



Evaluating the effect of a ketogenic diet in a Leigh syndrome mouse model

L Willemse

 **orcid.org/ 0000-0002-0802-8310**

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Promoter: Prof R Louw

Co-promoter: Dr K Terburgh

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Sole Companion

*We were born alone,
We will die alone.
Yet in life's subtlety,
You are your own quiet company.*

*There is one you, and always will be—
Unique in existence, shackled or free.
Belief in yourself is wildly underrated;
You captain your course, self-directed.*

*Pick yourself up, stumble or fall—
You'll conquer mountains, colossal or small.
A fundamental truth, a fact so true:
The first and last witness is always **You**.*

- A poem by Luciano Willemse

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ABSTRACT

Mitochondrial diseases (MDs) are a heterogeneous group of metabolic disorders with clinical manifestations resulting from impaired energy production and tissue-specific vulnerabilities. Leigh syndrome (LS), a mitochondrial disorder, is a severe paediatric neurodegenerative disorder primarily associated with complex I (CI) deficiency. CI dysfunction in LS is typically linked to mutations in nuclear DNA-encoded subunits of CI like NDUF54, which plays a critical role in CI assembly and stability. Defects in this subunit leads to mitochondrial dysfunction and neurodegeneration. With current MD treatments typically focusing on symptom management, there is an urgent need for innovative therapeutic strategies that target the underlying metabolic and mitochondrial dysfunctions. This thesis investigates the therapeutic potential of dietary interventions to alleviate mitochondrial dysfunction in LS, using the preclinical *Ndufs4* knockout (KO) mouse model, which recapitulates hallmark features of LS.

In Phase A of the study, a preliminary investigation was conducted in *Ndufs4* wild-type (WT) and heterozygous (HET) mice to evaluate the impact of a ketogenic diet (KD) on mTOR activity, redox balance, and systemic metabolism. Given the known association between alcohol consumption and redox dysregulation, chronic alcohol administration was used to simulate the redox and metabolic alterations observed in MD. Western blot analyses of liver samples revealed that the KD significantly suppressed mTOR complex 1 (mTORC1) activity, a key regulator of cellular metabolism. Metabolomic profiling using multi-platform approaches, such as LC-MS/MS, ¹H-NMR, and GC-TOF-MS, demonstrated that the KD induced significant alterations in metabolic pathways, particularly affecting amino acid metabolism and the tricarboxylic acid (TCA) cycle. Flow cytometry assessments further showed a reduction in oxidative stress markers, although prolonged alcohol exposure appeared to blunt some of the KD benefits. These findings underscored the complex interaction between dietary composition and redox homeostasis.

Building upon Phase A of the study, the dietary intervention was modified in Phase B to a high-fat diet (HFD) enriched with omega-3 (ω -3) polyunsaturated fatty acids (PUFAs) derived from fish oil. In this phase, both phenotypic and biochemical parameters were assessed in a *Ndufs4* KO mouse after dietary intervention. Phenotypic evaluations, included locomotor activity tests, clasping tests, grip strength measurements, and survival assessments. These evaluations revealed that mice fed the ω -3 PUFA-enriched HFD exhibited significant improvements in clasping symptoms and an extended lifespan compared to those on a normal diet. Biochemical analyses of whole brain tissues indicated that signalling pathways such as mTOR, PGC1- α , SIRT1 and TNF- α was unaffected by the diet. However, systemic inflammation in serum was reduced by the diet intervention as indicated by a decrease in IL-6 and TNF cytokines. Comprehensive metabolomic analyses further indicated that the HFD reprogrammed metabolic

pathways involving the TCA cycle, branched-chain amino acid metabolism, and lipid metabolism.

In conclusion, the findings of this thesis imply that dietary interventions using a PUFA-enriched HFD offers a promising, safer, and more effective strategy for mitigating LS pathology than a standard KD. The HFD may alleviate some of the metabolic impairments underlying LS through the attenuation of systemic inflammation and alteration of metabolic pathways. These results provide a compelling rationale for further investigation into dietary interventions as complementary therapeutic strategies for MDs. Future studies are needed to investigate the precise molecular mechanisms underlying this PUFA enriched HFD and to evaluate the potential for clinical translation.

Keywords: Mitochondrial diseases, Leigh syndrome, complex I deficiency, *Ndufs4* knockout mouse model, ketogenic diet, high-fat diet, polyunsaturated fatty acids, metabolic reprogramming, systemic inflammation, redox homeostasis

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LIST OF SYMBOLS AND UNITS

Symbols

*	Asterisk	©	Copyright
-/-	Homozygous knockout genotype	®	Registered
~	Approximately	♀	Female
+/-	Heterozygous knockout genotype	♂	Male
+/+	Homozygous wild-type genotype	Ø	Diameter
<	Less-than	™	Trademark
>	Greater-than	ΔpH_m	Mitochondrial pH gradient
±	Plus-minus	Δpmf	Proton motive force
×	Times	$\Delta \psi_m$	Mitochondrial membrane potential
↑	Increase	#	Number
↓	Decrease		

Units

%	Percentage	mM	Millimolar
×g	Relative centrifugal force	mol	Mole
°C	Degrees Celsius	ms	millisecond
Abs	Absorbance	ng	Nanogram
amu	Atomic mass unit	nm	Nanometre
eV	Electron volt	nmol	Nanomole
g	Gram	pH	Potential of hydrogen; negative of base 10 logarithm of H ⁺ molar concentration
h	Hours	ppm	Parts per million
Hz	Hertz	psi	Pound-force per square inch;
kb	Kilobase	sec	Seconds
kDa	Kilodaltons	V	Volt
L	Litres	v/v	Volume of solute per volume of solvent
M	Molarity; moles solute per litre of solvent	w/v	Weight of solute per volume of solvent
m/z	Mass-to-charge ratio	µg	Microgram
mg	Milligram	µL	Microlitre
min	Minutes	µM	Micromolar
mL	Millilitre		
mm	Millimetre		

LIST OF ABBREVIATIONS AND LATIN TERMS

Abbreviations

¹ H-NMR	Proton nuclear magnetic resonance
3'	3-prime end; 3-prime hydroxyl group of the polynucleotide chain
3-PBA	3-Phenylbutyric acid
5'	5-prime end; 5-prime phosphate group of the polynucleotide chain
AAV	Adeno-Associated Virus
ADP	Adenosine diphosphate
AI	Artificial Intelligence
AMDIS	Automated Mass Spectral Deconvolution and Identification System
AMP	Adenosine 5'-monophosphate
ANN	Artificial Neural Network
ANOVA	Analysis of variance
Asp	Aspartic acid
ATP	Adenosine triphosphate
ATPAF2	ATP Synthase Mitochondrial F1 Complex Assembly Factor 2
AVMA	American Veterinary Medical Association
BCAA	Branched-chain amino acid
BCKDH	Branched-chain α -keto acid dehydrogenase
BN-PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pairs
BSTFA	O-bis(trimethylsilyl)trifluoro acetamide
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
Cat.	Catalogue
CBA	Cytometric bead array
CI	Complex I; NADH: ubiquinone oxidoreductase; or confidence interval
CII	Complex II; succinate: ubiquinone oxidoreductase
CIII	Complex III; ubiquinol: ferricytochrome c oxidoreductase
CIV	Complex IV
CNS	Central nervous system
CO ₂	Carbon dioxide
CoA	Coenzyme A
Cre/loxP	Cre: the P1 gene product; loxP (locus of crossing (x) over P1): the recombination site
CS	Citrate synthase
CV	Complex V; ATP synthase; or coefficient of variance (depending on the context)
CV%	Percentage coefficient of variance
Cyt c or C	Cytochrome c
DMKG	Dimethyl ketoglutarate
DMPA	<i>N, N</i> -dimethyl-L-phenylalanine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D-value	Effect size value of practical significance
e ⁻	Electron
EC	Enzyme commission
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol-bis (β -aminoethyl ether)- <i>N,N,N',N'</i> -tetra acetic acid
EI	Electron impact
ES	Effect size
ESI	Electrospray ionisation
EtBr	Ethidium bromide
ETC	Electron transport chain
ETF	Electron-transferring flavoprotein
ETFDH	Electron-transferring-flavoprotein dehydrogenase
ETF: QO	Electron transfer flavoprotein: ubiquinone oxidoreductase
FACS	Fluorescence-Activated Cell Sorting
FAD	Flavin adenine dinucleotide
FDR	False discovery rate
Fe-S	Iron-sulphur
FMN	Flavin mononucleotide
GABA	γ -Aminobutyric acid
GC-TOF-MS	Gas chromatography time-of-flight mass spectrometry
Gln	Glutamine
Glu	Glutamate
H-strand	Heavy strand
H ⁺	Hydrogen ion; proton
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
HET	Heterozygous
HFD	High-fat diet
HIF1 α	Hypoxia inducible factor 1 alpha
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
ICV	Intracerebroventricular
ID	Identification number
IFN γ	Interferon gamma
IL-12p70	Interleukin-12 p70
IL-6	Interleukin-6
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IS	Internal standard
IV	Intravenous
KD	Ketogenic diet
KIC	α -Ketoisocaproic acid
KO	Knock-out
L-strand	Light strand
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
Leu	Leucine
LS	Leigh syndrome
Lys	Lysine
MCU	Mitochondrial calcium uniporter
MD	Mitochondrial disease
m-FDH	Mitochondrial flavoprotein dehydrogenase

mPTP	Mitochondrial permeable transition pore
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mtO ₂ ⁻	Mitochondrial superoxide
mTOR	Mammalian/Mechanistic target of rapamycin
mTORC1	Mammalian/Mechanistic target of rapamycin complex 1
n	Number of; amount
N module	NADH binding module
NAD(P) ⁺	Oxidized nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NaN ₃	Sodium azide
ND	Normal Diet
nDNA	Nuclear DNA
NDUF	NADH: ubiquinone oxidoreductase (core) subunit
NDUFAF	NADH: ubiquinone oxidoreductase complex assembly factor
NDUFS	NADH dehydrogenase (ubiquinone) iron-sulphur protein
NFκB	Nuclear factor kappa B
NH ₄ ⁺	Ammonium cation
NMN	Nicotinamide mononucleotide
NRF	National Research Foundation of South Africa
NWU	North-West University
O ₂	Oxygen
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
P	Postnatal day
P module	Proton pumping module
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCDDP	Preclinical Drug Development Platform
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pro	Proline
PUFA	Polyunsaturated fatty acid
p-value	Probability value
Q	Ubiquinone; coenzyme Q
Q module	Ubiquinone binding module
QC	Quality control
QH ₂	Ubiquinol; reduced coenzyme Q
R ²	Coefficient of determination
Redox	Reduction-oxidation
RET	Reverse electron transport
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species

rRNA	Ribosomal ribonucleic acid
RSA	Republic of South Africa
RT	Retention time
S/N	Signal-to-noise ratio
S6K1	ribosomal protein S6 kinase beta-1
SAVC	South African Veterinary Council
SC	Super complex
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SOP	Standard operating procedure
SPF	Specific pathogen-free
TCA	Tricarboxylic acid cycle
TLR	Toll-like receptor
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TNF- α	Tumour necrosis factor alpha
tRNA	Transfer ribonucleic acid
UPLC-HDMS	Ultra-performance liquid chromatography-high-definition mass spectrometry
UPRmt	Mitochondrial unfolded protein response
USA	United States of America
UV	Ultraviolet
v	Version; relating to software
VDAC	Voltage-dependent anion channel
VIP	Variable important in projection
VUmc	Vrije Universiteit (VU) university medical centre
WES	Whole exome sequencing
WT	Wild type
α KG	α -Ketoglutaric acid
β -Ala	Beta-alanine

Latin terms

<i>In vivo</i>	In life; a process within a living organism
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CHAPTER 1

Introduction

1.1. Introduction and research rationale

Mitochondrial diseases (MDs), which arise primarily from defects in the oxidative phosphorylation (OXPHOS) system, represent one of the most challenging groups of genetic metabolic disorders due to poorly understood mechanisms and a lack of curative therapies (Lake *et al.*, 2016; Mootha & Chinnery, 2018; Rahman, 2020). Complex I (CI) deficiency is the most common cause of OXPHOS defects (1 in 10 000 births) and is thus regarded as the most prevalent form of inborn errors of metabolism (Gorman *et al.*, 2015). This high prevalence is due to CI's structural complexity, which comprises 45 subunits, and its role in electron transfer, increasing its susceptibility to genetic and biochemical disruptions (Stroud *et al.*, 2016). This deficiency frequently manifests as Leigh syndrome (LS), a severe paediatric neurometabolic disorder characterised by progressive encephalopathy, neurodegeneration, and premature lethality (Ruhoy & Saneto, 2014; Sofou *et al.*, 2014). This syndrome is the most prevalent paediatric presentation of MD, affecting approximately 1 in 40 000 live births (Lim & McFarland, 2019). The *Ndufs4* knockout (KO) mouse, a validated model of CI deficiency, recapitulates key LS features, including fatal encephalomyopathy and region-specific brain lesions, like the brainstem and basal ganglia, making it a valuable tool for studying disease progression and therapeutic interventions (Esterhuizen *et al.*, 2017; Kruse *et al.*, 2008; Van De Wal *et al.*, 2022).

Primary diagnostic approaches for MD include genetic testing like whole-exome sequencing and biochemical assays that measure enzyme activity in fibroblasts or tissue biopsies (Lake *et al.*, 2016; Rahman, 2020). However, the heterogeneity of MD, which is driven by over 350 associated genes, manifest in diverse clinical presentations and overlapping symptoms with other neurometabolic disorders. This complicates efficient and robust diagnosis and may exacerbate underdiagnosis and misdiagnosis. These diagnostic challenges further complicate the timely identification of MDs, emphasising the need for continuous research into novel therapeutic strategies. Currently, there is no curative treatment for MD, management primarily focuses on symptom relief using vitamins, antioxidants or palliative care (Lim & McFarland, 2019; Parikh *et al.*, 2015). Therapeutic approaches often include vitamin and nutraceutical supplementation, such as CoQ10 and thiamine (Iqbal *et al.*, 2022; Maksoud *et al.*, 2021; Mantle & Hargreaves, 2022). However, these strategies have shown limited efficacy due to poor bioavailability and symptom-specific targeting (Parikh *et al.*, 2017). Nonetheless, they remain attractive therapeutic options because they are non-invasive, cost-effective, and low-risk compared to pharmacological, gene therapy and mitochondrial transplantation-based approaches (Aldossary *et al.*, 2022; El-Hattab *et al.*, 2017; van de Wal *et al.*, 2022). Furthermore, various novel therapeutic strategies have recently been developed and evaluated, with others still under investigation. One such therapeutic approach includes pharmacological therapies like rapamycin, a potent inhibitor of the mammalian/mechanistic target of rapamycin (mTOR) that influences energy metabolism, which has shown promise in the treatment of LS in

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the *Ndufs4* KO mouse model (Johnson *et al.*, 2013; van de Wal *et al.*, 2022). However, this treatment approach faces translational barriers as it may lead to potential off-target mTOR effects and immunosuppression risks (Barriocanal-Casado *et al.*, 2019). Additionally, gene therapy and mitochondrial transplantation have shown promise as therapeutic strategies due to their potential to improve mitochondrial function and alleviate disease symptoms in various MD models (Corrà *et al.*, 2022; Di Donfrancesc *et al.*, 2022; Ulger *et al.*, 2022). However, these approaches are still in their experimental phases and may hold potential safety and efficacy concerns (Gorman *et al.*, 2016; Di Donfrancesc *et al.*, 2022).

Dietary interventions such as the ketogenic diet (KD), defined as a high-fat, low carbohydrate diet, have emerged as promising, affordable, non-invasive, and safe treatment options for MD. The KD has shown potential to enhance mitochondrial biogenesis, inhibit mTOR activity, and improve the redox balance through ketone body metabolism (El-Hattab *et al.*, 2017; McDaniel *et al.*, 2011; Poff *et al.*, 2017). As such, the KD may potentially address multiple pathomechanisms of LS, including NADH/NAD⁺ imbalance and bioenergetic failure. This diet has demonstrated neuroprotective effects in various MD models, such as *Surf1*^{-/-}, mitochondrial myopathy rodents and patients, as well as other neurodegenerative disorders like Alzheimer's and Parkinson's disease (Ahola-Erkkilä *et al.*, 2010; Ahola *et al.*, 2016; Jiang *et al.*, 2022; Lee & Chiang, 2021; Zweers *et al.*, 2021). In these models, the KD was successful in improving seizure control and reducing inflammation. These findings underscore the therapeutic potential of dietary interventions like the KD as a treatment option for MD. However, the application of a KD in LS models, such as the *Ndufs4* KO mice model, remains to be investigated.

In this study, we initially conducted a preliminary investigation to assess the effect of a KD combined with chronic alcohol administration, a known redox disruptor, in healthy *Ndufs4* wild-type/heterozygous (WT/HET) mice. Alcohol was chosen to mimic LS-associated features, such as metabolic dysregulation and NADH/NAD⁺ imbalance due to its well described disruption of redox homeostasis and mitochondrial function (Feldstein & Bailey, 2011; Razvodovsky *et al.*, 2009; Zakhari, 2013). Evaluating the KD in WT/HET mice under alcohol-induced metabolic stress provided a proof-of-concept model to evaluate diet's efficacy during controlled redox disruption. Building on these findings, we then evaluated the KD's effect in a validated preclinical LS mouse model using *Ndufs4* KO mice (Kruse *et al.*, 2008; van de Wal *et al.*, 2022). Accordingly, the study and thesis were divided into two phases: Phase A focussed on assessing a KD's ability to mitigate alcohol-induced redox stress, providing insight for Phase B's focus on LS-specific pathophysiology in *Ndufs4* KO mice. We hypothesised that a KD could alleviate metabolic and redox dysregulation in both models, in both phases, offering potential translational benefits for LS patients.

1.2. Aim and objectives

1.2.1. Aim

This study aimed to evaluate the therapeutic potential of a ketogenic diet in the *Ndufs4* knockout mouse model of Leigh syndrome.

1.2.2. Objectives

To achieve the overarching research aim, the objectives were structured into two phases: Phase A, evaluating the effects of a KD in *Ndufs4* WT and HET mice, and Phase B, investigating the impact of a PUFA-enriched high-fat diet (HFD) in *Ndufs4* KO and WT mice.

1.2.2.1. Phase A: Evaluate the effects of a KD in alcohol-exposed *Ndufs4* WT/HET mice

1. Animal breeding, genotyping, and selection:
 - 1.1 Interbreeding of *Ndufs4* HET mice to obtain WT and HET mice.
 - 1.2 Confirm the genotype (WT, HET, or KO) of offspring at postnatal day (P) 21 using PCR analysis.
2. Dietary intervention:
 - 2.1 Randomly assign WT and HET mice to four experimental groups: either a normal diet (ND) or a KD, with or without alcohol administration.
 - 2.2 Initiate dietary intervention at P23/P24 and maintain it until P47.
 - 2.3 Euthanize mice at P47 and collect urine, blood, and liver samples for downstream analyses.
3. Evaluation of mTORC1 activity and urinary metabolism:
 - 3.1 Assess hepatic mTORC1 activity by measuring the S6K1 phosphorylation status using Western blot analysis.
 - 3.2 Conduct metabolomic profiling of urine samples using a multi-platform approach, including LC-MS/MS, ¹H-NMR, and GC-TOF-MS.
4. Assessment of systemic inflammation:
 - 4.1 Quantify inflammatory markers in serum using the CBA Mouse Inflammation Kit and flow cytometry.
5. Comparative analysis of experimental outcomes:
 - 5.1 Perform data preprocessing, normalisation, and quality control procedures.
 - 5.2 Determine the cellular redox status by assessing redox-related metabolic indicators through integrating findings from metabolomics analyses.

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5.3 Conduct statistical analyses to identify significant metabolic and molecular alterations.

5.4 Interpret findings using pathway enrichment analysis and relevant literature to elucidate potential mechanisms.

1.2.2.2. *Phase B: Evaluation of the therapeutic effect of a PUFA-supplemented HFD in *Ndufs4* KO mice*

1. Animal breeding, genotyping, and selection:
 - 1.1 Interbreeding of *Ndufs4* HET mice to obtain WT and KO mice
 - 1.2 Confirm the genotype (WT, HET, or KO) of offspring at P21 via PCR analysis.
2. Dietary intervention:
 - 2.1 Randomly assign KO mice for phenotypic assessments into two groups: KO mice on either a ND or an HFD.
 - 2.2 Randomly assign mice for biochemical evaluation into four groups: WT on either an ND or HFD, and KO mice on either an ND or HFD.
 - 2.3 Maintain dietary intervention until natural death for phenotypic assessments and for three weeks in biochemical evaluation groups.
3. Phenotypic characterisation of *Ndufs4* KO mice:
 - 3.1 Monitor body weight and growth patterns, visualised through growth curve analysis.
 - 3.2 Assess survival by determining lifespan following dietary intervention.
 - 3.3 Evaluate disease severity using routine clasping tests.
 - 3.4 Assess locomotor function using the open-field test.
 - 3.5 Measure grip strength via the wire-grid hang test.
4. Molecular and inflammatory pathway analysis:
 - 4.1 Evaluate the effects of ND and HFD on key signalling pathways, including mTORC1, SIRT1, PGC-1 α and TNF- α , using Western blot analysis.
 - 4.2 Quantify systemic inflammation via inflammatory marker analysis in serum using the CBA Mouse Inflammation Kit and flow cytometry.
5. Metabolomic and lipidomic analysis of brain tissue:
 - 5.1 Perform targeted metabolomics on redox-related metabolites using GC-MS/MS.
 - 5.2 Conduct untargeted metabolomics using GC-TOF-MS.
 - 5.3 Assess brain lipidomic alterations via UPLC-HDMS.

6. Comparative analysis of phenotypic and biochemical findings:
 - 6.1 Perform data preprocessing, normalisation, and quality control procedures.
 - 6.2 Conduct statistical analyses to identify significant phenotypic, metabolic, and molecular alterations between groups.
 - 6.3 Interpret findings using pathway enrichment analysis and existing literature to elucidate mechanistic insights.

1.3. Research design

This study is a single project conducted at North-West University (NWU), utilising HET B6.129S4-*Ndufs4*^{tm1.1Rpa}/J mice, which were interbred to obtain homozygous *Ndufs4* KO, WT, and HET mice. Tail snips were collected for PCR-based genotyping to confirm their genotype. The study was divided into Phase A and Phase B, with the research design illustrated in Figure 1-1 and discussed in detail below.

In Phase A, *Ndufs4* WT and HET mice (n = 10 per dietary group) underwent an overnight fast in individual metabolic cages to facilitate the collection of 12-hour urine (U) samples following the three-week dietary intervention. Afterward, the mice were euthanized, before serum and liver tissue were collected. Liver tissue was immediately snap-frozen in liquid nitrogen, while serum was snap-frozen after isolation from the blood.

For Phase B of the study, the KD was modified to a HFD enriched with PUFAs after novel literature indicated that *Ndufs4* KO mice can't tolerate a full KD (Bornstein *et al.*, 2022). This part of the study focused on the phenotypic investigation of *Ndufs4* KO mice (n = 12 per dietary group; ND and HFD) throughout the dietary intervention until natural death. Additionally, KO (n = 14) and WT (n = 14) mice per dietary group were included for biochemical analyses post-intervention. These mice were euthanized before serum and brain tissue were collected.

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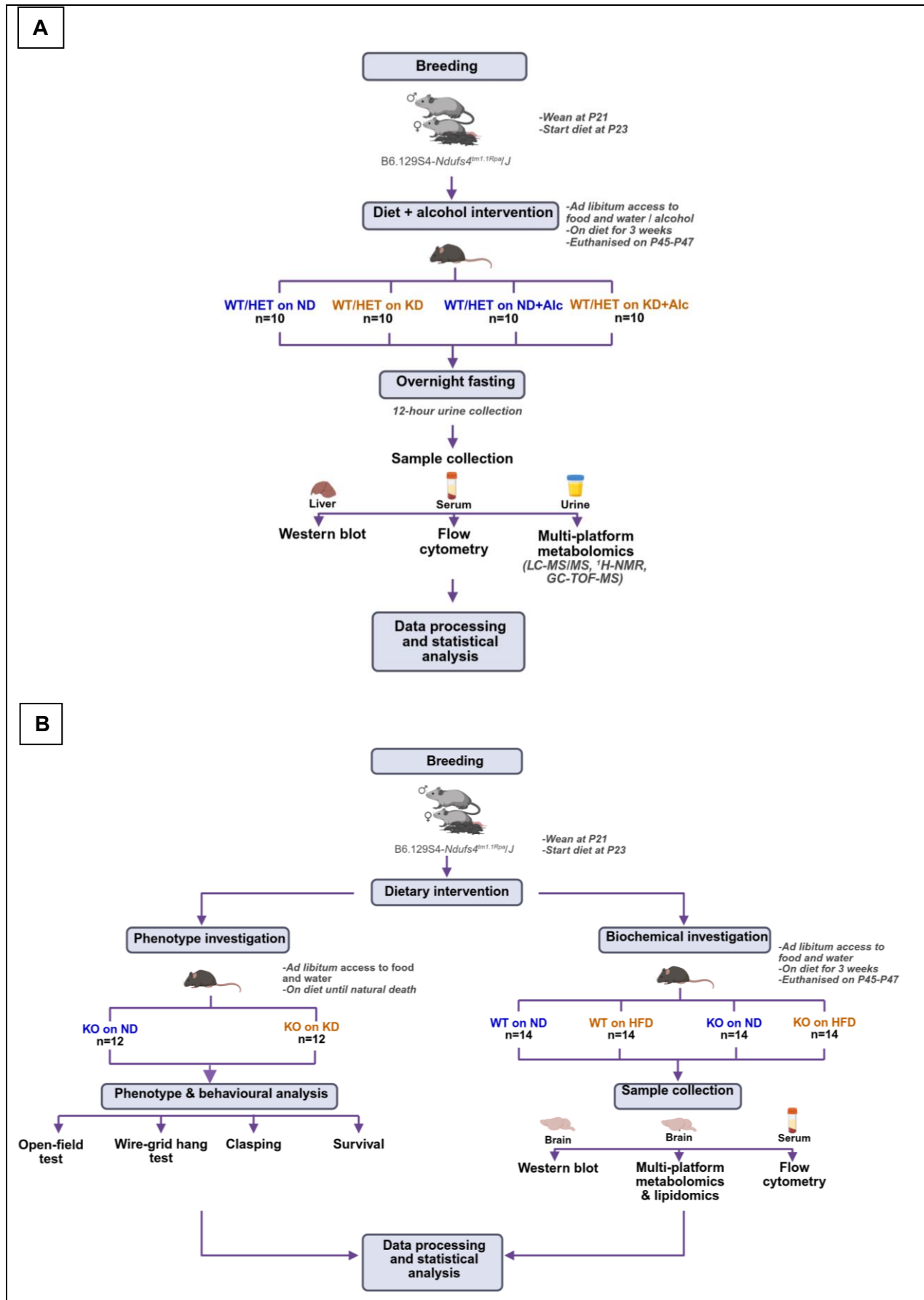


Figure 1-1: Schematic illustration of the research design for (A) Phase A and (B) Phase B.
Abbreviations: WT, wild type; HET, heterozygous; KO, knock-out; ND, normal diet; KD, ketogenic diet;

HFD, high-fat diet; LC-MS/MS, liquid chromatography tandem mass spectrometry; gas chromatography time-of-flight mass spectrometry; ¹H-NMR, proton nuclear magnetic resonance.

1.4. Thesis structure

This thesis follows the chapter format in accordance with NWU guidelines and is structured into six chapters, each with its own reference list. The study's findings (Chapters 4 and 5) are presented in their published form, with minor style and formatting adjustments to align with the thesis requirements. These adjustments include modifying the referencing style, updating table and figure numbers, and converting American English to British English. Declarations from the articles are not included in these chapters. Supplementary information from the published papers can be found in the Appendix.

Chapter 1 outlines the research rationale, aim, objectives, and study design. It also provides an overview of the thesis structure, followed by details on research outcomes, ethical approval, financial support, as well as author and co-author contributions.

Chapter 2 presents a literature review on key topics relevant to this thesis. It covers mitochondrial structure and function, MD, CI deficiency-related LS, the *Ndufs4* KO mouse model, as well as diagnostic and treatment approaches for MD and NDUF54-related CI deficiency LS.

Chapter 3 details the materials and methods used in this study, covering animal handling, sampling, genotyping, dietary composition and intervention, phenotypic assessments, SDS-PAGE and Western blotting, flow cytometry, multi-platform metabolomics, lipidomics, data processing, and statistical analyses.

Chapter 4 provides the first peer-reviewed paper from this study, published in *Metabolomics*. This paper presents the molecular and metabolic data from Phase A, focusing on *Ndufs4* WT and HET mice on the different dietary protocols. It also explores the hypothesis that a KD may mitigate some of the redox and metabolic consequences of chronic alcohol consumption.

Chapter 5 presents the second peer-reviewed paper from this study, published in *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease*. This paper investigates the phenotypic, behavioural, molecular, and metabolic differences between *Ndufs4* KO and WT mice under different dietary protocols. It explores systemic inflammation, mechanistic pathways, and metabolic alterations, highlighting the therapeutic potential of a PUFA-enriched HFD in enhancing survival, reducing disease severity, and alleviating systemic inflammation. Additionally, it demonstrates the metabolic rescue potential of a PUFA-enriched HFD in the *Ndufs4* mouse model of LS.

Chapter 6 summarises the research findings and presents the concluding remarks. It concludes with a discussion of the study's strengths, limitations, and future research directions.

Appendices A–F contain the genotyping results, full published articles resulting from this study, along with their online supplementary information. The language editing certificate, a requirement from the NWU, is attached as Appendix F, concluding the thesis.

1.5. Research outputs

This study made significant contributions to the field of natural sciences and MD research through the publication of two peer-reviewed articles in high-impact journals. Additionally, it included three presentations at national and international conferences, along with a 3-minute presentation at the FameLab South Africa (SA) 2024 regionals, national semi-final and national final.

1.5.1. Published peer-reviewed article I (Chapter 4, Appendix B)

Willemse L., Terburgh K. & Louw R. 2023. A ketogenic diet alters mTOR activity, systemic metabolism and potentially prevents collagen degradation associated with chronic alcohol consumption in mice. *Metabolomics*, 19(5):43

The article (<https://doi.org/10.1007/s11306-023-02006-w>) was published in the journal: *Metabolomics* (<https://link.springer.com/journal/11306>) with an impact factor of 3.5 (2023). *Metabolomics* is a peer-reviewed journal that focuses on the study of metabolite profiles and their applications in understanding biological systems, disease mechanisms, and various environmental and clinical conditions. Author guidelines can be accessed at: <https://www.springer.com/journal/11306/submission-guidelines#Instructions%20for%20Authors>

1.5.2. Published peer-reviewed article II (Chapter 5, Appendix D)

Willemse L., Terburgh K. & Louw R. 2025. The therapeutic potential of a polyunsaturated fatty acid-enriched high-fat diet in Leigh syndrome: Insights from a preclinical model. *Biochim. Biophys. Acta Mol. Basis Dis.*, 1871(6):167873.

This article (<https://doi.org/10.1016/j.bbadis.2025.167873>) was published in the journal: *Biochimica et Biophysica Acta – Molecular Basis of Disease* (<https://www.journals.elsevier.com/bbamolecular-basis-of-disease>) with an impact factor of 4.2 (2024). *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease* publishes research on the molecular and cellular mechanisms underlying various diseases, focusing on the biochemistry, biophysics, and molecular biology of disease processes. The author guidelines can be accessed at: <https://www.elsevier.com/journals/biochimica-et-biophysica-acta-molecular-basisof-disease/0925-4439/guide-for-authors>

1.5.3. Additional scientific contributions

1.5.3.1. Conferences (presented by the author underlined in the reference)

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Willemse L., Terburgh K. & Louw R. 2022, 23-26 January. Metabolic consequences of the inhibition of the mammalian target of rapamycin complex in mice fed a ketogenic diet and ethanol. *Poster presentation*. 27th Virtual Congress of the South African Society for Biochemistry and Molecular Biology 2022 (SASBMB 2022), Online.

Willemse L., Terburgh K. & Louw R. 2024, 7-10 July. Evaluating the effect of a high ω -3 ketogenic diet on survival, mTOR activity, inflammation, and systemic metabolism in a Leigh syndrome mouse model. *Oral presentation*. 28th SASBMB Congress 2024, Polokwane, South Africa.

Willemse L., Terburgh K. & Louw R. 2024, 16-18 October. A ketogenic diet enriched with omega-3 fatty acids alleviates primary mitochondrial disease in mice. *Poster presentation*. 10th Bordeaux Neurocampus International Conference 2024, Bordeaux, France.

Willemse L., Terburgh K. & Louw R. 2025, 13-14 January. Evaluating the effect of a ketogenic diet in a Leigh syndrome mouse model. *Oral presentation*. Workshop: Mitochondrial disease research in South Africa: from gene editing to gene therapy, Potchefstroom, South Africa.

1.5.3.2. Competitions (presented by the author underlined in the reference)

Willemse L., Terburgh K. & Louw R. 2024, 19 April. Evaluating the effect of a diet high in fish oil in a neurodegenerative mouse model. *3-minute oral presentation*. FameLab SA 2024 Regional Heat, Online.

Willemse L., Terburgh K. & Louw R. 2024, 10 September. Evaluating the effect of a diet high in fish oil in a neurodegenerative mouse model. *3-minute oral presentation*. FameLab SA 2024 National Semi-final, Online.

Willemse L., Terburgh K. & Louw R. 2024, 19 September. Evaluating the effect of a diet high in fish oil in a neurodegenerative mouse model. *3-minute oral presentation*. FameLab SA 2024 National Final, Pretoria, South Africa.

1.6. Declarations

1.6.1. Ethics approval

Ethics approval for this study (NWU-00516-20-A5) was obtained from the AnimCare animal research ethics committee of NWU and complied with national legislation.

1.6.2. Financial support

This research was funded by the National Research Foundation of South Africa (NRF, Grant No. 120829 and North-West University [NWU]). The views and conclusions presented in this work are those of the authors and do not necessarily reflect those of the NRF or NWU.

1.6.3. Author contributions

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Data Collection: The datasets used in this study were generated by L. Willemse. The author was responsible for the ethics application, sample collection, genotyping, animal handling, phenotypic and behavioural assessments, as well as all the analytical work.

Images and figures: All images and figures in this thesis were created by the author unless indicated otherwise.

Article I presented in Chapter 4:

L. Willemse conceptualised the research and designed the methodology with R. Louw, as well as conducting the investigation, performing experimental analysis, processing and curating the data, and writing the original draft. L. Willemse also contributed to data visualisation. R. Louw supervised the project, managed administration, secured funding, and contributed to visualisation, and manuscript review and editing. K. Terburgh curated data and contributed to manuscript review and editing.

Article II presented in Chapter 5:

L. Willemse along with R. Louw designed the research plan and methodology. L. Willemse performed formal analysis, performed the data processing, curated the data, and wrote the original draft. L. Willemse also contributed to data visualisation. R. Louw supervised the project, managed administration, secured funding, data visualisation, and manuscript review and editing. K. Terburgh curated the data and contributed to manuscript review and editing.

Additional contributions:

As part of the PhD program, L. Willemse contributed to additional research beyond the scope of this thesis. This included laboratory work for the Rooibos study conducted by the Cape Peninsula University of Technology (CPUT) in collaboration with the NWU. For this study, L. Willemse was responsible for the randomisation, preparation, and analysis of baseline and Rooibos-treated human plasma samples (n = 520) using LC-MS/MS and GC-TOF-MS, along with quality control samples (n = 162 for LC-MS/MS and n = 90 for GC-TOF-MS). This work involved experimental analysis, data alignment, and pre-treatment. Additionally, L. Willemse contributed to the writing and editing of a non-peer-reviewed, public science communication article titled “*Nurturing Nutrition*” (https://www.linkedin.com/feed/update/urn:li:activity:7296179354261176320?utm_source=share&utm_medium=member_desktop&rcm=ACoAAC3pDeoBNdZgAYdJhAFNd1dei0dNxfqY0rI) for the International Coalition for Cannabis Education (ICEC), a non-governmental organisation (NGO) and non-profit organisation (NPO). This was an expert-panelled short article as part of an international NGO/NPO education outreach on lipid-related dietary health.

All authors involved signed the declaration on this page:

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As a co-author/researcher, I hereby approve and give consent that the above-mentioned articles and data can be used for the Ph.D. of L. Willemse. I declare that my role in the study, as indicated above, is a representation of my actual contribution.

				
Willemse, L.		Terburgh, K.		Louw, R.

1.7. References

- Ahola-Erkkilä, S., Carroll, C.J., Peltola-Mjösund, K., Tulkki, V., Mattila, I., Seppänen-Laakso, T., ... Suomalainen, A. 2010. Ketogenic diet slows down mitochondrial myopathy progression in mice. *Hum. Mol. Genet.*, 19(10):1974-1984.
- Ahola, S., Auranen, M., Isohanni, P., Niemisalo, S., Urho, N., Buzkova, J., ... Muurinen, T. 2016. Modified Atkins diet induces subacute selective ragged-red-fiber lysis in mitochondrial myopathy patients. *Mol. Med.*, 8(11):1234-1247.
- Aldossary, A.M., Tawfik, E.A., Alomary, M.N., Alsudir, S.A., Alfahad, A.J., Alshehri, A.A., ... Alzaydi, M.M. 2022. Recent advances in mitochondrial diseases: From molecular insights to therapeutic perspectives. *Saudi Pharm. J.*, 30(8):1065-1078.
- Barriocanal-Casado, E., Hidalgo-Gutiérrez, A., Raimundo, N., González-García, P., Acuña-Castroviejo, D., Escames, G. & López, L.C. 2019. Rapamycin administration is not a valid therapeutic strategy for every case of mitochondrial disease. *eBioMedicine*, 42:511-523.
- Bornstein, R., James, K., Stokes, J., Park, K.Y., Kayser, E.-B., Snell, J., ... Johnson, S.C. 2022. Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy. *Neurobiol. Dis.*, 163:105594.
- Corrà, S., Cerutti, R., Balmaceda, V., Viscomi, C. & Zeviani, M. 2022. Double administration of self-complementary AAV9 NDUF54 prevents Leigh disease in *Ndufs4*^{-/-} mice. *Brain*, 145(10):3405-3414.
- Di Donfrancesco, A., Massaro, G., Di Meo, I., Tiranti, V., Bottani, E. & Brunetti, D. 2022. Gene therapy for mitochondrial diseases: Current status and future perspective. *Pharmaceutics*, 14(6):1287.
- El-Hattab, A.W., Zarante, A.M., Almannai, M. & Scaglia, F. 2017. Therapies for mitochondrial diseases and current clinical trials. *Mol. Genet. Metab.*, 122(3):1-9.
- Esterhuizen, K., Van der Westhuizen, F.H. & Louw, R. 2017. Metabolomics of mitochondrial disease. *Mitochondrion*, 35:97-110.
- Feldstein, A.E. & Bailey, S.M. 2011. Emerging role of redox dysregulation in alcoholic and nonalcoholic fatty liver disease. *Antioxid. Redox Signal.*, 15(2):421-424.
- Gorman, G.S., Schaefer, A.M., Ng, Y., Gomez, N., Blakely, E.L., Alston, C.L., ... Chinnery, P.F. 2015. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Ann. Neurol.*, 77(5):753-759.
- Iqbal, S., Shah, M.A., Rasul, A., Shah, S., Shah, G.M., Irfan, M., ... Althobaiti, N.A. 2022. Therapeutic role of nutraceuticals in mitochondrial disorders. *Role. Phytonutr. Metab. Disord.*, 313-358.
- Jiang, Z., Yin, X., Wang, M., Chen, T., Wang, Y., Gao, Z. & Wang, Z. 2022. Effects of Ketogenic Diet on Neuroinflammation in Neurodegenerative Diseases. *Aging Dis.*, 13(4):1146-1165.

CHAPTER 1

- Johnson, S.C., Yanos, M.E., Kayser, E.-B., Quintana, A., Sangesland, M., Castanza, A., ... Kaeberlein, M. 2013. mTOR Inhibition Alleviates Mitochondrial Disease in a Mouse Model of Leigh Syndrome. *Science*, 342(6165):1524-1528.
- Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab.*, 7(4):312-320.
- Lake, N.J., Compton, A.G., Rahman, S. & Thorburn, D.R. 2016. Leigh syndrome: one disorder, more than 75 monogenic causes. *Ann. Neurol.*, 79(2):190-203.
- Lee, I.-C. & Chiang, K.-L. 2021. Clinical diagnosis and treatment of leigh syndrome based on SURF1: genotype and phenotype. *Antioxidants*, 10(12):1950.
- Lim, A.Z. & McFarland, R. 2019. Leigh Syndrome. *Diagnosis and Management of Mitochondrial Disorders*:151-167.
- Maksoud, R., Balinas, C., Holden, S., Cabanas, H., Staines, D. & Marshall-Gradisnik, S. 2021. A systematic review of nutraceutical interventions for mitochondrial dysfunctions in myalgic encephalomyelitis/chronic fatigue syndrome. *J. Transl. Med.*, 19:1-11.
- Mantle, D. & Hargreaves, I.P. 2022. Mitochondrial dysfunction and neurodegenerative disorders: role of nutritional supplementation. *Int. J. Mol. Sci.*, 23(20):12603.
- Mootha, V.K. & Chinnery, P.F. 2018. Oxygen in mitochondrial disease: can there be too much of a good thing? *J. Inherit. Metab. Dis.*, 41:761-763.
- Parikh, S., Goldstein, A., Karaa, A., Koenig, M.K., Anselm, I., Brunel-Guitton, C., ... Enns, G.M. 2017. Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet. Med.*, 19(12):1380-1380.
- Parikh, S., Goldstein, A., Koenig, M.K., Scaglia, F., Enns, G.M., Saneto, R., ... DiMauro, S. 2015. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet. Med.*, 17(9):689-701.
- Poff, A.M., Koutnik, A.P. & Egan, B. 2020. Nutritional ketosis with ketogenic diets or exogenous ketones: features, convergence, and divergence. *Curr. Sports Med. Rep.*, 19(7):251-259.
- Rahman, S. 2020. Mitochondrial disease in children. *J. Intern. Med.*, 287(6):609-633.
- Razvodovsky, Y.E., Smirnov, V.Y. & Doroshenko, E.M. 2009. Effect of amino acids compositions on the level of free amino acids of the liver under chronic alcohol intoxication. *Health & Ecol. Issues*, (4):126-131.
- Ruhoy, I.S. & Saneto, R.P. 2014. The genetics of Leigh syndrome and its implications for clinical practice and risk management. *Appl. Clin. Genet.*, :221-234.
- Sofou, K., De Coo, I.F., Isohanni, P., Ostergaard, E., Naess, K., De Meirleir, L., ... Lönnqvist, T. 2014. A multicenter study on Leigh syndrome: disease course and predictors of survival. *Orphanet J. Rare Dis.*, 9:1-16.
- Stroud, D.A., Surgenor, E.E., Formosa, L.E., Reljic, B., Frazier, A.E., Dibley, M.G., ... Thorburn, D.R. 2016. Accessory subunits are integral for assembly and function of human mitochondrial complex I. *Nature*, 538(7623):123-126.
- Ulger, O. & Kubat, G.B. 2022. Therapeutic applications of mitochondrial transplantation. *Biochimie*, 195:1-15.
- Van De Wal, M.A., Adjobo-Hermans, M.J., Keijer, J., Schirris, T.J., Homberg, J.R., Wieckowski, M.R., ... Quintana, A. 2022. Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention. *Brain*, 145(1):45-63.
- Zakhari, S. 2013. Alcohol metabolism and epigenetics changes. *Alcohol Res.*, 35(1):6.

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CHAPTER 2

Literature review

2.1. Introduction

This chapter offers a comprehensive overview of the key concepts underpinning the thesis. It begins with a generalised introduction to mitochondria and their essential components, emphasising the oxidative phosphorylation (OXPHOS) system, particularly the first enzyme complex (CI) and the NDUFS4 subunit of CI. The discussion then transitions to mitochondrial dysfunction and the associated diseases that emerge from such dysfunction. Initially, it presents general characteristics and pathophysiology of mitochondrial diseases (MD), before narrowing the focus to the central themes of the study: CI dysfunction, NDUFS4 deficiency, and Leigh syndrome (LS). Subsequently, the chapter provides an extensive overview of the whole-body *Ndufs4* knockout (KO) mouse model of CI deficiency, highlighting potential therapeutic interventions, including pharmacological, nutraceutical, and dietary strategies.

2.2. Mitochondrial biology

2.2.1. Structure and dynamics

Mitochondria are double-membrane-bound organelles comprising an outer mitochondrial membrane (OMM), a folded inner mitochondrial membrane (IMM), an intermembrane space (IMS), and the mitochondrial matrix (Mannella, 2006; Vasava & Mashiyava, 2016; Chen *et al.*, 2023). The OMM is permeable to small molecules due to voltage-dependent anion channels (VDACs) and serves as a hub for signalling interactions with the cytosol. In contrast, the IMM is impermeable and folded into cristae, which house the electron transport chain (ETC) complexes that are essential for oxidative phosphorylation (OXPHOS) (Nunnari & Suomalainen, 2012; Prasai, 2017; Ježek *et al.*, 2023; Rahman & Rahman, 2018). The matrix contains enzymes for the tricarboxylic acid (TCA) cycle, fatty acid oxidation, and mtDNA replication, making it the biochemical hub (Figure 2-1) for metabolic, catabolic and anabolic reactions (Nunnari & Suomalainen, 2012; Hong *et al.*, 2024; Rath *et al.*, 2021).

Mitochondria undergo continuous processes of fusion and fission, which are regulated by dynamin-related GTPases, such as MFN1/2 (fusion) and Drp1 (fission) (Nunnari & Suomalainen, 2012). Fusion enables content mixing and repair, while fission facilitates mitochondrial turnover, transport, and the separation of damaged organelle components. Damaged mitochondria are removed through mitophagy, a selective autophagy process involving PINK1/Parkin signalling (Quinn *et al.*, 2020; Rüb *et al.*, 2017; Song *et al.*, 2022; Zhu *et al.*, 2022). These processes ensure mitochondrial quality control and adaptability for various metabolic demands.

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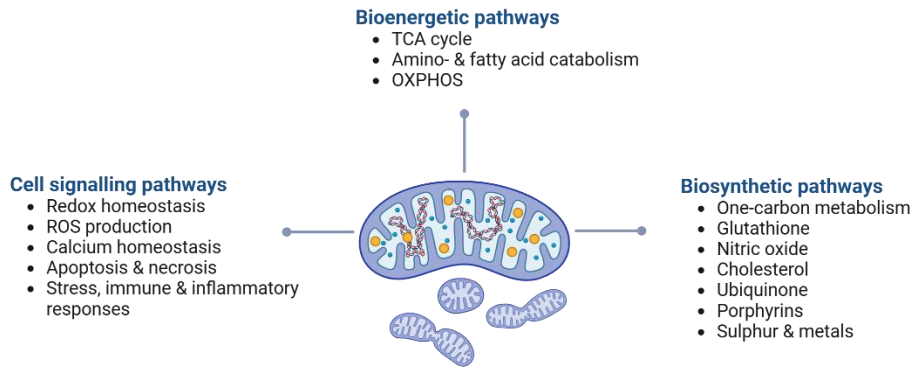


Figure 2-1: Mitochondrion roles and functions. Mitochondria serve as the cell's bioenergetic and metabolic hubs, playing central roles in both anabolic and catabolic processes. Beyond energy production, they actively monitor and regulate cellular health by coordinating with other subcellular compartments to maintain homeostasis and functionality. *Abbreviations: OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid.*

2.2.2. Mitochondrial genetics and regulation

Mitochondria possess their own double-stranded circular mitochondrial DNA (mtDNA), which is ~16.5 kb in length and consists of 13 OXPHOS subunits, 2 rRNAs, and 22 tRNAs (Chinnery & Hudson, 2013; Rath *et al.*, 2021; Ferreira & Rodriguez, 2024). The two strands of mtDNA are distinguished as heavy strand (H-strand) and light strand (L-strand), and the H-strand contains most of the protein-coding genes, while the L-strand encodes a small number of tRNAs (Rath *et al.*, 2021; Ferreira & Rodriguez, 2024). Unlike nuclear DNA (nDNA), mtDNA lacks introns and histones, is maternally inherited, and is structured as nucleoids attached to the IMM. The displacement loop (D-loop), a noncoding region of mtDNA, contributes to transcription and replication regulation by its control elements, such as the heavy- and light-strand promoters [HSP1, HSP2, and LSP] (Basu *et al.*, 2020). Over 99% of mitochondrial proteins, including those for mtDNA replication, transcription, and repair, are nuclear-encoded, highlighting the organelle's dependence on nDNA (Calvo *et al.*, 2016; Ferreira & Rodriguez, 2024). Tissue-specific mtDNA copy numbers (e.g., higher in energy-demanding cells like cardiomyocytes) optimise OXPHOS capacity (Dickinson *et al.*, 2013; Greaves *et al.*, 2012; Hernández, 2023).

Mitochondrial function is regulated at genetic, transcriptional, and post-translational levels. Nuclear-mitochondrial interaction, termed mitonuclear communication, involves retrograde (mitochondria-to-nucleus) and anterograde (nucleus-to-mitochondria) signalling. Among the primary regulators are peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), a master coactivator of mitochondrial biogenesis, and AMPK, a sensor of cellular energy status and an inducer of catabolic programs (Rath *et al.*, 2021). Stress responses, such as the mitochondrial unfolded protein response (UPR_{mt}), preserve proteostasis during dysfunction, while post-translational modifications such as phosphorylation and acetylation control enzyme activity (Naresh & Haynes, 2019).

2.3. The OXPHOS system

2.3.1. Electron transport chain

The mitochondrial oxidative phosphorylation (OXPHOS) system comprises five multi-subunit complexes, namely complex I (CI, NADH-ubiquinone oxidoreductase), complex II (CII, succinate-ubiquinone oxidoreductase), complex III (CIII, ubiquinol-cytochrome c oxidoreductase), complex IV (CIV, cytochrome c oxidase) and complex V (CV, F1F0-ATP synthase). The complexes are embedded in the IMM, working in concert with mobile electron carriers, ubiquinone (Q) and cytochrome c (cyt c), to generate ATP (Hatefi, 1985; Koopman *et al.*, 2013; Fernández-Vizarra *et al.*, 2022). Electrons derived from NADH or FADH₂ (produced via glycolysis, fatty acid β -oxidation, and the TCA cycle) enter the ETC and flow through CI or CII, respectively, to Q, forming ubiquinol (QH₂) (McDonald *et al.*, 2018; Zhao *et al.*, 2019). QH₂ transfers electrons to CIII, which transports them to cyt c for transfer to CIV, where molecular oxygen is reduced to water (Blaza *et al.*, 2014; Zhao *et al.*, 2019). This electron flux drives proton pumping across the IMM, creating an electrochemical gradient (Δpmf) consisting of the mitochondrial membrane potential ($\Delta\Psi_m$) and the proton concentration gradient (ΔpH) that powers ATP synthesis by CV. This Δpmf is also crucial for the regulation of other critical processes, such as calcium buffering and ROS homeostasis (Koopman *et al.*, 2013; Zhao *et al.*, 2019).

Contrary to the "random collision" model, OXPHOS complexes assemble into super complexes (SCs) such as the respirasome (CI+CIII₂+CIV₁₋₄), which enhance electron transfer efficiency, reduce ROS leakage, and stabilise individual complexes (Boekema & Braun, 2007; Lobo-Jarne & Ugalde, 2018; Milenkovic *et al.*, 2017; Zhao *et al.*, 2019). Cardiolipin, an IMM-specific phospholipid, is critical for SC formation and structural integrity (Arnarez *et al.*, 2016; Fernández-Vizarra *et al.*, 2022).

2.3.2. Complex I

CI is the largest of the OXPHOS enzymes (~980 kDa); it is an L-shaped protein with a hydrophilic matrix arm and a hydrophobic membrane arm. It is composed of 45 subunits (14 conserved catalytic core subunits and 31 accessory subunits) that are organised into three functional modules (Formosa *et al.*, 2018). These modules (Figure 2-2) include the N-module, which catalyses NADH oxidation through flavin mononucleotide (FMN) and Fe-S clusters, the Q-module, which reduces ubiquinone using electrons from the N-module, and the P-module, which translocates 4 H⁺ per NADH oxidised, contributing ~40% of Δpmf (Fiedorczuk *et al.*, 2016; Formosa *et al.*, 2018; Hoefs *et al.*, 2012; Papa *et al.*, 2010). CI assembly is a systematic process that requires chaperones such as NDUFAF1-6 and Fe-S clusters. Mutations in assembly factors, such as NDUFS4 and NDUFA12, destabilise CI and cause OXPHOS dysfunction (Adjobo-Hermans *et al.*, 2020; Fernandez-Vizarra & Zeviani, 2021; Stroud *et al.*,

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2016). NDUFS4 is essential in CI assembly, stability, and function. The protein is nuclear encoded and must undergo post translational modification to enable importation into mitochondria and integration into CI (Kahlhöfer *et al.*, 2017; Papa *et al.*, 2010; Scacco *et al.*, 2003). CI couples NADH oxidation to proton pumping, driving ATP synthesis while maintaining redox balance by regenerating NAD^+ . It also regulates ROS production and under physiological conditions, low levels of ROS from CI's Fe-S clusters act as signalling molecules, modulating pathways like hypoxia response and inflammation (Mills *et al.*, 2016; Vernis *et al.*, 2017).

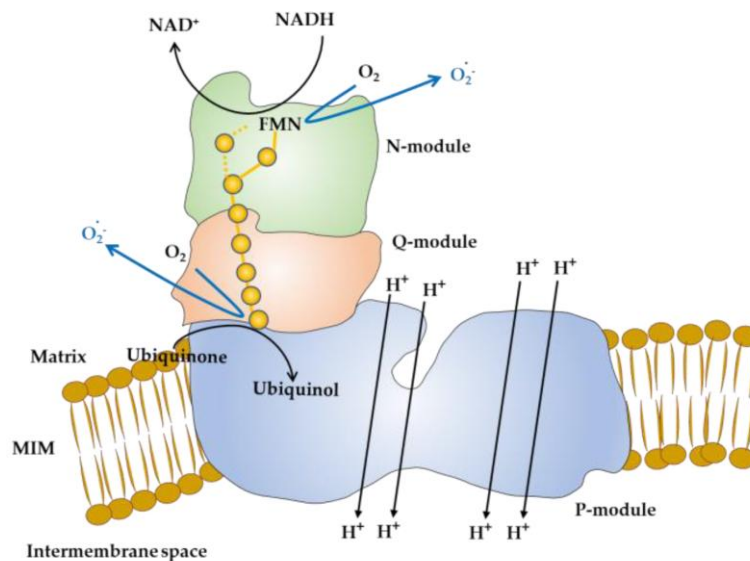


Figure 2-2: Diagram of mammalian CI. Complex I adopts an L-shaped architecture divided into three functional modules: the N-module (green), the Q-module (pink), and the P-module (blue). The N- and Q-modules form the hydrophilic arm that projects into the mitochondrial matrix, while the P-module comprises the hydrophobic arm embedded within the inner mitochondrial membrane. During catalysis, NADH is oxidized by FMN, and the resulting electrons travel through a chain of seven iron–sulphur clusters (yellow spheres) before reducing a ubiquinone molecule. *Abbreviations: FMN, flavin mononucleotide; O_2 , oxygen; H^+ , hydrogen; MIM, mitochondrial inner membrane; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydride.* Reprinted from Signorile and de Signorile (2023), <https://doi.org/10.3390/antiox12020221>, with permission in accordance with the Creative Commons Attribution Licence (CC BY) terms.

2.3.3. Dysfunctional OXPHOS and disease implications

CI dysfunction impairs ATP synthesis, leading to an increase in ROS through electron leakage at Fe-S clusters or FMN, and depleting NAD^+ levels, resulting in an impaired redox status (Distelmaier *et al.*, 2009; Quinlan *et al.*, 2011). Pathogenic mutations in CI subunits such as NDUFS4 and NDUFV1 or assembly factors destabilise SCs, worsening energy deficits and oxidative damage (Formosa *et al.*, 2018; López-Fabuel *et al.*, 2016).

OXPHOS dysfunction (Figure 2-3) is a hallmark of mitochondrial disorders. Defects in complexes such as CI and other OXPHOS components have been implicated in pathologies such as Leigh syndrome (LS). This is a severe neurodegenerative disorder, which is characterised by bilateral basal ganglia lesions, lactic acidosis, and developmental

deterioration, most commonly caused by deficiencies in CI or CIV (Lake *et al.*, 2016; McCormick *et al.*, 2023). Mutations in nuclear-encoded CI subunits like NDUF51, NDUF54 and NDUFV1 or assembly factors disrupt electron transport, leading to a decrease in energy production and toxic ROS accumulation that damage neurons and cardiomyocytes. Like LS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) – often caused by mtDNA mutations such as m.3243A>G – interferes with CI/CIII interactions, resulting in stroke-like episodes and muscle weakness (El-Hattab *et al.*, 2015).

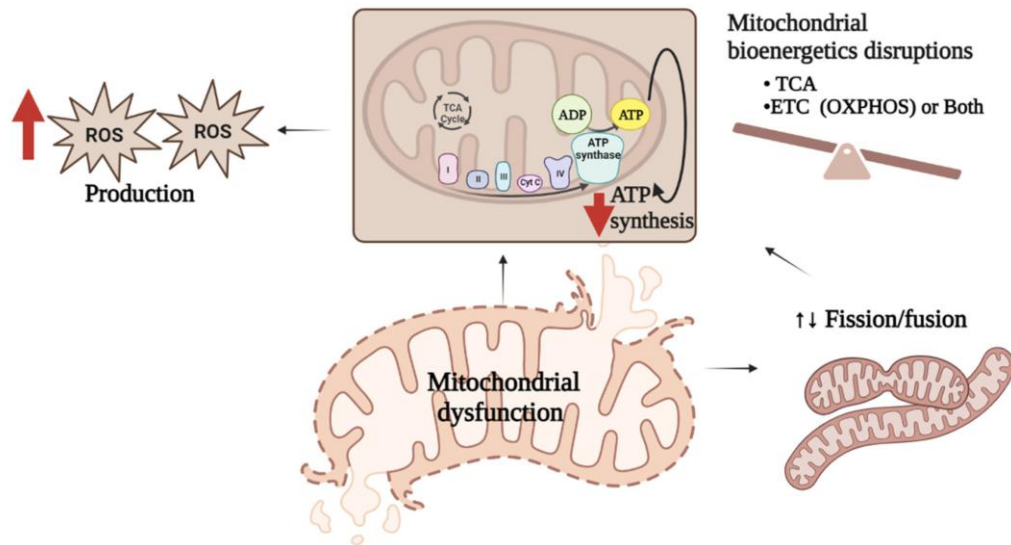


Figure 2-3: OXPHOS and mitochondrial dysfunction. The diagram illustrates the interconnectedness of the OXPHOS system and mitochondrial dysfunction. Mitochondrial dysfunction is often characterised with disruptions in mitochondrial bioenergetics, leading to a reduction in ATP synthesis and increased ROS production. *Abbreviations:* CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; ADP, adenosine triphosphate; ATP, adenosine triphosphate; ETC, electron transport chain; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle. Reprinted from Sultana *et al.* (2023), <https://doi.org/10.3390/biology12071019>, with permission in accordance with the Creative Commons Attribution Licence (CC BY) terms.

2.4. Mitochondrial dysfunction and disease

2.4.1. General underpinnings of mitochondrial dysfunction

MDs encompass a diverse group of disorders that are characterised by defects in the OXPHOS system, which impair cellular energy production. MDs are usually classified into two main categories, namely primary and secondary MDs (Valenti & Vacca, 2022). Primary MDs are either caused by pathogenic mutations in OXPHOS subunits, assembly factors, or genes involved in OXPHOS regulation. Secondary MDs often result from genetic defects unrelated to OXPHOS function, as well as environmental factors or other hereditary non-mitochondrial diseases (Gorman *et al.*, 2016; Howell *et al.*, 2005; Niyazov *et al.*, 2016). Both primary and secondary MDs often lead to a wide range of clinical symptoms (Figure 2-4), affecting multiple organ systems or a single organ in certain cases. Primary MDs are usually linked to disorders in

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oxidative phosphorylation, such as LS, MELAS and Alpers-Huttenlocher syndrome (Niyazov *et al.*, 2016), with the estimated prevalence of primary MDs in adults occurring in approximately 1 in 4300 individuals (Ghezzi & Zeviani, 2018; Gorman *et al.*, 2015). Secondary MDs have become increasingly linked to chronic conditions such as hypertension, diabetes, cancer, as well as neurodegenerative diseases like Alzheimer's and Parkinson's (Keshavan & Rahman, 2018; Parikh *et al.*, 2019).

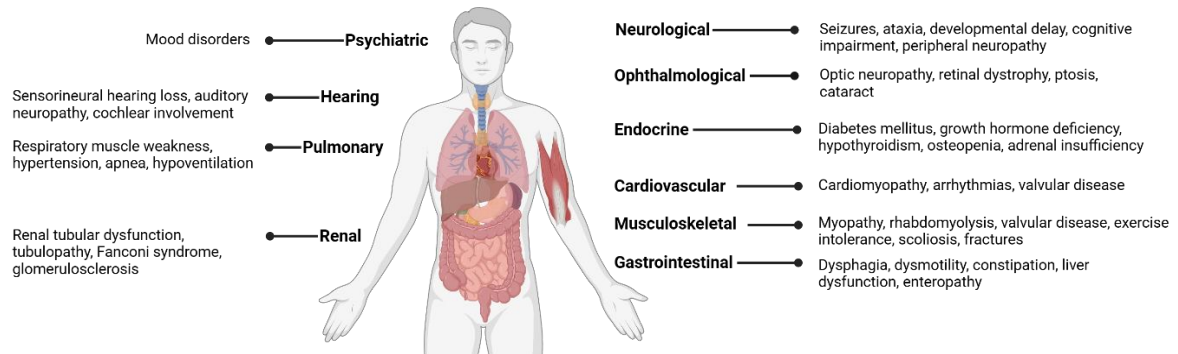


Figure 2-4: Clinical features of MD. Body systems affected by MD and their associated spectrum of clinical symptoms. MD can manifest at any age, impacting either a single organ or multiple organ systems, with a wide range of severity. *The clinical features listed here are derived and adapted from Parikh et al. (2017) and Rahman (2020).*

Although the impairment of the OXPHOS system is a critical feature of MDs, mitochondrial dysfunction extends beyond the impairment of the OXPHOS system and the ETC. Mitochondrial dysfunction also plays a pivotal role in apoptosis, calcium homeostasis, and cellular stress signalling (Gorman *et al.*, 2016; Perrone *et al.*, 2023; Wu *et al.*, 2019). The mitochondria regulate apoptosis via the intrinsic pathway, including but not limited to, cytochrome c release and BCL-2 family proteins (Kroemer, 2003; Lossi, 2022). However, mitochondrial dysfunction caused by nDNA and/or mtDNA mutations or ROS overproduction, can lead to apoptosis dysregulation, resulting in neurodegeneration, cardiomyopathies or other complications (Lemasters *et al.*, 1999). Additionally, studies have shown an increase in apoptotic susceptibility in LS, which present with severe neurodegeneration, further underscoring dysregulation of apoptosis and cellular damage beyond energy deficits in MDs (Carrozzo *et al.*, 2004).

Furthermore, the mitochondria also play an essential role in buffering cytosolic calcium (Ca^{2+}) via the mitochondrial calcium uniporter (MCU). Recent research revealed that altered mitochondrial Ca^{2+} uptake (Figure 2-5) may lead to various dysfunctions in numerous diseases including MDs (Perrone *et al.*, 2023). In addition, mutations in calcium regulatory proteins, such as MICU1, have also been linked with various neuromuscular phenotypes in certain MDs (Giorgi *et al.*, 2012; Perrone *et al.*, 2023). Moreover, disruptions in calcium handling have also been shown to contribute to stroke-like episodes in MELAS, further emphasising calcium dysregulation's role in MD (Giorgi *et al.*, 2012; Perrone *et al.*, 2023).

Although diagnostic approaches, such as next-generation sequencing, have improved our understanding of MDs (Chin *et al.*, 2024), clinical diagnosis remains challenging. These challenges are often due to the genetic heterogeneity of MDs, with over 340 genes being implicated in MDs, including both mtDNA and nDNA (Alston *et al.*, 2021; Gusic & Prokisch, 2021; Mayr *et al.*, 2015). Many of these genes impact OXPHOS enzymes and other processes associated with the OXPHOS system. Additionally, MDs often manifest with a wide range of neurological and muscular symptoms, including neuropathy, ataxia, myopathies, ophthalmoplegia and many more (Alston *et al.*, 2017; Smeitink *et al.*, 2001), often overlapping with other chronic conditions, leading to misdiagnosis and underdiagnosis. Moreover, for pathogenic effects arising from mtDNA mutations to manifest, a specific threshold of mutant mtDNA needs to be surpassed (Alston *et al.*, 2021; Ishikawa & Nakada, 2021). As such, the presence of both wild-type (WT) and mutant mtDNA further complicates the relationship between genotype and phenotype.

Currently, no curative treatment option for MD exists. Disease management strategies usually focus on preventing life-threatening complications, dietary optimisation, and supportive care (Parikh *et al.*, 2017; Rahman, 2020). Lastly, the heterogeneity of MDs further complicates therapeutic strategies, as treatment efficacy tends to vary among MD patients, making optimised therapeutic approaches challenging.

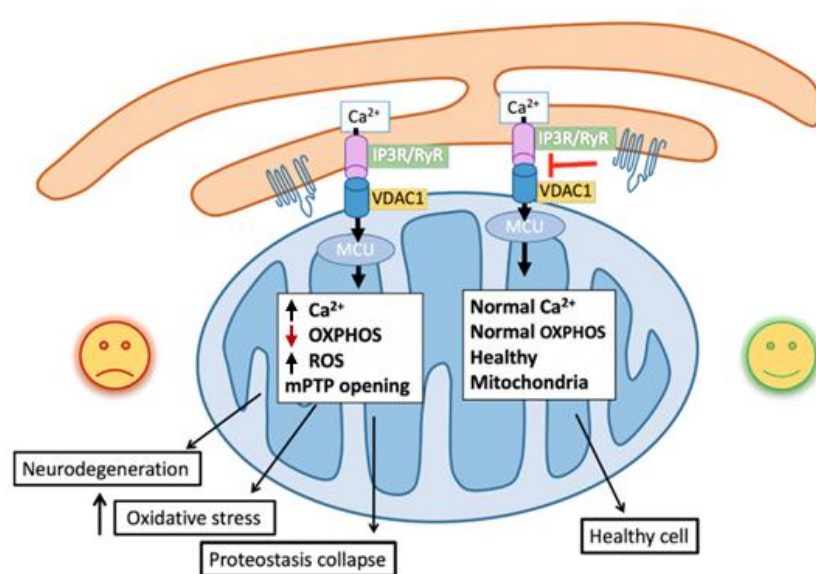


Figure 2-5: Mitochondria and calcium handling. Increased calcium release and accumulation caused by dysfunctions in the mitochondria may lead to a decrease in OXPHOS, and an increase in ROS production as well as the mitochondrial permeable transition pore (mPTP) opening. This further leads to a cascading effect resulting in increased oxidative stress and potential neurodegeneration. *Abbreviations:* Ca^{2+} , calcium; OXPHOS, oxidative phosphorylation; MCU, mitochondrial calcium uniporter; VDAC1, voltage-dependent anion channel 1; IP3R/RyR, IP₃ receptor/ryanodine receptor. Reprinted and adapted from Ryan *et al.* (2020), <https://doi.org/10.3390/ijms21239153>, with permission in accordance with the Creative Commons Attribution Licence (CC BY) terms.

2.4.2. CI deficiency and LS

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Isolated CI deficiency (OMIM 252010) is the most common form of mitochondrial disorders and is highly prevalent in children, accounting for approximately 30% of cases (Distelmaier *et al.*, 2009; Ghezzi & Zeviani, 2018; Loeffen *et al.*, 2000). Mutations linked to CI deficiency have been identified in all seven mtDNA-encoded subunits and in 19 of the 37 nDNA-encoded subunits (Hoefs *et al.*, 2012; Ng *et al.*, 2020). Additionally, mutations in 10 of the 14 CI assembly factors have also been reported. Notably, roughly 70-80% of these cases are linked to mutations in nDNA, often resulting in greater clinical presentations compared to mtDNA mutations (Alston *et al.*, 2021; Saneto, 2020; Swalwell *et al.*, 2011). CI deficiency disrupts the OXPHOS system, reducing ATP production and increasing ROS production, leading to cellular damage. This dysfunction significantly contributes to the pathology observed in MDs, typically affecting tissues such as brain, heart, and muscle (Rodenburg, 2016).

CI deficiency is frequently associated with LS (OMIM 256000), a neurodegenerative disorder which often results in bilateral necrotic lesions in specific brain areas, mitochondrial dysfunction, as well as locomotor and cognitive decline (Adjobo-Hermans *et al.*, 2020; Lim *et al.*, 2022; Rahman *et al.*, 1996). LS symptoms are usually apparent during infancy or early childhood and can include hypotonia, respiratory distress, failure to thrive, nystagmus, psychomotor regression, dystonia, ataxia, and spasticity. Additionally, elevated lactic acid levels are observed in approximately 90% of cases, with frequent multisystemic involvement, such as the heart, liver, and kidneys (Koene *et al.*, 2011; Lake *et al.*, 2016; Lim *et al.*, 2022; Rodenburg, 2016). The prognosis for patients afflicted with isolated CI deficiency is generally poor, with approximately 50% of affected children not surviving beyond two years of age, and 75% dying before the age of ten (Koopman *et al.*, 2013; Loeffen *et al.*, 2000). MRI of LS patients frequently reveal lesions in the basal ganglia, brainstem, thalamus, and cerebellum (Rahman *et al.*, 2017).

The most common autosomal causes of LS include mutations in the nuclear *NDUFS4* gene, which encodes a non-enzymatic accessory subunit of CI. Several loss-of-function mutations in this gene have been identified (Budde *et al.*, 2000; Lamont *et al.*, 2017; Ortigoza-Escobar *et al.*, 2016; Papa, 2002). Moreover, compared to mutations of nDNA, mtDNA mutations in CI subunits often result in a wide range of symptoms and affected organs. The onset of clinical symptoms often varies and can manifest during early childhood to late adolescence. The variability of symptom onset is largely influenced by heteroplasmy due to the proportion of mutant versus WT mtDNA present in cells (Distelmaier *et al.*, 2009; Loeffen *et al.*, 2000; Lim *et al.*, 2022). CI deficiency often leads to an altered redox status, disrupted mitochondrial membrane potential, increased ROS production, and disruption of cellular calcium homeostasis (Distelmaier *et al.*, 2009; Valsecchi *et al.*, 2012; Yin *et al.*, 2024).

To better understand the consequences of *NDUFS4* deficiency, numerous patient-derived cell models and animal models have been established. Notably, the whole-body *Ndufs4* knockout

(KO) mouse model, established by Palmiter and colleagues (Kruse *et al.*, 2008), remains the most well-characterised and widely used preclinical model for studying human CI dysfunction.

2.5. The *Ndufs4* KO mouse model

2.5.1. Model background and description

The *Ndufs4* KO mouse model was created by the removal of the first 17 amino acids of the NDUFS4 protein via Cre/loxP-mediated deletion of exon 2 of the *Ndufs4* gene (JAX, 2020b; Kruse *et al.*, 2008). This deletion interferes with CI assembly and stability, reducing CI's function and activity (Kruse *et al.*, 2008; Yin *et al.*, 2024). Unlike the catalytic subunits, the NDUFS4 protein is an accessory subunit that is essential for CI stability (Stroud *et al.*, 2016), highlighting its potential as a viable model for studying CI deficiency. Additionally, although there are other mouse models of CI deficiency, such as the *Ndufv1* KO mice and *Ndufs6* KO, some of these models either exhibit milder phenotypes or embryonic lethality, limiting postnatal LS studies (Hong *et al.*, 2014; Koopman *et al.*, 2010; Yin *et al.*, 2024).

Furthermore, the loss of the NDUFS4 protein causes severe reductions of the CI subunit across various tissues that have a high energy demand, such as brain, liver and muscle (Adjobo-Hermans *et al.*, 2020; JAX, 2017; Kruse *et al.*, 2008; van de Wal *et al.*, 2022). In addition, the pathology observed in this model closely resembles that of human LS [Figure 2-6] (Quintana *et al.*, 2010). As such, this makes the *Ndufs4* KO mouse model a valuable tool for studying isolated CI deficiency and pathologies related to defects of CI and other MDs (van de Wal *et al.*, 2022).

Moreover, heterozygous (HET) *Ndufs4* mice have been shown to remain healthy for a minimum duration of one-year, while homozygous *Ndufs4* KO mice present with severe phenotypic manifestations due to the absence of NDUFS4 (Jax, 2017, Kruse *et al.*, 2008). However, even though the CI deficiency in this model leads to reduced ATP production in the OXPHOS system, residual activity via CIII interactions and NDUFAF2 upregulation are evident in this model, as well as in patient-derived fibroblasts (Calvaruso *et al.*, 2011; Adjobo-Hermans *et al.*, 2020). Other CI models, such as the *Ndufs2* KO mouse model, usually lack this type of compensatory mechanism. Additionally, *Ndufs4* KO mice are favoured over other CI models due to their reproducible LS-like pathology, postnatal viability, and its translational utility, which enables therapeutic testing (Ferrari *et al.*, 2017; van de Wal *et al.*, 2022).

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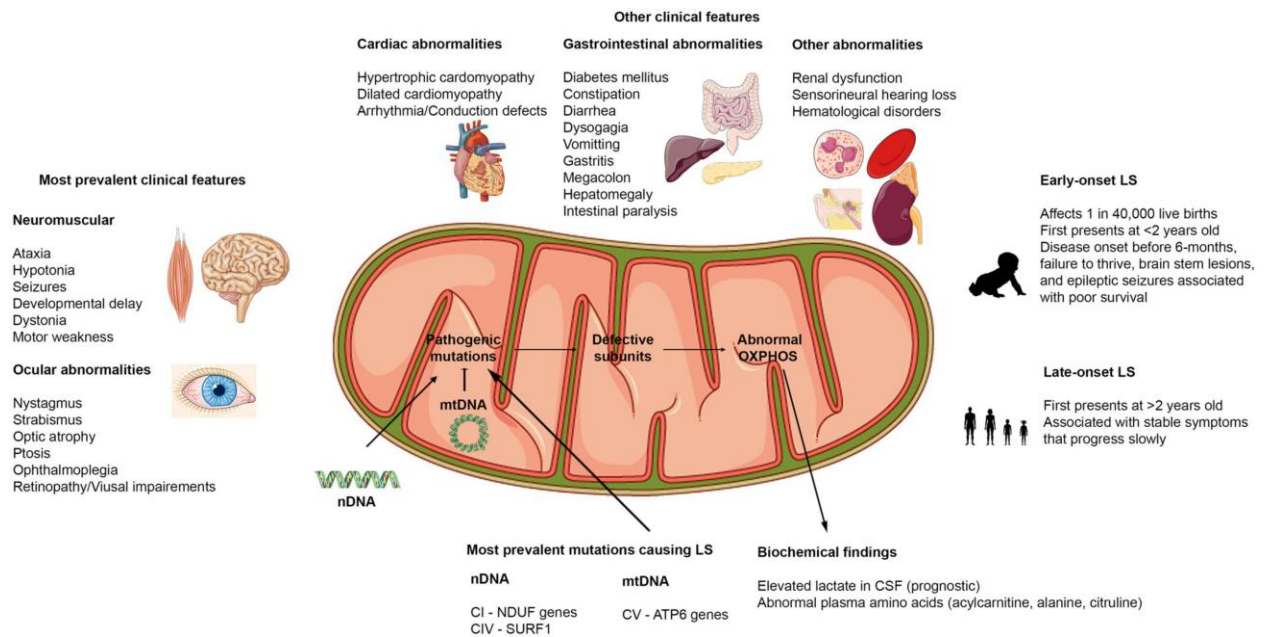


Figure 2-6: Clinical characteristics of LS. The figure illustrates the clinical features most often observed in LS, with the brain, muscle and eyes being predominantly affected. The clinical features also include cardiovascular, gastrointestinal, and other abnormalities. LS is most often associated with pathogenic mutations in either nDNA or mtDNA leading to irregularities in the OXPHOS system. *Reprinted from Bakare et al. (2021), <https://doi.org/10.3389/fphys.2021.693734>, with permission in accordance with the Creative Commons Attribution Licence (CC BY) terms.*

2.5.2. Phenotypic characteristics of the *Ndufs4* KO mouse model

CI deficiency caused by the loss of the NDUF54 protein in the *Ndufs4* KO mouse model results in a progressive neurometabolic disorder that closely mimics the phenotype of human LS. Like human LS, symptoms usually start off being mild, progressing and worsening as the mice get older. At the early stage, postnatal day (P) 0 to 18, behavioural or developmental differences are not evident compared to WT/HET littermates (Kruse *et al.*, 2008). However, subtle retinal ganglion cell abnormalities have been detected as early as P16 (Yu *et al.*, 2015; Song *et al.*, 2017). Despite CI deficiency, *Ndufs4* KO mice are relatively active, indicating potential compensatory mechanisms like glycolysis, fatty acid oxidation and bypassing CI via the electron transfer flavoprotein: ubiquinone oxidoreductase (ETF: QO) pathway (Alam *et al.*, 2015; Terburgh *et al.*, 2019). These adaptive mechanisms may delay symptom onset but are yet to be fully elucidated in future studies.

Furthermore, as the mice progress to the early disease phase during P18-P26, growth retardation and transient alopecia are observed due to potential systemic inflammation (Jin *et al.*, 2014). Additionally, vision loss is also evident during this stage as these mice tend to have a predisposition to cataracts and retinal degeneration (Song *et al.*, 2017). While macrophage activation could explain the systemic inflammation resulting in alopecia (Jin *et al.*, 2014), its role in later brain lesions is unclear.

When the mice reach the mid-stage of the disease around P26-P38, neurological deterioration becomes more evident, with symptoms such as motor deterioration, ataxia, seizures, and hypothermia (Kruse *et al.*, 2008; Quintana *et al.*, 2010; Shil *et al.*, 2021). In addition to increased neurological deterioration, respiratory and cardiovascular irregularities have also been observed during this stage (Quintana *et al.*, 2010). However, it is to be noted that nearly all studies using this model make use of male mice. Although unreported in *Ndufs4* KO female mice, female KO mice of other neurodegenerative disorders have exhibited delayed neurological decline due to the potential neuroprotective effects of oestrogen (Pozzi *et al.*, 2006; Suzuki *et al.*, 2006). Additionally, a transcriptomic study using *Ndufs4* KO mice reported that untreated *Ndufs4* KO mice exhibited Alzheimer's-like transcriptomic changes in brain tissue. Mice were treated with a mild complex I inhibitor, CP2, where female KO mice showed a greater reversal of these changes toward WT expression levels compared to males (Gao *et al.*, 2024). This suggests an intrinsic sex-dependent response to mitochondrial dysfunction.

As the mice progress to the late stage of the disease after P38, terminal neurodegeneration worsens. During this stage, lesions in the brainstem become more prominent, along with weight loss and respiratory failure (Jin *et al.*, 2014; Kruse *et al.*, 2008; Quintana *et al.*, 2010; Quintana *et al.*, 2012). Feeding difficulties, which have been reported in *Ndufs4* KO Drosophila, may also be an underrecognised feature during the late stage of the disease in murine models (Foriel *et al.*, 2018). The disease results in the eventual death of KO mice as the symptoms progress towards P50-P60. However, the survival times often vary between different research environments (Jin *et al.*, 2014; Johnson *et al.*, 2013; Kruse *et al.*, 2008; Miller *et al.*, 2021; Quintana *et al.*, 2010; Quintana *et al.*, 2012), with fewer studies (van Rensburg *et al.*, 2024; Stokes *et al.*, 2022; Yoon *et al.*, 2022) reporting housing conditions that may affect phenotype severity.

The *Ndufs4* KO mouse model's predictable disease progression enables therapeutic testing at various stages of the disease, highlighting its validity as a preclinical model. Although most studies focus on survival (van de Wal *et al.*, 2022), future studies need to focus on quality-of-life metrics such as motor function, vision, inflammation and other abnormalities present in this disease.

2.5.3. Biochemical consequences of an *Ndufs4*-CI deficiency

2.5.3.1. Energy repercussions

Mitochondria isolated from *Ndufs4* KO mice have demonstrated that the loss of the NDUFS4 protein reduces CI-driven respiration of the ETC by ~50% in brain, skeletal muscle, and liver tissues (Kruse *et al.*, 2008). Additionally, a structural cryogenic electron microscopy (cryo-EM) study revealed that the deletion of NDUFS4 destabilises CI, hindering its assembly and electron transport efficacy, reducing ATP production (Yin *et al.*, 2024).

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Studies have shown that despite CI dysfunction, increases in CII and citrate synthase (CS) activity are evident in some *Ndufs4* KO tissues (Alam *et al.*, 2015; Calvaruso *et al.*, 2011; de Haas *et al.*, 2017; Kruse *et al.*, 2008; Terburgh *et al.*, 2019; Valsecchi *et al.*, 2012). Additionally, CIV levels have also been reported to be increased in the *Ndufs4* KO brain, potentially compensating for CI deficiency (Martin-Perez *et al.*, 2020). Adjobo-Hermans *et al.* (2020) reported no significant differences in the assembly factors or subunit levels for CII–CV in the *Ndufs4* KO mice, except for an increase in the CV assembly factor ATPAF₂ and higher CII subunit levels in the heart and kidneys. Moreover, CIII activity has also been shown to decrease in certain tissues such as the liver and brain but not universally (de Haas *et al.*, 2017; Kruse *et al.*, 2008; Terburgh *et al.*, 2019). Studies conducted on synaptic mitochondria in the brain have shown a reduction of approximately 26% in ATP production, while non-synaptic mitochondria maintain normal levels (Balsa *et al.*, 2020; Kayser *et al.*, 2016; Manjeri *et al.*, 2016). Hippocampal mitochondria in *Ndufs4* KO mice have also been reported to exhibit reduced synaptophysin expression, further linking ATP deficits to synaptic dysfunction (Shil *et al.*, 2021).

ATP levels have been reported to remain relatively stable in skeletal muscle, cardiomyocytes, fibroblasts, bone marrow cells, and thymocytes due to compensatory glycolysis and fatty acid oxidation (Alam *et al.*, 2015; Bertaux *et al.*, 2018; Karamanlidis *et al.*, 2013; Kruse *et al.*, 2008; Valsecchi *et al.*, 2012). Additionally, studies have reported potential metabolic reprogramming in the *Ndufs4* KO brain, including disrupted TCA cycle intermediates and increased glycolysis, mirroring Alzheimer's disease signatures (Goa *et al.*, 2024; Shil *et al.*, 2021).

2.5.3.2. ROS overproduction

The loss of NDUF54 leads to the structural instability of CI, resulting in the disruption of electron transport efficiency in the ETC, increasing the likelihood of electron leakage and superoxide (O₂^{•-}) production at Fe-S clusters (Yin *et al.*, 2024). Additionally, an alteration of enzyme kinetics in *Ndufs4* KO mitochondria may indicate electron shunting to alternative pathways, increasing ROS generation during the oxidation of NADH (Yin *et al.*, 2024).

An increase in ROS levels has been observed in the entire brain according to some studies (Johnson *et al.*, 2013; Lee *et al.*, 2019). Furthermore, specific brain regions such as the cerebral cortex and vestibular nuclei have also been reported to show elevated ROS levels during later disease stages (de Haas *et al.*, 2017; Liu *et al.*, 2017; Johnson *et al.*, 2020). The findings indicate a potential correlation between ROS levels and neurodegeneration. In addition, an increase in mitochondrial superoxide (mtO₂^{•-}) and cytosolic hydrogen peroxide (H₂O₂) contribute to oxidative damage, synaptic loss, and neuroinflammation (Liu *et al.*, 2015; Quintana *et al.*, 2010). ROS overproduction in *Ndufs4* KO neurons have also been linked to perturbations in the TCA cycle intermediates, such as succinate accumulation, which may drive reverse electron transport (RET) at CI (Peruzzotti-Jametti *et al.*, 2024; Yin *et al.*, 2024; Zhang *et al.*, 2018).

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Pre-symptomatic *Ndufs4* KO brains show no explicit oxidative damage, indicating that ROS accumulation may be a secondary consequence of CI dysfunction and metabolic stress (Johnson *et al.*, 2020). Antioxidants, such as N-acetyl cysteine amide (NACA) and KH176 (sonlicromanol), have demonstrated to reduce ROS in acute models but fail to improve survival in *Ndufs4* KO mice (Liu *et al.*, 2015; de Haas *et al.*, 2017). It has also been reported that NADPH deficiency is a significant factor in impairing glutathione recycling and amplifying redox stress independent of ROS (Balsa *et al.*, 2020). These findings further suggest that ROS may not be the primary driver of pathology.

Subunit-specific ROS phenotypes exist across various CI models. Knockdown of other CI subunits, such as NDUFA8 and NDUFAF1, present with different ROS profiles, with NDUFA8 deficiency causing the highest mtO₂⁻ elevation (Bennet *et al.*, 2023). This variability may explain the inconsistent findings of ROS production in *Ndufs4* and other CI deficient models. Additionally, mitochondria-targeted antioxidants, like MitoQ, partially reduce ROS in *NDUFAF1*-deficient cells but not in *NDUFA8* KO cells, further underscoring the reported variability between subunit-specific ROS phenotypes (Bennet *et al.*, 2023).

2.5.3.3. Calcium dysregulation

Ca²⁺ regulation plays a crucial role in mitochondrial function during both normal and pathological states (Kostic *et al.*, 2018). The $\Delta\Psi_m$ is essential for Ca²⁺ regulation as it provides the electrochemical driving force needed for Ca²⁺ uptake via the MCU (Stefani *et al.*, 2011; vanden Hoven & Santini, 2004). Although not directly reported in the *Ndufs4* KO mouse model, studies have shown that CI dysfunction destabilises the $\Delta\Psi_m$, reducing the organelle's capacity to buffer cytosolic calcium (Koopman *et al.*, 2010; Valsecchi *et al.*, 2012). Ca²⁺ overload in neurons has been reported to trigger excitotoxicity and activate calpain proteases, worsening neurodegeneration (Giorgi *et al.*, 2018). Additionally, irregularities in Ca²⁺ signalling have also been observed in neurons and astrocytes of various models of neurodegeneration (Baev *et al.*, 2022). Moreover, the mitochondria of *Ndufs4* KO neurons and fibroblasts have also shown abnormalities such as morphology swelling and cristae loss, potentially impairing calcium handling (Quintana *et al.*, 2010; Shil *et al.*, 2021; Valsecchi *et al.*, 2013). Neuronal calcium handling may be compromised due to reduced ATP availability for Ca²⁺ dependent ATPase pumps (Clapham, 2007; Kostic *et al.*, 2018). Elevated ROS and chronic neuroinflammation in *Ndufs4* KO mice have been shown to activate NF- κ B and IKK- α pathways, which may further exacerbate calcium dysregulation (Martin-Perez *et al.*, 2020; Gao *et al.*, 2024). In addition, treatment with rapamycin in the *Ndufs4* KO mouse model has shown to reduce phosphorylation of protein kinase C, improve calcium handling, and delay neurodegeneration (Martin-Perez *et al.*, 2020).

The ablation of *Ndufs4* in Neuro-2a cells has demonstrated to shorten neurites and disrupt extracellular regulated kinase (ERK) signalling, which regulates Ca²⁺ related synaptic

plasticity (Shil *et al.*, 2021). Additionally, glucose-induced hyperlactatemia in older *Ndufs4* KO mice has been correlated with Ca^{2+} overload in neurons, further increasing metabolic stress and dysfunction (Stokes *et al.*, 2022). Leukocyte-mediated inflammation in *Ndufs4* KO mice has also been shown to increase chemokines, which may alter neuronal Ca^{2+} fluctuations through cytokine receptors (Stokes *et al.*, 2021; Stokes *et al.*, 2022). In addition, recent transcriptomic analysis has revealed an Alzheimer's-like Ca^{2+} dysregulation in *Ndufs4* KO brains, including mitochondrial transport impairment and ROS-facilitated Ca^{2+} leakage (Gao *et al.*, 2024).

2.5.3.4. *Inflammation and apoptosis*

Inflammation is one of the distinguishing features with regards to the pathophysiology reported in *Ndufs4* KO models (van de Wal *et al.*, 2022). Glutamatergic and GABAergic neurons (Figure 2-7) play a critical role in the LS phenotype. GABAergic neurons in the cortex, hippocampus, basal ganglia and olfactory bulbs, have shown specific vulnerability to neuroinflammation (Aguilar *et al.*, 2022; Bolea *et al.*, 2019; Shil *et al.*, 2021). Similarly, glutamatergic neurons in the thalamus exhibit neurotoxicity due to glutamate metabolism dysregulation in astrocytes, which downregulate GLT-1 transporters (Gella *et al.*, 2020; Johnson *et al.*, 2020; Shil *et al.*, 2021). It has been observed that *Ndufs4* KO mice exhibit elevated inflammatory markers, such as IL-6, IFN γ , IL-12p70, in serum, brain, liver, spleen, and skin (Hanaford *et al.*, 2024; Jin *et al.*, 2014; Johnson *et al.*, 2013). These inflammatory markers have been correlated with fatty acid and lactate accumulation, as well as ROS overproduction (Yu *et al.*, 2015). Additionally, liver metabolic dysfunction has been observed to worsen systemic inflammation via impaired fatty acid oxidation and mitochondrial stress (Jin *et al.*, 2014). Elevated inflammatory cytokines, such as IFN γ , has been observed in the brainstem of *Ndufs4* KO mice, contributing to disease onset and progression of LS (Hanaford *et al.*, 2024).

Neurodegeneration of brain specific regions, such as olfactory bulb and vestibular nucleus, exhibit microglial activation and astrogliosis proportional to disease severity (Quintana *et al.*, 2010; van de Wal *et al.*, 2022). Inflammation of the retina in *Ndufs4* KO mice have also been reported to drive ganglion cell loss, correlating to innate immune dysregulation (Jiang *et al.*, 2019; Yu *et al.*, 2015). Moreover, the hyperactivation of the mammalian/mechanistic target of rapamycin (mTOR) in symptomatic stages has been reported to promote hypoxia-inducible factor alpha (HIF-1 α) signalling, metabolic rewiring, and NF- κ B-mediated cytokine release, further contributing to inflammation (Johnson *et al.*, 2020; Johnson *et al.*, 2013; Martin-Perez *et al.*, 2020). Inflammatory cytokines like TNF- α have also been reported to directly impair glutamatergic synaptic plasticity by promoting NMDA receptor internalisation, exacerbating neuroinflammation (Aguilar *et al.*, 2022). In addition, peripheral immune cells, such as CD11b $^{+}$ monocytes, Gr-1 $^{+}$ granulocytes, and F4/80 $^{+}$ cell populations, have been found to penetrate the central nervous system (CNS), exacerbating neuroinflammation and lesion formation (Stokes *et al.*, 2022).

Apoptotic pathways have also been correlated with the inflammatory pathology observed in *Ndufs4* KO mice as caspase-8 activation, involved in extrinsic apoptosis, precedes neuronal necrosis in affected brain regions (Quintana *et al.*, 2010). Additionally, mitochondrial dysfunction results in Bcl-2 family imbalances, increasing Bax/Bak-mediated permeability of the outer membrane (Singh *et al.*, 2019). Moreover, another study found that interferon-gamma (IFN γ) also play a role in the inflammatory pathogenesis of NDUFS4-related LS (Hanaford *et al.*, 2024). The treatment with anti-IFN γ has also been shown to hold therapeutic potential in attenuating neuroinflammation and disease progression in *Ndufs4* KO models (Hanaford *et al.*, 2024). In addition, CP2, which is a mild CI inhibitor, has been shown to partially rescue Alzheimer's-like transcriptomic signatures in female *Ndufs4* KO mice by reducing inflammatory genes, such as *Tnf* and *Il1b*, and normalising microglial activation (Gao *et al.*, 2024). CP2 also restored GABAergic interneuron populations in this study by suppressing astrocyte-derived complement C3, which is responsible for synaptic elimination. As such, understanding the mechanisms surrounding inflammation is crucial to further develop treatment approaches to reduce inflammation and enhance therapeutic outcomes in MDs.

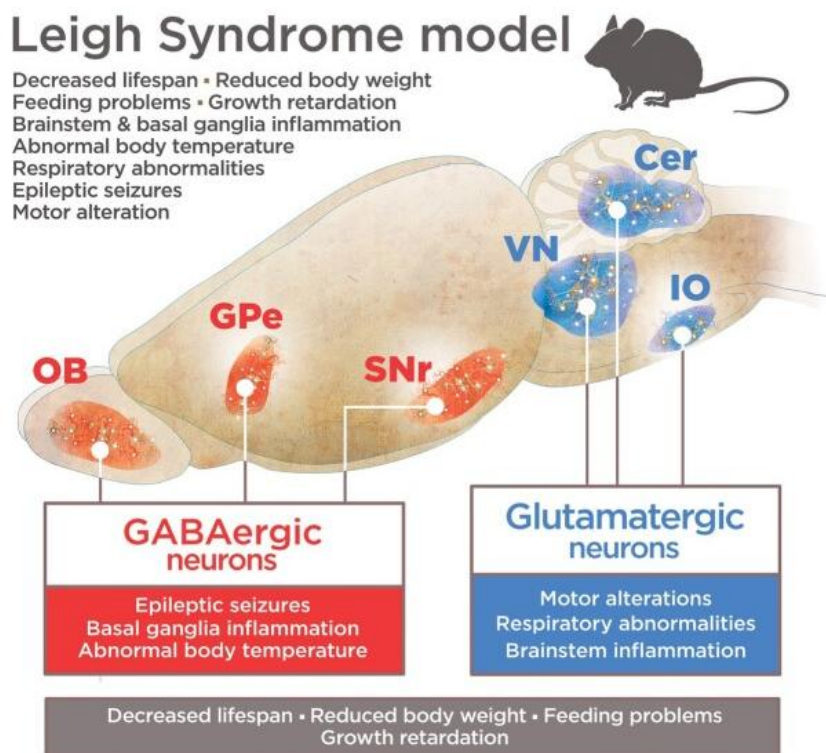


Figure 2-7: Neuroinflammation in *Ndufs4* KO mice. *Ndufs4* KO mice present with inflammation and neurodegeneration in various brain regions. These features are prominent GABAergic and glutamatergic neurons, contributing to the neuropathophysiology of this disease. Abbreviations: Cer, cerebellum; GPe, external globus pallidus; IO, inferior olive; OB, olfactory bulb; SNr, substantia nigra pars reticulata; VN, vestibular nuclei. Reprinted and modified from van de Wal *et al.* (2022), DOI: 10.1093/brain/awab426, with permission in accordance with the Creative Commons Attribution Non-Commercial Licence terms (<https://creativecommons.org/licenses/by-nc/4.0/>).

2.5.3.5. Redox status

CI deficiency impairs NAD⁺ production, altering the NADH/NAD⁺ ratio and mitochondrial redox regulation (Kahlhöfer *et al.*, 2017; Yin *et al.*, 2024). In neurons, reductions of mitochondrial NAD⁺ have been reported in a compartment-specific manner, with mitochondrial NAD⁺ levels declining earlier and more severely than cytosolic NAD⁺ loss (Kulkarni *et al.*, 2019). A study using hypoxic conditions in *Ndufs4* KO mice reported an improvement in cerebral NAD⁺ levels through reducing lactate/pyruvate ratios (Grange *et al.*, 2021). This further highlights an altered redox state as a pathological feature of this disease and as a potential therapeutic target. A CI deficiency destabilises the ETC, increasing electron leakage and superoxide production (Yin *et al.*, 2024). Additionally, dysfunction disrupts NADPH production by altering mitochondrial one-carbon metabolism, impairing glutathione regulations and increasing oxidative stress (Esterhuizen *et al.*, 2017). Redox imbalances due to NDUF54 knockdown drive lipid peroxidation and ferroptosis in UVB-exposed skin, reducing ferroptosis through the inhibition of the MAPK pathway and upregulation of glutathione peroxidase 4 (GPX4) (Teng *et al.*, 2025). The overexpression of GPX4, which is crucial for redox modulation, has been reported to mitigate ferroptosis and restore redox balance in *Ndufs4* KO mice (Teng *et al.*, 2025). A study also found that glycerol-3-phosphate biosynthesis improves cytosolic NAD⁺ in *Ndufs4* KO mice but not mitochondrial redox stress in xenograft tumours, revealing compartment- and model-specific redox failures (Liu *et al.*, 2021). Also, leukocyte-mediated inflammation in *Ndufs4* KO mice has been correlated with elevated chemokines, such as CXCL10, which may indirectly disrupt mitochondrial calcium and redox homeostasis.

A disrupted NADH/NAD⁺ ratio, resulting in a reduction of NAD⁺ levels, can alter energy metabolism. An altered NADH/NAD⁺ ratio has been shown to inhibit branched-chain α -keto acid dehydrogenase (BCKDH), resulting in the accumulation of branched-chain amino acids (BCAAs), such as leucine, isoleucine, and valine in *Ndufs4* KO mouse brain and induced pluripotent stem cells (Terburgh *et al.*, 2021; Goolab *et al.*, 2025; Yoon *et al.*, 2022). In addition, the transport of mitochondria-specific proline may buffer redox stress; however, it has been shown to deplete glycolytic intermediates in tissues like muscle (Liu *et al.*, 2021). Moreover, sub-cellular metabolomics has also revealed that mitochondrial BCAA accumulation precedes whole-cell metabolic shifts, further highlighting the impact of redox dysregulation (van der Walt *et al.*, 2021).

2.5.3.6. Metabolic alterations

Ndufs4 KO mouse tissues have shown to exhibit elevated glycolysis, leading to an increase in lactate and pyruvate levels, as well as cellular acidosis (Esterhuizen *et al.*, 2017; Kruse *et al.*, 2008; Schleifer *et al.*, 2019). In addition, the glycerophosphate shuttle and proline cycle activity increase to recycle cytosolic NADH, reducing redox stress, but worsening lactic acidosis (Liu *et al.*, 2021; Zieliński *et al.*, 2016). When *Ndufs4* KO mice are exposed to chronic hypoxic conditions, it reduces systemic lactate levels through improving NAD⁺/NADH ratios (Jain *et al.*,

2016). NADH accumulation due to a CI deficiency has been reported to inhibit citrate synthase and isocitrate dehydrogenase, resulting in the buildup of TCA intermediates such as fumarate and succinate, as well as an impairment of ATP synthesis (Alam *et al.*, 2015; Piroli *et al.*, 2020; Piroli *et al.*, 2016). Glutamine anaplerosis and increased glutaminolysis has been observed as a critical compensatory mechanism in various models of mitochondrial dysfunction (Chen *et al.*, 2018). Additionally, *Ndufs4* KO mice also exhibit alterations in glutamine metabolism in tissue like the brain and has been linked to neurodegenerative processes observed in the *Ndufs4* KO mouse model and other MD models (Bornstein *et al.*, 2023; Terburgh *et al.*, 2021). It has also been observed that glutamine metabolism is dysregulated in organs like the liver and pancreas, further impairing mitochondrial function in *Ndufs4* KO models (Yang *et al.*, 2020).

In *Ndufs4* KO mice, BCAAs such as leucine, isoleucine, and valine have been observed to accumulate in serum and tissues (Terburgh *et al.*, 2021; van de Wal *et al.*, 2022). This accumulation indicates a potential impairment of mitochondrial catabolism of BCAAs, which usually enter the TCA cycle via branched-chain α -keto acids (BCKAs). An elevation of BCAAs and BCKAs in the brain and skeletal muscle indicate an impairment of their degradation. This may contribute to neurodegeneration and metabolic stress observed in this model and other disorders of mitochondrial dysfunction (Khaled *et al.*, 2023; Yoo *et al.*, 2022). Additionally, it has been reported that BCKDH, an enzyme that converts BCKAs into acetyl-CoA and succinyl-CoA, shows reduced activity in *Ndufs4* KO models (Mills *et al.*, 2022). This may be due to an increase of BCKDH inhibition, leading to a build-up of potential toxic BCAA intermediates, which may contribute to neurological symptoms. Moreover, an altered BCAA metabolism in *Ndufs4* KO mice may be linked to neuroinflammation and excitotoxicity due to the function of BCAAs in neurotransmitter homeostasis, as is observed in other MD models (Yoo *et al.*, 2022). Leucine and isoleucine contribute to glutamate synthesis, which may exacerbate excitotoxic neuronal damage in LS pathology (Lake *et al.*, 2016). BCAAs also influence the redox balance and may worsen oxidative stress if their metabolism becomes dysregulated, resulting in the exacerbation of mitochondrial dysfunction.

2.5.3.7. Molecular pathways and mechanisms

CI deficiency impairs oxidative phosphorylation, leading to reduced ATP production and altered cellular energy status. This reduction in energy production can potentially inhibit mTORC1 activity, as mTORC1 is sensitive to nutrient and energy levels (Haissaguerre *et al.*, 2014). However, chronic CI deficiency in *Ndufs4* KO mice may contribute to mTORC1 hyperactivation via ROS-mediated stabilisation of Rheb-GTPase, which may further contribute to disease pathology (Johnson *et al.*, 2020; Johnson *et al.*, 2013; Zong *et al.*, 2024). Additionally, the inhibition of mTOR has been observed to maintain neuronal ATP levels in neuronal cell models, even when oxidative phosphorylation is reduced in various disease models (Zheng *et al.*, 2016).

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This makes mTOR a potential therapeutic target for MDs like LS, as rapamycin, an mTOR inhibitor, which has been reported to extend lifespan and reduce neurodegeneration in *Ndufs4* KO mice (Johnson *et al.*, 2013, Johnson *et al.*, 2020).

Moreover, PI3K γ , a leukocyte-specific isoform, has also been found to be indirectly linked to the pathophysiology of CI deficiency (Stokes *et al.*, 2022). Although PI3K γ is not directly regulated by CI, it has shown to promote leukocyte infiltration and microglial activation, which in turn causes neuroinflammation in *Ndufs4* KO mice (Aguilar *et al.*, 2022; Stokes *et al.*, 2022). The pharmacological inhibition of PI3K γ has shown the potential to reduce lesion formation and improve survival, suggesting its role as a secondary therapeutic target (Stokes *et al.*, 2022).

Furthermore, PGC-1 α , a master regulator of mitochondrial biogenesis, is another molecular pathway that may be indirectly compromised in CI deficiency. Impaired CI function reduces the NAD⁺/NADH ratio, lowering the activity of NAD⁺-dependent sirtuins, such as SIRT1 and SIRT3, which deacetylate and activate PGC-1 α (Lombard *et al.*, 2011; Sack & Finkel, 2012; Kane & Sinclair, 2018; Lee *et al.*, 2019). This leads to a repetitive cycle where mitochondrial dysfunction lowers NAD⁺, leading to a reduction in SIRT1/3 activity, further impairing PGC-1 α -driven biogenesis (Abu Shelbayeh *et al.*, 2023; Ventura-Clapier *et al.*, 2008). In the *Ndufs4* KO mouse model, PGC-1 α overexpression via bezafibrate treatment has demonstrated to restore neuronal development (Inka *et al.*, 2021), highlighting its potential as a therapeutic target.

Ndufs4-CI deficiency also influences HIF- α signalling through paradoxical brain hyperoxia during systemic hypoxia (Grange *et al.*, 2021; Wang *et al.*, 2024). In the whole-body *Ndufs4* KO mouse model, this oxygen imbalance exacerbates NAD⁺ depletion and neurodegeneration. Additionally, therapies such as HIF-2 α inhibition have shown the capability to mimic hypoxic conditions, normalise brain oxygen concentration, and improve redox balance, enhancing survival and reducing lesions in this mouse model (Wang *et al.*, 2024). Similarly, chronic hypoxia has also shown promise in improving NAD⁺ levels and alleviating neuropathology, positioning HIF- α and oxygen sensing as potential therapeutic targets (Grange *et al.*, 2021).

In summary, NDUF54-related CI deficiency affects several key molecular regulators. These alterations further contribute to the complex pathophysiology observed in *Ndufs4* KO models, providing insights into mechanisms underlying MDs like LS.

2.6. Diagnosis and treatment of MDs, including NDUF54-related CI deficiency

2.6.1. Diagnosis of MDs

Diagnosis of MDs is very challenging due to their genetic heterogeneity and limited awareness among general medical practitioners (Mavraki *et al.*, 2023). There is no single test that can detect the large number of genes involved in MDs to effectively establish a diagnosis (Alston *et al.*, 2017). Furthermore, the multisystemic pattern of MDs is likely to result in various clinical

presentations that do not always lead towards the same initial diagnosis, thereby further complicating clinical evaluation (Distelmaier *et al.*, 2009; Ghezzi & Zeviani, 2018). This complexity demands a more comprehensive and integrative diagnostic approach.

In the past few years, advancement in genetic testing has transformed diagnostic approaches from a "biopsy first" policy to genome-scale studies via next-generation sequencing (NGS) technologies. NGS enables the simultaneous assessment of both mtDNA and nuclear-encoded genes, enhancing diagnostic sensitivity and output (Chin *et al.*, 2024; Mavraki *et al.*, 2023). Whole exome sequencing (WES) has also become a widely used tool for molecular diagnosis due to its capability to assess a broad range of genes related to MDs. Additionally, blood tests are most often used to assess mtDNA heteroplasmy, even though muscle is more reliable in terms of verifying heteroplasmy levels with regards to tissue-specific expression (Koopman *et al.*, 2016; Rodenburg, 2016). For diagnosing CI deficiency, biochemical analyses are often performed on mitochondria isolated from muscle or skin biopsies. Muscle biopsies are generally needed for a definitive assessment since mitochondrial function can significantly differ in tissues like fibroblasts, compared to muscle (Koopman *et al.*, 2016). In addition, spectrophotometric assays, blue native polyacrylamide gel electrophoresis (BN-PAGE), and immunodetection are also used to assess the activity and structural integrity of OXPHOS complexes (Hoefs *et al.*, 2012; Valsecchi *et al.*, 2010).

The strengths of these diagnostic techniques lie in their ability to provide detailed insights into mitochondrial function and genetic mutations (Mavraki *et al.*, 2023). However, the invasiveness of muscle biopsies and expression heterogeneity variability within tissues for mtDNA heteroplasmy highlights potential drawbacks. Future diagnostic areas may include the introduction of novel technologies to enhance diagnostic specificity and reduce the invasiveness of tests. This can include the use of single cell sequencing and advanced imaging techniques (Alston *et al.*, 2021; Saneto, 2020). Additionally, existence of non-invasive biomarkers for mitochondrial disease would also significantly improve detection as well as early treatment (Ghezzi & Zeviani, 2018). Furthermore, emerging techniques such as multi-omics approaches are gaining attention for their potential in diagnosing mitochondrial disorders (Hong *et al.*, 2023; Wen *et al.*, 2025). Artificial intelligence (AI) and machine learning algorithms, such as artificial neural networks (ANNs), are also being investigated for their potential in predicting disease progression and improving diagnostic strategies (Giulivi *et al.*, 2023).

2.6.2. Treatment strategies for MDs

2.6.2.1. *Therapeutic interventions: Pharmacological approaches, gene therapy and mitochondrial transplantation*

Different therapeutic strategies (Figure 2-8) have been explored for treatment of CI deficiency, including pharmacologic agents, gene therapy, and mitochondrial targeted therapy (Bottani *et*

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al., 2020; van de Wal *et al.*, 2022). These strategies tend to alleviate symptoms and aid disease management by targeting specific pathways, however, no curative treatment exists. Given the inflammatory phenotype associated with CI deficiency, anti-inflammatory therapeutic strategies have been investigated. These include toll-like receptor (TLR) antagonists and systemic anti-inflammatory drugs, which have been reported to reduce both systemic and brain inflammation mediated by TLR pathways in the *Ndufs4* KO mouse model (Jin *et al.*, 2014). TLR antagonist treatment has also been shown to lengthen lifespan of mice by approximately 30 to 90 days, with therapeutic effects being largely due to reduced inflammation and improved mitochondrial function. These approaches hold potential for direct clinical translation. However, combination therapies are often needed, further complicating clinical translation due to potential drug interactions and long-term administration challenges (Jin *et al.*, 2014; Zhang *et al.*, 2020). Moreover, PI3Ky inhibition has also shown promise as a therapeutic treatment option in *Ndufs4* KO mice with regards to their potential in reducing inflammation (Stokes *et al.*, 2022). The treatment with PI3Ky inhibitors, such as p110 γ and IPI-549, increased survival from a median of 60 days to around 110 days, and reduced neuroinflammation outside of lesions.

Hypoxia therapy has also been shown as a promising therapeutic approach in *Ndufs4* KO mice (Falk, 2025; Jain *et al.*, 2016; Jain *et al.*, 2019). The advantages of hypoxia therapy is the fact that it can induce metabolic reprogramming, leading to increased mitochondrial biogenesis and glycolytic flux. This results in improved survival and disease phenotypes observed within the model, as demonstrated by studies that have manipulated mitochondrial function using hypoxia (Falk, 2025; Grange *et al.*, 2021; Jain *et al.*, 2016; Wang *et al.*, 2024). Normoxia-treated KO mice were observed to live up to around 60 days, while hypoxia-treated mice were observed to live between 170 days and 270 days, these mice eventually die from cardiac disease (Ferrari *et al.*, 2017; Jain *et al.*, 2016). However, the application of this therapeutic approach in humans is limited due to the risk of harm in the form of tissue hypoxia and the lack of control of hypoxic exposure in clinical settings (Falk, 2025; Grange *et al.*, 2021; Jain *et al.*, 2019; Lee *et al.*, 2019; Wang *et al.*, 2024).

Inhibition of the mTORC1 pathway has also been explored in *Ndufs4* KO mice. mTOR inhibition has shown the ability to delay neurological decline through enhancing mitophagy and metabolic regulation, attenuating ETC dysfunction. These observations are supported by evidence showing the role of mTOR signalling in autophagy and mitochondrial dynamics in the *Ndufs4* KO mouse model (Johnson *et al.*, 2013; Martin-Perez *et al.*, 2020). These studies also evaluated the potential of inhibiting the mTOR pathway, including the inhibition of S6K, a downstream protein of the mTORC1 pathway. Systemic or liver-specific S6K1 disruption through mTOR inhibitors like rapamycin has been shown to enhance neuronal survival and extended lifespan in *Ndufs4* KO mice (Ito *et al.*, 2017; Johnson *et al.*, 2013). However, S6K1 disruption in neurons or adipocytes did not produce similar benefits, indicating tissue-specific

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effects. In addition, PKC inhibitor treatment recapitulated several therapeutic effects of rapamycin, including reduced neuroinflammation and prevention of temporary alopecia in *Ndufs4* KO mice (Martin-Perez *et al.*, 2020). The chronic administration of rapamycin lowered lactate and glycolytic intermediate levels, reduced neurological decline and increased neuronal survival but not ETC activity (Johnson *et al.*, 2013; Stokes *et al.*, 2022). In these studies, rapamycin-treated *Ndufs4* KO mice survived up to approximately 80 days and 100 days or more, respectively, depending on the dosage and treatment timing. This treatment approach holds potential in managing disease progression of CI deficiency, though off-target effects like immunosuppression are a concern (Barriocanal-Casado *et al.*, 2019).

Furthermore, the drug acarbose, which is used for diabetes, has also been considered as an alternative pharmacological treatment strategy (Bitto *et al.*, 2022). Acarbose treatment has shown the ability to increase median survival from 50 days to 100 days or more, and improve disease phenotypes in *Ndufs4* KO mice in an mTOR independent manner by altering the gut microbiome. This aligns with evidence that highlights the importance of the gut-brain axis in MD (Bitto *et al.*, 2022; Qiao *et al.*, 2024). This approach holds significant potential to offer benefits through microbiome modulation; however, clinical application may be limited due to gastrointestinal side effects and variability of gut-microbiome responses across individuals (Zachos *et al.*, 2024).

A recent study used a lipid-selenium conjugate drug to target mitochondria in TMBIM6/NDUFS4-deficient mouse models (Chang *et al.*, 2024). This therapeutic approach demonstrated the ability to suppress mitophagy-mediated necroptosis and reduce malate and fumarate, improving mitophagy and decreasing pathological mitochondrial fission. This aligns with evidence regarding the involvement of mitophagy in maintaining mitochondrial health (Chang *et al.*, 2024; Zhang *et al.*, 2020). However, further research is needed to determine its safety and efficacy in humans. A phase 2b clinical trial assessed the safety and efficacy of the oral drug sonlicromanol in adult patients with Leber hereditary optic neuropathy, a primary MD caused by the m.3243A>G mutation (Smeitink *et al.*, 2024). Although the trial initially failed to achieve statistically significant improvements in attention, potential benefits were observed in areas like fatigue, balance, pain, and quality of life. These findings suggest that sonlicromanol may offer a promising therapeutic option for this type of MD, but its efficacy in other MDs remains to be investigated.

Gene therapy using viral vectors has also shown promise in the treatment of CI deficiency in *Ndufs4* KO mice by directly correcting underlying genetic abnormalities (Corrà *et al.*, 2022; Di Meo *et al.*, 2017; Hanaford *et al.*, 2022). This is consistent with research attesting to the therapeutic efficacy of gene therapy in the correction of genetic mutations in mitochondrial disease (Di Meo *et al.*, 2017; Hanaford *et al.*, 2022; Silva-Pinheiro *et al.*, 2020). One study employed AAV-delivered therapy with an AAV2/9 vector encoding the coding sequence of the

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human NDUFS4 [*hNDUFS4*] (Di Meo *et al.*, 2017). Intravenous (IV) administration corrected CI deficiency in peripheral tissues, while intracerebroventricular (ICV) delivery partially corrected brain deficiencies, consistent with findings on the importance of targeted gene delivery in mitochondrial diseases (Di Meo *et al.*, 2017). A different study using B-CMV-*hNDUFS4* vector treatment significantly prolonged the survival of *Ndufs4* KO mice, from a median survival time of 55 days to 100 days, with some of the treated mice surviving over a year (Silva-Pinheiro *et al.*, 2020).

Mitochondrial transplantation therapy has advanced as a treatment strategy for mitochondrial disorders such as LS (Clemente-Suárez *et al.*, 2023; McCully *et al.*, 2022; Nakai *et al.*, 2024). This technique enables the transfer of healthy mitochondria into diseased cells, potentially restoring mitochondrial bioenergetics. This is further supported with evidence demonstrating the therapeutic effectiveness of mitochondrial transplantation to improve mitochondrial function in disease models (McCully *et al.*, 2022; McCully *et al.*, 2023; Zhang *et al.*, 2020). The average survival extension from mitochondrial transfer therapy in cell cultures and mouse models ranges from a median of 50 to 180 days, depending on the disease model and timing of the intervention (McCully *et al.*, 2023; Nakai *et al.*, 2024). Additionally, the administration of isolated mitochondria from *Ndufs4* WT mice has been reported to extend lifespan, improve neurological function and increase energy expenditure of *Ndufs4* KO mice (Nakai *et al.*, 2024). This technique may offer a novel strategy to directly address mitochondrial dysfunction; however, immune rejection, scalability, and the durability of transplanted mitochondria *in vivo* are significant challenges in clinical application (D'Amato *et al.*, 2023).

In summary, these therapeutic strategies highlight significant progress in addressing the complexities of MDs like LS. The pharmacological targeting of inflammatory and metabolic pathways, as well as the advancements of gene therapy and mitochondrial transplantation, underscore the potential of these approaches for meaningful clinical interventions. These approaches still face significant limitations, ranging from cost-effectiveness, invasiveness, tissue specificity and delivery, to safety concerns (Clemente-Suárez *et al.*, 2023; D'Amato *et al.*, 2023; Donfrancesco *et al.*, 2022; Herring *et al.*, 2024). This emphasises the need for continued refinement, innovation and the development of feasible clinical translatable therapies.

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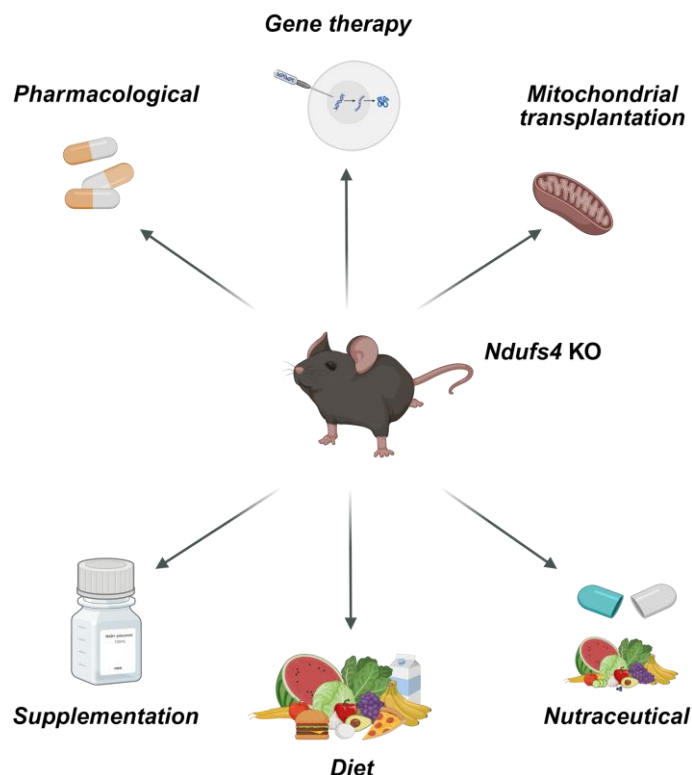


Figure 2-8: Therapeutic interventions in the *Ndufs4* KO mouse model. Various therapeutic strategies have been assessed in the treatment of CI-deficiency in LS mouse models. These therapies range from pharmacological administration, gene therapy and mitochondrial transplantation to vitamin supplementation, nutraceuticals and dietary intervention. *Abbreviations: KO, knockout.*

2.6.2.2. Therapeutic interventions: vitamin supplementation, nutraceuticals and dietary interventions

In addition to pharmacological, genetic, and mitochondrial transfer therapies, other therapies for mitochondrial disorders have emerged as alternative strategies to improve mitochondrial function and alleviate disease symptoms (van de Wal *et al.*, 2022). These therapies include vitamin supplements, nutraceuticals, and dietary intervention. These approaches offer cost-effective and non-invasive strategies that can complement existing therapies and improve patient outcomes (Makkar *et al.*, 2020; Maksoud *et al.*, 2021).

Vitamin and cofactor supplementation, such as riboflavin, CoQ10, vitamins B1, B12, C, E, K, and L-carnitine, have been shown to enhance mitochondrial function by supporting oxidative phosphorylation, improving energy production, and reducing oxidative stress (de Haas *et al.*, 2017; Distelmaier *et al.*, 2009; Iqbal *et al.*, 2022; Koene *et al.*, 2011; Neugebauer *et al.*, 2025). Furthermore, sirtuin activation and calcium homeostasis supplementation approaches have been explored to boost mitochondrial resilience. Antioxidants like Trolox, a vitamin E analogue, has shown potential in reducing ROS as well as improving CI assembly in models like *Ndufs4* KO mice (de Haas *et al.*, 2017; Koopman *et al.*, 2008). In these mice, antioxidant supplementation was observed to result in a modest lifespan extension from a median of 50

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days to 180 days, depending on the treatment and timing. Additionally, vatiquinone, a vitamin E-derivative that has been shown to target ferroptosis, was also evaluated in *Ndufs4* KO mice (Kayser *et al.*, 2025). Where this treatment demonstrated the ability to reduce seizure risk, it showed no significant impact on survival or disease progression. Although these approaches do not completely rescue CI deficiency, they provide non-invasive therapeutic options that may support mitochondrial health in MDs (de Haas *et al.*, 2017; Distelmaier *et al.*, 2009; Koene *et al.*, 2011; Koopman *et al.*, 2008; Neugebauer *et al.*, 2025).

A study by Lee *et al.* (2019), that evaluated the effect of NMN, a NAD⁺ precursor in the *Ndufs4* KO mouse model, showed that this supplementation extended lifespan and reduced lactic acidosis by restoring the NAD⁺ redox balance. This treatment also inhibited HIF1- α in the skeletal muscle without any effects in the brain (Lee *et al.*, 2019). NMN supplementation also increased levels of α -KG, a critical factor in HIF1 α degradation, specifically in muscle tissue. The same study also evaluated the effect of dimethyl ketoglutarate (DMKG), a cell-permeable α -KG analogue, which significantly improved survival in *Ndufs4* KO mice (Lee *et al.*, 2019). DMKG demonstrated the enhancement of compensatory CII-respiration via the TCA cycle and inhibited hypoxia signalling, as shown by reductions in HIF1 α and lactate dehydrogenase A (LDHA) levels, without affecting the NAD⁺ levels within the brain. Additionally, α -KG and malate supplementation, which serve as alternative substrates for CI, have also been proposed as a therapeutic approach in mitochondrial dysfunction. This strategy bypasses partial CI deficiencies, maintaining electron transport and mitochondrial respiration (Kayser *et al.*, 2016).

Furthermore, a study evaluating the impact of iron on MD progression in the *Ndufs4* KO mouse model (Kelly *et al.*, 2023), found that iron supplementation worsens symptoms while iron restriction delays symptom onset, reduces neuroinflammation, and prolongs lifespan. These findings highlighted an underlying relationship between iron homeostasis dysregulation and MD pathology (Kelly *et al.*, 2023). The mice that received an iron restricted diet displayed improved survival from a median of 55 days to 75 days compared to mice receiving a diet with normal iron levels. These results revealed an iron overload in the livers of sick mice along with an altered expression of iron-regulated genes. The results underscore the potential of iron chelation as a therapeutic intervention for mitochondrial disease.

The ketogenic diet (KD), defined as a low-carbohydrate, high-fat diet, has emerged as a therapeutic strategy for MDs with OXPHOS defects (Huang *et al.*, 2022; Qu *et al.*, 2021; Zweers *et al.*, 2021). By providing ketones as an alternative fuel to glucose, the KD may alleviate energy deficits in tissues with impaired mitochondrial function, while mitigating oxidative stress and reducing inflammation (Qu *et al.*, 2021; Zweers *et al.*, 2021). In addition, KDs have demonstrated significant therapeutic potential in delaying neurological symptoms like seizures and improving motor coordination in other models of MD (Qu *et al.*, 2021). These include models such as Alpers-Huttenlocher syndrome in clinical patients (Joshi *et al.*, 2009), early-

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onset ophthalmoplegia in clinical patients (Laugel *et al.*, 2007), and mitochondrial myopathy in rodent models and clinical patients (Ahola-Erkkilä *et al.*, 2010; Ahola *et al.*, 2016). Other models also investigated include clinical patients afflicted with CI dysfunction induced epileptic seizures (Zweers *et al.*, 2021).

The KD's potential to inhibit the mTOR pathway also represents a key mechanism with therapeutic implications for MDs (McDaniel *et al.*, 2021). A study using a kainic acid-induced epileptic model observed an improvement in seizure control that continued even after the dietary intervention was stopped (McDaniel *et al.*, 2021). The inhibition of mTOR may mitigate pathological processes, such as neuronal hyperexcitability and mitochondrial dysfunction, which are common in various mitochondrial disorders (Zhu *et al.*, 2022). In addition to mTOR inhibition, the KD has also been reported to enhance mitochondrial biogenesis and redox homeostasis (Gano *et al.*, 2014; Zhu *et al.*, 2022). In rodent models, the KD has demonstrated to increase PGC-1 α , a master regulator of mitochondrial biogenesis (Gano *et al.*, 2014; Zhu *et al.*, 2022). From these findings, it is evident that dietary modulation like a KD may hold therapeutic potential in the treatment of mitochondrial disorders. However, the application of KDs in the context of NDUFS4-related CI deficiency, particularly in models such as the *Ndufs4* KO mouse model of LS, has yielded mixed results.

A study done using the *Ndufs4* KO mouse model of LS, observed that a KD was successful in inducing ketosis and partially alleviated seizures but failed to improve cachexia, motor deficits, or lifespan (Bornstein *et al.*, 2022). Additionally, *Ndufs4* KO mice were unable to fully tolerate the standard KD formulation, and as such, dietary adaptations were necessary. This raises an important consideration, while all KDs are high-fat diets (HFDs), not all HFDs are ketogenic. Therapeutic HFDs may not need to focus on strict carbohydrate restriction but can instead prioritise fatty acid composition to target mitochondrial dysfunction. In addition, while strict carbohydrate restriction in KDs can induce ketosis, it may also strain glucose-dependent pathways in tissues afflicted with CI deficiency. Modified HFDs may bypass this by improving fatty acid composition, rather than carbohydrate restriction, and directly enhance mitochondrial function and redox balance.

Moreover, when evaluating the effectiveness of dietary interventions – such as HFDs, like the KD or others for the treatment of MD like LS – it is essential to consider several key factors that may influence treatment outcomes. Firstly, the timing of dietary intervention relative to disease onset is critical, as early interventions may offer greater therapeutic benefit (Qu *et al.*, 2021). Secondly, the dietary modification, like the type of fats used, for example, the ratio of omega-3 (ω -3) to omega-6 (ω -6) polyunsaturated fatty acids (PUFAs), and micronutrient content, must be accurately tailored to the specific metabolic and mitochondrial deficits of the disease model of interest (de Oliveira *et al.*, 2017; Nathan *et al.*, 2019; Qu *et al.*, 2021). Finally, dietary interventions must not only be employed to solely induce ketosis but must also address inherent

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mitochondrial dysfunction, particularly in conditions like LS (Qu *et al.*, 2021). This could potentially explain why a KD has been shown to be effective in some MD models but not in LS models, such as the *Ndufs4* KO mouse model, along with the differences in human and animal models.

While the standard KD usually consists of medium-chain triglycerides (MCTs) or long-chain fatty acids to produce ketones, the specific fatty acid composition of the diet is an essential factor that can impact mitochondrial function (Barzegar *et al.*, 2021). Although MCTs in some KDs are less prone to promoting inflammation, saturated long-chain fatty acids (e.g., palmitate) commonly found in animal-based KD formulations can exacerbate mitochondrial dysfunction and oxidative stress (Ferreira *et al.*, 2025; Mitchelson *et al.*, 2025; Nishida *et al.*, 2024). Studies have demonstrated that not all fatty acids are equally effective in influencing and supporting mitochondrial health (Rohrbach, 2009; Sullivan *et al.*, 2018). Additionally, PUFAs, such as ω -3 and ω -6 fatty acids, have shown the capability to modulate mitochondrial membrane fluidity, enhance mitochondrial biogenesis, reduce oxidative stress and enhance neuromuscular health (Herbst *et al.*, 2014; Pollicino *et al.*, 2023; Rohrbach, 2009; Stanley *et al.*, 2012). Unlike saturated fats often used in standard KDs, PUFAs, like ω -3 fatty acids, have been shown to reduce lipid peroxidation and inflammation, which is a critical aspect of CI deficiency (Herbst *et al.*, 2014; Pollicino *et al.*, 2023). This positions PUFA-enriched HFDs as a potential alternative to standard KDs in MD models. Moreover, studies employing PUFA supplementation and PUFA-enriched HFDs suggest that PUFAs may enhance therapeutic efficacy (Liu *et al.*, 2020; Shirooie *et al.*, 2018). In addition, these studies have shown that PUFAs have the potential to improve mitochondrial membrane fluidity, biogenesis, and redox balance while modulating mTOR signalling (Liu *et al.*, 2020; Shirooie *et al.*, 2018). Although PUFA-supplemented HFDs remain to be evaluated in LS models, their therapeutic potential in neurodegenerative and metabolic disorders position them as potential candidates (Liu *et al.*, 2020; Pollicino *et al.*, 2023; Stanley *et al.*, 2012). The failure of standard KDs in the *Ndufs4* KO model emphasises a critical gap. While ketosis may alleviate energy deficits and seizures, it does not address LS-specific pathologies like neuroinflammation or lipid peroxidation. The optimisation of HFDs that prioritises PUFA composition may fill this gap by addressing various LS-specific pathologies without the metabolic stress of strict carbohydrate restriction. Also, the brain's high lipid content emphasises its sensitivity to fatty acid composition. As such, PUFA-enriched HFDs may be beneficial for neuronal membranes and potentially suppress neuroinflammation in LS models, as standard KDs high in saturated fats lack these neuroprotective effects.

In summary, while the various therapeutic strategies discussed show promise in the treatment of CI deficiency, a definitive cure remains elusive. Pharmacological, gene therapy and mitochondrial transplantation interventions show the potential to enhance or potentially restore mitochondrial function and reduce inflammation, offering valuable insights to therapeutic

advancements. However, their limitations include cost-effectiveness, invasiveness and safety concerns (D'Amato *et al.*, 2023; Herring *et al.*, 2024). On the contrary, vitamin and co-factor supplementation, nutraceuticals, and dietary therapies like HFDs, offer non-invasive, safer and more affordable strategies to enhance mitochondrial function, prolong lifespan, and alleviate symptoms (Makkar *et al.*, 2020; Maksoud *et al.*, 2021). However, while the KD has succeeded as a therapeutic approach in other MD models, application in *Ndufs4* KO model of LS has not been as successful. The relative effectiveness of standard KDs in *Ndufs4*-associated LS underscores the need for optimising dietary interventions like HFDs beyond carbohydrate restriction. Additionally, non-ketogenic PUFA-enriched HFD formulations may mitigate the poor tolerance of standard KDs observed in *Ndufs4* KO mice by eliminating reliance on ketosis (Bornstein *et al.*, 2022). These dietary formulations could leverage PUFAs' dual role in mitochondrial health and anti-inflammatory signalling while targeting LS-specific mechanisms, such as redox imbalance and neuroinflammation, without depending on ketosis. While KDs depend on ketosis to bypass potential defects in glycolysis, PUFA-enriched HFDs may operate along ketosis-independent pathways, which may enhance mitochondrial membrane fluidity, redox state, and improve energy metabolism. This underscores the potential of PUFA-enriched HFDs as a promising therapeutic strategy for CI deficiencies, aligning with this study's focus on evaluating their application in the *Ndufs4* KO mouse model of LS.

2.7. References

- Abu Shelbayeh, O., Arroum, T., Morris, S. & Busch, K.B. 2023. PGC-1 α Is a Master Regulator of Mitochondrial Lifecycle and ROS Stress Response. *Antioxidants*, 12(5), 1075.
- Adjobo-Hermans, M.J.W., de Haas, R., Willems, P.H.G.M., Wojtala, A., van Emst-de Vries, S.E., Wagenaars, J.A., ... Koopman, W.J.H. 2020. NDUFS4 deletion triggers loss of NDUFA12 in *Ndufs4*^{-/-} mice and Leigh syndrome patients: A stabilizing role for NDUFAF2. *Biochim. Biophys. Acta, Bioenerg.*, 1861(8):148213.
- Ahola-Erkkilä, S., Carroll, C.J., Peltola-Mjösund, K., Tulkki, V., Mattila, I., Seppänen-Laakso, T., ... Suomalainen, A. 2010. Ketogenic diet slows down mitochondrial myopathy progression in mice. *Hum. Mol. Genet.*, 19(10):1974-1984.
- Ahola, S., Auranen, M., Isohanni, P., Niemisalo, S., Urho, N., Buzkova, J., ... Muurinen, T. 2016. Modified Atkins diet induces subacute selective ragged-red-fiber lysis in mitochondrial myopathy patients. *EMBO Mol. Med.*, 8(11):1234-1247.
- Alam, M.T., Manjeri, G.R., Rodenburg, R.J., Smeitink, J.A.M., Notebaart, R.A., Huynen, M., ... Koopman, W.J.H. 2015. Skeletal muscle mitochondria of *NDUFS4*^{-/-} mice display normal maximal pyruvate oxidation and ATP production. *Biochim. Biophys. Acta, Bioenerg.*, 1847(6):526-533.
- Alston, C.L., Rocha, M.C., Lax, N.Z., Turnbull, D.M. & Taylor, R.W. 2017. The genetics and pathology of mitochondrial disease. *J. Pathol.*, 241(2):236-250.
- Alston, C.L., Stenton, S.L., Hudson, G., Prokisch, H. & Taylor, R.W. 2021. The genetics of mitochondrial disease: dissecting mitochondrial pathology using multi-omic pipelines. *J. Pathol.*, 254(4):430-442.

CHAPTER 2

- Arnarez, C., Marrink, S.J. & Periole, X. 2016. Molecular mechanism of cardiolipin-mediated assembly of respiratory chain supercomplexes. *Chem. Sci.*, 7(7):4435-4443.
- Baev, A.Y., Vinokurov, A.Y., Novikova, I.N., Dremin, V.V., Potapova, E.V. & Abramov, A.Y. 2022. Interaction of Mitochondrial Calcium and ROS in Neurodegeneration. *Cells*, 11(4):706.
- Balsa, E., Perry, E.A., Bennett, C.F., Jedrychowski, M., Gygi, S.P., Doench, J.G. & Puigserver, P. 2020. Defective NADPH production in mitochondrial disease complex I causes inflammation and cell death. *Nat. Commun.*, 11(1):2714.
- Barriocanal-Casado, E., Hidalgo-Gutiérrez, A., Raimundo, N., González-García, P., Acuña-Castroviejo, D., Escames, G. & López, L.C. 2019. Rapamycin administration is not a valid therapeutic strategy for every case of mitochondrial disease. *eBioMedicine*, 42:511-523.
- Barzegar, M., Afghan, M., Tarmahi, V., Behtari, M., Rahimi Khamaneh, S. & Raeisi, S. 2021. Ketogenic diet: overview, types, and possible anti-seizure mechanisms. *Nutr. Neurosci.*, 24(4):307-316.
- Basu, U., Bostwick, A.M., Das, K., Dittenhafer-Reed, K.E. & Patel, S.S. 2020. Structure, mechanism, and regulation of mitochondrial DNA transcription initiation. *J. Biol. Chem.*, 295(52):18406-18425.
- Bennett, N.K., Lee, M., Orr, A.L. & Nakamura, K. 2023. Systems-level analyses dissociate genetic regulators of reactive oxygen species and energy production. *Proc. Natl Acad. Sci. USA.*, 121(3):e2307904121.
- Bertaux, A., Cabon, L., Brunelle-Navas, M.N., Bouchet, S., Nemazanyy, I. & Susin, S.A. 2018. Mitochondrial OXPHOS influences immune cell fate: lessons from hematopoietic AIF-deficient and NDUFS4-deficient mouse models. *Cell Death Dis.*, 9(6):581.
- Bitto, A., Grillo, A.S., Stanaway, I.B., Nguyen, B.M.G., Ying, K., Tung, H., ... Kaeberlein, M. 2022. Acarbose suppresses symptoms of mitochondrial disease in a mouse model of Leigh Syndrome. *Nat. Metab.* 5, 955–967.
- Blaza, J.N., Serreli, R., Jones, A.J., Mohammed, K. & Hirst, J. 2014. Kinetic evidence against partitioning of the ubiquinone pool and the catalytic relevance of respiratory-chain supercomplexes. *PNAS*, 111(44):15735-15740.
- Boekema, E.J. & Braun, H.-P. 2007. Supramolecular structure of the mitochondrial oxidative phosphorylation system. *J. Biol. Chem.*, 282(1):1-4.
- Bolea, I., Gella, A., Sanz, E., Prada-Dacasa, P., Menardy, F., Bard, A.M., ... Quintana, A. 2019. Defined neuronal populations drive fatal phenotype in a mouse model of Leigh syndrome. *ellife*, 8:e47163.
- Bornstein, R., Mulholland, M.T., Sedensky, M., Morgan, P. & Johnson, S.C. 2023. Glutamine metabolism in diseases associated with mitochondrial dysfunction. *Mol. Cell. Neurosci.*, 126:103887.
- Bornstein, R., James, K., Stokes, J., Park, K.Y., Kayser, E.B., Snell, J., ... Johnson, S.C. 2022. Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy. *Neurobiol. Dis.*, 163:105594.
- Bottani, E., Lamperti, C., Prigione, A., Tiranti, V., Persico, N. & Brunetti, D. 2020. Therapeutic Approaches to Treat Mitochondrial Diseases: "One-Size-Fits-All" and "Precision Medicine" Strategies. *Pharmaceutics*, 12(11):1083.
- Budde, S.M., van den Heuvel, L.P., Janssen, A.J., Smeets, R.J., Buskens, C.A., DeMeirleir, L., ... Smeitink, J.A. 2000. Combined enzymatic complex I and III deficiency associated with mutations in the nuclear encoded NDUFS4 gene. *Biochem. Biophys. Res. Commun.*, 275(1):63-68.
- Calvaruso, M.A., Willems, P., van den Brand, M., Valsecchi, F., Kruse, S., Palmiter, R., ... Nijtmans, L. 2011. Mitochondrial complex III stabilizes complex I in the absence of NDUFS4 to provide partial activity. *Hum. Mol. Genet.*, 21(1):115-120.

CHAPTER 2

- Calvo, S.E., Clauser, K.R. & Mootha, V.K. 2016. MitoCarta2. 0: an updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res.*, 44(D1):D1251-D1257.
- Carrozzo, R., Rizza, T., Stringaro, A., Pierini, R., Mormone, E., Santorelli, F.M., ... Matarrese, P. 2004. Maternally-inherited Leigh syndrome-related mutations bolster mitochondrial-mediated apoptosis. *J. Neurochem.*, 90(2):490-501.
- Chang, X., Zhou, H., Hu, J., Ge, T., He, K., Chen, Y., ... Fan, X. 2024. Targeting mitochondria by lipid-selenium conjugate drug results in malate/fumarate exhaustion and induces mitophagy-mediated necroptosis suppression. *Int. J. Biol. Sci.*, 20(14):5793.
- Chen, Q., Kirk, K., Shurubor, Y.I., Zhao, D., Arreguin, A.J., Shahi, I., ... D'Aurelio, M. 2018. Rewiring of Glutamine Metabolism Is a Bioenergetic Adaptation of Human Cells with Mitochondrial DNA Mutations. *Cell Metab.*, 27(5):1007-1025.e1005.
- Chen, W., Zhao, H. & Li, Y. 2023. Mitochondrial dynamics in health and disease: mechanisms and potential targets. *Signal Transduct. Target Ther.*, 8(1):333.
- Chin, H.L., Lai, P.S. & Tay, S.K.H. 2024. A clinical approach to diagnosis and management of mitochondrial myopathies. *Neurotherapeutics*, 21(1):e00304.
- Chinnery, P.F. & Hudson, G. 2013. Mitochondrial genetics. *Br. Med. Bull.*, 106(1):135-159.
- Clapham, D.E. 2007. Calcium Signaling. *Cell*, 131(6):1047-1058.
- Clemente-Suárez, V.J., Redondo-Flórez, L., Beltrán-Velasco, A.I., Ramos-Campo, D.J., Belinchón-deMiguel, P., Martínez-Guardado, I., ... Tornero-Aguilera, J.F. 2023. Mitochondria and brain disease: a comprehensive review of pathological mechanisms and therapeutic opportunities. *Biomedicines*, 11(9):2488.
- Corrà, S., Cerutti, R., Balmaceda, V., Viscomi, C. & Zeviani, M. 2022. Double administration of self-complementary AAV9 NDUFS4 prevents Leigh disease in *Ndufs4*^{-/-} mice. *Brain*, 145(10):3405-3414.
- D'Amato, M., Morra, F., Di Meo, I. & Tiranti, V. 2023. Mitochondrial Transplantation in Mitochondrial Medicine: Current Challenges and Future Perspectives. *Int. J. Mol. Sci.*, 24(3):1969.
- de Haas, R., Das, D., Garanto, A., Renkema, H.G., Greupink, R., van den Broek, P., ... Smeitink, J.A. 2017. Therapeutic effects of the mitochondrial ROS-redox modulator KH176 in a mammalian model of Leigh Disease. *Sci. Rep.*, 7(1):11733.
- de Oliveira, M.R., Nabavi, S.F., Nabavi, S.M. & Jardim, F.R. 2017. Omega-3 polyunsaturated fatty acids and mitochondria, back to the future. *Trends Food Sci. Technol.*, 67:76-92.
- De Stefani, D., Raffaello, A., Teardo, E., Szabò, I. & Rizzuto, R. 2011. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature*, 476(7360):336-340.
- Di Donfrancesco, A., Massaro, G., Di Meo, I., Tiranti, V., Bottani, E. & Brunetti, D. 2022. Gene therapy for mitochondrial diseases: Current status and future perspective. *Pharmaceutics*, 14(6):1287.
- Di Meo, I., Marchet, S., Lamperti, C., Zeviani, M. & Viscomi, C. 2017. AAV9-based gene therapy partially ameliorates the clinical phenotype of a mouse model of Leigh syndrome. *Gene. Ther.*, 24(10):661-667.
- Dickinson, A., Yeung, K., Donoghue, J., Baker, M., Kelly, R.D., McKenzie, M., ... St John, J.C. 2013. The regulation of mitochondrial DNA copy number in glioblastoma cells. *Cell Death Differ.*, 20(12):1644-1653.
- Distelmaier, F., Koopman, W.J., Van Den Heuvel, L.P., Rodenburg, R.J., Mayatepek, E., Willems, P.H. & Smeitink, J.A. 2009. Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease. *Brain*, 132(4):833-842.
- El-Hattab, A.W., Adesina, A.M., Jones, J. & Scaglia, F. 2015. MELAS syndrome: Clinical manifestations, pathogenesis, and treatment options. *Mol. Genet. Metab.*, 116(1):4-12.

CHAPTER 2

- Esterhuizen, K., van der Westhuizen, F.H. & Louw, R. 2017. Metabolomics of mitochondrial disease. *Mitochondrion*, 35:97-110.
- Falk, M.J. 2025. Small-molecule hypoxia therapy in mitochondrial disease. *Cell*, 188(6):1462-1465.
- Fernandez-Vizarra, E. & Zeviani, M. 2021. Mitochondrial disorders of the OXPHOS system. *FEBS Letters*, 595(8):1062-1106.
- Fernández-Vizarra, E., Callegari, S., Garrabou, G. & Pacheu-Grau, D. 2022. Editorial: Mitochondrial OXPHOS System: Emerging Concepts and Technologies and Role in Disease. *Front. Cell. Dev. Biol.*, 10:924272.
- Ferrari, M., Jain, I.H., Goldberger, O., Rezoagli, E., Thoonen, R., Cheng, K.H., ... Zapol, W.M. 2017. Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome. *Proc. Natl. Acad. Sci. USA*, 114(21):E4241-e4250.
- Ferreira, T. & Rodriguez, S. 2024. Mitochondrial DNA: Inherent Complexities Relevant to Genetic Analyses. *Genes*, 15(5):617.
- Ferreira, Y.A.M., Estadella, D., Pisani, L.P. 2025. Effect of Different Fatty Acid Types on Mitochondrial Dysfunction Associated With Brown and Beige Adipose Tissue. *Nutr. Rev.*, 15:nuaf048.
- Fiedorczuk, K., Letts, J.A., Degliesposti, G., Kaszuba, K., Skehel, M. & Sazanov, L.A. 2016. Atomic structure of the entire mammalian mitochondrial complex I. *Nature*, 538(7625):406-410.
- Foriel, S., Beyrath, J., Eidhof, I., Rodenburg, R.J., Schenck, A. & Smeitink, J.A.M. 2018. Feeding difficulties, a key feature of the Drosophila NDUF54 mitochondrial disease model. *Dis. Model Mech.*, 11(3):dmm032482.
- Formosa, L.E., Dibley, M.G., Stroud, D.A. & Ryan, M.T. 2018. Building a complex complex: Assembly of mitochondrial respiratory chain complex I. *Semin. Cell Dev. Biol.*, 76:154-162.
- Gano, L.B., Patel, M. & Rho, J.M. 2014. Ketogenic diets, mitochondria, and neurological diseases. *J. Lipid Res.*, 55(11):2211-2228.
- Gao, H., Jensen, K., Nesbit, J., Ostroot, M., Baloni, P., Funk, C. & Trushina, E. 2024. *Ndufs4* knockout induces transcriptomic signatures of Alzheimer's Diseases that are partially reversed by mitochondrial complex I inhibitor. *bioRxiv*:2024.2002.2020.581247.
- Gella, A., Prada-Dacasa, P., Carrascal, M., Urpi, A., González-Torres, M., Abian, J., ... Quintana, A. 2020. Mitochondrial Proteome of Affected Glutamatergic Neurons in a Mouse Model of Leigh Syndrome. *Front. Cell Dev. Biol.*, 8:660.
- Ghezzi, D. & Zeviani, M. 2018. Human diseases associated with defects in assembly of OXPHOS complexes. *Essays Biochem.*, 62(3):271-286.
- Giorgi, C., Danese, A., Missiroli, S., Patergnani, S. & Pinton, P. 2018. Calcium Dynamics as a Machine for Decoding Signals. *Trends in Cell Biology*, 28(4):258-273.
- Giorgi, C., Agnoletto, C., Bononi, A., Bonora, M., De Marchi, E., Marchi, S., ... Pinton, P. 2012. Mitochondrial calcium homeostasis as potential target for mitochondrial medicine. *Mitochondrion*, 12(1):77-85.
- Giulivi, C., Zhang, K. & Arakawa, H. 2023. Recent advances and new perspectives in mitochondrial dysfunction. *Sci. Rep.*, 13(1):7977.
- Goolab, S., Terburgh, K., du Plessis, C., Scholefield, J. & Louw, R. 2025. CRISPR-Cas9 mediated knockout of NDUF54 in human iPSCs: A model for mitochondrial complex I deficiency. *Biochim. Biophys. Acta - Mol. Basis Dis.*, 1871(2):167569.

CHAPTER 2

- Gorman, G.S., Schaefer, A.M., Ng, Y., Gomez, N., Blakely, E.L., Alston, C.L., ... Chinnery, P.F. 2015. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Ann. Neurol.*, 77(5):753-759.
- Gorman, G.S., Chinnery, P.F., DiMauro, S., Hirano, M., Koga, Y., McFarland, R., ... Turnbull, D.M. 2016. Mitochondrial diseases. *Nat. Rev. Dis.*, 2(1):1-22.
- Grange, R.M.H., Sharma, R., Shah, H., Reinstadler, B., Goldberger, O., Cooper, M.K., ... Zapol, W.M. 2021. Hypoxia ameliorates brain hyperoxia and NAD(+) deficiency in a murine model of Leigh syndrome. *Mol. Genet. Metab.*, 133(1):83-93.
- Greaves, L.C., Reeve, A.K., Taylor, R.W. & Turnbull, D.M. 2012. Mitochondrial DNA and disease. *J. Pathol.*, 226(2):274-286.
- Gusic, M. & Prokisch, H. 2021. Genetic basis of mitochondrial diseases. *FEBS Letters*, 595(8):1132-1158.
- Haissaguerre, M., Saucisse, N. & Cota, D. 2014. Influence of mTOR in energy and metabolic homeostasis. *Mol. Cell. Endocrinol.*, 397(1):67-77.
- Hanaford, A.R., Khanna, A., James, K., Truong, V., Liao, R., Chen, Y., ... Johnson, S.C. 2024. Interferon-gamma contributes to disease progression in the *Ndufs4*(-/-) model of Leigh syndrome. *Neuropathol Appl. Neurobiol.*, 50(3):e12977.
- Hatefi, Y. 1985. The mitochondrial electron transport and oxidative phosphorylation system. *Annu. Rev. Biochem.*, 54:1015-1069.
- Herbst, E.A., Paglialunga, S., Gerling, C., Whitfield, J., Mukai, K., Chabowski, A., ... Holloway, G.P. 2014. Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle. *J. Physiol.*, 592(6):1341-1352.
- Hernández, C.L. 2023. Mitochondrial DNA in Human Diversity and Health: From the Golden Age to the Omics Era. *Genes*, 14(8):1534.
- Herring, W.L., Gallagher, M.E., Shah, N., Morse, K.C., Mladsi, D., Dong, O.M., ... Andemariam, B. 2024. Cost-Effectiveness of Lovotibeglogene Autotemcel (Lovo-Cel) Gene Therapy for Patients with Sickle Cell Disease and Recurrent Vaso-Occlusive Events in the United States. *Pharmacoeconomics*, 42(6):693-714.
- Hoefs, S.J.G., Rodenburg, R.J., Smeitink, J.A.M. & van den Heuvel, L.P. 2012. Molecular base of biochemical complex I deficiency. *Mitochondrion*, 12(5):520-532.
- Hong, S., Kim, S., Kim, K. & Lee, H. 2023. Clinical Approaches for Mitochondrial Diseases. *Cells*, 12(20):2494.
- Hong, W.L., Huang, H., Zeng, X. & Duan, C.Y. 2024. Targeting mitochondrial quality control: new therapeutic strategies for major diseases. *Mil. Med. Res.*, 11(1):59.
- Howell, N., Elson, J.L., Chinnery, P.F. & Turnbull, D.M. 2005. mtDNA mutations and common neurodegenerative disorders. *Trends in Genetics*, 21(11):583-586.
- Huang, L., Li, H., Zhong, J., Yang, L., Chen, G., Wang, D., ... Sun, D. 2022. Efficacy and Safety of the Ketogenic Diet for Mitochondrial Disease With Epilepsy: A Prospective, Open-labeled, Controlled Study. *Front. Neurol.*, 13:880944.
- Inak, G., Rybak-Wolf, A., Lisowski, P., Pentimalli, T.M., Jüttner, R., Glažar, P., ... Prigione, A. 2021. Defective metabolic programming impairs early neuronal morphogenesis in neural cultures and an organoid model of Leigh syndrome. *Nat. Commun.*, 12(1):1929.
- Iqbal, S., Shah, M.A., Rasul, A., Shah, S., Shah, G.M., Irfan, M., ... Khan, A.H. 2022. Chapter 10 - Therapeutic role of nutraceuticals in mitochondrial disorders. In: Khan, H., Akkol, E.K. & Daglia, M., eds. *Role Phytonutr. Metab. Dis.*, 313-358.

CHAPTER 2

- Ishikawa, K. & Nakada, K. 2021. Attempts to understand the mechanisms of mitochondrial diseases: The reverse genetics of mouse models for mitochondrial disease. *Biochim. Biophys. Acta Gen. Subj.*, 1865(3):129835.
- Ito, T.K., Lu, C., Khan, J., Nguyen, Q., Huang, H.Z., Kim, D., ... Kaeberlein, M. 2017. Hepatic S6K1 Partially Regulates Lifespan of Mice with Mitochondrial Complex I Deficiency. *Front. Genet.*, 8:113.
- Jain, I.H., Zazzeron, L., Goli, R., Alexa, K., Schatzman-Bone, S., Dhillon, H., ... Mootha, V.K. 2016. Hypoxia as a therapy for mitochondrial disease. *Science*, 352(6281):54-61.
- Jain, I.H., Zazzeron, L., Goldberger, O., Marutani, E., Wojtkiewicz, G.R., Ast, T., ... Mootha, V.K. 2019. Leigh Syndrome Mouse Model Can Be Rescued by Interventions that Normalize Brain Hyperoxia, but Not HIF Activation. *Cell Metab.*, 30(4):824-832.e823. 10.1016/j.cmet.2019.07.006
- JAX. 2017. Mouse strain datasheet - 027058. <https://www.jax.org/strain/027058> Date of access: 10 Mar. 2017.
- JAX. 2020b. Mouse strain datasheet - 027058. <https://www.jax.org/strain/027058>. Date of access: 8 Jan. 2020.
- Ježek, P., Jabůrek, M., Holendová, B., Engstová, H. & Dlasková, A. 2023. Mitochondrial Cristae Morphology Reflecting Metabolism, Superoxide Formation, Redox Homeostasis, and Pathology. *Antioxid. Redox Signal.*, 39(10-12):635-683.
- Jiang, D., Xiong, G., Feng, H., Zhang, Z., Chen, P., Yan, B., ... Lian, Q. 2019. Donation of mitochondria by iPSC-derived mesenchymal stem cells protects retinal ganglion cells against mitochondrial complex I defect-induced degeneration. *Theranostics*, 9(8):2395-2410.
- Jin, Z., Wei, W., Yang, M., Du, Y. & Wan, Y. 2014. Mitochondrial Complex I Activity Suppresses Inflammation and Enhances Bone Resorption by Shifting Macrophage-Osteoclast Polarization. *Cell Metab.*, 20(3):483-498.
- Johnson, S.C., Kayser, E.B., Bornstein, R., Stokes, J., Bitto, A., Park, K.Y., ... Morgan, P.G. 2020. Regional metabolic signatures in the Ndufs4(KO) mouse brain implicate defective glutamate/ α -ketoglutarate metabolism in mitochondrial disease. *Mol. Genet. Metab.*, 130(2):118-132.
- Johnson, S.C., Yanos, M.E., Kayser, E.B., Quintana, A., Sangesland, M., Castanza, A., ... Kaeberlein, M. 2013. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science*, 342(6165):1524-1528.
- Joshi, C.N., Greenberg, C.R., Mhanni, A.A. & Salman, M.S. 2009. Ketogenic diet in Alpers-Huttenlocher syndrome. *Pediatr. Neurol.*, 40(4):314-316.
- Kahlhöfer, F., Kmita, K., Wittig, I., Zwicker, K. & Zickermann, V. 2017. Accessory subunit NUYM (NDUFS4) is required for stability of the electron input module and activity of mitochondrial complex I. *Biochim. Biophys. Acta, Bioenerg.*, 1858(2):175-181.
- Kane, A.E. & Sinclair, D.A. 2018. Sirtuins and NAD(+) in the Development and Treatment of Metabolic and Cardiovascular Diseases. *Circ Res*, 123(7):868-885. 10.1161/circresaha.118.312498
- Karamanlidis, G., Lee, C.F., Garcia-Menendez, L., Kolwicz, S.C., Jr., Suthammarak, W., Gong, G., ... Tian, R. 2013. Mitochondrial complex I deficiency increases protein acetylation and accelerates heart failure. *Cell Metab.*, 18(2):239-250. 10.1016/j.cmet.2013.07.002
- Kayser, E.-B., Mulholland, M., Olkhova, E.A., Chen, Y., Coulson, H., Cairns, O., ... Johnson, S.C. 2025. Evaluating the efficacy of vatiquinone in preclinical models of Leigh syndrome and GPX4 deficiency. *Orphanet Journal of Rare Diseases*, 20(1):65.
- Kayser, E.B., Sedensky, M.M. & Morgan, P.G. 2016. Region-Specific Defects of Respiratory Capacities in the Ndufs4(KO) Mouse Brain. *PLoS One*, 11(1):e0148219. 10.1371/journal.pone.0148219

CHAPTER 2

- Kelly, C.J., Couch, R.K., Ha, V.T., Bodart, C.M., Wu, J., Huff, S., ... Grillo, A.S. 2023. Iron status influences mitochondrial disease progression in Complex I-deficient mice. *elife*, 12, 10.7554/eLife.75825
- Keshavan, N. & Rahman, S. 2018. Natural history of mitochondrial disorders: a systematic review. *Essays Biochem*, 62(3):423-442. 10.1042/ebc20170108
- Khaled, M.L., Ren, Y., Kundalia, R., Alhaddad, H., Chen, Z., Wallace, G.C., ... Smalley, I. 2023. Branched-chain keto acids promote an immune-suppressive and neurodegenerative microenvironment in leptomeningeal disease. *bioRxiv*, 10.1101/2023.12.18.572239
- Koene, S., Willems, P.H., Roestenberg, P., Koopman, W.J. & Smeitink, J.A. 2011. Mouse models for nuclear DNA-encoded mitochondrial complex I deficiency. *Journal of inherited metabolic disease*, 34:293-307.
- Koopman, W.J., Distelmaier, F., Smeitink, J.A. & Willems, P.H. 2013. OXPHOS mutations and neurodegeneration. *Embo j*, 32(1):9-29. 10.1038/emboj.2012.300
- Koopman, W.J., Verkaart, S., van Ernst-de Vries, S.E., Grefte, S., Smeitink, J.A., Nijtmans, L.G. & Willems, P.H. 2008. Mitigation of NADH: ubiquinone oxidoreductase deficiency by chronic Trolox treatment. *Biochim. Biophys. Acta, Bioenerg.*, 1777(7-8):853-859.
- Koopman, W.J., Beyrath, J., Fung, C.W., Koene, S., Rodenburg, R.J., Willems, P.H. & Smeitink, J.A. 2016. Mitochondrial disorders in children: toward development of small-molecule treatment strategies. *EMBO Mol. Med.*, 8(4):311-327.
- Kostic, M., Katoshevski, T. & Sekler, I. 2018. Allosteric Regulation of NCLX by Mitochondrial Membrane Potential Links the Metabolic State and Ca^{2+} Signaling in Mitochondria. *Cell Reports*, 25(12):3465-3475.e3464.
- Kroemer, G. 2003. Mitochondrial control of apoptosis: an introduction. *Biochemical and Biophysical Research Communications*, 304(3):433-435.
- Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab*, 7(4):312-320. 10.1016/j.cmet.2008.02.004
- Kulkarni, C.A. & Brookes, P.S. 2019. Cellular Compartmentation and the Redox/Nonredox Functions of NAD(⁺). *Antioxid Redox Signal*, 31(9):623-642. 10.1089/ars.2018.7722
- Lake, N.J., Bird, M.J., Isohanni, P. & Paetau, A. 2015. Leigh syndrome: neuropathology and pathogenesis. *J Neuropathol Exp Neurol*, 74(6):482-492. 10.1097/nen.0000000000000195
- Lake, N.J., Compton, A.G., Rahman, S. & Thorburn, D.R. 2016. Leigh syndrome: One disorder, more than 75 monogenic causes. *Ann Neurol*, 79(2):190-203. 10.1002/ana.24551
- Lamont, R.E., Beaulieu, C.L., Bernier, F.P., Sparkes, R., Innes, A.M., Jackel-Cram, C., ... Lemire, E.G. 2017. A novel NDUFS4 frameshift mutation causes Leigh disease in the Hutterite population. *Am J Med Genet A*, 173(3):596-600. 10.1002/ajmg.a.37983
- Laugel, V., This-Bernd, V., Cormier-Daire, V., Speeg-Schatz, C., de Saint-Martin, A. & Fischbach, M. 2007. Early-onset ophthalmoplegia in Leigh-like syndrome due to NDUFV1 mutations. *Pediatric neurology*, 36(1):54-57.
- Lee, C.F., Caudal, A., Abell, L., Nagana Gowda, G.A. & Tian, R. 2019. Targeting NAD(⁺) Metabolism as Interventions for Mitochondrial Disease. *Sci Rep*, 9(1):3073. 10.1038/s41598-019-39419-4
- Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., ... Herman, B. 1999. Mitochondrial Dysfunction in the Pathogenesis of Necrotic and Apoptotic Cell Death. *Journal of Bioenergetics and Biomembranes*, 31(4):305-319.
- Liang, H. & Ward, W.F. 2006. PGC-1 α : a key regulator of energy metabolism. *Advances in Physiology Education*, 30(4):145-151.

CHAPTER 2

- Lim, A.Z., Ng, Y.S., Blain, A., Jiminez-Moreno, C., Alston, C.L., Nesbitt, V., ... McFarland, R. 2022. Natural History of Leigh Syndrome: A Study of Disease Burden and Progression. *Ann. Neurol.*, 91(1):117-130.
- Liu, L., MacKenzie, K.R., Putluri, N., Maletić-Savatić, M. & Bellen, H.J. 2017. The Glia-Neuron Lactate Shuttle and Elevated ROS Promote Lipid Synthesis in Neurons and Lipid Droplet Accumulation in Glia via APOE/D. *Cell Metab*, 26(5):719-737.e716. 10.1016/j.cmet.2017.08.024
- Liu, L., Zhang, K., Sandoval, H., Yamamoto, S., Jaiswal, M., Sanz, E., ... Bellen, H.J. 2015. Glial lipid droplets and ROS induced by mitochondrial defects promote neurodegeneration. *Cell*, 160(1-2):177-190. 10.1016/j.cell.2014.12.019
- Liu, R., Chen, L., Wang, Y., Zhang, G., Cheng, Y., Feng, Z., ... Liu, J. 2020. High ratio of ω -3/ ω -6 polyunsaturated fatty acids targets mTORC1 to prevent high-fat diet-induced metabolic syndrome and mitochondrial dysfunction in mice. *The Journal of Nutritional Biochemistry*, 79:108330.
- Liu, S., Fu, S., Wang, G., Cao, Y., Li, L., Li, X., ... Jiang, H. 2021. Glycerol-3-phosphate biosynthesis regenerates cytosolic NAD(+) to alleviate mitochondrial disease. *Cell Metab*, 33(10):1974-1987.e1979. 10.1016/j.cmet.2021.06.013
- Lobo-Jarne, T. & Ugalde, C. 2018. Respiratory chain supercomplexes: Structures, function and biogenesis. *Semin Cell Dev Biol*, 76:179-190. 10.1016/j.semcdb.2017.07.021
- Loeffen, J.L., Smeitink, J.A., Trijbels, J.M., Janssen, A.J., Triepels, R.H., Sengers, R.C. & van den Heuvel, L.P. 2000. Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. *Hum Mutat*, 15(2):123-134. 10.1002/(sici)1098-1004(200002)15:2<123::Aid-humu1>3.0.Co;2-p
- Lombard, D.B., Tishkoff, D.X. & Bao, J. 2011. Mitochondrial sirtuins in the regulation of mitochondrial activity and metabolic adaptation. *Handb Exp Pharmacol*, 206:163-188. 10.1007/978-3-642-21631-2_8
- Lopez-Fabuel, I., Le Douce, J., Logan, A., James, A.M., Bonvento, G., Murphy, M.P., ... Bolaños, J.P. 2016. Complex I assembly into supercomplexes determines differential mitochondrial ROS production in neurons and astrocytes. *Proceedings of the National Academy of Sciences*, 113(46):13063-13068.
- Lossi, L. 2022. The concept of intrinsic versus extrinsic apoptosis. *Biochemical Journal*, 479(3):357-384.
- Makkar, R., Behl, T., Bungau, S., Zengin, G., Mehta, V., Kumar, A., ... Oancea, R. 2020. Nutraceuticals in Neurological Disorders. *Int J Mol Sci*, 21(12), 10.3390/ijms21124424
- Maksoud, R., Balinas, C., Holden, S., Cabanas, H., Staines, D. & Marshall-Gradisnik, S. 2021. A systematic review of nutraceutical interventions for mitochondrial dysfunctions in myalgic encephalomyelitis/chronic fatigue syndrome. *Journal of Translational Medicine*, 19(1):81.
- Manjeri, G.R., Rodenburg, R.J., Blanchet, L., Roelofs, S., Nijtmans, L.G., Smeitink, J.A., ... Willems, P.H. 2016. Increased mitochondrial ATP production capacity in brain of healthy mice and a mouse model of isolated complex I deficiency after isoflurane anesthesia. *J Inherit Metab Dis*, 39(1):59-65. 10.1007/s10545-015-9885-x
- Mannella, C.A. 2006. Structure and dynamics of the mitochondrial inner membrane cristae. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1763(5):542-548.
- Martin-Perez, M., Grillo, A.S., Ito, T.K., Valente, A.S., Han, J., Entwisle, S.W., ... Villén, J. 2020. PKC downregulation upon rapamycin treatment attenuates mitochondrial disease. *Nat Metab*, 2(12):1472-1481. 10.1038/s42255-020-00319-x
- Mavraki, E., Labrum, R., Sergeant, K., Alston, C.L., Woodward, C., Smith, C., ... Fratter, C. 2023. Genetic testing for mitochondrial disease: the United Kingdom best practice guidelines. *European Journal of Human Genetics*, 31(2):148-163.
- Mayr, J.A., Haack, T.B., Freisinger, P., Karall, D., Makowski, C., Koch, J., ... Ahting, U. 2015. Spectrum of combined respiratory chain defects. *Journal of inherited metabolic disease*, 38:629-640.

CHAPTER 2

- McCormick, E.M., Keller, K., Taylor, J.P., Coffey, A.J., Shen, L., Krotoski, D., ... Rahman, S. 2023. Expert Panel Curation of 113 Primary Mitochondrial Disease Genes for the Leigh Syndrome Spectrum. *Ann. Neurol.*, 94(4):696-712.
- McCully, J.D., del Nido, P.J. & Emani, S.M. 2022. Therapeutic mitochondrial transplantation. *Current Opinion in Physiology*, 27:100558.
- McCully, J.D., Del Nido, P.J. & Emani, S.M. 2023. Mitochondrial transplantation: the advance to therapeutic application and molecular modulation. *Frontiers in Cardiovascular Medicine*, 10:1268814.
- McDonald, A.E., Pichaud, N. & Darveau, C.A. 2018. "Alternative" fuels contributing to mitochondrial electron transport: Importance of non-classical pathways in the diversity of animal metabolism. *Comp Biochem Physiol B Biochem Mol Biol*, 224:185-194.
- Milenkovic, D., Blaza, J.N., Larsson, N.-G. & Hirst, J. 2017. The Enigma of the Respiratory Chain Supercomplex. *Cell Metab.*, 25(4):765-776.
- Miller, H.C., Louw, R., Mereis, M., Venter, G., Boshoff, J.D., Mienie, L., ... van der Westhuizen, F.H. 2021. Metallothionein 1 Overexpression Does Not Protect Against Mitochondrial Disease Pathology in Ndufs4 Knockout Mice. *Mol. Neurobiol.*, 58(1):243-262.
- Mitchelson, K.A.J., O'Connell, F., Wynne, K., Matallanas, D., O'Sullivan, J., Roche, HM. 2025. Saturated fat exacerbates mitochondrial dysfunction through remodelling of ATP production and inflammation in Barrett's oesophagus compared to monounsaturated fat, particularly in contrast to oesophageal adenocarcinoma. *Neoplasia*, 66:101173.
- Nakai, R., Varum, S., Field, R.L., Shi, H., Giwa, R., Jia, W., ... Saneto, R.P. 2024. Mitochondria transfer-based therapies reduce the morbidity and mortality of Leigh syndrome. *Nature Metabolism*, 6(10):1886-1896.
- Naresh, N.U. & Haynes, C.M. 2019. Signaling and Regulation of the Mitochondrial Unfolded Protein Response. *Cold Spring Harb Perspect Biol*, 11(6).
- Nathan, J., Bailur, S., Datay, K., Sharma, S. & Khedekar Kale, D. 2019. A Switch to Polyunsaturated Fatty Acid Based Ketogenic Diet Improves Seizure Control in Patients with Drug-resistant Epilepsy on the Mixed Fat Ketogenic Diet: A Retrospective Open Label Trial. *Cureus*, 11(12):e6399.
- Neugebauer, J., Reinson, K., Bellusci, M., Park, J.H., Hikmat, O., Bertini, E., ... Darin, N. 2025. Current global vitamin and cofactor prescribing practices for primary mitochondrial diseases: Results of a European reference network survey. *Journal of Inherited Metabolic Disease*, 48(1):e12805.
- Ng, Y.S., Thompson, K., Loher, D., Hopton, S., Falkous, G., Hardy, S.A., ... Taylor, R.W. 2020. Novel MT-ND Gene Variants Causing Adult-Onset Mitochondrial Disease and Isolated Complex I Deficiency. *Front Genet*, 11:24.
- Nishida, R., Nukaga, S., Kawahara, I., Miyagawa, Y., Goto, K., Nakashima, C., ... Kuniyasu, H. 2024. Differential Effects of Three Medium-Chain Fatty Acids on Mitochondrial Quality Control and Skeletal Muscle Maturation. *Antioxidants*, 13(7), 821.
- Niyazov, D.M., Kahler, S.G. & Frye, R.E. 2016. Primary Mitochondrial Disease and Secondary Mitochondrial Dysfunction: Importance of Distinction for Diagnosis and Treatment. *Mol Syndromol*, 7(3):122-137.
- Nunnari, J. & Suomalainen, A. 2012. Mitochondria: in sickness and in health. *Cell*, 148(6):1145-1159. 10.1016/j.cell.2012.02.035
- Ortigoza-Escobar, J.D., Oyarzabal, A., Montero, R., Artuch, R., Jou, C., Jiménez, C., ... Pérez-Dueñas, B. 2016. Ndufs4 related Leigh syndrome: A case report and review of the literature. *Mitochondrion*, 28:73-78. 10.1016/j.mito.2016.04.001
- Papa, S. 2002. The NDUFS4 nuclear gene of complex I of mitochondria and the cAMP cascade. *Biochim. Biophys. Acta, Bioenerg.*, 1555(1):147-153.

CHAPTER 2

- Papa, S., Scacco, S., De Rasmio, D., Signorile, A., Papa, F., Panelli, D., ... Larizza, M. 2010. cAMP-dependent protein kinase regulates post-translational processing and expression of complex I subunits in mammalian cells. *Biochim. Biophys. Acta, Bioenerg.*, 1797(6-7):649-658. 10.1016/j.bbabi.2010.03.013
- Parikh, S., Karaa, A., Goldstein, A., Bertini, E.S., Chinnery, P.F., Christodoulou, J., ... Rahman, S. 2019. Diagnosis of 'possible' mitochondrial disease: an existential crisis. *J. Med. Genet.*, 56(3):123-130.
- Parikh, S., Goldstein, A., Karaa, A., Koenig, M.K., Anselm, I., Brunel-Guitton, C., ... Chinnery, P.F. 2017. Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet. Med.*, 19(12).
- Perrone, M., Patergnani, S., Di Mambro, T., Palumbo, L., Wieckowski, M.R., Giorgi, C. & Pinton, P. 2023. Calcium Homeostasis in the Control of Mitophagy. *Antioxid. Redox Signal.*, 38(7-9):581-598.
- Peruzzotti-Jametti, L., Willis, C.M., Hamel, R., Krzak, G., Reisz, J.A., Prag, H.A., ... Pluchino, S. 2024. Mitochondrial reverse electron transport in myeloid cells perpetuates neuroinflammation. *bioRxiv:2024.2001.2003.574059*.
- Piroli, G.G., Manuel, A.M., Smith, H.H., McCain, R.S., Walla, M.D. & Frizzell, N. 2020. Succination of Dihydrolipoyllysine Succinyltransferase (DLST) Exacerbates Mitochondrial ATP Deficiency in a Mouse Model of Leigh Syndrome. *bioRxiv:2020.2001.2009.900514*.
- Piroli, G.G., Manuel, A.M., Clapper, A.C., Walla, M.D., Baatz, J.E., Palmiter, R.D., ... Frizzell, N. 2016. Succination is Increased on Select Proteins in the Brainstem of the NADH dehydrogenase (ubiquinone) Fe-S protein 4 (Ndufs4) Knockout Mouse, a Model of Leigh Syndrome. *Mol. Cell Proteomics*, 15(2):445-461.
- Pollicino, F., Veronese, N., Dominguez, L.J. & Barbagallo, M. 2023. Mediterranean diet and mitochondria: new findings. *Exp. Gerontol.*, 176:112165.
- Pozzi, S., Benedusi, V., Maggi, A. & Vegeto, E. 2006. Estrogen action in neuroprotection and brain inflammation. *Ann. NY Acad. Sci.*, 1089:302-323.
- Prasai, K. 2017. Regulation of mitochondrial structure and function by protein import: A current review. *Pathophysiology*, 24(3):107-122.
- Qiao, L., Yang, G., Wang, P. & Xu, C. 2024. The potential role of mitochondria in the microbiota-gut-brain axis: Implications for brain health. *Pharmacol. Res.*, 209:107434.
- Qu, C., Keijer, J., Adjobo-Hermans, M.J.W., van de Wal, M., Schirris, T., van Karnebeek, C., ... Koopman, W.J.H. 2021. The ketogenic diet as a therapeutic intervention strategy in mitochondrial disease. *Int. J. Biochem. Cell Biol.*, 138:106050.
- Quinlan, C.L., Treberg, J.R. & Brand, M.D. 2011. Chapter 3 - Mechanisms of Mitochondrial Free Radical Production and their Relationship to the Aging Process. *Handbook Biol. Aging.*, 47-61.
- Quinn, P.M.J., Moreira, P.I., Ambrósio, A.F. & Alves, C.H. 2020. PINK1/PARKIN signalling in neurodegeneration and neuroinflammation. *Acta Neuropathol. Commun.*, 8(1):189.
- Quintana, A., Kruse, S.E., Kapur, R.P., Sanz, E. & Palmiter, R.D. 2010. Complex I deficiency due to loss of Ndufs4 in the brain results in progressive encephalopathy resembling Leigh syndrome. *Proc. Natl. Acad. Sci. USA*, 107(24):10996-11001.
- Quintana, A., Zanella, S., Koch, H., Kruse, S.E., Lee, D., Ramirez, J.M. & Palmiter, R.D. 2012. Fatal breathing dysfunction in a mouse model of Leigh syndrome. *J. Clin. Invest.*, 122(7):2359-2368.
- Rahman, J. & Rahman, S. 2018. Mitochondrial medicine in the omics era. *Lancet*, 391(10139):2560-2574.
- Rahman, J., Noronha, A., Thiele, I. & Rahman, S. 2017. Leigh map: A novel computational diagnostic resource for mitochondrial disease. *Ann. Neurol.*, 81(1):9-16.

CHAPTER 2

- Rahman, S. 2020. Mitochondrial disease in children. *J. Intern. Med.*, 287(6):609-633.
- Rath, S., Sharma, R., Gupta, R., Ast, T., Chan, C., Durham, T.J., ... Mootha, V.K. 2021. MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations. *Nucleic Acids Res.*, 49(D1):D1541-d1547.
- Rodenburg, R.J. 2016. Mitochondrial complex I-linked disease. *Biochim. Biophys. Acta, Bioenerg.*, 1857(7):938-945.
- Rohrbach, S. 2009. Effects of dietary polyunsaturated fatty acids on mitochondria. *Curr. Pharm. Des.*, 15(36):4103-4116.
- Rüb, C., Wilkening, A. & Voos, W. 2017. Mitochondrial quality control by the Pink1/Parkin system. *Cell Tissue Res.*, 367:111-123.
- Sack, M.N. & Finkel, T. 2012. Mitochondrial metabolism, sirtuins, and aging. *Cold. Spring. Harb. Perspect. Biol.*, 4(4):287-298.
- Saneto, R.P. 2020. Mitochondrial diseases: expanding the diagnosis in the era of genetic testing. *J. Transl. Genet. Genom.*, 4:384-428.
- Saxton, R.A. & Sabatini, D.M. 2017. mTOR signaling in growth, metabolism, and disease. *Cell*, 168(6):960-976.
- Scacco, S., Petruzzella, V., Budde, S., Vergari, R., Tamborra, R., Panelli, D., ... Papa, S. 2003. Pathological mutations of the human NDUFS4 gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex. *J. Biol. Chem.*, 278(45):44161-44167.
- Schleifer, G., Marutani, E., Ferrari, M., Sharma, R., Skinner, O., Goldberger, O., ... Zapol, W.M. 2019. Impaired hypoxic pulmonary vasoconstriction in a mouse model of Leigh syndrome. *Am. J. Physiol. Lung. Cell. Mol. Physiol.*, 316(2):L391-L399.
- Shil, S.K., Kagawa, Y., Umaru, B.A., Nanto-Hara, F., Miyazaki, H., Yamamoto, Y., ... Owada, Y. 2021. Ndufs4 ablation decreases synaptophysin expression in hippocampus. *Sci. Rep.*, 11(1):10969.
- Shirooie, S., Nabavi, S.F., Dehpour, A.R., Belwal, T., Habtemariam, S., Argüelles, S., ... Nabavi, S.M. 2018. Targeting mTORs by omega-3 fatty acids: A possible novel therapeutic strategy for neurodegeneration? *Pharmacol. Res.*, 135:37-48.
- Silva-Pinheiro, P., Cerutti, R., Luna-Sanchez, M., Zeviani, M. & Viscomi, C. 2020. A single intravenous injection of AAV-PHP. B-hNDUFS4 ameliorates the phenotype of Ndufs4^{-/-} mice. *Mol. Ther. Methods Clin. Dev.*, 17:1071-1078.
- Smeitink, J., van den Heuvel, L. & DiMauro, S. 2001. The genetics and pathology of oxidative phosphorylation. *Nat. Rev. Genet.*, 2(5):342-352.
- Smeitink, J., van Es, J., Bosman, B., Janssen, M.C.H., Klopstock, T., Gorman, G., ... Renkema, H. 2024. Phase 2b program with sonlicromanol in patients with mitochondrial disease due to m.3243A>G mutation. *Brain*, 148(3):896-907.
- Song, C., Pan, S., Zhang, J., Li, N. & Geng, Q. 2022. Mitophagy: A novel perspective for insighting into cancer and cancer treatment. *Cell Prolif.*, 55(12):e13327.
- Song, L., Yu, A., Murray, K. & Cortopassi, G. 2017. Bipolar cell reduction precedes retinal ganglion neuron loss in a complex 1 knockout mouse model. *Brain Res.*, 1657:232-244.
- Stanley, W.C., Khairallah, R.J. & Dabkowski, E.R. 2012. Update on lipids and mitochondrial function: impact of dietary n-3 polyunsaturated fatty acids. *Curr. Opin. Clin. Nutr. Metab. Care*, 15(2):122-126.

CHAPTER 2

- Stokes, J.C., Bornstein, R.L., James, K., Park, K.Y., Spencer, K.A., Vo, K., ... Johnson, S.C. 2022. Leukocytes mediate disease pathogenesis in the Ndufs4(KO) mouse model of Leigh syndrome. *JCI Insight*, 7(5), 10.1172/jci.insight.156522
- Stroud, D.A., Surgenor, E.E., Formosa, L.E., Reljic, B., Frazier, A.E., Dibley, M.G., ... Ryan, M.T. 2016. Accessory subunits are integral for assembly and function of human mitochondrial complex I. *Nature*, 538(7623):123-126.
- Sullivan, E.M., Pennington, E.R., Green, W.D., Beck, M.A., Brown, D.A. & Shaikh, S.R. 2018. Mechanisms by which dietary fatty acids regulate mitochondrial structure-function in health and disease. *Adv. Nutr.*, 9(3):247-262.
- Suzuki, S., Brown, C.M. & Wise, P.M. 2006. Mechanisms of neuroprotection by estrogen. *Endocrine*, 29(2):209-215.
- Swalwell, H., Kirby, D.M., Blakely, E.L., Mitchell, A., Salemi, R., Sugiana, C., ... Lamont, P.J. 2011. Respiratory chain complex I deficiency caused by mitochondrial DNA mutations. *Eur. J. Hum. Genet.*, 19(7):769-775.
- Teng, Y., Cui, H., Xu, D., Tang, H., Gu, Y., Tang, Y., ... Fan, Y. 2025. Specific Knockdown of the NDUFS4 Gene Reveals Important Roles of Ferroptosis in UVB-induced Photoaging. *Inflammation*, 48(1):223-235.
- Terburgh, K., Lindeque, Z., Mason, S., van der Westhuizen, F. & Louw, R. 2019. Metabolomics of Ndufs4^{-/-} skeletal muscle: Adaptive mechanisms converge at the ubiquinone-cycle. *Biochim. Biophys. Acta - Mol. Basis Dis.*, 1865(1):98-106.
- Terburgh, K., Coetzer, J., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021. Aberrant BCAA and glutamate metabolism linked to regional neurodegeneration in a mouse model of Leigh syndrome. *Biochim. Biophys. Acta - Mol. Basis Dis.*, 1867(5):166082.
- Valenti, D. & Vacca, R.A. 2022. Primary and Secondary Mitochondrial Diseases: Etiologies and Therapeutic Strategies. *J. Clin. Med.*, 11(14):4209.
- Valsecchi, F., Koopman, W.J., Manjeri, G.R., Rodenburg, R.J., Smeitink, J.A. & Willems, P.H. 2010. Complex I disorders: causes, mechanisms, and development of treatment strategies at the cellular level. *Dev. Disabil. Res. Rev.*, 16(2):175-182.
- Valsecchi, F., Monge, C., Forkink, M., de Groof, A.J., Benard, G., Rossignol, R., ... Willems, P.H. 2012. Metabolic consequences of NDUFS4 gene deletion in immortalized mouse embryonic fibroblasts. *Biochim. Biophys. Acta*, 1817(10):1925-1936.
- van de Wal, M.A.E., Doornbos, C., Bibbe, J.M., Homberg, J.R., van Karnebeek, C., Huynen, M.A., ... Koopman, W.J.H. 2022. Ndufs4 knockout mice with isolated complex I deficiency engage a futile adaptive brain response. *Biochim. Biophys. Acta Proteins Proteom.*, 1873(1):141055.
- van de Wal, M.A.E., Doornbos, C., Bibbe, J.M., Homberg, J.R., van Karnebeek, C., Huynen, M.A., ... Koopman, W.J.H. 2025. Ndufs4 knockout mice with isolated complex I deficiency engage a futile adaptive brain response. *Biochim. Biophys. Acta Proteins Proteom.*, 1873(1):141055.
- van Rensburg, D.J., Lindeque, Z., Harvey, B.H. & Steyn, S.F. 2024. Ndufs4 KO mice: A model to study comorbid mood disorders associated with mitochondrial dysfunction. *Pharmacol Biochem Behav*, 234:173689. 10.1016/j.pbb.2023.173689
- vanden Hoven, R.N. & Santini, J.M. 2004. Arsenite oxidation by the heterotroph *Hydrogenophaga* sp. str. NT-14: the arsenite oxidase and its physiological electron acceptor. *Biochim. Biophys. Acta, Bioenerg.*, 1656(2):148-155.
- Vasava, A. & Mashiyava, P. 2016. Electron transport chain: role in reactive oxygen species production and aging. *Sch. J. Agric. Vet. Sci.*, 3(5):378-388.

CHAPTER 2

- Ventura-Clapier, R., Garnier, A. & Veksler, V. 2008. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1 α . *Cardiovasc. Res.*, 79(2):208-217.
- Vernis, L., El Banna, N., Baïlle, D., Hatem, E., Heneman, A. & Huang, M.-E. 2017. Fe-S Clusters Emerging as Targets of Therapeutic Drugs. *Oxid. Med. Cell. Longev.*, 2017(1):3647657.
- Wang, H., Miranda, M., Marutani, E., Lichtenegger, P., Wojtkiewicz, G.R., Ichinose, F. & Mootha, V.K. 2024. Therapeutic hypoxia for mitochondrial disease via enhancement of hemoglobin affinity and inhibition of HIF-2 α . *J. Clin. Invest.*, 134(23):e185569.
- Wen, H., Deng, H., Li, B., Chen, J., Zhu, J., Zhang, X., ... Zhou, Y. 2025. Mitochondrial diseases: from molecular mechanisms to therapeutic advances. *Signal Transduct Target Ther.*, 10(1):9.
- Yang, L., Garcia Canaveras, J.C., Chen, Z., Wang, L., Liang, L., Jang, C., ... Rabinowitz, J.D. 2020. Serine Catabolism Feeds NADH when Respiration Is Impaired. *Cell Metab.*, 31(4):809-821.e806.
- Yin, Z., Agip, A.A., Bridges, H.R. & Hirst, J. 2024. Structural insights into respiratory complex I deficiency and assembly from the mitochondrial disease-related *ndufs4*(-/-) mouse. *Embo j*, 43(2):225-249.
- Yoo, H.-S., Shanmugalingam, U. & Smith, P.D. 2022. Potential roles of branched-chain amino acids in neurodegeneration. *Nutrition*, 103-104:111762.
- Yoon, J.-Y., Daneshgar, N., Chu, Y., Chen, B., Hefti, M., Vikram, A., ... Dai, D.-F. 2022. Metabolic rescue ameliorates mitochondrial encephalo-cardiomyopathy in murine and human iPSC models of Leigh syndrome. *Clin. Transl. Med.*, 12(7):e954.
- Yu, A.K., Song, L., Murray, K. & Cortopassi, G. 2015. Rapamycin rescues the innate immune/inflammatory response in the retina of the *Ndufs4* mouse. *Investigative Ophthalmology & Visual Science*, 56(7):5541-5541.
- Zachos, K.A., Gamboa, J.A., Dewji, A.S., Lee, J., Brijbassi, S. & Andreazza, A.C. 2024. The interplay between mitochondria, the gut microbiome and metabolites and their therapeutic potential in primary mitochondrial disease. *Front. Pharmacol.*, 15:1428242.
- Zhang, H., Gong, G., Wang, P., Zhang, Z., Kolwicz, S.C., Rabinovitch, P.S., ... Wang, W. 2018. Heart specific knockout of *Ndufs4* ameliorates ischemia reperfusion injury. *J. Mol. Cell. Cardiol.*, 123:38-45.
- Zhang, L., Zhang, Z., Khan, A., Zheng, H., Yuan, C. & Jiang, H. 2020. Advances in drug therapy for mitochondrial diseases. *Ann. Transl. Med.*, 8(1):17.
- Zhao, R.Z., Jiang, S., Zhang, L. & Yu, Z.B. 2019. Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int. J. Mol. Med.*, 44(1):3-15.
- Zhu, D., Li, X. & Tian, Y. 2022. Mitochondrial-to-nuclear communication in aging: an epigenetic perspective. *Trends Biochem Sci.*, 47(8):645-659.
- Zieliński Ł, P., Smith, A.C., Smith, A.G. & Robinson, A.J. 2016. Metabolic flexibility of mitochondrial respiratory chain disorders predicted by computer modelling. *Mitochondrion*, 31:45-55.
- Zong, Y., Li, H., Liao, P., Chen, L., Pan, Y., Zheng, Y., ... Gao, J. 2024. Mitochondrial dysfunction: mechanisms and advances in therapy. *Signal Transduct Target Ther.*, 9(1):124.
- Zweers, H., van Wegberg, A.M., Janssen, M.C. & Wortmann, S.B. 2021. Ketogenic diet for mitochondrial disease: a systematic review on efficacy and safety. *Orphanet J. Rare Dis.*, 16:1-15.

CHAPTER 3

Materials and methods

3.1. Introduction

The study was divided into two phases: Phase A, which served as a preliminary investigation, and Phase B, which utilized the pre-clinically relevant *Ndufs4* KO mouse model. Comprehensive details are provided in this chapter on animal handling, dietary intervention protocols, DNA isolation procedures, PCR-based genotype confirmation, and sample collection. Furthermore, through the methods chapter, it is made clear which methodologies were applied specifically to Phase A and/or Phase B. Additionally, this chapter describes the phenotypic assessments conducted, along with methodologies for SDS-PAGE and Western blotting, flow cytometry, and multi-platform metabolomic (LC-MS/MS, ¹H-NMR, GC-TOF-MS, and GC-MS/MS) and lipidomic analyses (UPLC-HDMS) for the characterisation of urinary and brain tissue-specific metabolic profiles.

3.2. Animals and sample collection

3.2.1. Animals, dietary intervention and sample sizes

Whole-body *Ndufs4* knockout (KO), wild-type (WT), and heterozygous (HET, B6.129S4-*Ndufs4*^{tm1.1Rpa}/J) mice were generated from HET breeding pairs (JAX stock #027058) obtained from Jackson Laboratory (JAX; ME, USA). In Phase A of the study, male WT and HET mice were used interchangeably as healthy controls due to the absence of phenotypic differences between these two genotypes (Kruse *et al.*, 2008; van de Wal *et al.*, 2022). The decision to use only male mice in this preliminary phase was based on well-established differences in hormonal cycles, tissue-specific metabolic activity, and overall metabolic profiles between males and females (de Souza *et al.*, 2022; Ruoppolo *et al.*, 2018). Phase B of the study made use of both male and female, WT and KO mice. Genotype identification was confirmed via polymerase chain reaction (PCR) analysis using tail snips, following the method outlined in Section 3.3, to ensure proper genotype designation for study inclusion.

The animals were bred and housed in the specific pathogen-free (SPF) unit of the vivarium (SAVC reg. no. FR15/13458) at the Pre-Clinical Drug Development Platform (PCDDP; NWU, RSA), with ethical approval obtained from the NWU Animal Research Ethics Committee (approval number NWU-00516-20-A5). Mice were housed under controlled environmental conditions with a temperature of 22 ± 1°C, humidity maintained at 55 ± 10%, and a 12:12 hour light/dark cycle. The animals had *ad libitum* access to food and water. To improve animal welfare, enclosures included soft sawdust bedding, paper towels for nesting, and polycarbonate mouse houses or tubes. All animal handling and procedures adhered to the ethical guidelines for research and drug testing in South Africa, as stipulated by national legislation. Only personnel authorized by the South African Veterinary Council (SAVC) under vivarium supervision handled the animals. The minimum sample size required per group (n = 10) was

determined in collaboration with Dr Mari van Reenen (former Biostatistician at the Focus Area Human Metabolomics, NWU).

3.2.2. Animal identification and numbering

On postnatal day (P) 21, ear punches were performed on all mice for individual identification and tail snips were collected for genotyping. Mice were weaned and separated from their mothers on P23. Male and female mice were housed in separate cages, with WT and KO mice also housed separately. To support thermoregulation, KO mice included in the survival cohort were housed with a healthy HET mouse (Stokes *et al.*, 2022).

3.2.3. Animal number, dietary groups and diet composition

In Phase A, 40 male mice were randomly assigned to one of four dietary intervention groups ($n = 10$ per group) starting at P23. The diets provided included: (i) a control diet (normal diet, ND) consisting of standard laboratory chow (RB2005, LabChef, NutritionHub, South Africa) with drinking water; (ii) a ND supplemented with 5% (v/v) alcohol in drinking water for the first week and 10% (v/v) alcohol thereafter; (iii) a KD diet (KD) with drinking water; or (iv) a KD (prepared in-house with the guidance of NutritionHub, South Africa) with alcohol supplementation similar to the second group. The control diet was composed of 20% protein, 75% carbohydrates, and 5% fat, while the KD consisted of 8.2% protein, 91.7% fat, and <0.1% carbohydrates (Table C-1). The dietary interventions lasted approximately three weeks, and mice were weighed three times per week to monitor growth. Mice were euthanized on P46-47, following the dietary intervention, and were placed in metabolic cages the day before euthanasia to collect fasting urine samples.

Phase B involved 80 mice and focused on both phenotypic and biochemical assessments. Mice were fed either a control diet or a polyunsaturated fatty acid (PUFA)-enriched high-fat diet (HFD), referred onwards as HFD only, supplemented with PUFAs derived from fish oil (Table 5-1) starting at P23. For phenotypic analyses, 24 KO mice (12 male, 12 female) were randomly assigned to two groups: control-fed (ND) KO and HFD-fed KO mice. For biochemical analyses, mice were allocated to one of four treatment groups ($n = 14$ per group): ND-fed WT, HFD-fed WT, ND-fed KO, and HFD-fed KO. The ND (D23050803i, Research Diets, USA) contained 20% protein, 70% carbohydrates, and 10% fat, while the HFD (D09020506i, Research Diets, USA) contained 20% protein, 20% carbohydrates, and 60% fat. Both diets were isocaloric and were administered *ad libitum*. Unlike Phase A, where the ND and KD were not nutritionally or calorically matched, Phase B ensured that both diets were balanced in terms of protein, vitamin, and mineral content, in addition to being isocaloric.

3.2.4. Humane endpoints and euthanasia

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Pain and distress in animals were assessed throughout the duration of each phase of the study using a humane endpoint score sheet (SOP_Viv_Anim 27: Determining pain and distress in laboratory rodents). Mice selected for euthanasia were humanely euthanized between P45-47, a time window chosen to correspond with the late stage of the disease model. This age range was determined based on prior survival curves and phenotypic assessments performed at our facility (Miller *et al.*, 2021). Mice used for the phenotypic investigation were maintained on the experimental diet until they reached the endpoint of natural death. For both phases, mice were euthanized via exsanguination in accordance with the American Veterinary Medical Association (AVMA) guidelines (AVMA, 2020). In Phase A, mice were fasted for 16-18 hours in metabolic cages to collect urine before euthanasia, whereas Phase B mice were euthanized without a prior fasting period. The euthanasia procedure was conducted by a trained research animal technician, with euthanasia taking place between 10:00 and 11:00 AM to minimise variations in physiological states related to nocturnal feeding behaviours and the circadian cycle (Griffin, 2006).

3.2.5. Sampling and tissue collection

In Phase A of the study, urine was collected over 12 hours from individual mice in metabolic cages during an overnight fasting period, while water was freely available. To inhibit bacterial growth, the separating cone, collection funnel, and collection tubes were pretreated with 100 μ L of a 0.1% (w/v) sodium azide (NaN_3) solution before starting the collection process. Following urine collection, mice were euthanized by exsanguination at a consistent time window (10:00–11:00 AM). In both Phase A and Phase B, brain, blood, and liver tissue were harvested immediately after euthanasia. These tissues were rapidly snap-frozen in liquid nitrogen to stop metabolic activity and subsequently stored at -80°C for further analysis.

3.3. DNA extraction and genotyping

3.3.1. Reagents

For DNA extraction and amplification, the following reagents were used: the Quick-DNA™ MiniPrep Kit (cat #D4069, Inqaba Biotech™), the Phire™ Tissue Direct PCR Master Mix Kit (cat #F-170L, Thermo Fisher Scientific), and specific primers for *Ndufs4* [*Ndufs4* 1060, cat #S32E6; *Ndufs4* Rev, cat #S3424, Inqaba Biotech™]. Amplicon separation and visualisation were performed using agarose (cat #8100, CONDA Pronadisa), ethidium bromide (EtBr) as a nucleic acid stain (cat #46065, Sigma-Aldrich®), 10× Bionic™ Buffer (cat #B6185, Sigma-Aldrich®), nuclease-free PCR-grade water (H_2O ; cat #129114, Qiagen), and GeneRuler™ 100 bp DNA ladder (cat #SM0323, Thermo Fisher Scientific).

3.3.2. Genomic DNA isolation

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Genomic DNA was extracted from approximately 25 mg of mouse tail snips following the manufacturer's protocol using the Quick-DNA™ MiniPrep Kit. The concentration and purity of the isolated DNA were then assessed by measuring absorbance at 260 nm for DNA and 280 nm for protein using the NanoDrop® 1000 UV-Vis spectrophotometer (software version 3.8.1, Thermo Fisher Scientific). Based on these measurements, DNA samples were adjusted to a concentration of 25 ng/μL for subsequent analysis (Section 3.3.3).

3.3.3. Polymerase chain reaction

A DNA fragment spanning exon 2 (the loxP-flanked 'excision region') of the mutant *Ndufs4* allele was amplified by PCR, with primers located in the adjacent introns as described in the original *Ndufs4*^{tm1.1Rpa/J} genotyping protocol (JAX, 2017; Kruse *et al.*, 2008). The 10 μL reaction mixture consisted of 1× Phire™ Tissue Direct PCR Master Mix, nuclease-free PCR-grade water, 25 ng of template DNA, and 0.5 μM each of the forward (*Ndufs4* 1060) and reverse (*Ndufs4* Rev) primers designed to bind intronic sequences flanking exon two of *Ndufs4*. Amplification was conducted on a T100™ thermal cycler (Bio-Rad) with the following thermal profile: initial denaturation at 98 °C for 5 minutes to activate the DNA polymerase, followed by 34 cycles of denaturation at 98 °C for 5 seconds, annealing at 57.3 °C for 5 seconds, and extension at 72 °C for 20 seconds. A final elongation step was performed at 72 °C for 1 minute.

3.3.4. Agarose gel electrophoresis

A 1% (w/v) agarose gel was prepared using 1× Bionic™ Buffer with 5 μg/mL ethidium bromide as a nucleic acid stain. Following the preparation, amplicons were subsequently separated by electrophoresis on the agarose gel. Diluted PCR products (10 μL, 30% v/v) and a GeneRuler™ 100 bp DNA ladder were loaded onto the gel. The electrophoresis was initiated at 40 V for 15 minutes, followed by an increase to 70 V for approximately 1.5 hours, employing a Sub-Cell® GT horizontal electrophoresis apparatus (Bio-Rad). Following electrophoresis, the gel was visualized and photographed under ultraviolet (UV) light using the ChemiDoc™ MP system (Bio-Rad). The resulting images were analysed with Image Lab software (version 5.2.1). Genotypes (Figure A-1) were determined based on amplicon sizes: the presence of two intact *Ndufs4* allele segments (1229 bp) indicated a WT genotype (*Ndufs4*^{+/+}); two shortened allele segments (429 bp) indicated a KO genotype (*Ndufs4*^{-/-}), which lacks the second exon of *Ndufs4*; and the presence of both intact and shortened bands identified HET carriers (*Ndufs4*^{+/-}).

3.4. Phenotypic evaluation

Growth curves were generated for all mice across both study phases, with Phase A using 40 mice and Phase B using 80 mice. Since *Ndufs4* KO mice typically have a lifespan of only 2–3 months, we assessed whether HFD treatment could extend their survival age while also evaluating potential improvements in key phenotypic manifestations in Phase B. These include

clasping, locomotor impairments, ataxia, and weight loss (Kruse *et al.*, 2008; Quintana *et al.*, 2010). Selected phenotypic and behavioural assessments were then conducted to evaluate these manifestations including survival analysis, monitoring of clasping on-set, open-field tests and wire-grid hang tests. A total of 24 mice from Phase B were included in these phenotypic assessments.

3.4.1. Growth curves

All mice in both Phase A (n = 40) and Phase B (n = 80) were weighed five times per week at approximately the same time each day. Weighing commenced once the mice were assigned to their respective groups and cages. Mice were weighed to generate growth curves over the 3-week dietary intervention for each phase until euthanised (P45-47). Data analysis for the growth rate was carried out using Excel 2021 (Microsoft 365).

3.4.2. Survival

The day of natural death for selected mice was recorded to evaluate the effectiveness of HFD in extending the lifespan of *Ndufs4* KO mice used in Phase B. Survival analyses were conducted using the minimum necessary number of mice (n = 10 per group) to ensure statistical significance while prioritising animal welfare and minimising animal suffering. Mice included in the survival analysis were weighed five times per week and monitored daily to determine the precise day at which they naturally died. Locomotor activity and ataxia assays were conducted on the same mice to assess the impact of the HFD on disease phenotypes. From P45 onward, food pellets were placed on the floor of the cage, and water bottles with extended spouts were provided to facilitate access to food and water, ensuring that deaths did not result from starvation. The date of natural death was recorded for each mouse throughout Phase B, followed by the survival analyses which was performed using the Log-rank (Mantel-Cox) test.

3.4.3. Locomotor activity

Locomotor activity was evaluated using an open-field test (Figure 3-1) in conjunction with automated tracking software, specifically Ethovision XT14 by Noldus Information Technology BV. The open-field test was conducted during the early (P28–30), mid (P38–40), and late (P48–50) stages of the disease. To acclimate the mice to the testing environment, their home cages were placed in the testing room for at least 15 minutes. During this time, the mice were left undisturbed, and the experimenter remained out of sight. Each mouse was individually placed at the centre of one of four 0.5 m² black opaque arena chambers. The four arena chambers allowed for the simultaneous assessment of four mice at a time. The mice were allowed to explore the arena freely for five minutes, adhering to the protocol outlined by Seibenhener and Wooten (2015). To maintain consistency across trials, any droppings were removed from the arena, and it was thoroughly disinfected with 70% ethanol to eliminate any residual odours. The

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Ethovision software tracked and recorded both horizontal and vertical activity throughout the five-minute session. Following the test, the mice were returned to their home cages.

This automated system enabled continuous, objective behavioural monitoring, providing greater sensitivity than manual observation while minimising investigator bias. The following parameters were measured: cumulative time spent in the centre of the arena (in seconds), mean velocity (movement speed in cm/s), and total distance travelled (in cm). These measures were used as indicators of overall locomotor activity.



Figure 3-1: Photograph of the segmented chambers of the open-field test. Four segmented arena chambers used for the open-field test, allowing the simultaneous assessment of exploratory behaviour and locomotor activity in four mice from each group at a time during the dietary intervention.

3.4.4. Grip strength

Pronounced ataxia is exhibited by *Ndufs4* KO mice, characterised by splayed limbs, impaired balance, frequent falls, slow movement alterations, and an unresponsive reaction to physical stimuli, as previously noted by (Kruse *et al.*, 2008). The wire-grid hang test (Figure 3-2) was used to quantify this phenotypic feature and was conducted during the early (P28–30), mid (P38–40), and late (P48–50) stages of the disease.

The method described by Carlson *et al.* (2010) was used as the basis for the test procedure, which evaluates muscular strength in all limbs against gravity. This straightforward and cost-effective test eliminates the need for prior acclimatisation of the mice. To prevent escape, the edges of the wire cage were encircled with a 25 cm cardboard enclosure. Each mouse was then positioned on the wire grid inside this enclosure, where it could grasp the grid with all four limbs. The grid was inverted above a plastic cage containing soft sawdust and paper towels, which had been placed at a height of approximately 35 cm above the bedding. Safety in the event of a fall was ensured by this setup, while also allowing voluntary descent.

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The duration of grip was recorded using a stopwatch, with a maximum time of 180 seconds. Each mouse underwent three trials, separated by a 10-minute rest period. Mice that fell off the grid within 10 seconds were retested to avoid potential biases related to pre-existing grip issues. Prior to the wire grid hang test, each mouse was weighed, and the mean holding time across the three trials was calculated. The physical impulse (Fdt) was then determined by multiplying the holding time by the gravitational force acting on the mouse [$Fdt \text{ (N s)} = \text{body mass (g)} \times 0.00980665 \text{ N/g} \times \text{holding time (s)}$], which represents the minimum sustained force the animal applied to counteract gravity (Carlson *et al.*, 2010).



Figure 3-2: The wire-grid hang test setup as depicted. Mice were positioned on the lid of the cage, which was then inverted. The duration for which the animal could maintain its grip, with a maximum limit of 180 seconds, was measured.

3.5. SDS-PAGE and western blotting

3.5.1. Reagents

For protein extraction and isolation, the following were utilized: RIPA buffer (cat #R0278, Sigma Aldrich), Pierce™ protease and phosphatase inhibitor mini tablets (Cat #A32959, Thermo Fisher) and the Pierce™ universal nuclease for cell lysis (cat #88700, Thermo Fisher). Protein quantification was accomplished using bicinchoninic acid (BCA) solution (cat #B9643, Sigma Aldrich), copper sulphate (CuSO_4 , cat #2284, Sigma Aldrich) and bovine serum albumin (BSA, cat #P0914, Sigma Aldrich).

Protein separation via SDS-PAGE was performed using the TGX Stain-Free™ FastCast™ acrylamide starter kit, 10% (cat #1610182, Bio-Rad), tetramethylethylenediamine (TEMED, cat #T7024, Sigma Aldrich), 10% (w/v) ammonium persulfate (APS, cat #A3678, Sigma Aldrich), 10x Tris/Glycine/SDS (cat #1610732, Bio-Rad), Precision Plus Protein™ dual xtra prestained protein standard (cat #1610377, Bio-Rad), 4x Laemmli sample buffer (cat #1610737, Bio-Rad), 355 mM β -mercaptoethanol (cat #M3148, Sigma Aldrich), and Milli-Q water.

Western blot and immunodetection was achieved using Trans-Blot Turbo Mini 0.2 μm nitrocellulose transfer packs (cat #1704158, Bio-Rad), 10x tris-buffered saline (TBS) with 1%

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(w/v) casein (cat #1610782, Bio-Rad), Tween®20 (cat #P1379, Merck), phospho-p70 S6 kinase 1 (cat #9234S, Cell Signalling Technologies), phospho-mTOR (cat #5536S, Cell Signalling Technologies), phospho-p70 S6 kinase (cat #9234S, Cell Signalling Technologies), phospho-4E-BP1 (cat #9459S, Cell Signalling Technologies), p70S6K1 (cat #9202S, Cell Signalling Technologies), 4E-BP1 (cat #9644S, Cell Signalling Technologies), SirT1 (cat #9475S, Cell Signalling Technologies), mTOR (cat #ab32028, Abcam), PGC1- α (cat #ab54481, Abcam), TNF- α (cat #A0046, Cape Biologix), β -actin (cat #ab6276, Abcam), goat anti-mouse IgG H&L (HRP) (cat # ab97023, Abcam) and goat anti-rabbit IgG H&L (HRP) (cat #ab97051, Abcam) and Western ECL substrate (cat #170-5060, Bio-Rad).

3.5.2. Protein extraction and isolation

The tissue extraction and isolation method described was applied in both Phase A and Phase B. To maintain tissue integrity, all preparations were performed on ice. Tissue samples were finely minced on a chilled steel surface using scalpel blades, weighed, and homogenised using a Potter-Elvehjem homogenizer to generate a 10% (w/v) homogenate in ice-cold RIPA buffer containing 1X protease and phosphatase inhibitors as well as nuclease. Homogenisation involved approximately 10–20 strokes until the mixture was uniform. The homogenate was transferred into microcentrifuge tubes and centrifuged at 600 $\times$ g for 10 minutes at 4°C. The supernatant was then carefully separated into fresh tubes and stored at –80°C until further analysis. Before downstream applications, the samples were subjected to at least two cycles of freezing and thawing.

3.5.3. Protein quantification

Protein concentrations of the samples ($n = 4$ per group) used in each phase were determined using the bicinchoninic acid (BCA) assay, originally described by Smith *et al.* (1985). The working reagent was prepared by combining BCA solution with CuSO₄ in a 50:1 ratio. BSA served as the standard for generating a calibration curve, with concentrations ranging from 0 to 20 μ g in 4 μ g increments. Sample volumes of 2 μ L for liver tissue extracts and brain tissue extracts in each phase were added to each well, with Milli-Q water used to adjust the total volume to 20 μ L. Next, 200 μ L of the BCA:CuSO₄ working solution was added to each well, and the 96-well microplate was incubated at 37°C for 30 minutes. Absorbance readings at 562 nm were recorded using a Synergy HT microplate reader (BioTek Instruments), and data were processed with Gen5™ Data Analysis Software (BioTek Instruments).

3.5.4. SDS-PAGE

Protein samples ($n = 4$ per group) in each phase were separated using SDS-PAGE, following the Laemmli method (Laemmli, 1970). The TGX Stain-Free™ FastCast™ acrylamide starter kit, 10% was used for gel preparation, along with TEMED, 10% (w/v) APS, and a Mini-PROTEAN® Tetra Cell system (cat #1658004, Bio-Rad). The gel (1 mm) was cast according to the

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manufacturer's protocol, and 1x Tris/Glycine/SDS running buffer was used. Protein concentrations were adjusted to 20 µg/µL for liver samples in Phase A and 50 µg/µL for brain samples in Phase B. The protein samples and protein standard were mixed with 1x Laemmli sample buffer, 355 mM β-mercaptoethanol, and Milli-Q water. The mixtures were heated at 72°C for 10 minutes and centrifuged at 16 000 ×g for 5 minutes to remove debris. A volume of 20 µL from each sample and the protein ladder was loaded into individual wells, and electrophoresis was conducted at 300 V for 22 minutes in Phase A and 200 V for 30 minutes in Phase B.

3.5.5. Western blot and immunodetection

Protein transfer from SDS-PAGE gels to nitrocellulose membranes was performed for each phase using the Trans-Blot Turbo Transfer System (cat #1704155, Bio-Rad) and 0.2 µm nitrocellulose transfer packs. Transfers were conducted at a constant current of 1.3 A and 25 V according to the manufacturer's guidelines. Following transfer, membranes were imaged using the ChemiDoc™ MP Imaging System (Bio-Rad) with a stain-free protocol. Membranes were then blocked in 1x TBS with 1% (w/v) casein for 1 hour.

In Phase A, membranes were incubated overnight at 4°C with primary antibodies targeting phospho-p70 S6 kinase 1 and the housekeeping protein β-actin, at 4 °C overnight. Secondary antibody incubation with either the HRP conjugated anti-mouse or anti-rabbit antibodies was performed at room temperature for 1 hour. Protein bands were visualized using Western ECL substrate and the ChemiDoc™ MP imaging system. Densitometric analysis was subsequently performed with Image Lab™ software version 6.1 (Bio-Rad) to calculate the density ratios of phospho-p70 S6 kinase relative to β-actin.

In Phase B, membranes were incubated overnight at 4°C with primary antibodies against phospho-mTOR, phospho-p70 S6 kinase, phospho-4E-BP1, p70SK1, 4E-BP1, SirT1, mTOR, PGC1-α, and TNF-α, with the housekeeping protein, β-actin serving as the loading control. Membranes were then incubated with either HRP conjugated anti-mouse or anti-rabbit secondary antibodies for 1 hour at room temperature. Chemiluminescent detection was performed using a Western ECL substrate, and images were captured using the ChemiDoc™ MP imaging system (Bio-Rad) as in Phase A. Quantitative analysis was conducted using Image Lab™ software version 6.1, and density ratios were determined by normalising target proteins to either their total protein levels or β-actin, where relevant. This was then plotted on a bar graph to observe the relative intensity differences between each group for each phase of the study.

3.6. Cytometric bead array and flow cytometry

The serum concentrations of pro-inflammatory biomarkers, including IL-6, IL-10, MCP-1, TNF, IFN-γ, and IL-12, were measured in both phases using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (cat #552364, BD Biosciences) according to the manufacturer's

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protocol. Serum samples were thawed and aliquoted (50 μ L) for CBA analysis, which was conducted according to the manufacturer's protocol (Manual, 2008) and previously described methodology (Lian *et al.*, 2019; Williams *et al.*, 2013). A serial dilution of cytokine standards was prepared to generate a standard curve for cytokine quantification, ranging from 0 to 5000 pg/mL with a dilution factor of 2.

Seven distinct sets of capture beads, each conjugated with a specific antibody targeting one of the cytokines measured, were combined with flow cytometry to enable multiplex cytokine detection. A 50 μ L volume of phycoerythrin (PE)-conjugated detection reagent and 50 μ L of the mixed capture beads were added to each sample and standard. This mixture was incubated for 2 hours at room temperature, protected from light. During incubation, samples were briefly vortexed (~1 second) every 30 minutes to enhance the interaction between the capture beads and cytokines. This process allowed the formation of sandwich complexes consisting of capture beads, cytokines, and the PE-detection antibody. During flow cytometric analysis, laser excitation of these complexes produced fluorescence signals proportional to the cytokine concentrations. After incubation, samples were washed, and the pellet was resuspended in 300 μ L of wash buffer for flow cytometric detection.

Samples and standards were analysed on a BD Accuri C6 Plus flow cytometer (BD Biosciences) in Phase A and a BD FACSLyric™ flow cytometry system (BD Biosciences) in Phase B. Prior to data acquisition on the day of analysis, instrument performance was verified using an automated quality control (QC) procedure with Cytometer Setup and Tracking beads (cat #661414, BD Biosciences). The instruments passed QC assessment on both analysis days. In Phase A, data collection utilized a BD Biosciences template, setting forward scatter (FSC) and side scatter (SSC) thresholds at 500 000 to exclude debris and background noise. Gating strategies involved FSC versus SSC dot plots and histograms displaying event counts against allophycocyanin (APC) to define bead populations. Cytokine concentrations were visualized using dot plots of PE versus APC, illustrating distinct bead populations and concentration shifts. Similarly, in Phase B, data collection employed a BD Biosciences template with FSC and SSC thresholds adjusted to 10 000 for debris exclusion and noise minimisation. Gating strategies mirrored those of Phase A, utilising histograms that plotted event counts (with 2 000 beads captured) against CBA red to define bead populations. Cytokine concentrations were then visualized through dot plots of PE versus CBA red, highlighting distinct bead populations and their respective concentration changes.

Data from all samples were exported as Flow Cytometry Standard (FCS) files for downstream analysis. Standard curves were generated, and cytokine concentrations (pg/mL) were calculated through regression analysis. Phase A data were processed using FCS Express software (De Novo, version 6), while FlowJo software (version 10.8) was used for data processing and analyses in both Phase A and B. Subsequently, outlier removal and statistical

tests, including independent samples t-tests and the Mann-Whitney U test, were performed using Excel 365, IBM SPSS Statistics 25, and GraphPad Prism version 9.

3.7. Multi-platform metabolomics and lipidomics

In Phase A, targeted metabolomics using liquid chromatography–tandem mass spectrometry (LC-MS/MS), semi-targeted profiling with ¹H-NMR, and untargeted metabolomics with gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) were conducted on mouse urine samples (n = 10 per group). In Phase B, targeted gas chromatography–tandem mass spectrometry (GC-MS/MS), untargeted GC-TOF-MS, and untargeted lipidomic analysis using UPLC-HDMS were performed on brain tissue samples (n = 14 per group). All urine and brain tissue samples were prepared and analysed individually within their respective phases, with pooling restricted to QC sample preparation. Metabolomic profiling methods adhered to protocols previously established and validated for mouse urine and brain tissues by the Mitochondria Research Group at North-West University (Lindeque *et al.*, 2013; Mels *et al.*, 2011; Miller *et al.*, 2021).

3.7.1. Reagents

During sample preparation across both phases, the following reagents were utilized: QuantiChrom™ creatinine assay kit (cat #DICT-500, BioAssay Systems), sodium hydroxide (NaOH, cat #221465, Sigma-Aldrich), 0.3 mM ethylenediaminetetraacetic acid (EDTA, cat #03685, Sigma-Aldrich); 0.1% (v/v) , dimethyl sulfoxide (DMSO, cat #D2650, Sigma-Aldrich); 0.0004% (v/v) Tween®20 (cat #P1379, Merck), 3 mM picric acid (cat #80456, Sigma-Aldrich, methanol (cat #2304, Honeywell, Burdick & Jackson, HPLC grade), water (H₂O; cat #BJ365CS, Honeywell, Burdick & Jackson, HPLC grade), chloroform (cat #0494, Honeywell, Burdick & Jackson, HPLC grade), 1-butanol (cat #34867 , Honeywell, Burdick & Jackson, HPLC grade), potassium phosphate monobasic anhydrous (KH₂PO₄; cat #229806, Merck), sodium azide (NaN₃, cat #S2002, Sigma-Aldrich), potassium hydroxide (KOH; #P1767, Merck) and deuterium oxide (D₂O; cat #435767, Merck).

For various analytical standards, the following internal standards were employed: *N, N* dimethyl-L-phenylalanine (DMPA; cat #273910, Sigma-Aldrich, 99%), 3-phenylbutyric acid (3-PBA; cat #116807, Sigma-Aldrich®, 98%), 3-(trimethylsilyl) propionic acid (TSP; cat #269913, Merck), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (cat #850360P, Avanti Polar Lipids), 1,2-diheptadecanoyl-sn-glycero-3-phosphate (cat #830856P, Avanti Polar Lipids), and 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (cat #830456P, Avanti Polar Lipids).

Additionally, the following stable isotopes were also utilized: L-isoleucine-d₁₀ (cat #DLM-14 1-0.1, Cambridge Isotope Laboratories, 98%), L-valine-d₈ (Cat. #E161P, Cambridge Isotope Laboratories, 99%), L-phenylalanine-ring-d₅ (cat #E151P, Eurisotop, 98%) L- carnitine-methyl-

d_3 -HCl, octanoyl-L-carnitine-8,8,8- d_3 -HCl, and octadecanoyl-L-carnitine-18,18,18- d_3 -HCl obtained from Vrije Universiteit university medical center (VUmc).

During sample derivatization and analysis, the following reagents were used: acetyl chloride (cat #00990, Fluka analytical, $\geq 99\%$), 1-butanol (cat #281549, Sigma-Aldrich®, 99.8%), methoxyamine hydrochloride (cat #226904, Aldrich, 98%), O-bis(trimethylsilyl) trifluoroacetamide with 1% tert-butyldimethylchlorosilane [TMCS] (BSTFA; cat #B-023, Supelco), pyridine anhydrous (cat #270970, Sigma-Aldrich®, 99.8%), N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with 1% TMCS (cat #M-108, Supelco), acetonitrile (ACN; cat #BJ015CS, Honeywell, Burdick & Jackson, $>99\%$ HPLC grade), water (H_2O ; cat #BJ365CS, Honeywell, Burdick & Jackson, HPLC grade), formic acid (cat #06440, Sigma-Aldrich®, 98% for mass spectrometry) and leucine enkephalin (Leu Enk, cat #186006013, Waters).

3.7.2. Addition of internal standards

In both Phase A and Phase B, known quantities of internal standards (IS) were added to urine and tissue samples at the start of sample preparation to account for potential variations during processing. For Phase A, LC-MS/MS sample aliquots were supplemented with a mixture of amino acid and acylcarnitine isotopes to mitigate ion suppression effects during analysis. For GC-TOF-MS analyses in Phase A and GC-TOF-MS and GC-MS/MS analyses in Phase B, 3-PBA was selected as the IS for urine and brain tissue, respectively. DMPA was used as the IS for LC-MS/MS analyses. DMPA was also used as an IS for ^1H -NMR analyses, along with TSP in Phase A. Brain lipid extracts for UPLC-HDMS lipidomic analyses in Phase B were spiked with 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-glycero-3-phosphate, and 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) fatty acid ISs. For tissue samples, ISs were added relative to the tissue mass as part of the water used in monophasic extraction (Section 3.7.4). Given the minimal preparation required for urine samples, LC-MS/MS aliquots were concurrently supplemented with DMPA and isotopic standards. The selection of ISs was based on their absence from murine samples, reliable derivatization properties, optimal elution times, and consistent detectability across multiple metabolomic platforms (Van Aardt *et al.*, 2016).

3.7.3. Urine sample preparation

3.7.3.1. Urinary creatinine concentration

Urinary creatinine concentrations in Phase A were measured using spectrophotometry based on a modified Jaffe reaction, following the manufacturer's protocol for the QuantiChrom™ Creatinine Assay Kit. In this assay, creatinine reacts with picric acid under alkaline conditions to produce a yellow-orange complex, with the increase in absorbance measured at 510 nm. For each sample, 10 μ L of urine was combined with 200 μ L of a working solution containing 0.1 M

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NaOH, 0.3 mM EDTA, 0.1% (v/v) DMSO, 0.0004% (v/v) Tween®20, and 3 mM picric acid. Reactions were performed in triplicate on a 96-well plate, and absorbance was recorded at 1-minute intervals over a 5-minute period using a Synergy™ HT Multi-detection microplate reader. Creatinine concentrations were quantified using a standard curve ranging from 0 to 50 mg%. All creatinine measurements for urine samples analysed in multi-platform metabolomic assays were performed across two 96-well plates.

3.7.3.2. *Urine deproteinisation*

Urine samples corresponding to 0.0625 μ mol of creatinine were measured and transferred into individual microcentrifuge tubes designated for each analytical platform. Internal standards were added (Section 3.7.2). Urine samples were then deproteinised for LC-MS/MS, ^1H -NMR and GC-TOF-MS analyses. Deproteinisation was achieved by adding methanol in a 3:1 (v/v) ratio of methanol to urine. The mixture was briefly vortexed for 15 seconds, incubated at -20°C for 20 minutes, and centrifuged at $15\,000 \times g$ for 10 minutes. The resulting supernatants were aliquoted for both for LC-MS/MS and GC-TOF-MS analyses, then transferred into glass vials and dried under a nitrogen gas stream at 37°C . To remove any residual solvent, 80 μL of methanol was added, and the drying process was repeated. Fully dried urine extracts were stored at -80°C until analysis.

3.7.4. **Metabolite and lipid extraction from brain tissue**

3.7.4.1. **Monophasic brain extracts for metabolomics**

For brain samples used in Phase B, a monophasic solvent ratio of 3:1:1 (v/v) methanol:chloroform:water was employed, assuming an 80% water content in wet brain tissue, as reported by Gonzalez-Riano *et al.* (2016). This ratio was chosen to ensure a monophasic extract and prevent solvent phase separation, as discussed by Gullberg *et al.* (2004). To standardise metabolite concentrations across samples, solvent volumes were adjusted based on tissue weight, thereby reducing matrix-induced ion suppression and ensuring consistent concentrations, as noted by Kapoore and Vaidyanathan (2016), and Boysen *et al.* (2018). Typically, the final monophasic solution was prepared at a concentration of 5% (w/v). Throughout the procedure, samples were kept on ice whenever possible, and all solvents were maintained at 4°C unless otherwise specified.

On the extraction day, brain tissue samples stored at -80°C in 2 mL safe-lock microcentrifuge tubes were prepared for metabolite extraction. To enhance mechanical disruption, a single cold steel bead (3 mm diameter) was added to each tube, following the method outlined by Gullberg *et al.* (2004). Solvents were added based on tissue weight: 12 $\mu\text{L}/\text{mg}$ of methanol (-20°C), 3.2 $\mu\text{L}/\text{mg}$ of water containing ISs, and 4 $\mu\text{L}/\text{mg}$ of chloroform (-20°C), with the goal of achieving a final 3-PBA concentration of 25 ppm. Homogenisation was performed for 5 minutes at 30 Hz using a Retsch MM 400 vibration mill, ensuring uniform sample disruption while processing

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each tube individually to prevent contamination. After bead removal, samples were vortexed for 1 minute, incubated on ice for 10 minutes, and centrifuged at 20 000 ×g for 15 minutes at 4°C to separate proteins and tissue debris.

The monophasic supernatant was carefully transferred to fresh microcentrifuge tubes, avoiding disturbance of the pellet. Aliquots were prepared for downstream analyses. For GC-TOF and GC-QQQ platforms, 200 µL of the supernatant per sample was transferred to glass vials, dried under a gentle nitrogen stream in a fume hood, capped tightly, and stored at –80°C until derivatization.

3.7.4.2. Lipid extracts for lipidomics

For the homogenisation and lipid extraction of brain tissue, snap-frozen samples were weighed and placed into 2 mL centrifuge tubes containing 3 mm steel beads. Lipid extraction was conducted using a modified single-phase butanol/methanol (BUME) method, following the method described by previous studies (Alshehry *et al.*, 2015; Löfgren *et al.*, 2012; Reichl *et al.*, 2020). A cold BUME solution (3:1, v/v), pre-mixed with diheptadecanoyl (C17:0) fatty acid IS (Section 3.7.2), was added at a ratio of 4 µL per mg of brain tissue. Homogenisation was performed using a vibration mill set at 30 Hz for 20 minutes, followed by centrifugation at 4 000 ×g for 10 minutes. The upper supernatant layer was carefully transferred to clean tubes and centrifuged again at 12 000 ×g for 10 minutes to remove residual debris. Aliquots of 50 µL from the clarified extract were placed into 2 mL autosampler glass vials, dried using a SpeedVac vacuum concentrator, and stored at –80°C until further processing. The volumes analysed on the various analytical platforms during each phase are shown in Table 3-1.

Table 3-1: Volumes analysed on different analytical platforms during Phase A and B

Analytical platform	Sample type	Extract volume (µL)	Final sample volume (µL)	Injection volume (µL)	Mass IS analysed (ng)
LC-MS/MS	Urine	20-250	100	1	25
¹ H-NMR	Urine	54	60	N/A	50
GC-TOF-MS	Urine	20-250	100	1	25
	Brain	200	100	1	25
GC-MS/MS	Brain	200	100	1	25
UPLC-HDMS	Brain	50	100	1	20

The table outlines key parameters for each platform, including sample type (column 2), initial sample extract aliquot (column 3), the final volume of each prepared sample prior to analysis (column 4), injected sample volume for chromatographic platforms (column 5), and the resulting IS mass in the analysed sample volume (column 6). For chromatographic platforms, the on-column IS mass was determined by multiplying the final IS concentration in the sample by the injection volume.

3.7.5. Quality control samples

For Phase A, urine QC samples were prepared for each mouse group (ND, ND + Alc, KD, and KD + Alc) by pooling equal amounts of sample extracts from the respective groups. These pooled QC samples were aliquoted, stored, and prepared using the same protocols as the experimental samples for each analytical platform. QC samples were analysed at specific intervals to ensure instrument stability and reproducibility: for GC-TOF-MS, QC samples were analysed at the beginning, after every six experimental samples, and at the end of each batch; for LC-MS/MS, QC samples were analysed at the beginning, after every five experimental samples, and at the end of each batch; and for ¹H-NMR analysis, QC samples were incorporated at the beginning, middle, and end of the analytical sequence. Additionally, for LC-MS/MS, an in-house standard mixture containing all target metabolites was analysed at the end of each batch. This mixture included a QC sample spiked with the standard to monitor system performance and method consistency.

For Phase B, QC samples for metabolomic and lipidomic analyses of brain extracts were prepared by pooling equal amounts of sample extracts from each dietary group (WT-ND, WT-HFD, KO-ND, and KO-HFD). QC samples were analysed at consistent intervals to monitor analytical performance. Specifically, QC samples were analysed after every seven experimental samples during GC-TOF-MS and GC-MS/MS analyses and after every six samples during UPLC-HDMS analyses.

3.7.6. Metabolomics and lipidomic analyses

3.7.6.1. LC-MS/MS sample preparation and analyses (Phase A)

Urine samples (Section 3.7.3) for LC-MS/MS analysis in Phase A were randomly assigned for preparation and analysis. The samples were prepared by drying 200-250 µL of urine (containing 0.0625 µmol creatinine) along with internal standards and isotopes under a nitrogen gas stream at 37 °C. Derivatization was performed by adding ~300 µL of a butanol: acetyl chloride mixture to each sample, followed by incubation at 50 °C for 1 hour. The derivatized samples were subsequently dried under nitrogen at 37 °C and reconstituted in 50 µL of a water: acetonitrile solution (50:50, v/v) containing 0.1% formic acid. The reconstituted samples were vortexed thoroughly, transferred to 250 µL glass inserts, and loaded into an Agilent® 1200 series auto-sampler for analysis.

LC-MS/MS analysis was performed following the method by Mels *et al.* (2011), with minor modifications. A 1200 series liquid chromatograph coupled to a 6410 triple quadrupole mass spectrometer (Agilent Technologies, USA) was used. Chromatographic separation was achieved using a Zorbax SB-Aq C18 reverse-phase column (150 mm × 2.1 mm × 1.8 µm, Agilent Technologies) maintained at 35 °C, with a flow rate of 0.2 mL/min. A guard column was employed to protect the analytical column from particulate contamination. The mobile phases

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consisted of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The chromatographic gradient was as follows. First, an initial 5% mobile phase B, maintained for 1 minute, then a gradual increase to 23% mobile phase B over 7 minutes, followed by a 4-minute isocratic hold. A further increase to 100% mobile phase B at 15 minutes, was held for 5 minutes. Finally, the gradient returned to 5% mobile phase B over 1 minute and equilibrated for 7 minutes (post-run).

The column effluent was directed to the mass spectrometer, operating in positive electrospray ionisation (ESI) mode. Source parameters included a capillary voltage of 3 500 V, nitrogen as the drying gas (7.5 L/min at 300 °C), and a nebulizer pressure of 30 psi. Data acquisition utilized the multiple reaction monitoring (MRM) mode with an electron multiplier voltage of 300 V and a dwell time of 45 ms per compound. To minimise salt exposure to the mass spectrometer, eluent prior to 1.4 minutes was diverted to waste. The MRM transitions (Table 3-2) for all butyl esters were distributed across two-time windows to ensure sufficient data points per peak.

Data acquisition and processing were performed using Agilent® MassHunter Workstation software (version B02.01 for data acquisition, version B06.00 for qualitative and quantitative analysis) and MassHunter Optimizer software (version B02.01 for MRM configuration).

Table 3-2: MRM parameters for metabolites and internal standard isotopes monitored

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (eV)
Asparagine	189.1	144.1	89	8
Ornithine	189.2	70.2	74	20
Histidine	212.1	110.1	89	16
Taurine	126	107.9	93	10
Cystine	353	130	118	16
Glycine	132.1	76.1	65	4
Serine	162.1	60.1	94	12
Glutamine	203.1	84.1	89	20
Lysine-d ₄	203.2	84.1	89	20
Threonine	176.1	74.1	94	12
β-Alanine	146.2	72.1	90	10
Alanine	146.1	44.2	80	15
Hydroxy-Proline	188.1	68.1	120	35
Citrulline	232.2	70.1	89	32
γ-Aminobutyric acid	160.1	87.1	79	8
Carnitine-(methyl-d ₃)	221.2	103.1	127	16
Carnitine	218.2	103	132	16
Proline	172.1	70.2	94	20
Creatine	188.1	90.1	104	16
Arginine	231.2	70.1	103	40
Valine-d ₈	182.2	80.2	103	12
Valine	174.2	72.2	89	12
Acetylcarnitine	260.2	85.1	122	24

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Cystathionine	335.2	190.1	127	16
Methionine	206.1	104.1	94	8
Tyrosine	238.2	136.1	94	12
3-OH-Kynurenine	265.4	136	120	10
Isoleucine-d ₁₀	198.2	96.2	108	12
Isoleucine	188.2	86.2	89	8
Leucine	188.2	86.2	89	8
Homocystine	381.2	192.1	95	8
Phenylalanine-(ring-d ₅)	227.2	125.1	108	16
Phenylalanine	222.2	120.1	108	16
Aspartic acid	246.2	144.1	98	12
Glutamic acid	260.2	84.1	89	24
Tryptophan	261.2	244.1	94	8
Butyrylcarnitine	288.2	85.1	110	20
Decanoylcarnitine	372.3	85.1	125	28
Cysteine	178.1	76	180	20
Hexanoylcarnitine	316.2	85.1	137	24
DodecanoylCarnitine	400.3	85.1	155	28
Homocysteine	192	90	161	12
Tetradecanoylcarnitine	428.4	85.1	150	28
Octanoylcarnitine-d ₃	347.3	85.1	136	24
Octanoylcarnitine	344.3	85.1	115	28
Isovalerylcarnitine	302.2	85.1	105	24
Palmitoylcarnitine	456.4	85.1	160	36
Octadecanoylcarnitine	484	85.1	155	36
Octadecanoylcarnitine-d ₃	487.5	85.1	160	36

Conditions for butylated compounds and deuterium-labelled internal standards (IS) are detailed together with their precursor- and product ions. *Abbreviations: m/z, mass to charge ratio; V, voltage; eV, electron volts.*

3.7.6.2. ¹H-NMR sample preparation and analyses (Phase A)

The urine samples (Section 3.7.3) in Phase A were centrifuged at 12 000 × g for 5 minutes at 25°C to remove particulates. A chemical shift standard buffer solution (10%) was prepared following the protocol described by Dona *et al.* (2014). The buffer consisted of 1.5 M KH₂PO₄ 100 ppm trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid, and 13 ppm NaN₃ in D₂O, with the pH adjusted to 7.4. For each sample, 54 µL of the urine sample ultrafiltrate and 6 µL of the 10% buffer solution were measured using an eVol® digital syringe. The resulting supernatant was then transferred into 2 mm NMR tubes (Bruker), mixed thoroughly by repeated aspiration and purging, and then prepared for analysis. The syringe underwent a washing sequence after each sample to prevent contamination. The NMR tubes were assembled using the Bruker MATCH system and loaded into a SampleXpress autosampler.

¹H-NMR spectroscopy was conducted using a miniaturized method adapted from Mason *et al.* (2018). Urine samples were analyzed at 500 MHz in 2 mm MATCH tubes using a 5 mm triple-resonance inverse (TXI) probe head optimized for ¹H detection. The sample temperature was

maintained at 300 K throughout the analysis. Spectra were acquired using 128 transients, with 32K data points and a spectral width of 6000 Hz (12.0 ppm). The NOSEY pre-saturation pulse sequence was applied to suppress the water resonance at 4.70 ppm via single-frequency irradiation during a 4-second relaxation delay. The 90° excitation pulse was set to 10 µs, with an acquisition time of 2.7 seconds and a receiver gain of 64.

Each sample underwent four dummy scans, followed by 128 acquisition scans, resulting in a total run time of 15 minutes and 45 seconds per sample. Automated shimming was performed using the deuterium signal, followed by locking, probe tuning and matching, and pulse calibration. Data acquisition and initial processing were conducted using Bruker Topspin (v3.5), with additional data processing performed using Bruker AMIX (v3.9.14).

3.7.6.3. GC-TOF-MS sample preparation and analyses (Phase A and B)

In both Phase A and Phase B, derivatization was performed using a standardized two-step oximation and silylation procedure. Oximation involved adding 50 µL of methoxyamine hydrochloride (15-20 mg/mL in pyridine) to the dried extracts, brief vortexing, and incubating at 60°C for 1 hour. Silylation followed by adding 50 µL of BSTFA (99:1, v/v) with TMCS and incubating at 60°C for an additional hour. The derivatized samples (100 µL) were then transferred to 250 µL tapered glass inserts within 2 mL vials and loaded onto an autosampler for analysis.

In Phase A, dried and thawed urine samples (20–250 µL) containing 0.0625 µmol creatinine (Section 3.7.3), dried under nitrogen at 37°C, and derivatized as described before. For Phase B, brain extracts (200 µL) as prepared in Section 3.7.4.1 were processed similarly. The final derivatized sample volumes (100 µL) were then transferred to 250 µL pulled-point glass inserts for GC-TOF-MS analysis.

Samples were analysed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped with an Agilent 7693 autosampler and coupled to a LECO Pegasus® HT time-of-flight mass spectrometer. Injection (1 µL) was performed in splitless mode using a Phenomenex Zebron GC FocusLiner® inlet liner. The inlet temperature was maintained at 250°C, and helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. Chromatographic separation was achieved on a Restek Rxi-5Sil MS column (30 m × 250 µm × 0.25 µm) with an oven temperature program that began at 40°C, held for 1 minute, and then increased at a rate of 7°C/min until reaching 120°C. The temperature was subsequently increased at a rate of 10°C/min to 230°C, followed by a final increase of 13°C/min to 300°C, where it was held for 2.5 minutes before being cooled to 40°C. The transfer line and ion source temperatures were maintained at 225°C and 200°C, respectively. Mass spectrometry operated in electron impact (EI) ionisation mode at 70 eV with a detector voltage of ~1500 V. Data acquisition was performed at 20 spectra/second, scanning an m/z range of 50–950, with a solvent delay of 480

seconds. Peaks were detected using five apexing masses with a signal-to-noise ratio threshold of greater than 20.

Brain extract samples were analysed using an Agilent 8890 gas chromatograph coupled to a Pegasus® BT TOF-MS. Injection (1 μ L) was performed at a 1:5 split ratio, with the inlet temperature set at 270°C. Helium served as the carrier gas at a constant flow rate of 1 mL/min. Chromatographic separation utilized a Restek Rxi-5MS column (29.69 m $\times$ 0.25 mm $\times$ 0.25 μ m) with an oven temperature program starting at 70°C, held for 1 minute, followed by an increase of 10°C/min to 320°C, where it was held for 3 minutes. The transfer line and ion source temperatures were maintained at 270°C and 250°C, respectively. Mass spectrometry operated in EI ionisation mode at 70 eV. Data acquisition occurred at 20 spectra/second after a 300-second solvent delay, scanning an m/z range of 50–850.

For Phase A, data acquisition and processing were performed using LECO ChromaTOF software (v4.5x), and (v5.55) in Phase B. ChromaTOF software with peak alignment was performed using the Statistical Compare feature. Peaks were aligned based on mass spectra and retention times, and spectral matching was conducted against the NIST11 and in-house mass spectral libraries using a similarity threshold of 80% or greater.

Metabolite identification in both phases adhered to confidence levels as described by Schymanski *et al.* (2014). Features assigned as Level 1 were identified by matching spectral and retention time data to reference standards. Features assigned as Level 2 were tentatively identified based on structural evidence, while those labelled as Level 3 were assigned based on matching molecular mass or fragmentation spectra. All unidentified features were excluded from the dataset to ensure data integrity.

3.7.6.4. GC-MS/MS sample preparation and analyses (Phase B)

For the targeted quantification of redox-related metabolites in Phase B, brain extract samples (Section 3.7.4.1) were resuspended in 80 μ L of pyridine. The samples were then derivatized by adding 20 μ L of MTBSTFA (99:1, v/v) to each sample and incubated at 22°C for 10 minutes. After derivatization, the 100 μ L mixture was transferred into 250 μ L tapered glass inserts within 2 mL glass vials for subsequent analysis using an autosampler.

The analysis was performed using a GC-MS/MS system comprising a 7890B gas chromatograph with a 7010B triple quadrupole mass spectrometer equipped with an electron impact ionisation source. The GC-MS/MS system also included a 7693 autosampler (Agilent® Technologies), and data acquisition was managed using Agilent MassHunter Workstation software (version B10.00), as previously described by Jooste *et al.* (2024).

Chromatographic separation was accomplished using a Rxi®-1MS capillary column (30 m $\times$ 0.32 mm $\times$ 0.25 μ m). A 1 μ L aliquot of the derivatized sample was injected in split mode with a 10:1 split ratio, while the front inlet temperature was maintained at 250°C. Helium served as the

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carrier gas at a constant flow rate of 1 mL/min, with a post-run flow rate of 1.2 mL/min. The GC oven temperature was programmed as follows: an initial hold at 70°C for 1 minute, followed by a temperature increase at 12°C/min to 135°C with a hold time of 1.5 minutes. The temperature then increased at a rate of 2°C/min to 145°C and held for 1.5 minutes, followed by a rapid increase at 23°C/min to a final temperature of 300°C, which was held for 1 minute.

The ion source was maintained at 250°C, and electron impact ionisation was performed at 70 eV. Nitrogen was used as the collision gas at a flow rate of 1.5 mL/min. Mass spectrometric analysis was carried out in dynamic multiple reaction monitoring (dMRM) mode, with transitions optimized using reference standards (Table 3-3).

To enhance detection sensitivity, the dMRM analysis was split into three distinct methods, each requiring a separate sample injection to ensure all target compounds were analysed. For reliable metabolite identification, two specific transitions were monitored for each metabolite, with identification confirmed through both spectral matching and retention time comparison, adhering to established guidelines (Schymanski *et al.*, 2014; Sumner *et al.*, 2007).

Data was acquired and processed using the Agilent® MassHunter Workstation software (vB02.01 for data acquisition, vB06.00 for qualitative and quantitative analysis) and MassHunter Optimizer software (vB02.01 for MRM configuration).

Table 3-3: Dynamic multiple reaction monitoring (dMRM) conditions for compounds analyzed via GC-MS/MS

Compound name	Derivative type	Quantifier / qualifier	Precursor ion (m/z)	Product ion (m/z)	CE (V)	Retention time (min)	Method
2-Hydroxy-3-methylbutyrate	Di-tBDMS	Quantifier	261.3	147.1	6	9.51	1
2-Hydroxy-3-methylbutyrate	Di-tBDMS	Qualifier	289.3	147.1	7	9.52	1
2-Hydroxy-3-methylvalerate	Di-tBDMS	Qualifier	275.3	147.2	7	11.50	3
2-Hydroxy-3-methylvalerate / 2-Hydroxy-4-methylvalerate	Di-tBDMS	Quantifier	303.3	147.1	10	11.50	3
2-Hydroxyadipate	Tri-tBDMS	Quantifier	447.4	245.2	7	14.88	2
2-Hydroxyadipate	Tri-tBDMS	Qualifier	245.2	147.1	7	14.88	2
2-Hydroxyglutarate	Tri-tBDMS	Quantifier	433.4	73.0	16	14.43	1
2-Hydroxyglutarate	Tri-tBDMS	Qualifier	433.4	147.1	16	14.43	1
2-Keto-3-methylbutyrate	Di-tBDMS	Quantifier	287.2	147.0	11	11.09	3
2-Keto-3-	Di-tBDMS	Qualifier	287.2	189.1	11	11.10	3

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methylobutyrate							
2-Keto-3-methylobutyrate	Mono-tBDMS	Quantifier	129.1	75.0	5	4.72	1
2-Keto-3-methylobutyrate	Mono-tBDMS	Qualifier	174.2	159.0	6	4.71	1
2-Keto-3-methylvalerate	Mono-tBDMS	Quantifier	188.2	159.0	5	5.65	1
2-Keto-3-methylvalerate	Mono-tBDMS	Qualifier	143.2	75.1	6	5.65	1
2-Keto-3-methylvalerate / 2-keto-4-methylvalerate	Di-tBDMS	Quantifier	301.3	189.2	10	11.7	3
2-Keto-3-methylvalerate / 2-keto-4-methylvalerate	Di-tBDMS	Qualifier	301.3	147.2	10	11.7	3
2-Ketoadipate	Tri-tBDMS	Quantifier	445.4	375.4	13	14.96	2
2-Ketoadipate	Tri-tBDMS	Qualifier	375.3	147.1	6	14.96	2
2-Ketoglutarate	Di-tBDMS	Quantifier	215.3	73.1	10	12.59	2
2-Ketoglutarate	Di-tBDMS	Qualifier	317.2	157.0	7	12.60	2
2-Ketoglutarate	Tri-tBDMS	Quantifier	375.3	147.1	15	14.53	2
2-Ketoglutarate	Tri-tBDMS	Qualifier	431.3	147.2	17	14.54	2
3-Hydroxybutyrate	Di-tBDMS	Quantifier	233.2	147.1	7	9.35	2
3-Hydroxybutyrate	Di-tBDMS	Qualifier	275.2	147.1	6	9.35	2
3-Hydroxydecanoate	Di-tBDMS	Quantifier	313.4	147.0	7	13.84	3
3-Hydroxydecanoate	Di-tBDMS	Qualifier	359.3	147.0	9	13.84	3
3-Hydroxyisovalerate	Di-tBDMS	Quantifier	289.3	147.1	7	11.22	3
3-Hydroxyisovalerate	Di-tBDMS	Qualifier	289.3	189.2	6	11.22	3
3-Hydroxypalmitate	Di-tBDMS	Quantifier	443.5	147.1	11	16.08	3
3-Hydroxypalmitate	Di-tBDMS	Qualifier	327.4	73.0	15	16.16	3
3-Phenylbutyric acid_IS	Mono-tBDMS	Quantifier	221.3	105.1	5	10.38	1, 2 & 3
3-Phenylbutyric acid_IS	Mono-tBDMS	Qualifier	105.3	77.0	20	10.38	1, 2 & 3
Acetoacetate	Di-tBDMS	Quantifier	199.2	73.1	11	10.55	2
Acetoacetate	Di-tBDMS	Qualifier	273.2	147.2	15	10.34	2
Aconitate	Tri-tBDMS	Quantifier	327.2	147.1	12	15.00	2
Aconitate	Tri-tBDMS	Qualifier	461.4	73.1	17	15.00	2
C19_IS	Mono-tBDMS	Quantifier	355.5	131.2	12	15.79	1, 2 & 3
C19_IS	Mono-tBDMS	Qualifier	355.5	75.1	12	15.79	1, 2 & 3
Citrate	Tetra-tBDMS	Quantifier	591.5	431.4	12	15.90	2

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Citrate	Tetra-tBDMS	Qualifier	459.4	73.1	13	15.90	2
Isocitrate	Tetra-tBDMS	Quantifier	403.4	147.0	8	15.97	2
Isocitrate	Tetra-tBDMS	Qualifier	591.6	459.3	14	15.97	2
Lactate	Di-tBDMS	Quantifier	261.2	147.2	6	7.79	1
Lactate	Di-tBDMS	Qualifier	233.3	147.2	5	7.78	1
Aspartate	Tri-tBDMS	Quantifier	390.3	73.0	13	14.16	1
Aspartate	Tri-tBDMS	Qualifier	418.3	390.2	7	14.16	1
Malate	Tri-tBDMS	Quantifier	287.0	73.1	10	13.96	1
Malate	Tri-tBDMS	Qualifier	419.4	73.1	5	13.97	1
Oxaloacetate	Tri-tBDMS	Quantifier	417.4	147.0	16	14.08	1
Oxaloacetate	Tri-tBDMS	Qualifier	417.4	73.1	16	14.08	1
Pyruvate	Di-tBDMS	Quantifier	259.2	189.2	6	8.42	1
Pyruvate	Di-tBDMS	Qualifier	259.2	147.1	7	8.42	1

Conditions for derivatized compounds and internal standards (IS) are detailed together with their derivative type, quantifier, qualifier, precursor- and product ions. Abbreviations: m/z, mass to charge ratio; V, voltage; min, minutes.

3.7.6.5. UPLC-HDMS sample preparation and analyses (Phase B)

Before lipidomic profiling, the brain lipid extracts and ISs (described in Section 3.7.4.2) were dried and subsequently reconstituted in 100 μ L of methanol/acetonitrile (2:1, v/v). The reconstituted samples were transferred into 250 μ L tapered glass inserts placed within the same vials to prepare them for analysis.

An untargeted lipidomic analysis was conducted using a Synapt G2-Si system. This system integrated ultra-performance liquid chromatography (UPLC) with high-definition mass spectrometry (HDMS) (Waters). Chromatographic separation utilized a Waters Acquity UPLC CSH C18 column (2.1 $\times$ 100 mm, 1.7 μ m). A gradient elution protocol, similar to that described by Reichl *et al.* (2020), was applied, employing two mobile phases: solvent A (60% acetonitrile, 40% water, 10 mM ammonium formate, and 0.1% formic acid) and solvent B (90% isopropyl alcohol, 10% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid). Initially, the gradient began at 40% B and was held constant for 4 minutes, followed by a linear transition to 70% B over 20 minutes. The condition of 70% B was maintained for 12 minutes before rapidly increasing to 99% B within 20 seconds. Re-equilibration to 40% B was carried out for 4 minutes, resulting in a total runtime of 40 minutes. The flow rate was maintained at 0.4 mL/min, with a column temperature of 45°C, and an injection volume of 1 μ L. The cooled autosampler, held at 4°C, introduced the samples into the system.

Mass spectrometric detection was performed using the HDMS, equipped with an ESI source. Analyses included both positive and negative ionisation modes for all samples. Throughout the

analyses, leucine enkephalin was used as the lock-mass to ensure accurate mass measurements by providing a constant reference point, allowing for real-time calibration and correction of mass spectrometry data.

Data acquisition was managed with MassLynx software (version 5, Waters). Progenesis QI software (version 6, Waters), combined with the Lipid Maps database, facilitated peak alignment by matching mass spectra and retention times.

3.8. Data processing and statistical analyses

3.8.1. Data pre-processing and pre-treatment

This study adhered to established in-house protocols and consulted the relevant literature for data processing and normalisation. Initially, spectral data from each metabolomics platform were individually assessed for accurate peak selection and alignment, then extracted into matrices to assess batch effects and data integrity.

The data preprocessing workflow encompassed several critical steps: eliminating inconsistent features, removing contaminant peaks, excluding inconsistent samples, applying internal standards for post-analysis normalisation to mitigate systematic and technical variations, utilising QC samples to identify and remove inconsistent features, and imputing missing values to preserve data structure. Subsequently, data transformation was performed to enhance normality, facilitating better comparability of metabolite intensities across samples prior to statistical analysis.

Outlier samples were identified and removed using confidence intervals for principal component analysis (PCA) scores, with confirmation via cluster analysis. The matrices were further refined by filtering aligned data to retain only features present in at least 80% of each group. Additionally, data were filtered using a coefficient of variation (CV) cut-off of 25% for targeted analyses and 50% for untargeted analyses in QC samples for LC-MS/MS, ¹H-NMR, GC-TOF-MS, and GC-MS/MS platforms.

Urine data underwent pre-acquisition normalisation to creatinine content. Post-acquisition normalisation for both urine samples in Phase A and brain tissue samples in Phase B was accomplished using stable isotopes, including valine-d₈, isoleucine-d₁₀, phenylalanine-d₅, L-carnitine-d₃, octanoylcarnitine-d₃, octadecanoylcarnitine-d₃, and the IS DMPA for LC-MS/MS analyses; DMPA and TSP for ¹H-NMR analyses; 3-PBA for GC-TOF-MS and GC-MS/MS; and diheptadecanoyl (C17:0) fatty acid internal standards for UPLC-HDMS.

3.8.2. Data quality check

To ensure data quality in both phases of the study, repeatability and, where applicable, batch effects were evaluated. Scatter plots were generated with sample run order on the x-axis and

total signal intensity on the y-axis. Total signal per sample was calculated by summing the relative peak intensities of all metabolites, post-IS normalisation. This visualisation facilitated the assessment of QC sample distribution and detection of inter-batch variations. Additionally, the CV% for each metabolite was calculated within each QC sample. These CV% values were plotted, with the x-axis representing CV% ranges and the y-axis indicating the percentage of metabolites within those ranges.

3.8.3. Statistical analyses

For statistical analyses, multivariate and univariate methods were employed across both phases. PCA score plots were utilized to visually compare all groups, assessing within-group variances and the potential for data to distinguish between groups. Independent samples t-tests, one-way ANOVAs, and two-way ANOVAs were conducted to identify statistical differences between groups. Specifically, one-way ANOVA followed by post-hoc Tukey's tests were utilized to pinpoint significantly altered compounds between groups. In Phase A, two-way ANOVA assessed the interaction between diet and alcohol, while in Phase B, it evaluated the interaction between diet and genotype. Significant interactions were further examined using post-hoc Tukey's tests. A significance threshold of $p < 0.05$ was applied after implementing the Benjamini-Hochberg false discovery rate (FDR) correction, alongside an effect size cut-off of Cohen's d (d-value) > 0.6 for metabolomics and > 0.8 for lipidomics. Analyses were performed using MetaboAnalyst 5.0 (Pang *et al.*, 2021; Xia & Wishart, 2016) for PCA and one-way ANOVA, as well as IBM SPSS Statistics 27 and GraphPad Prism 9 for two-way ANOVA, multiple t-tests and subsequent post-hoc tests.

3.9. References

- Alshehry, Z.H., Barlow, C.K., Weir, J.M., Zhou, Y., McConville, M.J. & Meikle, P.J. 2015. An Efficient Single Phase Method for the Extraction of Plasma Lipids. *Metabolites*, 5(2):389-403.
- Boysen, A.K., Heal, K.R., Carlson, L.T. & Ingalls, A.E. 2018. Best-matched internal standard normalisation in liquid chromatography–mass spectrometry metabolomics applied to environmental samples. *Anal. Chem.*, 90(2):1363-1369.
- Carlson, C.G., Rutter, J., Bledsoe, C., Singh, R., Hoff, H., Bruemmer, K., ... McCarthy, L. 2010. A simple protocol for assessing inter-trial and inter-examiner reliability for two noninvasive measures of limb muscle strength. *J. Neurosci. Methods.*, 186(2):226-230.
- de Souza, G.O., Wasinski, F. & Donato Jr, J. 2022. Characterization of the metabolic differences between male and female C57BL/6 mice. *Life Sci.*, 301:120636.
- Dona, A.C., Jiménez, B., Schäfer, H., Humpfer, E., Spraul, M., Lewis, M.R., ... Nicholson, J.K. 2014. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal. Chem.*, 86(19):9887-9894.
- Gonzalez-Riano, C., Garcia, A. & Barbas, C. 2016. Metabolomics studies in brain tissue: A review. *J. Pharm. Biomed. Anal.*, 130:141-168.

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Griffin, J.L. 2006. Understanding mouse models of disease through metabolomics. *Curr. Opin. Chem. Biol.*, 10(4):309-315.

Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M. & Moritz, T. 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. *Anal. Biochem.*, 331(2):283-295.

JAX. 2017. Mouse strain datasheet - 027058. <https://www.jax.org/strain/027058> Date of access: 10 Mar. 2017.

Jooste, T., 2024. Establishing a GC-MS/MS method to quantify redox markers in NDUFS4 knockout mice. Potchefstroom: North-West University.

Kapoor, R.V. & Vaidyanathan, S. 2016. Towards quantitative mass spectrometry-based metabolomics in microbial and mammalian systems. *Phil. Trans. A Math. Phys. Eng. Sci.*, 374(2079):20150363.

Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab.*, 7(4):312-320.

Lian, G., Li, X., Zhang, L., Zhang, Y., Sun, L., Zhang, X., ... Zhang, T. 2019. Macrophage metabolic reprogramming aggravates aortic dissection through the HIF1 α -ADAM17 pathway. *eBioMedicine*, 49:291-304.

Lindeque, J.Z., Hidalgo, J., Louw, R. & van der Westhuizen, F.H. 2013. Systemic and organ specific metabolic variation in metallothionein knockout mice challenged with swimming exercise. *Metabolomics*, 9:418-432.

Löfgren, L., Ståhlman, M., Forsberg, G.-B., Saarinen, S., Nilsson, R. & Hansson, G.I. 2012. The BUME method: A novel automated chloroform-free 96-well total lipid extraction method for blood plasma [S]. *J. Lipid Res.*, 53(8):1690-1700.

Manual, I. 2008. Mouse Inflammation Kit.

Mason, S., Terburgh, K. & Louw, R. 2018. Miniaturized 1 H-NMR method for analyzing limited-quantity samples applied to a mouse model of Leigh disease. *Metabolomics*, 14:1-12.

Mels, C.M., Jansen van Rensburg, P., van der Westhuizen, F.H., Pretorius, P.J. & Erasmus, E. 2011. Increased Excretion of C4-Carnitine Species after a Therapeutic Acetylsalicylic Acid Dose: Evidence for an Inhibitory Effect on Short-Chain Fatty Acid Metabolism. *Int. Sch. Res. Notices*, 2011(1):851870.

Miller, H.C., Louw, R., Mereis, M., Venter, G., Boshoff, J.-D., Mienie, L., ... Domínguez-Martínez, A. 2021. Metallothionein 1 overexpression does not protect against mitochondrial disease pathology in *Ndufs4* knockout mice. *Mol. Neurobiol.*, 58:243-262.

Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., Barrette, M., ... Xia, J. 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. *Nucleic Acids Res.*, 49(W1):W388-W396.

Quintana, A., Kruse, S.E., Kapur, R.P., Sanz, E. & Palmiter, R.D. 2010. Complex I deficiency due to loss of *Ndufs4* in the brain results in progressive encephalopathy resembling Leigh syndrome. *Proc. Natl. Acad. Sci.*, 107(24):10996-11001.

Reichl, B., Eichelberg, N., Freytag, M., Gojo, J., Peyrl, A. & Buchberger, W. 2020. Evaluation and optimization of common lipid extraction methods in cerebrospinal fluid samples. *J. Chromatogr. B.*, 1153:122271.

Ruoppolo, M., Caterino, M., Albano, L., Pecce, R., Di Girolamo, M.G., Crisci, D., ... Campesi, I. 2018. Targeted metabolomic profiling in rat tissues reveals sex differences. *Sci. Rep.*, 8(1):4663.

CHAPTER 3

- Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P. & Hollender, J. 2014. Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ. Sci. Technol.*, 48, 2097–2098.
- Seibenhener, M.L. & Wooten, M.C. 2015. Use of the open field maze to measure locomotor and anxiety-like behavior in mice. *J. Vis. Exp.* (96):52434.
- Smith, P.e., Krohn, R.I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., ... Klenk, D. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, 150(1):76-85.
- Stokes, JC., Bornstein, RL., James, K., Park, KY., Spencer, KA., Vo, K., ... Johnson SC. 2022. Leukocytes mediate disease pathogenesis in the Ndufs4(KO) mouse model of Leigh syndrome. *JCI Insight.*, 8;7(5):e156522.
- Sumner, L.W., Amberg, A., Barrett, D., Beale, M.H., Beger, R., Daykin, C.A., ... Griffin, J.L. 2007. Proposed minimum reporting standards for chemical analysis: chemical analysis working group (CAWG) metabolomics standards initiative (MSI). *Metabolomics*, 3:211-221.
- Van Aardt, W.J., Le Roux, J.M., Lindeque, J.Z., Mason, S. & Louw, R. 2016. The effect of temperature on the respiration and metabolism of the African burrowing scorpion (*Opisthophthalmus latimanus*). *Comp. Biochem. Physiol. Part D Genomics Proteomics.*, 20:50-56.
- van de Wal, M.A.E., Adjobo-Hermans, M.J.W., Keijer, J., Schirris, T.J.J., Homberg, J.R., Wieckowski, M.R., ... Koopman, W.J.H. 2022. Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention. *Brain*, 145(1):45-63.
- Williams, A., Steffens, F., Reinecke, C. & Meyer, D. 2013. The Th1/Th2/Th17 cytokine profile of HIV-infected individuals: a multivariate cytokinomics approach. *Cytokine*, 61(2):521-526.
- Xia, J. & Wishart, D.S. 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Curr. Protoc. Bioinformatics.*, 55(1):14.10.

CHAPTER 4

A ketogenic diet alters mTOR activity, systemic metabolism and potentially prevents collagen degradation associated with chronic alcohol consumption in mice

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Chapter 4: Scope

The scope of this chapter of the study was on investigating the combined and isolated effects of a ketogenic diet (KD) and chronic alcohol consumption on mTOR pathway activity, systemic metabolism, and redox/inflammatory states in mice. This chapter aimed to clarify how a KD interacts with chronic alcohol consumption to influence mTOR signalling, metabolic reprogramming, while addressing gaps in understanding their combined effects on redox balance and metabolism.

Status

The manuscript presented as Chapter 4 has been published.

Candidate contribution

In this phase of the study, candidate L. Willemse, in collaboration with R. Louw, conceptualised the study's design and methodological framework. With authorisation from the PCDDP Vivarium personnel, L. Willemse was responsible for feeding and weighing the animals. Moreover, the candidate executed all experimental procedures, which encompassed monitoring animal weight, performing genotyping, conducting Western blotting, and carrying out GC-TOF-MS, LC-MS/MS, ¹H-NMR analyses, as well as flow cytometry. Data processing, analysis, curation, and visualisation were also managed by L. Willemse, with subsequent review and editing by K. Terburgh. Additionally, the original manuscript draft was written by L. Willemse and later revised and edited with further input from K. Terburgh and R Louw.

Abstract

Introduction: A ketogenic diet (KD), which is a high fat, low carbohydrate diet has been shown to inhibit the mammalian target of rapamycin (mTOR) pathway and alter the redox state. Inhibition of the mTOR complex has been associated with the attenuation and alleviation of various metabolic and- inflammatory diseases such as neurodegeneration, diabetes, and metabolic syndrome. Various metabolic pathways and signalling mechanisms have been explored to assess the therapeutic potential of mTOR inhibition. However, chronic alcohol consumption has also been reported to alter mTOR activity, the cellular redox- and inflammatory state. Thus, a relevant question that remains is what effect chronic alcohol consumption would have on mTOR activity and overall metabolism during a KD-based intervention.

Objectives: The aim of this study was to evaluate the effect of alcohol and a KD on the phosphorylation of the mTORC1 target p70S6K, systemic metabolism as well as the redox- and inflammatory state in a mouse model.

Methods: Mice were fed either a control diet with/without alcohol or a KD with/without alcohol for three weeks. After the dietary intervention, samples were collected and subjected towards western blot analysis, multi-platform metabolomics analysis and flow cytometry.

Results: Mice fed a KD exhibited significant mTOR inhibition and reduction in growth rate. Alcohol consumption alone did not markedly alter mTOR activity or growth rate but moderately increased mTOR inhibition in mice fed a KD. In addition, metabolic profiling showed alteration of several metabolic pathways as well as the redox state following consumption of a KD and alcohol. A KD was also observed to potentially prevent bone loss and collagen degradation associated with chronic alcohol consumption, as indicated by hydroxyproline metabolism.

Conclusion: This study sheds light on the influence that a KD alongside alcohol intake can exert on not just mTOR, but also their effect on metabolic reprogramming and the redox state.

Keywords: Ketogenic diet, mTOR, Metabolomics, Redox, Western Blot, Metabolic Reprogramming

4.1. Introduction

A ketogenic diet (KD), defined as a high fat, low carbohydrate diet, has been shown to attenuate various disorders that affect brain function and neurological metabolism, as well as refractory epilepsy (Vining, 1999), mitochondrial disease (Sperl, 1997), diabetes (Qu *et al.*, 2021) and glucose transporter defects (Bolla *et al.*, 2019). More recently, the KD has been one of the most significantly researched approaches with regards to weight loss and as a strategy to manage obesity (Paoli, 2014). Several mechanisms have been assessed regarding the potential therapeutic effects of a KD, including, but not limited to, ketosis and ketone bodies (Freeman & Kossoff, 2010), inflammatory pathways (Dupuis *et al.*, 2015), oxidative stress regulation (Jin *et al.*, 2014) and the mammalian target of rapamycin (mTOR) pathway (McDaniel *et al.*, 2011). Of these potential mechanisms, the disease ameliorating effects of the mTOR signalling pathway has been of particular interest, resulting in extensive research thereof (Genzer *et al.*, 2016). The mTOR pathway is considered a central control hub for growth and metabolism (Wullschlegel *et al.*, 2006), activated by phosphoinositide-3 kinase (PI3K)/ protein kinase B (Akt) signalling during conditions of ample nutrient and growth factor availability, and inhibited by AMP-activated protein kinase (AMPK) during energy deficiency (Jewell & Guan, 2013). mTOR is a protein kinase that integrates energy, nutrient, and growth signals to modulate several cellular processes, and exists in two distinct functional complexes namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The former is considered the primary regulatory complex with regard to cellular homeostasis, stress responses, autophagy, and energy metabolism (Laplane & Sabatini, 2013; Morita *et al.*, 2015; Morita *et al.*, 2013) and also plays a particularly significant role in managing the Krebs cycle (TCA cycle), mitochondrial biogenesis, as well as oxidative stress (Morita *et al.*, 2015; Zhao *et al.*, 2017). As such, mTORC1 has been a target of interest in the development of therapeutic intervention strategies for several disorders such as epilepsy, cancer, neurodegeneration, diabetes, and mitochondrial diseases (Hay & Sonenberg, 2004; Johnson *et al.*, 2013; McDaniel *et al.*, 2011; Xie *et al.*, 2016). When the AMPK or Akt pathway is stimulated by growth factors, nutrients, or insulin signalling, it leads to mTORC1 activation and phosphorylation of the downstream protein, p70S6K, to regulate protein synthesis, cell growth and proliferation (Hay & Sonenberg, 2004). The phosphorylation of p70S6K is one of the best characterised downstream effects of mTOR, as such, the measurement of the activity of this protein makes it a suitable target for monitoring mTOR inhibition (Hay & Sonenberg, 2004). Inhibition of mTORC1 ultimately leads to the alleviation of several of these diseases, thereby implicating distinct metabolic pathways and signalling cascades. Accordingly, administration of the potent mTOR inhibitor, rapamycin, has been reported to prevent epilepsy, disrupt tumour metabolism as well as impair mitochondrial disease progression in various animal models (Johnson *et al.*, 2013; Xie *et al.*, 2016; Zeng *et al.*, 2008). Various studies have also evaluated the inhibitory effect of a KD on mTOR activity in rodent

models of mitochondrial disease, epilepsy, and longevity (McDaniel *et al.*, 2011; Roberts *et al.*, 2017; Singh *et al.*, 2018). Together, these findings suggest a credible relationship between a KD and mTOR activity. Additionally, alcohol consumption is known to affect ketone body metabolism, alter the cellular redox state, and impair mTOR phosphorylation (Feldstein & Bailey, 2011; Lang *et al.*, 2003; Veech *et al.*, 1972; Zakhari, 2006). Numerous studies have also evaluated the effect of a KD on alcohol withdrawal symptoms as well as alcohol addiction in both rodents and humans (Blanco-Gandía *et al.*, 2021; Bornebusch *et al.*, 2021; Dencker *et al.*, 2018; Wiers *et al.*, 2021). In these studies researchers observed that a KD decreases alcohol consumption, as well as alleviating alcohol withdrawal symptoms. Moreover, an increase in worldwide alcohol consumption has been observed, where alcohol use disorder (AUD) has been linked to around 5% of global deaths (Rehm & Imtiaz, 2016). Considering this, the effect of chronic alcohol consumption on the mTOR axis and overall metabolism during a KD-based intervention for weight management and disease treatment comes into question. To address this concept, the current study aimed to first investigate the isolated and combined effects of chronic alcohol consumption and a KD on the mTORC1 phosphorylation state, systemic metabolism, and redox state in a healthy mouse model. For this study the mice consuming the normal diet along with alcohol consumption served as a type of control for a redox state shift towards a more increased NADH/NAD⁺ ratio. This in return was used as a reference point in evaluating whether a ketogenic diet could prevent or reverse this shift in the redox state. Following the dietary intervention, mouse liver tissue was used to assess p70S6K1, a downstream protein of mTOR phosphorylation, whereas multiplatform-based metabolomics analyses of mouse urinary samples were utilized to evaluate systemic metabolic and redox fluctuations within the various dietary groups.

4.2. Methods and materials

4.2.1. Animals, dietary intervention and sampling

Male mice, heterozygous (HET) or wild-type (WT) of equal numbers for *Ndufs4*^{tm1.1Rpa/J}, were used interchangeably in this study since both genotypes can serve as healthy controls (Kruse *et al.*, 2008) due to there being no difference in phenotype between the two genotypes (van de Wal *et al.*, 2022). Original *Ndufs4* HET breeding pairs (B6.129S4-*Ndufs4*^{tm1.1Rpa/J}) were acquired from Jackson Laboratory (JAX; ME, USA, stock #027058). Only male mice were used in this study due to the known differences in hormone cycles, tissue-specific metabolism as well as metabolic profiles between sexes (Ruoppolo *et al.*, 2018; Terburgh *et al.*, 2021). Standard polymerase chain reaction genotyping was used as described previously (Valsecchi *et al.*, 2010) to distinguish *Ndufs4* mouse genotypes and confirm that mice of the correct genotype were used for the study. Mice were housed and bred at the specific pathogen-free (SPF) unit of the Vivarium (SAVC reg. #FR15/13458) of the Preclinical Drug Development Platform (PCDDP,

NWU) under the following conditions: a day/night cycle of 12 h; temperature of $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$; and relative humidity of $55\% \pm 10\%$. After postnatal day (P) 23, a total of 40 mice were randomly assigned to one of four dietary intervention groups ($n=10$ animals per group), with ad libitum access to either; i) a control diet, referred to in this study as a normal diet (ND) in this study (standard laboratory chow, RB2005, LabChef, NutritionHub) and drinking water; ii) a control diet (ND) with 5% (v/v) alcohol in drinking water for the first week and 10% (v/v) alcohol in drinking water for the remainder of the study; iii) a KD and drinking water; or iv) a KD with 5% (v/v) alcohol in drinking water for the first week and 10% (v/v) alcohol in drinking water for the remainder of the study. The control diet (ND) contained (% of total kcal) 20% protein, 75% carbohydrate, and 5% fat; whereas the KD contained 8.2% protein, 91.7% fat, and $<0.1\%$ carbohydrate. Detailed information regarding the ingredient composition of the KD can be found in the Appendix (Table C-1). These dietary interventions lasted approximately three weeks, with mice weighed three times a week (around the same time intervals) to assess growth rates. Animals were euthanised on postnatal day P46-47, after completing the three-week dietary intervention. Mice were placed in metabolic cages the day before euthanasia to collect fasting urinary samples. Mice were euthanised via exsanguination as described by the American Veterinary Medical Association (AVMA) (AVMA, 2020) by a research animal technician (PCDDP, NWU, RSA) after a 16-18 hour fasting period at the same time of day (10:00 to 11:00 AM) to reduce inter-animal physiological variation because of the differences in nocturnal feeding habits as well as that of the stages of the circadian cycle (Griffin & Nicholls, 2006). Finally, after blood and liver tissues were collected, all samples were swiftly snap-frozen in liquid nitrogen to quench metabolic activity, and subsequently stored at -80°C .

The AnimCare animal research ethics committee of North-West University approved (NWU-00516-20-A5) the animal protocols used in this study. All animals were maintained, and all procedures performed, in accordance with the code of ethics in research, training, and testing of drugs in South Africa and complied with national legislation.

4.2.2. SDS-PAGE and western blotting of liver pS6K1

Brain tissue (~30 mg) was homogenised in RIPA lysis buffer (~300 μl) through sonication using three pulses of three seconds. Subsequently, homogenates were obtained, and protein concentrations were determined using the BCA assay. Homogenates were kept on ice throughout the procedure, which were subsequently vortexed, and centrifuged at 1000 $\times g$ before heat denaturation for ten min at 100°C . For each dietary group, aliquots (20 μg) of the protein were separated via a sodium dodecyl sulphate–polyacrylamide gel (SDS-PAGE), run at room temperature. Afterwards, the gel was transferred to nitrocellulose and blocked. After that, the membranes were incubated with phospho-p70S6 kinase primary antibodies (Cell Signaling Technologies) at 4°C overnight. Next, the membranes were incubated with secondary antibodies at room temperature. Lastly, chemiluminescent detection was achieved using a

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Western ECL substrate (Bio-Rad, Johannesburg, RSA) and a ChemiDoc™ MP imaging system (Bio-Rad). Finally, the results were analysed via Image Lab™ v. 6.1 software (Bio-Rad,) in order to attain the density ratio of the target proteins relative to β -actin, used as the control protein. Detailed information regarding the procedures and antibody dilutions are described in Chapter 3 and Appendix C.

4.2.3. Determination of urinary creatinine concentration

The creatinine concentration of each urinary sample was spectrophotometrically quantified using the QuantiChrom™ Creatinine Assay Kit (BioAssay Systems). In this assay, creatinine reacts with picric acid in an alkaline solution forming a yellow orange adduct. The increase in absorbance of this adduct is measured at a wavelength of 510 nm. For the assay, 10 μ l of mouse urine was mixed with 200 μ l working solution (0.1 M sodium hydroxide (NaOH); 0.3 mM ethylenediaminetetraacetic acid (EDTA); 0.1% (v/v) DMSO; 0.0004% (v/v) Tween®20; and 3 mM picric acid), in duplicate, in a 96-well plate. The samples were then analysed via a Synergy™ HT Multi-detection microplate reader (Biotek® Instruments) by assessing the increase in linear trend of absorbance at 510 nm for 5 min, in 1 min intervals. Absolute quantities were determined by making use of a standard curve, ranging from 0-50 mg%.

4.2.4. Metabolomics sample preparation

For all the metabolomics analyses, a pre-determined volume (~20-250 μ l) of urine containing 0.0625 μ mole creatinine was added to microcentrifuge tubes, with the addition of internal standards (ISs). Urine samples were analysed via untargeted (gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and nuclear magnetic resonance (^{1} H-NMR)) and targeted (liquid chromatography-tandem mass spectrometry (LC-MS/MS)) techniques. Due to the small volumes of some of the KD + Alc. urine samples obtained, only 6 biological replicates could be used for this group. For GC-TOF-MS analyses, 16,14 μ l IS mixture, which contained 3-phenylbutyrate (100 ppm), norleucine (100 ppm) and N,N-dimethylphenylalanine (50 ppm), was added per sample. For LC-MS/MS analyses, 25 μ l IS mixture was added. This mixture consisted of N,N-dimethylphenylalanine, ring- ^{2} H₅-phenylalanine, ^{2} H₁₀-isoleucine, ^{2} H₈-valine, L-Carnitine-(methyl-d₃), ^{2} H₃-octanoylcarnitine and ^{2} H₃-octadecanoylcarnitine, each with a final concentration of 2.5 ppm. After the addition of the IS mixture, mouse urine samples were deproteinised by adding three volumes of ice-cold methanol. The samples were then vortexed, incubated for 20 min at -20°C, centrifuged at 15 000 xg for 10 min and finally dried under a gentle stream of nitrogen gas at 37°C. Additionally, another 80 μ l of methanol was added to all dried samples and then dried again to guarantee that the samples were completely dry and all water removed prior to derivatization. For ^{1} H-NMR analyses, urine samples did not undergo deproteinisation as samples were dried and then resuspended in sterile, filtered water and centrifuged to remove macromolecules and other insoluble particulates prior to ^{1} H-NMR

analyses. Also, ISs such as 3-trimethylsilyl propionic acid (TSP) were added during NMR sample preparation. LC-MS samples were butylated, while GC-MS samples were oximated and silylated prior to analysis. Additional details regarding sample preparation are described in Chapter 3 and Appendix C.

4.2.5. LC-MS/MS analyses

For targeted LC-MS/MS analyses, urinary samples were analysed using a 1200 series liquid chromatograph coupled to a 6410-triple quadrupole-mass spectrometer (Agilent Technologies) as previously described (Terburgh *et al.*, 2019). The chromatographic separation was accomplished by injecting samples (1 µl) on a C18 Zorbax SB-Aq (150 mm x 2.1 mm x 1.8 µm) reverse phase column (Agilent Technologies), kept at 35°C, with a flow rate of 0.2 ml/min during the entire run. Furthermore, the mobile phases used comprised of H₂O (solvent A) and acetonitrile (solvent B). Both mobile phases were acidified with 0.1% formic acid to enable protonation of metabolites (Cech & Enke, 2001). The chromatographic gradient started at a 5% mobile phase B and was maintained for 1 min, it was then followed with an increase to 23% of mobile phase B over a duration of 7 min. In addition, an isocratic hold of around 4 min was maintained at a 23% mobile phase B. Subsequently, mobile phase B was increased to 100% at 15 min and was kept there for 5 min. Lastly, the gradient declined to a 5% mobile phase B after 1 min and was maintained for 7 min (post-run) to guarantee that the column is in equilibrium before proceeding to the next analysis. The effluent coming from the liquid chromatograph was then diverted to the mass spectrometer, which made use of the positive electrospray ionisation (ESI) mode using the following source parameters: capillary voltage of 3 500 V; nitrogen drying gas at a flow rate of 7.5 l/min at 300°C; and nebuliser pressure of 30 psi. The spectra were acquired via the multiple reaction monitoring (MRM) mode with an electron multiplier voltage (EMV) of 300 V and a dwell time of 45 ms for all compounds, with the MRM parameters set up for specific amino acids, -derivatives, acylcarnitines, and isotopes (Table 3-2).

4.2.6. GC-TOF-MS analyses

For untargeted GC-TOF-MS analyses, samples were analysed via a 7890A gas chromatograph (Agilent Technologies) joined to a Pegasus HT time-of-flight mass spectrometer (Leco). Samples (1 µl) were injected using a split ratio of 1:10. The temperature of the inlet was 250°C throughout the sample injection. Compounds were chromatographically separated using a DB-5 MS capillary column (30 m x 250 µm x 0.25 µm) (Restek), and helium was used as a carrier gas at a constant flow rate of 1.5 ml/min. For mass spectrometry, the separated compounds were subjected to electron impact ionisation (EI) (70 eV), with the ion source temperature set at 200°C. After a solvent delay of 250 seconds, mass spectra were acquired (50-950 m/z) at a scan rate of 20 spectra/second. A predictable peak width of 3 seconds and a signal-to-noise (S/N) ratio of > 20 were used to detect peaks with 5 apexing masses.

4.2.7. Miniaturised ¹H-NMR spectroscopy

¹H-NMR spectroscopy was carried out according to a miniaturised method previously described (Mason *et al.*, 2018). Urine samples were analysed at 500 MHz in 2 mm NMR MATCH tubes, using the 5 mm triple-resonance inverse (TXI) probe head, which has been adjusted for ¹H evaluation. During analysis a constant sample temperature was sustained at 300 K. ¹H spectra were obtained as 128 transients in 32K data points with a spectral width of 6000 Hz (12.0 ppm). A pulse sequence program NOESY-presat was used to suppress the H₂O resonance at 4.70 ppm, by saturating the H₂O resonance by single-frequency irradiation through a 4 second relaxation delay, at a 90° excitation pulse of 10 µs. An acquisition time of 2.7 seconds and a receiver gain of 64 were set, correspondingly. The number of dummy scans (n = 4) and scans (n = 128) produced a run time of 15 minutes and 45 seconds per sample. Shimming was carried out for each sample automatically using the deuterium signal, then locked, probe tuned and matched, followed by pulse calibration. NMR analysis and processing was accomplished using Bruker Topspin (v 3.5), and additional processing was performed using Bruker AMIX (v 3.9.14).

4.2.8. Inflammatory assay

To determine the inflammatory status in different dietary groups, a BD Cytometric bead array mouse inflammation kit (BD Biosciences, #552364) and Accuri C6 flow cytometer (BD Biosciences) were used, according to the manufacturer instructions and the method discussed in Chapter 3 and Appendix C. The levels of selected inflammatory cytokines, namely, interleukin 6 (IL6), interleukin 10 (IL10), monocyte chemoattractant protein 1 (MCP1), tumour necrosis factor (TNF), interferon gamma (IFN-γ) and interleukin 12p70 (IL12p70) were determined.

4.2.9. Data processing and statistical analysis

Data analysis for the growth rate, western blot analysis and inflammation assay was carried out using Excel 2021 (Microsoft 365). The average weight over the duration of the study was plotted on a bar graph for each group to visualise the growth rate. For the western blot results, the relative intensity of the target protein (p70S6K1) was normalised using the relative intensity of the control protein, β-actin, which was then plotted on a bar graph to observe the relative intensity differences between each group. The data obtained following the inflammation assay was pre-filtered manually using the FCAP Array software (v3.0), after which each cytokine was assigned to its relative peak intensity followed by manual clustering for each cytokine. The inflammation data of each cytokine's relative concentrations were first plotted on a line graph to obtain a R² value ≤ 1, then plotted on a bar graph to observe the differences of the cytokine concentrations between each group. The significant difference between groups for the growth rate, western blot and inflammation assay results were determined using a one-way ANOVA followed by a post-hoc Tukey's test. The spectral data acquired from each metabolomics platform were extracted into matrices. Each data matrix was separately examined regarding

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correct peak selection and alignment, as well as batch effects and data integrity. The matrices were also pre-processed by which the aligned data was filtered to retain only features with an 80% presence within each group, along with removing data using a 50% cut-off coefficient of variation (CV) for quality-control samples in each group for GC-TOF-MS, LC-MS/MS and ^1H -NMR. Urine data was normalised pre-acquisition to creatinine content (mg/g creatinine) and post-acquisition to tropic acid (GC-TOF-MS), isotope (LC-MS/MS) internal standards and TSP for data obtained via ^1H -NMR analysis. LC metabolites were normalised using stable isotopes such as, valine- d_8 , isoleucine- d_{10} , phenylalanine- d_5 , free carnitine- d_3 , octanoylcarnitine- d_3 , and octadecanoylcarnitine- d_3 if there was a significant metabolite correlation to these isotopes. Batch corrections were not considered necessary, since QCs displayed no notable batch effects. Following this, log-transformation, mean centring and pareto scaling were applied for data pre-treatment and confidence intervals for PCA scores, confirmed by cluster analysis, were used for outlier detection. The data underwent both multivariate and univariate analyses. Initially, all groups (normal diet vs normal diet with alcohol vs KD vs KD with alcohol) were compared visually using PCA scores plots to observe within group variances and whether the data could partition animals into the different groups. Following this, univariate analysis using one-way ANOVA, along with a post-hoc Tukey's test, were applied to shortlist differentiating compounds between groups for each respective platform. A two-way ANOVA was used to evaluate diet and alcohol's interaction effects for the various compounds for each group, where a significant interaction was evaluated by a subsequent post-hoc Tukey's test. Metabolites (features) were deemed significant if they had a false discovery rate-corrected p-value (q-value) of ≤ 0.05 , using a post-hoc Tukey's test, and a Cohen's d-value of ≥ 0.8 (Ellis & Steyn, 2003). Both the PCA and one-way ANOVA were performed in MetaboAnalyst 5.0 (Xia *et al.*, 2015), whereas the two-way ANOVA, along with a subsequent post-hoc Tukey's test, was carried out using IBM SPSS Statistics 27 and Graphpad Prism 9. Lastly, if spectral features had high identification confidence levels, they were assigned metabolite identities (Schymanski *et al.*, 2014). Commercial and in-house spectral libraries, in conjunction with public databases, were used to identify dataset features through spectral and retention time matching. The significant features identities were confirmed using each platform's relative standards as well as isotopes (Schymanski *et al.*, 2014). These features were also subjected to pathway analysis using a manual approach, to identify pathways of significance.

4.3. Results and Discussion

4.3.1. Growth curves

Growth curves (Figure 4-1) were constructed to evaluate the effect of a KD and chronic alcohol (Alc.) consumption on animal body weight over a period of three weeks. Mice on a KD had a significantly reduced growth rate relative to those on a control diet (ND), with the final average

body weight being 22 ± 2 g for the ND and ND + Alc. groups and 15 ± 2 g for the KD and KD + Alc. groups. Therefore, it is evident that diet *alone* (i.e., a KD) had a significant ($p < 0.001$) effect on murine body mass, while chronic alcohol consumption did not. The available literature on the effect of alcohol on body weight is conflicting, as studies show either an increase, decrease or no difference at all of alcohol consumption on body weight (Huang *et al.*, 2012; Perides *et al.*, 2005; Traversy & Chaput, 2015). On the contrary, several studies have confirmed that a KD reduces body weight gain, possibly due to mTOR inhibition (Bueno *et al.*, 2013; Roberts *et al.*, 2017; Santos *et al.*, 2012).

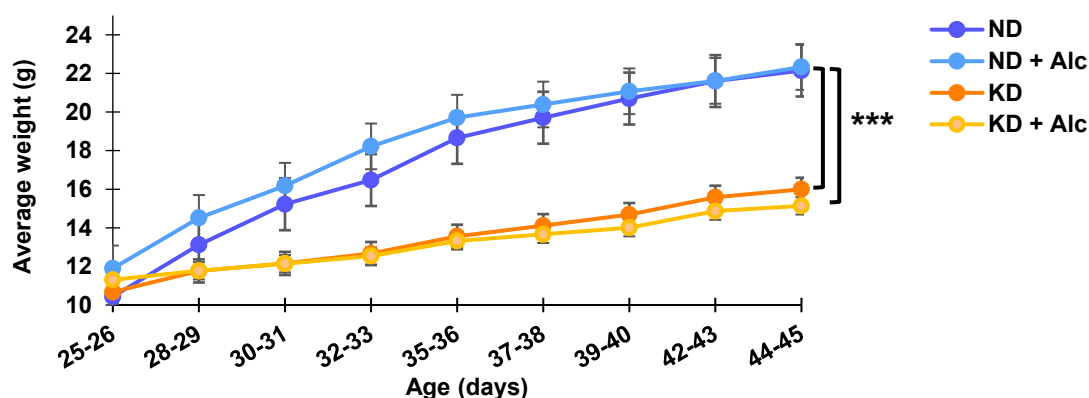


Figure 4-1: Consumption of a KD in mice leads to lower body weight gain. Growth curves for dietary groups on a normal diet without (ND) and with alcohol intake (ND + Alc.) and on a ketogenic diet without (KD) and with alcohol intake (KD + Alc.). Average weight $\pm$ standard deviation ($n=10$) per group is given as a function of animal age. Mice were weighed three times a week. Asterisks (***) obtained via a post-hoc Tukey's test, indicate significant differences between ND vs KD and ND vs KD + Alc. groups.

4.3.2. mTOR activity

The mTOR substrate, S6K1 (Thr389), is frequently used as a viable indicator of mTORC1 activity (Ikenoue *et al.*, 2009). Therefore, we examined liver S6K1 phosphorylation status in the various groups using western blot analysis. Liver pS6K1 levels (Figure 4-2) were significantly diminished ($p < 0.05$) in the KD and KD + Alc. groups compared to mice on a normal diet (ND). Hence, we confirm that a KD significantly inhibits mTOR pathway activity in mice, an observation supported by previous research (McDaniel *et al.*, 2011). Chronic alcohol consumption alone (ND + Alc. group), however, did not significantly alter murine S6K1 phosphorylation status, with pS6K1 levels only trending lower. Additionally, no significant difference was observed between the KD + Alc. group and the KD group. In addition, no significant interaction was observed between diet and alcohol for mTOR activity. As such, it does not seem that chronic alcohol consumption alone or alcohol consumed along with a KD has a significant impact on mTOR activity. A KD significantly lowered growth rates and levels of liver pS6K1, a downstream substrate of mTORC1 phosphorylation, in mice. These results support previous studies showing that a KD inhibits mTOR phosphorylation (McDaniel *et al.*, 2011) and has a considerable impact on the management of weight and obesity (Bueno *et al.*, 2013; Roberts *et al.*, 2017; Santos *et al.*, 2012). In contrast, chronic alcohol consumption did not

markedly affect growth rates or S6K1 phosphorylation when consuming a normal or KD, despite previous reports of its potential inhibitory effect on the mTOR pathway (Foster, 2010). These results are in agreement with previous studies that did not notice any significant changes in growth from alcohol consumption (Perides *et al.*, 2005). However, since the literature regarding chronic alcohol consumption's effect on weight is conflicting (Huang *et al.*, 2012; Perides *et al.*, 2005; Traversy & Chaput, 2015), further research is warranted. It is well known that mTORC1 activation is essential for growth (Wullschleger *et al.*, 2006), with reduced phosphorylation of this pathway correlated to impaired cell growth (Laplante & Sabatini, 2012). Thus, the data confirms that a KD reduces growth rate, possibly through mTOR inhibition, while chronic alcohol consumption does not markedly impact mTOR activity or body weight in mice. Moreover, the results obtained from the study indicate that the combination of a KD and chronic alcohol consumption does not significantly inhibit mTOR to a greater extent than a KD alone. Even though phospho-p70S6k is usually normalised against the total p70S6k, we derived our normalisation approach using β -actin from previous studies making use of the same antibody (Hsin *et al.*, 2021; Wu *et al.*, 2021). Moreover, previous studies that have also made use of a ketogenic diet and assessed its effect on mTORC1, have confirmed that total expression of S6K is relatively stable in comparison to alterations in the phosphorylated form (Bornstein *et al.*, 2022; McDaniel *et al.*, 2011; Roberts *et al.*, 2017).

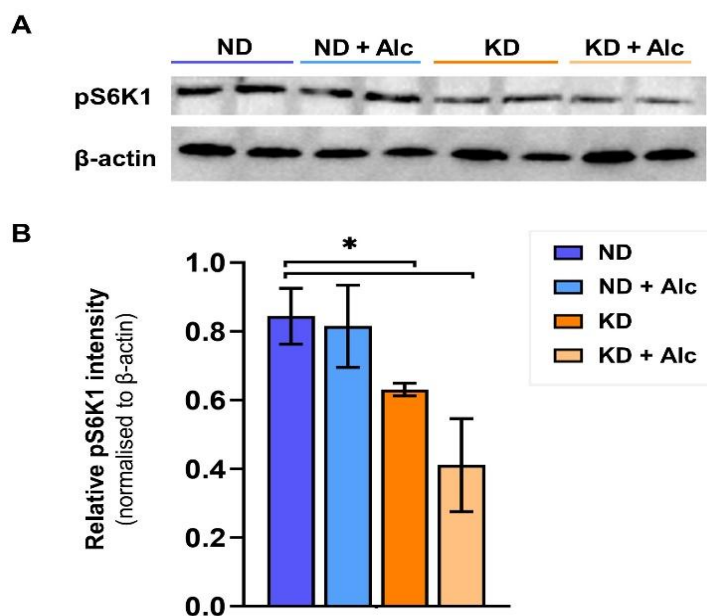


Figure 4-2: A KD inhibits S6K1 phosphorylation in mice. (A) Representative Western blot image and (B) quantitative summary of pS6K1 and β -actin following separation by SDS-page. Average β -actin (~42 kDa) was used as control protein of pS6K1 (~75 kDa). Data represents the average relative pS6K1 intensity $\pm$ standard deviation (n=4) per group. ND indicates normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. Asterisks (* $p < 0.05$) obtained via a post-hoc Tukey's test, indicate significant differences between ND vs KD and ND vs KD + Alc. groups.

4.3.3. Metabolic alterations

Next, we investigated the systemic effect of a KD and chronic alcohol consumption on murine metabolism via GC-TOF-MS, LC-MS/MS and $^1\text{H-NMR}$ spectroscopy. ANOVA with post-hoc Tukey's identified numerous metabolites significantly differing among the four dietary intervention groups ($n=6-10$ per group, Table C-2). Multivariate principal component analysis (PCA; Figure 4-3A) and a heatmap (Figure 4-3B) of these discriminatory metabolites revealed a distinctive metabolic separation between animal groups on a KD and control diet. However, chronic alcohol consumption could only partially separate animal groups on the same diet, indicating that diet (i.e., a KD) had a greater impact on metabolism than alcohol consumption. When comparing the urinary metabolomes of the different dietary intervention groups to controls (ND), significant alterations were observed in amino acid and TCA cycle metabolism. Subsequent pathway analysis revealed that the most significantly altered urinary metabolites were involved in amino acid metabolism (Figure 4-4), with branched-chain amino acid and one-carbon metabolism markedly affected, and the tricarboxylic acid (TCA) cycle (Figure 4-5). Figure 4-4 and Figure 4-5 illustrate the practical (effect size d -value) and statistical (p -value) significance of these metabolic alterations in the KD, KD + Alc. and ND + Alc. groups when respectively compared to controls (ND). Other metabolites that also displayed significant interaction effects were creatine, alloxanoic acid and isobutyric acid. Additional information on metabolites with an interactive effect can be found in Appendix C (Table C-3 and Figure C-3). All significantly altered features are described in the Supplementary material.

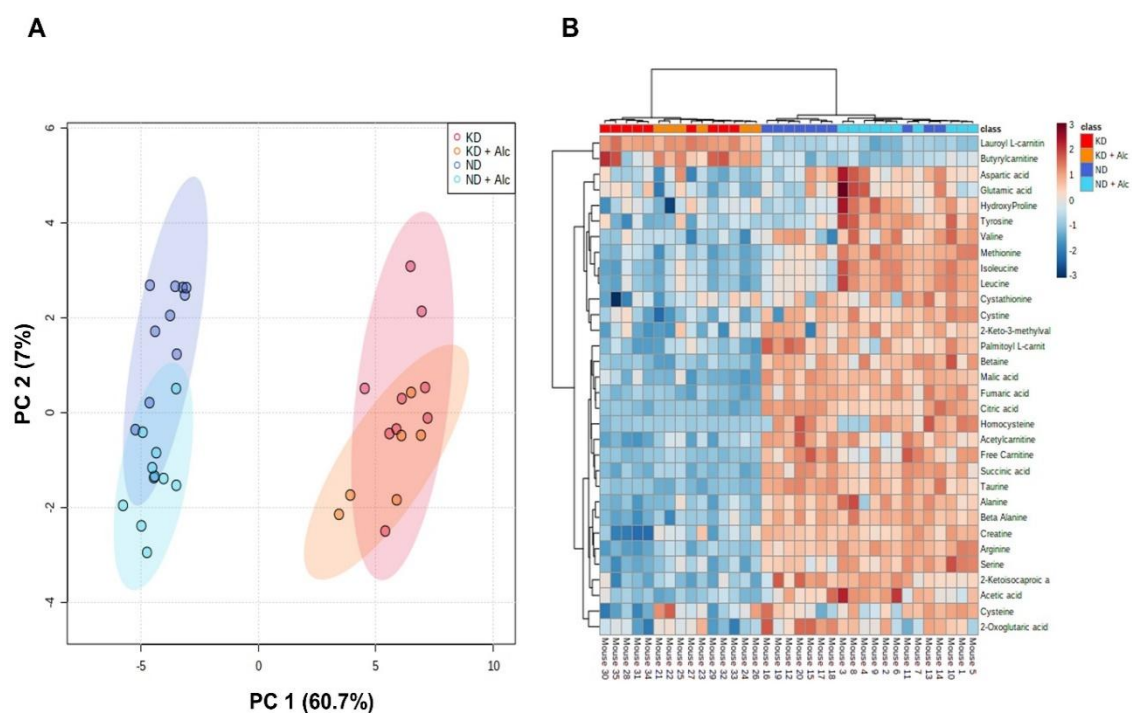


Figure 4-3: A KD and chronic alcohol consumption leads to metabolic alterations in mice. (A) PCA scores plot illustrates the natural separation of all significantly altered metabolites when the different groups ($n=6$ to 10 per group) were compared. (B) Heatmap of main metabolites altered in different groups

(n=6 to 10 per group) following dietary intervention. Each metabolite was normalised to its respective internal standard (based on the relative metabolomics platform) and subsequently log transformed, as well as auto-scaled using MetaboAnalyst 5.0.

4.3.3.1. Amino acid metabolism

Several essential (leucine, isoleucine, arginine, and methionine), non-essential (alanine, aspartate and serine) and non-proteinogenic (homocysteine and cystathionine) amino acids were markedly ($p < 0.001$ and $d > 0.8$) reduced in the urine of animals consuming a KD with and without alcohol when compared to controls (ND). Additionally, leucine and isoleucine catabolites, 2-ketoisocaproic acid and 2-keto-3-methylvaleric acid along with choline catabolite, betaine, were also significantly lower in these mice (KD and KD + Alc.) compared to controls. Contrastingly, animals chronically consuming alcohol while on a normal diet (ND vs ND + Alc.) excreted markedly higher ($p < 0.001$ and $d > 0.8$) levels of essential amino acids, leucine, isoleucine, arginine, and methionine along with proline's post translational product, 4-hydroxyproline (Figure 4-5). The latter serves as a biomarker for bone resorption and is used to detect osteoporosis (Barbul, 2008; Kuo & Chen, 2017). Interestingly, 4-hydroxyproline levels were unaltered in mice consuming alcohol while on a KD. Overall, a KD more dramatically altered amino acid metabolism than alcohol consumption. When the amino acid metabolites were evaluated for an interactive effect between diet and alcohol only a few metabolites were observed to have interaction, namely, taurine (conditionally essential), cysteine (non-essential) and 4-hydroxyproline. Mice fed a KD diet (KD and KD + Alc.) excreted significantly lower levels of several amino acids, including the essential amino acids, leucine, isoleucine, arginine, and methionine along with several other amino acids that form part of one-carbon metabolism (homocysteine, serine, cystathionine, and betaine). This reduction in amino acid levels could, in part, reflect decreased protein intake, since a KD is inherently low in protein (8.2% of calories from protein in KD vs 20% in ND). However, it was recently shown that a KD with at least 5% of calories derived from protein does not induce a protein restriction response (Hu *et al.*, 2018). We therefore postulate that the marked reduction observed in mouse urinary one-carbon metabolites while on a KD, reflect a perturbation in this pathway. In support, others have shown that a KD results in the downregulation of a subset of genes involved in one-carbon metabolism in mouse liver (Pissios *et al.*, 2013) and an inhibition of one-carbon metabolism in HepG2 cells and mice (Hsu *et al.*, 2022). This inhibitory effect on one-carbon metabolism has been ascribed to the reduced mTOR signalling associated with a KD (Hsu *et al.*, 2022). Chronic alcohol intake during a ND significantly increased the excretion of the same essential amino acids lowered by a KD, likely reflecting altered protein turnover (Bernal *et al.*, 1993; Lang *et al.*, 2010). However, alcohol consumption did not markedly alter the effect of a KD on amino acid metabolism. In addition, one of the most significant metabolic alterations resulting uniquely from chronic alcohol consumption, was an increase in urinary 4-hydroxyproline levels. 4-Hydroxyproline is a major part of collagen, with urinary levels thereof serving as a bone-resorption biomarker of collagen

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turnover (Barbul, 2008; Kuo & Chen, 2017). Hence, the observed elevation in 4-hydroxyproline may reflect increased collagen degradation, a phenomenon previously linked to chronic alcohol consumption (Preedy *et al.*, 1991). Importantly, animals chronically consuming alcohol while on a KD, did not display any alterations in 4-hydroxyproline levels. Thus, a KD seems to play a protective and preventative role in alcohol-induced collagen degradation. However, further research is warranted, as we only assessed 4-hydroxyproline, while several other biomarkers of collagen turnover exist (Preedy *et al.*, 1991).

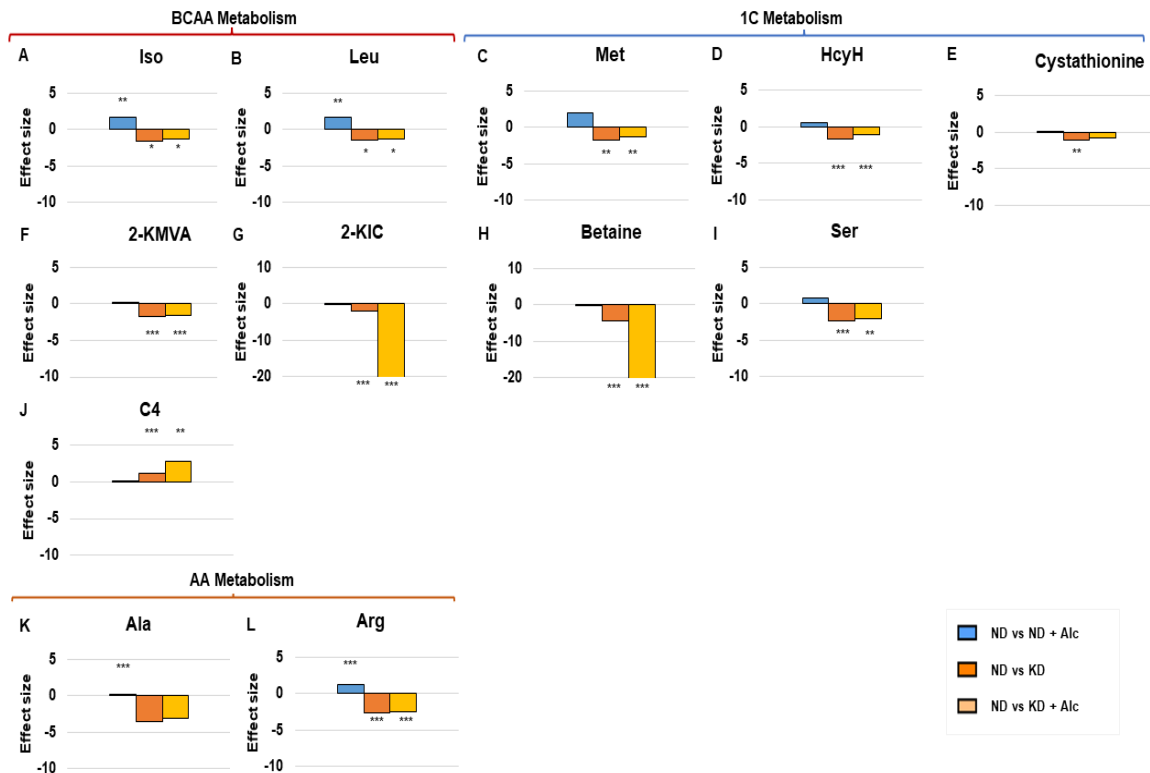


Figure 4-4: A KD lowers urinary amino acid levels irrespective of alcohol consumption. Bar graphs depict Cohen's d-values (effect size) for each metabolite when relative abundance between the different mouse groups (n=6 to 10 per group) were compared to the control group (ND). Leu indicates leucine; Ile, isoleucine; 2-KIC, 2-ketoisocaproic acid; 2-KMVA, 2-keto-3-methylvaleric acid; C4, butyryl-carnitine; Arg, arginine; Ala, alanine; Met, Methionine; HcyH, homocysteine; Ser, serine; ND, normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. *p < 0.05, ** p < 0.01, ***p < 0.001 vs. the control group, obtained via a post-hoc Tukey's test.

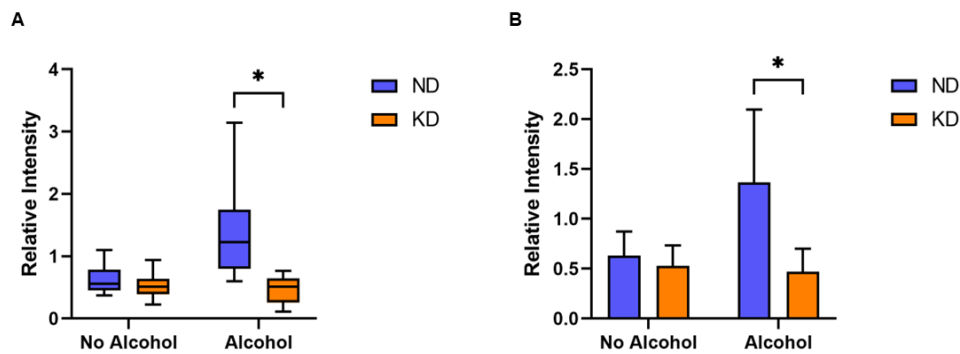


Figure 4-5: A KD prevents increased urinary 4-hydroxyproline excretion observed during chronic alcohol intake. (A) Boxplots depict the relative 4-hydroxyproline intensity for dietary groups (n=6-10 per group) on a normal diet without (ND) and with alcohol intake (ND + Alc.) and on a ketogenic diet without (KD) and with alcohol intake (KD + Alc.). For each boxplot the length of the box represents the 25th to 75th percentile inter-quartile range, the interior horizontal line represents the median, and the vertical lines stemming from the box extend to minimum and maximum values. (B) Bar graphs show the relative intensities for 4-hydroxyproline when the interaction of diet and alcohol was compared. Asterisks (* $p < 0.05$) obtained via a post-hoc Tukey's test, indicate significant differences between the ND vs ND + Alc. groups.

4.3.3.2. TCA cycle metabolism

TCA cycle intermediates, malic acid ($p < 0.001$ and $d > 0.8$; Figure 4-6A), fumaric acid ($p < 0.001$ and $d > 0.8$; Figure 4-6B), succinic acid ($p < 0.01$ and $d > 0.8$; Figure 4-6C), α -ketoglutaric acid ($p < 0.001$ and $d > 0.8$; Figure 4-6D) and citric acid ($p < 0.001$ and $d > 0.8$; Figure 4-6E) were significantly decreased in the KD and KD + Alc. groups compared to controls. It was also noted that for the KD + Alc. group, α -ketoglutaric acid and citric acid showed the greatest practical significance when the effect size was assessed compared to the control. Other studies using a KD have made similar observations and link this decrease in TCA cycle intermediates to the inhibitory effect of a KD on mTOR activity (Longo *et al.*, 2019; Ramanathan & Schreiber, 2009). This corresponds to the mTOR inhibition we observed in mice on a KD via Western blotting. Additionally, chronic alcohol consumption did, however, seem to exacerbate citric acid, α -ketoglutaric acid, succinic acid depletion in mice on a KD. In accord, previous studies have also shown that chronic alcohol consumption can lead to decreased levels of TCA cycle intermediates (Chen *et al.*, 2012). Hence, it is evident that the metabolism of the TCA cycle is significantly altered by a KD and altered to an even greater extent when alcohol is consumed in conjunction, which ultimately results in alterations of energy metabolism and the redox state (Auta *et al.*, 2017).

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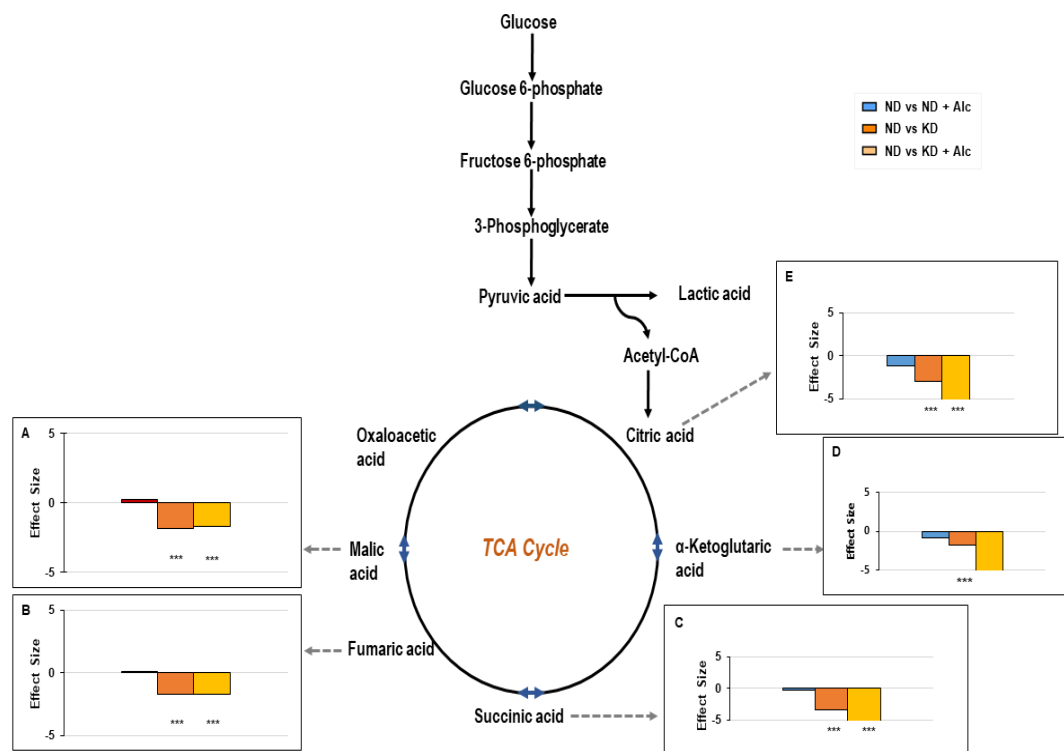


Figure 4-6: Alterations of TCA cycle intermediates are more prominent with dietary interventions than chronic alcohol consumption when the different mouse groups (n=6-10 per group) were compared. Bar graphs reveal Cohen's d-values (effect size; $d > 0.8$) for the significant metabolites when the relative abundance between the different mice groups were compared to the control group (ND). ND indicates normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. *** $p < 0.001$ vs. the control group, obtained via a post-hoc Tukey's test. *Redox state evaluation via metabolic indicators of NADH/NAD⁺ ratio*

To evaluate the effect that either a KD or chronic alcohol consumption has on the redox status in mice, metabolic indicators of NADH/NAD⁺ ratio were assessed in urine (Figure 4-7), as it is difficult to detect NAD⁺ and NADH by conventional metabolomics (Smith *et al.*, 2019). Metabolic indicators that are usually used include alanine, leucine, and isoleucine in relation to glutamate (Smith *et al.*, 2019). Seeing that an elevated NADH/NAD⁺ ratio drives the formation of amino acids from glutamic acid [i.e., an increase in amino acid production from glutamic acid is correlated to increased NADH levels] (Smith *et al.*, 2019), the ratio of various amino acids relative to glutamic acid were assessed. The ratios of alanine ($p < 0.001$; Figure 4-7A), isoleucine ($p < 0.001$; Figure 4-7B) and leucine ($p < 0.01$; Figure 4-7C) relative to glutamic acid were significantly decreased in the group consuming a KD solely. The consumption of alcohol on a ND lead to trended higher levels of amino acid ratios, indicating a more reduced redox state in mice. However no statistically significant differences were observed for the groups consuming alcohol on either the control diet (ND + Alc.) or a KD (KD + Alc.). Thus, it is evident from the mentioned findings that mice consuming a KD had a more oxidative redox state (i.e., a decreased level of NADH relative to NAD⁺) compared to the control diet (ND), while the other groups had no significant alteration in redox state. The mentioned amino acid metabolite ratios indicated a significantly decreased NADH/NAD⁺ ratio in mice on a KD compared to controls

(ND). Thus, confirming that a KD increases the availability of NAD^+ through altering the NADH/NAD^+ ratio. This alteration in redox balance could be related to the observed perturbations in amino acid and energy metabolism in mice on a KD diet. No significant alterations in NADH/NAD^+ ratio were seen following alcohol consumption in the normal diet group (ND + Alc.), indicating that diet *alone* (i.e., KD) had a greater impact on the redox state than alcohol consumption. However, chronic alcohol consumption along with a KD, prevents any favourable changes towards a decreased NADH/NAD^+ ratio, which would be observed when a KD is consumed alone. Previous research has shown that the metabolism of alcohol results in redox state changes due to an increase in the NADH/NAD^+ ratio (Cederbaum, 2012). On the contrary, other studies have shown that a KD can decrease the NADH/NAD^+ ratio, thereby altering the redox state (Elamin *et al.*, 2017; Xin *et al.*, 2018). As such, our findings provide insight with regards to changes in the redox state when alcohol is consumed together with a KD.

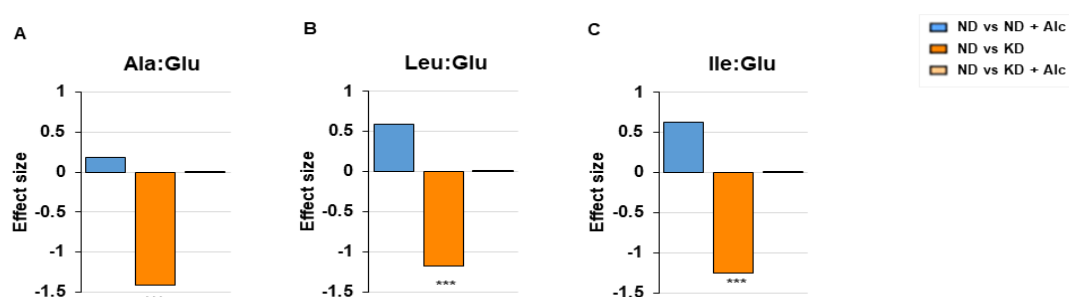


Figure 4-7: A KD results in a more oxidative redox state in mice. Bar graphs (A-C) depict Cohen's d-values (effect size) of alterations in metabolic indicators of NADH/NAD^+ ratio, when the different dietary groups were compared to controls. Positive and negative effect size values respectively indicate increased and decreased levels in the experimental group vs the control group. An elevated NADH/NAD^+ ratio drives the formation of amino acids from glutamic acid. Ala indicates alanine; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; ND, normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. Asterisks (***) obtained via a post-hoc Tukey's test, indicate significant differences between the ND vs KD group.

4.3.4. Inflammatory status of mouse serum

Several serum inflammatory biomarkers were measured in the different dietary groups. All experimental groups displayed significantly increased ($p < 0.05$; Figure 4-8) levels of IL-6 relative to the control group. The ND + Alc. group had the highest increase in IL-6, followed by the KD group and then the KD + Alc. group. This finding suggests that both alcohol and a KD has a pro-inflammatory effect. However, no significant alterations in IFN or MCP-1 levels were observed among the dietary groups, while marked intragroup variation limited our use of the other measured inflammatory cytokines (Figure C-4). Although several studies have described the anti-inflammatory properties of a KD (Ciaffi *et al.*, 2021; Dupuis *et al.*, 2015), others have also observed signs of increased inflammation associated with a KD in mice (Garbow *et al.*,

2011) and human subjects (Rosenbaum *et al.*, 2019). Thus, no conclusive assumption can be made with regards to a KD and chronic alcohol consumption's impact on inflammation and further research is required. Moreover, seeing that existing literature also shows conflicting evidence with regards to the effect a KD and alcohol consumption has on inflammation (Ciaffi *et al.*, 2021; Dupuis *et al.*, 2015; Garbow *et al.*, 2011; Rosenbaum *et al.*, 2019). Considering our inconclusive observations, along with conflicting literature reports, further research on the inflammatory effects of a KD and alcohol consumption is warranted.

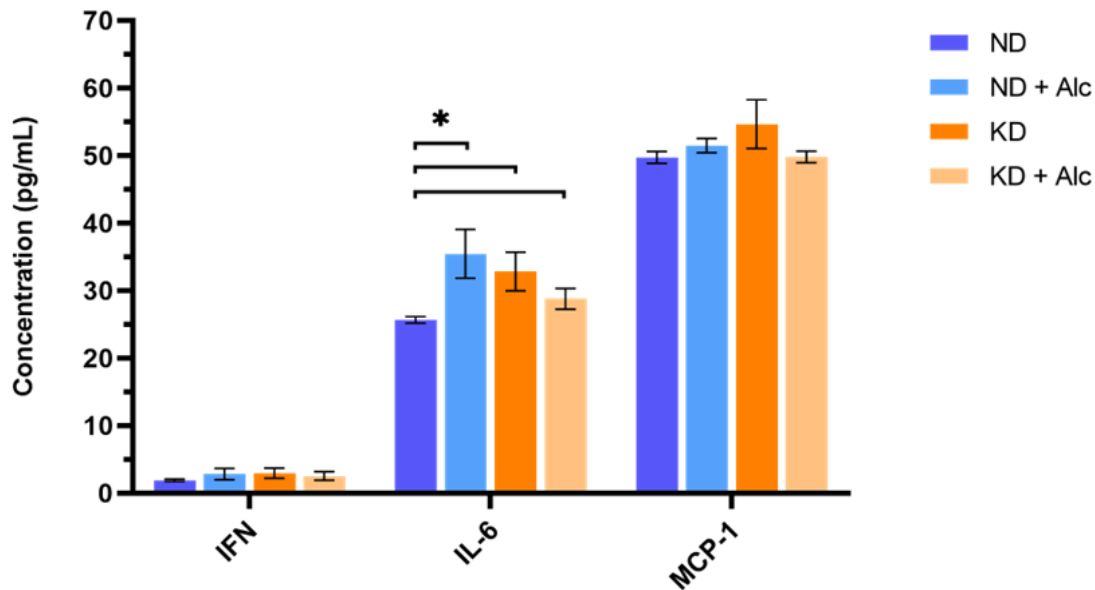


Figure 4-8: The concentration levels (pg/mL) of pro-inflammatory biomarkers as measured in the serum of mice from all four dietary groups (n = 6 per group). The error bars represent the standard error. ND indicates normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. *p < 0.05 vs. the control group, obtained via a post-hoc Tukey's test.

4.4. Conclusion

The therapeutic potential of a KD and its mechanisms of action for conditions such as, epilepsy, obesity, diabetes, inflammation, and mitochondrial disease has gained immense attention in recent years (Bolla *et al.*, 2019; Dupuis *et al.*, 2015; Freeman & Kossoff, 2010; Jin *et al.*, 2014; Qu *et al.*, 2021; Vining, 1999). The regulatory effect that a KD has on the mTOR pathway is of great importance (Genzer *et al.*, 2016) and has been evaluated in various disease models (Genzer *et al.*, 2016; Johnson *et al.*, 2013; McDaniel *et al.*, 2011; Roberts *et al.*, 2017; Singh *et al.*, 2018; Tian *et al.*, 2019; Xie *et al.*, 2016; Zeng *et al.*, 2008). Since alcohol consumption reportedly alters the cellular redox state and impedes mTOR phosphorylation (Feldstein & Bailey, 2011; Lang *et al.*, 2003; Zakhari, 2006), we aimed to establish the effect that chronic alcohol consumption has on the mTOR pathway and systemic metabolism during a KD-based intervention. This was achieved by investigating the isolated and combined effects of chronic alcohol consumption and a KD on growth rate, S6K1 phosphorylation, urinary metabolism,

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metabolic indicators of redox state, and serum inflammatory markers in a healthy mouse model. This study, however, did have its limitations. Firstly, alcohol and food intake were not monitored accordingly when mice were given ad libitum access to alcohol drinking water and food. As such, it is possible that the mice on a KD and those on a KD with chronic alcohol consumption consumed less food than that of the control diet, especially since a KD has previously been linked to increased satiety and decreased food intake (Paoli *et al.*, 2015). In addition, this could also lead to the mice consuming less vitamins and trace minerals, which in return could account for difference in growth (Churuangsuk *et al.*, 2019; Kenig *et al.*, 2019). Additionally, the KD used in this study was not an isocaloric KD, as the total calorie content was not identical for the ND and KD. Moreover, the KD used in this study was also more protein-restricted when compared to the ND. Lastly, consideration needs to be taken when a similar dietary strategy is applied to humans, seeing that the mice were around three weeks old when they were put on the dietary intervention. Children might respond differently than adults when put on a ketogenic dietary intervention (Kim *et al.*, 2019).

In conclusion, this study shows that a KD attenuates mTOR signalling and weight gain, as well as decreases the excretion of amino acid and TCA cycle metabolites in mice. Also, the KD seems to drive energy metabolism towards a more oxidative redox state by decreasing the NADH/NAD⁺ ratio through metabolic regulation. In addition, the study's results regarding a KD's effect on inflammation were inconclusive due to limited sample volume and the fact that only one pro-inflammatory marker, IL-6, was significantly increased. As such, more research regarding a KD's effect on inflammation is warranted. Furthermore, we provide evidence that a KD could decrease or prevent bone loss and collagen degradation, by decreasing structural protein degradation even during chronic alcohol consumption. Although findings of previous studies have shown that a KD alone does not lead to potential bone loss and degradation (Bisschop *et al.*, 2003), our study is the first to report that a KD could potentially reduce bone loss and collagen degradation during chronic alcohol consumption. As such, it is evident that a KD indeed has some therapeutic potential, especially for conditions that lead to an unfavourable redox state, increase in structural protein degradation, as well as disorders in which attenuation of mTOR leads to improved disease outcome. However, the potential benefits of a KD could be overturned when alcohol is consumed chronically in conjunction with a KD, as alcohol consumption seems to prevent the shift towards a more oxidative redox state and lead to a potential decrease in NAD⁺ levels. Thus, any intervention using a KD for the management of conditions like obesity, diabetes, epilepsy, and other metabolic disorders, should take note of the factors that could potentially hinder the therapeutic effects of a KD, such as chronic alcohol consumption, especially where the redox state is of concern. Further research is warranted making use of multiple protein markers to monitor mTOR activity, as well as applying multi-

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platform metabolomics on other tissues such blood, brain, and muscle to evaluate tissue specific differences in systemic metabolism, to further assess the therapeutic potential of a KD. Lastly, any study looking at the effect of a KD should also consider the total protein and total caloric content of the diet, as well as the effects it has on the dietary intervention.

4.5. References

- American Veterinary Medical Association. AVMA guidelines for the euthanasia of animals: 2020 edition, <https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf>; 2020 [accessed: 12 April 2020].
- Auta, J., Zhang, H., Pandey, S.C. & Guidotti, A. 2017. Chronic Alcohol Exposure Differentially Alters One-Carbon Metabolism in Rat Liver and Brain. *Alcohol Clin. Exp. Res.*, 41(6):1105-1111.
- Barbul, A. 2008. Proline precursors to sustain Mammalian collagen synthesis. *J. Nutr.*, 138(10):2021s-2024s.
- Bernal, C.A., Vazquez, J.A. & Adibi, S.A. 1993. Leucine metabolism during chronic ethanol consumption. *Metabolism*, 42(9):1084-1086.
- Bisschop, P.H., De Sain-Van Der Velden, M.G., Stellaard, F., Kuipers, F., Meijer, A.J., Sauerwein, H.P. & Romijn, J.A. 2003. Dietary carbohydrate deprivation increases 24-hour nitrogen excretion without affecting postabsorptive hepatic or whole body protein metabolism in healthy men. *J. Clin. Endocrinol. Metab.*, 88(8):3801-3805.
- Blanco-Gandía, M.D.C., Ródenas-González, F., Pascual, M., Reguilón, M.D., Guerri, C., Miñarro, J. & Rodríguez-Arias, M. 2021. Ketogenic Diet Decreases Alcohol Intake in Adult Male Mice. *Nutrients*, 13(7):2167. Bolla, A.M., Caretto, A., Laurenzi, A., Scavini, M. & Piemonti, L. 2019. Low-Carb and Ketogenic Diets in Type 1 and Type 2 Diabetes. *Nutrients*, 11(5):962.
- Bornebusch, A.B., Mason, G.F., Tonetto, S., Damsgaard, J., Gjedde, A., Fink-Jensen, A. & Thomsen, M. 2021. Effects of ketogenic diet and ketone monoester supplement on acute alcohol withdrawal symptoms in male mice. *Psychopharmacology (Berl)*, 238(3):833-844.
- Bornstein, R., James, K., Stokes, J., Park, K.Y., Kayser, E.B., Snell, J., ... Johnson, S.C. 2022. Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy. *Neurobiol. Dis.*, 163:105594.
- Bueno, N.B., de Melo, I.S., de Oliveira, S.L. & da Rocha Ataíde, T. 2013. Very-low-carbohydrate ketogenic diet v. low-fat diet for long-term weight loss: a meta-analysis of randomised controlled trials. *Br J. Nutr.*, 110(7):1178-1187.
- Cech, N.B. & Enke, C.G. 2001. Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom. Rev.*, 20(6):362-387.
- Cederbaum, A.I. 2012. Alcohol metabolism. *Clinics in liver disease*, 16(4):667-685.
- Chen, W.X., Xu, C.Q., Chen, S.H., Xu, G.Y. & Ye, H.Z. 2012. Citric acid reduces the decline in P300 amplitude induced by acute alcohol consumption in healthy adults. *J. Zhejiang Univ. Sci. B*, 13(5):395-401.
- Churuangasuk, C., Griffiths, D., Lean, M.E.J. & Combet, E. 2019. Impacts of carbohydrate-restricted diets on micronutrient intakes and status: A systematic review. *Obes. Rev.*, 20(8):1132-1147.
- Ciaffi, J., Mitselman, D., Mancarella, L., Brusi, V., Lisi, L., Ruscitti, P., ... Ursini, F. 2021. The Effect of Ketogenic Diet on Inflammatory Arthritis and Cardiovascular Health in Rheumatic Conditions: A Mini Review. *Front. Med. (Lausanne)*, 8:792846.

Chapter 4

- Dencker, D., Molander, A., Thomsen, M., Schlumberger, C., Wortwein, G., Weikop, P., ... Fink-Jensen, A. 2018. Ketogenic Diet Suppresses Alcohol Withdrawal Syndrome in Rats. *Alcohol Clin. Exp. Res.*, 42(2):270-277.
- Dupuis, N., Curatolo, N., Benoist, J.F. & Auvin, S. 2015. Ketogenic diet exhibits anti-inflammatory properties. *Epilepsia*, 56(7):e95-98.
- Elamin, M., Ruskin, D.N., Masino, S.A. & Sacchetti, P. 2017. Ketone-Based Metabolic Therapy: Is Increased NAD(+) a Primary Mechanism? *Front. Mol. Neurosci.*, 10:377.
- Ellis, S.M. & Steyn, H.S. 2003. Practical significance (effect sizes) versus or in combination with statistical significance (p-values): research note. *Manag. Dyn.*, 12(4):51-53.
- Feldstein, A.E. & Bailey, S.M. 2011. Emerging role of redox dysregulation in alcoholic and nonalcoholic fatty liver disease. *Antioxid. Redox Signal*, 15(2):421-424.
- Foster, D.A. 2010. Reduced mortality and moderate alcohol consumption: the phospholipase D-mTOR connection. *Cell Cycle*, 9(7):1291-1294.
- Freeman, J.M. & Kossoff, E.H. 2010. Ketosis and the ketogenic diet, 2010: advances in treating epilepsy and other disorders. *Adv. Pediatr.*, 57(1):315-329.
- Garbow, J.R., Doherty, J.M., Schugar, R.C., Travers, S., Weber, M.L., Wentz, A.E., ... Crawford, P.A. 2011. Hepatic steatosis, inflammation, and ER stress in mice maintained long term on a very low-carbohydrate ketogenic diet. *Am. J. Physiol. Gastrointest. Liver Physiol.*, 300(6):G956-967.
- Genzer, Y., Dadon, M., Burg, C., Chapnik, N. & Froy, O. 2016. Effect of dietary fat and the circadian clock on the expression of brain-derived neurotrophic factor (BDNF). *Mol. Cell Endocrinol.*, 430:49-55.
- Griffin, J.L. & Nicholls, A.W. 2006. Metabolomics as a functional genomic tool for understanding lipid dysfunction in diabetes, obesity and related disorders. *Pharmacogenomics*, 7(7):1095-1107.
- Hay, N. & Sonenberg, N. 2004. Upstream and downstream of mTOR. *Genes Dev.*, 18(16):1926-1945.
- Hsin, I.L., Shen, H.P., Chang, H.Y., Ko, J.L. & Wang, P.H. 2021. Suppression of PI3K/Akt/mTOR/c-Myc/mtp53 Positive Feedback Loop Induces Cell Cycle Arrest by Dual PI3K/mTOR Inhibitor PQR309 in Endometrial Cancer Cell Lines. *Cells*, 10(11), 2916.
- Hsu, F.Y., Liou, J.Y., Tang, F.Y., Sou, N.L., Peng, J.H. & Chiang, E.I. 2022. Ketogenic Diet Consumption Inhibited Mitochondrial One-Carbon Metabolism. *Int. J. Mol. Sci.*, 23(7),
- Hu, S., Wang, L., Yang, D., Li, L., Togo, J., Wu, Y., ... Speakman, J.R. 2018. Dietary Fat, but Not Protein or Carbohydrate, Regulates Energy Intake and Causes Adiposity in Mice. *Cell Metab.*, 28(3):415-431.e414.
- Huang, C., Titus, J.A., Bell, R.L., Kapros, T., Chen, J. & Huang, R. 2012. A mouse model for adolescent alcohol abuse: stunted growth and effects in brain. *Alcohol Clin. Exp. Res.*, 36(10):1728-1737.
- Ikenoue, T., Hong, S. & Inoki, K. 2009. Monitoring mammalian target of rapamycin (mTOR) activity. *Methods Enzymol.*, 452:165-180.
- Jewell, J.L. & Guan, K.L. 2013. Nutrient signaling to mTOR and cell growth. *Trends Biochem. Sci.*, 38(5):233-242.
- Jin, Z., Berthiaume, J.M., Li, Q., Henry, F., Huang, Z., Sadhukhan, S., ... Zhang, G.F. 2014. Catabolism of (2E)-4-hydroxy-2-nonenal via ω - and ω -1-oxidation stimulated by ketogenic diet. *J. Biol. Chem.*, 289(46):32327-32338.
- Johnson, S.C., Yanos, M.E., Kayser, E.B., Quintana, A., Sangesland, M., Castanza, A., ... Kaeberlein, M. 2013. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science*, 342(6165):1524-1528.

Chapter 4

- Kenig, S., Petelin, A., Poklar Vatovec, T., Mohorko, N. & Jenko-Pražnikar, Z. 2019. Assessment of micronutrients in a 12-wk ketogenic diet in obese adults. *Nutrition*, 67-68:110522.
- Kim, S.H., Shaw, A., Blackford, R., Lowman, W., Laux, L.C., Millichap, J.J. & Nordli, D.R., Jr. 2019. The ketogenic diet in children 3 years of age or younger: a 10-year single-center experience. *Sci. Rep.*, 9(1):8736.
- Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab*, 7(4):312-320. 10.1016/j.cmet.2008.02.004
- Kuo, T.R. & Chen, C.H. 2017. Bone biomarker for the clinical assessment of osteoporosis: recent developments and future perspectives. *Biomark Res.*, 5:18.
- Lang, C.H., Lynch, C.J. & Vary, T.C. 2010. Alcohol-induced IGF-I resistance is ameliorated in mice deficient for mitochondrial branched-chain aminotransferase. *J. Nutr.*, 140(5):932-938.
- Lang, C.H., Frost, R.A., Deshpande, N., Kumar, V., Vary, T.C., Jefferson, L.S. & Kimball, S.R. 2003. Alcohol impairs leucine-mediated phosphorylation of 4E-BP1, S6K1, eIF4G, and mTOR in skeletal muscle. *Am. J. Physiol Endocrinol. Metab.*, 285(6):E1205-1215.
- Laplanche, M. & Sabatini, D.M. 2012. mTOR signaling in growth control and disease. *Cell*, 149(2):274-293.
- Laplanche, M. & Sabatini, D.M. 2013. Regulation of mTORC1 and its impact on gene expression at a glance. *J. Cell Sci.*, 126(Pt 8):1713-1719.
- Longo, R., Peri, C., Cricri, D., Coppi, L., Caruso, D., Mitro, N., ... Crestani, M. 2019. Ketogenic Diet: A New Light Shining on Old but Gold Biochemistry. *Nutrients*, 11(10):2497.
- Mason, S., Terburgh, K. & Louw, R. 2018. Miniaturized (1)H-NMR method for analyzing limited-quantity samples applied to a mouse model of Leigh disease. *Metabolomics*, 14(6):74.
- McDaniel, S.S., Rensing, N.R., Thio, L.L., Yamada, K.A. & Wong, M. 2011. The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. *Epilepsia*, 52(3):e7-11.
- Morita, M., Gravel, S.P., Hulea, L., Larsson, O., Pollak, M., St-Pierre, J. & Topisirovic, I. 2015. mTOR coordinates protein synthesis, mitochondrial activity and proliferation. *Cell Cycle*, 14(4):473-480.
- Morita, M., Gravel, S.P., Chénard, V., Sikström, K., Zheng, L., Alain, T., ... Sonenberg, N. 2013. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. *Cell Metab.*, 18(5):698-711.
- Paoli, A. 2014. Ketogenic diet for obesity: friend or foe? *Int. J. Environ. Res. Public Health*, 11(2):2092-2107.
- Paoli, A., Bosco, G., Camporesi, E.M. & Mangar, D. 2015. Ketosis, ketogenic diet and food intake control: a complex relationship. *Front. Psychol.*, 6:27.
- Perides, G., Tao, X., West, N., Sharma, A. & Steer, M.L. 2005. A mouse model of ethanol dependent pancreatic fibrosis. *Gut*, 54(10):1461-1467.
- Pissios, P., Hong, S., Kennedy, A.R., Prasad, D., Liu, F.F. & Maratos-Flier, E. 2013. Methionine and choline regulate the metabolic phenotype of a ketogenic diet. *Mol. Metab.*, 2(3):306-313.
- Preedy, V.R., Baldwin, D.R., Keating, J.W. & Salisbury, J.R. 1991. Bone collagen, mineral and trace element composition, histomorphometry and urinary hydroxyproline excretion in chronically-treated alcohol-fed rats. *Alcohol Alcohol.*, 26(1):39-46.
- Qu, C., Keijer, J., Adjobo-Hermans, M.J.W., van de Wal, M., Schirris, T., van Karnebeek, C., ... Koopman, W.J.H. 2021. The ketogenic diet as a therapeutic intervention strategy in mitochondrial disease. *Int. J. Biochem. Cell Biol.*, 138:106050.

Chapter 4

- Ramanathan, A. & Schreiber, S.L. 2009. Direct control of mitochondrial function by mTOR. *Proc. Natl. Acad. Sci. USA*, 106(52):22229-22232.
- Rehm, J. & Imtiaz, S. 2016. A narrative review of alcohol consumption as a risk factor for global burden of disease. *Subst. Abuse Treat. Prev. Policy*, 11(1):37.
- Roberts, M.N., Wallace, M.A., Tomilov, A.A., Zhou, Z., Marcotte, G.R., Tran, D., ... Lopez-Dominguez, J.A. 2017. A Ketogenic Diet Extends Longevity and Healthspan in Adult Mice. *Cell Metab*, 26(3):539-546.e535.
- Rosenbaum, M., Hall, K.D., Guo, J., Ravussin, E., Mayer, L.S., Reitman, M.L., ... Leibel, R.L. 2019. Glucose and Lipid Homeostasis and Inflammation in Humans Following an Isocaloric Ketogenic Diet. *Obesity (Silver Spring)*, 27(6):971-981.
- Ruoppolo, M., Caterino, M., Albano, L., Pecce, R., Di Girolamo, M.G., Crisci, D., ... Campesi, I. 2018. Targeted metabolomic profiling in rat tissues reveals sex differences. *Sci. Rep.*, 8(1):4663.
- Santos, F.L., Esteves, S.S., da Costa Pereira, A., Yancy, W.S., Jr. & Nunes, J.P. 2012. Systematic review and meta-analysis of clinical trials of the effects of low carbohydrate diets on cardiovascular risk factors. *Obes. Rev.*, 13(11):1048-1066.
- Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P. & Hollender, J. 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ. Sci. Technol.*, 48(4):2097-2098.
- Singh, A., Mettler, T., Oh, H. & Kim, D.-Y. 2018. The Ketogenic Diet Attenuates Both Hyperactivity in mTOR pathway and Astrogliosis Through Regulation of AMPK signaling in the Epileptic Brain. *FASEB J.*, 32(S1):805.811-805.811.
- Smith, H.Q., Li, C., Stanley, C.A. & Smith, T.J. 2019. Glutamate Dehydrogenase, a Complex Enzyme at a Crucial Metabolic Branch Point. *Neurochem. Res.*, 44(1):117-132.
- Sperl, W. 1997. Diagnosis and therapy of mitochondriopathies. *Wiener Klinische Wochenschrift*, 109(3):93-99.
- Terburgh, K., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021. Cross-comparison of systemic and tissue-specific metabolomes in a mouse model of Leigh syndrome. *Metabolomics*, 17:1-14.
- Terburgh, K., Lindeque, Z., Mason, S., Van der Westhuizen, F. & Louw, R. 2019. Metabolomics of Ndufs4^{-/-} skeletal muscle: Adaptive mechanisms converge at the ubiquinone-cycle. *Biochim. Biophys. Acta - Mol. Basis Dis.*, 1865(1):98-106.
- Tian, T., Li, X. & Zhang, J. 2019. mTOR Signaling in Cancer and mTOR Inhibitors in Solid Tumor Targeting Therapy. *Int. J. Mol. Sci.*, 20(3).
- Traversy, G. & Chaput, J.P. 2015. Alcohol Consumption and Obesity: An Update. *Curr. Obes. Rep.*, 4(1):122-130.
- Valsecchi, F., Koopman, W.J., Manjeri, G.R., Rodenburg, R.J., Smeitink, J.A. & Willems, P.H. 2010. Complex I disorders: causes, mechanisms, and development of treatment strategies at the cellular level. *Dev. Disabil. Res. Rev.*, 16(2):175-182.
- van de Wal, M.A.E., Adjubo-Hermans, M.J.W., Keijer, J., Schirris, T.J.J., Homberg, J.R., Wieckowski, M.R., ... Koopman, W.J.H. 2022. Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention. *Brain*, 145(1):45-63.
- Veech, R.L., Guynn, R. & Veloso, D. 1972. The time-course of the effects of ethanol on the redox and phosphorylation states of rat liver. *Biochem J*, 127(2):387-397.
- Vining, E.P. 1999. Clinical efficacy of the ketogenic diet. *Epilepsy Res.*, 37(3):181-190.

Chapter 4

- Wiers, C.E., Vendruscolo, L.F., van der Veen, J.W., Manza, P., Shokri-Kojori, E., Kroll, D.S., ... Volkow, N.D. 2021. Ketogenic diet reduces alcohol withdrawal symptoms in humans and alcohol intake in rodents. *Sci. Adv.*, 7(15):eabf6780.
- Wu, D., Yu, H.Q., Xiong, H.J., Zhang, Y.J., Lin, X.T., Zhang, J., ... Xie, C.M. 2021. Elevated Sodium Pump $\alpha 3$ Subunit Expression Promotes Colorectal Liver Metastasis via the p53-PTEN/IGFBP3-AKT-mTOR Axis. *Front. Oncol.*, 11:743824.
- Wullschleger, S., Loewith, R. & Hall, M.N. 2006. TOR signaling in growth and metabolism. *Cell*, 124(3):471-484.
- Xia, J., Sinelnikov, I.V., Han, B. & Wishart, D.S. 2015. MetaboAnalyst 3.0--making metabolomics more meaningful. *Nucleic Acids Res.*, 43(W1):W251-257.
- Xie, J., Wang, X. & Proud, C.G. 2016. mTOR inhibitors in cancer therapy. *F1000Res*, 5:F1000 Faculty Rev-2078.
- Xin, L., Ipek, Ö., Beaumont, M., Shevlyakova, M., Christinat, N., Masoodi, M., ... Cuenoud, B. 2018. Nutritional Ketosis Increases NAD(+)/NADH Ratio in Healthy Human Brain: An in Vivo Study by (31)P-MRS. *Front. Nutr.*, 5:62.
- Zakhari, S. 2006. Overview: how is alcohol metabolized by the body? *Alcohol Res. Health*, 29(4):245-254.
- Zeng, L.H., Xu, L., Gutmann, D.H. & Wong, M. 2008. Rapamycin prevents epilepsy in a mouse model of tuberous sclerosis complex. *Ann. Neurol.*, 63(4):444-453.
- Zhao, D., Yang, J. & Yang, L. 2017. Insights for Oxidative Stress and mTOR Signaling in Myocardial Ischemia/Reperfusion Injury under Diabetes. *Oxid. Med. Cell Longev.*, 2017:6437467.

CHAPTER 5

The therapeutic potential of a polyunsaturated fatty acid-enriched high-fat diet in Leigh syndrome: Insights from a preclinical model

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Chapter 5: Scope

The scope of this chapter of the study was focussed on evaluating the therapeutic potential of a polyunsaturated fatty acid (PUFA)-enriched high-fat diet (HFD) in the *Ndufs4* knockout (KO) mouse model of Leigh syndrome, focusing on lifespan, motor function, systemic inflammation, and neurometabolic/lipidomic profiles. The scope included administering a PUFA-HFD or a normal diet (ND) to mice starting at weaning (postnatal day 23) and assessing phenotypical and biochemical outcomes until natural death or euthanasia after three weeks on the dietary intervention. The study's phenotypic and behavioural evaluations entailed survival analysis, grip-strength and locomotor activity assays, symptom evaluation and monitoring of weight. The biochemical evaluations entailed western blotting to assess mechanistic signalling pathways like mTORC1 and SIRT1, flow cytometry to assess systemic inflammation, and brain tissue metabolomics/lipidomics to identify metabolic disruptions.

Status

The manuscript presented as Chapter 5 has been submitted and is under review.

Candidate contribution

In this phase of the study, candidate L. Willemse, together with R. Louw, developed the study design and framework. Under the authorisation of the PCDDP Vivarium personnel, L. Willemse was responsible for the feeding, weighing, and the behavioural and phenotypic assessments of the animals. Furthermore, the candidate executed all experimental protocols, including monitoring mice weight, performing clasping tests, the wire-grid hang test, the open-field test, and tracking mice survival. In addition, L. Willemse conducted genotyping, Western blotting, flow cytometry, as well as analyses using GC-TOF-MS, GC-MS/MS, and UPLC-HDMS. The candidate also managed data processing, analysis, curation, and visualisation, which were subsequently reviewed and refined by K. Terburgh. Finally, the original manuscript draft was written by L. Willemse and later revised and edited with the assistance of K. Terburgh and R. Louw.

Abstract

Introduction: Leigh syndrome is often caused by *Ndufs4* mutations. The *Ndufs4* knockout (KO) mouse model recapitulates key disease features, including systemic inflammation, neurodegeneration, and motor deficits. While dietary interventions such as the ketogenic diet show promise in mitigating mitochondrial dysfunction, conflicting results highlight uncertainties regarding its efficacy. Here, we evaluate the therapeutic potential of a polyunsaturated fatty acid (PUFA)-enriched high-fat diet (HFD) in *Ndufs4* KO mice.

Methods: Dietary intervention began at postnatal day 23, with mice receiving either a normal diet (ND) or a HFD enriched with PUFAs. Phenotypic evaluation, including locomotor function, clasping behaviour, and survival, continued until natural death. In a second group of animals, biochemical analyses were conducted after three weeks on the diets, using Western blot to evaluate neurometabolic and inflammatory regulators, flow cytometry to quantify serum inflammation markers, and metabolic profiling to identify alterations in neurometabolism and the neurolipidome.

Results: The HFD significantly extended lifespan and improved clasping behaviour in *Ndufs4* KO mice but had no effect on locomotor activity or grip strength decline. While whole-brain mTOR (p70S6K1, 4E-BP1) and SIRT1 (PGC1- α , TNF- α) signalling pathways remained unaffected, the diet significantly reduced serum pro-inflammatory markers TNF and IL-6. Furthermore, the PUFA-enriched HFD partially restored disruptions in TCA cycle, ketone body, branched-chain amino acid, and lipid metabolism, indicating potential metabolic reprogramming.

Conclusion: Dietary interventions, such as a PUFA-enriched HFD, may alleviate systemic inflammation, partially correct metabolic imbalances, and mitigate specific disease phenotypes in Leigh syndrome, warranting further investigation into the underlying mechanisms and broader therapeutic applications.

Keywords: Mitochondrial disease, ketogenic diet, polyunsaturated fatty acids, mTOR, flow cytometry, metabolomics, lipidomics

5.1. Introduction

Mitochondrial diseases encompass a spectrum of disorders, primarily stemming from oxidative phosphorylation (OXPHOS) system defects. Affecting approximately 1 in 5000 births, these disorders are among the most prevalent inborn errors of metabolism (Gorman *et al.*, 2015; Schaefer *et al.*, 2004). A significant proportion of cases arise from deficiencies in complex I (CI) of the OXPHOS system (Koopman *et al.*, 2005; Papa *et al.*, 2012), which disrupt ATP production, elevate reactive oxygen species (ROS), and perturb redox homeostasis (Koopman *et al.*, 2005; Papa *et al.*, 2012). Clinically, most paediatric cases manifest as subacute necrotising encephalomyelopathy or Leigh syndrome (Lim *et al.*, 2022). Leigh syndrome, a severe neurodegenerative disorder, is characterised by symmetrical, bilateral brain lesions, leading to psychomotor regression and early mortality (Ruhoy & Saneto, 2014; Sofou *et al.*, 2014). To date, more than 80 pathogenic mutations, spanning mitochondrial and nuclear DNA, have been linked to the disease (Lim *et al.*, 2022). Among these, mutations in the *NDUFS4* gene, encoding a CI subunit, are particularly prominent (Bris *et al.*, 2017; Papa *et al.*, 2004).

The development of robust preclinical models has been instrumental in advancing our understanding of mitochondrial diseases like CI deficiency and exploring therapeutic strategies (Esterhuizen *et al.*, 2017). The *Ndufs4* knockout (KO) mouse model is widely recognized as one of the most accurate representations of CI deficiency-related Leigh syndrome, recapitulating its hallmark features, including growth retardation, locomotor dysfunction, ataxia, muscular hypotonia, and premature death (Kruse *et al.*, 2008). Studies using this model have identified critical disease-driving mechanisms, including NAD⁺ deficiency (Lee *et al.*, 2019; Yoon *et al.*, 2022), mTORC1 hyperactivation (Johnson *et al.*, 2013), and neuroinflammation (Hanaford & Johnson, 2022). However, despite these advances, curative therapies for CI deficiency remain elusive (van de Wal *et al.*, 2022).

Among emerging therapeutic approaches, the ketogenic diet (KD) has garnered attention as a metabolic intervention with potential to ameliorate mitochondrial dysfunction. Characterised by its high-fat and low-carbohydrate composition, the KD induces ketogenesis and promotes mitochondrial adaptations, including enhanced biogenesis, improved OXPHOS, and reduced oxidative stress (Jarrett *et al.*, 2008; Sullivan *et al.*, 2004). Moreover, the diet's ability to modulate NAD⁺ metabolism and suppress mTOR signalling further establishes it as a promising strategy for mitigating CI dysfunction (Elamin *et al.*, 2017; Johnson *et al.*, 2013; Willemse *et al.*, 2023). However, Bornstein *et al.* (2022) recently reported no benefits of a standard KD in *Ndufs4* KO mice. While this diet successfully induced dietary ketosis, it did not mitigate disease progression, and a strict KD was also poorly tolerated in this mouse model.

This raises the question of whether dietary composition — particularly the fatty acid profile — in KDs and other high-fat diets (HFDs) may affect treatment outcomes. As recently highlighted by

(Elisia *et al.*, 2024), the fat source in KD and HFD formulations may be a crucial determinant of treatment efficacy, since different fatty acids distinctly influence metabolic and inflammatory pathways. Notably, omega (ω)-3 polyunsaturated fatty acids (PUFAs), derived from fish oil, exhibit potent anti-inflammatory properties, mitigating neuroinflammation, regulating redox balance, and inhibiting mTOR activity (Chen *et al.*, 2018; Liu *et al.*, 2020; Shirooie *et al.*, 2018). These effects suggest that incorporating ω -3 PUFAs into KD or HFD formulations could hold synergistic therapeutic benefits for CI deficiency, where metabolic dysfunction and inflammation are central to disease progression.

In this study, we utilise the *Ndufs4* KO mouse model of Leigh syndrome to evaluate the therapeutic potential of a HFD enriched with PUFAs, derived from Menhaden fish oil. This approach was selected to circumvent the limitations of traditional and classic KDs and HFDs in mitochondrial disease models, particularly in terms of fatty acid composition. The diet's impact on motor function, ataxia and survival was assessed through phenotypic evaluations of locomotor activity, grip strength, clasping behaviour, and lifespan. Additionally, biochemical analyses were performed to examine the effects of the diet on mTORC1 and SIRT1 signalling pathways, systemic inflammation, as well as neurometabolic and -lipidomic profiles. Our findings provide new insights into the potential of a PUFA-enriched HFD as therapeutic strategy for neurodegenerative mitochondrial diseases like Leigh syndrome.

5.2. Materials and methods

5.2.1. Animal care and handling

Whole-body *Ndufs4* knockout (KO) and wild-type (WT) mice were bred from heterozygous crosses (B6.129S4-*Ndufs4*^{tm1.1Rpa}/J, JAX #027058) (Kruse *et al.*, 2008), with genotypes confirmed by PCR as previously described (Valsecchi *et al.*, 2012). Animals were housed in the specific pathogen-free unit of the Vivarium (SAVC reg. no. FR15/13458) at the North-West University (NWU) Pre-Clinical Drug Development Platform (RSA). The facility maintained controlled conditions (22 $\pm$ 1°C, 55 $\pm$ 10% humidity, 12:12 hour light/dark cycle), with ad libitum access to food and water. Enclosures were equipped with sawdust bedding, paper towels, and polycarbonate mouse houses. Animals were handled and their welfare maintained by South African Veterinary Council (SAVC)-authorized personnel in accordance with ethical guidelines outlined in the code of ethics for research, training, and drug testing in South Africa. The study was approved by the NWU Animal Research Ethics Committee (NWU-00516-20-A5) and complied with national legislation.

5.2.2. Dietary interventions

The experimental workflow, depicted in Figure 5-1, outlines the study's group allocation, dietary intervention, sample sizes, and analytical procedures. After weaning on postnatal day (P) 23,

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mice were randomly assigned to normal diet (ND) or a high-fat diet enriched with ω -3 dominant PUFAs from Menhaden fish oil, referred onwards as just HFD. Both sexes were equally represented in the diet groups and given ad libitum access to food. Table 1 summarizes the nutritional profile of the diets, with the ND (D23050803i, Research Diets, USA) containing 20% protein, 70% carbohydrates, and 10% fat; while the HFD (D09020506i, Research Diets, USA) contained 20% protein, 20% carbohydrates, and 60% fat. The 60% fat/20% carbohydrate composition was chosen to avoid the extreme fat ratios typical used in classic KDs and HFDs, as strict ketosis has proven insufficient as a therapeutic strategy in this model - *Ndufs4* KO mice do not tolerate a full KD and loses weight drastically once placed on this diet (Bornstein *et al.*, 2022). Therefore, the diet intervention in this study is designated as a PUFA-enriched HFD to distinguish it from conventional and classic high-fat, very-low-carbohydrate formulations. Food intake was monitored by weighing the food before and after providing it to the mice. The food was replaced every two to three days to ensure freshness, with the removed food being reweighed. Mice on the ND consumed approximately 3.0 grams of food per day, while those on the HFD consumed around 2.2 grams per day. Despite differences in food intake by weight, both groups had comparable daily caloric consumption (11.55 kcal/day for the ND vs. 11.53 kcal/day for the HFD) due to the higher caloric density of the HFD.

Phenotypic evaluations (Figure 5-1A) were conducted on KO mice (n = 12 per group) allowed to reach their natural endpoint, while biochemical evaluations (Figure 5-1B) were performed on KO and WT mice (n = 14 per group) euthanized at the late disease stage (P45–47). Following euthanasia, blood serum and brain tissues were collected. All samples were rapidly snap-frozen in liquid nitrogen to halt metabolic activity and were subsequently stored at -80°C for preservation.

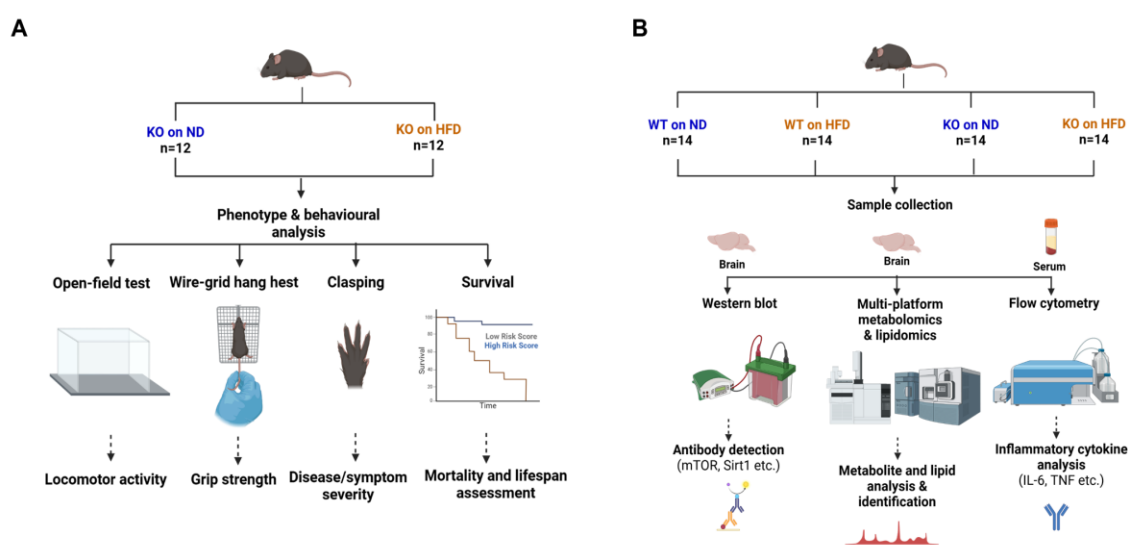


Figure 5-1: Experimental workflow. (A) Phenotypic and behavioural assessment and **(B)** biochemical analyses. Abbreviations: *KO*, knockout; *ND*, normal diet; *P*, postnatal day; *HFD*, high-fat diet; *WT*, wild-type.

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Table 5-1: Dietary nutritional profiles and ingredient composition

	ND			HFD		
Nutrients	g	% kcal	g per 100 g	g	% kcal	g per 100 g
Protein	19.20	20	19.24	26.20	20	26.23
Carbohydrates	67.30	70	71.11	26.30	20	31.50
Fat	4.30	10	5.21	34.90	60	35.89
Total		100	100		100	100
kcal/g	3.85			5.24		
Ingredients	g	kcal	g per 100 g	g	kcal	g per 100 g
Casein, 80 mesh	200.00	800	18.96	200.00	800	25.84
L-cystine	3.00	12	0.28	3.00	12	0.38
Corn starch	315.00	1260	29.86	0.00	0	0
Maltodextrin 10	35.00	140	3.32	125.00	500	16.15
Sucrose	350.00	1400	33.17	68.80	275	8.89
Cellulose, BW200	50.00	0	4.74	50.00	0	6.46
Soybean oil	25.00	225	2.37	25.00	225	3.23
Lard	20.00	180	1.90	122.50	1103	15.83
Fish oil (Menhaden)	0.00	0	0	122.50	1103	15.83
Mineral mix S10026	10.00	0	0.95	10.00	0	1.29
Dicalcium phosphate	13.00	0	1.23	13.00	0	1.68
Calcium carbonate	5.50	0	0.52	5.50	0	0.71
Potassium citrate, 1 H ₂ O	16.50	0	1.56	16.5	0	2.13
Vitamin mix V10001	10.00	40	0.95	10.00	40	1.29
Choline bitartrate	2.00	0	0.19	2.00	0	0.26
FD&C blue dye #1	0.05	0	0.001	0.00	0	0
FD&C red dye #40	0.00	0	0	0.05	0	0.01
Total	1055.05	4057	100	773.85	4057	100
Fatty acid composition	g	%	g per 100 g	g	%	g per 100 g
Saturated fatty acid (SFA)	10.10	23.50	0.95	74.60	30.00	9.64
Monounsaturated fatty acid (MUFA)	12.80	29.70	1.21	74.70	30.00	9.65
Polyunsaturated fatty acid (PUFA)	20.20	46.80	1.90	99.40	40.00	12.84
Omega-3/6 fatty acid composition	g	%	g per 100 g	g	%	g per 100 g
Omega-3 fatty acids	2.1	4.87	0.20	42	16.88	5.43
Omega-6 fatty acids	17.9	41.53	1.70	49	19.69	6.33

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Abbreviations: ND, normal diet; HFD, high-fat diet; kcal, kilocalories; and g, gram.

5.2.3. Phenotypic evaluation

Disease progression and survival in *Ndufs4* KO mice were phenotypically assessed as previously described (Kruse *et al.*, 2008; Quintana *et al.*, 2010) and detailed in Chapter 3 and Appendix E. Locomotor activity, ataxia, and grip strength were assessed at three disease stages — early (P28–30), mid (P38–40), and late (P48–50) — using open-field and wire-grid hang tests. The clasping test, a measure of motor function and neurological health (Guyenet *et al.*, 2010), was conducted daily, with natural death dates recorded.

5.2.4. SDS-PAGE and Western blotting

Brain tissue (~30 mg) was homogenized in RIPA buffer (~300 μ L) via sonication (three 3-s pulses), and protein concentrations determined using the BCA assay. Samples were heat denatured (10 min at 72°C), separated by SDS-PAGE (30 μ g protein), and transferred to nitrocellulose membranes. Membranes were blocked in 1% casein TBS and incubated overnight (4°C) with primary antibodies targeting (phospho-) mTOR, (phospho-) p70S6K1, phospho-) 4EBP1, (phospho-), SIRT1, PGC1- α , TNF- α and β -actin. Thereafter, HRP-conjugated secondary antibodies (goat anti-mouse and goat anti-rabbit) were applied at room temperature. Chemiluminescent detection was performed using a Western ECL substrate and the ChemiDoc™ MP imaging system (Bio-Rad). Band intensities were quantified with Image Lab software (v. 6.1) and normalised to total protein or β -actin. Detailed procedures and antibody information are provided in Chapter 3 and Appendix E.

5.2.5. Inflammation assay

Inflammatory profiles in mouse serum were measured using the BD Cytometric Bead Array Mouse Inflammation Kit, following the manufacturer's instructions. The assay utilized a multiplex bead array coupled with flow cytometry (BD FACSLyric, BD Biosciences) to detect interleukin (IL) 6, IL-10, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor (TNF), interferon gamma (IFN- γ), and IL-12p70. Cytokine concentrations (pg/mL) were calculated based on standard curves using FlowJo (v. 10.8) and FCS Express (v. 6, De Novo) for data analysis.

5.2.6. Metabolomics and lipidomics

Metabolomic and lipidomic profiling of brain tissue was performed using untargeted and targeted approaches on separately prepared samples. Polar and nonpolar metabolites were extracted using a monophasic methanol/water/chloroform (6:2:2) Bligh-Dyer method (Gullberg *et al.*, 2004), while lipids were extracted using a monophasic butanol/methanol (3:1) method (Löfgren *et al.*, 2016). Internal standards, 3-phenylbutyric acid for metabolomics and

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diheptadecanoyl (C17:0) fatty acids for lipidomics, were added during extraction. Samples were aliquoted and dried under nitrogen gas prior to analysis.

Untargeted metabolomics was performed on oximated and silylated samples using gas chromatography (Agilent 8890) coupled with time-of-flight mass spectrometry (LECO Pegasus BT). Targeted analysis of redox-related metabolites (Table 3-3) was conducted on silylated samples using gas chromatography (Agilent 7890B) coupled with triple quadrupole mass spectrometry (Agilent 7010B). Untargeted lipidomics was performed on samples resuspended in methanol/acetonitrile (2:1) using ultra-performance liquid chromatography coupled with high-definition mass spectrometry (Waters Synapt G2-Si).

Samples were randomized to minimise analytical variability, with quality control samples included to monitor instrument performance. Metabolites were identified by matching mass spectra to commercial and in-house libraries with an 80% similarity threshold to ensure high-confidence annotation.

Further details on sample preparation, analysis conditions, and data processing are provided in Chapter 3 and Appendix E.

5.2.7. Statistical analyses

Survival analysis was conducted using the Log-rank (Mantel-Cox) test. Independent sample t-tests, one-way, and two-way ANOVAs were used to assess group differences in phenotype, weight, and Western blot data. For flow cytometry data, outlier removal was followed by independent sample t-tests and Mann-Whitney U tests. These analyses were performed using Excel 365, IBM SPSS Statistics 25, and GraphPad Prism v. 9.

Metabolic data were processed using Excel 365, MetaboAnalyst v. 6.0 (Pang *et al.*, 2021; Xia *et al.*, 2009), and GraphPad Prism v. 9, while lipidomic data processing additionally utilized Progenesis Q1 v. 2.0. Multiple independent sample t-tests, one-way, and two-way ANOVAs with post hoc Tukey's tests were used to identify group differences. Statistical significance was set at $p < 0.05$, corrected for multiple testing using Benjamini-Hochberg false discovery rate (BH-FDR). Effect size thresholds were set at Cohen's $d > 0.6$ for metabolomics and > 0.8 for lipidomics.

5.3. Results

5.3.1. Phenotype and behavioural assessment

The Log-Rank test (Figure 5-2A) revealed a significant lifespan increase in KO-HFD (P72-95) compared to KO-ND mice (P45-60, $p < 0.001$). To assess possible disease symptom alleviation, limb clasping was monitored throughout the animals' lifespan (Figure 5-2B-C), while locomotor activity (Figure 5-2D-F) and grip strength (mean physical impulse, Figure 5-2G) were assessed

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at P30, P40, and P50. KO-HFD mice displayed a markedly delayed onset of limb clasp (P60-70) and significant improvements in clasp compared KO-ND mice (P42-50, $p < 0.0001$). However, open-field tests showed no improvement in locomotor activity, with cumulative duration, mean velocity, and total distance unaltered by the diet. Wire-grid hang tests revealed a temporary reduction in grip strength of KO-HFD mice at P30 ($p < 0.05$), with no differences observed at P40 or P50. Lastly, growth curves indicated that the HFD did not impact bodyweight in KO or WT animals, although KO mice on either diet exhibited reduced growth compared to WT.

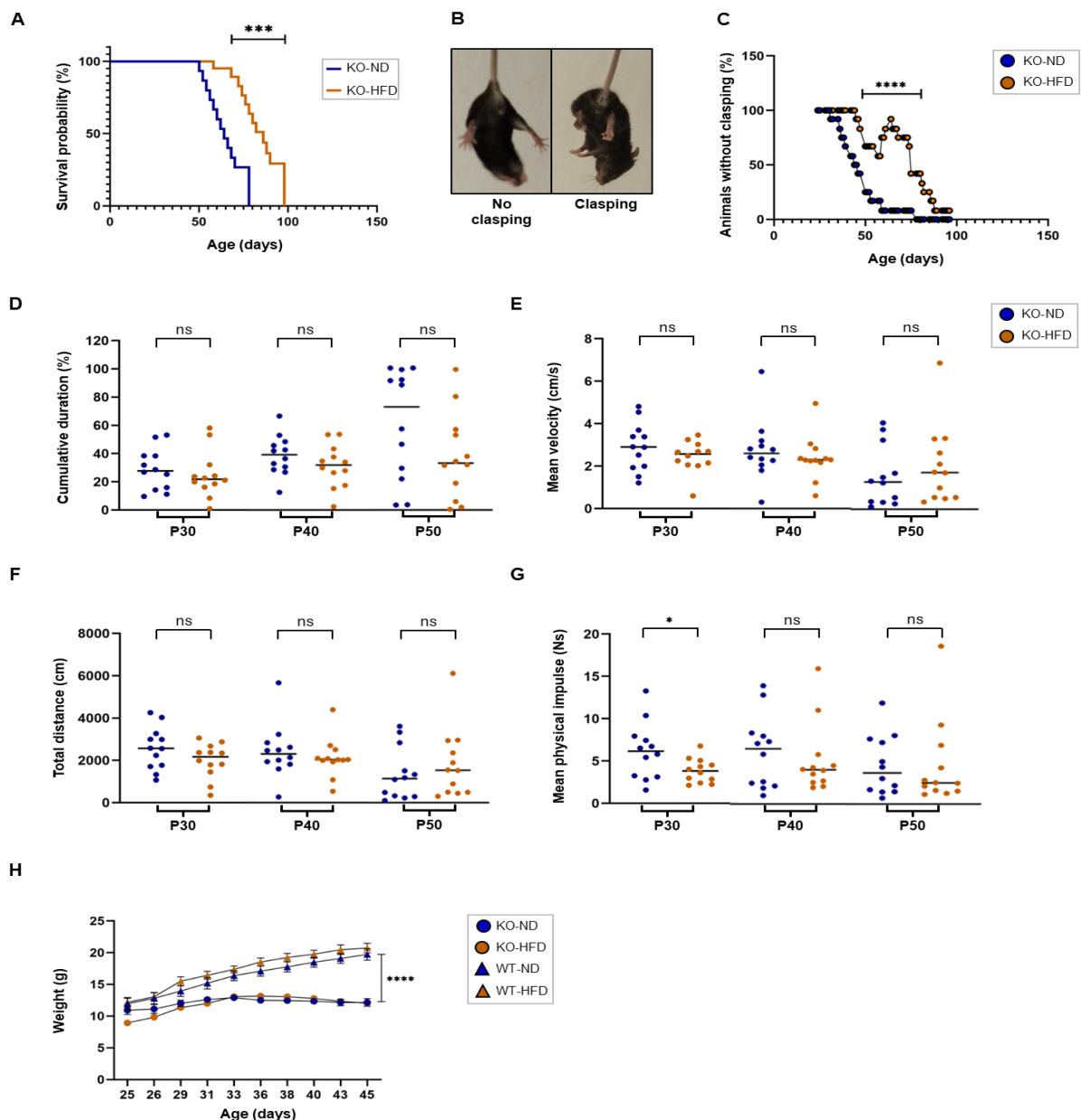


Figure 5-2: Phenotypic evaluation of *Ndufs4* KO and WT mice fed a HFD or ND. (A) Mantel-Cox survival curve and (B-C) clasp assessment of disease severity in KO mice on either a ND or HFD over time. Scatter plots depicting open-field test results, including (D) cumulative duration in centre over 5 min, (E) mean velocity, (F) total distance travelled, and (G) mean physical impulse at P30, P40 and P50 in KO mice on each diet. Horizontal bars represent the mean. (H) Growth curves indicating weight over time in WT and KO mice across dietary groups. Individual datapoints represent the mean $\pm$ SD. Brackets indicate

statistically significant differences where $p < 0.05$ (*), $p < 0.001$ (***), $p < 0.0001$ (****) and $p > 0.05$ (ns). $n = 12$ animals per group. Abbreviations: cm, centimetre; cm/s, centimetre per second; KO, knockout; Ns, Newton seconds; g, gram; and WT, wild-type.

5.3.2. Western blots and inflammation assay

To investigate the mechanisms underlying the beneficial effects of a HFD on longevity and clasping behaviour in KO mouse, protein levels of key metabolic and inflammatory regulators were assessed in brain tissue. Western blot analysis measured the phosphorylation status of mTOR and its substrates, p70S6K1 and 4E-BP1 (Figure 5-3B-D), as well as the levels of SIRT1, PGC1- α , and TNF- α (Figure 5-3E-H). Neither genotype nor diet significantly affected protein expression in the brain when correcting for multiple comparisons. However, observable trends included decreased levels of PGC1- α and increased levels of TNF- α in KO-ND compared to WT-ND brains, which were not altered by the HFD. Serum inflammation assays further revealed elevated TNF in KO-ND compared to WT-ND mice. A HFD markedly decreased TNF and IL-6 concentrations in KO mouse serum to levels below that of WT. However, no differences were observed for other cytokines (IFN- γ , MCP-1, IL-10, and IL-12p70) when mice from either genotype or dietary group were compared.

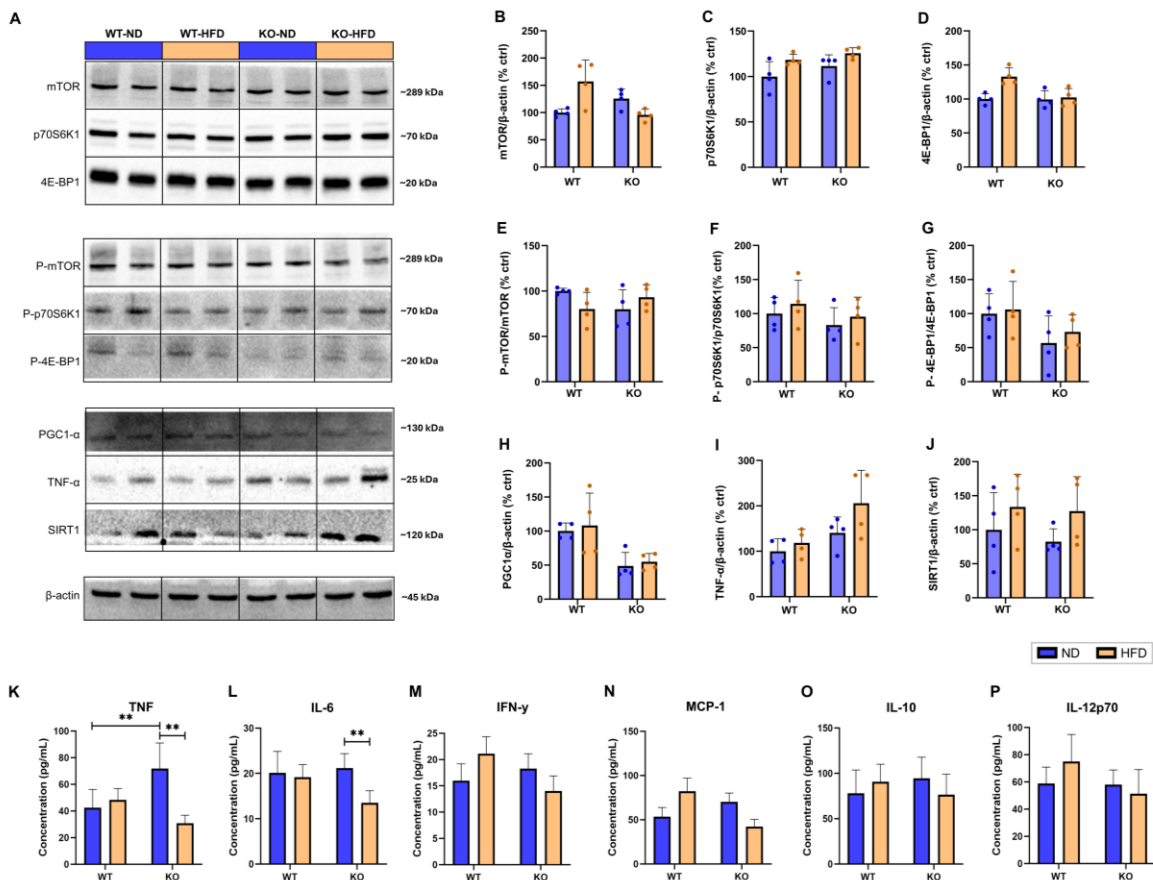


Figure 5-3: The effect of a HFD on metabolic and inflammatory regulators in *Ndufs4* KO and WT mice. (A-J) Western blot analysis of specific metabolic markers in WT and KO mouse brain tissue ($n = 4$ per group). Representative blot images from two biological replicates are shown. **(K-P)** Flow cytometry analysis of serum inflammation markers in WT and KO mice ($n = 14$ per

group). Bar graphs represent the mean $\pm$ SD; individual data points shown as dots. Brackets indicate statistically significant differences where $p < 0.05$ (*) or $p < 0.01$ (**). *Abbreviations: IFN- γ , interferon gamma; IL-6, interleukin 6; IL-10, interleukin 10; IL-12p70, interleukin 12p70; KO, knockout; MCP-1, monocyte chemoattractant protein 1; TNF(- α), tumor necrosis factor(-alpha); and WT, wild-type.*

5.3.3. Metabolic alterations

To determine whether a HFD alters the neurometabolome in *Ndufs4* KO mice, multi-platform metabolic profiling was done. Univariate analysis revealed significant alterations in tricarboxylic acid (TCA) cycle, ketone body, and branched-chain amino acid (BCAA) metabolism (Figure 5-4), with a HFD generally counteracting the metabolic changes observed in KO-ND vs WT-ND mice. In the TCA cycle, KO-ND brains showed a decrease in aspartate ($p < 0.05$) and oxaloacetate ($p < 0.05$), alongside increased alanine ($p < 0.0001$), lactate ($p < 0.001$), and succinate ($p < 0.001$) compared to WT-ND brains. Contrastingly, KO-HFD brains exhibited higher ($p < 0.05$) aspartate and oxaloacetate, along with lower ($p < 0.05$) alanine, lactate, and succinate. Similarly, the ketones, 3-hydroxybutyrate ($p < 0.05$) and acetoacetate ($p < 0.05$), were elevated in KO-ND compared to WT-ND brains, with the levels of 3-hydroxybutyrate ($p < 0.05$) decreased in KO-HFD brains. In BCAA metabolism, a significant increase was observed for leucine ($p < 0.0001$), isoleucine ($p < 0.0001$), valine ($p < 0.0001$) and glutamate ($p < 0.0001$), as well as a decrease in α -ketoisovalerate ($p < 0.05$) in KO-ND compared to WT-ND brains. These same metabolites, except for α -ketoisovalerate, were significantly decreased ($p < 0.05$, $p < 0.01$, $p < 0.01$ and $p < 0.01$) in KO-HFD compared to KO-ND brains.

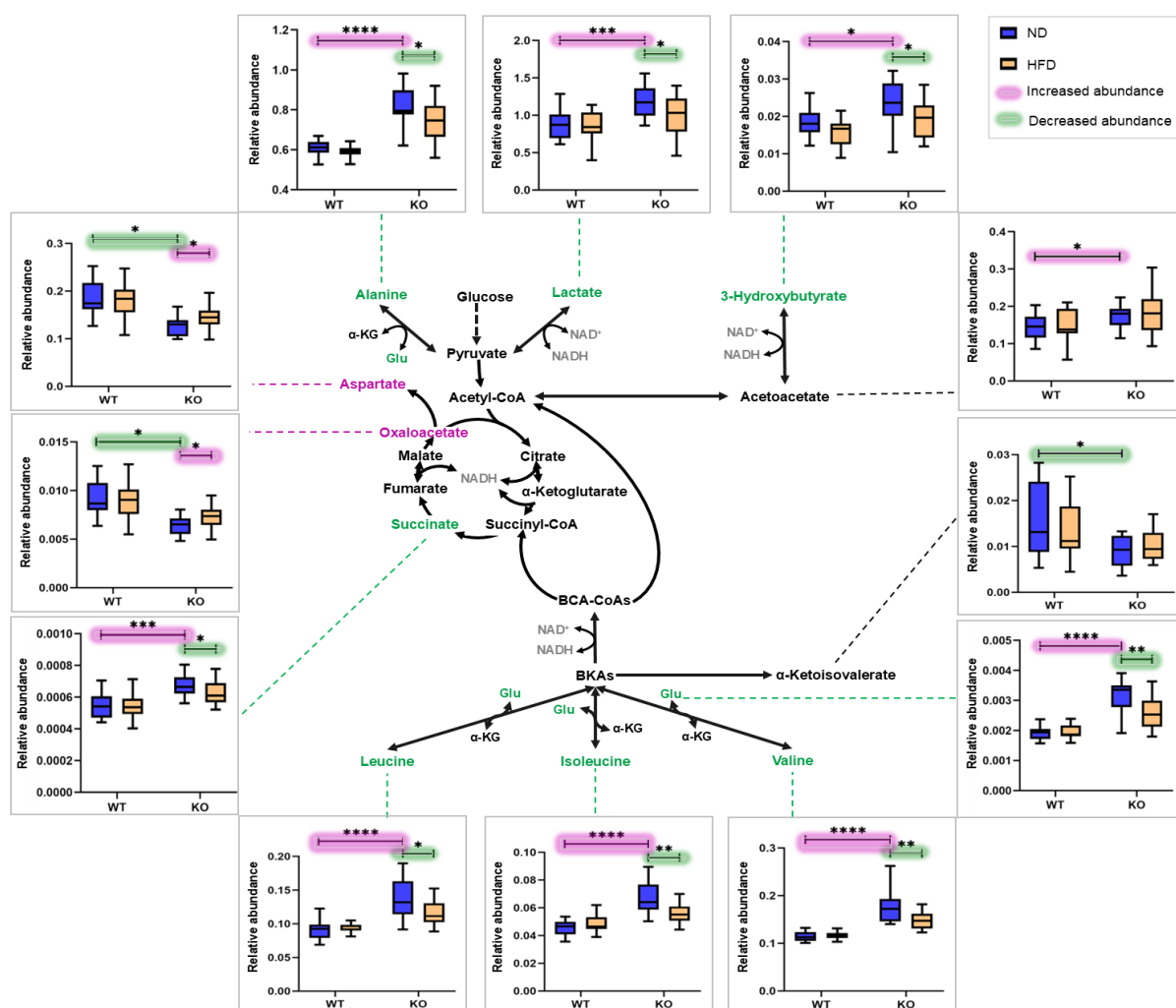


Figure 5-4: Metabolic alterations in brain tissue of *Ndufs4* KO and WT mice fed a HFD or ND. Brackets indicate statistically significant differences where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****). $n = 14$ per group. Abbreviations: α -KG, α -ketoglutarate; BCA-CoAs, branched-chain acyl-coenzyme As; BKAs; branched-chain keto acids; Glu, glutamate; KO, knockout; NAD^+ , oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ND, normal diet; HFD, high-fat diet; and WT, wild-type.

5.3.4. Lipid alterations

The effects of a HFD on the neurolipidome in *Ndufs4* KO mice was also evaluated. Univariate analysis revealed marked changes in phospholipid metabolism due to dietary intervention (Figure 5-5). When comparing WT and KO mice on a ND, KO brains exhibited a significantly higher abundance of diacylglycerol ($p < 0.05$), triacylglycerol ($p < 0.01$), phosphatidic acid ($p < 0.01$), phosphatidylglycerol ($p < 0.05$), phosphatidylinositol ($p < 0.0001$), phosphatidylserine ($p < 0.01$), phosphatidylethanolamine ($p < 0.05$), and phosphatidylcholine ($p < 0.05$). Moreover, when compared to KO-ND brains, significant reductions were noted in KO-HFD brain diacylglycerol ($p < 0.0001$), phosphatidic acid ($p < 0.01$), phosphatidylglycerol ($p < 0.0001$), phosphatidylinositol ($p < 0.0001$), phosphatidylserine ($p < 0.0001$), and phosphatidylcholine ($p < 0.0001$).

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0.0001). Conversely, triacylglycerol ($p < 0.01$) and phosphatidylethanolamine ($p < 0.0001$) levels were elevated in KO mice on a HFD compared to those on a ND.

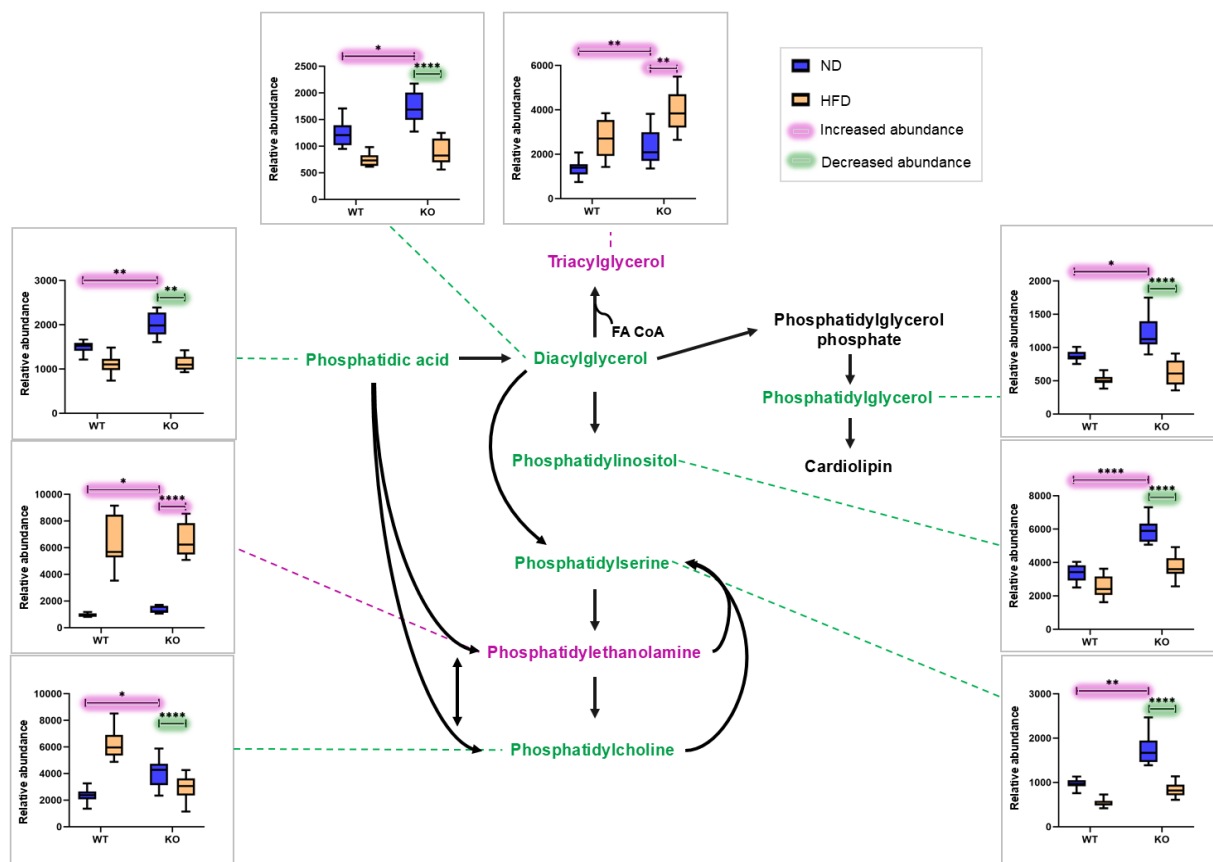


Figure 5-5: Phospholipid alterations in brain tissue of *Ndufs4* KO and WT mice fed a HFD or ND. Brackets indicate statistically significant differences where $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.0001$ (****). $n = 14$ per group. Abbreviations: FA CoA, fatty acyl-coenzyme A; KO, knockout; ND, normal diet; HFD, high-fat diet; and WT, wild-type.

5.4. Discussion

5.4.1. A PUFA-enriched HFD attenuates Leigh syndrome in *Ndufs4* KO mice

This study utilized *Ndufs4* KO mice to evaluate the therapeutic potential of a PUFA-enriched HFD for Leigh syndrome. A HFD significantly extended the lifespan of *Ndufs4* KO mice up to a maximum of 100 days (vs 72 days on a ND), while delaying the onset and reducing the severity of neurodegenerative clamping symptoms. These findings contrast with the report from Bornstein *et al.* (2022), who showed no benefits to overall disease progression or survival in the same mouse model using a standard KD. However, not all KDs and HFDs are identical; they vary in macronutrient composition, total caloric content, and fat source.

Bornstein *et al.* (2022) noted poor tolerance in *Ndufs4* KO mice on a full KD (90% fat, < 1% carbohydrates), opting for a gradual introduction to an adapted diet (80% fat, 8.5% carbohydrates). In this study, we further adjusted the diet to contain 60% fat and 20% carbohydrates, classifying it as a HFD that approaches the lower threshold for ketosis in mice. A

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more balanced macronutrient composition proved to be well tolerated in KO mice, although a transient decline in grip strength and body weight was observed during early stages (P30). This likely reflected an initial adaptation period, as grip strength and weight were comparable between KO-HFD and KO-ND groups by P40 and P50. While the HFD did not prevent weight loss in KO mice, it also did not exacerbate the phenotype, likely due to the isocaloric nature of the diets, as consistent caloric intake supports weight maintenance in such contexts (Pathak *et al.*, 2022; Roberts *et al.*, 2017; Zhou *et al.*, 2023).

Another key modification made to the diet was the supplementation of Menhaden fish oil, chosen for its high PUFA content. The source and composition of fatty acids in a KD and HFD are increasingly recognized as crucial factors influencing its therapeutic efficacy (Elisia *et al.*, 2024; Nathan *et al.*, 2019). Although no previous studies have directly examined the effects of PUFAs on mitochondrial disease models, PUFAs have shown to enhance mitochondrial function and alleviate disease severity in conditions associated with mitochondrial dysfunction, such as obesity (Borja-Magno *et al.*, 2023), aging (Afshordel *et al.*, 2015), Alzheimer's disease (Park *et al.*, 2020), and palmitate-induced mitochondrial impairment (Nisr *et al.*, 2020). Our findings align with these results, showing that HFD intervention mitigates Leigh syndrome in a preclinical model. However, the absence of histological data to directly visualise brain lesions represents a limitation of the current study. While improvements in clasping behaviour and changes in neuronal metabolite profiles suggest a potential mitigation of neurodegeneration, histological analyses are needed in follow-up studies to confirm these findings and provide a more detailed characterisation of the HFD's effects on brain morphology. Additionally, while our study demonstrates that the HFD attenuates disease progression in *Ndufs4* KO mice, we acknowledge that the mechanistic role of OXPHOS adaptations, particularly shifts in CI/CII substrate utilisation, remains unresolved. Direct measurement of mitochondrial respiratory activity would clarify whether the HFD enhances CII-driven ATP production or compensates for CI deficiency through alternative pathways. Future studies utilising high-resolution respirometry are needed to address this question.

Other therapeutic interventions tested in *Ndufs4* KO mice have produced mixed results in terms of symptomatic improvement and lifespan extension. The NAD⁺ precursor nicotinamide mononucleotide did not improve clinical symptoms but extended median lifespan to 110 days (Lee *et al.*, 2019). The ROS-redox modulator H176 improved motor function but failed to impact disease onset or severity (de Haas *et al.*, 2017). mTOR inhibitors such as rapamycin delayed disease onset, prevented neurological symptoms, and extended median lifespan to 111–114 days (Johnson *et al.*, 2013). Severe continuous hypoxic conditions-maintained body weight, improved motor function, reduced hypothermia, and extended lifespan to 70–80 days (Jain *et al.*, 2016). Similarly, acarbose treatment delayed disease onset and increased median lifespan

to 92 days (Bitto *et al.*, 2023). Our HFD intervention demonstrates comparable outcomes, improving neurological symptoms and extending median lifespan up to 75 days.

The HFD is highly translatable to clinical settings and offers a cost-effective adjunctive therapeutic option compared to pharmacological treatments like rapamycin, which, while effective, carries potential side effects (Barriocanal-Casado *et al.*, 2019; Li *et al.*, 2014). A HFD may offer a better safety profile, reducing the risk of adverse effects while providing metabolic benefits (Nathan *et al.*, 2019). Furthermore, combining a HFD with other interventions may yield greater benefits in *Ndufs4* KO mice by capitalising on the distinct advantages of each treatment. This positions a HFD as a promising option for combination therapy, particularly in complex diseases requiring a multi-targeted approach. However, while our findings highlight the therapeutic potential of a PUFA-enriched HFD, the specific contributions of individual fatty acids (e.g., ω -3 vs. ω -6 PUFAs) remain unclear, warranting further studies.

5.4.2. A HFD does not affect whole-brain mTOR or SIRT1 signalling

To explore the possible mechanisms underlying the therapeutic benefits of a HFD, we analysed key metabolic and inflammatory signalling pathways implicated in Leigh syndrome neuropathology, that are also known to be influenced by a KD. In the brains of *Ndufs4* KO mice, alterations in nutrient-sensing metabolic regulators, such as mTORC1 hyperactivation (Johnson *et al.*, 2013) and PGC-1 α reduction (Frambach *et al.*, 2020) have been reported; along with elevated levels of the pro-inflammatory molecules, NF- κ B (Martin-Perez *et al.*, 2020) and TNF- α (Aguilar *et al.*, 2022; Aguilar *et al.*, 2024).

Our findings revealed no evidence of mTOR pathway alterations in KO mice on either a ND or HFD, as indicated by unchanged levels of mTOR, its downstream targets (p70S6K1 and 4E-BP1), or their phosphorylated forms. In agreement, Bornstein *et al.* (2022) also reported no mTOR inhibition in the brains of *Ndufs4* KO mice following KD administration. Despite observable trends — reduced PGC-1 α and elevated TNF- α in the *Ndufs4* KO brain — these changes were not significantly modulated by the HFD. SIRT1, a central regulator of both proteins, also remained unchanged across genotypes and dietary conditions.

Overall, these findings suggest that the therapeutic benefits of a HFD do not arise from direct modulation of the mTOR/p70S6K1/4E-BP1 or SIRT1/PGC-1 α axes in the whole-brain, which regulate neuronal metabolism and inflammation. However, while these pathways were unaffected in whole-brain tissue, a HFD may influence signalling in specific brain regions or peripheral tissues. For instance, distinct responses may occur in more severely affected brain regions such as the olfactory bulbs (Quintana *et al.*, 2010), or peripheral tissues like the liver (Willemse *et al.*, 2023), where KDs with similar fat content resulted in mTOR inhibition (Liu *et al.*, 2020).

5.4.3. A HFD reduces systemic inflammation

Ndufs4 KO mice are known to exhibit profound systemic inflammation, characterised by elevated levels of pro-inflammatory cytokines (Aguilar *et al.*, 2022; Aguilar *et al.*, 2024; Peruzzotti-Jametti *et al.*, 2024; van de Wal *et al.*, 2022). To assess the impact of a HFD on the systemic inflammatory profile, we performed serum flow cytometry. The HFD intervention reversed the elevation of the pro-inflammatory cytokine TNF and significantly reduced IL-6 levels in KO serum. However, other cytokines (IFN γ , MCP-1, IL-10 or IL-12p70) remained unchanged by either genotype or diet. While the HFD significantly reduced IL-6 and TNF levels in KO mice compared to KO mice on an ND, baseline IL-6 levels did not differ between WT and KO mice on the ND. As such, the reduction observed in systemic inflammation could be mainly attributed to the reduction in TNF cytokine levels.

Although a HFD did not influence whole-brain inflammatory markers or mTOR signalling, as determined via western blot analysis, the diet effectively reduced inflammation on a systemic level. Improvements in systemic inflammation, clasping symptoms, and survival in HFD-fed KO mice align with the established anti-inflammatory properties of PUFAs (Calder, 2015). Long-chain ω -3 PUFAs are well known for their anti-inflammatory effects and are abundant in Menhaden fish oil, a key component of our HFD formulation. These findings suggest that dietary ω -3 PUFA supplementation might be a critical component of the HFD's therapeutic efficacy, likely acting through reduction of peripheral inflammation.

While this study focuses on systemic inflammation, future research should assess neuroinflammatory cytokines, such as brain TNF and IL-6, to better understand the direct effects of the HFD on central nervous system pathology. These investigations would require specialized protocols to isolate and quantify cytokines in brain tissue, which were beyond the scope of this preclinical study.

5.4.4. A HFD enhances brain energy metabolism by improving TCA cycling along with ketone body- and BCAA utilisation

Metabolic profiling revealed that the HFD intervention mitigated key neurometabolic derangements caused by CI deficiency, particularly in TCA cycle, ketone body, and BCAA pathways. Consistent with previous studies (Johnson *et al.*, 2020; Terburgh *et al.*, 2021), *Ndufs4* KO brains showed reduced aspartate and oxaloacetate levels, suggesting impaired TCA cycle anaplerosis and altered flux. Concurrent accumulation of lactate, alanine, and succinate further indicated disrupted TCA cycling and a shift toward anaerobic glycolysis — hallmark features of mitochondrial dysfunction (Esterhuizen *et al.*, 2017). The HFD markedly increased brain aspartate and oxaloacetate levels in KO mice, while reducing lactate, alanine, and succinate, suggesting improved mitochondrial substrate utilisation and TCA cycling. These findings align with reports that PUFAs can improve TCA cycle homeostasis in mice (Ahmed *et*

al., 2014; Liu *et al.*, 2021). However, the exact mechanism by which a HFD impacts TCA cycle dynamics in mitochondrial disease models requires further investigation.

Ndufs4 KO mice on a ND further exhibited significantly elevated levels of ketone bodies (3-hydroxybutyrate and acetoacetate), suggesting impaired utilisation of these alternative energy substrates. This accumulation may be exacerbated by altered inter-organ metabolic coordination, since the liver upregulates fatty acid β -oxidation and ketone body export as part of a compensatory mechanism to preserve hepatic bioenergetics during CI deficiency (Abrosimov *et al.*, 2024). Following the HFD intervention, KO mice showed a significant decrease in 3-hydroxybutyrate (3-HBA) levels compared to those on a ND. This reduction suggests enhanced fatty acid utilisation for energy, likely due to the high PUFA content of the HFD, which alters the fatty acid composition available for ketogenesis and may promote mitochondrial biogenesis and function (Cunnane, 2004; de Oliveira *et al.*, 2017; Jang *et al.*, 2023). Improved mitochondrial efficiency is linked to a favourable NAD⁺/NADH ratio, which regulates cellular metabolism and affects enzymes like 3-HBA dehydrogenase, potentially reducing 3-HBA production (Elamin *et al.*, 2017; Kemperman *et al.*, 2023; Xie *et al.*, 2020). While significant changes in mTOR, PGC1 α , or SIRT1 activity—key mitochondrial regulators—were not observed, alterations in lipid metabolism suggest potential mechanisms for the observed metabolic shifts and redox state (Ademowo *et al.*, 2017; de Oliveira *et al.*, 2017). This aligns with findings in mitochondrial disease models, where lipid dysregulation and inconsistent 3-HBA changes are common (Esterhuizen *et al.*, 2017; Terburgh *et al.*, 2021). ω -3-derived epoxyeicosanoids, known to enhance mitochondrial biogenesis and fusion while reducing inflammation, may play a role, however, their chemical instability likely diminishes their effectiveness and makes their effects harder to detect. Synthetic analogues like OMT-28, designed for mitochondrial myopathy treatment, demonstrate the therapeutic potential of these pathways (El-Sikhry *et al.*, 2016). As such, a HFD appears to improve mitochondrial metabolism and reduce 3-HBA in *Ndufs4* KO mice, though further studies are needed to clarify the underlying mechanisms and regulatory pathways.

Additionally, significant accumulation of BCAAs (leucine, isoleucine and valine) and glutamate was observed in KO mice on a ND. BCAA accumulation, another hallmark of mitochondrial dysfunction-related reductive stress (Esterhuizen *et al.*, 2017), has been reported in whole-brain mitochondria (van der Walt *et al.*, 2021) and discrete brain regions (Johnson *et al.*, 2020; Terburgh *et al.*, 2021; Yoon *et al.*, 2022) of *Ndufs4* KO mice. A HFD significantly reduced the levels of all three BCAAs and glutamate in KO brains, suggesting enhanced catabolism and utilisation of these substrates for energy production. Notably, BCAA accumulation is implicated in neuroinflammation (Gao *et al.*, 2024; Yoo *et al.*, 2022; Zhenyukh *et al.*, 2017) and, together with altered glutamate handling, may dysregulate glutamatergic- and γ -aminobutyric acid (GABA)ergic neurotransmitter metabolism (Dufour *et al.*, 2001; Terburgh *et al.*, 2021). Given

that *Ndufs4* KO mice exhibit neuroinflammation in neurodegeneration-prone regions (Aguilar *et al.*, 2022; Bolea *et al.*, 2019; Quintana *et al.*, 2010) and their neurological deficits are driven by glutamatergic- and GABAergic neurons (Bolea *et al.*, 2019), the HFD may target key pathogenic mechanisms by partially normalising brain BCAA and glutamate levels.

5.4.5. A HFD alters phospholipid metabolism

A HFD significantly altered the whole-brain lipid profile of *Ndufs4* KO mice. Compared to WT mice on a ND, KO mice displayed elevated levels of multiple lipid classes, including diacylglycerols (DAGs), triacylglycerols (TAGs), phosphatidylglycerols (PGs), phosphatidylserines (PSs), phosphatidylinositols (PIs), phosphatidylethanolamines (PEs), and phosphatidylcholines (PCs). These elevations likely reflect disrupted lipid homeostasis associated with mitochondrial dysfunction (Bathina & Das, 2023; Moschandrea *et al.*, 2024), which is consistent with the OXPHOS impairment in this mouse model. Notably, the HFD substantially reduced levels of most of these lipid classes in KO mice, except for TAGs and PEs, indicating a partial restoration of lipid balance. While the mechanism underlying the persistent elevation of TAGs and PEs in KO mice on both diets remains unclear, it may reflect an adaptation response to enhance energy storage and mitochondrial function under the stress of impaired OXPHOS. The further increase in these lipids with a HFD could suggest enhanced mitochondrial dynamics, promoting cellular resilience under stress. Since these lipidomic changes were accompanied by broader metabolic shifts and key physiological improvements in KO mice — including, reduced systemic inflammation, amelioration of clasping symptoms, and increased lifespan — our findings underscore the intricate interplay between lipid metabolism, the wider metabolome, and inflammation in this model.

Beyond these lipid-specific considerations, while the current study focused solely on the whole brain metabolome and lipidome, further research is needed to investigate region-specific and cell-specific changes in these pathways in mice fed the HFD. Furthermore, other lipid signalling pathways that are crucial for mitochondrial bioenergetics and inflammation should be explored on a systemic level as well.

5.5. Conclusion

This study demonstrates that a PUFA-enriched HFD offers significant therapeutic benefits in the *Ndufs4* KO mouse model of Leigh syndrome. This intervention extends lifespan, alleviates neurodegenerative symptoms, and reduces systemic inflammation, highlighting the potential role of PUFAs in modulating mitochondrial function and inflammation. Importantly, these findings emphasise that the composition of dietary fats in KDs and HFDs is a pivotal factor in determining their efficacy, distinguishing this approach from less effective KD and HFD formulations in the same model. While we observed no significant impact on whole-brain mTOR activity or key metabolic regulators such as PGC-1 α and SIRT1, the HFD enhanced TCA cycle

flux and improved ketone- and BCAA utilisation, reflecting improved energy metabolism in KO mouse brains. Furthermore, systemic inflammation markers, such as TNF and IL-6, were reduced by a HFD, suggesting that the diet may modulate inflammation at a systemic level.

A PUFA-enriched HFD offers a promising, cost-effective therapeutic option for the treatment of mitochondrial diseases with fewer side effects. Given the complex nature of Leigh syndrome and other mitochondrial diseases, combining HFDs with other interventions could enhance therapeutic efficacy while mitigating potential adverse effects. These findings emphasise the need for further research to explore the synergistic effects of multi-targeted therapies on region-specific brain metabolism and its translatability to clinical settings.

5.6. References

- Abrosimov, R., Baeken, M.W., Hauf, S., Wittig, I., Hajieva, P., Perrone, C.E. & Moosmann, B. 2024. Mitochondrial complex I inhibition triggers NAD⁺-independent glucose oxidation via successive NADPH formation, “futile” fatty acid cycling, and FADH₂ oxidation. *GeroScience*, 46(4):3635-3658.
- Ademowo, O.S., Dias, H.K.I., Burton, D.G.A. & Griffiths, H.R. 2017. Lipid (per) oxidation in mitochondria: an emerging target in the ageing process? *Biogerontology*, 18(6):859-879.
- Afshordel, S., Hagl, S., Werner, D., Röhner, N., Kögel, D., Bazan, N.G. & Eckert, G.P. 2015. Omega-3 polyunsaturated fatty acids improve mitochondrial dysfunction in brain aging--impact of Bcl-2 and NPD-1 like metabolites. *Prostaglandins Leukot. Essent. Fatty Acids*, 92:23-31.
- Aguilar, K., Comes, G., Canal, C., Quintana, A., Sanz, E. & Hidalgo, J. 2022. Microglial response promotes neurodegeneration in the Ndufs4 KO mouse model of Leigh syndrome. *Glia*, 70(11):2032-2044.
- Aguilar, K., Canal, C., Comes, G., Díaz-Clavero, S., Llanos, M.A., Quintana, A., ... Hidalgo, J. 2024. Interleukin-6-elicited chronic neuroinflammation may decrease survival but is not sufficient to drive disease progression in a mouse model of Leigh syndrome. *J. Inflamm. (Lond)*, 21(1):1.
- Ahmed, A.A., Balogun, K.A., Bykova, N.V. & Cheema, S.K. 2014. Novel regulatory roles of omega-3 fatty acids in metabolic pathways: a proteomics approach. *Nutr. Metab. (Lond)*, 11(1):6.
- Barriocanal-Casado, E., Hidalgo-Gutiérrez, A., Raimundo, N., González-García, P., Acuña-Castroviejo, D., Escames, G. & López, L.C. 2019. Rapamycin administration is not a valid therapeutic strategy for every case of mitochondrial disease. *eBioMedicine*, 42:511-523.
- Bathina, S. & Das, U.N. 2023. Role of Mitochondrial Dysfunction in Cellular Lipid Homeostasis and Disease. *Discov. Med.*, 35(178):653-663.
- Bitto, A., Grillo, A.S., Ito, T.K., Stanaway, I.B., Nguyen, B.M.G., Ying, K., ... Kaeberlein, M. 2023. Acarbose suppresses symptoms of mitochondrial disease in a mouse model of Leigh syndrome. *Nat. Metab.*, 5(6):955-967.
- Bolea, I., Gella, A., Sanz, E., Prada-Dacasa, P., Menardy, F., Bard, A.M., ... Quintana, A. 2019. Defined neuronal populations drive fatal phenotype in a mouse model of Leigh syndrome. *eLife*, (8):e47163.
- Borja-Magno, A.I., Furuzawa-Carballeda, J., Guevara-Cruz, M., Arias, C., Granados, J., Bourges, H., ... Gómez, F.E. 2023. Supplementation with EPA and DHA omega-3 fatty acids improves peripheral immune cell mitochondrial dysfunction and inflammation in subjects with obesity. *J. Nutr. Biochem.*, 120:109415.
- Bornstein, R., James, K., Stokes, J., Park, K.Y., Kayser, E.B., Snell, J., ... Johnson, S.C. 2022. Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy. *Neurobiol. Dis.*, 163:105594.

CHAPTER 5

- Bris, C., Rouaud, T., Desquirit-Dumas, V., Gueguen, N., Goudenege, D., Barth, M., ... Procaccio, V. 2017. Novel NDUFS4 gene mutation in an atypical late-onset mitochondrial form of multifocal dystonia. *Neurol. Genet.*, 3(6):e205.
- Calder, P.C. 2015. Functional Roles of Fatty Acids and Their Effects on Human Health. *J. Parenter. Enteral. Nutr.*, 39(1 Suppl):18s-32s.
- Chen, X., Chen, C., Fan, S., Wu, S., Yang, F., Fang, Z., ... Li, Y. 2018. Omega-3 polyunsaturated fatty acid attenuates the inflammatory response by modulating microglia polarization through SIRT1-mediated deacetylation of the HMGB1/NF- κ B pathway following experimental traumatic brain injury. *J. Neuroinflammation*, 15(1):116.
- Cunnane, S.C. 2004. Metabolism of polyunsaturated fatty acids and ketogenesis: an emerging connection. *Prostaglandins Leukot. Essent. Fatty Acids*, 70(3):237-241.
- de Haas, R., Das, D., Garanto, A., Renkema, H.G., Greupink, R., van den Broek, P., ... Smeitink, J.A. 2017. Therapeutic effects of the mitochondrial ROS-redox modulator KH176 in a mammalian model of Leigh Disease. *Sci. Rep.*, 7(1):11733.
- de Oliveira, M.R., Nabavi, S.F., Nabavi, S.M. & Jardim, F.R. 2017. Omega-3 polyunsaturated fatty acids and mitochondria, back to the future. *Trends Food Sci. Technol.*, 67:76-92.
- Dufour, F., Nalecz, K.A., Nalecz, M.J. & Nehlig, A. 2001. Modulation of absence seizures by branched-chain amino acids: correlation with brain amino acid concentrations. *Neurosci. Res.*, 40(3):255-263.
- El-Sikhry, H.E., Alsaleh, N., Dakarapu, R., Falck, J.R. & Seubert, J.M. 2016. Novel Roles of Epoxyeicosanoids in Regulating Cardiac Mitochondria. *PLoS One*, 11(8):e0160380.
- Elamin, M., Ruskin, D.N., Masino, S.A. & Sacchetti, P. 2017. Ketone-Based Metabolic Therapy: Is Increased NAD(+) a Primary Mechanism? *Front. Mol. Neurosci.*, 10:377.
- Elisia, I., Yeung, M., Kowalski, S., Shyp, T., Tee, J., Hollman, S., ... Krystal, G. 2024. A ketogenic diet rich in fish oil is superior to other fats in preventing NNK-induced lung cancer in A/J mice. *Sci. Rep.*, 14(1):5610.
- Esterhuizen, K., van der Westhuizen, F.H. & Louw, R. 2017. Metabolomics of mitochondrial disease. *Mitochondrion*, 35:97-110.
- Frambach, S.J.C.M., van de Wal, M.A.E., van den Broek, P.H.H., Smeitink, J.A.M., Russel, F.G.M., de Haas, R. & Schirris, T.J.J. 2020. Effects of clofibrate and KH176 on life span and motor function in mitochondrial complex I-deficient mice. *Biochim. Biophys. Acta Mol. Basis Dis.*, 1866(6):165727.
- Gao, Q., Bi, D., Li, B., Ni, M., Pang, D., Li, X., ... Zhu, C. 2024. The Association Between Branched-Chain Amino Acid Concentrations and the Risk of Autism Spectrum Disorder in Preschool-Aged Children. *Mol. Neurobiol.*, 61(8):6031-6044.
- Gorman, G.S., Schaefer, A.M., Ng, Y., Gomez, N., Blakely, E.L., Alston, C.L., ... McFarland, R. 2015. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Ann. Neurol.*, 77(5):753-759.
- Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M. & Moritz, T. 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. *Anal. Biochem.*, 331(2):283-295.
- Guyenet, S.J., Furrer, S.A., Damian, V.M., Baughan, T.D., La Spada, A.R. & Garden, G.A. 2010. A simple composite phenotype scoring system for evaluating mouse models of cerebellar ataxia. *J. Vis. Exp.*, (39):1787. Hanaford, A. & Johnson, S.C. 2022. The immune system as a driver of mitochondrial disease pathogenesis: a review of evidence. *Orphanet J. Rare Dis.*, 17(1):335.

CHAPTER 5

- Jain, I.H., Zazzeron, L., Goli, R., Alexa, K., Schatzman-Bone, S., Dhillon, H., ... Mootha, V.K. 2016. Hypoxia as a therapy for mitochondrial disease. *Science*, 352(6281):54-61. 1
- Jang, J., Kim, S.R., Lee, J.E., Lee, S., Son, H.J., Choe, W., ... Kang, I. 2023. Molecular Mechanisms of Neuroprotection by Ketone Bodies and Ketogenic Diet in Cerebral Ischemia and Neurodegenerative Diseases. *Int. J. Mol. Sci.*, 25(1):124.
- Jarrett, S.G., Milder, J.B., Liang, L.P. & Patel, M. 2008. The ketogenic diet increases mitochondrial glutathione levels. *J. Neurochem.*, 106(3):1044-1051.
- Johnson, S.C., Kayser, E.B., Bornstein, R., Stokes, J., Bitto, A., Park, K.Y., ... Morgan, P.G. 2020. Regional metabolic signatures in the Ndufs4(KO) mouse brain implicate defective glutamate/ α -ketoglutarate metabolism in mitochondrial disease. *Mol. Genet. Metab.*, 130(2):118-132.
- Johnson, S.C., Yanos, M.E., Kayser, E.B., Quintana, A., Sangesland, M., Castanza, A., ... Kaeberlein, M. 2013. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science*, 342(6165):1524-1528.
- Kemperman, R.H., Ganetzky, R.D. & Master, S.R. 2023. B-169 Beta-hydroxybutyrate/acetoacetate Ratio as Indicator for Mitochondrial Diseases Utilizing a Novel LC-MS/MS Based Ketone Body Panel. *Clin. Chem.*, 69(Supplement_1).
- Koopman, W.J., Verkaart, S., Visch, H.J., van der Westhuizen, F.H., Murphy, M.P., van den Heuvel, L.W., ... Willems, P.H. 2005. Inhibition of complex I of the electron transport chain causes O₂- mediated mitochondrial outgrowth. *Am. J. Physiol. Cell Physiol.*, 288(6):C1440-1450.
- Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab.*, 7(4):312-320.
- Lee, C.F., Caudal, A., Abell, L., Nagana Gowda, G.A. & Tian, R. 2019. Targeting NAD(+) Metabolism as Interventions for Mitochondrial Disease. *Sci. Rep.*, 9(1):3073.
- Li, J., Kim, S.G. & Blenis, J. 2014. Rapamycin: one drug, many effects. *Cell Metab.*, 19(3):373-379.
- Lim, A.Z., Ng, Y.S., Blain, A., Jiminez-Moreno, C., Alston, C.L., Nesbitt, V., ... McFarland, R. 2022. Natural History of Leigh Syndrome: A Study of Disease Burden and Progression. *Ann. Neurol.*, 91(1):117-130.
- Liu, R., Chen, L., Wang, Y., Zhang, G., Cheng, Y., Feng, Z., ... Liu, J. 2020. High ratio of ω -3/ ω -6 polyunsaturated fatty acids targets mTORC1 to prevent high-fat diet-induced metabolic syndrome and mitochondrial dysfunction in mice. *J. Nutr. Biochem.*, 79:108330.
- Liu, R., Chen, L., Wang, Z., Zheng, X., Hou, Z., Zhao, D., ... Liu, J. 2021. Omega-3 polyunsaturated fatty acids prevent obesity by improving tricarboxylic acid cycle homeostasis. *J. Nutr. Biochem.*, 88:108503.
- Löfgren, L., Forsberg, G.B. & Ståhlman, M. 2016. The BUMe method: a new rapid and simple chloroform-free method for total lipid extraction of animal tissue. *Sci. Rep.*, 6:27688.
- Martin-Perez, M., Grillo, A.S., Ito, T.K., Valente, A.S., Han, J., Entwisle, S.W., ... Villén, J. 2020. PKC downregulation upon rapamycin treatment attenuates mitochondrial disease. *Nat. Metab.*, 2(12):1472-1481.
- Moschandrea, C., Kondylis, V., Evangelakos, I., Herholz, M., Schneider, F., Schmidt, C., ... Pasparakis, M. 2024. Mitochondrial dysfunction abrogates dietary lipid processing in enterocytes. *Nature*, 625(7994):385-392.
- Nathan, J., Bailur, S., Datay, K., Sharma, S. & Khedekar Kale, D. 2019. A Switch to Polyunsaturated Fatty Acid Based Ketogenic Diet Improves Seizure Control in Patients with Drug-resistant Epilepsy on the Mixed Fat Ketogenic Diet: A Retrospective Open Label Trial. *Cureus*, 11(12):e6399.

CHAPTER 5

- Nisr, R.B., Shah, D.S. & Hundal, H.S. 2020. Mono- and Polyunsaturated Fatty Acids Counter Palmitate-Induced Mitochondrial Dysfunction in Rat Skeletal Muscle Cells. *Cell Physiol. Biochem.*, 54(5):975-993.
- Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., Barrette, M., ... Xia, J. 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. *Nucleic Acids Res.*, 49(W1):W388-W396.
- Papa, S., Petruzzella, V., Scacco, S., Vergari, R., Panelli, D., Tamborra, R., ... Santorelli, F.M. 2004. Respiratory complex I in brain development and genetic disease. *Neurochem. Res.*, 29(3):547-560.
- Papa, S., Rasmø, D.D., Technikova-Dobrova, Z., Panelli, D., Signorile, A., Scacco, S., ... Sardanelli, A.M. 2012. Respiratory chain complex I, a main regulatory target of the cAMP/PKA pathway is defective in different human diseases. *FEBS Lett*, 586(5):568-577.
- Park, Y.H., Shin, S.J., Kim, H.S., Hong, S.B., Kim, S., Nam, Y., ... Moon, M. 2020. Omega-3 Fatty Acid-Type Docosahexaenoic Acid Protects against A β -Mediated Mitochondrial Deficits and Pathomechanisms in Alzheimer's Disease-Related Animal Model. *Int. J. Mol. Sci.*, 21(11):3879.
- Pathak, S.J., Zhou, Z., Steffen, D., Tran, T., Ad, Y., Ramsey, J.J., ... Baar, K. 2022. 2-month ketogenic diet preferentially alters skeletal muscle and augments cognitive function in middle aged female mice. *Aging Cell*, 21(10):e13706.
- Peruzzotti-Jametti, L., Willis, C.M., Krzak, G., Hamel, R., Pirvan, L., Ionescu, R.B., ... Pluchino, S. 2024. Mitochondrial complex I activity in microglia sustains neuroinflammation. *Nature*, 628(8006):195-203.
- Quintana, A., Kruse, S.E., Kapur, R.P., Sanz, E. & Palmiter, R.D. 2010. Complex I deficiency due to loss of Ndufs4 in the brain results in progressive encephalopathy resembling Leigh syndrome. *Proc. Natl. Acad. Sci. USA*, 107(24):10996-11001.
- Roberts, M.N., Wallace, M.A., Tomilov, A.A., Zhou, Z., Marcotte, G.R., Tran, D., ... Lopez-Dominguez, J.A. 2017. A Ketogenic Diet Extends Longevity and Healthspan in Adult Mice. *Cell Metab.*, 26(3):539-546.e535.
- Ruhoy, I.S. & Saneto, R.P. 2014. The genetics of Leigh syndrome and its implications for clinical practice and risk management. *Appl. Clin. Genet.*, 7:221-234.
- Schaefer, A.M., Taylor, R.W., Turnbull, D.M. & Chinnery, P.F. 2004. The epidemiology of mitochondrial disorders--past, present and future. *Biochim. Biophys. Acta*, 1659(2-3):115-120.
- Shiraoie, S., Nabavi, S.F., Dehpour, A.R., Belwal, T., Habtemariam, S., Argüelles, S., ... Nabavi, S.M. 2018. Targeting mTORs by omega-3 fatty acids: A possible novel therapeutic strategy for neurodegeneration? *Pharmacol. Res.*, 135:37-48.
- Sofou, K., De Co, I.F., Isohanni, P., Ostergaard, E., Naess, K., De Meirleir, L., ... Darin, N. 2014. A multicenter study on Leigh syndrome: disease course and predictors of survival. *Orphanet J. Rare Dis.*, 9:52.
- Sullivan, P.G., Rippey, N.A., Dorenbos, K., Concepcion, R.C., Agarwal, A.K. & Rho, J.M. 2004. The ketogenic diet increases mitochondrial uncoupling protein levels and activity. *Ann. Neurol.*, 55(4):576-580.
- Terburgh, K., Coetzer, J., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021. Aberrant BCAA and glutamate metabolism linked to regional neurodegeneration in a mouse model of Leigh syndrome. *Biochim. Biophys. Acta Mol. Basis Dis.*, 1867(5):166082.
- Valsecchi, F., Monge, C., Forkink, M., de Groof, A.J.C., Benard, G., Rossignol, R., ... Willems, P.H.G.M. 2012. Metabolic consequences of NDUFS4 gene deletion in immortalized mouse embryonic fibroblasts. *Biochim. Biophys. Acta Bioenerg.*, 1817(10):1925-1936.
- van de Wal, M.A.E., Adjober-Hermans, M.J.W., Keijer, J., Schirris, T.J.J., Homberg, J.R., Wieckowski, M.R., ... Koopman, W.J.H. 2022. Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention. *Brain*, 145(1):45-63.

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- van der Walt, G., Lindeque, J.Z., Mason, S. & Louw, R. 2021. Sub-Cellular Metabolomics Contributes Mitochondria-Specific Metabolic Insights to a Mouse Model of Leigh Syndrome. *Metabolites*, 11(10):658.
- Willemse, L., Terburgh, K. & Louw, R. 2023. A ketogenic diet alters mTOR activity, systemic metabolism and potentially prevents collagen degradation associated with chronic alcohol consumption in mice. *Metabolomics*, 19(5):43.
- Xia, J., Psychogios, N., Young, N. & Wishart, D.S. 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res*, 37(Web Server issue):W652-660.
- Xie, N., Zhang, L., Gao, W., Huang, C., Huber, P.E., Zhou, X., ... Zou, B. 2020. NAD⁺ metabolism: pathophysiologic mechanisms and therapeutic potential. *Signal Transduct. Target. Ther.*, 5(1):227.
- Yoo, H.S., Shanmugalingam, U. & Smith, P.D. 2022. Potential roles of branched-chain amino acids in neurodegeneration. *Nutrition*, 103-104:111762.
- Yoon, J.Y., Daneshgar, N., Chu, Y., Chen, B., Hefti, M., Vikram, A., ... Dai, D.F. 2022. Metabolic rescue ameliorates mitochondrial encephalo-cardiomyopathy in murine and human iPSC models of Leigh syndrome. *Clin. Transl. Med.*, 12(7):e954.
- Zhenyukh, O., Civantos, E., Ruiz-Ortega, M., Sánchez, M.S., Vázquez, C., Peiró, C., ... Mas, S. 2017. High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation. *Free Radic. Biol. Med.*, 104:165-177.
- Zhou, Z., Kim, K., Ramsey, J.J. & Rutkowsky, J.M. 2023. Ketogenic diets initiated in late mid-life improved measures of spatial memory in male mice. *Geroscience*, 45(4):2481-24

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Summary and conclusions

6.1. Introduction

Complex I (CI) deficiency is the most frequent cause of mitochondrial diseases (MDs), which are heterogenous metabolic disorders caused by disorders of oxidative phosphorylation (OXPHOS). Leigh syndrome (LS), a severe childhood neurodegenerative disorder with symmetrical brain lesions and generalised energy impairment, is one of the frequent presentations of CI deficiency (Rahman *et al.*, 1996; Rodenburg, 2016). One of the primary models to examine MD aetiology and potential treatment is the *Ndufs4* knockout (KO) mouse model, which replicates LS disease and develops progressive encephalopathy, growth failure, and motor impairments (Alam *et al.*, 2015; Kruse *et al.*, 2008).

To date, no effective, curative therapies exist for most MDs. Therapeutic strategies investigated to date include pharmacological agents, supplementation, gene therapy, and dietary therapies, each at different stages of investigation. Pharmacological agents, such as idebenone, coenzyme Q₁₀, and sonlicromanol have been evaluated in clinical trials, though their efficacy remains uncertain (Sing *et al.*, 2021; Smeitink *et al.*, 2024; van de Wal *et al.*, 2022). Nutritional supplements like riboflavin show some promise in clinical utility for specific conditions (El-Hattab *et al.*, 2015; Parikh *et al.*, 2017; Mantle *et al.*, 2022), while gene therapy approaches are predominantly in the pre-clinical and early clinical phase of correcting genetic defects in MD (Corrà *et al.*, 2022; Di Donfrancesco *et al.*, 2022; Keshavan *et al.*, 2024). Dietary treatments are also under clinical evaluation but the evidence to support their effectiveness is limited (Ahola-Erkkilä *et al.*, 2010; Pfeffer & Chinnery, 2013). Overall, while these strategies offer hopeful therapeutic approaches, none have yet been proven to cure MDs, and there is an evident need for continued research and development in this field.

Inhibiting the mTOR pathway, a regulator of cellular metabolism and stress responses, is one promising therapeutic strategy for treating mitochondrial disorders. In a LS mouse model, the powerful mTOR inhibitor, rapamycin, increased survival and slowed disease progression (Johnson *et al.*, 2013). Beyond direct mTOR inhibitors like rapamycin, dietary interventions that indirectly modulate mTOR activity, such as calorie restriction, the ketogenic diet (KD) and other high-fat diets (HFDs), have also emerged as promising therapeutic strategies. KDs have been shown to exert neuroprotective effects by enhancing mitochondrial biogenesis, improving NADH/NAD⁺ ratios, and decreased oxidative stress (Elamin *et al.*, 2017; Sullivan *et al.*, 2004). This dietary approach has also shown to attenuate mTOR signalling (Liu *et al.*, 2020; McDaniel *et al.*, 2011; Willemse *et al.*, 2023). However, the therapeutic potential of polyunsaturated fatty acid (PUFA) supplementation or PUFA-enriched HFDs, distinct from the KD, remains unexplored in LS models. Modified KDs and HFDs enriched with PUFAs, which activate PPAR- γ , improve redox balance, and reduce inflammation (González-Pérez *et al.*, 2006; Nathan *et al.*,

2019), offer a synergistic approach to target multiple disease mechanisms, motivating their investigation here.

MDs, such as LS, are often characterised by disruptions in redox homeostasis, a crucial pathological mechanism contributing to disease progression. The possibilities of KDs and HFDs to reduce redox imbalances, improve metabolic regulation and offer therapeutic advantages was investigated in this thesis. As a preliminary investigation, a redox imbalance was induced by administering alcohol, a well-known disruptor of cellular redox homeostasis (Feldstein & Bailey, 2011; Lian *et al.*, 2013) to *Ndufs4* WT/HET mice. This model provides a simplified, reproducible approach to isolate the KD's impact on redox regulation and metabolism without the complexities of systemic mitochondrial dysfunction in LS. This preliminary study employed heterozygous *Ndufs4* KO mice to establish proof-of-concept that dietary modulation could counteract redox imbalance, a shared feature of both alcohol-induced stress and LS pathology. However, while we were busy with the KD experimental work (Chapter 4), a study was published that demonstrated that ketosis alone had limited efficacy in addressing the multifaceted pathology of LS, including neuroinflammation and persistent oxidative stress (Bornstein *et al.*, 2022). The study also reported that *Ndufs4* KO mice do not tolerate a full KD well.

Building on the findings from the KD (Chapter 4), the work progressed to a more clinically relevant model using *Ndufs4* KO mice, which present with severe mitochondrial dysfunction and neurodegeneration characteristics of LS. Due to the novel information from the Bornstein study (Bornstein *et al.*, 2022), the KD was modified for the second part of the study to a PUFA-enriched HFD in order to improve therapeutic potential by using PUFAs' dual capacity to inhibit mTOR, reduce inflammation and regulate the redox state (Chen *et al.*, 2018; Liu *et al.*, 2020; Shirooie *et al.*, 2018). Although this change was designed to target multiple disease mechanisms simultaneously, thereby overcoming the limitations of previous dietary treatments that focussed solely on metabolic reprogramming, it also avoided a full KD, which the *Ndufs4* KO mice cannot tolerate (Bornstein *et al.*, 2022). This study therefore underscores the importance of dietary composition, particularly the fatty acid profile, in optimising the efficacy of HFD-based interventions for mitochondrial diseases.

This thesis first investigated the effects of a KD on redox balance, metabolism, and mitochondrial function during chronic alcohol consumption in healthy *Ndufs4* WT/HET mice as a preliminary study. This was then followed by the investigation of the potential therapeutic impact of a PUFA-enriched HFD to mitigate MD pathology, with an emphasis on redox balance, metabolism, and inflammation in a clinically relevant CI-deficient LS mouse model.

6.2. Research findings summary

6.2.1. Objective 1 (Phase A): Animal breeding, genotyping, and dietary interventions

Whole-body *Ndufs4* wild-type (WT) and heterozygous (HET) mice were bred from HET crosses (B6.129S4-*Ndufs4*^{tm1.1Rpa}/J, JAX #027058) (Kruse *et al.*, 2008). Unlike homozygous KO mice, HET mice do not exhibit the LS phenotype; therefore, a redox imbalance had to be experimentally induced for the preliminary study using alcohol. Both WT and HET mice were used for this preliminary investigation due to the availability of HET mice, as numerous post-graduate studies at the NWU use the KO and WT mice, while the HET mice are rarely used. It was, therefore, much easier obtaining HET mice for the study than WT mice, although both genotypes present with similar metabolism and phenotypes (Hanaford *et al.* 2023; Ingraham *et al.*, 2010; Reynaud-Dulaurier *et al.*, 2020). To experimentally induce a redox imbalance, a hallmark of MD, we subjected WT and HET mice to chronic alcohol consumption alongside a KD dietary intervention (Willemse *et al.*, 2023). The genotypes of the mice were confirmed by PCR as previously described (Valsecchi *et al.*, 2012), after weaning around postnatal day 21 (P21). In this phase, male mice (n=10 per group) were assigned to one of four dietary intervention groups: i) a normal diet (ND) with drinking water; ii) ND with 5% (v/v) alcohol in drinking water for one week, followed by 10% (v/v) alcohol for the remainder of the study; iii) a KD with water; or iv) KD with 5% (v/v) alcohol for one week, followed by 10% (v/v) alcohol. Samples were handled and stored in a standardised manner to maintain integrity and data reliability. To minimise physiological variability due to nutritional status, feeding habits, and circadian rhythms, the mice were housed individually in metabolic cages and subjected to an overnight fast (12 hours) prior to urine collection. Animals were euthanised (P45-P47) via exsanguination to collect sufficient blood while avoiding the adverse effects of anaesthesia on the metabolome. Blood, liver and brain tissue samples were collected at a consistent time each day (between 10:00 and 11:00 AM). Following collection, urine and tissue samples were immediately snap-frozen and stored at -80 °C to preserve metabolites. Blood samples were clotted at room temperature for approximately 30-60 minutes before serum was collected, snap-frozen and stored at -80 °C. Throughout sample preparation, careful handling at low temperatures was maintained whenever feasible, and freeze-thaw cycles were minimised. Overall, these methodological approaches were successful in achieving the first research objective.

6.2.2. Objective 2 (Phase A): Evaluation of mTORC1 activity, inflammation and multi-platform metabolomics

The assessment of mTORC1 activity, inflammation and metabolomics were successfully performed in liver tissue and urine collected from *Ndufs4* WT/HET (n=10 per group) exposed to alcohol and/or a KD. The key findings of this phase were later published in Willemse *et al.* (2023). To evaluate mTORC1 activity, liver tissue samples were analysed using Western

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blotting to measure the phosphorylation of p70S6K, a downstream target of mTORC1, as detailed in Chapter 3. Mice fed a KD, exhibited significant inhibition of liver mTORC1 and a reduction in growth rate. These results coincide with previous studies showing that a KD inhibits mTOR phosphorylation in rat brain and liver (McDaniel *et al.*, 2011), and significantly affects management of weight and obesity (Bueno *et al.*, 2013; Roberts *et al.*, 2017). While alcohol consumption alone had no significant effect on mTORC1 activity or growth in our study (Willemse *et al.* 2023), it inhibited mTORC1 in KD-fed mice. This aligns with earlier findings that alcohol suppresses mTOR signalling (Foster *et al.*, 2010), highlighting a potential synergistic interaction between diet and mTOR activity.

To assess the effect of the KD and chronic alcohol consumption on the inflammation status, flow cytometry analysis was performed on serum obtained from collected blood samples. Serum inflammatory biomarkers were measured across dietary groups. However, due to intragroup variability and discrepancies with findings from several previous studies, no definitive conclusions could be drawn regarding the impact of a KD and chronic alcohol consumption on inflammation. These results underscore the need for further research to clarify these effects and address potential confounding factors, such as dietary composition and sample volume. These findings also highlight a critical gap addressed in Phase B by focusing on a PUFA-enriched diet with known anti-inflammatory properties (Calder, 2015; Marion-Letellier *et al.*, 2015).

For metabolic profiling, as detailed in Chapter 3, urine samples were collected from mice that had been placed in metabolic cages and fasted overnight. These samples were prepared for metabolomic analysis by adding internal standards and deproteinising with methanol. This process included vortexing, centrifugation, and drying under nitrogen gas to remove water. The study employed multiple analytical techniques for comprehensive metabolomic profiling: gas chromatography-time of flight mass spectrometry (GC-TOF-MS) was used for untargeted metabolomic analysis, while liquid chromatography-tandem mass spectrometry (LC-MS/MS) was utilised for targeted quantification of amino acids and acylcarnitines. Additionally, nuclear magnetic resonance (NMR) spectroscopy provided further insight into the metabolic profile without prior deproteinisation. For each platform, QC samples were added and demonstrated consistent repeatability and intermediate precision, with no significant batch effects observed. Metabolomic data revealed systemic metabolic changes and redox state alterations due to the dietary interventions. Metabolic profiling revealed modifications in several metabolic pathways, such as the TCA cycle, amino acid metabolism, as well as changes in redox state following KD and alcohol consumption. TCA cycle intermediates (malic acid, fumaric acid, succinic acid, α -ketoglutaric acid and citric acid) were found to be significantly decreased in urine of mice on a KD. Similarly, previous studies employing a KD have reported comparable findings, attributing the reduction in TCA cycle intermediates to the inhibitory effect of KD on mTOR activity (Longo, 2019; Ramanathan & Schreiber, 2009). Additionally, chronic alcohol consumption appeared to

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further exacerbate the depletion of TCA cycle intermediates (citric acid, α -ketoglutaric acid, and succinic acid) in mice maintained on a KD. Consistently, prior studies have demonstrated that chronic alcohol intake can reduce levels of TCA cycle intermediates (Chen *et al.*, 2012). Urinary levels of several amino acids, including essential (leucine, isoleucine, arginine, and methionine), non-essential (alanine, aspartate, and serine), and non-proteinogenic (homocysteine and cystathionine) amino acids, were significantly lower in animals fed a KD, both with and without alcohol, compared to those on a ND. Notably, the KD appeared to mitigate bone loss and collagen degradation associated with chronic alcohol consumption, as suggested by alterations in hydroxyproline metabolism. Alcohol consumption on a ND significantly elevated the urinary excretion of essential amino acids, including leucine, isoleucine, arginine, and methionine, as well as 4-hydroxyproline, a post-translational product of proline. In contrast, these levels remained unchanged in mice consuming alcohol while on a KD. Chronic alcohol consumption on a ND significantly elevated the excretion of essential amino acids that were otherwise reduced by a KD, potentially indicating disruptions in protein turnover (Bernal *et al.*, 1993; Lang *et al.*, 2010). As a key component of collagen and a urinary biomarker for bone resorption and collagen turnover (Barbul, 2008; Kuo & Chen, 2017), elevated urinary 4-hydroxyproline likely reflects increased collagen degradation, a process previously linked to chronic alcohol consumption (Preedy *et al.*, 1991).

Overall, the combination of Western blot analysis for mTORC1 activity and multi-platform metabolomics provided a comprehensive understanding of how a KD and alcohol consumption influence metabolic pathways and signalling mechanisms in the context of this phase of the study. The results of Phase A not only validated the KD's metabolic effects but also motivated the subsequent exploration of alternative dietary strategies, like the PUFA-HFD, to target inflammation and enhance therapeutic outcomes of *Ndufs4* KO mice in Phase B. Conclusively, the second objective of this phase of the research study was successfully accomplished.

The findings from Phase A demonstrated that a KD could inhibit mTOR activity, systemic metabolism and improve the redox state under alcohol induced redox stress. However, the inconsistencies of a KD to resolve inflammation, as well as its poor tolerability and failure to improve disease outcomes in *Ndufs4* KO mice model (Bornstein *et al.*, 2022) prompted the design of Phase B. After the completion of Phase A's objectives, we transitioned to the *Ndufs4* KO mouse model of LS and replaced the KD with a PUFA-enriched HFD. We speculated that ω -3 fatty acids would enhance mitochondrial function, reduce inflammation, and circumvent the limitations of standard KD formulations. This shift in dietary composition and disease stage allowed us to explore broader therapeutic mechanisms, as detailed in Chapter 5.

6.2.3. Objective 3 (Phase B): Breeding, genotyping, mice feeding and phenotypic evaluation

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Ndufs4 KO and wild-type (WT) mice were bred from HET crosses (Kruse *et al.*, 2008), as described in Section 6.2.1. Genotyping was performed according to Valsecchi *et al.* (2012), following the procedure outlined in Section 6.2.1. Phase A (Section 6.2.1-6.2.2) demonstrated that a KD modulates mTOR activity and mitigates alcohol-induced metabolic disruptions (Willemse *et al.*, 2023). In Phase B, we used the *Ndufs4* KO mouse model of LS to evaluate the therapeutic potential of a PUFA-enriched HFD. This change was motivated by the limitations identified in Phase A of the study, and the poor tolerability of strict KDs in the *Ndufs4* KO mouse model as reported by Bornstein and colleagues (Bornstein *et al.*, 2022) during the course of this study. To address these limitations, we replaced the KD with a PUFA-enriched HFD, supplemented with Menhaden fish oil, a source of ω -3 fatty acids. PUFA supplementation has demonstrated anti-inflammatory and mitochondrial-protective properties, and may also inhibit mTOR (Chen *et al.*, 2018; Liu *et al.*, 2020). Both male and female mice were used in this phase of the study. The phenotypic evaluation included two groups: i) KO mice on a control diet referred to as ND, and ii) KO mice on an HFD. The biochemical assessment included four groups: i) WT mice on a ND, ii) WT mice on an HFD, iii) KO mice on a ND, and iv) KO mice on an HFD.

Phenotypic evaluation of the *Ndufs4* KO mice involved several assessments to monitor disease progression and evaluate the effects of the HFD. These included: i) daily limb clasping tests to monitor neurodegeneration and motor dysfunction; ii) open-field tests for locomotor activity, assessing cumulative duration, mean velocity, and total distance travelled at early (P28–30), mid (P38–40), and late (P48–50) disease stages; iii) wire-grid hang tests to measure grip strength at the same disease stages; iv) survival assessment throughout the dietary intervention until natural death, and v) weight and growth rate. Survival and lifespan were monitored based on natural endpoints, and body weight was recorded periodically to assess the impact of the diet on growth. HFD improved clasping behaviour and survival but did not affect locomotor activity or grip strength in later stages. These results contrast with the findings of Bornstein *et al.* (2022), who observed no improvement in disease progression or survival with a standard KD in the same mouse model. These findings also underscore the importance of dietary composition over ketosis alone, as the PUFA-HFD used in our study avoided metabolic stress caused by extreme carbohydrate restriction, while retaining therapeutic benefits. Additionally, HFDs like the KDs are not uniform and can differ significantly in macronutrient composition, caloric density, and the type of fat used. While no previous studies have directly examined the impact of PUFAs on MD models, PUFAs have been shown to enhance mitochondrial function and reduce disease severity in other conditions related to mitochondrial dysfunction, including obesity (Borja-Magno *et al.*, 2023), aging (Afshordel *et al.*, 2015), Alzheimer's disease (Park *et al.*, 2020), and palmitate-induced mitochondrial damage (Nisr *et al.*, 2020). Lastly, although the HFD did not prevent weight loss in KO mice, it also did not worsen the phenotype. This outcome

is likely attributed to the isocaloric composition of the diets, as consistent caloric intake has been shown to support weight stability in similar contexts (Pathak *et al.*, 2022; Roberts *et al.*, 2017; Zhou *et al.*, 2023). Conclusively, our findings align with these studies, demonstrating that a HFD enriched with PUFAs attenuates disease symptoms such as clasping and extends lifespan in this mouse model, thus successfully achieving the third objective of the study.

6.2.4. Objective 4 (Phase B): Biochemical evaluation of signalling pathways, inflammation and neurometabolism

The biochemical evaluations performed using *Ndufs4* WT and KO mice encompassed several analyses, including Western blot assessments of key proteins in brain tissue, flow cytometry to assess systemic inflammation in blood serum, and both targeted and untargeted metabolomics and lipidomics in brain tissue, as detailed in Chapter 3. These methods were optimised for Phase B of the study, following insights gained from the initial Phase A pilot study. Biochemical analyses in Phase B revealed that the therapeutic potential of the PUFA-HFD's in *Ndufs4* KO mice was likely driven by distinct mechanisms, namely inflammation reduction and metabolic reprogramming, compared to that of the KD intervention used in Phase A.

Western blot analysis focused on key mechanistic protein pathways, including mTOR (a central regulator of cell growth and metabolism), its downstream targets p70S6K1 and 4E-BP1, SIRT1 (a protein deacetylase involved in metabolic regulation and stress responses), PGC-1 α (a critical regulator of mitochondrial biogenesis), as well as pro-inflammatory markers TNF- α (a cytokine). These analyses were employed to evaluate the effects of the HFD on these metabolic and inflammatory mechanisms. However, it was observed that the HFD intervention did not alter the mTOR pathway or the activity of key regulators such as p70S6K1 and 4E-BP1 in the brains of *Ndufs4* KO mice. Additionally, markers of inflammation and mitochondrial function, such as PGC-1 α and TNF- α , were also unaffected by the diet. In contrast, Phase A revealed hepatic mTORC1 inhibition via a KD (Willemse *et al.*, 2023), while the PUFA-enriched HFD used in Phase B had no significant effect on whole brain mTOR activity. This lack of effect is consistent with findings from Bornstein *et al.* (2022), who also reported no mTOR inhibition in *Ndufs4* KO mice brain following KD administration. These results suggest that the therapeutic benefits of the HFD are not due to direct modulation of these specific pathways in the whole brain. However, the effects might still be observed in more specific brain regions, such as the olfactory bulbs or brainstem, as reported by Quintana *et al.* (2010) compared to other brain regions (Terburgh, Lindeque, *et al.*, 2021), or peripheral tissues, such as the liver, where KDs have been shown to result in mTOR inhibition (Liu *et al.*, 2020). Therefore, follow-up research is warranted to investigate the effect of a PUFA-enriched HFD on signalling pathways in *Ndufs4* KO mice in different brain regions.

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Systemic inflammation in mouse serum was measured using flow cytometry. Various cytokines were measured, namely interleukin (IL) 6, IL-10, monocyte chemoattractant protein 1 (MCP-1), tumour necrosis factor (TNF), interferon gamma (IFN- γ), and IL-12p70. The HFD significantly reduced pro-inflammatory cytokines TNF- α and IL-6 in the serum of *Ndufs4* KO mice. This reduction in systemic inflammation was associated with improvements in survival and clasping behaviour. These findings align with the well-established anti-inflammatory properties of omega-3 (ω -3) PUFAs, as noted by Calder (2015), and support previous research reporting systemic inflammation in *Ndufs4* KO mice (Aguilar *et al.*, 2022; Aguilar *et al.*, 2024). The reduction in these cytokines suggests that the therapeutic effects of the HFD are partly mediated through a reduction in peripheral inflammation.

Targeted (GC-MS/MS) and untargeted (GC-TOF-MS) metabolomic and untargeted lipidomic (Synapt G2-Si) profiling were performed in mice brain to evaluate alterations following a HFD intervention. The HFD significantly improved brain energy metabolism in *Ndufs4* KO mice by enhancing TCA cycle activity and reducing lactate, alanine, and succinate accumulation, which indicates a partial restoration of the redox metabolism. Additionally, HFD reduced ketone body levels, suggesting an improved utilisation of fatty acids for energy. These findings are consistent with reports by Liu *et al.* (2021) and He *et al.* (2024), who found that ω -3 PUFAs enhance TCA cycle homeostasis in mice and pigs. The HFD also reduced the accumulation of branched-chain amino acids (BCAAs) and glutamate, both hallmark characteristics of mitochondrial dysfunction and neuroinflammation (Esterhuizen *et al.*, 2017; Gao *et al.*, 2024; Terburgh *et al.*, 2021). These findings mirror Phase A's observations that an HFD, like a KD, can mitigate alcohol-induced metabolic dysregulation.

Lastly, the HFD altered the lipid profile in the brains of *Ndufs4* KO mice, reducing levels of diacylglycerols, phosphatidylserines, and phosphatidylinositols, while triacylglycerols (TAGs) and phosphatidylethanolamines (PEs) were elevated. This suggests an adaptive response to mitochondrial dysfunction and oxidative stress. These changes in lipid metabolism are consistent with reports from Bathina and Das (2023) and Moschandrea *et al.* (2024), who noted disrupted lipid homeostasis in MD models. The elevation of TAGs and PEs under HFD may indicate enhanced mitochondrial dynamics, potentially contributing to cellular resilience under stress.

These findings suggest that the therapeutic effects of HFD in *Ndufs4* KO likely do not involve direct modulation of whole-brain signalling pathways such as mTOR, PGC-1 α and SIRT1. Instead, the HFD reduces systemic inflammation, enhances brain energy metabolism, and alters lipid metabolism, which collectively contribute to its beneficial effects. These results are in line with existing research on the beneficial effects of PUFAs and HFDs in mitochondrial dysfunction (Afshordel *et al.*, 2015) and inflammation (Calder, 2015), while providing new insights into the potential therapeutic effects of an HFD enriched with PUFAs. In conclusion,

these findings position the PUFA-HFD as a multi-modal therapeutic strategy: it combines the metabolic benefits of Phase A's KD with anti-inflammatory, metabolic and lipid-regulatory mechanisms to address LS pathology. Based on these evaluations and findings, it can be concluded that objective 4 was successfully accomplished.

6.3. Strengths, limitations, and prospects

6.3.1. Strengths

The initial phase of the study (Phase A) demonstrates significant strengths through its robust experimental design that examines the impact of a KD on mTOR activity, systemic metabolism, and redox states under the influence of chronic alcohol intake. This comprehensive approach provides valuable insights into the complicated interaction between nutritional intake, metabolic pathways, and extrinsic stressors such as alcohol intake. The controlled physiological variables used in this phase of the study, such as fasting mice before sample collection, the timing of euthanasia, and the use of metabolic cages further strengthen the reliability of the findings. The use of advanced methodologies, such as Western blotting to monitor protein signalling, metabolomics to offer detailed metabolic profiling, and flow cytometry to measure inflammatory markers, enhances the research capacity to link molecular alterations with system effects. The analysis of urine, blood, and liver tissue also highlighted the correlations between different physiological compartments. The findings underscore the KD's capability to reverse negative effects of chronic alcohol consumption, such as a perturbed metabolism and potential bone loss and collagen degradation, thereby broadening the therapeutic implication of KD for diseases beyond metabolic and neurological diseases. By focusing on mechanisms like mTOR inhibition and redox balance, key drivers of many chronic conditions, the study provides translational significance, presenting possible dietary therapies for alcohol-associated health conditions. Moreover, investigating the combined effects of KD and alcohol consumption on metabolic reprogramming and redox alteration represents a relatively unexplored field of investigation, adding to the novelty and scientific contribution of the study. This dietary intervention approach also addresses a realistic dietary approach where individuals consuming KDs may still consume alcohol, making the findings highly applicable to real-world scenarios.

One of the key strengths of Phase B of the study is its use of a well-established preclinical model, the *Ndufs4* KO mouse, which accurately replicates the clinical features of LS, including systemic inflammation and neurodegeneration. This allows for a relevant investigation into the effects of dietary interventions on mitochondrial dysfunction. The focus on non-pharmacological interventions like dietary strategies demonstrates the potential of therapeutic alternatives to drug development for rare diseases and MDs like LS, which often face challenges with regards to pharmaceutical research. The study employed the integration of phenotypic, biochemical, and molecular analyses, thus providing a comprehensive evaluation of the therapeutic effects of

an HFD. This multifaceted methodology enabled a thorough evaluation of both phenotypic outcomes and underlying biochemical mechanisms, providing robust evidence for the diet's efficacy in mitigating disease symptoms. Additional strengths included the optimisation of the dietary treatment with a high PUFA content, leveraging the anti-inflammatory and metabolic benefits of fish oil-based fats. This dietary approach highlights the potential of therapeutic strategies that go beyond macronutrient management by focussing on specific fatty acid compositions to enhance therapeutic efficacy. The fatty acid composition of HFDs, like a KD, is vital to its therapeutic efficacy because ω -3-rich sources provide alternate benefits in contrast to standard fats rich in Western diets, typically high in saturated and pro-inflammatory ω -6 fatty acids. Therefore, the nature and source of fatty acids are major contributors to the diet's potential in improving neurodegenerative disease outcomes (Elisia *et al.*, 2024). Additionally, the focus on a PUFA-enriched HFD addressed an important gap in the literature regarding dietary composition and its potential therapeutic benefits for MDs. The study also employed multi-platform metabolic and lipidomic profiling to detect metabolic shifts, such as increases in TCA cycle, ketone body, branched-chain amino acid and lipid metabolism, emphasising the diet's potential to reprogram neurometabolism. This study connects fundamental metabolic alterations with disease outcomes, strengthening its translational potential. Additionally, rigorous controls, including isocaloric diets and genotype-matched cohorts, minimised confounding variables and increased the validity of the findings. The significant findings regarding improvements in lifespan and specific metabolic pathways provide valuable insights into dietary strategies that could mitigate disease symptoms. Finally, the study's findings have a high translational potential, emphasising the feasibility and cost-effectiveness of dietary approaches in treating MDs. Furthermore, the study significantly contributes to MD research, which is often more underresearched compared to other diseases. The study's findings emphasise cost-effective and practical dietary approaches that could benefit underserved patients afflicted with rare diseases such as MDs.

6.3.2. Limitations

Despite its strengths, the study had several limitations. While the findings are promising, they are based on a preclinical animal model, which may not fully translate to human patients with LS due to differences in metabolism and disease manifestation. A key methodological limitation concerns the composition of the control diet. Both phases used a high-carbohydrate control diet (>70% carbs), with Phase B containing 34.5% sucrose. Elevated sucrose intake is known to adversely affect redox status, inflammation, and mitochondrial function—core variables in this study. Thus, some benefits attributed to the HFD may partially reflect reduced sucrose exposure rather than intrinsic effects of dietary fats. Furthermore, the control diet lacked matched saturated fat (e.g., lard) to isolate PUFA-specific effects. This design limits causal interpretation of the HFD's mechanisms and should be acknowledged as a confounder. The absence of

histological data to confirm attenuation of neurodegeneration, such as the reduction of brain lesions, limits the ability to directly correlate phenotypic and biochemical improvements. Additionally, the biochemical assessments were limited to whole-brain analyses, which may obscure region-specific effects of the HFD on signalling pathways and metabolic alterations. Another limitation of the study is the lack of direct mitochondrial respiration assays, such as CI and complex II (CII) activity, which are critical to understand whether the HFD enhances alternative OXPHOS pathways. Moreover, while the study assessed systemic inflammation, there was no investigation of liver and brain region-specific cytokine responses. Additionally, other peripheral organs, such as the liver, that were not investigated in Phase B of the study could have been significantly influenced by the HFD. The liver plays a pivotal role in metabolic regulation and ketone body production, and its response to the HFD may have contributed to the systemic anti-inflammatory effects and improved metabolic outcomes observed in *Ndufs4* KO mice. The study also did not address whether the HFD enhances compensatory shifts in CI/CII substrate use or whether it improves mitochondrial biogenesis, a fundamental aspect in the treatment of MD. Also, the lack of detailed investigation of mTOR activity, metabolomics and lipidomics in different brain regions, such as the olfactory bulbs or brainstem, obscures insights in determining the diet's precise therapeutic impact. This limitation further restricts the understanding of how the dietary intervention contributes to phenotype and behavioural improvements observed in this mouse model of LS. Furthermore, while the HFD improved systemic inflammation and metabolic profiles, it did not affect locomotor activity or grip strength, limiting to show functional improvement. The biochemical markers assessed in this study did not exhibit significant alterations, limiting the elucidation of the mechanistic underpinning of the HFD's therapeutic effect in the LS mouse model. However, many other markers and molecular mechanisms remain to be explored and are potential avenues for inquiry into the effects of the diet and how it works. These findings emphasise the necessity of comprehensive investigations to identify the specific therapeutic targets of an HFD in LS. Another limitation of the study is the fact that it did not investigate the stability or bioavailability of PUFA-derived metabolites, such as epoxyeicosanoids, which play a role in mitochondrial function and may possibly be crucial to the therapeutic effects of the HFD. Lastly, the lack of continuous assessment of neurometabolic and inflammatory changes over time limits the understanding of the effect of the diet, leaving unanswered questions about the underlying mechanisms driving the observed benefits. In addition, the timing of sample collection throughout the study may have missed transient metabolic changes and inflammatory responses that could provide crucial insights into early-stage therapeutic effects. Lastly, studies on long-term effects and potential side effects of comparable dietary interventions in human subjects should be considered.

6.3.3. Prospects

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There should be future recommendations based on the strengths and limitations of the study in various areas. Firstly, histological studies in animal models need to be employed to confirm neuroanatomical alterations, like reduced brain lesions, and correlated with behavioural outcomes. Mitochondrial CI/CII activity and OXPHOS responses could also be directly measured using high-resolution respirometry and shed light on whether the HFD influences alternative energy pathways. Future studies could also assess the impact of HFDs on mitochondrial biogenesis and activity, as an improvement in mitochondrial health could be a potential mechanism of this diet's therapeutic effect. In addition, further investigations should investigate the specific brain regions affected by the dietary intervention to better understand its localised effects on signalling pathways and metabolic alterations, particularly brain regions such as the olfactory bulb and brainstem, which are implicated in LS pathology and energy metabolism. Similarly, other peripheral tissues and organs, like the liver and the gut microbiome, should be explored as it may give insights on systemic metabolic adaptations that may contribute to the therapeutic effects of PUFA-enriched HFDs.

Secondly, other complementary techniques can be employed to obtain additional information from the mouse model. These techniques can include targeted lipidomic approaches for the separation of individual PUFAs, like ω -3 and ω -6, and their corresponding metabolites, such as resolvins and epoxyeicosanoids, which could elucidate their differential effects on mitochondrial function and inflammation. Future studies can also investigate the interaction between PUFA-derived metabolites and epigenetic changes as it can reveal potential and novel regulatory mechanisms through which HFDs may influence gene expression. In addition, broadening the metabolic range of metabolic profiling or employing more targeted studies, such as metabolic flux analyses, to validate these findings and provide further information on dynamic metabolic pathways and processes influenced by an HFD, clarifying its mechanisms of action. Furthermore, future studies could incorporate targeted and untargeted proteomics as a powerful tool to identify additional significant mechanistic pathways that may not have been captured in the current study. This could include spatial proteomics due to its capability to provide detailed information on protein localisation and interactions of different brain regions and cellular structures. By analysing the proteomic profiles of brain tissues and other relevant biological samples, future studies can uncover novel protein interactions and signalling pathways that could serve as potential therapeutic targets, thereby broadening the scope of dietary interventions for MDs. This approach could lead to a more comprehensive understanding of how dietary modifications influence disease pathology and promote metabolic health. The integration of advanced multiomic strategies, including metabolomics, lipidomics, and proteomics, offers promising avenues for deeper insights into metabolic reprogramming and potential therapeutic mechanisms. Additionally, the implementation of advanced imaging techniques, like magnetic resonance imaging spectroscopy (MRS), could be applied in

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preclinical models for non-invasive assessments of neurometabolic alterations in real-time. This could provide valuable insights into the therapeutic effects of HFDs on neurometabolism and function.

Beyond the aforementioned, studies focussing on the gut microbiome could assess how HFDs influence the composition and function of the gut microbiome, which may produce beneficial metabolites that potentially contribute to health outcomes. Targeted lipidomic approaches can also be used to detect the bioavailability and stability of PUFA-derived metabolites, which could shed light on their role in mitochondrial health and the gut microbiome. Moreover, personalised HFDs tailored to the individual metabolic profiles and gut microbiome of preclinical models may also enhance therapeutic outcomes. Also, investigating potential side effects and interactions of dietary therapies with other treatments in preclinical models will be crucial for developing comprehensive dietary strategies for managing MDs.

Lastly, further studies need to be done to validate the findings in humans, as the preclinical models do not always perfectly reflect human metabolic responses. Future clinical translational studies could evaluate the effect of PUFA-enriched HFDs on neurological health, motor coordination, and quality of life. This could provide critical insight in understanding the potential benefits of this dietary approach in LS patients. This can involve clinical trials examining the safety and efficacy of PUFA-enriched HFDs in LS patients with short-term and long-term measurements. Combining the HFD with exercise, pharmacological drugs or other therapeutic interventions that target mitochondrial dysfunction may enhance improvements of metabolic health and disease outcomes. Future studies should prioritise personalised and precision dietary strategies, using biochemical profiling to tailor HFDs to individual patient needs and enhance therapeutic efficacy. Moreover, long-term studies are needed to evaluate HFD benefits and identify potential adaptive mechanisms that may occur over time. Overall, this research can significantly contribute towards the development of non-pharmacological approaches for mitochondrial disease therapy, particularly through dietary intervention.

6.4. References

Afshordel, S., Hagl, S., Werner, D., Röhner, N., Kögel, D., Bazan, N.G. & Eckert, G.P. 2015. Omega-3 polyunsaturated fatty acids improve mitochondrial dysfunction in brain aging--impact of Bcl-2 and NPD-1 like metabolites. *Prostaglandins Leukot Essent Fatty Acids*, 92:23-31.

Aguilar, K., Comes, G., Canal, C., Quintana, A., Sanz, E. & Hidalgo, J. 2022. Microglial response promotes neurodegeneration in the Ndufs4 KO mouse model of Leigh syndrome. *Glia*, 70(11):2032-2044.

4

Aguilar, K., Canal, C., Comes, G., Díaz-Clavero, S., Llanos, M.A., Quintana, A., ... Hidalgo, J. 2024. Interleukin-6-elicited chronic neuroinflammation may decrease survival but is not sufficient to drive disease progression in a mouse model of Leigh syndrome. *J Inflamm (Lond)*, 21(1):1.

CHAPTER 6

- Ahola-Erkkilä, S., Carroll, C.J., Peltola-Mjösund, K., Tulkki, V., Mattila, I., Seppänen-Laakso, T., ... Suomalainen, A. 2010. Ketogenic diet slows down mitochondrial myopathy progression in mice. *Hum. Mol. Genet.*, 19(10):1974-1984.
- Alam, M.T., Manjeri, G.R., Rodenburg, R.J., Smeitink, J.A., Notebaart, R.A., Huynen, M., ... Koopman, W.J. 2015. Skeletal muscle mitochondria of NDUFS4^{-/-} mice display normal maximal pyruvate oxidation and ATP production. *Biochim. Biophys. Acta*, 1847(6-7):526-533.
- Barbul, A. 2008. Proline precursors to sustain Mammalian collagen synthesis. *J. Nutr.*, 138(10):2021s-2024s.
- Bathina, S. & Das, U.N. 2023. Role of Mitochondrial Dysfunction in Cellular Lipid Homeostasis and Disease. *Discovery Medicine*, 35(178):653-663.
- Bernal, C.A., Vazquez, J.A. & Adibi, S.A. 1993. Leucine metabolism during chronic ethanol consumption. *Metabolism*, 42(9):1084-1086.
- Borja-Magno, A.I., Furuzawa-Carballeda, J., Guevara-Cruz, M., Arias, C., Granados, J., Bourges, H., ... Gómez, F.E. 2023. Supplementation with EPA and DHA omega-3 fatty acids improves peripheral immune cell mitochondrial dysfunction and inflammation in subjects with obesity. *J. Nutr. Biochem.*, 120:109415.
- Bornstein, R., James, K., Stokes, J., Park, K.Y., Kayser, E.B., Snell, J., ... Johnson, S.C. 2022. Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy. *Neurobiol. Dis.*, 163:105594.
- Bueno, N.B., de Melo, I.S., de Oliveira, S.L. & da Rocha Ataíde, T. 2013. Very-low-carbohydrate ketogenic diet v. low-fat diet for long-term weight loss: a meta-analysis of randomised controlled trials. *Br J. Nutr.*, 110(7):1178-1187.
- Calder, P.C. 2015. Functional Roles of Fatty Acids and Their Effects on Human Health. *J. Parenter Enteral Nutr.*, 39(1 Suppl):18s-32s.
- Chen, W.X., Xu, C.Q., Chen, S.H., Xu, G.Y. & Ye, H.Z. 2012. Citric acid reduces the decline in P300 amplitude induced by acute alcohol consumption in healthy adults. *J. Zhejiang Univ. Sci. B*, 13(5):395-401.
- Chen, X., Chen, C., Fan, S., Wu, S., Yang, F., Fang, Z., ... Li, Y. 2018. Omega-3 polyunsaturated fatty acid attenuates the inflammatory response by modulating microglia polarization through SIRT1-mediated deacetylation of the HMGB1/NF-κB pathway following experimental traumatic brain injury. *J Neuroinflammation*, 15(1):116.
- Corrà, S., Cerutti, R., Balmaceda, V., Viscomi, C. & Zeviani, M. 2022. Double administration of self-complementary AAV9NDUFS4 prevents Leigh disease in Ndufs4^{-/-} mice. *Brain*, 145(10):3405-3414.
- Di Donfrancesco, A., Massaro, G., Di Meo, I., Tiranti, V., Bottani, E. & Brunetti, D. 2022. Gene Therapy for Mitochondrial Diseases: Current Status and Future Perspective. *Pharmaceutics*, 14(6),
- El-Hattab, A.W., Adesina, A.M., Jones, J. & Scaglia, F. 2015. MELAS syndrome: Clinical manifestations, pathogenesis, and treatment options. *Mol. Genet. Metab.*, 116(1-2):4-12.
- Elamin, M., Ruskin, D.N., Masino, S.A. & Sacchetti, P. 2017. Ketone-Based Metabolic Therapy: Is Increased NAD(+) a Primary Mechanism? *Front. Mol. Neurosci.*, 10:377.
- Elisia, I., Yeung, M., Kowalski, S., Shyp, T., Tee, J., Hollman, S., ... Krystal, G. 2024. A ketogenic diet rich in fish oil is superior to other fats in preventing NNK-induced lung cancer in A/J mice. *Sci. Rep.*, 14(1):5610.
- Esterhuizen, K., Van der Westhuizen, F.H. & Louw, R. 2017. Metabolomics of mitochondrial disease. *Mitochondrion*, 35:97-110.

CHAPTER 6

- Feldstein, A.E. & Bailey, S.M. 2011. Emerging role of redox dysregulation in alcoholic and nonalcoholic fatty liver disease. *Antioxid. Redox Signal*, 15(2):421-424.
- Foster, G.D., Wyatt, H.R., Hill, J.O., Makris, A.P., Rosenbaum, D.L., Brill, C., ... Klein, S. 2010. Weight and metabolic outcomes after 2 years on a low-carbohydrate versus low-fat diet: a randomized trial. *Ann. Intern. Med.*, 153(3):147-157.
- Gao, H., Jensen, K., Nesbit, J., Ostroot, M., Baloni, P., Funk, C. & Trushina, E. 2024. *Ndufs4* knockout induces transcriptomic signatures of Alzheimer's Diseases that are partially reversed by mitochondrial complex I inhibitor. *bioRxiv*:2024.2002.2020.581247.
- González-Pérez, A., Planagumà, A., Gronert, K., Miquel, R., López-Parra, M., Titos, E., ... Clària, J. 2006. Docosahexaenoic acid (DHA) blunts liver injury by conversion to protective lipid mediators: protectin D1 and 17S-hydroxy-DHA. *Faseb J.*, 20(14):2537-2539.
- Hanaford, A., Khanna, A., James, K., Truong, V., Liao, R., Chen, Y., ... Johnson, S. 2023. Interferon-gamma modestly contributes to disease progression in the *Ndufs4* (-/-) model of Leigh syndrome while IP10 is dispensable. *Neuropathol. Appl. Neurobiol.*, 50(3):e12977.
- He, W., Liu, X., Feng, Y., Ding, H., Sun, H., Li, Z. & Shi, B. 2024. Dietary fat supplementation relieves cold temperature-induced energy stress through AMPK-mediated mitochondrial homeostasis in pigs. *J. Anim. Sci. Biotechnol.*, 15(1):56.
- Hwang, H., Liu, R., Eldridge, R., Hu, X., Forghani, P., Jones, D.P. & Xu, C. 2023. Chronic ethanol exposure induces mitochondrial dysfunction and alters gene expression and metabolism in human cardiac spheroids. *Alcohol Clin. Exp. Res. (Hoboken)*, 47(4):643-658.
- Ingraham, C.A., Burwell, L.S., Skalska, J., Brookes, P.S., Howell, R.L., Sheu, S.S. & Pinkert, C.A. 2009. NDUF54: creation of a mouse model mimicking a Complex I disorder. *Mitochondrion*, 9(3):204-210.
- Johnson, S.C., Yanos, M.E., Kayser, E.B., Quintana, A., Sangesland, M., Castanza, A., ... Kaeberlein, M. 2013. mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science*, 342(6165):1524-1528.
- Keshavan, N., Minczuk, M., Viscomi, C. & Rahman, S. 2024. Gene therapy for mitochondrial disorders. *J. Inherit. Metab. Dis.*, 47(1):145-175.
- Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab.*, 7(4):312-320.
- Kuo, T.R. & Chen, C.H. 2017. Bone biomarker for the clinical assessment of osteoporosis: recent developments and future perspectives. *Biomark. Res.*, 5:18.
- Lang, C.H., Lynch, C.J. & Vary, T.C. 2010. Alcohol-induced IGF-I resistance is ameliorated in mice deficient for mitochondrial branched-chain aminotransferase. *J. Nutr.*, 140(5):932-938.
- Lian, M., Hua, J., Sheng, L. & Qiu, D.K. 2013. Prevalence and significance of autoantibodies in patients with alcoholic liver disease. *J. Dig. Dis.*, 14(7):396-401.
- Liu, R., Chen, L., Wang, Y., Zhang, G., Cheng, Y., Feng, Z., ... Liu, J. 2020. High ratio of ω -3/ ω -6 polyunsaturated fatty acids targets mTORC1 to prevent high-fat diet-induced metabolic syndrome and mitochondrial dysfunction in mice. *J. Nutr. Biochem.*, 79:108330.
- Liu, R., Chen, L., Wang, Z., Zheng, X., Hou, Z., Zhao, D., ... Liu, J. 2021. Omega-3 polyunsaturated fatty acids prevent obesity by improving tricarboxylic acid cycle homeostasis. *J. Nutr. Biochem.*, 88:108503.
- Longo, V.D. 2019. Programmed longevity, youthspan, and juvenology. *Aging Cell*, 18(1):e12843.
- Mantle, D. & Hargreaves, I.P. 2022. Mitochondrial Dysfunction and Neurodegenerative Disorders: Role of Nutritional Supplementation. *Int. J. Mol. Sci.*, 23(20):12603.

CHAPTER 6

- Marion-Letellier, R., Savoye, G. & Ghosh, S. 2015. Polyunsaturated fatty acids and inflammation. *IUBMB Life*, 67(9):659-667.
- McDaniel, S.S., Rensing, N.R., Thio, L.L., Yamada, K.A. & Wong, M. 2011. The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. *Epilepsia*, 52(3):e7-e11.
- Moschandrea, C., Kondylis, V., Evangelakos, I., Herholz, M., Schneider, F., Schmidt, C., ... Jaeckstein, M.Y. 2024. Mitochondrial dysfunction abrogates dietary lipid processing in enterocytes. *Nature*, 625(7994):385-392.
- Nathan, J., Bailur, S., Datay, K., Sharma, S. & Khedekar Kale, D. 2019. A Switch to Polyunsaturated Fatty Acid Based Ketogenic Diet Improves Seizure Control in Patients with Drug-resistant Epilepsy on the Mixed Fat Ketogenic Diet: A Retrospective Open Label Trial. *Cureus*, 11(12):e6399.
- Nisr, R.B., Shah, D.S. & Hundal, H.S. 2020. Mono- and Polyunsaturated Fatty Acids Counter Palmitate-Induced Mitochondrial Dysfunction in Rat Skeletal Muscle Cells. *Cell Physiol. Biochem.*, 54(5):975-993.
- Parikh, S., Goldstein, A., Karaa, A., Koenig, M.K., Anselm, I., Brunel-Guitton, C., ... Enns, G.M. 2017. Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med.*, 19(12):1380-1397.
- Park, Y.H., Shin, S.J., Kim, H.S., Hong, S.B., Kim, S., Nam, Y., ... Moon, M. 2020. Omega-3 Fatty Acid-Type Docosahexaenoic Acid Protects against A β -Mediated Mitochondrial Deficits and Pathomechanisms in Alzheimer's Disease-Related Animal Model. *Int. J. Mol. Sci.*, 21(11).
- Pathak, S.J., Zhou, Z., Steffen, D., Tran, T., Ad, Y., Ramsey, J.J., ... Baar, K. 2022. 2-month ketogenic diet preferentially alters skeletal muscle and augments cognitive function in middle aged female mice. *Aging Cell*, 21(10):e13706.
- Pfeffer, G. & Chinnery, P.F. 2013. Diagnosis and treatment of mitochondrial myopathies. *Ann. Med.*, 45(1):4-16.
- Preedy, V.R., Baldwin, D.R., Keating, J.W. & Salisbury, J.R. 1991. Bone collagen, mineral and trace element composition, histomorphometry and urinary hydroxyproline excretion in chronically-treated alcohol-fed rats. *Alcohol Alcohol.*, 26(1):39-46.
- Quintana, A., Kruse, S.E., Kapur, R.P., Sanz, E. & Palmiter, R.D. 2010. Complex I deficiency due to loss of Ndufs4 in the brain results in progressive encephalopathy resembling Leigh syndrome. *Proc. Natl. Acad. Sci. USA*, 107(24):10996-11001.
- Rahman, S., Blok, R.B., Dahl, H.H., Danks, D.M., Kirby, D.M., Chow, C.W., ... Thorburn, D.R. 1996. Leigh syndrome: clinical features and biochemical and DNA abnormalities. *Ann. Neurol.*, 39(3):343-351.
- Ramanathan, A. & Schreiber, S.L. 2009. Direct control of mitochondrial function by mTOR. *Proc. Natl. Acad. Sci. USA*, 106(52):22229-22232.
- Reynaud-Dulaurier, R., Benegiamo, G., Marrocco, E., Al-Tannir, R., Surace, E.M., Auwerx, J. & Decressac, M. 2020. Gene replacement therapy provides benefit in an adult mouse model of Leigh syndrome. *Brain*, 143(6):1686-1696.
- Roberts, M.N., Wallace, M.A., Tomilov, A.A., Zhou, Z., Marcotte, G.R., Tran, D., ... Lopez-Dominguez, J.A. 2017. A Ketogenic Diet Extends Longevity and Healthspan in Adult Mice. *Cell Metab.*, 26(3):539-546.e535.
- Rodenburg, R.J. 2016. Mitochondrial complex I-linked disease. *Biochim. Biophys. Acta*, 1857(7):938-945.
- Shirooie, S., Nabavi, S.F., Dehpour, A.R., Belwal, T., Habtemariam, S., Argüelles, S., ... Nabavi, S.M. 2018. Targeting mTORs by omega-3 fatty acids: A possible novel therapeutic strategy for neurodegeneration? *Pharmacol. Res.*, 135:37-48.

CHAPTER 6

- Singh, A., Faccenda, D. & Campanella, M. 2021. Pharmacological advances in mitochondrial therapy. *eBioMedicine*, 65,
- Smeitink, J., van Es, J., Bosman, B., Janssen, M.C.H., Klopstock, T., Gorman, G., ... Renkema, H. 2024. Phase 2b program with sonlicromanol in patients with mitochondrial disease due to m.3243A>G mutation. *Brain*, 148(3):896-907.
- Sullivan, P.G., Rippy, N.A., Dorenbos, K., Concepcion, R.C., Agarwal, A.K. & Rho, J.M. 2004. The ketogenic diet increases mitochondrial uncoupling protein levels and activity. *Ann. Neurol.*, 55(4):576-580. 10.1002/ana.20062
- Terburgh, K., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021. Cross-comparison of systemic and tissue-specific metabolomes in a mouse model of Leigh syndrome. *Metabolomics*, 17(12):101.
- Terburgh, K., Coetzer, J., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021. Aberrant BCAA and glutamate metabolism linked to regional neurodegeneration in a mouse model of Leigh syndrome. *Biochim. Biophys. Acta Mol. Basis Dis.*, 1867(5):166082.
- Thoudam, T., Gao, H., Jiang, Y., Huda, N., Yang, Z., Ma, J. & Liangpunsakul, S. 2024. Mitochondrial quality control in alcohol-associated liver disease. *Hepatol. Commun.*, 8(11).
- Valsecchi, F., Monge, C., Forkink, M., de Groof, A.J., Benard, G., Rossignol, R., ... Willems, P.H. 2012. Metabolic consequences of NDUFS4 gene deletion in immortalized mouse embryonic fibroblasts. *Biochim. Biophys. Acta*, 1817(10):1925-1936.
- van de Wal, M.A.E., Adjobo-Hermans, M.J.W., Keijer, J., Schirris, T.J.J., Homberg, J.R., Wieckowski, M.R., ... Koopman, W.J.H. 2022. Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention. *Brain*, 145(1):45-63.
- Zhou, Z., Kim, K., Ramsey, J.J. & Rutkowski, J.M. 2023. Ketogenic diets initiated in late mid-life improved measures of spatial memory in male mice. *GeroScience*, 45(4):2481-2494.

APPENDIX A

Genotyping results

APPENDIX A

A. Genotype verification

Figure A-1 provides an example of the gel electrophoresis results acquired for each animal used in this study. As discussed in Section 3.3.4, PCR genotype verification was performed DNA isolated from tail snips DNA, collected post-mortem.

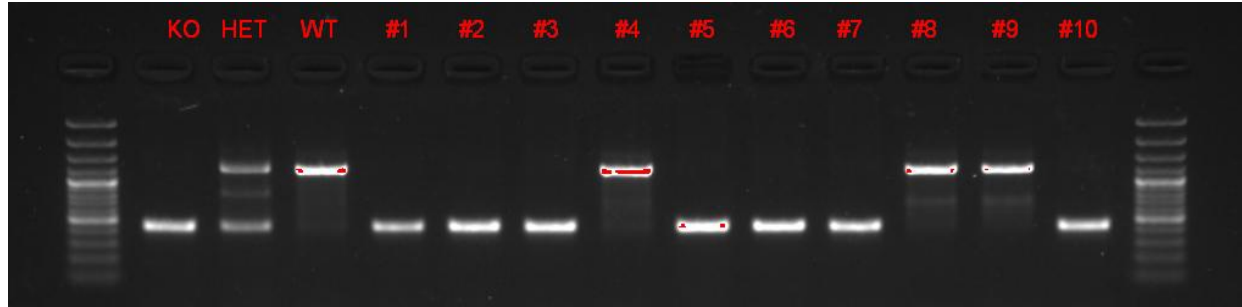


Figure A-1: Agarose gel depicting genotyping results. The photo depicts a 100-1000 bp DNA ladder (lane 1 and 15) along with *Ndufs4* amplicons of KO (lane 2), HET (lane 3), WT (lane 4) controls and sample DNA (lane 5-14). Homozygous KO (*Ndufs4*^{-/-}) DNA (lane 2) exhibit a fragmented *Ndufs4* amplicon around 429 bp, while homozygous WT (*Ndufs4*^{+/+}) DNA (lane 4) display an intact *Ndufs4* amplicon at 1229 bp. Heterozygous (*Ndufs4*^{+/-}) DNA (lane 3) display both the fragmented and intact bands. *Abbreviations: bp, base pairs.*

APPENDIX B

Published peer-reviewed article I



A ketogenic diet alters mTOR activity, systemic metabolism and potentially prevents collagen degradation associated with chronic alcohol consumption in mice

Luciano Willemse¹ · Karin Terburgh¹ · Roan Louw¹Received: 13 March 2023 / Accepted: 31 March 2023 / Published online: 19 April 2023
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Abstract

Introduction A ketogenic diet (KD), which is a high fat, low carbohydrate diet has been shown to inhibit the mammalian target of rapamycin (mTOR) pathway and alter the redox state. Inhibition of the mTOR complex has been associated with the attenuation and alleviation of various metabolic and inflammatory diseases such as neurodegeneration, diabetes, and metabolic syndrome. Various metabolic pathways and signalling mechanisms have been explored to assess the therapeutic potential of mTOR inhibition. However, chronic alcohol consumption has also been reported to alter mTOR activity, the cellular redox- and inflammatory state. Thus, a relevant question that remains is what effect chronic alcohol consumption would have on mTOR activity and overall metabolism during a KD-based intervention.

Objectives The aim of this study was to evaluate the effect of alcohol and a KD on the phosphorylation of the mTORC1 target p70S6K, systemic metabolism as well as the redox- and inflammatory state in a mouse model.

Methods Mice were fed either a control diet with/without alcohol or a KD with/without alcohol for three weeks. After the dietary intervention, samples were collected and subjected towards western blot analysis, multi-platform metabolomics analysis and flow cytometry.

Results Mice fed a KD exhibited significant mTOR inhibition and reduction in growth rate. Alcohol consumption alone did not markedly alter mTOR activity or growth rate but moderately increased mTOR inhibition in mice fed a KD. In addition, metabolic profiling showed alteration of several metabolic pathways as well as the redox state following consumption of a KD and alcohol. A KD was also observed to potentially prevent bone loss and collagen degradation associated with chronic alcohol consumption, as indicated by hydroxyproline metabolism.

Conclusion This study sheds light on the influence that a KD alongside alcohol intake can exert on not just mTOR, but also their effect on metabolic reprogramming and the redox state.

Keywords Ketogenic diet · Mtor · Metabolomics · Redox · Western blot · Metabolic reprogramming

Abbreviations

KD	Ketogenic diet
mTOR	Mammalian target of rapamycin
LC–MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
GC–TOF–MS	Gas chromatography time of flight mass spectrometry
¹ H–NMR	Proton nuclear magnetic resonance

1 Introduction

A ketogenic diet (KD), defined as a high fat, low carbohydrate diet, has been shown to attenuate various disorders that affect brain function and neurological metabolism, as well as refractory epilepsy (Vinnig, 1999), mitochondrial disease (Sperl, 1997), diabetes (Qu et al., 2021) and glucose transporter defects (Bolla et al., 2019). More recently, the KD has been one of the most significantly researched approaches with regards to weight loss and as a strategy to manage obesity (Paoli, 2014). Several mechanisms have been assessed regarding the potential therapeutic effects of a KD, including, but not limited to, ketosis and ketone bodies (Freeman et al., 2010), inflammatory pathways (Dupuis et al., 2015), oxidative stress regulation (Jin et al., 2014)

✉ Roan Louw
Roan.Louw@nwu.ac.za

¹ Human Metabolomics, Faculty of Natural and Agricultural Sciences, North-West University (Potchefstroom Campus), Private Bag X6001, Potchefstroom, South Africa

and the mammalian target of rapamycin (mTOR) pathway (McDaniel et al., 2011). Of these potential mechanisms, the disease ameliorating effects of the mTOR signalling pathway has been of particular interest, resulting in extensive research thereof (Genzer et al., 2016). The mTOR pathway is considered a central control hub for growth and metabolism (Wullschlegel et al., 2006), activated by phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) signalling during conditions of ample nutrient and growth factor availability, and inhibited by AMP-activated protein kinase (AMPK) during energy deficiency (Jewell and Guan, 2013). mTOR is a protein kinase that integrates energy, nutrient, and growth signals to modulate several cellular processes, and exists in two distinct functional complexes namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The former is considered the primary regulatory complex with regard to cellular homeostasis, stress responses, autophagy, and energy metabolism (Laplanche & Sabatini, 2013; Morita et al., 2013, 2015), and also plays a particularly significant role in managing the Krebs cycle (TCA cycle), mitochondrial biogenesis, as well as oxidative stress (Morita et al., 2015; Zhao et al., 2017). As such, mTORC1 has been a target of interest in the development of therapeutic intervention strategies for several disorders such as epilepsy, cancer, neurodegeneration, diabetes, and mitochondrial diseases (Hay & Sonenberg, 2004; Johnson et al., 2013; McDaniel et al., 2011; Xie et al., 2016). When the AMPK or Akt pathway is stimulated by growth factors, nutrients, or insulin signalling, it leads to mTORC1 activation and phosphorylation of the downstream protein, p70S6K, to regulate protein synthesis, cell growth and proliferation (Hay & Sonenberg, 2004). The phosphorylation of p70S6K is one of the best characterised downstream effects of mTOR, as such, the measurement of the activity of this protein makes it a suitable target for monitoring mTOR inhibition (Hay & Sonenberg, 2004). Inhibition of mTORC1 ultimately leads to the alleviation of several of these diseases, thereby implicating distinct metabolic pathways and signalling cascades. Accordingly, administration of the potent mTOR inhibitor, rapamycin, has been reported to prevent epilepsy, disrupt tumour metabolism as well as impair mitochondrial disease progression in various animal models (Johnson et al., 2013; Xie et al., 2016; Zeng et al., 2008). Various studies have also evaluated the inhibitory effect of a KD on mTOR activity in rodent models of mitochondrial disease, epilepsy, and longevity (McDaniel et al., 2011; Roberts et al., 2017; Singh et al., 2018). Together, these findings suggest a credible relationship between a KD and mTOR activity. Additionally, alcohol consumption is known to affect ketone body metabolism, alter the cellular redox state, and impair mTOR phosphorylation (Feldstein & Bailey, 2011; Lang et al., 2003; Veech et al., 1972; Zakhari, 2006). Numerous studies have also evaluated the effect of a KD on alcohol withdrawal symptoms as well as alcohol

addiction in both rodents and humans (Blanco-Gandía et al., 2021; Bornebusch et al., 2021; Dencker et al., 2018; Wiers et al., 2021). In these studies researchers observed that a KD decreases alcohol consumption, as well as alleviating alcohol withdrawal symptoms. Moreover, an increase in worldwide alcohol consumption has been observed, where alcohol use disorder (AUD) has been linked to around 5% of global deaths (Rehm & Imtiaz, 2016). Considering this, the effect of chronic alcohol consumption on the mTOR axis and overall metabolism during a KD-based intervention for weight management and disease treatment comes into question. To address this concept, the current study aimed to first investigate the isolated and combined effects of chronic alcohol consumption and a KD on the mTORC1 phosphorylation state, systemic metabolism, and redox state in a healthy mouse model. For this study the mice consuming the normal diet along with alcohol consumption served as a control for NADH-reductive stress, a redox state shift towards a more decreased NAD^+/NADH ratio. This was used to evaluate whether a ketogenic diet could prevent or even reverse this shift in the redox state. Following the dietary intervention, mouse liver tissue was used to assess p70S6K1, a downstream protein of mTOR phosphorylation, whereas multi-platform-based metabolomics analyses of mouse urinary samples were utilised to evaluate systemic metabolic and redox fluctuations within the various dietary groups.

2 Methods and materials

Complete details regarding the methods and materials used can be found in the Supplementary material.

2.1 Animals, dietary intervention and sampling

Equal numbers of male mice, heterozygous (HET) or wild-type (WT) for *Ndufs4*^{tm1.1Rpa} (Fig. S2), were used interchangeably in this study since both genotypes can serve as healthy controls (Kruse et al., 2008), due to there being no difference in phenotype between the two genotypes (van de Wal et al., 2022). All four treatment groups contained equal numbers of WT and heterozygote animals, while the metabolomics data showed no natural separation between the WT and heterozygote animals (Fig. S2). Original *Ndufs4* HET breeding pairs (B6.129S4-*Ndufs4*^{tm1.1Rpa}/J) were acquired from Jackson Laboratory (JAX; ME, USA, stock #027,058). Only male mice were used in this study due to the known differences in hormone cycles, tissue-specific metabolism as well as metabolic profiles between sexes (Ruoppolo et al., 2018; Terburgh et al., 2021). Standard polymerase chain reaction genotyping was used as described previously (Valsecchi et al., 2010) to distinguish *Ndufs4* mouse genotypes and confirm

that mice of the correct genotype were used for the study. Mice were housed and bred at the specific pathogen-free (SPF) unit of the Vivarium (SAVC reg. #FR15/13458) of the Preclinical Drug Development Platform (PCDDP, NWU) under the following conditions: a day/night cycle of 12 h; temperature of $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$; and relative humidity of $55\% \pm 10\%$. After postnatal day (P) 23, a total of 40 mice were randomly assigned to one of four dietary intervention groups ($n = 10$ animals per group), with ad libitum access to either; i) a control diet, referred to as a normal diet (ND) in this study (standard laboratory chow, RB2005, LabChef, NutritionHub) and drinking water; ii) ND with 5% (v/v) alcohol in drinking water for the first week and 10% (v/v) alcohol in drinking water for the remainder of the study; iii) a KD and drinking water; or iv) a KD with 5% (v/v) alcohol in drinking water for the first week and 10% (v/v) alcohol in drinking water for the remainder of the study. The ND contained (% of total kcal) 20% protein, 75% carbohydrate, and 5% fat; whereas the KD contained 8.2% protein, 91.7% fat, and $<0.1\%$ carbohydrate. Detailed information regarding the ingredient composition of the KD can be found in the Supplementary material (Table S1). These dietary interventions lasted approximately three weeks, with mice weighed three times a week (around the same time intervals) to assess growth rates. Animals were euthanised on postnatal day P46–47, after completing the three-week dietary intervention. Mice were placed in metabolic cages the day before euthanasia to collect fasting urinary samples. Mice were euthanised via exsanguination as described by the American Veterinary Medical Association (AVMA) (AVMA, 2020) by a research animal technician (PCDDP, NWU, RSA) after a 16–18 h fasting period at the same time of day (10:00 to 11:00 AM) to reduce inter-animal physiological variation because of the differences in nocturnal feeding habits as well as that of the stages of the circadian cycle (Griffin et al., 2006). Finally, after blood and liver tissues were collected, all samples were swiftly snap-frozen in liquid nitrogen to quench metabolic activity, and subsequently stored at -80°C .

The AnimCare animal research ethics committee of North-West University approved (NWU-00516-20-A5) the animal protocols used in this study. All animals were maintained, and all procedures performed, in accordance with the code of ethics in research, training, and testing of drugs in South Africa and complied with national legislation.

2.2 SDS-PAGE and western blotting of liver pS6K1

Liver tissue (~ 100 mg) was homogenised in HEPES lysis buffer (~ 1 ml) with a Glass/Teflon® Tight Fitting Potter–Elvehjem Homogeniser (Glas-Col) using 15 strokes.

Thereafter, liver homogenates were centrifuged with a Heraeus™ Multifuge™ X3 centrifuge (Thermo Fisher Scientific). Subsequently, supernatants were collected, and protein concentrations were determined using the BCA assay. Supernatants were kept on ice throughout the procedure, which were subsequently vortexed, and centrifuged at $1000 \times g$ before heat denaturation for five min at 100°C . For each dietary group, aliquots ($20 \mu\text{g}$) of the protein were separated via a sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE), run at room temperature. Afterwards, the gel was transferred to nitrocellulose and blocked. After that, the membranes were incubated with phospho-p70S6 kinase primary antibodies (Cell Signaling Technologies) at 4°C overnight. Next, the membranes were incubated with secondary antibodies at room temperature. Lastly, chemiluminescent detection was achieved using a Western ECL substrate (Bio-Rad, Johannesburg, RSA) and a ChemiDoc™ MP imaging system (Bio-Rad). Finally, the results were analysed via Image Lab™ v. 6.1 software (Bio-Rad,) in order to attain the density ratio of the target proteins relative to β -actin, used as the control protein. Detailed information regarding the procedures and antibody dilutions are described in the supplementary materials.

2.3 Determination of urinary creatinine concentration

The creatinine concentration of each urinary sample was spectrophotometrically quantified using the QuantiChrom™ Creatinine Assay Kit (BioAssay Systems). In this assay, creatinine reacts with picric acid in an alkaline solution forming a yellow orange adduct. The increase in absorbance of this adduct is measured at a wavelength of 510 nm. For the assay, $10 \mu\text{l}$ of mouse urine was mixed with $200 \mu\text{l}$ working solution (0.1 M sodium hydroxide (NaOH); 0.3 mM ethylenediaminetetraacetic acid (EDTA); 0.1% (v/v) DMSO; 0.0004% (v/v) Tween®20; and 3 mM picric acid), in duplicate, in a 96-well plate. The samples were then analysed via a Synergy™ HT Multi-detection microplate reader (Biotek® Instruments) by assessing the increase in linear trend of absorbance at 510 nm for 5 min, in 1 min intervals. Absolute quantities were determined by making use of a standard curve, ranging from 0 to $50 \text{ mg}\%$.

2.4 Metabolomics sample preparation

For all the metabolomics analyses, a pre-determined volume (~ 20 – $250 \mu\text{l}$) of urine containing $0.0625 \mu\text{mole}$ creatinine was added to microcentrifuge tubes, with the addition of internal standards (ISs). Urine samples were analysed via untargeted (gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy) and targeted [liquid

chromatography-tandem mass spectrometry (LC–MS/MS)] techniques. Due to the small volumes of some of the KD + A1c. urine samples obtained, only 6 biological replicates could be used for this group. For GC–TOF–MS analyses, 16.14 μ l IS mixture, which contained 3-phenylbutyrate (100 ppm), norleucine (100 ppm) and *N,N*-dimethylphenylalanine (50 ppm), was added per sample. For LC–MS/MS analyses, 25 μ l IS mixture was added. This mixture consisted of *N,N*-dimethylphenylalanine, ring- $^2\text{H}_5$ -phenylalanine, $^2\text{H}_{10}$ -isoleucine, $^2\text{H}_8$ -valine, L-Carnitine-(methyl- d_3), $^2\text{H}_3$ -octanoylcarnitine and $^2\text{H}_3$ -octadecanoylcarnitine, each with a final concentration of 2.5 ppm. After the addition of the IS mixture, mouse urine samples were deproteinised by adding three volumes of ice-cold methanol. The samples were then vortexed, incubated for 20 min at -20°C , centrifuged at 15 000 $\times g$ for 10 min and finally dried under a gentle stream of nitrogen gas at 37°C . Additionally, another 80 μ l of methanol was added to all dried samples and then dried again to guarantee that the samples were completely dry and all water removed prior to derivatisation. For ^1H -NMR analyses, urine samples did not undergo deproteinisation as samples were dried and then resuspended in sterile, filtered water and centrifuged to remove macromolecules and other insoluble particulates prior to ^1H -NMR analyses. Also, ISs such as 3-trimethylsilyl propionic acid (TSP) were added during NMR sample preparation. LC–MS samples were butylated, while GC–MS samples were oximated and silylated prior to analysis. Additional details regarding sample preparation are described in the Supplementary material.

2.5 LC–MS/MS analyses

For targeted LC–MS/MS analyses, urinary samples were analysed using a 1200 series liquid chromatograph coupled to a 6410-triple quadrupole-mass spectrometer (Agilent Technologies) as previously described (Terburgh et al., 2019). The chromatographic separation was accomplished by injecting samples (1 μ l) on a C18 Zorbax SB-Aq (150 mm $\times$ 2.1 mm $\times$ 1.8 μm) reverse phase column (Agilent Technologies), kept at 35°C , with a flow rate of 0.2 ml/min during the entire run. Furthermore, the mobile phases used comprised of H_2O (solvent A) and acetonitrile (solvent B). Both mobile phases were acidified with 0.1% formic acid to enable protonation of metabolites (Cech & Enke, 2001). The chromatographic gradient started at a 5% mobile phase B and was maintained for 1 min, it was then followed with an increase to 23% of mobile phase B over a duration of 7 min. In addition, an isocratic hold of around 4 min was maintained at a 23% mobile phase B. Subsequently, mobile phase B was increased to 100% at 15 min and was kept there for 5 min. Lastly, the gradient declined to a 5% mobile phase B after 1 min and was maintained for 7 min (post-run) to guarantee that the column is in equilibrium before proceeding

to the next analysis. The effluent coming from the liquid chromatograph was then diverted to the mass spectrometer, which made use of the positive electrospray ionisation (ESI) mode using the following source parameters: capillary voltage of 3 500 V; nitrogen drying gas at a flow rate of 7.5 l/min at 300°C ; and nebuliser pressure of 30 psi. The spectra were acquired via the multiple reaction monitoring (MRM) mode with an electron multiplier voltage (EMV) of 300 V and a dwell time of 45 ms for all compounds, with the MRM parameters set up for specific amino acids, -derivatives, acylcarnitines, and isotopes (Table S2).

2.6 GC–TOF–MS analyses

For untargeted GC–TOF–MS analyses, samples were analysed via a 7890A gas chromatograph (Agilent Technologies) joined to a Pegasus HT time-of-flight mass spectrometer (Leco). Samples (1 μ l) were injected using a split ratio of 1:10. The temperature of the inlet was 250°C throughout the sample injection. Compounds were chromatographically separated using a DB-5 MS capillary column (30 m $\times$ 250 μm $\times$ 0.25 μm) (Restek), and helium was used as a carrier gas at a constant flow rate of 1.5 ml/min. For mass spectrometry, the separated compounds were subjected to electron impact ionisation (EI) (70 eV), with the ion source temperature set at 200°C . After a solvent delay of 250 s, mass spectra were acquired (50–950 m/z) at a scan rate of 20 spectra/second. A predictable peak width of 3 s and a signal-to-noise (S/N) ratio of > 20 were used to detect peaks with 5 apexing masses.

2.7 Miniaturised ^1H -NMR spectroscopy

^1H -NMR spectroscopy was carried out according to a miniaturised method previously described (Mason et al., 2018). Urine samples were analysed at 500 MHz in 2 mm NMR MATCH tubes, using the 5 mm triple-resonance inverse (TXI) probe head, which has been adjusted for ^1H evaluation. During analysis a constant sample temperature was sustained at 300 K. ^1H spectra were obtained as 128 transients in 32 K data points with a spectral width of 6000 Hz (12.0 ppm). A pulse sequence program NOESY-presat was used to suppress the H_2O resonance at 4.70 ppm, by saturating the H_2O resonance by single-frequency irradiation through a 4 s relaxation delay, at a 90° excitation pulse of 10 μs . An acquisition time of 2.7 s and a receiver gain of 64 were set, correspondingly. The number of dummy scans ($n=4$) and scans ($n=128$) produced a run time of 15 min and 45 s per sample. Shimming was carried out for each sample automatically using the deuterium signal, then locked, probe tuned and matched, followed by pulse calibration. NMR analysis and processing was accomplished using Bruker Topspin (v

3.5), and additional processing was performed using Bruker AMIX (v 3.9.14).

2.8 Inflammatory assay

To determine the inflammatory status in different dietary groups, a BD Cytometric bead array mouse inflammation kit (BD Biosciences, #552,364) and Accuri C6 flow cytometer (BD Biosciences) were used, according to the manufacturer instructions and the method discussed in the Supplementary material. The levels of selected inflammatory cytokines, namely, interleukin 6 (IL6), interleukin 10 (IL10), monocyte chemoattractant protein 1 (MCP1), tumour necrosis factor (TNF), interferon gamma (IFN- γ) and interleukin 12p70 (IL12p70) were determined.

2.9 Data processing and statistical analysis

Data analysis for the growth rate, western blot analysis and inflammation assay was carried out using Excel 2021 (Microsoft 365). The average weight over the duration of the study was plotted on a bar graph for each group to visualise the growth rate. For the western blot results, the relative intensity of the target protein (p70S6K1) was normalised using the relative intensity of the control protein, β -actin, which was then plotted on a bar graph to observe the relative intensity differences between each group. The data obtained following the inflammation assay was pre-filtered manually using the FCAP Array software (v3.0), after which each cytokine was assigned to its relative peak intensity followed by manual clustering for each cytokine. The inflammation data of each cytokine's relative concentrations were first plotted on a line graph to obtain a R^2 value ≤ 1 , then plotted on a bar graph to observe the differences of the cytokine concentrations between each group. Significant differences between groups for the growth rate, western blot and inflammation assay results were determined using a one-way ANOVA followed by a post-hoc Tukey's test. The spectral data acquired from each metabolomics platform were extracted into matrices. Each data matrix was separately examined regarding correct peak selection and alignment, as well as batch effects and data integrity. The matrices were also pre-processed by which the aligned data was filtered to retain only features with an 80% presence within each group, along with removing data using a 50% cut-off coefficient of variation (CV) for quality-control samples in each group for GC-TOF-MS, LC-MS/MS and $^1\text{H-NMR}$. Urine data was normalised pre-acquisition to creatinine content (mg/g creatinine) and post-acquisition to tropic acid (GC-TOF-MS), isotope (LC-MS/MS) internal standards and TSP for data obtained via $^1\text{H-NMR}$ analysis. LC metabolites were normalised using stable isotopes such as, valine_d8, isoleucine_d10, phenylalanine_d5, free

carnitine_d3, octanoylcarnitine_d3, and octadecanoylcarnitine_d3 if there was a significant metabolite correlation to these isotopes. Batch corrections were not considered necessary, since QCs displayed no notable batch effects. Following this, log-transformation, mean centering and pareto scaling were applied for data pre-treatment and confidence intervals for PCA scores, confirmed by cluster analysis, were used for outlier detection. The data underwent both multivariate and univariate analyses. Initially, all groups (normal diet vs normal diet with alcohol vs KD vs KD with alcohol) were compared visually using PCA scores plots to observe within group variances and whether the data could partition animals into the different groups. Following this, univariate analysis using one-way ANOVA, along with a post-hoc Tukey's test, were applied to shortlist differentiating compounds between groups. A two-way ANOVA was used to evaluate diet and alcohol's interaction effects for the various compounds for each group, where a significant interaction was evaluated by a subsequent post-hoc Tukey's test. Metabolites (features) were deemed significant if they had a false discovery rate-corrected p-value (q-value) of ≤ 0.05 , using a post-hoc Tukey's test, and a Cohen's d-value of ≥ 0.8 (Ellis & Steyn, 2003). Both the PCA and one-way ANOVA were performed in MetaboAnalyst 5.0 (Xia et al., 2015), whereas the two-way ANOVA, along with a subsequent post-hoc Tukey's test, was carried out using IBM SPSS Statistics 27 and Graphpad Prism 9. Lastly, if spectral features had high identification confidence levels, they were assigned metabolite identities (Schymanski et al., 2014). Commercial and in-house spectral libraries, in conjunction with public databases, were used to identify dataset features through spectral and retention time matching. The significant features' identities were confirmed using each platform's relative standards as well as isotopes (Schymanski et al., 2014). These features were also subjected to pathway analysis using a manual approach, to identify pathways of significance.

3 Results and discussion

3.1 Growth curves

Growth curves (Fig. 1) were constructed to evaluate the effect of a KD and chronic alcohol (Alc.) consumption on animal body weight over a period of three weeks. Mice on a KD had a significantly reduced growth rate relative to those on a control diet (ND), with the final average body weight being 22 ± 2 g for the ND and ND + Alc. groups and 15 ± 2 g for the KD and KD + Alc. groups. Therefore, it is evident that diet alone (i.e., a KD) had a significant ($p < 0.001$) effect on murine body mass, while chronic alcohol consumption did not. The available literature on the effect of alcohol on body weight is conflicting, as studies show either an

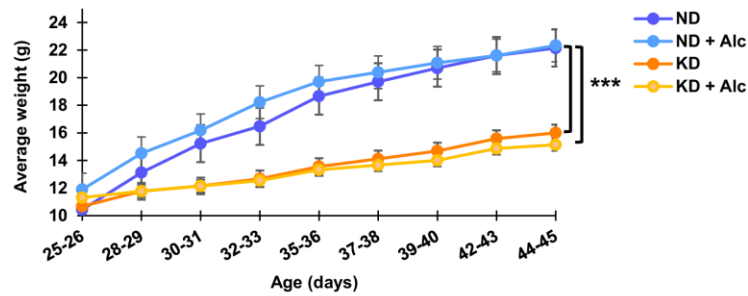


Fig. 1 Consumption of a KD in mice leads to lower body weight gain. Growth curves for dietary groups on a normal diet without (ND) and with alcohol intake (ND + Alc.) and on a ketogenic diet without (KD) and with alcohol intake (KD + Alc.). Average weight $\pm$ standard

deviation ($n=10$) per group is given as a function of animal age. Mice were weighed three times a week. Asterisks ($***p < 0.001$) indicate significant differences between ND vs KD and ND vs KD + Alc. groups as determined by post-hoc Tukey's test

increase, decrease or no difference at all of alcohol consumption on body weight (Traversy G, Chaput, 2015; Huang et al., 2012; Perides et al., 2005). On the contrary, several studies have confirmed that a KD reduces body weight gain, possibly due to mTOR inhibition (Bueno et al., 2013; Roberts et al., 2017; Santos et al., 2012).

3.2 mTOR activity

The mTOR substrate, S6K1 (Thr389), is frequently used as a viable indicator of mTORC1 activity (Ikenoue et al., 2009). Therefore, we examined liver S6K1 phosphorylation status in the various groups using western blot analysis. Liver pS6K1 levels (Fig. 2) were significantly diminished ($p < 0.05$) in the KD and KD + Alc. groups compared to mice on a normal diet (ND). Hence, we confirm that a KD significantly inhibits mTOR pathway activity in mice, an observation supported by previous research (McDaniel et al., 2011). Chronic alcohol consumption alone (ND + Alc. group), however, did not significantly alter murine S6K1 phosphorylation status, with pS6K1 levels only trending lower. Additionally, no significant difference was observed between the KD + Alc. group and the KD group. In addition, no significant interaction was observed between diet and alcohol for mTOR activity. As such, it does not seem that chronic alcohol consumption alone or alcohol consumed along with a KD has a significant impact on mTOR activity. A KD significantly lowered growth rates and levels of liver pS6K1, a downstream substrate of mTORC1 phosphorylation, in mice. These results support previous studies showing that a KD inhibits mTOR phosphorylation (McDaniel et al., 2011) and has a considerable impact on the management of weight and obesity (Bueno et al., 2013; Roberts et al., 2017; Santos et al., 2012). In contrast, chronic alcohol consumption did not markedly affect growth rates or S6K1 phosphorylation when consuming a normal or KD, despite

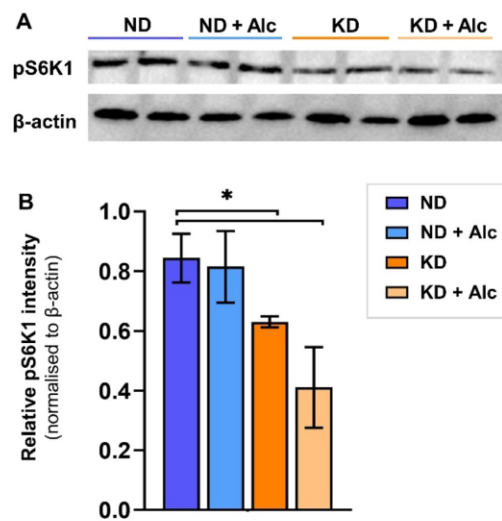


Fig. 2 A KD inhibits S6K1 phosphorylation in mice. **A** Representative Western blot image and **B** quantitative summary of pS6K1 and β -actin following separation by SDS-page. Average β -actin (~42 kDa) was used as control protein of pS6K1 (~75 kDa). Data represents the average relative pS6K1 intensity $\pm$ standard deviation ($n=4$) per group. ND indicates normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. Asterisks ($*p < 0.05$) indicate significant differences between ND vs KD and ND vs KD + Alc. Groups, as determined by post-hoc Tukey's test

previous reports of its potential inhibitory effect on the mTOR pathway (Foster, 2010). These results are in agreement with previous studies that did not notice any significant changes in growth from alcohol consumption (Perides et al., 2005). However, since the literature regarding chronic alcohol consumption's effect on weight is conflicting (Traversy

G, Chaput, 2015; Huang et al., 2012; Perides et al., 2005), further research is warranted. It is well known that mTORC1 activation is essential for growth (Wullschleger et al., 2006), with reduced phosphorylation of this pathway correlated to impaired cell growth (Laplanche et al., 2012). Thus, the data confirms that a KD reduces growth rate, possibly through mTOR inhibition, while chronic alcohol consumption does not markedly impact mTOR activity or body weight in mice. Moreover, the results obtained from the study indicate that the combination of a KD and chronic alcohol consumption does not significantly inhibit mTOR to a greater extent than a KD alone.

4 Metabolic alterations

Next, we investigated the systemic effect of a KD and chronic alcohol consumption on murine metabolism via GC-TOF-MS, LC-MS/MS and ¹H-NMR spectroscopy. ANOVA with post-hoc Tukey's test identified numerous metabolites significantly differing among the four dietary intervention groups (n = 6–10 per group, Table S3). Multivariate principal component analysis (PCA; Fig. 3A) and a heatmap (Fig. 3B) of these discriminatory metabolites revealed a

distinctive metabolic separation between animal groups on a KD and control diet. However, chronic alcohol consumption could only partially separate animal groups on the same diet, indicating that diet (i.e., a KD) had a greater impact on metabolism than alcohol consumption. When comparing the urinary metabolomes of the different dietary intervention groups to controls (ND), significant alterations were observed in amino acid and TCA cycle metabolism. Subsequent pathway analysis revealed that the most significantly altered urinary metabolites were involved in amino acid metabolism (Fig. 4), with branched-chain amino acid and one-carbon metabolism markedly affected, and the tricarboxylic acid (TCA) cycle (Fig. 5). Figures 4–5 illustrate the practical (effect size d-value) and statistical (p-value) significance of these metabolic alterations in the KD, KD + Alc. and ND + Alc. groups when respectively compared to controls (ND). Other metabolites that also displayed significant interaction effects were creatine, alloxanoic acid and isobutyric acid. Additional information on metabolites with an interactive effect can be found in the Supplementary material (Table S4 and Fig. S3). All significantly altered features are described in the Supplementary material.

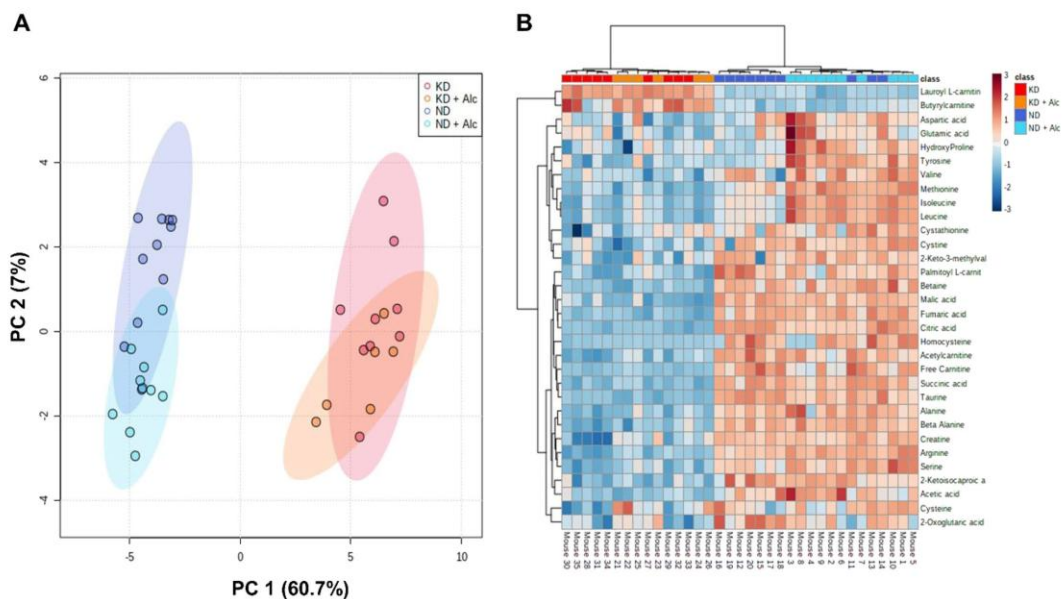


Fig. 3 A KD and chronic alcohol consumption leads to metabolic alterations in mice. **A** PCA scores plot illustrates the natural separation of all significantly altered metabolites when the different groups (n = 6 to 10 per group) were compared. **B** Heatmap of main metabolites altered in different groups (n = 6 to 10 per group) following dietary

intervention. Each metabolite was normalised to its respective internal standard (based on the relative metabolomics platform) and subsequently log transformed, as well as auto-scaled using MetaboAnalyst 5.0

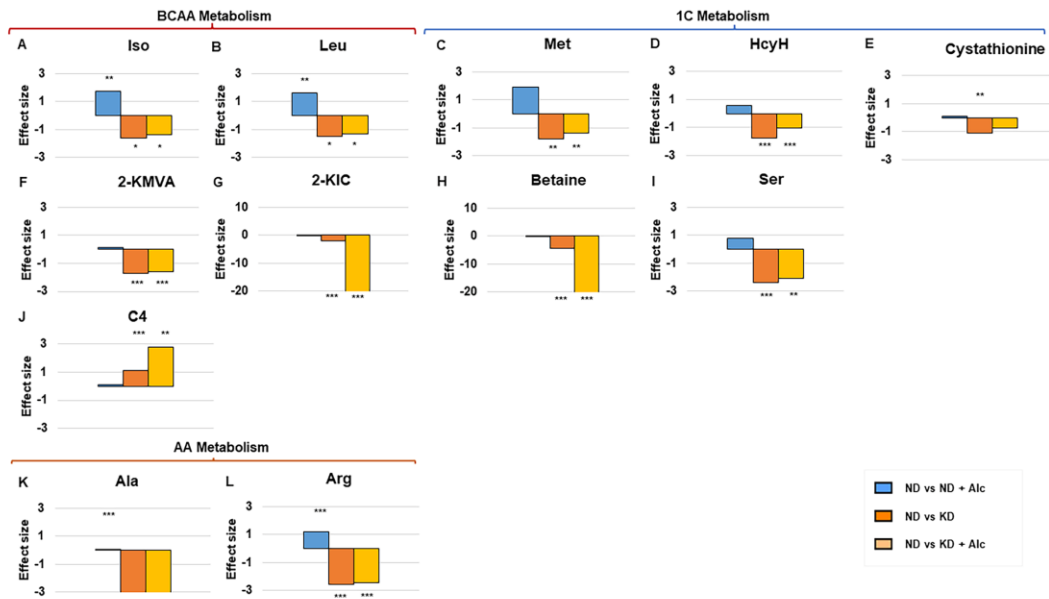


Fig. 4 A KD lowers urinary amino acid levels irrespective of alcohol consumption. Bar graphs depict Cohen's *d*-values (effect size) for each metabolite when relative abundance between the different mouse groups ($n=6$ to 10 per group) were compared to the control group (ND). *Leu* indicates leucine; *Ile*, isoleucine; *2-KIC*, 2-ketoisocaproic acid; *2-KMVA*, 2-keto-3-methylvaleric acid; *C4*, butyryl-carnitine;

Arg, arginine; *Ala*, alanine; *Met*, Methionine; *HcyH*, homocysteine; *Ser*, serine; ND, normal diet; ND+Alc., normal diet and alcohol; KD, ketogenic diet; KD+Alc., ketogenic diet and alcohol. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group, obtained via a post-hoc Tukey's test

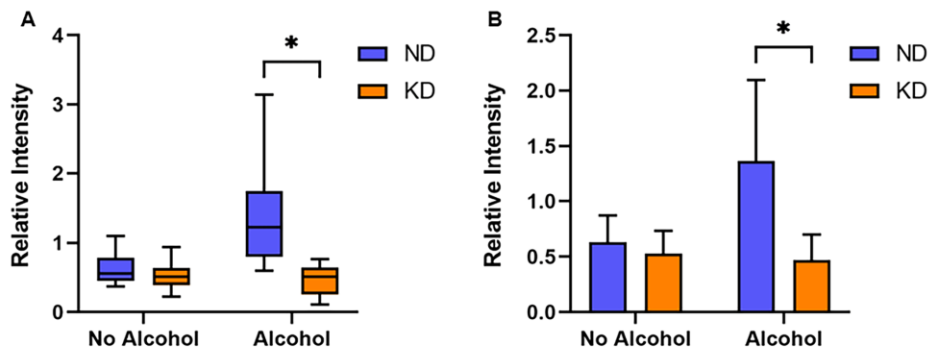


Fig. 5 A KD prevents increased urinary 4-hydroxyproline excretion observed during chronic alcohol intake. **A** Boxplots depict the relative 4-hydroxyproline intensity for dietary groups ($n=6$ – 10 per group) on a normal diet without (ND) and with alcohol intake (ND+Alc.) and on a ketogenic diet without (KD) and with alcohol intake (KD+Alc.). For each boxplot the length of the box represents the 25th to 75th percentile inter-quartile range, the interior horizontal

line represents the median, and the vertical lines stemming from the box extend to minimum and maximum values. **B** Bar graphs show the relative intensities for 4-hydroxyproline when the interaction of diet and alcohol was compared. Asterisks (* $p < 0.05$) indicate significant differences between the ND vs ND + Alc. groups as determined by post-hoc Tukey's test

4.1 Amino acid metabolism

Several essential (leucine, isoleucine, arginine, and methionine), non-essential (alanine, aspartate and serine) and non-proteinogenic (homocysteine and cystathionine) amino acids were markedly ($p < 0.001$ and $d > 0.8$) reduced in the urine of animals consuming a KD with and without alcohol when compared to controls (ND). Additionally, leucine and isoleucine catabolites, 2-ketoisocaproic acid and 2-keto-3-methylvaleric acid along with choline catabolite, betaine, were also significantly lower in these mice (KD and KD + Alc.) compared to controls. Contrastingly, animals chronically consuming alcohol while on a normal diet (ND vs ND + Alc.) excreted markedly higher ($p < 0.001$ and $d > 0.8$) levels of essential amino acids, leucine, isoleucine, arginine, and methionine along with proline's post translational product, 4-hydroxyproline (Fig. 5). The latter serves as a biomarker for bone resorption and is used to detect osteoporosis (Barbul, 2008; Kuo and Chen, 2017). Interestingly, 4-hydroxyproline levels were unaltered in mice consuming alcohol while on a KD. Overall, a KD more dramatically altered amino acid metabolism than alcohol consumption. When the amino acid metabolites were evaluated for an interactive effect between diet and alcohol only a few metabolites were observed to have interaction, namely, taurine (conditionally essential), cysteine (non-essential) and 4-hydroxyproline. Mice fed a KD diet (KD and KD + Alc.) excreted significantly lower levels of several amino acids, including the essential amino acids, leucine, isoleucine, arginine, and methionine along with several other amino acids that form part of one-carbon metabolism (homocysteine, serine, cystathionine, and betaine). This reduction in amino acid levels could, in part, reflect decreased protein intake, since a KD is inherently low in protein (8.2% of calories from protein in KD vs 20% in ND). However, it was recently shown that a KD with at least 5% of calories derived from protein does not induce a protein restriction response (Hu et al., 2018). We therefore postulate that the marked reduction observed in mouse urinary one-carbon metabolites while on a KD, reflect a perturbation in this pathway. In support, others have shown that a KD results in the downregulation of a subset of genes involved in one-carbon metabolism in mouse liver (Pissios et al., 2013) and an inhibition of one-carbon metabolism in HepG2 cells and mice (Hsu et al., 2022). This inhibitory effect on one-carbon metabolism has been ascribed to the reduced mTOR signalling associated with a KD (Hsu et al., 2022). Chronic alcohol intake during a ND significantly increased the excretion of the same essential amino acids lowered by a KD, likely reflecting altered protein turnover (Bernal et al., 1993; Lang et al., 2010). However, alcohol consumption did not markedly alter the effect of a KD on amino acid metabolism. In addition, one of the most significant metabolic alterations resulting uniquely from chronic

alcohol consumption, was an increase in urinary 4-hydroxyproline levels. 4-Hydroxyproline is a major part of collagen, with urinary levels thereof serving as a bone-resorption biomarker of collagen turnover (Barbul, 2008; Kuo and Chen, 2017). Hence, the observed elevation in 4-hydroxyproline may reflect increased collagen degradation, a phenomenon previously linked to chronic alcohol consumption (Preedy et al., 1991). Importantly, animals chronically consuming alcohol while on a KD, did not display any alterations in 4-hydroxyproline levels. Thus, a KD seems to play a protective and preventative role in alcohol-induced collagen degradation. However, further research is warranted, as we only assessed 4-hydroxyproline, while several other biomarkers of collagen turnover exist (Preedy et al., 1991).

4.2 TCA cycle metabolism

TCA cycle intermediates, malic acid ($p < 0.001$ and $d > 0.8$; Fig. 6A), fumaric acid ($p < 0.001$ and $d > 0.8$; Fig. 6B), succinic acid ($p < 0.01$ and $d > 0.8$; Fig. 6C), α -ketoglutaric acid ($p < 0.001$ and $d > 0.8$; Fig. 6D) and citric acid ($p < 0.001$ and $d > 0.8$; Fig. 6E) were significantly decreased in the KD and KD + Alc. groups compared to controls. It was also noted that for the KD + Alc. group, α -ketoglutaric acid and citric acid showed the greatest practical significance when the effect size was assessed compared to the control. Other studies using a KD have made similar observations and link this decrease in TCA cycle intermediates to the inhibitory effect of a KD on mTOR activity (Longo et al., 2019; Ramanathan & Schreiber, 2009). This corresponds to the mTOR inhibition we observed in mice on a KD via Western blotting. Additionally, chronic alcohol consumption did, however, seem to exacerbate citric acid, α -ketoglutaric acid and succinic acid depletion in mice on a KD. In accord, previous studies have also shown that chronic alcohol consumption can lead to decreased levels of TCA cycle intermediates (Chen et al., 2012). Hence, it is evident that the metabolism of the TCA cycle is significantly altered by a KD and altered to an even greater extent when alcohol is consumed in conjunction, which ultimately results in alterations of energy metabolism and the redox state (Auta et al., 2017).

4.3 Redox state evaluation via metabolic indicators of NADH/NAD⁺ ratio

To evaluate the effect that either a KD or chronic alcohol consumption has on the redox status in mice, metabolic indicators of NADH/NAD⁺ ratio were assessed in urine (Fig. 7), as it is difficult to detect NAD⁺ and NADH by conventional metabolomics (Smith et al., 2019). Metabolic indicators that are usually used include alanine, leucine, and isoleucine in relation to glutamate (Smith et al., 2019). Seeing that an elevated NADH/NAD⁺ ratio drives the formation of amino

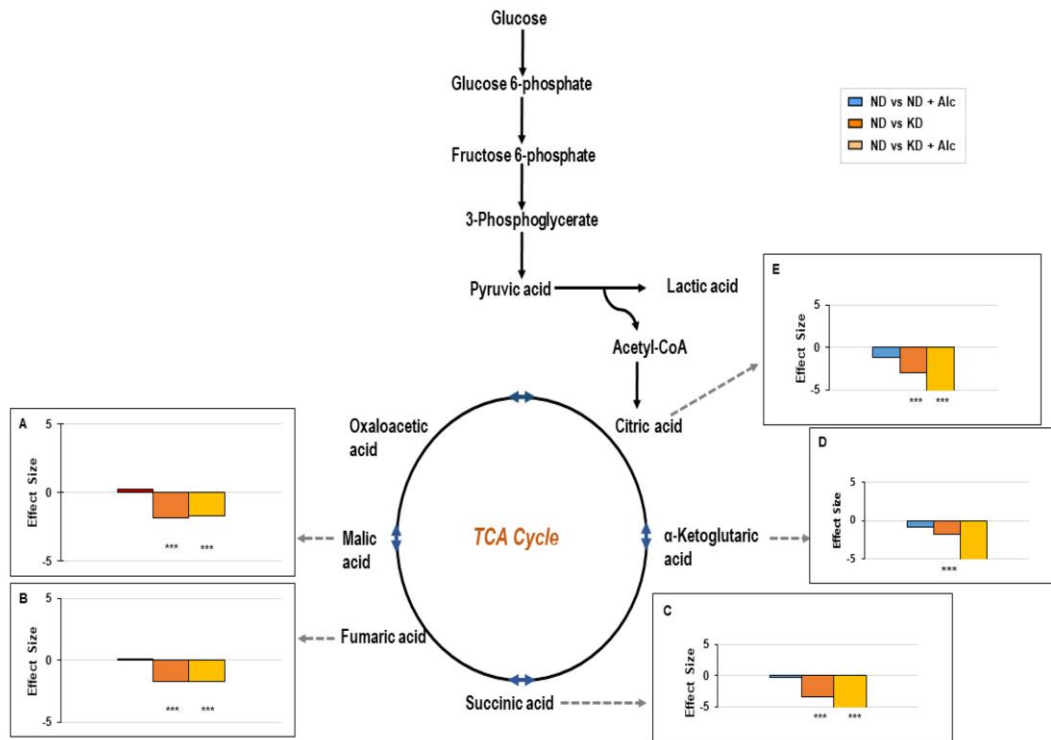


Fig. 6 Alterations of TCA cycle intermediates are more prominent with dietary interventions than chronic alcohol consumption when the different mouse groups ($n=6-10$ per group) were compared. Bar graphs reveal Cohen's d -values (effect size; $d>0.8$) for the significant metabolites when the relative abundance between the different

mice groups were compared to the control group (ND). ND indicates normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. *** $p<0.001$ vs. the control group, obtained via a post-hoc Tukey's test

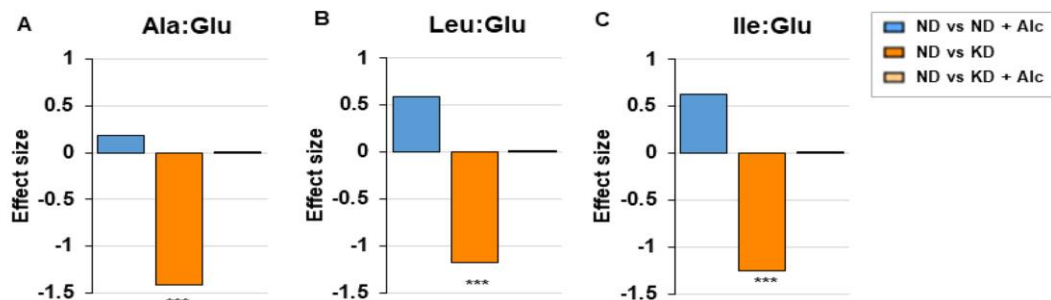


Fig. 7 A KD results in a more oxidative redox state in mice. Bar graphs (A–C) depict Cohen's d -values (effect size) of alterations in metabolic indicators of NADH/NAD⁺ ratio, when the different dietary groups were compared to controls. Positive and negative effect size values respectively indicate increased and decreased levels in the experimental group vs the control group. An elevated NADH/

NAD⁺ ratio drives the formation of amino acids from glutamic acid. Ala indicates alanine; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; ND, normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. Asterisks (*** $p \leq 0.001$) indicate significant differences between the ND vs KD group as determined by post-hoc Tukey's test

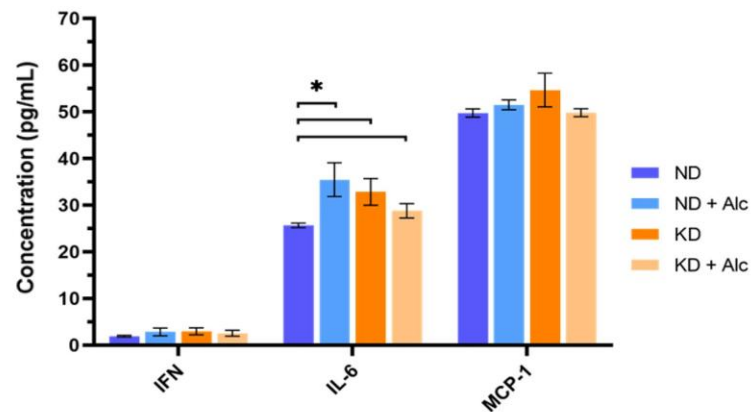
acids from glutamic acid (i.e., an increase in amino acid production from glutamic acid is correlated to increased NADH levels) (Smith et al., 2019), the ratio of various amino acids relative to glutamic acid were assessed. The ratios of alanine ($p < 0.001$; Fig. 8A), isoleucine ($p < 0.001$; Fig. 8B) and leucine ($p < 0.01$; Fig. 8C) relative to glutamic acid were significantly decreased in the group consuming a KD solely. The consumption of alcohol on a ND lead to trended higher levels of amino acid ratios, indicating a more reduced redox state in mice. However, no statistically significant differences were observed for the groups consuming alcohol on either the control diet (ND + Alc.) or a KD (KD + Alc.). Thus, it is evident from the mentioned findings that mice consuming a KD had a more oxidative redox state (i.e., a decreased level of NADH relative to NAD^+) compared to the control diet (ND), while the other groups had no significant alteration in redox state. The mentioned amino acid metabolite ratios indicated a significantly decreased NADH/ NAD^+ ratio in mice on a KD compared to controls (ND). Thus, confirming that a KD increases the availability of NAD^+ through altering the NADH/ NAD^+ ratio. This alteration in redox balance could be related to the observed perturbations in amino acid and energy metabolism in mice on a KD diet. No significant alterations in NADH/ NAD^+ ratio was seen following alcohol consumption in the normal diet group (ND + Alc.), indicating that diet alone (i.e., KD) had a greater impact on the redox state than alcohol consumption. However, chronic alcohol consumption along with a KD, prevents any favorable changes towards a decreased NADH/ NAD^+ ratio, which would be observed when a KD is consumed alone. Previous research has shown that the metabolism of alcohol results in redox state changes due to an increase in the NADH/ NAD^+ ratio (Cederbaum, 2012). On the contrary, other studies have shown that a KD can decrease the NADH/ NAD^+ ratio, thereby altering the redox state (Elamin et al., 2017; Xin et al., 2018). As such, our

findings provide insight with regards to changes in the redox state when alcohol is consumed together with a KD.

4.4 Inflammatory status of mouse serum

Several serum inflammatory biomarkers were measured in the different dietary groups. All experimental groups displayed significantly increased ($p < 0.05$; Fig. 8) levels of IL-6 relative to the control group. The ND + Alc. group had the highest increase in IL-6, followed by the KD group and then the KD + Alc. group. This finding suggests that both alcohol and a KD has a pro-inflammatory effect. However, no significant alterations in IFN or MCP-1 levels were observed among the dietary groups, while marked intragroup variation limited our use of the other measured inflammatory cytokines (Fig. S4). Although several studies have described the anti-inflammatory properties of a KD (Ciaffi et al., 2021; Dupuis et al., 2015), others have also observed signs of increased inflammation associated with a KD in mice (Garbow et al., 2011) and human subjects (Rosenbaum et al., 2019). Thus, no conclusive assumption can be made with regards to a KD and chronic alcohol consumption's impact on inflammation and further research is required. Moreover, seeing that existing literature also shows conflicting evidence with regards to the effect a KD and alcohol consumption has on inflammation (Ciaffi et al., 2021; Dupuis et al., 2015; Garbow et al., 2011; Rosenbaum et al., 2019). Considering our inconclusive observations, along with conflicting literature reports, further research on the inflammatory effects of a KD and alcohol consumption is warranted.

Fig. 8 The concentration levels (pg/mL) of pro-inflammatory biomarkers as measured in the serum of mice from all four dietary groups ($n = 6$ per group). The error bars represent the standard error. ND indicates normal diet; ND + Alc., normal diet and alcohol; KD, ketogenic diet; KD + Alc., ketogenic diet and alcohol. * $p < 0.05$ vs. the control group, obtained via a post-hoc Tukey's test,



5 Conclusion

The therapeutic potential of a KD and its mechanisms of action for conditions such as, epilepsy, obesity, diabetes, inflammation, and mitochondrial disease has gained immense attention in recent years (Vinnig, 1999; Qu et al., 2021; Bolla et al., 2019; Freeman et al., 2010; Dupuis et al., 2015; Jin et al., 2014). The regulatory effect that a KD has on the mTOR pathway is of great importance (Genzer et al., 2016) and has been evaluated in various disease models (Genzer et al., 2016; Johnson et al., 2013; McDaniel et al., 2011; Roberts et al., 2017; Singh et al., 2018; Tian et al., 2019; Xie et al., 2016; Zeng et al., 2008). Since alcohol consumption reportedly alters the cellular redox state and impedes mTOR phosphorylation (Feldstein & Bailey, 2011; Lang et al., 2003; Zakhari, 2006), we aimed to establish the effect that chronic alcohol consumption has on the mTOR pathway and systemic metabolism during a KD-based intervention. This was achieved by investigating the isolated and combined effects of chronic alcohol consumption and a KD on growth rate, S6K1 phosphorylation, urinary metabolism, metabolic indicators of redox state, and serum inflammatory markers in a healthy mouse model. This study, however, did have its limitations. Firstly, alcohol and food intake were not monitored accordingly when mice were given ad libitum access to alcohol drinking water and food. As such, it is possible that the mice on a KD and those on a KD with chronic alcohol consumption consumed less food than that of the control diet, especially since a KD has previously been linked to increased satiety and decreased food intake (Paoli et al., 2015). In addition, this could also lead to the mice consuming less vitamins and trace minerals, which in return could contribute to difference in growth (Churuangsuk et al., 2019; Kenig et al., 2019). Additionally, the KD used in this study was not an isocaloric KD, as the total calorie content was not identical for the ND and KD. Moreover, the KD used in this study was also more protein-restricted when compared to the ND. Lastly, consideration needs to be taken when a similar dietary strategy is applied to humans, since the mice were three weeks old when they were put on the dietary intervention. Children might respond differently than adults when put on a ketogenic dietary intervention (Kim et al., 2019).

In conclusion, this study shows that a KD attenuates mTOR signalling and weight gain, as well as decreases the excretion of amino acid and TCA cycle metabolites in mice. Also, the KD seems to drive energy metabolism towards a more oxidative redox state by increasing the NAD^+/NADH ratio through metabolic regulation. In addition, the study's results regarding a KD's effect

on inflammation were inconclusive due to limited sample volume and the fact that only one pro-inflammatory marker, IL-6, was significantly increased. As such, more research regarding a KD's effect on inflammation is warranted. Furthermore, we provide evidence that a KD could decrease or prevent bone loss and collagen degradation, by decreasing structural protein degradation even during chronic alcohol consumption. Although findings of previous studies have shown that a KD alone does not lead to potential bone loss and degradation (Bisschop et al., 2003), our study is the first to report that a KD could potentially reduce bone loss and collagen degradation during chronic alcohol consumption. As such, it is evident that a KD indeed has some therapeutic potential, especially for conditions that lead to an unfavourable redox state, increase in structural protein degradation, as well as disorders in which attenuation of mTOR leads to improved disease outcome. However, the potential benefits of a KD could be overturned when alcohol is consumed chronically in conjunction with a KD, as alcohol consumption seems to prevent the shift towards a more oxidative redox state and lead to a potential decrease in NAD^+ levels. Thus, any intervention using a KD for the management of conditions like obesity, diabetes, epilepsy, and other metabolic disorders, should take note of the factors that could potentially hinder the therapeutic effects of a KD, such as chronic alcohol consumption, especially where the redox state is of concern. Further research is warranted making use of multiple protein markers to monitor mTOR activity, as well as applying multi-platform metabolomics on other tissues such blood, brain, and muscle to evaluate tissue specific differences in systemic metabolism, to further assess the therapeutic potential of a KD. Lastly, any study looking at the effect of a KD should also consider the total protein and total caloric content of the diet, as well as the effects it has on the dietary intervention.

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Author contributions L.W. helped conceptualize the research; Methodology; Investigation, Formal analysis, Data curation, Writing—original draft, Visualisation. RL: Conceptualisation, Project administration, Supervision, Funding acquisition, Writing—review & editing, Visualisation. KT: Data curation, Writing—review & editing.

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Data availability The datasets are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no conflict of interest.

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References

- American Veterinary Medical Association. AVMA guidelines for the euthanasia of animals: 2020 edition. <https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf>; 2020 [accessed: 12 April 2020]
- Auta, J., Zhang, H., Pandey, S. C., & Guidotti, A. (2017). Chronic alcohol exposure differentially alters one-carbon metabolism in rat liver and brain. *Alcoholism, Clinical and Experimental Research*, 41(6), 1105–1111. <https://doi.org/10.1111/acer.13382>
- Barbul, A. (2008). Proline precursors to sustain mammalian collagen synthesis. *The Journal of Nutrition*, 138(10), 2021S–2024S. <https://doi.org/10.1093/jn/138.10.2021S>
- Bernal, C. A., Vazquez, J. A., & Adibi, S. A. (1993). Leucine metabolism during chronic ethanol consumption. *Metabolism*, 42(9), 1084–1086. [https://doi.org/10.1016/0026-0495\(93\)90262-M](https://doi.org/10.1016/0026-0495(93)90262-M)
- Bisschop, P. H., Sain-van der Velden, M. G. M., Stellaard, F., Kuipers, F., Meijer, A. J., Sauerwein, H. P., & Romijn, J. A. (2003). Dietary Carbohydrate deprivation increases 24-hour nitrogen excretion without affecting postabsorptive hepatic or whole body protein metabolism in healthy men. *The Journal of Clinical Endocrinology & Metabolism*, 88(8), 3801–3805. <https://doi.org/10.1210/jc.2002-021087>
- Blanco-Gandía, M. D. C., Ródenas-González, F., Pascual, M., Reguilón, M. D., Guerri, C., Miñarro, J., & Rodríguez-Arias, M. (2021). Ketogenic diet decreases alcohol intake in adult male mice. *Nutrients*. <https://doi.org/10.3390/nu13072167>
- Bolla, A. M., Caretto, A., Laurenzi, A., Scavini, M., & Piemonti, L. (2019). Low-carb and ketogenic diets in type 1 and type 2 diabetes. *Nutrients*, 11(5), 962. <https://doi.org/10.3390/nu11050962>
- Bornebusch, A. B., Mason, G. F., Tonetto, S., Damsgaard, J., Gjedde, A., Fink-Jensen, A., & Thomsen, M. (2021). Effects of ketogenic diet and ketone monoester supplement on acute alcohol withdrawal symptoms in male mice. *Psychopharmacology (berl)*, 238(3), 833–844. <https://doi.org/10.1007/s00213-020-05735-1>
- Bueno, N. B., Melo, I. S. V., de Oliveira, S. L., & da Rocha Ataíde, T. (2013). Very-low-carbohydrate ketogenic diet v low-fat diet for long-term weight loss: a meta-analysis of randomised controlled trials. *British Journal of Nutrition*, 110(7), 1178–1187. <https://doi.org/10.1017/S0007114513000548>
- Cech, N. B., & Enke, C. G. (2001). Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrometry Reviews*, 20(6), 362–387. <https://doi.org/10.1002/mas.10008>
- Chen, W. X., Xu, C. Q., Chen, S. H., Xu, G. Y., & Ye, H. Z. (2012). Citric acid reduces the decline in P300 amplitude induced by acute alcohol consumption in healthy adults. *Journal of Zhejiang University. Science. B*, 13(5), 395–401. <https://doi.org/10.1631/jzus.B1100230>
- Churuangsu, C., Griffiths, D., Lean, M. E. J., & Combet, E. (2019). Impacts of carbohydrate-restricted diets on micronutrient intakes and status: A systematic review. *Obesity Reviews*, 20(8), 1132–1147. <https://doi.org/10.1111/obr.12857>
- Ciaffi, J., Mitselman, D., Mancarella, L., Brusi, V., Lisi, L., Ruscitti, P., & Ursini, F. (2021). The effect of ketogenic diet on inflammatory arthritis and cardiovascular health in rheumatic conditions: A mini review. *Frontiers*. <https://doi.org/10.3389/fmed.2021.792846>
- Dencker, D., Molander, A., Thomsen, M., Schlumberger, C., Wortwein, G., Weikop, P., & Fink-Jensen, A. (2018). Ketogenic diet suppresses alcohol withdrawal syndrome in rats. *Alcoholism, Clinical and Experimental Research*, 42(2), 270–277. <https://doi.org/10.1111/acer.13560>
- Dupuis, N., Curatolo, N., Benoist, J. F., & Auvin, S. (2015). Ketogenic diet exhibits anti-inflammatory properties. *Epilepsia*, 56(7), e95–98. <https://doi.org/10.1111/epi.13038>
- Elamin, M., Ruskin, D. N., Masino, S. A., & Sacchetti, P. (2017). Ketone-based metabolic therapy: Is increased NAD(+) a primary mechanism? *Frontiers in Molecular Neuroscience*, 10, 377. <https://doi.org/10.3389/fnmol.2017.00377>
- Ellis, S. M., & Steyn, H. S. (2003). Practical significance (effect sizes) versus or in combination with statistical significance (p-values): Research note. *Journal of Southern African Studies*, 12(4), 51–53. <https://doi.org/10.10520/EJC69666>
- Feldstein, A. E., & Bailey, S. M. (2011). Emerging role of redox dysregulation in alcoholic and nonalcoholic fatty liver disease. *Antioxidants & Redox Signaling*, 15(2), 421–424. <https://doi.org/10.1089/ars.2011.3897>
- Foster, D. A. (2010). Reduced mortality and moderate alcohol consumption: the phospholipase D-mTOR connection. *Cell Cycle*, 9(7), 1291–1294. <https://doi.org/10.4161/cc.9.7.11145>
- Freeman, J., & Kossoff, E. (2010). Ketosis and the ketogenic diet, 2010: Advances in treating epilepsy and other disorders. *Advances in Pediatrics*, 57, 315–329. <https://doi.org/10.1016/j.yapd.2010.08.003>
- Garbow, J. R., Doherty, J. M., Schugar, R. C., Travers, S., Weber, M. L., Wentz, A. E., & Crawford, P. A. (2011). Hepatic steatosis, inflammation, and ER stress in mice maintained long term on a very low-carbohydrate ketogenic diet. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 300(6), G956–967. <https://doi.org/10.1152/ajpgi.00539.2010>
- Genzer, Y., Dadon, M., Burg, C., Chapnik, N., & Froy, O. (2016). Effect of dietary fat and the circadian clock on the expression of brain-derived neurotrophic factor (BDNF). *Molecular and Cellular Endocrinology*, 430, 49–55. <https://doi.org/10.1016/j.mce.2016.04.015>
- Griffin, J. L., & Nicholls, A. W. (2006). Metabolomics as a functional genomic tool for understanding lipid dysfunction in diabetes, obesity and related disorders. *Pharmacogenomics*, 7(7), 1095–1107. <https://doi.org/10.2217/14622416.7.7.1095>
- Hay, N., & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes and Development*, 18(16), 1926–1945. <https://doi.org/10.1101/gad.1212704>
- Hsu, F.-Y., Liou, J.-Y., Tang, F.-Y., Sou, N.-L., Peng, J.-H., & Chiang, E.-P.I. (2022). Ketogenic diet consumption inhibited mitochondrial one-carbon metabolism. *International Journal of Molecular Sciences*, 23(7), 3650. <https://doi.org/10.3390/ijms23073650>
- Hu, S., Wang, L., Yang, D., Li, L., Togo, J., Wu, Y., & Wang, G. J.Cm. (2018). Dietary fat, but not protein or carbohydrate regulates energy intake and causes adiposity in mice. *Cell Metabolism*, 28(3), 415–431. <https://doi.org/10.1016/j.cmet.2018.06.010>
- Huang, C., Titus, J. A., Bell, R. L., Kapros, T., Chen, J., & Huang, R. (2012). A mouse model for adolescent alcohol abuse: Stunted

- growth and effects in brain. *Alcoholism, Clinical and Experimental Research*, 36(10), 1728–1737. <https://doi.org/10.1111/j.1530-0277.2012.01759.x>
- Ikenoue, T., Hong, S., & Inoki, K. (2009). Monitoring mammalian target of rapamycin (mTOR) activity. *Methods in Enzymology*, 452, 165–180. [https://doi.org/10.1016/S0076-6879\(08\)03611-2](https://doi.org/10.1016/S0076-6879(08)03611-2)
- Jewell, J. L., & Guan, K.-L. (2013). Nutrient signaling to mTOR and cell growth. *Trends in Biochemical Sciences*, 38(5), 233–242. <https://doi.org/10.1016/j.tibs.2013.01.004>
- Jin, Z., Berthiaume, J. M., Li, Q., Henry, F., Huang, Z., Sadhukhan, S., & Zhang, G. F. (2014). Catabolism of (2E)-4-hydroxy-2-nonenal via ω - and ω -1-oxidation stimulated by ketogenic diet. *Journal of Biological Chemistry*, 289(46), 32327–32338. <https://doi.org/10.1074/jbc.M114.602458>
- Johnson, S. C., Yanos, M. E., Kayser, E. B., Quintana, A., Sangesland, M., Castanza, A., & Kaerberlein, M. (2013). mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. *Science*, 342(6165), 1524–1528. <https://doi.org/10.1126/science.1244360>
- Kenig, S., Petelin, A., Poklar Vatovec, T., Mohorko, N., & Jenko-Pražnikar, Z. (2019). Assessment of micronutrients in a 12-wk ketogenic diet in obese adults. *Nutrition*, 67–68, 110522. <https://doi.org/10.1016/j.nut.2019.06.003>
- Kim, S. H., Shaw, A., Blackford, R., Lowman, W., Laux, L. C., Millichap, J. J., & Nordli, D. R., Jr. (2019). The ketogenic diet in children 3 years of age or younger: A 10-year single-center experience. *Science and Reports*, 9(1), 8736. <https://doi.org/10.1038/s41598-019-45147-6>
- Kruse, S. E., Watt, W. C., Marcinek, D. J., Kapur, R. P., Schenkman, K. A., & Palmiter, R. D. (2008). Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metabolism*, 7(4), 312–320. <https://doi.org/10.1016/j.cmet.2008.02.004>
- Kuo, T.-R., & Chen, C.-H. (2017). Bone biomarker for the clinical assessment of osteoporosis: Recent developments and future perspectives. *Biomarker Research*, 5, 18–18. <https://doi.org/10.1186/s40364-017-0097-4>
- Lang, C. H., Frost, R. A., Deshpande, N., Kumar, V., Vary, T. C., Jefferson, L. S., & Kimball, S. R. (2003). Alcohol impairs leucine-mediated phosphorylation of 4E-BP1, S6K1, eIF4G, and mTOR in skeletal muscle. *American Journal of Physiology Endocrinology and Metabolism*, 285(6), E1205–E1215. <https://doi.org/10.1152/ajpendo.00177.2003>
- Lang, C. H., Lynch, C. J., & Vary, T. C. (2010). Alcohol-induced IGF-I resistance is ameliorated in mice deficient for mitochondrial branched-chain aminotransferase. *The Journal of Nutrition*, 140(5), 932–938. <https://doi.org/10.3945/jn.109.120501>
- Lapante, M., & Sabatini, D. M. (2012). mTOR Signaling in growth control and disease. *Cell*, 149(2), 274–293. <https://doi.org/10.1016/j.cell.2012.03.017>
- Lapante, M., & Sabatini, D. M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *Journal of Cell Science*, 126(Pt 8), 1713–1719. <https://doi.org/10.1242/jcs.125773>
- Longo, R., Peri, C., Crici, D., Coppi, L., Caruso, D., Mitro, N., & Crestani, M. (2019). Ketogenic diet: A new light shining on old but gold biochemistry. *Nutrients*, 11(10), 2497. <https://doi.org/10.3390/nu11102497>
- Mason, S., Terburgh, K., & Louw, R. (2018). Miniaturized (1)H-NMR method for analyzing limited-quantity samples applied to a mouse model of Leigh disease. *Metabolomics*, 14(6), 74. <https://doi.org/10.1007/s11306-018-1372-6>
- McDaniel, S., Rensing, N., Yamada, K., & Wong, M. (2011). The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. *Epilepsia*, 52, e7–11. <https://doi.org/10.1111/j.1528-1167.2011.02981.x>
- Morita, M., Gravel, S. P., Chénard, V., Sikström, K., Zheng, L., Alain, T., & Sonenberg, N. (2013). mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. *Cell Metabolism*, 18(5), 698–711. <https://doi.org/10.1016/j.cmet.2013.10.001>
- Morita, M., Gravel, S. P., Hulea, L., Larsson, O., Pollak, M., St-Pierre, J., & Topisirovic, I. (2015). mTOR coordinates protein synthesis, mitochondrial activity and proliferation. *Cell Cycle*, 14(4), 473–480. <https://doi.org/10.4161/15384101.2014.991572>
- Paoli, A. (2014). Ketogenic diet for obesity: Friend or foe? *International Journal of Environmental Research and Public Health*, 11(2), 2092–2107. <https://doi.org/10.3390/ijerph110202092>
- Paoli, A., Bosco, G., Camporesi, E. M., & Mangar, D. (2015). Ketosis, ketogenic diet and food intake control: A complex relationship. *Frontiers in Psychology*, 6, 27. <https://doi.org/10.3389/fpsyg.2015.00027>
- Perides, G., Tao, X., West, N., Sharma, A., & Steer, M. L. (2005). A mouse model of ethanol dependent pancreatic fibrosis. *Gut*, 54(10), 1461–1467. <https://doi.org/10.1136/gut.2004.062919>
- Pissios, P., Hong, S., Kennedy, A. R., Prasad, D., Liu, F. F., & Maratos-Flier, E. (2013). Methionine and choline regulate the metabolic phenotype of a ketogenic diet. *Mol Metab*, 2(3), 306–313. <https://doi.org/10.1016/j.molmet.2013.07.003>
- Preedy, V. R., Baldwin, D. R., Keating, J. W., & Salisburry, J. R. (1991). Bone collagen, mineral and trace element composition, histomorphometry and urinary hydroxyproline excretion in chronically-treated alcohol-fed rats. *Alcohol and Alcoholism*, 26(1), 39–46.
- Qu, C., Keijer, J., Adjubo-Hermans, M. J. W., van de Wal, M., Schirris, T., van Karnebeek, C., & Koopman, W. J. H. (2021). The ketogenic diet as a therapeutic intervention strategy in mitochondrial disease. *The International Journal of Biochemistry & Cell Biology*, 138, 106050. <https://doi.org/10.1016/j.biocel.2021.106050>
- Ramanathan, A., & Schreiber, S. L. (2009). Direct control of mitochondrial function by mTOR. *Proc Natl Acad Sci U S A*, 106(52), 22229–22232. <https://doi.org/10.1073/pnas.0912074106>
- Rehm, J., & Imtiaz, S. (2016). A narrative review of alcohol consumption as a risk factor for global burden of disease. *Substance Abuse Treatment, Prevention, and Policy*, 11(1), 37. <https://doi.org/10.1186/s13011-016-0081-2>
- Roberts, M. N., Wallace, M. A., Tomilov, A. A., Zhou, Z., Marcotte, G. R., Tran, D., & Lopez-Dominguez, J. A. (2017). A ketogenic diet extends longevity and healthspan in adult mice. *Cell Metabolism*, 26(3), 539–546.e535. <https://doi.org/10.1016/j.cmet.2017.08.005>
- Rosenbaum, M., Hall, K. D., Guo, J., Ravussin, E., Mayer, L. S., Reitman, M. L., & Leibel, R. L. (2019). Glucose and lipid homeostasis and inflammation in humans following an isocaloric ketogenic diet. *Obesity*, 27(6), 971–981. <https://doi.org/10.1002/oby.22468>
- Ruoppolo, M., Caterino, M., Albano, L., Pecce, R., Di Girolamo, M. G., Crisci, D., & Campesi, I. (2018). Targeted metabolomic profiling in rat tissues reveals sex differences. *Scientific Reports*, 8(1), 4663. <https://doi.org/10.1038/s41598-018-22869-7>
- Santos, F. L., Esteves, S. S., da Costa Pereira, A., Yancy, W. S., Jr., & Nunes, J. P. (2012). Systematic review and meta-analysis of clinical trials of the effects of low carbohydrate diets on cardiovascular risk factors. *Obesity Reviews*, 13(11), 1048–1066. <https://doi.org/10.1111/j.1467-789X.2012.01021.x>
- Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., & Hollender, J. (2014). Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science and Technology*, 48(4), 2097–2098. <https://doi.org/10.1021/es5002105>
- Singh, A., Mettler, T., Oh, H., & Kim, D.-Y. (2018). The ketogenic diet attenuates both hyperactivity in mTOR pathway and astrogliosis through regulation of AMPK signaling in the epileptic brain. *The FASEB Journal*, 32(8), 811–805. <https://doi.org/10.1096/fasebj.2018.32.1supplement.805.11>

- Smith, H. Q., Li, C., Stanley, C. A., & Smith, T. J. (2019). Glutamate dehydrogenase, a complex enzyme at a crucial metabolic branch point. *Neurochemical Research*, 44(1), 117–132. <https://doi.org/10.1007/s11064-017-2428-0>
- Sperl, W. (1997). Diagnosis and therapy of mitochondriopathies. *Wiener Klinische Wochenschrift*, 109(3), 93–99.
- Terburgh, K., Lindeque, J. Z., van der Westhuizen, F. H., & Louw, R. (2021). Cross-comparison of systemic and tissue-specific metabolomes in a mouse model of Leigh syndrome. *Metabolomics*, 17(12), 101. <https://doi.org/10.1007/s11306-021-01854-8>
- Terburgh, K., Lindeque, Z., Mason, S., van der Westhuizen, F., & Louw, R. (2019). Metabolomics of Ndufs4^{-/-}-skeletal muscle: Adaptive mechanisms converge at the ubiquinone-cycle. *Biochimica Et Biophysica Acta (BBA)—Molecular Basis of Disease*, 1865(1), 98–106. <https://doi.org/10.1016/j.bbadis.2018.10.034>
- Tian, T., Li, X., & Zhang, J. (2019). mTOR signaling in cancer and mTOR inhibitors in solid tumor targeting therapy. *International Journal of Molecular Sciences*, 20(3), 755. <https://doi.org/10.3390/ijms20030755>
- Traversy, G., & Chaput, J.-P. (2015). Alcohol consumption and obesity: An update. *Current Obesity Reports*, 4(1), 122–130. <https://doi.org/10.1007/s13679-014-0129-4>
- Valsecchi, F., Koopman, W. J., Manjeri, G. R., Rodenburg, R. J., Smeitink, J. A., & Willems, P. H. (2010). Complex I disorders: Causes, mechanisms, and development of treatment strategies at the cellular level. *Developmental Disabilities Research Reviews*, 16(2), 175–182. <https://doi.org/10.1002/ddrr.107>
- van de Wal, M. A. E., Adjobo-Hermans, M. J. W., Keijer, J., Schirris, T. J. J., Homberg, J. R., Wiecekowsky, M. R., & Koopman, W. J. H. (2022). Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention. *Brain*, 145(1), 45–63. <https://doi.org/10.1093/brain/awab426%JBRAIN>
- Veech, R. L., Guynn, R., & Veloso, D. (1972). The time-course of the effects of ethanol on the redox and phosphorylation states of rat liver. *The Biochemical Journal*, 127(2), 387–397. <https://doi.org/10.1042/bj1270387>
- Vining, E. P. (1999). Clinical efficacy of the ketogenic diet. *Epilepsy Research*, 37(3), 181–190. [https://doi.org/10.1016/s0920-1211\(99\)00070-4](https://doi.org/10.1016/s0920-1211(99)00070-4)
- Wiers, C. E., Vendruscolo, L. F., van der Veen, J. W., Manza, P., Shokri-Kojori, E., Kroll, D. S., & Volkow, N. D. (2021). Ketogenic diet reduces alcohol withdrawal symptoms in humans and alcohol intake in rodents. *Science Advances*. <https://doi.org/10.1126/sciadv.abf6780>
- Wullschlegel, S., Loewith, R., & Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell*, 124(3), 471–484. <https://doi.org/10.1016/j.cell.2006.01.016>
- Xia, J., Sinelnikov, I. V., Han, B., & Wishart, D. S. (2015). MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Research*, 43(W1), W251–257. <https://doi.org/10.1093/nar/gkv380>
- Xie, J., Wang, X., & Proud, C. G. (2016). mTOR inhibitors in cancer therapy. *F1000Research*. <https://doi.org/10.12688/f1000research.9207.1>
- Xin, L., Ipek, O., Beaumont, M., Shevlyakova, M., Christinat, N., Masoodi, M., & Cuenoud, B. (2018). Nutritional ketosis increases NAD(+)/NADH ratio in healthy human brain: An in vivo study by (31)P-MRS. *Frontiers in Nutrition*, 5, 62. <https://doi.org/10.3389/fnut.2018.00062>
- Zakhari, S. (2006). Overview: How is alcohol metabolized by the body? *Alcohol Research & Health*, 29(4), 245–254.
- Zeng, L. H., Xu, L., Gutmann, D. H., & Wong, M. (2008). Rapamycin prevents epilepsy in a mouse model of tuberous sclerosis complex. *Annals of Neurology*, 63(4), 444–453. <https://doi.org/10.1002/ana.21331>
- Zhao, D., Yang, J., & Yang, L. (2017). Insights for oxidative stress and mTOR signaling in myocardial ischemia/reperfusion injury under diabetes. *Oxidative Medicine and Cellular Longevity*, 2017, 6437467. <https://doi.org/10.1155/2017/6437467>

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APPENDIX C

Chapter 4: Supplementary information

A ketogenic diet alters mTOR activity, systemic metabolism and potentially prevents collagen degradation associated with chronic alcohol consumption in mice

Luciano Willemse¹, Karin Terburgh¹, Roan Louw¹

¹Human Metabolomics, Faculty of Natural and Agricultural Sciences, North-West University (Potchefstroom Campus), South Africa

C. Supplemental information

C.1. Materials and methods

C.1.1. Dietary composition

Table C-1: Nutritional profile and ingredient composition of the control and ketogenic diets

A		
Control diet nutritional value	"As is" (g/kg)	DM (g/kg)
Protein (min)	220	240
Moisture (max)	100	-
Oils and fats (min)	50	55
Linoleic acid (min)	12	14
Fiber (max)	40	45
Ash (max)	70	75
Ca:P ratio	1.1-2:1	1.1-2:1
Calcium (min)	12	14
Phosphorous (min)	7.5	8
Vitamin A (min)	16 000 (IU/kg)	16 000 (IU/kg)
Vitamin D (min)	2000 (IU/kg)	2000 (IU/kg)
Vitamin E (min)	100 (g/kg)	100 (g/kg)
Ingredients: Maize, wheat bran, soybean, soybean protein concentrate, fish meal, maize protein concentrate molasses, sucrose, calcium carbonate, sodium chloride, calcium phosphate		
B		
Ketogenic diet nutritional value	"As is" (g/kg)	DM (g/kg)
Protein (min)	121.5	135
Oils and fats (min)	750	790
Linoleic acid (min)	320	360
Fiber (max)	55	60
Ca:P ratio	1.1-2:1	1.1-2:1
Calcium (min)	12	14
Phosphorous (min)	7.5	8
Vitamin A (min)	16 000 (IU/kg)	16 000 (IU/kg)
Vitamin D (min)	2000 (IU/kg)	2000 (IU/kg)
Vitamin E (min)	100 (g/kg)	100 (g/kg)
Ingredients: Palm oil, butter, canola oil, casein, cellulose, dextrose, vitamin & mineral mix		
C		
	Control diet (%cal per 100g)	Ketogenic diet (%cal per 100g)
Protein	20	8.2
Fat	5	91.7
Carbohydrate	75	<0.1

Abbreviations: Cal, calories; DM, dry matter; g/kg, gram per kilogram.

C.1.2. SDS-PAGE analysis and western blotting

Liver tissue was homogenized in HEPES lysis (~1 mL per 100mg tissue) buffer via a 10 mL Glass/Teflon® Tight Fitting Potter-Elvehjem Homogeniser (Glas-Col, USA) using 15 strokes. Following homogenisation, the liver homogenates were centrifuged at 1000 x g for 10 min at 4 °C with a Heraeus™ Multifuge™ X3 centrifuge (Thermo Fisher Scientific, USA). Subsequently, the supernatants were collected, and the protein concentrations were determined with a BCA protein assay. Samples were prepped for SDS-PAGE by mixing the sample with 4X laemmli sample buffer and milli-Q water to a final volume of 25 µL, which was then boiled at 100°C for 5 min. For each dietary group, aliquots (20 µg) of the protein were separated via SDS-PAGE at 300V for 22 min using a 10% acrylamide gel, then transferred to nitrocellulose membranes (cat #230-4, Bio-Rad, USA), and blocked using 1% casein in TBST buffer (cat #161-0782, Bio-Rad, USA) for 1 h. After that, the membranes were incubated with phospho-p70 S6 kinase primary antibodies (cat #9234S, Cell Signalling Technologies, USA) and the control protein, β-actin (cat # ab6276, Abcam, UK), at 4 °C overnight. Afterwards, the membranes were incubated with secondary antibodies (cat # ab97051, Abcam, UK) at room temperature for 1 h. Then, chemiluminescent detection was achieved using a Western ECL substrate (cat #170-5060, Bio-Rad, USA) and the ChemiDoc™ MP imaging system (Bio-Rad, USA). Finally, the results were analysed via Image Lab™ v. 6.1 software (Bio-Rad, USA) in order to attain the density ratio of the target proteins relative to β-actin.

C.1.3. Metabolomics**C.1.3.1. Determination of urinary creatinine concentration**

Spectrophotometry was used to measure urinary creatinine via the Jaffe reaction method, which was adapted from the manufacturing protocol of the QuantiChrom™ Creatinine Assay Kit (DICT-500, BioAssay Systems, USA). In this assay, a reaction takes place between creatinine and picric acid, while in an alkaline solution forming a yellow orange adduct, of which the increase in absorbance is measured at wavelength of 510 nm. 10 µl of urine was mixed with a 200 µl working solution consisting of 0.1 M sodium hydroxide (NaOH), 0.3 mM ethylenediaminetetraacetic acid (EDTA); 0.1% (v/v) DMSO; 0.0004% (v/v) Tween®20, and 3 mM picric acid. The assay was done in triplicate using a 96-well plate. Consequently, samples were then analysed using a Synergy™ HT Multi-detection microplate reader by measuring the linearity of the increase in absorbance at 510 nm for 5 min, in 1 min intervals. Lastly, absolute quantities were then calculated using a standard curve that ranged from 0-50 mg%. All urine samples used in multi-platform metabolomic assays creatinine concentrations were determined on two 96-well plates.

C.1.3.2. Quality control samples

Urinary quality control (QC) samples were prepared for each mice group (ND, ND + Alc., KD and KD + Alc.) by pooling equal amounts of the relevant groups' sample extracts together. Afterwards, the QC samples were aliquoted, stored and prepared by the same means as the other samples for each analytical platform respectively. QC samples were analysed in the beginning, after every 6

(GC-TOF-MS) or 5 (LC-MS/MS) samples, and at the end of each batch, for both GC-TOF-MS and LC-MS/MS analyses. Also, an in-house standard mixture (which contained all the metabolites to be analysed) including a QC sample spiked with this mixture, was analysed at the end of each batch during LC-MS/MS analysis.

C.1.3.3. LC-MS/MS sample preparation and analysis

For LC-MS/MS sample preparation, a pre-planned volume (20 μ L to 250 μ L) of urine containing 0.0625 μ mole creatinine, as well as internal standards and isotopes were evaporated under a stream of nitrogen gas at 37 °C. For sample derivatization, N-butanol:acetyl chloride (~300 μ L) was added to each sample and which were then incubated at 50 °C for 1 h. Afterward, the butylated samples were dried under a stream of nitrogen gas at 37 °C. Lastly, the dried samples were reconstituted in a final volume of water:acetonitrile (50:50) (v/v) consisting of 0.1 % formic acid (50 μ L). Following this, the samples were then thoroughly vortexed to dissolve the dried compounds. The final volume of the prepped urine samples was then transferred to 250 μ L pulled point glass inserts. Finally, all the sample vials were loaded onto an Agilent© 1200 series auto sampler for LC-MS/MS analysis. LC-MS/MS analysis was performed as described by Mels et al. (2011) with slight adjustments. For LC-MS/MS analyses of amino acid-, carnitine- and acylcarnitine butyl esters a 1200 series liquid chromatograph coupled to a 6410-triple quadrupole-mass spectrometer (Agilent Technologies, USA) was used. The chromatographic separation was accomplished by injecting samples (1 μ L) on a C18 Zorbax SB-Aq (150 mm x 2.1 mm x 1.8 μ m) reverse phase column (Agilent Technologies), kept at 35°C, with a flow rate of 0.2 ml/min during the entire analysis run. Additionally, a guard column was also attached to the column to prevent entry of particulate matter. Furthermore, the mobile phases, which consisted of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) were used. The chromatographic gradient started at a 5% mobile phase B and was maintained for 1 min, it was then followed with an increase to 23% of mobile phase B over a duration of 7 min. In addition, an isocratic hold of around 4 min was maintained at a 23% mobile phase B. Subsequently, mobile phase B was increased to 100% at 15 min and was kept there for 5 min. Lastly, the gradient declined to a 5% mobile phase B after 1 min and was maintained for 7 min (post-run) to guarantee that the column is in equilibrium before proceeding to the next analysis. The effluent coming from the liquid chromatograph was then diverted to the mass spectrometer, which made use of the positive electrospray ionisation (ESI) mode using the following source parameters: capillary voltage of 3 500 V; drying gas (nitrogen) at a flow rate of 7.5 l/min at 300°C; and nebuliser pressure of 30 psi. The spectra were acquired via the multiple reaction monitoring (MRM) mode with an electron multiplier voltage (EMV) of 300 V and a dwell time of 45 ms for all compounds. During the analysis, three-time windows were used. The first window diverted eluent prior to the detection of the first analyte (< 1.4 min)] away from the column and towards the waste to limit the exposure of the mass spectrometer to salts in the sample. The MRMs of all the butyl esters were distributed among the

final two-time windows, thereby ensuring sufficient data points per compound peak. Last of all, Agilent®'s MassHunter Workstation Software (v B02.01; Data acquisition for 6400 Series Triple Quadrupole) and MassHunter Optimizer software (v B02.01) were used for data acquisition (with MRM configuration settings), while data extraction was accomplished using Agilent®'s MassHunter Workstation software (v B06.00; Qualitative Analysis and Quantitative Analysis).

C.1.3.4. GC-TOF-MS sample preparation and analysis

For GC-TOF-MS sample preparation (20 µl to 250 µl) of urine containing 0.0625 µmole creatinine, as well as internal standards dried under a stream of nitrogen gas at 37°C. Derivatization of the samples was accomplished through oximation and silylation. For oximation, methoxyamine hydrochloride (20 mg/mL in pyridine, 50 µL) was added and the samples were subsequently vortexed for 1 min to dissolve the dried compounds. Afterwards, the samples were then incubated at 60 °C for 1 hour. Next, the samples were silylated by adding BSTFA (50 µL) containing 1 % (v/v) TMCS and incubated at 60 °C for 1 hour. The final sample volume (100 µL) for each sample was then transferred to 250 µL pulled point glass inserts. Finally, each vial was loaded onto via a 7890A gas chromatograph (Agilent Technologies, USA) with an Agilent® 7693 autosampler coupled to a LECO Pegasus HT time-of-flight mass spectrometer with an electron impact (EI) ionisation source.

The GC-TOF-MS protocol described by Lindeque et al. (2013) was used for this study. The samples (1 µL) were then injected using the spitless mode *and* a Phenomenex Zebron GC FocusLiner® Inlet Liner for Agilent (split/splitless, single taper w/wool, 4 mm ID x 78.5 mm L x 6.3 mm OD) was fitted on the GC system. Throughout the entire analysis, the front inlet's temperature was kept at 250 °C and the carrier gas (helium) was kept at a constant flow rate of 1.5 mL/min. A Restek Rxi-5Sil MS (30m x 250 µm x 0.25 µm) column was used for chromatography analysis and the initial GC oven temperature was held at 40 °C for 1 min. Afterwards, the oven temperature was then increased by 7 °C/min until reaching 120 °C, followed by 10 °C/min until reaching 230 °C and finally 13 °C/min until it reached 300 °C, which was then maintained at that temperature for 2.5 min before being cooled to 40 °C with a total run time of ~ 35 min. During the entire analysis, the transfer line and ion source temperatures were respectively maintained at 225 °C and 200 °C. For the detection of metabolites, mass spectrometry was operated using electron impact (EI) ionisation (- 70 V) mode which allowed for the fragmentation of all eluting compounds. Using a solvent delay of 480 seconds, data was acquired at a rate of 20 spectra/second with a mass-to-charge (m/z) ratio scan range of 50–950 amu with a detector voltage of ~1 500 V. Peaks were detected by 5 apexing masses using a peak width of 3 seconds and a signal-to-noise (S/N) ratio of > 20. For data acquisition and extraction, the LECO Corporation ChromaTOF software (v 4.5x) was used. The software's Statistical Compare feature was used to align peaks, by automatic baseline removal via the "spanning" tracking method (offset of 1; just above the noise) as well as auto smoothing. Spectral matching was carried making use of the NIST11 commercial- as well as an in-house mass

spectral library to identify important analytes. For metabolite identification, an identification confidence level was ascribed to each feature as described by Schymanski et al. (2014). All the features that were unidentified were excluded from the dataset. Features were considered as a Level 3 if a tentative structure was obtained (based on either matching intact molecular mass or fragmentation spectra). In contrast, Level 2 features had greater structure identity based on additional evidence. For features labelled as Level 1, the structure was identified with the spectral and retention time corresponding with a reference standard.

C.1.3.5. Measuring inflammatory biomarkers in mice serum

The concentration levels of pro-inflammatory biomarkers of IL6, IL10, MCP1, TNF, IFN- γ and IL12 were measured using the BD Cytometric bead array (CBA) mouse inflammation kit (BD Biosciences, cat# 552364) according to the protocol of the kit, which made use of a multiplex bead array assay coupled with flow cytometry. The samples as well as standards were analysed via a BD Accuri C6 flow cytometer. Afterwards, standard curves were produced and then regression analysis was applied using FlowJo (version 10.8) and FCS Express software (De Novo, version 6) to determine the concentrations of the biomarkers (in pg/ml) of the mice serum samples.

C.1.3.6. Data processing and normalisation

For data processing and normalisation, in-house protocols were used along with the relevant literature recommendations. The stages that were followed are summarised in Figure C-1 and include: 1) The removal of inconsistent features; 2) Elimination of contaminant peaks; 3) Removal of inconsistent samples; 4) The use of internal standards for post-analysis normalisation, to minimise systematic and technical variations; 5) Removal of inconsistent features making use of QC samples; and 6) Missing value imputation (MVI) to replace missing values with a non-zero value (while maintaining the data structure). Following this, data was transformed (glog) to improve the data set normality for greater comparability of metabolite intensities between and within samples before applying statistical analysis. Lastly, the removal of outlier samples was also done with the data sets.

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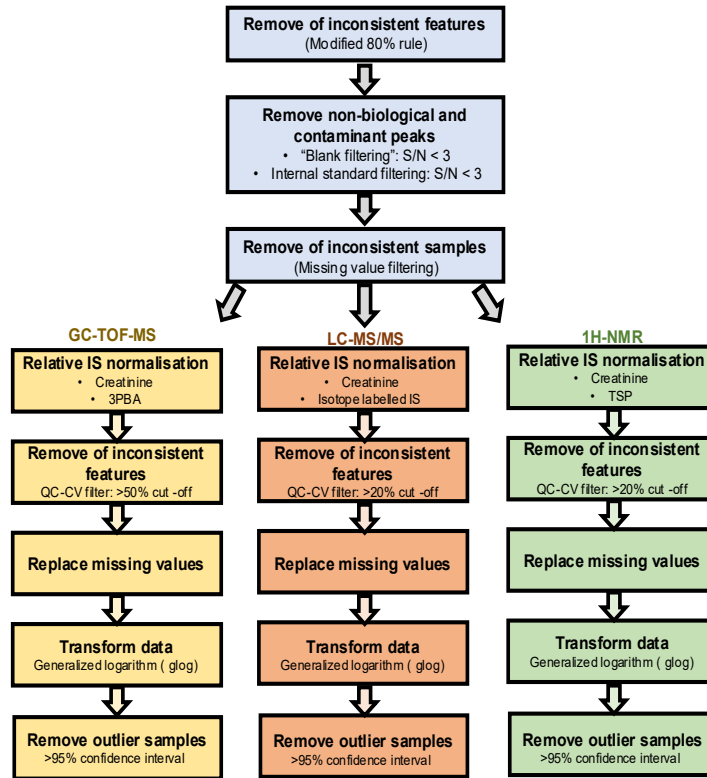
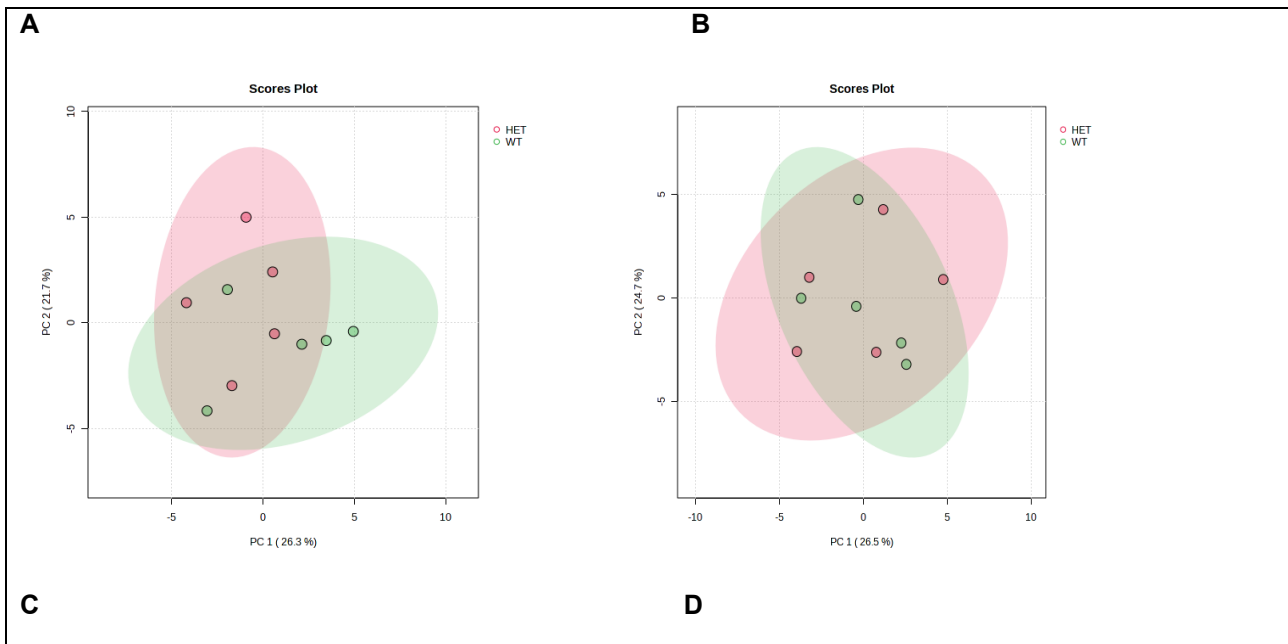


Figure C-1: Data processing and normalisation of extracted metabolomics data sets. The data processing stages involved the removal of inconsistent and contaminant peaks, removal of inconsistent samples, following analysis.

C.2. Results



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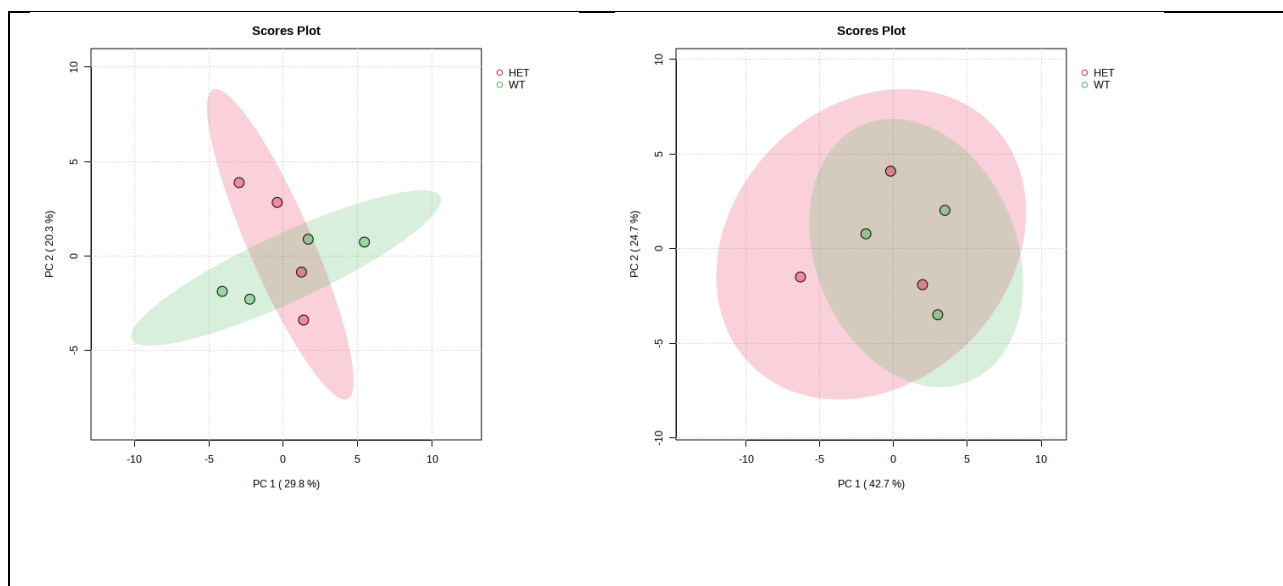


Figure C-2: PCA score plots. PCA scores plot illustrates minimum separation of significantly altered metabolites between (A) WT (n=5) and HET mice (n=5) on a normal diet, (B) WT (n=5) and HET mice (n=5) on a normal diet with alcohol consumption, (C) WT (n=4) and HET (n=4) mice on a ketogenic diet and (D) WT (n=3) and HET (n=3) mice on a ketogenic diet with alcohol consumption, when the different genotypes (n=6 to 10 per group) were compared. Each metabolite was normalised to its respective internal standard (based on the relative metabolomics platform) and subsequently log transformed, as well as auto-scaled using MetaboAnalyst 5.0.

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Table C-2: Significant discriminatory metabolites

KD vs ND											
Compound	p-value*	d-value	↑/ ↓	ID Level & Class	Platform	Compound	p-value*	d-value	↑/ ↓	ID Level & Class	Platform
2-Hydroxyadipic acid	p<0.001	1.45	↑	3; O	GC-TOF-MS	2-Hydroxyisobutyric acid	p<0.001	6.12	↓	3; O	GC-TOF-MS
2-Ketoisocaproic acid	p<0.001	2.17	↓	2; O	¹ H-NMR	2-Keto-3-methylvaleric acid	p<0.001	1.72	↓	3; O	GC-TOF-MS
2-Methylbutyrylglycine	p<0.001	1.64	↑	3; O	GC-TOF-MS	5-Hydroxyindole-3-acetic acid	p<0.001	5.04	↓	3	GC-TOF-MS
3-Ureidopropionic acid	0.004	1.46	↓	2; O	¹ H-NMR	Butyryl-L-Carnitine	p<0.001	1.13	↑	1; A, F	LC-MS/MS
4-Ketoglucose	p<0.001	2.88	↓	3; O	GC-TOF-MS	Cystathionine	0.010	1.10	↓	1; A	LC-MS/MS
Alanine	p<0.001	3.51	↓	1; A	LC-MS/MS	Cystine	0.001	1.80	↓	1; A	LC-MS/MS
Allantoin	p<0.001	1.63	↓	2; O	¹ H-NMR	D-Arabino-Hexonic acid	0.008	4.57	↓	3; O	GC-TOF-MS
Arginine	p<0.001	2.58	↓	1; A	LC-MS/MS	D-Gluconic acid	0.047	0.84	↑	3; O	GC-TOF-MS
b-Aminoisobutyric acid	0.048	1.18	↑	3; O	GC-TOF-MS	Dimethylamine	p<0.001	1.89	↓	2; O	¹ H-NMR
Betaine	p<0.001	4.30	↓	2; O	¹ H-NMR	Ethanolamine	p<0.001	4.53	↓	2; O	¹ H-NMR
Citric acid	p<0.001	3.02	↓	2; T	¹ H-NMR	Fumaric acid	0.006	1.70	↓	3; T	GC-TOF-MS
Dodecanoyl-L-Carnitine	p<0.001	4.37	↑	1; A, F	LC-MS/MS	GABA	p<0.001	2.97	↓	1; A	LC-MS/MS

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Hydroquinone	0.029	5.43	↓	3; O	GC-TOF-MS	Glutaric acid	p<0.001	15.66	↓	3; O	GC-TOF-MS
Hypoxanthine	0.035	6.02	↓	3; O	GC-TOF-MS	Homocysteine	p<0.001	1.73	↓	1; A	LC-MS/MS
Inositol	p<0.001	8.67	↓	3; O	GC-TOF-MS	Isocitric acid	p<0.001	5.86	↓	3; T	GC-TOF-MS
L-Carnitine	p<0.001	2.06	↓	1; A, F	LC-MS/MS	Isoleucine	0.026	1.59	↓	1; A	LC-MS/MS
Myo-Inositol	p<0.001	10.45	↓	3; O	GC-TOF-MS	Leucine	0.049	1.47	↓	1; A	LC-MS/MS
N-Valeryl glycine	0.001	3.53	↓	3; O	GC-TOF-MS	Gulonic acid	0.021	1.06	↑	3; O	GC-TOF-MS
Oxalic acid	0.004	1.01	↑	3; O	GC-TOF-MS	Malic acid	p<0.001	1.87	↓	3; T	GC-TOF-MS
Palmitoyl-L-Carnitine	0.040	1.97	↓	1; A, F	LC-MS/MS	Methionine	0.002	1.78	↓	1; A	LC-MS/MS
Phenylacetyl glycine	p<0.001	1.28	↓	2; O	¹ H-NMR	Methylamine	p<0.001	0.00	↓	2; O	¹ H-NMR
Succinic acid	p<0.001	3.46	↓	2; T	¹ H-NMR	Serine	p<0.001	2.71	↓	1; A	LC-MS/MS
Valeric acid	p<0.001	4.93	↓	3; O	GC-TOF-MS	Tricarballic acid	p<0.001	7.09	↓	3; O	GC-TOF-MS
α-Ketoglutaric acid	p<0.001	1.83	↓	2; O	¹ H-NMR	Trimethylamine	p<0.001	1.65	↓	2; O	¹ H-NMR
β-Alanine	p<0.001	3.51	↓	1; A	LC-MS/MS						
KD+Alc vs ND											
Compound	p-value*	d-value	↑/ ↓	ID Level & Class	Platform	Compound	p-value*	d-value	↑/ ↓	ID Level & Class	Platform

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2-Hydroxyisobutyric acid	0.003	1.73	↓	3; O	GC-TOF-MS	2-Hydroxyadipic acid	p<0.001	8.18	↑	3; O	GC-TOF-MS
2-Hydroxyvaleric acid	0.002	3.73	↑	3; O	GC-TOF-MS	2-Keto-3-methylvaleric acid	0.001	1.58	↓	3; O	GC-TOF-MS
2-Ketoisocaproic acid	p<0.001	6.40	↓	2; O	¹ H-NMR	3-Hydroxyadipic acid	p<0.001	4.73	↑	3; O	GC-TOF-MS
2-Methylbutyrylglycine	p<0.001	7.59	↑	3; O	GC-TOF-MS	4-Ketoglucose	p<0.001	1.68	↓	3; O	GC-TOF-MS
2-Pyrrolidone-5-carboxylic acid	0.012	2.90	↑	3; O	GC-TOF-MS	Butyryl-L-Carnitine	0.004	2.81	↑	1; A, F	LC-MS/MS
3-Hydroxybutyrate	0.022	606.94	↑	2; O	¹ H-NMR	Cystine	p<0.001	2.41	↓	1; A	LC-MS/MS
5-Hydroxyindole-3-acetic acid	p<0.001	1.35	↓	3; O	GC-TOF-MS	Dimethylamine	p<0.001	370.17	↓	2; O	¹ H-NMR
Alanine	p<0.001	3.16	↓	1; A	LC-MS/MS	Ethanolamine	p<0.001	1027.71	↓	2; O	¹ H-NMR
Allantoin	p<0.001	9.79	↓	2; O	¹ H-NMR	Fumaric acid	p<0.001	1.42	↓	3; T	GC-TOF-MS
Arginine	p<0.001	2.41	↓	1; A	LC-MS/MS	GABA	p<0.001	2.89	↓	1; A	LC-MS/MS
Betaine	p<0.001	5.39	↓	2; O	¹ H-NMR	Glutaric acid	p<0.001	2.60	↓	3; O	GC-TOF-MS
Citric acid	p<0.001	2.99	↓	2; T	¹ H-NMR	Homocysteine	p<0.001	1.71	↓	1; A	LC-MS/MS
D-Arabino-Hexonic acid	0.039	1.11	↓	3; O	GC-TOF-MS	Homocystine	p<0.001	1.07	↑	1; A	LC-MS/MS

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D-Gluconic acid	p<0.001	5.11	↑	3; O	GC-TOF-MS	Inositol	p<0.001	1.96	↓	3; O	GC-TOF-MS
Dodecanoyl-L-Carnitine	p<0.001	3.58	↑	1; A, F	LC-MS/MS	Isocitric acid	p<0.001	2.55	↓	3; T	GC-TOF-MS
Glyceric acid	0.005	1.56	↓	3; O	GC-TOF-MS	Isoleucine	0.041	1.37	↓	1; A	LC-MS/MS
Glycine	0.033	329.59	↓	2; A	¹ H-NMR	Leucine	0.050	1.33	↓	1; A	LC-MS/MS
L-Carnitine	p<0.001	1.99	↓	1; A, F	LC-MS/MS	Malic acid	p<0.001	1.71	↓	3; T	GC-TOF-MS
Gulonic acid	p<0.001	5.12	↑	3; O	GC-TOF-MS	Methionine	0.0096	1.79	↓	1; A	LC-MS/MS
Phenylacetyl glycine	p<0.001	12.81	↓	2; O	¹ H-NMR	Methylamine	p<0.001	0.00	↓	2; O	¹ H-NMR
Succinic acid	p<0.001	11.44	↓	2; T	¹ H-NMR	Myo-Inositol	p<0.001	1.82	↓	3; O	GC-TOF-MS
Tricarballic acid	0.014	1.37	↓	3; O	GC-TOF-MS	Serine	0.003	2.07	↓	1; A	LC-MS/MS
Valeric acid	p<0.001	2.26	↓	3; O	GC-TOF-MS	Trimethylamine	p<0.001	372.02	↓	2; O	¹ H-NMR
β-Alanine	p<0.001	3.55	↓	1; A	LC-MS/MS						
ND+Alc vs ND											
Compound	p-value*	d-value	↑/ ↓	ID Level & Class	Platform	Compound	p-value*	d-value	↑/ ↓	ID Level & Class	Platform
Acetic acid	0.038	0.74	↑	2; O	¹ H-NMR	3-Methyladipic acid	0.004	1.05	↓	3; O	GC-TOF-

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											MS
Allantoin	0.005	1.02	↓	2; O	¹ H-NMR	Hypoxanthine	0.003	1.02	↑	3; O	GC-TOF-MS
Arginine	0.001	1.23	↑	1; A	LC-MS/MS	Isoleucine	0.002	1.71	↑	1; A	LC-MS/MS
Dimethylamine	0.005	1.07	↓	2; O	¹ H-NMR	Leucine	0.003	1.61	↑	1; A	LC-MS/MS

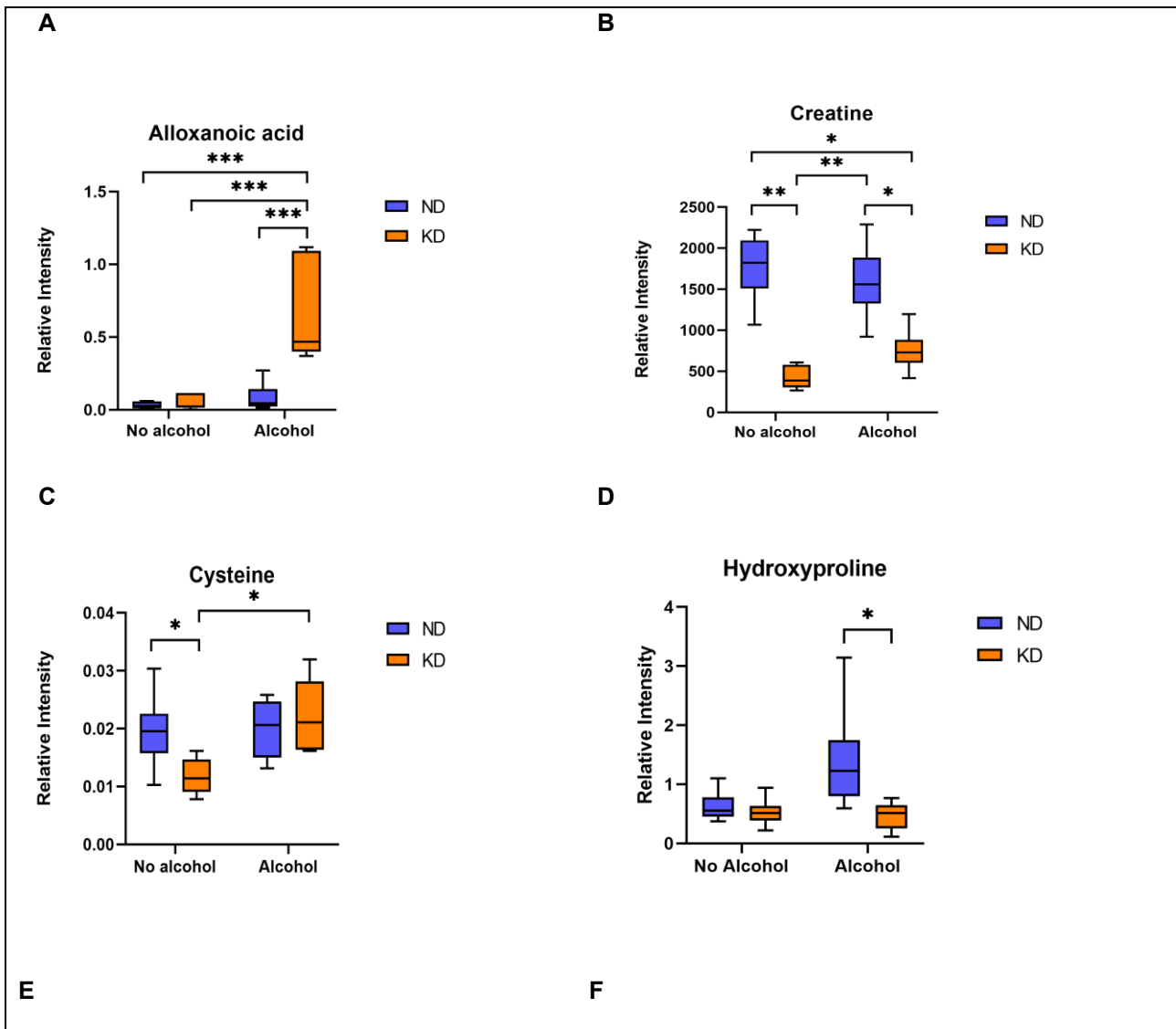
The significant discriminatory metabolites with an identification confidence Level of 1, 2 and 3 together with an FDR-corrected p-value <0.05 and d-value ≥0.8 between the different dietary groups, obtained via one-way ANOVA followed by a post-hoc Tukey test. Arrows indicate an increase (↑) or decrease (↓) relative to the metabolite levels of the control group. The identification (ID) level in conjunction with the relative metabolite class (amino acid, A, fatty acids, F; TCA cycle, T; or other, O) is listed for each metabolite. GABA, γ-aminobutyric acid. The relative platform used to identify each metabolite is also listed. GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography with tandem mass spectrometry; ¹H-NMR, hydrogen-1 nuclear magnetic resonance.

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Table C-3: The significant metabolites with an interaction between diet and alcohol

Interactions between diet and alcohol			
Compound	p-value*	ID Level	Class
Alloxanoic acid	p<0.001	3	A
Creatine	0.012	1	O
Cysteine	0.030	1	A
Hydroxyproline	0.042	1	A
Isobutyric acid	0.008	3	A
Taurine	0.045	2	A

An identification confidence Level of 1, 2 and 3 is given, together with an FDR-corrected p-value <0.05 and d-value ≥0.8, obtained via a two-way ANOVA followed by a post-hoc Tukey test. Arrows indicate an increase (↑) or decrease (↓) relative to the metabolite levels of the control group. The identification (ID) level in conjunction with the relative metabolite class (amino acid, A or other, O) is listed for each metabolite.



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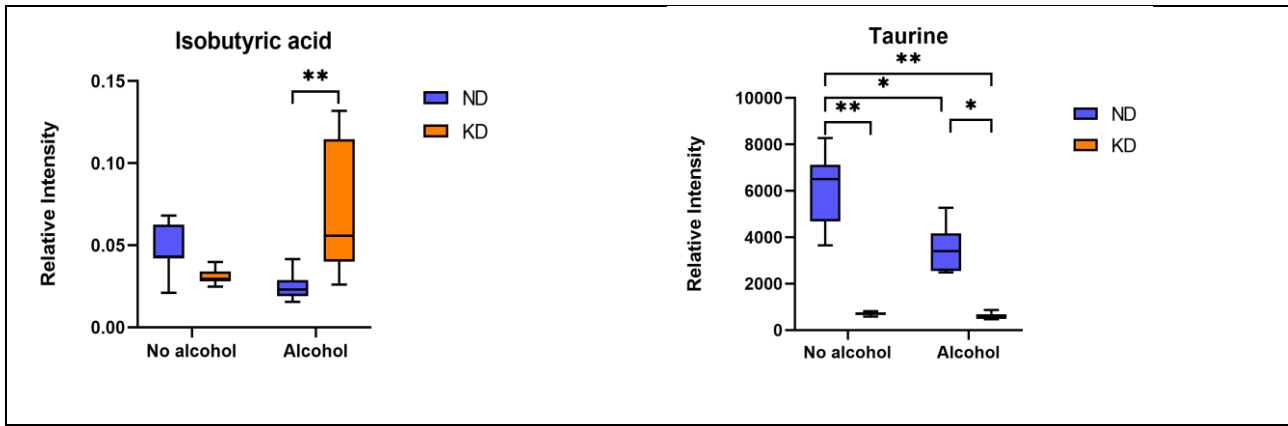


Figure C-3: Box plots indicate the relative urinary levels of the significant metabolites with an interaction between diet and alcohol (n=6-10 per group). * $p < 0.05$, ** $p < 0.01$ and * $p < 0.001$, obtained via a post-hoc Tukey's test.**

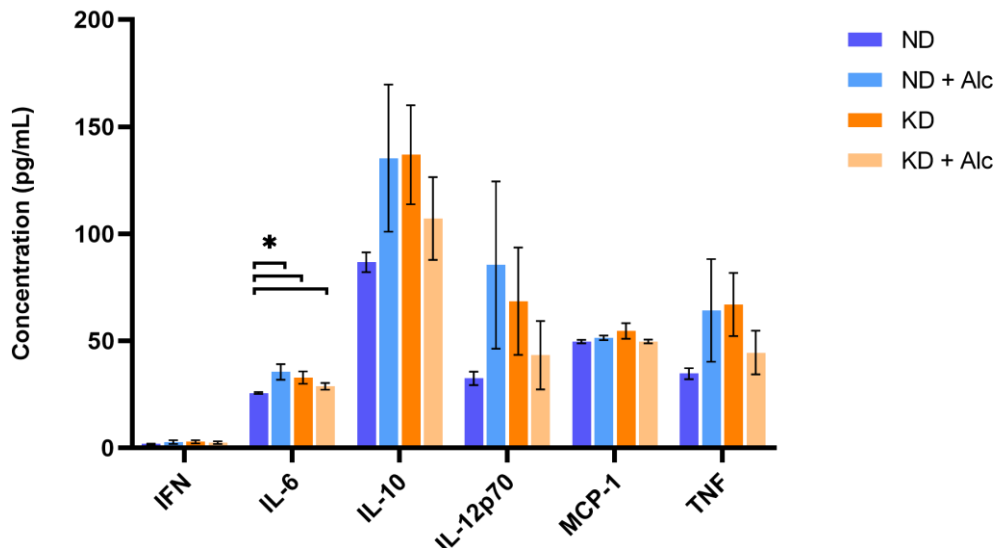


Figure C-4: Box plots indicate the relative urinary levels of the significant metabolites with an interaction between diet and alcohol (n=6-10 per group). * $p < 0.05$, ** $p < 0.01$ and * $p < 0.001$, obtained via a post-hoc Tukey's test.**

C.3. References

- Chen, J., Zhang, P., Lv, M., Guo, H., Huang, Y., Zhang, Z., & Xu, F. (2017). Influences of Normalisation Method on Biomarker Discovery in Gas Chromatography–Mass Spectrometry-Based Untargeted Metabolomics: What Should Be Considered? *Anal. Chem.*, 89(10), 5342-5348.
- Di Guida R, Engel J, Allwood JW, Weber RJ, Jones MR, Sommer U, Viant MR, Dunn WB. Non-targeted UHPLC-MS metabolomic data processing methods: a comparative investigation of normalisation, missing value imputation, transformation and scaling. *Metabolomics*. 2016;12:93.
- Lindeque, J., Hidalgo, J., Louw, R., & Westhuizen, F. (2013). Systemic and organ specific metabolic variation in metallothionein knockout mice challenged with swimming exercise. *Metabolomics*, 9, 418-432.
- Lindeque, J. Z., Jansen van Rensburg, P. J., Louw, R., van der Westhuizen, F. H., Florit, S., Ramírez, L., . . . Hidalgo, J. (2015). Obesity and metabolomics: metallothioneins protect against high-fat diet-induced consequences in metallothionein knockout mice. *OmicS*, 19(2), 92-103. doi:

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Mels, C. M., Jansen van Rensburg, P., van der Westhuizen, F. H., Pretorius, P. J., & Erasmus, E. (2011). Increased excretion of c4-carnitine species after a therapeutic acetylsalicylic Acid dose: evidence for an inhibitory effect on short-chain Fatty Acid metabolism. *ISRN Pharmacol.*, 2011, 851870.

Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., & Hollender, J. (2014). Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ. Sci. Technol.*, 48(4), 2097-2098.

Wanichthanarak, K., Fan, S., Grapov, D., Barupal, D. K., & Fiehn, O. (2017). Metabox: A Toolbox for Metabolomic Data Analysis, Interpretation and Integrative Exploration. *PLoS One*, 12(1), e0171046.

APPENDIX D

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The therapeutic potential of a polyunsaturated fatty acid-enriched high-fat diet in Leigh syndrome: Insights from a preclinical model

Luciano Willemse, Karin Terburgh, Roan Louw*

Mitochondria Research Group, Human Metabolomics, North-West University, Potchefstroom, South Africa

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ABSTRACT

Introduction: Leigh syndrome is often caused by *Ndufs4* mutations. The *Ndufs4* knockout (KO) mouse model recapitulates key disease features, including systemic inflammation, neurodegeneration, and motor deficits. While dietary interventions such as the ketogenic diet show promise in mitigating mitochondrial dysfunction, conflicting results highlight uncertainties regarding its efficacy. Here, we evaluate the therapeutic potential of a polyunsaturated fatty acid (PUFA)-enriched high-fat diet (HFD) in *Ndufs4* KO mice.

Methods: Dietary intervention began at postnatal day 23, with mice receiving either a normal diet (ND) or a HFD enriched with PUFAs. Phenotypic evaluation, including locomotor function, clasping behaviour, and survival, continued until natural death. In a second group of animals, biochemical analyses were conducted after three weeks on the diets, using Western blot to evaluate neurometabolic and inflammatory regulators, flow cytometry to quantify serum inflammation markers, and metabolic profiling to identify alterations in neurometabolism and the neurolipidome.

Results: The HFD significantly extended lifespan and improved clasping behaviour in *Ndufs4* KO mice but had no effect on locomotor activity or grip strength decline. While whole-brain mTOR (p70S6K1, 4E-BP1) and SIRT1 (PGC1- α , TNF- α) signalling pathways remained unaffected, the diet significantly reduced serum pro-inflammatory markers TNF and IL-6. Furthermore, the PUFA-enriched HFD partially restored disruptions in TCA cycle, ketone body, branched-chain amino acid, and lipid metabolism, indicating potential metabolic reprogramming.

Conclusion: Dietary interventions, such as a PUFA-enriched HFD, may alleviate systemic inflammation, partially correct metabolic imbalances, and mitigate specific disease phenotypes in Leigh syndrome, warranting further investigation into the underlying mechanisms and broader therapeutic applications.

1. Introduction

Mitochondrial diseases encompass a spectrum of disorders, primarily stemming from oxidative phosphorylation (OXPHOS) system defects. Affecting approximately 1 in 5000 births, these disorders are among the most prevalent inborn errors of metabolism [1,2]. A significant proportion of cases arise from deficiencies in complex I (CI) of the OXPHOS system [3,4], which disrupt ATP production, elevate reactive oxygen species (ROS), and perturb redox homeostasis [3,4]. Clinically, most paediatric cases manifest as subacute necrotizing encephalomyelopathy or Leigh syndrome [5]. Leigh syndrome, a severe neurodegenerative disorder, is characterized by symmetrical, bilateral brain lesions, leading to psychomotor regression and early mortality [6,7]. To date, >80 pathogenic mutations, spanning mitochondrial and nuclear DNA, have

been linked to the disease [5]. Among these, mutations in the *NDUFS4* gene, encoding a CI subunit, are particularly prominent [8,9].

The development of robust preclinical models has been instrumental in advancing our understanding of mitochondrial diseases like CI deficiency and exploring therapeutic strategies [10]. The *Ndufs4* knockout (KO) mouse model is widely recognized as one of the most accurate representations of CI deficiency-related Leigh syndrome, recapitulating its hallmark features, including growth retardation, locomotor dysfunction, ataxia, muscular hypotonia, and premature death [11]. Studies using this model have identified critical disease-driving mechanisms, including NAD⁺ deficiency [12,13], mTORC1 hyperactivation [14], and neuroinflammation [15]. However, despite these advances, curative therapies for CI deficiency remain elusive [16].

Among emerging therapeutic approaches, the ketogenic diet (KD)

* Corresponding author.

E-mail address: Roan.Louw@nwu.ac.za (R. Louw).

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has garnered attention as a metabolic intervention with potential to ameliorate mitochondrial dysfunction. Characterized by its high-fat and low-carbohydrate composition, the KD induces ketogenesis and promotes mitochondrial adaptations, including enhanced biogenesis, improved OXPHOS, and reduced oxidative stress [17,18]. Moreover, the diet's ability to modulate NAD^+ metabolism and suppress mTOR signalling further establishes it as a promising strategy for mitigating CI dysfunction [14,19,20]. However, Bornstein, James [21] recently reported no benefits of a standard KD in *Ndufs4* KO mice. While this diet successfully induced dietary ketosis, it did not mitigate disease progression, and a strict KD was also poorly tolerated in this mouse model.

This raises the question of whether dietary composition — particularly the fatty acid profile — in KDs and other high-fat diets (HFDs) may affect treatment outcomes. As recently highlighted by [22], the fat source in KD and HFD formulations may be a crucial determinant of treatment efficacy, since different fatty acids distinctly influence metabolic and inflammatory pathways. Notably, omega (ω)-3 polyunsaturated fatty acids (PUFAs), derived from fish oil, exhibit potent anti-inflammatory properties, mitigating neuroinflammation, regulating redox balance, and inhibiting mTOR activity [23–25]. These effects suggest that incorporating ω -3 PUFAs into KD or HFD formulations could hold synergistic therapeutic benefits for CI deficiency, where metabolic dysfunction and inflammation are central to disease progression.

In this study, we utilize the *Ndufs4* KO mouse model of Leigh syndrome to evaluate the therapeutic potential of a HFD enriched with PUFAs, derived from Menhaden fish oil. This approach was selected to circumvent the limitations of traditional and classic KDs and HFDs in mitochondrial disease models, particularly in terms of fatty acid composition. The diet's impact on motor function, ataxia and survival was assessed through phenotypic evaluations of locomotor activity, grip strength, clasping behaviour, and lifespan. Additionally, biochemical analyses were performed to examine the effects of the diet on mTORC1 and SIRT1 signalling pathways, systemic inflammation, as well as neurometabolic and -lipidomic profiles. Our findings provide new insights into the potential of a PUFA-enriched HFD as therapeutic strategy for neurodegenerative mitochondrial diseases like Leigh syndrome.

2. Methods & materials

2.1. Animal care and handling

Whole-body *Ndufs4* knockout (KO) and wild-type (WT) mice were bred from heterozygous crosses (B6.129S4-*Ndufs4*^{tm1.1Rpa/J}, JAX #027058) [11], with genotypes confirmed by PCR as previously described [26]. Animals were housed in the specific pathogen-free unit of the Vivarium (SAVC reg. no. FR15/13458) at the North-West University (NWU) Pre-Clinical Drug Development Platform (RSA). The facility maintained controlled conditions ($22 \pm 1^\circ\text{C}$, $55 \pm 10\%$ humidity, 12:12 h light/dark cycle), with ad libitum access to food and water. Enclosures were equipped with sawdust bedding, paper towels, and polycarbonate mouse houses. Animals were handled and their welfare maintained by South African Veterinary Council (SAVC)-authorized personnel in accordance with ethical guidelines outlined in the code of ethics for research, training, and drug testing in South Africa. The study was approved by the NWU Animal Research Ethics Committee (NWU-00516-20-A5) and complied with national legislation.

2.2. Dietary interventions

The experimental workflow, depicted in Fig. 1, outlines the study's group allocation, dietary intervention, sample sizes, and analytical procedures. After weaning on postnatal day (P) 23, mice were randomly assigned to normal diet (ND) or a high-fat diet enriched with ω -3 dominant PUFAs from Menhaden fish oil, referred onwards as just HFD. Both sexes were equally represented in the diet groups and given ad libitum access to food. Table 1 summarizes the nutritional profile of the diets, with the ND (D23050803i, Research Diets, USA) containing 20 % protein, 70 % carbohydrates, and 10 % fat; while the HFD (D09020506i, Research Diets, USA) contained 20 % protein, 20 % carbohydrates, and 60 % fat. The 60 % fat/20 % carbohydrate composition was chosen to avoid the extreme fat ratios typical used in classic KDs and HFDs, as strict ketosis has proven insufficient as a therapeutic strategy in this model - *Ndufs4* KO mice do not tolerate a full KD and loses weight drastically once placed on this diet [21]. Therefore, the diet intervention in this study is designated as a PUFA-enriched HFD to distinguish it from conventional and classic high-fat, very-low-carbohydrate formulations.

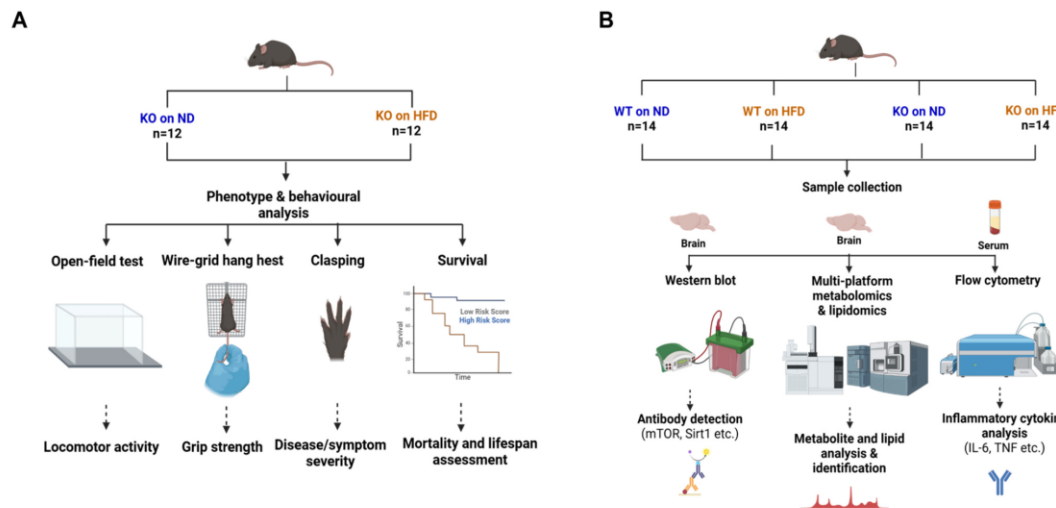


Fig. 1. Experimental workflow. (A) Phenotypic and behavioural assessment and (B) biochemical analyses. Abbreviations: KO, knockout; ND, normal diet; P, postnatal day; HFD, high-fat diet; WT, wild-type.

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Table 1
Dietary nutritional profiles and ingredient composition.

Nutrients	ND			HFD		
	g	% kcal	g per 100 g	g	% kcal	g per 100 g
Protein	19.20	20	19.24	26.20	20	26.23
Carbohydrates	67.30	70	71.11	26.30	20	31.50
Fat	4.30	10	5.21	34.90	60	35.89
Total		100	100		100	100
kcal/g	3.85			5.24		
Ingredients	g	kcal	g per 100 g	g	kcal	g per 100 g
Casein, 80 mesh	200.00	800	18.96	200.00	800	25.84
L-cystine	3.00	12	0.28	3.00	12	0.38
Corn starch	315.00	1260	29.86	0.00	0	0
Maltodextrin 10	35.00	140	3.32	125.00	500	16.15
Sucrose	350.00	1400	33.17	68.80	275	8.89
Cellulose, BW200	50.00	0	4.74	50.00	0	6.46
Soybean oil	25.00	225	2.37	25.00	225	3.23
Lard	20.00	180	1.90	122.50	1103	15.83
Fish oil (Menhaden)	0.00	0	0	122.50	1103	15.83
Mineral mix S10026	10.00	0	0.95	10.00	0	1.29
Dicalcium phosphate	13.00	0	1.23	13.00	0	1.68
Calcium carbonate	5.50	0	0.52	5.50	0	0.71
Potassium citrate, monohydrate	16.50	0	1.56	16.5	0	2.13
Vitamin mix V10001	10.00	40	0.95	10.00	40	1.29
Choline bitartrate	2.00	0	0.19	2.00	0	0.26
FD&C blue dye #1	0.05	0	0.001	0.00	0	0
FD&C red dye #40	0.00	0	0	0.05	0	0.01
Total	1055.05	4057	100	773.85	4057	100
Fatty acid composition	g	%	g per 100 g	g	%	g per 100 g
Saturated fatty acid (SFA)	10.10	23.50	0.95	74.60	30.00	9.64
Monounsaturated fatty acid (MUFA)	12.80	29.70	1.21	74.70	30.00	9.65
Polyunsaturated fatty acid (PUFA)	20.20	46.80	1.90	99.40	40.00	12.84
Omega-3/6 fatty acid composition	g	%	g per 100 g	g	%	g per 100 g
Omega-3 fatty acids	2.1	4.87	0.20	42	16.88	5.43
Omega-6 fatty acids	17.9	41.53	1.70	49	19.69	6.33

Abbreviations: ND, normal diet; HFD, high-fat diet; kcal, kilocalories; and g, gram.

Food intake was monitored by weighing the food before and after providing it to the mice. The food was replaced every two to three days to ensure freshness, with the removed food being reweighed. Mice on the ND consumed approximately 3.0 g of food per day, while those on the HFD consumed around 2.2 g per day. Despite differences in food intake by weight, both groups had comparable daily caloric consumption (11.55 kcal/day for the ND vs. 11.53 kcal/day for the HFD) due to the higher caloric density of the HFD.

Phenotypic evaluations (Fig. 1A) were conducted on KO mice ($n = 12$ per group) allowed to reach their natural endpoint, while biochemical evaluations (Fig. 1B) were performed on KO and WT mice ($n = 14$ per group) euthanized at the late disease stage (P45–47). Following euthanasia, blood serum and brain tissues were collected. All samples were rapidly snap-frozen in liquid nitrogen to halt metabolic activity and were subsequently stored at -80°C for preservation.

2.3. Phenotypic evaluation

Disease progression and survival in *Ndufs4* KO mice were phenotypically assessed as previously described [11,27] and detailed in the supplementary information (SI). Locomotor activity, ataxia, and grip strength were assessed at three disease stages — early (P28–30), mid (P38–40), and late (P48–50) — using open-field and wire-grid hang tests. The clasping test, a measure of motor function and neurological health [28], was conducted daily, with natural death dates recorded.

2.4. SDS-PAGE and Western blotting.

Brain tissue (~30 mg) was homogenized in RIPA buffer (~300 μL)

via sonication (three 3-s pulses), and protein concentrations determined using the BCA assay. Samples were heat denatured (10 min at 72°C), separated by SDS-PAGE (30 μg protein), and transferred to nitrocellulose membranes. Membranes were blocked in 1 % casein TBS and incubated overnight (4°C) with primary antibodies targeting (phospho-) mTOR, (phospho-) p70S6K1, (phospho-) 4EBP1, SIRT1, PGC1- α , TNF- α and β -actin. Thereafter, HRP-conjugated secondary antibodies (goat anti-mouse and goat anti-rabbit) were applied at room temperature. Chemiluminescent detection was performed using a Western ECL substrate and the ChemiDoc™ MP imaging system (Bio-Rad). Band intensities were quantified with Image Lab software (v. 6.1) and normalized to total protein or β -actin. Detailed procedures and antibody information are provided in the SI.

2.5. Inflammation assay

Inflammatory profiles in mouse serum were measured using the BD Cytometric Bead Array Mouse Inflammation Kit, following the manufacturer's instructions. The assay utilized a multiplex bead array coupled with flow cytometry (BD FACSLytic, BD Biosciences) to detect interleukin (IL) 6, IL-10, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor (TNF), interferon gamma (IFN- γ), and IL-12p70. Cytokine concentrations (pg/mL) were calculated based on standard curves using FlowJo (v. 10.8) and FCS Express (v. 6, De Novo) for data analysis.

2.6. Metabolomics and lipidomics

Metabolomic and lipidomic profiling of brain tissue was performed

using untargeted and targeted approaches on separately prepared samples. Polar and nonpolar metabolites were extracted using a monophasic methanol/water/chloroform (6:2:2) Bligh-Dyer method [29], while lipids were extracted using a monophasic butanol/methanol (3:1) method [30]. Internal standards, 3-phenylbutyric acid for metabolomics

and diheptadecanoyl (C17:0) fatty acids for lipidomics, were added during extraction. Samples were aliquoted and dried under nitrogen gas prior to analysis.

Untargeted metabolomics was performed on oximated and silylated samples using gas chromatography (Agilent 8890) coupled with time-of-

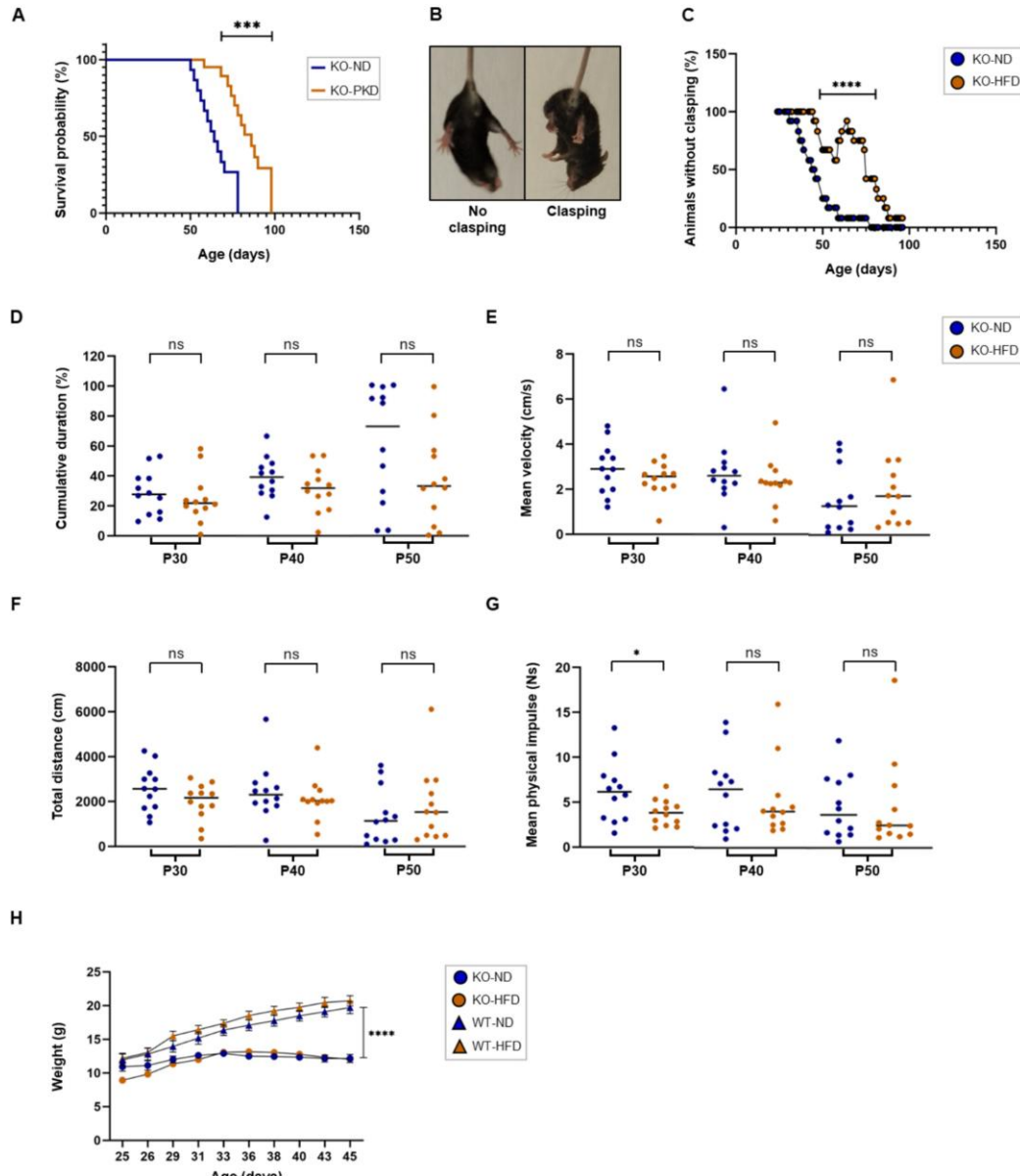


Fig. 2. Phenotypic evaluation of *Ndufs4* KO and WT mice fed a HFD or ND. (A) Mantel-Cox survival curve and (B–C) clasping assessment of disease severity in KO mice on either a ND or HFD over time. Scatter plots depicting open-field test results, including (D) cumulative duration in centre over 5 min, (E) mean velocity, (F) total distance traveled, and (G) mean physical impulse at P30, P40 and P50 in KO mice on each diet. Horizontal bars represent the mean. (H) Growth curves indicating weight over time in WT and KO mice across dietary groups. Individual datapoints represent the mean $\pm$ SD. Brackets indicate statistically significant differences where $p < 0.05$ (*), $p < 0.001$ (***), $p < 0.0001$ (****) and $p > 0.05$ (ns). $n = 12$ animals per group. Abbreviations: cm, centimeter; cm/s, centimeter per second; KO, knockout; Ns, Newton seconds; g, gram; and WT, wild-type.

flight mass spectrometry (LECO Pegasus BT). Targeted analysis of redox-related metabolites (Table S1) was conducted on silylated samples using gas chromatography (Agilent 7890B) coupled with triple quadrupole mass spectrometry (Agilent 7010B). Untargeted lipidomics was performed on samples resuspended in methanol/acetonitrile (2:1) using ultra-performance liquid chromatography coupled with high-definition mass spectrometry (Waters Synapt G2-Si).

Samples were randomized to minimize analytical variability, with quality control samples included to monitor instrument performance. Metabolites were identified by matching mass spectra to commercial and in-house libraries with an 80 % similarity threshold to ensure high-confidence annotation.

Further details on sample preparation, analysis conditions, and data processing are provided in the SI.

2.7. Statistical analyses

Survival analysis was conducted using the Log-rank (Mantel-Cox) test. Independent sample *t*-tests, one-way, and two-way ANOVAs were used to assess group differences in phenotype, weight, and Western blot data. For flow cytometry data, outlier removal was followed by independent sample *t*-tests and Mann-Whitney *U* tests. These analyses were performed using Excel 365, IBM SPSS Statistics 25, and GraphPad Prism v. 9.

Metabolic data were processed using Excel 365, MetaboAnalyst v. 6.0 [31,32], and GraphPad Prism v. 9, while lipidomic data processing additionally utilized Progenesis Q1 v. 2.0. Multiple independent sample *t*-tests, one-way, and two-way ANOVAs with post hoc Tukey's tests were used to identify group differences. Statistical significance was set at $p < 0.05$, corrected for multiple testing using Benjamini-Hochberg false discovery rate (BH-FDR). Effect size thresholds were set at Cohen's $d > 0.6$ for metabolomics and > 0.8 for lipidomics.

3. Results

3.1. Phenotype and behavioural assessment

The Log-Rank test (Fig. 2A) revealed a significant lifespan increase in KO-HFD (P72-95) compared to KO-ND mice (P45-60, $p < 0.001$). To assess possible disease symptom alleviation, limb claspings was monitored throughout the animals' lifespan (Fig. 2B-C), while locomotor activity (Fig. 2D-F) and grip strength (mean physical impulse, Fig. 2G) were assessed at P30, P40, and P50. KO-HFD mice displayed a markedly delayed onset of limb claspings (P60-70) and significant improvements in claspings compared KO-ND mice (P42-50, $p < 0.0001$). However, open-field tests showed no improvement in locomotor activity, with cumulative duration, mean velocity, and total distance unaltered by the diet. Wire-grid hang tests revealed a temporary reduction in grip strength of KO-HFD mice at P30 ($p < 0.05$), with no differences observed at P40 or P50. Lastly, growth curves indicated that the HFD did not impact bodyweight in KO or WT animals, although KO mice on either diet exhibited reduced growth compared to WT.

3.2. Western blots and inflammation assay

To investigate the mechanisms underlying the beneficial effects of a HFD on longevity and claspings behaviour in KO mouse, protein levels of key metabolic and inflammatory regulators were assessed in brain tissue. Western blot analysis measured the phosphorylation status of mTOR and its substrates, p70S6K1 and 4E-BP1 (Fig. 3B-D), as well as the levels of SIRT1, PGC1- α , and TNF- α (Fig. 3E-H). Neither genotype nor diet significantly affected protein expression in the brain when correcting for multiple comparisons. However, observable trends included decreased levels of PGC1- α and increased levels of TNF- α in KO-ND compared to WT-ND brains, which were not altered by the HFD.

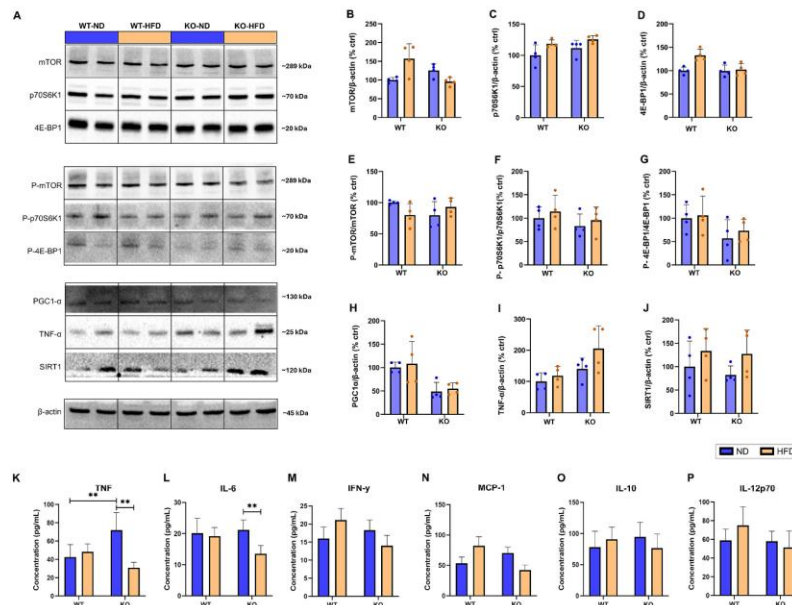


Fig. 3. The effect of a HFD on metabolic and inflammatory regulators in *Ndufs4* KO and WT mice. (A–J) Western blot analysis of specific metabolic markers in WT and KO mouse brain tissue ($n = 4$ per group). Representative blot images from two biological replicates are shown. (K–P) Flow cytometry analysis of serum inflammation markers in WT and KO mice ($n = 14$ per group). Bar graphs represent the mean $\pm$ SD; individual data points shown as dots. Brackets indicate statistically significant differences where $p < 0.05$ (*) or $p < 0.01$ (**). Abbreviations: IFN- γ , interferon gamma; IL-6, interleukin 6; IL-10, interleukin 10; IL-12p70, interleukin 12p70; KO, knockout; MCP-1, monocyte chemoattractant protein 1; TNF(α), tumor necrosis factor(α); and WT, wild-type.

Serum inflammation assays further revealed elevated TNF in KO-ND compared to WT-ND mice. A HFD markedly decreased TNF and IL-6 concentrations in KO mouse serum to levels below that of WTs. However, no differences were observed for other cytokines (IFN- γ , MCP-1, IL-10, and IL-12p70) when mice from either genotype or dietary group were compared.

3.3. Metabolic alterations

To determine whether a HFD alters the neurometabolome in *Ndufs4* KO mice, multi-platform metabolic profiling was done. Univariate analysis revealed significant alterations in tricarboxylic acid (TCA) cycle, ketone body, and branched-chain amino acid (BCAA) metabolism (Fig. 4), with a HFD generally counteracting the metabolic changes observed in KO-ND vs WT-ND mice. In the TCA cycle, KO-ND brains showed a decrease in aspartate ($p < 0.05$) and oxaloacetate ($p < 0.05$),

alongside increased alanine ($p < 0.0001$), lactate ($p < 0.001$), and succinate ($p < 0.001$) compared to WT-ND brains. Contrastingly, KO-HFD brains exhibited higher ($p < 0.05$) aspartate and oxaloacetate, along with lower ($p < 0.05$) alanine, lactate, and succinate. Similarly, the ketones, 3-hydroxybutyrate ($p < 0.05$) and acetoacetate ($p < 0.05$), were elevated in KO-ND compared to WT-ND brains, with the levels of 3-hydroxybutyrate ($p < 0.05$) decreased in KO-HFD brains. In BCAA metabolism, a significant increase was observed for leucine ($p < 0.0001$), isoleucine ($p < 0.0001$), valine ($p < 0.0001$) and glutamate ($p < 0.0001$), as well as a decrease in α -ketoisovalerate ($p < 0.05$) in KO-ND compared to WT-ND brains. These same metabolites, except for α -ketoisovalerate, were significantly decreased ($p < 0.05$, $p < 0.01$, $p < 0.01$ and $p < 0.01$) in KO-HFD compared to KO-ND brains.

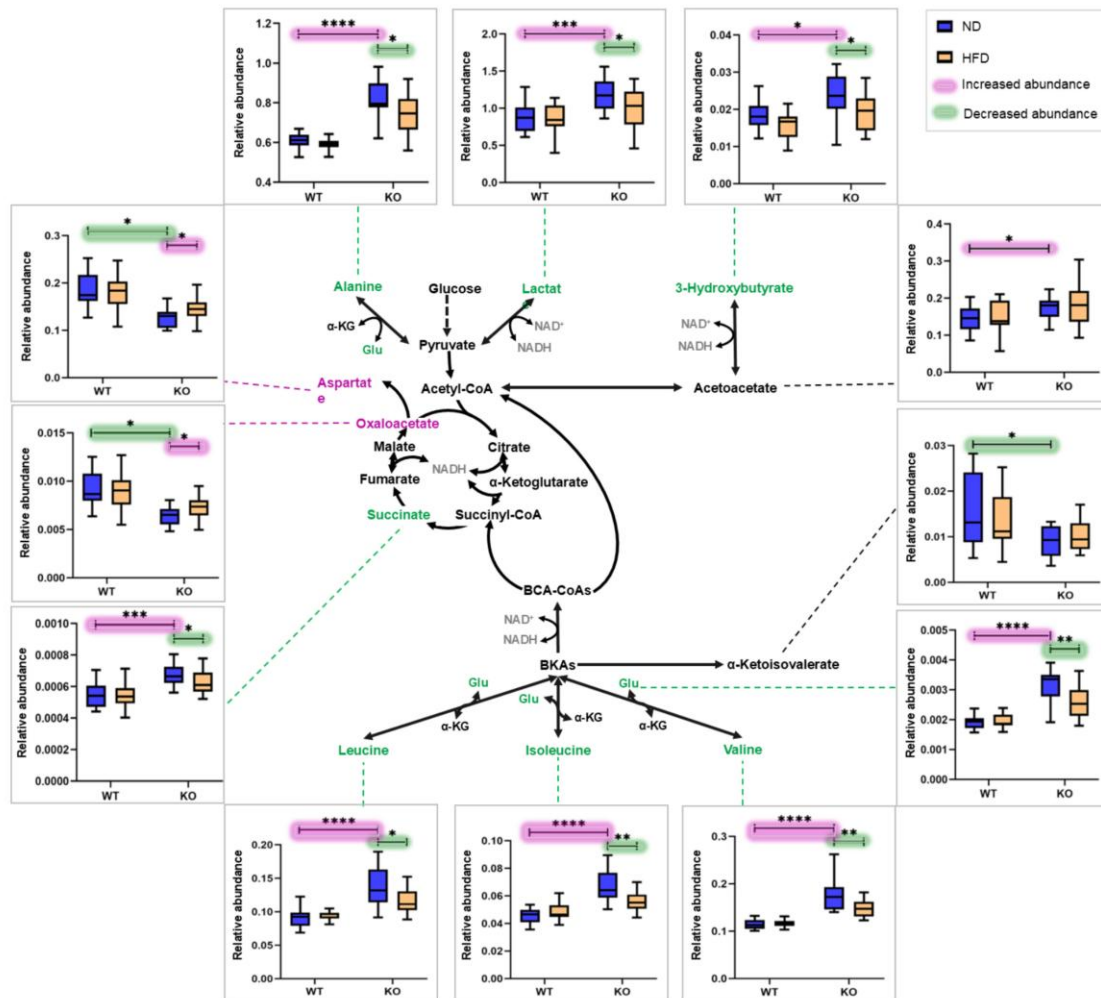


Fig. 4. Metabolic alterations in brain tissue of *Ndufs4* KO and WT mice fed a HFD or ND. Brackets indicate statistically significant differences where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****). $n = 14$ per group. Abbreviations: α -KG, α -ketoglutarate; BCA-CoAs, branched-chain acyl-coenzyme As; BKAAs, branched-chain keto acids; Glu, glutamate; KO, knockout; NAD $^{+}$, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ND, normal diet; HFD, high-fat diet; and WT, wild-type.

3.4. Lipid alterations

The effects of a HFD on the neurolipidome in *Ndufs4* KO mice was also evaluated. Univariate analysis revealed marked changes in phospholipid metabolism due to dietary intervention (Fig. 5). When comparing WT and KO mice on a ND, KO brains exhibited a significantly higher abundance of diacylglycerol ($p < 0.05$), triacylglycerol ($p < 0.01$), phosphatidic acid ($p < 0.01$), phosphatidylglycerol ($p < 0.05$), phosphatidylinositol ($p < 0.0001$), phosphatidylserine ($p < 0.01$), phosphatidylethanolamine ($p < 0.05$), and phosphatidylcholine ($p < 0.05$). Moreover, when compared to KO-ND brains, significant reductions were noted in KO-HFD brain diacylglycerol ($p < 0.0001$), phosphatidic acid ($p < 0.01$), phosphatidylglycerol ($p < 0.0001$), phosphatidylinositol ($p < 0.0001$), phosphatidylserine ($p < 0.0001$), and phosphatidylcholine ($p < 0.0001$). Conversely, triacylglycerol ($p < 0.01$) and phosphatidylethanolamine ($p < 0.0001$) levels were elevated in KO mice on a HFD compared to those on a ND.

4. Discussion

4.1. A PUFA-enriched HFD attenuates Leigh syndrome in *Ndufs4* KO mice

This study utilized *Ndufs4* KO mice to evaluate the therapeutic potential of a PUFA-enriched HFD for Leigh syndrome. A HFD significantly extended the lifespan of *Ndufs4* KO mice up to a maximum of 100 days (vs 72 days on a ND), while delaying the onset and reducing the severity of neurodegenerative clamping symptoms. These findings contrast with

the report from Bornstein, James [21], who showed no benefits to overall disease progression or survival in the same mouse model using a standard KD. However, not all KDs and HFDs are identical; they vary in macronutrient composition, total caloric content, and fat source.

Bornstein, James [21] noted poor tolerance in *Ndufs4* KO mice on a full KD (90 % fat, < 1 % carbohydrates), opting for a gradual introduction to an adapted diet (80 % fat, 8.5 % carbohydrates). In this study, we further adjusted the diet to contain 60 % fat and 20 % carbohydrates, classifying it as a HFD that approaches the lower threshold for ketosis in mice. A more balanced macronutrient composition proved to be well tolerated in KO mice, although a transient decline in grip strength and body weight was observed during early stages (P30). This likely reflected an initial adaptation period, as grip strength and weight were comparable between KO-HFD and KO-ND groups by P40 and P50. While the HFD did not prevent weight loss in KO mice, it also did not exacerbate the phenotype, likely due to the isocaloric nature of the diets, as consistent caloric intake supports weight maintenance in such contexts [33–35].

Another key modification made to the diet was the supplementation of Menhaden fish oil, chosen for its high PUFA content. The source and composition of fatty acids in a KD and HFD are increasingly recognized as crucial factors influencing its therapeutic efficacy [22,36]. Although no previous studies have directly examined the effects of PUFAs on mitochondrial disease models, PUFAs have shown to enhance mitochondrial function and alleviate disease severity in conditions associated with mitochondrial dysfunction, such as obesity [37], aging [38], Alzheimer's disease [39], and palmitate-induced mitochondrial impairment [40]. Our findings align with these results, showing that

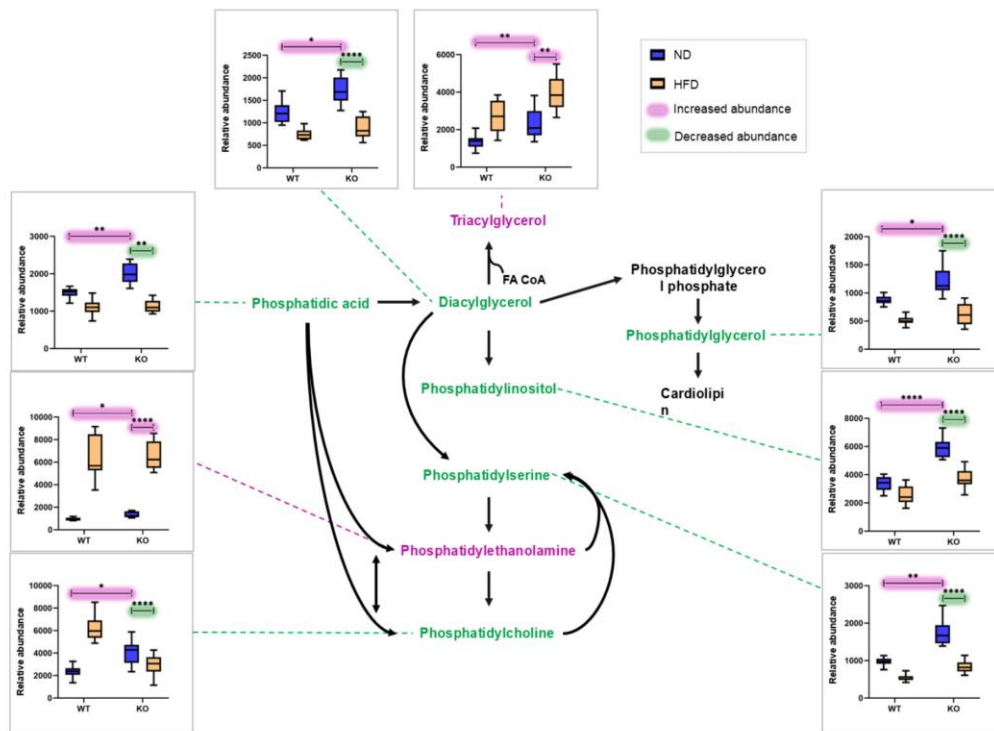


Fig. 5. Phospholipid alterations in brain tissue of *Ndufs4* KO and WT mice fed a HFD or ND. Brackets indicate statistically significant differences where $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.0001$ (****). $n = 14$ per group. Abbreviations: FA CoA, fatty acyl-coenzyme A; KO, knockout; ND, normal diet; HFD, high-fat diet; and WT, wild-type.

HFD intervention mitigates Leigh syndrome in a preclinical model. However, the absence of histological data to directly visualize brain lesions represents a limitation of the current study. While improvements in clasping behaviour and changes in neuronal metabolite profiles suggest a potential mitigation of neurodegeneration, histological analyses are needed in follow-up studies to confirm these findings and provide a more detailed characterisation of the HFD's effects on brain morphology. Additionally, while our study demonstrates that the HFD attenuates disease progression in *Ndufs4* KO mice, we acknowledge that the mechanistic role of OXPHOS adaptations, particularly shifts in CI/CII substrate utilization, remains unresolved. Direct measurement of mitochondrial respiratory activity would clarify whether the HFD enhances CII-driven ATP production or compensates for CI deficiency through alternative pathways. Future studies utilizing high-resolution respirometry are needed to address this question.

Other therapeutic interventions tested in *Ndufs4* KO mice have produced mixed results in terms of symptomatic improvement and lifespan extension. The NAD⁺ precursor nicotinamide mononucleotide did not improve clinical symptoms but extended median lifespan to 110 days [12]. The ROS-redox modulator H176 improved motor function but failed to impact disease onset or severity [41]. mTOR inhibitors such as rapamycin delayed disease onset, prevented neurological symptoms, and extended median lifespan to 111–114 days [14]. Severe continuous hypoxic conditions-maintained body weight, improved motor function, reduced hypothermia, and extended lifespan to 70–80 days [42]. Similarly, acarbose treatment delayed disease onset and increased median lifespan to 92 days [43]. Our HFD intervention demonstrates comparable outcomes, improving neurological symptoms and extending median lifespan up to 75 days.

The HFD is highly translatable to clinical settings and offers a cost-effective adjunctive therapeutic option compared to pharmacological treatments like rapamycin, which, while effective, carries potential side effects [44,45]. A HFD may offer a better safety profile, reducing the risk of adverse effects while providing metabolic benefits [36]. Furthermore, combining a HFD with other interventions may yield greater benefits in *Ndufs4* KO mice by capitalizing on the distinct advantages of each treatment. This positions a HFD as a promising option for combination therapy, particularly in complex diseases requiring a multi-targeted approach. However, while our findings highlight the therapeutic potential of a PUFA-enriched HFD, the specific contributions of individual fatty acids (e.g., ω -3 vs. ω -6 PUFAs) remain unclear, warranting further studies.

4.2. A HFD does not affect whole-brain mTOR or SIRT1 signalling

To explore the possible mechanisms underlying the therapeutic benefits of a HFD, we analysed key metabolic and inflammatory signalling pathways implicated in Leigh syndrome neuropathology, that are also known to be influenced by a KD. In the brains of *Ndufs4* KO mice, alterations in nutrient-sensing metabolic regulators, such as mTORC1 hyperactivation [14] and PGC-1 α reduction [46] have been reported; along with elevated levels of the pro-inflammatory molecules, NF- κ B [47] and TNF- α [48,49].

Our findings revealed no evidence of mTOR pathway alterations in KO mice on either a ND or HFD, as indicated by unchanged levels of mTOR, its downstream targets (p70S6K1 and 4E-BP1), or their phosphorylated forms. In agreement, Bornstein, James [21] also reported no mTOR inhibition in the brains of *Ndufs4* KO mice following KD administration. Despite observable trends — reduced PGC-1 α and elevated TNF- α in the *Ndufs4* KO brain — these changes were not significantly modulated by the HFD. SIRT1, a central regulator of both proteins, also remained unchanged across genotypes and dietary conditions.

Overall, these findings suggest that the therapeutic benefits of a HFD do not arise from direct modulation of the mTOR/p70S6K1/4E-BP1 or SIRT1/PGC-1 α axes in the whole-brain, which regulate neuronal metabolism and inflammation. However, while these pathways were

unaffected in whole-brain tissue, a HFD may influence signalling in specific brain regions or peripheral tissues. For instance, distinct responses may occur in more severely affected brain regions such as the olfactory bulbs [27], or peripheral tissues like the liver [20], where KDs with similar fat content resulted in mTOR inhibition [25].

4.3. A HFD reduces systemic inflammation

Ndufs4 KO mice are known to exhibit profound systemic inflammation, characterized by elevated levels of pro-inflammatory cytokines [16,48–50]. To assess the impact of a HFD on the systemic inflammatory profile, we performed serum flow cytometry. The HFD intervention reversed the elevation of the pro-inflammatory cytokine TNF and significantly reduced IL-6 levels in KO serum. However, other cytokines (IFN γ , MCP-1, IL-10 or IL-12p70) remained unchanged by either genotype or diet. While the HFD significantly reduced IL-6 and TNF levels in KO mice compared to KO mice on a ND, baseline IL-6 levels did not differ between WT and KO mice on the ND. As such, the reduction observed in systemic inflammation could be mainly attributed to the reduction in TNF cytokine levels.

Although a HFD did not influence whole-brain inflammatory markers or mTOR signalling, as determined via western blot analysis, the diet effectively reduced inflammation on a systemic level. Improvements in systemic inflammation, clasping symptoms, and survival in HFD-fed KO mice align with the established anti-inflammatory properties of PUFAs [51]. Long-chain ω -3 PUFAs are well known for their anti-inflammatory effects and are abundant in Menhaden fish oil, a key component of our HFD formulation. These findings suggest that dietary ω -3 PUFA supplementation might be a critical component of the HFD's therapeutic efficacy, likely acting through reduction of peripheral inflammation.

While this study focuses on systemic inflammation, future research should assess neuroinflammatory cytokines, such as brain TNF and IL-6, to better understand the direct effects of the HFD on central nervous system pathology. These investigations would require specialized protocols to isolate and quantify cytokines in brain tissue, which were beyond the scope of this preclinical study.

4.4. A HFD enhances brain energy metabolism by improving TCA cycling along with ketone body- and BCAA utilization

Metabolic profiling revealed that the HFD intervention mitigated key neurometabolic derangements caused by CI deficiency, particularly in TCA cycle, ketone body, and BCAA pathways. Consistent with previous studies [52,53], *Ndufs4* KO brains showed reduced aspartate and oxaloacetate levels, suggesting impaired TCA cycle anaplerosis and altered flux. Concurrent accumulation of lactate, alanine, and succinate further indicated disrupted TCA cycling and a shift toward anaerobic glycolysis — hallmark features of mitochondrial dysfunction [10]. The HFD markedly increased brain aspartate and oxaloacetate levels in KO mice, while reducing lactate, alanine, and succinate, suggesting improved mitochondrial substrate utilization and TCA cycling. These findings align with reports that PUFAs can improve TCA cycle homeostasis in mice [54,55]. However, the exact mechanism by which a HFD impacts TCA cycle dynamics in mitochondrial disease models requires further investigation.

Ndufs4 KO mice on a ND further exhibited significantly elevated levels of ketone bodies (3-hydroxybutyrate and acetoacetate), suggesting impaired utilization of these alternative energy substrates. This accumulation may be exacerbated by altered inter-organ metabolic coordination, since the liver upregulates fatty acid β -oxidation and ketone body export as part of a compensatory mechanism to preserve hepatic bioenergetics during CI deficiency [56]. Following the HFD intervention, KO mice showed a significant decrease in 3-hydroxybutyrate (3-HBA) levels compared to those on a ND. This reduction suggests enhanced fatty acid utilization for energy, likely due to the high PUFA

content of the HFD, which alters the fatty acid composition available for ketogenesis and may promote mitochondrial biogenesis and function [57–59]. Improved mitochondrial efficiency is linked to a favourable NAD^+/NADH ratio, which regulates cellular metabolism and affects enzymes like 3-HBA dehydrogenase, potentially reducing 3-HBA production [19,60,61]. While significant changes in mTOR, PGC1 α , or SIRT1 activity—key mitochondrial regulators—were not observed, alterations in lipid metabolism suggest potential mechanisms for the observed metabolic shifts and redox state [57,62]. This aligns with findings in mitochondrial disease models, where lipid dysregulation and inconsistent 3-HBA changes are common [10,53]. ω -3-derived epoxycosanoids, known to enhance mitochondrial biogenesis and fusion while reducing inflammation, may play a role, however, their chemical instability likely diminishes their effectiveness and makes their effects harder to detect. Synthetic analogues like OMT-28, designed for mitochondrial myopathy treatment, demonstrate the therapeutic potential of these pathways [63]. As such, a HFD appears to improve mitochondrial metabolism and reduce 3-HBA in *Ndufs4* KO mice, though further studies are needed to clarify the underlying mechanisms and regulatory pathways.

Additionally, significant accumulation of BCAAs (leucine, isoleucine and valine) and glutamate was observed in KO mice on a ND. BCAA accumulation, another hallmark of mitochondrial dysfunction-related reductive stress [10], has been reported in whole-brain mitochondria [64] and discrete brain regions [13,52,53] of *Ndufs4* KO mice. A HFD significantly reduced the levels of all three BCAAs and glutamate in KO brains, suggesting enhanced catabolism and utilization of these substrates for energy production. Notably, BCAA accumulation is implicated in neuroinflammation [65–67] and, together with altered glutamate handling, may dysregulate glutamatergic- and γ -aminobutyric acid (GABA)ergic neurotransmitter metabolism [53,68]. Given that *Ndufs4* KO mice exhibit neuroinflammation in neurodegeneration-prone regions [27,48,69] and their neurological deficits are driven by glutamatergic- and GABAergic neurons [69], the HFD may target key pathogenic mechanisms by partially normalizing brain BCAA and glutamate levels.

4.5. A HFD alters phospholipid metabolism

A HFD significantly altered the whole-brain lipid profile of *Ndufs4* KO mice. Compared to WT mice on a ND, KO mice displayed elevated levels of multiple lipid classes, including diacylglycerols (DAGs), triacylglycerols (TAGs), phosphatidylglycerols (PGs), phosphatidylserines (PSs), phosphatidylinositols (PIs), phosphatidylethanolamines (PEs), and phosphatidylcholines (PCs). These elevations likely reflect disrupted lipid homeostasis associated with mitochondrial dysfunction [70,71], which is consistent with the OXPHOS impairment in this mouse model. Notably, the HFD substantially reduced levels of most of these lipid classes in KO mice, except for TAGs and PEs, indicating a partial restoration of lipid balance. While the mechanism underlying the persistent elevation of TAGs and PEs in KO mice on both diets remains unclear, it may reflect an adaptation response to enhance energy storage and mitochondrial function under the stress of impaired OXPHOS. The further increase in these lipids with a HFD could suggest enhanced mitochondrial dynamics, promoting cellular resilience under stress. Since these lipidomic changes were accompanied by broader metabolic shifts and key physiological improvements in KO mice — including, reduced systemic inflammation, amelioration of clasp symptoms, and increased lifespan — our findings underscore the intricate interplay between lipid metabolism, the wider metabolome, and inflammation in this model.

Beyond these lipid-specific considerations, while the current study focused solely on the whole brain metabolome and lipidome, further research is needed to investigate region-specific and cell-specific changes in these pathways in mice fed the HFD. Furthermore, other lipid signalling pathways that are crucial for mitochondrial

bioenergetics and inflammation should be explored on a systemic level as well.

5. Conclusion

This study demonstrates that a PUFA-enriched HFD offers significant therapeutic benefits in the *Ndufs4* KO mouse model of Leigh syndrome. This intervention extends lifespan, alleviates neurodegenerative symptoms, and reduces systemic inflammation, highlighting the potential role of PUFAs in modulating mitochondrial function and inflammation. Importantly, these findings emphasize that the composition of dietary fats in KDs and HFDs is a pivotal factor in determining their efficacy, distinguishing this approach from less effective KD and HFD formulations in the same model. While we observed no significant impact on whole-brain mTOR activity or key metabolic regulators such as PGC-1 α and SIRT1, the HFD enhanced TCA cycle flux and improved ketone- and BCAA utilization, reflecting improved energy metabolism in KO mouse brains. Furthermore, systemic inflammation markers, such as TNF and IL-6, were reduced by a HFD, suggesting that the diet may modulate inflammation at a systemic level.

A PUFA-enriched HFD offers a promising, cost-effective therapeutic option for the treatment of mitochondrial diseases with fewer side effects. Given the complex nature of Leigh syndrome and other mitochondrial diseases, combining HFDs with other interventions could enhance therapeutic efficacy while mitigating potential adverse effects. These findings emphasize the need for further research to explore the synergistic effects of multi-targeted therapies on region-specific brain metabolism and its translatability to clinical settings.

CRedit authorship contribution statement

Luciano Willemse: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karin Terburgh:** Writing – review & editing, Supervision, Data curation. **Roan Louw:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Roan Louw reports financial support was provided by National Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2025.167873>.

Data availability

Data will be made available on request.

References

- [1] G.S. Gorman, et al., Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease, *Ann. Neurol.* 77 (5) (2015) 753–759.
- [2] Schaefer, A.M., et al., *The epidemiology of mitochondrial disorders—past, present and future*, *Biochim. Biophys. Acta*, 2004. 1659(2-3): p. 115–20.
- [3] W.J. Koopman, et al., Inhibition of complex I of the electron transport chain causes O₂⁻-mediated mitochondrial outgrowth, *Am. J. Phys. Cell Phys.* 288 (6) (2005) C1440–C1450.
- [4] S. Papa, et al., Respiratory chain complex I, a main regulatory target of the cAMP/PKA pathway is defective in different human diseases, *FEBS Lett.* 586 (5) (2012) 568–577.
- [5] A.Z. Lim, et al., Natural history of Leigh syndrome: a study of disease burden and progression, *Ann. Neurol.* 91 (1) (2022) 117–130.
- [6] K. Sofou, et al., A multicenter study on Leigh syndrome: disease course and predictors of survival, *Orphanet J. Rare Dis.* 9 (2014) 52.
- [7] I.S. Ruhoy, R.P. Saneto, The genetics of Leigh syndrome and its implications for clinical practice and risk management, *Appl. Clin. Genet.* 7 (2014) 221–234.
- [8] C. Bris, et al., Novel NDUFS4 gene mutation in an atypical late-onset mitochondrial form of multifocal dystonia, *Neurol. Genet.* 3 (6) (2017) e205.
- [9] S. Papa, et al., Respiratory complex I in brain development and genetic disease, *Neurochem. Res.* 29 (3) (2004) 547–560.
- [10] K. Esterhuizen, F.H. van der Westhuizen, R. Louw, Metabolomics of mitochondrial disease, *Mitochondrion* 35 (2017) 97–110.
- [11] S.E. Kruse, et al., Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy, *Cell Metab.* 7 (4) (2008) 312–320.
- [12] C.F. Lee, et al., Targeting NAD(+) metabolism as interventions for mitochondrial disease, *Sci. Rep.* 9 (1) (2019) 3073.
- [13] J.Y. Yoon, et al., Metabolic rescue ameliorates mitochondrial encephalomyopathy in murine and human iPSC models of Leigh syndrome, *Clin. Transl. Med.* 12 (7) (2022) e954.
- [14] S.C. Johnson, et al., mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome, *Science* 342 (6165) (2013) 1524–1528.
- [15] A. Hanaford, S.C. Johnson, The immune system as a driver of mitochondrial disease pathogenesis: a review of evidence, *Orphanet J. Rare Dis.* 17 (1) (2022) 335.
- [16] M.A.E. van de Wal, et al., Ndufs4 knockout mouse models of Leigh syndrome: pathophysiology and intervention, *Brain* 145 (1) (2022) 45–63.
- [17] P.G. Sullivan, et al., The ketogenic diet increases mitochondrial uncoupling protein levels and activity, *Ann. Neurol.* 55 (4) (2004) 576–580.
- [18] S.G. Jarrett, et al., The ketogenic diet increases mitochondrial glutathione levels, *J. Neurochem.* 106 (3) (2008) 1044–1051.
- [19] M. Elamin, et al., Ketone-based metabolic therapy: is increased NAD(+) a primary mechanism? *Front. Mol. Neurosci.* 10 (2017) 377.
- [20] L. Willemse, K. Terburgh, R. Louw, A ketogenic diet alters mTOR activity, systemic metabolism and potentially prevents collagen degradation associated with chronic alcohol consumption in mice, *Metabolomics* 19 (5) (2023) 43.
- [21] R. Bornstein, et al., Differential effects of mTOR inhibition and dietary ketosis in a mouse model of subacute necrotizing encephalomyelopathy, *Neurobiol. Dis.* 163 (2022) 105594.
- [22] I. Elisia, et al., A ketogenic diet rich in fish oil is superior to other fats in preventing NNK-induced lung cancer in a/J mice, *Sci. Rep.* 14 (1) (2024) 5610.
- [23] X. Chen, et al., Omega-3 polyunsaturated fatty acid attenuates the inflammatory response by modulating microglia polarization through SIRT1-mediated deacetylation of the HMGB1/NF- κ B pathway following experimental traumatic brain injury, *J. Neuroinflammation* 15 (1) (2018) 116.
- [24] S. Shirooie, et al., Targeting mTORs by omega-3 fatty acids: a possible novel therapeutic strategy for neurodegeneration? *Pharmacol. Res.* 135 (2018) 37–48.
- [25] R. Liu, et al., High ratio of ω -3/ ω -6 polyunsaturated fatty acids targets mTORC1 to prevent high-fat diet-induced metabolic syndrome and mitochondrial dysfunction in mice, *J. Nutr. Biochem.* 79 (2020) 108330.
- [26] F. Valsecchi, et al., Metabolic consequences of NDUFS4 gene deletion in immortalized mouse embryonic fibroblasts, *Biochim. Biophys. Acta Bioenerg.* 1817 (10) (2012) 1925–1936.
- [27] A. Quintana, et al., Complex I deficiency due to loss of Ndufs4 in the brain results in progressive encephalopathy resembling Leigh syndrome, *Proc. Natl. Acad. Sci. USA* 107 (24) (2010) 10996–11001.
- [28] S.J. Guenet, et al., A simple composite phenotype scoring system for evaluating mouse models of cerebellar ataxia, *J. Vis. Exp.* 39 (2010).
- [29] J. Gullberg, et al., Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomic studies with gas chromatography/mass spectrometry, *Anal. Biochem.* 331 (2) (2004) 283–295.
- [30] L. Löfgren, G.B. Forsberg, M. Ståhlman, The BUMe method: a new rapid and simple chloroform-free method for total lipid extraction of animal tissue, *Sci. Rep.* 6 (2016) 27688.
- [31] Z. Pang, et al., MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights, *Nucleic Acids Res.* 49 (W1) (2021) W388–W396.
- [32] J. Xia, et al., MetaboAnalyst: a web server for metabolomic data analysis and interpretation, *Nucleic Acids Res.* 37 (Web Server issue) (2009) W652–W660.
- [33] M.N. Roberts, et al., A ketogenic diet extends longevity and Healthspan in adult mice, *Cell Metab.* 26 (3) (2017) 539–546.e5.
- [34] S.J. Pathak, et al., 2-month ketogenic diet preferentially alters skeletal muscle and augments cognitive function in middle aged female mice, *Aging Cell* 21 (10) (2022) e13706.
- [35] Z. Zhou, et al., Ketogenic diets initiated in late mid-life improved measures of spatial memory in male mice, *Geroscience* 45 (4) (2023) 2481–2494.
- [36] J. Nathan, et al., A switch to polyunsaturated fatty acid based ketogenic diet improves seizure control in patients with drug-resistant epilepsy on the mixed fat ketogenic diet: a retrospective open label trial, *Cureus* 11 (12) (2019) e6399.
- [37] A.I. Borja-Magno, et al., Supplementation with EPA and DHA omega-3 fatty acids improves peripheral immune cell mitochondrial dysfunction and inflammation in subjects with obesity, *J. Nutr. Biochem.* 120 (2023) 109415.
- [38] Afshordel, S., et al., *Omega-3 polyunsaturated fatty acids improve mitochondrial dysfunction in brain aging—impact of Bcl-2 and NPD-1 like metabolites*, *Prostaglandins Leukot. Essent. Fat. Acids*, 2015. 92: p. 23–31.
- [39] Y.H. Park, et al., Omega-3 fatty acid-type docosahexaenoic acid protects against α -mediated mitochondrial deficits and Pathomechanisms in Alzheimer's disease-related animal model, *Int. J. Mol. Sci.* 21 (11) (2020).
- [40] R.B. Nisr, D.S. Shah, H.S. Hundal, Mono- and polyunsaturated fatty acids counter palmitate-induced mitochondrial dysfunction in rat skeletal muscle cells, *Cell. Physiol. Biochem.* 54 (5) (2020) 975–993.
- [41] R. de Haas, et al., Therapeutic effects of the mitochondrial ROS-redox modulator KH176 in a mammalian model of Leigh disease, *Sci. Rep.* 7 (1) (2017) 11733.
- [42] I.H. Jain, et al., Hypoxia as a therapy for mitochondrial disease, *Science* 352 (6281) (2016) 54–61.
- [43] A. Bitto, et al., Acarbose suppresses symptoms of mitochondrial disease in a mouse model of Leigh syndrome, *Nat. Metab.* 5 (6) (2023) 955–967.
- [44] J. Li, S.G. Kim, J. Blenis, Rapamycin: one drug, many effects, *Cell Metab.* 19 (3) (2014) 373–379.
- [45] E. Barriocanal-Casado, et al., Rapamycin administration is not a valid therapeutic strategy for every case of mitochondrial disease, *EBioMedicine* 42 (2019) 511–523.
- [46] S.J.C.M. Frambach, et al., Effects of clofibrate and KH176 on life span and motor function in mitochondrial complex I-deficient mice, *Biochim. Biophys. Acta (BBA) - Mol. Basis Dis.* 1866 (6) (2020) 165727.
- [47] M. Martin-Perez, et al., PKC downregulation upon rapamycin treatment attenuates mitochondrial disease, *Nat. Metab.* 2 (12) (2020) 1472–1481.
- [48] K. Aguilar, et al., Microglial response promotes neurodegeneration in the Ndufs4 KO mouse model of Leigh syndrome, *Glia* 70 (11) (2022) 2032–2044.
- [49] K. Aguilar, et al., Interleukin-6-elicited chronic neuroinflammation may decrease survival but is not sufficient to drive disease progression in a mouse model of Leigh syndrome, *J. Inflamm. (Lond)* 21 (1) (2024) 1.
- [50] L. Peruzzotti-Jametti, et al., Mitochondrial complex I activity in microglia sustains neuroinflammation, *Nature* 628 (8006) (2024) 195–203.
- [51] P.C. Calder, Functional roles of fatty acids and their effects on human health, *JPEN J. Parenter. Enteral Nutr.* 39 (1 Suppl) (2015) 18s–32s.
- [52] S.C. Johnson, et al., Regional metabolic signatures in the Ndufs4(KO) mouse brain implicate defective glutamate/ α -ketoglutarate metabolism in mitochondrial disease, *Mol. Genet. Metab.* 130 (2) (2020) 118–132.
- [53] K. Terburgh, et al., Aberrant BCAA and glutamate metabolism linked to regional neurodegeneration in a mouse model of Leigh syndrome, *Biochim. Biophys. Acta Mol. basis Dis.* 1867 (5) (2021) 166082.
- [54] R. Liu, et al., Omega-3 polyunsaturated fatty acids prevent obesity by improving tricarboxylic acid cycle homeostasis, *J. Nutr. Biochem.* 88 (2021) 108503.
- [55] A.A. Ahmed, et al., Novel regulatory roles of omega-3 fatty acids in metabolic pathways: a proteomics approach, *Nutr. Metab. (Lond.)* 11 (1) (2014) 6.
- [56] R. Abrosimov, et al., Mitochondrial complex I inhibition triggers NAD⁺-independent glucose oxidation via successive NADPH formation, “futile” fatty acid cycling, and FADH₂ oxidation, *GeroScience* 46 (4) (2024) 3635–3658.
- [57] M.R. de Oliveira, et al., Omega-3 polyunsaturated fatty acids and mitochondria, back to the future, *Trends Food Sci. Technol.* 67 (2017) 76–92.
- [58] J. Jang, et al., Molecular mechanisms of neuroprotection by ketone bodies and ketogenic diet in cerebral ischemia and neurodegenerative diseases, *Int. J. Mol. Sci.* 25 (1) (2023).
- [59] S.C. Cunnane, Metabolism of polyunsaturated fatty acids and ketogenesis: an emerging connection, *Prostaglandins Leukot. Essent. Fat. Acids* 70 (3) (2004) 237–241.
- [60] Kemperman, R.H., R.D. Ganetzky, and S.R. Master, B-169 Beta-hydroxybutyrate/ acetoacetate ratio as Indicator for mitochondrial diseases utilizing a novel LC-MS/MS based ketone body panel. *Clin. Chem.*, 2023. 69(Supplement 1).
- [61] N. Xie, et al., NAD⁺ metabolism: pathophysiological mechanisms and therapeutic potential, *Signal Transduct. Target. Ther.* 5 (1) (2020) 227.
- [62] O.S. Ademowo, et al., Lipid (per) oxidation in mitochondria: an emerging target in the ageing process? *Biogerontology* 18 (6) (2017) 859–879.
- [63] H.E. El-Sikhry, et al., Novel roles of Epoxycosanooids in regulating cardiac mitochondria, *PLoS One* 11 (8) (2016) e0160380.
- [64] G. van der Walt, et al., Sub-cellular metabolomics contributes mitochondria-specific metabolic insights to a mouse model of Leigh syndrome, *Metabolites* 11 (10) (2021) 658.
- [65] O. Zhenyukh, et al., High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation, *Free Radic. Biol. Med.* 104 (2017) 165–177.
- [66] H.S. Yoo, U. Shanmugalingam, P.D. Smith, Potential roles of branched-chain amino acids in neurodegeneration, *Nutrition* 103–104 (2022) 111762.

APPENDIX D

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[67]

Q. Gao, et al., The association between branched-chain amino acid concentrations and the risk of autism Spectrum disorder in preschool-aged children, *Mol. Neurobiol.* 61 (8) (2024) 6031–6044.

[68]

F. Dufour, et al., Modulation of absence seizures by branched-chain amino acids: correlation with brain amino acid concentrations, *Neurosci. Res.* 40 (3) (2001) 255–263.

[69]

I. Bolea, et al., Defined neuronal populations drive fatal phenotype in a mouse model of Leigh syndrome, *eLife* 8 (2019) e47163.

[70]

S. Bathina, U.N. Das, Role of mitochondrial dysfunction in cellular lipid homeostasis and disease, *Discov. Med.* 35 (178) (2023) 653–663.

[71]

C. Moschandrea, et al., Mitochondrial dysfunction abrogates dietary lipid processing in enterocytes, *Nature* 625 (7994) (2024) 385–392.

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Chapter 5: Supplementary information

E. Supplemental information

E.1. Materials and methods

E.1.1. Animal tagging and numbering

On postnatal day (P) 21, ear punches were administered to all mice for identification purposes before tail snips were collected for genotyping. The mice were weaned and separated from their mothers on P24. Male and female mice were separately housed. Wild-type (WT) and knockout (KO) were also separately housed. KO mice, which were included in the survival cohort, were housed in a cage along with a healthy heterozygous (HET) mouse to aid in maintaining body temperature homeostasis.

E.1.2. Animal monitoring and humane endpoints

Throughout the duration of the study, distress in the animals was monitored using a humane endpoint score sheet derived from the Standard Operating Procedure (SOP) of the Vivarium (SOP_Viv_Anim 27 - Determining Pain and Distress in Laboratory Rodents). The euthanasia procedure followed the guidelines outlined in the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2020 Edition). The implementation of these humane endpoint measures was the responsibility of a research animal technician, who also supervised the breeding of mice.

E.1.3. Genotyping

Genomic DNA was isolated from tail snips and liver tissue using the Zymo Research Quick-DNATM Miniprep Plus kit (Inqaba Biotech, D4068) and following the instructions of the manufacturer. Subsequently, the isolated DNA was quantified using a NanoDrop One Microvolume UV-Vis spectrometer (Thermo Fisher Scientific) and diluted to concentrations of 25 ng/μL in nuclease-free water (Qiagen, cat #129114).

A polymerase chain reaction (PCR) was conducted to validate the deletion of exon 2 of the *Ndufs4* gene. The PCR reaction utilized specific forward (5'-AGCCTGTTCTCATACCTCGG-3') and reverse (5'-TTGTGCTTACAGGTTCAAAGTGA-3') primers, designed for the *Ndufs4* gene as described by (Valsecchi *et al.*, 2012). The primers were used at a concentration of 0.5 μM each. The reaction mixture consisted of 1x Phire Tissue Direct PCR Mastermix (Thermo Fisher Scientific, F-170L), nuclease-free water, and 25 ng DNA. The thermal cycling conditions for the PCR reaction were as follows: an initial activation step at 98°C for 5 min, followed by 34 cycles of denaturation at 98°C for 5 seconds, annealing at 57.3°C for 5 seconds, and extension at 72°C for 20 seconds. After 34 cycles, a final extension step was performed at 72°C for 1 min, followed by cooling to 4°C until the samples were removed from the thermal cycler. To visualise the PCR products, a 1% (w/v) agarose gel was prepared in 1x Bionic™ Buffer (Merck, cat #B6185) containing 5 μg/mL ethidium bromide. Alongside the samples, a GeneRuler™ 100 bp DNA Ladder (Thermo Fisher Scientific, cat #SM0324) was loaded onto the gel. The samples were diluted 3:7 in nuclease-free

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water before loading. The gel was run at a starting voltage of 40 V for 10 min and subsequently increased to 75 V for 1 hr. Analysis of the DNA fragments on the agarose gel revealed distinct bands. A PCR product of 1229 bp indicated the presence of the WT allele, while a 429 bp product indicated a homozygote (*Ndufs4* KO). Heterozygotes were identified by the presence of both 1229 bp and 429 bp fragments.

E.1.4. Survival curves

To evaluate the efficacy of a polyunsaturated fatty acid-enriched high-fat diet (HFD) in extending the lifespan of *Ndufs4* KO mice, natural death dates were recorded. Survival analyses were performed using the minimum number of mice required to achieve statistical significance, prioritising animal welfare and minimising distress.

Mice in the survival cohort were weighed five times per week, and daily monitoring enabled precise determination of their natural expiration dates. Locomotor activity and ataxia assays were conducted on the same mice to assess the impact of the HFD on disease phenotypes. To prevent starvation-related deaths after P45, food pellets were placed on the cage floor, and water bottles with extended spouts were provided to ensure access.

Additionally, video recordings were conducted at P30, P40, and P50 to document phenotypic progression. Each session lasted at least 5 min, capturing the movement of the animals within their enclosure to evaluate additional phenotypic behavior associated with the disease.

E.1.5. Locomotor activity

Locomotor activity was assessed using an open-field test and automated tracking software (Ethovision XT14; Noldus Information Technology BV). Before testing, mice were habituated to the testing environment by placing their home cages in the testing room for at least 15 min, during which they were left undisturbed, with the experimenter out of sight. Each mouse was individually introduced into a black wooden arena (50 cm²) segmented into four sections. Mice were placed at the center of the arena and allowed to explore freely for 5 min, following the protocol described by (Seibenhener & Wooten, 2015). To ensure consistency, droppings were removed from the test chamber, and the arena was thoroughly cleaned with 70% ethanol after each trial to eliminate odor traces. Ethovision software recorded cumulative horizontal and vertical activity during the 5-min session. After the test, mice were returned to their housing cages.

This automated methodology enables continuous and unbiased behavioral monitoring, offering superior sensitivity compared to manual observation and minimizing the risk of investigator bias. Parameters measured included cumulative duration in the center of the arena (time in seconds spent in the center), mean velocity (movement speed in cm/s) and total distance traveled (in cm).

E.1.6. Wire-grid hang test

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Ndufs4 KO mice display pronounced ataxia, including splayed limbs, impaired balance leading to frequent falls, slow and ineffective movement corrections, and an unresponsive reaction to a firm nudge, as previously described by (Kruse *et al.*, 2008). To quantify this phenotypic manifestation, we employed the wire grid hang test.

The procedure followed that outlined by (Carlson *et al.*, 2010) for assessing muscular strength in all four limbs against gravitational force. This simple, cost-effective test does not require prior acclimatisation of the mice. To prevent the mice from escaping to the opposite side of the cage lid, a 25 cm cardboard box was attached to the edges of the wire cage. The mouse was placed on the wire grid inside the cardboard box, enabling it to grasp with all four limbs. The grid was then inverted above a plastic cage filled with soft sawdust bedding and paper towels, positioned approximately 35 cm above the bedding. This height ensured safety in the event of a fall, preventing injury while allowing voluntary descent.

Grip duration, up to a maximum of 180 seconds, was recorded using a stopwatch (Kruse *et al.*, 2008). Each mouse underwent three trials with a 10-min rest between trials. Mice that fell off the grid within 10 seconds were retested to ensure no pre-existing grip issues affected the results. The mean physical impulse was calculated by integrating the force applied, including both the gravitational force and the weight of the mouse, over the duration of the grip. This measure provided a precise estimate of the impulse during the sustained force application.

E.1.7. Euthanasia and sample collection

Following the completion of the dietary intervention on ~P45, the mice were humanely euthanized through exsanguination. The subsequent collection of tissues encompassed various vital organs and bodily systems, namely the blood, brain, quadriceps muscle, heart, liver, kidneys, caecum, and large intestines. Each tissue sample was then carefully placed into individual 1.5 mL microcentrifuge tubes and rapidly frozen in liquid nitrogen. Subsequently, the samples were stored at -80°C until analysis. In particular, the brain tissues utilized in this study were first sectioned on an ice-cold steel table, accurately weighed, and subsequently divided into 1.5 mL microcentrifuge tubes for further analyses.

E.1.8. SDS-PAGE and western blotting

Brain tissue samples (~30 mg) were homogenized in RIPA lysis buffer (~300 µL) using sonication with three pulses of three seconds each per sample. Subsequently, homogenates were obtained, and protein concentration was determined using a BCA protein assay. Samples were prepared for SDS-PAGE by mixing with 4X Laemmli sample buffer and milli-Q water to a final volume of 25 µL, followed by heating at 72°C for 10 min. For each dietary group, aliquots (30 µg) of protein were separated via SDS-PAGE using a 12% acrylamide gel at 200 V for 30 min, then transferred to nitrocellulose membranes (cat #230-4, Bio-Rad, USA) and blocked with 1% casein in TBST buffer (cat #161-0782, Bio-Rad, USA) for 1 hour. Subsequently, membranes were incubated overnight at

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4°C with primary antibodies including phospho-mTOR (cat #5536S), phospho-p70 S6 kinase (cat #9234S), phospho-4E-BP1 (cat #9459S), p70S6K1 (cat #9202S), 4E-BP1 (cat #9644S), SirT1 (cat #9475S) from Cell Signaling Technologies, USA, as well as mTOR (cat #ab32028) and PGC1- α (cat #ab54481) from Abcam, UK, and TNF- α (cat #A0046) from Cape Bioligix, Cape Town, and the housekeeping protein, β -actin (cat #ab6276, Abcam). Following primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies, anti-Rb (cat #ab97051, Abcam) and anti-Ms (cat #ab97023, Abcam), at room temperature for 1 hour. Chemiluminescent detection was performed using a Western ECL substrate (cat #170-5060, Bio-Rad, USA) and the ChemiDoc™ MP imaging system (Bio-Rad). Finally, Image Lab™ v. 6.1 software (Bio-Rad, USA) was used to analyze the results and obtain the density ratio of target proteins relative to β -actin.

E.1.9. Inflammation assay

The levels of pro-inflammatory biomarkers, including IL-6, IL-10, MCP-1, TNF, IFN- γ , and IL-12, were quantified using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (cat# 552364, BD Biosciences) in accordance with the manufacturer's instructions. The assay involved a multiplex bead array coupled with flow cytometry. Both samples and standards were analyzed using a BD Accuri C6 Flow Cytometer. Subsequently, standard curves were generated, and regression analysis was conducted utilising FlowJo (version 10.8) and FCS Express software (version 6, De Novo) to ascertain the concentrations (in pg/mL) of the biomarkers in the serum samples from mice.

E.1.10. Monophasic metabolite extraction

For brain samples, a final monophasic ratio of 3:1:1 (v/v) methanol:chloroform was used, based on the assumption of 80% water content in wet brain tissue (Gonzalez-Riano *et al.*, 2016). This ratio was selected to ensure a monophasic extract and prevent solvent partitioning (Gullberg *et al.*, 2004). To standardise concentrations across samples prior to analysis, solvent volumes were adjusted according to sample weight [pre-analysis normalisation] (Chen *et al.*, 2018). This approach aimed to minimise matrix-induced ion suppression and ensure the injection of an optimal sample volume, with consistent metabolite concentrations (Boysen *et al.*, 2018; Kapoore & Vaidyanathan, 2016). Typically, solvents were added to achieve a final monophasic solution at a 5% (w/v) concentration. Samples were kept on ice whenever possible, and all solvents were maintained at 4 °C, unless specified otherwise.

On the day of extraction, frozen brain samples, stored in 2 mL safe-lock microcentrifuge tubes, were prepared for metabolite extraction. A single cold steel bead (3 mm Ø) was added to each tube to enhance extraction efficiency, following the method of Gullberg *et al.* (2004). For each gram of pre-weighed frozen brain tissue, the following solvents were added: 12 μ L/mg methanol (at -20 °C), 3.2 μ L/mg water containing internal standards, and 4 μ L/mg chloroform (also at -20 °C). These solvent volumes aimed to achieve a final 3-PBA concentration of 25 ppm in the monophasic

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solution. The soft brain tissue was then homogenized for 5 min at 30 Hz using the Retsch MM 400 vibration mill, ensuring rapid and uniform homogenisation while maintaining sample integrity by processing each tissue sample in a separate tube (Wu *et al.*, 2017). Magnetic beads were carefully removed to avoid sample loss. The homogenized samples were vortexed for 1 min, incubated on ice for 10 min, and centrifuged at 20 000 xg for 15 min at 4 °C to remove proteins and tissue debris.

The monophasic supernatant was carefully transferred to a new microcentrifuge tube, avoiding disturbance of the pellet, and then divided for analysis across various platforms. For GC-TOF and GC-QQQ analysis, 200 µL of the supernatant for each sample was transferred to a glass vial. The samples were dried under a gentle nitrogen stream in a fume hood, tightly capped, and stored at -80 °C until derivatization.

E.1.11. GC-TOF-MS

For untargeted analysis of brain tissue extracts, we utilized GC-TOFMS with an Agilent 8890 gas chromatograph coupled to a Pegasus® BT time-of-flight (TOF) mass spectrometer, equipped with an electron impact ionisation source.

Samples were derivatized through a two-step oximation and silylation procedure. Oximation was carried out by adding 50 µL of methoxyamine hydrochloride (15 mg/mL in pyridine) to the dried extracts, followed by vortexing and incubation at 60°C for 60 min. Silylation was performed by adding 50 µL of N, O-bis(trimethylsilyl) trifluoroacetamide (99:1, v/v), and incubating at 60°C for an additional 60 min. The derivatized samples were then transferred to 250 µL tapered glass inserts within 2 mL glass vials and loaded onto the autosampler for analysis.

Chromatographic separation was achieved using an Rxi®-5MS capillary column (29.69 m x 0.25 mm x 0.25 µm, Restek). Sample (1 µL) was injected at a 1:5 split ratio, with the front inlet set to 270°C. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The GC oven temperature program started at 70°C for 1 min, followed by a ramp of 10°C/min to a final temperature of 320°C, held for 3 min. The transfer line and ion source temperatures were set at 270°C and 250°C, respectively, with compounds subjected to electron impact ionisation at 70 eV. Mass spectra were acquired at a scan rate of 20 spectra/s after a 300-second solvent delay, covering a range of 50-850 m/z.

Data acquisition and processing were performed using ChromaTOF software (v5.55, LECO Corporation). Peak alignment was achieved using the Statistical Compare package, aligning peaks based on their mass spectrum and retention times. Peak annotation was performed by comparing the spectral and retention time data to both commercial (NIST11, mainlib, and replib) and in-house libraries, with a similarity threshold of 80% or greater.

E.1.12. GC-MS/MS

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For targeted analysis of redox-related metabolites, we utilized GC-MS/MS with a 7890B GC coupled to a 7010B triple quadrupole mass spectrometer, equipped with an electron impact ionisation source, as outlined by Jooste et al. (2024). The system was integrated with a 7693 autosampler (Agilent® Technologies) and data acquisition was performed using Agilent MassHunter Workstation (vB10.00).

Samples were resuspended in 80 µL of pyridine and derivatized with 20 µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (99:1, v/v) at 22°C for 10 min. The derivatized mixture was then transferred to 250 µL tapered glass inserts in 2 mL glass vials for analysis using the autosampler.

Chromatographic separation was achieved using an Rxi®-1MS capillary column (30 m x 0.32 mm, 0.25 µm). Sample (1 µL) was injected at a 10:1 split ratio, with the front inlet temperature set at 250°C. Helium was used as the carrier gas with a constant flow rate of 1 mL/min and a post-run flow rate of 1.2 mL/min. The GC oven temperature program consisted of an initial 1-min hold at 70°C, followed by a ramp at 12°C/min to 135°C, where it was held for 1.5 min. A further ramp at 2°C/min reached 145°C, held for 1.5 min, and a final ramp of 23°C/min was applied to reach 300°C, held for 1 min. The ion source temperature was set to 250°C, and compounds were ionized using electron impact at 70 eV, with nitrogen as the collision gas at a flow rate of 1.5 mL/min. Mass spectrometry was conducted in dynamic multiple reaction monitoring (dMRM) mode, optimized with reference standards (Table 3-2).

To enhance sensitivity, dMRM analysis was divided into three separate analytical methods, requiring three individual sample injections to analyze all compounds. For robust metabolite identification, two distinct transitions were monitored per metabolite, enabling confirmation via spectral matching and retention time comparison (Schymanski *et al.*, 2014; Sumner *et al.*, 2007).

E.1.13. Lipid extraction and UPLC-HDMS analysis

For brain tissue homogenisation and lipid extraction, snap-frozen tissue was weighed and transferred into 2 mL centrifuge tubes containing one 3 mm tungsten beads each. A cold BUME solution (butanol/methanol, 3:1 v/v) containing diheptadecanoyl (C17:0) fatty acid internal standards were prepared. The internal standards included 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (cat #850360P), 1,2-diheptadecanoyl-sn-glycero-3-phosphate (cat #830856P), and 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (cat #830456P), all obtained from Avanti Polar Lipids (USA). This solution was added to the tissue samples at a volume of 4 µL per milligram of tissue. Samples were homogenized at 30 Hz for 20 minutes using a vibration mill, followed by centrifugation at 4 000 g for 10 minutes. The upper phase of the supernatant was carefully collected and transferred to new tubes, centrifuged again at 12 000 g for 10 minutes to remove any debris, and aliquoted (50 µL) into 2 mL autosampler glass vials. The sample aliquots were then dried using a SpeedVac vacuum concentrator and stored at -80°C. Prior to analysis,

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dried samples were resuspended in 100 µL of methanol/acetonitrile (2:1) and then transferred to 250 µL tapered glass inserts within the same 2 mL glass vials for lipidomic profiling.

Untargeted lipidomic analysis of brain lipid extracts was conducted using a Synapt G2-Si system, combining ultra-performance liquid chromatography (UPLC) with high-definition mass spectrometry (HDMS) (Waters). Chromatographic separation was performed on a Waters Acquity UPLC CSH C18 column (2.1 × 100 mm, 1.7 µm). A gradient elution was used with two mobile phases: solvent A (60% acetonitrile, 40% water, 10 mM ammonium formate, and 0.1% formic acid) and solvent B (90% isopropyl alcohol, 10% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid). The conditions began with 40% B, held for 4 min, followed by a linear gradient to 70% B over 20 min. The 70% B condition was maintained for 12 min before ramping to 99% B over 20 seconds. The system was re-equilibrated to 40% B for 4 min, giving a total run time of 40 min. The flow rate was set to 0.4 mL/min, and the column temperature was kept at 45°C, with an injection volume of 1 µL. Samples were introduced using a cooled autosampler maintained at 4°C.

Mass spectrometric detection was performed using the HDMS, equipped with an electrospray ionisation (ESI) source, with ion mobility mode disabled. Data acquisition was carried out using MassLynx software (version 5, Waters), and Progenesis Q1 (version 6, Waters) was used for peak alignment based on matching mass spectra and retention times.

E.2. References

- Boysen, A.K., Heal, K.R., Carlson, L.T. & Ingalls, A.E. 2018. Best-Matched Internal Standard Normalization in Liquid Chromatography-Mass Spectrometry Metabolomics Applied to Environmental Samples. *Anal. Chem.*, 90(2):1363-1369.
- Carlson, C.G., Rutter, J., Bledsoe, C., Singh, R., Hoff, H., Bruemmer, K., ... McCarthy, L. 2010. A simple protocol for assessing inter-trial and inter-examiner reliability for two noninvasive measures of limb muscle strength. *J. Neurosci. Methods.*, 186(2):226-230.
- Chen, S.S., Wang, L., Yu, I.K.M., Tsang, D.C.W., Hunt, A.J., Jérôme, F., ... Poon, C.S. 2018. Valorization of lignocellulosic fibres of paper waste into levulinic acid using solid and aqueous Brønsted acid. *Bioresour. Technol.*, 247:387-394.
- Gonzalez-Riano, C., Garcia, A. & Barbas, C. 2016. Metabolomics studies in brain tissue: A review. *J. Pharm. Biomed. Anal.*, 130:141-168.
- Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M. & Moritz, T. 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. *Anal Biochem*, 331(2):283-295. 10.1016/j.ab.2004.04.037
- Jooste, T., 2024. Establishing a GC-MS/MS method to quantify redox markers in NDUF54 knockout mice. Potchefstroom: North-West University.
- Kapoor, R.V. & Vaidyanathan, S. 2016. Towards quantitative mass spectrometry-based metabolomics in microbial and mammalian systems. *Philos. Trans. A Math. Phys. Eng. Sci.*, 374(2079),
- Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A. & Palmiter, R.D. 2008. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab.*, 7(4):312-320.

APPENDIX E

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P. & Hollender, J. 2014. Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ. Sci. Technol.*, 48(4):2097-2098.

Seibenhener, M.L. & Wooten, M.C. 2015. Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. *J. Vis. Exp.*, (96):e52434.

Sumner, L.W., Amberg, A., Barrett, D., Beale, M.H., Beger, R., Daykin, C.A., ... Viant, M.R. 2007. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*, 3(3):211-221.

Valsecchi, F., Monge, C., Forkink, M., de Groof, A.J., Benard, G., Rossignol, R., ... Willems, P.H. 2012. Metabolic consequences of NDUFS4 gene deletion in immortalized mouse embryonic fibroblasts. *Biochim. Biophys. Acta*, 1817(10):1925-1936.

Wu, H., Wen, S.P., Huang, H., Li, B.L., Wu, X.L., Gao, K.Y., ... Nie, Z.R. 2017. Effects of homogenization on precipitation of Al₃(Er,Zr) particles and recrystallization behavior in a new type Al-Zn-Mg-Er-Zr alloy. *Mater. Sci. Eng. A.*, 689:313-322.

APPENDIX F

Language editing certificate



084 365 4320 
editingexcellencepotch@gmail.com 

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