

Investigating tissue-specificity in a mouse model of Leigh Syndrome using metabolomics

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***" For from Him and through Him and to Him
are all things. To Him be the glory forever."***

- Romans 11:36 -

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ABSTRACT

Mitochondrial disease (MD) is one of the most challenging groups of inborn disorders featuring a tissue-specific vulnerability, governed by largely unknown mechanisms. The value of metabolomics in multi-systemic MD research has been increasingly recognised; however, minimally invasive biofluids are the sample type that is generally favoured and it remains unknown whether such systemic metabolomes clearly reflect tissue-specific metabolic alterations. Here, we mine and cross-compare metabolomes from *Ndufs4* knockout (KO) mice with global complex I (CI) deficiency-related Leigh syndrome (LS) — a complex neurometabolic MD defined by progressive focal lesions in specific brain regions. With this approach, the study aimed to identify and evaluate the extent of common and unique metabolic alterations associated with LS, on a brain regional and systemic level. Semi-targeted liquid chromatography-tandem mass spectrometry and untargeted gas chromatography time-of-flight mass spectrometry were performed on seven sample types from *Ndufs4* KO ($n \geq 19$) vs wild type (WT, $n \geq 20$) mice; which include one lesion-resistant and three lesion-prone brain regions; two skeletal muscles of different fibre type composition; and urine. To gain mechanistic insight into regional neurodegeneration, metabolic alterations in the four brain regions were investigated alongside regional respiratory chain enzyme activity. Enzyme assays confirmed significantly decreased (60–80%) CI activity in all investigated *Ndufs4* KO brain regions, with the lesion-resistant region displaying the highest residual CI activity (38% of WT). A higher residual CI activity and a less perturbed NADH/NAD⁺ ratio, correlate with less severe metabolic perturbations in *Ndufs4* KO brain regions. Moreover, less perturbed BCAA oxidation and increased glutamate oxidation seem to distinguish lesion-resistant from -prone KO brain regions, thereby identifying key areas of metabolism to target in future therapeutic intervention studies. The subsequent cross-comparison of all seven sample-type metabolomes further revealed widespread alterations in alanine, aspartate, glutamate, and arginine metabolism in *Ndufs4* KO mice. An adaptive rewiring of glutamate and nitrogen metabolism, along with increased oxidation of alternative respiratory chain ubiquinone cycle fuels, α -hydroxyglutarate, succinate, and glycerol-3-phosphate seem to be a system-wide response to CI deficiency. Brain region-specific metabolic signatures include the accumulation of BCAAs, proline, and glycolytic intermediates, whereas skeletal muscles display evidence of hyper-catabolism. Furthermore, we describe a systemic dysregulation in one-carbon metabolism and the tricarboxylic acid cycle, which was not clearly reflected in the *Ndufs4* KO brain. Altogether, our results provide novel insight into brain region-specific and systemic metabolic responses to LS, while confirming the value of urinary metabolomics when evaluating MD-associated metabolites but cautioning against mechanistic studies relying solely on systemic biofluids.

Keywords: *Ndufs4* knockout mice • Leigh syndrome • Complex I deficiency • Mitochondrial disease • Metabolomics • Brain regions • Urine • Skeletal muscle

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

LIST OF ABBREVIATIONS AND LATIN TERMS

Abbreviations	
1C	One-carbon
3'	3-prime end; 3-prime hydroxyl group of the polynucleotide chain
3-MH	3-Methylhistidine
3-PBA	3-Phenylbutyric acid
5'	5-prime end; 5-prime phosphate group of the polynucleotide chain
AC	Anterior cortex
ADP	Adenosine diphosphate
AHS	Alpers Huttenlocher syndrome
AM	<i>Ante meridiem</i> (Latin): before noon
AMDIS	Automated Mass Spectral Deconvolution and Identification System
AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
Asp	Aspartic acid
ATP	Adenosine triphosphate
ATPAF2	ATP Synthase Mitochondrial F1 Complex Assembly Factor 2
AVMA	American Veterinary Medical Association
BAIBA	β -aminoisobutyrate
BBA	<i>Biochimica et Biophysica Acta</i> (Latin): Journal of Biochemistry and Biophysics
BBD	γ -butyrobetaine dioxygenase
BCAA	Branched-chain amino acid
BCKDH	Branched-chain α -keto acid dehydrogenase
BHMT	Betaine-homocysteine methyltransferase
bp	Base pairs
BR	Brain regions
BS	Brainstem
BSTFA	O-bis(trimethylsilyl)trifluoro acetamide
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
Cat.	Catalogue
CB	Cerebellum
CI	Complex I; NADH:ubiquinone oxidoreductase; or confidence interval (depending on the context)
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
CPEO	Chronic progressive external ophthalmoplegia
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
DHAP	Dihydroxyacetone phosphate
DLST	Dihydrolipoyllysine-residue succinyltransferase
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
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
EI	Electron impact
ES	Effect size
ESI	Electrospray ionisation
ETF	Electron-transferring flavoprotein
ETFDH	Electron-transferring-flavoprotein dehydrogenase
FAD(H ₂)	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FDR	False discovery rate
Fe-S	Iron-sulfur
FMN	Flavin mononucleotide
F-ratio	Fisher ratio
G3P	Glycerol-3-phosphate
GABA	γ-Aminobutyric acid
GC-TOF-MS	Gas chromatography time-of-flight mass spectrometry
Gln	Glutamine
Glu	Glutamate
GLUT1	Glucose transporter 1
GnRH	Gonadotropin-releasing hormone
GPDH	Glycerol-3-phosphate dehydrogenase
GSH	Reduced glutathione
GSSG	Oxidised glutathione; glutathione disulfide
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
HIF1α	Hypoxia inducible factor 1 alpha
HK	Hexokinase
HMDB	Human Metabolome Database
HP	Hydroxyphenyl
HPLC	High-performance liquid chromatography
IBM	Inner boundary membrane
ID	Identification number
IJV	Internal jugular venous
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IS	Internal standard
JNK	c-Jun N-terminal kinases
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGDHC	2-Ketoglutarate dehydrogenase complex
KIC	α-Ketoisocaproate
KO	Knockout (homozygous; both alleles of interest are absent)
KSS	Kearns-Sayre syndrome

LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
Leu	Leucine
LHON	Leber's hereditary optic neuropathy
LS	Leigh syndrome
Lys	Lysine
M	Skeletal muscles
MD	Mitochondrial disease
ME	Maine
MEF	Mouse embryonic fibroblasts
MELAS	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERRF	Mitochondrial encephalopathy with ragged red fibres
MET-IDEA	Metabolomics Ion based Data Extraction Algorithm
m-FDH	Mitochondrial flavoprotein dehydrogenase
MIDD	Maternal-inherited diabetes and sensorineural hearing loss
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MSI	Metabolomics standards initiative
MSUD	Maple syrup urine disease
mtDNA	Mitochondrial DNA
mTORC(1)	Mammalian/mechanistic target of rapamycin complex (1)
n	Number of; amount
N module	NADH binding module
N/A	Not applicable or not available (depending on context)
NAD(P) ⁺	Oxidized nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
NAG	N-acetyl glutamate
NaN ₃	Sodium azide
NARP	Neuropathy, ataxia, and retinitis pigmentosa
ND	NADH dehydrogenase subunit or Not detected (depending on context)
nDNA	Nuclear DNA
NDUF	NADH:ubiquinone oxidoreductase (core) subunit
NDUFAF	NADH:ubiquinone oxidoreductase complex assembly factor
NDUFS	NADH dehydrogenase (ubiquinone) iron-sulfur protein
NH ₄ ⁺	Ammonium cation
NHREC	National Health Research Ethics Committee
NIH	National Institutes of Health NIH
NIST	National Institute of Standards and Technology
NMDR	National Metabolomics Data Repository
NRF	National Research Foundation of South Africa
NWU	North-West University
O ₂	Oxygen
OAA	Oxaloacetate
OB	Olfactory bulbs
OMIM	Online Mendelian Inheritance in Man
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
P	Postnatal day
P module	Proton pumping module
P5C	Pyrroline-5-carboxylate

P6C	Pyroline-6-carboxylate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCDDP	Preclinical Drug Development Platform
PCK	protein kinase C
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
pGlu	Pyroglutamate
pO ₂	Partial pressure of oxygen
PPP	Pentose phosphate pathway
Pro	Proline
PRODH	Proline dehydrogenase
PTFE	Polytetrafluoroethylene
p-value	Probability value; the probability of making a Type I error
Q	Ubiquinone; coenzyme Q
Q module	Ubiquinone binding module
QC	Quality control
QH ₂	Ubiquinol; reduced coenzyme Q
R ²	Coefficient of determination; indicated the linearity data
RC	Respiratory chain
Redox	Reduction-oxidation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
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
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SAVC	South African Veterinary Council
SC	Supercomplex
SCF	short chain fatty acids
SD	Standard deviation
SDH	Succinate dehydrogenase
SOL	Solei
SOP	Standard operating procedure
SPF	Specific pathogen-free
TCA	Tricarboxylic acid cycle
TIA	Technology Innovation Agency
TLR	Toll-like receptor
T _m	Melting temperature
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
tRNA	Transfer ribonucleic acid
U	Urine
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 center
WQ	White quadriceps
WT	Wild type; genetically unaltered sample (specific to this study)
α AA	α -Aminoadipic acid
α HG	α -Hydroxyglutaric acid
α KG	α -Ketoglutaric acid
β -Ala	Beta-alanine

Latin terms

<i>Ad libitum</i>	According to pleasure; feeding habits according to preference as food is always available
<i>De novo</i>	Anew; from the beginning
<i>Homo sapiens</i>	A species of primate to which modern humans belong
<i>In situ</i>	In the site of origin/natural place
<i>In vitro</i>	In the glass; a process outside of a living organism
<i>In vivo</i>	In life; a process within a living organism
<i>Mus musculus</i>	A species of rodent; commonly known as a house mouse
<i>Post-mortem</i>	After death

CHAPTER

Introduction



1

1.1 Research rationale

Oxidative phosphorylation (OXPHOS) system dysfunction results in primary mitochondrial disease (MD) — one of the most challenging groups of genetic metabolic disorders for which disease mechanisms are poorly grasped and effective treatment options are lacking (Lake *et al.*, 2016; Mootha & Chinnery, 2018; Rahman, 2020). As the predominant form of MD, respiratory chain complex I (CI) deficiency and its most prevalent paediatric manifestation, Leigh syndrome (LS), are at the forefront of MD research (Rahman *et al.*, 1996; Rodenburg, 2016). LS is a severe, often multi-systemic, neurometabolic disease characterised by progressive focal lesions in specific brain regions (Lake *et al.*, 2016; Rahman *et al.*, 2017). The most widely studied pre-clinical model of CI deficiency-related LS, the *Ndufs4* knockout (KO) mouse, develops a progressive encephalopathic phenotype similar to LS patients, which increasingly worsens until premature death (Kruse *et al.*, 2008; Quintana *et al.*, 2010). Research on *Ndufs4* KO mice revealed that specific neurodegeneration-prone brain regions drive the fatal phenotype (Bolea *et al.*, 2019; Quintana *et al.*, 2010), while others and peripheral tissues, especially skeletal muscle, lack strong phenotypic involvement (Alam *et al.*, 2015; Kruse *et al.*, 2008; Quintana *et al.*, 2010). However, the mechanisms behind this tissue- and brain region-specific vulnerability to global CI deficiency, remain elusive.

In this regard, the application of multi-platform metabolomics as a hypothesis-generating tool (Pinu *et al.*, 2019) could provide novel insight. The ability to investigate a variety of tissues and biofluids is considered a particular advantage of large-scale metabolomics. Yet, its application in MD research is still rather uncommon, with non-invasive (urine) or minimally invasive (blood) biofluids generally being the favoured sample type — despite uncertainties regarding their reflection of tissue-specific metabolic alterations (Esterhuizen *et al.*, 2017; Rahman & Rahman, 2018; Steele *et al.*, 2017). Although whole brain (Johnson *et al.*, 2013) and brain regional metabolomics (Johnson *et al.*, 2020) was recently done in young *Ndufs4* KO mice, no significant brain-regional metabolic alterations were evident prior to the onset of encephalopathy and lesion formation. However, since such young animals are considered a rather inappropriate model of LS (Finsterer, 2019), further investigations of more diseased *Ndufs4* KO mice are required.

Therefore, our group conducted the first large-scale metabolomics investigation of brain regions, skeletal muscles, and urine from a relatively large group of diseased *Ndufs4* KO mice. Our extensive analysis of skeletal muscle metabolomes identified several adaptive pathways that seem to explain the tissue's lack of phenotypic involvement (Terburgh *et al.*, 2019) [Appendix D]. However, brain regional metabolic alterations and their connection to skeletal muscle and urine metabolomes remain unexplored.

1.2 Aim and objectives

1.2.1 Aim

The aim of this study was to investigate multi-platform metabolomics data from brain regions, skeletal muscles, and urine of diseased *Ndufs4* KO and WT mice to i) gain mechanistic insight into regional neurodegeneration and ii) establish how well the systemic metabolome reflects tissue-specific metabolic alterations.

1.2.2 Objectives

As part of a larger project, the following objectives were formulated to achieve the abovementioned aim:

1. Collect brain regions, skeletal muscles, and urine from *Ndufs4* KO and WT mice:
 - 1.1 Interbreeding, genotyping, and selecting mice for the study.
 - 1.2 Euthanasia, sample collection and *post-mortem* genotype verification.
2. Perform multi-platform metabolic profiling of *Ndufs4* KO and WT brain regions, skeletal muscles, and urine:
 - 2.1 Perform semi targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS)
 - 2.2 Perform untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS)
3. Compare brain regional metabolomes from *Ndufs4* KO vs WT mice:
 - 3.1 Data preprocessing and pretreatment.
 - 3.2 Statistical analysis and discriminatory metabolite discovery.
 - 3.3 Biological interpretation via pathway analysis and literature study.
4. Compare tissue and urine metabolomes from *Ndufs4* KO vs WT mice:
 - 4.1 Data preprocessing and pretreatment.
 - 4.2 Statistical analysis and discriminatory metabolite discovery.
 - 4.3 Biological interpretation via pathway analysis and literature study.

1.3 Research design

This study forms part of a larger project at the North-West University (NWU), for which heterozygous (HET) B6.129S4-*Ndufs4*^{tm1.1Rpa}/J mice were interbred to obtain homozygous *Ndufs4* KO and WT mice. A relatively large group of late-stage diseased [postnatal day (P) P45-50] male animals were selected for tissue-specific investigations, including respiratory chain enzyme activity assays and metabolomics. The research design relevant to this study, is displayed in Figure 1-1 and discussed hereafter. *Ndufs4* KO (n≥19) and WT (n≥20) mice were fasted overnight in individual metabolic cages to collect 12-hour urine (U) samples. Thereafter, animals were euthanised, tissues were harvested, snap frozen in liquid nitrogen, and stored at – 80°C. This includes one lesion-resistant brain region (anterior cortex, AC) and three lesion-prone regions (brainstem, BS; cerebellum, CB; and olfactory bulbs, OB), as well as primarily

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glycolytic (white parts of the quadriceps femoris, WQ) and primarily oxidative (solei, SOL) skeletal muscles. Tail snips were also collected for *post-mortem* PCR-based genotype verification. For each biological replicate, each sample type (n=7) was prepared and analysed individually in sample type-specific randomised batches via semi-targeted LC-MS/MS and untargeted GC-TOF-MS. The resulting spectral datasets (n=14) were then preprocessed, pretreated, statistically analysed, and biologically interpreted using pathway analyses and existing literature. Brain-regional metabolomes were first assessed (alongside enzymatic data), after which all investigated tissue- and urine-specific metabolomes were cross-compared.

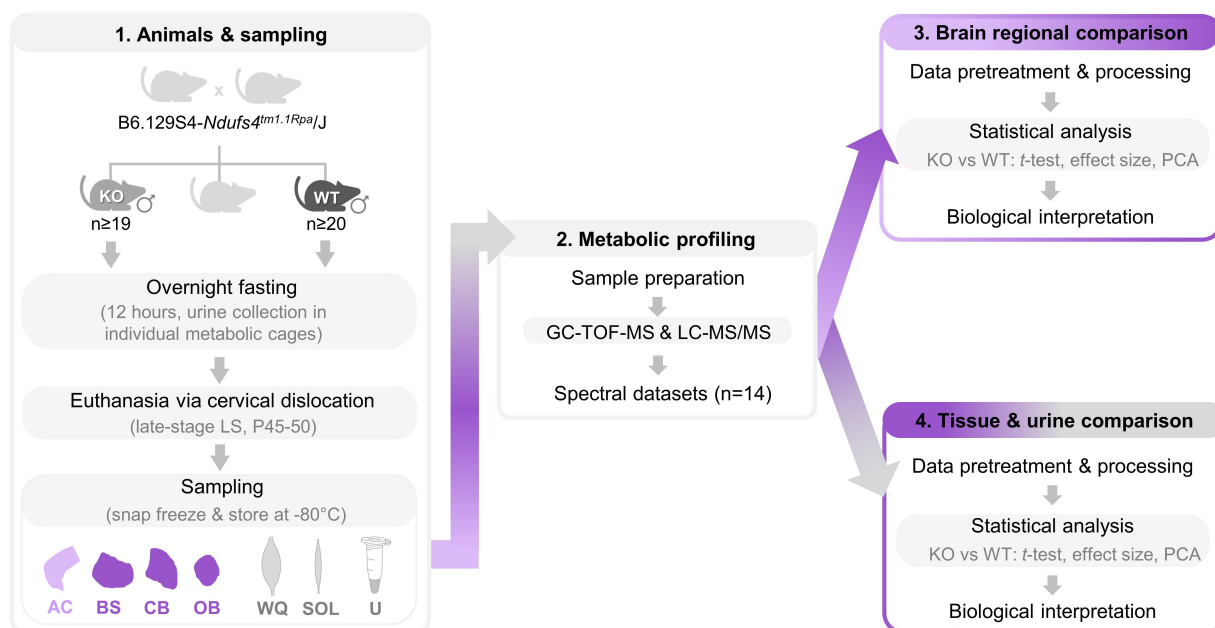


Figure 1-1: Schematic representation of the research design. Abbreviations: AC, anterior cortex; BS, brainstem; CB, cerebellum; GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; KO, *Ndufs4* KO; LS, Leigh syndrome; n, number; OB, olfactory bulbs; P, postnatal day; RC, respiratory chain; SOL, solei; U, urine; WT, wild type; WQ, white quadriceps.

1.4 Structure of the thesis

The thesis is written in chapter format and complies with the requirements of the NWU. The content is organised into six chapters, as outlined below, with individual reference lists provided for each chapter. The results of the study (Chapters 4 and 5) are presented as published, with slight style and formatting alterations¹ made to fit that of the thesis.

Chapter 1 provides the rationale behind the research, along with the aim, objectives, and research design. Subsequently, the structure of the thesis is given, followed by the research outcomes and declarations concerning ethics approval, financial support, as well as author and co-author contributions.

¹ This includes changing the referencing style, table and figure numbers, and American English words to British English. Abstracts, keywords, and declarations of the articles are also not presented in these chapters. Supplementary figures are displayed after the reference list of each chapter.

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Chapter 2 provides a literature review of the key topics relevant to this thesis, including mitochondrial characteristics and components, MD, CI-deficiency related LS, and the *Ndufs4* KO mouse model.

Chapter 3 presents detailed information on the materials and methods concerning animal handling, sampling, *post-mortem* genotype confirmation, multi-platform metabolomics, data processing and statistical analyses.

Chapter 4 comprises the first peer-reviewed paper resulting from the study, published in *Biochimica et Biophysica Acta (BBA) — Molecular Basis of Disease*. The paper provides brain-regional metabolic- and enzymatic data from *Ndufs4* KO vs WT mice, along with hypotheses on the underlying mechanisms taking place in CI deficient brain regions.

Chapter 5 contains the second peer-reviewed paper resulting from the study, published in *Metabolomics*. The paper describes similarities and differences among brain regional-, skeletal muscle-, and urine metabolomes in *Ndufs4* KO vs WT mice with some emphasis on MD-associated biofluid markers. The paper further provides postulated causes of the observed metabolic alterations, together with a comprehensive comparison of our findings with literature reports.

Chapter 6 provides a summary of the research findings and concluding remarks. The chapter closes with a description of the study's strengths, limitations, and research prospects.

Appendices: the thesis concludes with Appendices A-F consisting of supplementary information on the animal used (A), LC-MS/MS standards (B), metabolomics data quality (C), and publications referred to in the thesis (D-F). These publications include an article previously presented (D) in the author's MSc dissertation, which forms part of the larger project and is referred to throughout the thesis, as well as two research articles resulting from this study (E and F).

1.5 Research outputs

This study contributed to the field of natural science and MD research in the form of two peer-reviewed articles published in high-impact journals and four presentations at national and international conferences.

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

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.

The article (<https://doi.org/10.1016/j.bbadis.2021.166082>) was published in the journal: *Biochimica Biophysica Molecular Basis of Disease* (<https://www.journals.elsevier.com/bba-molecular-basis-of-disease>) with an impact factor of 5.187 (2020). The journal publishes research on disease processes and models of human disease, with focus on underlying pathological

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mechanisms contributing to disease understanding and/or treatment. Author guidelines can be accessed at: <https://www.elsevier.com/journals/biochimica-et-biophysica-acta-molecular-basis-of-disease/0925-4439/guide-for-authors>

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

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*, 17:101

The article (<https://doi.org/10.1007/s11306-021-01854-8>) was published in the journal: *Metabolomics* (<https://link.springer.com/journal/11306>) with an impact factor of 4.290 (2020). The journal publishes a broad scope of current research regarding the development and application of metabolomics platforms in man, animals, plants, and microbes. Author guidelines can be accessed at: <https://www.springer.com/journal/11306/submission-guidelines#Instructions%20for%20Authors>

1.5.3 Conference contributions

Terburgh, K., Lindeque, J.Z., Mason, S.W, van der Westhuizen, F.H. & Louw, R. 2019, 11-12 March. Metabolomics of *Ndufs4*^{-/-} skeletal muscle: adaptive mechanisms converge at the ubiquinone-cycle. Oral presentation. Metabolomics South Africa Symposium, Pretoria, South Africa.

Terburgh, K., Lindeque, J.Z., Mason, S.W, van der Westhuizen, F.H. & Louw, R. 2019, 23-27 June. Metabolomics of *Ndufs4*^{-/-} skeletal muscle: adaptive mechanisms converge at the ubiquinone-cycle. Oral presentation. 15th Annual Conference of the Metabolomics Society (Metabolomics 2019), The Hage, Netherlands.

Terburgh, K., Coetzer, J., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021, 4-5 March. Neurometabolomics of Leigh syndrome: Region-specific insights from the *Ndufs4* KO mouse brain. Poster presentation. The BRAIN Conference, Online.

Terburgh, K., Coetzer, J., Lindeque, J.Z., van der Westhuizen, F.H. & Louw, R. 2021, 22-24 June. Region-specific metabolomics of the Leigh syndrome brain: Insight into regional vulnerability to complex I deficiency. Poster presentation. 17th Annual Conference of the Metabolomics Society (Metabolomics 2021), Online.

1.6 Declarations

1.6.1 Ethics approval

The data used in this study were generated from samples collected for a larger project, approved by the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of North-West University in 2015 (ethics approval no. NWU-0001-15-A5). Subsequently, ethics approval was obtained for relevant sub-studies under this project (ethics approval no. NWU-

00001-15-S1 and NWU-00378-16-A5), and the current study presented here (ethics approval no. NWU-00509-20-A1).

1.6.2 Financial support

This work was supported by the National Research Foundation of South Africa (NRF, Grant No. 92736, 108146, 111479 and 121368), the Technology Innovation Agency of the Department of Science and Technology of South Africa (TIA, Grant No. Metabol. 01), and the NWU. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF, TIA or NWU.

1.6.3 Author contributions

Data collection: The datasets used in this study were generated as part of a larger study involving K. Terburgh, J. Coetzer, and W. Horak. Therefore, the author was involved in the initial sub-study ethics application, sample collection, genotyping, and analytical work. The author was also responsible for preparing and uploading all metabolomics datasets and metadata to the NIH Common Fund's National Metabolomics Data Repository (NMDR).

Images and figures: All images and figures in this thesis were personally generated by the author, unless referenced otherwise.

Paper I presented in Chapter 4: K. Terburgh, J. Coetzer and R. Louw designed the research plan with input from J.Z. Lindeque and F.H. vd Westhuizen. F.H. vd Westhuizen acquired the animal model and ethical approval with assistance from R. Louw. R. Louw and F.H. vd Westhuizen obtained funding for the study. J. Coetzer conducted experiments and data processing with assistance from K. Terburgh, R. Louw and J.Z. Lindeque. K. Terburgh analysed the data and wrote the manuscript with input from R. Louw, J.Z. Lindeque and F.H. vd Westhuizen.

Paper II presented in Chapter 5: K. Terburgh and R. Louw designed the research plan with input from J.Z. Lindeque. and F.H. vd Westhuizen. F.H. vd Westhuizen acquired the animal model and ethical approval with assistance from R. Louw. R. Louw and F.H. vd Westhuizen obtained funding for the study. K. Terburgh performed the data processing with assistance from R. Louw and J.Z. Lindeque. K. Terburgh analysed the data and wrote the manuscript with input from R. Louw, J.Z. Lindeque and F.H. vd Westhuizen.

Additional contributions: K. Terburgh further contributed to work beyond that presented in this thesis as part of the PhD programme. This includes laboratory work conducted for the African-PREDICT study (NWU). For this study, K. Terburgh randomised, prepared, and analysed human urine samples (n=1044) together with quality control samples (n=157) via LC-MS/MS. This work included method optimisation, data preprocessing, data quality assessment, and report writing. Additionally, K. Terburgh assisted with the writing of ethics- and grant applications beyond those relevant to the work presented in this thesis.

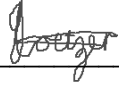
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All authors involved signed the declaration on this page:

As a co-author/researcher, I hereby approve and give consent that the mentioned articles and data may be used for the PhD thesis of K. Terburgh. I declare that my role in the study, as indicated above, is an accurate representation of my contribution.



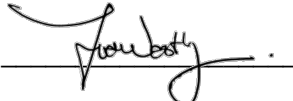
K. Terburgh



J. Coetzer



J.Z. Lindeque



F.H. van der Westhuizen



R. Louw

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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.

CHAPTER

Literature study



2

2.1 Introduction

This chapter provides an in-depth summary of the concepts central to the thesis. A generalised overview of mitochondrion and its key components is given, with focus on the oxidative phosphorylation (OXPHOS) system and its first enzyme complex (CI). Thereafter, the tissue-specificity and flexibility of mitochondria are emphasised. Next, mitochondrial disease (MD) and its tissue-specific nature is discussed, with the focus then shifting towards the main study topic — CI deficiency-related Leigh syndrome (LS) and recent insights from the most widely studied preclinical model thereof.

2.2 Mitochondria and their basic characteristics

2.2.1 Mitochondrial structure, function, and genetics

Mitochondria are highly adaptable cytoplasmic organelles found in large numbers (hundreds to thousands) in all nucleated eukaryotic cells. These organelles can exist in isolation or in big networks within a cell; where they cross-communicate with the nucleus and other sub-cellular compartments to help meet overall cellular needs and play a central role in a myriad of processes (summarised in Figure 2-1) involved in cellular energy production and homeostasis, metabolism, and signalling (Nunnari & Suomalainen, 2012; Prasai, 2017; Rahman & Rahman, 2018).

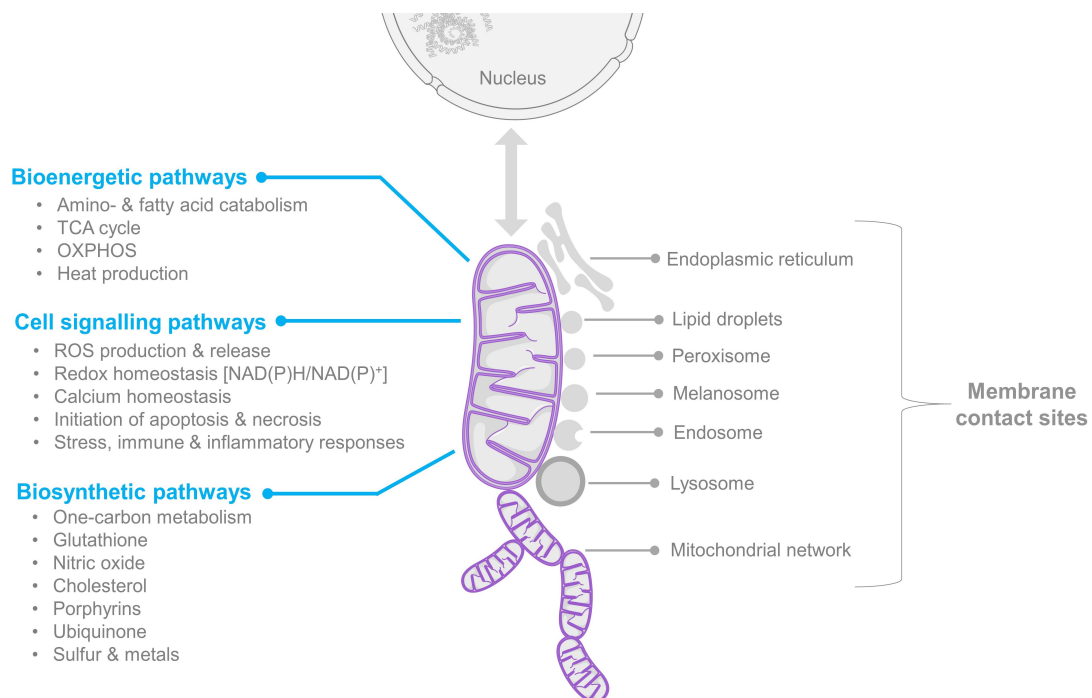


Figure 2-1: The many roles of the mitochondrion. Some mitochondrial roles are listed. Mitochondria are the bioenergetic and metabolic hubs of the cell, have major anabolic and catabolic functions, and constantly monitor and regulate cellular health and function by communicating with other subcellular compartments. *Abbreviations: OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid.*

Basic mitochondrial architecture (Figure 2-2) comprises two membranes, an outer (OMM) and inner (IMM) membrane that separate two aqueous sub-compartments, an inter-membrane space (IMS) and a central matrix. Some parts of the IMM run parallel to the OMM (inner boundary

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membrane or IBM) while other parts form invaginations (cristae) that extend into the matrix. The OMM is largely permeable as it contains porins or voltage-dependent anion channels (VDAC) and forms a central platform for cell signalling events and the establishment of membrane contact sites with other organelles. Contrastingly, the IMM is relatively impermeable, with the IBM housing several channel transporters that shuttle molecules across the membrane, while cristae house the oxidative phosphorylation (OXPHOS) system — responsible for the majority of cellular adenosine triphosphate (ATP) production (Lea & Hollenberg, 1989; Mannella, 2006; Vasava & Mashiyava, 2016).

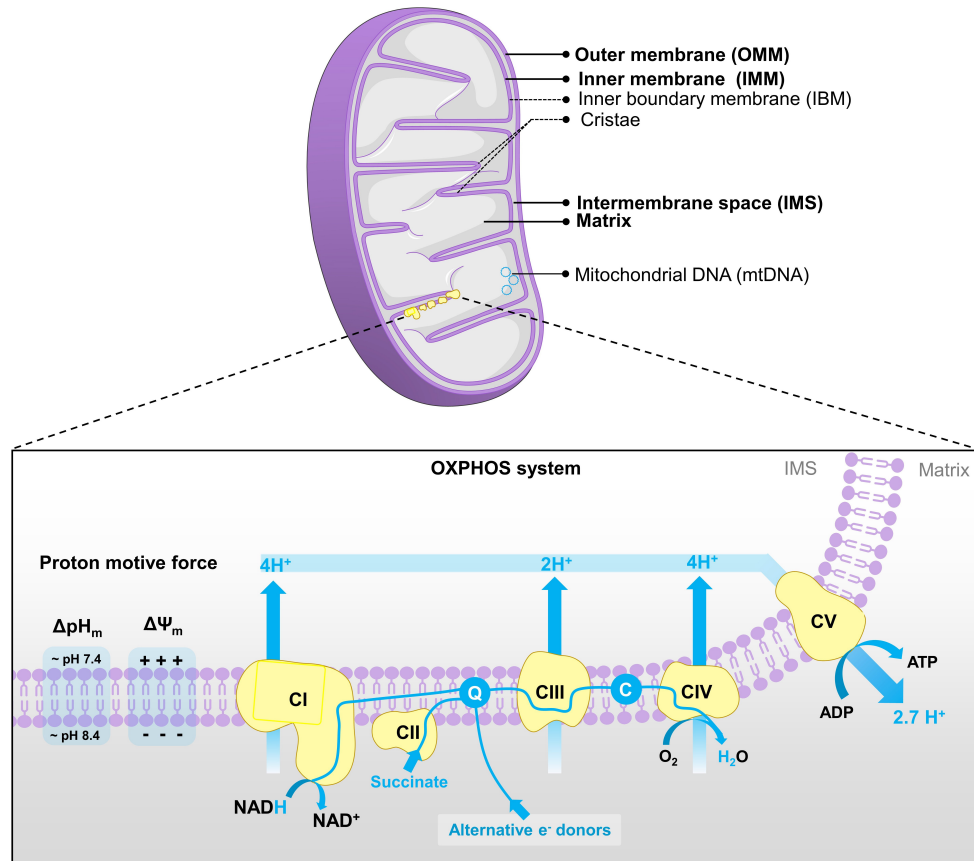


Figure 2-2: The mitochondrion and OXPHOS system. Mitochondria are enveloped by a double membrane separating the IMS and central matrix. Multiple mtDNA copies are anchored to the IMM in nucleoids at the matrix side. Both mtDNA and nDNA encode proteins required for mitochondrial (and OXPHOS system) function. The five complexes of the OXPHOS system are embedded in the IMM cristae, where electron flow (blue lines) through the respiratory chain (CI-CIV) to O_2 is coupled to proton (H^+) translocation (blue arrows) from the matrix to the IMS. This creates a pH gradient (ΔpH_m) and a membrane potential ($\Delta \Psi_m$) that form a proton motive force across the IMM, driving ATP synthesis via CV. *Abbreviations:* ADP, adenosine diphosphate; ATP, adenosine triphosphate; CI-V, complexes I to V; C, cytochrome c; e^- , electron; H^+ , protons; H_2O , water; NAD^+ , nicotinamide adenine dinucleotide (oxidised); $NADH$, nicotinamide adenine dinucleotide (reduced); pH, potential of hydrogen; O_2 , molecular oxygen; OXPHOS, oxidative phosphorylation; Q, ubiquinone.

The IMS and matrix are both protein-dense, with the matrix containing numerous anabolic and catabolic enzymes involved in metabolite biosynthesis and -signalling as well as the catabolism of nutrient-derived molecules (Nunnari & Suomalainen, 2012; Prasai, 2017; Rahman & Rahman, 2018). The mitochondrial matrix also houses the organelle's own double-stranded, circular genome (~16.5 kb) along with the machinery required for mitochondrial (mt) DNA transcription,

replication, and protein synthesis. Numerous mtDNA copies are found per mitochondrion, clustered into nucleoids, anchored to the IMM. mtDNA is maternally inherited, lacks introns, and contains a noncoding region that promotes transcription and replication (Chinnery & Hudson, 2013; Lee & Han, 2017; Shokolenko & Alexeyev, 2015; St John, 2014). While 99% of the mitochondrial proteome is encoded by nuclear(n) DNA and imported into mitochondria, 13 OXPHOS system subunits and 24 RNAs required for mitochondrial translation and protein synthesis are encoded by mtDNA (Rath *et al.*, 2021).

2.2.2 The OXPHOS system

The mitochondrial OXPHOS system (Figure 2-2) consists of five multi-subunit enzyme complexes, i.e., CI (NADH-ubiquinone oxidoreductase, EC 1.6.5.3), CII (succinate-ubiquinone oxidoreductase, EC 1.3.5.1), CIII (ubiquinol-cytochrome c oxidoreductase, EC 1.10.2.2), CIV (cytochrome c oxidase, EC 1.9.3.1), and CV (F_1F_0 -ATP synthase, EC 3.6.1.34). The first four complexes (I-IV), together with two mobile electron carriers, ubiquinone (Q) and cytochrome c (cyt C or C), form the respiratory chain (RC). Electron transport through the RC to molecular oxygen (O_2) maintains an electrochemical gradient across the IMM that drives the phosphorylation of adenosine diphosphate (ADP) via CV, producing ATP (Hatefi, 1985; Koopman *et al.*, 2013; Milenkovic *et al.*, 2017; Sherratt, 1991).

The electrons powering this process are derived from cytosolic and mitochondrial pathways of nutrient catabolism that fuel the production of either reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide ($FADH_2$). This includes glycolysis, fatty acid β -oxidation, glutaminolysis, branched-chain amino acid (BCAA) catabolism, and the tricarboxylic acid (TCA) cycle. Several metabolite shuttles can transfer cytosolic reducing equivalents to the mitochondrion, while recycling cytosolic NAD^+ , such as the malate-aspartate (Mal-Asp) shuttle and the glycerol-3-phosphate (G3P) shuttle. Electrons are ultimately donated to the IMM Q-pool by NADH-linked CI, $FADH_2$ -linked CII, or several other flavoproteins (Figure 2-3), which recycle mitochondrial NAD^+ or FAD in the process. Alternative flavoprotein electron donors are linked to the RC Q-pool, either directly, or indirectly via electron-transferring-flavoprotein dehydrogenase (ETF_{FDH}, EC 1.5.5.1), which obtains electrons from eleven different mitochondrial flavoproteins, including those involved in the catabolism of BCAAs, lysine, fatty acids, dimethylglycine, and sarcosine (Hatefi, 1985; McDonald & Gospodaryov, 2019; McDonald *et al.*, 2018; Watmough & Frerman, 2010).

In the Q-cycle, CIII oxidises reduced Q and donates the electrons to cyt C. Finally, reduced cyt C transfers the electrons to CIV, which in turn reduces O_2 to water (Cramer *et al.*, 2011; Quinlan *et al.*, 2011). As electrons move through CI, CIII, and CIV, these complexes pump protons (H^+) out of the matrix into the IMS — resulting in a pH gradient (ΔpH_m) and an electrical membrane potential ($\Delta \Psi_m$) over the IMM that creates a proton-motive force (in mV), propelling the flow of protons back into the matrix via CV and driving ATP synthesis. Protons are also capable of leaking

back into the matrix (without ATP production) to control the degree of proton motive force (Brand & Nicholls, 2011; Logan *et al.*, 2016; Mitchell, 1961). Additionally, some electrons can leak from the RC to partially reduce O_2 and produce reactive oxygen species (ROS), which function as important signalling molecules at low levels. This mitochondrial ROS production mainly occurs at redox centre sites [Q, flavin mononucleotide (FMN), FAD, and iron-sulfur (Fe–S)] within specific enzymes involved in electron donation and transport (Quinlan *et al.*, 2011; Zhao *et al.*, 2019).

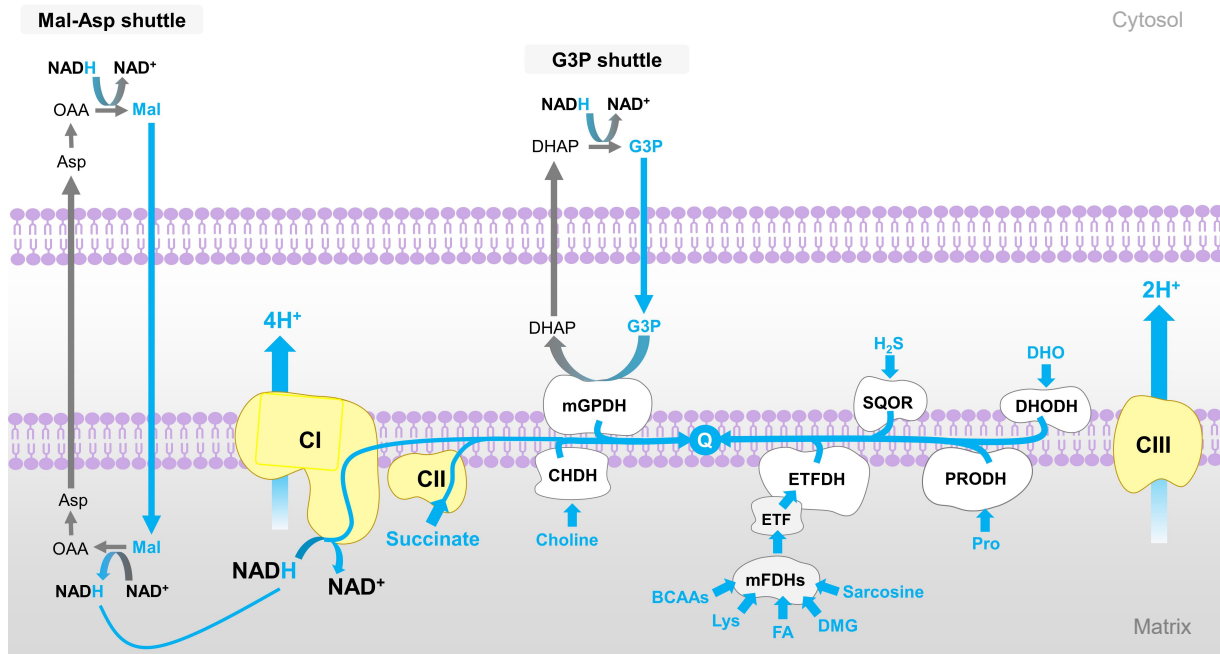


Figure 2-3: Redox shuttles and alternative IMM Q-pool electron donors. Cytosolic-derived electrons are transferred to the Q-pool while via the interconversion of oxaloacetate (OAA) and malate (Mal) in the cytosol and matrix in the Mal-Asp shuttle or dihydroxyacetone phosphate (DHAP) and glycerol-3-phosphate (G3P) in the cytosol and IMS in the G3P shuttle. Some alternative Q-pool electron donating flavoproteins are shown in white. Abbreviations: Asp, aspartate; BCAA, branched-chain amino acids; DMG, dimethylglycine; CHDH, choline dehydrogenase; DHODH, dihydroorotate dehydrogenase; ETFDH, electron-transferring-flavoprotein dehydrogenase; FA, fatty acids; Lys, lysine; mFDHs, mitochondrial flavoprotein dehydrogenases; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase; PRODH, proline dehydrogenase; SQR, sulfide-ubiquinone oxidoreductase

Although RC complexes are classically visualised as individual entities, it has been well established that complexes I, III, and IV associate and assemble into various supercomplexes [SCs] (Davies *et al.*, 2018; Gu *et al.*, 2016; Guo *et al.*, 2017; Letts *et al.*, 2019; Letts *et al.*, 2016; Wu *et al.*, 2016). It has also been shown that the characteristic IMM phospholipid, cardiolipin, plays a key role in SC formation (Arnarez *et al.*, 2016; Zhang *et al.*, 2002). Among the various SCs, $SCI+III_2+IV_{1-4}$ (the respirasome) is most abundant in eukaryotes (Javadov *et al.*, 2021; Milenkovic *et al.*, 2017), although $SCI+III_2$ is also prevalent and most conserved across kingdoms (Davies *et al.*, 2018). While the functional role of SC formation remains largely unknown, these higher order structures have been postulated to maintain the structural integrity of individual RC complexes, enhance electron transport efficiency, modulate ROS production, and prevent non-specific IMM protein aggregation (Javadov *et al.*, 2021; Letts & Sazanov, 2017; Lobo-Jarne & Ugalde, 2018; Milenkovic *et al.*, 2017; Wu *et al.*, 2016).

2.2.3 CI of the OXPHOS system

Among the mammalian RC complexes, CI is the main, most efficient electron donor that is responsible for generating most of the proton motive force (up to 40%) and plays a central role in the cycling of NADH and NAD⁺ (Hoefs *et al.*, 2012; Smeitink *et al.*, 2001a). CI (~980 kDa) is also the largest, most intricate, RC complex as it consists of 45 protein subunits organised into two arms — one embedded in the IMM and the other protruding into the matrix. As illustrated in Figure 2-4, the catalytic core of the L-shaped enzyme (shown in yellow) is made up of 14 central subunits that are organised into three functional modules. In the matrix arm, electrons are transferred from NADH to FMN in the tip of the NADH oxidation (N) module, and subsequently, moved through a chain of seven Fe-S clusters to the Q binding site in the ubiquinone reduction (Q) module. This is coupled to the pumping of four matrix-derived protons to the IMS via four antiporter-like motifs located in the proton pumping (P) module of the membrane arm. The core subunits are further encased by 31 accessory subunits (shown in grey) — some of which are known to be essential for CI assembly and function, while others are incompletely characterised. In addition, several assembly factors are also imperative for CI biogenesis. Among the various proteins required for CI assembly, the majority are encoded by nDNA [central subunits of the matrix arm (n=7), all accessory subunits (n=31) and assembly factors (n=~20)] with only seven central subunits of the membrane arm encoded by mtDNA (Agip *et al.*, 2019; Fiedorczuk *et al.*, 2016; Hirst & Roessler, 2016; Sazanov, 2015; Stroud *et al.*, 2016; Zhu *et al.*, 2016; Zickermann *et al.*, 2015).

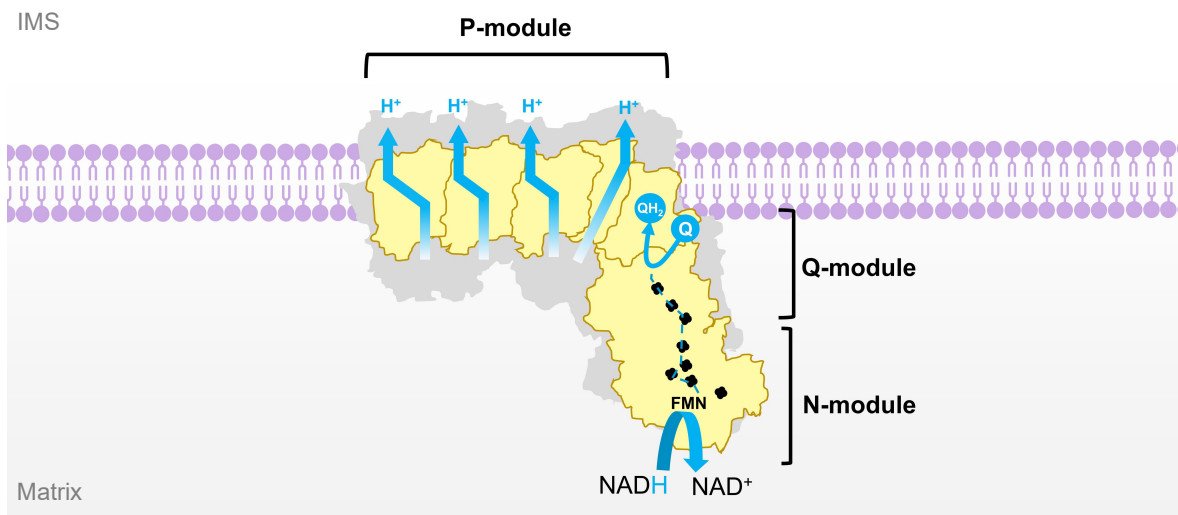


Figure 2-4: Mitochondrial complex I. The structure of mammalian complex I consists of 31 accessory subunits (grey) that envelop 14 central subunits (yellow), which are organised into three functionally distinct (N, Q and P) modules. The N module binds and oxidises NADH via a flavin mononucleotide (FMN), after which electrons pass along a chain of iron–sulfur clusters to reduce ubiquinone (Q), which is bound in the Q module. For each NADH oxidised, the P-module transfers four protons from the matrix to the IMS. *CI structure based on the illustration from Mammalian respiratory complex I through the lens of cryo-EM, Agip et al. (2019), 48:165–84; with permission of Annual Reviews, Inc. as conveyed through Copyright Clearance Center, Inc.*

The CI assembly process starts with the formation of separate submodules, which interact to form assembly intermediates corresponding to the functional modules of CI. In the final stages, a

complete Q/P subassembly (~830 kDa), lacking the N-module, is formed and stabilised by the assembly factor, NDUFAF2. Subsequently, the N-module is incorporated and assembly factors are removed (Guerrero-Castillo *et al.*, 2017; Lobo-Jarne & Ugalde, 2018; Mimaki *et al.*, 2012; Mukherjee & Ghosh, 2020; Stroud *et al.*, 2016). N module turnover is mostly independent from, and more rapid than, the rest of CI (Szczepanowska *et al.*, 2020), with the SCI+III₂ seemingly important for CI turnover regulation (Letts *et al.*, 2019). Furthermore, it seems that contact and crosstalk between complexes in the respirasome increase CI catalytic efficiency and regulate ROS production by CI (Reyes-Galindo *et al.*, 2019). In mammals, most CI (~90%) is localised in SCs with CIII₂+IV₁₋₄ or CIII₂, but it is still debated whether SC formation entails prior RC complex assembly or cooperative-assembly (Lobo-Jarne & Ugalde, 2018; Milenkovic *et al.*, 2017). Recent evidence supports the latter model as it was shown that CIII regulates CI and CIV maturation and SC formation, with the presence of active CIII required for the incorporation of the CI N module into the Q/P subassembly (Protasoni *et al.*, 2020).

2.3 The dynamic and tissue-specific nature of mitochondria

The preceding section provides a rather generalised overview of the mitochondrion and its key components relevant to this thesis. However, it is imperative to note that mitochondria are not uniform. Mitochondrial characteristics vary greatly among tissue- and cell types to meet specific functional, bioenergetic, and metabolic demands. This includes differences in mitochondrial number, morphology (overall shape and membrane ultrastructure), organisation, and cellular distribution, as well as mtDNA, protein, and lipid content (Giacomello *et al.*, 2020; Glancy *et al.*, 2020; Kappler *et al.*, 2019; Murphy *et al.*, 2016; Nunnari & Suomalainen, 2012; Prasai, 2017; Vasava & Mashiyava, 2016). Moreover, ongoing processes of mitochondrial fission and fusion, network formation and fragmentation, cristae remodelling, mitochondrial biogenesis, as well as movement through the cytoskeleton ensure that intracellular mitochondria can adapt to internal and external cues, as needed (Glancy & Balaban, 2011; Glancy *et al.*, 2020; Johnson *et al.*, 2007; Kappler *et al.*, 2019).

High energy-demand tissues generally have a higher mitochondrial abundance than others, with cellular mitochondrial content regulated to match bioenergetic demand (Koopman *et al.*, 2005; Prasai, 2017; Vasava & Mashiyava, 2016). Among energy-demanding tissues, cellular mtDNA levels (mtDNA/nDNA ratio) are highest in cardiac- and skeletal muscle, and lowest in the liver (heart>muscle>kidney>brain>liver). This mtDNA level also significantly correlates with respiratory capacity per cell (measured as CIV activity/nDNA) for all tissues, when considered together (Fernández-Vizarra *et al.*, 2011). In accord, cardiac- and skeletal muscle mitochondria contain a higher number of cristae per mitochondrial surface area, along with higher levels of RC CI-V and cyt C than other tissues (brain, liver, and kidney). Thereagainst, Q content is highest in skeletal muscle and liver. Furthermore, although the molar ratio of CII-IV are conserved among tissues, the ratio of Q and cyt C to RC complexes (CII:Q:CIII:cyt C:CIV) are considerably higher in the

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liver (1.1:134.7:3:9:6.5) and brain (1:57.9:3:34.8:7.5), respectively (Benard *et al.*, 2006). It has also been shown that some RC enzyme complex subunits are expressed in tissue-specific isoforms, which seem to have a regulatory role; e.g. tissue-specific isoforms of the CI accessory subunit, NDUFV3, correlate with differences in kinetic parameters, especially the substrate affinity of CI (Guerrero-Castillo *et al.*, 2017) and tissue-specific CIV subunit isoforms have been proposed to mediate the regulation of CIV activity, proton pumping efficiency, signalling, dimer formation, and SC formation (Sinkler *et al.*, 2017).

Furthermore, cardiac- and skeletal muscle mitochondria are highly connected and organised into a relatively stable mitochondrial reticulum, with mitochondria condensed near the sarcolemma and between myofibrils. Contrastingly, liver and kidney mitochondria are more irregular in shape and less compressed by the tissue structure, with liver mitochondria uniformly distributed through the cell and kidney mitochondria found in larger numbers in the tubules, where ion pumping is highly active. Neuronal mitochondria are generally found in heterogeneous mitochondrial networks and continually move from the cell body to synapses, with relatively more mitochondria found in the axon than the cell body. However, mitochondrial morphology and biochemical properties also differ within a cell, depending on their intracellular location (Benard *et al.*, 2006; Glancy *et al.*, 2020).

Functional differences between mitochondria are also evident on a proteomic and metabolic level. Cardiac- and skeletal muscle is optimised to convert fuel to ATP for contraction. While the heart is tailored for OXPHOS ATP production, primarily via fatty-acid oxidation (Johnson *et al.*, 2007); skeletal muscles are optimised for ATP production via OXPHOS or glycolysis, depending on myofiber- and activity type [i.e. continuous slow- or short duration contractions] (Glancy & Balaban, 2011). Skeletal muscles also have a high capacity for CI-linked substrates (Kappler *et al.*, 2019) and are major glutamine producers (Jang *et al.*, 2019). Contrastingly, the liver is a major glutamine consumer, optimised for urea cycle function and biosynthetic pathways (e.g., gluconeogenesis and ketogenesis) and prefers fuels like unsaturated fatty acids and succinate (Jang *et al.*, 2019; Johnson *et al.*, 2007; Kappler *et al.*, 2019). The kidneys are adapted for the metabolism of reabsorbed amino acids (Johnson *et al.*, 2007), uniquely use circulating citrate as a TCA cycle fuel, and play a key role in systemic redox- and one-carbon homeostasis by oxidising lactate to pyruvate, and producing the majority of glycine and serine, respectively (Jang *et al.*, 2019). Furthermore, the brain is optimised for neurotransmitter metabolism and ROS detoxification (Johnson *et al.*, 2007), with the majority of its energy derived from glucose (via glycolysis, TCA cycle and OXPHOS) and consumed by neurons, specifically at synapses (Dienel, 2019; Harris *et al.*, 2012). This is, however, an oversimplified comparison as many mitochondrial proteomic differences (Johnson *et al.*, 2007) and more than 700 instances of tissue-specific metabolite production/consumption were recently reported (Jang *et al.*, 2019).

2.4 Mitochondrial dysfunction

2.4.1 An overview of mitochondrial disease

Mitochondrial disease (MD) refers to a group of disorders, characterised by mitochondrial dysfunction, that results in a diverse spectrum of clinical phenotypes. MD is further classified as primary or secondary, depending on the causative factors. Pathogenic mutations directly affecting OXPHOS system biogenesis/function¹ result in primary MD; while other genetic and non-genetic causes result in secondary mitochondrial dysfunction (Niyazov *et al.*, 2016). The occurrence of secondary mitochondrial dysfunction has been described in an increasing number of common (hereditary and non-hereditary) diseases, including cancer, diabetes mellitus, cardiovascular disease, and neurodegenerative diseases, e.g., Alzheimer's and Parkinson's disease (Keshavan & Rahman, 2018; Parikh *et al.*, 2019; Thorburn, 2004). This thesis will focus on primary MD — the most common inborn error of metabolism with an estimated prevalence of 1 in 4 300² (Ghezzi & Zeviani, 2018; Keshavan & Rahman, 2018).

Primary MD can present at any age (ranging from neonatal to adulthood) and progressively affect almost any organ, often multiple organ systems, with varying levels of severity (Figure 2-5). Post-mitotic tissues with a high energetic and metabolic demand, especially the muscular- and central nervous systems, are most often affected (Keshavan & Rahman, 2018; Smeitink *et al.*, 2001b). Although several classic mitochondrial syndromes have been identified (Table 2-1), most patients present with symptoms that do not fall into these categories and many symptoms overlap with common diseases. Thus, clinically diagnosing MD is extremely challenging (Saneto, 2020; Suomalainen & Battersby, 2018).

The use of next-generation sequencing strategies has led to significant progress in the genetic diagnosis of MD, with pathogenic mutations identified in more than 300 different genes from mostly nuclear but also mitochondrial genomes. These pathogenic mutations can occur *de novo* during embryogenesis or follow a variety of inheritance patterns (sporadic, maternal, autosomal recessive, autosomal dominant, X linked dominant) due to the genetic duality and multi-copy nature of mitochondria. Generally, nDNA mutations follow an autosomal recessive inheritance pattern and result in 75% of the more severe infantile- and childhood onset MD cases, while mtDNA mutations are maternally inherited and account for more adulthood onset cases. However, there are exceptions to these generalisations and several factors complicate mtDNA related MD (Alston *et al.*, 2017; Alston *et al.*, 2021; Keshavan & Rahman, 2018).

¹ OXPHOS subunit, assembly factor or cofactor biosynthesis; mtDNA replication, transcription, and translation; mitochondrial biogenesis; etc. (Niyazov *et al.*, 2016).

² Determined in European populations. Prevalence studies in South-African populations are lacking due to a general lack of MD awareness and an understudied population despite reports that all South-African populations are affected by MD (Smuts *et al.*, 2010; van der Walt *et al.*, 2012).

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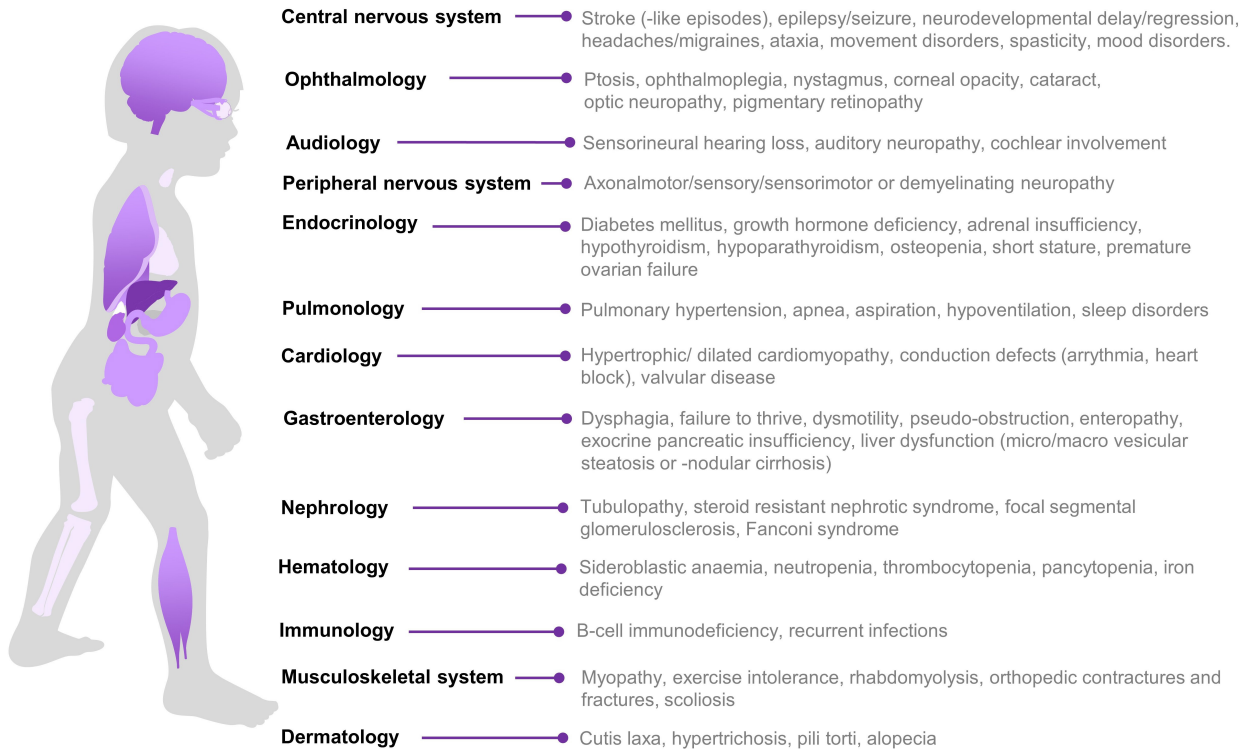


Figure 2-5: The complex manifestation of MD. Body systems affected by MD and the corresponding spectrum of possible clinical symptoms. MD can present at any age in any isolated organ or multiple organ systems, with varying levels of severity. *List of clinical features obtained and adapted from Parikh et al. (2017) and Rahman (2020).*

Table 2-1: Classic mitochondrial syndromes

Onset	Syndrome	Genome	Primary clinical features
Infantile/ childhood	Pearson	mtDNA	Sideroblastic anaemia, exocrine pancreatic dysfunction, pancytopenia
	Kearns-Sayre	mtDNA	progressive external ophthalmoplegia, retinitis pigmentosa, cerebellar ataxia, cardiac conduction block, diabetes, short stature, myopathy
	Leigh	mt/nDNA	Psychomotor delay, subacute relapsing encephalopathy, brainstem/cerebellar involvement, dystonia, seizures
	AHS	nDNA	Seizures, hepatopathy, psychomotor delay, gastrointestinal tract dysmotility, peripheral neuropathy, blindness
	Barth	nDNA	Dilated cardiomyopathy, cyclic neutropenia, myopathy
Adolescence/ adulthood	LHON	mtDNA	Optic atrophy
	NARP	mtDNA	Retinitis pigmentosa, peripheral neuropathy, ataxia
	MELAS	mtDNA	Metabolic strokes, seizures, migraine, blindness, dystonia, myopathy, short stature
	MIDD	mtDNA	Diabetes, sensorineural hearing loss
	MERRF	mtDNA	Myoclonus, myoclonic seizures, myopathy, sensorineural hearing loss, cerebellar ataxia, lipomatosis
	CPEO	mt/nDNA	External ophthalmoplegia, bilateral ptosis, myopathy
	Friedreich ataxia	nDNA	Progressive spinocerebellar ataxia, dysarthria, muscle weakness, diabetes, cardiomyopathy
	MNGIE	nDNA	Leukoencephalopathy, gastrointestinal tract dysmotility, ophthalmoplegia, cachexia, peripheral neuropathy

Abbreviations: CPEO, chronic progressive external ophthalmoplegia; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MIDD, maternal-inherited diabetes and sensorineural hearing loss; NARP, neuropathy, ataxia, and retinitis pigmentosa; MERRF, myoclonus, epilepsy with ragged red fibers; AHS, Alpers Huttenlocher syndrome; MNGIE: mitochondrial neurogastrointestinal encephalomyopathy. Adapted from Saneto (2020) as permitted by the Creative Commons Attribution License (CC BY), <https://creativecommons.org/licenses/by/4.0/>, with some additional primary features obtained from Chinnery (2000 [updated 2021]).

Patients with mtDNA mutations can be homoplasmic (100% mutant genomes) or, more commonly, heteroplasmic (mixture of mutant and wild type genomes). In the latter case, a specific mutational threshold (~60-80% mutant genome copies) must be breached before any pathogenic consequences are detected — with higher mutational loads often linked to more severe symptoms and earlier disease onset. Nevertheless, mutational threshold levels vary (among organs, patients, and mutation type) and there are numerous cases where mutational load and phenotype do not correlate. Furthermore, the mutational load can vary among a patient's organs, or individual cells of an organ and fluctuate over time. This also results in unpredictable maternal inheritance patterns as mutational loads generally differ significantly and randomly among mature oocytes and, therefore, descendants. Moreover, differences in nuclear genetic background and lifestyle lead to further variability among patients (Alston *et al.*, 2021; Craven *et al.*, 2017; Ishikawa & Nakada, 2021; Rossignol *et al.*, 2003; Saneto, 2020).

Hence, despite genetic advances, MDs remain challenging to understand, diagnose, and manage. Both mtDNA and nDNA mutations show poor genotype-phenotype correlations among patients with the same mutation, or biochemical defects affecting the same RC complex, resulting in vastly different clinical symptoms — or similar clinical symptoms resulting from different mutations. This makes it particularly difficult to diagnose MD cases, where causative defects cannot be identified or confirmed (e.g., novel putative pathogenic mutations). Moreover, within the body, the same mutation variably affects different cell- and tissue types (Alston *et al.*, 2021; Ishikawa & Nakada, 2021). It has been widely established that RC activity can be inhibited up to a critical value, called the biochemical threshold, before any effects on mitochondrial respiration or ATP synthesis are observed. This biochemical threshold value also differs among mutation- and tissues types (Rossignol *et al.*, 2003). Hence, due to the extreme genetic-, biochemical-, and clinical heterogeneity, MD pathomechanisms remain poorly understood — especially aspects related to mutation- and tissue specificity. Consequently, no effective or curative treatments are available for MD, with current care standards entailing dietary adjustments or specific co-factor/vitamin supplementation (Parikh *et al.*, 2017; Rahman, 2020; Thompson, 2021).

2.4.2 CI deficiency and Leigh syndrome

One-third of paediatric MD cases result from isolated CI deficiency (OMIM 252010), making it the most common form of MD (Alston *et al.*, 2017; Ghezzi & Zeviani, 2018; Kirby *et al.*, 1999; Smeitink *et al.*, 2001a). Clinically, the majority (>80%) of these cases present as Leigh(-like³) syndrome (LS) [OMIM 256000]. Although LS is associated with a variable spectrum of symptoms, it typically presents in early infancy/childhood with the most characteristic features including, symmetrical bilateral necrotic lesions in distinct brain regions, mitochondrial dysfunction, and a

³ Leigh-like syndrome is diagnosed when clinical features strongly resemble LS but key diagnostic criteria are lacking e.g. atypical lactate levels, neuroimaging or neuropathology (Rahman & Thorburn, 2015).

neurodegenerative phenotype. LS often results in respiratory failure and death within two years of disease onset. Symptoms largely correlate to lesion development and location — most commonly in the brainstem, cerebellum, and basal ganglia. However, other brain regions (thalamus, optic nerves, and spinal cord) and peripheral tissues (eyes, ears, heart, liver, kidneys, skin, musculoskeletal-, haematological-, endocrine- and gastrointestinal systems) are also often affected and the phenotype is usually multisystemic (Lake *et al.*, 2016; Loeffen *et al.*, 2000; Rahman *et al.*, 1996; Rodenburg, 2016).

While isolated CI deficiency has been linked to mutations in 38 genes [encoding all 14 core subunits, 13 of the accessory subunits, and 11 of the assembly factors of CI] (Saneto, 2020); mutations in more than 80 different genes are known to cause LS. This includes rarer defects in other OXPHOS components (CII-V and Q), pyruvate dehydrogenase, and several other proteins that affect mitochondrial function (Lake *et al.*, 2016; Rahman *et al.*, 2017; Ruhoy & Saneto, 2014). Some of the more severe forms of LS are caused by defects in the NDUFS4 [NADH dehydrogenase (ubiquinone) iron-sulfur protein 4] subunit of CI (Ortigoza-Escobar *et al.*, 2016). NDUFS4 is a non-enzymatic accessory subunit (18 kDa) of the CI N module, located near the border between the N- and Q-modules (Figure 2-6) where it interacts with several other N/Q-module accessory subunits, including NDUFA12 (Fiedorczuk *et al.*, 2016; Zhu *et al.*, 2016). Thus far, 13 loss-of-function mutations have been identified in the nuclear *Ndufs4* gene⁴, which is regarded a mutational hotspot of CI deficiency (Lamont *et al.*, 2017; Ortigoza-Escobar *et al.*, 2016; Papa, 2002). Consequently, several patient-derived cell models and animal models have been developed to gain insight into *Ndufs4* deficiency [reviewed by Breuer *et al.* (2013)]. Among these, the whole-body *Ndufs4* knockout (KO) mouse model developed by the Palmiter group (Kruse *et al.*, 2008) is currently the best characterised and most widely studied preclinical model of human CI deficiency and LS.

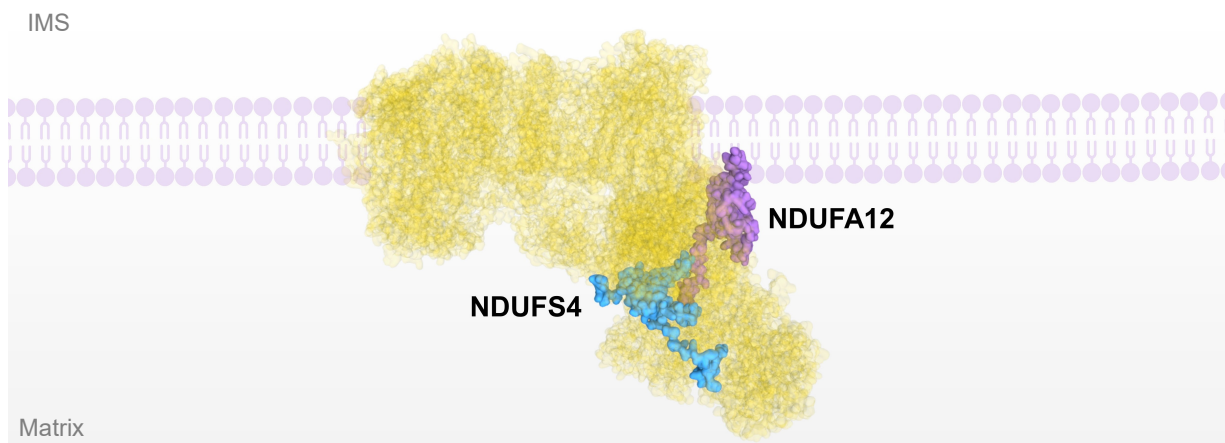


Figure 2-6: Localisation of NDUFS4 and NDUFA12 in mammalian CI. The NDUFS4 (blue) and NDUFA12 (purple) accessory subunits of CI link the N- and Q modules. NDUFS4 also interacts with several other subunits including NDUFA 6-12, and -9; NDUFV 1 and 3-10; as well as NDUFS 1-3, and -8. *PBD ID: 5LNK, Fiedorczuk et al. (2016), Mol* [D. Sehnal, S. Bittrich, M. Deshpande, R. Svobodová, K. Berka, V. Bazgier, S.*

⁴ Homo sapiens *Ndufs4*, NCBI gene ID: 4724

Velankar, S.K. Burley, J. Koča, A.S. Rose (2021) *Mol* Viewer: modern web app for 3D visualization and analysis of large biomolecular structures. Nucleic Acids Research. doi: 10.1093/nar/gkab314*, and RCSB PDB.

2.5 Insight from the *Ndufs4* KO mouse model

2.5.1 The effect of NDUF54 loss on CI assembly and activity

The whole-body *Ndufs4* KO mouse model was developed through the Cre/loxP-mediated excision of the second *Ndufs4* exon⁵, which encodes a mitochondrial targeting sequence and the first 17 amino acids of mature NDUF54. *Ndufs4* heterozygotes (HET) appear healthy for at least a year, while the synthesis of mature NDUF54 is ubiquitously inhibited in homozygous *Ndufs4* KO mice (JAX, 2017; Kruse *et al.*, 2008) — resulting in complete NDUF54 loss and a significant reduction in CI subunit levels in tissues (brain, diaphragm, liver, heart, kidney, and skeletal muscle). NDUF54 deficiency affects N- and Q-module subunit levels more severely than P-module subunits and causes near-total loss of NDUF12⁶, another N-module accessory subunit, which physically interacts with NDUF54 and together, link the N and Q modules (Adjobo-Hermans *et al.*, 2020). Consequently, N- to Q-module attachment is destabilised with N- (200 kDa) and Q/P- (830 kDa) module subassemblies, but not holo-CI, detected in *Ndufs4* KO tissues and mouse embryonic fibroblasts (MEFs). Although these CI subassemblies are inactive under blue-native-PAGE conditions, residual CI activity is maintained *in situ* (Adjobo-Hermans *et al.*, 2020; Calvaruso *et al.*, 2012). Similar findings were reported in *Ndufs4*-mutated patient fibroblasts (Adjobo-Hermans *et al.*, 2020; Breuer *et al.*, 2013; Lobo-Jarne & Ugalde, 2018; Scacco *et al.*, 2003).

Residual CI activity, despite NDUF54 loss, has been ascribed to the stabilisation of CI subassemblies through their higher order association with CIII, as SCI-830+III₂ retains CI activity in *Ndufs4* KO skeletal muscle (Calvaruso *et al.*, 2012). However, more recent data suggest that CI stabilisation may result from an adaptive increase in the levels of the assembly factor paralog for NDUF12, NDUF12, which associates with CI-830 subassemblies, in free (CI-830) and SC (SCI-830+III₂ and SCI-830+III₂+IV) forms, in *Ndufs4* KO MEFs and patient fibroblasts. This stabilisation is proposed to sustain, without fully restoring, *in situ* CI activity possibly in concert with MIM lipids (Adjobo-Hermans *et al.*, 2020).

Nevertheless, residual CI activity varies among patients and tissues, ranging from 15-75% of control values in *Ndufs4*-mutated patient fibroblasts and 9-44% of control values in *Ndufs4* KO mouse tissues [lung (9%) < pancreas (17%) < liver (19%) < kidney (25%) < brain (26%) < skeletal muscle (29%) < heart (44%)] (Calvaruso *et al.*, 2012). CI subunit levels are also decreased to a greater extent in the *Ndufs4* KO mouse brain and diaphragm than other tissues, which was

⁵ Mus musculus *Ndufs4*, NCBI gene ID: 17993

⁶ Contrastingly, NDUF12 absence does not result in NDUF54 loss (Adjobo-Hermans *et al.*, 2020).

suggested to be related to the phenotypic vulnerability of these tissues (Adjobo-Hermans *et al.*, 2020).

2.5.2 Phenotypic manifestation of *Ndufs4*-related CI deficiency

NDUFS4 loss and CI deficiency in mice result in a progressive neurometabolic phenotype that closely resembles human LS. Some overlapping symptoms between NDUFS4-LS patients and *Ndufs4* KO mice include abnormal brain images, growth retardation, failure to thrive, lethargy, visual problems, locomotor impairment, ataxia, muscular hypotonia, breathing irregularities, and apnoeic episodes (Quintana *et al.*, 2012). The stages of disease progression (Figure 2-7) generally correspond to animal age/postnatal day [P] (Quintana *et al.*, 2010; Quintana *et al.*, 2012). However, variable survival rates (ranging from ~P55-75) are reported among facilities (Jin *et al.*, 2014; Johnson *et al.*, 2013; Kruse *et al.*, 2008; Miller *et al.*, 2021; Quintana *et al.*, 2010; Quintana *et al.*, 2012).

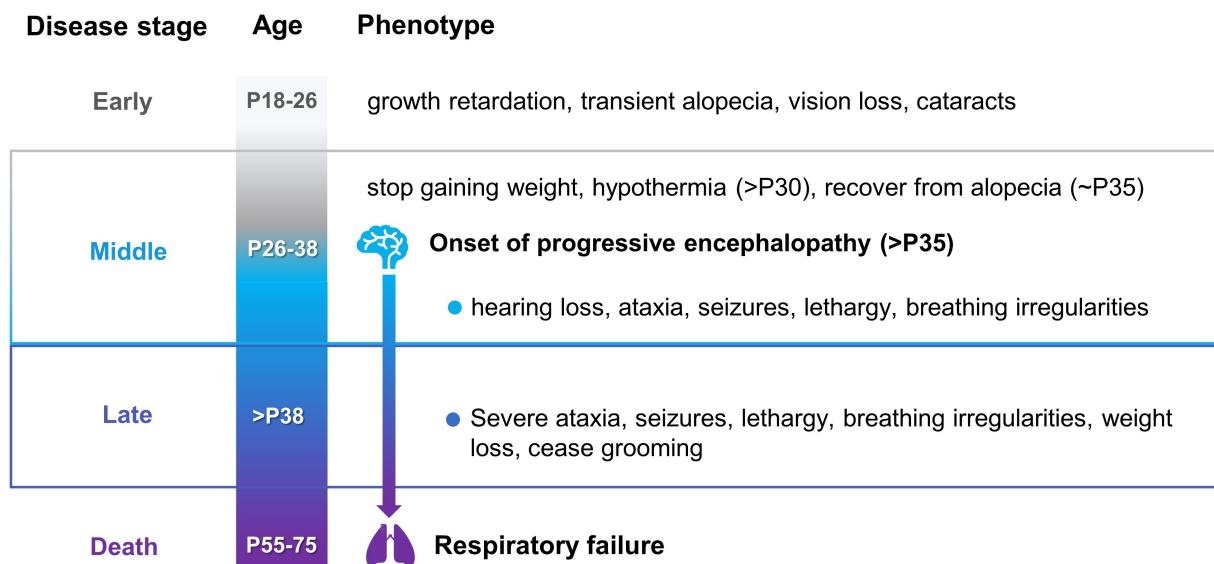


Figure 2-7: The progression of the LS phenotype in *Ndufs4* KO mice. *Ndufs4* KO mice appear normal at birth but start to display symptoms of encephalopathy at P35, which progressively worsen until premature death between P55-75. Abbreviations: P, postnatal day

Initially (<P18), *Ndufs4* KO mice appear phenotypically indistinguishable from control (HET/WT) littermates (Kruse *et al.*, 2008); however, some pups may experience early intermittent breathing irregularities [P14] (Quintana *et al.*, 2012), and display pathological changes in retinal ganglion cells [~P16] (Song *et al.*, 2017; Yu *et al.*, 2015b). During the early disease stage (P18-26), *Ndufs4* KO mice display growth retardation, transient alopecia, vision loss, and cataracts. Subsequently, mice stop gaining weight, become more lethargic, and experience decreased body temperature or spontaneous hypothermic events throughout the middle disease stage (P26-38). At ~P35, mice recover from alopecia and start displaying symptoms of encephalopathy — including hearing loss, deterioration of motor ability, ataxia, and (epileptic) seizures. The animals also exhibit decreased heart rates and breathing irregularities, with lower breathing rates prominent in the mid- and late disease stage. To avoid confusion, it must be noted that *Ndufs4* KO mice are often referred to as

“pre-symptomatic” prior to the onset of the encephalopathic phenotype (<P35). Thereafter, symptoms progressively worsen with *Ndufs4* KO mice losing weight and no longer grooming at the late disease stage (>P38), after which brain-regional lesions also become prominent (Jin *et al.*, 2014; Kruse *et al.*, 2008; Quintana *et al.*, 2010; Quintana *et al.*, 2012). Although not observed in *Ndufs4* KO mice at the middle disease stage (Kruse *et al.*, 2008), feeding difficulties observed in *Ndufs4* KO *drosophila* suggest it may be an overlooked late-stage feature of *Ndufs4* KO models (Foriel *et al.*, 2018). Finally, *Ndufs4* KO mice die prematurely, usually due to respiratory failure (Kruse *et al.*, 2008; Quintana *et al.*, 2010; Quintana *et al.*, 2012). As death occurs before sexual maturity is reached, the *Ndufs4* KO model must be bred from HET crosses (JAX, 2017).

2.5.3 Tissue-specific vulnerability to *Ndufs4*-related CI deficiency

Subsequent research on *Ndufs4* KO mice has revealed a tissue-specific vulnerability to CI deficiency, with unknown causal mechanisms. The disease most severely manifests in the brain, with specific brain regions being more vulnerable to the *Ndufs4* KO than others. The olfactory bulbs first display histopathological changes, followed by the brainstem (especially the vestibular nucleus) and cerebellum, although the striatum component of the basal ganglia is also often affected at later stages. Thereagainst, the mid- and forebrain appear to be unaffected (Chen *et al.*, 2017; Kruse *et al.*, 2008; Quintana *et al.*, 2010; Quintana *et al.*, 2012; Song & Cortopassi, 2015). Vulnerable brain regions display focal progressive lesions with spongiform neurodegeneration, vascular proliferation, gliosis, aberrant mitochondrial morphology in nerve terminals, and necrosis (Kruse *et al.*, 2008; Quintana *et al.*, 2010). Among tissues, skeletal muscle seems relatively unaffected phenotypically (Alam *et al.*, 2015; Chouchani *et al.*, 2014; Kruse *et al.*, 2008) and studies on conditional *Ndufs4* KO mice confirmed that NDUF54 loss in peripheral tissues [cardiac- (Chouchani *et al.*, 2014; Karamanlidis *et al.*, 2013; Sterky *et al.*, 2012), liver-, hematopoietic-, and myeloid- (Jin *et al.*, 2014) specific *Ndufs4* KOs] lead to mild phenotypes with longer survival rates. Peripheral tissues do, however, contribute to disease pathology as the adeno-associated virus-based improvement of *Ndufs4* deficiency, either solely in the brain (only partial correction) or peripheral organs, had no clinical benefit, whereas correcting both, improved the clinical phenotype and significantly prolonged the lifespan in *Ndufs4* KO mice (Di Meo *et al.*, 2017).

From conditional glial- and neuronal- (Quintana *et al.*, 2010; Quintana *et al.*, 2012; Ramadasan-Nair *et al.*, 2019; Sterky *et al.*, 2012), and neuronal subtype- (Bolea *et al.*, 2019; Chen *et al.*, 2017; Choi *et al.*, 2017; Johnson *et al.*, 2020) *Ndufs4* KO mice, it was clear that neurons predominately drive the fatal LS phenotype with specific symptoms linked to specific neuronal subtypes. NDUF54 loss in glutamatergic neurons mainly affected the brainstem and cerebellum, resulting in inflammation, motor- and respiratory deterioration, decreased neuronal firing, and early death (Bolea *et al.*, 2019). Glutamatergic neurons were also identified as key drivers of volatile anaesthetic hypersensitivity observed in *Ndufs4* KO mice (Zimin *et al.*, 2018; Zimin *et al.*, 2016).

In contrast, NDUFS4 loss in γ -aminobutyrate (GABA)ergic neurons mainly affect the basal ganglia and olfactory bulbs, resulting in more restricted inflammation, reduced weight gain, hypothermia, severe temperature-induced epileptic seizures, and early death (Bolea *et al.*, 2019). However, no brain regional differences in neuronal populations could be identified in *Ndufs4* KO mice prior to lesion formation [P29-31] (Johnson *et al.*, 2020). Furthermore, although not contributing to neuronal loss, other brain cells also impact disease pathology, e.g., NDUFS4 loss in dopaminergic neurons results in decreased dopamine levels and non-motor symptoms of Parkinson's disease (Choi *et al.*, 2017); striatal medium spiny neurons contribute to motor deficits (Chen *et al.*, 2017a); and gonadotropin-releasing hormone (GnRH) neurons exhibit impaired GnRH production and secretion of gonadotrophs and testosterone, leading to pubertal delays (Kagawa *et al.*, 2020). Taken together, specific brain regions and neuronal populations clearly play distinct roles in driving the LS phenotype; however, the major factors underlying regional neurodegeneration remain elusive and the contribution of systemic metabolic alterations to this neurometabolic disease cannot be ruled out (Di Meo *et al.*, 2017; Ito *et al.*, 2017; Lee *et al.*, 2019).

2.5.4 Cellular metabolic consequences of *Ndufs4*-related CI deficiency

CI plays a central role in cellular metabolism, as its NADH oxidation-, Q reduction- and proton pumping actions contribute to the levels and balance of key metabolic modulators, i.e., the ratios of ADP/ATP, NADH/NAD⁺ and ROS production (Desquiret-Dumas *et al.*, 2019). By way of its effects on these metabolic mediators and metabolism, CI is also intrinsically linked to the immune system and inflammatory responses. In the next sections, the effect of NDUFS4 loss on these parameters, and tissue-specific metabolism, will be discussed.

2.5.4.1 OXPHOS, O₂ consumption, and ATP production

The loss of NDUFS4 does not seem to strongly affect the assembly or function of OXPHOS complexes, other than CI, although literature reports are somewhat variable (Alam *et al.*, 2015; Calvaruso *et al.*, 2012; de Haas *et al.*, 2017; Kruse *et al.*, 2008; Terburgh *et al.*, 2019; Valsecchi *et al.*, 2012). No significant differences in CII-CV enzyme activities were seen among *Ndufs4* KO tissues (Calvaruso *et al.*, 2012). However, some studies have described elevations in CII and/or CS activity, which have been linked to a compensatory increase in CII-mediated fuelling of the OXPHOS system (Alam *et al.*, 2015; Kruse *et al.*, 2008). Furthermore, reduced CIII activity has also been reported in tissues from *Ndufs4* KO mice (de Haas *et al.*, 2017; Kruse *et al.*, 2008; Terburgh *et al.*, 2019) and some NDUFS4 deficient patient fibroblasts (Breuer *et al.*, 2013; Budde *et al.*, 2000; Ugalde *et al.*, 2004). Martin-Perez *et al.* (2020) also reported increased CIV levels in the *Ndufs4* KO brain; however, this was not evident in pre-symptomatic brain regions (Johnson *et al.*, 2020). In agreement, Adjobo-Hermans *et al.* (2020) saw no significant differences in CII-V assembly factor- or subunit levels, except for increased levels of CV assembly factor, ATPAF2, in all tissues and average CII subunits in the heart and kidneys.

Similarly, CII-IV driven oxygen consumption is not significantly affected, while CI driven respiration is decreased by ~50% in intact brain, skeletal muscle, and liver of *Ndufs4* KO mice (Kruse *et al.*, 2008). On a brain regional level, CI-driven respiration is markedly decreased in non-synaptic mitochondria from lesion-prone and -resilient regions, while only synaptosomes from lesion-prone brain regions displayed marked reductions (Kayser *et al.*, 2016). Furthermore, although *Ndufs4* KO brain mitochondria exhibit a ~26% decrease in maximal CI-driven ATP production and pyruvate oxidation (Manjeri *et al.*, 2016), whole brain ATP levels are unaltered in *Ndufs4* KO mice (Balsa *et al.*, 2020). The *Ndufs4* KO also does not seem to alter ATP production in immortalised embryonic fibroblasts (Valsecchi *et al.*, 2012), skeletal muscle mitochondria (Alam *et al.*, 2015; Kruse *et al.*, 2008), cardiomyocytes (Karamanlidis *et al.*, 2013), bone marrow cells, or thymocytes (Bertaux *et al.*, 2018).

Nevertheless, the initial (P26-37) normal whole-body O₂ consumption observed in *Ndufs4* KO mice (Kruse *et al.*, 2008), decreases with age (~50% at P45), while cerebral oxygen partial pressure (PO₂) increases — both correlating with disease progression (Jain *et al.*, 2019). It was subsequently confirmed that brain O₂ consumption is decreased and PO₂ is elevated in cerebral internal jugular venous (IJV) blood of *Ndufs4* KO mice; however, this was not reflected in arterial blood (Grange *et al.*, 2021). In accord with these observations of brain hyperoxia, early (P30) or late (P55) onset treatment with severe, chronic (but not intermittent or mild) hypoxia⁷, restores cerebral PO₂, rescues the neurological phenotype, prevents brain lesion formation, and significantly extends lifespan (~5 fold) in *Ndufs4* KO mice, while high ambient O₂ tensions exacerbate the phenotype (Ferrari *et al.*, 2017; Jain *et al.*, 2019; Jain *et al.*, 2016).

2.5.4.2 Reductive stress

At the late stage of LS (P66-75), *Ndufs4* KO brain and peripheral tissues (skeletal muscle, heart, liver) display significantly increased NADH levels, decreased NAD⁺ levels, and an increased NADH/NAD⁺ ratio (Lee *et al.*, 2019). Grange *et al.* (2021) subsequently established that NAD⁺ depletion is evident in the brainstem and cerebellum prior to lesion formation, as well as the lesion-resistant cerebrum of *Ndufs4* KO mice (~P38) — with hypoxia treatment not only normalising PO₂, but also NAD⁺ levels. In agreement, hypoxia treatment restored the elevated NADH/NAD⁺ ratio in the lungs of late stage diseased *Ndufs4* KO mice (Schleifer *et al.*, 2018).

NAD⁺ and its other forms [NAD(P)⁺/NAD(P)H] are central oxidoreductase cofactors for many metabolic reactions shown to be perturbed in *Ndufs4* KO mice (Sections 2.5.4.4). In addition, NAD⁺ also acts as a (co)substrate for signalling enzymes that modulate metabolism and transcriptional responses, such as the family of deacetylases, called sirtuins [SIRTs] (Klimova & Kristian, 2019; Xie *et al.*, 2020). In accord, protein hyperacetylation is seen in the peripheral

⁷ Mice continuously breathing normobaric 11% O₂ (severe hypoxia) or 17% O₂ (mild hypoxia) instead of 21% oxygen (Ferrari *et al.*, 2017; Jain *et al.*, 2019; Jain *et al.*, 2016).

tissues (heart, skeletal muscle, and liver), brain (Karamanlidis *et al.*, 2013; Lee *et al.*, 2019); and brainstem (Piroli *et al.*, 2020) of *Ndufs4* KO mice. Some significantly hyperacetylated mitochondrial proteins include the antioxidant enzyme, superoxide dismutase 2 (SOD2), and glutamate dehydrogenase (GDH) in the *Ndufs4* KO brain (Lee *et al.*, 2019) and pyruvate dehydrogenase (PDH) subunits (along with decreased PDH activity) in susceptible neurons⁸ of *Ndufs4* KO mice (Gella *et al.*, 2020) — all of which are known targets of the NAD⁺-dependent mitochondrial deacetylase, SIRT3.

Furthermore, Yang *et al.* (2020) recently demonstrated that a maladaptive increase in the fraction of NADH produced by folate-mediated serine catabolism, despite the redox imbalance, exacerbates NADH accumulation in *Ndufs4* KO mice and contributes to the progression of spasticity. Others have also confirmed that reductive stress impacts survival in *Ndufs4* KO mice. Normalising NAD⁺ redox balance in peripheral tissues (skeletal muscle, heart and liver) of whole-body *Ndufs4* KO mice, through treatment with an NAD⁺ precursor, resulted in a significant lifespan extension without affecting neurological symptoms (Lee *et al.*, 2019); while regenerating NAD⁺ levels in the brain of brain-specific *Ndufs4* KO mice, without altering ATP production⁹, prevents neuroinflammatory lesion formation and markedly extends lifespan without improving ataxia (McElroy *et al.*, 2020). Thus, redox imbalance and reductive stress clearly play a key role in the LS pathology.

2.5.4.3 Oxidative stress

Several studies have reported elevated markers of oxidative stress or ROS levels in the *Ndufs4* KO whole brain (Johnson *et al.*, 2013; Lee *et al.*, 2019); olfactory bulbs (Liu *et al.*, 2015; Quintana *et al.*, 2010); cerebral cortex and external capsule (de Haas *et al.*, 2017); retinas (Song *et al.*, 2017); as well as bone marrow cells and thymocytes (Bertaux *et al.*, 2018). Contrastingly, many others have reported no elevations in oxidative stress markers or ROS levels in *Ndufs4* KO fibroblasts and neuron cultures (Kruse *et al.*, 2008); whole brain, skeletal muscle, and bone marrow-derived macrophages (Miller *et al.*, 2021); the heart (Chouchani *et al.*, 2014; Karamanlidis *et al.*, 2013); most brain regions [except olfactory bulbs] (Kayser *et al.*, 2016), brainstem (Piroli *et al.*, 2016); olfactory bulbs and motor cortex (Felici *et al.*, 2014); as well as forebrain and midbrain mitochondria (Jain *et al.*, 2019). In accord, the reported effects of antioxidant treatments in *Ndufs4* KO mice are varied. Treatment with the antioxidant, N-acetyl cysteine amide, delayed disease onset and reduced clinical symptoms but did not extend lifespan (Liu *et al.*, 2015); while neither treatment with a Trolox derivative, KH176, (de Haas *et al.*, 2017), nor metallothionein 1 overexpression (Miller *et al.*, 2021) improved disease severity or survival. Balsa *et al.* (2020) recently postulated that cytosolic redox imbalance, caused by NADPH deficiency, as opposed to ROS, may underlie neurodegeneration and inflammation in *Ndufs4* KO mice, since reduced

⁸ Glutamatergic neurons in the brainstem vestibular nucleus (Gella *et al.*, 2020).

⁹ Through the conditional expression of the yeast NADH dehydrogenase, ND1, in the brain (McElroy *et al.*, 2020).

serine-driven mitochondrial one-carbon metabolism in *Ndufs4* KO brain mitochondria, and a decrease in NADPH and reduced glutathione (GSH) levels in the *Ndufs4* KO brain, correlate with c-Jun N-terminal kinases (JNK) pathway activation and increased expression of inflammatory markers. In contrast, it was shown that chronically elevated ROS levels trigger JNK and sterol regulatory element binding (SREBP) activity in neurons, which result in glial lipid droplet accumulation, causing a disturbed neuroprotective lipid/lactic acid shuttle mechanism between neurons and glia in lesion-prone brain regions of *Ndufs4* KO mice prior to neurodegeneration (Liu *et al.*, 2017; Liu *et al.*, 2015). Hence, aberrant ROS production may play a role in the LS disease pathology, but this role is currently unclear due to discrepancies among studies, which have called into question whether systemic oxidative stress is truly a central driver of the LS disease pathology (Miller *et al.*, 2021; Piroli *et al.*, 2016).

2.5.4.4 Glycolysis and the TCA cycle

Kruse *et al.* (2008) first reported normal or slightly elevated serum lactate levels in young (P18) and older (>P40) *Ndufs4* KO mice along with normal blood glucose levels. Contrastingly, others subsequently reported significantly elevated serum lactate in suckling pups (P22); elevated blood plasma lactate and 2-hydroxybutyrate in older (P50-65) mice (Jain *et al.*, 2016); as well as higher lactate/pyruvate ratios in IJV plasma relative to arterial plasma (Grange *et al.*, 2021). Despite some discrepancies, these observations likely reflect the known NADH/NAD⁺ imbalance in *Ndufs4* KO mice, as increased lactate production and an elevated lactate/pyruvate ratio indicates cytosolic redox state (Esterhuizen *et al.*, 2017; Go *et al.*, 2020; Munnich *et al.*, 1996).

In accord, intracerebral lactate accumulation is evident in *Ndufs4* KO mice, prior to lesion formation, with lactate levels increasing from the middle- to late disease stage but decreasing with lesion development — possibly due to neuronal loss and decreased energy demand (Takahashi *et al.*, 2019). Neurometabolomics in pre-symptomatic (P30) *Ndufs4* KO mice further confirmed lactate accumulation in the whole brain prior to lesion formation, along with decreased levels of specific neurotransmitters (GABA and dopamine) and metabolic alterations in major energy producing pathways, including increased glycolytic intermediates and pyruvate; lower levels of most free amino acids, some free fatty acids, nucleotides, and their catabolic products (Johnson *et al.*, 2013). In agreement, Lee *et al.* (2019) further reported an upregulation of the transcription factor subunit, hypoxia inducible factor 1 alpha (HIF1α), and its targets, lactate dehydrogenase (LDH) and glucose transporter 1 (GLUT1, SLC2A1) in the whole brain of *Ndufs4* KO mice. This was postulated to reflect increased brain glycolysis, resulting from a pseudo hypoxic state due to ROS-induced HIF1α accumulation.

Thereagainst, several other studies provide conflicting evidence. No signs of an upregulation of glycolytic capacity were evident in *Ndufs4* KO brain regional synaptosomes (Kayser *et al.*, 2016) and brain regional metabolomics in pre-symptomatic (P29-31) *Ndufs4* KO mice, revealed little metabolic differences between genotypes, with only markedly lower brainstem, glutamate levels

and higher reduced-glutathione (GSH) levels reported in the brainstem, olfactory bulbs, and resilient cortex. Furthermore, no significant metabolic differences were observed between *Ndufs4* KO lesion-prone and -resistant brain regions (Johnson *et al.*, 2020). Additionally, neither glucose nor glutamine's relative contribution to (or handling within) the TCA cycle seems to be markedly altered in the *Ndufs4* KO brain or most peripheral tissues. However, a marked decrease was seen in glutamine anaplerosis in the pancreas and liver, as well as the circulatory glutamine turnover flux in *Ndufs4* KO mice (Yang *et al.*, 2020).

In contrast, others provide evidence for an affected TCA cycle in the brain. Significant fumarate accumulation was seen in *Ndufs4* KO lesion-prone brain regions prior to disease symptoms, but not at the late (~P55) disease stage (Piroli *et al.*, 2016). This fumarate accumulation was further linked to increased succination of protein thiols in VDAC 1-2 and the DLST¹⁰ component of the α -ketoglutarate dehydrogenase complex (KGDHC). The former is proposed to catalyse dysfunctional ATP/ADP translocation, while the latter was shown to reduce KGDHC activity, succinyl-CoA production, and protein succinylation. Interestingly, resistant brain regions and skeletal muscle show no changes in protein succination, suggesting a role in regional neurodegeneration (Piroli *et al.*, 2020; Piroli *et al.*, 2016). Furthermore, altered mitochondrial (TCA cycle) metabolism and reduced itaconate production have been suggested to impair microglial ability to resolve inflammation in *Ndufs4* KO mice (McCain *et al.*, 2021).

2.5.4.5 Inflammation

Ndufs4 KO mice exhibit progressive microglial activation in brain regions, with the olfactory bulbs and vestibular nucleus of the brainstem, first and most severely affected (Quintana *et al.*, 2010). Several studies have reported a correlation between the degree of gliosis, disease severity and neurodegeneration in *Ndufs4* KO mice and LS patients (Bolea *et al.*, 2019; Lake *et al.*, 2015; Liu *et al.*, 2017; Liu *et al.*, 2015; Quintana *et al.*, 2010). Nevertheless, widespread inflammation has been reported in affected brain regions (Bolea *et al.*, 2019; Felici *et al.*, 2014; Jain *et al.*, 2016; Miller *et al.*, 2021; Quintana *et al.*, 2010), whole brain (Johnson *et al.*, 2013), retina (Jiang *et al.*, 2019; Yu *et al.*, 2015a), serum, bone marrow-, spleen-, liver-, and skin cells (Jin *et al.*, 2014) of *Ndufs4* KO mice. Hyperactivation of the mechanistic target of rapamycin (mTOR) — a key mediator of cell metabolism, HIF1 α signalling, and inflammation — was reported in the brain of diseased (>P45) *Ndufs4* KO mice (Johnson *et al.*, 2013), although not evident in brain regions at the pre-symptomatic stage (Johnson *et al.*, 2020). Jin *et al.* (2014) subsequently demonstrated that transient alopecia in *Ndufs4* KO suckling neonates is associated with toll-like receptor 2/4-mediated systemic inflammation, which seems to result from defective fatty acid metabolism in the liver. The consequent accumulation of fatty acids and lactate in circulation was proposed to induce ROS overproduction that leads to inflammation and osteopetrosis in *Ndufs4* KO mice. In

¹⁰ Dihydrolipoyllysine-residue succinyltransferase (DLST)

agreement, the whole-body or liver-specific disruption of a prominent mTOR pathway effector, S6K1, was shown to enhance neuronal survival and moderately extend lifespan in *Ndufs4* KO mice, while the same benefits were not seen with neuron- or adipocyte-specific S6K1 disruption (Ito *et al.*, 2017).

It was further shown that chronic systemic treatment with the mTOR inhibitor, rapamycin, inhibits signalling through both mTOR complexes in the *Ndufs4* KO brain via the downregulation of protein kinase C (PKC), resulting in reduced neuroinflammation, glial activation, glycolytic intermediates, and lactate levels, while delaying neurological deficits and markedly extending lifespan (~2 fold) — without rescuing RC function or altering oxidative stress markers (Johnson *et al.*, 2013; Martin-Perez *et al.*, 2020). In support, systemic rapamycin treatment also suppresses innate immune- and inflammatory responses in retinal ganglion cells of *Ndufs4* KO mice (Yu *et al.*, 2015a), while treatment of *Ndufs4* KO mice with PKC inhibitors provide the same benefits as rapamycin treatment, in addition to preventing transient alopecia (Martin-Perez *et al.*, 2020).

On a metabolic level, the beneficial effects of rapamycin treatment was recently ascribed to the restoration of a seemingly dysfunctional glutamine/glutamate/ α -ketoglutarate metabolic axis in the brain of *Ndufs4* KO mice (Johnson *et al.*, 2020). Interestingly, α -ketoglutarate/malate was previously shown to maintain normal CI-driven respiration in *Ndufs4* KO whole brain mitochondria via an unknown mechanism (Kayser *et al.*, 2016) and supplementation with a cell-permeable α -ketoglutarate analogue, significantly increased survival in *Ndufs4* KO mice (Lee *et al.*, 2019). The latter treatment was suggested to maximise compensatory CII-driven respiration via the TCA cycle and suppress hypoxia signalling as it lowered HIF1 α and LDHA levels in *Ndufs4* KO brain without affecting the brain NAD⁺ pool (Lee *et al.*, 2019).

2.5.5 Recent research at the NWU

As part of a larger project at the NWU, we recently investigated the tissue considered to be phenotypically least affected in *Ndufs4* KO mice — skeletal muscle. This metabolomics study identified several adaptive pathways in *Ndufs4* KO skeletal muscle that seem to increase electron transport to the Q-pool and/or recycle NAD⁺ via CII, the G3P cycle, the ETF system, and the putative proline cycle (Terburgh *et al.*, 2019) [Appendix D]. In support, Zielinski *et al.* (2016) predicted, via computational modelling, that some of the same Q-linked pathways may maintain ATP production in CI-deficient human cardiomyocytes. Alam *et al.* (2015) further confirmed normal ATP production and pyruvate oxidation in *Ndufs4* KO skeletal muscle (P31-34), while demonstrating (via flux measurements and mathematical modelling) a shift in isocitrate dehydrogenase flux with a greater portion directed towards a bifurcated TCA cycle i.e., an increased flux through reactions fuelling CII, and a decreased flux through NAD⁺-consuming reactions. In further agreement, the therapeutic potential of two CII-linked Q-cycle fuels (succinate prodrugs and an α -ketoglutarate conjugate) have recently been described in CI-deficient human cells and *Ndufs4* KO mice (Ehinger *et al.*, 2016; Lee *et al.*, 2019). It was also demonstrated that

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using G3P as a fuel could completely restore respiration required for ATP synthesis in patient fibroblasts with CI assembly defects (Desquiret-Dumas *et al.*, 2019). Furthermore, Liu *et al.* (2021) recently confirmed that increased G3P synthesis and shuttle activity is an important endogenous NAD⁺-regeneration pathway during CI inhibition that suppresses neuroinflammation, and extends lifespan when enhanced in *Ndufs4* KO mice.

Altogether, these observations warrant further metabolic investigations of brain regions from diseased *Ndufs4* KO mice, which are considered a more appropriate model of LS than the pre-symptomatic mice (Finsterer, 2019) used for previous neurometabolomics investigations (Johnson *et al.*, 2020). Hence, in this study, we further investigate brain regional metabolic alterations in diseased *Ndufs4* KO mice and cross-compare these alterations with that found in skeletal muscle and urine to obtain a more holistic overview of LS pathophysiology.

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CHAPTER

Materials and methods



3

3.1 Introduction

This chapter provides information on the materials and methods used to achieve the study objectives. This includes details regarding animal handling and sampling, PCR-based genotype confirmation, and the metabolomics analyses (GC-TOF-MS and LC-MS/MS) used to investigate urinary and tissue-specific metabolomes.

3.2 Animals and sampling

3.2.1 Animal handling

Male whole-body homozygous *Ndufs4* KO mice, along with age- and sex-matched controls (WT) were bred, supplied, and housed at the Vivarium (SAVC reg. #FR15/13458; SANAS GLP compliance #G0019; AAALAC file #1717) of the Preclinical Drug Development Platform (NWU). One sex-group was used to minimise metabolic variation due to differences in hormone cycles, tissue-specific metabolism, and metabolic profiles between sexes (Ruoppolo *et al.*, 2018; Wells *et al.*, 2016). *Ndufs4* KO mice were born from heterozygous crosses¹², with original breeding trios obtained from Jackson Laboratory (B6.129S4-*Ndufs4*^{tm1.1Rpa}/J, JAX stock #027058). Refer to Mereis (2018) for details regarding colony establishment and breeding. Animals were group housed in polysulphone type II long, individually ventilated cages (396 x 215 x 172 mm) under temperature- ($22 \pm 1^\circ\text{C}$), humidity- ($55 \pm 10\%$), and light-controlled (12:12 hour light/dark cycle) conditions with standard laboratory chow (Rodent Breeder, #RM1845, LabChef, Nutritionhub) and water provided *ad libitum*. Animals were assigned identification numbers at wean age (P21-23) using an ear punch numbering system (Figure 3-1), after which tail snips were collected for genotyping purposes (Section 3.3). In addition, litter numbers were assigned to record parental lineage. All protocols and experiments were approved by the AnimCare animal research ethics committee (NHREC reg. #AREC-130913-015) of the NWU (ethics approval #NWU-0001-15-A5 and #NWU-00378-16-A5). 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.

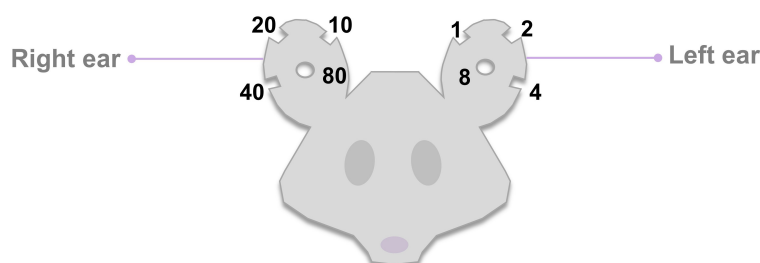


Figure 3-1: Front view depiction of the ear punching system Animal identification numbers are indicated by the sum of digits denoted by holes or notches in the right ear (ranging from 10 to 90) and left ear (ranging from 1 to 9). For example, a hole in each ear indicates an identification number of 88.

¹² *Ndufs4* KO mice typically die before sexual maturity is reached and are considered infertile (JAX, 2017).

3.2.2 Sample size and animal groups

The minimum sample size required per group ($n=20$) was determined in collaboration with Dr Mari van Reenen (Statistical consultant, NWU). However, as shown in Table 3-1, the ultimate sample size slightly varied, depending on animal and sample-type availability as well as outlier removal. More detailed information on the animals used in this study can be found in Appendix A (Section A.2).

Table 3-1: Animal numbers after outlier removal

Sample type	<i>Ndufs4</i> KO (number)	WT (number)
Brain regions (BR)	21	25
Skeletal muscles (M)	19	21
Urine (U)	23	30

3.2.3 Humane endpoints and euthanasia

Animal pain and distress were monitored throughout the study using a humane endpoint score sheet (SOP_Viv_Anim 27: Determining pain and distress in lab rodents). Animals that were excluded from the study (heterozygotes/females) were euthanised at P30 — after genotyping but prior to encephalopathy in *Ndufs4* KO mice. In these humane endpoint cases, euthanasia was conducted via anaesthetic overdose (SOP_Viv_Anim 1: Euthanasia of rodents, procedure 12.3). Animals included in the study were euthanised between P45-50, as this age range was selected to represent the late stage of LS, based on previous survival curves and phenotypic analyses performed at our facilities (Miller *et al.*, 2021). For these animals, euthanasia was conducted via cervical dislocation, as described by the AVMA (2013), to avoid the known adverse effects of anaesthesia on *Ndufs4* KO mice (Quintana *et al.*, 2012; Roelofs *et al.*, 2014).

3.2.4 Sampling and tissue dissection

12-hour urine (U) samples were collected from individual mice in metabolic cages during overnight fasting with *ad libitum* access to water. The separating cone, collection funnel, and -tubes were all treated with sodium azide [NaN_3 ; 0.1% (w/v); WT: 100 μL and KO: 50 μL] prior to sample collection to prevent bacterial growth. *Ndufs4* KO mice produced limited urine volumes, therefore, urine droplets that failed to reach the collection tube were also collected by using a pipette to gently mix the droplets with NaN_3 solution ($\sim 200\text{--}250$ μL) and transfer the mixture to the collection tube. After urine collection, mice were euthanised via cervical dislocation at the same time of day (8:00-9:00 AM). The brain was immediately removed from the skull, rinsed with saline solution (SABAX PBS; 0.9% NaCl (w/v), #7634, Adcock Ingram), and dissected into the regions of interest (anterior cortex, AC; brainstem, BS; cerebellum, CB; and olfactory bulbs, OB). Concurrently, the white muscle portions of quadriceps femoris (WQ), as well as solei (SOL) muscles, were collected from both hind limbs. Thereafter, tail snips were collected for *post-mortem* genotype confirmation as discussed in Section 3.3. All samples were snap frozen in liquid nitrogen upon collection (within 15 minutes *post-mortem* for tissues) to “quench” metabolic activity and stored at -80 °C until further use.

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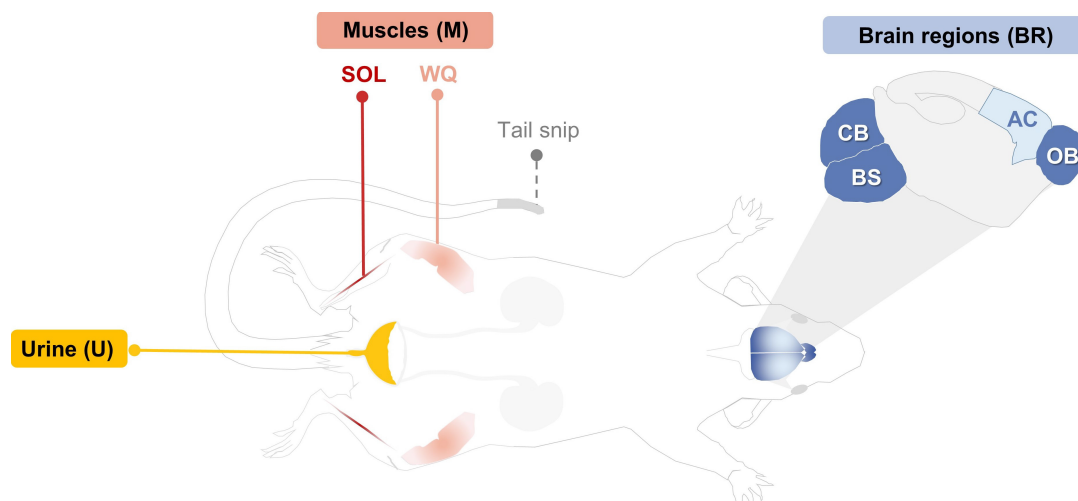


Figure 3-2: Sample types collected from *Ndufs4* mice. Urine samples were collected overnight in individual metabolic cages. After euthanasia, one lesion-resistant brain region (anterior cortex, AC) and three lesion-prone brain regions i.e., the brainstem (BS), cerebellum (CB), and olfactory bulbs (OB) were collected along with primarily oxidative (solei, SOL) and glycolytic (white quadriceps, WQ) muscle types. Tails snips were collected for genotypic purposes. **Confirming the *Ndufs4* genotypes**

As part of the initial colony establishment and breeding program managed by Mereis (2018), the C57BL6/J genetic background of the *Ndufs4* mouse strain was verified via whole genome genotyping at Jackson Laboratory (ME, USA) with all subsequent breeding pairs and litters genotyped according to the standard polymerase chain reaction (PCR) technique described by Valsecchi *et al.* (2012). The same technique was employed on tail snips of mice used in this study to confirm that samples of the correct genotype were collected.

3.3.1 Reagents

For DNA isolation and amplification, the ZR Genomic DNA™-Tissue MiniPrep Kit (Cat. #D3050, Inqaba Biotech™), Phire™ Tissue Direct PCR Master Mix Kit (Cat. #F-170L; Thermo Fisher Scientific), and specific *Ndufs4* primers (Table 3-2) [*Ndufs4* 1060, Cat. #S32E6; *Ndufs4* Rev, Cat. #S3424, Inqaba Biotech™] were used. For amplicon separation and visualisation, agarose (Cat. #8100; CONDA pronadisa), ethidium bromide nucleic acid stain (EtBr; Cat. #46065, Sigma-Aldrich®), 10× Bionic™ Buffer (Cat. #B6185, Sigma-Aldrich®), nuclease-free PCR grade water (H₂O; Cat. #129114, Qiagen), GeneRuler™ 100 bp DNA ladder (Cat. #SM0241, Thermo Fisher Scientific), and 6× DNA loading dye (Cat. #SM0242, Thermo Fisher Scientific) were used.

Table 3-2: Additional information on specific *Ndufs4* PCR primers

Primer	Sequence (5' – 3')	Size (bases)	T _m (°C)
<i>Ndufs4</i> 1060 (Forward)	AGCCTGTTCTCATACCTCGG	20	62.45
<i>Ndufs4</i> Rev (Reverse)	TTGTGCTTACAGGTTCAAAGTGA	23	59.2

Abbreviations: 5'-3', polynucleotide direction from 5'-end to 3'-end; T_m, melting temperature.

3.3.2 Genomic DNA isolation and quantification

Genomic DNA was isolated from mouse tail snips (~ 25 mg) according to manufacturer instructions using the ZR Genomic DNA™-Tissue MiniPrep Kit. Subsequently, DNA (260 nm) and protein (280 nm) absorbance were measured using the NanoDrop® 1000 UV-Visual

spectrophotometer (operating software v 3.8.1, Thermo Fisher Scientific) to determine DNA concentration and purity¹³. Thereafter, DNA samples were diluted to 25 ng/μL for further processing (Section 3.4.2)

3.3.3 DNA amplification by polymerase chain reaction

A section of the isolated DNA, spanning the site of excision in the mutant *Ndufs4* gene, was amplified via PCR. The PCR reaction mixture (10 μL) contained 1× Phire™ Tissue Direct PCR Master Mix, nuclease-free PCR grade water, 25 ng DNA and 0.5 μM forward (*Ndufs4* 1060) and reverse (*Ndufs4* Rev) primers corresponding to the introns flanking *Ndufs4* exon two (Table 3-2). PCR amplification was done using a T100™ thermal cycler (Bio-Rad) under the following conditions: 98 °C for 5 minutes to ensure DNA polymerase activation; 34 cycles of: 98 °C for 5 seconds, 57.3 °C for 5 seconds, and 72 °C for 20 seconds to allow DNA denaturation, primer annealing and DNA elongation, respectively; followed by a final incubation at 72 °C for 1 minute.

3.3.4 DNA characterisation by agarose gel electrophoresis

Amplicons were separated by electrophoresis on a 1% (w/v) agarose gel containing 5 μg/mL ethidium bromide as a nucleic acid stain in 1× Bionic™ Buffer. Diluted PCR products¹⁴ (10 μL, 30%, v/v) together with a GeneRuler™ 100 bp DNA ladder¹⁵ were loaded on the gel and run at 40 V (15 minutes) followed by 70 V (~ 1.5 hours) using a Sub-Cell® GT horizontal electrophoresis apparatus (Bio-Rad). Amplicon separation was visualised and photographed under ultraviolet (UV) light using the ChemiDoc™ MP system (Bio-Rad) with Image Lab software (v 5.2.1). Mouse genotypes were distinguished based upon amplicon size, with bands corresponding to two intact *Ndufs4* allele segments (1229 bp) indicating a WT (*Ndufs4*^{+/+}); two shortened allele segments (429 bp) indicating a KO (*Ndufs4*^{-/-}) without the second *Ndufs4* exon; and the presence of both an intact and shortened band indicating a HET carrier (*Ndufs4*^{+/-}). An example electrophoretogram can be found in Appendix B (Section B.1).

3.4 Multi-platform metabolomics of urine and tissues

Untargeted GC-TOF-MS and semi-targeted LC-MS/MS were performed on each of the seven sample types collected from *Ndufs4* KO (n≥19) and WT (n≥20) mice. These urine and tissue samples were individually prepared and analysed, with pooling only done for quality control (QC) sample preparation. Metabolic profiling was performed according to previously described methods (Lindeque *et al.*, 2013; Mels *et al.*, 2011) that were previously standardised at the Mitochondria Research Laboratory (NWU) for applicability to mouse urine, brain regions, and muscle types.

¹³ An A260/A280 ratio of ~1.8 was considered optimal.

¹⁴ Contains loading dye from the Phire™ Tissue Direct PCR Master Mix.

¹⁵ Contains a 2:1:3 ratio of DNA ladder : 6× DNA loading dye : nuclease-free water.

3.4.1 Reagents

During sample preparation, the following reagents were used: QuantiChrom™ Creatinine Assay Kit (Cat. #DICT-500, BioAssay Systems), methanol (Cat. #2304, Honeywell, Burdick & Jackson, HPLC grade), water (H₂O; Cat. #BJ365CS, Honeywell, Burdick & Jackson, HPLC grade), and chloroform (Cat. #0494, Honeywell, Burdick & Jackson, HPLC grade). Internal standards: *N, N*-dimethyl-L-phenylalanine (DMPA; Cat. #273910, Sigma-Aldrich®, 99%), 3-phenylbutyric acid (3-PBA; Cat. #116807, Sigma-Aldrich®, 98%), tropic acid (Cat. #93540, Fluka, ≥99%). Stable isotopes: L-arginine_4,4,5,5-d₄·HCl (Cat. #DLM-6038, 93%), L-asparagine_C¹³4-N¹⁵2 : H₂O (Cat. #CNLM-3819-H), L-citrulline_4,4,5,5-d₄ (Cat. #DLM-6039, 93%), L-glutamine_C¹³5-N¹⁵2 (Cat. #CNLM-1275-H), L-isoleucine_d₁₀ (Cat. #DLM-141-0.1, 98%), and L-lysine-4,4,5,5_d₄·2HCl (Cat. #DLM-2640, 96-98%) purchased from Cambridge Isotope Laboratories; L-valine_d₈ (Cat. #E161P, 99%), and L-phenylalanine_ring_d₅ (Cat. #E151P, 98%) purchased from Eurisotop; and L-carnitine_methyl-d₃·HCl, acetyl-L-carnitine_methyl-d₃·HCl, octanoyl-L-carnitine_8,8,8-d₃·HCl, dodecanoyl-L-carnitine_10,10,10-d₃·HCl, and octadecanoyl-L-carnitine_18,18,18-d₃·HCl obtained from VUmc¹⁶.

During sample derivatisation and analysis, the following reagents were used: acetyl chloride (Cat. #00990, Fluka analytical, ≥99%), 1-butanol (Cat. #281549, Sigma-Aldrich®, 99.8%), methoxyamine hydrochloride (Cat. #226904, Aldrich, 98%), O-bis(trimethylsilyl) trifluoroacetamide (BSTFA; Cat. #33027, Supelco, 98%), trimethylchlorosilane (TMCS; Cat. #92360, Fluka analytical, 98%), pyridine anhydrous (Cat. #270970, Sigma-Aldrich®, 99.8%), acetonitrile (ACN; Cat. #BJ015CS, Honeywell, Burdick & Jackson, >99% HPLC grade), water (H₂O; Cat. #BJ365CS, Honeywell, Burdick & Jackson, HPLC grade), and formic acid (Cat. #06440, Sigma-Aldrich®, 98% for mass spectrometry).

3.4.2 Addition of internal standards

Known quantities of internal standards (IS) were added to urine and tissues at the start of sample preparation to account for biases that may arise during the process. Additionally, sample aliquots intended for LC-MS/MS were supplemented with a mixture of amino acid¹⁷ and acylcarnitine¹⁸ isotopes to account for ion suppression effects during analysis. For GC-TOF-MS, tropic acid and 3-phenylbutyric acid were selected as ISs for urine and tissues, respectively, while *N, N*-dimethylphenylalanine (DMPA) was selected as IS for LC-MS/MS. For tissues, these internal standards were added according to tissue mass, as part of the water used for monophasic extraction (Section 3.4.4). Since urine samples required minimum preparation, LC aliquots were simultaneously supplemented with a mixture of DMPA and isotopes. The ISs described here were

¹⁶ Isotopes were supplied by Dr Brink at the VU medical center, hence no Cat. # are available.

¹⁷ Consisting of valine_d₈, isoleucine_d₁₀, phenylalanine_d₅, lysine_d₄, glutamine_C¹³5-N¹⁵2, asparagine_C¹³4-N¹⁵2, arginine_d₄ and citrulline_d₄.

¹⁸ Consisting of free carnitine_d₃, octanoylcarnitine_d₃, dodecanoylcarnitine_d₃ and octadecanoylcarnitine_d₃, with the urine IS mixture additionally containing acetylcarnitine_d₃, but not free- or dodecanoyl carnitine isotopes.

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selected due to their natural absence from murine samples, repeatable derivatisation, favourable elution time and consistent detection via multiplatform metabolomics (van Aardt et al., 2016). Separate IS mixtures were prepared for each sample type, with the concentration (ppm) of these mixtures calculated to allow the analysis of 30 ng LC-IS and 50 ng GC-IS for BR; 25 ng LC-IS and GC-IS for M; as well as 2.5 ng LC-IS and 50 ng GC-IS for urine, as indicated in Table 3-3.

Table 3-3: Metabolomics conditions for the different sample types and analytical platforms

Tissue type	Initial sample volume (µL)	Final sample volume (µL)	Injection volume (µL)	Mass IS analysed (ng)	Mass isotope analysed (ng)
GC-TOF-MS					
BR	200	100	1	50	N/A
OB	150	50	1	50	N/A
WQ	40	100	1	25	N/A
SOL	200	50	1	25	N/A
Urine	20-250*	100	1 (1:10 split)	50	N/A
LC-MS/MS					
BR	100 (57)	50	2	30	2.5
OB	75 (57)	25	2	30	2.5
WQ	50	100	1	25	2.5
SOL	50	25	2.5	25	2.5
Urine	20-250*	100	4	2.5	2.5

*For each platform, the table displays the initial volume of urine or tissue extract aliquoted (column 2), the final volume of prepared sample (column 3), the sample volume injected on-column (column 4), and the theoretical mass IS (column 5) or isotope (column 6) in the analysed sample volume. *initial urine aliquot volumes vary depending on sample creatinine content.*

3.4.3 Urine sample preparation

3.4.3.1 Determining urinary creatinine concentration

Urinary creatinine concentration was spectrophotometrically determined using the QuantiChrom™ Creatinine Assay Kit, based on the kinetic reaction originally described by Jaffe (1886). Assays were performed, in triplicate, in a 96-well UV microtiter plate on a Synergy™ HT microplate reader (Biotek® Instruments) with Gen5™ software (v 1.11.5). Each 210 µL reaction consisted of 10 µL urine and 200 µL working solution containing 3 mM picric acid, 0.1 M sodium hydroxide, 0.3 mM EDTA, 0.1% (v/v) DMSO, and 0.0004% (v/v) Tween®20. The linear increase in absorbance, due to the formation of a yellow/orange adduct between creatinine and picric acid, was measured at 510 nm for 5 minutes, in 1-minute intervals. Each plate also contained a creatinine dilution series ranging from 0-50 mg% used to construct a standard curve ($R^2 > 0.99$) and calculate absolute creatinine quantities via least mean squares linear regression.

3.4.3.2 Deproteinising urine

Predetermined volumes of urine, corresponding to 0.0625 µmole creatinine, were transferred to separate microcentrifuge tubes for each analytical platform and supplemented with internal standards (Section 3.4.2). Samples were then deproteinised by the addition of methanol [3:1 (v/v) methanol:urine], vortex mixing (15 seconds), incubation at -20°C (20 minutes), and centrifugation at 15 000 ×g (10 minutes). The resulting supernatants were then transferred to glass vials and evaporated to dryness under a stream of nitrogen gas at 37°C. Finally, additional methanol

(80 μ L) was added, and samples were dried again to ensure complete evaporation. Dried urine samples were then stored at -80°C.

3.4.4 Metabolite extraction from tissues

For each mouse tissue, a monophasic metabolite extract was prepared and divided into three aliquots — for LC-MS/MS and GC-TOF-MS analysis as well as QC sample preparation. Brain extracts destined for LC-MS/MS were then further processed to obtain polar phases of biphasic extracts for analysis. Due to the limited quantities of SOL and OB samples, different liquid/liquid extraction volumes were used for these tissues.

3.4.4.1 Monophasic brain and muscle extracts

Monophasic metabolite extracts were prepared from brain regions (BR) and skeletal muscles (M) using a modified Bligh–Dyer method (Gullberg *et al.*, 2004) with a solvent ratio of 3:1:1 methanol/water/chloroform. Solvent volumes were added according to wet tissue mass, as indicated in Table 3-4, while considering the approximate tissue water content (0.75-0.80 μ L/mg tissue). Methanol and water, containing internal standards (Section 3.4.2), were added to pre-minced and -weighed tissues in safe-lock microcentrifuge tubes (1.5 mL, Eppendorf®). Next, tissues were pulverised for 2 minutes (BR:20 Hz, M:30 Hz) in the presence of 3 mm $\varnothing$ tungsten carbide and/or 5 mm $\varnothing$ stainless-steel beads¹⁹ using a Retsch® MM 400 vibration mill. Thereafter, chloroform was added, and homogenates were vortex mixed, incubated on ice (10 minutes), and centrifuged at 20 000 $\times$ g (4 °C; BR:15 minutes, M:10 minutes). The resulting supernatants were then transferred to new tubes and stored at -80°C. For BR, however, a portion (BR:100 μ L; OB:75 μ L) of each monophasic extract was further processed to obtain a polar metabolite fraction for LC as described hereafter.

Table 3-4: Solvent volumes used for monophasic metabolite extraction

Tissue type	Methanol (μ L/ mg tissue)	Water-IS mix (μ L/ mg tissue)	Chloroform (μ L/ mg tissue)
BR	12	3.2	4
OB	24	7.2	8
WQ	6	1.25	2
SOL	60	19.25	20

Due to the size difference among tissues, different liquid/liquid extraction volumes were used to obtain sufficient sample volumes — most notably for the quantity-limited SOL and OB. Here, BR refer to the AC, BS, and CB.

3.4.4.2 Biphasic brain extracts for liquid chromatography

For each BR, biphasic extracts were prepared from monophasic aliquots using a step-wise method (Wu *et al.*, 2008). Additional volumes of chloroform (BR:40 μ L; OB:30 μ L) and water (BR:34 μ L; OB:25.5 μ L), containing stable isotopes, were added to monophasic extracts to obtain a final biphasic ratio of 2:1.8:2 (v/v) methanol/water/chloroform. The samples were then vortex mixed, incubated on ice (10 minutes) and centrifuged at 20 000 $\times$ g (15 minutes, 4 °C). Finally,

¹⁹ ~7:1, w/w. For BR and SOL, 3 mm $\varnothing$ beads were used while 5 and 3 mm $\varnothing$ beads were used for WQ.

the resulting top (aqueous) phase of each extract was transferred to a new tube and stored at -80°C.

3.4.4.3 Tissue extract aliquots

After metabolite extraction, each sample was respectively aliquoted into glass vials (2 mL, Agilent©) for LC, GC, and QC sample preparation (Section 3.4.5). The aliquot volumes for each sample type and platform are given in Table 3-3 (column 2). Volume-limited samples (OB and SOL) were transferred to microvials (1.1 mL, Separations) for GC, and inserts (250 µL with polymer feet, Separations) for LC, to maximise recovery for small sample volumes. Thereafter, only M extract aliquots intended for LC were supplemented with stable isotopes, since BR polar metabolite fractions (57 µL) already contained isotopes. Finally, all sample and QC aliquots were evaporated to dryness under a stream of nitrogen gas at 37°C and stored at -80°C.

3.4.5 Quality control samples

A QC sample was prepared for each of the seven sample types by pooling equal volumes of the relevant *Ndufs4* KO and WT extracts from that sample type. Since two different BR extract types were used, two pooled QC samples were prepared for each BR — one from monophasic extracts (for GC) and the other from biphasic extracts (for LC). The relevant pooled QC sample, for each sample type and platform, was then aliquoted and handled in the same manner as other samples. For each analytical platform, urine samples were analysed in three randomised batches over three days, while the six tissue sample types were respectively analysed, each in two randomised batches over two days. QC samples were included into these sample batches and analysed at the beginning, intermittently (every 5-8 samples), and at the end of each batch to monitor metabolomics data integrity. Additionally, an in-house standard mixture consisting of all semi-targeted metabolites (2.5 ppm, see Appendix C for details) and a QC sample spiked with this standard mixture, were analysed after each LC batch.

3.4.6 Metabolomics analyses

3.4.6.1 GC-TOF-MS sample preparation and analysis

For GC-TOF-MS, samples were derivatised via a two-step reaction consisting of oxymation and silylation. First, the dried urine and tissue samples were supplemented with 50 µL (25 µL for OB and SOL) methoxyamine hydrochloride (20 mg/mL in pyridine), vortex mixed, and incubated at 60°C (60 minutes). Next, 50 µL (25 µL for OB and SOL) *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA):trimethylchlorosilane (TMCS) [99:1 (v/v)] was added to samples, which were then vortex mixed, incubated at 60°C (60 minutes). After reaching room temperature, samples were then transferred to glass inserts (BR and WQ: 250 µL tapered; urine: flat bottom) and loaded onto an Agilent© 7693 auto sampler for GC-TOF-MS analysis.

GC-TOF-MS was performed as described by Lindeque *et al.* (2013), with slight modifications. The GC-TOF-MS system consisted of an Agilent© 7890A series gas chromatograph coupled to a

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LECO Pegasus HT time-of-flight mass analyser. A sample volume of 1 μL was injected for all sample types, with the injector operating in split (1:10 ratio) and spitless mode for urine and tissues, respectively. The front inlet temperature was kept at 250 $^{\circ}\text{C}$ and helium was used as carrier gas at a constant flow rate of 1.5 mL/minute. Chromatography was performed on a Rxi[®]-5Sil MS capillary column (Restek, 28.6 m x 250 μm x 0.25 μm) using the GC oven temperature program provided in Table 3-5.

Table 3-5: Gas chromatography oven temperature program

Parameter	Target temperature ($^{\circ}\text{C}$)	Rate ($^{\circ}\text{C}/\text{min}$)	Hold time (min)
Initial temperature:	70 (BR:40)		1
1 st temperature ramp	120	7	-
2 nd temperature ramp	230	10	-
3 rd temperature ramp	300	13	1 (BR:2)
Return to initial	70 (BR:40)		-

Parentheses indicate conditions for BR that differed from that for M and urine samples.

The eluting compounds were then subjected to electron impact ionisation (70 eV), with the transferline and ion source temperatures held at 225 $^{\circ}\text{C}$ and 200 $^{\circ}\text{C}$, respectively. After a solvent delay (urine: 230 seconds, M: 250 seconds, BR: 480), mass spectra within the scan range of 50–950 m/z were acquired at a scan rate of 20 spectra/second. Peaks with 5 apexing masses were detected using an expected peak width of 3 seconds and a signal-to-noise ratio of > 20. The LECO Corporation ChromaTOF software (v 4.5x) was used for data acquisition and extraction, which included automatic baseline removal via the “spanning” tracking method (offset of 1; right above the noise) and auto smoothing, with the software’s Statistical Compare feature used to align peaks. Dataset features were identified via spectral and retention time matching using commercial (NIST11) and in-house spectral libraries together with public databases and metabolite identities were assigned to spectral features, with high confidence levels (Schymanski *et al.*, 2014; Sumner *et al.*, 2007). For metabolite cross comparisons among sample types, the data extraction tool, MET-IDEA [Metabolomics Ion based Data Extraction Algorithm] (Broeckling *et al.*, 2006), was additionally used to ensure that important features, unique to one or more sample types, were also extracted from other datasets where possible.

3.4.6.2 LC-MS/MS sample preparation and analysis

For LC-MS/MS, samples were butylated prior to their analysis. First, dried urine and tissue samples were reconstituted in 300 μL (200 μL for OB and SOL) 1-butanol:acetyl chloride (4:1, v/v). Tissue samples were then incubated at 50 $^{\circ}\text{C}$ for 60 minutes, while urine samples were incubated at 65 $^{\circ}\text{C}$ for 15 minutes. Thereafter, the samples were evaporated to dryness under a stream of nitrogen gas at 37 $^{\circ}\text{C}$ and reconstituted in a final volume (25 μL for OB and SOL; 50 μL for BR, and 100 μL for WQ and urine) of water:acetonitrile (50:50, v/v), containing 0.1% formic acid. Finally, the vortex-mixed samples were transferred to 250 μL tapered glass inserts and loaded onto an Agilent© 1200 series auto sampler for LC-MS/MS analysis.

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LC-MS/MS was performed as described by Mels *et al.* (2011), with slight modifications. The LC-MS/MS system consisted of an Agilent® 1200 series LC front end coupled to an Agilent® 6410 series triple quadrupole mass analyser. The column temperature was kept at 35°C and 30°C for urine and tissue samples, respectively, while water (A) and acetonitrile (B) — both containing 0.1% formic acid — were used as mobile phases. A sample volume of 1 µL, 2 µL, 2.5 µL and 4 µL, were respectively injected for WQ, BR, SOL, and urine samples. Chromatography was performed on a C18 Zorbax SB-Aq reverse phase column (2.1mm x 150 mm x 3.5 µm, Agilent®) fitted with a Phenomenex guard column according to the gradient-elution program given in Table 3-6.

Table 3-6: Liquid chromatography gradient elution program

Urine					Tissues				
Time (min)	% A	% B	Hold time (min)	Flow rate (mL/min)	Time (min)	%A	% B	Hold time (min)	Flow rate (mL/min)
Initial	95	5	1	0.25	Initial	95	5	1	0.30
7	82	18		0.25	3	80	20	3	0.30
16	0	100	5	0.25	13	0	100	5	0.30
24	95	5	24	0.25	18.5	95	5	1.5	0.35
Post run	95	5	10	0.25	Post run	95	5	10	0.35

A= water (0.1% formic acid), B= acetonitrile (0.1% formic acid).

The resulting effluent was transferred to the mass spectrometer via positive electrospray ionisation under the following source conditions: capillary voltage of 3 500 V; nitrogen source gas at 300 °C with a 7.5 L/minute flow rate; and nebuliser pressure of 30 psi. Spectra were acquired using the multiple reaction monitoring (MRM) mode at an electron multiplier voltage of 300 V and a dwell time of 45 milliseconds for all compounds. The specific MRM parameters for each semi-targeted metabolite is given in Appendix C, Table 3-7. Data acquisition and extraction was done using Agilent®'s MassHunter Workstation software (Data acquisition for 6400 Series Triple Quadrupole, v B02.01; Qualitative Analysis and Quantitative Analysis software, v B06.00).

Table 3-7: MRM conditions for compounds measured via LC-MS/MS

Compound	Precursor ion (m/z)	Product ion (m/z)	F (V)	CE (V)
Amino acids and derivatives				
1-Methyl-L-Histidine	226.4	124.2	120	15
3-Alanine	146.2	72.1	90	10
3-Hydroxykynurenine	281.2	152.1	108	12
3-Aminoisobutyric acid	160.1	30.1	100	15
3-Methyl-L-Histidine	226.4	96.2	130	25
4-Aminobutyric acid	160.1	87.1	79	8
4-Hydroxy-L-proline	188.1	68.1	120	35
5-Hydroxy-L-lysine	219.4	82.2	100	20
Creatine	188.1	90.1	104	16
Kynurenine	265.4	136	120	10
L-2-Aminoadipic acid	274.2	98.1	113	24
L-Alanine / L-Sarcosine	146.1	44.2	80	15
L-Arginine	231.2	70.1	103	40
L-Arginine- <i>d</i> ₄	235.2	74.2	108	32
L-Asparagine	189.1	144.1	89	8
L-Asparagine- ¹³ C ₄ , ¹⁵ N ₂	195.2	148.1	89	8

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Compound	Precursor ion (m/z)	Product ion (m/z)	F (V)	CE (V)
L-Aspartic acid	246.2	144.1	98	12
L-Citrulline	232.2	70.1	89	32
L-Citrulline-d ₄	236.2	74.1	108	28
L-Cystathionine	335.2	190.1	127	16
L-Cystine	353.0	130.0	118	16
L-Glutamic acid	260.2	84.1	89	24
L-Glutamine- ¹³ C ₅ , ¹⁵ N ₂	210.2	89.1	89	20
L-Glycine	132.1	76.1	65	4
L-Histidine	212.1	110.1	89	16
L-Isoleucine / L-Leucine	188.2	86.2	89	8
L-Isoleucine-d ₁₀	198.2	96.2	108	12
L-Isothreonine	176.1	74.1	94	12
L-Lysine / L-Glutamine	203.1	84.1	89	20
L-Lysine-d ₄	207.2	88.2	118	16
L-Methionine	206.1	104.1	94	8
L-Ornithine	189.2	70.2	74	20
L-Phenylalanine	222.2	120.1	108	16
L-Phenylalanine-(ring-d ₅)	227.2	125.1	108	16
L-Pipecolic acid	186.1	84.1	108	16
L-Proline	172.1	70.2	94	20
L-Pyroglutamic acid	186.1	84.1	108	16
L-Serine	162.1	60.1	94	12
L-Threonine	176.1	158.1	94	5
L-Tryptophan	261.2	244.1	94	8
L-Tyrosine	238.2	136.1	94	12
L-Valine	174.2	72.2	89	12
L-Valine-d ₈	182.2	80.2	103	12
N, N, N-Trimethylglycine	174.2	58.2	130	45
N, N-Dimethylglycine	160.3	58.2	90	20
N, N-Dimethyl-L-phenylalanine	250.1	148.1	103	20
N-Acetylaspartic acid	288.2	144.1	98	16
N-Acetylglutamic acid	302.2	186.1	113	12
Taurine	126.0	107.9	93	10
Acylcarnitines				
Acetyl-L-carnitine (C2)	260.2	85.1	122	24
Acetyl-L-carnitine-d ₃	263.2	85.1	127	20
Butyryl-L-carnitine (C4)	288.2	85.1	110	20
L-Carnitine (C0)	218.2	103.0	132	16
L-Carnitine-(methyl-d ₃)	221.2	103.1	127	16
Decanoyl-L-carnitine (C10)	372.3	85.1	125	28
Dodecanoyl-L-carnitine (C12)	400.3	85.1	155	28
Dodecanoyl-L-carnitine-d ₃	403.4	85.1	160	32
Hexadecanoyl-L-carnitine (C16)	456.4	85.1	160	36
Hexanoyl-L-carnitine (C6)	316.2	85.1	137	24
Isovaleryl-L-carnitine (C5)	302.2	85.1	105	24
Octadecanoyl-L-carnitine (C18)	484.0	85.1	155	36
Octadecanoyl-L-carnitine-d ₃	487.5	85.1	160	36
Octanoyl-L-carnitine (C8)	344.3	85.1	115	28
Octanoyl-L-carnitine-d ₃	347.3	85.1	136	24
Propionyl-L-carnitine (C3)	274.2	85.1	127	20
Tetradecanoyl-L-carnitine (C14)	428.4	85.1	150	28

MRM conditions for butylated compounds and isotope-labelled internal standards are detailed together with their precursor- and product ions. The dwell time for all compounds was 45 seconds. Abbreviations: CE, collision energy voltage; F, fragmentor voltage; m/z, mass to charge ratio; V, Voltage.

3.4.7 Data preprocessing and pretreatment

Spectral data matrices were individually inspected (correct peak detection and alignment, batch precision and data integrity) and preprocessed. Data preprocessing steps were performed in

Excel (Microsoft 365) and included data filtering, missing value imputation, and normalisation. To all datasets, a missing value filter (modified 80% rule) was applied to remove features that were not detected in 80% of both²⁰ *Ndufs4* KO and WT group samples. Tissue data was normalised post-acquisition to tissue mass (ng/mg tissue) using internal standards, 3-phenylbutyric acid (for GC) and *N*, *N*-dimethylphenylalanine (for LC). Urine data was normalised pre-acquisition to creatinine content (mg/g creatinine) and post-acquisition to tropic acid (for GC) or isotope (for LC) internal standards. For tissue and urine data, LC-MS/MS metabolites were also normalised to their own isotope, or an isotope to which they strongly correlated, to account for ion suppression effects. Subsequently, missing value imputation was done. For initial brain regional comparisons missing values were replaced using the k-nearest neighbour (KNN) algorithm, which imputes a value corresponding to the average of other peaks in the sample that have a similar signal intensity (Di Guida *et al.*, 2016). For the subsequent sample type cross-comparison, missing values in all datasets were replaced with a small value assumed to be the detection limit i.e., half of the minimum positive value in the original data. Next, a QC variance filter was applied to remove features from the dataset with a CV-value above 25% (for LC) or 50% (for GC) in the QC samples. However, metabolites of interest for data cross comparisons²¹ were included in the dataset if QC CV values were 10% above these cut off limits for important metabolite identification. Thereafter, the ratios of specific metabolite pairs were calculated and a Fisher (F) ratio noise filter (Pierce *et al.*, 2006) was applied prior to statistical analysis to remove noisy features with an F ratio of <0.05. Finally, the data was log transformed and outliers were detected (PCA Hotelling's T^2 ellipses, loadings plot and biplots). Data processing was done using Excel (Microsoft 365), Statistica v 13.0 (TIBCO Software Inc.), and MetaboAnalyst v 3.0/v 4.0 [Xia Lab (Chong *et al.*, 2018; Xia *et al.*, 2015)]. The metabolomics data obtained in this study can be accessed at the NIH Common Fund's NMDR website, the Metabolomics Workbench (<https://www.metabolomicsworkbench.org>) where it has been assigned project IDs: PR000721 (Terburgh *et al.*, 2019), PR001003 (Terburgh *et al.*, 2021a), and PR001187 (Terburgh *et al.*, 2021b).

3.4.8 Statistical analyses

For each sample type, univariate analyses were used to determine discriminatory features between *Ndufs4* KO and WT metabolic profiles. Statistical and practical significance were determined using the student's t-test and effect size (ES, Cohen's *d*-value) calculations, respectively. False discovery rate (FDR) corrected *p*-values were calculated in MetaboAnalyst, with an FDR-*p* of ≤ 0.05 considered statistically significant. Principal component analysis (PCA) and volcano plots were used to visually investigate the clustering, covariance, and discriminatory power of the important features the brain-regional datasets. PCA was performed in

²⁰ If a feature was not detected in $\geq 80\%$ of one group, but detected in $\geq 80\%$ of the other group, the feature was kept in the dataset.

²¹ i.e., metabolites significantly altered in one or more sample types.

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MetaboAnalyst on a glog-transformed dataset comprising only the important metabolites identified on each platform. Brain-regional and other sample cross-comparisons were then based on the qualitative determination of shared and unique discriminatory metabolites and the relative comparison of the magnitude of change (ES) in the levels of shared discriminatory metabolites. For clarification, it must be noted that the use of ANOVA to cross-compare sample types was not a statistically sound option because the seven different sample types were not blocked together but analysed separately in sample-type specific batches, and metabolites were not absolutely (but relatively) quantified. For each sample type, pathway analysis was also performed on significantly altered metabolites using MetaboAnalyst. Compound labels were matched to HMDB and KEGG IDs, with *mus musculus* (mouse) KEGG as the selected pathway library. A hypergeometric test was the selected over-representation analysis method for pathway enrichment analysis, while relative betweenness centrality was the selected node importance measure for pathway topology analysis.

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CHAPTER

Aberrant BCAA and glutamate metabolism linked to regional neurodegeneration in a mouse model of Leigh syndrome



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4.1 Introduction

Effective oxidative phosphorylation (OXPHOS) is central to the proper functioning of mitochondria — the metabolic hubs of the cell. In the OXPHOS system, electron transport through respiratory chain (RC) complexes I, III and IV maintain a proton gradient across the inner mitochondrial membrane, which drives the bulk of cellular adenosine triphosphate (ATP) production via ATP synthase. The electrons powering this process are derived from nutrient catabolism and donated to the RC at the ubiquinone (Q) pool via complex I (CI), CII and other flavoproteins (McDonald *et al.*, 2018; Milenkovic *et al.*, 2017; Sherratt, 1991). Among these, CI is the main and most efficient electron donor that is responsible for regenerating NAD⁺. CI dysfunction (OMIM 252010) is the most common form of mitochondrial disease (MD) (Ghezzi & Zeviani, 2018; Smeitink *et al.*, 2001) and most often presents as Leigh syndrome (LS) in children, affecting ~1 in 40 000 new-borns (Rahman *et al.*, 1996; Rodenburg, 2016). LS is linked to more than 80 different genetic defects (Lake *et al.*, 2016; Rahman *et al.*, 2017), with many mutations affecting NDUF54 — a key subunit for CI assembly, stability and activity (Calvaruso *et al.*, 2012; Valsecchi *et al.*, 2010) — accounting for some of the more severe forms of the disease (Ortigoza-Escobar *et al.*, 2016). CI deficiency and LS portray genetic and clinical heterogeneity along with poorly understood pathogenic mechanisms and lack effective or curative treatments. Although LS is associated with a highly variable spectrum of symptoms, the most characteristic features include symmetrical bilateral necrotic lesions in distinct brain regions, mitochondrial dysfunction, and a neurodegenerative phenotype (Lake *et al.*, 2015; Lake *et al.*, 2016; Rahman *et al.*, 1996).

The whole-body *Ndufs4* knockout (KO) mouse model of CI deficiency, develops a progressive phenotype closely resembling human LS at about postnatal day (P) 35, which increasingly worsen until premature death (~P55-75). Symptoms include developmental delay, failure to thrive, lethargy, ophthalmoplegia, locomotor impairment, hearing loss, encephalomyopathy, respiratory deficits and epileptic seizures (Kruse *et al.*, 2008; Quintana *et al.*, 2010). Research on *Ndufs4* KO mice have revealed a tissue-specific vulnerability to CI deficiency with unknown causal mechanisms. The disease most severely manifests in the brain, with specific brain regions, such as the olfactory bulbs, cerebellum, and brainstem, being more vulnerable to the *Ndufs4* KO than others (Chen *et al.*, 2017; Quintana *et al.*, 2010; Quintana *et al.*, 2012). Vulnerable brain regions display focal progressive lesions with spongiform neurodegeneration, vascular proliferation, gliosis, aberrant mitochondrial morphology in nerve terminals and necrosis (Kruse *et al.*, 2008; Quintana *et al.*, 2010). From studies on conditional *Ndufs4* KO mice [neuronal- and glial- (Bolea *et al.*, 2019; Quintana *et al.*, 2010; Ramadasan-Nair *et al.*, 2019; Sterky *et al.*, 2012), cardiac- (Chouchani *et al.*, 2014; Karamanlidis *et al.*, 2013; Sterky *et al.*, 2012); liver-, hematopoietic- and myeloid- (Jin *et al.*, 2014) specific *Ndufs4* KOs] the origin of the fatal phenotype was shown to be predominantly neuronal. However, the major factors underlying regional neurodegeneration remain elusive. A recent investigation in pre-symptomatic (P29-31) *Ndufs4* KO mice found no significant brain regional differences in neuronal

populations, DNA damage, CIV levels, mTOR activity or metabolism between *Ndufs4* KO and wild type (WT) mice prior to lesion formation (Johnson *et al.*, 2020). However, we hypothesise that as LS progresses the metabolism of lesion-prone brain regions will be more perturbed than that of unaffected regions. In this regard, the application of metabolomics as a hypothesis-generating tool could provide novel insight into the mechanisms behind LS and regional neurodegeneration and identify areas of metabolism to further investigate in future targeted studies.

Therefore, we investigated brain regional bioenergetics and metabolism in a relatively large group of late stage diseased (P45-50) *Ndufs4* KO and WT mice. A combination of spectrophotometric enzyme activity assays and multi-platform metabolomics techniques were applied to investigate four brain regions that include three lesion-prone regions (brainstem, BS; cerebellum, CB; and olfactory bulbs, OB) and one lesion-resistant region (anterior cortex, AC). Here, we report distinct metabolic profiles that correlate with the level of residual CI activity in different *Ndufs4* KO brain regions. Furthermore, we show that the fate of metabolites involved in brain glutamate handling and nitrogen flux may play a central role in regional vulnerability to CI deficiency. Altogether, our results provide a better understanding of the pathomechanism of LS and pave the way for the future development of effective treatment options.

4.2 Materials and methods

4.2.1 Animals and sampling

Male whole-body homozygous *Ndufs4* KO (*Ndufs4*^{-/-}) mice along with age- and sex-matched controls (WT, *Ndufs4*^{+/+}) were used. Due to known differences in hormone cycles, tissue-specific metabolism, and metabolic profiles between sexes (Ruoppolo *et al.*, 2018; Wells *et al.*, 2016) only one sex-group was used in order to minimise sex-related metabolic variation. KO mice were born from heterozygous crosses (*Ndufs4*^{+/-}; B6.129S4-*Ndufs4*^{tm1.1Rpa}/J) obtained from Jackson Laboratory (JAX stock #027058). *Ndufs4* genotypes were confirmed by polymerase chain reaction using tail snips. The animals were bred and housed at the specific pathogen-free unit of the Vivarium (SAVC reg. no. FR15/13458) of the Preclinical Drug Development Platform (PCDDP; NWU, RSA) and approval (NWU-00378-16-A5) for this study was obtained from the Animal Research Ethics Committee of the NWU. Animals were group housed under temperature- (22 ± 1°C), humidity- (55 ± 10%) and light-controlled (12:12 hour light/dark cycle) conditions with standard laboratory chow (Rodent Breeder, #RM1845, LabChef, Nutritionhub) and water provided *ad libitum*. Based on survival curves and phenotypic analyses [results previously reported (Miller *et al.*, 2021)], an age range from P45-50 was selected to represent the late stage of LS. Mice were euthanised between P45-50 via cervical dislocation at the same time of day (8:00-9:00 AM) after overnight (12 hours) fasting. The brain was removed and rinsed with ice-cold saline solution (SABAX PBS; 0.9% NaCl (w/v), #7634, Adcock Ingram) to remove surrounding blood. The brain regions of interest, namely the AC, BS, CB, and OB, were then dissected, snap-frozen in liquid nitrogen (within 15 minutes post-mortem) and stored at -80°C until used.

4.2.2 Enzyme assays

4.2.2.1 Methods

The activities of the RC complexes I-IV, as well as citrate synthase (CS) were measured in freeze-thawed post-700 ×g supernatants of all brain regions for *Ndufs4* KO (n≥11) and WT (n≥9) mice. Analyses were performed in triplicate for all assays based on methods described before (Janssen *et al.*, 2007; Luo *et al.*, 2008; Rahman *et al.*, 1996; Shepherd & Garland, 1969).

4.2.2.2 Data processing

RC enzyme activities were expressed as mean ± standard deviation normalised to both protein content and CS. Although results were similar for these normalisation methods, the latter is not reported here as we observed significantly elevated CS activity in all KO brain regions. Extreme outliers were removed using box-plots based on Tukey's method (Tukey, 1977) and data symmetry was assessed based on standardised skewness values. Data processing was done using Excel 2013 (Microsoft) and SPSS Statistics (v.25.0; IBM).

4.2.2.3 Statistical analyses

A two-way mixed analysis of variance (ANOVA) was done for each variable (between-subjects factor: genotype; within-subjects factor: brain region). If the assumption of equal variance was severely violated, ANOVA was done on power transformed data. For significant interaction effects, paired t-tests (brain regional differences within a genotype) and independent samples t-tests (genotypic differences within a brain region) along with effect size (Cohen's *d*-values) calculations were done. In the case of insignificant interaction effects and significant main effects, pairwise comparisons based on marginal means were calculated together with ηp^2 effect sizes (effect of genotype) or Cohen's *d*-values (brain regional differences). Values of $p < 0.05$ were considered statistically significant and effect sizes were interpreted as follows; $\eta p^2 \geq 0.14$ or $d \geq 0.8$ indicated a large effect; $\eta p^2 \geq 0.06$ or $d \geq 0.5$ indicated a medium effect; and $\eta p^2 \geq 0.01$ or $d \geq 0.3$ indicated a small effect (Ellis & Steyn, 2003; Kirk, 1996).

4.2.3 Metabolic profiling

4.2.3.1 Methods

Multi-platform metabolomics was done on four different brain regions of *Ndufs4* KO (n=21) and WT (n=25) mice as previously described (Esterhuizen *et al.*, 2019; Lindeque *et al.*, 2013) via untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). For each mouse, each brain region was extracted and analysed individually, yielding a respective 21 (KO) and 25 (WT) biological replicates per brain region. Metabolite extraction was done using a stepwise Bligh–Dyer extraction method (Wu *et al.*, 2008) to obtain a monophasic (methanol/water/chloroform, 6:2:2) and biphasic (methanol/water/chloroform, 2:1.8:2) extract for each of the 184 individual samples. Monophasic extracts were used for GC-TOF-MS, while the polar phases of biphasic extracts were used for LC-MS/MS. Frozen brain tissues were weighed and homogenised in the presence of internal standards

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using a vibration mill. Internal standards, N, N-dimethyl-L-phenylalanine (DMPA) and 3-phenylbutyric acid (3-PBA), were added according to tissue mass as part of the water used for extraction. For each brain region, a quality control (QC) sample was prepared from pooled aliquots of all sample extracts from that specific brain region. Thereafter, all samples were transferred to vials, dried under nitrogen and derivatised prior to their analysis. For GC-TOF-MS, samples were oximated and silylated, while LC-MS/MS samples were supplemented with stable isotopes and butylated.

4.2.3.2 Data processing

Spectral data was extracted into matrices, individually inspected (correct peak detection and alignment, batch precision and data integrity) and preprocessed (data filtering, missing value imputation and normalisation). Metabolites were normalised to tissue mass through normalisation with 3-PBA (GC-TOF-MS data) or DMPA (LC-MS/MS data). For LC-MS/MS data, metabolites were normalised to either their own isotope (where possible) or an isotope with a similar retention time prior to DMPA normalisation. No significant batch effects were evident thus no batch corrections were required. Finally, data pretreatment (glog transformation and mean centering) and outlier detection (PCA Hotelling's T^2 ellipses, loadings plot and biplots) were done. Data processing was done using Excel 2013 (Microsoft), SPSS Statistics (v.25.0; IBM) and MetaboAnalyst 3.0 (Xia et al., 2015). The metabolomics data obtained in this study (Terburgh et al., 2021) can be accessed at the Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (<https://www.metabolomicsworkbench.org>) where it has been assigned Project ID: PR001003.

4.2.3.3 Statistical analyses

Univariate analyses were used to determine significantly discriminatory features between *Ndufs4* KO and WT metabolic profiles. The student's t-test was carried out to identify features with significantly altered intensities and a false discovery rate (FDR) corrected *p*-value of <0.05 was considered statistically significant. Effect sizes (Cohen's *d*-value) of ≥ 0.8 were considered practically significant and used complimentary to the t-test. Principal component analysis (PCA) and volcano plots were used to visually investigate the clustering, covariance, and discriminatory power of the important features. PCA was performed in MetaboAnalyst 3.0 (Xia et al., 2015) on a glog-transformed dataset comprising only the important metabolites identified on each platform. Dataset features were identified via spectral and retention time matching using commercial and in-house spectral libraries together with public databases and metabolite identities were assigned to spectral features, with high confidence levels (Schymanski et al., 2014; Sumner et al., 2007).

4.3 Results and discussion

4.3.1 RC enzyme activities are differentially affected in *Ndufs4* KO brain regions

Table 4-1 summarises the average activities of RC complexes for the different brain regions. Compared to WT, a differential and significant reduction in CI activity was observed in the *Ndufs4* KO brain regions in the following order: OB (86% reduction) > BS (75%) > CB (72%) > AC (62%). The OB — the first and one of the most severely affected brain regions in KO mice (Quintana *et al.*, 2010) — showed the lowest residual CI activity (14%), while the lesion-resistant AC exhibited the highest (38%). Several others have proposed that residual CI activity levels greatly affect the extent of CI deficiency's downstream consequences (Alam *et al.*, 2015; Distelmaier *et al.*, 2009; Dubinsky, 2009; Kühn *et al.*, 2015; Pathak & Davey, 2008; Verkaart *et al.*, 2007). Our metabolic data support the notion of such a biochemical threshold effect, as brain regions with lower residual CI activity displayed more apparent metabolic perturbations (Section 4.3.2). Additionally, CIV and CS activities were slightly (~10-20%) but significantly increased in all *Ndufs4* KO brain regions, except the BS. Since this was not paralleled by increased CII and CIII activity, mitochondrial biogenesis was not considered the sole mechanism behind these observations. Although not evident in brain regions at the pre-symptomatic stage (Johnson *et al.*, 2020), increased CIV levels were reported for *Ndufs4* KO whole brain (Martin-Perez *et al.*, 2020), while increased CS (and CII) activity in other *Ndufs4* KO tissues have been linked to a compensatory increase in CII-mediated fuelling of the OXPHOS system (Alam *et al.*, 2015; Ingraham *et al.*, 2009; Kruse *et al.*, 2008). Our data could reflect similar adaptive responses in mitochondrial energy metabolism. When comparing different brain regions, CII, CIII (except for OB), CIV, and CS activities were all higher ($p < 0.01$) in the lesion-prone regions than the AC for both genotypes — suggesting that regions with greater requirements for OXPHOS driven energy production, may be more vulnerable to neurodegeneration in RC deficiencies, as previously proposed (Johnson *et al.*, 2020; Pickrell *et al.*, 2011). Furthermore, innately lower CIII levels in the OB might render this brain region particularly vulnerable to *Ndufs4* deficiency, since CIII is known to form a functional, albeit unstable, supercomplex with CI subassemblies in *Ndufs4* KO mice (Calvaruso *et al.*, 2012).

Table 4-1: RC enzyme activities in brain regions of *Ndufs4* KO and WT mice

Vmax (mU/mg)	AC			BS			CB			OB		
	WT	KO	KO/WT	WT	KO	KO/WT	WT	KO	KO/WT	WT	KO	KO/WT
CI	27 ± 2	10 ± 1****	0.38	35 ± 4	9 ± 3****	0.25	37 ± 3	10 ± 1****	0.28	23 ± 3	3 ± 1****	0.14
CII	33 ± 3	35 ± 2	1.05	42 ± 5	38 ± 4	0.91	44 ± 4	49 ± 5*	1.10	39 ± 3	38 ± 6	0.97
CIII	266 ± 30	248 ± 15	0.93	291 ± 27	306 ± 46	1.05	294 ± 18	301 ± 19	1.02	204 ± 30	197 ± 18	0.97
CIV	647 ± 36	704 ± 31**	1.09	925 ± 102	944 ± 101	1.02	893 ± 58	1070 ± 53***	1.20	808 ± 53	925 ± 108**	1.14
CS	352 ± 26	398 ± 26***	1.13	459 ± 20	467 ± 25	1.02	398 ± 25	485 ± 24****	1.22	410 ± 32	450 ± 34**	1.10

Enzyme activities normalised to mg protein. Average values (±SD) obtained from WT (n≥9) and KO (n≥11) animals. Asterisks indicate statistically significant differences between KO and WT: *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$. Abbreviations: mU; nmol/min; Vmax, maximal activity.

4.3.2 Metabolic alterations are graded in *Ndufs4* KO brain regions

Multivariate PCA (Figure 4-1) of the important discriminatory metabolites revealed a distinct metabolic profile in each brain region capable of separating genotype clusters, to a graded degree, ranging from partial separation in the AC to complete separation in the OB. Similarly, on a univariate level (Table 4-S1; Figure 4-S1), the degree of metabolic perturbations in *Ndufs4* KO brain regions gradually increased from the AC to the BS, CB, and OB. This graded perturbation of brain metabolism mostly corresponded to the graded loss of CI activity we report in Section 4.3.1. In accord, established metabolic indicators of NADH/NAD⁺ redox balance revealed a similarly graded increase in the NADH/NAD⁺ ratio of *Ndufs4* KO brain regions compared to WTs (Figure 4-S2). The ratios of alanine, leucine and isoleucine relative to glutamic acid were increased in *Ndufs4* KO brain regions (OB>CB>BS>AC), while the pyruvic acid:lactic acid and pyruvic acid:acetyl-carnitine ratios were respectively decreased and increased only in the lesion-prone regions. An increased NADH/NAD⁺ ratio drives the formation of amino acids (from α -ketoacids and glutamic acid) and lactic acid (from pyruvic acid) by an equilibrium reaction, while the pyruvic acid:acetyl-carnitine ratio is indicative of NAD⁺-dependent acetyl-CoA formation from pyruvate (Clarke *et al.*, 2013; Go *et al.*, 2020; Hansford & Cohen, 1978; Williamson *et al.*, 1967). Furthermore, generally similar metabolic alterations were found in the different *Ndufs4* KO brain regions (when compared to WTs), albeit with varying degrees of practical and statistical significance. Figure 4-2 depicts some of the most significantly increased (magenta) and decreased (green) metabolites common to all four *Ndufs4* KO brain regions (n=12) along with those alterations exclusive to either the lesion-prone (n=5) or -resistant (n=6) regions. The OB was most severely affected by the *Ndufs4* KO as this region displayed the most significant and largest number of total (n=49) and unique (n=23) metabolic alterations. Furthermore, unlike other brain regions, most of these important metabolites were increased, with a greater number being carbohydrates and lipids. Box plots of the significantly altered metabolites can be found in the supplementary information (Figure 4-S3). A more detailed discussion of the metabolic alterations in the different brain regions, and our hypotheses on them, follows.

4.3.2.1 BCAA metabolism is strongly perturbed in lesion-prone *Ndufs4* KO brain regions

In the lesion-prone *Ndufs4* KO brain regions, all three branched-chain amino acids (BCAAs) were significantly increased (Figure 4-3), while their catabolic by-products, butyryl(C4)- and isovaleryl(C5)-carnitine, were decreased compared to WTs. Although the resilient *Ndufs4* KO AC showed the same trends, the effect size of these alterations was markedly lower with only valine reaching statistical and practical significance. Hence, the lower residual CI activity and greater NADH/NAD⁺ redox imbalance in the three lesion-prone brain regions seems to result in greater congestion of BCAA catabolism. BCAA accumulation is often reported in mitochondrial disease studies and believed to result from an inhibition of the NAD⁺-dependent reaction catalysed by

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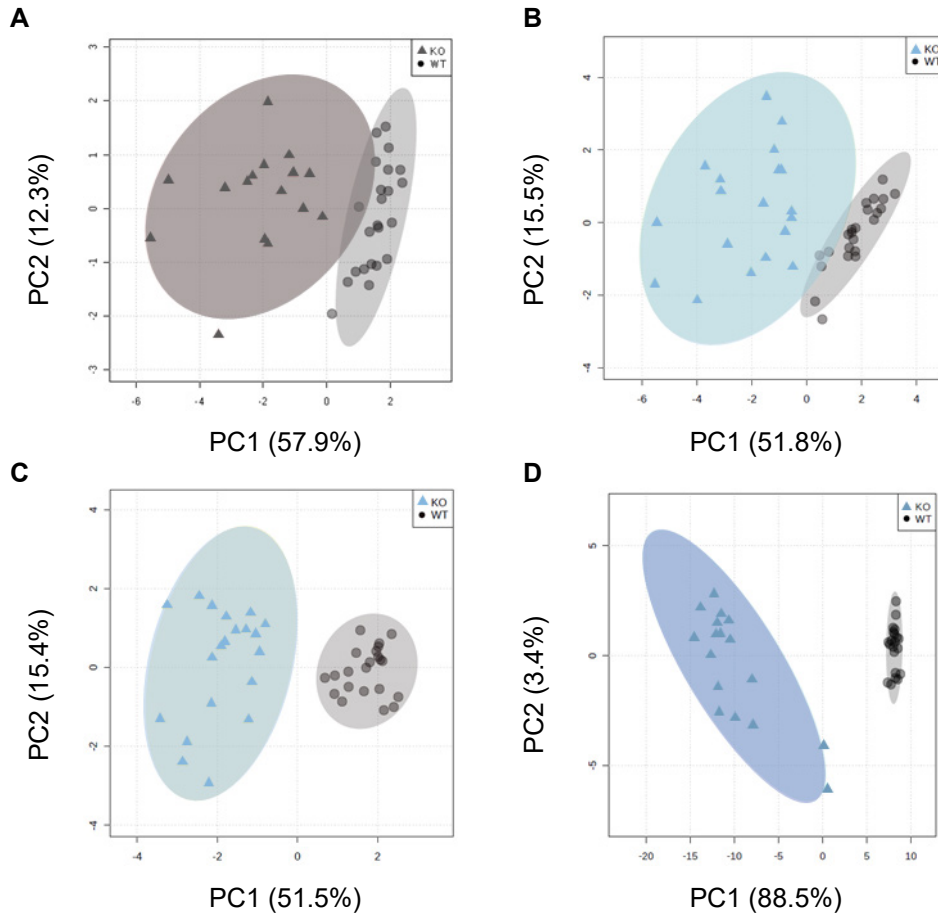


Figure 4-1: Discriminative power of important metabolites altered in *Ndufs4* KO mouse brain regions. Principle component analysis (PCA) scores plots of metabolites significantly altered ($p < 0.05$, $d \geq 0.8$) in the A: anterior cortex, B: brainstem, C: cerebellum and D: olfactory bulbs of KO mice ($\blacktriangle$, $n=21$) compared to WT mice ($\bullet$, $n=25$).

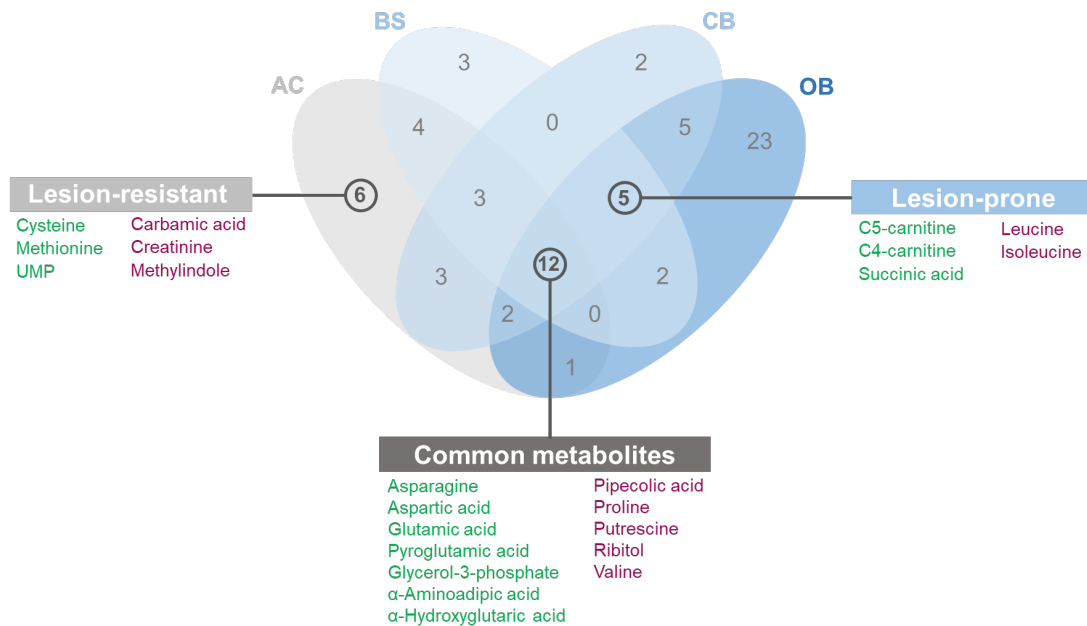


Figure 4-2: Venn diagram of important metabolites altered in brain regions of *Ndufs4* KO mice. Green and magenta respectively indicate metabolites significantly ($p < 0.05$, $d \geq 0.8$) decreased or increased in KO mice ($n=21$) compared to WT mice ($n=25$). Among the significantly altered metabolites, 12 were common to all brain regions, 6 were unique to the lesion-resistant AC and 5 were common to the lesion-prone brain regions. Abbreviations: AC, anterior cortex; BS, brainstem; CB, cerebellum; OB, olfactory bulbs; UMP, uridine monophosphate.

branched-chain α -keto acid dehydrogenase (BCKDH) due to redox imbalance (Clarke *et al.*, 2013; Esterhuizen *et al.*, 2017; Falk *et al.*, 2008; Lozoya *et al.*, 2018; Vergano *et al.*, 2014). Notably, there is considerable metabolic and phenotypic similarity between *Ndufs4* deficiency and BCKDH deficiency (Maple syrup urine disease, OMIM 248600). In the case of Maple syrup urine disease (MSUD), insufficient branched-chain α -keto acid (BCKA) decarboxylation results in the increased conversion of BCKAs and glutamic acid (Glu) back to BCAAs and α -ketoglutaric acid (α KG), leading to BCAA accumulation and decreased acyl-CoA product formation. This, in turn, results in Glu and aspartic acid (Asp) depletion and disruption of energy metabolism, with the accumulation of lactate, lysine and alanine often evident. The MSUD pathology is largely driven by the accumulation of leucine (Leu) and its ketoacid, α -ketoisocaproate (KIC), resulting in neurological and developmental delay, encephalopathy, feeding difficulty and seizures (Arany & Neinast, 2018; Bifari & Nisoli, 2017; Muelly *et al.*, 2013; Yudkoff, 2017; Yudkoff *et al.*, 2005; Zinnanti *et al.*, 2009). While Leu is capable of regulating food intake and body weight through its activation of the mammalian target of rapamycin (mTOR) complex 1 (Lorin *et al.*, 2013; Son *et al.*, 2019), neuronal network dysfunction, encephalopathy and the occurrence of seizures in MSUD models (Dodd *et al.*, 1992; Görtz *et al.*, 2003) and an epilepsy model exposed to high Leu concentrations (Dufour *et al.*, 2001) have been strongly linked to alterations in glutamatergic- and γ -aminobutyric acid (GABA)ergic neurotransmitter concentrations and/or transmission. Since Leu was significantly elevated in the lesion-prone brain regions of *Ndufs4* KO mice, but not in the AC ($p>0.05$; $d=0.43$), we postulate that this BCAA could also be a central driver of the LS pathology. This hypothesis is further strengthened by the fact that glutamatergic- and GABAergic neuronal populations were shown to drive most symptoms in conditional *Ndufs4* KO mice (Bolea *et al.*, 2019) and mTOR pathway hyperactivation was reported in whole brain of diseased ($P>45$) *Ndufs4* KO mice (Johnson *et al.*, 2013). However, the hypothesised connection between these observations and regional Leu accumulation, needs to be further tested. In this regard, the evaluation of brain regional mTOR activity in diseased *Ndufs4* KO mice is also required.

4.3.2.2 *Glu metabolism maintains TCA-cycle anaplerosis in the lesion-resistant AC of Ndufs4 KO mice*

As with MSUD cases, glutamine (Gln) levels were not markedly altered in *Ndufs4* KO brain regions, while Glu and Asp levels were significantly decreased (Figure 4-4). However, Glu and Asp levels were more prominently decreased in the *Ndufs4* KO AC, where BCAA oxidation was least perturbed. These reductions could reflect an adaptive increase in Gln-Glu- α KG anaplerosis, as was recently reported to take place during OXPHOS deficiency (Chen *et al.*, 2018). This mechanism provides α KG to the TCA cycle, which, depending on the degree of OXPHOS impairment, either favours an energy-generating oxidative flux (moderate impairment) or an anabolic reductive flux (severe impairment) directed toward acetylation and lipid synthesis. The oxidative α KG flux results in increased oxaloacetate-derived Asp efflux from mitochondria and cataplerosis to alanine, thereby

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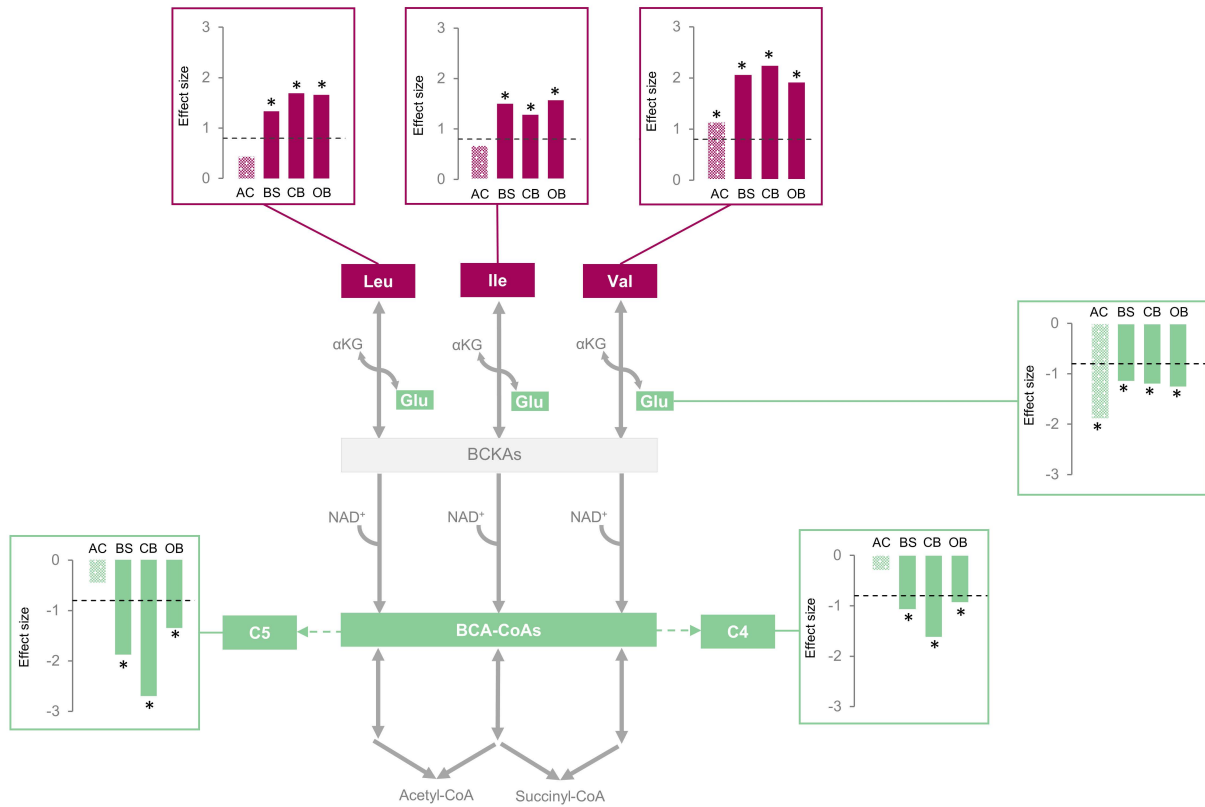


Figure 4-3: BCAA related metabolic alterations in *Ndufs4* KO brain regions. Alterations are more significant in the three vulnerable regions (solid bars) than the AC (dotted bars). Bar graphs depict Cohen's *d*-values (y-axis) for each metabolite when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate increased or decreased metabolites levels in KO mice. Dashed lines mark large practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (FDR-corrected p -value < 0.05). Abbreviations: BCAAs, branched-chain amino acids; BCA-CoAs, branched-chain acyl-CoA derivatives; BCKAs, branched-chain α -keto acids; C4, butyryl-carnitine; C5, isovaleryl-carnitine; FAD/FADH₂, oxidised/reduced flavin adenine dinucleotide; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; NAD⁺, oxidized nicotinamide adenine dinucleotide; Val; valine; α KG, α -ketoglutaric acid.

fuelling cytosolic NAD⁺ regeneration (via malate dehydrogenase) and supporting glycolysis (Chen *et al.*, 2018). All KO brain regions also displayed significantly decreased levels of another α KG precursor, α -hydroxyglutaric acid (α HG), which can additionally fuel the Q-pool independently of CI via electron-transferring flavoprotein (ETF) – ETF ubiquinone oxidoreductase system-linked α HG dehydrogenase. We, therefore, hypothesise that the marked decrease in Glu, Asp and α HG levels in *Ndufs4* KO brain regions could point to a compensatory increase in α KG flux to the TCA cycle, with the AC possibly better utilising the energy-generating oxidative flux.

In the central nervous system (CNS), Glu and Asp are competitively utilised for neurotransmitter- and bioenergetic purposes and require *de novo* synthesis to maintain internal pools (Cooper & Jeitner, 2016; Griffin & Bradshaw, 2017). BCAAs are regarded a key nitrogen source for *de novo* Glu synthesis in the CNS (Sperringer *et al.*, 2017; Yudkoff *et al.*, 1983), with Leu providing the majority (30-50%) of Glu and Gln α -amino groups (Smith *et al.*, 1987; Yudkoff *et al.*, 2005). Together, these metabolites form part of an intricate transport system that regulates brain glutamate handling and nitrogen flux between astrocytes and neurons (Suh *et al.*, 2019; Yudkoff, 2017). In the putative

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Leu-Glu cycle, astrocytes produce Glu and KIC from Leu and α KG, KIC is then transported to neurons where it, together with Glu, is used to produce α KG and Leu. Similarly, in the Glu-Gln cycle, astrocytes produce Gln from Glu (and NH_4^+ and ATP) and transport it to neurons where it is preferentially used to produce Glu (and NH_4^+). Alterations in either Leu/KIC or α KG/Glu ratios could disrupt these transport systems in the *Ndufs4* KO brain. Remarkably, the beneficial effects of rapamycin was recently ascribed to the restoration of a seemingly dysfunctional Gln/Glu/ α KG metabolic axis in pre-symptomatic *Ndufs4* KO mice (Johnson *et al.*, 2020); while α KG/malate was also shown to maintain normal CI-driven respiration in *Ndufs4* KO whole brain mitochondria via an unknown mechanism (Kayser *et al.*, 2016) and supplementation with a cell-permeable α -KG analogue significantly increased survival in *Ndufs4* KO mice (Lee *et al.*, 2019). Altogether, we hypothesise that the degree to which BCAA catabolism is congested and the compensatory capacity of increased α KG oxidation may be central to regional neurodegeneration in *Ndufs4* deficiency.

Additionally, all *Ndufs4* KO brain regions also displayed increased levels of N-acetylglutamic acid (NAG), putrescine and proline (Pro) along with decreased levels of pyroglutamate (pGlu), citrulline and ornithine. Both Pro and pGlu levels were more significantly altered in the lesion-prone brain regions. Pro accumulation could reflect a shift in flux through the redox regulatory Pro cycle, favouring NAD(P)^+ and Pro synthesis, which reportedly plays an important role in the regulation of mTOR signalling (Karna *et al.*, 2020; Oka *et al.*, 2013; Sahu *et al.*, 2016). Gln-derived Pro biosynthesis was also shown to be induced in response to increased oxidation of Gln (and glucose) carbons in the TCA cycle and functions to prevent reactive oxygen species generation when TCA cycle activity surpasses OXPHOS capacity (Schwörer *et al.*, 2020). The need for such a vent of mitochondrial redox stress, might be greater in lesion-prone brain regions. Similarly, larger reductions in pGlu levels could also reflect a greater requirement for, and production of, the Glu-derived antioxidant, glutathione in lesion-prone brain regions. In agreement with our results, brain regions of pre-symptomatic *Ndufs4* KO mice reportedly display elevated levels of reduced glutathione along with a decreased trend in the levels of a metabolite cluster including, pGlu, Glu, Asp and most urea cycle intermediates (Johnson *et al.*, 2020). The urea cycle-related metabolic alterations in *Ndufs4* KO brain regions could result from altered Glu and ammonia levels, as suggested to occur due to Gln metabolic rewiring during OXPHOS deficiency (Chen *et al.*, 2018). The induction of urea cycle function has also been proposed to occur in brain endothelial cells when the removal of excess nitrogen is required (Griffin & Bradshaw, 2017), however, with the lack of NAG synthase (Caldovic & Tuchman, 2003) and complete urea cycle function in the CNS, further targeted studies are required.

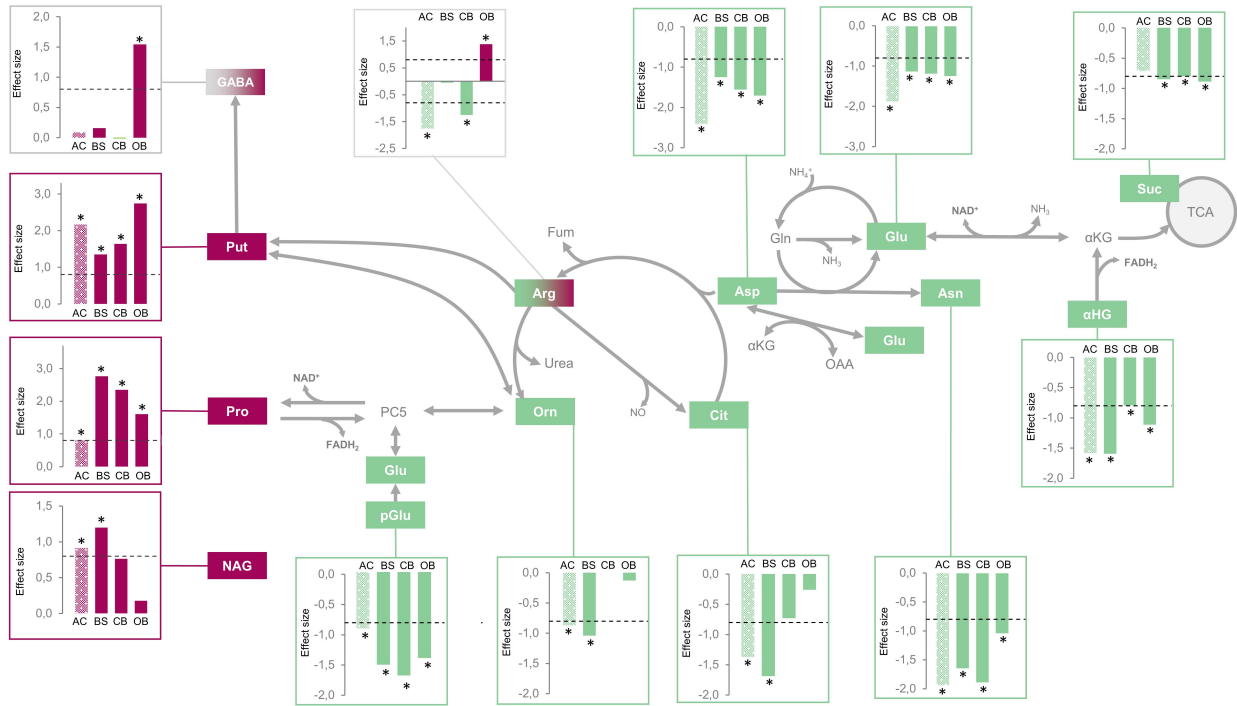


Figure 4-4: Glutamate related metabolic alterations in *Ndufs4* KO brain regions. Bar graphs depict Cohen's d-values (y-axis) for each metabolite when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate an increase or decrease in KO metabolite levels. Dashed lines mark practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (FDR-corrected p -value < 0.05). Abbreviations: A-CoA, acetyl-coA; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Car-P, carbamoyl-phosphate; Cit, citrulline; $FADH_2$, reduced flavin adenine dinucleotide; Fum, fumarate; GABA, γ -aminobutyric acid; Gln, glutamine; Glu, glutamic acid; Glyco, glycolic acid; GSA, glutamyl- γ -semialdehyde; Hpro, hydroxyproline; NAD^+ , oxidised nicotinamide adenine dinucleotide; NAG, N-acetylglutamic acid; NH_3 , ammonia; NO, nitric oxide, OAA, oxaloacetic acid; Orn, Ornithine; P5C, pyrroline-5-carboxylate; pGlu, pyroglutamic acid; Pro, proline; Put, putrescine; Suc, succinic acid; α KG, α -ketoglutaric acid.

4.3.2.3 Olfactory bulb lysine, carbohydrate and lipid metabolism are greatly perturbed in *Ndufs4* KO mice

A trend of increased levels of lysine and its metabolites, pipecolic acid, carnitine, and acetyl-carnitine, along with decreased α -amino adipic acid (α AA) were seen in all *Ndufs4* KO brain regions (Figure 4-5). Like Leu, lysine is an important precursor for *de novo* Glu synthesis in the CNS, with its catabolism influenced by NAD^+ (and α KG) bioavailability (Papes *et al.*, 2001). Our data suggests a block in lysine catabolism, possibly due to disturbed redox balance, along with the preferred formation of pipecolic acid from pyrroline-6-carboxylate (P6C). The latter reaction could function to replenish $NAD(P)^+$ levels via pyrroline-5-carboxylate (P5C) reductase, an enzyme that also catalyses Pro synthesis from P5C. These alterations in lysine metabolism were most significant in the KO OB, in which arginine and γ -aminobutyric acid (GABA) levels were uniquely elevated (Figure 4-4). Lysine is known to block arginase-mediated urea synthesis from arginine and compete for uptake with arginine at the mitochondrial ornithine carriers in the brain (Sauer *et al.*, 2010). Since both arginine and lysine can function as activators of mTOR signalling (Li & Yan, 2019) and affect glutamatergic neurotransmission (Kondoh *et al.*, 2010), their significant accumulation in the OB might explain why it is the predominantly affected brain region in *Ndufs4* KO mice.

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Furthermore, pyruvic acid and alanine levels trended higher in all *Ndufs4* KO brain regions, while lactic acid was increased only in the lesion-prone regions. These alterations were graded, with the OB most severely affected. The OB also showed increased levels of other glycolysis-and pentose phosphate pathway (PPP)-related intermediates such as glucose, glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate, which was not seen in other regions. In most *Ndufs4* KO brain regions, we additionally observed markedly increased erythronic acid²² and ribitol levels — both known to accumulate in the brain due to impaired glycolysis that shunts glucose to the PPP (Xu *et al.*, 2016). The interpretation of these results is, however, complicated by the fact that *Ndufs4* KO mice exhibit inflammatory cell activation and infiltration in the affected brain regions at the late stage of LS (Bolea *et al.*, 2019; Felici *et al.*, 2014; Jain *et al.*, 2016; Johnson *et al.*, 2013; Quintana *et al.*, 2010). Known metabolic alterations in activated inflammatory cells include a switch away from OXPHOS towards glycolysis with increased flux through the PPP, lactic acid and lipid production (Kelly & O'Neill, 2015). Nevertheless, *Ndufs4* KO mice at our facility exhibit minimal inflammation at this age [P46-51] (Miller *et al.*, 2021), while others report similar trends in glycolytic intermediates and lactic acid in brain regions of pre-symptomatic *Ndufs4* KO mice (Johnson *et al.*, 2020) and intracerebral lactic acid accumulation prior to neurodegeneration (Takahashi *et al.*, 2019). These alterations could result from *Ndufs4* deficiency-induced hyperacetylation as pyruvate dehydrogenase (PDH) subunit hyperacetylation and resulting decreases in PDH activity were recently reported in mitochondria from affected neuronal populations in *Ndufs4* KO mice (Gella *et al.*, 2020). Neuro-inflammation and BCAA accumulation could further exacerbate these metabolic alterations in lesion-prone brain regions as high levels of BCAAs can inhibit PDH (Li *et al.*, 2017) and increase the probability of neurodegeneration by influencing the immune properties of microglial cells and decreasing neuroprotective functions (De Simone *et al.*, 2013). All KO brain regions also displayed trended higher levels of palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1); along with decreased 9,10-dihydroxystearic acid. These alterations were, again, most pronounced in the OB. Decreased levels of C18:0 and increased C18:1 suggests an increased flux through stearyl-CoA desaturase, which can regenerate NAD⁺ but also dysregulate neuro-inflammation (Liu *et al.*, 2010; Liu *et al.*, 2011; Ng & Say, 2018) and lipid metabolism/homeostasis, as also seen with C16:0 accumulation (Ackerman *et al.*, 2018; Astarita *et al.*, 2011; Fanning *et al.*, 2019; Qin & Kojima, 2019). In agreement with our results, previous studies on *Ndufs4* KO mice report decreased free fatty acids in whole brain (Johnson *et al.*, 2013) along with increased lipid synthesis and a disturbance in the neuroprotective lipid/lactic acid shuttle mechanism between neurons and glia in lesion-prone regions (Liu *et al.*, 2017; Liu *et al.*, 2015).

Further alterations were seen in dihydroxyacetone phosphate (DHAP) and glycerol-3-phosphate (G3P) — metabolites that connect glycolysis, lipid metabolism and OXPHOS. G3P levels were significantly decreased in all KO brain regions, in a graded manner (AC>BS>CB>OB), suggesting a

²² unreliably detected in the brainstem (BS)

key role for G3P oxidation in fuelling respiration during CI deficiency. DHAP levels were elevated only in lesion-prone KO brain regions, with its accumulation noticeably greater in the OB, as reported for pre-symptomatic *Ndufs4* KO mice (Johnson *et al.*, 2020). This likely reflects upstream perturbations in glycolysis due to redox imbalance. DHAP was also recently proposed to be a central mTOR activator that transmits glucose availability to mTORC1 and plays a critical role in *de novo* lipogenesis (Orozco *et al.*, 2020). Since the interconversion of DHAP and G3P in the G3P shuttle functions to fuel respiration independently of CI and regenerate cytosolic NAD⁺ (McDonald *et al.*, 2018), this redox shuttle may play an important role in the response to CI deficiency. Moreover, with G3P shuttle activity questioned in the brain and cytosolic G3P dehydrogenase expression (responsible for DHAP reduction) generally considered low/absent, it would be interesting to investigate G3P shuttle activity in different *Ndufs4* KO brain regions.

Altogether, greater perturbations in lysine, carbohydrate, and lipid metabolism in the *Ndufs4* KO OB — which displayed the lowest residual CI activity (14% vs 38% in the AC) — support the hypothesis that brain regions with more severe levels of CI deficiency and a greater NADH/NAD⁺ redox imbalance, exhibit a preference for reductive carboxylation, acetylation, and lipid synthesis.

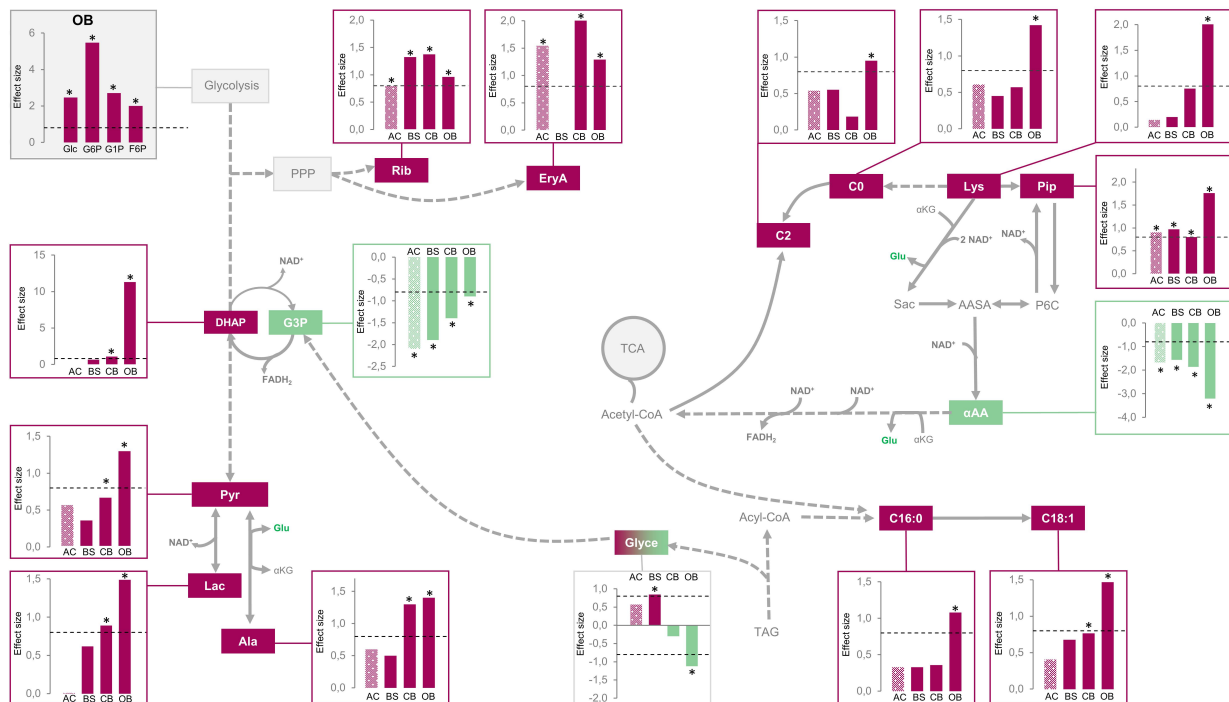


Figure 4-5: Lysine, carbohydrate and lipid related metabolic alterations in *Ndufs4* KO brain regions. Bar graphs depict Cohen's d-values (y-axis) for each metabolite when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate an increase or decrease in KO metabolite levels. Dashed lines mark practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (FDR-corrected p -value < 0.05). Abbreviations: AASA, aminoadipic acid semialdehyde; Ala, alanine; C0, carnitine; C16:0, palmitic acid; C18:1, oleic acid; C2, acetyl-carnitine; DHAP, dihydroxyacetone phosphate; EryA, erythronic acid; F6P, fructose-6-phosphate; FADH₂, reduced flavin adenine dinucleotide; G1P, glucose-1-phosphate; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; Glc, glucose; Glu, glutamic acid; Glyce, glycerol; Lac, lactic acid; Lys, lysine; NAD⁺, oxidised nicotinamide adenine dinucleotide; P6C, pyrroline-6-carboxylate; Pip, pipercolic acid; PPP, pentose phosphate pathway; Pyr, pyruvic acid; Rib, Ribitol; Sac, saccharopine; TCA, tricarboxylic acid cycle; αAA, α-aminoadipic acid; αKG, α-ketoglutaric acid.

4.4 Conclusion

Here, we uncover a link between the severity of CI deficiency and the degree of metabolic perturbations in different *Ndufs4* KO brain regions. Our metabolic data suggest that the related level of NADH/NAD⁺ redox imbalance impacts brain regional capacity to bypass CI deficiency and maintain homeostasis. In *Ndufs4* KO mice, an adaptive increase in Glu, α HG and G3P oxidation seem to drive energy-generation in the lesion-resistant AC, which maintained the highest level of residual CI activity. These adaptive mechanisms appear less effective in lesion-prone brain regions with more severe levels of CI deficiency. Moreover, we hypothesise that the marked accumulation of possible mTOR signalling regulators (such as Leu, Pro and DHAP) in lesion-prone brain regions implicate this signalling pathway in regional neurodegeneration. Future validation studies should, therefore, focus on measuring mTOR pathway activity in brain regions of diseased *Ndufs4* KO mice. Furthermore, we highlight the metabolic and phenotypic similarity between *Ndufs4* deficiency and MSUD, thereby pinpointing Leu catabolism as a promising therapeutic target for the treatment of shared clinical symptoms such as neurological and developmental delay, encephalopathy, feeding difficulty and seizures. Altogether, we identify key areas of brain metabolism to further investigate in the context of LS such as the metabolic sources and fate of α KG in the TCA cycle and the Leu/Glu/Gln transport systems between astrocytes and neurons. Although previous *in vivo* glucose and glutamine tracing experiments have shown a relative lack of changes between *Ndufs4* KO and WT mice (McElroy *et al.*, 2020; Yang *et al.*, 2020), our results suggest that brain region-specific measurements of those and other metabolic fuels could reveal a different picture, especially at the level of astrocytes and neurons.

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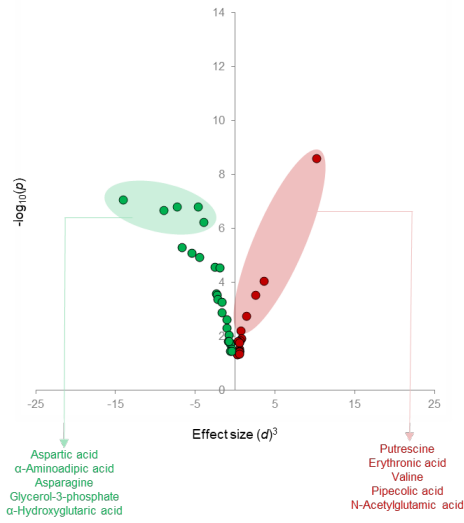
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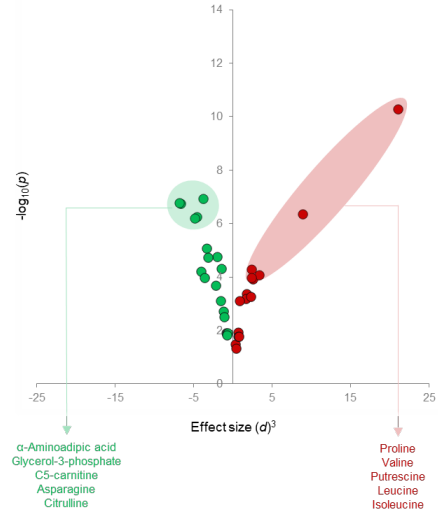
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4.6 Supplementary data

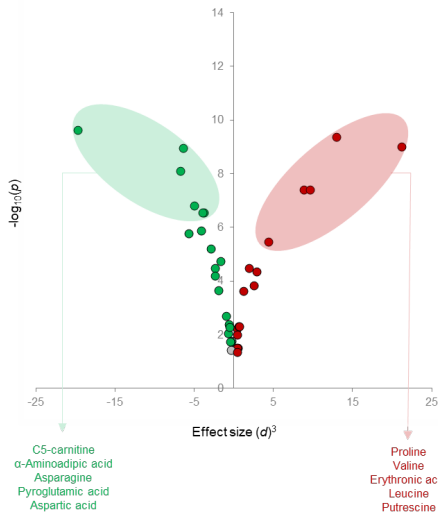
A



B



C



D

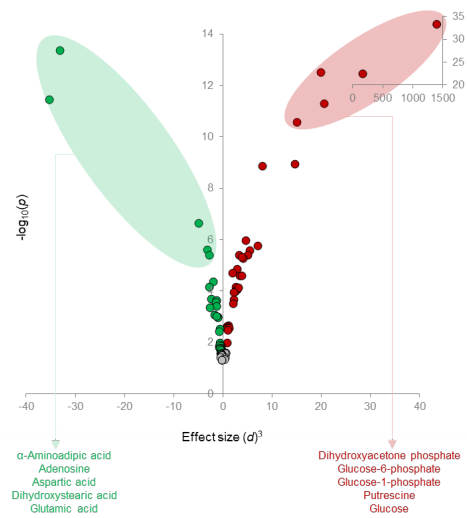


Figure 4-S1: Important metabolic alterations in *Ndufs4* KO brain regions. Volcano plots depicting multi-platform metabolomics datasets of **A.** anterior cortex, **B.** brainstem, **C.** cerebellum and **D.** olfactory bulbs from *Ndufs4* KO (n=21) mice compared to WT (n=25). Green and red symbols respectively indicate metabolites significantly lower or higher in *Ndufs4* KO mice compared to WT ($p < 0.5$ and $d \geq 0.8$). Practically insignificant ($d < 0.8$) features are shown in grey. Statistical significance (y-axis) depicted as negative logarithm to the base 10 ($-\log_{10}$) of p-values. Effect sizes (x-axis), calculated using Cohen's d , were raised to the third power (d^3). The five most significantly decreased and increased metabolites are highlighted and listed according to rank.

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Table 4-S1: Significant metabolites discriminating between the *Ndufs4* wild type and knockout mice in the selected brain regions. The significant discriminatory metabolites with an identification confidence Level of 1, 2 or 3 together with an FDR-corrected *p*-value <0.05 and *d*-value ≥0.8. Compounds are ordered in decreasing statistical (FDR-corrected *p*-value) significance. Arrows indicate an increase (↑) or decrease (↓) relative to the wild type metabolite levels. The identification (ID) level is given for each metabolite along with a designated metabolite class (amino acid, A; carbohydrates, C; fatty acids, F; TCA cycle, T; or other, O).

Anterior Cortex											
Compound	↑/↓	<i>p</i> -value*	<i>d</i> -value	ID Level	Class	Compound	↑/↓	<i>p</i> -value*	<i>d</i> -value	ID Level	Class
Putrescine	↑	2.56E-09	2.17	3	A	Serine	↓	4.83E-03	1.02	1	A
Aspartic acid	↓	8.73E-09	2.41	1	A	Pipecolic acid	↑	6.33E-03	0.90	1	A
α-Aminoadipic acid	↓	1.55E-07	1.67	1	A	N-Acetylglutamic acid	↑	1.17E-02	0.91	1	A
Asparagine	↓	1.55E-07	1.94	1	A	Methylindole	↑	1.42E-02	0.82	3	A
Glycerol-3-phosphate	↓	2.06E-07	2.08	3	C,F	Uridine 5'-monophosphat	↓	1.50E-02	0.95	3	O
α-Hydroxyglutaric acid	↓	5.71E-07	1.58	3	T	5-Oxoproline / pyroglutamic acid	↓	1.50E-02	0.93	3	A
Glutamic acid	↓	4.98E-06	1.89	1	A	(9S,10S)-Dihydroxystearic acid	↓	1.50E-02	0.85	3	F
Arginine	↓	7.97E-06	1.76	1	A	Propionylcarnitine	↓	1.68E-02	0.85	1	A,F
Hydroxy-Proline	↓	1.17E-05	1.66	1	A	Creatinine	↑	1.75E-02	0.78	1	O
Citrulline	↓	2.74E-05	1.37	1	A	Ornithine	↓	1.89E-02	0.87	1	A
Dihydroxyacetone phosphate	↓	2.80E-05	1.24	3	F	Arachidonic acid	↑	1.89E-02	0.78	3	F
Erythronic acid	↑	8.94E-05	1.54	3	C	Methionine	↓	2.86E-02	0.77	1	A
Threose	↓	2.63E-04	1.34	2	C	Proline	↑	2.91E-02	0.80	1	A
Glycolic acid	↓	2.91E-04	1.33	3	F	Carbamic acid	↑	2.91E-02	0.79	3	O
Cysteine	↓	5.50E-04	1.20	1	A	Ribitol	↑	3.44E-02	0.79	3	C
Valine	↑	1.77E-03	1.12	1	A						
Brainstem											
Compound	↑/↓	<i>p</i> -value*	<i>d</i> -value	ID Level	Class	Compound	↑/↓	<i>p</i> -value*	<i>d</i> -value	ID Level	Class
Proline	↑	5.17E-11	2.76	1	A	Leucine	↑	1.06E-04	1.33	1	A
α-Aminoadipic acid	↓	1.18E-07	1.56	1	A	3-methyl Histidine	↑	1.25E-04	1.37	1	A
Glycerol-3-phosphate	↓	1.74E-07	1.89	3	C,F	Dihydroxyacetone phosphate	↓	2.10E-04	1.29	3	F
Isovalerylcarnitine	↓	1.83E-07	1.88	1	A,F	N-Acetylglutamic acid	↑	4.29E-04	1.20	1	A
Valine	↑	4.47E-07	2.07	1	A	Ribitol	↑	5.59E-04	1.32	3	C
Asparagine	↓	5.61E-07	1.65	1	A	Taurine	↑	6.51E-04	1.20	1	A
Citrulline	↓	6.52E-07	1.69	1	A	Pipecolic acid	↑	8.16E-04	0.97	1	A
Hydroxy-Proline	↓	8.87E-06	1.49	1	A	Propionylcarnitine	↓	8.16E-04	1.15	1	A,F
Aspartic acid	↓	1.75E-05	1.25	1	A	Butyrylcarnitine	↓	1.95E-03	1.06	1	A,F
25-OH-cholesterol	↓	1.86E-05	1.47	2	F	Ornithine	↓	3.16E-03	1.04	1	A
Glutamic acid	↓	5.05E-05	1.14	1	A	Threose	↑	1.21E-02	0.90	2	C
Putrescine	↑	5.25E-05	1.35	3	A	Succinic acid	↓	1.31E-02	0.85	1	T
α-Hydroxyglutaric acid	↓	6.16E-05	1.59	3	T	Glycolic acid	↓	1.51E-02	0.89	3	F
Isoleucine	↑	8.52E-05	1.50	1	A	Glycerol	↑	1.67E-02	0.90	3	C,F
5-Oxoproline / pyroglutamic acid	↓	1.06E-04	1.53	3	A						

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Cerebellum											
Compound	↑/↓	p-value*	d-value	ID Level	Class	Compound	↑/↓	p-value*	d-value	ID Level	Class
Isovalerylcarnitine	↓	2.27E-10	2.70	1	A,F	Alanine	↑	3.38E-05	1.25	1	A
Proline	↑	4.20E-10	2.35	1	A	Isoleucine	↑	4.56E-05	1.28	1	A
Valine	↑	1.00E-09	2.25	1	A	Ribitol	↑	1.47E-04	1.33	3	C
α-Aminoadipic acid	↓	1.10E-09	1.86	1	A	Arginine	↓	2.19E-04	1.25	1	A
Asparagine	↓	7.82E-09	1.89	1	A	Phosphoglycerol (enol)	↑	2.41E-04	0.97	3	F
Leucine	↑	4.03E-08	1.69	1	A	Threose	↓	2.07E-03	1.01	2	C
Erythronic acid	↑	4.03E-08	1.69	3	C	Isothreonine / Homoserine	↓	4.09E-03	0.85	1	A
5-Oxoproline / pyroglutamic acid	↓	1.51E-07	1.85	3	A	2,2-dimethylpropan-1-imine	↑	4.85E-03	0.86	3	O
Aspartic acid	↓	2.87E-07	1.56	1	A	Lactic acid	↑	4.85E-03	0.91	1	C
Serine	↓	2.87E-07	1.58	1	A	α-Hydroxyglutaric acid	↓	5.10E-03	0.88	3	T
Butyrylcarnitine	↓	1.29E-06	1.61	1	A,F	Glycine	↓	8.92E-03	0.89	1	A
Hydroxy-Proline	↓	1.69E-06	1.78	1	A	Pipecolic acid	↑	5.75E-03	0.75	1	A
Putrescine	↑	3.44E-06	0.90	3	A	N-Acetylglutamic acid	↑	1.02E-02	0.76	1	A
Glycerol-3-phosphate	↓	6.13E-06	1.72	3	C,F	Succinic acid	↓	1.84E-02	0.89	1	T
Glutamic acid	↓	1.84E-05	1.19	1	A	Oleic acid	↑	3.17E-02	0.77	1	F
Propionylcarnitine	↓	3.38E-05	1.33	1	A,F	Arachidonic acid	↑	4.55E-02	0.76	3	F
Olfactory bulbs											
Compound	↑/↓	p-value*	d-value	ID Level	Class	Compound	↑/↓	p-value*	d-value	ID Level	Class
Phosphoglycerol (enol)	↑	4.37E-34	11.29	3	F	Arginine	↑	7.13E-05	1.38	1	A
Glucose-6-phosphate	↑	2.54E-23	5.47	2	C	Oleic acid	↑	7.36E-05	1.47	1	F
α-Aminoadipic acid	↓	4.24E-14	3.21	1	A	Carnitine	↑	8.57E-05	1.42	1	A,F
Glucose-1-phosphate	↑	3.03E-13	2.71	3	C	Erythronic acid	↑	1.11E-04	1.29	3	C
Adenosine	↓	3.54E-12	3.28	1	O	Isovalerylcarnitine	↓	2.00E-04	1.35	1	A,F
Putrescine	↑	5.02E-12	2.74	3	A	Pyruvic acid	↑	2.10E-04	1.30	2	C
Glucose	↑	2.64E-11	2.46	2	C	α-Hydroxyglutaric acid	↓	2.23E-04	1.11	3	T
Lysine	↑	1.12E-09	2.44	1	A	Threose	↓	2.73E-04	1.14	2	C
Fructose-6-phosphate	↑	1.32E-09	2.00	3	C	Formylglycine	↑	3.07E-04	1.27	3	A
Aspartic acid	↓	2.24E-07	1.71	1	A	2,5-Pyrazinediol	↓	9.69E-04	1.10	3	O
Leucine	↑	1.07E-06	1.66	1	A	Asparagine	↓	1.03E-03	1.04	1	A
Valine	↑	1.68E-06	1.92	1	A	N-Acetylaspartic acid	↓	1.05E-03	1.03	1	A
Alanine	↑	1.89E-06	1.55	1	A	Threonine	↑	2.14E-03	1.01	1	A
3-((carboxymethyl)(methyl)amino)-3-oxopropanoic acid	↓	2.51E-06	1.47	3	O	Lyxose	↑	2.35E-03	0.96	2	C

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Pipecolic acid	↑	2.59E-06	1.76	1	A	Ribitol	↑	2.75E-03	0.96	3	C
Cystathionine	↑	4.01E-06	1.71	1	A	Palmitic acid	↑	2.81E-03	1.08	1	F
Lactic acid	↑	4.05E-06	1.49	1	C	Pantothenic acid	↓	2.97E-03	0.87	3	O
Ethyl-9,10-dihydroxyoctadecanoate	↓	4.05E-06	1.42	3	F	Glycerol-3-phosphate	↓	3.75E-03	0.92	3	C,F
γ-Aminobutyric acid	↑	4.89E-06	1.55	3	A	Xylo-hexos-5-ulose	↓	1.02E-02	0.85	3	C
Proline	↑	5.46E-06	1.60	1	A	Acetylcarnitine	↑	1.06E-02	0.95	1	A,F
2,2-dimethylpropan-1-imine	↑	1.41E-05	1.42	3	O	Succinic acid	↓	1.24E-02	0.89	1	T
D-Mannonic acid, γ-lactone	↑	2.03E-05	1.25	2	C	Butyrylcarnitine	↓	1.40E-02	0.92	1	A,F
Isoleucine	↑	2.50E-05	1.57	1	A	Beta-Alanine	↓	1.61E-02	0.90	1	A
Glutamic acid	↓	4.42E-05	1.25	1	A	Stearic acid	↓	1.81E-02	0.87	1	F
5-Oxoproline / pyroglutamic acid	↓	6.93E-05	1.42	3	A						
*FDR-adjusted											

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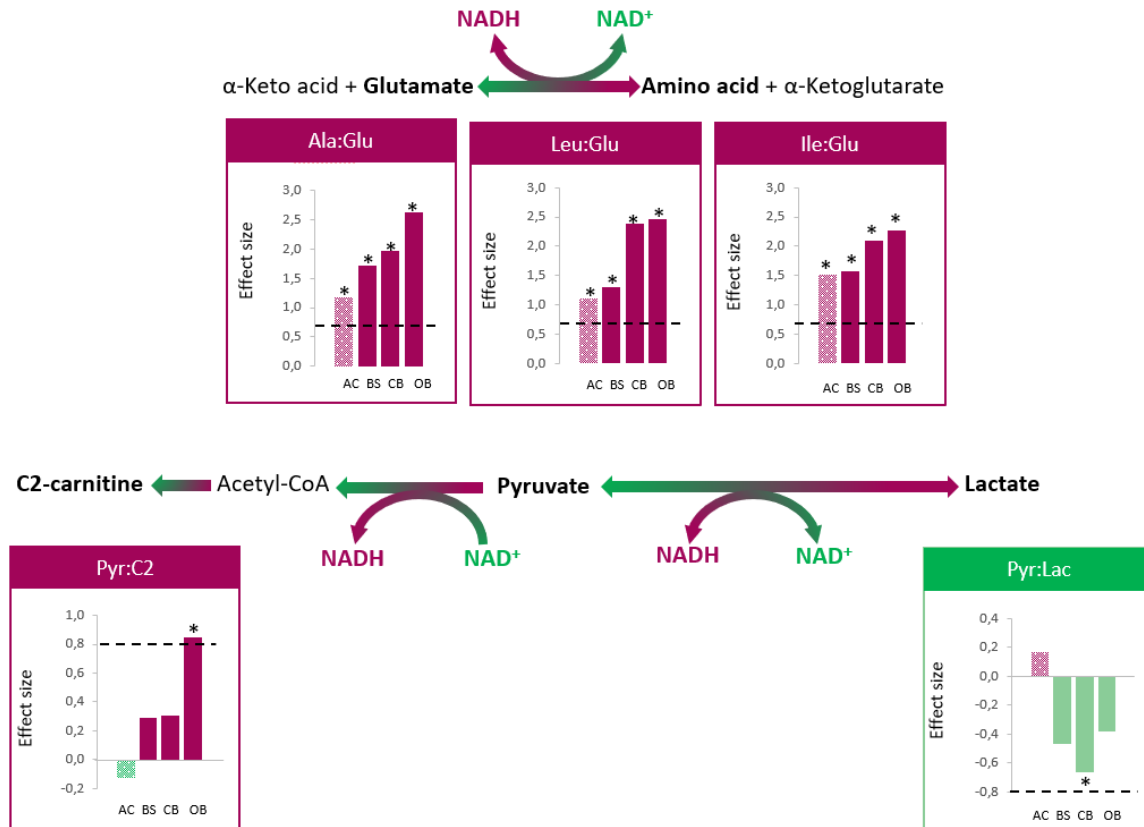


Figure 4-S2: Metabolic indicators of NADH/NAD⁺ ratio in *Ndufs4* KO vs WT brain regions. Bar graphs depict Cohen's *d*-values (y-axis) for each metabolite ratio when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate an increase or decrease in KO metabolite ratios. Dashed lines mark practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (p -value < 0.05). An increased NADH/NAD⁺ ratio drives the formation of amino acids (from α -ketoacids and glutamic acid) and lactic acid (from pyruvic acid) by an equilibrium reaction, while the pyruvic acid:acetyl-carnitine ratio is indicative of NAD⁺-dependent acetyl-coA formation from pyruvate. Abbreviations: Ala, alanine; C2, acetyl-carnitine; Glu, glutamic acid; Ile, isoleucine; Lac, lactic acid; Leu, leucine; Pyr, pyruvic acid.

■ WT ■ KO

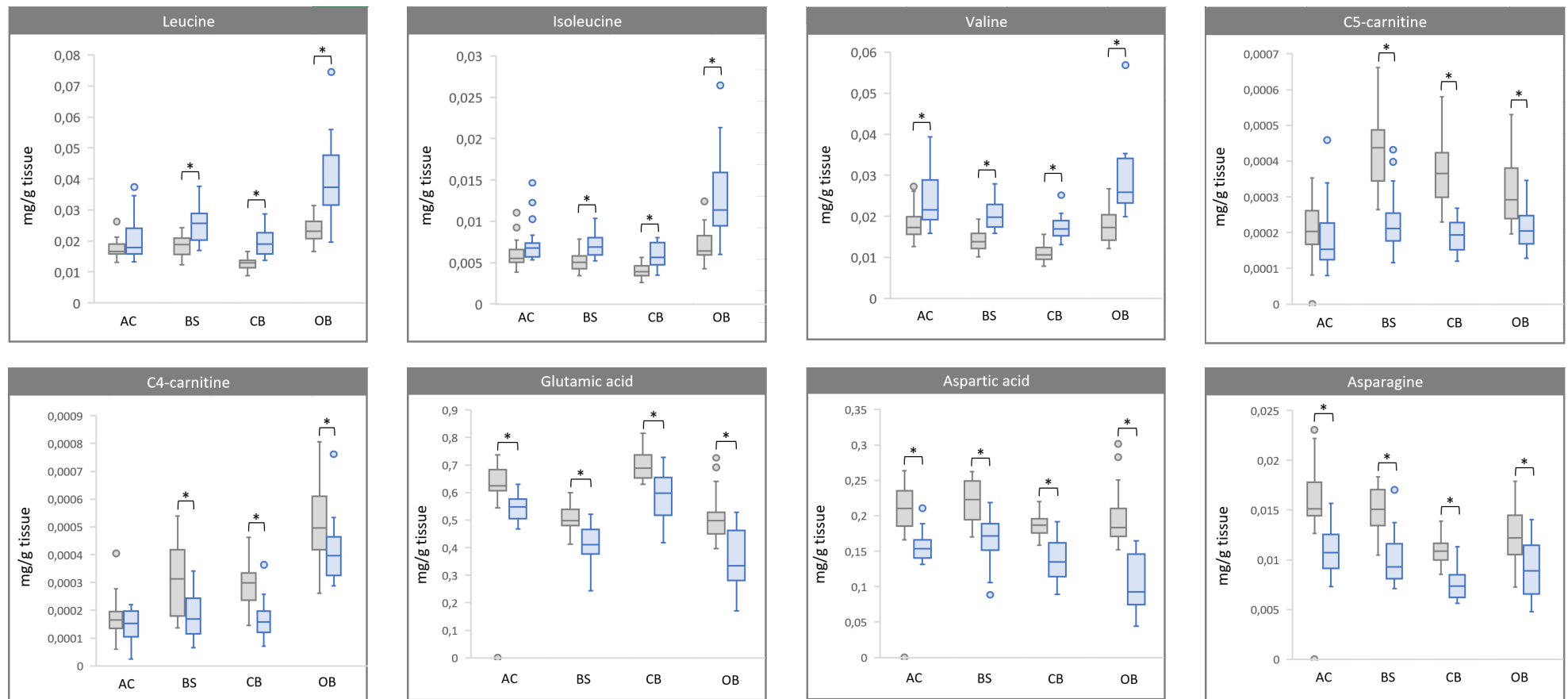


Figure 4-S3: Relative abundance of metabolites in *Ndufs4* KO and WT brain regions. Box plots depict the relative abundance of each metabolite in mg/g tissue for all biological replicates per genotype (WT: n=25; KO: n=21). Grey and blue boxes respectively indicate WT and *Ndufs4* KO groups. The length of the box represents 25th to 75th percentile inter-quartile range, interior horizontal line represents median, and vertical lines stemming from the box extend to minimum and maximum values. Circles indicate outlier values. Asterisks indicate statistical and practical significance (FDR-corrected p -value < 0.05 ; $d \geq 0.8$).

■ WT ■ KO

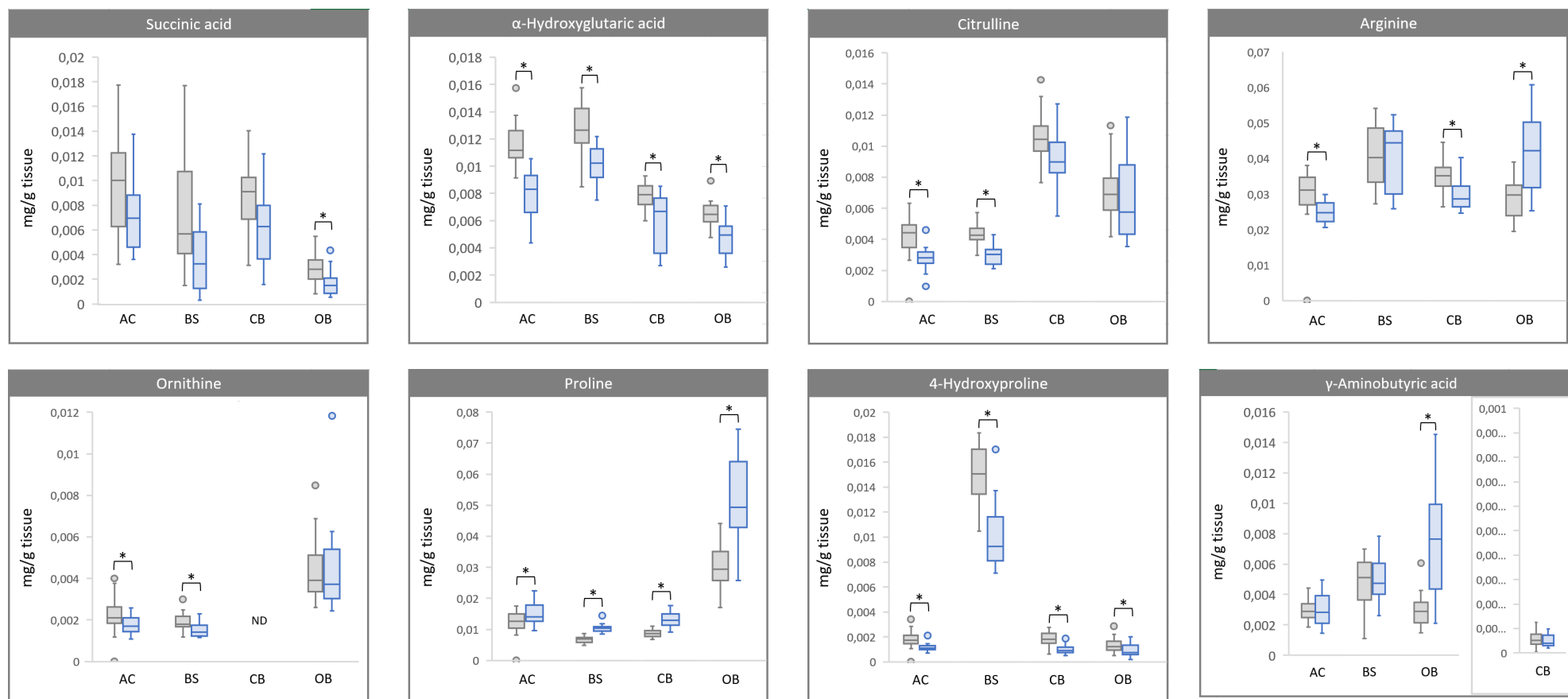


Figure 4-S3: Relative abundance of metabolites in *Ndufs4* KO and WT brain regions (Continued)

■ WT ■ KO

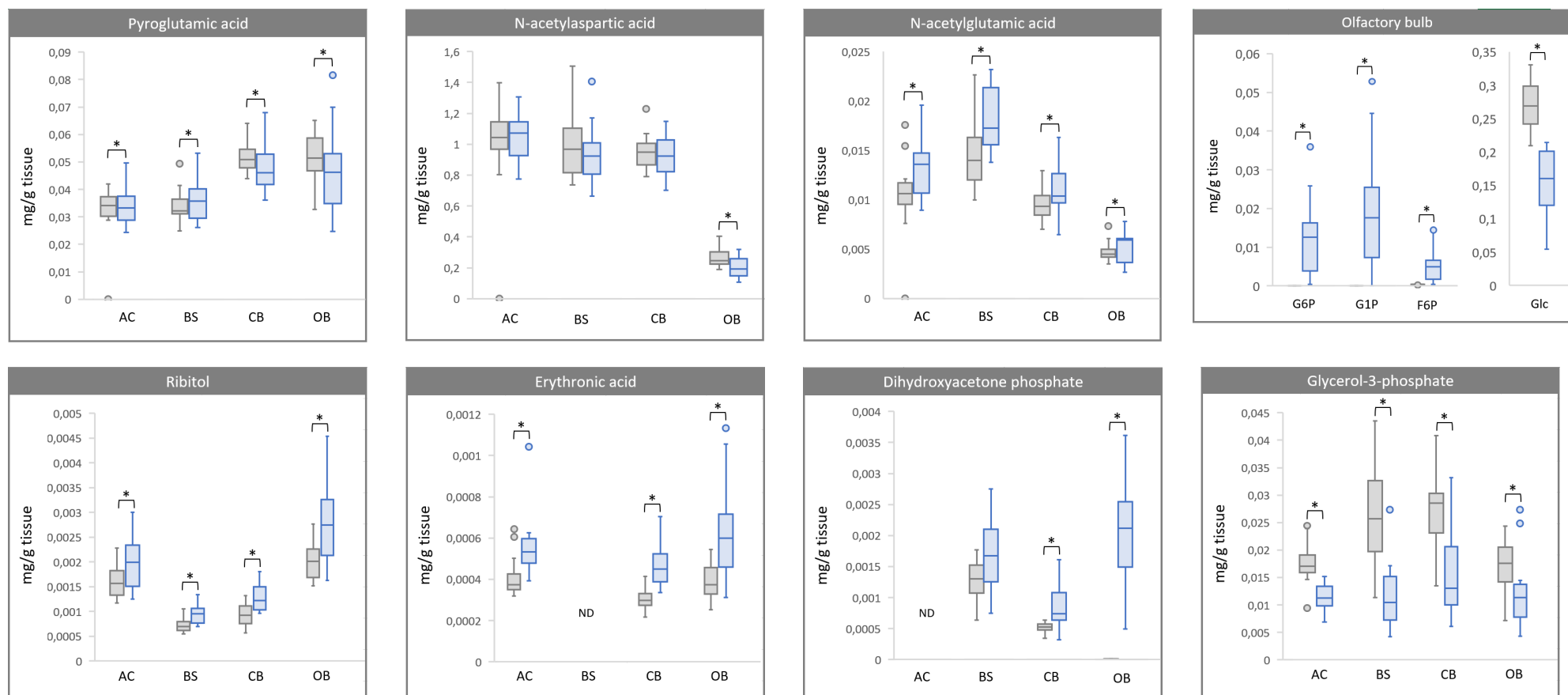


Figure 4-S3: Relative abundance of metabolites in *Ndufs4* KO and WT brain regions (Continued)

■ WT ■ KO

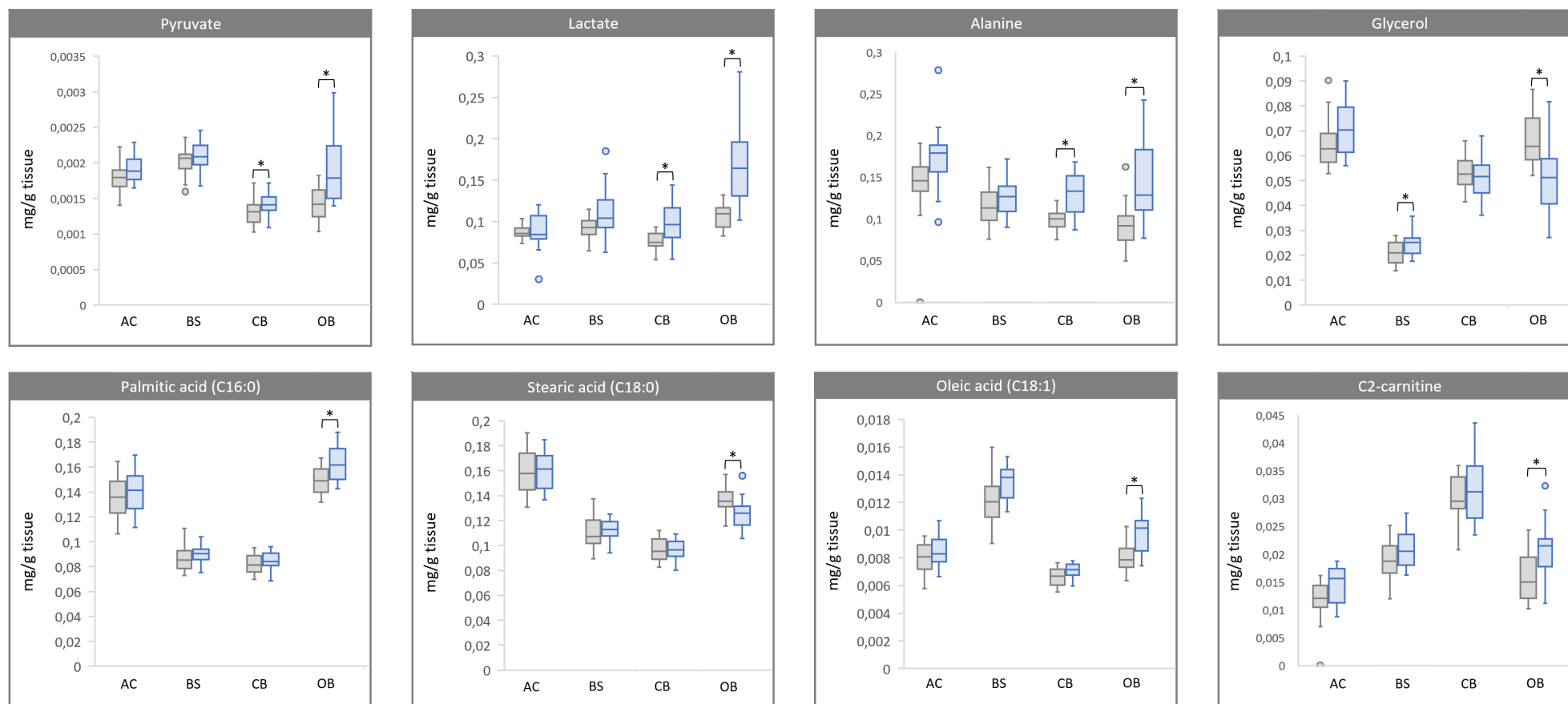


Figure 4-S3: Relative abundance of metabolites in *Ndufs4* KO and WT brain regions (Continued)

■ WT ■ KO

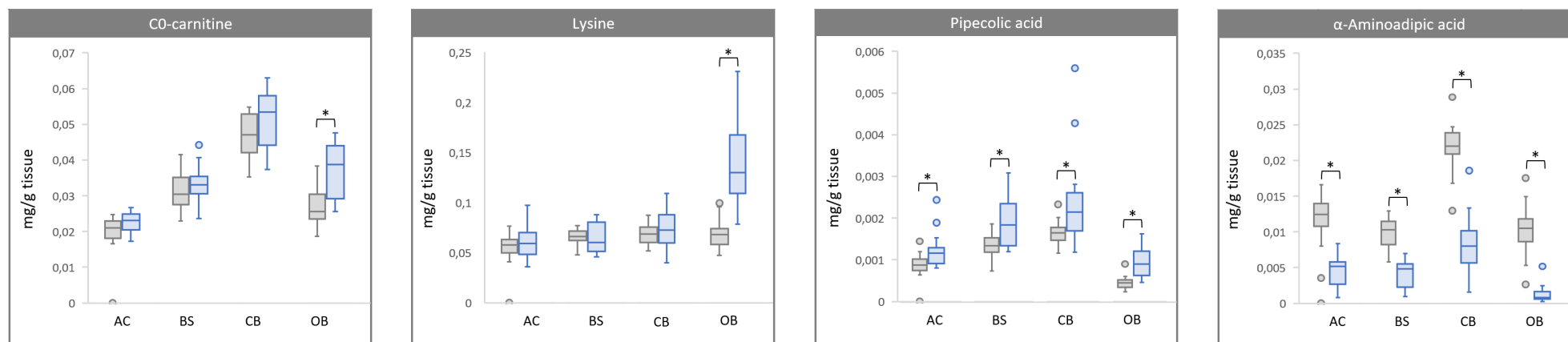


Figure 4-S3: Relative abundance of metabolites in *Ndufs4* KO and WT brain regions (Continued)

CHAPTER

Cross-comparison of systemic and tissue-specific metabolomes in a mouse model of Leigh syndrome

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5

5.1 Introduction

A clear understanding of the pathologies and treatment of primary mitochondrial diseases (MDs), which results from a dysfunction of the oxidative phosphorylation (OXPHOS) system, remain a great challenge. Largely unknown mechanisms govern a tissue-specific vulnerability to MD-causing mutations and an extreme heterogeneity in genotype-phenotype correlations among patients (Lake *et al.*, 2016; Mootha & Chinnery, 2018; Rahman, 2020). As the most common form of MD, respiratory chain complex I (CI) deficiency and its most prevalent paediatric manifestation, Leigh syndrome (LS, OMIM # 256000), are at the forefront of MD research (Rahman *et al.*, 1996; Rodenburg, 2016). The *Ndufs4* knockout (KO) mouse model of LS suffers from all-pervading CI deficiency, exhibits progressive focal lesions in specific brain regions, and displays symptoms similar to human patients. Symptoms include developmental delay, failure to thrive, locomotor impairment, encephalomyopathy and respiratory deficits, which progressively worsen until premature death (Kruse *et al.*, 2008; Quintana *et al.*, 2010). Studies on *Ndufs4* KO mice have revealed that specific neuronal populations (linked to lesion-prone brain regions) drive most symptoms (Bolea *et al.*, 2019), while peripheral tissues, like skeletal muscle, seem relatively unaffected phenotypically (Alam *et al.*, 2015; Chouchani *et al.*, 2014; Kruse *et al.*, 2008). However, the contribution of systemic metabolic alterations to this neurometabolic disease cannot be ruled out (Bolea *et al.*, 2019; Di Meo *et al.*, 2017; Ito *et al.*, 2017; Lee *et al.*, 2019).

Metabolomics present a powerful tool with which to analyse the downstream effects of multi-systemic diseases like MD in a holistic manner (Rahman & Rahman, 2018). Although the application of metabolomics in the field of MD research is relatively novel, it has made significant progress in recent years — especially with the increased availability of well characterised MD models (Esterhuizen *et al.*, 2017). Non-invasive (urine) or minimally invasive (blood) systemic samples are generally favoured for metabolomics investigations of MD. However, it remains inconclusive whether such samples are optimal to study and/or monitor MD, and how well systemic metabolomes reflect tissue-specific metabolic alterations (Esterhuizen *et al.*, 2017; Steele *et al.*, 2017). A few metabolomics studies have been performed on *Ndufs4* KO tissues (Emmerzaal *et al.*, 2020; Jin *et al.*, 2014; Johnson *et al.*, 2020; Johnson *et al.*, 2013; Terburgh *et al.*, 2021a; Terburgh *et al.*, 2019b), however, the link between systemic and brain regional metabolomes remains unexplored. Here, we integrate and cross-compare tissue and urine metabolomes from *Ndufs4* KO vs wild type (WT) mice to establish whether differences exist between brain region-specific and systemic metabolic profiles in LS. Skeletal muscle metabolomes were included in this comparison to show the contribution of peripheral tissues. Seven sample types from individual late stage diseased *Ndufs4* KO and WT mice were extensively investigated via untargeted and semi-targeted multiplatform metabolomics. These samples include one lesion-resistant brain region (anterior cortex); three lesion-prone brain

regions (brainstem, cerebellum, and olfactory bulbs); urine collected overnight; the primarily glycolytic white parts of the quadriceps femoris; and the primarily oxidative solei.

5.2 Materials and methods

5.2.1 Animals and housing

Male whole-body homozygous *Ndufs4* KO mice, along with age- and sex-matched controls (WT) were used. Only one sex-group was used in order to minimise sex-related metabolic variation due to known differences in hormone cycles, tissue-specific metabolism and metabolic profiles between sexes (Ruoppolo *et al.*, 2018; Wells *et al.*, 2016; Wu & Gao, 2015). KO mice were born from heterozygous crosses (B6.129S4-*Ndufs4*^{tm1.1Rpa}/J) obtained from Jackson Laboratory (JAX stock #027058). *Ndufs4* genotypes were confirmed by polymerase chain reaction using tail snips. The animals were bred and housed at the specific pathogen-free unit of the Vivarium (SAVC reg. no. FR15/13458) of the Preclinical Drug Development Platform (PCDDP; NWU, RSA) and approval (NWU-00378-16-A5) for this study was obtained from the Animal Research Ethics Committee of the NWU. Animals were group housed under temperature- ($22 \pm 1^\circ\text{C}$), humidity- ($55 \pm 10\%$) and light-controlled (12:12 hour light/dark cycle) conditions with standard laboratory chow (Rodent Breeder, #RM1845, LabChef, Nutritionhub) and water provided *ad libitum*. Samples were collected between P45-50 as this age range was selected to represent the late stage of LS, based on our previous survival curves and phenotypic analyses (Miller *et al.*, 2021).

5.2.2 Sample collection

Urine was collected from individual mice in metabolic cages during overnight (12 hours) fasting with *ad libitum* access to water. Since *Ndufs4* KO mice yielded limited urine volumes, urine droplets that failed to reach the collection tube were also collected. Hence, the separating cone, collection funnel and -tubes were all treated with sodium azide (NaN_3 ; 0.1% (w/v); WT: 100 μL and KO: 50 μL) to prevent bacterial growth. After urine collection, mice were euthanised via cervical dislocation at the same time of day (8:00-9:00 AM). The brain was immediately removed from the skull, rinsed with saline solution (SABAX PBS; 0.9% NaCl (w/v), #7634, Adcock Ingram) and dissected into the regions of interest (anterior cortex, brainstem, cerebellum, and olfactory bulbs). Concurrently, the white muscle portions of quadriceps femoris, as well as solei muscles, were collected from both hind limbs. All samples were snap frozen in liquid nitrogen upon collection (within 15 min post-mortem for tissues) and stored at -80°C until use.

5.2.3 Metabolic profiling

5.2.3.1 Sample analysis

Untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and semi-targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed on seven sample types from *Ndufs4* KO ($n \geq 19$) and WT ($n \geq 20$) mice. Tissue extracts were individually prepared and analysed as previously described (Terburgh *et al.*, 2021a; Terburgh *et*

al., 2019b), while detailed descriptions on urine sample preparation and analysis are given in the supplementary information. In short, frozen tissues were weighed and homogenised in the presence of internal standards added according to tissue mass. Monophasic tissue extracts were prepared via modified Bligh–Dyer (Gullberg *et al.*, 2004) (methanol/water/chloroform, 3:1:1), with additional step-wise extraction (Wu *et al.*, 2008) to obtain polar phases of biphasic brain extracts (methanol/water/chloroform, 2:1.8:2) for LC-MS/MS. Tissue extracts were divided between the platforms, with stable isotopes added to LC-MS/MS samples. Pre-determined volumes of urine, containing 0.0625 μ mole creatinine, were supplemented with internal standards (included stable isotopes for LC-MS/MS samples) and deproteinised using methanol. Urine and tissue extracts were then dried under nitrogen and derivatised prior to their analysis. For GC-TOF-MS, samples were oximated and silylated, while LC-MS/MS samples were butylated.

5.2.3.2 Data processing

Spectral data matrices were individually inspected (correct peak detection and alignment, batch precision and data integrity) and preprocessed (data filtering, half-minimum missing value imputation and normalisation). Tissue data was normalised post-acquisition to tissue mass (ng/mg tissue) using internal standards, 3-phenylbutyric acid (GC-TOF-MS) and *N*, *N*-dimethylphenylalanine (LC-MS/MS). Additionally, LC-MS/MS metabolites were also normalised to isotope internal standards to account for ion suppression effects. Urine data was normalised pre-acquisition to creatinine content (mg/g creatinine) and post-acquisition to tropic acid (GC-TOF-MS) or isotope (LC-MS/MS) internal standards. Metabolites detected via LC-MS/MS were normalised to their own isotopes, where available, or an isotope with a similar retention time to which the metabolite strongly correlated in QC samples. Finally, the data was log transformed and outliers were detected (PCA Hotelling's T^2 ellipses, loadings plot and biplots). Data processing was done using Excel (Microsoft 365), Statistica v 13.0 (TIBCO Software Inc.) and MetaboAnalyst v 4.0 [Xia Lab (Chong *et al.*, 2018)]. The metabolomics data obtained in this study can be accessed at the Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (<https://www.metabolomicsworkbench.org>) where it has been assigned project IDs: PR000721 (Terburgh *et al.*, 2019a), PR001003 (Terburgh *et al.*, 2021b), and PR001187 (Terburgh *et al.*, 2021c).

5.2.3.3 Statistical analyses

For each sample type, univariate analyses were used to determine discriminatory features between *Ndufs4* KO and WT metabolic profiles. Statistical and practical significance were determined using the student's t-test and effect size (ES, Cohen's *d*-value) calculations, respectively. False discovery rate (FDR) corrected *p*-values were calculated in MetaboAnalyst (Chong *et al.*, 2018), with an FDR-*p* of ≤ 0.05 considered statistically significant. Dataset features were identified via spectral and retention time matching using commercial and in-house spectral libraries together with public databases and metabolite identities were assigned to spectral

features, with high confidence levels (Table 5-S1) ranging from level one to three, as defined by Schymanski *et al.* (2014). For each sample type, pathway analysis was performed on significantly altered metabolites using MetaboAnalyst (Chong *et al.*, 2018). Compound labels were matched to HMDB and KEGG IDs, with *mus musculus* (mouse) KEGG as the selected pathway library. A hypergeometric test was the selected over-representation analysis method for pathway enrichment analysis, while relative betweenness centrality was the selected node importance measure for pathway topology analysis.

5.3 Results and discussion

5.3.1 Cross-comparison of urine- and tissue metabolomes

Several significant metabolic alterations were observed in the urine and tissue-specific metabolomes of *Ndufs4* KO vs WT mice, with the number of discriminatory metabolites (and their significance level) generally lower in the muscles. Figure 5-1 (A-J) illustrates the main similarities and differences among the metabolic alterations seen in the seven *Ndufs4* KO sample types when compared to their WT counterparts. Reductions in the tissue content and renal excretion of 4-hydroxyproline and 2-aminoadipate were among the most significant and consistent alterations observed in *Ndufs4* KO mice compared to WT mice (Figure 5-2, Figure 5-S1). Furthermore, the levels of citrulline, 2-hydroxyglutarate, succinate, glutamate, pyroglutamate, aspartate, butyrylcarnitine and isovalerylcarnitine also trended lower in most *Ndufs4* KO sample types, with varying levels of significance. The accumulation of branched-chain amino acids (BCAAs), proline, and arachidonate were unique to the four brain regions, although arachidonate could not be detected in other sample types. Additionally, the *Ndufs4* KO urine metabolome contained 51 uniquely altered metabolites (directionality of alterations also considered), many of which could only be detected in urine samples. Combined pathway over-representation and topology analysis (Figure 5-3, Table 5-S2) further revealed that alanine, aspartate, and glutamate metabolism were strongly affected ($\text{FDR-}p < 0.05$, $\text{impact} > 0.2$) in most *Ndufs4* KO sample types, with the significance level again relatively lower in the muscles. *Ndufs4* KO brain regions and urine further displayed significantly enriched arginine, proline, and BCAA metabolism. Although lesion-resistant and -prone brain regions display generally similar metabolic alterations in *Ndufs4* KO vs WT mice, the practical and statistical significance level of these alterations vary. In our previous work (Terburgh *et al.*, 2021a) we reported a graded reduction in CI activity in *Ndufs4* KO brain regions, with the lesion resistant anterior cortex displaying the highest level of residual CI activity and the olfactory bulbs displaying the lowest [AC (38% of WT) > CB (28%) > BS (25%) > OB (14%)]. Furthermore, we showed that a higher residual CI activity and a less perturbed NADH/NAD⁺ balance correlate with milder metabolic perturbations in *Ndufs4* KO brain regions. Thus, there seems to be a critical threshold (related to the level of redox imbalance and its effect on metabolism) that, when breached, likely limits brain regional capacity to bypass CI deficiency

and maintain homeostasis. Glycolysis/gluconeogenesis was markedly enriched in the *Ndufs4* KO olfactory bulbs (FDR- $p < 0.05$), with moderate enrichment ($p < 0.05$) also evident in the other two lesion-prone brain regions. The *Ndufs4* KO olfactory bulbs additionally displayed marked enrichment of pantothenate and CoA biosynthesis, which was also reflected in the urine metabolome. Moreover, glycine, serine and threonine metabolism were prominently enriched in the cerebellum, urine, and white quadriceps, while glutathione metabolism was more enriched in the anterior cortex and brainstem of *Ndufs4* KO mice. *Ndufs4* KO urine also displayed markedly enriched beta-alanine-, glutamine and glutamate-, glyoxylate and dicarboxylate-, tricarboxylic acid (TCA) cycle-, histidine- and butanoate metabolism. In the next sections, we take a deeper look into some of the most prominent similarities and differences among the various *Ndufs4* KO metabolomes.

5.3.2 Metabolites strongly altered in all *Ndufs4* KO metabolomes

5.3.2.1 4-Hydroxyproline

Significant reductions in the levels of proline's post-translational product, 4-hydroxyproline, were evident in all *Ndufs4* KO sample types investigated (Figure 5-1 B). Since urinary 4-hydroxyproline commonly serves as a bone-resorption biomarker of collagen turnover, decreased renal excretion thereof suggests decreased degradation of collagen from the bone matrix in *Ndufs4* KO mice. In accord, Cl deficiency was shown to cause an intrinsic defect in osteoclastogenesis, leading to decreased bone resorption and increased bone mass (osteopetrosis) in *Ndufs4* KO mice (Jin *et al.*, 2014). However, the significant reductions in brain and muscle 4-hydroxyproline levels we report here imply that other sources may also contribute to reduced 4-hydroxyproline excretion. As 4-hydroxyproline is a known pyruvate, glucose, and glycine precursor (Wu *et al.*, 2011), these reductions may reflect its increased utilisation in *Ndufs4* KO tissues. Thus, while urinary 4-hydroxyproline may serve as a biomarker for osteopetrosis associated with Cl deficiency, its metabolism is also strongly affected in *Ndufs4* KO tissues and warrants further investigation.

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Figure 5-1: Cross-comparison of metabolic alterations in *Ndufs4* KO mice. Heatmap of effect size (Cohen's d) values for each metabolite when relative abundance is compared between *Ndufs4* KO ($n \geq 19$) and WT ($n \geq 20$) mice. Metabolic alterations are compared across seven sample types and grouped according to metabolic pathways (A-J). Shades of purple and green respectively indicate increased and decreased abundance in *Ndufs4* KO mice and correspond to the level of practical significance as indicated

CHAPTER 5

in the key. Asterisks indicate statistical significance (FDR-adjusted $p < 0.05$). The first column indicates the analytical platform used for detection. Abbreviations: AC; anterior cortex; BS, brainstem; CB; cerebellum; GC; GC-TOF-MS; LC; LC-MS/MS; ND, not (reliably) detected; OB, olfactory bulbs; SOL, solei; U, urine; WQ, white quadriceps.

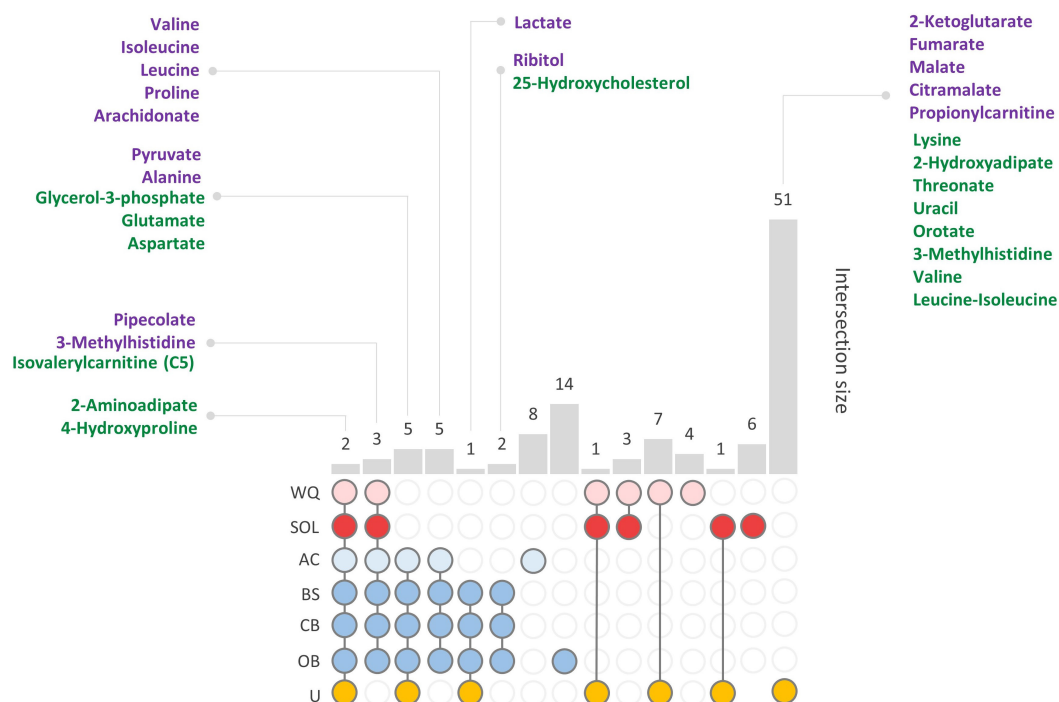


Figure 5-2: Intersections of importantly altered metabolites among *Ndufs4* KO sample types. Rows indicate different sample types, while columns correspond to one segment in a Venn diagram. Colour filled cells (in columns) indicate the associated samples in a set with empty cells not part of that intersection. Bars indicate the number of unique metabolic alterations per set intersection. Purple and green text respectively indicate significantly ($p < 0.05$) increased or decreased metabolite levels in *Ndufs4* KO ($n \geq 19$) mice compared to WT ($n \geq 20$). Only a few of the relevant metabolic alterations unique to urine are displayed. Abbreviations: AC; anterior cortex; BS, brainstem; CB; cerebellum; OB, olfactory bulbs; SOL, solei; U, urine; WQ, white quadriceps.

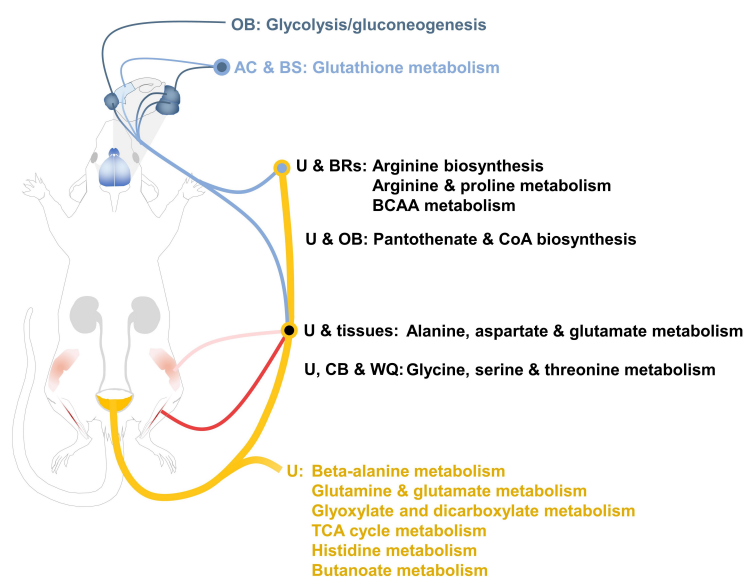


Figure 5-3: Affected metabolic pathways in *Ndufs4* KO mice. Significantly enriched pathways identified via pathway over-representation (FDR-adjusted $p < 0.05$) analysis. Coloured lines represent sample types; while dots indicate intersection of lines and signify commonly affected pathways among sample types. Colours and abbreviations: AC; anterior cortex (light blue); BRs, all brain regions (medium blue); BS, brainstem (dark blue); CB; cerebellum (dark blue); OB, olfactory bulbs (dark blue); SOL, solei (red); U, urine (yellow); WQ, white quadriceps (light peach).

5.3.2.2 2-Aminoadipate and lysine catabolism

The precursor for 2-ketoadipate in the lysine catabolic pathway, 2-aminoadipate, is another metabolite markedly reduced in all *Ndufs4* KO sample types (Figure 5-1 C). The renal excretion of other 2-ketoadipate precursors (5-hydroxylysine, lysine, and tryptophan) and alternative products (2-hydroxyadipate and glutarate) were also significantly reduced in *Ndufs4* KO mice. Additionally, the lysine catabolite, pipecolate, markedly accumulated in brain regions (and muscles) although not measured in urine. As lysine catabolism is dependent on nicotinamide adenine dinucleotide (NAD⁺) bioavailability, redox imbalance is one of the main, and well documented, factors linked to MD-related perturbations in this pathway (Esterhuizen *et al.*, 2017). Lysine levels were, however, not as consistently altered with its accumulation only evident in the *Ndufs4* KO olfactory bulbs (and muscles). The degree of lysine accumulation in the olfactory bulbs, further correlated with the accumulation of its metabolite, carnitine, which was also elevated in other brain regions and urine. These observations may point to increased proteolysis in *Ndufs4* KO tissues, as the release of trimethyllysine from cognate proteins is required for endogenous carnitine synthesis, which takes place mainly in the liver, kidneys, and brain but not muscles (Vaz & Wanders, 2002). In support, some of the most significant alterations in *Ndufs4* KO urine were centred around ascorbate (Figure 5-1 J), a cofactor required for two dioxygenase reactions in the carnitine biosynthesis pathway. Although urinary ascorbate levels only trended lower, markedly decreased renal excretion of glucarate and gluconate (metabolites from the glucuronate pathway that diverge into the murine ascorbate synthesis pathway) as well as threonate and oxalate (breakdown products of ascorbate) may reflect increased ascorbate demand in *Ndufs4* KO mice. The enzymes that catalyse these dioxygenase reactions, ϵ -N-trimethyllysine dioxygenase (TMLD, EC 1.14.11.8) and γ -butyrobetaine dioxygenase (BBD, EC 1.14.11.1), also convert 2-ketoglutarate to succinate and perhaps present a favourable alternative route to fuel the ubiquinone (Q)-pool via CII. Furthermore, alterations in ascorbate metabolism may be more evident in the urine metabolome since the expression of BBD is high in the kidneys, moderate in the liver, but low in the brain (Vaz & Wanders, 2002). Marked elevation in brain and muscle levels of 3-methylhistidine (3-MH) and the 3-MH/histidine ratio further support the notion of elevated proteolysis in *Ndufs4* KO tissues (Figure 5-1 D). However, the renal excretion of histidine and its methylated derivatives were markedly reduced. These discrepancies confirm previous concerns regarding the use of urinary 3-MH as an index of myofibrillar protein turnover (Shargill *et al.*, 1984; van Goudoever & Matthews, 2017) as several hundred widespread histidine-methylated proteins were recently identified (Davydova *et al.*, 2021; Ning *et al.*, 2016; Wilkinson *et al.*, 2019) and considerable amounts of urinary 3-MH was shown to originate from non-muscle sources (Millward & Bates, 1983; Nishizawa *et al.*, 1977; Wassner & Li, 1982). Nevertheless, the results reported here not only reveal widespread perturbations in the metabolism of lysine and tryptophan, but also suggest elevated proteolysis and carnitine biosynthesis in a subset of *Ndufs4* KO tissues.

Hence, the future investigation of tissue-specific protease activity in *Ndufs4* KO mice is recommended.

5.3.3 Biofluid markers commonly associated with MD

In *Ndufs4* KO urine, significant elevations were seen in biofluid markers commonly associated with MD i.e., pyruvate, alanine, lactate, and the lactate/pyruvate ratio (FDR- $p < 0.05$, ES $d = 1.20$) — which reflects cytosolic redox state (Esterhuizen *et al.*, 2017; Go *et al.*, 2020; Munnich *et al.*, 1996). These alterations were similar in the brain, which also displayed elevated glycolytic- and pentose phosphate pathway intermediates, with clear differences seen in the degree of metabolite accumulation among regions (Figure 5-1 J). The *Ndufs4* KO olfactory bulbs were most significantly affected, while lactate accumulation was only evident in the neurodegeneration-prone brain regions. In agreement, Johnson and colleagues report that pre-symptomatic *Ndufs4* KO mice display significantly elevated levels of pyruvate and lactate along with higher trends in alanine levels in whole brain (Johnson *et al.*, 2013) and trended higher levels of glycolytic intermediates and lactate in brain regions, with significant lactate elevation only seen in the olfactory bulbs (Johnson *et al.*, 2020). Contrastingly, reports of blood lactate levels in *Ndufs4* KO mice vary, with normal, slight, and significant elevation observed (Jain *et al.*, 2016; Jin *et al.*, 2014; Kruse *et al.*, 2008). Considering recent reports of lactate (and not glucose) being the primary circulating TCA substrate for most tissues (except the brain) with the kidneys playing a key role in systemic redox homeostasis by oxidising lactate to pyruvate (Jang *et al.*, 2019), we suggest that excretory (urine) rather than circulating biofluids may be more appropriate for MD-marker monitoring as the lack of homeostatic control mechanisms in urine allows magnification of metabolic alteration occurring in blood (Wu & Gao, 2015). Similarly, although blood glucose levels are reportedly unaltered in *Ndufs4* KO mice (Kruse *et al.*, 2008), we observed decreased renal excretion of glucose, ribitol, several monosaccharides and sugar acids, suggesting increased glucose utilisation in *Ndufs4* KO tissues. In agreement, pyruvate, lactate, and alanine levels all trended lower in *Ndufs4* KO skeletal muscles, possibly indicating increased release thereof to fuel gluconeogenesis. Taken with the accumulation of glycolytic- and pentose phosphate pathway intermediates that we, and others, observed in the *Ndufs4* KO brain as well as reports of upregulated glucose transporter 1 (GLUT1, SLC2A1) protein levels in the *Ndufs4* KO brain (Lee *et al.*, 2019) and pro-glycolysis genes in the *Ndufs4* KO liver (Jin *et al.*, 2014), we postulate that glucose production, uptake and utilisation is elevated in *Ndufs4* KO mice.

In agreement with our observations, muscle lactate levels in patients with mitochondrial disease, was shown to be lower at rest but elevated during exercise (Lindholm *et al.*, 2004; Löfberg *et al.*, 2001). Another possible explanation for this discrepancy between *Ndufs4* KO muscle and brain metabolomes is the activity of the glycerol-3-phosphate (G3P) shuttle, which is one of the most important, highly active redox regulatory shuttles in skeletal muscle that has limited activity in the brain (Liu *et al.*, 2021; Schiaffino & Reggiani, 2011). Increased G3P synthesis (and shuttle activity)

is known to reduce muscle lactate build-up (Alfadda *et al.*, 2004) and was recently shown to be an important endogenous NAD⁺-regeneration pathway during CI inhibition that suppresses neuroinflammation and extends lifespan when enhanced in *Ndufs4* KO mice (Liu *et al.*, 2021). Interestingly, mice with cytosolic G3P dehydrogenase (cGPD; EC 1.1.1.8) deficiency display increased dihydroxy acetone phosphate (DHAP) and decreased G3P in muscle (MacDonald & Marshall, 2000; MacDonald & Marshall, 2001), similar to what we observed in *Ndufs4* KO brain regions — suggesting that there may be an increased need for this shuttle in brain during CI deficiency. In agreement, lower levels of G3P and an increased trend in DHAP levels could also be seen in the whole-brain metabolomics data from pre-symptomatic *Ndufs4* KO mice (Johnson *et al.*, 2013). Although we could not detect these metabolites in muscles, urinary G3P excretion was markedly decreased in *Ndufs4* KO mice. In addition, TCA cycle-linked metabolites (succinate and 2-hydroxyglutarate) which, like G3P, can fuel the Q-pool independently of CI, were consistently and significantly decreases in *Ndufs4* KO brain regions and urine. The accumulation of TCA cycle intermediates, 2-ketoglutarate, fumarate, and malate in biofluids is another metabolic signature characteristic of MD, that suggests TCA cycle congestion. Although these metabolites were significantly elevated in *Ndufs4* KO urine, they were not markedly altered in the various brain regions (Figure 5-1 F). Others have also reported early-stage elevations in fumarate levels in affected brain regions (but not muscle) of *Ndufs4* KO mice, which was not evident in brain regions of older mice (Piroli *et al.*, 2020; Piroli *et al.*, 2016). Overall, urine proves to be a fitting biofluid with which to monitor classic MD biomarkers that reflect the redox state in affected brain regions. However, with inconsistencies between tissue and urine levels of TCA cycle intermediates observed, questions arise regarding the extent of TCA cycle congestion in individual *Ndufs4* KO tissues.

5.3.4 Inconsistent alterations between systemic and brain region-specific metabolomes

5.3.4.1 BCAA catabolism

BCAAs, valine, leucine, and isoleucine markedly accumulated in *Ndufs4* KO lesion-prone brain regions, with elevations also evident in the lesion-resistant anterior cortex (Figure 5-1 G). The degree of these elevations correlated with reductions in leucine's catabolic by-product, isovaleryl-carnitine (C5). Although these alterations were not observed in whole brain or brain regions of pre-symptomatic mice (Johnson *et al.*, 2020; Johnson *et al.*, 2013), perturbations in BCAA catabolism have been reported in MD cases and, as with lysine, is believed to result from the redox imbalance associated with MD (Esterhuizen *et al.*, 2017). Several tissues jointly control BCAA catabolism, with skeletal muscle and liver playing a central role (Holeček, 2018). The first step in BCAA catabolism primarily occurs in skeletal muscles, which then release most branched-chain keto acids (along with glutamine and alanine) into circulation to be taken up by other tissues (Harper *et al.*, 1984). BCAA levels were not significantly altered in skeletal muscles, but trended lower in the glycolytic white quadriceps, while the renal excretion of BCAAs (and their metabolites)

were markedly decreased in *Ndufs4* KO mice. Taken together, these observations suggest that an increased influx of BCAA in the brain, together with a restriction in the NAD⁺-dependent step of BCAA catabolism, may result in elevated BCAA levels. Furthermore, we postulate that BCAA catabolism could be enhanced in a subset of *Ndufs4* KO tissues, possibly to fuel succinyl-CoA production and the Q-pool via electron-transferring-flavoprotein dehydrogenase (ETFDH, EC 1.5.5.1). Such a tissue-specific difference in BCAA handling has also been proposed to take place in insulin resistant individuals (Newgard, 2012).

5.3.4.2 Aspartate, glutamate, and arginine metabolism

Glutamate (Glu), pyroglutamate, and aspartate levels were significantly decreased in *Ndufs4* KO brain regions and urine (Figure 5-1 A). Among these, the degree of aspartate reduction was greater in the brain, in which asparagine levels were also markedly lower. In accord, pre-symptomatic *Ndufs4* KO mice display trended lower brain-regional levels of a metabolite cluster including Glu, pyroglutamate, aspartate, and ornithine as well as decreased trends in whole brain levels of 3-aminobutyrate and most free amino acids, (Johnson *et al.*, 2020). We additionally observed significant reductions in glutamine (Gln) and 3-aminobutyrate abundance in *Ndufs4* KO urine but not brain regions. Furthermore, alterations in aspartate and Glu metabolism were consistent in *Ndufs4* KO urine and white quadriceps, although less significant in the muscles. These observations could reflect a systemic increase in Gln and Glu consumption, a phenomenon which has recently been linked to compensatory mechanisms that function to decrease mitochondrial redox stress during OXPHOS deficiency. Enhanced Gln-derived anaplerosis of the TCA cycle was shown to regenerate NAD⁺ via aspartate efflux from mitochondria, however, when the level of OXPHOS deficiency exceeds a certain threshold, Gln starts fuelling acetylation and lipogenesis via reductive carboxylation (Chen *et al.*, 2018). Similarly, enhanced Gln-derived proline biosynthesis was shown to regenerate NAD(P)⁺ levels when TCA cycle activity surpasses OXPHOS capacity (Schwörer *et al.*, 2020). While the former mechanism may be a systemic adaption, the latter seems to be exclusive to the *Ndufs4* KO brain, where proline levels were uniquely elevated (Figure 5-1 B). The marked decrease in urine and muscle proline levels further confirm that this putative phenomenon is not systemic. On the contrary, Yang and colleagues recently reported a marked decrease in circulatory Gln turnover flux and Gln anaplerosis in the pancreas and liver of *Ndufs4* KO mice, while Gln's contribution to (or handling within) the TCA cycle did not markedly alter in other tissues (Yang *et al.*, 2020). Our results further indicate a significant decrease in the renal excretion of N-acetylglutamate (NAG) and other urea cycle intermediates (carbamate, ornithine, citrulline, arginine, and urate) in *Ndufs4* KO mice, with the urinary alanine/Glu- and Glu/Gln ratios elevated (FDR- $p < 0.05$, ES $d > 1.2$). Although citrulline levels consistently trended lower in *Ndufs4* KO brain regions (and muscles), the degree and direction of other alterations in arginine metabolism were inconsistent. Contrastingly, most of these metabolites were moderately elevated in both glycolytic and oxidative muscles. Taken

together with the alterations described in previous sections, this might reflect increased muscle proteolysis and amino acid catabolism along with increased ammonia transfer to the liver. Elevated levels of NAG (brain regions) and N-acetylglutamate (muscles) further suggest increased protein acetylation in *Ndufs4* KO tissues.

5.3.4.3 Lipid metabolism

The levels of glycerol along with several carnitine- and glycine conjugated medium chain fatty acids (hexanoyl-, octanoyl- and decanoylcarnitine; butyryl-, valeryl- and hexanoylglycine) were decreased in *Ndufs4* KO urine (and muscle). Glycerol levels were variably affected among *Ndufs4* KO brain regions, while the levels of fatty acids, palmitate (C16:0), oleate (C18:1), and arachidonate (C20:4, ω -6) consistently trended higher (Figure 5-1 E). Cytosolic acetyl-CoA accumulation is known to stimulate lipogenesis, cholesterol synthesis, and protein acetylation (Pietrocola *et al.*, 2015; Saggerson, 2008). Significant elevations were seen in the renal excretion and tissue content of acetylcarnitine, which suggest acetyl-CoA accumulation in *Ndufs4* KO mice, as this membrane permeant storage form of acetyl-CoA serves to dispose of or replenish it as needed (Schroeder *et al.*, 2012). Additionally, the olfactory bulbs, which was most markedly affected, displayed decreased levels of pantothenate (a CoA component) and its precursor, beta-alanine — likely reflecting increased CoA demand. In lesion-prone brain regions, significant reductions were also evident in the levels of 25-hydroxycholesterol, which like arachidonate, has been described to play a regulatory role in cholesterol metabolism (Demetz *et al.*, 2014; Kilsdonk *et al.*, 1995). Interestingly, increasing cholesterol production, export, and utilisation was recently shown to attenuate elevated NAD(P)H levels in CI deficient fibroblast (Schirris *et al.*, 2021). Recent reports of protein hyper-acetylation (Gella *et al.*, 2020; Piroli *et al.*, 2020) and increased lipid synthesis in lesion-prone brain regions (Liu *et al.*, 2017; Liu *et al.*, 2015) as well as elevated levels of triacylglycerol and free fatty acids in the serum of *Ndufs4* KO mice (Jin *et al.*, 2014) further support the notion of acetyl-CoA accumulation and increased lipogenesis in *Ndufs4* KO brain regions. Taken together with the observations discussed in previous sections, the data points to a systemic mismatch between the amount of acetyl-CoA generated via catabolic reactions in *Ndufs4* KO tissues and the processing capacity of the TCA cycle.

5.3.4.4 Choline-, one-carbon-, and nucleotide metabolism

Decreased levels of intermediates in the metabolism of choline to glycine i.e., trimethylglycine (betaine), dimethylglycine and methylglycine (sarcosine) along with methionine, and cystathionine were among the most significant alterations observed in *Ndufs4* KO urine (Figure 5-1 H). Although not as evident in the brain, these alterations were mirrored in the *Ndufs4* KO white quadriceps. Choline-derived betaine remethylates homocysteine to methionine in the methionine cycle, with the subsequent oxidation of dimethylglycine and sarcosine both feeding the Q-pool via ETFDH and ultimately producing glycine. Betaine mainly serves as a methyl donor in the liver, where betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) is highly expressed (Szegedi *et al.*,

2008; Teng *et al.*, 2011). In addition to the liver, renal metabolism is also thought to play an important role in systemic one-carbon (1C) homeostasis, as the kidneys produce the bulk of systemic serine and glycine (Jang *et al.*, 2019). Decreased renal excretion of glycine, serine, histidine, threonine, and cystine in *Ndufs4* KO mice further suggests significant perturbations in hepatic and renal 1C metabolism. Despite discrepancies among *Ndufs4* KO brain regions, betaine and serine levels consistently trend lower (except in the olfactory bulbs) with glycine markedly elevated in the brainstem and reduced in the cerebellum.

Marked reductions in some purine and pyrimidine intermediates were also evident in the olfactory bulbs and anterior cortex (Figure 5-1 I). In agreement, significantly decreased renal excretion of purine- and pyrimidine catabolism end-products, urate and beta-alanine, were observed in *Ndufs4* KO mice along with decreased levels of orotate, uracil, 3-ureidopropionate, and thymine catabolite, 3-amino-isobutyrate. In accordance, nucleotides and products of nucleotide catabolism were also decreased in whole brain of pre-symptomatic *Ndufs4* KO mice (Johnson *et al.*, 2013). As purine and pyrimidine nucleotides are important precursors for the synthesis of nucleotide cofactors such as NAD⁺ and S-adenosyl methionine (SAM), these alterations are likely linked to perturbations in redox balance and 1C metabolism. Several studies have functionally linked 1C metabolism and serine catabolism, specifically, to the OXPHOS system and deficiencies thereof (Bao *et al.*, 2016; Lucas *et al.*, 2018; Nikkanen *et al.*, 2016); with a maladaptive increase in the fraction of NADH produced by folate-mediated serine catabolism recently reported in *Ndufs4* KO mice (Yang *et al.*, 2020). Altogether, our data shows that serine-, 1C-, and nucleotide- metabolism is markedly perturbed in *Ndufs4* KO mice, with the methylation cycle most dramatically affected. While these alterations in the urine metabolome likely reflect hepatic and renal metabolism, 1C-metabolism linked perturbations are also evident in *Ndufs4* KO brain regions and muscles.

5.3.5 Contributions of gut microbial metabolism

Several metabolic alterations in *Ndufs4* KO urine seemed to reflect altered gut microbial metabolism (Figure 5-S2). This includes significantly elevated levels of 2,3-butandiol, 2,6-dihydroxybenzoate, citramalate and propionylcarnitine along with decreased levels of 3-hydroxyphenyl (HP) propanoate, 4-HP-glycolate, 4-HP-acetate, 4-HP-pyruvate. 2,3-Butandiol is known to accumulate in the urine of MD patients and thought to reflect high systemic levels of glucose that is further metabolised by gut microbes (Elshaghabe *et al.*, 2016; Esterhuizen *et al.*, 2019; Venkataraman *et al.*, 2014). Gut microbiota are also major producers of short chain fatty acids (SCFs), especially propionate and butyrate (Jang *et al.*, 2019). Therefore, markedly altered SCF acyl-conjugates in *Ndufs4* KO mice may be related to intestinal metabolism. It was recently shown that gut microbiota can metabolise host-derived lactate to SCFs, predominantly propionate, which can then cross back into the host (Scheiman *et al.*, 2019). Since propionylcarnitine levels were decreased in most *Ndufs4* KO brain regions (and muscles), the

urinary accumulation of propionylcarnitine is likely related to lactate accumulation in *Ndufs4* KO mice and gut-derived elevations in propionate. In support, increased urinary citramalate excretion is also seen with propionyl-CoA accumulation (in propionyl-CoA carboxylase deficiency) and is associated with intestinal dysbiosis (Greter *et al.*, 1980). Furthermore, reduced urinary excretion of 4-HP-acetate and 4-HP-pyruvate may reflect perturbations in host-microbial co-metabolism of aromatic amino acids (Li *et al.*, 2021). This is supported by decreased levels of phenylalanine, tryptophan, kynurenine, and 5-hydroxy-indole exclusively in *Ndufs4* KO urine. Alterations in gut microbiota have been reported in a variety of disease phenotypes with this host-gut microbiota interaction recently linked to changes in mitochondrial function and redox status (Yardeni *et al.*, 2019). Accordingly, our data indicates that altered microbial compositions or metabolism likely contribute to metabolic observations in biofluids of LS patients. Furthermore, it must be noted that when working with mouse models, microbiota transfer between mice via coprophagy could influence metabolomes when genotypes are cohoused — as was done in this study. Although the ingestion of feces by self-grooming is considered more probable, the transfer of microbiota between genotypes cannot be ruled out and measures should be taken to avoid this (Brinkman *et al.*, 2013; Laukens *et al.*, 2016). Together, these observations also highlight the necessity of future studies investigating host-bacterial co-metabolism and the gut-brain axis in LS.

5.4 Conclusions

Here, we integrate and cross-compare multi-platform metabolomics data from urine and tissues of late stage diseased *Ndufs4* KO vs WT mice to better understand how systemic biofluids reflect tissue-specific metabolic alterations in LS. We confirm that biofluid markers associated with MD (elevated alanine, pyruvate, and lactate) and several other metabolic signatures in urine reflect metabolic alterations in the LS brain, thereby verifying the value of urine for the monitoring of these metabolic markers. In addition to glycolytic and TCA cycle intermediates, it would be advantageous to investigate (and validate) whether the following urinary metabolites could serve as biomarkers of LS and MD: amino acids and derivatives related to alanine, aspartate and glutamate-; arginine and proline-; BCAA-; lysine-; histidine-; and choline metabolism; as well as sugar derivatives; metabolites related to the G3P shuttle; and gut microbial metabolism. Widespread reductions were evident in the abundance of several metabolites capable of fuelling Q-pool linked dehydrogenases (other than CI) in *Ndufs4* KO mice, therefore, we hypothesise that tissue-specific differences in these dehydrogenases could influence metabolic flexibility and the consequences of CI deficiency in different tissues. Our data further reveals that impaired BCAA catabolism, increased proline biosynthesis, and the accumulation of glycolytic intermediates are brain-specific metabolic perturbations in LS. Contrastingly, elevated levels of TCA cycle intermediates in urine, and significant reductions in one-carbon metabolism intermediates, are not clearly reflected in the *Ndufs4* KO brain and appear to be systemic of origin. Furthermore, the discrepancies we report between urinary and tissue-specific metabolomes highlight the

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shortcomings of only using systemic biofluids as an investigative tool for multi-systemic diseases. Although non- or minimally invasive systemic samples contain valuable information, these metabolomes should be carefully interpreted in conjunction with tissue-specific data — especially when aiming to elucidate disease mechanisms. In this regard, having reliable MD models and access tissue samples that cannot be obtained from patients, is vital to improve our understanding of complex MD processes. Additionally, future sex-specific studies, targeting the areas of metabolism identified here, should be a priority. Almost all aspects of metabolism and energy homeostasis are regulated in a sex-specific manner, which influences disease pathogenesis. Sex hormones and -chromosomes along with differences in tissue-specific gene regulation and gut microbiota between sexes play a central role in this metabolic sexual dimorphism (Chella Krishnan *et al.*, 2018; Clegg & Mauvais-Jarvis, 2018). Indeed, many metabolic pathways affected in male *Ndufs4* KO mice, have been shown to display sexual dimorphism in murine models and/or humans. This includes glucose and lipid metabolism (Baars *et al.*, 2018; Chella Krishnan *et al.*, 2018; Clegg & Mauvais-Jarvis, 2018; Lundsgaard & Kiens, 2014), amino acid and acylcarnitine metabolism (Ruoppolo *et al.*, 2018; Wu & Gao, 2015), branched-chain amino acid metabolism (Vignoli *et al.*, 2018), and bone metabolism (Choi *et al.*, 2021).

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5.6 Supplementary data

Table 5-S1: Metabolite identification levels.

Metabolite	Platform	ID level						
		WQ	SOL	AC	BS	CB	OB	U
1-Methylhistidine	LC	1	1	1	1	1	1	1
2,3-Butandiol	GC	ND	ND	ND	ND	ND	ND	3
2,6-Dihydroxybenzoate	GC	ND	ND	ND	ND	ND	ND	3
25-Hydroxycholesterol	GC	ND	ND	2	2	2	2	ND
2-Aminoadipate	LC	1	1	1	1	1	1	1
2-Hydroxyadipate	GC	ND	ND	ND	ND	ND	ND	3
2-Hydroxyglutarate	GC	1	1	3	3	3	3	1
2-Ketoglutarate	GC	ND	ND	ND	ND	ND	3	1
3-(3-Hydroxyphenyl)propanoate	GC	ND	ND	ND	ND	ND	ND	3
3-Aminobutyrate (GABA)	LC	1	1	1	1	1	1	1
3-Aminoisobutyrate (BAIBA)	LC	ND	3	ND	ND	ND	ND	3
3-Hydroxymethylglutarate	GC	ND	ND	ND	ND	ND	ND	3
3-Methylhistidine	LC	1	1	1	1	1	1	1
3-Ureidopropionate	GC	ND	ND	ND	ND	ND	ND	3
4-hydroxymandelate	GC	ND	ND	ND	ND	ND	ND	3
4-Hydroxyphenylacetate	GC	ND	ND	ND	ND	ND	ND	1
4-Hydroxyphenylpyruvate	GC	ND	ND	ND	ND	ND	ND	3
5-Hydroxy-indole	GC	ND	ND	ND	ND	ND	ND	3
5-Hydroxylysine	LC	ND	ND	ND	ND	ND	ND	1
9S,10S-Dihydroxystearate	GC	ND	ND	3	3	3	3	ND
Acetylcarnitine (C2)	LC	1	1	1	1	1	1	1
Adenosine	GC	ND	ND	1	1	ND	1	ND
Alanine	LC	1	1	1	1	1	1	1
Arachidonate (C20:4, ω -6)	GC	ND	ND	1	1	1	1	ND
Arginine	LC	1	1	1	1	1	1	1
Ascorbate	GC	ND	ND	ND	ND	ND	ND	3
Asparagine	LC	1	1	1	1	1	1	1
Aspartate	LC	1	1	1	1	1	1	1
Beta-alanine	LC	ND	ND	1	1	1	1	1
Butyrylcarnitine (C4)	LC	1	1	1	1	1	1	1
Butyrylglycine (C4)	GC	ND	ND	ND	ND	ND	ND	3
Carbamate	GC	ND	ND	3	3	3	3	3
Carnitine (C0)	LC	1	1	1	1	1	1	1
Citramalate	GC	ND	ND	ND	ND	ND	ND	3
Citrulline	LC	1	1	1	1	1	1	1
Creatine	LC	1	1	1	1	1	1	1
Creatinine	GC	3	3	3	3	3	3	ND
Cystathionine	LC	1	1	1	1	1	1	1
Cysteine	GC	ND	ND	1	1	1	1	ND
Cystine	LC	ND	ND	1	1	ND	ND	1
Decanoylcarnitine (C10)	LC	ND	ND	ND	ND	ND	ND	1
Dihydroxyacetone phosphate (DHAP)	GC	ND	ND	3	3	3	3	ND
Dimethylglycine	LC	1	1	ND	ND	ND	ND	1
Erythronate	GC	ND	ND	3	ND	3	3	ND
Erythrose	GC	ND	ND	2	2	2	2	2
Formylglycine	GC	ND	ND	3	3	3	3	ND
Fructose-6-phosphate	GC	ND	ND	3	3	ND	3	ND
Fumarate	GC	1	1	1	1	1	1	1
Glucarate	GC	ND	ND	ND	ND	ND	ND	3
Glucose	GC	2	2	3	2	3	2	2
Glucose-1-phosphate	GC	ND	ND	ND	3	ND	3	ND
Glucose-6-phosphate	GC	ND	ND	ND	ND	ND	2	ND
Glutamate	LC	1	1	1	1	1	1	1
Glutamine	LC	1	1	1	1	1	1	1
Glutarate	GC	ND	ND	ND	ND	ND	ND	3
Glycerate	GC	1	3	ND	ND	ND	ND	1
Glycerol	GC	1	3	3	3	3	3	3
Glycerol-3-phosphate (G3P)	GC	ND	ND	3	3	3	3	3

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Metabolite	Platform	ID level						
		WQ	SOL	AC	BS	CB	OB	U
Glycine	LC	1	1	1	1	1	1	1
Glycolate	GC	3	ND	3	3	3	3	ND
Gulonate	GC	ND	ND	ND	ND	ND	ND	3
Hexanoylcarnitine (C6)	LC	1	1	ND	ND	ND	1	1
Hexanoylglycine (C6)	GC	ND	ND	ND	ND	ND	ND	3
Histidine	LC	1	1	1	1	1	1	1
Hydroxymalonate (Tartronate)	GC	ND	ND	ND	ND	ND	ND	3
Hydroxyproline	LC	1	1	1	1	1	1	1
Isoleucine	GC	1	1	1	1	1	1	ND
Isoleucine-leucine	LC	1	1	1	1	1	1	1
Isothreonine	LC	ND	ND	1	1	1	1	1
Isovalerylcarnitine (C5)	LC	1	1	1	1	1	1	1
Isovalerylglycine (C5)	GC	ND	ND	ND	ND	ND	ND	3
Kynurenine	LC	ND	ND	ND	ND	ND	ND	1
Lactate	GC	1	1	1	1	1	1	1
Leucine	GC	1	1	1	1	1	1	ND
Lysine	LC	1	1	1	1	1	1	1
Malate	GC	1	1	2	2	2	2	1
Malonate	GC	ND	ND	2	2	2	1	3
Mannonate	GC	ND	ND	2	2	2	2	1
Methionine	LC	1	1	1	1	1	1	1
Methylglycine (sarcosine)	LC	1	1	ND	ND	ND	ND	1
Methylsuccinate	GC	ND	ND	ND	ND	ND	ND	3
N-acetylaspartate	LC	1	1	1	1	1	1	ND
N-acetylglutamate	LC (GC for U)	1	1	1	1	1	1	3
Octanoylcarnitine (C8)	LC	1	1	1	1	1	1	1
Oleate (C18:1)	GC	3	3	1	1	1	1	ND
Ornithine	LC	1	1	1	1	1	1	1
Orotate	GC	ND	ND	ND	ND	ND	ND	3
Oxalate	GC	1	1	1	1	1	1	1
Palmitate (C16:0)	GC	1	1	1	1	1	1	ND
Pantothenate (vitamin B5)	GC	ND	ND	3	3	3	3	ND
Phenylalanine	LC	1	1	1	1	1	1	1
Pipecolate	LC	1	1	1	1	1	1	ND
Proline	LC	1	1	1	1	1	1	1
Propionylcarnitine (C3)	LC	1	1	1	1	1	1	1
Putrescine	GC	ND	ND	3	3	3	3	ND
Pyroglutamate	GC	3	1	1	1	1	1	1
Pyruvate	GC	1	2	2	2	2	2	1
Ribitol	GC	ND	ND	3	3	3	3	3
Ribonate	GC	ND	ND	ND	ND	ND	ND	3
Ribose	GC	ND	ND	3	3	ND	ND	2
Serine	LC	1	1	1	1	1	1	1
Sorbose	GC	ND	ND	ND	ND	ND	ND	2
Stearate (C18:0)	GC	1	1	1	1	1	1	ND
Succinate	GC	1	1	1	1	1	1	1
Taurine	LC	1	1	1	1	1	1	ND
Threonate	GC	ND	ND	ND	ND	ND	ND	3
Threonine	LC	1	1	1	1	1	1	1
Threose	GC	ND	ND	2	2	2	2	ND
Trimethylglycine (betaine)	LC	1	1	1	1	1	1	1
Tryptophan	LC	1	1	1	1	1	1	1
UMP	GC	ND	ND	3	3	ND	3	ND
Uracil	GC	ND	1	1	1	1	1	1
Urate	GC	ND	ND	ND	ND	ND	ND	1
Urea	GC	3	1	3	3	3	3	3
Valerylglycine (C5)	GC	ND	ND	ND	ND	ND	ND	3
Valine	LC	1	1	1	1	1	1	1

Identification levels were assigned according to the classification system of Schymanski et al. (2014), i.e., level 1= confirmed structures (reference standard); level 2=probable structure (library spectrum match); level 3=putative candidate (structure, substituent, class) Abbreviations: AC, anterior cortex; BS, brainstem; CB, cerebellum; GC, GC-TOF-MS; LC, LC-MS/MS; ND, not (reliably) detected; OB, olfactory bulbs; SOL, solei; U, urine; WQ, white quadriceps

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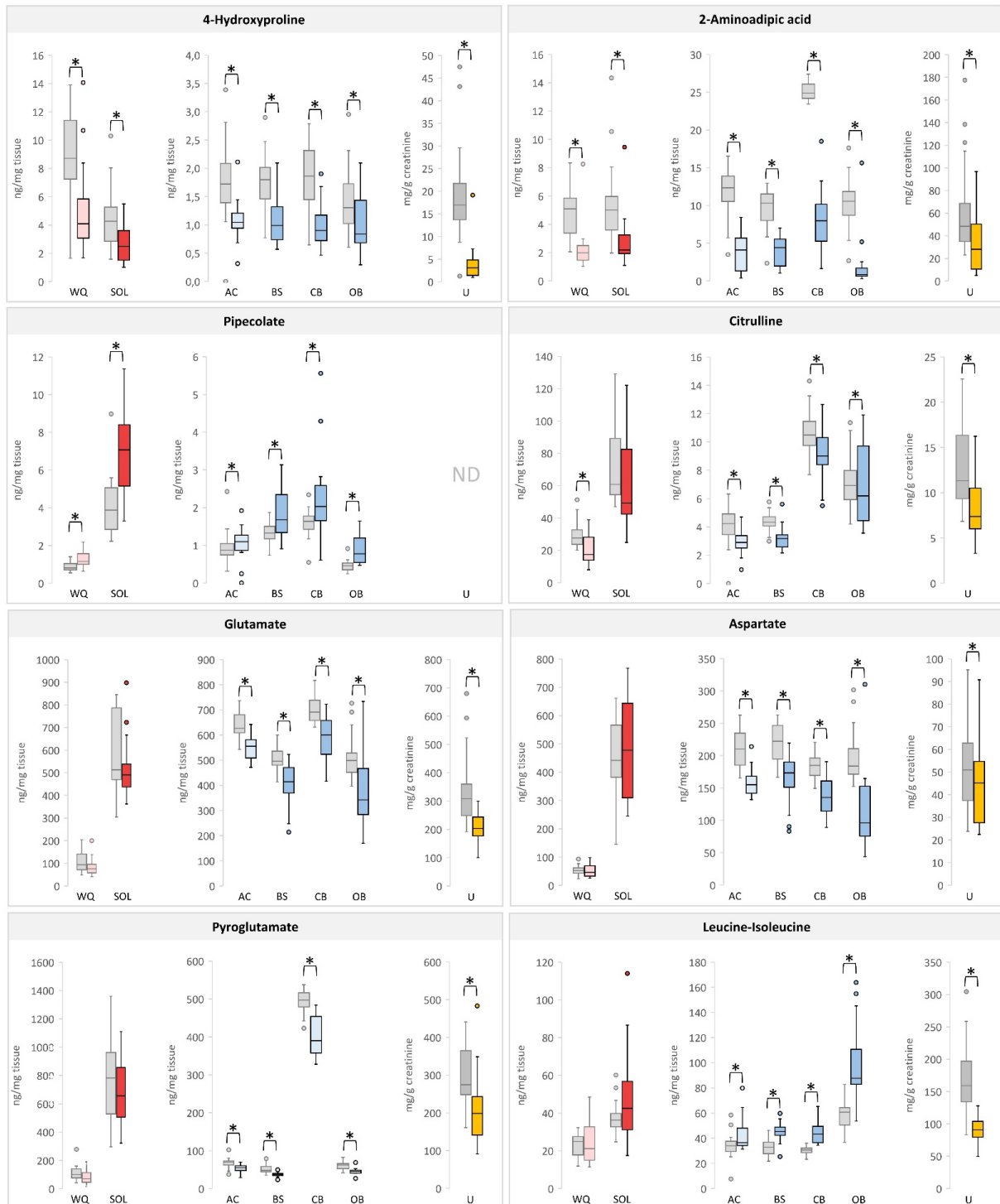


Figure 5-S1: Relative abundance of some metabolites measured in *Ndufs4* KO vs WT sample types. Box plots depict the relative abundance of each metabolite in ng/mg tissue (for tissues) or mg/g creatinine (for urine) for all biological replicates per genotype before outlier removal and log transformation. Grey and coloured boxes respectively indicate WT and *Ndufs4* KO groups. The length of the box represents 25th to 75th percentile inter-quartile range, interior horizontal line represents median, and vertical lines stemming from the box extend to minimum and maximum values. Circles indicate outlier values. Asterisks indicate statistical significance (FDR- $p < 0.05$).

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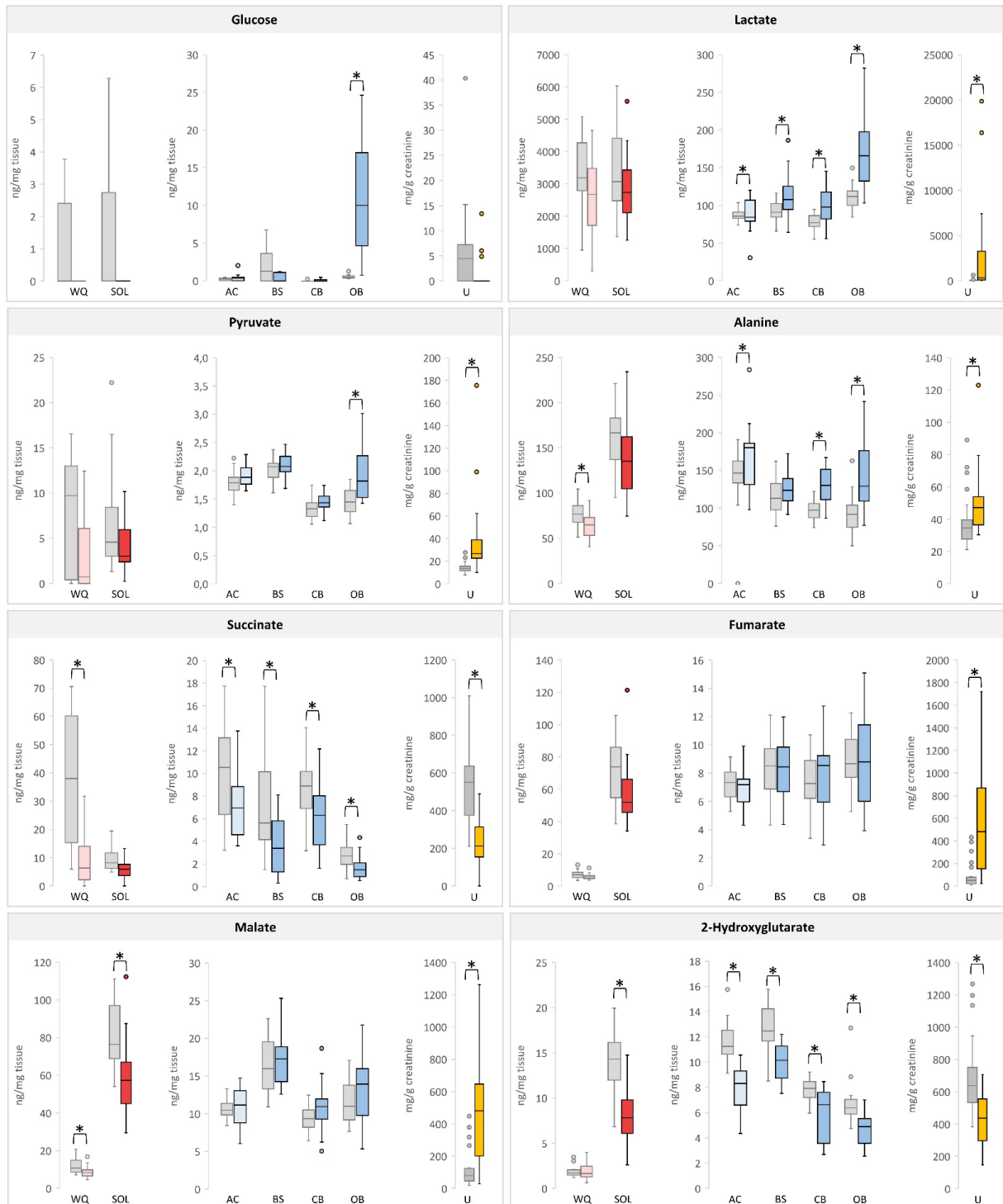


Figure 5-S1: Relative abundance of some metabolites measured in *Ndufs4* KO vs WT sample types (continued)

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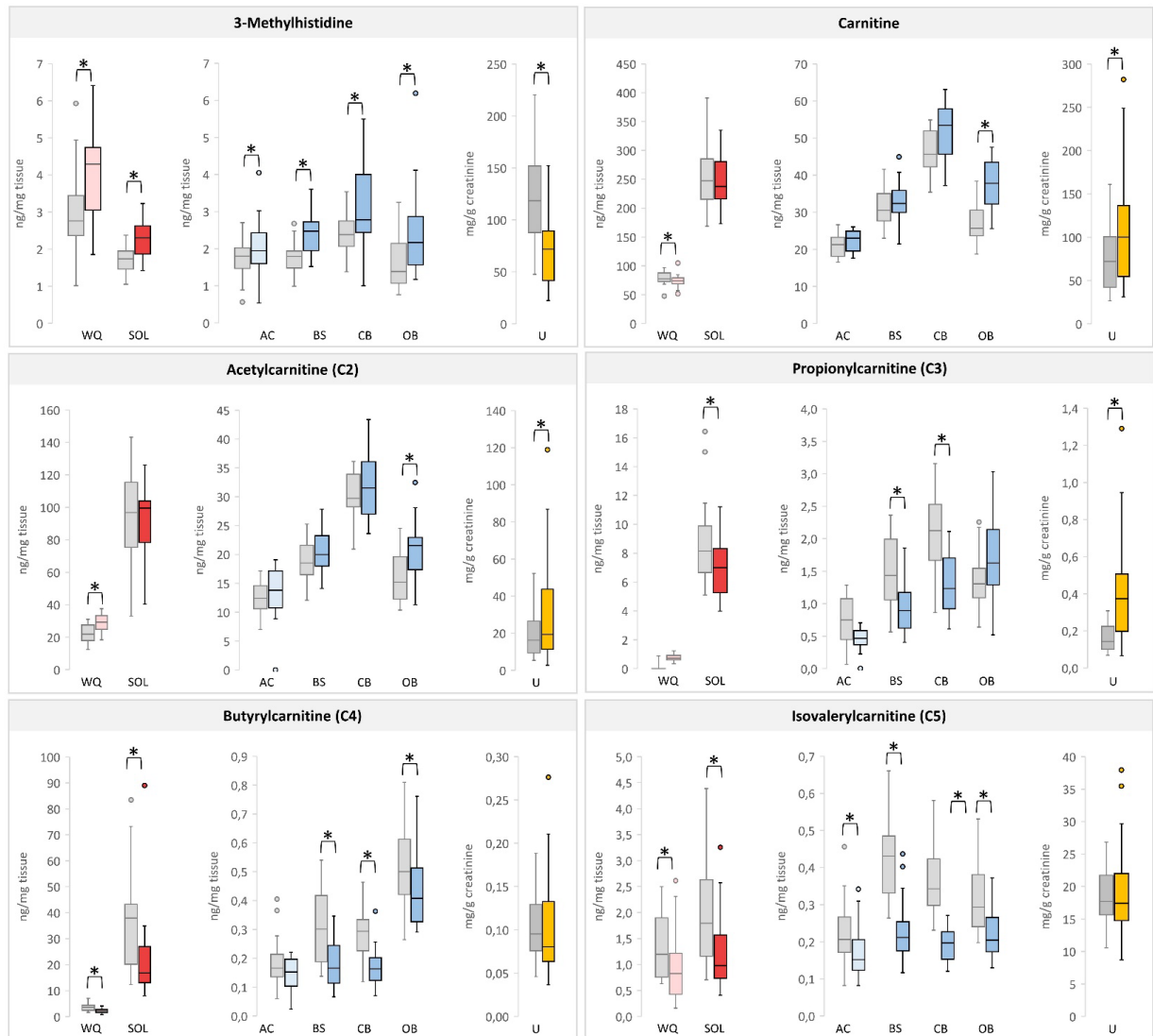


Figure 5-S1: Relative abundance of some metabolites measured in *Ndufs4* KO vs WT sample types (continued)

Intestinal microbiota		WQ	SOL	AC	BS	CB	OB	U	KEY	d-value	≥2.0	≥1.2	≥0.8	≥0.5	≥0.2	* FDR-adjusted <i>p</i> <0.05	ND Not detected	
GC	4-hydroxymandelate	ND	ND	ND	ND	ND	ND	-1.86*		↑								
GC	4-Hydroxyphenylacetate	ND	ND	ND	ND	ND	ND	-0.99*		↑								
GC	4-Hydroxyphenylpyruvate	ND	ND	ND	ND	ND	ND	-0.88*		↓								
GC	3-(3-Hydroxyphenyl)propanoate	ND	ND	ND	ND	ND	ND	-0.66*										
GC	2,6-Dihydroxybenzoate	ND	ND	ND	ND	ND	ND	1.08*										
GC	2,3-Butandiol	ND	ND	ND	ND	ND	ND	1.41*										
GC	Citramalate	ND	ND	ND	ND	ND	ND	1.79*										
LC	Propionylcarnitine (C3)	-0.44	-0.78	-0.45	-1.22	-1.39	0.36	1.35*										

Figure 5-S2: Metabolic alterations in *Ndufs4* KO mice related to intestinal microbiota. Heatmap of effect size (Cohen's *d*) values for each metabolite when relative abundance is compared between *Ndufs4* KO ($n \geq 19$) and WT ($n \geq 20$) mice. Shades of purple and green respectively indicate increased and decreased abundance in *Ndufs4* KO mice and correspond to the level of practical significance as indicated in the key. Asterisks indicate statistical significance (FDR-adjusted $p < 0.05$). The first column indicates the analytical platform used for detection. Abbreviations: AC; anterior cortex; BS, brainstem; CB; cerebellum; GC; GC-TOF-MS; LC; LC-MS/MS; ND, not (reliably) detected; OB, olfactory bulbs; SOL, solei; U, urine; WQ, white quadriceps

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Table 5-S2: Pathway over-representation and topology analysis results

Sample type	Total	Expected	Hits	p-value	FDR-p	Impact
White quadriceps (WQ)						
Glycine serine and threonine metabolism	34	0,72	7	3,5E-06	3,0E-04	0,29
Aminoacyl-tRNA biosynthesis	48	1,02	7	3,9E-05	1,7E-03	0,00
Alanine aspartate and glutamate metabolism	28	0,59	5	2,2E-04	6,1E-03	0,20
Arginine biosynthesis	14	0,30	3	2,7E-03	5,7E-02	0,23
Lysine degradation	25	0,53	3	1,5E-02	2,5E-01	0,14
Glyoxylate and dicarboxylate metabolism	32	0,68	3	2,8E-02	4,0E-01	0,30
Solei (SOL)						
Arginine biosynthesis	14	0,20	4	3,1E-05	1,6E-03	0,14
Aminoacyl-tRNA biosynthesis	48	0,70	6	3,9E-05	1,6E-03	0,17
Alanine aspartate and glutamate metabolism	28	0,41	4	5,6E-04	1,6E-02	0,09
Lysine degradation	25	0,37	3	5,1E-03	1,1E-01	0,14
Arginine and proline metabolism	38	0,56	3	1,6E-02	2,8E-01	0,23
Histidine metabolism	16	0,23	2	2,2E-02	3,0E-01	0,22
Glycine serine and threonine metabolism	34	0,50	2	8,6E-02	1,0E+00	0,27
Urine (U)						
Aminoacyl-tRNA biosynthesis	48	2,39	17	1,4E-11	1,2E-09	0,17
Arginine biosynthesis	14	0,70	9	1,9E-09	8,0E-08	0,48
Glycine serine and threonine metabolism	34	1,69	10	2,6E-06	7,2E-05	0,72
Alanine aspartate and glutamate metabolism	28	1,39	9	3,7E-06	7,9E-05	0,67
Valine leucine and isoleucine biosynthesis	8	0,40	4	3,4E-04	5,3E-03	0,00
Arginine and proline metabolism	38	1,89	8	3,8E-04	5,3E-03	0,43
Glyoxylate and dicarboxylate metabolism	32	1,59	7	7,1E-04	8,5E-03	0,23
Pantothenate and CoA biosynthesis	19	0,95	5	1,8E-03	1,9E-02	0,05
D-Glutamine and D-glutamate metabolism	6	0,30	3	2,1E-03	1,9E-02	0,50
Citrate cycle (TCA cycle)	20	1,00	5	2,3E-03	1,9E-02	0,21
beta-Alanine metabolism	21	1,05	5	2,9E-03	2,2E-02	0,50
Butanoate metabolism	15	0,75	4	5,1E-03	3,6E-02	0,03
Histidine metabolism	16	0,80	4	6,5E-03	4,2E-02	0,22
Phenylalanine tyrosine and tryptophan biosynthesis	4	0,20	2	1,4E-02	8,3E-02	0,50
Cysteine and methionine metabolism	33	1,64	5	2,1E-02	1,2E-01	0,30
Nitrogen metabolism	6	0,30	2	3,2E-02	1,7E-01	0,00
Pyrimidine metabolism	39	1,94	5	4,1E-02	1,9E-01	0,14
Glycerolipid metabolism	16	0,80	3	4,2E-02	1,9E-01	0,37
Glutathione metabolism	28	1,39	4	4,7E-02	2,1E-01	0,12
Ascorbate and aldarate metabolism	10	0,50	2	8,5E-02	3,5E-01	0,00
Pentose phosphate pathway	22	1,10	3	9,3E-02	3,5E-01	0,05
Pyruvate metabolism	22	1,10	3	9,3E-02	3,5E-01	0,21
Anterior cortex						
Aminoacyl-tRNA biosynthesis	48	1,08	11	1,7E-09	1,5E-07	0,17
Arginine biosynthesis	14	0,32	6	2,2E-07	9,3E-06	0,48
Arginine and proline metabolism	38	0,86	8	8,6E-07	2,4E-05	0,51
Alanine aspartate and glutamate metabolism	28	0,63	6	2,2E-05	4,6E-04	0,42
Glutathione metabolism	28	0,63	5	2,9E-04	4,9E-03	0,04
Valine leucine and isoleucine biosynthesis	8	0,18	3	5,5E-04	7,6E-03	0,00
Histidine metabolism	16	0,36	3	4,8E-03	5,8E-02	0,00
Glycine serine and threonine metabolism	34	0,77	4	6,2E-03	6,5E-02	0,21
Pantothenate and CoA biosynthesis	19	0,43	3	8,0E-03	7,4E-02	0,00
Glyoxylate and dicarboxylate metabolism	32	0,72	3	3,3E-02	2,8E-01	0,04
Cysteine and methionine metabolism	33	0,75	3	3,6E-02	2,8E-01	0,12
Butanoate metabolism	15	0,34	2	4,3E-02	3,0E-01	0,00
Glycerolipid metabolism	16	0,36	2	4,9E-02	3,1E-01	0,04
Valine leucine and isoleucine degradation	40	0,90	3	5,9E-02	3,5E-01	0,00
Citrate cycle (TCA cycle)	20	0,45	2	7,3E-02	4,1E-01	0,08

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Sample type	Total	Expected	Hits	p-value	FDR-p	Impact
Brainstem (BS)						
Aminoacyl-tRNA biosynthesis	48	1,12	9	5,9E-07	5,0E-05	0,00
Arginine biosynthesis	14	0,33	5	8,7E-06	3,7E-04	0,41
Alanine aspartate and glutamate metabolism	28	0,65	6	2,6E-05	7,4E-04	0,42
Arginine and proline metabolism	38	0,88	6	1,6E-04	3,4E-03	0,44
Glutathione metabolism	28	0,65	5	3,4E-04	5,7E-03	0,12
Valine leucine and isoleucine biosynthesis	8	0,19	3	6,0E-04	8,3E-03	0,00
Histidine metabolism	16	0,37	3	5,2E-03	5,5E-02	0,00
Glycerolipid metabolism	16	0,37	3	5,2E-03	5,5E-02	0,28
Glycolysis / Gluconeogenesis	26	0,60	3	2,1E-02	1,9E-01	0,10
Glyoxylate and dicarboxylate metabolism	32	0,74	3	3,6E-02	3,0E-01	0,11
Butanoate metabolism	15	0,35	2	4,6E-02	3,5E-01	0,00
Valine leucine and isoleucine degradation	40	0,93	3	6,3E-02	4,1E-01	0,00
Fructose and mannose metabolism	18	0,42	2	6,4E-02	4,1E-01	0,04
Pantothenate and CoA biosynthesis	19	0,44	2	7,0E-02	4,2E-01	0,00
Citrate cycle (TCA cycle)	20	0,46	2	7,7E-02	4,3E-01	0,08
Primary bile acid biosynthesis	46	1,07	3	8,8E-02	4,5E-01	0,06
Pyruvate metabolism	22	0,51	2	9,1E-02	4,5E-01	0,21
Cerebellum (CB)						
Aminoacyl-tRNA biosynthesis	48	1,21	12	3,9E-10	3,2E-08	0,17
Arginine biosynthesis	14	0,35	5	1,3E-05	5,6E-04	0,42
Valine leucine and isoleucine biosynthesis	8	0,20	4	2,2E-05	6,3E-04	0,00
Alanine aspartate and glutamate metabolism	28	0,71	6	4,3E-05	9,1E-04	0,42
Glycine serine and threonine metabolism	34	0,86	5	1,3E-03	2,1E-02	0,52
Arginine and proline metabolism	38	0,96	5	2,1E-03	3,0E-02	0,28
Histidine metabolism	16	0,40	3	6,6E-03	7,8E-02	0,00
Glyoxylate and dicarboxylate metabolism	32	0,81	4	7,5E-03	7,8E-02	0,15
Cysteine and methionine metabolism	33	0,83	4	8,3E-03	7,8E-02	0,30
Pantothenate and CoA biosynthesis	19	0,48	3	1,1E-02	9,1E-02	0,00
Valine leucine and isoleucine degradation	40	1,01	4	1,6E-02	1,3E-01	0,01
Glycolysis / Gluconeogenesis	26	0,66	3	2,6E-02	1,8E-01	0,10
Glutathione metabolism	28	0,71	3	3,1E-02	2,0E-01	0,12
Butanoate metabolism	15	0,38	2	5,3E-02	3,2E-01	0,00
Citrate cycle (TCA cycle)	20	0,50	2	8,9E-02	5,0E-01	0,08
Olfactory bulbs (OB)						
Aminoacyl-tRNA biosynthesis	48	1,59	12	1,3E-08	1,1E-06	0,00
Alanine aspartate and glutamate metabolism	28	0,93	8	1,6E-06	6,6E-05	0,59
Valine leucine and isoleucine biosynthesis	8	0,27	4	6,8E-05	1,9E-03	0,00
Arginine and proline metabolism	38	1,26	7	1,7E-04	3,5E-03	0,42
Arginine biosynthesis	14	0,46	4	8,4E-04	1,4E-02	0,42
Glycolysis / Gluconeogenesis	26	0,86	5	1,3E-03	1,8E-02	0,10
Pantothenate and CoA biosynthesis	19	0,63	4	2,9E-03	3,5E-02	0,03
Butanoate metabolism	15	0,50	3	1,2E-02	1,1E-01	0,03
Starch and sucrose metabolism	15	0,50	3	1,2E-02	1,1E-01	0,34
Histidine metabolism	16	0,53	3	1,4E-02	1,1E-01	0,00
Glycerolipid metabolism	16	0,53	3	1,4E-02	1,1E-01	0,28
Biosynthesis of unsaturated fatty acids	36	1,20	4	2,9E-02	2,0E-01	0,00
Lysine degradation	25	0,83	3	4,7E-02	3,1E-01	0,14
Glutathione metabolism	28	0,93	3	6,3E-02	3,7E-01	0,03
Neomycin kanamycin and gentamicin biosynthesis	2	0,07	1	6,5E-02	3,7E-01	0,00

CHAPTER

Summary and conclusions



6

6.1 Introduction

Primary complex I (CI) deficiency is the leading cause of mitochondrial disease (MD) that most often manifests as Leigh syndrome (LS) in children — a devastating, often heterogenous and multisystemic, neurometabolic disease for which there is no effective treatment (Rahman *et al.*, 1996; Rodenburg, 2016). LS typically features a neurodegenerative phenotype with symmetrical bilateral necrotic lesions in specific brain regions (Lake *et al.*, 2016; Rahman *et al.*, 2017). The *Ndufs4* KO mouse model of CI-deficiency related LS displays progressive focal lesion in the olfactory bulbs, brainstem, and cerebellum, whereas other brain regions (like the anterior cortex) and peripheral tissues (like skeletal muscle) seem relatively unaffected phenotypically (Alam *et al.*, 2015; Bolea *et al.*, 2019; Kruse *et al.*, 2008; Quintana *et al.*, 2010). While *Ndufs4* KO blood (Grange *et al.*, 2021; Jain *et al.*, 2016; Jin *et al.*, 2014; Kruse *et al.*, 2008) and brain (Johnson *et al.*, 2020; Johnson *et al.*, 2013) metabolomes have been somewhat explored, the mechanisms underlying this tissue-specific vulnerability to global CI deficiency remain elusive and an integrated, system-wide analysis of metabolomes in diseased *Ndufs4* KO mice is lacking.

Therefore, this study aimed to mine and cross compare multi-platform metabolomics data from brain regions, skeletal muscles, and urine in diseased *Ndufs4* KO and WT mice to gain mechanistic insight into regional neurodegeneration and establish how well the urinary metabolome, which is often used for MD investigations/monitoring in patients, reflects tissue-specific metabolic alterations. In the following sections, the findings and conclusions drawn from each of the four main research objectives are summarised. Thereafter, the strengths, limitations, and prospects of the research are discussed.

6.2 Summary of the research findings

6.2.1 Objective 1: Collect brain regions, skeletal muscles, and urine from *Ndufs4* KO and WT mice

Heterozygous *Ndufs4* mice were successfully interbred to obtain *Ndufs4* KO and WT animals for the larger project, of which this study forms part. This process proved to be lengthy, as *Ndufs4* KO mice seemed to be born at a rate lower than the expected Mendelian ratio (Mereis, 2018). Additional factors that made sample collection for this study particularly time-consuming, is the waiting period of 45-50 days required for late-stage disease progression, the fact that only male animals were sought, and the relatively large animal numbers (approximately 19) required per group. Nevertheless, the breeding- and genotyping strategy was effectively managed to maximise the number of animals obtained in the time set out for sample collection. Furthermore, litters were successfully genotyped — after weaning and *post-mortem* — using the selected PCR technique, with results similar to that reported by Valsecchi *et al.* (2012); thereby confirming that samples of the correct genotype were collected. Another vital aspect of the study design was ensuring metabolic data integrity through appropriate (and homogenous) pre-analytical sample handling

and storage conditions. First, to reduce physiological variation due to nutritional status/feeding habits and circadian rhythm, animals fasted overnight (12 hours) in individual metabolic cages during urine collection, after which tissue samples were collected at the same time of day (8:00-9:00 AM). Second, due to the anaesthetic hypersensitivity of *Ndufs4* KO mice (Quintana *et al.*, 2012; Roelofs *et al.*, 2014), animals were euthanised via cervical dislocation to prevent adverse effects. Third, to minimise metabolite degradation, all tissue samples were swiftly snap frozen after collection and stored at -80 °C, with a total 15-minute time limit selected from euthanasia to tissue snap-freezing. Samples were also handled at low temperatures during preparation, where possible, with freeze-thaw cycles kept to a minimum. Taken together, the first research objective was successfully achieved.

6.2.2 Objective 2: Perform multi-platform metabolic profiling of *Ndufs4* KO and WT brain regions, skeletal muscles, and urine

Multi-platform metabolomics was successfully performed on seven different sample types from *Ndufs4* KO ($n \geq 19$) vs WT ($n \geq 20$) mice, including one lesion-resistant brain region (anterior cortex), three lesion-prone brain regions (brainstem, cerebellum, olfactory bulbs), primarily glycolytic (white quadriceps) and oxidative (solei) skeletal muscles, and urine. These analyses were performed by several individuals, including the author, as part of a larger project. As detailed in Chapter 3, both untargeted GC-TOF-MS and semi-targeted LC-MS/MS were done on a single sample from each animal. Samples of the same type (e.g., all cerebella) were blocked together in randomised batches containing quality control (QC) samples and analysed over two to three days on each platform, for each sample type. The data quality assessments (Appendix C) subsequently performed in this study, confirmed the suitability of all fourteen datasets. For each sample type and platform, QC samples showed good repeatability and intermediate precision, with no significant batch effects evident. Urinary samples, which required minimal preparation, showed the lowest variance, while the two quantity-limited tissues, the olfactory bulbs and solei, exhibited slightly higher variance than other sample types, perhaps due to the particular conditions required for their preparation and analysis — such as the use of larger extraction volumes per sample weight; different derivatisation solvent volumes; different vials/inserts for sample preparation; and larger injection volumes or sample concentration. Conclusively, the second research objective was adequately met.

6.2.3 Objective 3: Compare brain regional metabolomes from *Ndufs4* KO vs WT mice

The eight brain-regional metabolic datasets were pretreated, preprocessed, and statistically analysed as described in Chapter 3 (Section 3.4.7-8). Thereafter, the metabolic data was examined and interpreted alongside brain-regional RC enzymatic data. The results (Chapter 4) were published in the peer-reviewed journal, *Biochimica et Biophysica Acta (BBA) — Molecular Basis of Disease* (Terburgh *et al.*, 2021a).

Contrary to that reported for brain regions of pre-symptomatic mice (Johnson *et al.*, 2020), we observed numerous significant brain-regional metabolic alterations between diseased *Ndufs4* KO vs WT mice. Many of these metabolic alterations were similar among lesion-resistant and -prone brain regions, however, the practical and statistical significance level of these alterations in *Ndufs4* KO mice largely varied. When taken together, the metabolic and enzymatic data revealed a link between the severity of CI deficiency, the related level of NADH/NAD⁺ imbalance, and the degree of metabolic perturbations in *Ndufs4* KO brain regions. In the lesion-resistant anterior cortex, which maintained the highest level of residual CI activity (38% of WT), an adaptive increase in glutamate-, α -hydroxyglutarate-, and glycerol-3-phosphate (G3P) oxidation seems to drive energy-generation. However, this adaptive mechanism appears less effective in lesion-prone brain regions with more severe levels of CI deficiency — as reductive carboxylation, as well as the accumulation of lactate, branched-chain amino acids (BCAAs), and other possible mTOR regulators (proline and dihydroxyacetone phosphate) seemingly take precedence. Among the lesion-prone regions, the olfactory bulbs — which are known to be the first and one of the most severely affected brain regions (Quintana *et al.*, 2010) — displayed the lowest residual CI activity (14% of WT) and the largest number of metabolic alterations, the majority of which were related to carbohydrate and lipid metabolism. In accord, others (Chen *et al.*, 2018; Gaude *et al.*, 2018) have also identified enhanced glutamine/glutamate/ α -ketoglutarate anaplerosis of the tricarboxylic acid cycle (TCA), as a key adaptive response to oxidative phosphorylation (OXPHOS) deficiency that, depending on the degree of deficiency, either generates energy, recycles NAD⁺, and supports glycolysis (oxidate flux = moderate) or results in acetylation and increased lipid synthesis (reductive flux = severe). Furthermore, Schwörer *et al.* (2020) also recently demonstrated that enhanced Gln-derived proline biosynthesis is induced in response to increased oxidation of Gln (and glucose) carbons in the TCA cycle to replenish NAD(P)⁺ and prevent reactive oxygen species (ROS) generation when TCA cycle activity surpasses OXPHOS capacity (Schwörer *et al.*, 2020). Additionally, some of these metabolic alterations may be related to the neuro-inflammation observed in diseased *Ndufs4* KO mice. The activity of mitochondrial G3P dehydrogenase and the G3P shuttle was recently shown to modulate the balance between the beneficial and detrimental effects of the inflammatory response by controlling its induction and suppression through the regulation of glucose oxidation and oxidative metabolism, and in turn, acetyl-CoA production, histone acetylation, and the induction of inflammatory mediator-encoding genes (Langston *et al.*, 2019). Altered levels of acetyl-CoA, α -hydroxyglutarate, and succinate are also known to influence innate- and adaptive immune systems responses (Martínez-Reyes & Chandel, 2020); while high levels of BCAAs can increase the probability of neurodegeneration by influencing the immune properties of microglial cells and decreasing neuroprotective functions (De Simone *et al.*, 2013). Altogether, the data shows that the fate of metabolites, which form part of an intricate transport system that regulates brain BCAA, glutamate, and nitrogen handling, may play a vital role in regional vulnerability to CI deficiency. This hypothesis aligns with recent MD

research identifying glutamatergic and GABAergic neurons as central drivers of CI deficiency-related encephalopathy in *Ndufs4* KO mice (Bolea *et al.*, 2019); and attributing the beneficial effects of rapamycin treatment to the restoration of dysfunctional glutamate/ α -ketoglutarate metabolism in the *Ndufs4* KO brain (Johnson *et al.*, 2020). Moreover, by further highlighting the metabolic and phenotypic similarity between *Ndufs4* deficiency and a primary defect in BCAA catabolism, where alterations in glutamatergic- and GABAergic neurotransmission also plays a role (Dodd *et al.*, 1992; Dufour *et al.*, 2001; Görtz *et al.*, 2003), we pinpoint BCAA-, and particularly leucine, catabolism as a promising target for the treatment of shared clinical symptoms, including neurological and developmental delay, encephalopathy, feeding difficulty, and seizures. Therefore, the third objective of the study was fittingly achieved.

6.2.4 Objective 4: Compare tissue and urine metabolomes from *Ndufs4* KO vs WT mice

The fourteen metabolomics datasets from brain regions, skeletal muscles, and urine were pretreated, preprocessed, and statistically analysed as described in Chapter 3 (Section 3.4.7-8). Subsequently, the datasets were integrated, cross-compared, and extensively investigated. The results (Chapter 5) were published in the peer-reviewed journal, *Metabolomics* (Terburgh *et al.*, 2021b).

Brain regions, skeletal muscles, and urine of *Ndufs4* KO mice displayed altered alanine, aspartate, glutamate, and urea cycle metabolism. Hence, an adaptive rewiring of glutamate and nitrogen metabolism seems to be a whole-body response to CI deficiency. Contrary to the brain, *Ndufs4* KO skeletal muscle metabolomes seem to indicate a hyper-catabolic state with most metabolite levels, including glucose, alanine, pyruvate, lactate, TCA cycle intermediates, and proline lowered. Further evidence suggests heightened muscle proteolysis, amino acid catabolism, and ammonia transfer to the liver for gluconeogenesis. The accumulation of brain regional BCAAs, proline, and glycolytic intermediates were not mirrored in urine or skeletal muscles, with the urinary excretion of these metabolites significantly decreased. The urinary metabolome did, however, clearly reflect several brain region-specific alterations, including elevated alanine, pyruvate, and lactate (in lesion-prone regions) — which are considered classic MD biofluid markers. Additionally, signs of the metabolism of high systemic levels of glucose (to 2,3-butanediol) and lactate (to propionylcarnitine) by gut microbiota, as well as altered host-microbial co-metabolism of aromatic amino acids were seen in *Ndufs4* KO urine.

Interestingly, Desquiret-Dumas *et al.* (2019) recently demonstrated in patient fibroblasts that a metabolic distinction could be made between a CI functional defect, with and without, CI disassembly. A purely functional CI defect results in overall hyper-catabolism, to sustain OXPHOS; while a severe assembly defect results in increased glycolysis (at the expense of OXPHOS) with lactate accumulation, CI ROS overproduction by assembly intermediates, and AMPK-dependant pyruvate dehydrogenase (PDH) inactivation that diminishes pyruvate oxidation. While the former seems to reflect skeletal muscle metabolism in *Ndufs4* KO mice, the

latter metabolic alterations were more prominent in lesion-prone brain regions, especially the olfactory bulbs, which had the lowest residual CI activity and the lowest innate CIII levels — the RC enzyme shown to be essential for supercomplex (SC) formation and CI assembly (Protasoni *et al.*, 2020). Since pyruvate oxidation and ATP production is normal in *Ndufs4* KO skeletal muscle (Alam *et al.*, 2015) but diminished in the brain (Manjeri *et al.*, 2016), it is tempting to hypothesise that differences in the level of CI disassembly in tissues may also play a role in the tissue-specific metabolic response and vulnerability to CI deficiency.

We further hypothesise that, in a subset of *Ndufs4* KO tissues, enhanced BCAA and proline catabolism possibly fuel the Q-pool via electron-transferring-flavoprotein dehydrogenase (ETF_{FDH}) and proline dehydrogenase, respectively. Similarly, system-wide reductions in the levels of alternative Q-pool fuels, including succinate, α -hydroxyglutarate, and glycerol-3-phosphate, suggest increased use thereof to maintain bioenergetics in *Ndufs4* KO tissues. Taken together with our previous observations in skeletal muscle (Terburgh *et al.*, 2019) [Appendix D] and supporting data from others (as discussed in Chapter 2, Section 2.5.5), we postulate that tissue-specific differences in the content and capacity of alternative electron donors and redox-regulatory shuttles play an important role in a tissue's ability to restore homeostasis and bypass CI deficiency. Furthermore, the G3P shuttle may be particularly relevant to the LS phenotype, since it regulates NAD⁺ and lactate levels (Alfadda *et al.*, 2004) and is highly active in skeletal muscle but has limited activity in the brain (Liu *et al.*, 2021; Schiaffino & Reggiani, 2011). Similarly, we (Terburgh *et al.*, 2019) and others (Zielinski *et al.*, 2016) identified the proline cycle as another important redox shuttle involved in direct Q-pool reduction in CI deficient skeletal- and cardiac muscle. The significant accumulation of dihydroxyacetone phosphate and proline in brain regions suggests that the proline cycle and G3P shuttle may not function effectively in the brain.

Among the discrepancies observed between urinary and brain region-specific metabolomes, the most prominent were marked elevations in urinary TCA cycle intermediates and reductions in one-carbon (1C) metabolites, which seemed to reflect hepatic and renal metabolism. Nevertheless, several metabolic alterations point to a systemic mismatch between the amount of acetyl-CoA generated via catabolic reactions in *Ndufs4* KO tissues and the processing capacity of the TCA cycle. Furthermore, some 1C-metabolism linked perturbations were also evident in *Ndufs4* KO brain regions and skeletal muscles.

Altogether, our work supports the notion that a cell's biochemical threshold for CI deficiency may rely upon its capacity for, and the efficacy of, adaptive responses that compensate for reduced NADH oxidation (reductive stress) and electron transport to the RC Q pool. As mentioned in Chapter 2 (Section 2.5.4), chronic, but not intermittent, hypoxia has been the most effective treatment in *Ndufs4* KO mice to date (Ferrari *et al.*, 2017; Jain *et al.*, 2019; Jain *et al.*, 2016). While the exact mechanism behind its benefits is not entirely clear, a transition from acute to chronic hypoxia is associated with a shift in cellular respiration away from CI-driven (pyruvate

oxidation) to ETF- (fatty acid oxidation) and CII- (glutamine oxidation) driven respiration (Fuhrmann *et al.*, 2019). Hence, such metabolic reprogramming could be linked to the ability of hypoxia treatment to replenish NAD⁺ levels and improve oxygen consumption in *Ndufs4* KO mice (Grange *et al.*, 2021; Schleifer *et al.*, 2018). Moreover, differences in the level of residual CI activity and disassembly may also impact tissue-specific metabolic responses to CI deficiency, which in turn, could be related to innate differences in CI and RC content, organisation, assembly, maintenance, or variances in spare respiratory capacity. Additionally, this study demonstrates the value of urinary metabolomics in MD investigations, while highlighting the importance of integrating systemic biofluid- and tissue-specific metabolomes when aiming to elucidate MD mechanisms.

6.3 Strengths, limitations, and prospects of the research

6.3.1 Strengths

This discovery-phase study demonstrates the remarkable value of multiplatform metabolomics in the quest to better understand the underlying pathophysiology of complex multisystemic diseases, like MD. As the first cross-comparison of large-scale tissue- and urine-specific metabolomes in *Ndufs4* KO vs WT mice, this study provides a more holistic overview of how CI deficiency affects metabolism along with information on the suitability of specific sample types for MD metabolomics applications. A great strength of the research design is the application of more than one analytical platform on the same individual biological sample; the analysis of seven different sample types; and the relatively large sample size used ($n \geq 19$ per group). These aspects of the research design expand metabolome coverage and increase the credibility of the results. It is also noteworthy that, as part of the larger project, our group is the first to report data on urinary-, brain regional-, and skeletal muscle-specific metabolic and enzymatic alterations in *Ndufs4* KO mice. Contrary to Johnson *et al.* (2020), who recently described no significant alterations in brain-regional metabolomes of early aged pre-symptomatic (P29–31) *Ndufs4* KO mice, we studied late-stage diseased (P45–50) *Ndufs4* KO mice, which are considered to be a more appropriate model of LS (Finsterer, 2019). A further comparison of our urinary data to previous studies in blood (Grange *et al.*, 2021; Jain *et al.*, 2016; Jin *et al.*, 2014; Kruse *et al.*, 2008) suggests that the urinary metabolome, as an excretory systemic biofluid, provides additional valuable information on proximal and blood-perfusing organs that are lacking or understated in blood — possibly due to homeostatic control mechanisms. Moreover, the ease of collection and stability of urine makes it a particularly advantageous sample type (Wu & Gao, 2015). In accord, the analysis of our results led to novel insight into regional neurodegeneration, inter-organ relationships, and systemic metabolic alterations in CI deficiency-related LS — thereby identifying key, tissue-specific areas of metabolism to further investigate and possibly target, to promote patient health.

6.3.2 Limitations

Despite its strengths, the research design also had limitations. First, as this was a hypothesis-generating study, our findings need to be further investigated in a more targeted manner. Second, the exclusion of female animals from the study is a shortcoming, although this was done to minimise inter-group hormonal and metabolic differences, since comparing sex-specific metabolic alterations was beyond the scope of this study. As discussed in Chapter 5 (Section 5.4), many areas of metabolism shown to be altered in this study (including glucose-, lipid-, amino acid-, acylcarnitine-, BCAA-, and bone metabolism) have the potential to display sexual dimorphism. Such metabolic sexual dimorphism is influenced by sex-specific differences in hormones and chromosomes, tissue-specific gene regulation, and gut microbiota (Chella Krishnan *et al.*, 2018; Clegg & Mauvais-Jarvis, 2018), which may affect pathogenic mechanisms or responses to treatment options in LS. Although no sex-specific differences in disease phenotype or animal survival have been reported for *Ndufs4* KO mice (Kayser *et al.*, 2016; Kruse *et al.*, 2008; Lee *et al.*, 2019; Miller *et al.*, 2021; Quintana *et al.*, 2010), there is a slight (unexplained) male predominance among LS patients (Marin *et al.*, 2013; Rahman *et al.*, 1996) and future sex-specific studies are needed. Third, only animal age was used to define the endpoint for inclusion in the study, which in retrospect, resulted in considerable biological variation among *Ndufs4* KO animals on a metabolic level. Hence, we recommend using phenotype scoring in future work to determine uniform disease progression and animal endpoints. Fourth, while established metabolic markers of brain regional NADH/NAD⁺ ratio was used, direct NAD(H) measurements were not performed as our previous attempts using commercial kits were inaccurate. Fifth, cell type-specific metabolic differences in *Ndufs4* KO vs WT mice may have been masked by brain regional metabolomes, seeing that glutamatergic- and GABAergic neurons were recently shown to play a more important role in the *Ndufs4* KO phenotype than other brain cells (Bolea *et al.*, 2019).

Furthermore, the research also entailed some shortcomings inherent to metabolomics and animal model studies. It is currently impossible to truly measure the whole metabolome, thus, metabolomics produces biased results (Christians *et al.*, 2011). By employing the same profiling techniques in seven different sample types, using multiple platforms, we attempted to reduce some of this inherent bias, nevertheless, it cannot be avoided. In this respect, our cross-comparison of metabolic alterations in various *Ndufs4* KO sample types contained some missing values due to innate differences in the presence and/or concentration of metabolites in these samples. Moreover, some of the important features in our untargeted datasets could not be identified and were, therefore, not considered during cross-comparison, pathway analysis, or biological interpretations. In addition, inherent metabolic differences between mice and humans may also limit the translatability of some of the findings. It must also be noted that the findings obtained from this mouse model are not necessarily applicable to all mitochondrial

encephalopathies, as *Ndufs4*-mutations only account for a small portion of MD cases, including CI deficiency and LS.

6.3.3 Prospects

Our observations warrant future flux studies at the level of brain regions, neurons, and glia that target brain BCAA, glutamate, and nitrogen handling; the metabolic sources and fate of α -ketoglutarate in the TCA cycle; and the leucine/glutamate/glutamine transport systems between astrocytes and neurons. However, these areas of metabolism should also be holistically targeted in future investigations since BCAA, glutamate, and nitrogen metabolism are jointly controlled by different tissues. Future studies should also determine whether the correlation we observed between brain-regional residual CI activity and metabolic perturbation, result from the related degree of reductive stress or whether differences in CI and/or SC disassembly levels may also dictate this metabolic response. Furthermore, it should be established whether an increased flux through alternative electron donors is a beneficial whole-body adaptive response to CI deficiency or whether this may be maladaptive in some tissues, like the brain, as these electron donors could contribute to oxidative stress. Moreover, our identification of altered gut-microbial metabolism in the urine of *Ndufs4* KO mice warrant further in-depth investigations of host-bacterial co-metabolism and the gut-brain axis in LS, especially considering the important role that host-gut microbiota interactions seem to play in disease phenotypes associated with alterations in mitochondrial function and/or redox status (Yardeni *et al.*, 2019). It would also be advantageous for future studies to investigate (and validate) whether any of the urinary metabolites we identified could serve as adequate biomarkers of LS and MD. This includes amino acids and derivatives related to alanine, aspartate and glutamate-; arginine and proline-; BCAA-; lysine-; histidine-; and choline metabolism; as well as sugar derivatives; metabolites related to the G3P shuttle; and gut microbial metabolism. With regards to therapeutic approaches for *Ndufs4*-related LS, we suggest that preliminary strategies should focus on regenerating NAD⁺ levels, stimulating alternative Q-pool electron donors, and improving oxygen consumption. Enhancing the activity of endogenous redox shuttles such as the G3P and proline cycles, especially in the brain, is a key therapeutic avenue to pursue. The combined supplementation of NAD⁺ precursors and alternative Q-pool fuels (e.g., α -hydroxyglutarate, succinate, dimethylglycine, etc.) may be a preliminary treatment option for patients. However, the cell membrane-permeability and uptake of such compounds across the blood-brain barrier should be studied, with their combined effects clinically verified. In particular, the effects of dietary supplementation vs restriction of BCAAs and proline should be investigated considering their reduced urinary excretion and brain regional accumulation.

6.4 References

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APPENDIX

Animal information



APPENDIX A

A.1 Post-mortem genotype verification

Figure A-1 provides an example of the gel electrophoresis results obtained for each animal used in this study. As discussed in Section 3.3.4, PCR genotype verification was performed on tail snip-derived DNA, collected *post-mortem*.

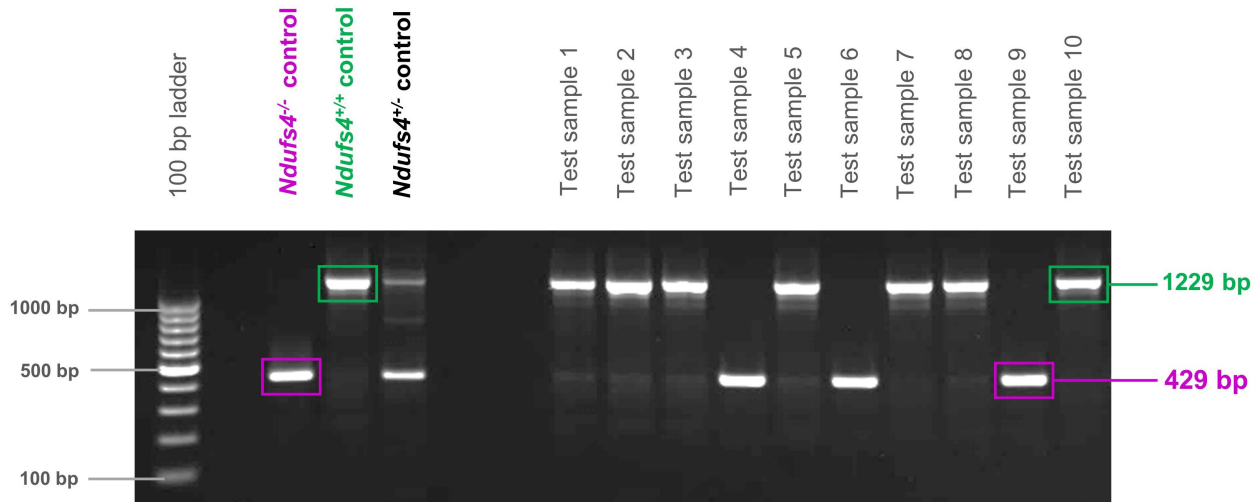


Figure A-1: Example agarose gel electrophoretogram portraying genotyping results. The photo depicts a 100-1000 bp DNA ladder (lane 1) along with *Ndufs4* amplicons of control (lanes 3-5) and test sample DNA. Homozygous KO (*Ndufs4*^{-/-}) DNA (lane 3) display a disrupted *Ndufs4* amplicon (429 bp, magenta) from which an 800 bp region containing exon two was removed, while homozygous WT (*Ndufs4*^{+/+}) DNA (lane 4) display an intact *Ndufs4* amplicon (1229 bp, green) containing exon two. Heterozygous (*Ndufs4*^{+/-}) DNA (lane 5) display both the disrupted and intact bands. *Abbreviations: bp, base pairs.*

A.2 Animal information

Table A-1 lists the identification- and litter numbers of the animals used for metabolomics investigations along with their age at euthanasia and the sample types analysed from each mouse.

Table A-1: Animals used for metabolomics analyses

KO			WT		
ID_litter number	Age (days)	Sample type	ID_litter number	Age (days)	Sample type
68_1o	46	all	8_1v	48	all
10_1v	48	all	54_2c	47	all
56_2d	46	all	61_2e	47	all
69_2f	48	all	80_2g	48	all
37_3j	48	all	81_2g	48	all
44_3k	46	all	62_2t	48	all
86_3q	49	all	9_3d	47	all
2_3s	49	all	42_3k	46	all
54_4a	46	all	57_3l	46	all
2_4i	48	all	83_3p	49	all
8_4j	48	all	88_3q	49	all
34_4o	47	all	48_3z	46	all
42_4q	49	all	62_4b	46	all
50_4s	45	all	63_4b	46	all
89_1r	46	U & BR	95_1t	48	U & BR
15_1w	48	U & BR	2_1u	46	U & BR

APPENDIX A

KO			WT		
ID_litter number	Age (days)	Sample type	ID_litter number	Age (days)	Sample type
45_3k	46	U & BR	82_2g	48	U & BR
1_4i	48	U & BR	27_1x	47	BR&M
96_2i	46	U & M	59_2e	46	BR&M
28_3g	49	U & M	2_3c	47	BR&M
20_4m	47	U & M	9_3t	49	BR&M
70_1o	46	BR&M	19_3u	49	BR&M
10_1f	46	BR	35_3x	49	BR&M
4_1v	48	BR	40_3y	49	BR&M
52_2r	49	M	14_1w	48	BR
60_2e	46	U	12_1f	46	U
67_2f	48	U	49_2a	47	U
18_2l	45	U	53_2c	46	U
33_2o	45	U	91_2i	46	U
72_4d	56	U	65_2u	48	U
74_4d	56	U	11_3d	47	U
			26_3g	49	U
			65_3n	46	U
			68_3n	46	U
			81_3p	49	U
			87_3q	49	U
			97_4h	48	U
			98_4h	48	U
			9_4j	49	U
			16_4m	47	U
			27_4n	47	U
			29_4n	47	U
			37_4p	49	U

Abbreviations: BR, brain regions (anterior cortex, brainstem, cerebellum, and olfactory bulbs); ID, identification; KO, knockout; M, skeletal muscles (solei and white quadriceps); P, postnatal day; U, urine; WT, wild type

APPENDIX

LC-MS/MS standards



APPENDIX B

Table B-1: Content of LC standard mixture

Compound	Purity (%)	Catalogue number	Supplier
1-Methyl-L-histidine	≥ 98	67520	Sigma-Aldrich®
3-Alanine	≥ 99	37009	BDH biochemical
3-Hydroxy-DL-kynurenine	≥ 98	H1771	Sigma-Aldrich®
3-Methyl-L-histidine	99	M9005	Sigma-Aldrich®
4-Aminobutyric acid	≥ 99	A5835	Sigma-Aldrich®
Acetyl-L-carnitine hydrochloride	99	360929	Sigma-Aldrich®
Butyryl-L-carnitine	≥ 97	42623	Sigma-Aldrich®
Creatine monohydrate	≥ 99	27900	Fluka™
Decanoyl-L-carnitine	≥ 97	50637	Sigma-Aldrich®
DL-3-Aminoisobutyric acid	≥ 98	8290	Fluka™
DL-Isoleucine	99	298689	Sigma-Aldrich®
Dodecanoyl-L-carnitine	≥ 97	39953	Sigma-Aldrich®
Hexanoyl-L-carnitine	≥ 97	07439	Sigma-Aldrich®
Isovaleryl-L-carnitine	≥ 97	51371	Sigma-Aldrich®
L-2-Aminoadipic acid	≥ 98	A7275	Sigma-Aldrich®
L-Alanine	≥ 99	5130	Fluka™
L-Arginine	≥ 99	11010	Fluka™
L-Asparagine anhydrous	≥ 99	11150	Fluka™
L-Aspartic acid	≥ 99	11190	Fluka™
L-Carnitine hydrochloride	≥ 98	C0283	Sigma-Aldrich®
L-Citrulline	≥ 99	27510	Fluka™
L-Cystathionine	≥ 98	30055	Fluka™
L-Cysteine	≥ 99	30090	Fluka™
L-Cystine	≥ 99	30200	Fluka™
L-Glutamic acid	≥ 98	G-8415	Sigma-Aldrich®
L-Glutamine	≥ 99	49419	Fluka™
L-Glycine	≥ 99	G7126	Sigma-Aldrich®
L-Histidine	≥ 99	53320	Fluka™
L-Homocysteine	≥ 98	H6010	Sigma-Aldrich®
L-Homoserine / L-Isothreonine	≥ 98	H6515	Sigma-Aldrich®
L-Leucine	≥ 98	L8000	Sigma-Aldrich®
L-Lysine	≥ 98	62840	Fluka™
L-Methionine	≥ 99	64320	Fluka™
L-Ornithine hydrochloride	99	O830-5	Sigma-Aldrich®
L-Phenylalanine	≥ 99	78020	Fluka™
L-Pipecolic acid	99	P2519	Sigma-Aldrich®
L-Proline	≥ 99	P0380	Sigma-Aldrich®
L-Pyroglutamic acid	99	F-3634	Sigma-Aldrich®
L-Sarcosine	98	S-9881	Sigma-Aldrich®
L-Serine	≥ 99	84960	Fluka™
L-Threonine	≥ 98	89180	Fluka™
L-Tryptophan	≥ 99	93659	Fluka™
L-Tyrosine	≥ 99	93830	Fluka™
L-Valine	≥ 98	V0500	Sigma-Aldrich®
N, N, N-trimethylglycine anhydrous	≥ 98	61962	Fluka™
N,N-Dimethylglycine	≥ 99	D1156	Sigma-Aldrich®
N-Acetylaspartic acid	≥ 99	00920	Sigma-Aldrich®
N-Acetyl-L-glutamic acid	99	855642	Sigma-Aldrich®
Octadecanoyl-L-carnitine	≥ 97	61229	Sigma-Aldrich®
Octanoyl-L-carnitine	≥ 97	50892	Sigma-Aldrich®
Palmitoyl-L-carnitine	≥ 97	61251	Sigma-Aldrich®
Propionyl-L-carnitine	≥ 97	42602	Sigma-Aldrich®
Taurine	≥ 99	86330	Fluka™
Tetradecanoyl-L-carnitine	≥ 97	61367	Sigma-Aldrich®
Trans-4-Hydroxy-L-proline	≥ 99	41875	Sigma-Aldrich®

C.1. Data quality assessment

The fourteen datasets obtained from the semi-targeted and untargeted metabolomics analysis of brain regions, skeletal muscles, and urine were inspected prior to further processing to ensure good data quality. The repeatability (intra-batch variation) and intermediate precision (inter-batch variation) with which metabolites were detected in QC sample replicates were first inspected. Figure C-1 depicts the intra- and inter-batch coefficient of variance (CV) distribution for metabolites detected in QC samples via semi-targeted LC-MS/MS [in brain regions (A-D), skeletal muscles (I-J), and urine (K)] and untargeted GC-TOF-MS [in brain regions (E-H), skeletal muscles (L-M), and urine (N)]. For targeted analyses, the acceptable limit of precision is typically set at a CV-value between 15-25%, with values <15% indicating good data quality for highly targeted absolute quantification (FDA, 2001). Although semi-targeted relative quantification was done in this study, more than 80% of metabolites had intra- and inter-batch CV-values below 10% for all sample types, except the quantity-limited olfactory bulbs (OB) and solei (SOL), for which most metabolites still had CV-values below 25%. For untargeted analyses, a CV cut-off value between 30-50% is typically used (Dunn *et al.*, 2012), with high quality data containing CV-values of <30% for ~70% of features (Gika *et al.*, 2014). In accordance, most features detected via untargeted analysis had intra- and inter-batch CV-values below 50% in all sample types, with 88% of urinary features and 56-86% of tissue-specific features displaying CV-values below 30%. Next, the scatter and clustering of QC samples across batches were investigated for each sample type and platform. Sequential total signal (sum of all relative peak intensities for each sample) scatter plots (Figure C-2) showed no significant drifts or trends in the data, with adequate horizontal alignment of QC samples seen for all datasets. Furthermore, multivariate principal component analysis [PCA] (Figure C-3) of QC and test samples (WT and KO) further confirmed the absence of obvious batch effects in the datasets, with tight QC sample clustering indicating relatively little technical variance. Therefore, most visible differences in test sample scattering and clustering can be ascribed to inherent biological variance.

As expected, the CV distribution and clustering of QC samples reveal greater technical variance during untargeted analysis, which can in part also be attributed to the fact that the silylation reaction used for sample derivatisation is not quenched but continues over time (Erban *et al.*, 2007; Fiehn, 2008). This can lead to increased variation among samples, throughout a batch as varying levels of compound derivatisation or decomposition can occur during the waiting period prior to sample analysis. While samples at the beginning of the batch might stand in the autosampler for a few minutes to hours prior to analysis, those at the end of the batch are subjected to much longer waiting times (e.g., ~16 hours). Furthermore, urinary QC samples displayed less intra- and inter-batch variation than tissues, with the highest variance seen in the quantity-limited OB and SOL QC samples. While the former observation likely reflects the greater stability of, and minimum sample preparation required for urine, the latter could be attributed to the fact that quantity-limited samples

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required different extraction and derivatisation solvent volumes; different vials/inserts for sample preparation, and larger injection volumes or sample concentration than other tissues. Nevertheless, the metabolomics techniques employed proved to yield adequately repeatable and precise data with good quality, for all sample types.

APPENDIX C

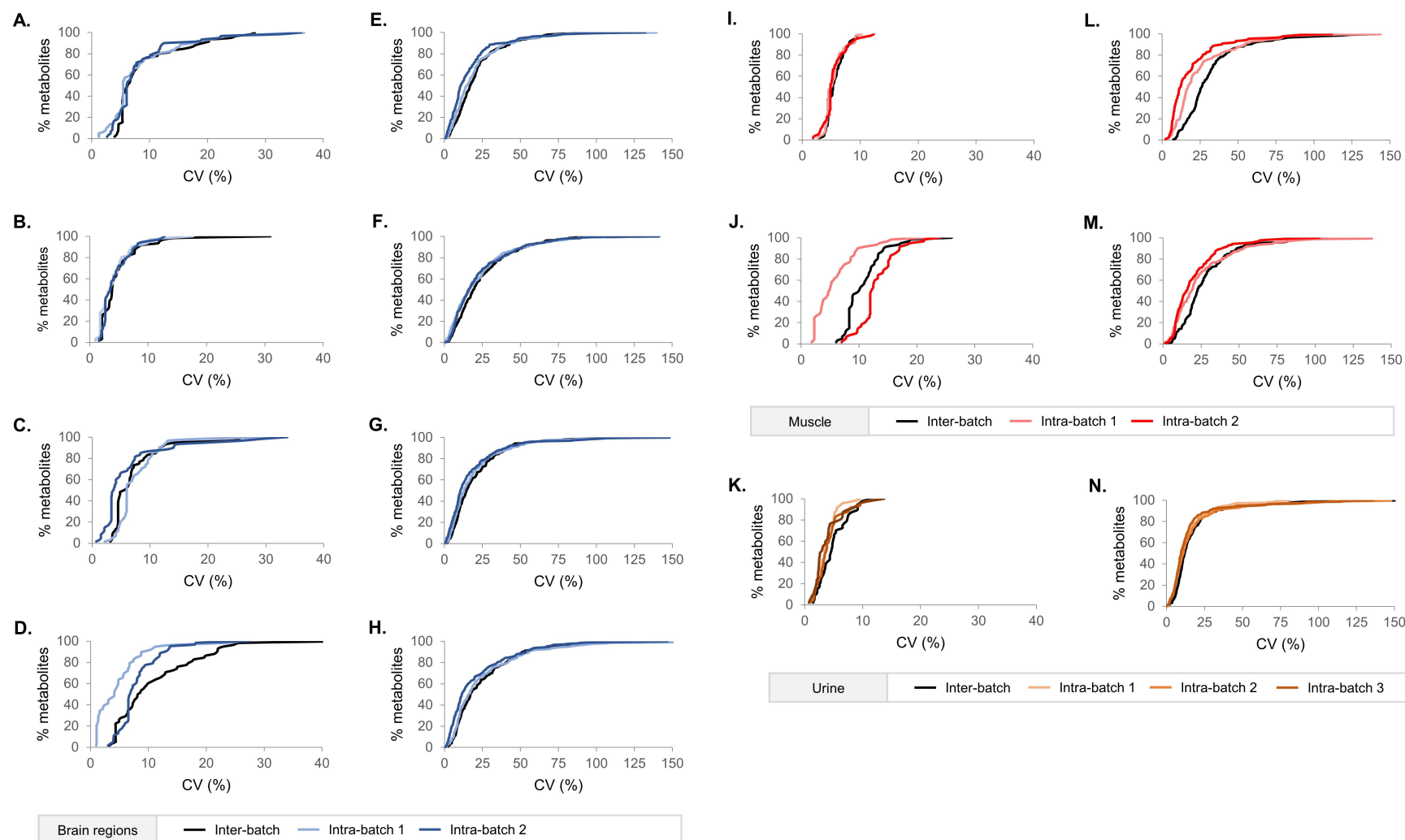


Figure C-1: Coefficient of variance distribution of QC samples. Graphs depict the intra- and inter-batch coefficient of variance (CV%) distribution for metabolites measured in QC samples from brain regions (blue), muscles (red) and urine (orange). Brain regional results for LC-MS/MS and GC-TOF-MS are respectively given in graphs A and E for the anterior cortex; B and F for the brainstem; C and G for the cerebellum; and D and H for the olfactory bulbs. Muscle-specific results for LC-MS/MS and GC-TOF-MS are respectively given in graphs I and L for the white quadriceps, as well as J and M for the soleus. Urinary results for LC-MS/MS and GC-TOF-MS are respectively given in graphs K and N. *Abbreviations: QC, quality control.*

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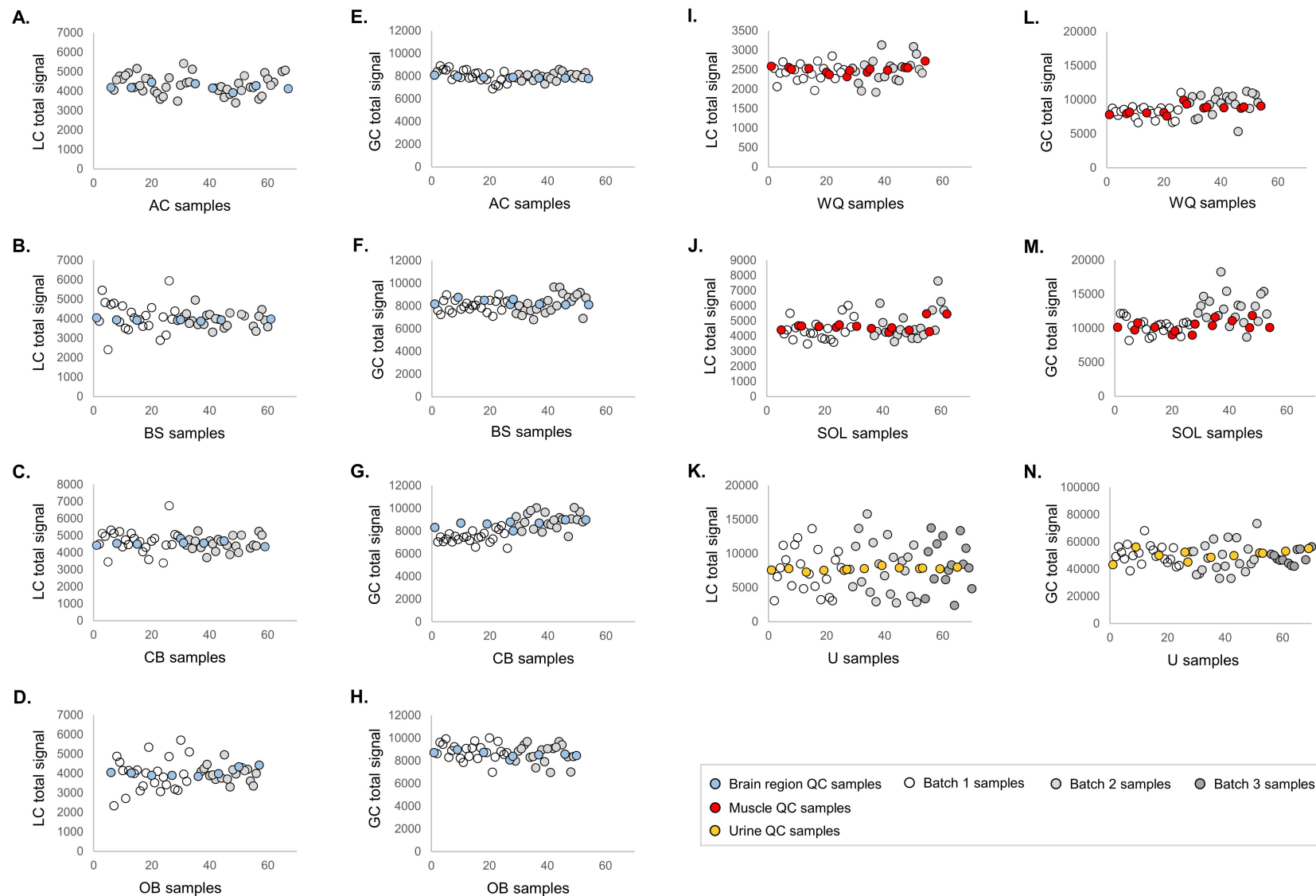


Figure C-2: Sequential total signal scatter plots of all samples. Scatter plots for LC-MS/MS analysis of brain regions (A-D), muscles (I-J) and urine (K) as well as GC-TOF-MS analysis of brain regions (E-H), muscles (L-M) and urine (N) are given. The x-axis specifies the samples in run order, while the y-axis specifies the total signal obtained for each sample i.e., the sum of the relative peak intensities of all metabolites measured in the sample. *Abbreviations: AC; anterior cortex; BS, brainstem; CB; cerebellum; GC; GC-TOF-MS; LC; LC-MS/MS; OB, olfactory bulbs; QC*

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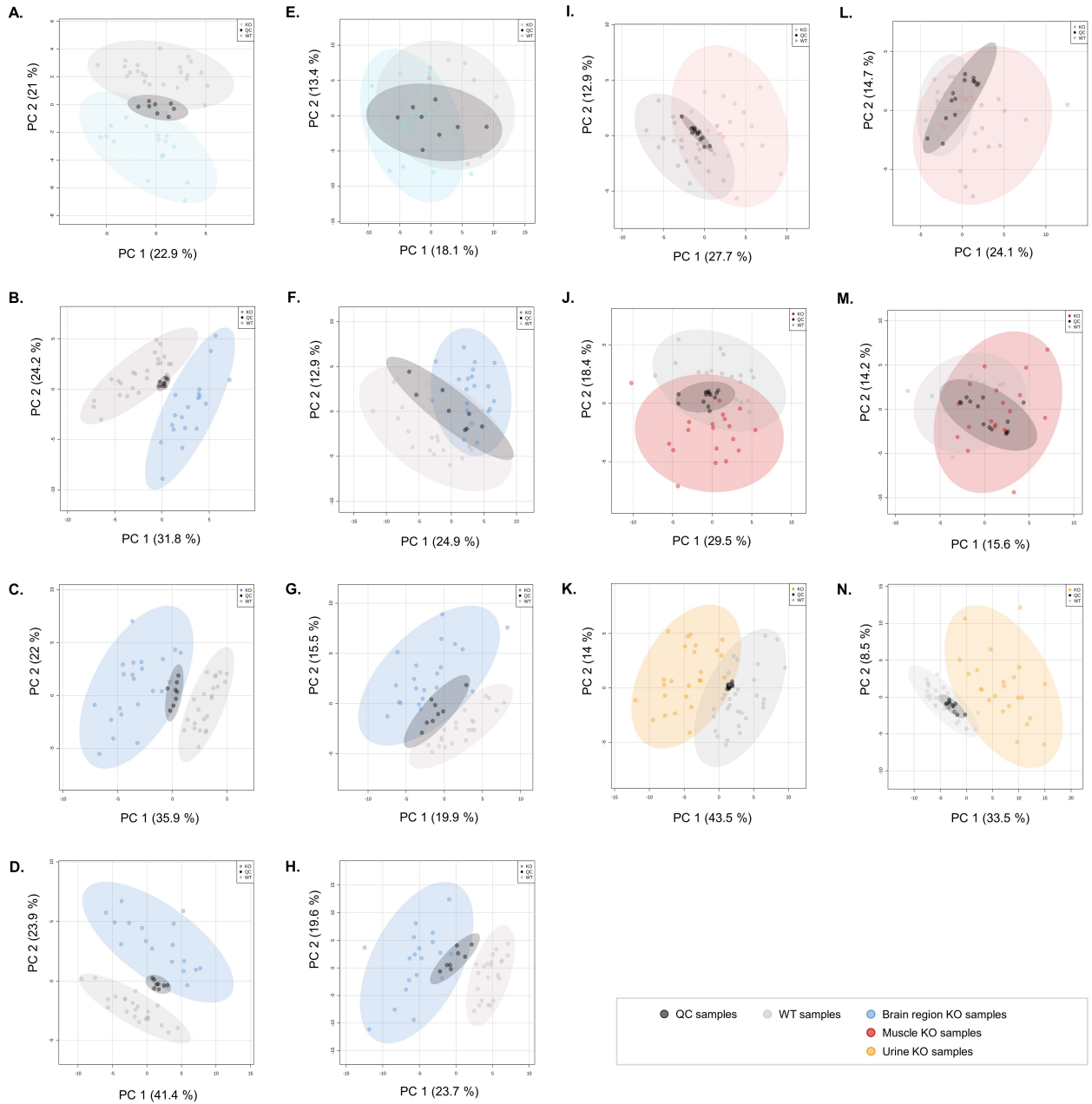


Figure C-3: Visual inspection of multivariate data clustering in all samples. PCA scores plots (PC1 vs PC2) are given for LC-MS/MS analysis of brain regions (A-D), muscles (I-J), and urine (K) as well as GC-TOF-MS analysis of brain regions (E-H), muscles (L-M), and urine (N). Black, grey, and coloured (blue=brain regions, red=muscles, and yellow=urine) data points respectively indicate QC, WT and KO samples. Tight clustering of QCs is visualised for the anterior cortex (A and E), brainstem (B and F), cerebellum (C and G), olfactory bulbs (D and H), white quadriceps (I and L), solei (J and M), and urine (K and N). Data was log transformed, mean centered and autoscaled. *Abbreviations: KO, Ndufs4 knockout; PCA, principal component analysis; QC, quality control; WT, wild type*

C.2. References

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APPENDIX

Previously published article on *Ndufs4* skeletal muscle.

This article was previously presented in the author's MSc dissertation and is only given here to convey additional information relevant to this thesis and the larger study it forms part of.





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Metabolomics of *Ndufs4*^{-/-} skeletal muscle: Adaptive mechanisms converge at the ubiquinone-cycle



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ABSTRACT

Leigh syndrome is one of the most common childhood-onset neurometabolic disorders resulting from a primary oxidative phosphorylation dysfunction and affecting mostly brain tissues. *Ndufs4*^{-/-} mice have been widely used to study the neurological responses in this syndrome, however the reason why these animals do not display strong muscle involvement remains elusive. We combined biochemical strategies and multi-platform metabolomics to gain insight into the metabolism of both glycolytic (white quadriceps) and oxidative (soleus) skeletal muscles from *Ndufs4*^{-/-} mice. Enzyme assays confirmed severely reduced (80%) CI activity in both *Ndufs4*^{-/-} muscle types, compared to WT. No significant alterations were evident in other respiratory chain enzyme activities; however, *Ndufs4*^{-/-} solei displayed moderate decreases in citrate synthase (12%) and CIII (18%) activities. Through hypothesis-generating metabolic profiling, we provide the first evidence of adaptive responses to CI dysfunction involving non-classical pathways fueling the ubiquinone (Q) cycle. We report a respective 48 and 34 discriminatory metabolites between *Ndufs4*^{-/-} and WT white quadriceps and soleus muscles, among which the most prominent alterations indicate the involvement of the glycerol-3-phosphate shuttle, electron transfer flavoprotein system, CII, and proline cycle in fueling the Q cycle. By restoring the electron flux to CIII via the Q cycle, these adaptive mechanisms could maintain adequate oxidative ATP production, despite CI deficiency. Taken together, our results shed light on the underlying pathogenic mechanisms of CI dysfunction in skeletal muscle. Upon further investigation, these pathways could provide novel targets for therapeutic intervention in CI deficiency and potentially lead to the development of new treatment strategies.

1. Introduction

Mitochondria play a vital role in a myriad of processes involved in metabolism, cellular bioenergetics, and metabolic cell signaling [1–4].

The mitochondrial oxidative phosphorylation (OXPHOS) system, which comprises complexes I–IV and ATP synthase (CV), is the major contributor to cellular energy production and sustains most mitochondrial functions. The dysfunction of complex I (CI) (OMIM 252010) of the

Abbreviations: ¹H NMR, Proton nuclear magnetic resonance spectroscopy; 1-Mhis, 1-Methylhistidine; 2-AA, 2-Amino adipate; 2-Mhis, 2-Methylhistidine; 5MTHF, 5-Methyl-tetrahydrofolate; Ala, Alanine; Arg, Arginine; Asn, Asparagine; Asp, Aspartate; BAIBA, β-Aminoisobutyrate; β-Ala, Beta-Alanine; C0, Carnitine; C1, complex I; C2, Acetyl-carnitine; CII, complex II; C3, Propionyl-carnitine; CIII, complex III; C4, Butyryl-carnitine; CIV, complex IV; C5, Isovaleryl-carnitine; C6, Hexanoyl-carnitine; C8, Octanoyl-carnitine; C12, Dodecanoyl-carnitine; C14, Myristoyl-carnitine; C16, Palmitoyl-carnitine; C18, Stearoyl-carnitine; c-GPDH, Cytosolic glycerol-3-phosphate dehydrogenase; Cit, Citrulline; CS, Citrate synthase; Cyt c, Cytochrome c; DHAP, Dihydroxyacetone phosphate; DMG, N, N-Dimethylglycine; ETF, Electron transfer flavoprotein; ETF-QO, Electron transfer flavoprotein:ubiquinone oxidoreductase; FAD, Oxidized flavin adenine dinucleotide; FADH₂, Reduced flavin adenine dinucleotide; GC–TOF–MS, Gas chromatography time-of-flight mass spectrometry; GP, Glycerol-3-phosphate; Gln, Glutamine; Glu, Glutamate; Gly, Glycine; Hcys, Homocysteine; Hpro, Hydroxyproline; LC–MS/MS, Liquid chromatography tandem mass spectrometry; Lys, Lysine; Met, Methionine; m-FDH(s), Mitochondrial flavoprotein dehydrogenase(s); m-GPDH, Mitochondrial glycerol-3-phosphate dehydrogenase; NAA, N-Acetylaspartate; NAD⁺, Oxidized nicotinic amide adenine dinucleotide; NADH, Reduced nicotinic amide adenine dinucleotide; NAG, N-Acetylglutamate; *Ndufs4*, NADH dehydrogenase (ubiquinone) iron-sulphur protein 4; *Ndufs4*^{-/-}, Homozygous *Ndufs4* knockout; Orn, Ornithine; OXPHOS, Oxidative phosphorylation; P-5-C, Pyrroline-5-carboxylate; pGlu, Pyroglutamate; Phe, Phenylalanine; Pip, Pipecolate; Pro, Proline; ProDH, Proline dehydrogenase; PYCR, Pyrroline-5-carboxylate reductase; Q, Ubiquinone (oxidized coenzyme Q10); QH₂, Ubiquinol (reduced coenzyme Q10); RC, Respiratory chain; SAH, S-Adenosylhomocysteine; SAM, S-Adenosylmethionine; Sar, Sarcosine; Ser, Serine; Tau, Taurine; THFA, Tetrahydrofolate; Thr, Threonine; TMG, Trimethylglycine; Trp, Tryptophan; WT, Wild type (*Ndufs4*^{+/+})

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OXPHOS system, is the most common defect in mitochondrial energy metabolism, often resulting in progressive, severe multi-system deterioration. Due to the extreme complexity and heterogeneous nature (genetically and clinically) of human CI deficiency, the underlying pathogenic mechanisms thereof remain poorly understood [5,6].

Current studies on mouse models of CI dysfunction, such as the whole-body *Ndufs4*^{-/-} (knockout) mouse, attempt to better comprehend mitochondrial disease. The nuclear DNA-encoded NDUF54 protein is essential to CI assembly, stability, and activity [7,8]. *Ndufs4*^{-/-} mice present with symptoms similar to human CI deficiency at around postnatal day (P) 35 and develop a progressive Leigh-like phenotype, including developmental delays, failure to thrive, lethargy, ophthalmoplegia, locomotor impairment, hearing loss, as well as progressive ataxia, and necrotizing encephalomyopathy leading to early death (P50–60) [9–11]. Since the development of whole-body *Ndufs4*^{-/-} mice, several conditional knockouts (neuronal, glial, cardiac, hematopoietic, and hepatic) have been developed in order to gain insight into tissue-specific consequences of CI dysfunction (reviewed by Toracco et al. [12]). However, due to the main interest in the predominant neurological phenotype of this disease model, limited information is available on skeletal muscle-specific consequences of the *Ndufs4* knockout.

A recent study by Fariel and colleagues [13] highlighted the involvement of skeletal muscle in *Ndufs4* knockout pathology. The authors reported similarities between the phenotypes of skeletal muscle-specific and whole-body *Ndufs4* knockout *Drosophila*, such as reduced lifespan, feeding difficulties, locomotor impairment, and climbing defects. Given the important, often underappreciated, role of the muscular system in health and disease [14], as well as its integration and interdependence with the nervous system [15], it is imperative to elucidate the effects of a *Ndufs4* knockout on muscle metabolism. Skeletal muscle not only facilitates breathing and locomotion but also plays an essential role in various systemic, energy-demanding processes such as whole-body protein metabolism, glucose, and fatty acid consumption, thermoregulation, immunity, as well as the maintenance of adequate bone strength and density [14–16]. As *Ndufs4*^{-/-} mice display weight loss [9], a significant decrease in body fat [11], decreases in body temperature [9], as well as systemic inflammation and osteoporosis [10], it can be hypothesized that aberrant skeletal muscle metabolism might also play an important role in murine *Ndufs4*^{-/-} phenotypes.

We aimed to gain insight into the consequences of a *Ndufs4*^{-/-} on skeletal muscle bioenergetics and metabolism by combining biochemical strategies (respiratory chain enzyme assays) and multi-platform metabolic profiling (LC-MS/MS, GC-TOF-MS, and NMR analyses). Since the metabolic properties and subpopulations of muscle mitochondria depend on muscle fiber type composition [16], both glycolytic (white quadriceps; primarily type IIB myofibers) and oxidative (soleus; primarily type I myofibers) muscles were investigated. To the best of our knowledge, this is the first report of fiber type-specific enzymatic and metabolic data in *Ndufs4*^{-/-} skeletal muscles. We report a metabolic signature consisting of 48 compounds in white quadriceps and 34 compounds in soleus muscles that distinguish whole-body *Ndufs4* knockout mice from wild types. These metabolic alterations provide insight into skeletal muscle-specific changes in metabolic pathways that result from whole-body mitochondrial dysfunction. These pathways could possibly be targeted in therapeutic interventions to promote muscle health in mitochondrial disorders and improve locomotion, metabolism, and quality of life.

2. Materials and methods

Detailed information regarding the described methods can be found in the supplementary data (Appendix A).

2.1. Animals and sampling

Male whole-body *Ndufs4* knockout mice (B6.129S4-*Ndufs4*^{tm1.1Rpa}/J; referred to as *Ndufs4*^{-/-}) along with age- and sex-matched controls (*Ndufs4*^{+/+}; referred to as WT) were used. *Ndufs4*^{-/-} mice were born from heterozygous (*Ndufs4*^{+/+}) crosses obtained from Jackson Laboratory (ME, USA) (JAX stock #027058, <https://www.jax.org/strain/027058>). It should be noted that this mouse strain is BL6/J-derived and, therefore, potentially contains the *Nnt*^{C57BL/6J} mutation. *Ndufs4* genotypes were confirmed by polymerase chain reaction (PCR) using tail snips. The animals were bred and housed at the specific pathogen-free (SPF) unit of the Vivarium (SAVC reg. no. FR15/13458) of the Pre-Clinical Drug Development Platform (PCDDP, NWU, RSA) and approval (NWU-00378-16-A5) for this study was obtained from the Animal Research Ethics Committee of the NWU. Animals were group housed under temperature- (22 ± 1 °C), humidity- (55 ± 10%) and light-controlled (12:12-h light/dark cycle) conditions with standard laboratory chow (Laboratory animal food: Rodent Breeder, Cat. #RM1845, LabChef, Nutritionhub) and water provided ad libitum. Mice were euthanized between postnatal day (P) 45–50 via cervical dislocation at the same time of day (8:00–9:00 AM) after overnight (12-h) fasting. From both hind limbs, white muscle portions of quadriceps femoris, as well as soleus muscles, were collected. Tissues were snap-frozen in liquid nitrogen (within 15 min *post-mortem*) and stored at –80 °C until used.

2.2. Enzyme assays

The enzyme activities of complex I (EC 1.6.5.3), complex II (EC 1.3.5.1), complex III (EC 1.10.2.2), and complex IV (EC 1.9.3.1) of the respiratory chain, as well as citrate synthase (EC 2.3.3.1), were measured in 700 × g supernatants of white quadriceps and soleus muscle homogenates. Analyses were performed on *Ndufs4*^{-/-} (n = 12) and WT (n = 10) samples via standard operating procedures based on existing methods [17–19].

2.3. Metabolic profiling

Metabolic profiles were obtained from the white quadriceps and soleus muscles of *Ndufs4*^{-/-} (n = 19) and WT (n = 20) mice using a multi-platform metabolomics approach. The analytical techniques were performed as previously described and comprised of targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) [20], untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) [21], and proton nuclear magnetic resonance (¹H NMR) spectroscopy [22]. However, ¹H NMR spectroscopy could not be performed on soleus muscles, due to limited sample quantity. The data obtained in this study [23] can be accessed at the NIH Common Fund's Data Repository and Coordinating Center (supported by NIH grant, U01-DK097430) website, the Metabolomics Workbench, <http://www.metabolomicsworkbench.org>.

2.3.1. Extract preparation and derivatization

Muscle tissues were finely minced and homogenized in the presence of internal standards using a vibration mill. Metabolite extraction was achieved using a modified monophasic Bligh–Dyer extraction method [24] with a solvent ratio of 3:1:1 (methanol:water:chloroform). Details regarding the exact solvent volumes used for white quadriceps (~80 mg) and soleus (~5 mg) muscles can be found in the supplementary data (Appendix A). The water used for extraction contained a mixture of internal standards consisting of *N*, *N*-dimethyl-*L*-phenylalanine and 3-phenylbutyric acid. Quality control (QC) samples were prepared from aliquots of all samples within a tissue type. Tissue extracts were divided into several vials (one for each analytical platform) and dried under nitrogen. Preparation per platform, in short, was: reconstitution in water for ¹H NMR; oximation and silylation for

GC-TOF-MS; as well as the addition of stable isotopes and butylation for LC-MS/MS.

2.4. Data processing

All data processing were done using Excel 2013 (Microsoft Inc.) and MetaboAnalyst 3.0 [25].

2.4.1. Enzyme assays

Enzyme activities were normalized to total protein (mg) content, reflecting tissue mass. Pretreatment of data involved generalized logarithm (glog) transformation.

2.4.2. Metabolic profiling

Spectral data obtained from the analytical platforms were extracted into matrices. Data matrices were individually inspected (correct peak picking and alignment, batch effect, and data integrity) as well as preprocessed (data filtering, missing value imputation, and normalization). Metabolites were normalized to tissue mass. This was achieved through normalization with either *N*, *N*-dimethyl-L-phenylalanine (GC-TOF-MS and LC-MS/MS data) or 3-phenylbutyric acid (¹H NMR data). In addition, metabolites measured with LC-MS/MS were further normalized to stable isotopes, which were added to samples before derivatization. Each metabolite was either normalized to its own isotope (where possible) or to an isotope with a similar retention time to which the metabolite strongly correlated. No batch corrections were needed as the QCs showed no significant batch effects. Thereafter, data pretreatment (glog transformation and mean centering) and outlier detection (principal component analysis (PCA) and heatmaps) were done.

2.5. Statistical analyses

2.5.1. Enzyme assays

Differences between *Ndufs4*^{-/-} and WT enzyme activities were regarded as statistically significant with a Student's *t*-test *p*-value of < 0.05.

2.5.2. Metabolic profiling

Significantly discriminatory features between *Ndufs4*^{-/-} and WT metabolic profiles were determined via univariate analyses. Statistically important differences (Student's *t*-test *p*-values) were inspected at a 5% and 10% significance level, and false discovery rate-adjusted *p*-values were calculated. The cut-off threshold for practical significance [26] was set at an effect size *D*-value of ≥ 0.5. The important features were visually investigated regarding their clustering, covariance, and discriminatory power via PCA as well as volcano plots. PCA was performed in MetaboAnalyst 3.0 [25] on a log-transformed and autoscaled dataset — comprising only the important metabolites identified on each platform. Finally, metabolite identities were assigned to spectral features, with high confidence levels [27,28]. Data set features were identified via spectral and retention time matching using commercial and in-house spectral libraries together with public databases.

3. Results and discussion

3.1. Respiratory chain enzyme activities in *Ndufs4*^{-/-} muscles of different fiber type composition

The average (± SD) mitochondrial enzyme activities (respiratory chain complexes I-IV and citrate synthase) in *Ndufs4*^{-/-} (*n* = 12) and WT (*n* = 10) white quadriceps and soleus muscles are summarized in Table 1. Enzyme activities were normalized to total protein (mg) content, rather than citrate synthase (CS) content, in order to focus on the relative enzyme capacity per tissue mass — thereby emphasizing the relative mitochondrial content of the two muscle fiber types, which was

relevant to our metabolic results. In addition, Alam and colleagues [29] recently called into question whether CS activity could be considered a measure of mitochondrial volume in *Ndufs4*^{-/-} mice. Through experimental and computational strategies, the authors reported significantly increased (72%) CS activity in *Ndufs4*^{-/-} muscles — suggested to reflect an increase in Krebs cycle enzyme levels rather than mitochondrial content. With regard to our data, no significant difference in CS activity was found in *Ndufs4*^{-/-} white quadriceps muscles compared to WTs; whereas a moderate decrease (12%, *p* = 0.0149) in CS activity was observed in *Ndufs4*^{-/-} soleus muscles. Therefore, since a statistically significant difference was detected between WT and *Ndufs4*^{-/-} soleus samples, normalization to CS would more than likely skew enzymatic data and lead to a distorted picture of the results. The lower *Ndufs4*^{-/-} soleus CS activity we report, is in stark contrast to the 72% increase in CS activity previously reported for *Ndufs4*^{-/-} (P35) hind limb muscles [29] — the difference could possibly be ascribed to the age difference between the mice studied or differences in the experimental procedures employed. However, several studies [9,30–32] on other *Ndufs4*^{-/-} tissues have reported no alterations in CS activity, similar to the results we report here. This discrepancy should thus be investigated further.

Our data revealed a severe reduction (80%) in rotenone-sensitive CI activity (*p* < 0.0001) in both *Ndufs4*^{-/-} white quadriceps and soleus muscles, compared to WTs. These results correspond with that reported for *Ndufs4*^{-/-} skeletal muscle [7,29]. Although CI activity is reduced by the same factor in both muscles, glycolytic fibers have approximately 2–3 times fewer mitochondria than oxidative fibers [16]. Accordingly, with a *Ndufs4* knockout, our data demonstrate that glycolytic fibers display a much lower residual CI activity (4.58 ± 1.67 nmol/min/mg) than oxidative fibers (14.74 ± 6.67 nmol/min/mg). As evident from our metabolomics data (discussed in Section 3.4), this difference in residual CI activity reflects a biochemical threshold effect with the metabolic consequences of CI deficiency considerably more apparent in white quadriceps compared to soleus muscles. In agreement, model predictions by Alam and colleagues [29] (on entire hind limb *Ndufs4*^{-/-} muscle) suggested that CI activity could be inhibited up to a critical value (90% of normal levels) before major metabolic consequences become apparent.

We found no significant differences in CII–CIV activities in *Ndufs4*^{-/-} white quadriceps compared to WTs. However, in *Ndufs4*^{-/-} soleus muscles, CIII activity was moderately decreased (18%, *p* < 0.0001). CIII has been shown to form a functional, albeit unstable, supercomplex with CI subassemblies in *Ndufs4*^{-/-} muscle [7] and slight decreases in CIII activity have been reported in other tissues from *Ndufs4*^{-/-} mice [9,31] as well as patients [30,33–36]. Literature reports on respiratory chain (RC) enzyme activities in *Ndufs4*^{-/-} mice are quite diverse, with multiple studies [7,9,29–31] reporting variations in CII–CIV activity. This variation can possibly be attributed to tissue-specific differences, variances in sample preparation resulting in different amounts of functional enzyme (especially in the case of CI and supercomplexes thereof, due to its instability), as well as differences in the age of the mice at which point they were studied.

3.2. Statistical significance and discriminative power of *Ndufs4*^{-/-} metabolic profiles

At 95% and 90% significance, univariate statistical analyses revealed a respective 48 (Table A.2) and 34 (Table A.3) discriminatory metabolites between *Ndufs4*^{-/-} and WT white quadriceps and soleus muscles. Volcano plots (Fig. 1 A and B) depict the practical significance of all analytes detected by multi-platform metabolomics in white quadriceps and soleus muscles respectively. Significantly altered metabolites are indicated as decreased (green) or increased (red), while insignificant alterations are shown in gray. The five most decreased and increased metabolites are highlighted with ellipses and listed according to rank. As evident in the volcano plots, the majority of important

Table 1
Mitochondrial respiratory chain enzyme activities in skeletal muscles from WT and *Ndufs4*^{-/-} mice.

Enzyme activity	White quadriceps			Soleus		
(nmol/min/mg)	WT	<i>Ndufs4</i> ^{-/-}	<i>Ndufs4</i> ^{-/-} /WT	WT	<i>Ndufs4</i> ^{-/-}	<i>Ndufs4</i> ^{-/-} /WT
CI	22.9 ± 5.9	4.58 ± 1.7*	0.20	81.2 ± 15.1	14.7 ± 6.7*	0.19
CII	29.9 ± 4.2	31.3 ± 5.0	1.05	63.1 ± 9.8	63.6 ± 10.3	1.01
CIII	430.2 ± 57.1	455.6 ± 47.8	1.06	1430.1 ± 105.1	1170.7 ± 97.4*	0.82
CIV	275.1 ± 32.4	273.8 ± 30.6	1.00	1033.1 ± 168.7	1077.0 ± 199.1	1.04
CS	233.4 ± 48.4	229.7 ± 50.6	0.98	636.7 ± 51.5	562.9 ± 75.3*	0.88

Enzyme activities normalized to mg protein. Average values (± SD) were obtained from 12 *Ndufs4*^{-/-} and 10 WT animals. Significant differences in *Ndufs4*^{-/-} enzyme activity relative to WT are indicated with an asterisk (*p < 0.05). Abbreviations: CI, Complex I; CII, Complex II; CIII, Complex III; CIV, Complex IV; CS, Citrate synthase; WT, Wild type (*Ndufs4*^{+/+}).

metabolites were decreased in *Ndufs4*^{-/-} muscles, compared to WT, with the white quadriceps (Fig. 1A) displaying a more pronounced metabolic profile. The highest relative fold change was attributed to decreased levels of N, N-dimethylglycine (DMG), creatinine, and 2-aminoadipate (2AA), while the most prominent increases in metabolite levels (common to both muscles) were seen in lysine (Lys) and its catabolic product — pipecolate (Pip), as well as N-acetylaspartate (NAA). Multivariate PCA (Fig. 2) of significant metabolites revealed a partial separation in the clustering of *Ndufs4*^{-/-} and WT groups in both muscles, indicating a distinct metabolic profile in *Ndufs4*^{-/-} muscle.

3.3. Common metabolic alterations in *Ndufs4*^{-/-} skeletal muscles of different fiber type composition

Numerous alterations in the levels of amino acids, acylcarnitines, and organic acids were evident in *Ndufs4*^{-/-} skeletal muscles. The Venn diagram in Fig. 3 illustrates the number of similarities, as well as differences, observed between *Ndufs4*^{-/-} metabolic profiles in the two muscles. Among the metabolites differing between genotypes, the majority (n = 26) were common to both white quadriceps and soleus muscles. Taken together, these adaptations revealed a similar bioenergetic flexibility in skeletal muscles of different fiber type composition. The most prominent changes in metabolite levels could be organized into several non-classical pathways involved in oxidative metabolism and redox regulation as depicted in Fig. 4. These pathways seem to participate in an attempt to increase (and restore) the electron flux to the RC via the ubiquinone (Q) pool, fueling CIII and allowing oxidative ATP production while bypassing CI in the process. A more in-depth discussion of the adaptive mechanisms involved in *Ndufs4*^{-/-} skeletal muscle follows.

3.3.1. Glycolysis, triglyceride catabolism and the glycerol-3-phosphate (GP) shuttle

The accumulation of pyruvate and its derivatives, lactate, and alanine is considered a hallmark feature of mitochondrial metabolic dysfunction attributed to a disturbed redox balance (decreased NAD⁺/

NADH ratio) [37]. In agreement with this hallmark, Johnson and colleagues [11] reported the accumulation of pyruvate, lactate, and glycolytic intermediates in *Ndufs4*^{-/-} whole-brain (P30). However, in *Ndufs4*^{-/-} skeletal muscles, we observed significantly decreased levels of pyruvate, glycerol, and alanine along with significantly (white quadriceps) and moderately (soleus) decreased lactate levels. A possible mechanistic explanation for these observations is the activity of the glycerol-3-phosphate (GP) shuttle. Like lactate dehydrogenase, the GP shuttle rapidly re-oxidizes cytosolic NADH produced by glycolysis. However, an important difference between these redox regulatory mechanisms is the oxidative ATP producing capacity of the GP shuttle. Lying at the intersection of glycolysis, fatty acid metabolism, and oxidative phosphorylation, the GP shuttle serves as an alternative electron donor to the RC at the ubiquinone (Q) pool by transferring reducing equivalents from the cytosol to mitochondria. In this shuttle, the cytosolic enzyme, glycerol-3-phosphate dehydrogenase (c-GPDH, EC 1.1.1.8), oxidizes NADH to NAD⁺ during the synthesis of GP from dihydroxyacetone phosphate (DHAP) — formed from either glycolysis or the phosphorylation of glycerol derived from triglyceride catabolism. GP can then be used as a substrate for the synthesis of either pyruvate, triglycerides, or phospholipids, however, in the case of the GP shuttle, GP is converted back to DHAP via the inner-membrane bound mitochondrial form of GPDH (m-GPDH) which reduces FAD to FADH₂. FADH₂ then reduces ubiquinone (Q) to ubiquinol (QH₂). The resulting DHAP is then recycled to again oxidize cytosolic NADH. Although understanding of the structure, function, and regulation of this system is still limited (reviewed by McDonald et al. [38]), several studies have suggested the important role of m-GPDH in thermogenesis and have shown it to be an important site of ROS production. Among the various metabolite shuttles responsible for the transfer of reducing equivalents from the cytosol to mitochondria, the GP shuttle is the most important in skeletal muscle [16]. Therefore, we postulate that the metabolic alterations we observed indicate the diversion of the glycolytic and triglyceride catabolic fluxes towards the GP shuttle in an attempt to not only restore the redox balance but also electron delivery to the RC at the Q pool. This mechanism could also possibly be involved in aberrant

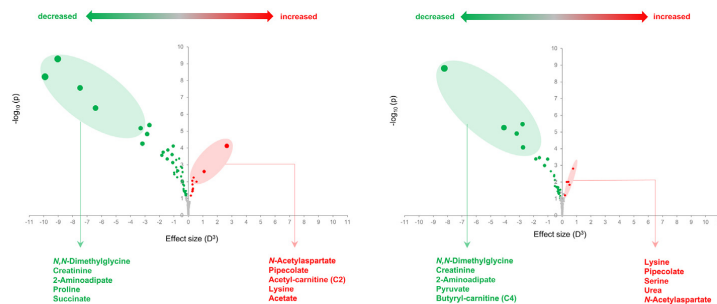


Fig. 1. Univariate analyses indicating the practical significance of important skeletal muscle metabolites. Volcano plots depicting multi-platform metabolomics results of A: white quadriceps muscles and B: soleus muscles from *Ndufs4*^{-/-} mice (n = 19) compared to WT (n = 20). The x-axis specifies the effect size raised to the third power (D³) and the y-axis specifies the negative logarithm to the base 10 of the t-test p-values. Green and red symbols respectively indicate metabolites significantly lower or higher in *Ndufs4*^{-/-} mice compared to WT (p < 0.1 and D ≥ 0.5). The five most significantly decreased and increased metabolites are highlighted and listed according to rank.

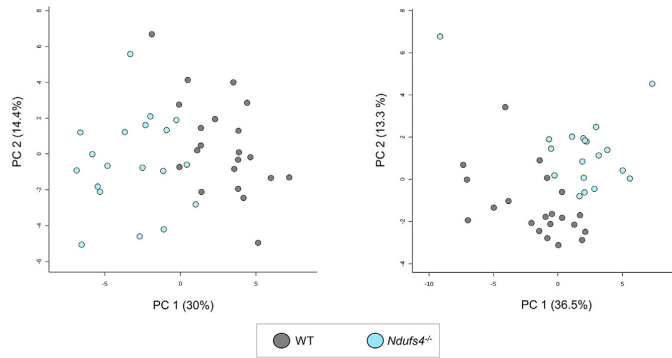


Fig. 2. Multivariate analyses indicating the discriminative power of important skeletal muscle metabolites. Principal component analysis (PCA) scores plots depicting significant metabolites ($p < 0.1$, $D \geq 0.5$) identified in A: white quadriceps muscles and B: soleus muscles. Partial separation in the clustering of *Ndufs4*^{-/-} ($n = 19$, blue) and WT ($n = 20$, gray) groups are visible in both cases. White quadriceps: PC1 and PC2 account for 30.0% and 14.4% of variation respectively. Soleus: PC1 and PC2 account for 36.5% and 13.3% of variation respectively.

ROS production, a feature often reported in mitochondrial disease.

3.3.2. β -oxidation, amino acid catabolism, and the electron transfer flavoprotein (ETF/ETF-QO) system

Both *Ndufs4*^{-/-} skeletal muscle types displayed dramatic decreases in the levels of *N*, *N*-dimethylglycine (DMG), fatty acid acylcarnitines of even (C0, C4, C6, C8, C12, and C16) and odd (C3 and C5) chain length, as well as 2-aminoadipate (2-AA). Although previous metabolomics investigations on mitochondrial disease have reported disturbances in one-carbon metabolism, the catabolism of fatty acids, branched-chain amino acids (BCAA), as well as the ketogenic amino acid, lysine, the underlying mechanisms remain unclear [37]. We postulate that these diverse metabolic alterations point to several pathways that converge at the electron transfer flavoprotein (ETF/ETF-QO) system. The ETF/ETF-QO system functions as an electron transfer pathway conducting electrons from eleven mitochondrial flavoprotein dehydrogenases (m-FDHs) to the Q pool of the RC. These m-FDHs include enzymes involved in mitochondrial one-carbon metabolism (dimethylglycine dehydrogenase, EC 1.5.99.2; and sarcosine dehydrogenase, EC 1.5.99.1), fatty acid β -oxidation (short-chain acyl-CoA dehydrogenase, EC 1.3.99.2; medium-chain acyl-CoA dehydrogenase, EC 1.3.99.3; and long-chain acyl-CoA dehydrogenase, EC 1.3.99.13), as well as branched-chain amino acid catabolism (isovaleryl-CoA dehydrogenase, EC 1.3.99.10; and isobutyryl-CoA dehydrogenase) and lysine catabolism (glutaryl-CoA dehydrogenase, EC 1.3.99.7) (reviewed by Watmough

et al. [39]). Therefore we hypothesize that, in *Ndufs4*^{-/-} skeletal muscle, there is an increased flux through these m-FDHs supplying the ETF/ETF-QO system with electrons in order to compensate for decreased electron transfer from CI. This increased flux, in part, could lead to the observed decreased metabolite levels. However, this switch in flux cannot effectively compensate for the CI defect as all of these FADH₂ producing pathways also require NAD⁺ as a co-factor. NADH levels could build up due to CI inactivity as well as these compensatory reactions themselves. The hypothesized result of the altered redox state and shortage of NAD⁺ is seen in the lysine catabolic pathway. Lysine is normally catabolized to 2-AA via the saccharopine pathway, which requires NADP⁺ and two NAD⁺ molecules. The subsequent conversion of 2-AA to glutaryl-CoA (substrate for ETF/ETF-QO system) requires an additional NAD⁺ molecule. In addition to decreased 2-AA levels, we observed the accumulation of lysine and its alternative catabolite, pipecolate (Pip), in *Ndufs4*^{-/-} skeletal muscles — indicating that an alternative non-NAD⁺-consuming catabolic route is preferred, resulting in an even larger reduction in 2-AA levels. Altogether, the data suggest that β -oxidation and amino acid catabolism might fuel the Q cycle via the ETF/ETF-QO system, even though a disturbed redox balance might limit the efficacy of some of the reactions.

When comparing previous metabolomics studies on *Ndufs4* knockout mice with our current observations, similar ambiguous effects on lipid metabolism are noticeable. In *Ndufs4*^{-/-} liver, markedly reduced levels of fat droplets and free fatty acids were reported [11];

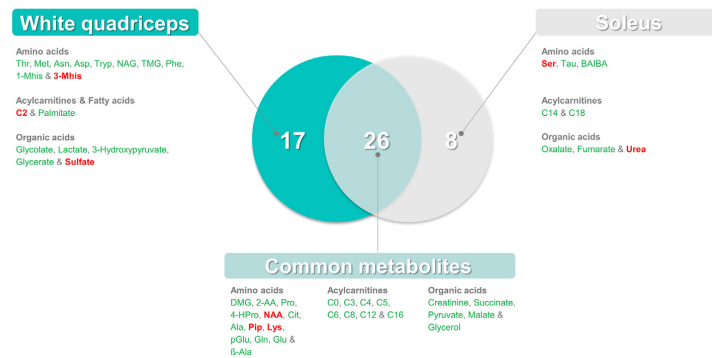


Fig. 3. Venn diagram of important metabolites discriminating *Ndufs4*^{-/-} and WT groups in skeletal muscles. Green and red respectively indicate metabolites significantly ($p < 0.1$ and $D \geq 0.5$) lower or higher in *Ndufs4*^{-/-} mice ($n = 19$) compared to WT ($n = 20$). Among the important metabolites identified, 26 were common to both muscles, while several alterations were unique to the quadriceps ($n = 17$) and soleus ($n = 8$). Important metabolites ($n = 5$) identified in white quadriceps muscles by NMR spectroscopy were excluded from the comparison as soleus samples were not analyzed on that analytical platform due to limited sample quantity. Abbreviations: 1-Mhis, 1-Methylhistidine; 2-AA, 2-Aminoadipate; 2-Mhis, 2-Methylhistidine; Ala, Alanine; Asn, Asparagine; Asp, Aspartate; BAIBA, β -aminoisobutyrate; β -Ala, Beta-alanine; C0, Carnitine; C2, Acetyl-carnitine; C3, Propionyl-carnitine; C4, Butyryl-carnitine; C5, Isovaleryl-carnitine; C6, Hexanoyl-carnitine; C8, Octanoyl-carnitine; C12, Dodecanoyl-carnitine; C14, Myristoyl-carnitine; C16, Palmitoyl-carnitine; C18, Stearoyl-carnitine; Cit, Citrulline; DMG, *N*, *N*-Dimethylglycine; Gln, Glutamine; Glu, Glutamate; Hpro, Hydroxyproline; Lys, Lysine; Met, Methionine; NAA, *N*-Acetylglutamate; pGlu, Pyroglutamate; Phe, Phenylalanine; Pip, Pipecolate; Pro, Proline; Ser, Serine; Tau, Taurine; Thr, Threonine; TMG, Trimethylglycine; Trp, Tryptophan.

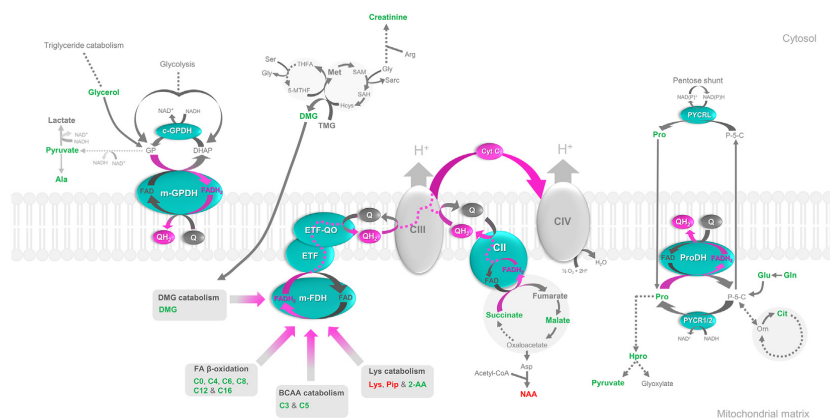


Fig. 4. Common metabolic alterations in *Ndufs4*^{-/-} skeletal muscles. *Ndufs4*^{-/-} quadriceps and soleus skeletal muscles both show a metabolic shift towards several non-classical pathways involved in oxidative metabolism and redox regulation, in an attempt to restore electron delivery to the respiratory chain via the ubiquinone (Q) pool. Green and red indicate metabolite levels significantly lower or higher, respectively, in *Ndufs4*^{-/-} mice (n = 19) compared to WT (n = 20). Pink arrows indicate electron flow. Dashed lines reflect multi-step reactions not fully shown. Multi-platform metabolic profiling of *Ndufs4*^{-/-} skeletal muscles indicates (from left to right): 1) a shift in glycolysis and triglyceride catabolism towards the glycerol-3-phosphate shuttle, which re-oxidizes cytosolic NADH while serving as an electron donor to the respiratory chain via the mitochondrial enzyme glycerol-3-phosphate dehydrogenase (m-GPDH); 2) a shift in flux through several mitochondrial flavoprotein dehydrogenases (m-FDH) involved in one-carbon metabolism (dimethylglycine dehydrogenase), fatty acid β -oxidation (short-, medium- and long-chain acyl-CoA dehydrogenase), and amino acid catabolism (isobutyryl-, isovaleryl- and glutaryl-CoA dehydrogenase) which supply electrons to the respiratory chain via the electron transfer flavoprotein (ETF/ETF-QO) system; 3) a shift in flux through the Krebs cycle to increase electron delivery to the respiratory chain via complex II (CII; succinate dehydrogenase); and 4) increased mitochondrial cycling of proline and pyrroline-5-carboxylate (P-5-C) to directly provide electrons to the respiratory chain via proline dehydrogenase and sustain NAD⁺ levels. Abbreviations: Enzymes in blue: ETF, Electron transfer flavoprotein; ETF-QO, Electron transfer flavoprotein:ubiquinone oxidoreductase; m-FDH(s), Mitochondrial flavoprotein dehydrogenase(s); m-GPDH, Mitochondrial glycerol-3-phosphate dehydrogenase; c-GPDH, Cytosolic glycerol-3-phosphate dehydrogenase; CII, Complex II or succinate dehydrogenase; ProDH, Proline dehydrogenase; PYCR, Pyrroline-5-carboxylate reductase; Cyt c, Cytochrome c. Electron carriers: FAD, Oxidized flavin adenine dinucleotide; FADH₂, Reduced flavin adenine dinucleotide; NAD⁺, Oxidized nicotinamide adenine dinucleotide; NADH, Reduced nicotinamide adenine dinucleotide; Q, Ubiquinone (oxidized coenzyme Q); QH₂, Ubiquinol (reduced coenzyme Q). Metabolites: 2-AA, 2-Aminoacidipate; 5-MTHF, 5-Methyltetrahydrofolate; Ala, Alanine; Arg, Arginine; Asp, Aspartate; C0, Carnitine; C12, Dodecanoyl-carnitine; C16, Palmitoyl-carnitine; C3, Propionyl-carnitine; C4, Butyryl-carnitine; C5, Isovaleryl-carnitine; C6, Hexanoyl-carnitine; C8, Octanoyl-carnitine; Cit, Citrulline; DHAP, Dihydroxyacetone phosphate; DMG, N, N-Dimethylglycine; GP, Glycerol-3-phosphate; Gln, Glutamine; Glu, Glutamate; Gly, Glycine; Hcys, Homocysteine; Hpro, Hydroxyproline; Lys, Lysine; Met, Methionine; NAA, N-Acetylaspartate; Orn, Ornithine; P-5-C, Pyrroline-5-carboxylate; Pip, Pipecolate; Pro, Proline; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; Sar, Sarcosine; Ser, Serine; THFA, Tetrahydrofolate.

while significantly increased blood hydroxy-acylcarnitine (C4:0, C16:0, and C18:1) levels were reported in a comparable *Ndufs4* knockout mouse model (*Ndufs4*^{flx/flx}) [32]. The latter observation was postulated to reflect a blockage in the NAD⁺-dependent (third) step of β oxidation. However, upon further investigation, *Ndufs4*^{flx/flx} liver and heart revealed no alterations in carnitine species, while skeletal muscle displayed no detectable lipid accumulation via Oil Red O staining [32]. These observations could fit into our combined hypotheses of decreased lipogenesis and increased lipolysis due to the activity of the GP shuttle, as well as increased fatty acid β oxidation, albeit inefficient due to a redox imbalance.

3.3.3. One-carbon metabolism, the methylation cycle, and the transsulfuration pathway

The altered redox state, as well as the postulated use of DMG by the ETF/ETF-QO system, seem to contribute to the disruption of one-carbon metabolism, the methylation cycle, and the transsulfuration pathway. These disturbances were more prominent in white quadriceps muscles which, along with decreased levels of DMG, displayed significantly decreased levels of trimethylglycine (betaine), methionine, threonine, and the accumulation of choline. Trimethylglycine (TMG) originates from the NAD⁺ dependent catabolism of choline, which takes place in mitochondria. DMG is then produced from TMG in a folate-independent cytosolic reaction during which homocysteine is re-methylated to methionine. NAD⁺ shortage could, therefore, cause choline build up and a further reduction in DMG levels. As DMG also has the ability to yield one-carbon units via folate-dependent mitochondrial reactions

involving dimethylglycine dehydrogenase (which produces sarcosine) and sarcosine dehydrogenase (which produces glycine), this metabolite links one-carbon metabolism to the ETF/ETF-QO system.

Furthermore, in both *Ndufs4*^{-/-} skeletal muscle types, dramatic decreases were seen in creatinine levels along with slightly (not significantly) reduced creatine levels. Creatine biosynthesis takes place chiefly in the liver and kidneys, however, a small amount of skeletal muscle creatine stores have been shown to be produced in the muscles themselves [40]. Creatine biosynthesis is a two-step reaction involving the conversion of arginine and glycine to guanidinoacetate, which is then methylated via SAM to form creatine and SAH. A disturbance in one-carbon metabolism could lead to lowered SAM available for methylation reactions, therefore dramatic decreases in creatinine could reflect the disrupted methylation of guanidinoacetate to creatine. In agreement, significantly reduced levels of 1-methylhistidine (1-Mhis) and increased levels of histamine in *Ndufs4*^{-/-} white quadriceps could also reflect disturbed methylation reactions. On the whole, the observed metabolic alterations indicate disturbances in one-carbon metabolism and methylation reactions, possibly due to a disturbed redox balance and derangements in DMG metabolism.

3.3.4. The Krebs cycle and mitochondrial complex II (succinate dehydrogenase)

We observed significantly decreased levels of succinate and malate in both muscles along with significant (soleus) and moderate (white quadriceps) reductions in fumarate levels. A similar observation was made in *Ndufs4*^{-/-} (P35) hind limb skeletal muscle by Alam and

colleagues [29], who reported decreased succinate levels along with an increase in flux (10–11%) through reactions providing substrates to CII and a slight decrease in the other (generally NAD^+ consuming) reactions which mainly fuel CI. The reduction in metabolite levels we observed could, therefore, reflect an increased use of succinate to fuel CII and the respiratory chain via the Q pool together with a decreased re-supply thereof due to a congested Krebs cycle. In addition, we observed increased levels of *N*-acetylaspartate (NAA) which is hypothesized to be an alternative storage and transport form of acetate and has been suggested to play a role in lipid metabolism and posttranslational protein modification [41]. Although the physiological functions of NAA are still poorly understood, it was recently discovered that NAA metabolism is not exclusive to the brain as previously thought. Aberrant NAA metabolism has been reported in mitochondrial disease, however, to the best of our knowledge this is the first report thereof in skeletal muscle. NAA is synthesized from aspartate and acetyl-CoA by aspartate *N*-acetyltransferase (Asp-NAT, EC 2.3.1.17). In skeletal muscle, the expression of *Nat8l* mRNA (encoding Asp-NAT) has been observed, although levels were reported to be negligible [41]. We, therefore, hypothesize that the rate of acetyl-CoA synthesis from sources like β -oxidation and lysine catabolism (which fuel the ETF/ETF-QO system) exceeds the rate at which the Krebs cycle can utilize the molecule, therefore acetyl-CoA accumulates and is converted to NAA [41].

3.3.5. Proline metabolism and the proline cycle

Although there is still much to be learned about the precise mechanism and functions of the proline cycle, the vital role of this relatively novel pathway in health and disease is becoming increasingly clear (reviewed by Phang et al. [42]). Some of the most striking metabolic alterations we observed in *Ndufs4*^{-/-} skeletal muscles included the reduction of proline, hydroxyproline (Hpro), citrulline, glutamate, and glutamine levels. These alterations could reflect aberrant proline metabolism, which is strongly associated with the pentose phosphate pathway, urea cycle, and Krebs cycle. The mitochondrial flavin-dependent enzyme, proline dehydrogenase (ProDH, EC 1.5.99.8), together with the cytosolic (PYCRL) and mitochondrial (PYCRL2) isoforms of pyrroline-5-carboxylate reductase (EC 1.5.1.2) are responsible for the interconversions of proline and pyrroline-5-carboxylate (P-5-C) constituting the proline cycle. Proline can directly act as an alternative source of electron delivery to the RC Q pool, through its catabolism to P-5-C via ProDH. P-5-C can also be synthesized from either glutamate or ornithine while its conversion back to proline regenerates NAD(P)^+ . We, therefore, postulate that the adaptive increased cycling of proline and P-5-C might serve to sustain QH_2 and NAD(P)^+ levels. In addition to energy production and redox homeostasis, proline metabolism has been shown to be involved in ROS production, the initiation of apoptosis, autophagy, as well as protein and nucleotide synthesis while proline's post-translational product, hydroxyproline, has recently been recognized as a substrate for the synthesis of pyruvate, glucose, and glycine (reviewed by Wu et al. [43]). Taken together, proline metabolism might play a central role in several metabolic pathway derangements often reported in mitochondrial disease.

3.4. Fiber type-specific metabolic alterations in *Ndufs4*^{-/-} skeletal muscles

When looking at the difference between the important metabolites identified in *Ndufs4*^{-/-} muscles (Fig. 3), seventeen metabolites were uniquely altered in the white quadriceps while eight alterations were unique to the soleus. This comparison excludes the five important metabolites detected with NMR spectroscopy as soleus samples were not analyzed on that analytical platform due to limited sample quantity. Altogether, glycolytic fibers (white quadriceps) seemed to show more pronounced metabolic disturbances in response to CI deficiency with NAA accumulation being more than two-fold higher than in soleus muscles, along with an additional accumulation of acetyl-CoA derivatives, acetate, and acetyl-carnitine (C2). In addition, *Ndufs4*^{-/-} white

quadriceps muscles showed significant 3-methylhistidine accumulation, which is indicative of myofibrillar protein breakdown [44]. This observation agrees with literature reports of glycolytic fibers showing more pronounced atrophy than oxidative fibers in pathological conditions affecting muscle protein balance, and aging [16]. Taken together with the greater disturbance in methylation reactions we observed in white quadriceps muscles (discussed in Section 3.3.3), these alterations could indicate more profound disruptions of the redox balance (NAD^+/NADH ratio), Krebs cycle congestion and possible acetyl-CoA accumulation in more glycolytic muscle fibers. In addition to less dramatic metabolic alterations than in the white quadriceps, we saw significantly increased levels of serine along with decreased levels of taurine and pyroglutamate in *Ndufs4*^{-/-} soleus muscles. These alterations could indicate a shift in glycolysis towards serine production, which could be used for glutathione synthesis — a phenomenon often described in mitochondrial disease due to increased electron leak and ROS production. As oxidative fibers have been shown to have a greater supply of substrates, oxygen, and more mitochondria (with greater oxidative capacity) [16], it can be postulated that mitochondrial ROS levels — due to the CI deficiency — are higher in *Ndufs4*^{-/-} soleus muscles and therefore the need for glutathione ROS scavenging is greater. Furthermore, our results suggest that BCAA catabolism might be more dramatically affected in soleus muscles as we observed moderately (but not significantly) increased levels of valine and leucine, significant decreases in β -aminoisobutyrate (BAIBA) levels, and much greater decreases in C5- and C3-carnitine levels than in the white quadriceps muscles. Hence, the data could point to a more effectively functioning Krebs cycle and a better metabolic adaption of soleus muscles to the CI defect.

4. Conclusions

We report the first fiber type-specific enzymatic and metabolic data in *Ndufs4*^{-/-} skeletal muscles. Enzyme assays revealed a severe reduction (80%) in CI activity in both glycolytic (white quadriceps) and oxidative (soleus) muscles from whole-body *Ndufs4*^{-/-} mice. Such an extreme reduction in CI activity would greatly reduce the electron flux to the rest of the respiratory chain (RC). As an adaptive response in both tissues, our metabolic data suggest that alternative fuels and non-classical pathways serve to sustain the RC ubiquinol (QH_2) pool, thereby restoring electron flux to CIII through the ubiquinone (Q) cycle. However, despite these metabolic adaptations in skeletal muscle, a disturbed redox balance still seems to result in congested mitochondrial pathways. Metabolic and bioenergetic disturbances due to CI deficiency seem to be more evident in glycolytic fibers, possibly due to their innately lower mitochondrial content. Some of the most significant metabolic alterations we observed in *Ndufs4*^{-/-} muscles implicate the involvement of the glycerol-3-phosphate (GP) shuttle, the electron transfer flavoprotein (ETF/ETF-QO) system, complex II of the RC, and the proline cycle in electron delivery to the Q pool. Some of these mechanisms might also serve to restore NAD^+ levels (GP shuttle and proline cycle), while others could contribute to redox imbalance (NAD^+ consuming reactions fueling ETF/ETF-QO and the Krebs cycle) in CI deficiency. Altogether, the observed adaptive mechanisms could explain the apparent lack of an obvious muscle phenotype in *Ndufs4*^{-/-} mice, as well as the reported ability of skeletal muscle to maintain normal ATP production, despite CI deficiency [9,29]. However, the hypotheses generated in this study need to be followed up/validated in more targeted metabolic studies on *Ndufs4*^{-/-} mice. For now, it remains unclear whether these adaptive responses are capable of sustaining energy homeostasis when challenged with exercise or nutrient deprivation. A comparison of these and related metabolic pathways in other tissues (e.g. the brain) could possibly provide answers to tissue-specific phenotypes and novel insight in targeted therapeutic interventions.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

The AnimCare animal research ethics committee of North-West University approved (NWU-00378-16-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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2018.10.034>.

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APPENDIX D

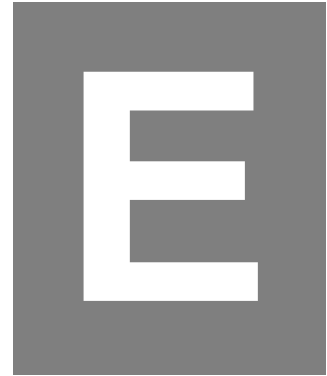
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APPENDIX

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Aberrant BCAA and glutamate metabolism linked to regional neurodegeneration in a mouse model of Leigh syndrome

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ABSTRACT

The dysfunction of respiratory chain complex I (CI) is the most common form of mitochondrial disease that most often presents as Leigh syndrome (LS) in children — a severe neurometabolic disorder defined by progressive focal lesions in specific brain regions. The mechanisms underlying this region-specific vulnerability to CI deficiency, however, remain elusive. Here, we examined brain regional respiratory chain enzyme activities and metabolic profiles in a mouse model of LS with global CI deficiency to gain insight into regional vulnerability to neurodegeneration. One lesion-resistant and three lesion-prone brain regions were investigated in *Ndufs4* knockout (KO) mice at the late stage of LS. Enzyme assays confirmed significantly decreased (60–80%) CI activity in all investigated KO brain regions, with the lesion-resistant region displaying the highest residual CI activity (38% of wild type). A higher residual CI activity, and a less perturbed NADH/NAD⁺ ratio, correlate with less severe metabolic perturbations in KO brain regions. Moreover, less perturbed BCAA oxidation and increased glutamate oxidation seem to distinguish lesion-resistant from -prone KO brain regions, thereby identifying key areas of metabolism to target in future therapeutic intervention studies.

1. Introduction

Effective oxidative phosphorylation (OXPHOS) is central to the proper functioning of mitochondria — the metabolic hubs of the cell. In the OXPHOS system, electron transport through respiratory chain (RC) complexes I, III and IV maintain a proton gradient across the inner mitochondrial membrane, which drives the bulk of cellular adenosine triphosphate (ATP) production via ATP synthase. The electrons

powering this process are derived from nutrient catabolism and donated to the RC at the ubiquinone (Q) pool via complex I (CI), CII and other flavoproteins [1–3]. Among these, CI is the main and most efficient electron donor that is responsible for regenerating NAD⁺. CI dysfunction (OMIM 252010) is the most common form of mitochondrial disease (MD) [4,5] and most often presents as Leigh syndrome (LS) in children, affecting ~1 in 40,000 newborns [6,7]. LS is linked to more than 80 different genetic defects [8,9], with many mutations affecting NDUFS4

Abbreviations: 3-PBA, 3-phenylbutyric acid; AC, anterior cortex; ANOVA, analysis of variance; Asp, aspartic acid; ATP, adenosine triphosphate; BCAA, branched-chain amino acid; BCKDH, branched-chain α -keto acid dehydrogenase; BS, brainstem; C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; CB, cerebellum; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CNS, central nervous system; CS, citrate synthase; DHAP, dihydroxyacetone phosphate; DMPA, *N,N*-dimethyl-*L*-phenylalanine; DNA, deoxyribonucleic acid; ETF, electron-transferring flavoprotein; FDR, false discovery rate; G3P, glycerol-3-phosphate; GABA, γ -aminobutyric acid; GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; Gln, glutamine; Glu, glutamic acid; HET, heterozygous; KIC, α -ketoisocaproate; KO, knockout; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Leu, leucine; LS, Leigh syndrome; MD, mitochondrial disease; MSUD, maple syrup urine disease; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NAD(P)⁺, nicotinamide adenine dinucleotide phosphate (oxidized form); NAD⁺, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NAG, *N*-acetylglutamic acid; NDUFS4, NADH dehydrogenase [ubiquinone] iron-sulfur protein 4; NH₄⁺, ammonium cation; NMDR, national metabolomics data repository; OAA, oxaloacetate; OB, olfactory bulbs; OXPHOS, oxidative phosphorylation; P, postnatal day; P5C, pyrroline-5-carboxylate; P6C, pyrroline-6-carboxylate; PBS, phosphate-buffered saline; PCA, principal component analysis; PCR, polymerase chain reaction; pGlu, pyroglutamate; PPP, pentose phosphate pathway; Pro, proline; Q, ubiquinone; QC, quality control; RC, respiratory chain; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; WT, wild type; α AA, α -amino adipic acid; α HG, α -hydroxyglutaric acid; α KG, α -ketoglutaric acid.

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— a key subunit for CI assembly, stability and activity [10,11] — accounting for some of the more severe forms of the disease [12]. CI deficiency and LS portray genetic and clinical heterogeneity along with poorly understood pathogenic mechanisms and lack effective or curative treatments. Although LS is associated with a highly variable spectrum of symptoms, the most characteristic features include symmetrical bilateral necrotic lesions in distinct brain regions, mitochondrial dysfunction and a neurodegenerative phenotype [6,9,13].

The whole-body *Ndufs4* knockout (KO) mouse model of CI deficiency, develops a progressive phenotype closely resembling human LS at about postnatal day (P) 35, which increasingly worsen until premature death (~P55–75). Symptoms include developmental delay, failure to thrive, lethargy, ophthalmoplegia, locomotor impairment, hearing loss, encephalomyopathy, respiratory deficits and epileptic seizures [14,15]. Research on *Ndufs4* KO mice have revealed a tissue-specific vulnerability to CI deficiency with unknown causal mechanisms. The disease most severely manifests in the brain, with specific brain regions, such as the olfactory bulbs, cerebellum and brainstem, being more vulnerable to the *Ndufs4* KO than others [14,16,17]. Vulnerable brain regions display focal progressive lesions with spongiform neurodegeneration, vascular proliferation, gliosis, aberrant mitochondrial morphology in nerve terminals and necrosis [14,15]. From studies on conditional *Ndufs4* KO mice [neuronal- and glial- [14,18–20], cardiac- [20–22]; liver-, hematopoietic- and myeloid- [23] specific *Ndufs4* KOs] the origin of the fatal phenotype was shown to be predominantly neuronal. However, the major factors underlying regional neurodegeneration remain elusive. A recent investigation in pre-symptomatic (P29–31) *Ndufs4* KO mice found no significant brain regional differences in neuronal populations, DNA damage, CIV levels, mTOR activity or metabolism between *Ndufs4* KO and wild type (WT) mice prior to lesion formation [24]. However, we hypothesize that as LS progresses the metabolism of lesion-prone brain regions will be more perturbed than that of unaffected regions. In this regard, the application of metabolomics as a hypothesis-generating tool could provide novel insight into the mechanisms behind LS and regional neurodegeneration and identify areas of metabolism to further investigate in future targeted studies.

Therefore, we investigated brain regional bioenergetics and metabolism in a relatively large group of late stage diseased (P45–50) *Ndufs4* KO and WT mice. A combination of spectrophotometric enzyme activity assays and multi-platform metabolomics techniques were applied to investigate four brain regions that include three lesion-prone regions (brainstem, BS; cerebellum, CB; and olfactory bulbs, OB) and one lesion-resistant region (anterior cortex, AC). Here, we report distinct metabolic profiles that correlate with the level of residual CI activity in different *Ndufs4* KO brain regions. Furthermore, we show that the fate of metabolites involved in brain glutamate handling and nitrogen flux may play a central role in regional vulnerability to CI deficiency. Altogether, our results provide a better understanding of the pathomechanism of LS and pave the way for the future development of effective treatment options.

2. Materials and methods

2.1. Animals and sampling

Male whole-body homozygous *Ndufs4* KO (*Ndufs4*^{−/−}) mice along with age- and sex-matched controls (WT, *Ndufs4*^{+/+}) were used. Due to known differences in hormone cycles, tissue-specific metabolism and metabolic profiles between sexes [25,26] only one sex-group was used in order to minimise sex-related metabolic variation. KO mice were born from heterozygous crosses (*Ndufs4*^{+/+}; B6.129S4-*Ndufs4*^{tm1.1Rpa/J}) obtained from Jackson Laboratory (JAX stock #027058). *Ndufs4* genotypes were confirmed by polymerase chain reaction using tail snips. The animals were bred and housed at the specific pathogen-free unit of the Vivarium (SAVC reg. no. FR15/13458) of the Pre-Clinical Drug Development Platform (PCDDP; NWU, RSA) and approval (NWU-00378-16-

A5) for this study was obtained from the Animal Research Ethics Committee of the NWU. Animals were group housed under temperature- (22 ± 1 °C), humidity- (55 ± 10%) and light-controlled (12:12 h light/dark cycle) conditions with standard laboratory chow (Rodent Breeder, #RM1845, LabChef, Nutritionhub) and water provided ad libitum. Based on survival curves and phenotypic analyses (results previously reported [27]), an age range from P45–50 was selected to represent the late stage of LS. Mice were euthanized between P45–50 via cervical dislocation at the same time of day (8:00–9:00 AM) after overnight (12h) fasting. The brain was removed and rinsed with ice-cold saline solution (SABAX PBS; 0.9% NaCl (w/v), #7634, Adcock Ingram) to remove surrounding blood. The brain regions of interest, namely the AC, BS, CB and OB, were then dissected, snap-frozen in liquid nitrogen (within 15 min postmortem) and stored at −80 °C until used.

2.2. Enzyme assays

2.2.1. Methods

The activities of the RC complexes I–IV, as well as citrate synthase (CS) were measured in freeze-thawed post-700 ×g supernatants of all brain regions for *Ndufs4* KO (*n* ≥ 11) and WT (*n* ≥ 9) mice. Analyses were performed in triplicate for all assays based on methods described before [6,28–30].

2.2.2. Data processing

RC enzyme activities were expressed as mean ± standard deviation normalized to both protein content and CS. Although results were similar for these normalization methods, the latter is not reported here as we observed significantly elevated CS activity in all KO brain regions. Extreme outliers were removed using box-plots based on Tukey's method [31] and data symmetry was assessed based on standardized skewness values. Data processing was done using Excel 2013 (Microsoft) and SPSS Statistics (v.25.0; IBM).

2.2.3. Statistical analyses

A two-way mixed analysis of variance (ANOVA) was done for each variable (between-subjects factor: genotype; within-subjects factor: brain region). If the assumption of equal variance was severely violated, ANOVA was done on power transformed data. For significant interaction effects, paired *t*-tests (brain regional differences within a genotype) and independent samples *t*-tests (genotypic differences within a brain region) along with effect size (Cohen's *d*-values) calculations were done. In the case of insignificant interaction effects and significant main effects, pairwise comparisons based on marginal means were calculated together with η^2 effect sizes (effect of genotype) or Cohen's *d*-values (brain regional differences). Values of *p* < 0.05 were considered statistically significant and effect sizes were interpreted as follows; η^2 ≥ 0.14 or *d* ≥ 0.8 indicated a large effect; η^2 ≥ 0.06 or *d* ≥ 0.5 indicated a medium effect; and η^2 ≥ 0.01 or *d* ≥ 0.3 indicated a small effect [32,33].

2.3. Metabolic profiling

2.3.1. Methods

Multi-platform metabolomics was done on four different brain regions of *Ndufs4* KO (*n* = 21) and WT (*n* = 25) mice as previously described [34,35] via untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). For each mouse, each brain region was extracted and analyzed individually, yielding a respective 21 (KO) and 25 (WT) biological replicates per brain region. Metabolite extraction was done using a stepwise Bligh–Dyer extraction method [36] to obtain a monophasic (methanol/water/chloroform, 6:2:2) and biphasic (methanol/water/chloroform, 2:1.8:2) extract for each of the 184 individual samples. Monophasic extracts were used for GC-TOF-MS, while the polar phases of biphasic extracts were used for LC-MS/MS.

Frozen brain tissues were weighed and homogenized in the presence of internal standards using a vibration mill. Internal standards, *N*, *N*-dimethyl-*L*-phenylalanine (DMPA) and 3-phenylbutyric acid (3-PBA), were added according to tissue mass as part of the water used for extraction. For each brain regions, a quality control (QC) sample was prepared from pooled aliquots of all sample extracts from that specific brain region. Thereafter, all samples were transferred to vials, dried under nitrogen and derivatized prior to their analysis. For GC-TOF-MS, samples were oximated and silylated, while LC-MS/MS samples were supplemented with stable isotopes and butylated.

2.3.2. Data processing

Spectral data was extracted into matrices, individually inspected (correct peak detection and alignment, batch precision and data integrity) and preprocessed (data filtering, missing value imputation and normalization). Metabolites were normalized to tissue mass through normalization with 3-PBA (GC-TOF-MS data) or DMPA (LC-MS/MS data). For LC-MS/MS data, metabolites were normalized to either their own isotope (where possible) or an isotope with a similar retention time prior to DMPA normalization. No significant batch effects were evident thus no batch corrections were required. Finally, data pretreatment (glog transformation and mean centering) and outlier detection (PCA Hotelling's T^2 ellipses, loadings plot and biplots) were done. Data processing was done using Excel 2013 (Microsoft), SPSS Statistics (v.25.0; IBM) and MetaboAnalyst 3.0 [38]. The metabolomics data obtained in this study [37] can be accessed at the Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (<https://www.metabolomicsworkbench.org>) where it has been assigned Project ID: PR001003.

2.3.3. Statistical analyses

Univariate analyses were used to determine significantly discriminatory features between *Ndufs4* KO and WT metabolic profiles. The student's *t*-test was carried out to identify features with significantly altered intensities and a false discovery rate (FDR) corrected *p*-value of <0.05 was considered statistically significant. Effect sizes (Cohen's *d*-value) of ≥ 0.8 were considered practically significant and used complementary to the *t*-test. Principal component analysis (PCA) and volcano plots were used to visually investigate the clustering, covariance and discriminatory power of the important features. PCA was performed in MetaboAnalyst 3.0 [38] on a glog-transformed dataset comprising only the important metabolites identified on each platform. Dataset features were identified via spectral and retention time matching using commercial and in-house spectral libraries together with public databases and metabolite identities were assigned to spectral features, with high confidence levels [39,40].

3. Results and discussion

3.1. RC enzyme activities are differentially affected in *Ndufs4* KO brain regions

Table 1 summarizes the average activities of RC complexes for the different brain regions. Compared to WT, a differential and significant reduction in CI activity was observed in the *Ndufs4* KO brain regions in the following order: OB (86% reduction) > BS (75%) > CB (72%) > AC (62%). The OB — the first and one of the most severely affected brain regions in KO mice [14] — showed the lowest residual CI activity (14%), while the lesion-resistant AC exhibited the highest (38%). Several others have proposed that residual CI activity levels greatly affect the extent of CI deficiency's downstream consequences [41–46]. Our metabolic data support the notion of such a biochemical threshold effect, as brain regions with lower residual CI activity displayed more apparent metabolic perturbations (Section 3.2). Additionally, CIV and CS activities were slightly (~10–20%) but significantly increased in all *Ndufs4* KO brain regions, except the BS. Since this was not paralleled by increased CII and CIII activity, mitochondrial biogenesis was not considered the sole mechanism behind these observations. Although not evident in brain regions at the pre-symptomatic stage [24], increased CIV levels were reported for *Ndufs4* KO whole brain [47], while increased CS (and CII) activity in other *Ndufs4* KO tissues have been linked to a compensatory increase in CII-mediated fueling of the OXPHOS system [15,42,48]. Our data could reflect similar adaptive responses in mitochondrial energy metabolism. When comparing different brain regions, CII, CIII (except for OB), CIV, and CS activities were all higher ($p < 0.01$) in the lesion-prone regions than the AC for both genotypes — suggesting that regions with greater requirements for OXPHOS driven energy production, may be more vulnerable to neurodegeneration in RC deficiencies, as previously proposed [24,49]. Furthermore, innately lower CIII levels in the OB might render this brain region particularly vulnerable to *Ndufs4* deficiency, since CIII is known to form a functional, albeit unstable, supercomplex with CI subassemblies in *Ndufs4* KO mice [10].

3.2. Metabolic alterations are graded in *Ndufs4* KO brain regions

Multivariate PCA (Fig. 1) of the important discriminatory metabolites revealed a distinct metabolic profile in each brain region capable of separating genotype clusters, to a graded degree, ranging from partial separation in the AC to complete separation in the OB. Similarly, on a univariate level (Table S2; Fig. S3), the degree of metabolic perturbations in *Ndufs4* KO brain regions gradually increased from the AC to the BS, CB and OB. This graded perturbation of brain metabolism mostly corresponded to the graded loss of CI activity we report in Section 3.1. In accord, established metabolic indicators of NADH/NAD⁺ redox balance revealed a similarly graded increase in the NADH/NAD⁺ ratio of *Ndufs4* KO brain regions compared to WT (Fig. S4). The ratios of alanine, leucine and isoleucine relative to glutamic acid were increased in *Ndufs4* KO brain regions (OB > CB > BS > AC), while the pyruvic acid:lactic acid and pyruvic acid:acetyl-carnitine ratios were respectively

Table 1
RC enzyme activities in brain regions of *Ndufs4* KO and WT mice.

Vmax (mU/mg)	AC			BS			CB			OB		
	WT	KO	KO/ WT	WT	KO	KO/ WT	WT	KO	KO/ WT	WT	KO	KO/ WT
CI	27 ± 2	10 ± 1****	0.38	35 ± 4	9 ± 3****	0.25	37 ± 3	10 ± 1****	0.28	23 ± 3	3 ± 1****	0.14
CII	33 ± 3	35 ± 2	1.05	42 ± 5	38 ± 4	0.91	44 ± 4	49 ± 5*	1.10	39 ± 3	38 ± 6	0.97
CIII	266 ± 30	248 ± 15	0.93	291 ± 27	306 ± 46	1.05	294 ± 18	301 ± 19	1.02	204 ± 30	197 ± 18	0.97
CIV	647 ± 36	704 ± 31**	1.09	925 ± 102	944 ± 101	1.02	893 ± 58	1070 ± 53****	1.20	808 ± 53	925 ± 108**	1.14
CS	352 ± 26	398 ± 26***	1.13	459 ± 20	467 ± 25	1.02	398 ± 25	485 ± 24****	1.22	410 ± 32	450 ± 34**	1.10

Enzyme activities normalized to mg protein. Average values (±SD) obtained from WT ($n \geq 9$) and KO ($n \geq 11$) animals. Asterisks indicate statistically significant differences between KO and WT: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Abbreviations: mU; nmol/min; Vmax, maximal activity.

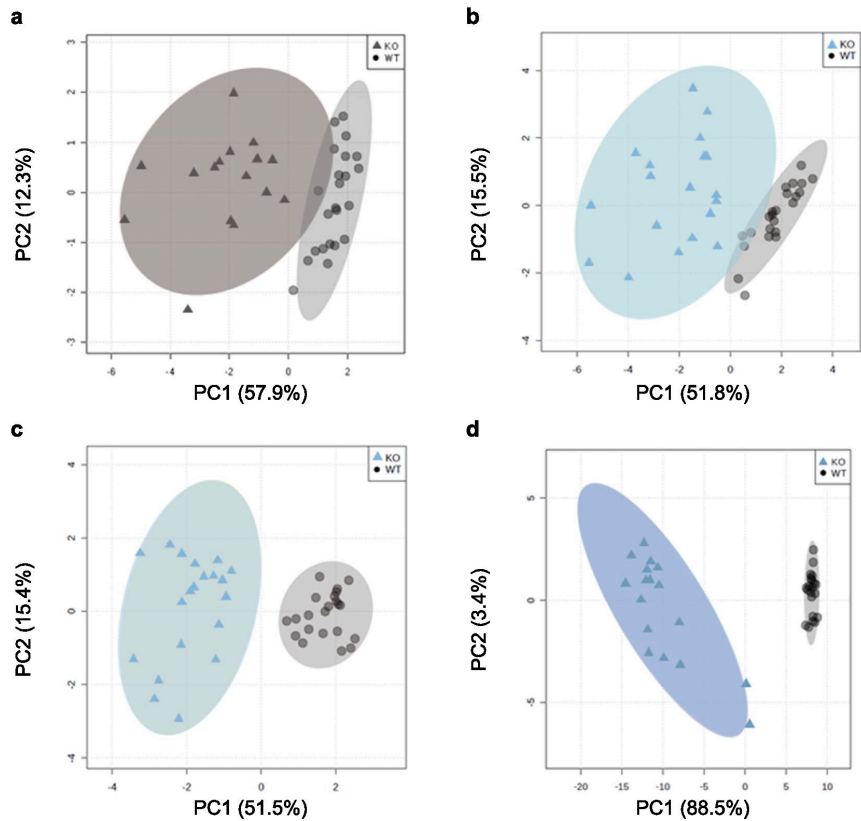


Fig. 1. Discriminative power of important metabolites altered in *Ndufs4* KO mouse brain regions. Principle component analysis (PCA) scores plots of metabolites significantly altered ($p < 0.05$, $d \geq 0.8$) in the a: anterior cortex, b: brainstem, c: cerebellum and d: olfactory bulbs of KO mice ($\blacktriangle$, $n = 21$) compared to WT ($\bullet$, $n = 25$).

decreased and increased only in the lesion-prone regions. An increased NADH/NAD⁺ ratio drives the formation of amino acids (from α -ketoacids and glutamic acid) and lactic acid (from pyruvic acid) by an equilibrium reaction, while the pyruvic acid:acetyl-carnitine ratio is

indicative of NAD⁺-dependent acetyl-coA formation from pyruvate [50–53]. Furthermore, generally similar metabolic alterations were found in the different *Ndufs4* KO brain regions (when compared to WT), albeit with varying degrees of practical and statistical significance. Fig. 2

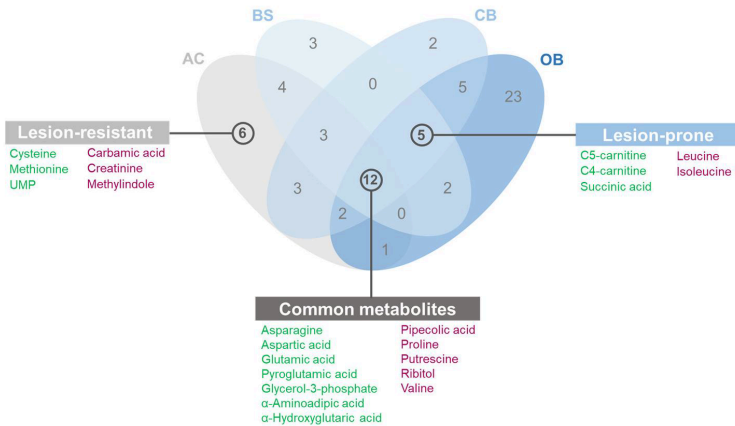


Fig. 2. Venn diagram of important metabolites altered in brain regions of *Ndufs4* KO mice. Green and magenta respectively indicate metabolites significantly ($p < 0.05$, $d \geq 0.8$) decreased or increased in KO ($n = 21$) mice compared to WT ($n = 25$). Among the significantly altered metabolites, 12 were common to all brain regions, 6 were unique to the lesion-resistant AC and 5 were common to the lesion-prone brain regions. Abbreviations: AC, anterior cortex; BS, brainstem; CB, cerebellum; OB, olfactory bulbs.

depicts some of the most significantly increased (magenta) and decreased (green) metabolites common to all four *Ndufs4* KO brain regions ($n = 12$) along with those alterations exclusive to either the lesion-prone ($n = 5$) or -resistant ($n = 6$) regions. The OB was most severely affected by the *Ndufs4* KO as this region displayed the most significant and largest number of total ($n = 49$) and unique ($n = 23$) metabolic alterations. Furthermore, unlike other brain regions, most of these important metabolites were increased, with a greater number being carbohydrates and lipids. Box plots of the significantly altered metabolites can be found in the SI (Fig. S5). A more detailed discussion of the metabolic alterations in the different brain regions, and our hypotheses on them, follows.

3.2.1. BCAA metabolism is strongly perturbed in lesion-prone *Ndufs4* KO brain regions

In the lesion-prone *Ndufs4* KO brain regions, all three branched-chain amino acids (BCAAs) were significantly increased (Fig. 3), while their catabolic by-products, butyryl(C4)- and isovaleryl(C5)-carnitine, were decreased compared to WTs. Although the resilient *Ndufs4* KO AC showed the same trends, the effect size of these alterations was markedly lower with only valine reaching statistical and practical significance. Hence, the lower residual CI activity and greater NADH/NAD⁺ redox imbalance in the three lesion-prone brain regions seems to result in greater congestion of BCAA catabolism. BCAA accumulation is often reported in mitochondrial disease studies and believed to result from an inhibition of the NAD⁺-dependent reaction catalyzed by branched-chain α -keto acid dehydrogenase (BCKDH) due to redox imbalance [52,54–57]. Notably, there is considerable metabolic and phenotypic similarity between *Ndufs4* deficiency and BCKDH deficiency (Maple syrup urine disease, OMIM 248600). In the case of Maple syrup urine disease (MSUD), insufficient branched-chain α -keto acid (BCKA) decarboxylation results in the increased conversion of BCKAs and glutamic acid (Glu) back to BCAAs and α -ketoglutaric acid (α KG), leading to BCAA accumulation and decreased acyl-CoA product formation. This, in turn, results in Glu and aspartic acid (Asp) depletion and disruption of energy metabolism, with the accumulation of lactate, lysine and alanine often evident. The MSUD pathology is largely driven by the accumulation of leucine (Leu) and its ketoacid, α -ketoisocaproate (KIC), resulting in neurological and developmental delay, encephalopathy, feeding difficulty and seizures [58–63]. While Leu is capable of regulating food intake and body weight through its activation of the mammalian target of rapamycin (mTOR) complex 1 [64,65], neuronal network dysfunction, encephalopathy and the occurrence of seizures in MSUD models [66,67] and an epilepsy model exposed to high Leu concentrations [68] have been strongly linked to alterations in glutamatergic- and γ -aminobutyric acid (GABA)ergic neurotransmitter concentrations and/or transmission. Since Leu was significantly elevated in the lesion-prone brain regions of *Ndufs4* KO mice, but not in the AC ($p > 0.05$; $d = 0.43$), we postulate that this BCAA could also be a central driver of the LS pathology. This hypothesis is further strengthened by the fact that glutamatergic- and GABAergic neuronal populations were shown to drive most symptoms in conditional *Ndufs4* KO mice [19] and mTOR pathway hyperactivation was reported in whole brain of diseased ($P > 45$) *Ndufs4* KO mice [69]. However, the hypothesized connection between these observations and regional Leu accumulation, needs to be further tested. In this regard, the evaluation of brain regional mTOR activity in diseased *Ndufs4* KO mice is also required.

3.2.2. Glu metabolism maintains TCA cycle anaplerosis in the lesion-resistant AC of *Ndufs4* KO mice

As with MSUD cases, glutamine (Gln) levels were not markedly altered in *Ndufs4* KO brain regions, while Glu and Asp levels were significantly decreased (Fig. 4). However, Glu and Asp levels were more prominently decreased in the *Ndufs4* KO AC, where BCAA oxidation was least perturbed. These reductions could reflect an adaptive increase in Gln-Glu- α KG anaplerosis, as was recently reported to take place during

OXPHOS deficiency [70]. This mechanism provides α KG to the TCA cycle, which, depending on the degree of OXPHOS impairment, either favours an energy-generating oxidative flux (moderate impairment) or an anabolic reductive flux (severe impairment) directed toward acetylation and lipid synthesis. The oxidative α KG flux results in increased oxaloacetate-derived Asp efflux from mitochondria and cataplerosis to alanine, thereby fuelling cytosolic NAD⁺ regeneration (via malate dehydrogenase) and supporting glycolysis [70]. All KO brain regions also displayed significantly decreased levels of another α KG precursor, α -hydroxyglutaric acid (α HG), which can additionally fuel the Q-pool independently of CI via electron-transferring flavoprotein (ETF) – ETF ubiquinone oxidoreductase system-linked α HG dehydrogenase. We, therefore, hypothesize that the marked decrease in Glu, Asp and α HG levels in *Ndufs4* KO brain regions could point to a compensatory increase in α KG flux to the TCA cycle, with the AC possibly better utilizing the energy-generating oxidative flux.

In the central nervous system (CNS), Glu and Asp are competitively utilized for neurotransmitter- and bioenergetic purposes and require de novo synthesis to maintain internal pools [71,72]. BCAAs are regarded a key nitrogen source for de novo Glu synthesis in the CNS [73,74], with Leu providing the majority (30–50%) of Glu and Gln α -amino groups [61,75]. Together, these metabolites form part of an intricate transport system that regulates brain Glu handling and nitrogen flux between astrocytes and neurons [60,76]. In the putative Leu-Glu cycle, astrocytes produce Glu and KIC from Leu and α KG, KIC is then transported to neurons where it, together with Glu, is used to produce α KG and Leu. Similarly, in the Glu-Gln cycle, astrocytes produce Gln from Glu (and NH₄⁺ and ATP) and transport it to neurons where it is preferentially used to produce Glu (and NH₄⁺). Alterations in either Leu/KIC or α KG/Glu ratios could disrupt these transport systems in the *Ndufs4* KO brain. Remarkably, the beneficial effects of rapamycin was recently ascribed to the restoration of a seemingly dysfunctional Gln/Glu/ α KG metabolic axis in pre-symptomatic *Ndufs4* KO mice [24]; while α KG/malate was also shown to maintain normal CI-driven respiration in *Ndufs4* KO whole brain mitochondria via an unknown mechanism [77] and supplementation with a cell-permeable α -KG analogue significantly increased survival in *Ndufs4* KO mice [78]. Altogether, we hypothesize that the degree to which BCAA catabolism is congested and the compensatory capacity of increased α KG oxidation may be central to regional neurodegeneration in *Ndufs4* deficiency.

Additionally, all *Ndufs4* KO brain regions also displayed increased levels of *N*-acetylglutamic acid (NAG), putrescine and proline (Pro) along with decreased levels of pyroglutamate (pGlu), citrulline and ornithine. Both Pro and pGlu levels were more significantly altered in the lesion-prone brain regions. Pro accumulation could reflect a shift in flux through the redox regulatory Pro cycle, favoring NAD(P)⁺ and Pro synthesis, which reportedly plays an important role in the regulation of mTOR signaling [79–81]. Gln-derived Pro biosynthesis was also shown to be induced in response to increased oxidation of Gln (and glucose) carbons in the TCA cycle and functions to prevent reactive oxygen species generation when TCA cycle activity surpasses OXPHOS capacity [82]. The need for such a vent of mitochondrial redox stress, might be greater in lesion-prone brain regions. Similarly, larger reductions in pGlu levels could also reflect a greater requirement for, and production of, the Glu-derived antioxidant, glutathione in lesion-prone brain regions. In agreement with our results, brain regions of pre-symptomatic *Ndufs4* KO mice reportedly display elevated levels of reduced glutathione along with a decreased trend in the levels of a metabolite cluster including, pGlu, Glu, Asp and most urea cycle intermediates [24]. The urea cycle-related metabolic alterations in *Ndufs4* KO brain regions could result from altered Glu and ammonia levels, as suggested to occur due to Gln metabolic rewiring during OXPHOS deficiency [70]. The induction of urea cycle function has also been proposed to occur in brain endothelial cells when the removal of excess nitrogen is required [71], however, with the lack of NAG synthase [83] and complete urea cycle function in the CNS, further targeted studies are required.

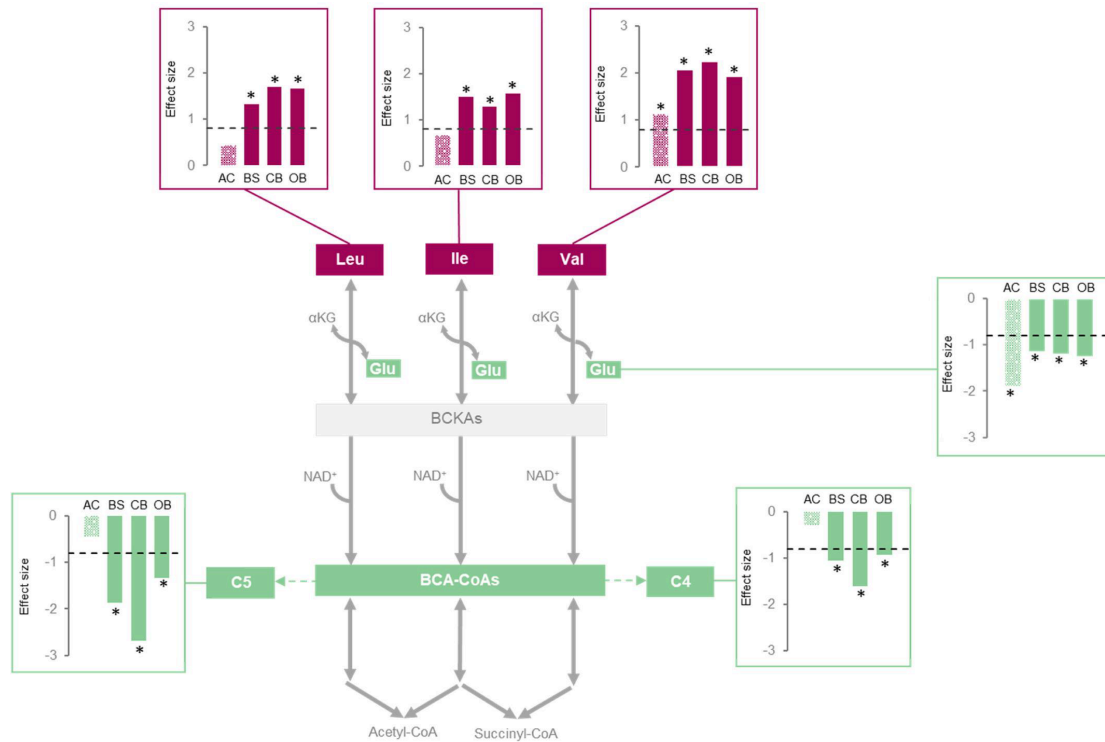


Fig. 3. BCAA related metabolic alterations in *Ndufs4* KO brain regions. Alterations are more significant in the three vulnerable regions (solid bars) than the AC (dotted bars). Bar graphs depict Cohen's *d*-values (y-axis) for each metabolite when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate increased or decreased metabolites levels in KO mice. Dashed lines mark large practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (FDR-corrected p-value < 0.05). Abbreviations: BCAAs, branched-chain amino acids; BCA-CoAs, branched-chain acyl-CoA derivatives; BCKAs, branched-chain α -keto acids; C4, butyryl-carnitine; C5, isovaleryl-carnitine; FAD/FADH₂, oxidized/reduced flavin adenine dinucleotide; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; NAD⁺, oxidized nicotinamide adenine dinucleotide; Val, valine; α KG, α -ketoglutaric acid.

3.2.3. Olfactory bulb lysine, carbohydrate and lipid metabolism are greatly perturbed in *Ndufs4* KO mice

A trend of increased levels of lysine and its metabolites, pipecolic acid, carnitine and acetyl-carnitine, along with decreased α -amino adipic acid (α AA) were seen in all *Ndufs4* KO brain regions (Fig. 5). Like Leu, lysine is an important precursor for de novo Glu synthesis in the CNS, with its catabolism influenced by NAD⁺ (and α KG) bioavailability [84]. Our data suggests a block in lysine catabolism, possibly due to disturbed redox balance, along with the preferred formation of pipecolic acid from pyrroline-6-carboxylate (P6C). The latter reaction could function to replenish NAD(P)⁺ levels via pyrroline-5-carboxylate (PC5) reductase, an enzyme that also catalyzes Pro synthesis from P5C. These alterations in lysine metabolism were most significant in the KO OB, in which arginine and γ -aminobutyric acid (GABA) levels were uniquely elevated (Fig. 4). Lysine is known to block arginase-mediated urea synthesis from arginine and compete for uptake with arginine at the mitochondrial ornithine carriers in the brain [85]. Since both arginine and lysine can function as activators of mTOR signaling [86] and affect glutamatergic neurotransmission [87], their significant accumulation in the OB might explain why it is the predominantly affected brain region in *Ndufs4* KO mice.

Furthermore, pyruvic acid and alanine levels trended higher in all *Ndufs4* KO brain regions, while lactic acid was increased only in the lesion-prone regions. These alterations were graded, with the OB most severely affected. The OB also showed increased levels of other

glycolysis- and pentose phosphate pathway (PPP)-related intermediates such as glucose, glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate, which was not seen in other regions. In most *Ndufs4* KO brain regions, we additionally observed markedly increased erythronic acid¹ and ribitol levels — both known to accumulate in the brain due to impaired glycolysis that shunts glucose to the PPP [88]. The interpretation of these results is, however, complicated by the fact that *Ndufs4* KO mice exhibit inflammatory cell activation and infiltration in the affected brain regions at the late stage of LS [14,19,69,89,90]. Known metabolic alterations in activated inflammatory cells include a switch away from OXPHOS toward glycolysis with increased flux through the PPP, lactic acid and lipid production [91]. Nevertheless, *Ndufs4* KO mice at our facility did not exhibit severe inflammation at this age (P46–51) [27], while others report similar trends in glycolytic intermediates and lactic acid in brain regions of pre-symptomatic *Ndufs4* KO mice [24] and intracerebral lactic acid accumulation prior to neurodegeneration [92]. These alterations could result from *Ndufs4* deficiency-induced hyperacetylation as pyruvate dehydrogenase (PDH) subunit hyperacetylation and resulting decreases in PDH activity were recently reported in mitochondria from affected neuronal populations in *Ndufs4* KO mice [93]. Neuro-inflammation and BCAA accumulation could further exacerbate these metabolic alterations in lesion-prone

¹ Unreliably detected in the brainstem (BS).

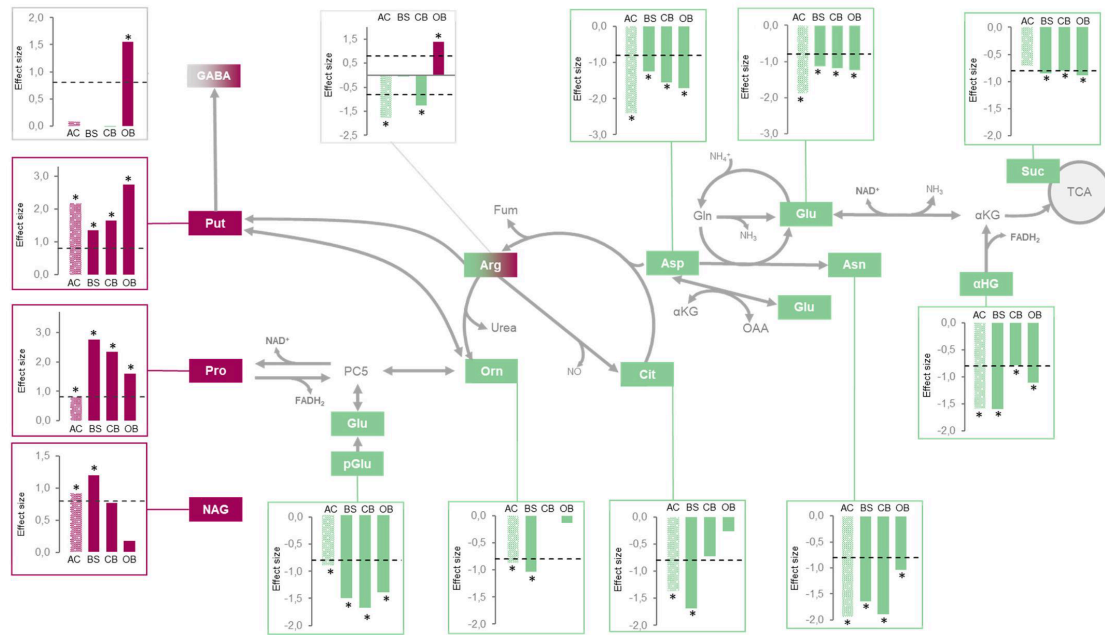


Fig. 4. Glutamate related metabolic alterations in *Ndufs4* KO brain regions. Bar graphs depict Cohen's *d*-values (y-axis) for each metabolite when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate an increase or decrease in KO metabolite levels. Dashed lines mark practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (FDR-corrected p -value < 0.05). Abbreviations: Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cit, citrulline; FADH₂, reduced flavin adenine dinucleotide; Fum, fumarate; GABA, γ -aminobutyric acid; Gln, glutamine; Glu, glutamic acid; NAD⁺, oxidized nicotinamide adenine dinucleotide; NAG, N-acetylglutamic acid; NH₃, ammonia; NO, nitric oxide; OAA, oxaloacetic acid; Orn, Ornithine; P5C, pyrroline-5-carboxylate; pGlu, pyroglutamic acid; Pro, proline; Put, putrescine; Suc, succinic acid; α KG, α -ketoglutaric acid.

brain regions as high levels of BCAAs can inhibit PDH [94] and increase the probability of neurodegeneration by influencing the immune properties of microglial cells and decreasing neuroprotective functions [95]. All KO brain regions also displayed trended higher levels of palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1); along with decreased 9,10-dihydroxystearic acid. These alterations were, again, most pronounced in the OB. Decreased levels of C18:0 and increased C18:1 suggests an increased flux through stearoyl-CoA desaturase, which can regenerate NAD⁺ but also dysregulate neuro-inflammation [96–98] and lipid metabolism/homeostasis, as also seen with C16:0 accumulation [99–102]. In agreement with our results, previous studies on *Ndufs4* KO mice report decreased free fatty acids in whole brain [69] along with increased lipid synthesis and a disturbance in the neuro-protective lipid/lactic acid shuttle mechanism between neurons and glia in lesion-prone regions [103,104].

Further alterations were seen in dihydroxyacetone phosphate (DHAP) and glycerol-3-phosphate (G3P) — metabolites that connect glycolysis, lipid metabolism and OXPHOS. G3P levels were significantly decreased in all KO brain regions, in a graded manner (AC > BS > CB > OB), suggesting a key role for G3P oxidation in fueling respiration during CI deficiency. DHAP levels were elevated only in lesion-prone KO brain regions, with its accumulation noticeably greater in the OB, as reported for pre-symptomatic *Ndufs4* KO mice [24]. This likely reflects upstream perturbations in glycolysis due to redox imbalance. DHAP was also recently proposed to be a central mTOR activator that transmits glucose availability to mTORC1 and plays a critical role in de novo lipogenesis [105]. Since the interconversion of DHAP and G3P in the G3P shuttle functions to fuel respiration independently of CI and regenerate cytosolic NAD⁺ [3], this redox shuttle may play an important role in the response to CI deficiency. Moreover, with G3P shuttle activity

questioned in the brain and cytosolic G3P dehydrogenase expression (responsible for DHAP reduction) generally considered low/absent, it would be interesting to investigate G3P shuttle activity in different *Ndufs4* KO brain regions.

Altogether, greater perturbations in lysine, carbohydrate and lipid metabolism in the *Ndufs4* KO OB — which displayed the lowest residual CI activity (14% vs 38% in the AC) — support the hypothesis that brain regions with more severe levels of CI deficiency and a greater NADH/NAD⁺ redox imbalance, exhibit a preference for reductive carboxylation, acetylation and lipid synthesis.

4. Conclusion

Here, we uncover a link between the severity of CI deficiency and the degree of metabolic perturbations in different *Ndufs4* KO brain regions. Our metabolic data suggest that the related level of NADH/NAD⁺ redox imbalance impacts brain regional capacity to bypass CI deficiency and maintain homeostasis. In *Ndufs4* KO mice, an adaptive increase in Glu, α HG and G3P oxidation seem to drive energy-generation in the lesion-resistant AC, which maintained the highest level of residual CI activity. These adaptive mechanisms appear less effective in lesion-prone brain regions with more severe levels of CI deficiency. Moreover, we hypothesize that the marked accumulation of possible mTOR signaling regulators (such as Leu, Pro and DHAP) in lesion-prone brain regions implicate this signaling pathway in regional neurodegeneration. Future validation studies should, therefore, focus on measuring mTOR pathway activity in brain regions of diseased *Ndufs4* KO mice. Furthermore, we highlight the metabolic and phenotypic similarity between *Ndufs4* deficiency and MSUD, thereby pinpointing Leu catabolism as a promising therapeutic target for the treatment of shared clinical symptoms

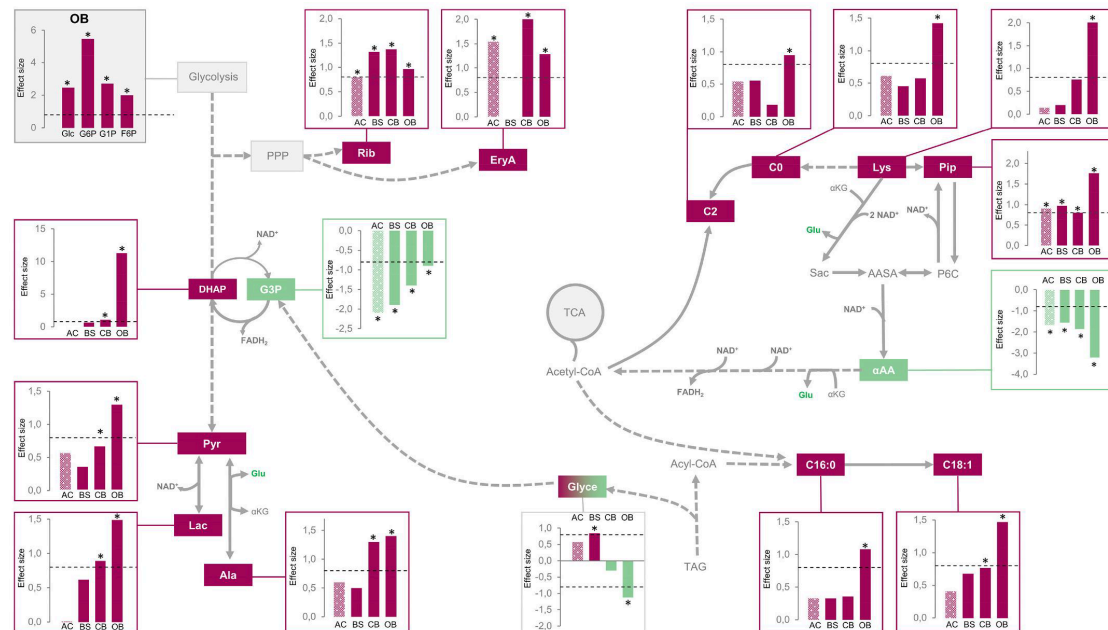


Fig. 5. Lysine, carbohydrate and lipid related metabolic alterations in *Ndufs4* KO brain regions. Bar graphs depict Cohen's *d*-values (y-axis) for each metabolite when relative abundance is compared between KO and WT mice in different brain regions (x-axis). Magenta and green bars respectively indicate an increase or decrease in KO metabolite levels. Dashed lines mark practical significance ($d \geq 0.8$), while asterisks indicate statistical significance (FDR-corrected *p*-value < 0.05). Abbreviations: AASA, aminoadipic acid semialdehyde; Ala, alanine; C0, carnitine; C16:0, palmitic acid; C18:1, oleic acid; C2, acetyl-carnitine; DHAP, dihydroxyacetone phosphate; EryA, erythronic acid; F6P, fructose-6-phosphate; FADH₂, reduced flavin adenine dinucleotide; G1P, glucose-1-phosphate; G3P, glyceral-3-phosphate; G6P, glucose-6-phosphate; Glc, glucose; Glu, glutamic acid; Glyce, glycerol; Lac, lactic acid; Lys, lysine; NAD⁺, oxidized nicotinamide adenine dinucleotide; P6C, pyrroline-6-carboxylate; PPP, pentose phosphate pathway; Pyr, pyruvic acid; Rib, Ribitol; Sac, saccharopine; TCA, tricarboxylic acid cycle; αAA, α-aminoadipic acid; αKG, α-ketoglutaric acid.

such as neurological and developmental delay, encephalopathy, feeding difficulty and seizures. Altogether, we identify key areas of brain metabolism to further investigate in the context of LS such as the metabolic sources and fate of αKG in the TCA cycle and the Leu/Glu/Gln transport systems between astrocytes and neurons. Although previous *in vivo* glucose and glutamine tracing experiments have shown a relative lack of changes between *Ndufs4* KO and WT mice [106,107], our results suggest that brain region-specific measurements of those and other metabolic fuels could reveal a different picture, especially at the level of astrocytes and neurons.

CCRediT authorship contribution statement

K.T., J.C. and R.L. designed the research plan with input from J.Z.L. and F.vd.W. F.vd.W. acquired the animal model and ethical approval with assistance from R.L. R.L. and F.vd.W. obtained funding for the study. J.C. conducted experiments and data processing with assistance from K.T., R.L. and J.Z.L. K.T. analysed the data and wrote the manuscript with input from R.L., J.Z.L. and F.vd.W.

Declaration of competing interest

The authors 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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Ethical approval

The AnimCare animal research ethics committee of North-West University approved (NWU-00378-16-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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbadis.2021.166082>.

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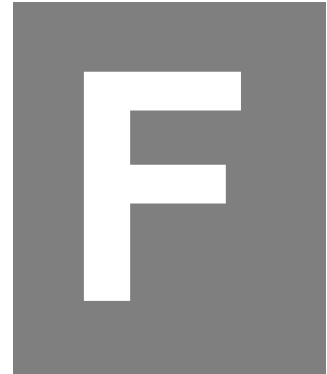
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APPENDIX

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article II





Cross-comparison of systemic and tissue-specific metabolomes in a mouse model of Leigh syndrome

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Abstract

Introduction The value of metabolomics in multi-systemic mitochondrial disease research has been increasingly recognized, with the ability to investigate a variety of biofluids and tissues considered a particular advantage. Although minimally invasive biofluids are the generally favored sample type, it remains unknown whether systemic metabolomes provide a clear reflection of tissue-specific metabolic alterations.

Objectives Here we cross-compared urine and tissue-specific metabolomes in the *Ndufs4* knockout mouse model of Leigh syndrome—a complex neurometabolic MD defined by progressive focal lesions in specific brain regions—to identify and evaluate the extent of common and unique metabolic alterations on a systemic and brain regional level.

Methods Untargeted and semi-targeted multi-platform metabolomics were performed on urine, four brain regions, and two muscle types of *Ndufs4* KO ($n \geq 19$) vs wildtype ($n \geq 20$) mice.

Results Widespread alterations were evident in alanine, aspartate, glutamate, and arginine metabolism in *Ndufs4* KO mice; while brain-region specific metabolic signatures include the accumulation of branched-chain amino acids, proline, and glycolytic intermediates. Furthermore, we describe a systemic dysregulation in one-carbon metabolism and the tricarboxylic acid cycle, which was not clearly reflected in the *Ndufs4* KO brain.

Conclusion Our results confirm the value of urinary metabolomics when evaluating MD-associated metabolites, while cautioning against mechanistic studies relying solely on systemic biofluids.

Keywords *Ndufs4* knockout mice · Brain regions · Metabolomics · Mitochondrial disease · Complex I deficiency · Leigh syndrome

Abbreviations

1C	One-carbon	FDR	False discovery rate
3-MH	3-methylhistidine	G3P	Glycerol-3-phosphate
BCAA	Branched-chain amino acids	GC-TOF-MS	Gas chromatography time-of-flight mass spectrometry
BBD	γ -butyrobetaine dioxygenase	GLUT1	Glucose transporter 1
BHMT	Betaine-homocysteine methyltransferase	HP	Hydroxyphenyl
CI	Complex I	KO	Knockout
CII	Complex II	LC-MS/MS	Liquid chromatography-tandem mass spectrometry
CoA	Coenzyme A	LS	Leigh syndrome
DHAP	Dihydroxyacetone phosphate	MD	Mitochondrial disease
ES	Effect size	NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
ETFDH	Electron-transferring-flavoprotein dehydrogenase	NAG	N-acetyl glutamate
		OXPHOS	Oxidative phosphorylation
		P	Postnatal day
		Q	Ubiquinone
		SAM	S-adenosyl methionine
		SCF	Short chain fatty acids

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TCA Tricarboxylic acid
WT Wild type

1 Introduction

A clear understanding of the pathologies and treatment of primary mitochondrial diseases (MDs), which results from a dysfunction of the oxidative phosphorylation (OXPHOS) system, remain a great challenge. Largely unknown mechanisms govern a tissue-specific vulnerability to MD-causing mutations and an extreme heterogeneity in genotype-phenotype correlations among patients (Lake et al., 2016; Mootha and Chinnery, 2018; Rahman, 2020). As the most common form of MD, respiratory chain complex I (CI) deficiency and its most prevalent pediatric manifestation, Leigh syndrome (LS, OMIM # 256000), are at the forefront of MD research (Rahman et al., 1996; Rodenburg, 2016). The *Ndufs4* knock-out (KO) mouse model of LS suffers from all-pervading CI deficiency, exhibits progressive focal lesions in specific brain regions, and displays symptoms similar to human patients. Symptoms include developmental delay, failure to thrive, locomotor impairment, encephalomyopathy and respiratory deficits, which progressively worsen until premature death (Kruse et al., 2008; Quintana et al., 2010). Studies on *Ndufs4* KO mice have revealed that specific neuronal populations (linked to lesion-prone brain regions) drive most symptoms (Bolea et al., 2019), while peripheral tissues, like skeletal muscle, seem relatively unaffected phenotypically (Alam et al., 2015; Chouchani et al., 2014; Kruse et al., 2008). However, the contribution of systemic metabolic alterations to this neurometabolic disease cannot be ruled out (Bolea et al., 2019; Di Meo et al., 2017; Ito et al., 2017; Lee et al., 2019).

Metabolomics present a powerful tool with which to analyze the downstream effects of multi-systemic diseases like MD in a holistic manner (Rahman and Rahman, 2018). Although the application of metabolomics in the field of MD research is relatively novel, it has made significant progress in recent years—especially with the increased availability of well characterized MD models (Esterhuizen et al., 2017). Non-invasive (urine) or minimally invasive (blood) systemic samples are generally favored for metabolomics investigations of MD. However, it remains inconclusive whether such samples are optimal to study and/or monitor MD, and how well systemic metabolomes reflect tissue-specific metabolic alterations (Esterhuizen et al., 2017; Steele et al., 2017). A few metabolomics studies have been performed on *Ndufs4* KO tissues (Emmerzaal et al., 2020; Jin et al., 2014; Johnson et al., 2020; Johnson et al., 2013; Terburgh et al., 2021a; Terburgh et al., 2019b), however, the link between systemic and brain regional metabolomes remains unexplored. Here, we integrate and cross-compare tissue and urine metabolomes

from *Ndufs4* KO vs wildtype (WT) mice to establish whether differences exist between brain region-specific and systemic metabolic profiles in LS. Skeletal muscle metabolomes were included in this comparison to show the contribution of peripheral tissues. Seven sample types from individual late stage diseased *Ndufs4* KO and WT mice were extensively investigated via untargeted and semi-targeted multiplatform metabolomics. These samples include one lesion-resistant brain region (anterior cortex); three lesion-prone brain regions (brainstem, cerebellum, and olfactory bulbs); urine collected overnight; the primarily glycolytic white parts of the quadriceps femoris; and the primarily oxidative solei.

2 Materials and methods

2.1 Animals and housing

Male whole-body homozygous *Ndufs4* KO mice, along with age- and sex-matched controls (WT) were used. Only one sex-group was used in order to minimize sex-related metabolic variation due to known differences in hormone cycles, tissue-specific metabolism and metabolic profiles between sexes (Ruoppolo et al., 2018; Wells et al., 2016; Wu and Gao, 2015). KO mice were born from heterozygous crosses (B6.129S4-*Ndufs4*^{tm1.1Rpa/J}) obtained from Jackson Laboratory (JAX stock #027058). *Ndufs4* genotypes were confirmed by polymerase chain reaction using tail snips. The animals were bred and housed at the specific pathogen-free unit of the Vivarium (SAVC reg. no. FR15/13458) of the Pre-Clinical Drug Development Platform (PCDDP; NWU, RSA) and approval (NWU-00378-16-A5) for this study was obtained from the Animal Research Ethics Committee of the NWU. Animals were group housed under temperature- ($22 \pm 1^\circ\text{C}$), humidity- ($55 \pm 10\%$) and light-controlled (12:12 hour light/dark cycle) conditions with standard laboratory chow (Rodent Breeder, #RM1845, LabChef, Nutritionhub) and water provided *ad libitum*. Samples were collected between P45-50 as this age range was selected to represent the late stage of LS, based on our previous survival curves and phenotypic analyses (Miller et al., 2021).

2.2 Sample collection

Urine was collected from individual mice in metabolic cages after overnight (12 hours) fasting with *ad libitum* access to water. Since *Ndufs4* KO mice yielded limited urine volumes, urine droplets that failed to reach the collection tube were also collected. Hence, the separating cone, collection funnel and -tubes were all treated with sodium azide (NaN_3 ; 0.1% (w/v); WT: 100 μL and KO: 50 μL) to prevent bacterial growth. After urine collection, mice were euthanized via cervical dislocation at the same time of day (8:00-9:00

AM). The brain was immediately removed from the skull, rinsed with saline solution (SABAX PBS; 0.9% NaCl (w/v), #7634, Adcock Ingram) and dissected into the regions of interest (anterior cortex, brainstem, cerebellum, and olfactory bulbs). Concurrently, the white muscle portions of quadriceps femoris, as well as solei muscles, were collected from both hind limbs. All samples were snap frozen in liquid nitrogen upon collection (within 15 min post-mortem for tissues) and stored at -80°C until use.

2.3 Metabolic profiling

2.3.1 Sample analysis

Untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and semi-targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed on seven sample types from *Ndufs4* KO ($n \geq 19$) and WT ($n \geq 20$) mice. Tissue extracts were individually prepared and analyzed as previously described (Terburgh et al., 2021a; Terburgh et al., 2019b), while detailed descriptions on urine sample preparation and analysis are given in the supplementary information. In short, frozen tissues were weighed and homogenized in the presence of internal standards added according to tissue mass. Monophasic tissue extracts were prepared via modified Bligh–Dyer (Gullberg et al., 2004) (methanol/water/chloroform, 3:1:1), with additional step-wise extraction (Wu et al., 2008) to obtain polar phases of biphasic brain extracts (methanol/water/chloroform, 2:1.8:2) for LC-MS/MS. Tissue extracts were divided between the platforms, with stable isotopes added to LC-MS/MS samples. Pre-determined volumes of urine, containing $0.0625 \mu\text{mol}$ creatinine, were supplemented with internal standards (included stable isotopes for LC-MS/MS samples) and deproteinized using methanol. Urine and tissue extracts were then dried under nitrogen and derivatized prior to their analysis. For GC-TOF-MS, samples were oximated and silylated, while LC-MS/MS samples were butylated.

2.3.2 Data processing

Spectral data matrices were individually inspected (correct peak detection and alignment, batch precision and data integrity) and preprocessed (data filtering, half-minimum missing value imputation and normalization). Tissue data was normalized post-acquisition to tissue mass (ng/mg tissue) using internal standards, 3-phenylbutyric acid (GC-TOF-MS) and *N*, *N*-dimethylphenylalanine (LC-MS/MS). Additionally, LC-MS/MS metabolites were also normalized to isotope internal standards to account for ion suppression effects. Urine data was normalized pre-acquisition to creatinine content (mg/g creatinine) and post-acquisition to tropic acid (GC-TOF-MS) or isotope (LC-MS/MS) internal

standards. Metabolites detected via LC-MS/MS were normalized to their own isotopes, where available, or an isotope with a similar retention time to which the metabolite strongly correlated in QC samples. Finally, the data was log transformed and outliers were detected (PCA Hotelling's T^2 ellipses, loadings plot and biplots). Data processing was done using Excel (Microsoft 365), Statistica v 13.0 (TIBCO Software Inc.) and MetaboAnalyst v 4.0 (Xia Lab (Chong et al., 2018)). The metabolomics data obtained in this study can be accessed at the Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (<https://www.metabolomicsworkbench.org>) where it has been assigned project IDs: PR000721 (Terburgh et al., 2019a), PR001003 (Terburgh et al., 2021b), and PR001187 (Terburgh et al., 2021c).

2.3.3 Statistical analyses

For each sample type, univariate analyses were used to determine discriminatory features between *Ndufs4* KO and WT metabolic profiles. Statistical and practical significance were determined using the student's *t*-test and effect size (ES, Cohen's *d*-value) calculations, respectively. False discovery rate (FDR) corrected *p*-values were calculated in MetaboAnalyst (Chong et al., 2018), with an FDR-*p* of ≤ 0.05 considered statistically significant. Dataset features were identified via spectral and retention time matching using commercial and in-house spectral libraries together with public databases. Metabolite identities were assigned to spectral features with high confidence levels (Table S2) ranging from level one to three, as defined by Schyman-ski et al. (2014). For each sample type, pathway analysis was performed on significantly altered metabolites using MetaboAnalyst (Chong et al., 2018). Compound labels were matched to HMDB and KEGG IDs, with *mus musculus* (mouse) KEGG as the selected pathway library. Hypergeometric test was the selected over-representation analysis method for pathway enrichment analysis, while relative betweenness centrality was the selected node importance measure for pathway topology analysis.

3 Results and discussion

3.1 Cross-comparison of urine- and tissue metabolomes

Several significant metabolic alterations were observed in the urine and tissue-specific metabolomes of *Ndufs4* KO vs WT mice, with the number of discriminatory metabolites (and their significance level) generally lower in the muscles. Figure 1A–J illustrates the main similarities and differences among the metabolic alterations seen in the seven

APPENDIX F

A Aspartate & glutamate metabolism							
LC Glutamine	WQ	SOL	AC	BS	CB	OB	U
LC Glutamate	-0.61	-0.10	0.00	0.33	-0.14	-0.49	-2.40
GC Pyroglutamate	-0.58	-0.32	-1.78	-1.09	-1.13	-1.26	-1.35
LC 3-Aminobutyrate (GABA)	-0.56	-0.12	-0.94	-1.53	-1.71	-0.72	-0.86
LC Aspartate	-0.23	0.45	-0.24	-0.27	-0.42	-0.48	-2.09
LC Asparagine	-0.31	0.03	-2.33	-1.21	-1.58	-1.72	-0.51
LC N-acetylglutamate	-0.84	0.72	-1.81	-1.65	-1.88	-1.05	-0.49
LC N-acetylglutamate	-0.75	0.08	0.94	1.22	0.78	0.17	-2.86
LC N-acetylglutamate	1.63	0.99	-0.03	-0.27	-0.19	-1.03	ND
B Arginine & proline metabolism							
GC Carbamate	ND	ND	0.87	-0.08	-0.22	0.65	-0.95
LC Ornithine	0.27	0.78	-0.82	-1.01	-0.26	-0.14	-0.73
LC Citrulline	-1.04	-0.56	-1.26	-1.66	-0.72	-0.27	-1.00
LC Arginine	0.37	0.68	-1.66	-0.03	-1.18	1.37	-1.34
GC Urea	0.28	0.69	0.20	0.40	0.33	0.26	0.09
GC Putrescine	ND	ND	1.23	1.35	0.53	2.69	ND
LC Proline	-1.80	-0.40	0.79	2.75	2.38	1.59	-1.94
LC Hydroxyproline	-1.32	-1.04	-1.65	-1.44	-1.75	-0.73	-3.02
LC Creatine	0.28	0.40	0.68	-0.05	-0.07	0.31	-0.62
GC Creatinine	-2.15	-1.60	-0.76	-0.23	-0.48	-0.03	ND
C Lysine & tryptophan metabolism							
LC Lysine	0.83	1.25	0.08	-0.16	0.15	2.43	-0.57
LC Carnitine (C0)	-0.68	-0.10	0.66	0.46	0.53	1.41	0.67
LC Picoalate	1.13	1.56	0.99	1.02	0.46	1.75	ND
LC 5-Hydroxylysine	ND	ND	ND	ND	ND	ND	-0.64
LC 2-Aminoadipate	-1.88	-1.47	-1.66	-1.54	-1.90	-3.22	-0.98
GC 2-Hydroxyadipate	ND	ND	ND	ND	ND	ND	-2.75
LC Tryptophan	-0.34	-0.01	0.01	-0.01	-0.25	0.61	-0.56
LC Kynurenine	ND	ND	ND	ND	ND	ND	-0.57
GC 5-Hydroxy-indole	ND	ND	ND	ND	ND	ND	-1.44
GC Glutamate	ND	ND	ND	ND	ND	ND	-1.26
D Histidine metabolism							
LC Histidine	0.34	1.01	0.16	0.41	-0.16	0.34	-1.11
LC 3-Methylhistidine	0.83	1.14	0.75	1.40	0.51	1.01	-1.17
LC 1-Methylhistidine	-0.73	-0.34	-0.06	-0.29	-0.55	-0.11	-1.10
E Lipid metabolism							
GC Glycerol	-0.88	-0.7	0.56	0.86	-0.29	-1.16	-1.27
GC Palmitate (C16:0)	-0.60	-0.49	0.38	0.33	0.36	1.06	ND
GC Stearate (C18:0)	-0.30	-0.09	0.12	0.16	0.00	-0.89	ND
GC Oleate (C18:1)	-0.29	-0.31	0.46	0.68	0.77	1.44	ND
GC Arachidonate (C20:4, ω-6)	ND	ND	0.82	0.59	0.70	0.73	ND
GC 25-Hydroxycholesterol	ND	ND	-0.22	-1.47	-0.75	-0.68	ND
GC 9S,10S-Dihydroxystearate	ND	ND	-0.83	-0.65	0.49	-0.56	ND
LC Acetylcarnitine (C2)	1.03	0.04	0.54	0.55	0.18	0.93	0.55
LC Propionylcarnitine (C3)	-0.44	-0.78	-0.45	-1.22	-1.39	0.36	1.35
LC Butyrylcarnitine (C4)	-1.34	-0.99	-0.45	-1.09	-1.61	-0.89	-0.11
GC Butyrylglycine (C4)	ND	ND	ND	ND	ND	ND	-1.58
GC Valerylglutamate (C5)	ND	ND	ND	ND	ND	ND	-1.94
LC Hexanoylcarnitine (C6)	-0.94	-0.46	ND	ND	ND	-0.63	-0.48
GC Hexanoylglycine (C6)	ND	ND	ND	ND	ND	ND	-1.42
LC Octanoylcarnitine (C8)	-0.96	-0.06	0.08	0.21	0.11	-0.04	-1.12
LC Decanoylcarnitine (C10)	ND	ND	ND	ND	ND	ND	-0.97
GC Malonate	ND	ND	0.05	-0.19	-0.33	-0.81	0.34
GC Hydroxymalonate (Tartartrate)	ND	ND	ND	ND	ND	ND	-1.50
F TCA cycle metabolism							
GC 2-Ketoglutarate	ND	ND	ND	ND	ND	0.20	0.87
GC 2-Hydroxyglutarate	-0.17	-1.40	-1.58	-1.59	-0.79	-1.10	-1.09
GC Succinate	-0.92	-0.40	-0.75	-0.85	-0.78	-0.79	-0.60
GC Fumarate	-0.51	-0.64	-0.24	0.02	0.14	0.01	1.71
GC Malate	-0.91	-1.01	0.04	0.29	0.43	0.34	1.40
GC Citramalate	ND	ND	ND	ND	ND	ND	1.79
G BCAA metabolism							
LC Valine	-0.24	0.36	0.94	1.73	2.15	1.51	-1.47
LC Isoleucine-leucine	-0.12	0.31	0.75	1.65	1.91	1.63	-2.13
GC Isoleucine	-0.28	0.33	0.62	1.50	1.43	1.54	ND
GC Leucine	0.16	0.29	0.43	1.33	2.07	1.66	ND
LC Isovalerylarnitine (C5)	-0.62	-0.97	-0.64	-1.80	-2.73	-1.36	0.03
GC Isovalerylglutamate (C5)	ND	ND	ND	ND	ND	ND	-1.30
GC Methylsuccinate	ND	ND	ND	ND	ND	ND	0.76
GC 3-Hydroxymethylglutamate	ND	ND	ND	ND	ND	ND	-0.66
H One-carbon metabolism							
LC Glycine	0.06	0.37	-0.43	0.81	-0.87	0.10	-1.10
LC Methionine	-0.78	0.01	-0.39	-0.13	-0.49	0.01	-1.71
LC Trimethylglycine (betaine)	-0.61	0.24	-0.47	-0.23	-0.70	0.09	-2.53
LC Dimethylglycine	-2.49	-2.45	ND	ND	ND	ND	-2.78
LC Methylglycine (sarcosine)	-0.80	-0.36	ND	ND	ND	ND	-3.33
LC Threonine	-0.92	0.17	0.02	0.38	-0.06	0.60	-1.06
LC Serine	-0.04	0.65	-1.04	-0.27	-1.34	0.26	-1.51
LC Cystathionine	-0.73	-0.40	0.45	0.22	-0.58	1.70	-1.55
GC Cysteine	ND	ND	-1.19	0.32	-0.17	-0.01	ND
LC Cystine	ND	ND	-0.31	0.08	ND	ND	-0.82
GC Formylglycine	ND	ND	0.78	0.08	-0.01	1.22	ND
LC Taurine	0.37	-0.30	0.36	1.20	0.58	0.15	ND
LC Phenylalanine	-0.32	0.24	-0.10	0.23	-0.22	0.39	-1.20
LC Isothreonine	ND	ND	0.23	0.06	-0.85	0.17	-0.69
GC Glycolate	-1.13	ND	-1.25	-0.89	-0.66	0.01	ND
GC Glycerate	-0.79	0.28	ND	ND	ND	ND	-1.00
I Pyrimidine & purine metabolism							
GC Orotate	ND	ND	ND	ND	ND	ND	-1.52
GC UMP	ND	ND	-0.99	-0.42	ND	0.62	ND
GC Uracil	ND	-0.10	-0.42	-0.43	0.07	0.42	-1.71
GC 3-Ureidopropionate	ND	ND	ND	ND	ND	ND	-0.99
LC Beta-alanine	-0.51	-0.32	-0.15	0.37	-0.40	-0.91	-1.32
GC Pantothenate (vitamin B5)	ND	ND	0.34	0.18	-0.14	0.88	ND
LC 3-Aminoisobutyrate (BAIBA)	ND	0.33	ND	ND	ND	ND	-1.27
GC Adenosine	ND	ND	-0.15	-0.19	ND	-0.94	ND
GC Urate	ND	ND	ND	ND	ND	ND	-0.83
J Glycolysis & sugar derivatives							
GC Glucose	-0.50	-0.54	0.24	0.06	0.60	2.45	-0.78
GC Glucose-1-phosphate	ND	ND	ND	0.71	ND	2.79	ND
GC Glucose-6-phosphate	ND	ND	ND	ND	ND	8.48	ND
GC Fructose-6-phosphate	ND	ND	0.54	0.81	ND	3.82	ND
GC Dihydroxyacetone phosphate (DHAP)	ND	ND	1.22	0.65	0.20	5.19	ND
GC Glycerol-3-phosphate (G3P)	ND	ND	-2.11	-1.89	-1.43	-0.92	-1.06
GC Pyruvate	-0.51	-0.54	0.60	0.35	0.66	1.31	1.40
GC Lactate	-0.52	-0.50	0.01	0.62	0.80	1.48	1.34
LC Alanine	-0.87	-0.52	0.63	0.55	1.25	1.35	1.00
GC Ribitol	ND	ND	0.79	1.32	1.37	0.92	-0.84
GC Ribose	ND	ND	0.16	0.13	ND	ND	-0.59
GC Ribonate	ND	ND	ND	ND	ND	ND	-2.95
GC Sorbose	ND	ND	ND	ND	ND	ND	-1.28
GC Erythrose	ND	ND	-1.24	-0.31	-0.99	-0.37	-2.69
GC Erythronate	ND	ND	1.50	ND	2.13	1.26	ND
GC Threonate	ND	ND	0.80	0.90	-0.28	1.26	ND
GC Threonate	ND	ND	ND	ND	ND	ND	-3.45
GC Mannonate	ND	ND	0.05	0.45	-0.55	1.23	0.21
GC Oxalate	-0.23	-0.51	-0.73	-0.29	0.25	0.24	-1.72
GC Glucuronate	ND	ND	ND	ND	ND	ND	-0.87
GC Gulonate	ND	ND	ND	ND	ND	ND	-1.62
GC Ascorbate	ND	ND	ND	ND	ND	ND	-0.45

KEY	d-value				* FDR-adjusted $p < 0.05$
	↑	≥2.0	≥1.2	≥0.8	≥0.5
	↓	≥2.0	≥1.2	≥0.8	≥0.5
	ND	ND	ND	ND	ND

Fig. 1 Cross-comparison of metabolic alterations in *Ndufs4* KO mice. Heatmap of effect size (Cohen's *d*) values for each metabolite when relative abundance is compared between *Ndufs4* KO ($n \geq 19$) and WT ($n \geq 20$) mice. Metabolic alterations are compared across seven sample types and grouped according to metabolic pathways (A-J). Shades of purple and green respectively indicate increased and decreased abundance in *Ndufs4* KO mice and correspond to the level of practical significance as indicated in the key. Asterisks indicate statistical significance (FDR-adjusted $p < 0.05$). The first column indicates the analytical platform used for detection. Abbreviations: AC Anterior cortex, BS Brainstem, CB Cerebellum, GC GC-TOF-MS, LC LC-MS/MS, ND Not (reliably) detected, OB Olfactory bulbs, SOL Solei, U Urine, WQ white quadriceps

Ndufs4 KO sample types when compared to their WT counterparts. Reductions in the tissue content and renal excretion of 4-hydroxyproline and 2-aminoadipate were among the most significant and consistent alterations observed in *Ndufs4* KO mice compared to WTs (Figs. 2, S1). Furthermore, the levels of citrulline, 2-hydroxyglutarate, succinate, glutamate, pyroglutamate, aspartate, butyrylcarnitine and isovalerylcarnitine also trended lower in most *Ndufs4* KO sample types, with varying levels of significance. The accumulation of branched-chain amino acids (BCAAs), proline, and arachidonate were unique to the four brain regions, although arachidonate could not be detected in other sample types. Additionally, the *Ndufs4* KO urine metabolome contained 51 uniquely altered metabolites (directionality of alterations also considered), many of which could only be detected in urine samples. Combined pathway over-representation and topology analysis (Fig. 3, Table S3) further revealed that alanine, aspartate, and glutamate metabolism were strongly affected (FDR- $p < 0.05$, impact > 0.2) in most *Ndufs4* KO sample types, with the significance level again relatively lower in the muscles. *Ndufs4* KO brain regions and urine further displayed significantly enriched arginine, proline, and BCAA metabolism. Although lesion-resistant and -prone brain regions display generally similar metabolic alterations in *Ndufs4* KO vs WT mice, the practical and statistical significance level of these alterations vary. In our previous work (Terburgh et al., 2021a) we report a graded reduction in CI activity in *Ndufs4* KO brain regions, with the lesion resistant anterior cortex displaying the highest level of residual CI activity and the olfactory bulbs displaying the lowest [AC (38% of WT) > CB (28%) > BS (25%) > OB (14%)]. Furthermore, we showed that a higher residual CI activity and a less perturbed NADH/NAD⁺ balance correlate with milder metabolic perturbations in *Ndufs4* KO brain regions. Thus, there seems to be a critical threshold (related to the level of redox imbalance and its effect on metabolism) that, when breached, likely limits brain regional capacity to bypass CI deficiency and maintain homeostasis. Glycolysis/gluconeogenesis was markedly enriched in the *Ndufs4* KO olfactory bulbs (FDR- $p < 0.05$), with moderate enrichment ($p < 0.05$) also evident in the other two

lesion-prone brain regions. The *Ndufs4* KO olfactory bulbs additionally displayed marked enrichment of pantothenate and CoA biosynthesis, which was also reflected in the urine metabolome. Moreover, glycine, serine and threonine metabolism were prominently enriched in the cerebellum, urine, and white quadriceps, while glutathione metabolism was more enriched in the anterior cortex and brainstem of *Ndufs4* KO mice. Additionally, *Ndufs4* KO urine also displayed markedly enriched beta-alanine-, glutamine and glutamate-, glyoxylate and dicarboxylate-, tricarboxylic acid (TCA) cycle-, histidine- and butanoate metabolism. In the next sections, we take a deeper look into some of the most prominent similarities and differences among the various *Ndufs4* KO metabolomes.

3.2 Metabolites strongly altered in all *Ndufs4* KO metabolomes

3.2.1 4-Hydroxyproline

Significant reductions in the levels of proline's post-translational product, 4-hydroxyproline, were evident in all *Ndufs4* KO sample types investigated (Fig. 1-B). Since urinary 4-hydroxyproline commonly serves as a bone-resorption biomarker of collagen turnover, decreased renal excretion thereof suggests decreased degradation of collagen from the bone matrix in *Ndufs4* KO mice. In accord, CI deficiency was shown to cause an intrinsic defect in osteoclastogenesis, leading to decreased bone resorption and increased bone mass (osteopetrosis) in *Ndufs4* KO mice (Jin et al., 2014). However, the significant reductions in brain and muscle 4-hydroxyproline levels we report here imply that other sources may also contribute to reduced 4-hydroxyproline excretion. As 4-hydroxyproline is a known pyruvate, glucose, and glycine precursor (Wu et al., 2011), these reductions may reflect its increased utilization in *Ndufs4* KO tissues. Thus, while urinary 4-hydroxyproline may serve as a biomarker for osteopetrosis associated with CI deficiency, its metabolism is also strongly affected in *Ndufs4* KO tissues and warrants further investigation.

3.2.2 2-Aminoadipate and lysine catabolism

The precursor for 2-ketoadipate in the lysine catabolic pathway, 2-aminoadipate, is another metabolite markedly reduced in all *Ndufs4* KO sample types (Fig. 1-C). The renal excretion of other 2-ketoadipate precursors (5-hydroxylysine, lysine, and tryptophan) and alternative products (2-hydroxyadipate and glutarate) were also significantly reduced in *Ndufs4* KO mice. Additionally, the lysine catabolite, pipecolate, markedly accumulated in brain regions (and muscles) although not measured in urine. As lysine catabolism is dependent on nicotinamide adenine dinucleotide

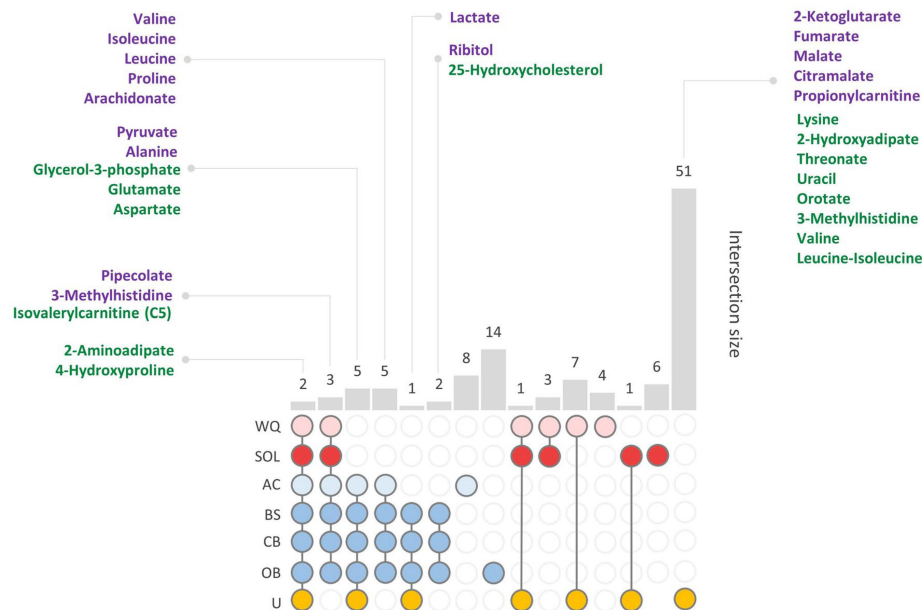


Fig. 2 Intersections of importantly altered metabolites among *Ndufs4* KO sample types. Rows indicate different sample types, while columns correspond to one segment in a Venn diagram. Color filled cells (in columns) indicate the associated samples in a set with empty cells not part of that intersection. Bars indicate the number of unique metabolites per set intersection. Purple and green text respec-

tively indicate significantly ($p < 0.05$) increased or decreased metabolite levels in *Ndufs4* KO ($n \geq 19$) mice compared to WT ($n \geq 20$). Only a few of the relevant metabolic alterations unique to urine are displayed. Abbreviations: AC Anterior cortex, BS Brainstem, CB Cerebellum, OB Olfactory bulbs, SOL Solei, U Urine, WQ White quadriceps

(NAD⁺) bioavailability, redox imbalance is one of the main, and well documented, factors linked to MD-related perturbations in this pathway (Esterhuizen et al., 2017). Lysine levels were, however, not as consistently altered with its accumulation only evident in the *Ndufs4* KO olfactory bulbs (and muscles). The degree of lysine accumulation in the olfactory bulbs, further correlated with the accumulation of its metabolite, carnitine, which was also elevated in other brain regions and urine. These observations may point to increased proteolysis in *Ndufs4* KO tissues, as the release of trimethyllysine from cognate proteins is required for endogenous carnitine synthesis, which takes place mainly in the liver, kidneys, and brain but not muscles (Vaz and Wanders, 2002). In support, some of the most significant alterations in *Ndufs4* KO urine were centered around ascorbate (Fig. 1-J), a cofactor required for two dioxygenase reactions in the carnitine biosynthesis pathway. Although urinary ascorbate levels only trended lower, markedly decreased renal excretion of glucarate and gluconate (metabolites from the glucuronate pathway that diverge into the murine ascorbate synthesis pathway) as well as threonate and oxalate (breakdown products of ascorbate) may reflect increased ascorbate demand

in *Ndufs4* KO mice. The enzymes that catalyze these dioxygenase reactions, ϵ -N-trimethyllysine dioxygenase (TMLD, EC 1.14.11.8) and γ -butyrobetaine dioxygenase (BBD, EC 1.14.11.1), also convert 2-ketoglutarate to succinate and perhaps present a favorable alternative route to fuel the ubiquinone (Q)-pool via CII. Furthermore, alterations in ascorbate metabolism may be more evident in the urine metabolome since the expression of BBD is high in the kidneys, moderate in the liver, but low in the brain (Vaz and Wanders, 2002). Marked elevation in brain and muscle levels of 3-methylhistidine (3-MH) and the 3-MH/histidine ratio further support the notion of elevated proteolysis in *Ndufs4* KO tissues (Fig. 1-D). However, the renal excretion of histidine and its methylated derivatives were markedly reduced. These discrepancies confirm previous concerns regarding the use of urinary 3-MH as an index of myofibrillar protein turnover (Shargill et al., 1984; van Goudoever and Matthews, 2017) as several hundred widespread histidine-methylated proteins were recently identified (Davydova et al., 2021; Ning et al., 2016; Wilkinson et al., 2019) and considerable amounts of urinary 3-MH was shown to originate from non-muscle sources (Millward and Bates, 1983; Nishizawa et al., 1977;

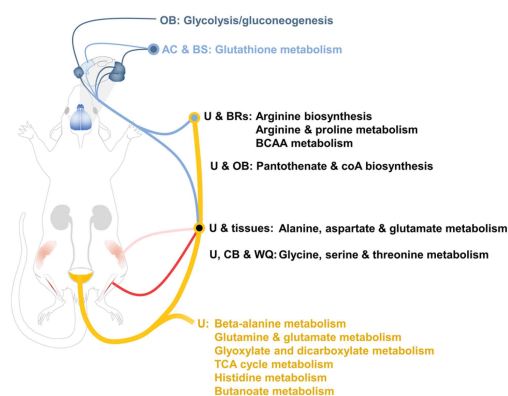


Fig. 3 Affected metabolic pathways in *Ndufs4* KO mice. Significantly enriched pathways identified via pathway over-representation (FDR-adjusted $p < 0.05$) analysis. Colored lines represent sample types; while dots indicate intersection of lines and signify commonly affected pathways among sample types. Colors and abbreviations: AC Anterior cortex (light blue), BRs All brain regions (medium blue), BS Brainstem (dark blue), CB Cerebellum (dark blue), OB Olfactory bulbs (dark blue), SOL Solei (red), U Urine (yellow), WQ White quadriceps (light peach)

Wassner and Li, 1982). Nevertheless, the results reported here not only reveal widespread perturbations in the metabolism of lysine and tryptophan, but also suggest elevated proteolysis and carnitine biosynthesis in a subset of *Ndufs4* KO tissues. Hence, the future investigation of tissue-specific protease activity in *Ndufs4* KO mice is recommended.

3.3 Biofluid markers commonly associated with MD

In *Ndufs4* KO urine, significant elevations were seen in biofluid markers commonly associated with MD i.e., pyruvate, alanine, lactate and the lactate/pyruvate ratio (FDR- $p < 0.05$, ES $d = 1.20$)—which reflects cytosolic redox state (Esterhuizen et al., 2017; Go et al., 2020; Munnich et al., 1996). These alterations were similar in the brain, which also displayed elevated glycolytic- and pentose phosphate pathway intermediates, with clear differences seen in the degree of metabolite accumulation among regions (Fig. 1–J). The *Ndufs4* KO olfactory bulbs were most significantly affected, while lactate accumulation was only evident in the neurodegeneration-prone brain regions. In agreement, Johnson and colleagues report that pre-symptomatic *Ndufs4* KO mice display significantly elevated levels of pyruvate and lactate along with higher trends in alanine levels in whole brain Johnson et al. (2013) and trended higher levels of glycolytic intermediates and lactate in brain regions, with significant lactate elevation only seen in the olfactory bulbs (Johnson et al., 2020). Contrastingly, reports of blood

lactate levels in *Ndufs4* KO mice vary, with normal, slight, and significant elevation observed (Jain et al., 2016; Jin et al., 2014; Kruse et al., 2008). Considering recent reports of lactate (and not glucose) being the primary circulating TCA substrate for most tissues (except the brain) with the kidneys playing a key role in systemic redox homeostasis by oxidizing lactate to pyruvate (Jang et al., 2019), we suggest that excretory (urine) rather than circulating biofluids may be more appropriate for MD-marker monitoring as the lack of homeostatic control mechanisms in urine allows magnification of metabolic alteration occurring in blood (Wu and Gao, 2015). Similarly, although blood glucose levels are reportedly unaltered in *Ndufs4* KO mice (Kruse et al., 2008), we observed decreased renal excretion of glucose, ribitol, several monosaccharides and sugar acids, suggesting increased glucose utilization in *Ndufs4* KO tissues. In agreement, pyruvate, lactate, and alanine levels all trended lower in *Ndufs4* KO skeletal muscles, possibly indicating increased release thereof to fuel gluconeogenesis. Taken with the accumulation of glycolytic- and pentose phosphate pathway intermediates that we, and others, observed in the *Ndufs4* KO brain as well as reports of upregulated glucose transporter 1 (GLUT1, SLC2A1) protein levels in the *Ndufs4* KO brain (Lee et al., 2019) and pro-glycolysis genes in the *Ndufs4* KO liver (Jin et al., 2014), we postulate that glucose production, uptake and utilization is elevated in *Ndufs4* KO mice.

In agreement with our observations, muscle lactate levels in patients with mitochondrial disease, was shown to be lower at rest but elevated during exercise (Lindholm et al., 2004; Löfberg et al., 2001). Another possible explanation for this discrepancy between *Ndufs4* KO muscle and brain metabolomes is the activity of the glycerol-3-phosphate (G3P) shuttle, which is one of the most important, highly active redox regulatory shuttles in skeletal muscle that has limited activity in the brain (Liu et al., 2021; Schiaffino and Reggiani, 2011). Increased G3P synthesis (and shuttle activity) is known to reduce muscle lactate build-up (Alfadda et al., 2004) and was recently shown to be an important endogenous NAD^+ -regeneration pathway during CI inhibition that suppresses neuroinflammation and extends lifespan when enhanced in *Ndufs4* KO mice (Liu et al., 2021). Interestingly, mice with cytosolic G3P dehydrogenase (cGPD; EC 1.1.1.8) deficiency display increased dihydroxy acetone phosphate (DHAP) and decreased G3P in muscle (MacDonald and Marshall, 2000, 2001), similar to what we observed in *Ndufs4* KO brain regions—suggesting that there may be an increased need for this shuttle in brain during CI deficiency. In agreement, lower levels of G3P and an increased trend in DHAP levels could also be seen in the whole-brain metabolomics data from pre-symptomatic *Ndufs4* KO mice (Johnson et al., 2013). Although we could not detect these metabolites in muscles, urinary G3P excretion was markedly

decreased in *Ndufs4* KO mice. In addition, TCA cycle-linked metabolites (succinate and 2-hydroxyglutarate) which, like G3P, can fuel the Q-pool independently of CI, were consistently and significantly decreases in *Ndufs4* KO brain regions and urine. The accumulation of TCA cycle intermediates, 2-ketoglutarate, fumarate, and malate in biofluids is another metabolic signature characteristic of MD, that suggests TCA cycle congestion. Although these metabolites were significantly elevated in *Ndufs4* KO urine, they were not markedly altered in the various brain regions (Fig. 1-F). Others have also reported early-stage elevations in fumarate levels in affected brain regions (but not muscle) of *Ndufs4* KO mice, which was not evident in brain regions of older mice (Piroli et al., 2020; Piroli et al., 2016). Overall, urine proves to be a fitting biofluid with which to monitor classic MD biomarkers that reflect the redox state in affected brain regions. However, with inconsistencies between tissue and urine levels of TCA cycle intermediates observed, questions arise regarding the extent of TCA cycle congestion in individual *Ndufs4* KO tissues.

3.4 Inconsistent alterations between systemic and brain region-specific metabolomes

3.4.1 BCAA catabolism

BCAAs, valine, leucine, and isoleucine markedly accumulated in *Ndufs4* KO lesion-prone brain regions, with elevations also evident in the lesion-resistant anterior cortex (Fig. 1-G). The degree of these elevations correlated with reductions in leucine's catabolic by-product, isovalerylcarnitine (C5). Although these alterations were not observed in whole brain or brain regions of pre-symptomatic mice (Johnson et al., 2020; Johnson et al., 2013), perturbations in BCAA catabolism have been reported in MD cases and, as with lysine, is believed to result from the redox imbalance associated with MD (Esterhuizen et al., 2017). Several tissues jointly control BCAA catabolism, with skeletal muscle and liver playing a central role (Holeček, 2018). The first step in BCAA catabolism primarily occurs in skeletal muscles, which then release most branched-chain keto acids (along with glutamine and alanine) into circulation to be taken up by other tissues (Harper et al., 1984). BCAA levels were not significantly altered in skeletal muscles, but trended lower in the glycolytic white quadriceps, while the renal excretion of BCAAs (and their metabolites) were markedly decreased in *Ndufs4* KO mice. Taken together, these observations suggest that an increased influx of BCAA in the brain, together with a restriction in the NAD⁺-dependent step of BCAA catabolism, may result in elevated BCAA levels. Furthermore, we postulate that BCAA catabolism could be enhanced in a subset of *Ndufs4* KO tissues, possibly to fuel succinyl-CoA production and the Q-pool via

electron-transferring-flavoprotein dehydrogenase (ETFDH, EC 1.5.5.1). Such a tissue-specific difference in BCAA handling has also been proposed to take place in insulin resistant individuals (Newgard, 2012).

3.4.2 Aspartate, glutamate, and arginine metabolism

Glutamate (Glu), pyroglutamate, and aspartate levels were significantly decreased in *Ndufs4* KO brain regions and urine (Fig. 1-A). Among these, the degree of aspartate reduction was greater in the brain, in which asparagine levels were also markedly lower. In accord, pre-symptomatic *Ndufs4* KO mice display trended lower brain-regional levels of a metabolite cluster including Glu, pyroglutamate, aspartate, and ornithine as well as decreased trends in whole brain levels of 3-aminobutyrate and most free amino acids, (Johnson et al., 2020). We additionally observed significant reductions in glutamine (Gln) and 3-aminobutyrate abundance in *Ndufs4* KO urine but not brain regions. Furthermore, alterations in aspartate and Glu metabolism were consistent in *Ndufs4* KO urine and white quadriceps, although less significant in the muscles. These observations could reflect a systemic increase in Gln and Glu consumption, a phenomenon which has recently been linked to compensatory mechanisms that function to decrease mitochondrial redox stress during OXPHOS deficiency. Enhanced Gln-derived anaplerosis of the TCA cycle was shown to regenerate NAD⁺ via aspartate efflux from mitochondria, however, when the level of OXPHOS deficiency exceeds a certain threshold, Gln starts fueling acetylation and lipogenesis via reductive carboxylation (Chen et al., 2018). Similarly, enhanced Gln-derived proline biosynthesis was shown to regenerate NAD(P)⁺ levels when TCA cycle activity surpasses OXPHOS capacity (Schwörer et al., 2020). While the former mechanism may be a systemic adaption, the latter seems to be exclusive to the *Ndufs4* KO brain, where proline levels were uniquely elevated (Fig. 1B). The marked decrease in urine and muscle proline levels further confirm that this putative phenomenon is not systemic. On the contrary, Yang et al. (2020) recently reported a marked decrease in circulatory Gln turnover flux and Gln anaplerosis in the pancreas and liver of *Ndufs4* KO mice, while Gln's contribution to (or handling within) the TCA cycle did not markedly alter in other tissues. Our results further indicate a significant decrease in the renal excretion of N-acetylglutamate (NAG) and other urea cycle intermediates (carbamate, ornithine, citrulline, arginine, and urate) in *Ndufs4* KO mice, with the urinary alanine/Glu- and Glu/Gln ratios elevated (FDR-*p*<0.05, ES *d*>1.2). Although citrulline levels consistently trended lower in *Ndufs4* KO brain regions (and muscles), the degree and direction of other alterations in arginine metabolism were inconsistent. Contrastingly, most of these metabolites were moderately elevated in both glycolytic and oxidative muscles. Taken

together with the alterations described in previous sections, this might reflect increased muscle proteolysis and amino acid catabolism along with increased ammonia transfer to the liver. Elevated levels of NAG (brain regions) and N-acetylglutamate (muscles) further suggest increased protein acetylation in *Ndufs4* KO tissues.

3.4.3 Lipid metabolism

The levels of glycerol along with several carnitine- and glycine conjugated medium chain fatty acids (hexanoyl-, octanoyl- and decanoylcarnitine; butyryl-, valeryl- and hexanoylglycine) were decreased in *Ndufs4* KO urine (and muscle). Glycerol levels were variably affected among *Ndufs4* KO brain regions, while the levels of fatty acids, palmitate (C16:0), oleate (C18:1), and arachidonate (C20:4, ω -6) consistently trended higher (Fig. 1-E). Cytosolic acetyl-CoA accumulation is known to stimulate lipogenesis, cholesterol synthesis and protein acetylation (Pietrocola et al., 2015; Saggerson, 2008). Significant elevations were seen in the renal excretion and tissue content of acetylcarnitine, which suggest acetyl-CoA accumulation in *Ndufs4* KO mice, as this membrane permeant storage form of acetyl-CoA serves to dispose of or replenish it as needed (Schroeder et al., 2012). Additionally, the olfactory bulbs, which was most markedly affected, displayed decreased levels of pantothenate (a CoA component) and its precursor, beta-alanine—likely reflecting increased CoA demand. In lesion-prone brain regions, significant reductions were also evident in the levels of 25-hydroxycholesterol, which like arachidonate, has been described to play a regulatory role in cholesterol metabolism (Demetz et al., 2014; Kilsdonk et al., 1995). Interestingly, increasing cholesterol production, export, and utilization was recently shown to attenuate elevated NAD(P)H levels in CI deficient fibroblast (Schirris et al., 2021). Recent reports of protein hyper-acetylation (Gella et al., 2020; Piroli et al., 2020) and increased lipid synthesis in lesion-prone brain regions (Liu et al., 2017; Liu et al., 2015) as well as elevated levels of triacylglycerol and free fatty acids in the serum of *Ndufs4* KO mice (Jin et al., 2014) further support the notion of acetyl-CoA accumulation and increased lipogenesis in *Ndufs4* KO brain regions (Chen et al., 2018; Mullen et al., 2014; Yang et al., 2014). Taken together with the observations discussed in previous sections, the data points to a systemic mismatch between the amount of acetyl-CoA generated via catabolic reactions in *Ndufs4* KO tissues and the processing capacity of the TCA cycle.

3.4.4 Choline-, one-carbon-, and nucleotide metabolism

Decreased levels of intermediates in the metabolism of choline to glycine i.e., trimethylglycine (betaine), dimethylglycine and methylglycine (sarcosine) along with methionine,

and cystathionine were among the most significant alterations observed in *Ndufs4* KO urine (Fig. 1-H). Although not as evident in the brain, these alterations were mirrored in the *Ndufs4* KO white quadriceps. Choline-derived betaine remethylates homocysteine to methionine in the methionine cycle, with the subsequent oxidation of dimethylglycine and sarcosine both feeding the Q-pool via ETFDH and ultimately producing glycine. Betaine mainly serves as a methyl donor in the liver, where betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) is highly expressed (Szegedi et al., 2008; Teng et al., 2011). In addition to the liver, renal metabolism is also thought to play an important role in systemic one-carbon (1C) homeostasis, as the kidneys produce the bulk of systemic serine and glycine (Jang et al., 2019). Decreased renal excretion of glycine, serine, histidine, threonine, and cystine in *Ndufs4* KO mice further suggests significant perturbations in hepatic and renal 1C metabolism. Despite discrepancies among *Ndufs4* KO brain regions, betaine and serine levels consistently trend lower (except in the olfactory bulbs) with glycine markedly elevated in the brainstem and reduced in the cerebellum.

Marked reductions in some purine and pyrimidine intermediates were also evident in the olfactory bulbs and anterior cortex (Fig. 1-I). In agreement, significantly decreased renal excretion of purine- and pyrimidine catabolism end-products, urate and beta-alanine, were observed in *Ndufs4* KO mice along with decreased levels of orotate, uracil, 3-ureidopropionate, and thymine catabolite, 3-amino-isobutyrate. In accordance, nucleotides and products of nucleotide catabolism were also decreased in whole brain of pre-symptomatic *Ndufs4* KO mice (Johnson et al., 2013). As purine and pyrimidine nucleotides are important precursors for the synthesis of nucleotide cofactors such as NAD⁺ and S-adenosyl methionine (SAM), these alterations are likely linked to perturbations in redox balance and 1C metabolism. Several studies have functionally linked 1C metabolism and serine catabolism, specifically, to the OXPHOS system and deficiencies thereof (Bao et al., 2016; Lucas et al., 2018; Nikkanen et al., 2016); with a maladaptive increase in the fraction of NADH produced by folate-mediated serine catabolism recently reported in *Ndufs4* KO mice (Yang et al., 2020). Altogether, our data shows that serine-, 1C-, and nucleotide- metabolism is markedly perturbed in *Ndufs4* KO mice, with the methylation cycle most dramatically affected. While these alterations in the urine metabolome likely reflect hepatic and renal metabolism, 1C-metabolism linked perturbations are also evident in *Ndufs4* KO brain regions and muscles.

3.5 Contributions of gut microbial metabolism

Several metabolic alterations in *Ndufs4* KO urine seemed to reflect altered gut microbial metabolism (Fig. S2). This

includes significantly elevated levels of 2,3-butandiol, 2,6-dihydroxybenzoate, citramalate, and propionylcarnitine along with decreased levels of 3-hydroxyphenyl (HP) propanoate, 4-HP-glycolate, 4-HP-acetate, and 4-HP-pyruvate. 2,3-Butandiol is known to accumulate in the urine of MD patients and thought to reflect high systemic levels of glucose that is further metabolized by gut microbes (Elshagabee et al., 2016; Esterhuizen et al., 2019; Venkataraman et al., 2014). Gut microbiota are also major producers of short chain fatty acids (SCFs), especially propionate and butyrate (Jang et al., 2019). Therefore, markedly altered SCF acyl-conjugates in *Ndufs4* KO mice may be related to intestinal metabolism. It was recently shown that gut microbiota can metabolize host-derived lactate to SCFs, predominantly propionate, which can then cross back into the host (Scheiman et al., 2019). Since propionylcarnitine levels were decreased in most *Ndufs4* KO brain regions (and muscles), the urinary accumulation of propionylcarnitine is likely related to lactate accumulation in *Ndufs4* KO mice and gut-derived elevations in propionate. In support, increased urinary citramalate excretion is also seen with propionyl-CoA accumulation (in propionyl-CoA carboxylase deficiency) and is associated with intestinal dysbiosis (Greter et al., 1980). Furthermore, reduced urinary excretion of 4-HP-acetate and 4-HP-pyruvate may reflect perturbations in host-microbial co-metabolism of aromatic amino acids (Li et al., 2021). This is supported by decreased levels of phenylalanine, tryptophan, kynurenine, and 5-hydroxy-indole exclusively in *Ndufs4* KO urine. Alterations in gut microbiota have been reported in a variety of disease phenotypes with this host-gut microbiota interaction recently linked to changes in mitochondrial function and redox status (Yardeni et al., 2019). Accordingly, our data indicates that altered microbial compositions or metabolism likely contribute to metabolic observations in biofluids of LS patients. Furthermore, it must be noted that when working with mouse models, microbiota transfer between mice via coprophagy could influence metabolomes when genotypes are cohoused—as was done in this study. Although the ingestion of feces by self-grooming is considered more probable, the transfer of microbiota between genotypes cannot be ruled out and measures should be taken to avoid this (Brinkman et al., 2013; Laukens et al., 2016). Together, these observations highlight the necessity of future studies investigating host-bacterial co-metabolism and the gut-brain axis in LS.

4 Conclusions

Here, we integrate and cross-compare multi-platform metabolomics data from urine and tissues of late stage diseased *Ndufs4* KO vs WT mice to better understand how systemic biofluids reflect tissue-specific metabolic alterations in

LS. We confirm that biofluid markers associated with MD (elevated alanine, pyruvate, and lactate) and several other metabolic signatures in urine reflect metabolic alterations in the LS brain, thereby verifying the value of urine for the monitoring of these metabolic markers. In addition to glycolytic and TCA cycle intermediates, it would be advantageous to investigate (and validate) whether the following urinary metabolites could serve as biomarkers of LS and MD: amino acids and derivatives related to alanine, aspartate and glutamate-; arginine and proline-; BCAA-; lysine-; histidine-; and choline metabolism; as well as sugar derivatives; metabolites related to the G3P shuttle; and gut microbial metabolism. Widespread reductions were evident in the abundance of several metabolites capable of fueling Q-pool linked dehydrogenases (other than CI) in *Ndufs4* KO mice, therefore, we hypothesize that tissue-specific differences in these dehydrogenases could influence metabolic flexibility and the consequences of CI deficiency in different tissues. Our data further reveals that impaired BCAA catabolism, increased proline biosynthesis, and the accumulation of glycolytic intermediates are brain-specific metabolic perturbations in LS. Contrastingly, elevated levels of TCA cycle intermediates in urine, and significant reductions in one-carbon metabolism intermediates, are not clearly reflected in the *Ndufs4* KO brain and appear to be systemic of origin. Furthermore, the discrepancies we report between urinary and tissue-specific metabolomes highlight the shortcomings of only using systemic biofluids as an investigative tool for multi-systemic diseases. Although non- or minimally invasive systemic samples contain valuable information, these metabolomes should be carefully interpreted in conjunction with tissue-specific data—especially when aiming to elucidate disease mechanisms. In this regard, having reliable MD models and access tissue samples that cannot be obtained from patients, is vital to improve our understanding of complex MD processes. Additionally, future sex-specific studies, targeting the areas of metabolism identified here, should be a priority. Almost all aspects of metabolism and energy homeostasis are regulated in a sex-specific manner, which influences disease pathogenesis. Sex hormones and -chromosomes along with differences in tissue-specific gene regulation and gut microbiota between sexes play a central role in this metabolic sexual dimorphism (Chella Krishnan et al., 2018; Clegg and Mauvais-Jarvis, 2018). Indeed, many metabolic pathways affected in male *Ndufs4* KO mice, have been shown to display sexual dimorphism in murine models and/or humans. This includes glucose and lipid metabolism (Baars et al., 2018; Chella Krishnan et al., 2018; Clegg and Mauvais-Jarvis, 2018; Lundsgaard and Kiens, 2014), amino acid and acylcarnitine metabolism (Ruoppolo et al., 2018; Wu and Gao, 2015), branched-chain amino acid metabolism (Vignoli et al., 2018), and bone metabolism (Choi et al., 2021).

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Author contributions KT and RL designed the research plan with input from JZL and FvdW. FvdW acquired the animal model and ethical approval with assistance from RL. RL and FvdW obtained funding for the study. KT performed the data processing with assistance from RL and JZL. KT analyzed the data and wrote the manuscript with input from RL, JZL and FvdW.

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Data availability The metabolomics data obtained in this study can be accessed at the Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (<https://www.metabolomicsworkbench.org>) where it has been assigned project IDs: PR000721 (Terburgh et al., 2019a), PR001003 (Terburgh et al., 2021b), and PR001187 (Terburgh et al., 2021c).

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval The AnimCare animal research ethics committee of North-West University approved (NWU-0001-15-A5 and NWU-00378-16-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.

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