \[5, 6\]. This study confirms and builds upon these findings.

Our first major finding was the relative increase in strength on a low fat diet. This was an unexpected finding, and notably not aligned to any changes in muscle lipid. A low fat diet is commonly recommended to LSM patients, and it has been demonstrated to decrease liver size
Liver dysfunction and physical function has not been adequately studied, however there is growing evidence to suggest that liver dysfunction causes impaired muscle protein synthesis and decline of muscle strength [21], which could be rectified by a low fat diet [21]. A second major finding was establishing that L-carnitine alone and increased MCFAs alone could both produce reductions in IMCL. This has translational applications for clinical trials and therapy as L-carnitine can already be readily purchased in capsule form as a dietary supplement, and is simpler to medicate than a complex dietary program.

Fig 6. Lipidomics analysis of whole quadriceps of MCFAs + L-carnitine fed Nf1<sup>-/-</sup> mice confirms dietary intervention can alter lipid metabolism. (A) Principal-component analysis of LC-MS data showed group clustering and separation of lipid data between WT, Nf1<sup>-/-</sup> mice fed standard chow and Nf1<sup>-/-</sup> fed MCFA + L-carnitine chow. The first two components captured >60% of the variance across the dataset (PC1: 40%, PC2: 21.6%). (B) Heat map analysis of the top 50 lipid species selected based on fold change suggests increased TG and DG species. (C) Total DG, TG and PG are increased in whole quadriceps of Nf1<sup>-/-</sup> fed MCFA + L-carnitine chow. Total acylcarnitines are elevated in Nf1<sup>-/-</sup> mice, which were exacerbated upon MCFA dietary enrichment and carnitine supplementation. n = 8 quadriceps muscle samples analyzed for all lipidomics studies. Data presented as group mean ± SEM fold change compared to WT. p-values were assessed by ANOVA. *** p < 0.0001 * p < 0.003. CE; Cholesterol esters, DG; Diglycerides, TG; triglycerides, LPC; Lysophosphatidylcholine, LPE; Lysophosphatidylethanolamine, PC; Phosphatidylcholine, PE; Phosphatidylethanolamine, PG; Phosphatidylglycerol, PI; Phosphatidylinositol, PS; Phosphatidylserine.

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Lastly, the reappearance of IMCL following halting of the modified diet suggests that any clinical therapy will need to be ongoing to maintain benefits in individuals with NF1.

Moving forward, L-carnitine supplementation represents a promising therapeutic intervention for individuals with NF1 and concerns about muscle strength and fatigue. L-carnitine is a well-established treatment for LSM’s, including primary carnitine deficiency [11]. These patients can show a rapid reversal of clinical symptoms within one month [22–24] and, consistent with our murine data, L-carnitine needs to be maintained to provide ongoing symptomatic relief [9, 25]. While it can be challenging to extrapolate clinical timelines from animal data, the effects in our model were seen rapidly—as early as 4 weeks in the mice.

Nevertheless, the mechanism of L-carnitine on lipid metabolism and the lipidome has not been extensively studied, particularly in NF1-deficient muscle. The capacity of L-carnitine to reduce IMCL in Nf1Prx1−/− mice suggests a redirection from storage pathways to lipolysis. This

Table 3. GC-MS analysis reveals significant increase of fatty acids in whole quadriceps of modified diet fed Nf1Prx1−/− mice. n = 8 quadriceps muscle samples per group were analyzed. Data presented as group mean ± SEM fold change compared to WT. P-values were assessed by ANOVA.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Fold increase in Nf1−/− Standard muscle</th>
<th>P value</th>
<th>Fold increase in Nf1−/− MCFAs + L-carnitine</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitoleic Acid (C16:1)</td>
<td>5.12</td>
<td>&lt;0.001</td>
<td>20.51</td>
<td>0.001</td>
</tr>
<tr>
<td>Oleic Acid (C18:1n9c)</td>
<td>5.91</td>
<td>&lt;0.001</td>
<td>14.35</td>
<td>0.006</td>
</tr>
<tr>
<td>cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3)</td>
<td>1.86</td>
<td>0.002</td>
<td>14.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Myristoleic Acid (C14:1)</td>
<td>2.15</td>
<td>0.003</td>
<td>7.91</td>
<td>0.002</td>
</tr>
<tr>
<td>Myristic Acid (C14:0)</td>
<td>3.16</td>
<td>0.001</td>
<td>7.07</td>
<td>0.001</td>
</tr>
<tr>
<td>Palmitic Acid (C16:0)</td>
<td>3.54</td>
<td>&lt;0.001</td>
<td>5.95</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Fig 7. Acylcarnitines are elevated in Nf1Prx1−/− mice compared to WT. C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2 are significantly increased in whole quadriceps muscle samples of Nf1Prx1−/− mice compared to WT, which are further exacerbated (excluding C18:2) in modified diet fed Nf1Prx1−/− mice. n = 8 quadriceps muscle samples per group were analyzed. Data presented as group mean ± SEM. p-values were assessed by one way ANOVA. **** p<0.0001 *** p<0.003. C14:0 and C14:1; Tetradecanoylcarnitines, C16:0 and C16:1; hexadecanoylcarnitines, C18:0 and C18:1; octadecanoylcarnitines and C18:2; octadecadienoylcarnitine.

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is supported by an increased fatty acid content in muscle of mice fed the modified diet, as shown by GC-MS data. In cultured adipocytes, the addition of L-carnitine supplementation has been shown to stimulate lipolysis by the induction of lipolytic gene expression, including hormone sensitive lipase, carnitine palmitoyltransferase Ia (CPT-1a) and Acyl-CoA oxidase, and suppression of adipogenic genes, including PPARγ [26]. In a zebrafish model, L-carnitine supplementation similarly resulted in significantly increased CPT1 and decreased fatty acid synthase (FAS) expression [27]. The latter is particularly notable as Nf1Prx1<sup>−/−</sup> muscle exhibits increased FAS expression and decreased CPT1 levels [5].

While the lipidomics analysis revealed a range of changes within the Nf1Prx1<sup>−/−</sup> genotype, of particular note was the increase in acylcarnitines (particularly C16 and C18:1). Substantially elevated acylcarnitines have been previously associated with disorders of mitochondrial fatty acid oxidation and organic academia’s [28, 29]. Furthermore, elevated plasma C16 and C18:1 acylcarnitines are the formal diagnostic criteria for carnitine-acylcarnitine translocase deficiency [30], and support diagnosis of carnitine palmitoyltransferase II deficiency [31]. The acylcarnitine levels were further increased with dietary modification. Thus one possibility is that aberrant NF1-Ras signaling may lead to downstream changes in CPT1 activity as the primary enzyme that exchanges the CoA moiety from long-chain acyl-CoAs for carnitine to generate acylcarnitines [32].

While the Nf1Prx1<sup>−/−</sup> mouse model has proven useful for biochemical analysis, it remains challenging as a model to study muscle function. The mouse features partial-to-complete fusion of the hip joint [4, 33], which impairs locomotion and causes secondary reductions in loading and strength over time. The Nf1Prx1<sup>−/−</sup> hind limb muscles were proportionally smaller than the forelimb muscle when compared to WT mice (S2 Fig). Prior grip strength tests primarily utilize the forelimb muscles. In contrast, the in situ strength and fatigue testing focused on the hind limb TA muscle, which presents the greatest reduction in muscle size (-83%) compared to WT TA. Additionally, the variability of hip fusion affected our ability position their hind limbs, and subsequently altered the dorsal location of the mouse for in situ muscle physiology analysis. The resultant variability in the in situ muscle physiology data was challenging to draw definitive conclusions from. Indeed a post-hoc power analysis suggested n ≥ 54 mice are required to achieve 80% power for a genotype/diet effect, which is not feasible due to severity of the model and poor breeding of the strain.

In conclusion, data from this preclinical study supports the concept that NF1 features a metabolic dysregulation that can be ameliorated by dietary intervention. In particular, L-carnitine supplementation appears to be a feasible and promising option for human trials as it is inexpensive and well-tolerated. Such clinical studies would likely be superior options to examine functional effects on muscle in a longitudinal manner than the challenging Nf1Prx1<sup>−/−</sup> mouse model. The lipidome analysis suggests that Nf1 produces profound changes in the lipidome that can be altered by dietary intervention suggestive of an increase in muscle lipolysis. Moreover, the murine data suggests some evidence for dysregulated carnitine metabolism in Nf1, however this will need to be further addressed using clinical samples. While this study has focused specifically on muscle, these data also raise the possibility that the Nf1 regulation of metabolism may affect other tissues, e.g. bone [34] and could have broader implications for the treatment of the condition.

**Supporting information**

S1 Fig. Nf1Prx1<sup>−/−</sup> mice demonstrated significant deficits in muscle size and force production compared to WT. (A) Nf1Prx1<sup>−/−</sup> TA muscle wet weight was reduced by 81% compared to WT and (B) Nf1Prx1<sup>−/−</sup> TA force was reduced by 83% compared to WT. N = 10 in situ TA
muscles per group. Data presented as group mean ± SEM.

(TIF)

S2 Fig. Cartilaginous fusion of the hip joints is a characteristic feature of the Nf1Prx1-/- mouse model that varies between complete and partial fusion. (A) Lower extremity representative X-ray images of Nf1Prx1-/- mice to demonstrate leg position variability in dorsal placement due to variability in hip fusion. (B) Hind limb muscle wet weight is reduced by 61–83% in the Nf1Prx1-/- mouse model compared to WT.

(TIF)

S1 File. Complete description of lipidomics materials and methods.

(PDF)

S2 File. Full lipidomics data set.

(XLSX)

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References


Chapter 3 Prologue

In the previous chapter we build upon our previous work that suggests dietary intervention could be used to treat Nf1 muscle weakness and fatigue. Since our original publication in this research area, a number of families with a child suffering from NF1 muscle weakness and fatigue have actively contacted us about their decision to self-administer L-carnitine supplementation to their child.

In Chapter 3, a qualitative approach is taken to explore the decision making process of families that used L-carnitine supplementation for their child’s muscle weakness and fatigue, and the outcome of their decision. We aimed to capture personal experiences, which may not always be expressed in a clinical setting.
Chapter 3

Parents’ experiences using research into L-carnitine supplementation to treat their child’s muscle weakness and fatigue in neurofibromatosis type 1

Chapter 3 is currently under review for publication in the journal *PLOS ONE*, and has been included here in manuscript format.

Reference: Emily R Vasiljevski, Karen Scott, David Stevenson, Aaron Schindeler. Parents’ experiences using research into L-carnitine supplementation to treat their child’s muscle weakness and fatigue in neurofibromatosis type 1. *PlosONE* (currently under review)
Parents’ experiences using research into L-carnitine supplementation to treat their child’s muscle weakness and fatigue in neurofibromatosis type 1

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Conflicts of interest

Authors have no conflicts of interest to declare.

Keywords: Neurofibromatosis type 1 (NF1), muscle weakness, fatigue, childhood, L-carnitine
1. Abstract

Poor muscle tone, muscle weakness and fatigue affect many children with neurofibromatosis type 1 (NF1), and can have a considerable impact on pediatric quality of life. This is likely due to an underlying metabolic myopathy, which can be ameliorated in a preclinical mouse model by L-carnitine supplementation. Anecdotally, dissemination of this preclinical research has resulted in a number of parents electing to supplement their child with L-carnitine. We aimed to investigate the decision making process of these parents, and the outcome of their decision. A parent-reported, semi-structured questionnaire and interview was conducted with parents that decided to administer L-carnitine supplementation to their children with NF1-associated muscle weakness and fatigue. Questionnaire data were organized into a case series, and interview transcripts were thematically analyzed. Three themes were identified: (1) factors influencing parents’ decision to self-administer their child with L-carnitine (primary muscle concerns and psychosocial repercussions, ineffective treatment strategies, research accessibility and availability, and professional medical communication); (2) outcomes of L-carnitine supplementation (physical improvements, side effects, future treatment and sharing of health findings); and (3) areas that warrant further guidance (L-carnitine dosage, research accessibility, disseminating research and clinical trial interest). Utilizing a qualitative approach to understand the parents’ decision making process and outcome of their decision enables a more in depth understanding of their personal experiences, which cannot be easily captured in a clinical setting. This study demonstrates the complex interplay between NF1 muscular symptomology, a considerable lack of therapeutic options and the use of experimental medication. Future research exploring the combination of L-carnitine supplementation and physical therapy could unlock a more effective treatment strategy for muscle weakness and fatigue in NF1.
2. Introduction

Neurofibromatosis type 1 (NF1) is a complex and multifaceted genetic disorder that is clinically diagnosed by the presence of two or more of the following criteria: ≥6 café-au-lait macules, ≥2 neurofibromas or a plexiform neurofibroma, inguinal or axillary freckling, ≥2 Lisch nodules, optic glioma, a distinctive osseous lesion or a first-degree relative with NF1. NF1 is inherited in an autosomal-dominant manner with an incidence of 1:3000, however half of these individuals represent an NF1 pathogenic variant of de novo origin. Inter-patient variability in disease onset and progression and clinical variability within an individual at different life stages make it challenging to understand the impact of NF1 on quality of life.

Qualitative research plays a critical role in capturing the experiences of individuals with NF1, and its effect on health and well-being. Several studies illuminate the existence of muscular symptoms, including fatigue (sometimes characterized by experiences of sudden exhaustion), muscle weakness, coordination and balance difficulties, and fine and gross motor impairments, which negatively impact quality of life (1-5). Findings of muscle functional impairment in NF1 have also been reported in a magnitude of clinical studies (6-10). In a recent clinical study, strength deficits ranging from 3-43% were observed across 15 upper and lower limb muscle groups, including grip strength, hip abduction and plantarflexion (11). A study of self-concept revealed that ≥30% of children and adolescents with NF1 report a low self-concept for their physical abilities. Despite this, a lack of treatment options mean that muscular symptoms are frequently overlooked.

In 2018, our team used a preclinical mouse model to test a medium-chain fatty acid enriched diet augmented with L-carnitine supplementation (12). The dietary intervention was designed to reduce intramyocellular lipid (IMCL) accumulation, a characteristic feature of NF1 deficient muscle and other metabolic myopathies (13). We reported a significant reduction in IMCL and
functional improvements in strength after 8 weeks of treatment (12). The dissemination of these preclinical findings via scientific publication resulted in a number of families showing interest in or independently trialing L-carnitine supplementation for their child’s NF1 related muscle weakness and fatigue.

L-carnitine is an over-the-counter nutraceutical supplement that is of low cost (~$30 a month) and readily available. It is frequently recommended to patients with impairments in L-carnitine synthesis, transport or long-chain fatty acid metabolism due to its vital role in shuttling long-chain fatty acids through the mitochondrial membrane for beta-oxidation.

In this study, we examine the decision made by parents to self-supplement their children with L-carnitine for the alleviation of NF1 muscle weakness and fatigue, and assess the outcomes of their decision. A qualitative approach was chosen to enable an in-depth exploration of personal experiences, which may not always be expressed in a clinical setting. This will inform us about how parents utilize scientific information to make informed decisions around experimental medications.

3. Methods

3.1. Participant selection and setting

The number of families currently using L-carnitine for the therapeutic relief of their child’s NF1 muscular symptoms is unknown, however prior to the start of this study, eight parents had actively contacted us (including by email or telephone) regarding this topic. All originally contacted us because of our 2018 preclinical research study: “Dietary intervention rescues myopathy associated with neurofibromatosis type 1” (12). Since then, several parents have also shared their personal experiences with L-carnitine supplementation. This research study is
focused on parental perceptions of L-carnitine supplementation for the treatment of NF1 muscular symptoms, therefore parents who administered L-carnitine to their child for purposes other than muscular symptoms were excluded.

Ethics approval was obtained by the Sydney Children’s Hospitals Network Human Research Ethics Committee (2019/ETH00538). Only the parents who had actively contacted us were initially invited to partake in this research study. Others were recruited through a passive form of snowballing sampling: participants passed on the researchers’ contact details and interested parents contacted the researchers (14). All participants provided written informed consent.

3.2. Data collection

A parent-reported, semi-structured questionnaire was distributed to the parent who initially contacted us via email. It consisted of 36 items that fell under four broad areas that attempted to quantify some aspects surrounding their decision to supplement their child with L-carnitine and the outcome: general information, clinical features of NF1, medical history of muscle weakness and fatigue, and L-carnitine and other supplementation.

The parent who completed the questionnaire was interviewed. Parent-reported, semi-structured interviews were conducted by EV, previously trained by KS. Open-ended semi-structured questions were used as outlined in S1 Table, and frequently elaborated on according to individual context. There was no time limit for answers and comments, which likely led to further expressions of personal meanings. The interviews were recorded on Zoom Video Communication and then transcribed using NVivo transcription (NVivo12 software). The interview transcripts were reviewed against the original audio recordings for accuracy by EV.
3.3. Analysis

Questionnaire results are outlined in a case series description about each child. Interview transcripts were thematically analyzed by EV, as described by Braun and Clarke (2006). The data was read and re-read in an active way, searching for meanings and patterns. Initial ideas and the reasons they were interesting for the study were noted. Then, as many initial codes as relevant were produced and organized into meaningful groups. The different codes were sorted into potential themes and sub-themes. To decrease potential bias and increase the credibility of results, the transcripts were re-analyzed independently by KS. Any convergent and divergent aspects of the researchers’ analyses were discussed until consensus was reached to determine the final thematic categorization that best represented the results. Illustrative quotations were identified to represent themes and sub-themes.

4. Results

4.1. Recruitment and participant characteristics

Of the eight families approached, four met the criteria for participation and agreed to take part in the study (50%), two did not meet the criteria as they had not initiated L-carnitine supplementation due to uncertainty around dosing (25%), and two did not respond (25%). Four additional families contacted us throughout the course of the study regarding L-carnitine supplementation, however none initiated L-carnitine supplementation for NF1-associated muscle weakness and fatigue at the time of the study and were thus excluded.

The four families who participated in the study completed the questionnaire and interview. These families’ characteristics are provided in Table 1. The interviewed parents were 50% female, and USA was the country of origin for 75%. The children were aged 6-17 years old,
and 75% were male. Parents reported a range of other NF1 manifestations in their children, including café-au-lait macules (100%), optic glioma (75%), inguinal or axillary freckling (75%) and Lisch nodules (50%). Pseudonyms have been created to protect the identities of the parents and children. The audio interviews lasted from 24 to 31 minutes (average 27 minutes).

4.2. Case series.

Julie

Julie complained of muscle weakness and fatigue prior to L-carnitine supplementation. Julie’s muscular symptoms were noticed by Isabelle on a daily basis. These symptoms had concerned Isabelle since Julie was five years old, and had perceptively worsened. Compared to other children of the same age and sex, Isabelle believed Julie was considerably weaker and fatigued quicker. She supplemented Julie with 1000mg acetyl L-carnitine orally daily. Julie has now been receiving this supplementation for roughly three years (14-17 years of age). On one occasion she tried 2000mg, however Julie developed pungent urine and the decision was made to reduce the dose back to 1000mg. She confessed to missing some doses, or being particularly slack for periods of time, and on those occasions Julie’s muscular symptoms returned. With L-carnitine supplementation Isabelle perceived Julie had increased muscle tone, strength and energy, was more alert and engaged, had decreased daytime napping and reduced autistic behavior. These improvements were noticed ‘immediately’ (an exact time frame was not indicated).

Russel

Russel complained about fatigue, but he did not report issues with muscle weakness. Despite this, Therese noticed he experienced both weakness and fatigue on a daily basis, since one year
of age. She recognized that Russel was considerably weaker and had less energy than other children of the same age and sex. An acylcarnitine profile test result demonstrated a high esterified/free carnitine ratio (ratio not provided). Therese provided Russel with 833mg L-carnitine and 500mg acetyl L-carnitine daily, and he has received these supplements for around two and a half years (8.5-11 years of age). Therese noticed with carnitine supplementation that Russel has increased energy, and is more alert and engaged. Improvements were observed within one week of the initial start. Therese also perceived Russel’s muscle strength to have improved, however she attributed this to physical therapy. This was because his initial physical therapy report noted muscle weakness despite being on carnitine for eight months, and then subsequent reports demonstrated improved strength with regular physical therapy training sessions.

_Sam_

Sam similarly complained about fatigue, but did not report issues with muscle weakness. However, Peter perceived Sam to struggle with general muscle weakness, particularly upper body exercises, for example, climbing on monkey bars or the fire pole in the playground. Peter noticed Sam to be only moderately tired (roughly 2-3 times a week). However, it was obvious to him that Sam was weaker and tired quicker than other children of the same age and sex, which became evident at 2-3 years of age. Two months prior to his fifth birthday, Peter supplemented Sam with 500mg L-carnitine daily for two weeks. He increased the dose to 1000mg, and two vomiting episodes occurred in the span of one week leading to cessation of treatment. Before discontinuation, Peter noticed Sam had improved muscle strength, increased energy and decreased daytime napping. Peter noticed these benefits within 2 weeks of the initial start of supplementation. These improvements were not sustained following treatment cessation, and Peter was interested in restarting the treatment for Sam (almost 6 years of age) at the original dose of 500mg.
Roger

Roger frequently complained about fatigue, but not muscle weakness. Nevertheless, David noticed that Roger was weaker and got tired before other children of the same age and sex, and frequently required rest breaks. This became evident to David early on, but he could not determine an age. Roger is currently involved in physical therapy and follows a healthy, balanced Mediterranean diet. Roger commenced 400mg L-carnitine supplementation, which has now been sustained for around two years (6-8 years of age). In the last several months David increased Roger’s dosage to 600mg without complications. David noted improved muscle strength, improved coordination, improved balance and increased energy and decreased day time naps. These improvements were noticed within one month of supplementation. However, he also attributed some improvements, including balance and coordination and possibly strength, to physical therapy that started prior to L-carnitine supplementation.

4.3. Thematic analysis

Three themes were identified in the data: (1) factors influencing parents’ decision to self-administer L-carnitine to their child (primary muscle concerns and psychosocial repercussions, ineffective treatment strategies, research accessibility and availability and professional medical communication), (2) outcomes of L-carnitine supplementation (physical improvements, side effects, future treatment and sharing of health findings) and (3) areas that warrant further guidance (L-carnitine dosage, research accessibility, disseminating research and clinical trial interest). Illustrative quotations are provided in Table 2, and the conceptual patterns and relationships among all themes are shown in Fig 1.
Factors influencing parents’ decision to self-administer their child with L-carnitine.

Primary muscle concerns and psychosocial repercussions

Fatigue, muscle weakness, low muscle tone, and poor motor coordination were the primary muscle concerns mentioned by parents. Fatigue was particularly concerning for parents as it was prominent in their child’s day-to-day living and had a significant impact on their quality of life. One parent reported that their child’s muscular symptoms resulted in behavioral difficulties, and another was concerned about the repercussions on their child’s psychosocial functioning. This added to the concerns of primary muscular symptoms.

Ineffective treatment strategies

The absence of an effective therapy for NF1 muscular symptoms was contributory to the parents’ attempt to find a treatment. The only treatment option that was targeted towards NF1 muscular symptoms was physical therapy. Half of the children were actively engaged with this approach, however they did not find this ameliorated fatigue, which was their most prominent concern. Other vitamins and supplements, including Vitamins B and C, CoQ10 and curcumin had been tried. These supplements were sometimes tried prior to L-carnitine, and sometimes concurrently, however were reported to provide no added benefit or alleviation of muscle weakness and fatigue.

Research accessibility and availability

All parents expressed that they were motivated to try L-carnitine in their child after reading the original, preclinical research article by Summers, et al. 2018 (12). Being able to access scientific research online or at scientific conferences was vital to learning about this research in all cases. All parents acknowledged that they had a scientific background or a professional role in research governance, and this aided in their ability to understand and interpret scientific
literature. After reading about the pre-clinical research, they all conducted further personal research, sometimes using medical databases, such as Google Scholar and PubMed. The majority of participants read research articles regarding L-carnitine in the context of autism spectrum disorder. They also investigated the safety profile of L-carnitine, and deemed it to not pose an unreasonable risk.

*Professional medical communication*

Key medical professionals, including scientific academics, dieticians, pediatricians, neurologist and NF specialists were sometimes engaged by parents in the decision making process. This was to seek more information about dosage and receive an external opinion about self-supplementing their child with L-carnitine. In all cases the parents’ made the decision to self-supplement their child, and their decision was supported by medical professionals.

**Outcomes of L-carnitine supplementation**

*Physical improvement*

Subjectively by parents, L-carnitine supplementation was found to be beneficial, and improve physical abilities in all children. The parents expressed that their children had greater endurance, being able to persist for longer during activities or exercise. The improvements were frequently reported as striking. In cases where supplementation was stopped, muscle weakness and fatigue returned.

*Side effects*

L-carnitine supplementation was stopped in one case because of two episodes of nausea and vomiting. However, the parent speculated that the dose of 1000mg was potentially too high for
his child (>50 mg/kg), and the parent was interested in re-commencing L-carnitine supplementation, albeit at a lower dose.

**Future treatment**

Apart from the parent intending to re-start L-carnitine, all other parents continued L-carnitine supplementation for their children. All remain actively involved with the scientific literature, and are open to new therapeutic options. There is interest in MEK inhibitors, and stem cell and gene therapy. Those parents who have tried physical therapy for their child remain engaged with this in conjunction with L-carnitine supplementation. In some cases, curcumin was noted as a current supplement or a supplement of future interest. However, their focus on curcumin pertained to managing their child’s tumor burden rather than muscle strength or fatigue.

**Sharing of health findings**

All participants described actively sharing their health findings with other NF1 families and medical professionals. NF1 families are well connected via social media sites, such as Facebook and WhatsApp, as well as blog sites. They frequently share research findings and personal experiences. Similarly, they share their findings with their medical professionals.

**Areas that warrant further guidance**

**L-carnitine dosage**

All parents expressed some degree of uncertainty regarding the optimal dose for their child, and were frequently curious about whether increasing the dose would result in a greater functional outcome.
Research accessibility

The majority of parents mentioned that research accessibility was an issue for other NF families in their network. Some tried to alleviate this by sharing articles they had access to and often relay a lay understanding of their findings.

Disseminating research

A majority of parents expressed some concern around sharing their health findings with other NF1 families, with one parent concerned that he could jeopardize his job position if he shared any illegitimate scientific research. When parents shared their health findings, they tried to remain neutral and not elicit or provoke a negative response from other parents of children with NF1. Occasionally other parents in their network exhibited conflicting views on the use of experimental medication, and this rarely resulted in some tension and argument with parents engaged in this study.

Clinical trial interest

All parents were interested in whether there was a clinical trial of L-carnitine supplementation for NF1 muscular symptoms. This was mainly because they wanted more information about an optimum dose and dosage strategy, e.g. single dose vs. split daily doses. Further, they were curious about how the efficacy of L-carnitine supplementation could be measured, and whether efficacy was limited to NF1 muscular symptoms, e.g. potential for cognitive or behavioral benefits.
5. Discussion

Self-medication is commonly practiced worldwide for a variety of reasons including easy access to medications/supplements, as well as limited knowledge of risks and management of illness (15-17). In our study, parents expressed concern regarding their child’s NF1 muscular symptomology because it significantly impacted their child’s day-to-day living. This is consistent with a prior study showing that children with NF1 have reduced participation in physical activities and skill-based activities, and often spend limited time with friends (18). It is likely that NF1 muscular symptoms are a contributing factor to the below-average social functioning in children with NF1, although the precise causes are still not well understood (19).

Aside from this study, the NF1 muscle phenotype has previously been implicated with compromised social functioning, with children’s physical difficulties limiting their ability to do things their peers could (e.g. join sport teams) leading to a sense of isolation (1).

Parents who made the decision to supplement their children with L-carnitine and were recruited to this study all had professional backgrounds that enabled them to access and interpret scientific findings related to NF1. This is consistent with other studies showing that educated parents are more likely to self-medicate their children (20, 21).

Many individuals in this study expressed the feeling of educating their medical professionals about NF1 muscular symptomology, for which they felt some degree of frustration. This highlights the critical role of NF1 specialists to understand all dimensions of NF1, as general practitioners likely lack the in-depth understanding of all of the medical complications associated with rare disorders. It is particularly important as less educated individuals rely on their medical providers for health findings and guidance.

All families perceived an improvement in their children’s symptoms within a short period after initiating L-carnitine supplementation. Patients with primary carnitine deficiency have also
been shown to exhibit an immediate response to L-carnitine supplementation (22, 23). Further, the parents in this study who either ceased L-carnitine supplementation or were negligent for periods of time, noted that muscle weakness and fatigue returned, suggesting that L-carnitine supplementation if effective would need to continue for sustained therapeutic relief. Additionally, three out of the four parents increased their child’s dosage throughout the course of treatment; however, in two instances this resulted in side-effects, such as pungent urine and vomiting episodes, and in once instance this resulted in cessation of treatment. Thus there is a need to develop evidence-based guidelines for dosing in children and adults with NF1.

Of the four families involved in this study, two were actively engaged with physical therapy in conjunction with L-carnitine. Both claimed that the two interventions worked well together to improve functional outcomes. In the medical literature, there have been only two exercise based regimens designed to influence the motor phenotype in NF1 (18, 24). The first study assessed a plyometric training program in children, which resulted in improved jumping and throwing performance; however, the sample size was small (n=3) and there was no control (24). The second involved a home-based resistive exercise program, and resulted in improved upper extremity function; however, no improvement was observed in physical activity participation. Although the latter study involved a control (n=16), functional assessment was limited to two questionnaires: the Pediatrics Outcomes Data Collection Instrument (PODCI) and Children's Assessment of Participation and Enjoyment (CAPE) (18). It is possible that an underlying metabolic myopathy in NF1 limits the capacity of exercise base regimens, which could be rectified by L-carnitine supplementation. Further investigation into the benefits of physical therapy in conjunction with L-carnitine supplementation is critical to ascertain the capacity of a combinatory approach for the treatment of NF1 muscular symptoms.

In NF1, the relationship between muscle weakness and fatigue is not well understood. Investigating this linkage could provide clues about the disease-specific mechanism underlying
the muscular symptoms of the condition. In spinal muscular atrophy, it has been demonstrated that physiological fatigue (measured by the difference in distance travelled in the 6th minute compared to the 1st minute of the 6 minute walk test) is correlated with the degree of weakness. This suggests that in spinal muscular atrophy the disease mechanism accounts for both muscle weakness and fatigue (25). In NF1, it is possible that a heightened state of oxidative stress (disrupted redox signaling and control) in skeletal muscle may cause muscle weakness and accelerate the rate of fatigue (26, 27). Therefore, the potent antioxidant nature of L-carnitine would enable physical exercise and improvement, making physical therapy and L-carnitine a plausible combinatory approach.

Our study has several limitations. Firstly, this was a parent-reported, retrospective study and recall bias has likely occurred. Secondly, there was a limited number of parents involved in this research who all demonstrated a scientific background, and it is possible that we did not reach data saturation. A comprehensive review by Guest et al. (2006) found that most main themes in qualitative data can be identified within six interviews, and all themes are identified (known as ‘data saturation’) by ten to twelve interviews (28). Therefore, some factors influencing the decision making process of parents’, outcomes and areas warranting further investigation may not have been captured by this research. Thirdly, the questionnaire utilized in this study had not previously been validated, and frequently the questionnaire items were not correctly completed and unanswered.

In summary, our work yields some insights into the decision making process of parents’ to try to improve their NF1 child’s physical abilities by providing L-carnitine supplementation. It raises the distinct possibility that the benefits of L-carnitine supplementation for NF1 could be further improved by physical therapy. Thus the potential additive or synergistic effects by combining L-carnitine and exercise remain a key area for future study.
6. References


7. Figures and Tables

Figure 1. Thematic schema representing the conceptual patterns and relationships among the decision making process of parents to treat their children that display NF1-associated muscle weakness and fatigue with L-carnitine supplementation, outcomes of parents’ decision to self-medicate their child, and areas that warrant further guidance.
Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>NF1 families</th>
<th>001</th>
<th>002</th>
<th>003</th>
<th>004</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Country of origin</strong></td>
<td>USA</td>
<td>USA</td>
<td>USA</td>
<td>Switzerland</td>
</tr>
<tr>
<td><strong>Parent sex</strong></td>
<td>Female</td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td><strong>Parent pseudonym</strong></td>
<td>Isabelle</td>
<td>Therese</td>
<td>Peter</td>
<td>David</td>
</tr>
<tr>
<td><strong>Child sex</strong></td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td><strong>Child pseudonym</strong></td>
<td>Julie</td>
<td>Russel</td>
<td>Sam</td>
<td>Roger</td>
</tr>
<tr>
<td><strong>Child age (y)</strong></td>
<td>17</td>
<td>11</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td><strong>Other reported NF1 diagnostic criteria</strong></td>
<td>Café-au-lait macules, optic glioma, and inguinal or axillary freckling</td>
<td>Café-au-lait macules, optic glioma, inguinal or axillary freckling, and Lisch nodules</td>
<td>Café-au-lait macules, inguinal or axillary freckling, and Lisch nodules</td>
<td>Café-au-lait macules, and optic glioma</td>
</tr>
<tr>
<td>Theme</td>
<td>Sub-theme</td>
<td>Quotations</td>
<td></td>
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<tr>
<td>Factors influencing parents’ decision to self-administer their child with L-carnitine</td>
<td>Primary muscle concerns and psychosocial repercussions</td>
<td>“She fell asleep in the middle of her birthday party. She was napping a lot. She just did not have the energy to participate fully in life.” (Isabelle) “He’s always had low muscle tone” (Therese) “He tended to be a bit clumsier than his peers, you know, since he started walking.” (Peter) “It was really hard to get him to do anything or go anywhere. And I think it resulted in behavioural problems.” (Therese)</td>
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<tr>
<td>Ineffective treatment strategies</td>
<td>“I’d been trying different things. I tried giving her CoQ12. Well, I tried giving her the B complex and nothing really touched it.” (Isabelle) “I had always been told that there was nothing you could do about that except for physical therapy” (Therese)</td>
<td></td>
<td></td>
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<tr>
<td>Research accessibility and availability</td>
<td>“I’m a scientist myself, I have my doctoral degree, and so information is very accessible...”</td>
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</table>
to me, I have no difficulty reading them [journal articles]” (Isabelle)

“I may have seen you guys present some of the data at a CTF [Children’s Tumor Foundation] meeting” (Peter)

“I like to use google scholar because I like to see the original research articles” (Therese)

“I read the levels they were giving the children taking into account how big the children were, if there were any safety concerns” (Isabelle)

“[It] just paints a picture that this is something that has been helpful with some of the same behavioural and cognitive difficulties that my daughter was experiencing” (Isabelle)

“Lot of Facebook groups... When an interesting new study comes up, usually somebody posts it and then that’s where I can learn about it” (Therese)

“I asked some researchers” (David)

“One of our collaborators is a neurologist with expertise in NF1 so I sort of ran the idea by her as well as Sam is seen down at the NF1 specialty clinic... so we talked to the geneticist who see[s] him down there. So we just sort of
<table>
<thead>
<tr>
<th>Outcomes of L-carnitine supplementation</th>
<th>Physical improvements</th>
<th>Side effects</th>
<th>Future treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ran it by those folks and they seemed on board with it.” (Peter)</td>
<td>“It was a dramatic, dramatic improvement... he had no energy in the afternoon to getting 10 000 steps a day” (Therese)</td>
<td>“When we increased the dosage (from 500mg to 1000mg), he had two vomiting episodes” (Peter)</td>
<td>“I’m happy with the way things are going now, but I'm always open to reading new things” (Therese)</td>
</tr>
<tr>
<td>“He seemed to be persisting on physical activities or exercises longer during the summer, we were swimming quite a bit” (Peter)</td>
<td>“Weeks that would go by, we would only get it in a few times and she actually started getting a worsening of symptoms” (Isabelle)</td>
<td></td>
<td>“I'm really excited that the MEK inhibitors are likely going to be approved this year. And I'm hoping that one day that will lead to a preventative treatment so that that pathway...”</td>
</tr>
<tr>
<td>Areas that warrant further guidance</td>
<td>L-carnitine dosage</td>
<td>Research accessibility</td>
<td></td>
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<tr>
<td>Sharing of health findings</td>
<td>&quot;that's overactive in NF can be muted by long term treatment using this effective therapy&quot; (Isabelle)</td>
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<td></td>
<td>&quot;Physical therapy is helping him with the balance and... coordination. I would say improvements in different areas.” (David)</td>
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<td></td>
<td>&quot;If I think it’s relevant or if I think it has a practical application, then I will post it to other parents of kids who have NF1” (Therese)</td>
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<tr>
<td></td>
<td>&quot;I probably share it with at least a couple, my NF specialist and paediatrician” (Isabelle)</td>
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<td></td>
<td>&quot;You know, we talked about what would be an appropriate dose with his paediatrician and the geneticist, and the idea was that going higher would be more likely to show a clinical effect.” (Peter)</td>
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<td></td>
<td>&quot;The trickiest was how much do you give to him” (David)</td>
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<td></td>
<td>&quot;One thing that I find very annoying is that there are a lot of articles I can’t read because they aren’t accessible” (Therese)</td>
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</tbody>
</table>
**Supplementary Table 1.** Interview guide for semi-structured interviews

<table>
<thead>
<tr>
<th>Semi-structured interview questions:</th>
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<tbody>
<tr>
<td>1. Why did you look for treatments for muscle weakness and fatigue?</td>
</tr>
<tr>
<td>2. Which of your child’s physical symptoms prompted you to look for treatments?</td>
</tr>
<tr>
<td>3. Which symptom(s) had the greatest impact on your child’s quality of life?</td>
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<tr>
<td>4. Which information prompted you to start using L-carnitine supplements?</td>
</tr>
<tr>
<td>5. Where did you find it? Websites/Blogs/Social Media/Data bases?</td>
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<tr>
<td>6. Which source(s) of information was most helpful?</td>
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<tr>
<td>7. How did you judge whether to trust the information?</td>
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<tr>
<td>8. How did you decide it would be safe to use the information to treat your child?</td>
</tr>
<tr>
<td>9. Did you speak to anyone about taking L-carnitine? Did family/friends/doctors/researchers influence your decision?</td>
</tr>
</tbody>
</table>
10. What other supplements/therapies/interventions have you tried for NF1?

11. How did you learn about these other supplements/therapies/interventions?

12. How did it/they compare to L-carnitine supplementation?

13. Are you still looking for treatments for NF1?

14. How do you look for online health information? Do you find it easy?

15. How do you judge whether you can trust online health information? Do you find it easy?

16. Do you ever stop looking because you are confused by what you read?

17. Does anyone help you look for online health information?

18. What help would you like to help you look for online health information?

19. Do you share the online health information you find with other parents or friends?
   Talk to them about it? Show them? Send it to them? Why/why not? Do they ever agree or disagree with the information? How do they feel? How does that make you feel?

20. Do you share the online health information you find with doctors? Talk to them about it? Show them? Send it to them? Why/why not? Do they ever agree or disagree with the information? How do they feel? How does that make you feel?

21. What prompted you to contact us about medicating your child with L-carnitine?

22. Do you know anybody else with a child with NF1 who is taking L-carnitine supplements? If so, would you be willing to tell them about this study and pass on our contact details, so if they would like to take part in the study, they can reach us?
Chapter 4 Prologue

In Chapter 3 we hint towards the potential of L-carnitine supplementation for the treatment of childhood muscle weakness and fatigue in NF1. While continued work in this area could be insightful, a clinical trial represents the most robust approach to ascertain the safety, feasibility and efficacy of L-carnitine supplementation.

In Chapter 4 we present our results from the first clinical trial to test L-carnitine supplementation for NF1-associated muscle weakness and fatigue. We hypothesise that L-carnitine supplementation is safe and feasible in this cohort, and changes in strength and endurance measures may be observed. We also conduct plasma acylcarnitine profiling to assess for evidence of a secondary carnitine deficiency.
Chapter 4

L-carnitine supplementation for muscle weakness and fatigue in children with neurofibromatosis type 1: A Phase 2a clinical trial

Chapter 3 is currently under review for publication in the journal *American Journal of Clinical Nutrition*, and has been included here in manuscript format.

Title: L-carnitine supplementation for muscle weakness and fatigue in children with neurofibromatosis type 1: A Phase 2a clinical trial

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Conflict of Interest: We declare no competing interests.

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Clinical Trial Registry number: ACTRN 12618002021257 (Study protocol: https://anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=12618002021257).
1. Abstract

**Background:** Reduced muscle tone, muscle weakness and fatigue can impact considerably on quality of life for children with neurofibromatosis type 1 (NF1). Human muscle biopsies and mouse models of NF1 deficiency in muscle show intramyocellular lipid accumulation, and preclinical data has indicated that L-carnitine supplementation can ameliorate this phenotype.

**Objective:** To examine whether daily L-carnitine supplementation is safe and feasible, and will improve muscle strength and reduce fatigue in children with NF1.

**Methods:** A 12-week, Phase 2a trial was conducted using 1000mg daily oral levocarnitine tartrate supplementation. Recruited children were between 8-12 years old with a clinical diagnosis of NF1, history of muscle weakness and fatigue, and naïve to L-carnitine. Primary outcomes were safety (self-reporting, biochemical testing) and compliance. Secondary outcomes included plasma acylcarnitine profiles, functional measures (muscle strength, long jump, handwriting speed, 6 minute walk test (6MWT)), and parent-reported questionnaires (PedsQL™, CBCL/6-18).

**Results:** Six children completed the trial with no self-reported adverse events. Biochemical tests for kidney and liver function were normal, and the average compliance was 95%. Plasma acylcarnitine levels were low, but within a range not clinically linked to carnitine deficiency. For strength measures there was a mean 53% increase in dorsiflexion strength (95% CI 8·89-60·75; p=0·02) and mean 66% increase in plantarflexion strength (95% CI 12·99-134·1; p=0·03). In terms of muscle performance, there was a mean 10% increase in long jump distance (95% CI 2·97-16·03; p=0·01) and 6MWT distance (95% CI 5·88-75·45; p=0·03). Comparison with the 1000 Norms data showed a significant improvement in Z-score for all of these measures. Parent reports showed no negative impact on quality of life, and the perceived benefits led to the majority of individuals remaining on L-carnitine after the study.
**Conclusion:** 12 weeks of L-carnitine supplementation is safe and feasible in children with NF1, and a Phase 3 trial should confirm the efficacy of treatment.

**Keywords:** Neurofibromatosis type 1, NF1, muscle weakness, fatigue, children, L-carnitine, supplementation.
2. Introduction

Neurofibromatosis type 1 (NF1) is the most common autosomal dominant genetic disorder, with a birth incidence of 1:3000 globally (1). NF1 is caused by inactivating mutations in the *NF1* gene located on chromosome 17q11.2 (2). The *NF1* gene encodes neurofibromin, a RAS-specific GTPase activating protein (GAP) that modulates the biological activity of RAS proteins (2), and thus *NF1* is classified as a tumor suppressor gene. While tumors are often the focus of clinical management at all stages of life, children with NF1 can be challenged by reductions in lean tissue mass, global muscle weakness, and problems in fine and gross motor functioning (3-5). They also express higher levels of physical and cognitive fatigue (6). In a study of self-concept, approximately 30% of children and adolescents with NF1 reported a low self-concept for physical and sporting abilities (7). There are currently no effective interventions for managing the physical limitations associated with NF1.

Summers et al. (2018) showed that double inactivation of *Nf1* in murine muscle leads to intramyocellular lipid accumulation, which was also observed in NF1 patient muscle biopsies (8). This phenotype was reminiscent of metabolic myopathies, a series of conditions that are often managed by L-carnitine supplementation and/or dietary enrichment with medium-chain fatty acids. Treatment of the *Nf1 Prx1*−/− mouse with this intervention led to a decrease in the accumulation of long-chain fats in the muscle, leading to a 45% increase in grip strength following a 12-week treatment.

L-carnitine is a vital molecular component of several energy producing pathways (9). Greater than 95% of the body’s total carnitine is localized in skeletal muscle, where it is necessary for the transport of long-chain fatty acids through the mitochondrial membrane for beta-oxidation (10). Normally, the body’s requirements for carnitine are met by the consumption of meat, but endogenous synthesis and increased renal absorption efficiency can contribute to whole-body carnitine homeostasis. Impairments in L-carnitine synthesis, transport or metabolism can result
in primary or secondary deficiencies, which can in turn lead to elevated levels of intramyocellular lipid in muscle biopsies (11). Carnitine deficiency often results in muscle weakness and increased fatigue.

L-carnitine supplementation is frequently recommended to patients with carnitine deficiency syndromes. Primary carnitine deficiency responds dramatically to oral carnitine therapy, with a complete reversal of clinical symptoms within a month (12, 13). Patients with other disorders that feature a secondary carnitine deficiency, such as kidney disease and dialysis patients, and very long chain acyl-CoA dehydrogenase deficiency can also receive benefits from carnitine replacement therapy (14, 15).

L-carnitine supplementation has never been examined as a clinical treatment for muscle weakness or fatigue in the context of NF1. Hence, this study represents the first proof-of-concept trial to examine compliance, safety, and efficacy of levocarnitine tartrate treatment in children with NF1-associated muscle weakness and fatigue. To explore evidence for a secondary carnitine deficiency or other metabolic deficit, patients were assessed in terms of their plasma acylcarnitine profile. Our hypotheses were that: (1) daily 1000 mg levocarnitine tartrate supplementation (2 divided doses) in children with NF1-associated muscle weakness and fatigue would be safe, feasible and acceptable to families; (2) changes in strength and endurance measures may be detectable and (3) plasma acylcarnitine profiling may show evidence of a secondary carnitine deficiency.

3. Subjects and methods

3.1. Study design and participants

This open-label, single-arm, single center, Phase 2a clinical trial was designed to assess the safety and compliance of L-carnitine supplementation in children with NF1. The trial was
registered on the Australian New Zealand Clinical Trials Registry with ACTRN number 12618002021257 (Study protocol: https://anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=12618002021257). The study was approved and monitored by the Sydney Children’s Hospital Network Human Research Ethics Committee (reference no. HREC/18/SCHN/288).

Participants were children between 8 and 12 years of age that fulfilled: (i) the National Institutes of Health Consensus Conference diagnostic criteria for NF1, (ii) reported a history of muscle weakness and fatigue, which was confirmed by NF1 specialists from the Neurogenetics/ Neuromuscular Clinic at The Children’s Hospital at Westmead, and (iii) were naïve to L-carnitine supplementation. The clinic provides NF1 specialist services to children in the Greater Sydney Metropolitan Region. Prior to the initial assessment, a medical history of all participants was obtained from the parent(s), and the participant’s medical file. Participants were excluded from the study if they met any of the following criteria (i) severe cognitive impairment (ii) insufficient English, (iii) seizures, (iv) skeletal abnormalities, e.g. tibial bowing and pseudarthrosis, acute foot or lower limb injuries, e.g. fracture and ankle sprain, or (v) incapacity to comply with a research protocol, e.g. prolonged absence. Written informed consent was obtained from all parents and assent from children as developmentally appropriate.

3.2. Procedures

All participants were allocated a daily dose of 1000 mg Levocarnitine tartrate (Musashi, Vitaco Health Australia Pty Ltd). Hard capsules (500mg) were consumed twice daily for 12 weeks. The families were instructed to provide the children with the capsules at breakfast and dinner time, roughly 10 hours apart.
L-carnitine is an over-the-counter nutraceutical supplement taken to improve fatty acid oxidation and energy production. L-carnitine supplementation is used to treat primary carnitine deficiency. However, as L-carnitine supplementation had never been previously clinically trialed in NF1, we started the first three participants one month apart. As there were no adverse events in these children, the remaining three participants were started on L-carnitine supplementation at fortnightly intervals.

L-carnitine was dispensed from the Pharmacy Department of The Children’s Hospital at Westmead. Functional assessments and questionnaires were carried out at the Kids Research Clinical Research Centre, The Children’s Hospital at Westmead. All functional assessments were conducted in the same order at three different time points; Baseline, 6- and 12-weeks post treatment taken by a trained clinical evaluator. Questionnaires were completed by the parents at baseline and 12-weeks post treatment. Blood was collected and analyzed by the Pathology Department of The Children’s Hospital at Westmead. Blood samples were collected at baseline and 12-weeks post treatment, and urine was collected at 12 weeks.

Participants were given the option to continue L-carnitine supplementation at their own cost after the study endpoint (12 weeks), and were followed up after 3 months. The participants were asked whether they chose to continue L-carnitine treatment, and what their regimen was. They were also asked to complete the same set of questionnaires after this period.

### 3.3. Outcome measures

The primary outcome measures for this study were safety and compliance. Safety was primarily analyzed by adverse event self-reporting. A weekly phone call was made to each participant family to ensure any safety concerns or adverse events were expressed. Additionally, there were three in-person consultations for each participant, which were scheduled prior to each
functional assessment. Biochemical safety assessments included plasma liver function (Supp. Table 1), urine chemistry (Supp. Table 2), circulating triglycerides and cholesterols, and acylcarnitine profiling (Supp. Table 3). At the final consultation, participants returned any remaining capsules. The number of remaining capsules were counted to assess their compliance. The intervention was declared safe and feasible if: (i) no more than 1 of the 6 participants withdrew due to experiencing an adverse event attributable to treatment, and (ii) at least 4 of the 6 participants were able to complete at least 75% of the prescribed dose of treatment and comply with study requirements.

Secondary outcomes were functional assessments, including body fat, measures of muscle strength (grip, dorsiflexion and plantarflexion), gait, power (long jump), fine motor function (hand writing speed test), gross motor function (6 minute walk test) and quality of life (Pediatric Quality of Life and Child Behaviour Checklist for ages 6-18). Body fat was measured using the MC-780MA Tanita Tokyo body composition analyzer. The MC-780MA body composition analyzer divides the human body into five sections; left leg, right leg, trunk, left arm and right arm, measuring impedance by a tetrapolar 8-point tactile electrode at 50 kHz. Maximal isometric strength of 3 muscle groups involved in prime movements, including grip strength, ankle dorsiflexion and plantarflexion were tested using hand-held dynamometry by a trained clinical evaluator (Citec; CIT Tehnics, Harren, The Netherlands). To meet the independence requirements for statistical analysis, the measurements from only the dominant limb were included for each participant (16). Gait was assessed by heel and toe walking, which was scored on a 3-point scale of difficulty: “no”, “some”, and “yes” to further examine dorsiflexion and plantarflexion strength. Power was measured by a standing long jump on a padded mat. Fine motor endurance was evaluated by the Wallen, et al (1996) Handwriting Speed Test that gives a raw score in letters per minute, and gross motor endurance by the 6 minute-walk-test (6MWT), which was completed barefoot on a point-to-point, 25 metre long,
flat, straight, hard surfaced track. Quality of life was assessed by parent reported questionnaires, including The Child Behaviour Checklist for ages 6-18 or CBCL/6-18 (©ASEBA 2020), and Pediatric Quality of Life (PedsQL™) modules; generic 4·0 (physical, psychosocial and total summary scores on a scale ranging from 0 to 100, with lower scores indicating worse health state) and neuromuscular 3·0 (neuromuscular disease, communication, family resources and neuromuscular total summary scores).

3.4. Statistical analysis

We analyzed treatment effect by calculating average % change from baseline compared to 12-week post treatment for all outcome measures for each participant (n=6), and confidence intervals for improvement were also calculated. Spearman’s rank-order correlation efficiency was applied to determine a weight specific dosage effect. Participant functional assessments from baseline and 12-week post treatment were compared to reference data to generate Z-score changes (negative Z-score is indicative of motor impairment based on age and sex-matched normative data). Hand-dynamometry strength measures, long jump and 6MWT were compared to reference data obtained from the 1000 Norms Project, an observational study investigating outcome measures of self-reported health and physical function in 1000 healthy individuals aged 3 to 101 years (17). Handwriting speed was compared to normative data adapted from Cermak (1989) and Wechsler (1974). Height, weight and body mass index (BMI) Z-scores were generated using USCDC2000 reference data.
Role of the funding source

This study was funded by the Children’s Tumor Foundation (US). The funding source had no involvement in the following: study design, collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

4. Results

Between June and August 2019, a total of six participants were recruited at the Neurogenetics/Neuromuscular clinic located in The Children’s Hospital at Westmead. All participants fulfilled clinical diagnostic criteria for NF1, a medical history of muscle weakness and fatigue and were naïve to L-carnitine supplementation. Demographics and clinical characteristics for the six participants are summarized in Table 1. Four (67.7%) were male and 2 (33.3%) were female. The mean age was 10.7 years (SD 1.2), and BMI Z-score was -0.32. All participants were assigned the pre-determined daily dose of 1000mg levocarnitine tartrate (n=6). The mean calculated daily weight-specific dose was 31.6 mg/kg/day (SD 10.5), based on weight measurements collected at baseline. All participants completed the pre-determined treatment duration of 12 weeks. All bottles of L-carnitine supplements were returned at the completion of the study.

There were no side-effects or adverse events reported throughout the duration of this study. Kidney and liver function were normal, and circulating fats was nominal following 12 weeks of L-carnitine supplementation. There were no withdrawals, and all participants completed ≥ 84% of the treatment course (calculated as a percentage; number of capsules taken/168*100), with a mean compliance of 95 ± 6.2%. Therefore, 1000mg/daily levocarnitine tartrate treatment was deemed safe and feasible.
Acylcarnitine profiling revealed no abnormalities in plasma acylcarnitine levels (Table 2). Mean baselines levels of acylcarnitines included; total carnitine 39·3 (Ref: 5-106, SD 9·2), free carnitine 31·8 (Ref: 3-60, SD 9·6), acetyl-carnitine 6 (Ref: 2-39, SD 1·5), propionylcarnitine 0·4 (Ref: 0·12-0·97, SD 0·2) and isovaleryl carnitine 0·1 (Ref: 0·00-0·22, SD 0·1). Following 12 weeks of L-carnitine supplementation, levels of acylcarnitines were; total carnitine 54·5 (Ref: 5-106, SD 6·8), free carnitine 44·5 (Ref: 3-60, SD 5·6), acetyl-carnitine 8·3 (Ref: 2-39, SD 1·8), propionylcarnitine 0·7 (Ref: 0·12-0·97, SD 0·3) and isovaleryl carnitine 0·2 (Ref: 0·00-0·22, SD 0·1).

Bioelectrical impedance analysis was performed to assess the effect of L-carnitine supplementation on body fat mass (kg) or amount (% body weight), due to its primary role in fat metabolism. There was a mean reduction in fat mass by 0·8% (SD 4·5, p=0·76), and mean reduction in fat amount by 2·8% (SD 4·4, p=0·21), neither statistically significant (Figure 1A). The average BMI Z-score decreased from -0·32 at baseline to -0·41 at 12-weeks post treatment. Following 12 weeks of L-carnitine supplementation there was no significant improvement in grip strength (95% CI -2·49-10·15; p= 0·18), however there was a mean 53% increase in dorsiflexion strength (95% CI 8·89-60·75; p=0·02) and a mean 66% increase in plantarflexion strength (95% CI 12·99-134·1; p=0·03) (Figure 1B). Standing long jump distance was significantly greater on average by 10% after the treatment course (95% CI 2·97-16·03; p=0·01) (Figure 1C). A mean 10% improvement was also observed for the 6MWT after 12-weeks of L-carnitine supplementation (95% CI 5·88-75·45; p=0·03). To note, the 6MWT of participant two was excluded due to cramping during testing time. Furthermore, handwriting speed increased by 15% on average, however due to the considerable variability in participant performance this failed to reach statistical significance (95% CI -7·38-20·24; p=0·28) (Figure 1C).
Functional outcome measures collected throughout the study were compared to age and sex matched normative data. NF1 participants performed below average on every outcome measure at baseline, with the exception of participant six who generated a positive Z score for dorsiflexion strength and handwriting speed, and participants three and four who also had a positive Z score for handwriting speed (Figure 2). After 12 weeks of L-carnitine supplementation there was an average Z-score improvement of dorsiflexion strength from -1.57 to -0.32 (95% CI 0.17-2.33; p=0.03) and plantarflexion strength from -1.98 to -0.15 (95% CI 0.08-3.59; p=0.04). Long jump Z-score improved from -2.05 to -1.5 (95% CI 0.15-0.95; p=0.02), and 6MWT from -3.2 to -2.38 (95% CI 0.34-1.3; p=0.01) (Figure 2).

Comparison of PedsQL™ scores between baseline and 12-weeks post treatment showed a trend towards improvement on the physical health summary domain of the generic module (median 46.9, IQR 42.2-60.9 to median 71.9, IQR 51.6-87.5) (Figure 3, Supp. Table 4). Due to varied parent perception when scoring their child, there was large variability in the starting scores resulting in statistical significance being unattainable for this outcome measure. Other domains, including psychosocial health summary, neuromuscular disease summary, communication summary, and family resources summary scores showed no significant differences suggesting that L-carnitine supplementation does not interfere with participant quality of life.

Participants were scored to the CBCL/6-18 syndrome scales, including anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior and aggressive behavior at baseline and 12-weeks post treatment. Majority of the children (4/6) fell within the clinical range of >2 syndrome scales at baseline. This was reduced to 2/6 children following 12-weeks post treatment (Supp. Table 5). However, no statistically significant differences could be detected due to sample size and interparticipant variability (Figure 4).
All participants were followed up 3 months after completion of the trial. Three families continued supplementation with L-carnitine. One family ceased treatment, however recommenced after fatigue symptoms returned. Two families did not continue treatment; with both families recognizing that NF1 cognitive and social behaviours were confounding their view of L-carnitine supplementation for their child’s muscle weakness and fatigue, and one family awaiting the published trial results to re-consider L-carnitine.

5. Discussion

The primary goal of this Phase 2a, proof of concept clinical trial was to establish the safety and feasibility of L-carnitine as a therapeutic intervention for NF1 muscle weakness and fatigue. Critically, there were no side effects of L-carnitine supplementation or adverse events reported and no abnormalities seen in liver and kidney function tests. These data are consistent with L-carnitine being well tolerated in both children and adolescents, as it is part of a normal diet (albeit in lower amounts). The safety of L-carnitine supplementation has previously been demonstrated in a number of different pediatric cohorts, including primary carnitine deficiency, autism spectrum disorder (ASD) and Rett syndrome (18, 19) (20). However, this represents its first trial in a pediatric NF1 population. The high compliance rate of 95%, with no withdrawals suggest that daily L-carnitine supplementation is a well-received therapeutic approach to treat muscle weakness and fatigue in NF1 children.

A daily dose of 1000mg was predetermined for all participants. Future studies could better control for weight-specific dose, which varied from 17·7 to 46·5 mg/kg/day in our cohort. Correlation analysis revealed no association between dose and functional outcomes, although this analysis had limited power. In prior pediatric studies, a 50mg/kg/day dose of L-carnitine supplementation is commonly practiced with high safety and proven efficacy and this likely
represents a suitable starting dose for those naïve to the therapy. Anecdotal reports and clinical studies suggest that dose escalation could merely increase the incidence and severity of side effects, such as nausea and vomiting (19, 21).

The use of L-carnitine in NF1 children with muscle weakness who suffer from seizures remains a question for future study. Such individuals were excluded from recruitment due to contraindications with L-carnitine, although these risks are poorly substantiated. A review of encephalopathy patients on valproic acid found no data suggesting that seizures were worsened by L-carnitine supplementation (22). Therefore, future studies may include removing this as an exclusion criterion.

It has been suggested that NF1 could benefit from being described as a lipid-storage metabolic myopathy (11). This concept is supported by the potential efficacy of L-carnitine seen in this trial. The efficacy of L-carnitine supplementation has already been demonstrated in some secondary mitochondrial disorders (20, 23, 24). This category of conditions includes spinal muscular atrophy (25), Parkinson’s disease (26), Rett syndrome (27) and ASD (28). Further mechanistic studies and mitochondrial function analysis may reveal that NF1 has features of a secondary mitochondrial disorder, although it will be important to rule out a primary mitochondrial disease) in cases of NF1 by gene panel testing of oxidative phosphorylation related genes.

It was speculated that individuals enrolled in the study may show clinical deficiency in carnitine that could explain their muscle weakness and/or fatigue. While plasma acylcarnitine profiling revealed no clinical deficiency, many individuals were towards the lower end of the normal range. Comparison to a control cohort would be necessary to assess for a secondary carnitine deficiency in NF1 children. More importantly, plasma carnitine concentrations do not always reflect the carnitine concentration observed in skeletal muscle. For example, hemodialysis patients frequently exhibit normal plasma carnitine, but have a low muscle
carnitine concentration (29, 30). However, muscle biopsies are highly invasive and would represent a major barrier to trial recruitment.

Our study has several limitations. Children with NF1 and muscle weakness and fatigue are a poorly defined subcohort yet represent the precise group that would be most engaged with finding a muscle-targeted therapy. Thus, while this trial design did not use a randomized or placebo-controlled design, it captures a stratification of the NF1 community most likely to adopt routine L-carnitine supplementation. Moreover, this limitation was balanced by comparing individual cases before and after therapy, and to a normative group from the 1000 Norms Project data set. As previously noted, the lack of an L-carnitine dose normalized to weight is another limitation, but one that was necessary within the practicalities of commercially available carnitine preparations. Indeed, this has proved advantageous to families looking to sustain their supplement use after the study endpoint. Finally, from the initial conception this study aimed to examine safety and compliance within a small cohort and was not designed to completely accommodate the genetic and phenotypic heterogeneity of children with NF1 and was not powered towards functional outcomes. Therefore, it is difficult to distinguish whether function improvement is an effect of the treatment, a placebo effect or the effect of a natural history. Hence it was always anticipated that this would represent a precursor to a larger multi-center trial.

In summary, our data demonstrate that 1000mg daily levocarnitine tartrate is safe and feasible in children with NF1-associated muscle weakness and fatigue. Efficacy data suggest improvements in muscle strength and energy levels. However, we propose that a multi-site, randomized, double-blind placebo controlled trial with a consistent dosage regimen of 50mg/kg/day would be the optimal approach to firmly establish the efficacy of L-carnitine supplementation. A greater childhood age range, that could be compared to age-, sex-, height- and weight- matched controls, would also improve future study data.
Contributors
EV, JB, PB and AS contributed to the design of the study. EV, GD, AM, KJ, AB and CM participated in recruitment and collection and assessment of data, and JB, JNB and MM contributed the 1000 Norms data. EV did the statistical analysis, with support from Sydney Children’s Hospital Network (SCHN) Statistician, Ms. Liz Barnes. All authors participated in the writing and editing of the manuscript.

Acknowledgements
We would like to acknowledge the Sydney Children’s Hospital Network (SCHN) Statistician, Ms. Liz Barnes for her support in statistical design and analysis. We would also like to acknowledge the Clinical Research Centre of Kids Research, and the Pharmacy, Pathology and Biochemical Genetics Departments of The Children’s Hospital at Westmead who assisted with start-up, day-to-day running, biochemical testing and analysis of results. Further, we acknowledge the US based Children’s Tumor Foundation (CTF) for their ongoing financial support. Finally, we acknowledge the six families involved in the clinical trial. Without their engagement and active co-operation this research would not have been possible.

Data sharing
Data collected for the study will not be made available to others.
6. References


7. Figures and Tables

Figure 1: Percentage change following 12 weeks of L-carnitine supplementation.

Percentage change from baseline group mean in A) body fat, B) strength measures and C) other functional outcomes, including long jump, 6 minute walk test (MWT) and handwriting speed test. n=6 NF1 children, at 12 weeks one child did not complete the 6MWT due to abdominal cramping. % change calculated by (12 weeks value – baseline value)/baseline value*100. Data presented as group mean + SD. p-values were assessed by Paired T test of baseline values and 12-weeks post treatment values. * p<0.03. Each symbol denotes participants 1-6.
Figure 2: Z-score analysis of patient outcome measures compared at baseline and 12 weeks post treatment to age and gender matched normative data. Z-score comparison of A) grip strength, B) dorsiflexion strength, C) plantarflexion strength, D) long jump, E) 6 minute walk (MWT), and F) handwriting speed. A-D & F) N=6 NF1 children, E) N=5 NF1 children. Z-score calculated by sample value – normative (age and gender matched) mean/ SD. Data present as group mean ± SD. p-values were assessed by Paired T test. *p< 0·05 and **p< 0·01. Normative data was collected through the 1000 Norms project. n=8 age and sex matched normative, n=10 9y male normative for A-E. Normative data was adapted from Cermak (1989)
and Wechsler (1974) for F. Dotted line at 0 represents where NF1 children would have a comparable Z score to age and sex matched normative data. Each symbol denotes participants 1-6.

**Figure 3: Box plots of PedsQL™ domain scores.** A) generic 4∙0 core module domains, including physical health summary, psychosocial health summary and total scores, and B) neuromuscular 3∙0 module domains, including about my child’s neuromuscular disease, communication, about our family resources and total scores. Data are presented as median and interquartile range at baseline and 12-weeks post treatment, n=6.
Figure 4: CBCL/6-18 syndrome scale scores. Raw scores of A) anxious/depressed, B) withdrawn/depressed, C) somatic complaints, D) social problems, E) thought problems, F) attention problems, G) rule-breaking, and H) aggressive behavior. Data are presented for each participant at baseline and 12-weeks post treatment. Each symbol denotes participants 1-6 (n=6)
Table 1: Baseline characteristics of participants

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Table 2: Acylcarnitine profile test results were all within reference range. Total carnitine, free carnitine, acetyl-carnitine, propionylcarnitine and isovaleryl carnitine results are representatively displayed for each of the participants (1-6) at baseline (0w) and following 12 weeks of L- carnitine supplementation (12w). The mean and SD were calculated at baseline and 12 weeks of supplementation.

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The data is presented as mean ± standard deviation (SD).
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<th>0.10</th>
<th>0.21</th>
<th>0.07</th>
<th>0.09</th>
<th>0.23</th>
<th>0.22</th>
<th>0.19</th>
<th>0.18</th>
<th>0.01</th>
<th>0.19</th>
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</table>


**Supplementary Table 1:** Liver function test procedures with reference range and units

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Reference range</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>35-51</td>
<td>g/L</td>
</tr>
<tr>
<td>Bilirubin Total</td>
<td>0-10</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Bilirubin Direct</td>
<td>0-10</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Aspart Transaminase (AST)</td>
<td>0-50</td>
<td>U/L</td>
</tr>
<tr>
<td></td>
<td>0-49 (M, 12y)</td>
<td>U/L</td>
</tr>
<tr>
<td></td>
<td>0-41 (F, 12y)</td>
<td>U/L</td>
</tr>
<tr>
<td>Alanine Transaminase (ALT)</td>
<td>0-36</td>
<td>U/L</td>
</tr>
<tr>
<td>Gamma-Glutamyl Transferase (GGT)</td>
<td>0-45</td>
<td>U/L</td>
</tr>
<tr>
<td>Alkaline Phosphatase (ALP)</td>
<td>120-440 (M, 9y)</td>
<td>U/L</td>
</tr>
<tr>
<td></td>
<td>130-530 (M, 10-12y)</td>
<td>U/L</td>
</tr>
<tr>
<td></td>
<td>100-460 (F, 10-12y)</td>
<td>U/L</td>
</tr>
</tbody>
</table>

**Supplementary Table 2:** Urine chemistry tested with units

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random urine sodium</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Random urine potassium</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Random urine creatinine</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Random urine urea</td>
<td>mmol/l</td>
</tr>
</tbody>
</table>

**Supplementary Table 3:** Acylcarnitines included in the acylcarnitine profile test with reference range and units

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Reference range</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total carnitine</td>
<td>5-106</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Free carnitine</td>
<td>3-60</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Acetylcarnitine</td>
<td>2-39</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Propionylcarnitine</td>
<td>0.12-0.97</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Tiglyl-or 3methylcrotonylcarnitine</td>
<td>0.00-0.11</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Carnitine</td>
<td>Value</td>
<td>Unit</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Butyrylcarnitine</td>
<td>0.00041</td>
<td>µmol/l</td>
</tr>
<tr>
<td>3-Hydroxybutyrylcarnitine</td>
<td>0.00-0.29</td>
<td>µmol/l</td>
</tr>
<tr>
<td>2-Methybutyryl-or carboxymethylcarnitine</td>
<td>0.00-0.22</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Isovalerylcarnitine</td>
<td>0.00-0.20</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Hexanoylcarnitine</td>
<td>0.00-0.10</td>
<td>µmol/l</td>
</tr>
<tr>
<td>3-Hydroxyisovalerylcarnitine</td>
<td>0.00-0.28</td>
<td>µmol/l</td>
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<tr>
<td>Octanoylcarnitine</td>
<td>0.00-2.40</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Malonylcarnitine</td>
<td>0.00-0.16</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Decadienoylcarnitine</td>
<td>0.00-0.37</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Decenoylecarnitine</td>
<td>0.00-0.49</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Decanoylcarnitine</td>
<td>0.00-0.22</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Glutarylcarnitine</td>
<td>0.00-0.53</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Dodecenoylcarnitine</td>
<td>0.00-0.62</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Dodecanoylcarnitine</td>
<td>0.00-0.08</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Methylglutarylcarnitine</td>
<td>0.00-0.79</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Tetradecenoylcarnitine</td>
<td>0.00-0.34</td>
<td>µmol/l</td>
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<tr>
<td>Tetradecanoylcarnitine</td>
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<td>Hexadecanoylcarnitine</td>
<td>0.00-0.11</td>
<td>µmol/l</td>
</tr>
<tr>
<td>3-Hydroxyhexadecanoyl carnitine</td>
<td>0.00-0.02</td>
<td>µmol/l</td>
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<tr>
<td>3-Hydroxyoctadecanoyl carnitine</td>
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</tr>
<tr>
<td>3-Hydroxyoctadecenoyl carnitine</td>
<td>0.00-0.08</td>
<td>µmol/l</td>
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</table>
Supplementary Table 4: PedsQL domain scores. Scores are displayed for each participant (1-6) at baseline (0w) and following 12 weeks of L-carnitine supplementation (12w). 0 = low score and 100 = high score. Median (interquartile range) have been displayed to better represent the data.

<table>
<thead>
<tr>
<th>Domain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Median (interquartile range)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0w</td>
<td>12w</td>
<td>0w</td>
<td>12w</td>
<td>0w</td>
<td>12w</td>
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<tr>
<td>40.6</td>
<td>50</td>
<td>46.9</td>
<td>100</td>
<td>25</td>
<td>6.25</td>
<td>65.6</td>
<td>87.5</td>
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<tr>
<td>(2) Psychosocial health summary</td>
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<td>50</td>
<td>61.7</td>
<td>81.7</td>
<td>33.3</td>
<td>23.3</td>
<td>85</td>
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<tr>
<td>(1) + (2)</td>
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<tr>
<td>31.5</td>
<td>50</td>
<td>56.5</td>
<td>88</td>
<td>30.4</td>
<td>17.4</td>
<td>78.3</td>
<td>91.3</td>
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</table>

Note: Values are median (interquartile range).
<table>
<thead>
<tr>
<th></th>
<th>(3) Neuromuscular disease summary</th>
<th>(4) Communication summary</th>
<th>(5) Family resources summary</th>
<th>(3) + (4) + (5)</th>
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<td>74.3</td>
<td>(48.6-77.9)</td>
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<td>(56.3-98.8)</td>
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<td>82.4</td>
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<td>92.5</td>
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<td>(56.3-98.8)</td>
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<td>(78.8-98.8)</td>
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<td>92.5</td>
<td>82.5</td>
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<tr>
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<td></td>
<td>(78.8-98.8)</td>
<td>(58-87.5)</td>
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</table>
## Supplementary Table 5: CBCL syndrome scores

Scores are displayed for each participant (1-6) at baseline (0w) and following 12 weeks of L-carnitine supplementation (12w). Score reduction indicates normalisation. C = clinical range, B = borderline clinical range.

<table>
<thead>
<tr>
<th>Syndrome scale</th>
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<th>4</th>
<th>5</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0w</td>
<td>12w</td>
<td>0w</td>
<td>12w</td>
<td>0w</td>
<td>12w</td>
</tr>
<tr>
<td>(1) Anxious/depressed</td>
<td>10 - B</td>
<td>11 - C</td>
<td>12 - C</td>
<td>6</td>
<td>12 - C</td>
<td>14 - C</td>
</tr>
<tr>
<td>(2) Withheld/depressed</td>
<td>9 - C</td>
<td>7 - B</td>
<td>4</td>
<td>0</td>
<td>7 - C</td>
<td>5 - B</td>
</tr>
<tr>
<td>(3) Somatic complaints</td>
<td>5 - B</td>
<td>8 - C</td>
<td>9 - C</td>
<td>1</td>
<td>11 - C</td>
<td>2</td>
</tr>
<tr>
<td>(4) Social problems</td>
<td>10 - C</td>
<td>12 - C</td>
<td>9 - C</td>
<td>8 - B</td>
<td>13 - C</td>
<td>14 - C</td>
</tr>
<tr>
<td></td>
<td>5 - 12 - C</td>
<td>11 - C</td>
<td>18 - C</td>
<td>11 - B</td>
<td>15 - C</td>
<td>12 - B</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>(5) Thought problems</td>
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<td>(6) Attention problems</td>
<td>4</td>
<td>2</td>
<td>15 - C</td>
<td>2</td>
<td>6</td>
<td>7</td>
</tr>
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<td>(7) Rule breaking</td>
<td>5</td>
<td>6</td>
<td>4</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>(8) Aggressive behaviors</td>
<td>25 - C</td>
<td>15 - B</td>
<td>3</td>
<td>10</td>
<td>0</td>
<td>0</td>
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</table>
1. Summary of novel findings

Less than a decade ago, the concept of NF1 having a significant impact on weakness and fatigue was still controversial. Since this time, there have been numerous clinical studies that have rigorously described the muscle phenotype. However, it was only recent preclinical studies using genetically modified mouse models that gave clues to the underlying mechanism. This thesis continues to build upon this research and its practical implications.

In Chapter 2 the \( \text{Nf1}_{\text{Prx1}}^{-/-} \) mouse model is used to evaluate a number of modified diets and dietary supplement therapies. A medium-chain fatty acid enriched diet augmented with L-carnitine supplementation resulted in a rapid reduction of intramyocellular lipid droplets within a minimal 4-week period, which had previously only been assessed after 8 weeks of dietary intervention (1). This effect was not sustained following reversion to standard chow, consistent with later qualitative parent reports. Further, lipidomics analysis revealed substantially elevated fatty acids and total neutral lipids (including diglycerides and triglycerides) in the muscle suggesting that dietary intervention can redirect lipids from storage pathways to lipolytic pathways. Indeed several studies suggest that MCFAs are funneled into oxidative pathways (versus storage) possibly due to enhanced cellular uptake and entry into mitochondria for oxidation (2-4). It was also demonstrated in this thesis work that \( \text{Nf1} \)-deficient muscle exhibits substantively elevated acylcarnitines (particularly C16 and C18:1), which have been previously associated with disorders of mitochondrial fatty acid oxidation (5, 6). Nevertheless, NF1 patient samples are required to confirm this aberrant carnitine metabolism in humans. Perhaps the most clinically-relevant finding from this body of work is that L-carnitine supplementation alone is sufficient to rescue the Nf1 lipid storage phenotype in the murine model.
Dissemination of our preclinical research resulted in a number of families actively contacting us about their decision to commence L-carnitine supplementation in their children. In Chapter 3 we employed a qualitative research approach to capture the decision making process of parents who self-supplemented their child’s NF1 muscular symptoms with L-carnitine. Thematic analysis revealed three compelling factors that contributed to their decision and were common to all participants. These were: primary muscle concerns and psychosocial repercussions, ineffective treatment strategies, and research availability and accessibility. Of particular note, parents who self-supplemented their child all had a professional background that enabled them to access and interpret scientific findings related to NF1. This was consistent with other studies that show educated parents can be more likely to self-medicate their children (7, 8). The outcome of their decision hinted at the potential therapeutic value of L-carnitine supplementation for NF1 muscular symptoms in patients. Additionally, being involved in physical therapy in conjunction with L-carnitine supplementation appeared to improve treatment outcome. Previously, exercise-based interventions have received little attention in the medical literature (9, 10), but have not yet been assessed in combination with nutraceutical therapy such as L-carnitine.

In Chapter 4, we conducted a Phase 2a clinical trial of 1000 mg daily levocarnitine tartrate for childhood, NF1-associated muscle weakness and fatigue. For the first time, we demonstrated the safety and feasibility of 12-weeks L-carnitine supplementation in a cohort of NF1 children. There were no adverse events or side effects reported over the course of study. Kidney and liver function tests returned normal results at the study end-point. Further, parent-reported questionnaire data indicated that the treatment did not interfere with quality of life. We found preliminary evidence for improved muscle strength and energy in the children following carnitine therapy. This supports the notion put forward by us in 2018 that NF1 patient treatment could benefit from disease
categorisation as a lipid-storage metabolic myopathy (11). At the 3-month follow up, four out of six participants were found to be continuing L-carnitine supplementation voluntarily, suggesting parental perception of improvement matched our clinical findings.

2. NF1 muscle pathology could feature a secondary mitochondrial disorder

In NF1, impaired motor function (including muscle weakness), poor coordination and poor balance have historically been attributed to dysfunction in the central nervous system (12-14). However this concept was fundamentally challenged by the phenotypes of muscle-specific mouse models of NF1. These feature a primary muscle phenotype independent of neurological and cognitive deficits. Moreover, findings of this thesis suggest a secondary mitochondrial disorder in addition to a lipid storage phenotype.

An early seminal study of Nf1 mutant fruit flies (D. melanogaster) gave the first indications of the NF1 gene product having an important influence on cellular metabolism (15). Nf1 mutant mitochondria, featuring Nf1 inactivation, displayed a reduced ADP-stimulated respiration rate and derived ATP synthesis rate, during the metabolism of NADH-linked complex I substrates pyruvate and malate. Further investigation revealed that this was likely due to increased superoxide anion production in contrast to reduced scavenging enzymes activity, which showed no significant differences. Notably, fly survivorship improved by 50% upon addition of antioxidants to fly feed, highlighting the potential of supplementation for mitochondrial disorders. Additional mechanistic investigation revealed that overexpressing Nf1 resulted in increased mitochondrial complex I activity and reduced mitochondrial ROS production. One possible mechanism of this could be the direct cAMP-activated PKA modification of complex 1 polypeptides by neurofibromin (15).
The RAS/MAPK cascade has been linked to the direct regulation of mitochondria and mitochondrial dynamics. The RAF kinase, a downstream effector of RAS, interact with A-kinase anchor protein (AKAP) complexes, which can localize PKA and enhance the efficiency of cAMP signal-transducing pathways to target mitochondria and regulate mitochondrial dynamics and activity (16) (17). Additionally, RAF kinases, particularly C-RAF, have been found on the surface of mitochondria. Although very little about the exact mechanisms are known, it is speculated that they could directly control some aspects of mitochondrial membrane physiology, as well as metabolism (18).

Further insight into the role of NF1 for mitochondrial function in mammals can be obtained from the investigation of preclinical Nf1 mouse models. Transcriptional analysis of 2-month old Nf1<sup>-/-</sup> muscle revealed a downregulation in NDUFAF4, a mitochondrial assembly protein involved in the assembly of complex I. However, quantitative metabolic activity assays of Nf1<sup>-/-</sup> muscle revealed a 2-fold increase in activity levels of succinate dehydrogenase (SDH) or respiratory complex II (19). Perhaps, this upregulation could be due to a compensatory mechanism triggered by the downregulation of complex I (20). In contrast, 2-day old Nf1<sup>MyoD<sup>-/-</sup> muscle demonstrated no difference in SDH activity compared to WT (19). Although 4-day old Nf1<sup>MyoD<sup>-/-</sup> muscle contained some abnormal (large or elongated) mitochondria, electron microscopy images revealed many mitochondria to have a normal morphology. The key finding from these EM studies was significant intramyocellular lipid accumulation. This suggests that aberrant long-chain fat metabolism may precede mitochondrial dysfunction. Indeed, patients with primary FAO deficiencies frequently exhibit secondary OXPHOS defects. Secondary OXPHOS defects could be due to the build-up of toxic intermediates (21-23) or direct interactions between FAO proteins and OXPHOS proteins that disrupt the stability and function of the OXPHOS apparatus (21, 24).
In cancer, a number of studies suggest the RAS oncogene promotes anaerobic glycolysis, and lipid synthesis through the pentose phosphate pathway (25, 26). It is well known that the RAS/MAPK cascade regulates the expression of Hypoxia Inducible Factor (HIF-1α) in tumour tissues, and accumulation of HIF-1α leads to increased anaerobic glycolysis (27). RAS also mediates the PI3K/mTOR pathway (28). mTOR, the downstream effector of the PI3K/mTOR pathway, activates a transcription program mediated by ribosomal protein S6 kinase beta-1 (S6K1). S6K1 affects metabolic gene targets of HIF-1α, and sterol-regulatory element-binding protein (SREBP1 and SREBP2) that promote the transcription of multiple key enzymes including fatty acid synthase (FAS) (29, 30) (28). Indeed, FAS expression is upregulated in Nf1\textsubscript{Prx1}\textsuperscript{−/−} muscle, and is a plausible mechanism underlying aberrant lipolysis and lipid storage.

Despite significant research demonstrating a relationship between lipid storage and mitochondrial disorder in NF1, there remains a poor understanding of the underlying molecular mechanisms. Prior studies with insect and murine models can report conflicting results, for example Nf1\textsubscript{MyoD}\textsuperscript{−/−} mice demonstrate minuscule evidence of mitochondrial disorder in comparison to Nf1 mutant flies and Nf1\textsubscript{Prx1}\textsuperscript{−/−} mice, and often preclinical models examine homozygous deletion rather than haploinsufficiency. Therefore, analysing NF1-deficient muscle biopsies could be particularly insightful and help distinguish whether a mitochondrial disorder exists (31). One case report in the literature reporting an NF1 patient with Complex I deficiency demonstrates the difficulty in identifying mitochondrial disorder in NF1 (32). Guthrie card analysis, mitochondrial gene sequencing, acylcarnitine and urinary organic analysis failed to detect the mitochondrial deficit, and was only identified upon analysis of mitochondrial activity in muscle. However, unlike other muscle disorders such as SMA or DMD, NF1 muscle biopsies are not routinely taken to confirm myopathy. This challenge also affects other conditions, including autism spectrum disorder (33).
Collecting muscle biopsies in a clinical trial setting is likely to be an infeasible approach due to the highly invasive procedure, which can cause serious discomfort, decrease recruitment rate and increase trial costs. Further, opportunistic collection of NF1 muscle during day surgery, e.g. scoliosis could lead to confounding results due to interactions with adjacent pathological tissues. With the advancement in stem cell technology, an attractive option could be to collect peripheral blood mononuclear cells (PBMCs) from NF1 myopathy patients, and reprogram them to skeletal muscle for the study of mitochondrial function (34). Seahorse metabolic analyzers could be used to interrogate key cellular functions, including mitochondrial respiration and the location of any OXPHOS defect in the electron transport chain.

3. The limitations of mouse models

The Nf1Prx1−/− mouse model variably exhibits distorted morphology of the femoral neck and the femoral head skeletal structure, resulting in abnormal hip development including partial or complete fusion in some animals. This phenomenon can also be observed at the elbow joint. Nf1Prx1−/− mice that exhibit complete fusion have significantly impaired locomotion, and this causes secondary reductions in loading and strength over time. One possibility is that this phenotypic variability could be due to the variable efficiency of Cre expression and/or recombination, or variability in germline recombination (35). Moreover, increased cortical bone porosity, reduced bone mineralization, abnormal vascularisation and hyper proliferation of adipocytes all contribute to confounding bias of muscle analyses. Indeed numerous studies have demonstrated tissue-to-tissue communication or cross talk between skeletal muscle and adipose tissue, and skeletal muscle and bone. For example, during exercise skeletal muscle releases various myokines that can cause
beiging of subcutaneous white adipose tissue (scWAT) (36, 37), and the bone hormone osteocalcin has been shown to increase nutrient uptake and catabolism in myofibres (38). These limitations are the likely explanation for the significant variability observed in the in situ muscle physiology data featured in Chapter 2 of this thesis, and implicate being able to draw definitive conclusions about the effect of dietary treatments on muscle function.

Due to the limitations of the $Nf1_{Prx1^-}$ mouse model it is imperative that a mouse model of Nf1 adult muscle be developed. The $Nf1_{MyoD^-}$ mouse model was developed using the MyoD promoter to restrict $Nf1$ gene ablation to skeletal muscle. However, the failure of the mice to thrive and early mortality precludes the use in long-term dietary studies (19). More recently, $Nf1$ has been inactivated in the prenatal myogenic lineage either under the $Lbx1$ promoter or $Myf5$ promoter, in an attempt to develop an $Nf1$ muscle-specific mouse model that survives into adulthood (39). Nevertheless, neither model exhibits intramyocellular lipid accumulation, a characteristic feature of human NF1 muscle biopsies. An inducible approach has been tried previously to develop a muscle-specific knockout mouse model (40). However, this approach targeted satellite cells, which poorly integrated into adult muscle.

Alternatively, advances in systemic gene delivery using viral vectors may be a utility in the creation of muscle-targeted knockouts in adulthood. An AdCre viral system was previously used to create local knockouts in bone (41), however Adenovirus can have pro-inflammatory effects and can be challenging to delivery systemically without side-effects. Indeed, Adeno-associated viruses (AAVs) are now more widely employed as gene therapy vectors. We suggest that an AAV-Cre approach could be attempted to create muscle specific knockouts, either by local restricted delivery to individual muscles, or targeting using muscle specific promoters. One recent study has shown bone-targeting using bone-specific promoters and AAV8 (42). In muscle, the most
promising AAV variant is serotype 9 (AAV9), however high vector doses affect the liver (43). Recently, AAVpo1 has been found to efficiently transduce muscle with less effect on the liver (44). Studying the inactivation of Nf1 in adult muscle will provide insight into the role of Nf1 and muscle metabolism, independent to muscle development.

4. Design of a Phase 3 clinical trial for L-carnitine supplementation in NF1

Based on the Phase 2a clinical trial described in this thesis, L-carnitine supplementation shows considerable clinical promise for the treatment of NF1-associated muscle weakness and fatigue. Progressing this data to a Phase 3 clinical trial would enable the collection of more information on effectiveness and possible side effects of L-carnitine. A randomised, double-blind, placebo-controlled trial of L-carnitine supplementation, which encompasses larger numbers and a dose normalised to body weight represents the optimal trial design moving forward.

Randomisation and masking (double-blinded) are required to minimise bias in trial conduct and data interpretation, and ensure a gold standard for the evaluation of L-carnitine supplementation in NF1 (45). The implementation of a placebo concurrent control group is the most suitable control arm in a Phase 3 trial of L-carnitine supplementation, despite the Declaration of Helsinki arguing that a placebo controlled group is unethical. Alternatively, they encourage an active-treatment concurrent control group (46). However, there is currently no other treatment option for muscle weakness and fatigue in NF1. Further, non-treatment is not life-threatening, and L-carnitine supplementation could be offered to those in the placebo arm on completion of the study. The FDA support the use of placebo controlled groups, and additional scientific discussion is necessary to determine a consensus on future recommendations of control groups in clinical trials.
A Phase 3 clinical trial could also benefit from an additional control group; a dose-comparison concurrent control. This control group would address parental concerns around optimum dosing, and the common perception around increasing dose to improve functional outcome, which is particularly problematic due to the safety of L-carnitine supplementation. In our Phase 2a clinical trial the mean dosage was 30mg/kg/day and maximum dosage was ~50mg/kg/day. Therefore we propose these two doses in a Phase 3 clinical trial of L-carnitine supplementation for the treatment of NF1 muscular symptoms. This will ensure that safety and feasibility are minimally compromised, and a dose comparison can be established.

Implementing a weight-adjusted dose will enable expansion of the recruitment age to 8-17 year old children. In order to recruit large enough numbers for a Phase 3 clinical trial we recommend incorporating multiple sites worldwide. This will also assess whether L-carnitine supplementation will be effective when a broader range of providers and patients use the intervention. A post hoc power analysis and sample size estimate of the primary outcome measures, including grip strength, long jump and handwriting speed test, from our Phase 2a clinical trial, estimate that 87-113 children are needed to achieve a significant power of 95% calculated by G*Power (47). These calculations are based on children aged between 8-12 years of age, and assumes 13-17 year old children would respond similarly. NF1 patients who display muscular symptoms (low muscle tone, muscle weakness and fatigue) should remain the primary subject of investigation, as they remain the class of patients to whom the trials findings will be applied.

Expanding to a multi-centre clinical trial may require a more stringent inclusion/exclusion criteria to ensure participant eligibility. Most notably, muscular symptoms will need to be clearly demarcated, and could include fulfilling a simple criteria. For example, displaying strength deficits
across ≥2/3 muscle movements; grip, plantarflexion and hip abduction measured using hand-held dynamometry, and a reduced 6MWT distance or long jump compared to normative data.

The Phase 3 clinical trial must maintain an ongoing safety evaluation of L-carnitine supplementation (according to good clinical practice guidelines). Safety should primarily be addressed through self-reporting, and although weekly phone calls throughout the entirety of the study are not feasible in a multi-centre trial involving over a hundred participants it could be implemented initially, e.g. weekly for the first two weeks after enrolment. Compliance should also be assessed at the end of the trial to determine any discrepancies in functional improvement.

All participants should be functionally assessed at baseline to ensure each research arm are equivalent prior to treatment, or can be corrected for after treatment. Treatment efficacy has been demonstrated within a 12 week period, and a single, 12 week time point can be maintained to reduce clinical trial burden and effectively ascertain a treatment effect in a Phase 3 trial. However, the post-trial follow up should not be after 6 months to better assess long-term benefits or delayed safety concerns (48).

It is important that the Phase 3 trial involves the collection of quality of life data. Parent-reported questionnaires should be collected due to the anticipated variability in participant age. The PedsQL Neurofibromatosis type 1 module should be included as it incorporates a specific domain on fine motor skills, which was not assessed in our Phase 2a clinical trial. This module was designed to be collected in conjunction with the PedsQL generic module, which addresses physical functioning (gross motor skills), (49). Finally, the PedsQL fatigue module would also be insightful, and has been previously used to assess fatigue in NF1 (50).
Findings from this thesis suggest that physical therapy in conjunction with L-carnitine supplementation could contribute to functional improvement. Whilst a two-by-two factorial design is not feasible, collecting data on the participant’s physical activity throughout the Phase 3 trial could provide some insight into the potential for a combinatory therapy. Self-monitoring physical activity using paper diaries to record exercise behaviours would be the most feasible approach. Asking participants to record their daily exercise type, duration and intensity would enable the analysis of physical therapy whilst taking L-carnitine supplements.

L-carnitine supplementation is a safe, inexpensive and feasible treatment option for NF1-associated muscle weakness and fatigue. Subsequently, many families have elected to self-supplement their child’s NF1 muscle weakness and fatigue with L-carnitine independent of a RCT. Therefore, as an alternative to a Phase 3 RCT trial, the effect of L-carnitine supplementation could be assessed in conjunction with family’s local NF specialists, and presented as an observational study. Observational studies are considered to have an inferior ranking of “strength of evidence” compared to RCT, however there is a growing body of evidence, which suggests that observational studies can be just as acceptable in informing clinical decisions (51, 52).

5. Concluding remarks

Over the past decade, there has been a growing clinical appreciation of the impact of muscle weakness and fatigue on children with NF1. This may also extend into adult populations. Our research has been at the forefront of understanding the underlying mechanism and implementing clinically relevant treatment protocols. Early work performed by Summers et al. utilized mouse models such as the \( NfI_{MyoD}^{+/-} \) and \( NfI_{Prx1}^{+/-} \) strains and showed not only a fundamental metabolic dysfunction leading to intramyocellular lipid accumulation, but also that this could be rescued by
dietary intervention. This thesis reveals insights into the time lines for these dietary interventions, detailed metabolomics analysis, and compared a variety of treatment formulations. This then led into clinical studies that assessed qualitatively children who were self-supplemented with L-carnitine by their parents as well as a Phase 2a clinical trial for 1000mg daily levocarnitine tartrate in children with NF1 aged 8-12 years old. These data support the safety and feasibility of L-carnitine therapy and strongly suggest some functional benefit. The extent of these benefits and the broad implementation of this intervention as health policy are important goals for future work.

6. References


