

Investigation into the protection of mitochondrial disease pathology by metallothionein overexpression

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ABSTRACT

Mitochondrial diseases (MD), such as Leigh Syndrome (LS), present with severe neurological and muscular phenotypes in patients, but have no known cure and limited treatment options. Based on their neuroprotective effects against other neurodegenerative diseases *in vivo* and their positive impact as an antioxidant against complex I deficiency *in vitro*, we investigated the potential protective effect of metallothioneins (MT) in a whole-body *Ndufs4* (complex I) knockout mouse model (with a very similar phenotype to LS) crossed with an *Mt1* overexpressing mouse model (*TgMt1*). Despite subtle reductions in the expression of neuroinflammatory markers GFAP and IBA1 in the vestibular nucleus and hippocampus, no improvement was observed in survival, growth, locomotor activity, balance or motor coordination in the *Mt1* overexpressing *Ndufs4*^{-/-} mice. Furthermore, at a cellular level, no differences were detected in the metabolomics profile, enzyme activity or protein oxidation levels in the brain and quadriceps from these mice. These outcomes, together with linked studies showed no differences in the expression of selected one-carbon (1C) metabolism and oxidative stress metabolism genes in the brain and quadriceps, nor in the ROS levels of macrophages derived from these mice. Therefore, we conclude that MT1 overexpression does not protect against the impaired motor activity or improve survival in these complex I deficient mice. The unexpected absence of increased oxidative stress and metabolic redox imbalance in this MD model may explain these observations. However, tissue-specific observations such as the mildly reduced inflammation in the hippocampus and vestibular nucleus, as well as differential MT1 expression in these tissues, may yet reveal a tissue-specific role for MT1 or other MT isoforms (MT2 and MT3) in these mice.

Keywords: *Ndufs4* knockout mice, Metallothionein, Transgenic metallothionein overexpressing mice, Mitochondrial disease, Leigh Syndrome, Phenotyping, Oxidative stress

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

SYMBOLS

*	Asterisk
-/-	Homozygous knockout genotype; both alleles of interest are absent from the specific gene locus
~	Approximately
+/-	Heterozygous knockout genotype; one allele of interest is absent from the specific gene locus, while the other is present
+/+	Homozygous wild-type genotype; both alleles of interest are present at the specific gene locus
<	Less-than
>	Greater-than
±	Plus-minus
×	Times
↑	Increase
↓	Decrease
I	One (Roman numeral)
II	Two (Roman numeral)
III	Three (Roman numeral)
IV	Four (Roman numeral)
Ø	Diameter
V	Five (Roman numeral)
α	Alpha
β	Beta
Δ	Delta
λ	Wavelength
ν	Reaction velocity

UNITS

%	Percentage
°C	Degrees Celsius
μg	Microgram
μL	Microlitre
μM	Micromolar
amu	Atomic mass unit
AU	Arbitrary units
cm	Centimetres
eV	Electron volt
g	Gram
h	Hours
Hz	Hertz
Kb	Kilobase
kDa	Kilodaltons
L	Litres
M	Molarity; moles solute per litre solvent

m/z	Mass-to-charge ratio
mg	Milligram
MHz	Megahertz
min	Minutes
mL	Millilitre
mm	Millimetre
mM	Millimolar
N	Normality; molar concentration divided by an equivalence factor
ng	Nanogram
nm	Nanometre
nmol	Nanomole
Ns	Newton second
pH	Potential of hydrogen; the negative of the base 10 logarithm of the H ⁺ molar concentration
ppm	Part per million
psi	Pound-force per square inch; unit of pressure
rpm	Revolutions per minute
s	Seconds
UCS	Units citrate synthase
V	Volt
v/v	Volume of solute per volume of solvent
w/v	Weight of solute per volume of solvent
x g	Relative centrifugal force
η^2	Eta squared
η_p^2	Partial eta squared

ABBREVIATIONS

1C	One-carbon
2-AAP	2-Acetamidophenol
3-PBA	3-Phenylbutyric acid
4-PBA	4-Phenylbutyric acid
5'	5-prime end; 5-prime phosphate group of the polynucleotide chain
<i>ad libitum</i>	Without restraint (Latin); food and water are available at all times
ADP	Adenosine diphosphate
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis-inducing factor
AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
APS	Ammonium persulphate
ARE	Antioxidant response element
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BBA	<i>Biochimica et Biophysica Acta</i> (Latin): Journal of Biochemistry and Biophysics
BCA	Bicinchoninic acid
BCAA	Branched-chain amino acid
BH-FDR	Benjamini-Hochberg adjustment to control the rate of false discovery
BMDM	Bone marrow-derived macrophages

BN-PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pairs
BSA	Bovine serum albumin
BSTFA	O-bis(trimethylsilyl) trifluoroacetamide
C	Cytochrome c; or complex (depending on the context)
C0	Carnitine
C12	Dodecanoylcarnitine
C14	Myristoylcarnitine
C16	Palmitoylcarnitine
C18	Stearoylcarnitine
C2	Acetylcarnitine
C3	Propionylcarnitine
C4	Butanoylcarnitine
C5	Isovalerylcarnitine
C6	Hexanoylcarnitine
C8	Octanoylcarnitine
Ca ²⁺	Calcium(II) ion
cAMP	Cyclic adenosine monophosphate
CAN	Acetonitrile
Cd ²⁺	Cadmium(II) ion
cG3PDH	Cytosolic glycerol-3-phosphate dehydrogenase
cHCl	Concentrated hydrochloric acid
CI	Complex I; NADH:ubiquinone oxidoreductase; EC 1.6.5.3
CII	Complex II; succinate:ubiquinone oxidoreductase; EC 1.3.5.1
CIII	Complex III; ubiquinol:ferricytochrome c oxidoreductase; EC 1.10.2.2
CIV	Complex IV; ferrocytochrome-c:oxygen oxidoreductase; EC 1.9.3.1
CNS	Central nervous system
CO ₂	Carbon dioxide
CoA	Coenzyme A
COX17	Cytochrome c oxidase copper chaperone 17
CS	Citrate synthase
CT or C _T	Threshold cycle
Cu	Copper
Cu ⁺	Copper(I) ion
CuSO ₄	Copper(II) sulphate
CV	Complex V; ATP synthase; EC 3.6.1.3. or coefficient of variance (depending on the context)
CV%	Percentage coefficient of variance
cyt c	Cytochrome c
D ₂ O	Deuterium oxide
DAAM	Digiscan Animal Activity Monitor
DCIP	2,6-Dichloroindophenol
<i>de novo</i>	Anew, from the beginning (Latin)
DH	Dehydrogenase
DHF	Dihydrofolate
DMG	Dimethylglycine
DMPA	N, N-dimethyl-L-phenylalanine
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenylhydrazone
DNPH	2,4-Dinitrophenylhydrazine
dNTP	Deoxyribonucleotide triphosphate
dTMP	Thymidine monophosphate
DTNB	2,2'-Dinitro-5,5'-dithiobenzoic acid
dUMP	Uridine monophosphate
d-value	Effect size value of practical significance
e ⁻	Electron
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
ES	Effect size
ESI	Electrospray ionisation
<i>et al.</i>	<i>et alii</i> (Latin): and others
ETF	Electron transfer flavoprotein
ETS	Electron transfer system
FAD	Oxidised flavin adenine dinucleotide
FADH ₂	Reduced flavin adenine dinucleotide
FASBMB	Federation of African Societies of Biochemistry and Molecular Biology
FeS	Iron-sulfur
FMN	Oxidised flavin mononucleotide
FMNH ₂	Reduced flavin mononucleotide
G3P	Glycerol-3-phosphate
GABA	γ-Aminobutyrate
GC	Gas chromatography
GC-TOF-MS	Gas chromatography time-of-flight mass spectrometry
GFAP	Glial fibrillary acidic protein
Glog	Generalised logarithm
GP	Glycerophosphate
GPC	Glycerophosphocholine
GRE	Glucocorticoid response elements
GSH	Reduced glutathione
GSSG	Oxidised glutathione; glutathione disulphide
GTP	Guanosine triphosphate
H ⁺	Hydrogen ion; proton
H ₂ O	Water
H ₂ O ₂	hydrogen peroxide
HCl	Hydrochloric acid
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPLC	High-performance liquid chromatography
Hq	Harlequin
HRP	Horse radish peroxidase
HSP-70	Heat shock protein-70
IBA1	Ionised calcium binding adapter molecule 1
ICP	Inductively coupled plasma
ID	Identification number

IL12	Interleukin-12
IL6	Interleukin-6
IMP	Inosine monophosphate
<i>in vitro</i>	Performed or taking place outside a living organism (Latin)
<i>in vivo</i>	Occurring or made to occur within a living organism (Latin)
IS	Internal standard
KG	α -Ketoglutarate
KH ₂ PO ₄	Potassium phosphate monobasic
KO OVER	<i>Ndufs4</i> knockout <i>Mt1</i> overexpressing sample (specific to this study)
KO	<i>Ndufs4</i> knockout sample (specific to this study)
KOH	Potassium hydroxide
KP _i	Potassium phosphate
LAIFKO	Liver-specific apoptosis-inducing factor knock out
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
loxP	Locus of X(cross)-over in P1
LS	Leigh Syndrome
M	Mean
MAIFKO	Muscle-specific apoptosis-inducing factor knock out
MD	Mitochondrial disease
MELAS	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
mG3PDH	Mitochondrial glycerol-3-phosphate dehydrogenase
MIDD	Maternally inherited diabetes and deafness
MnSOD	Manganese superoxide dismutase
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRE	Metal-responsive elements
MRI	Magnetic resonance imaging
MRL	Mitochondria research laboratory
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MT	Metallothionein
mtDNA	Mitochondrial deoxyribonucleic acid
MTF-1	Metal-responsive transcription factor
MTHF	Methylenetetrahydrofolate
mTOR	Mammalian/mechanistic target of rapamycin
n	Number of; amount
N module	NADH binding module
NAA	N-Acetylaspartate
NAD ⁺	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP ⁺	Oxidised nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAG	N-Acetylglutamate
NaN ₃	Sodium azide
NaOH	Sodium hydroxide
ND	NADH dehydrogenase subunit
nDNA	Nuclear deoxyribonucleic acid
NDS	Normal donkey serum
NDUF	NADH:ubiquinone oxidoreductase (core) subunit

<i>Ndufs4</i>	NADH dehydrogenase (ubiquinone) iron-sulfur protein 4 gene; NADH:ubiquinone oxidoreductase subunit S4 gene
NDUFS4	NADH dehydrogenase (ubiquinone) iron-sulfur protein 4; NADH:ubiquinone oxidoreductase subunit S4
<i>Ndufs4</i> ^{-/-}	Ndufs4 knockout mouse
NesKO	<i>Ndufs4</i> knockout mouse model in the neurons and glia
NMR	Nuclear magnetic resonance
NNT	Nicotinamide nucleotide transhydrogenase
NOESY	Nuclear overhauser effect spectroscopy
NRF	National Research Foundation of South Africa
NWU	North-West University
O ₂	Oxygen
O ₂ ^{-•}	Superoxide anion radical
OAA	Oxaloacetic acid
OMIM	Online Mendelian Inheritance in Man
OMP	Orotidine monophosphate
OVER	<i>Mt1</i> overexpressing sample (specific to this study)
OXPPOS	Oxidative phosphorylation
P module	Proton pumping module
P	Postnatal day
P/N	Part number
P5C	Pyrroline-5-carboxylate
PARP	Poly[adenine diphosphate (ADP)-ribose] polymerase
PBS	Physiological buffered saline
PBST	Physiological buffered saline with 0.2% (v/v) Triton-X
PC	Phosphocholine
PCA	Principal component analysis
PCDDP	Preclinical Drug Development Platform
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PRPP	Phosphoribosyl pyrophosphate
PTFE	Polytetrafluoroethylene
p-value	Significance value
PVDF	Polyvinylidene difluoride
Q module	Ubiquinone binding module
Q	Ubiquinone
QC	Quality control
qPCR	Quantitative polymerase chain reaction; real-time polymerase chain reaction
Redox	Reduction-oxidation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT	Retention time
RT-PCR	Reverse transcription polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SASBMB	South African Society for Biochemistry and Molecular Biology
SAVC	South African Veterinary Council

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SOP	Standard operating procedure
STAT	Signal transducers and activators of transcription
T	Thionin
TBS	Tris buffered saline
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
THF	Tetrahydrofolate
TMCS	Trimethylchlorosilane
TMG	Trimethylglycine
TNF α	Tumor necrosis factor alpha
tRNA	Transfer ribonucleic acid
TXI	Triple-resonance inverse
UMP	Uridine monophosphate
UQ	Ubiquinone
USA	United States of America
UV	Ultraviolet
VDAC	Voltage dependent anion channel subunit 1
vs	Versus
WT	Wild-type; genetically unaltered sample (specific to this study)
Zn	Zinc
Zn ²⁺	Zinc(II) ion
β 2m	β -2-microglobulin gene

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND, MOTIVATION AND PROBLEM STATEMENT

Mitochondrial diseases (MDs) are a heterogeneous group of disorders that present with both multisystemic or single organ clinical symptoms, have a prevalence of 1 in 4300 and currently have very limited treatment options with no known cures (Gorman *et al.*, 2015). Isolated complex I (CI) deficiency is the most common childhood MD which presents in ~80% of children as Leigh Syndrome (LS), an MD characterised by a muscular and neurological phenotype that is associated with increased oxidative stress and inflammation, decreased energy production, altered redox status, disturbance of the mitochondrial membrane potential and altered intracellular Ca^{2+} homeostasis (Distelmaier *et al.*, 2009; Reinecke *et al.*, 2009; Valsecchi *et al.*, 2010; Rodenburg, 2016). With no known cure and death at ~3 years of age, LS treatment options are urgently sought after, yet remain lacking. Current treatment strategies focus on promoting sirtuin activity, stabilising cellular Ca^{2+} homeostasis and increasing ROS scavenging, with potent antioxidants like Trolox (a vitamin E analogue) and variants of this molecule showing great promise for treatment of CI dysfunction (Koopman *et al.*, 2008; Distelmaier *et al.*, 2009; Koene *et al.*, 2011; de Haas *et al.*, 2017).

Metallothioneins (MTs) and their protective effects against the damaging consequences of reactive oxygen species (ROS) have been widely reported in both mitochondrial and neurodegenerative diseases. While Lindeque *et al.* (2010) reviewed the involvement of MTs in mitochondrial function and disease, others (Juarez-Rebollar *et al.*, 2017) have reviewed the important neuroprotective role of MT1 and 2 *in vivo* in brain disorders, such as focal and traumatic brain injury, epilepsy, ischemia and neurodegenerative diseases like Parkinson's disease and Alzheimer's disease (Carrasco *et al.*, 2000b; Giralt *et al.*, 2002; Ebadi *et al.*, 2005; Hidalgo *et al.*, 2006; Miyazaki *et al.*, 2007; Kim *et al.*, 2012; Eidizadeh *et al.*, 2015). Overexpression of MTs and exogenous treatment with MT1 and 2 in these mouse models have demonstrated improved tissue repair and neurological outcomes with decreased inflammatory responses and reduced proinflammatory cytokines, decreased ROS, neuro-regeneration and reduced apoptotic cell death (West *et al.*, 2004; Santos *et al.*, 2012). The role of MTs as free radical scavengers, their mechanisms for preventing additional ROS formation (Taylor *et al.*, 2003) and their cooperation with the glutathione redox cycle (Quesada *et al.*, 1996a; Cai *et al.*, 2006b) make these highly inducible small endogenous peptides greatly appealing as potential therapeutic effectors for patients with LS and other MD phenotypes. With limited, but promising *in vitro* data in support of this potential (Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006), the lack of *in vivo* MD models have been limiting to effectively explore this potential (Lindeque *et al.*, 2010).

To investigate the potential protective effect of MT1 on MD pathology *in vivo*, we cross-bred the *Ndufs4* whole-body knockout mouse developed by Kruse *et al.* (2008) - the first and best characterised genetic model of CI deficiency which presents very similarly to LS in humans - with the MT1 overexpressing transgenic mouse (*TgMt1*) developed by Palmiter *et al.* (1993). It is plausible that overexpression of *Mt1* in the *Ndufs4*^{-/-} mice could increase the age of survival (typically only 2-3 months of age), as well as improve phenotypic motor activity deficiencies in these mice, which typically present with weight loss, ataxia and loss of balance beginning at postnatal day (P) 35 (Kruse *et al.*, 2008). These phenotype investigations were enhanced by metabolomics analyses using brain and skeletal muscle tissue, since metabolomics allows for a comprehensive evaluation of the metabolites and their underlying interactions, that are sensitive to small changes in the redox status of the cells and theoretically should relate closely to the phenotype of the animals (Nicholson & Lindon, 2008). Along with a selected set of biochemical and histochemical analyses in the brain and quadriceps, this study provides the first comprehensive *in vivo* data to better evaluate the potential of *Mt1* overexpression as a protective adaptive response in a primary MD phenotype.

This study formed part of a larger study at the Mitochondria Research Laboratory (MRL) of the North-West University (NWU) consisting of one PhD and six MSc projects. This project focused specifically on the phenotype, biochemical consequences and metabolomics investigation of the brain and quadriceps from *Mt1* overexpressing *Ndufs4*^{-/-} mice. Projects performed by others developed the breeding strategy and methods used here and investigated the bioenergetics in fibroblasts from these mice (Mereis, 2018). They also explored the mRNA gene expression of selected one-carbon (1C) metabolism and oxidative stress metabolism genes (using the same brain and quadriceps samples from this thesis) (Mienie, 2020), and investigated ROS and inflammation in bone marrow-derived macrophages (BMDMs) from the same mice (Boshoff, 2020). Metabolomics investigations were also performed by others using urine, selected brain regions (Coetzer, 2020) and muscle types (Terburgh, 2019) in *Ndufs4* mice only. As the last piece of the larger study, this thesis reports on the culmination of these results to draw the final conclusions on whether *Mt1* overexpression does indeed exert a protective effect on neurological symptoms or motor activity *in vivo* in *Ndufs4*^{-/-} mice.

1.2 AIM, OBJECTIVES AND EXPERIMENTAL STRATEGY

The main *aim* of this study was to determine whether *Mt1* overexpression has a protective effect on the mitochondrial function of a CI deficient mouse model; as well as whether it has a significant effect on the disease phenotype progression in these mice.

The *objectives*, as demonstrated in the experimental strategy (Figure 1-2), were to:

1. Obtain *TgMt1* and *Ndufs4*^{-/-} mouse strains and to cross-breed them in order to obtain four genotypes (both mouse lines are back-crossed on the C57BL/6J line), namely, WT, OVER, KO and KO OVER, as depicted in Figure 1-1.

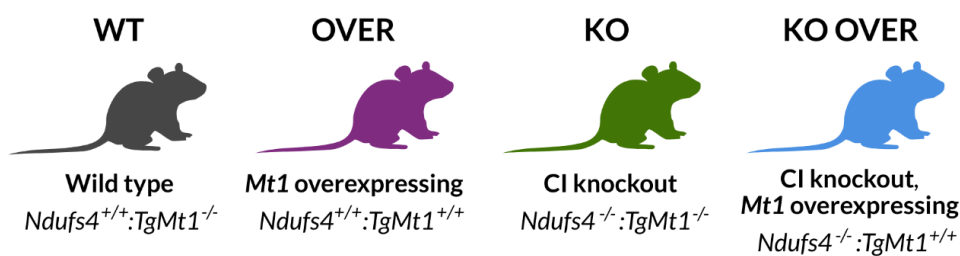


Figure 1-1: The four genotypes investigated in this study, WT, OVER, KO and KO OVER. The colours depicted here correspond to the colours used in the graphs throughout this thesis for each respective genotype.

2. Confirm the genotypes of the mice using the following methods:
 - Confirm deletion of exon 2 of *Ndufs4* (in tail snips and liver tissue) using PCR and gel electrophoresis.
 - Confirm insertion of the *Mt1* transgene (in liver tissue) using real-time quantitative PCR (qPCR).
 - Detect and quantify NADH dehydrogenase iron-sulfur protein 4 (NDUFS4) expression (in the brain and quadriceps) using SDS-PAGE and western blots.
 - Determine the expression of *Mt1* relative to β -2-microglobulin (β 2m) (in the brain and quadriceps) by quantifying mRNA using reverse transcription PCR (RT-PCR).
3. Compare the mitochondrial-related phenotype of the KO OVER mice to that of the WT, KO and OVER mice by evaluating the survival rate, growth curves, locomotor activity, motor coordination and balance of the animals at P30 and P50. The phenotypic evaluations included:
 - Construction of growth curves by weighing mice three times a week
 - Determination of the survival rate by allowing KO and KO OVER mice to die naturally without euthanasia
 - Open field testing to evaluate locomotor activity

- Wire grid hang test and rotarod to evaluate motor coordination
 - Balance beam to test balance
 - Video footage of the mice demonstrating the phenotypic changes as they aged
4. The key biochemical parameters in each of the four genotypes were investigated, using the whole brain (hereafter referred to as brain) and quadriceps (hereafter referred to as quads), in order to evaluate the effect of MT overexpression in the *Ndufs4*^{-/-} mice. The following biochemical tests were performed:
- Spectrophotometric measurement of the electron transfer system (ETS) enzyme activity of complexes I, II, III and IV in addition to citrate synthase (CS).
 - Immunohistochemistry of fixed-frozen brain samples using stains for glial fibrillary acidic protein (GFAP), ionised calcium binding adapter molecule 1 (IBA1) and MT.
 - Analysis of protein oxidation in the brain and quads
5. Metabolomics profiling was performed on the brain and quads using the following metabolic platforms:
- Nuclear magnetic resonance (NMR) spectroscopy
 - Liquid chromatography tandem mass spectrometry (LC-MS/MS)
 - Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS)

1.3 STRUCTURE OF THESIS

This thesis is presented in chapter format, as required by the NWU for the completion of the degree Philosophiae Doctor (Biochemistry). The thesis is presented in six chapters.

Chapter 1: The rationale, motivation, problem statement, aims and objectives are presented together with the experimental strategy. In addition, the structure of the thesis is laid out together with the research outputs and signed declarations from all co-authors.

Chapter 2: A detailed literature study is presented on the mitochondrion, the mitochondrial genome, the oxidative phosphorylation (OXPHOS) system and specifically CI, after which the focus is shifted towards mitochondrial disease, LS and the cellular consequences thereof. The diagnosis and treatment of CI dysfunction is then discussed together with MTs and their role in oxidative stress and metabolism. Finally, information on the mouse models used in this study is provided.

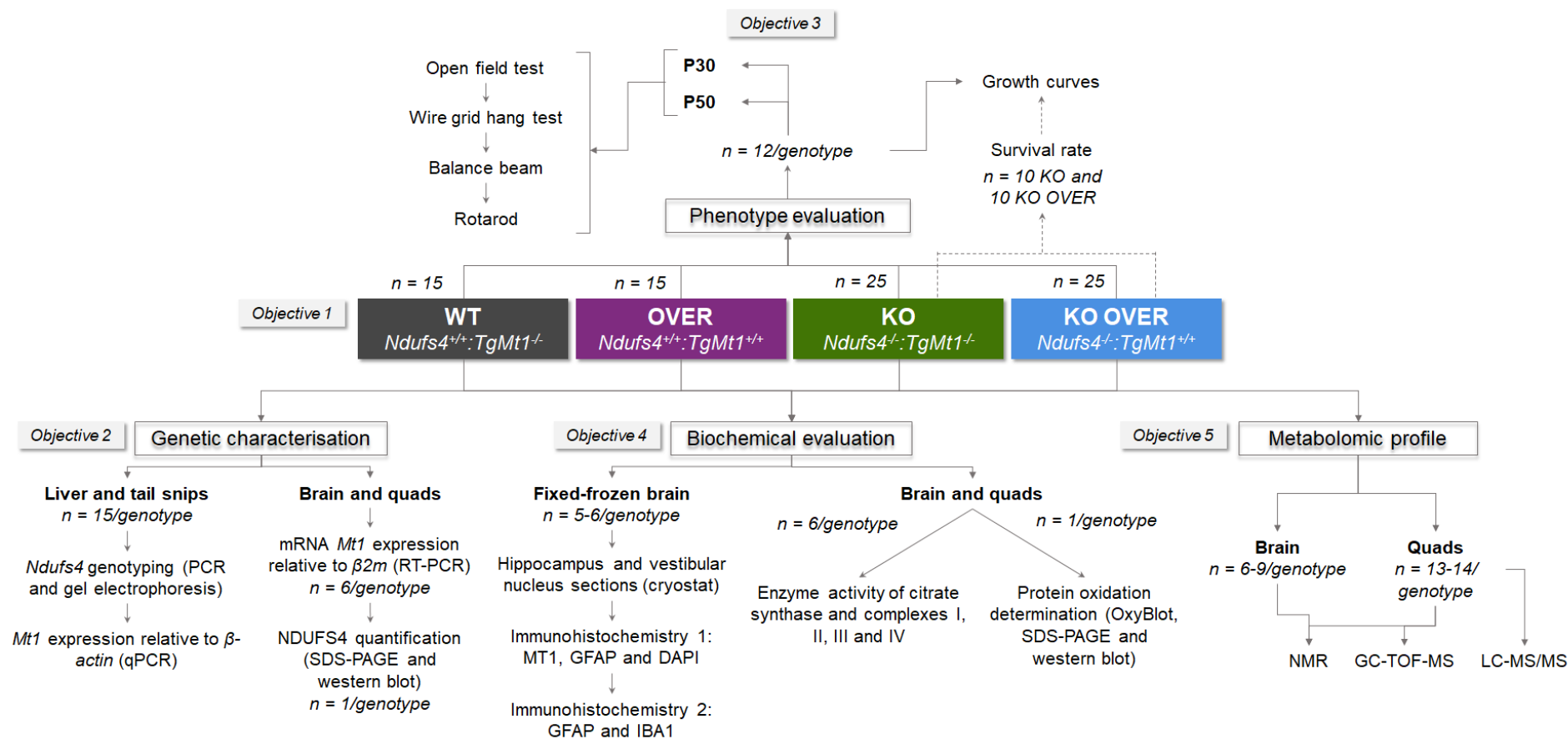


Figure 1-2: Schematic representation of the experimental strategy. The five objectives of this study are displayed together with the techniques that will be used to achieve them. The number of mice used per genotype are indicated as n. *Abbreviations:* DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA1, ionised calcium binding adapter molecule 1; GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; P, postnatal day.

Chapter 3: Details on all materials, methods and instrumentation used, to reach the objectives set out, are presented. This is followed by the data analysis procedures and statistical analyses performed.

Chapter 4: The biochemical results and discussion are presented. This includes the genetic characterisation of the mice, phenotyping results, immunohistochemistry images, ETS enzyme activities and the protein oxidation blots.

Chapter 5: The metabolomics results and discussion are presented. The quality of the data obtained from the three metabolomic platforms is first investigated, followed by identification of the discriminatory metabolites in the brain and quads. Finally, detailed descriptions are provided on the metabolic pathways that appear to be affected by these discriminatory metabolites.

Chapter 6: A summary of all the data is given here together with a description of how the results obtained, via the various approaches used in this study, link together and how this relates to the aim of the study. Final conclusions are then provided together with the strengths, limitations and future prospects of the study.

Supplementary Information:

Appendix A: All animals used in this study are listed together with their ID, date of birth, gender, age and the analyses that their tissues were used for. An example of the gel electrophoresis photo for the *Ndufs4* genotyping is also shown.

Appendix B: Supplementary phenotyping data is presented where all phenotyping results were stratified according to gender.

Appendix C: Supplementary immunohistochemistry images and protein oxidation results are shown together with the ETS enzyme activity data, which was normalised according to protein only and to CS only (as opposed to a combination of the two). Correlation analyses between the phenotyping results, RNA data and ETS enzyme activities is also displayed.

Appendix D: The multiple reaction monitoring (MRM) parameters for the LC-MS/MS analyses are listed.

Appendix E: The submitted peer-reviewed article from this study is inserted.

Appendix F: The language editing certificate is shown.

1.4 RESEARCH OUTPUTS

This study contributes to new knowledge in the fields of natural and health sciences, and specifically, MD research in the form of a submitted peer-reviewed research article and an oral presentation at an international conference.

1.4.1 Peer-reviewed articles (Appendix E)

The article, “Metallothionein 1 overexpression does not protect against mitochondrial disease pathology in *Ndufs4* knockout mice”, was submitted to the journal of *Molecular Neurobiology*, with a five-year impact factor of 4.643, and is currently under review. The authors listed are: Hayley Christy Miller, Roan Louw, Michelle Mereis, Gerda Venter, John-Drew Boshoff, Liesel Mienie, Mari van Reenen, Marianne Venter, Jeremie Zander Lindeque, Adán Domínguez-Martínez, Albert Quintana, Francois Hendrikus van der Westhuizen. The submitted article can be found in Appendix E.

Other publications from the author that resulted indirectly from techniques and expertise developed during the study period are:

- AUCAMP, J., BRONKHORST, A.J., PETERS, D.L., VAN DYK, H.C., VAN DER WESTHUIZEN, F.H. & PRETORIUS, P.J. 2017. Kinetic analysis, size profiling and bioenergetic association of DNA released by selected cell lines in vitro. *Cellular and Molecular Life Sciences*, pp. 1-19.
- AUCAMP, J., VAN DYK, H.C., BRONKHORST, A.J. & PRETORIUS, P.J. 2017. Valproic acid alters the content and function of the cell-free DNA released by hepatocellular carcinoma (HepG2) cells in vitro. *Biochimie*, 140:93-105.
- ENOGIERU, A.B., HAYLETT, W.L., MILLER, H.C., VAN DER WESTHUIZEN, F.H., HISS, D.C. & EKPO, O.E. 2019. Attenuation of Endoplasmic Reticulum Stress, Impaired Calcium Homeostasis, and Altered Bioenergetic Functions in MPP⁺-Exposed SH-SY5Y Cells Pretreated with Rutin. *Neurotoxicity Research*, 36:764-776.
- WENTZEL, J., LEWIES, A., MILLER, H.C. & DU PLESSIS, L. 2018. The antimicrobial peptide nisin Z induces selective toxicity and apoptotic cell death in cultured melanoma cells. *Biochimie*, 144:28-40.

1.4.2 Oral presentation

MILLER, H.C., VAN DER WESTHUIZEN, F.H., LOUW, R. & QUINTANA, A. 2018, 8-11 July. Protection of mitochondrial disease pathology by metallothionein overexpression: a phenotype study. Oral presentation at the South African Society for Biochemistry and Molecular Biology (SASBMB) - Federation of African Societies of Biochemistry and Molecular Biology (FASBMB) 2018 Conference, North-West University, Potchefstroom, South Africa.

1.5 AUTHOR CONTRIBUTIONS

Genetic characterisation in Chapter 3: M. Mereis developed and optimised the methods used to genetically characterise the mice, including, the *Ndufs4* genotyping, *Mt1* relative copy number determination, NDUFS4 quantification and RNA methods.

Metabolomics in Chapter 3: K. Terburgh and J. Coetzer developed and optimised the methods used to perform the NMR, LC-MS/MS and GC-TOF-MS analyses in the brain and quads.

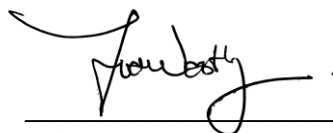
Peer-reviewed paper in Appendix E: All authors reviewed, edited and approved the final manuscript version for submission and all authors were involved with the design of the methodology. F.H. van Der Westhuizen, R. Louw and A. Quintana were involved with study design and supervision. H.C. Miller, F.H. van Der Westhuizen and R. Louw were responsible for the original manuscript draft. A. Quintana provided the resources for the immunohistochemistry work and was responsible for the imaging of the slides together with A. Domínguez-Martínez. G. Venter and J.D. Boshoff were responsible for the macrophage and inflammation analyses performed. L. Mienie and M. Venter were responsible for the RNA analyses of selected genes. M. Mereis developed the breeding strategy used in this study and optimised and developed the methods used for genetic characterisation of the mouse model. J.Z. Lindeque assessed the quality of the GC-TOF-MS data and determined the ID level of discriminatory metabolites. M. van Reenen supervised and assisted with all statistical analyses. H.C. Miller was responsible for study design, visualization of data, genetic characterisation of the model, phenotyping analyses, immunohistochemistry analyses, protein oxidation analyses and metabolomics.

All authors involved signed the declaration on this page:

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



H.C. Miller



F.H. van der Westhuizen



R. Louw



G. Venter



M. Pretorius



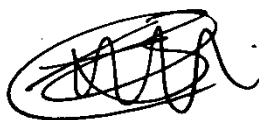
M. van Reenen



M. Mereis



L. Mienie



J.D. Boshoff



A. Quintana



J.Z. Lindeque



K. Terburgh



J. Coetzer

CHAPTER 2: LITERATURE STUDY

2.1 INTRODUCTION

In this literature study, a brief overview will be given of the mitochondrion, OXPHOS system and MD, followed by a more in depth look at CI and CI-linked MDs. The cellular consequences of CI deficiency will be discussed together with the current treatment strategies employed. Thereafter, the focus will be shifted to MTs and their potential as therapeutic agents in MDs. As this is a mouse model study, the specific mouse models used, will also be discussed.

2.2 MITOCHONDRIA AND THE OXPHOS SYSTEM

2.2.1 *Structure and function of the mitochondrion*

Mitochondria are intracellular organelles that can be found in all eukaryotic cells (Scheffler, 2007) where each cell contains on average 2000 mitochondria (von Knebel Doeberitz & Wentzensen, 2008). They consist of an outer membrane, an inner membrane (which is folded to form cristae), the intermembrane space (located between the two membranes) and the mitochondrial matrix (encapsulated by the inner membrane). These membranes contain numerous transport systems, which selectively regulate the transport of metabolites across these barriers. Embedded in the inner mitochondrial membrane are five enzymatic complexes (I–V) and two electron carriers, collectively known as the OXPHOS system, which allow the transfer of electrons to produce ATP (DiMauro & Schon, 2003). Within the mitochondrial matrix, enzymes can be found which form part of the Krebs cycle, a process that makes use of intermediates produced by the metabolism of carbohydrates (via glycolysis), fatty acids and amino acids, and utilises them for pyruvate oxidation and to maintain energy balance. Besides their fundamentally important role in energy metabolism and homeostasis, mitochondria are also responsible for cellular signalling involving calcium ions, ROS, apoptosis, cellular stress responses and maintaining the redox status (NADH/NAD⁺ ratio) (Koopman *et al.*, 2013).

2.2.2 *The OXPHOS system*

The OXPHOS system is displayed in Figure 2-1 and is discussed in more detail below, together with the ETS, alternative electron sources and tissue-specific differences in the ETS.

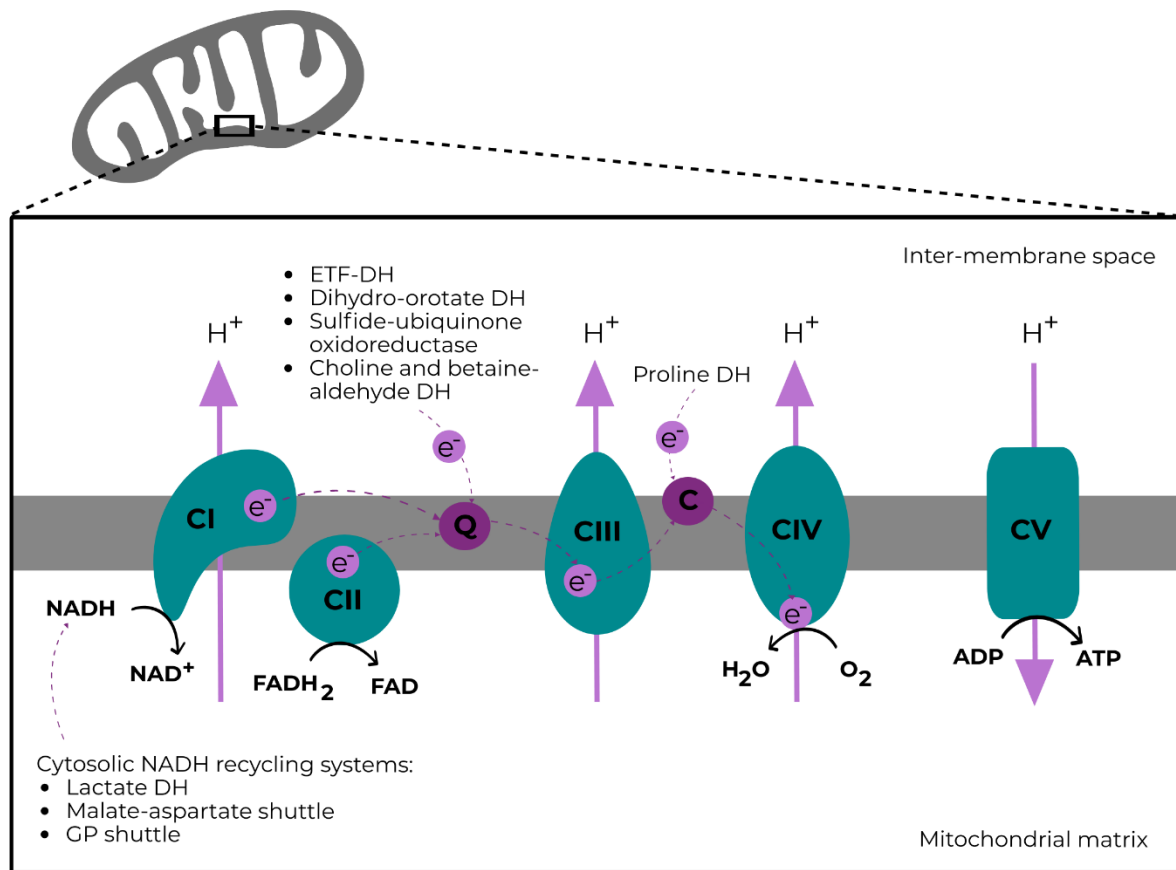


Figure 2-1: The OXPHOS system and alternative electron sources. A magnification of a section of the mitochondrial inner membrane. Electrons (indicated in light purple circles) are transported through the ETS with the concurrent transfer of hydrogen ions to the intermembrane space. The resulting proton motive force drives the conversion of ADP to ATP. *Abbreviations: ETF, electron-transferring flavoprotein; C, cytochrome c; DH, dehydrogenase; GP, glycerophosphate; Q, ubiquinone.*

2.2.2.1 Electron transfer system

The ETS is comprised of four protein complexes, namely, 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) and CIV (cytochrome c oxidase; EC 1.9.3.1), and two electron carriers, namely, ubiquinone (UQ, also known as coenzyme Q) and cytochrome c (cyt c). A distinction can be made between the OXPHOS system and the ETS due to the inclusion of a fifth protein complex in the OXPHOS system, namely, CV (F₁F₀-ATP synthase; EC 3.6.1.34). CIV and V are able to form dimers, and CI, CIII and CIV have been shown to interact with each other in different ways to form supercomplexes in the inner mitochondrial membrane of the mitochondria (Garone *et al.*, 2018).

Glucose, fatty acids and amino acids are used as metabolic fuels via glycolysis, pyruvate oxidation, β -oxidation, glutaminolysis, branched-chain amino acid (BCAA) catabolism and the Krebs cycle to produce NADH (reduced nicotinamide adenine dinucleotide) and FADH₂ (reduced

flavin adenine dinucleotide). As NADH and FADH₂ are oxidised to NAD⁺ (oxidised nicotinamide adenine dinucleotide) and FAD (oxidised flavin adenine dinucleotide) by NADH dehydrogenase (CI) and succinate dehydrogenase (CII) respectively, and electrons are transferred to UQ reducing it to ubiquinol. From ubiquinol, electrons are transferred to CIII followed by reduction of cyt c. Finally, electrons are transferred from cyt c to CIV and ultimately to molecular oxygen (with subsequent release of water) (Hatefi, 1985; Smeitink *et al.*, 2001; Koopman *et al.*, 2013).

2.2.2.2 Alternative electron sources

NADH dehydrogenase and succinate dehydrogenase, discussed above, form part of the classical method of entry to the ETS, but alternative sources can also transfer electrons, either directly or indirectly, to UQ. Electrons can enter the ETS via the following ways: dehydrogenases, oxidoreductases, quinones or mobile cytochrome carriers. Electron-transferring flavoprotein (ETF) dehydrogenase, dihydro-orotate dehydrogenase and sulfide-ubiquinone oxidoreductase transfer electrons to UQ by accepting electrons from the oxidation of fatty acids, converting dihydro-orotate to orotate (during pyrimidine synthesis) and oxidising amino acid derived sulphides, respectively. The choline dehydrogenase and betaine-aldehyde dehydrogenase system convert choline to betaine with production of NADH, in addition to dimethylglycine (DMG) dehydrogenase which oxidises DMG to sarcosine with concurrent transfer of electrons to UQ (as discussed in more detail in Sections 5.4.6 and 5.5.3.1) (McDonald *et al.*, 2018). Proline dehydrogenase (a flavoenzyme) uses proline as a substrate to generate NADH and succinate in downstream reactions, but it is also able to donate electrons directly to UQ independent of CI and II (Hancock *et al.*, 2016), as further discussed in Section 5.4.2.2.

Cytosolic NADH recycling systems also exist for converting NADH to NAD⁺ and these include lactate dehydrogenase (discussed in Section 5.4.1.1), the malate-aspartate shuttle (discussed in Section 5.4.1) and the glycerophosphate (GP) shuttle. The GP shuttle consists of two enzymes, namely, NAD⁺-linked cytosolic glycerol-3-phosphate dehydrogenase (cG3PDH) and the inner membrane bound FAD-linked mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH). Dihydroxyacetone phosphate (generated by glycolysis) is converted to glycerol-3-phosphate (G3P) by cG3PDH with oxidation of NADH to NAD⁺. G3P is then transported to mG3PDH where it is oxidised back to dihydroxyacetone phosphate, with concurrent reduction of FAD to FADH₂ and transfer of these electrons to UQ (McDonald *et al.*, 2018).

2.2.2.3 Oxidative phosphorylation

The chemiosmotic theory, initially described by Mitchell (1961), explains the proton pumping across the inner mitochondrial membrane. As electrons are transferred through the ETS,

hydrogen ions are pumped from the mitochondrial matrix into the intermembrane space by CI, CIII and CIV, resulting in an electrochemical gradient and mitochondrial membrane potential (collectively known as the proton motive force) across the inner mitochondrial membrane. This proton motive force is then used by CV to pump protons back into the mitochondrial matrix, with subsequent phosphorylation of ADP to ATP (i.e. OXPHOS). Proton leak back into the mitochondrial matrix also occurs without ATP production. The respiratory chain thus allows energy to be produced by oxidising various substrates and utilising them to produce a proton circuit that drives the OXPHOS system (Brand & Nicholls, 2011).

2.2.2.4 Tissue-specific differences

A study on the ETS of various rat tissues performed by Benard *et al.* (2006) demonstrated tissue-specific differences, specifically in brain and muscle, which are of particular importance in this thesis. They showed that the number of cristae within a given mitochondrial surface area; the maximum CIII, CIV and CS activity; and the quantity of CI units were all greater in muscle than in brain tissue. The density of the mitochondrial matrix was increased in the brain compared to muscle. Another finding of theirs was that the ratio of CII/UQ/CIII/cyt c/CIV differed between brain (1:58:3:35:8) and muscle (1:58:3:11:7) where muscle had a lower ratio of cyt c and CIV content than brain tissue. The OXPHOS capacity between different tissues thus varies significantly due to differences in the quantity, activity and stoichiometry of its components as well as the proportion of the proton motive force that is used by systems other than CV (Koopman *et al.*, 2010).

2.3 COMPLEX I

2.3.1 Structure, function and assembly

Mammalian CI consists of 44 subunits, 14 of which are fundamental subunits essential for catalytic function and the remaining 30 subunits, which are nDNA-encoded accessory subunits, the function of which is unknown for many of them. Of the 14 core subunits, seven are hydrophobic mtDNA-encoded subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) and seven are hydrophilic nDNA-encoded subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8), where ND stands for NADH dehydrogenase and NDUF stands for NADH dehydrogenase ubiquinone flavoprotein (Brandt, 2006; Balsa *et al.*, 2012; Fiedorczuk *et al.*, 2016). Fully assembled CI has a mass of approximately 969 kDa and is the largest of the ETS enzyme complexes, contributing 40% of the energy produced by proton translocation in the ETS (Koopman *et al.*, 2010).

Studies across multiple organisms have shown that CI has an L-shaped conformation consisting of two arms of equal size, with one arm embedded in the mitochondrial inner membrane and the

other arm projecting out into the mitochondrial matrix. It is made up of three functional modules, namely, N, Q and P, which are involved in NADH oxidation, UQ reduction and proton pumping respectively. The N and Q modules form part of the matrix arm whilst the P module forms part of the membrane bound arm of CI. The N module contains a flavin mononucleotide (FMN) which accepts electrons from NADH to form FMNH₂. These electrons are then transferred to the Q module by utilising the eight iron-sulfur (FeS) clusters contained in the seven hydrophilic subunits mentioned above. The P module is then lastly used as the Fe-S clusters are oxidised to donate their electrons to UQ and produce ubiquinol (QH₂), which can freely donate its electrons to CIII. The mechanism linking the electron transfer through CI to the translocation of protons by the P module is not yet fully elucidated but the stoichiometry appears to indicate four protons to two electrons (4H⁺/2e⁻). CI redox reactions may induce conformational changes between an active and inactive state (associated with formational changes of the ND3 subunit) in CI to regulate its activity (Brandt, 2006; Koopman *et al.*, 2010; Hirst, 2013; Koopman *et al.*, 2016).

The assembly of CI is a multifaceted process where specific subunits of each of the N, Q and P modules are first preassembled, and with the help of assembly chaperones and other accessory subunits, these preassembled modules are then combined to form the complete holo-enzyme. As mentioned in Section 2.2.2.1, CI has also been shown to form supercomplexes within the inner mitochondrial membrane (rather than exist on its own) with complexes III and IV to stabilise the assembly of the protein complexes, minimise the distance travelled by substrates, enhance electron transfer and reduce ROS production (Schagger & Pfeiffer, 2000; Vogel *et al.*, 2007; Acin-Perez *et al.*, 2008).

2.3.2 NDUFS4

The non-enzymatic and nDNA-encoded NDUFS4 accessory subunit plays a key role in this study. This 18 kDa protein has been found to be essential for the proper assembly and functioning of CI and appears to be inserted during a late phase of the assembly process (Papa *et al.*, 1996; Vogel *et al.*, 2007). It has been demonstrated that cAMP-dependent phosphorylation of the C-terminal of the NDUFS4 protein, using protein kinase A, plays a role in the mitochondrial import and development of mature NDUFS4, stimulating the catalytic activity of CI and biosynthesis of both mitochondrial and nuclear encoded subunits of CI as well as other respiratory proteins (De Rasmio *et al.*, 2008; Papa *et al.*, 2010). Absence of NDUFS4 prevents proper assembly of CI and allows the formation of a subcomplex of 830 kDa, while maintaining low CI activity (Scacco *et al.*, 2003). Calvaruso *et al.* (2012) demonstrated that loss of NDUFS4 impacts the N module causing loss of the NADH dehydrogenase electron-accepting portion of the enzyme and that this affects CI activity in all tissues, but to varying degrees. The residual CI activity found in these tissues was attributed to stabilization of the CI subcomplex by formation of the supercomplex with CIII. It is

also worth mentioning that *Ndufs4* mutations have been shown to not only affect complex I, but to also decrease complex III activity, which further supports the role of NDUFS4 in stabilization of the CI/CIII supercomplex (Budde *et al.*, 2000; Breuer *et al.*, 2013).

2.4 MITOCHONDRIAL GENOME

Mitochondria contain their own DNA, namely, mitochondrial DNA (mtDNA), which can be found within the mitochondrial matrix and is solely maternally inherited (Giles *et al.*, 1980). MtDNA is a double-stranded circular DNA molecule, which lacks introns and consists of ~16.6 kilobase pairs in humans and ~16.3 kilobase pairs in mice. MtDNA contains 37 genes which encode 13 polypeptides for OXPHOS (the remaining 79 genes required are encoded by nDNA), two ribosomal RNA (rRNA) molecules and 22 transfer RNA (tRNA) molecules and transcription is promoted by a non-coding section called the displacement-loop (Anderson *et al.*, 1981; Bibb *et al.*, 1981; Chinnery & Hudson, 2013). Contrary to nDNA, multiple copies (ranging from hundreds to thousands) of mtDNA exist within each cell, and are typically arranged into protein-DNA macrocomplexes (called nucleoids) that consist of five to seven mtDNA copies and are associated with the inner mitochondrial membrane (Wang & Bogenhagen, 2006).

MtDNA has a mutation rate approximately ten times higher than in nDNA, assumingly due to its lack of protective histones, lack of DNA repair mechanisms and impaired DNA replication and repair caused by oxidative damage from ROS (due to the close proximity of mtDNA to the ETS – a source of ROS) (Brown *et al.*, 1979; Richter *et al.*, 1988). These mtDNA variants may exist as point mutations in either protein-coding, tRNA or rRNA genes, or as mtDNA rearrangements which involves insertions and deletions. When all of the copies of mtDNA within a cell are of identical sequence, it is known as *homoplasmy*, but a state of *heteroplasmy* can also occur when there are different frequencies of variations in mtDNA copies causing both normal and variant mtDNA to exist together in one cell. Patients with mitochondrial disease can be homoplasmic for an mtDNA mutation, but more often, they are heteroplasmic and thus have a combination of mutant and wild-type mtDNA. The *threshold effect* is known to occur in these heteroplasmic patients where there appears to be a threshold of mutated mtDNA required before the biochemical defect becomes evident. This threshold is typically believed to be ~60-90% of mutated mtDNA, but differs between various tissues and individuals and the heteroplasmy levels of an individual are also known to fluctuate over time (Johns, 1995; Rossignol *et al.*, 2003; Tuppen *et al.*, 2010). It is believed that as many as ~1 in 200 healthy persons have pathogenic mutations at very low levels of heteroplasmy (Elliott *et al.*, 2008; Ye *et al.*, 2014).

2.5 MITOCHONDRIAL DISEASE AND COMPLEX I

2.5.1 Introduction to mitochondrial disease

MDs are a diverse group of disorders that are caused by dysfunction of the OXPHOS system and result in multisystemic or single organ clinical symptoms. They are classified as primary MDs when caused by a pathogenic mutation that encodes for the OXPHOS subunits, assembly factors or pathways influencing OXPHOS biogenesis. Secondary MDs, however, refer to MDs caused by genetic defects that are unrelated to OXPHOS function or biogenesis, as well as to MDs caused by environmental factors or other hereditary non-MDs. Examples of secondary MDs include common non-communicable diseases such as hypertension, diabetes, AIDS and other neurodegenerative diseases (e.g. Alzheimer's disease and Parkinson's disease) where compounding evidence of mitochondrial dysfunction has been reported (Howell *et al.*, 2005; Chinnery & Hudson, 2013; Gorman *et al.*, 2016; Koopman *et al.*, 2016; Craven *et al.*, 2017).

The prevalence of MDs in adults is estimated at 1 in 4300 and there are currently very few effective treatments and no known cures (Gorman *et al.*, 2015). MDs typically affect organs with high energy demands such as the brain, skeletal muscle and heart. Due to the complexity of mitochondrial genetics, in addition to the interplay between mitochondrial and nuclear mutations, there are many challenges to understanding the mechanisms and pathophysiology of MDs. The multiple possible inheritance patterns also add complexity where nDNA mutations are typically inherited in an autosomal recessive manner, but can also be autosomal dominant, X-linked or *de novo*, in contrast to mtDNA mutations which can be maternally inherited or sporadic and the mutation presents as homoplasmic or heteroplasmic (Wallace, 1999; Craven *et al.*, 2017; Suomalainen & Battersby, 2018). However, many advancements in the identification of MD-causing variants have been made in recent years, such as the defects of 281 genes in MD patients (including 36 genes from the mitochondrial genome) which were reported by Mayr *et al.* (2015) to affect OXPHOS enzymes; assembly; DNA, RNA and protein synthesis; substrates; co-factors; and homeostasis.

Due to the large genetic heterogeneity in mitochondrial diseases, there is a lack of correlation between genotype and phenotype as the clinical symptoms are very broad, affecting single or multiple organs, differing in severity and have varied age of onset. Due to their high metabolic requirements, muscle and brain are frequently affected, either exclusively, or, forming part of a multisystemic clinical presentation. In the case of muscle, myopathy and chronic progressive external ophthalmoplegia may occur, with the histopathological features of ragged-red fibres (abnormal accumulation of mitochondria in the sarcolemma of muscle fibres) and cyt c oxidase negative staining. Common neurological symptoms include ataxia, neuropathy, seizures,

sensorineural deafness and dementia and are characterised by atrophy, cortical lesions and neuronal cell loss (Smeitink *et al.*, 2001; Koopman *et al.*, 2016; Alston *et al.*, 2017).

2.5.2 Isolated complex I deficiency and Leigh Syndrome

Isolated CI deficiency (OMIM 252010) is reported as the most common of the MDs – accounting for as many as one-third of MD cases (Kirby *et al.*, 1999; Loeffen *et al.*, 2000; Distelmaier *et al.*, 2009; Ghezzi & Zeviani, 2018). Genetic alterations have been reported for all seven mtDNA-encoded subunits and in 19 of the 37 nDNA-encoded subunits of CI as well as in 10 of 14 CI assembly factors. Most cases (70 - 80%) are caused by nDNA mutations, and isolated CI deficiency caused by nDNA mutations appear to present with much more consistent clinical symptoms than mtDNA mutations (Swalwell *et al.*, 2011; Alston *et al.*, 2017). According to a retrospective study by Koene *et al.* (2012), children (typically less than one year old) most commonly presented with hypotonia (60% of patients) together with the following symptoms (where no one symptom appeared in more than 35% of patients): respiratory abnormalities, failure to thrive, nystagmus (abnormal eye movement), eating problems, psychomotor deterioration, dystonia (abnormal tonicity of the muscle characterised by lengthy, repetitive unintentional muscle contractions), ataxia (lack of balance and coordination with unsteadiness of gait) and spasticity. Increased lactic acid was seen in 90% of patients and heart, liver and kidney involvement were only seen in 24% of cases. The most indicative signs of nDNA encoded CI deficiency were brainstem involvement, optic atrophy and LS. Other less common clinical presentations of CI deficiency include neonatal cardiomyopathy, fatal infantile lactic acidosis, leukodystrophy and other indeterminate encephalomyopathies. The prognosis for CI deficiency is very poor with 50% of children dying before the age of two and 75% patient deaths before the age of ten years (Loeffen *et al.*, 2000; Koene *et al.*, 2012; Koopman *et al.*, 2013; Rodenburg, 2016).

Almost all children with isolated CI deficiency develop LS (OMIM 256000), an early-onset neurodegenerative disorder characterised by motor and cognitive impairments in conjunction with lactic acidemia or involuntary and uncontrollable movements (including the symptoms described above for isolated CI deficiency), with death typically occurring by three years of age. MRI scans of the brain of LS patients reveal bilateral, symmetrical focal hyperintensities in the basal ganglia, brainstem, thalamus, diencephalon, cerebellum and spinal cord. Pregnancies of children born with LS are typically normal with no prenatal indications of the disease (Loeffen *et al.*, 2000; Koene *et al.*, 2012; Lake *et al.*, 2016; Rodenburg, 2016). The clinical symptoms all mainly appear to be related to the nervous system and skeletal muscle – tissues with high energy demands – but there is also multisystemic involvement due to the nature of the defect and the bioenergetic thresholds of different tissues in individuals with different genetic backgrounds (Distelmaier *et al.*, 2009). LS is not exclusive to CI deficiency (despite occurring in ~80% of children with CI

deficiency) and has also been reported in defects of complexes II, III, IV and V as well as UQ biosynthesis faults, pyruvate dehydrogenase deficits, and defects in proteins that result in secondary MDs. Astoundingly, pathogenic mutations in more than 75 genes (in both nDNA and mtDNA) have been associated with LS (Lake *et al.*, 2016; Rodenburg, 2016). Mutations in *Ndufs4* (OMIM 602694) are reported as one of the most common autosomal recessive causes of LS and with 13 mutations in *Ndufs4* currently associated with CI deficiency, it is considered a mutational 'hotspot'. These mutations are all considered loss of function alleles and include frameshift, nonsense and splicing mutations as well as a multi-exon deletion (Budde *et al.*, 2000; Lamont *et al.*, 2017).

Mutations in the nDNA-encoded CI subunits are typically inherited in an autosomal recessive manner (both parents being heterozygous carriers of the mutation), but X-linked inheritance has also been reported. However, in a study by Thorburn *et al.* (2004), for ~50% of patients with CI deficiency, no genetic defect was discovered, and the cause of the disease was thus unknown. In the case of mtDNA mutations, there is a wider range of symptoms and affected organs, and onset is much more variable, presenting any time from early childhood to late adolescence, presumably due to the large role played by the levels of heteroplasmy (Loeffen *et al.*, 2000; Thorburn *et al.*, 2004; Distelmaier *et al.*, 2009).

2.5.3 Cellular consequences of complex I deficiency

Besides the decreased energy production (and thus altered ATP/ADP ratio) due to CI deficiency, numerous other primary and secondary cellular functions are also affected. The main biochemical consequences include an altered redox status, disturbance of the mitochondrial membrane potential, increased ROS and altered intracellular Ca^{2+} homeostasis (Distelmaier *et al.*, 2009; Reinecke *et al.*, 2009; Valsecchi *et al.*, 2010; Rodenburg, 2016).

2.5.3.1 Reactive oxygen species

ROS are chemically reactive species that contain oxygen and include superoxide ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). In the mitochondrion, superoxide can be produced when there is ineffective transfer of electrons between the subunits of the ETS as well as when the electron carriers (UQ and cyt c) are not effectively transferred through the inner mitochondrial membrane. The resulting build-up of electrons are increasingly leaked to oxygen and result in the formation of ROS. This is attenuated when there is an upregulated supply of reducing equivalents to the ETS. Mitochondrial sources of ROS include CI, CII, CIII, α -ketoglutarate (KG) dehydrogenase, G3PDH, ETF UQ oxidoreductase, isoform p66^{Shc} and the pyruvate/proton transporter. Outside of the mitochondria, there are also numerous other sources of ROS within the endoplasmic

reticulum, peroxisomes, cytosol, plasma membrane and extracellular space (Reinecke *et al.*, 2009; Koopman *et al.*, 2013).

CI is believed to be one of the main sources of mitochondrial ROS and as such, ROS is believed to play a central role in CI deficiency. Increased ROS production has been reported in numerous studies of OXPHOS deficiencies and CI dysfunction, however, there are also several reports where no increase in superoxide production was detected or where no correlation could be drawn between increased superoxides and oxidative damage or altered metabolic homeostasis (Hirst *et al.*, 2008; Reinecke *et al.*, 2009). An example of this can be seen in studies by Iuso *et al.* (2006) and Piccoli *et al.* (2006) where no ROS production was found in *Ndufs4* mutant fibroblasts, contrary to Verkaart *et al.* (2007) who reported increased ROS (which inversely correlated with CI activity) in 19 out of 21 patient fibroblasts with inherited isolated CI deficiency. There thus appear to be several factors affecting ROS production in OXPHOS disorders, including the severity of the disease, the source of the defect, and the mechanisms protecting the cells against the harmful effects of ROS that may differ between individuals (Reinecke *et al.*, 2009).

Under normal physiological conditions, mitochondrial and cytosolic ROS levels are regulated by antioxidant systems and play an important role in redox signalling. These ROS clearing mechanisms include enzymatic systems, such as manganese superoxide dismutase (MnSOD)/SOD2, glutathione peroxidase, glutathione reductase and thioredoxin reductase, as well as non-enzymatic systems (e.g. glutathione, vitamin C and E, carotenoids and flavonoids) and this steady state of redox balance is referred to as *oxidative stress* [as defined by Cutler *et al.* (2005)]. ROS signalling pathways have been linked to cell proliferation, apoptosis, metabolism and differentiation, anti-inflammatory and antioxidant responses, iron homeostasis and DNA-damage responses. Another positive influence of ROS is their ability to assist in the regulation of mitophagy by discarding of dysfunctional mitochondria (Koopman *et al.*, 2013). Westermann (2012) proposed a model where mitochondria can adopt three morphological states, namely, fragmented, normal and hyperfused and that these states were closely linked with low, normal and high OXPHOS activity, respectively. Distelmaier *et al.* (2009) then showed *in vitro* that the transition between these three states may be regulated in a ROS-dependent manner and that ROS thus also play a role in regulating mitochondrial morphology and function.

Increased oxidative stress occurs when there is excessive or uncontrolled ROS production and this leads to oxidative damage of DNA (nuclear and mitochondrial), proteins and lipids (Reinecke *et al.*, 2009; Koopman *et al.*, 2013). As mentioned above, the close proximity of mtDNA to the ETS allows ROS to have a genotoxic effect. This uncontrolled ROS production together with increased mtDNA mutations and the subsequent decrease in OXPHOS function can result in a vicious cycle where the pathology of mitochondrial disorders are attenuated (Indo *et al.*, 2007).

2.5.3.2 Mitochondrial membrane potential

Due to the protein damage that may be caused by increased oxidative stress, the mitochondrial membrane integrity may be affected leading to impaired maintenance of the mitochondrial membrane potential. However, there is conflicting evidence in support of this. In nine out of 15 isolated CI deficiency fibroblast patient cell lines, Distelmaier *et al.* (2009) found a significantly depolarised membrane potential compared to controls, contrary to Koopman *et al.* (2010) who did not see a significant effect on the membrane potential of rotenone-induced CI deficient fibroblasts. It was however agreed that very small changes in membrane potential can cause decreased ATP production.

2.5.3.3 Calcium homeostasis and ATP synthesis

Perturbation of the ATP/ADP ratio due to the decreased flux through the ETS, the effects of ROS, disturbances in the mitochondrial membrane potential (as discussed above) and altered redox status, also lead to secondary cellular consequences in calcium (Ca^{2+}) homeostasis. Ca^{2+} acts as a key signalling messenger in cells, in addition to operating as a secondary messenger to enhance ATP synthesis. Many ATPases on the membranes of intracellular Ca^{2+} stores are dependent on mitochondrial ATP supply (Distelmaier *et al.*, 2009; Reinecke *et al.*, 2009; Koopman *et al.*, 2010). Willems *et al.* (2008) proposed that decreased CI activity results in depolarization of the mitochondrial membrane potential, which in turn leads to a decreased ATP supply. This results in decreased fuelling of the Ca^{2+} intracellular store ATPases, which disturbs Ca^{2+} homeostasis and reduces the Ca^{2+} content of these stores. Since mitochondrial Ca^{2+} uptake is an essential trigger for ATP production via OXPHOS during cell activation, this in turn further impacts cellular energy supply in CI deficiency.

2.5.3.4 Altered redox status and metabolism

One of the key consequences of the electron transfer perturbation in CI deficiency, is the disturbance of the redox balance of nicotinamide nucleotides and flavin coenzymes which have major regulatory effects in numerous other metabolic reactions that rely on the ETS. This affects electron flow through the ETS, electron leakage and uncoupling. This inhibited flow of electrons during OXPHOS dysfunction may lead to disruptions in ATP production, however, numerous compensatory mechanisms are present within the cell and are capable of supplying the cell with ATP produced elsewhere and thus preventing fatal OXPHOS failure. Typically, a larger problem is the redox and metabolite imbalance that occurs due to over-oxidation of electron carriers downstream of OXPHOS inhibition, and over-reduction upstream. OXPHOS dysfunction may also

lead to cyt c release into the cytosol thus triggering apoptosis of cells that were not yet meant to die or failing to trigger cell death in those cells that should.

Commonly known systemic consequences of OXPHOS deficiencies include elevated organic acids, especially lactate and Krebs cycle intermediates. The elevated lactate is believed to be caused by a shift in the equilibrium of the pyruvate-lactate reaction where pyruvate is converted to lactate by lactate dehydrogenase with the concurrent conversion of NADH to NAD⁺. This shift occurs when there is a build-up of pyruvate, due to an OXPHOS or Krebs cycle blockage, and is used to recycle NAD⁺ so that glycolysis can proceed in an attempt to restore ATP levels. Similarly, alanine levels are also consistently reported as elevated in patients with OXPHOS disorders since the elevated pyruvate disturbs the equilibrium of the reversible alanine aminotransferase reaction and leads to conversion of pyruvate to alanine. Proline has also been found to be increased in cases of OXPHOS dysfunction where elevated lactate inhibits proline oxidation. Furthermore, elevated NADH also acts as an inhibitor for three Krebs cycle enzymes, namely, CS, isocitrate synthase and KG dehydrogenase, which leads to the accumulation of Krebs cycle intermediates. BCAA metabolism is also impacted by the increased NADH levels as it leads to inhibition of the branched-chain α -ketoacid dehydrogenase complex in the leucine, isoleucine and valine catabolic pathways, which in turn leads to elevated 2-ketoisocaproic acid, 2-keto-3-methylvaleric acid, 2-ketoisovaleric acid and 2-hydroxycaproic acid. Abnormal citrulline levels and altered metabolites in the phenylalanine and tyrosine catabolic pathways have also been reported in addition to increased citric acid and isoleucine, while discrepancies have been observed in literature for many other metabolites. Other pathways also impacted by altered redox state include the 1C metabolism, folate metabolism and transsulfuration pathways, which are also essential for pyrimidine and purine synthesis. NADPH biosynthesis, serine and glycine all form integral parts of these pathways, and as such, elevated glycine and serine levels are typically reported in mitochondrial disease (Smeitink *et al.*, 2006; Esterhuizen *et al.*, 2017).

Zielinski *et al.* (2016) made use of a computer model to hypothetically predict the metabolic pathways activated in different MDs. When simulating CI deficiency, they found that metabolism of serine, ketone bodies and leucine became inactive, whilst glucose, lactate, fatty acids and other BCAAs remained unaffected. The principal mechanisms appearing to compensate for the decreased NADH oxidation, by reducing the quinone pool, was the GP shuttle and the cycle between proline dehydrogenase and pyrroline-5-carboxylate reductase, which both utilised NADH to produce NAD⁺. When blocking the GP shuttle, increased lactate was seen, suggesting that the shuttle alleviated lactic acidosis to a certain degree. Beta-oxidation of fatty acids then increased, producing NADH and reducing the quinone pool via ETF as well as increasing levels of acetyl-CoA and thus compensating for its loss in the Krebs cycle. Upon inhibition of the proline cycle,

trans-2-enoyl- CoA reductase and acyl-CoA dehydrogenase (β -oxidation enzymes) were used to reduce the quinone pool and oxidise NADPH (obtained from nicotinamide nucleotide transhydrogenase (NNT), glutamate dehydrogenase and isocitrate dehydrogenase 2). When additionally inhibiting NNT, steps of the Krebs cycle were reversed and pyruvate was converted to oxaloacetate, followed by malate. Pyruvate was also converted into malate using the forward steps of the Krebs cycle (via citrate) and the elevated malate levels formed from both directions was then transported to the cytosol. By splitting, or bifurcating, the Krebs cycle in this way, its ability to metabolise acetyl-CoA from the β -oxidation of fatty acids was reduced, but the cycling between trans-2-enoyl- CoA reductase and acyl-CoA dehydrogenase continued with limited NADPH due to the inability of the Krebs cycle to now metabolise KG (which is produced by glutamate dehydrogenase and isocitrate dehydrogenase in exchange for glutamate and isocitrate, respectively).

2.5.4 Diagnosis and treatment of complex I diseases

Diagnosis and treatment of MDs proves challenging due to the general lack of awareness within the general medical community as well as the complicated genotype-phenotype relationship in these patients. When an MD is suspected, following a detailed family and medical history, clinical assessment and laboratory testing, a diagnosis may be confirmed by genetic and/or biochemical analyses. Due to the large number of possible genes that may be responsible for MDs, whole exome sequencing is commonly used for a molecular diagnosis. These genetic tests are typically performed in blood and thus mtDNA heteroplasmy levels cannot be accurately tested. Using muscle tissue, mtDNA heteroplasmy levels can be determined. CI deficiency is biochemically diagnosed by isolating mitochondria from muscle or skin biopsies and performing spectrophotometric, BN-PAGE and immunodetection assays to determine OXPHOS enzyme activities and structure. When specific diagnostic and disease criteria are met, the decision will then be made to perform the more invasive muscle biopsy procedure to obtain a clear diagnosis, as fibroblasts have been shown to differ from results obtained from muscle in some patients (Valsecchi *et al.*, 2010; Hoefs *et al.*, 2012; Koopman *et al.*, 2016; Rodenburg, 2016).

Therapies currently used for CI deficiency include antioxidants, vitamin and cofactor supplements (such as riboflavin, UQ, vitamin B1, B12, C, E, K and L-carnitine) and dietary adjustments (ketogenic diet), and while some offer symptom relief and assist with disease management, none offer a cure. These treatment strategies focus on promoting sirtuin activity, stabilising cellular Ca^{2+} homeostasis and increased ROS scavenging, with potent antioxidants like Trolox (a vitamin E analogue) showing great promise for treatment of CI dysfunction by increasing the amount of fully assembled CI and reducing ROS levels (Koopman *et al.*, 2008; Distelmaier *et al.*, 2009; Koene *et al.*, 2011; de Haas *et al.*, 2017). Other promising treatments that have been tested in mouse

models include rapamycin (an mTOR inhibitor) (Johnson *et al.*, 2013; Johnson *et al.*, 2015), nicotinamide riboside and nicotine mononucleotide (NAD⁺ precursors) (Cerutti *et al.*, 2014; Lee *et al.*, 2019), and a poly[adenine diphosphate (ADP)-ribose] polymerase (PARP)-1 inhibitor (Felici *et al.*, 2014). The latter two treatments increase intracellular NAD⁺ levels and are thus believed to improve mitochondrial function by shifting the metabolic status (Koopman *et al.*, 2016).

The role of MTs, molecules of particular importance in this thesis, as free radical scavengers, their mechanisms for preventing additional ROS formation (Taylor *et al.*, 2003) and their cooperation with the glutathione redox cycle (Quesada *et al.*, 1996a; Cai *et al.*, 2006b), make these highly inducible small endogenous peptides greatly appealing as potential therapeutic effectors for patients with LS and other MD phenotypes.

2.6 METALLOTHIONEINS

2.6.1 Structure and function

MTs, first discovered by Margoshes and Vallee (1957), are low molecular weight intracellular proteins (6-7 kDa) of 61-68 amino acids in length, consisting of 20 cysteine residues and lacking any aromatic amino acids (Coyle *et al.*, 2002). These amino acids are distributed into an α - and a β -domain, separated by a cysteine-free central “spacer”. The cysteine sulfhydryl groups allow MTs to bind 7-12 metal ions, the type of metal determining the number of metal ions capable of binding as well as the tertiary structure of the protein (Rigby & Stillman, 2004). Four MT isoforms have been identified, of which MT1 and MT2 are expressed in all major organs, MT3 is expressed in the central nervous system (CNS) and MT4, which is expressed in squamous epithelial cells (Babula *et al.*, 2012). MT3 has seven additional amino acids and is mainly expressed in neurons but can also be expressed in glia. MT1 and MT2 are highly similar, ubiquitous isoforms that can easily be induced by many similar factors, in the same organs, and display closely related functions. They are generally considered to be equivalent proteins since they have also always been discovered and isolated together at both protein and mRNA levels (Artells *et al.*, 2013). Recent studies have shown that there are, however, some functional differences between MT1 and MT2 and that there is a distinction between their metal binding capacities. MT1 appears to favour Cd²⁺, while MT2 seems to favour Zn²⁺ and is much less suitable for Cu⁺ coordination than MT1 (Artells *et al.*, 2013; Comes *et al.*, 2019).

MTs have many functions, the most important of which is the detoxification of heavy metals, maintenance of zinc and copper homeostasis and transport of essential metals. Other functions include free radical scavenging, apoptosis, regulation of intracellular redox balance and anti-inflammatory processes (Palmiter, 1998; Maret, 2000). Of particular relevance to this study is that

MTs have been strongly associated with the prevention of oxidative stress and cell death in MDs. In addition, MTs have been found to be intrinsically overexpressed in cells in response to primary mitochondrial deficiencies, supposedly in an attempt to reverse the oxidative damage incurred or to prevent further damage (Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006; Lindeque *et al.*, 2010). What has made this observation particularly exciting, when considering possible therapeutic applications, is that expression of MTs can be strongly induced by a number of modulators, including zinc, cadmium, copper, glucocorticoids (such as corticosterone and dexamethasone) (Penkowa, 2006) and cytokines such as IL6 (Poulsen *et al.*, 2005) and TNF α (Quintana *et al.*, 2007).

2.6.2 Localisation of metallothioneins

MTs are cytoplasmic proteins that have been found to localise to the nucleus and to the mitochondrial intermembrane space, the exact mechanism of which is not yet known (Lindeque *et al.*, 2010). Evidence of mitochondrial localisation was shown in an *in vitro* investigation of MT-overexpressing HeLa cells (both with and without stressor interventions) where the MT (sub)isoform MT1B, but not MT2A, was exclusively localised to mitochondria (Reinecke *et al.*, 2006; Lindeque *et al.*, 2010).

The exact mechanism by which MTs enter the mitochondria is not known, especially since MTs lack any mitochondrial targeting sequences, but various hypotheses have been proposed. Since molecules of up to ~10 kDa are able to enter the outer membrane of the mitochondria, it is possible that the MT molecule of ~7 kDa would be able to diffuse into the mitochondria as a result of equilibrium between mitochondrial and cytosolic MT, however, while MTs have been observed to be imported into the intermembrane space of liver mitochondria, their localisation was glaringly absent in the heart mitochondria of a cardiac-specific MT overexpressing transgenic mouse model. This indicated the presence of a more controlled mechanism for MT import – with tissue specificity (Zhou & Kang, 2000; Ye *et al.*, 2001). The structural similarity between COX17 and MT, in addition to its lack of a mitochondrial targeting sequence, could also indicate that MT may follow the same import mechanism into the mitochondria as COX17 (Ye *et al.*, 2001). Another proposed mechanism suggests that the mitochondrial outer membrane import and assembly machinery recognises proteins with repetitive cysteine residues and is able to import them into the intermembrane space by a disulphide exchange system. However, for this to occur, MTs must lose their conformation and release all metals, resulting in loss of their function as metal transporters (Mesecke *et al.*, 2005; Moltó *et al.*, 2007).

Despite the absence of MTs in heart mitochondria that was reported by Zhou and Kang (2000), their MT overexpressing mice still showed better mitochondrial protection against doxorubicin

than control mice. This indicated that the localisation of MTs to the intermembrane space is not reliant on MT concentration and that MTs have distant mechanisms by which they are able to protect mitochondria, possibly by preventing oxidative stress in the cytoplasm (Zhou & Kang, 2000; Ye *et al.*, 2001). Relevant indirect mechanisms by which MTs interact with the mitochondria are discussed below in Sections 2.6.3 and 2.6.4.

2.6.3 Metallothioneins and oxidative stress

As discussed in Section 2.5.3.1, MDs can lead to the production of excessive mitochondrial ROS, which has been shown to induce MT expression (Andrews, 2000; Kondoh *et al.*, 2001; Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006). Furthermore, increased ROS has also been shown to stimulate apoptosis (Kondo *et al.*, 1997). Due to the high susceptibility of mtDNA to oxidative damage as a result of its close proximity to the ROS producing-sites, in addition to ROS-induced apoptosis, this protective role of MTs may play a vital role in cell survival.

Various mechanisms for this protective effect have been suggested. Increased ROS has been shown to reversibly oxidise certain regions of CI (attenuating further ROS production) (Taylor *et al.*, 2003), thus, the transport of induced MTs to the mitochondrial intermembrane space may reduce those regions again and protect CI (Lindeque *et al.*, 2010). Another mechanism by which MTs can indirectly protect mitochondrial structures is via the GSH/GSSG system, as demonstrated in Figure 2-2. During increased oxidative stress, GSH (reduced glutathione) becomes depleted. MTs have been shown to interact with glutathione, and despite being able to scavenge free radicals much better than GSH, this function of MTs only becomes activated during times of increased oxidative stress due to their physiological levels and localisation. Under these stressed conditions, the shift towards MT as the primary antioxidant allows GSH to be conserved and less glutathione disulphide (GSSG) to be formed, allowing glutathione to protect against ROS in the areas within the mitochondrion where MTs cannot reach (Quesada *et al.*, 1996b; Wu & Kang, 1998; Ding *et al.*, 2002; Cai *et al.*, 2006a). Furthermore, MTs are able to reduce GSSG to GSH and to bind glutathione which results in structural changes that allow the transfer of metal ions between glutathione and MTs (Ferreira *et al.*, 1993; Maret, 1994). MTs have been shown to not only scavenge ROS but to also prevent the formation of ROS by modifying a nuclear-encoded subunit of CIV during oxidative stress which, in turn, increases CIV ability to use molecular oxygen (Merten *et al.*, 2005).

2.6.4 Metallothioneins and energy metabolism

The role of MTs as regulators of metal homeostasis further links them to energy metabolism due to the hundreds of enzymes within a cell that depend on metal ion cofactors (especially Zn²⁺ and

Cu⁺) for their function and structure (Coyle *et al.*, 2002; Moltó *et al.*, 2007). While MTs may be able to interact directly with complexes I, III and IV as well as cyt c, due to their localisation in the intermembrane space, they are unable to enter the mitochondrial matrix. However, MTs may indirectly interact with mitochondrial proteins and enzymes due to the donation of metal ions (such as Zn²⁺ and Cu⁺) across the inner mitochondrial membranes by GSH (as discussed above) or transporters (Costello *et al.*, 2004; Mehta *et al.*, 2006).

The OXPHOS system, in addition to many other enzymes, has been shown to be inhibited by heavy metals like zinc, copper and cadmium. By removing zinc from the inhibitory sites where it binds to enzymes, MTs are able to restore enzyme activity without any metal ion transfer at the catalytic site. In this manner, excessive zinc which leads to enzyme inhibition can be alleviated by overexpressed thioneins which bind the zinc, re-activate the enzymes and thus minimise oxidative stress (Jacob *et al.*, 1998; Maret *et al.*, 1999; Lindeque *et al.*, 2010).

Another mechanism that has been proposed for MTs and energy metabolism *in vitro*, is their ability to bind ATP and GTP, possibly to supply the energy that is required to release certain metal ions to acceptors. These conformational changes, as a result of binding of these purine nucleotides, have been proposed to play a determining role in the localisation of MTs within the nucleus and mitochondria (Jiang *et al.*, 1998; Maret *et al.*, 2002; Lindeque *et al.*, 2010).

2.6.5 Expression and regulation of metallothionein genes

The expression of *Mt1* and *Mt2* is controlled by the TATA box (the main promoter element), metal-responsive elements (MRE), antioxidant-response elements (ARE, regulated in response to the redox status) and glucocorticoid response elements (GRE), which can all be found in the regulatory region of the MT gene. MREs are activated by the metal-responsive transcription factor (MTF-1), an important metalloregulator protein that is reversibly activated by free intracellular Zn and has also been shown to be activated by oxidative stress. Cytokine signalling can also mediate the activation of elements by signal transducers and activators of transcription (STAT) (Dalton *et al.*, 1996; West *et al.*, 2008; Ruttkay-Nedecky *et al.*, 2013; Juarez-Rebollar *et al.*, 2017). The regulation of the MT gene and its interaction with Zn and oxidative stress is shown in Figure 2-2.

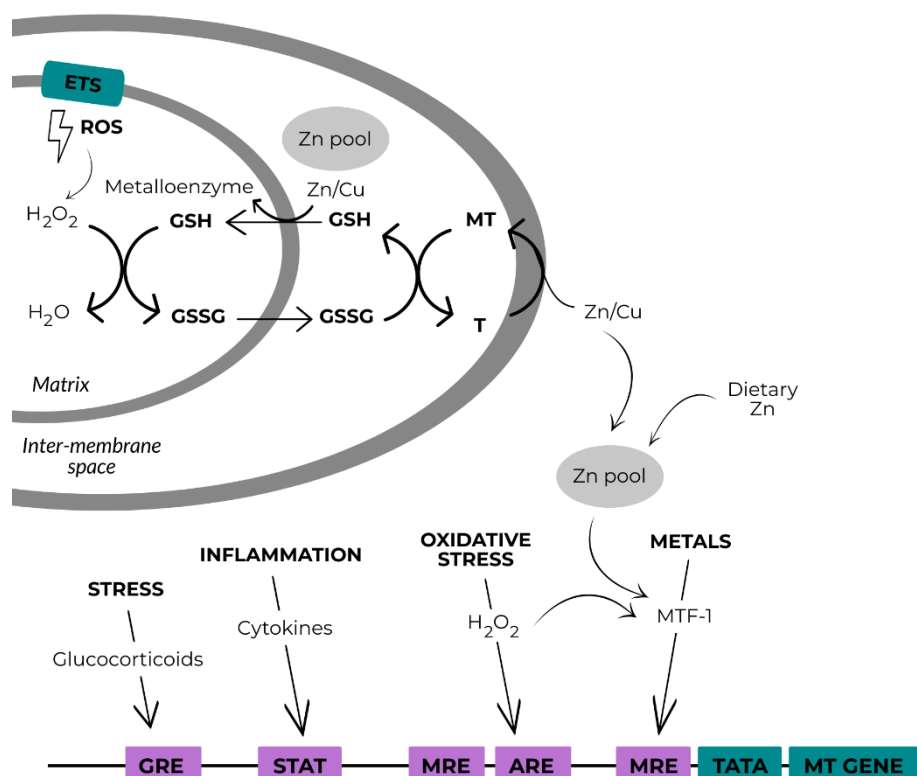


Figure 2-2: The regulation and expression of the MT gene and its interaction with zinc and oxidative stress. Stress, inflammation, oxidative stress and metals upregulate the transcription of MT. MTF-1 is activated by Zn and oxidative stress. Abbreviations: ARE, antioxidant-response elements; Cu, copper; ETS, electron transport system; GSH, reduced glutathione; GSSG, glutathione disulphide; GRE, glucocorticoid response elements; H_2O_2 , hydrogen peroxide; MRE, metal-responsive elements; MT, metallothionein; MTF-1, metal-responsive transcription factor; ROS, reactive oxygen species; STAT, signal transducers and activators of transcription; T, thionein; Zn, zinc.

2.7 MOUSE MODELS

2.7.1 Mitochondrial disease

2.7.1.1 Complex I disease models

With CI dysfunction being one of the most common mitochondrial diseases, various nDNA CI disease mouse models (both genetic and using chemical inhibition) exist to investigate the biological consequences. A common CI chemical inhibitor that has been used by this institution (Lindeque, 2011), and others (Koene *et al.*, 2011), is rotenone, which inhibits the transfer of electrons from the FeS clusters in CI to UQ (Koopman *et al.*, 2007a; Koopman *et al.*, 2007b). However, a disadvantage when using chemical inhibitors like rotenone, is the off-target effects that are not ordinarily seen in patients (Caboni *et al.*, 2004).

Genetic models include the Harlequin (Hq) mice (Klein *et al.*, 2002), muscle-specific and liver-specific apoptosis-inducing factor knock out (MAIFKO and LAIFKO) mice (Joza *et al.*, 2005; Pospisilik *et al.*, 2007) and *Ndufs4* whole-body knockout mice (Kruse *et al.*, 2008). The first three models target the apoptosis-inducing factor (AIF), which is a flavoprotein that plays a role in apoptosis and assists neuronal cell survival by preserving mitochondrial structure, and seems to play a significant role in the biogenesis and maintenance of CI (Cheung *et al.*, 2006; Benit *et al.*, 2008). However, to date, in human patients with LS or isolated CI deficiency, no pathological mutations in the AIF gene have been reported (Koene *et al.*, 2011; Bano & Prehn, 2018), and thus despite the decrease seen in CI activity from these mice, they do not serve as an ideal model of CI deficiency.

2.7.1.2 *Ndufs4* knockout model

On the other hand, the *Ndufs4*^{-/-} mouse model developed by the Palmiter group exhibits very similar clinical symptoms to human LS patients, with the *Ndufs4*^{-/-} mice presenting with reversible hair loss, visual and hearing defects, epilepsy, failure to thrive, ataxia (a less common symptom in human patients), breathing irregularities, loss of balance and lethargy until eventual death between P50 and P75 (Kruse *et al.*, 2008; Quintana *et al.*, 2012; Johnson *et al.*, 2013; Jain *et al.*, 2016). As the best characterised genetic model of CI deficiency, this model became commercially available in ~2013 and was thus used in this study. The *Ndufs4*^{-/-} model was produced by using Cre-lox recombination to delete exon 2 of the *Ndufs4* gene (which was flanked with *loxP* sites). The heterozygous mice - which present with a healthy phenotype indistinguishable from that of wild-type (WT) mice - were interbred to obtain the *Ndufs4*^{-/-} mice used in this study (since homozygous mice die before reaching sexual maturity) and were backcrossed on a C57BL6/J genetic background. Deletion of exon 2 results in a frameshift mutation (since it encodes the first 17 amino acids of the protein) and thus causes a lack of mature NDUFS4 (Kruse *et al.*, 2008; JAX, 2020b). Quintana *et al.* (2010) created a similar *Ndufs4* knockout mouse model to Kruse *et al.* (2008), but they only inactivated *Ndufs4* in the neurons and glia (so called *NesKO* mice). Astoundingly, the *NesKO* mice showed almost identical symptoms to the whole-body *Ndufs4* knockouts, thus indicating a predominant role of the CNS in these CI deficient mice and a neurological origin for the disease.

2.7.1.3 Brain region differences in *Ndufs4* knockout mice

Like patients with LS, the *Ndufs4*^{-/-} mice also display brain region specific neurodegeneration and inflammation, despite the loss of the NDUFS4 subunit in all brain cells. From approximately P35, the cerebellum, olfactory bulb and brain stem (especially the vestibular nucleus) begin to display histologic flaws and spongiform lesions not seen in the remainder of the mid- and forebrain. The

mitochondria from these more degenerative-prone areas also displayed decreased OXPHOS capacities compared to less susceptible areas. Due to the increasing energetic deficiency observed in *Ndufs4*^{-/-} synaptic mitochondria with age, it was hypothesised that the respiratory capacity of neurons in the more vulnerable regions gradually deteriorate at synaptic terminals (while less susceptible regions maintain normal OXPHOS capacity). Their inability to meet the increasing energy demands may bring about the region-specific neurodegeneration that is observed and contribute to the progression of the disease (Kayser *et al.*, 2016). Furthermore, Quintana *et al.* (2012) showed that mitochondrial dysfunction in the vestibular nucleus plays a critical role in the cause of death and breathing irregularities in *Ndufs4*^{-/-} mice. Selective inactivation of *Ndufs4* in the vestibular nucleus, in isolation, led to breathing abnormalities and premature death, while restoration of *Ndufs4* in the vestibular nucleus of the whole-body *Ndufs4* knockouts amended the breathing deficiencies and increased the lifespan of the mice.

2.7.2 Metallothionein

2.7.2.1 Metallothionein mouse models

Numerous nDNA targeted *Mt* mouse models exist for both *Mt* knockout (*Mt1+2* KO and *Mt3* KO) and *Mt* overexpressing mice (*Mt1*; heart-specific *Mt2A*; and *Mt3* overexpressing) (Michalska & Choo, 1993; Palmiter *et al.*, 1993; Masters *et al.*, 1994; Erickson *et al.*, 1995; Erickson *et al.*, 1997; Kang *et al.*, 1997). The *Mt1+2* as well as the *Mt3* knockout mouse models have previously been investigated at this institution (Pretorius, 2011; Lindeque *et al.*, 2013), while a model of particular interest to this study is the *Mt1* overexpressing transgenic mouse (*TgMt1*), also developed by the Palmiter group. The transgene was created using an *Mt1* gene that was modified with an additional two nucleotides located nine nucleotides upstream of the translational initiation codon and combining it with two regulatory DNA regions (10 kb and 7 kb) that flank *Mt1* and *Mt2*. In a heterozygous *TgMt1* mouse, this results in 56 copies of randomly inserted transgene, in addition to its two endogenous *Mt1* genes. However, the absolute mRNA expression levels of the *TgMt1* gene was determined to be approximately half that of the endogenous *Mt1* gene on a per gene basis, thus indicating that only half of the theoretical 56 transgene copies are physiologically expressed. Mice were then backcrossed on a C57BL6/J genetic background, which allowed for cross-breeding with the *Ndufs4*^{-/-} mice. These *TgMt1* mice showed position-independent and copy number-dependent expression of completely functional MT1, which could be induced by metals and hormones (Palmiter *et al.*, 1993; JAX, 2020a).

2.7.2.2 Tissue specific differences in metallothionein expression

Characterisation of the *TgMt1* mouse model by Iszard *et al.* (1995) established that the MT1 protein concentration was also increased in all major organs (and could further be increased by MT inducers), albeit at different levels, with a 10- to 20-fold increase in the pancreas, liver and stomach, and a two- to six-fold increase in the heart, lungs, kidneys, intestines, uterus, testes and spleen. The MT1 concentration in the brain was 1.4 times greater than controls. Surprisingly, they observed gender specific differences in the mRNA and protein levels of MT1 in the liver of both control and *TgMt1* mice, with significantly greater MT concentrations in females compared to males. However, these gender differences were not observed in any of the other tissues investigated.

While no data could be found regarding the MT levels in skeletal muscle of *TgMt1* mice, previous reports show increased MT in both rodent and humans models of skeletal muscle atrophy (Summermatter *et al.*, 2017). An *in vivo* study investigating the effect of MT deficiency on acute spinal cord injury concluded that the absence of MT does not worsen an oxidative stress response nor does it influence muscle atrophy (DeRuisseau *et al.*, 2009). *Mt1+2* KO mice that did not express MT1 or MT2 were shown to have increased muscle hypertrophy and a favoured increase in fast glycolytic type IIb muscle fibres (that are more prone to atrophy) and thus, deficiency of MT1+2 led to increased muscle strength (Summermatter *et al.*, 2017).

Three of the four known MT isoforms are expressed in the brain, namely, *Mt1*, *Mt2* and *Mt3*. When investigating the expression of MT genes in the mouse brain, it was shown that the expression of these three isoforms were not remarkably different, but that at the mRNA level, *Mt1* is expressed at the highest levels followed by *Mt3* and *Mt2*, which were expressed at 70% and 50% of that of *Mt1*, respectively. In these non-transgenic mice, it was also shown that all three MT isoforms were naturally expressed in multiple brain regions, namely, the cortex, olfactory bulb, caudate, hippocampus, cerebellum, thalamus and brain stem, with the highest MT mRNA levels observed in the olfactory bulb and up to two-fold differences in the MT expression of different regions. The mRNA levels showed localisation of *Mt1* (but not *Mt3*) in the glial cells of the cerebellum and localisation of *Mt3* (but not *Mt1*) in the granular cell layer of the dentate gyrus in the hippocampus. Furthermore, it was shown that *Mt1* is predominantly expressed in glial cells while *Mt3* is preferentially expressed in neurons (Choudhuri *et al.*, 1995).

When studying the regional brain distribution of MT in *TgMt1* mice specifically, Ono *et al.* (1997) found that male *TgMt1* mice had significantly greater levels of MT in whole brain samples, and two-fold greater levels in the cerebellum, compared to male controls. Female *TgMt1* mice had significantly greater levels of MT compared to female controls in all brain regions studied, with the

greatest increase in the cerebellum (3.5-fold) and the smallest increase in the cortex (two-fold). In addition, the whole brain samples of *TgMt1* female mice had significantly increased MT levels compared to male mice. Control mice with normal MT expression showed uniform MT levels between the different brain regions. Although the various isoforms could not be distinguished in Ono *et al.* (1997), MT3 was believed to constitute ~65% of total brain MT. They also observed brain region specific accumulation of zinc (that did not correlate with MT1 overexpression levels), especially in the males, with the greatest levels found to be in the hippocampus (which is rich in Znergic neurons. They attributed the gender differences to potential hormonal effects of oestrogen and/or testosterone on *Mt* mRNA transcription and MT protein expression.

2.7.2.3 Neuroprotection in *TgMt1* mice

Many studies have shown the important neuroprotective role of MT1 and 2 *in vivo* in brain disorders, such as focal and traumatic brain injury, epilepsy, ischemia and neurodegenerative diseases like Parkinson's disease and Alzheimer's disease (Carrasco *et al.*, 2000b; Giralt *et al.*, 2002; Ebadi *et al.*, 2005; Hidalgo *et al.*, 2006; Miyazaki *et al.*, 2007; Kim *et al.*, 2012; Eidizadeh *et al.*, 2015). Overexpression of MTs and exogenous treatment with MT1 and 2 in these mouse models have shown improved tissue repair and neurological outcomes with decreased inflammatory responses and reduced proinflammatory cytokines, decreased ROS, neuro-regeneration and reduced apoptotic cell death (West *et al.*, 2004; Santos *et al.*, 2012). A compromised balance in the maintenance of essential metals in the brain, as can occur in *Ndufs4*^{-/-} mice (that also exhibit a predominantly neurological phenotype), can lead to oxidative stress and cellular dysfunction (Ono *et al.*, 1997) and the *TgMt1* mice should thus, in theory, be an ideal model for testing the neuroprotective role of MT1. As mentioned earlier and as shown by numerous other studies, since MTs are endogenously produced in humans and are highly inducible by several effectors, they are an exciting therapeutic target for not only primary MDs but also in numerous other diseases.

CHAPTER 3: METHODS AND MATERIALS

3.1 INTRODUCTION

To achieve the objectives set out for this study, numerous methods and procedures were employed. Animals were bred to obtain mice of the four genotypes required for this study. Every new litter of pups was genotyped by obtaining tail snips at ~P17. Mice of the required genotypes were then weighed three times a week and included in either the survival analysis or the phenotype evaluations which were performed at ~P30 and ~P50. The phenotype evaluations included the wire grid hang test, rotarod, open field test and balance beam. Following the phenotype tests at P50, mice were euthanised and their organs were harvested and stored at -80°C. The whole brain and both quads of these mice were used for further biochemical evaluations, metabolomic profiling as well as for genetic characterisation of the model. The biochemical evaluation and genetic characterisation included: *Mt1* DNA and mRNA relative copy number determination, NDUFS4 quantification using SDS-PAGE and western blot, immunohistochemistry and ETS enzyme activity determination. The metabolomic profiling was performed across three different analytical platforms, namely, NMR, LC-MS/MS and GC-TOF-MS.

3.2 ANIMALS

3.2.1 *Animal care and handling*

All mice used were housed and bred at the Vivarium of the Pre-Clinical Drug Development Platform (PCDDP, SAVC reg. no. FR15/13458) of the North-West University (NWU). The mice were housed in individually ventilated cages at constant temperature ($22 \pm 1^\circ\text{C}$), humidity ($55 \pm 10\%$) and a 12:12-h light/dark cycle. Food and water were provided *ad libitum*. For environment enrichment, all cages were supplied with shredding paper as nesting material as well as red polycarbonate mouse houses or tubes. 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. Ethical approval for this study was obtained from the AnimCare ethics committee of the NWU with ethics approval numbers NWU-00364-16-A5 and NWU-0001-15-A5. Only persons with SAVC authorisation could handle the animals, under the supervision of the Vivarium.

3.2.2 Animal identification and numbering

At ~P18, all mice received ear punches (for identification) and tail snips were obtained. These tail snips were used to genotype the mice using the methods described in Sections 3.3.1 and 3.3.2. At ~P23, mice were weaned from their mothers and females and males were separated from one another. All KO and KO OVER mice were separated from WT and OVER mice (regardless of gender) to prevent the KO and KO OVER mice from being subjected to any stress or aggression that may be initiated by the healthy mice.

3.2.3 Sample size and animal groups

The mouse strains used to produce the required genotypes included B6.129S4-*Ndufs4*^{tm1.1Rpa/J} (*Ndufs4*) and B6.Cg-Tg(Mt1)174Bri/J (*TgMt1*), both obtained from Jackson Laboratory (ME, USA), which were then backcrossed on a C57BL6/J genetic background. Details on the breeding strategy and establishment of the mouse colonies can be obtained in Mereis (2018). In total, 80 mice (of both genders) were used in this study, as detailed in Table A1 and Table A2. This included 12 mice per genotype for the phenotyping analyses and 10 mice per genotype (of KO and KO OVER mice only) for the survival analyses. An additional three mice per genotype (12 extra mice in total) were also included, which were not phenotypically analysed. The tissues of these 80 mice were then used for the various biochemical and metabolomics analyses, as summarised in Table 3-1.

Table 3-1: Number of animals included in this study from each genotype

ASSAY	WT	OVER	KO	KO OVER	TOTAL
Survival analysis	0	0	10	10	20
Phenotyping assays	12	12	12	12	48
Immunohistochemistry	5	6	5	5	21
Biochemical analyses	6	6	6	6	24
Metabolomics (brain)	9	6	8	9	32
Metabolomics (quads)	14	13	13	13	53

3.2.4 Euthanasia and humane endpoints

Throughout the study, a humane endpoint score sheet from the SOP of the Vivarium (SOP_Viv_Anim 27 – Determining Pain and Distress in Lab rodents) was applied to monitor distress during the lifetime of the animals. Furthermore, any mice that were excluded from the study (since they do not have the correct genotype, such as the heterozygote mice) were euthanised at P30 (unless an older mouse was required for optimisation of a specific method) and used for optimisation and standardisation of methods. P30 was selected as the humane

endpoint since all mice had been genotyped by this age and KO mice started developing the mitochondrial disease phenotype after P30 (according to Jackson Laboratories, however, the heterozygote *Ndufs4* mice appear normal for at least a year). In both cases mentioned, euthanasia was conducted *via* cervical dislocation.

Mice included in the study were euthanised at P46-51 (excluding those used to construct survival curves) as significant symptoms of neuropathy became clear and differences between KO and WT mice were more detectable, thus allowing for the number of animals required for statistical significance to be minimised. These mice were euthanised by a registered animal technician via cervical dislocation as described by the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals* (AVMA) (2013 Edition) as this was determined (by Mr. Hylton Bunting, PCDDP, NWU) as the most appropriate method for allowing the harvesting of drug-free, undamaged brain tissue (in conjunction with all other tissues that were harvested). These humane endpoint aspects were enacted on by a research animal technician (PCDDP) overseeing the breeding of these mice.

3.3 GENETIC CHARACTERISATION OF THE MODEL

When establishing the mouse colonies used in this study (at the PCDDP), whole genome genotyping was performed by Mereis (2018), where the C57BL6/J genetic background of the *Ndufs4*^{+/-} and *TgMt1* mouse strains were verified using a 1500 SNP panel at Jackson Laboratory (ME, USA).

As mentioned above, tail snips were obtained from all mice at ~P18 and were used to determine the genotype of all new litters of mice, to allow for phenotypic evaluation of mice of the desired genotypes only. It was only necessary to genotype the *Ndufs4* gene of the tail snips (Section 3.3.2), and not the *TgMt1* gene, since the breeding pairs were set up in such a manner that the *TgMt1* genotype was known for all litters. All genotypes were confirmed a second time, following euthanasia of the animals, to ensure that all mice included in this study had been correctly genotyped and that no mice had been accidentally swapped during phenotype evaluations. This was done by following the same methods used when genotyping the tail snips (Section 3.3.2), in addition to confirming the *TgMt1* relative copy number (Section 3.3.3), but this time using liver tissue.

To further genetically characterise the model, NDUFS4 was quantified in the brain and quads to confirm deletion of NDUFS4 in all knockout mice. The sample preparation for the liver, brain and quad tissues is described in Section 3.5.5. All methods used for the genetic characterisation of this model were optimised within the Mitochondria Research Laboratory by Mereis (2018).

3.3.1 DNA isolation

DNA was isolated from the tail snips and liver tissue according to manufacturer instructions using the Zymo Research Quick-DNA™ Miniprep Plus kit (Inqaba Biotech, #D4068). The isolated DNA was quantified using a NanoDrop One Microvolume UV-Vis spectrometer (Thermo Fisher Scientific) and diluted to 25 ng/μL (for further processing in Section 3.3.2) and 5 ng/μL (for further processing in Section 3.3.3) in nuclease-free water (Qiagen, #129114).

3.3.2 *Ndufs4* genotyping

A PCR reaction was performed to confirm deletion of exon 2 of *Ndufs4* using specific forward (5'-AGCCTGTTCTCATACCTCGG-3') and reverse (5'-TTGTGCTTACAGGTTCAAAGTGA-3') primers (0.5 μM each) designed for the *Ndufs4* gene, as described by Valsecchi *et al.* (2012), along with 1x Phire Tissue Direct PCR Mastermix (Thermo Fisher Scientific, #F-170L), nuclease-free water and 25 ng DNA. Thermal cycling conditions consisted of an activation step of 98°C for 5 min, followed by 34 cycles of 98°C for 5 s, 57.3°C for 5 s and 72°C for 20 s, and then finally, following the 34 repeats, a step of 72°C for 1 min and then 4°C until the samples were removed from the thermal cycler. A 1% (w/v) agarose gel was prepared in 1x Bionic™ Buffer (Sigma-Aldrich, #B6185) with 5 μg/mL ethidium bromide. A GeneRuler™ 100 bp DNA Ladder (Thermo Fisher Scientific, #SM0241) was loaded on the gel alongside the samples, which were diluted 3:10 in nuclease-free water, and the gel was run at 6 V/cm. When analysing the DNA fragments, a 1229 bp band indicated a wild-type, a 429 bp band indicated a homozygote (*Ndufs4*^{-/-} knockout) and the presence of both bands indicated an *Ndufs4*^{+/-} heterozygote, an example of which is shown in Figure A1.

3.3.3 *TgMt1* relative copy number determination

A qPCR reaction using Taqman® Gene Expression Assays (Thermo Fisher Scientific) was performed to confirm the insertion of the *Mt1* transgene, according to manufacturer's instructions (TaqMan Gene Expressions Assay Protocol, P/N 4333458). This was done by relatively quantifying the *TgMt1* gene (Mm00496660_g1) and the *β-actin* (Mm02619580_g1) reference gene using the 2^{-ΔΔCT} method (Applied Biosystems User Bulletin No. 2, P/N 4303859). This involved separate reactions for each gene, in triplicate, containing 1x Taqman Gene Expression Master Mix (#4369016), 1x Taqman Gene Expression Assay (*TgMt1* or *β-actin*), nuclease-free water and 20 ng DNA. The cycling conditions included a step of 95°C for 10 min to initiate enzyme activation followed by 40 cycles of 95°C for 15s followed by 60°C for 1 min. The expected copy number for a wild-type was 2, for a heterozygote was 58 and for a homozygote was 114, as discussed in Section 2.7.2.

3.3.4 NDUFS4 quantification

To confirm that the protein levels, and not only the DNA levels, of NDUFS4 were decreased in the KO and KO OVER mice, brain and quads from one mouse per genotype were used to quantify NDUFS4 via SDS-PAGE and western blot.

3.3.4.1 Protein isolation

Tissues were kept on ice throughout preparation. Samples were chopped on an ice-cold steel table using scalpel blades, weighed and then homogenised using a Potter-Elvehjem homogenizer to create a 10% (w/v) homogenate in ice-cold Zheng buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 0.1 mM EGTA, pH 7.2). Approximately 15-20 strokes were used until the homogenate was evenly dispersed. The homogenate was transferred to a microcentrifuge tube and centrifuged at 600 x g for 10 min at 4°C. The supernatant was then carefully transferred to a clean tube and stored at -80°C. Prior to downstream processing, these samples were subjected to at least two freeze-thaw cycles.

3.3.4.2 Protein quantification

The bicinchoninic (BCA) assay, first described by Smith *et al.* (1985), was used to determine the protein concentration of the samples. A BCA:CuSO₄ solution was prepared by adding 1 part CuSO₄ (Sigma Aldrich, #2284) to 50 parts BCA (Sigma Aldrich, #B9643). Bovine serum albumin (BSA, Sigma Aldrich, #P0914) was used to create a standard curve with a range of 0-20 µg in increments of 4. An effective sample volume of 0.5 µL and 1 µL was added for the brain and quads respectively. The samples and BSA standard were topped up with Milli-Q water to a final volume of 20 µL. Of the BCA:CuSO₄ solution, 200 µL was added per well and the 96-well microtiter plate was incubated at 37°C for 30 min. The absorbance at 562 nm was then measured using a Synergy HT (BioTek Instruments) microplate reader and Gen5™ Data Analysis Software (BioTek Instruments).

3.3.4.3 SDS-PAGE

Protein samples were separated by SDS-PAGE analysis using the Laemmli Method (Laemmli, 1970). The TGX Stain-Free™ FastCast™ Acrylamide 1 Starter Kit, 10% (Bio-Rad Laboratories, #1610182) was used in conjunction with tetramethylethylenediamine (TEMED, Sigma Aldrich, #T7024), 10% (w/v) ammonium peroxodisulfate (APS, Sigma Aldrich, #A3678) and a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, #1658004). Manufacturer's instructions were followed to cast a 1 mm SDS-PAGE gel and a running buffer of 1x Tris/Glycine/SDS (10x; Bio-Rad Laboratories, #1610732) was used. The protein samples were diluted to 40 µg/µL for the

brain and 50 µg/µL for the quads. To the samples and the Precision Plus Protein™ Dual Xtra Prestained Protein Standard (Bio-Rad Laboratories, #1610377), 1x Laemmli Sample Buffer (Bio-Rad Laboratories, #1610737), 355 mM β-mercaptoethanol (Sigma Aldrich, #M3148) and Milli-Q water were added. The samples were boiled at 100°C for 5 min followed by centrifugation at 16 000 x g for 5 min to collect cell debris. Of each sample and ladder, 20 µL was added per well and the gel was then run at 200V for 30 min.

3.3.4.4 Western blot and immunodetection of NDUFS4

The TransBlot Turbo Blotting System (Bio-Rad Laboratories, #1704155) was used to transfer the proteins from the SDS-PAGE gel to the PVDF membrane (TransBlot Turbo Transfer Pack, 0.2 µM mini, Bio-Rad Laboratories, #1704156), as described in Bulletin #10016505 (Bio-Rad Laboratories). At the end of the transfer, the blot was imaged using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) with a stain-free protocol. The blot was thereafter blocked in 1x TBS with 1% (w/v) Casein (Bio-Rad Laboratories, #1610782) for 1.5 hours. For this step and all other steps that followed, the blot was gently agitated on a platform shaker at 26 rpm. Once the membrane had been blocked, it was incubated at 4°C overnight with primary antibodies for NDUFS4 (mouse monoclonal antibody, Abcam, #ab87399) and VDAC1 (rabbit polyclonal antibody, Abcam, #ab15895) which were diluted 1:1000 in 1x TBS with 1% (w/v) Casein. On the following day, three wash steps of 5 min each were carried out using washing buffer which consisted of 1x TBS (10x: Bio-Rad Laboratories, #1706435) with 0.001% Tween20 (10%: Bio-Rad Laboratories, #1662404). The HRP-conjugated secondary antibodies directed against mouse (Abcam, #ab97023) for NDUFS4 and against Rabbit (Abcam, #ab97051) for VDAC1 were diluted 1:10 000 in 1x TBS with 1% (w/v) Casein and incubated separately for 1 hour each, with three wash steps after each incubation. Imaging was then performed using the Clarity™ Western ECL Substrate (Bio-Rad Laboratories, #1705060), the ChemiDoc™ MP Imaging System and ImageLab™ software (version 5.2.1) with a Chemi Hi resolution protocol.

3.4 RNA ANALYSIS FOR *Mt1* QUANTIFICATION

Since it was not possible to quantify MT1 at the protein level, the mRNA levels of *Mt1*, in both the brain and quads of six mice per genotype, were determined using RT-PCR.

3.4.1 RNA isolation

Total RNA was isolated using TRIzol™ Reagent (Ambion, #15596026, Thermo Fisher Scientific) according to manufacturer instructions (MAN0001271, Rev A.0) as first described by Chomczynski (1993). The brain and quad aliquots that were set aside for RNA analysis in Section 3.5.5, were used to create a 10% (w/v) homogenate in TRIzol reagent. Samples were incubated

for 5 min after which 20% (v/v) chloroform (Honeywell Burdick & Jackson, #049) was added. Samples were gently inverted for 2-3 min followed by centrifugation for 15 min at 12 000 x g and 4°C, which allowed the mixture to separate into a lower red phenol-chloroform phase and a colourless upper aqueous phase. The aqueous phase containing the RNA was carefully transferred to a new tube and 50% (v/v) isopropanol (Merck, #109634), per volume TRIzol used for lysis, was added and incubated for 10 min. Following centrifugation for 10 min at 12 000 x g and 4°C, the total RNA precipitate formed a white gel-like pellet. The supernatant was discarded and the pellet resuspended 1:1 - according to the TRIzol volume used for lysis - in 75% (v/v) ethanol (Sigma Aldrich, #E7023). This was then vortexed and centrifuged for 5 min at 7500 x g and 4°C, after which the supernatant was discarded and the tube containing the RNA pellet was left to air-dry for ~30 min. Once the pellet turned clear, it was resuspended in 30 µL RNase-free water, heated at 60°C for 10 min and then stored at -80°C.

Isolated total RNA was purified using the MEGAclean™ Transcription Clean-Up Kit (Thermo Fisher Scientific, #AM1908) according to manufacturer instructions (1908M, Rev. C) and quantified using the NanoDrop One Microvolume UV-Vis spectrometer (Thermo Fisher Scientific).

3.4.2 Relative quantification of *Mt1* and *β2m*

The TaqMan® RNA-to-C_T™ 1-Step Kit (Applied Biosystems, #4392938) was used to quantify both *Mt1* (Mm00496660_g1) and *β2m* (Mm00437762_m1) TaqMan Gene Expression Assays (Applied Biosystems, #4332078) in triplicate, in separate reactions, according to manufacturer instructions (P/N 4393463, Rev. D). Each reaction, per gene, consisted of 1x TaqMan RT Enzyme Mix, 1x TaqMan RT-PCR Mix, 1x TaqMan Gene Expression Assay, nuclease-free water and 50 ng RNA template. The 7300 Real-Time PCR System (Applied Biosystems) cycling conditions included a reverse transcription step of 48°C for 15 min, a polymerase activation step of 95°C for 10 min and then 40 cycles of 95°C for 15 s along with 60°C for 1 min. The *Mt1* mRNA copy number relative to *β2m* was quantified using the 2^{-ΔCT} method (Applied Biosystems User Bulletin No. 2, P/N 4303859) (Livak & Schmittgen, 2001).

3.5 PHENOTYPIC EVALUATION

To determine whether or not overexpression of *Mt1* in the *Ndufs4*^{-/-} mice would increase the age of survival (typically only 2-3 months of age), as well as whether any improvements could be seen in hallmark phenotypic motor activity deficiencies of these mice (Kruse *et al.*, 2008; Quintana *et al.*, 2010), which typically present with weight loss, ataxia and loss of balance beginning at P35, selected phenotypic evaluations were performed. All mice included in this study were used to construct growth curves (n = 68). Of these mice, 20 were used for survival analysis and the

remaining 48 mice were used to perform locomotor activity and ataxia phenotype tests. These tests were performed on mice from the four genotypes at two different ages – younger than P30 and older than P46 – to obtain a clear distinction between the KO mice presenting with neurological symptoms and those that did not yet demonstrate symptoms.

For the mice that underwent the locomotor activity and ataxia assays, the timeline is shown in Figure 3-1. Between P25 and P30, mice underwent locomotor activity and ataxia assays over two consecutive days. On the first day, and in this order, mice were habituated to the open field testing cage for 15 min, weighed, underwent three wire grid hang tests trials, three balance beam training runs and two rotarod training runs (at 5 rpm). On the following day, once again in this order, mice underwent the open field test trial, two balance beam trials and three rotarod trials. On both days, adequate rest time was given between all activities and trials, as discussed below. Between P46 and P51, this was repeated on the same mice over two consecutive days. Directly following the second day's locomotor activity and ataxia assays, mice were euthanised via cervical dislocation and multiple tissues were harvested and snap frozen in liquid nitrogen.

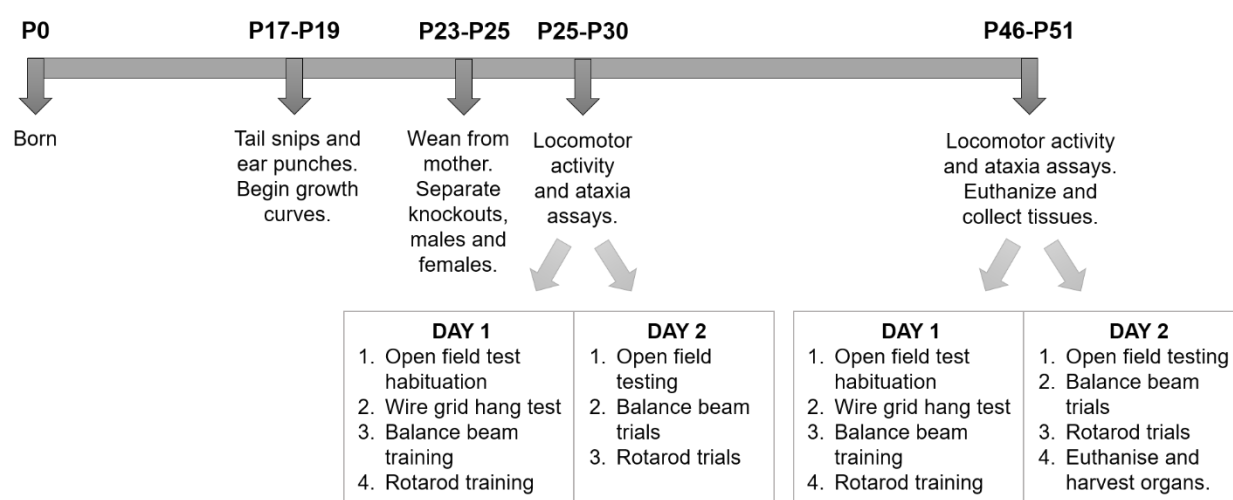


Figure 3-1: Timeline indicating all analyses performed on the 48 mice that underwent phenotypic evaluations. P = Postnatal day.

3.5.1 Growth curves

All 68 mice that were included in the phenotyping and survival analyses of this study were weighed three times a week at approximately the same time each day. The weighing of the mice began once they had received their ear punches for identification.

3.5.2 Survival curves

The day at which selected mice naturally died (all other mice were euthanised at P46-51) was recorded to establish how effective the overexpression of *Mt1* was in prolonging the life span of the KO mice. Survival analyses were only performed on the minimum possible number of mice, while maintaining statistical significance, to prevent unnecessary suffering.

The mice included in the survival analysis were weighed three times a week and monitored daily to ascertain the postnatal day number when they naturally died. They did not undergo any of the locomotor activity or ataxia assays, to minimise the number of factors that may play a role in their survival as well as to minimise the stress that these mice were subjected to. From P50 onwards, food pellets were placed on the floor of the cage and water bottles with longer spouts were used to ensure that the KO and KO OVER mice were able to reach the food and water and that deaths did not occur due to starvation.

In addition, two mice per genotype ($n = 8$) were video recorded at P30, P45 and P60 (every 15 days until natural death occurred) to document the progression of the phenotype. They were video recorded for approximately 30s where they were observed walking around in the cage, being picked up, placed on the cage lid and then returned to the cage. This was used to demonstrate the other phenotypic behaviours that typically occur in these mice.

3.5.3 Locomotor activity

Locomotor activity was evaluated using an open field test. Horizontal and vertical locomotor activity was determined using a Digiscan Animal Activity Monitor (DAAM, AccuScan Instruments), generously loaned to us by Dr. De Wet Wolmarans (North-West University, Department of Pharmacology, Potchefstroom, South Africa). Individual mice were placed in a monitoring cage (42 x 42 x 30 cm), divided into four segments, equipped with a grid of invisible infrared beams (as shown in Figure 3-2). The locomotor activity was then able to be measured as the laser beams were broken with the movement of the mouse. The monitor cages were surrounded by a series of horizontal infrared light beams (16 beams spaced 2.5 cm apart), with one set of beams at ground level and a second set 10 cm above the first. This array of infrared beams enabled the computerised collection of all locomotor activity by a digital analyser that effectively determines the position of the animal 100 times/s (AccuScan Instruments Incorporated, 2000). Two mice could be measured per cage simultaneously by placing them in opposite segments. Horizontal activity represented the total number of horizontal beam “breaks” that occurred during the given time period, whereas vertical activity represented the total number of vertical beam “breaks” that occurred during the given time period (Wolmarans *et al.*, 2013).

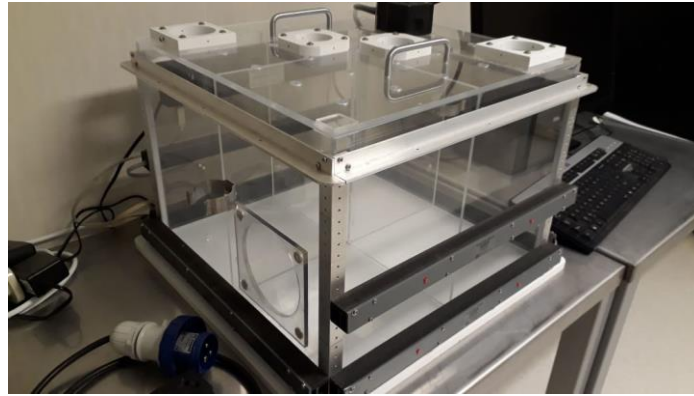


Figure 3-2: Photograph of the DAAM open field test instrumentation used. The cage was split into four quadrants to allow for two mice to be tested at the same time. A series of horizontal infrared beams, one at ground level and the second 10 cm above, detects the position of the animal in real-time.

One day prior to open field testing, mice were habituated to the environment by placing the mice in their respective corners of the cage chamber and leaving the mice undisturbed (with experimenter out of sight) for at least 15 min. Thereafter, the mice were returned to their housing cages overnight. All droppings were removed from the test chamber, but the chamber was not cleaned to allow the trace odours to remain for the following day and thus allow the environment to be more familiar for the mouse during the final testing. On the day of the analysis, the same mouse was placed in the same corners of the chamber as the previous day and once again left undisturbed (with experimenter out of sight) for at least 15 min to acclimate the mice. Thereafter, all cumulative horizontal and vertical activity was recorded by the analyser of the DAAM over a period of 5 minutes. The mice were then returned to their housing cages. This automated method provides continual computerised monitoring of the animal's behaviour that is more sensitive than simple observation and, most importantly, does not include the risks of investigator bias.

Following each experiment (i.e. before a new mouse was placed in the cage), the cage was cleaned with 70% ethanol to eliminate odour traces. The following parameters were obtained from the 5 min measurement period: total distance travelled (cm), rest time (the length of time, in s, that the animal stood still), movement time (the length of time, in s, that the animal was active) and the vertical episode count (the number of times that the animal reared up on its hind legs).

3.5.4 Ataxia assays

The *Ndufs4*^{-/-} mice develop severe ataxia with splayed legs, lack of balance and falling over, slow and awkward at correcting their movement and unresponsiveness to a firm nudge (Kruse *et al.*, 2008). To measure this phenotype, the wire grid hang test, rotarod and balance beam were used. This instrumentation was developed and built by the Instrument Making Department of the NWU, according to Deacon (2013) and Luong *et al.* (2011).

3.5.4.1 Wire grid hang test

The wire hang test was conducted similarly to that described by Carlson *et al.* (2010) and allowed for the non-invasive measurement of the ability of mice to use muscle strength, in all four limbs, to oppose gravitational force. It is an easy and inexpensive test to perform and the mice do not require acclimatization before the time. This test was performed after the open field test habituation on the first day. The wire cage lid from the housing cage was modified by fixing a 25 cm long cardboard box to its edges to prevent the mouse from climbing over to the other side of the cage lid during the test. The mouse was then placed on the wire grid (inside the four boxed walls created by the cardboard) so that it was able to grip with all four limbs (as photographed in Figure 3-3), and the grid was then flipped upside down above the plastic cage which was filled with soft sawdust bedding and paper towels. The grid was then raised so that the bottom end of the cardboard barrier was in line with the rim of the plastic cage (thus maintaining the grid at a height of approximately 35 cm above the sawdust bedding and paper towels). If the grid is placed higher than 35 cm, the mice may injure themselves when they fall, and if it is too low, they may fall voluntarily. The length of the time that the animal was able to hold onto the grid was recorded (up to a maximum of 180 s) using a stopwatch (Kruse *et al.*, 2008). Each animal underwent three trials with a resting period of 10 min between trials. Mice that fell off the grid in under 10 s were immediately retested as these falls were typically caused by the animal not having a good enough grip before the grid was flipped.



Figure 3-3: Illustration of the wire grid hang test. Mice were placed on the cage lid after which the grid was flipped upside down and the amount of time that the animal was able to hold on for (up to a maximum of 180 s) was recorded. Image obtained from SOP DMD_M.2.1.005 (Carlson, 2014).

Each mouse was weighed prior to the wire grid hang test and the mean holding time of the three trials was determined. From this, the physical impulse (Fdt) was calculated by multiplying the holding time by the gravitational force of the mouse [$Fdt \text{ (N s)} = \text{body mass (g)} \times 0.00980665 \text{ N/g} \times \text{holding time (s)}$] and represents the minimum total sustained force that was applied by the animal to oppose the gravitational force (Carlson *et al.*, 2010).

3.5.4.2 Balance beam

The balance beam was constructed according to the specifications described by Luong *et al.* (2011) and is photographed in Figure 3-4. This included a 1 m long beam, situated approximately 50 cm above a tabletop, with a dark box containing nesting material to attract the mice. At the start of the beam, there was a light (60W bulb) to avert the animals. The width of the wooden beam was 12 mm and paper towels were placed thickly on either side of the beam to cushion the fall of any mice. The amount of time that it took each mouse to cross the centre 80 cm of the beam was measured using two infrared beams (one to start and one to stop the timer when the beam was broken). This data was recorded on a USB drive. Nudging by the experimenter was sometimes required with mice that stopped halfway (due to random spontaneous behaviour) to maintain consistency between different mice. A video camera was positioned on a tripod to record their performance and the number of times that they slipped or needed to be nudged. The video camera was used purely for qualitative analysis of the posture and behaviour of the mice, and not for quantitative analysis.

One day prior to first testing (following the open field test habituation and wire grid hang test), the mice were placed in their housing cage in the testing room and were left undisturbed for 10 min. The main fluorescent light of the room was switched off, the main red light was switched on (mice are believed to not be able to see red) and the light bulb (positioned on the balance beam) was switched on prior to this 10 min acclimation period. The mouse was then placed on the light end of the balance beam (behind the sensor), facing towards the dark box. For the first training session, the mouse was held close to the base of the tail and gently pushed forward until it entered the dark box. For the second and third training sessions, the mouse tended to be more inclined to move towards the dark box by itself, but some nudging was often required along most of the balance beam to direct the mouse towards the dark box. If the mouse turned around on the beam, it was picked up and turned around again so that it was oriented in the correct direction. The nudging was used to prevent the mouse from turning around or from trying to climb down the side of the balance beam. Once in the dark box, the mouse was left undisturbed for at least 15 s before returning it to its cage. Three such training sessions were carried out with a minimum of 5 min resting periods between each session. The dark box and beam were cleaned of mouse droppings, but no detergents were used so that trace odours could remain and so that the mice were more familiar with the environment in the following days' trials. When this was repeated at >P46, it was only necessary to do two training sessions since the mice appeared to cross the balance beam more easily due to their training and trials at <P30.

The following day (after completing the open field testing), the exact same procedure was followed as for the balance beam training session, except that the software was switched on to record the

time taken for each mouse to cross the beam and a video camera was set up to record (with an identifier in the frame to allow for identification of each mouse when viewing the footage later on). For testing, mice were required to successively cross the beam twice (thus duplicate measurements), with nudging if necessary. At least 10 min rest was given between each trial. The beam and box were then cleaned of mouse droppings and wiped with 70% ethanol followed by water. For the KO and KO OVER mice, the video camera and software were setup to record on both the training day and the trial day, so that if the mice proved unable to successfully cross the balance beam on the second day, the data from the training day could still be used.



Figure 3-4: Photograph of the balance beam. Mice were placed on the light end of the balance beam and the length of time taken to cross the centre 80 cm of the beam was measured as the mice made their way to the dark box containing nesting material at the other end.

3.5.4.3 Rotarod

The rotarod was used to assess the motor coordination and balance in the mice. The rotarod was constructed similarly to that described by Deacon (2013), as shown in Figure 3-5, with a rotating wooden drum of ~3 cm in diameter and barriers (flanges of 25 cm in diameter) set 3.5 cm apart to prevent the mice from leaving the rod. At <P30, the mice were still able to turn around while on the rod, but at >P46 the mice turned around on the rod less frequently. Paper towels were placed thickly beneath the rod (which was 50 cm high to prevent mice from voluntarily jumping off the apparatus) to cushion the fall. An infrared beam was set up just above the rod. When pressing “ready” the rod rotated at 5 rpm. The mouse could then be positioned on the rod and once “start” was pressed; the rod began to accelerate (as long as the mouse was breaking the infrared beam)

and the animal continued to walk on the rod. As soon as the animal fell off the rod, the infrared beam was restored, the rod stopped rotating and the instrument recorded the length of time that the mouse had remained on the rod as well as the speed (in rpm) at which the mouse fell off.



Figure 3-5: Photograph of the rotarod. Mice were placed on the rod as it rotated at 5 rpm. The speed of rotation then increased from 5 to 40 rpm over a period of 5 min. When the animal fell off the rod, the infrared beam above the rod was restored, the rod stopped rotating and the latency to fall (in seconds) was recorded.

On the day prior to first testing (following the balance beam training), the wheel was set to rotate at 5 rpm and the mouse was then positioned on the rotarod (holding it by the tail and facing it away from the direction of rotation so that it was forced to walk forward), facing away from the experimenter. The mouse was then allowed to walk on the rotarod for 60 s (measured using a stopwatch) at 5 rpm before being returned to its cage. Mice underwent this training twice with a minimum resting time in between of 5 min. The area was cleaned of any mouse droppings. At >P46 only one training session of 60 s was required since the mice were already trained at <P30.

On the testing day (following the balance beam trials), the video camera was set up – facing the rotarod – to record each run. The rod was then set to rotate at 5 rpm at which time the mouse was placed on the rod, followed by acceleration to a maximum of 40 rpm over a period of 300 s. The latency to fall and speed at which the mice fell was recorded by the instrument. Three trials occurred per mouse, with a rest period of 15 min between trials. Mice that fell off the rod in under 10 s were retested immediately. If the mouse slipped off the rod but managed to climb back on, the run ended there since the rod stops turning when the infrared beam was restored. The rod was thereafter wiped clean with 70% ethanol and all mouse droppings in the area were removed

(Kruse *et al.*, 2008; Quintana *et al.*, 2010; Quintana *et al.*, 2012; Johnson *et al.*, 2013; de Haas *et al.*, 2016).

3.5.5 Euthanasia and sample collection

Directly after completing day two of the P50 phenotype evaluations, mice were euthanised via cervical dislocation and the tissues harvested in the following order: brain, quads, heart, lungs, liver and kidneys. Each tissue was then placed into a separate 1.5 mL microcentrifuge tube and immediately snap frozen in liquid nitrogen, with the exception of selected brain samples which were fixed as described in Section 3.6.1. Tissues were then stored at -80°C until sample preparation. The brain and quad tissues used in this study were chopped on an ice-cold steel table, weighed and then aliquoted into 1.5 mL microcentrifuge tubes for all other analyses. The aliquots set aside for protein analysis were immediately homogenised as described in Section 3.3.4.1. All other aliquots were placed on dry ice and again stored at -80°C until further use.

3.6 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on fixed-frozen brain samples obtained upon euthanasia of the mice following the phenotypic evaluations. All reagents and materials were generously supplied by the *Institut de Neurosciences* at the Autonomous University of Barcelona (Barcelona, Spain) where the analyses were performed by the author, after shipping all samples on dry ice from the NWU, with the help of Dr. Albert Quintana.

3.6.1 Fixing of brain tissue

A 4% (w/v) paraformaldehyde (PFA) solution (Sigma Aldrich, #P6148) was prepared by heating 1x PBS (Gibco, #18912014) to 60°C on a magnetic stirrer and adding the paraformaldehyde powder. Whilst stirring, 0.5 M NaOH was added dropwise until the paraformaldehyde powder had completely dissolved and the cloudy solution had cleared. Once cooled, the pH of the solution was adjusted to 7.4 using 0.5 M HCl.

The brain samples were carefully dissected to prevent damage to the tissue and were immediately placed in ice cold 4% PFA solution. The samples were kept on ice and stored at 4°C overnight. Thereafter, the brain samples were washed twice with 1x PBS and then stored in a 30% (w/v) sucrose in 1x PBS solution. The vials were inverted a few times and then left at room temperature overnight. Once the brain sample had sunk to the bottom of the vial, it was dried using paper towels, placed on aluminium foil and covered with crushed dry ice for 5 min. Thereafter, the frozen brain sample was placed in an ice cold 15 ml falcon tube and stored at -80°C until shipping and further analysis.

3.6.2 Slicing of brain tissue

Anti-freeze solution was made up using 40% (v/v) ethylene glycol (Sigma Aldrich, #102466), 30% (v/v) glycerol (Sigma Aldrich, #G7757) and 0.03 M PBS and the pH set to between 7.2 and 7.4.

A Leica CM3050S cryostat was used to cut 30 μ m tissue sections. The fixed-frozen brain samples were thawed in the chamber at -21°C, whilst the cryostat sample temperature was set to -19°C. The samples were fixed to the cryostat using OCT (Tissue-Tek, Sakura, #4583). All sections were immediately placed in ice cold 24-well plates containing anti-freeze solution and these plates were stored at -20°C. Sagittal sections were first obtained from one mouse per genotype to determine areas of interest. Thereafter, four mice per genotype were used where dorsal sections were obtained of the vestibular nuclei and the hippocampus (Franklin & Paxinos, 2008).

3.6.3 Staining for GFAP, IBA1 and MT

For the hippocampal and vestibular nuclei regions, six floating sections each were carefully rinsed in 1x PBS by placing them on an orbital platform shaker at 50 rpm for 5 min. This was repeated two more times using fresh PBS. A PBST solution was prepared by adding 0.2% (v/v) Triton-X100 to PBS. A 10% NDS-PBST solution was prepared by adding 10% (v/v) normal donkey serum (NDS) to the PBST solution. Following the three PBS wash steps, the floating sections were blocked by placing them in the 10% NDS-PBST solution on the shaker for 1 hour at 50 rpm. The primary and secondary antibodies were prepared as shown in Table 3-2 for the two immunohistochemistries that were performed.

The primary antibodies were prepared in 1% NDS-PBST, the floating sections added to the primary antibody dilution and the plates incubated on a shaker at 45 rpm and 4°C. For immunohistochemistry 1, the sections were incubated overnight, while for immunohistochemistry 2 they were incubated for three days. Thereafter, the sections were washed three times in PBST on a shaker at 50 rpm for 5 min. The secondary antibodies were then prepared in 1% NDS-PBST and the floating sections were incubated with the secondary antibodies for 1 hour on the shaker (50 rpm) at room temperature. Lastly, the sections were again washed three times in PBS by placing on the shaker for 5 min.

The tissue sections were then mounted onto Superfrost Ultra Plus microscope slides (Menzel-Glaser, Thermo Fisher Scientific) and once dry, the cover slip was placed over the sections using DAPI fluoromount-G (EMS, #17984-24) mounting solution. Slides were imaged using a Nikon Eclipse 90i upright epifluorescence microscope.

Table 3-2: List of primary and secondary antibodies used to perform the immunohistochemistry analyses

Antibody (species)	Primary/secondary	Supplier and catalogue number	Concentration
<i>Immunohistochemistry 1</i>			
IBA1 (Rabbit)	Primary	Wako, #01919741	1:1000
GFAP (Chicken)	Primary	Abcam, #Ab4674	1:2000
555 Donkey anti-Rabbit	Secondary	Thermo Fisher Scientific, #A31572	1:500
488 Goat anti-Chicken	Secondary	Abcam, #Ab150173	1:500
<i>Immunohistochemistry 2</i>			
MT* (Mouse)	Primary	Dako, #M0639	1:500
GFAP (Rabbit)	Primary	Thermo Fisher Scientific, #PA1-10019	1:5000
488 Donkey anti-Mouse	Secondary	Thermo Fisher Scientific, #A21202	1:500
555 Donkey anti-Rabbit	Secondary	Thermo Fisher Scientific, #A31572	1:500

* The MT antibodies used were generously provided by Prof. Juan Hidalgo from the *Institut de Neurosciencies* at the Autonomous University of Barcelona.

3.7 ELECTRON TRANSPORT SYSTEM ENZYME ANALYSIS

The brain and quads from six mice per genotype were used and 600 x g supernatants were created as described in Section 3.3.4.1, and the protein concentration within each sample was determined as described in Section 3.3.4.2. These samples were then further used to determine the enzyme activity of CS as well as complexes I, II, III and IV.

3.7.1 Reagents, instrument and sample preparation

The following reagents were obtained from Sigma Aldrich: Trizma base (#T1503), 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB, #D8130), Triton X-100 (#T9284), acetyl-CoA trillithium salt (Sigma-Roche #10101907001), oxaloacetic acid (OAA, #O4126), potassium phosphate monobasic (#P5655), potassium phosphate dibasic (#P2222), NADH dipotassium salt (#N4505), BSA (Sigma-Roche #10775835001), coenzyme Q1 (#C7956), 2,6-dichloroindophenol sodium salt hydrate (DCIP, #D1878), rotenone (#R8875), adenosine 5'-triphosphate disodium salt (ATP, Sigma-Roche #A2383), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, #E1644), sodium azide (#S2002), succinate (#S7501), decylubiquinone (#D7911), dimethyl sulfoxide (DMSO, #C6164), absolute ethanol (#E7023) and cyt c (#C7752). Tween 20 (#822184) was obtained from Merck.

The following reagents were prepared and stored in aliquots at -20°C. For CS, a 1M Tris.HCl stock solution was made up using Trizma base and concentrated HCl (cHCl) and the pH set to 8.1. Phosphate buffer (KPi buffer), with a different pH for each enzyme complex, was prepared by mixing 0.5 M potassium phosphate monobasic with 0.5 M potassium phosphate dibasic in different ratios. The following two solutions were made up and the pH adjusted using 2 M KOH: 250 mM EDTA (pH 7.0) and 1 M succinate (pH 7.8). A 1 mM rotenone solution and 10 mM decylubiquinone solution were prepared in DMSO, 10 mM coenzyme Q1 stocks were prepared in absolute ethanol, a 10% (v/v) BSA solution was made up in Milli-Q water and a 4 mM cyt c solution was prepared in 10 mM KPi buffer (pH 7.8).

Clear 96-well F-bottom microtiter plates (Greiner Bio-One International, #655101), a Synergy HT microplate reader and Gen5™ Data Analysis Software (BioTek Instruments) were used for all analyses. All reactions were carried out at a temperature of 37°C and in a final reaction volume of 200 µL. All reagents were kept on ice on the day of the analysis and all 600 x g supernatant samples were thawed on ice and mixed well before use. Samples were diluted for each enzyme complex as shown in Table 3-3. All reactions were performed in triplicate.

Table 3-3: 600 x g supernatant effective sample volume per reaction for OXPHOS complexes

Enzyme	Brain (µL)	Quads (µL)
CS	0.75	1.5
CI	1.5	3
CII	3.75	7.5
CIII	0.42	0.83
CIV	0.38	1

3.7.2 Citrate synthase

The following stock solutions were prepared fresh on the day of the analysis: 1 mM DTNB in 1 M Tris.HCl, 10% (v/v) Triton X-100 and 3 mM Acetyl Co-A each in Milli-Q water, and 2 mM OAA in 0.1 M Tris.HCl.

The CS reaction master mix was made up as follows in Milli-Q water: 0.1 mM DTNB, 0.025% Triton X-100 and 0.3 mM Acetyl-CoA. The diluted 600 x g supernatant was added to each well followed by the reaction master mix. Following a 10 min pre-incubation period at 37°C, the pre-heated OAA was added to each well to give a final concentration of 0.5 mM. The linear rate increase at 412 nm over 5 min in 1 min intervals was recorded (Shepherd & Garland, 1969; Robinson *et al.*, 1987).

3.7.3 Complex I

The following stock solutions were prepared fresh on the day of the analysis in Milli-Q water: 2 mM NADH and 5 mM DCIP. The DCIP was heated to 30°C for 20 min to dissolve all crystals.

The CI (NADH dehydrogenase) reaction master mix was made up as follows in Milli-Q water: 25 mM KPi buffer (pH 7.6), 70 µM Coenzyme Q1, 0.35% BSA and 60 µM DCIP. CI was measured in both the presence and absence of rotenone. For each sample, 1 µM rotenone was added to triplicate wells and DMSO (to the equivalent volume of rotenone) was added to a separate set of triplicate wells. To the rotenone or DMSO, the 600 x g supernatant samples were added along with the CI master mix. Following a 10 min pre-incubation period at 37°C, pre-heated NADH was added to each well to give a final concentration of 0.2 mM. The linear rate increase at 340 nm over 5 min in 1 min intervals was recorded (Rodenburg, 2011).

3.7.4 Complex II

The following stock solutions were prepared fresh on the day of the analysis in Milli-Q water: 0.1 M ATP (pH adjusted to 7.8 with 2 M KOH) and 100 mM sodium azide.

The CII (succinate dehydrogenase) reaction master mix was made up as follows in Milli-Q water: 80 mM KPi buffer (pH 7.8), 0.2% BSA, 2 mM EDTA, 0.2 mM ATP, 80 µM DCIP, 80 µM decylubiquinone and 0.3 mM sodium azide. The diluted 600 x g supernatant was added to each well followed by the CII master mix. Following a 10 min pre-incubation period at 37°C, the pre-heated succinate was added to each well to give a final concentration of 10 mM. The linear rate increase at 600 nm over 5 min in 1 min intervals was recorded (Rodenburg, 2011).

3.7.5 Complex III

A 2% (v/v) Tween 20 solution was prepared fresh in Milli-Q water on the day of the analysis. Furthermore, 10 mM decylubiquinol was made up by reducing decylubiquinone by firstly making up a 10 mM decylubiquinone solution in ethanol. To 250 µL decylubiquinone in ethanol, a few grains of potassium borohydride were added, and the mixture was then acidified by adding 10 µL 0.1N HCl/ethanol and vortexed until it became clear. Thereafter, 10 µL 3 N HCl/ethanol was added, the tube was centrifuged at 10 000 x g for 1 min and the supernatant was transferred to a clean tube for further use.

The CIII (ubiquinol-cyt c reductase) reaction master mix was made up as follows in Milli-Q water: 50 mM KPi buffer (pH 7.8), 1 mM EDTA, 0.04% Tween 20, 3 mM sodium azide and 300 µM decylubiquinol. This reaction mix was then pre-heated to 37°C. To each well, 50 µM cyt c was added together with the diluted 600 x g supernatant. Following a 10 min pre-incubation period at

37°C, the pre-heated CIII master mix was added to each well. The linear rate increase at 550 nm over 5 min in 1 min intervals was recorded (Rodenburg, 2011).

3.7.6 Complex IV

For CIV it was necessary to reduce the cyt c. This was done using potassium phosphate buffer and sodium ascorbate as described by Spinazzi *et al.* (2012). A 500 µM solution of reduced cyt c was then prepared.

The CIV (cyt c oxidase) reaction master mix was made up as follows in Milli-Q water: 20 mM KPi buffer (pH 7.4) and 70 µM reduced cyt c. This reaction mix was then pre-heated to 37°C. To each well, 10 mM KPi buffer (pH 7.4) was added together with the diluted 600 x g supernatant. Following a 10 min pre-incubation period at 37°C, the pre-heated CIV master mix was added to each well. The linear rate increase at 550 nm over 5 min in 1 min intervals was recorded (Rodenburg, 2011).

3.7.7 Enzyme activity data analysis

The protein content was divided by the volume of sample used in the analysis to obtain the protein concentration expressed as µg/µL. The mean velocity (*v*) of each enzymatic reaction was determined using the Gen5™ software (BioTek instruments) which calculates the gradient of the linear trend line over three to four readings, of each enzyme, where those readings producing the highest *R*² values were selected. These mean *v* values were then multiplied by the dilution factor used and substituted into the formulae displayed in Table 3-4 to obtain the enzyme activity normalised to both protein and units citrate synthase (UCS) and expressed as nmol/min/UCS.

Table 3-4: Equations used to determine the enzyme activity

Enzyme	Equation
CS	$\text{nmol/min} = \frac{v_1}{7465 \text{ Abs/mM}} \times \frac{0.2}{\text{CS } \mu\text{L}} \times 1000$
UCS	$\text{nmol/min/mg} = \frac{v_1}{7465 \text{ Abs/mM}} \times \frac{0.2}{\mu\text{L protein} \times ([\text{protein}] \div 1000)} \times 1000$
CI	$\text{nmol/min/UCS} = \frac{v_1 - v_2^*}{12712 \text{ Abs/mM}} \times \frac{0.2}{\mu\text{L protein} \times ([\text{protein}] \div 1000)} \times \frac{1000}{\text{UCS}}$
CII	$\text{nmol/min/UCS} = \frac{v_1}{12712 \text{ Abs/mM}} \times \frac{0.2}{\mu\text{L protein} \times ([\text{protein}] \div 1000)} \times \frac{1000}{\text{UCS}}$
CIII	$\text{nmol/min/UCS} = \frac{v_1}{4180 \text{ Abs/mM}} \times \frac{0.2}{\mu\text{L protein} \times ([\text{protein}] \div 1000)} \times \frac{1000}{\text{UCS}}$
CIV	$\text{nmol/min/UCS} = \frac{v_1}{4180 \text{ Abs/mM}} \times \frac{0.2}{\mu\text{L protein} \times ([\text{protein}] \div 1000)} \times \frac{1000}{\text{UCS}}$

[protein] represents the concentration of protein in µg/µL.

* For CI, *v*₁ represents the mean velocity of the reactions with DMSO and *v*₂ represents the mean velocity of the reactions with rotenone. Extinction coefficients indicated in the equations were determined for the appropriate chromogenic reagents and volumes in the microtiter plate.

3.8 PROTEIN OXIDATION

As discussed in Section 2.5.3.1, oxygen free radicals are produced by the ETS and the cellular redox system, as well as by immune responses, and during mitochondrial OXPHOS dysfunction oxidative stress commonly occurs which can in turn damage nucleic acids, lipids and proteins within the cell. This can lead to oxidative modifications where carbonyl groups are added to the side chains of proteins, resulting in changes in their biochemical activity. This, in turn, affects enzymatic activity, DNA binding activities of transcription factors and susceptibility to proteolytic degradation. To evaluate the potential effect of oxidative stress on the proteins of the mice used in this study, the OxyBlot™ Protein Oxidation Kit (Merck, #S7150) was used according to manufacturer instructions.

Protein lysates (600 x g), from Section 3.3.4.1, of one mouse per genotype were used for both brain and quads. Protein quantification was performed as described in Section 3.3.4.2 and an SDS-PAGE gel and western blot was performed as described in Sections 3.3.4.3 and 3.3.4.4 respectively, with changes in the method of sample preparation. To derivatise the carbonyl side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone), the following 20 µL reactions were carried out in two separate tubes for each sample: 20 µg protein sample, 6% (w/v) SDS and 10 µL of either 1x 2,4-dinitrophenylhydrazine (DNPH) (for the derivatised sample) or 1x Derivatization-Control Solution (for the negative control of each sample) was added. The two tubes per sample were then incubated at room temperature for 20 min after which 7.5 µL Neutralization Solution was added to both tubes together with 1 µL β-mercaptoethanol (Sigma Aldrich, #M3148) (to reduce the samples) and 2 or 4 µL 1N NaOH (for the brain and quads respectively, to adjust the pH). To both the derivatised sample and the negative control, 4 µL sample buffer, containing 1x Laemmli Sample Buffer (Bio-Rad Laboratories, #1610737) together with 355 mM β-mercaptoethanol, was added. Thereafter, the samples were processed as described in Sections 3.3.4.3 (at 115V) and 3.3.4.4 using 1:500 of the OxyBlot 1° Antibody for the overnight incubation. The following day, after the three wash steps, the blot was incubated with 1:1000 VDAC1 (rabbit polyclonal antibody, Abcam, #ab15895) for 2 hours. The HRP-conjugated secondary antibody directed against Rabbit (Abcam, #ab97051, 1:10 000) was used to visualise the oxyblot and VDAC bands.

The relative intensities of the oxidised proteins and VDAC were quantified using ImageLab version 5.2.1 (Bio-Rad Laboratories) with background correction. The samples were normalised according to VDAC and for the oxidised proteins, the negative control volume intensity was subtracted from the sample volume intensity.

3.9 STATISTICAL ANALYSIS OF BIOCHEMICAL DATA

Due to the small sample sizes in this study, vertical scatter plots were used to depict most data and non-formal testing was used for normality and homogeneity of variance (Morgan, 2017). IBM SPSS Statistics 25 was used for all statistical analysis, while GraphPad Prism version 5 and Excel 365 (Microsoft) were used to construct all graphs. The survival analysis was performed using the Log Rank (Mantel-Cox) test.

The mean (M), standard deviation (SD) and standard error (SE) were reported for all results along with effect sizes. For eta squared (η^2), an effect size of 0.01 was considered small, 0.06 as medium and anything greater than 0.14 as a large and practically significant effect size (Field, 2009). Outliers in the phenotyping data were identified using boxplots in SPSS (and removed) as those data points greater than three inter-quartile ranges from the mean. Where indicated on graphs, the error bars were 95% confidence intervals, and as such, the error bars of data points, which did not overlap at all, were considered to be statistically significant

Given the small sample sizes, it is unlikely that hypothesis tests will have sufficient power to detect departures from normality. Furthermore, failure to reject does not prove normality, even for larger samples. Thus, considering that most standard parametric tests are fairly robust to violations of the normality assumption, the following approach was used: Standardised skewness and kurtosis values were evaluated against established thresholds (Field, 2009) and if violations were considered severe, a transformation was applied. Given the type of data, a log transformation was considered appropriate and due to some zero observations, a shifted log was implemented with shift parameter equal to 1 in the case of the mRNA results (Section 4.3.3). Finally, model residuals were inspected.

For the phenotyping data, a factorial mixed ANOVA was performed for each variable with genotype as the between-subject factors, gender as the covariate and postnatal day age (P30 or P50) as the within-subjects factor. Equal variances were assessed as part of the ANOVA model and if violated, spread-and-level plots were used to assess the severity and identify the appropriate power transformation. If the violation was severe, the power transformation was applied, and the ANOVA model reconstructed on the transformed data. Sphericity was assessed based on Greenhouse-Geisser's epsilon, if this value was greater than 0.9 sphericity was assumed, since epsilon values close to 1 are indicative of sphericity. All phenotyping tests had sphericity values greater than 0.9 and thus sphericity could be assumed. Pairwise tests were also implemented when interaction effects were significant based on omnibus test p-value and partial eta squared effect sizes (η_p^2).

For the biochemical data obtained from the brain and quads, a two-way ANOVA was applied similarly to above. Despite the brain and quad tissues being obtained from the same mouse, independent analyses were performed as it has been shown in MDs that these tissues are affected in different ways. Lindeque et al. (2013) showed that MTs affected the metabolism of the brain and muscle in different ways, and Kruse et al. (2008) also showed that *Ndufs4*^{-/-} mice had differences in the mitochondria from the brain compared to the muscle. Thus, for mRNA and enzyme data, there were no within-subject factors and only two between-subject factors were assessed, namely, genotype and tissue. Due to the small sample sizes (n = 6), outliers were not removed, however the data was log transformed to ensure homogeneity of variance. Where interaction effects were significant, independent t-tests were performed.

When significant interactions were obtained for the mixed or factorial ANOVAs, the data was split according to age or tissue type and a one-way ANOVA was performed along with Games-Howell post hoc tests to determine the statistically significant differences within each age group or tissue type, respectively. When no significant interactions were obtained, the mixed or factorial ANOVA results were interpreted as is along with Sidak's correction for multiple testing to determine significant differences in the main effects.

3.10 METABOLOMICS

Samples were subjected to three different metabolomics platforms for metabolic investigation, namely, NMR, LC-MS/MS and GC-TOF-MS. For the quads, 13-14 samples per genotype were analysed on all three platforms in two separate batches. For the brain, 6-9 samples per genotype were analysed using NMR and GC-TOF-MS only in one batch. Brain samples were not analysed using the LC-MS/MS due to their high apolar metabolite content. Due to the amount of tissue required for all the biochemical assays performed, especially the immunohistochemistry, which made use of whole brain samples, fewer brain samples were available for metabolic profiling than quads samples, and in some specific samples, not enough tissue remained for the mass required by the metabolic profiling methods. All methods used were developed and described in detail by Terburgh (2019) and will only be briefly described here. All samples were randomised and analysed in one batch for the brain samples and two batches for the quad samples.

3.10.1 Instrumentation

The NMR instrumentation included a 500 MHz Bruker Avance™ III HD NMR spectrometer and an eVol® NMR digital syringe. For spectral processing and metabolite identification, Bruker Topspin (version 3.1) and Bruker AMIX (version 3.9.12) software were used respectively. To confirm metabolite identities, Bruker pH 7.0 and in-house spectral libraries were used.

The LC-MS/MS instrumentation included an Agilent© 1200 Series LC Front End with binary pump which was coupled to an Agilent© 6410 Series Triple Quadrupole mass analyser. The electrospray ionisation (ESI) source of the mass analyser was operated in positive ionisation mode. Data acquisition was performed using Agilent© MassHunter Workstation Software (version B02.01; Data acquisition for 6400 Series Triple Quadrupole) and MassHunter Optimiser software (version B02.01), configured to the MRM setting, and data extraction was performed using Agilent© MassHunter Workstation software (version B06.00; Qualitative Analysis and Quantitative Analysis).

The GC-TOF-MS instrumentation included an Agilent© 7890A series gas chromatograph, an Agilent© 7693 autosampler coupled to a LECO Pegasus HT time-of-flight mass analyser and an electron impact (EI) ionisation source. For data acquisition and extraction, the LECO Corporation ChromaTOF® software (version 4.5x) was used. The NIST11 commercial library and an in-house mass spectral library was used for spectral matching to identify important analytes.

3.10.2 Metabolite extraction and internal standards

For metabolite extraction, the following reagents were purchased from Honeywell, Burdick & Jackson: Methanol (#2304), HPLC-grade water (#BJ365CS) and chloroform (#0494). The internal standards *N, N*-dimethyl-L-phenylalanine (DMPA; #273910) and 3-phenylbutyric acid (3-PBA; #116807) were obtained from Sigma Aldrich.

For metabolite extraction, a modified monophasic Bligh-Dyer method (Gullberg *et al.*, 2004) was used with a solvent ratio of 3:1:1 (methanol:water:chloroform). Approximately 70 mg and 80 mg of tissue was weighed out, for the brain and quad aliquots, respectively, in Section 3.5.5. When calculating the solvent volumes, it was assumed that the samples consisted of 80% and 75% water for the brain and quads, respectively (Terburgh, 2019).

In order to account for variance that occurs during the metabolite extraction process, a known volume of internal standard (IS) mixture was added to each sample. This IS mixture consisted of compounds not naturally found in mouse muscle and brain tissue and that could be easily detected on all platforms, namely DMPA and 3-PBA, as first described by van Aardt *et al.* (2016). The concentrations (ppm) of internal standards in the mixture were calculated to allow the analysis of 50 ng DMPA in NMR samples, 25 ng DMPA in LC samples, and 25 ng 3-PBA in GC-TOF-MS samples (see Table 3-5).

On the day of metabolite extraction, methanol and the water plus IS mixture were added according to the exact mass of tissue weighed. Tungsten carbide and stainless steel beads (~7:1 (w/w), Brain: 3 mm Ø; Quads: 5 mm Ø and 3 mm Ø) were added to each tube and the tissues were then

pulverised by shaking in a Retsch MM 400 vibration mill for 2 min at 20 Hz and 30 Hz for the brain and quad samples respectively. After removing the beads, chloroform was added and the samples were vortexed and incubated on ice for 10 min. The homogenates were centrifuged at 20 000 x g for 10 min at 4°C and the supernatant was then carefully transferred to a clean tube. The extracts were then aliquoted for NMR (as shown by the extract volume column in Table 3-5) and for quality control (QC) samples and were then stored at -80°C. The QC sample was made up by pooling 50 µL from each of the other samples, creating aliquots and then preparing the QC samples in the same manner as all the other samples on all analytical platforms.

Table 3-5: Volumes measured on various metabolomics platforms

Analytical platform	Tissue type	Extract volume (µL)	Final sample volume (µL)	Injection volume (µL)	Mass IS analysed (ng)
<i>NMR</i>	Brain	140	140	N/A	50
	Quads	70	70	N/A	
<i>LC-MS/MS</i>	Quads	50	100	1	25
<i>GC-TOF-MS</i>	Brain	200	100	1	25
	Quads	40	100	1	

* For each platform, the table displays the initial tissue extract aliquot used (column 3), the final volume of each prepared sample (column 4), the sample volume injected on-column for chromatographic platforms (column 5), and the resulting mass IS in the analysed sample volume (column 6). For chromatographic platforms, the on-column IS mass was calculated from the final IS concentration in the sample and the injection volume.

On a separate day, the extracts were aliquoted (according to the extract volume column in Table 3-5) into 2 mL microvials (Agilent 5190-9062) using preslit PTFE caps (Agilent 5183-2076) for the vials used for LC-MS/MS and PTFE caps (Agilent 5182-0717) for vials used for GC-TOF-MS.

3.10.3 NMR

For NMR analysis the following reagents were purchased from Sigma Aldrich: Potassium phosphate monobasic anhydrous (KH₂PO₄; #229806), HPLC-grade water (#BJ365CS), sodium azide (NaN₃, #S2002) and potassium hydroxide (KOH; #P1767). From Merck, deuterium oxide (D₂O; #435767) and 3-(Trimethylsilyl) propionic acid (#269913) were obtained.

On the day of analysis, the NMR aliquots were evaporated under a stream of nitrogen gas at 37°C and then reconstituted in HPLC-grade water according to the final sample volume (column 4) in Table 3-5. The samples were centrifuged at 12 000 x g for 5 min at 25°C to remove any particles and the supernatant was then transferred to 2 mL glass vials (Agilent).

A chemical shift standard buffer solution (10%) was prepared as described by Dona *et al.* (2014), containing 1.5 M KH_2PO_4 , 100 ppm trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid and 13 ppm NaN_3 in D_2O (pH adjusted to 7.4). Of the sample ultra-filtrate and 10% buffer solution, 54 μL and 6 μL respectively were aspirated using an eVol NMR digital syringe and the total 60 μL was purged into a 2 mm NMR tube (100 mm in length), where it was then aspirated and purged to ensure thorough mixing. Thereafter, a wash sequence was initiated to clean the syringe before loading the next sample. The NMR tubes were then assembled using the Bruker MATCH system and loaded onto a SampleXpress autosampler.

As described by Terburgh (2019), the samples (maintained at a constant temperature of 300 K) were then measured in the 2 mm NMR MATCH tubes at 500 MHz, with the 5 mm triple-resonance inverse (TXI) probe head optimised for ^1H observation (spectra acquired as 128 transients in 32K data points with a spectral width of 6000 Hz, 12.0 ppm). The pulse sequence program NOESY-presat was used to suppress the H_2O resonance at 4.70 ppm, the acquisition time was set to 2.7 s and the receiver gain was set to 64. The run time per sample was 15 minutes and 45 seconds and each sample was automatically shimmed on the deuterium signal, locked, probe tuned and matched, and pulse calibrated. NMR analysis and processing were performed using Bruker Topspin (v 3.5), and further processing conducted using Bruker AMIX (v 3.9.14).

3.10.4 LC-MS/MS

The following reagents were purchased from Sigma Aldrich for the LC-MS/MS analysis: 1-butanol (#281549) and formic acid (#06440). From Honeywell, Burdick & Jackson, acetonitrile (ACN; BJ015CS) was obtained and from Fluka analytical, acetyl chloride (#00990) was obtained.

As mentioned previously, none of the brain samples were analysed using the LC-MS/MS. When aliquoting the LC-MS/MS quad samples, an additional stable isotope IS mixture ([final] = 2.5 ppm), made up in water, was added to each sample to account for variances during derivatisation and ionisation. The amino acids in the isotope mixture were purchased from Cambridge Isotope Laboratories and included: valine_ d_8 (#E161P), isoleucine_ d_{10} (#DLM-141-0.1), phenylalanine_ d_5 (#E151P), lysine_ d_4 (#DLM-2640), glutamine_ $\text{C}^{13}\text{5-N}^{15}\text{2}$ (#CNLM-1275-H), asparagine_ $\text{C}^{13}\text{4-N}^{15}\text{2}$, arginine_ d_4 (#DLM-6038) and citrulline_ d_4 (#DLM-6039). The acylcarnitines in the isotope mixture were obtained from VUmc and included: free carnitine_ d_3 , octanoylcarnitine_ d_3 , dodecanoylcarnitine_ d_3 and octadecanoylcarnitine_ d_3 . The samples were then evaporated under a stream of nitrogen gas at 37°C and frozen at -80°C until the day of analysis.

On the day of analysis, samples were derivatised via butylation by adding 300 μL butanolic hydrochloric acid [4:1 (v/v) N-butanol:acetyl chloride], vortexing and then incubating at 50°C for 1

hour. Samples were then once again completely evaporated under a stream of nitrogen gas at 37°C and reconstituted in a final volume (as indicated by the final sample volume column in Table 3-5) of water:acetonitrile (50:50, v/v) containing 0.1% formic acid. After thoroughly vortexing the samples, they were transferred to pulled point glass inserts (Agilent 5183-2085) so that each vial could be loaded on the Agilent 1200 series autosampler for LC-MS/MS analysis.

The LC-MS/MS was performed as described by Terburgh (2019). The column used for separation was a C18 Zorbax SB-Aq reverse phase (Agilent, 2.1 mm x 150 mm x 3.5 µm). Sample injection volume was 1 µL and the temperature of the column was maintained at 30°C throughout. To enhance metabolite separation, a mobile phase chromatographic gradient was used, at a flow of 0.3 mL/min, where solvent A consisted of water with 0.1% (v/v) formic acid and solvent B consisted of acetonitrile with 0.1% (v/v) formic acid. The gradient changes in solvent B and the time intervals were as follows: 5% for 1 min; increased to 20% over 2 min and then maintained for 3 min; increased to 100% over 7 min (after which the flow rate was adjusted to 0.35 mL/min) and then maintained for 5 min; lastly, decreased to 5% over 0.5 min and maintained for 1.5 min. An additional 10 min post-run was also performed to ensure equilibration of the column, thus giving a total run time of 30 min per sample. The mass spectrometer was operated in MRM mode, the parameters of which are shown in Table D8. The nitrogen gas ESI temperature was maintained at 300 °C (with a flow rate of 7.5 L/min); nebuliser pressure was kept at 30 psi and the capillary voltage at 3500 V.

3.10.5 GC-TOF-MS

The following reagents were obtained from Sigma Aldrich for GC-TOF-MS analysis: Hexane (#296090), methoxyamine hydrochloride (#226904) and pyridine anhydrous (#270970). O-bis(trimethylsilyl) trifluoroacetamide (BSTFA; #33027) was obtained from Supelco and trimethylchlorosilane (TMCS; #92360) from Fluka analytical.

When aliquoting the samples for GC-TOF-MS, an additional internal standard mix of 2-acetamidophenol (2-AAP; #A7000) and 4-phenylbutyric acid (4-PBA; #P21005) obtained from Sigma Aldrich was added to each sample to account for variances during derivatisation, such that a final concentration will be injected of 40 ppm for the brain and 25 ppm for the quads. Samples were then evaporated under a stream of nitrogen gas at 37°C and frozen at -80°C until the day of analysis.

On the day of analysis, the samples were derivatised, using freshly prepared reagents, first by oximation and then by silylation (Venter *et al.*, 2016; Esterhuizen *et al.*, 2018). A 20 mg/mL methoxyamine hydrochloride in pyridine was made up and 50 µL was added to each sample, the

samples were vortexed and then incubated at 60°C for 1 hour. Thereafter, 1% (v/v) TMCS was added to BSTFA and 50 µL of this 1% TMCS/BSTFA solution was added to each sample, the samples vortexed and then once again incubated at 60°C for 1 hour. After trimethylsilylation, the samples were transferred to flat-bottom inserts (Agilent 5181-3377) and each vial was loaded onto the Agilent 7693 autosampler for GC-TOF-MS analysis.

The GC-TOF-MS protocol described by Terburgh (2019) was used as described below. A Rxi-5Sil MS capillary column (Restek, 28.6 m x 250 µm x 0.25 µm) was used and 1 µL of the derivatised samples were injected in splitless mode with a 78.5 mm long split/splitless single taper deactivated FocusLiner with glass wool (Phenomenex, outside diameter 6.3 mm, inside diameter 4.0 mm), at 250°C. The GC oven temperature settings, using helium as carrier gas and a constant flow rate of 1.5 mL/min, were as follows: 70°C for 1 min; increased 7°C/min up to 120°C; increased 10°C/min up to 230°C; increased 13°C/min up to 300°C and held for 1 min; lastly, cooled to 70°C. This gave a total run time of 25.5 minutes per samples, throughout which the transfer line and ion source temperatures were held at 225°C and 200°C, respectively. Eluting compounds were fragmented by operating the mass spectrometer in EI ionisation (70V) mode. There was an initial solvent delay of 250 s, after which data (mass-to-charge (m/z) ratio range of 50-950 amu) was acquired at a rate of 20 spectra/s. Peaks with five apexing masses were detected using an expected peak width of 3 s and a signal-to-noise ratio of > 20.

3.10.6 Data analysis

3.10.6.1 Data pre-processing

The data generated from all three platforms was cleaned using Excel 365 (Microsoft) to filter out any metabolites that were not consistently and accurately measured, for missing value imputation and for normalisation to internal standards.

For the targeted LC-MS/MS data, all features with an area of less than 1000 (the limit of quantification), were removed. Thereafter, each dataset (from all three platforms) was grouped according to genotypes and QCs. Firstly, a missing value filter was applied to remove any features that were detected in less than 80% of the samples of a given genotype. Secondly, a QC variance filter was applied to remove any features with a coefficient of variance percentage (CV %) of more than 50% within the QC samples. Since the QCs were identical samples and were normalised to internal standards prior to QC filtering, features with such high variances were considered to be unstable and thus unreliable.

Missing value imputation was thereafter performed by replacing all empty data points with a value assumed to be the minimum detection limit (half of the smallest positive value in the original data), to allow for easier downstream statistical processing.

Normalisation to internal standards was then performed to account for differences between samples and to allow for batch comparisons. The LC-MS/MS data was normalised to stable isotopes (which were added prior to derivatisation) and then to DMPA (which was added during the initial monophasic metabolite extraction) to compensate for variation during both sample preparation, derivatisation and analytical errors. Stable isotopes were not available for all metabolites, so Pearson correlations between metabolites and isotope peak areas were performed using IBM SPSS Statistics version 25, and metabolites without their own isotopes were normalised to the isotope with which they best correlated. For NMR and GC-TOF-MS data, only one IS was used to normalise peak areas, namely DMPA and 3-PBA respectively.

3.10.6.2 Data pre-treatment

MetaboAnalyst 4.0 (Chong *et al.*, 2018) was used to perform data pre-treatment. The data was transformed using a generalised logarithm (glog) to create a more normal data distribution as well as to reduce the differences between metabolites with very low and high abundances. Auto-scaling was also performed to further reduce scaling differences between low and high abundance metabolites by centering around zero, rather than around the mean, and dividing by the SD of each variable (van den Berg *et al.*, 2006). Outlier detection was performed using principle component analysis (PCA) and heat maps. Any data points lying outside of the 95% confidence interval ellipsis of the PCA were excluded from further analysis.

3.10.6.3 Data quality checks

To assess the quality of the data, the repeatability and batch effects (in the case of the quad samples) were investigated. Scatter plots were constructed where the run order of the samples was indicated on the x-axis and the total signal obtained for each sample was indicated on the y-axis. To calculate the total signal of each sample, the relative peak intensities (after IS normalisation) of all metabolites measured in the sample were added together. This allowed a visual inspection of the distribution of all QCs (coloured dots) as well as differences between samples within different batches (where applicable). The distribution of the QCs was assessed by calculating the CV% for each metabolite within each QC and these were then plotted on a scatter plot with the CV% range on the x-axis and the % of metabolites (within that CV% range) on the y-axis.

The data from each platform was furthermore processed on MetaboAnalyst 4.0 where PCAs were constructed (as described in Section 3.10.6.2), but the data was separated into two groups for each platform, namely samples and QCs. Any batch effects or platform drift will result in noteworthy variation in the observed values from one analysis of the QC sample to the next. This variation is only detrimental if it is comparable to or exceeds the variation between experimental samples. By comparing the samples and QCs on a PCA, which projects the variation of a data source and allows for comparison between the variation from other data sources, the severity of shifts and drifts become apparent and indicate whether data correction is required. All data quality checks are shown in Section 5.2.

3.10.6.4 Statistical analysis of metabolomics data

When analysing the data, both statistical and practical significance were assessed, as they respectively determine whether results are probable due to chance and aid in understanding the biological magnitude of the differences observed (Sullivan & Feinn, 2012). Parametric univariate analyses were performed on the transformed and scaled data. This included independent t-tests with a significance level of 5%, to assess the differences between the averages of each genotype, and Cohen's d-value, a measure of effect size. The cut-off for significant effect sizes was set to 0.8. Whenever multiple significance tests are performed, the probability of false discoveries increases. To correct for multiple testing and to increase the probability of an accurate assessment of significance, the Benjamini-Hochberg adjustment to control the rate of false discovery (BH-FDR) was applied (Benjamini & Hochberg, 1995). For all platforms, features with both adjusted p-values <0.05 and d-values >0.8 were identified and combined for the brain and quads. The discriminatory metabolite data was autoscaled for the brain and quads respectively and PCAs were constructed in MetaboAnalyst 4.0 to inspect the data clustering, covariance and discriminatory power. Lastly, metabolite identities were assigned to features using mass spectra and retention times and matching them to commercial and in-house spectral libraries as well as public databases. The confidence of the metabolite identities were ranked according to Schymanski *et al.* (2014). For NMR and LC-MS/MS, level one identification was achieved, while for GC-TOF-MS level one to three identification was obtained.

CHAPTER 4: BIOCHEMICAL RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this chapter, the results of Objectives 1-4 (which excludes the metabolomics data – objective 5) will be presented together with a discussion thereof. The genetic characterisation results are shown first where the absence or presence of *Ndufs4* and *Mt1* DNA levels were determined using liver samples from all mice included in this study, followed by the *Ndufs4* mRNA levels in the brain and quads and finally, NDUFS4 protein determination. The phenotyping results are thereafter discussed by firstly displaying the growth and survival curves, followed by the locomotor activity and ataxia assays. Thereafter, the other biochemical assays are shown, including the immunohistochemistry images, enzyme activity assays and protein oxidation results.

4.2 ANIMAL NUMBERS AND SAMPLES

The total number of animals included in this study was shown in Table 3-1, while a detailed breakdown of all animals, their gender and the number of days that they lived is shown in Tables A1 and A2. The tissues used for analyses included tail snips, liver, brain and quads.

4.3 GENETIC CHARACTERISATION

Genetic characterisation of the mice included in this study was performed as described in Section 3.3 and included the genotyping (*Ndufs4* and *Mt1*) of tail snips followed by a second confirmation using liver tissue obtained after euthanising the animals. The protein levels of NDUFS4 in one mouse per genotype was also quantified in the brain and quads.

4.3.1 *Ndufs4* and *Mt1* at the DNA level

Liver tissue from all animals included in the phenotyping section of this study were used to confirm the correct genotype (as initially determined using tail snips). This was done as described in Sections 3.3.1 and 3.3.2 and involved PCR and gel electrophoresis (results not shown); an example photo of a gel is shown in Figure A1. Results obtained from the liver samples, that differed from the original genotype that was determined using the tail snips, were excluded from all further analyses. Thus, the genotypes of all mice included in this study were confirmed. As described in Section 3.3.3, the *Mt1* copy number was also determined using qPCR. The scatter plot shown in Figure 4-1 depicts the greatly increased *Mt1* copy number in the OVER and KO OVER mice relative to the other two genotypes. This served as confirmation that the *Mt1* genotype was correct for all mice included in this study. The expected copy number in the *TgMt1* mice is

114, but since relative quantification was performed here as opposed to absolute quantification, the copy numbers obtained varied.

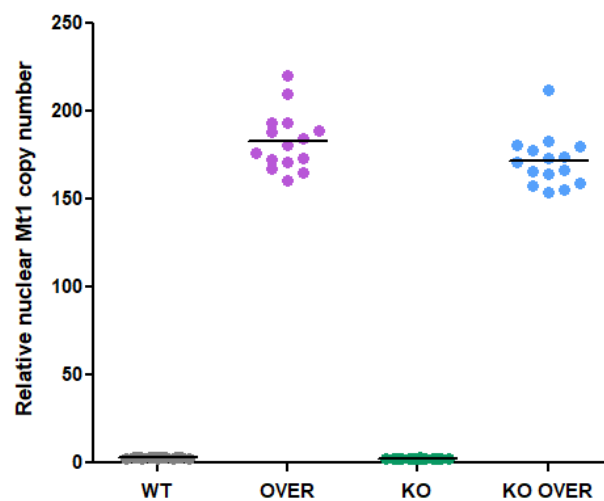


Figure 4-1: Vertical scatter plot depicting the nuclear *Mt1* copy number in liver samples, relative to β -actin, for all samples included in this study. The horizontal lines indicate the mean (n = 60).

4.3.2 *NDUFS4* protein confirmation

The model was further characterised by confirming the absence of NDUFS4 in the KO and KO OVER mice by SDS-PAGE and western blot, as described in Section 3.3.4. The model was also previously validated by others from the MRL of the NWU (Mereis, 2018; Terburgh, 2019; Coetzer, 2020; Mienie, 2020). The absence of NDUFS4 in the KO and KO OVER genotypes is clearly depicted in Figure 4-2. The decreased VDAC expression seen in the quads of the KO OVER mice was believed to have occurred due to inefficient transfer during the transfer of proteins from the SDS-gel to the PVDF membrane. This decrease was not seen in any other validations performed by others within the group or by this author.

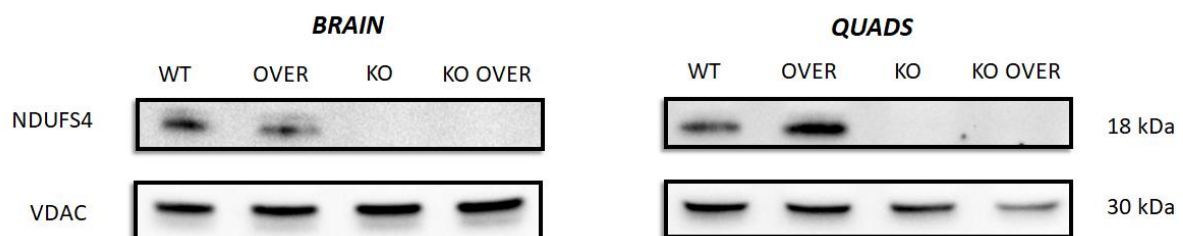


Figure 4-2: Western blot of NDUFS4 and VDAC1 following separation by SDS-PAGE in both brain and muscle tissue (n = 4).

4.3.3 *Mt1* mRNA results

To further confirm the model, the mRNA levels of *Mt1* relative to $\beta 2m$ were determined in the brain and quads of six mice per genotype. Vertical scatter plots of the brain and quad mRNA data is shown in Figure 4-3A and B.

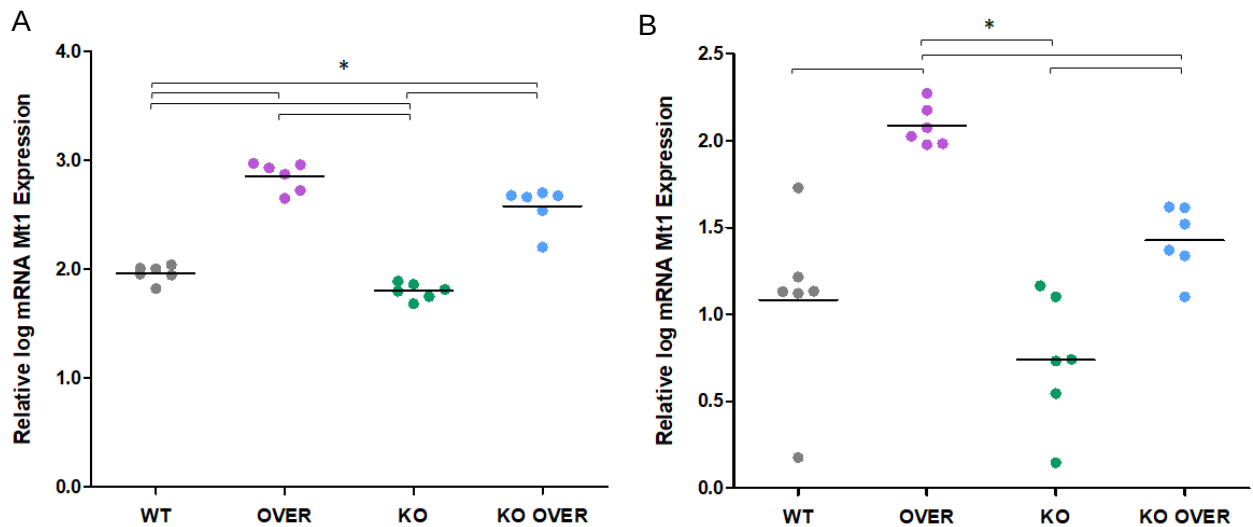


Figure 4-3: mRNA *Mt1* expression relative to β -2-microglobulin in the (A) brain and (B) quads (n = 24). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects), while controlling for gender (covariate), on relative mRNA *Mt1* expression. The results from this ANOVA were as follows:

- There was a non-significant interaction effect between genotype and tissue, $F(3,40) = 1.337$, $p = 0.276$, $\eta_p^2 = 0.093$.
- There was a non-significant main effect of gender on the relative mRNA *Mt1* expression, $F(1,40) = 2.062$, $p = 0.159$, $\eta_p^2 = 0.050$.
- There was a significant main effect of genotype on the relative mRNA *Mt1* expression, $F(3,40) = 54.767$, $p < 0.001$, $\eta_p^2 = 0.808$. Sidak's correction for multiple testing showed that the mRNA *Mt1* levels in the OVER and KO OVER mice were both significantly greater than in the WT and KO mice, and furthermore that the levels were greater in the OVER mice compared to the KO OVER mice (all p-values < 0.001). There was no significant difference between WT and KO mice ($p = 0.15$).
- There was also a significant main effect of tissue on the relative mRNA *Mt1* levels, $F(1,40) = 180.576$, $p < 0.001$, $\eta_p^2 = 0.822$. The mRNA *Mt1* expression in the brain ($M = 2.298$, $SD = 0.457$) was significantly greater than in the quads ($M = 1.333$, $SD = 0.596$).

As seen in Figure 4-3A and B, the variation in the brain data was much less than that seen in the quad data, but due to the small sample sizes, outliers could not be excluded. As desired, the OVER and KO OVER mice showed significantly increased *Mt1* expression at the mRNA level, thus confirming that which was seen at the DNA level (Figure 4-1) and further validating the characterisation of this mouse model. Contrary to Ono *et al.* (1997) but in agreement with Iszard *et al.* (1995) (as discussed in Section 2.7.2.2), gender did not appear to affect the MT mRNA levels in the brains of the OVER and KO OVER mice. While different brain regions were not investigated here, the WT vs OVER results correspond with that reported by Manso *et al.* (2016) who reported significantly increased *Mt1* mRNA in numerous brain areas in *TgMt1* mice that they investigated in an Alzheimer's disease model.

However, contrary to the multiple reports of increased expression of MTs in MDs in response to increased ROS, as reviewed by Lindeque *et al.* (2010) and reported by others (Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006), the KO mice did not show a significant increase in mRNA *Mt1* expression compared to WT mice and by visual inspection appeared to lie beneath the WT mice. A similar observation was made for the OVER and KO OVER mice where the *Mt1* expression was significantly decreased in the KO OVER mice. These results thus suggest *TgMt1*-induced *Mt1* overexpression only in the two tissues investigated, with an apparent downregulated *Mt1* expression in the *Ndufs4*^{-/-} mice. As shown later in this thesis, increased oxidative stress does not appear to be as evident in the KO and KO OVER mice as expected, and could thus explain the lack of *Mt1* induction shown here. It does not, however, explain the downregulation that was seen. As discussed in Section 2.6.5, inflammation, oxidative stress and zinc homeostasis are all factors that affect the transcription of *Mt1*, and since these are all also known cellular consequences of CI dysfunction, their interactions with the MT regulatory elements may result in the apparent downregulation in KO and KO OVER mice, but further investigation is warranted. In Section 3.6, however, increased MT1 expression in specific brain regions is shown in the KO compared to WT mice, thus also indicating the presence of a tissue-specific expression profile that may be overlooked in whole tissues – as was used here.

4.4 PHENOTYPIC EVALUATION

As detailed in Section 3.5, 20 mice (ten KO and ten KO OVER) underwent survival analysis and were not subjected to any motor activity analyses, while 48 other mice (n = 12 per genotype) underwent the locomotor activity and ataxia assays. The results of these 68 mice are shown here, regardless of gender, but to determine whether or not gender may have played a role in the phenotype and lifespan of the mice, all data was also split according to gender as shown in Appendix B. In Section 4.4.4 and 4.4.5, line graphs were used as opposed to bar graphs, even though the genotypes are independent entities, to depict the interaction between genotype and

age. The error bars on these line graphs also represented 95% confidence intervals, and as such, genotypes where error bars did not overlap indicated statistically significant differences between P30 and P50.

4.4.1 Growth curves

Due to the failure to thrive phenotype reported in *Ndufs4*^{-/-} mice (Kruse *et al.*, 2008), the weights of all mice included in the phenotyping and survival curve analyses were recorded and are shown in Figure 4-4. The error bars depicted on the growth curves are at 95% confidence intervals so that ages where the error bars did not overlap could be considered to be statistically significant differences.

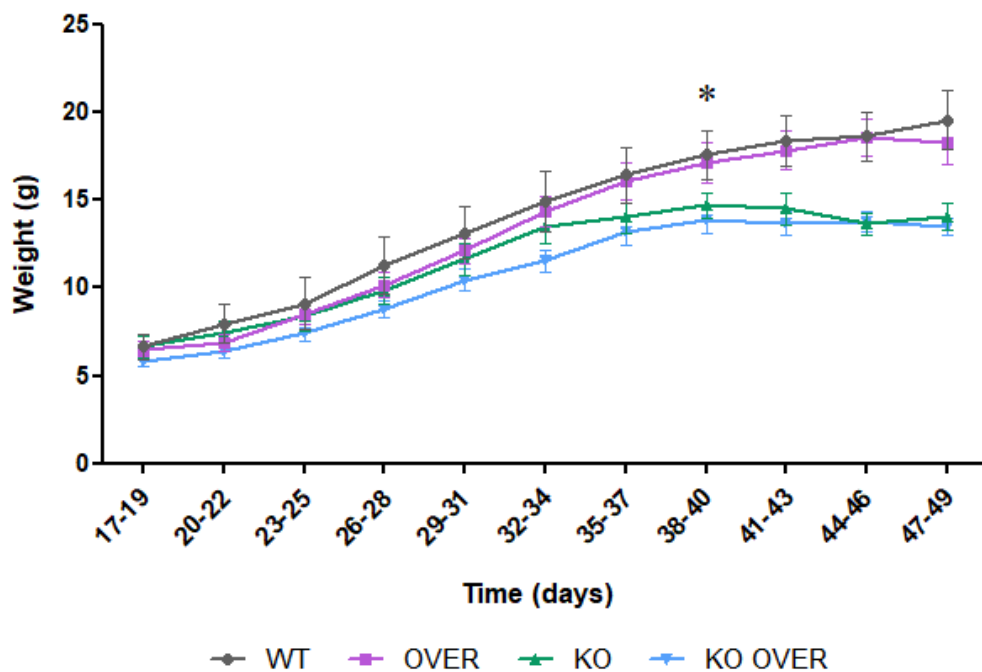


Figure 4-4: Growth curves of WT (n = 12), OVER (n = 12), KO (n = 22) and KO OVER (n = 22) mice, included in phenotyping and survival analyses, indicating their weight over time. Error bars represent the 95% confidence interval of the mean at each time interval.

From P38 onwards, the WT and OVER mice had weighed significantly more on average than the KO and KO OVER mice, but no distinction could be made between the KO and KO OVER mice or between the WT and OVER mice. At P32-34, the KO mice weighed significantly more than the KO OVER mice, but thereafter, the weights of the KO and KO OVER mice remained similar. This may suggest that *Mt1* overexpression results in decreased body weight during the early-mid stages of CI dysfunction, but since no differences were observed between WT and OVER mice, this is unlikely and can most likely be attributed to biological variation. The weights obtained for the OVER and KO mice corresponded with that observed by others (Kruse *et al.*, 2008; Felici *et*

al., 2014; de Haas *et al.*, 2016; Manso *et al.*, 2016). When stratifying the data based on gender (Figure B3), similar results were obtained for both males and females to that observed in Figure 4-4, with no significant differences between the KO and KO OVER mice or between the WT and OVER mice. It thus appears that the overexpression of *Mt1* did not have a significant effect on the body weight of *Ndufs4*^{-/-} mice and thus did not improve their failure to thrive.

4.4.2 Survival analysis

As described in Section 3.5.2, ten KO and ten KO OVER mice were left to die naturally and the age at which they died recorded. As described in Section 3.9, a Log Rank (Mantel-Cox) test was performed to determine differences in the survival rate of KO and KO OVER mice (as shown in Figure 4-5). The survival distributions were however not statistically significant, $\chi^2(1) = 1.153$, $p = 0.283$.

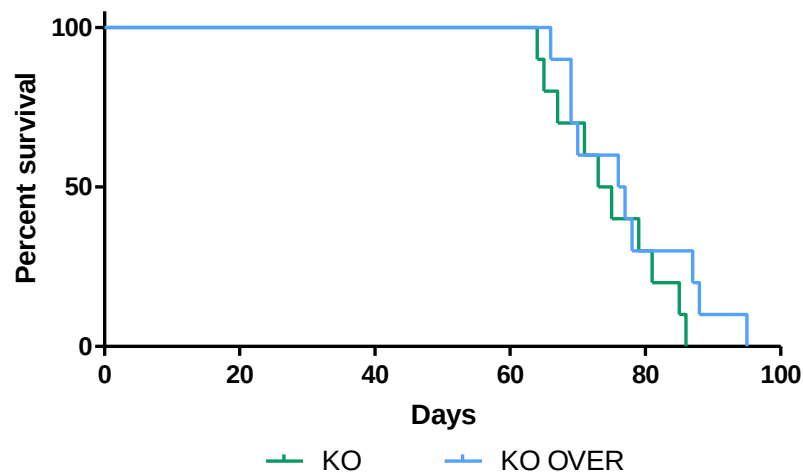


Figure 4-5: Mantel-Cox survival curve of KO OVER mice compared to KO mice (n = 10/genotype) over time.

Survival curves were also constructed where the KO and KO OVER mice were stratified according to gender (shown in Figure B4). Log Rank (Mantel-Cox) tests on both male and female survival rates also did not show significant survival distributions for KO and KO OVER mice (Appendix B2). Survival analyses thus indicate that the overexpression of *Mt1* does not lead to a statistically significant increase in longevity in the *Ndufs4*^{-/-} mice. While MTs have increasingly been shown to provide a survival advantage in neurological diseases and injury (Hidalgo *et al.*, 2001), this protective effect does not appear to be evident in the case of *Ndufs4* deficiency. The mean number of days that these KO and KO OVER mice (74.6 and 77.5 days respectively) survived was slightly higher than the range reported in other studies for *Ndufs4*^{-/-} mice (~P50 to P75) (Quintana *et al.*, 2012; Johnson *et al.*, 2013; Felici *et al.*, 2014; Jain *et al.*, 2016). The genetic background against which the mice were backcrossed may have played a role in the differences seen in the survival age and phenotyping results that we obtained compared to other studies.

Different genetic backgrounds bring about different phenotypic responses and may thus result in the disease phenotype presenting earlier or more severely than other strains (Schauwecker, 2011).

4.4.3 Physical appearance and behaviour observation

As described in Sections 3.5.2, 3.5.4.2 and 3.5.4.3, to visually depict the tests performed on the animals as well as to demonstrate animal behaviour, videos of the animals were recorded at different ages during testing. While videos were recorded for the balance beam test and rotarod test for all mice at both P30 and P50, only one mouse per genotype (of similar age and same gender) was randomly selected for the videos included here for demonstrative purposes only. In some instances, for the sake of time, only OVER mice were included in the video as a representative of both WT and OVER mice since no phenotypic differences were seen between mice from these two genotypes.

A comparison of the behavioural traits in mice of the different genotypes as well as the progression of the disease as the mice aged can be seen at <http://bit.ly/micebehaviour>.

The balance beam and rotarod videos of mice from the various genotypes at P50 can be seen at <http://bit.ly/balance50> and <http://bit.ly/rotarod50>, respectively.

4.4.4 Locomotor activity

Open field testing was used to evaluate locomotor activity, as described in Section 3.5.3, since the KO mice had been shown to have normal locomotor activity up to ~P30, after which the mice became lethargic (Kruse *et al.*, 2008). Figure 4-6A and B depict vertical scatter plots of the total distance travelled (in cm) and Figure 4-8A and B depict the total time rested (in s.), during the 5 minute observation period, at P30 and P50, respectively. All data was also stratified according to gender as shown in Figure B6 and Figure B7.

4.4.4.1 Total distance travelled

As discussed in Section 3.9, a mixed ANOVA was performed to determine the effect of genotype (the between-factor subjects) and postnatal day age (the within-factor subjects) on the locomotor activity, while controlling for gender (covariate). For the total distance travelled, there was a non-significant interaction between age and gender, $F(1,40) = 0.921$, $p = 0.343$, $\eta^2 = 0.023$. There was however a significant effect for the interaction between age and genotype, $F(3,40) = 3.794$, $p = 0.017$, $\eta^2 = 0.222$, as can be seen by the crossing of the lines in Figure 4-7.

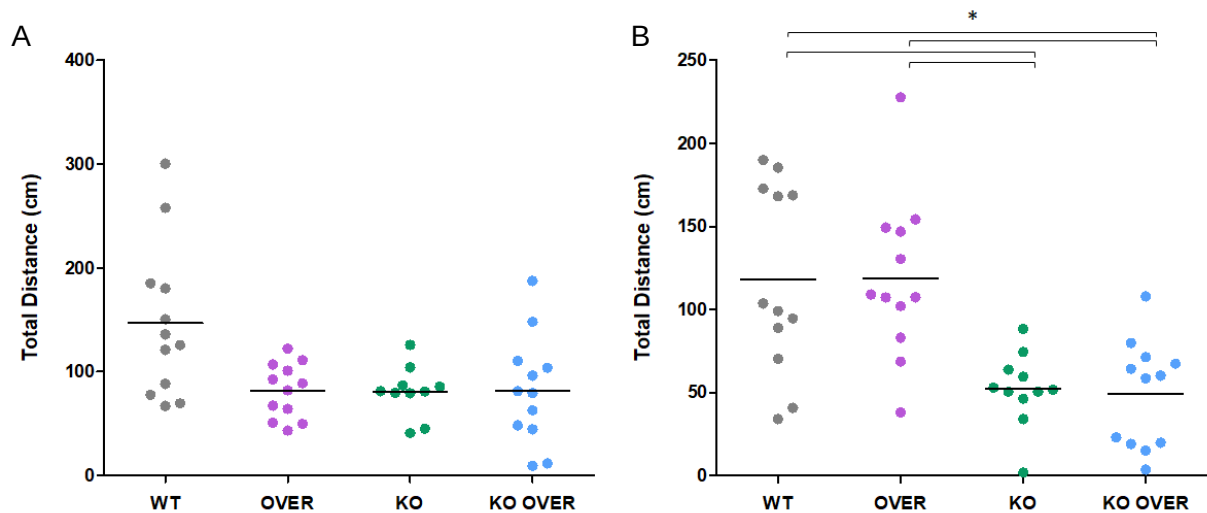


Figure 4-6: Vertical scatter plots depicting the total distance travelled by mice of the four genotypes (n = 12/genotype) at (A) P30 and (B) P50 over 5 min. Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

Games-Howell post-hoc tests indicated no significant differences between any of the genotypes at P30, however, at P50 the following significant differences were found:

- WT mice travelled on average 66.99 cm further than KO mice (SE = 17.83, $p = 0.009$) and 74.18 cm further than KO OVER mice (SE = 18.28, $p = 0.004$).
- OVER mice travelled on average 64.38 cm further than KO mice (SE = 16.59, $p = 0.008$) and 71.57 cm further than KO OVER mice (SE = 17.07, $p = 0.004$).

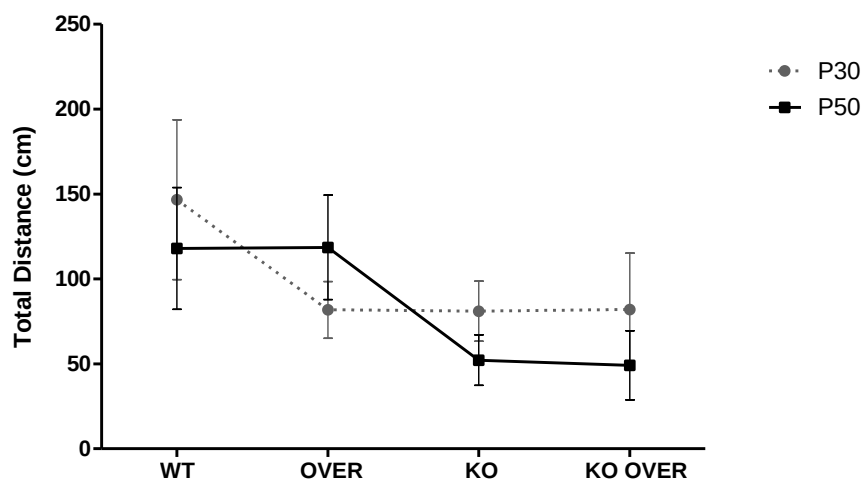


Figure 4-7: Line graph depicting the mean distance travelled by mice of the four genotypes (n = 12/genotype) at P30 and P50 over 5 min. Error bars represent 95% confidence interval of the mean.

4.4.4.2 Rest time

For the total rest time, there was no interaction effect between age and gender, $F(1,43) = 0.072$, $p = 0.790$, $\eta_p^2 = 0.002$, nor between age and genotype, $F(3,43) = 2.351$, $p = 0.086$, $\eta_p^2 = 0.141$, however the effect size of the interaction between age and genotype was large. Rest time did not differ significantly between P30 and P50, $F(1,43) = 0.019$, $p = 0.891$, $\eta_p^2 = 0.000$ (interaction effect depicted in Figure 4-9), nor between males and females, $F(1,43) = 0.479$, $p = 0.492$, $\eta_p^2 = 0.011$. There was however a significant main effect of rest time on genotype, $F(3,43) = 13.391$, $p < 0.001$, $\eta_p^2 = 0.483$ where Sidak's correction for multiple testing revealed the following significant differences:

- WT mice rested on average 28.422 s less than KO mice (SE = 7.734, $p = 0.004$) and 43.032 s less than KO OVER mice (SE = 7.748, $p < 0.001$).
- OVER mice rested on average 22.635 s less than KO mice (SE = 7.788, $p = 0.034$) and 37.245 s less than KO OVER mice (SE = 7.748, $p < 0.001$).

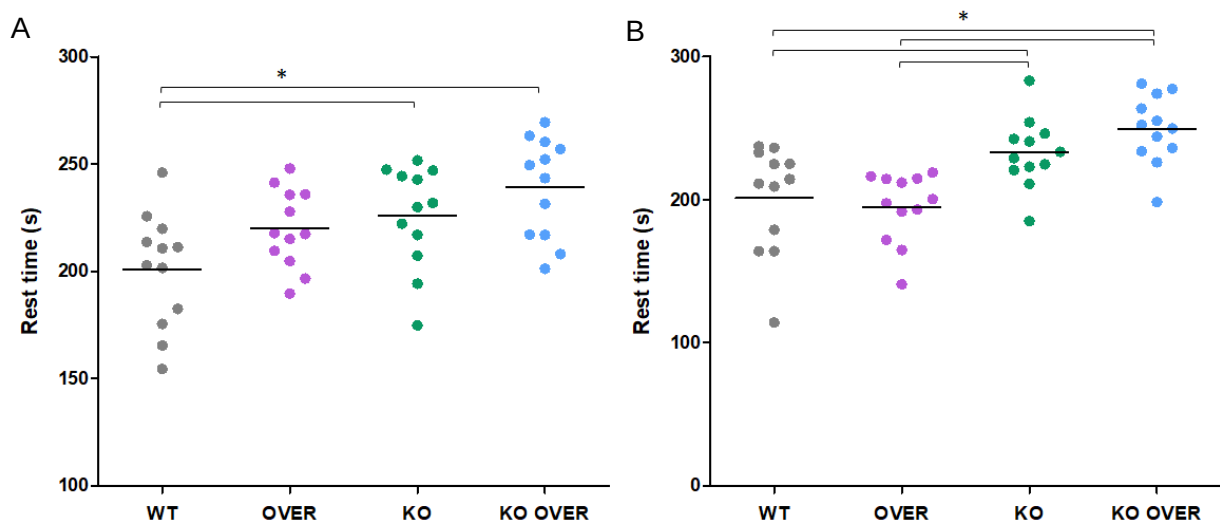


Figure 4-8: Vertical scatter plots depicting the rest time of mice of the four genotypes ($n = 12/\text{genotype}$) at (A) P30 and (B) P50 over 5 min. Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

While open field testing typically takes place over much longer periods of time (such as 24-72 hours) when observing animal behaviour, the severe muscle phenotype observed in the *Ndufs4*^{-/-} mice allowed for a much shorter observation period (5 min) to obtain statistically significant differences between the WT/OVER and KO/KO OVER mice, similar to that seen in Kruse *et al.* (2008) at P30 and P50.

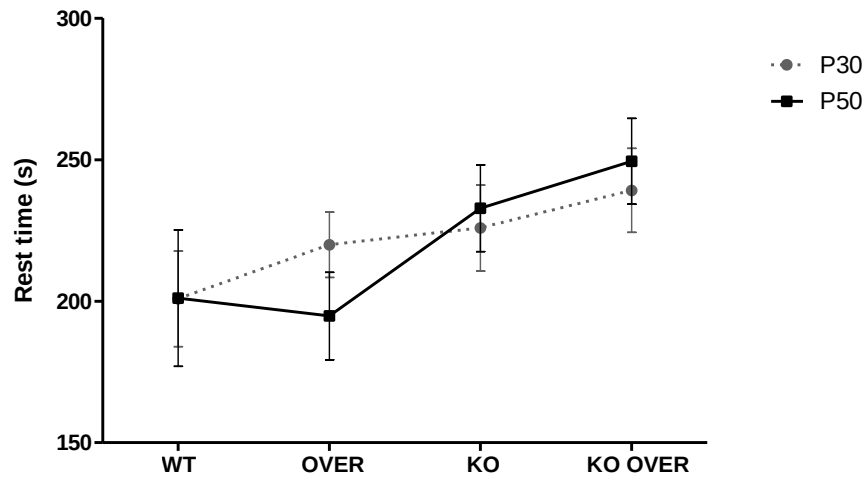


Figure 4-9: Line graph depicting the rest time (in seconds) of mice of the four genotypes (n = 12/genotype) at P30 and P50 over 5 min. Error bars represent 95% confidence interval of the mean.

4.4.5 Ataxia assays

Due to the reports of worsening ataxia and impaired motor activity in *Ndufs4*^{-/-} mice as the phenotype progresses, motor assessments were performed on our mice at P30 and P50.

4.4.5.1 Wire grid hang test

To test the muscle strength of the mice, the wire grid hang test was performed, as described in Section 3.5.4.1. Vertical scatter plots depicting the mean physical impulse are displayed in Figure 4-10A and B for P30 and P50, respectively. All data was also stratified according to gender as shown in Figure B8.

As discussed in Section 3.9, a mixed ANOVA was performed to determine the effect of genotype (the between-factor subjects) and postnatal day age (the within-factor subjects) on the mean physical impulse while controlling for gender (covariate). The interaction effect of age and gender was not significant, $F(1,43) = 1.081$, $p = 0.304$, $\eta_p^2 = 0.025$, while the interaction effect of age and genotype was however significant, $F(3,43) = 21.572$, $p < 0.001$, $\eta_p^2 = 0.601$ (interaction effect depicted in Figure 4-11). Games-Howell post-hoc tests indicated no significant differences between any of the genotypes at P30, however, at P50 the following significant differences were found:

- WT mice were able to hold on an average 12.747 s longer than KO mice (SE = 3.131, $p = 0.003$) and 16.695 s longer than KO OVER mice (SE = 2.563, $p < 0.001$).
- OVER mice were able to hold on an average 15.227 s longer than KO mice (SE = 3.424, $p = 0.001$) and 19.175 s longer than KO OVER mice (SE = 2.913, $p < 0.001$).

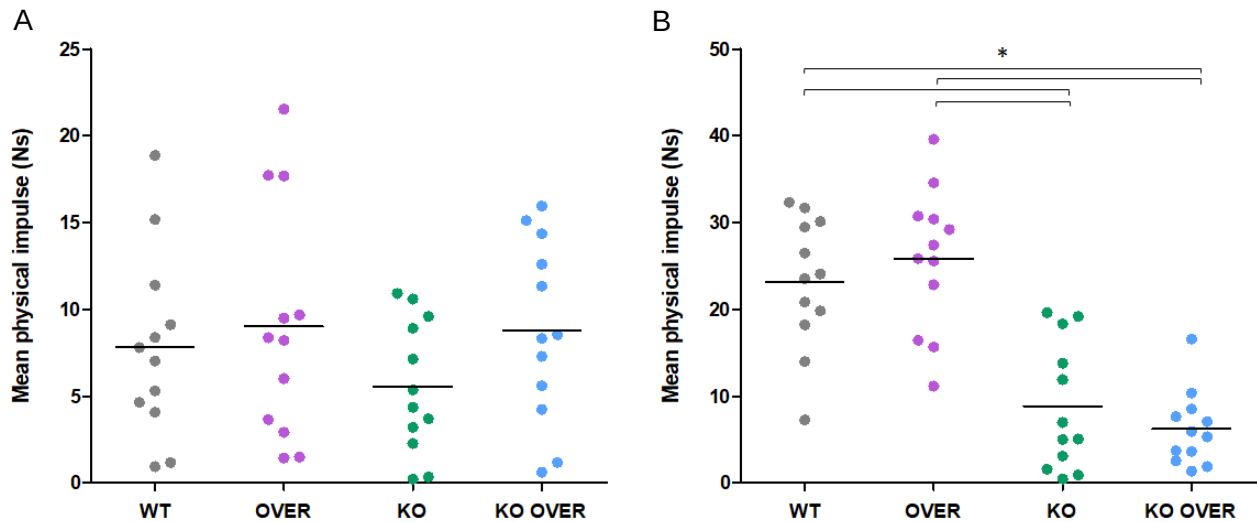


Figure 4-10: Vertical scatter plots depicting the mean physical impulse (in Newton second) of mice of the four genotypes (n = 12/genotype) at (A) P30 and (B) P50. Horizontal bar indicates the mean and brackets indicate statistically significant differences where p < 0.05 (*).

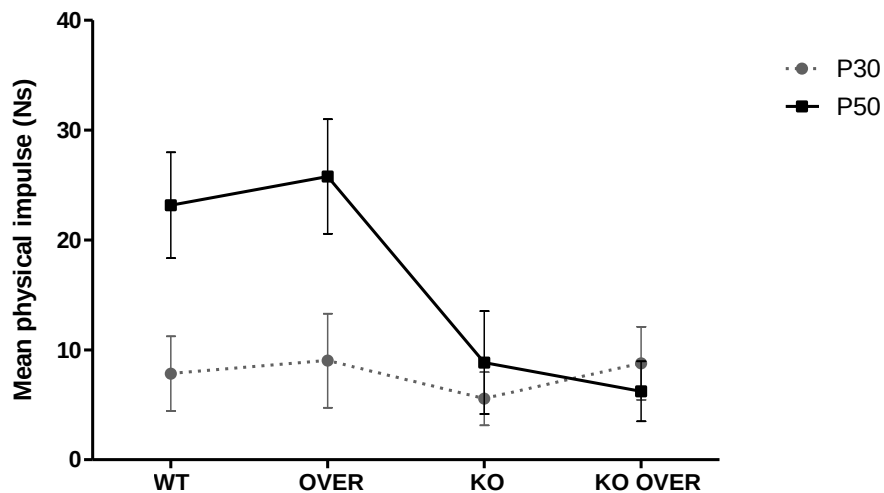


Figure 4-11: Line graph depicting the mean physical impulse (in Newton second) of mice of the four genotypes (n = 12/genotype) at P30 and P50 over 5 min. Error bars represent 95% confidence interval of the mean.

Kruse *et al.* (2008) performed the same test on the KO mice, but the results for this were, however, not reported other than indicating a worsening of ataxia in the KO mice. While absolute values of mean physical impulse could not be obtained from other studies on KO or OVER mice, the relative differences between the WT and KO mice – where KO mice showed poor muscle strength and the inability to hold on for as long as WT mice – in other grip and strength tests (Felici *et al.*, 2014; de Haas *et al.*, 2016) were similar to those seen for our wire grid hang test.

4.4.5.2 Balance beam

To test the balance ability of the mice, the balance beam test was performed, as described in Section 3.5.4.2. Vertical scatter plots depicting the amount of time it took mice to cross the balance beam are displayed in Figure 4-12A and B for P30 and P50, respectively. All data was also stratified according to gender as shown in Figure B9.

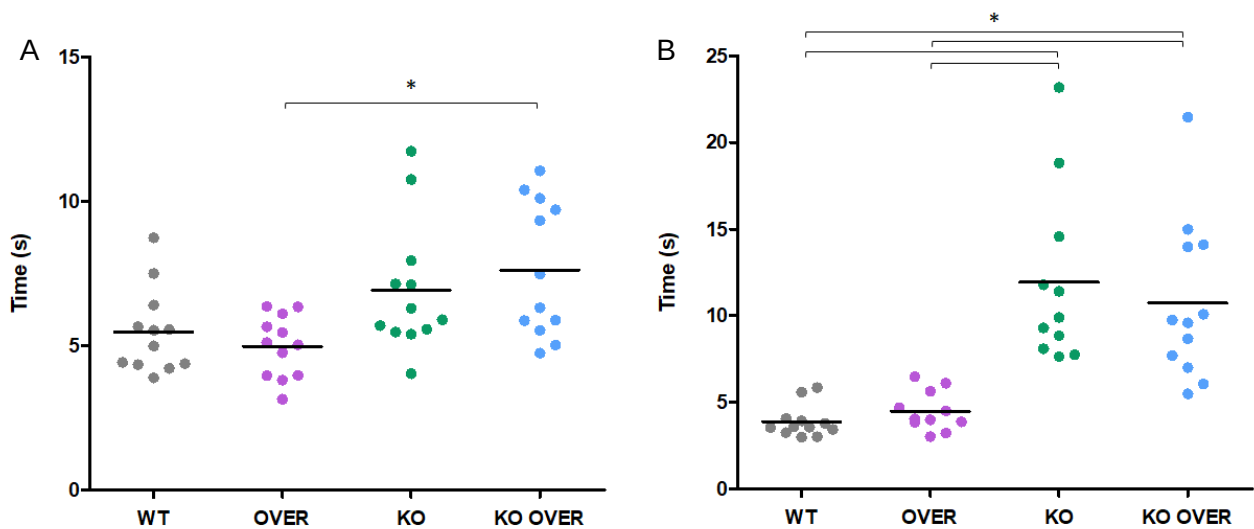


Figure 4-12: Vertical scatter plots depicting the time taken (in seconds) for mice of the four genotypes (n = 12/genotype) to cross a 0.8 m balance beam at ages (A) P30 and (B) P50. Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

As discussed in Section 3.9, a mixed ANOVA was performed to determine the effect of genotype (the between-factor subjects) and postnatal day age (the within-factor subjects) on the balance of the mice, while controlling for gender (covariate). The interaction effect of age and gender was not significant, $F(1,41) = 0.016$, $p = 0.899$, $\eta_p^2 < 0.001$, but the interaction effect of age and genotype was however significant, $F(3,41) = 9.077$, $p < 0.001$, $\eta_p^2 = 0.399$ (interaction effect depicted in Figure 4-13). Games-Howell post-hoc tests indicated the following significant differences between genotypes:

- OVER mice at P30 crossed the balance beam on average 2.641 s faster than KO OVER mice (SE = 0.742, $p = 0.013$).
- WT mice at P50 crossed the balance beam on average 8.427 s faster than KO mice (SE = 1.640, $p = 0.002$) and 7.328 s faster than KO OVER mice (SE = 1.386, $p = 0.001$).
- OVER mice at P50 crossed the balance beam on average 7.815 s faster than KO mice (SE = 1.655, $p = 0.004$) and 6.716 s faster than KO OVER mice (SE = 1.404, $p = 0.003$).

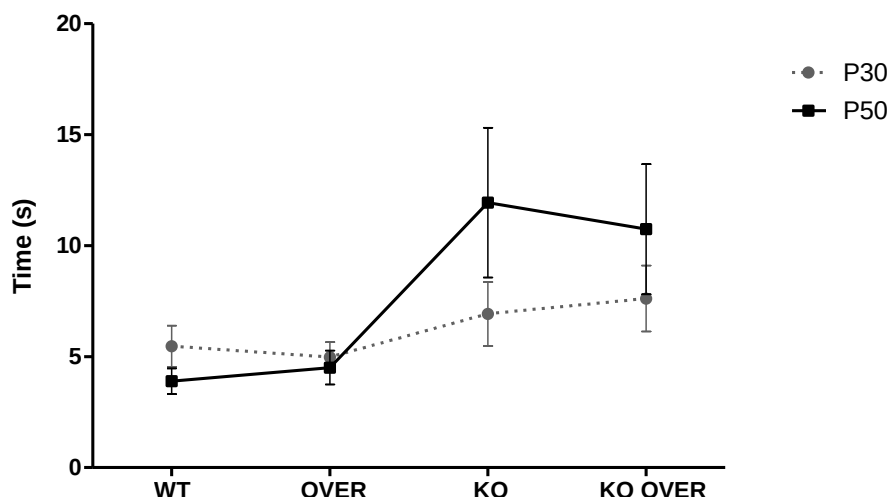


Figure 4-13: Line graph depicting the time taken (in seconds) by mice of the four genotypes (n = 12/genotype) to cross a balance beam at P30 and P50 over 5 min. Error bars represent 95% confidence interval of the mean.

Similarly to the wire grid hang test, studies with comparable balance beam values could not be found, but the relative differences seen between the WT and KO mice was comparable to that reported in similar studies (Kruse *et al.*, 2008; Quintana *et al.*, 2010; Felici *et al.*, 2014). The variation in these results was very high, especially for the KO and KO OVER mice at P50. One aspect to the variation was that this test relied on animal behaviour, which could not be predicted, and which will inherently vary between different animals. Some of the mice, regardless of the training, would tend to stop halfway across the beam and require nudging to continue, so not all mice seamlessly crossed the beam at a fixed pace. Other mice would be more inquisitive and would slow down to sniff the beam or possibly look for an alternative way of getting off the beam. Secondly, not all KO or KO OVER mice displayed the same severity of the disease at the same age, and thus performed better or worse than other mice from the same genotype and of the same age, depending on their phenotype at that stage.

4.4.5.3 Rotarod

To test the motor coordination of the mice, the rotarod was used, as described in Section 3.5.4.3. Vertical scatter plots depicting the amount of time the mice were able to stay on the accelerating rod are displayed in Figure 4-14A and B for P30 and P50, respectively. All data was also stratified according to gender as shown in Figure B10.

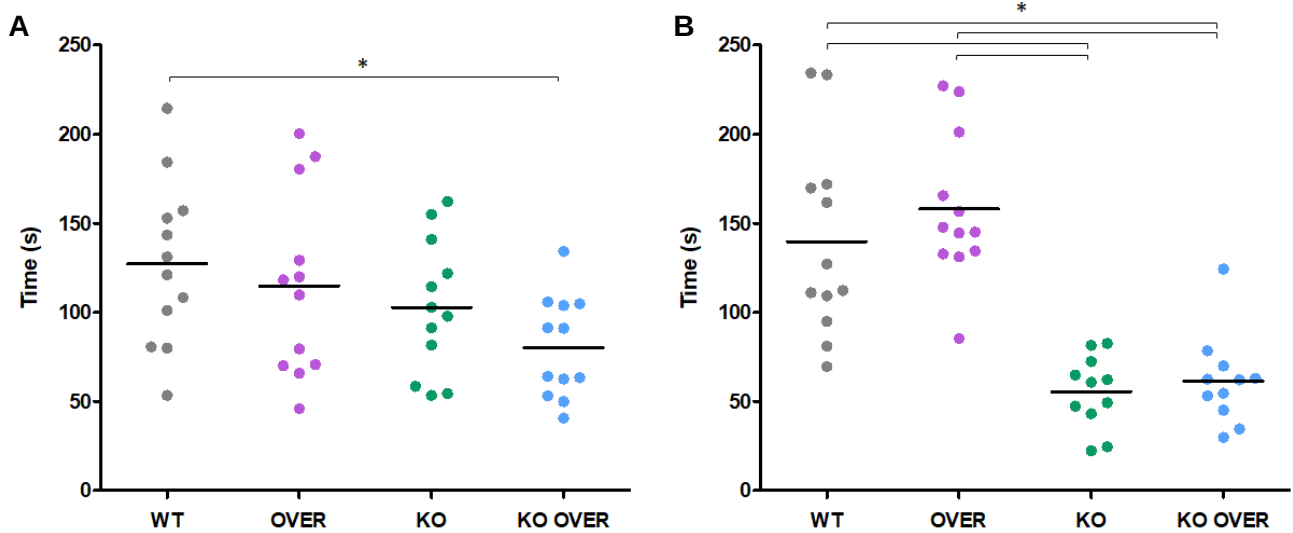


Figure 4-14: Vertical scatter plots depicting the latency to fall (in seconds) for mice of the four genotypes (n = 12/genotype) at (A) P30 and (B) P50. Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

As discussed in Section 3.9, a mixed ANOVA was performed to determine the effect of genotype (the between-factor subjects) and postnatal day age (the within-factor subjects) on the latency to fall from the rotarod, while controlling for gender (covariate). The interaction effect of age and gender was not significant, $F(1,41) = 0.156$, $p = 0.695$, $\eta_p^2 = 0.004$, while the interaction effect of age and genotype was significant, $F(3,41) = 6.333$, $p = 0.001$, $\eta_p^2 = 0.317$ (interaction effect depicted in Figure 4-15). Games-Howell post-hoc tests indicated the following significant differences between genotypes:

- WT mice at P30 latency to fall from the rotarod was on average 46.935 s slower than KO OVER mice (SE = 15.731, $p = 0.036$).
- WT mice at P50 latency to fall from the rotarod was on average 80.823 s slower than KO mice (SE = 16.862, $p = 0.002$) and 78.095 s slower than KO OVER mice (SE = 17.605, $p = 0.002$).
- OVER mice at P50 latency to fall from the rotarod was on average 98.403 s slower than KO mice (SE = 14.217, $p < 0.001$) and 95.676 s slower than KO OVER mice (SE = 15.091, $p < 0.001$).

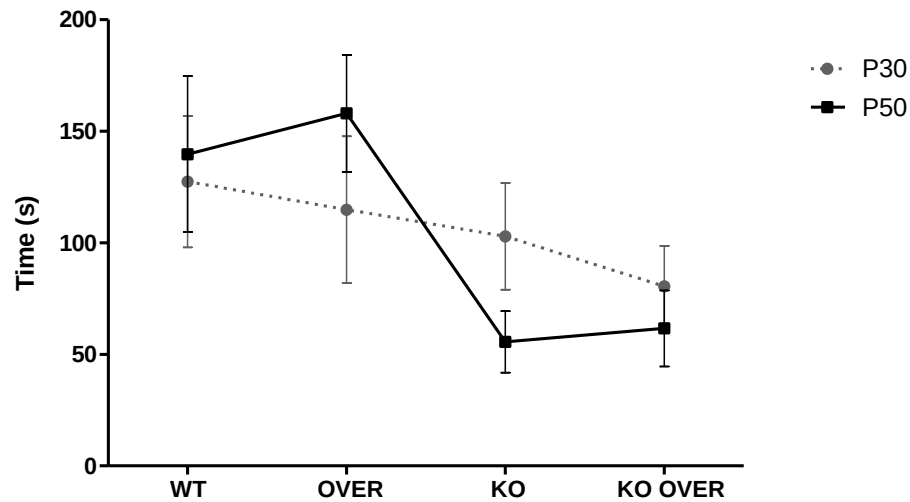


Figure 4-15: Line graph depicting the latency to fall (in seconds) of mice of the four genotypes (n = 12/genotype) at P30 and P50 over 5 min. Error bars represent 95% confidence interval of the mean.

It is important to note that the latency to fall times recorded did not necessarily mean that the mice fell off the rotarod. Due to the design of the apparatus, with the infrared beam above the rod determining when the timer stopped, mice would on occasion slip below the level of the infrared beam (which then caused the rod to stop rotating) and because of the rod being stationary again, specific mice would sporadically be able to climb back onto the rod. However, the latency to fall time was recorded as that time when they slipped to below the infrared beam. Had the rod not stopped rotating at this time, it is highly improbable that the mice would still have been able to climb back onto the rod but that they would indeed have fallen.

While very few significant differences were seen at P30 (other than that between WT and KO OVER) due to the great variability in the data, the P50 results obtained showed a clear decreased latency to fall in the KO and KO OVER mice. The P50 values differed slightly from those reported in Felici *et al.* (2014), de Haas *et al.* (2016), Kruse *et al.* (2008) and Quintana *et al.* (2010) where KO and NesKO (*Ndufs4* knockout in neurons and glia only) mice at P40-P50 were mostly unable to remain on an immobile rod long enough for the rotarod experiment to be conducted, and the latency to fall time lay closer to zero than to the average of ~50 s seen here. These results were, however, similar to those rotarod values reported in Jain *et al.* (2016) and Johnson *et al.* (2013) at P40 and P50. Whilst the diameter of the rod used in all these studies was the same as that used here, the width of the rod differed (depending on the rotarod supplier) and the varying widths may thus have played a role in the differences observed in the latency to fall times.

There were no differences seen between WT and OVER mice, corresponding to the results reported by Manso *et al.* (2016), however much higher latency to fall values were in general reported in our study. The rotarod apparatus and methods used here may explain the differences

seen in the absolute values reported, since our mice did not start on an immobile rod but rather on a rod that was rotating slowly at 4 rpm, and the rod used by Manso *et al.* (2016) had a thinner diameter in addition to being longer, thus making it harder for the mice. Both the *Ndufs4*^{-/-} and *TgMt1* mice results thus indicate that the difficulty level of the rotarod designed for our study, according to Deacon (2013), may have been lower, and allowed the mice to remain on the rod for longer than that reported by other studies, as it accelerated. Despite the differences in absolute values, relative comparisons between the genotypes (including WT mice and the results at P30) yielded the same results as those seen in all the above-mentioned studies.

4.4.6 Conclusion

The variation observed in the phenotyping analyses was high, but this was not unexpected due to the behavioural differences between mice. Loos *et al.* (2015) showed that despite standardising the environment, mice from genetically inbred mouse strains still inherently showed significant phenotypic variability and spontaneous behaviour. Since the interaction effect of gender was not found to be significant in any of the locomotor or ataxia assays, as also shown by the lack of improvement in variation in the graphs stratified by gender in Appendix B, gender did not appear to play a significant role in the motor phenotype of the mice. This concurs with Alam *et al.* (2015) who also used both male and female *Ndufs4*^{-/-} mice and saw similar results between the two genders.

From all the phenotyping results, very clear deductions could be made. At P30, the *Ndufs4*^{-/-} motor deficient phenotype may have begun in selected mice with some presenting with symptoms earlier than other mice of the same age, but a definite impairment in motor activity was not yet evident. At P50, the KO and KO OVER mice performed significantly worse than the WT and OVER mice according to growth, locomotor activity, grip strength, balance and motor coordination. The key comparison between the KO and KO OVER mice, however, did not yield the expected results since no significant differences were observed between these two genotypes in any of the above-mentioned assays, nor was the lifespan of the KO OVER mice found to be significantly increased.

While it is possible that subtler differences were present between the KO and KO OVER mice, the phenotyping tests used here, and the unavoidable behavioural variation may not have been robust enough to depict these subtler differences. It can however be concluded that the overexpression of *Mt1* did not appear to significantly improve the lifespan, balance, ataxia or muscle strength in the *Ndufs4*^{-/-} mice, nor were any obvious behavioural improvements visible when handling the mice.

4.5 IMMUNOHISTOCHEMISTRY

As described in Section 3.6, the hippocampus and vestibular nucleus were sectioned and immunohistochemistry involving glial fibrillary acidic protein (GFAP) and ionised calcium binding adapter molecule 1 (IBA1), markers of astrocyte reactivity and microglial activation (which is associated with inflammation) respectively, was performed on fixed frozen brain samples from mice of the four genotypes. In addition, staining for MT was also performed. These brain regions were selected based on reports of vacuolation and activation of astrocytes and microglial cells in the vestibular nucleus of *Ndufs4*^{-/-} mice, resulting in fatal breathing deficits (Quintana *et al.*, 2010; Quintana *et al.*, 2012), and multiple reports on the importance of MT expression in the hippocampus (Carrasco *et al.*, 2000b; Mocchegiani *et al.*, 2004; Manso *et al.*, 2016). Initial sagittal sections of our samples also demonstrated glial cell reactivity in these two brain regions. We performed two different immunohistochemistries in the hippocampus and vestibular nucleus with MT1, GFAP and DAPI staining, as shown in A-D and I-L respectively, and GFAP and IBA1 staining, as shown in E-H and M-P, respectively.

4.5.1 Astrocytes and microglia

GFAP is a hallmark in astrocytes, which are a main type of glial cells in the CNS. Astrocytes play vital roles in brain development, ion and neurotransmitter maintenance, energy homeostasis and they modulate neuronal signalling. During trauma or disease, astrocytes change in morphology and function which results in reactive astrogliosis and the consequential altered expression of multiple genes. Cytokines are known to trigger astrocyte activation. Reactive gliosis can lead to both negative and positive responses – inhibiting neuronal regeneration while increasing resilience against cellular stress by creating a glial scar (a physical barrier) to limit damage or injury. When astrocytes become reactive, GFAP is upregulated and there is hypertrophy of the cellular processes (as shown in Figure 4-16), but they remain within the same volume of brain tissue (Hol & Pekny, 2015).

IBA1 measures microglial activation. Microglia have been shown to have both positive and detrimental roles in inflammatory processes. Microglia are activated when scavenging for foreign material, damaged cells or DNA fragments, which in turn activates phagocytes and promotes regrowth of neural tissue following inflammation (Shapiro *et al.*, 2009). Astroglial and microglial reactivity are early and common features of neuropathology and glial reactivity is usually a result of neuronal cell death. CI dysfunction can also lead to the presence of ROS which can in turn activate glial cells. In a study by Quintana *et al.* (2010), hypertrophic IBA1-positive microglial cells were detected in the vestibular nucleus of early stage *Ndufs4*^{-/-} mice, whilst in the middle stage, activated microglial cells with highly reactive morphological changes (hypertrophy and thickened,

short processes) were abundant. In late stage animals, enhanced microglial activation was observed where dense infiltrates were present, suggesting highly activated/phagocytic microglial status. WT animals did not show astroglial activation and microglial cells maintained the morphology of a typical non-reactive state.

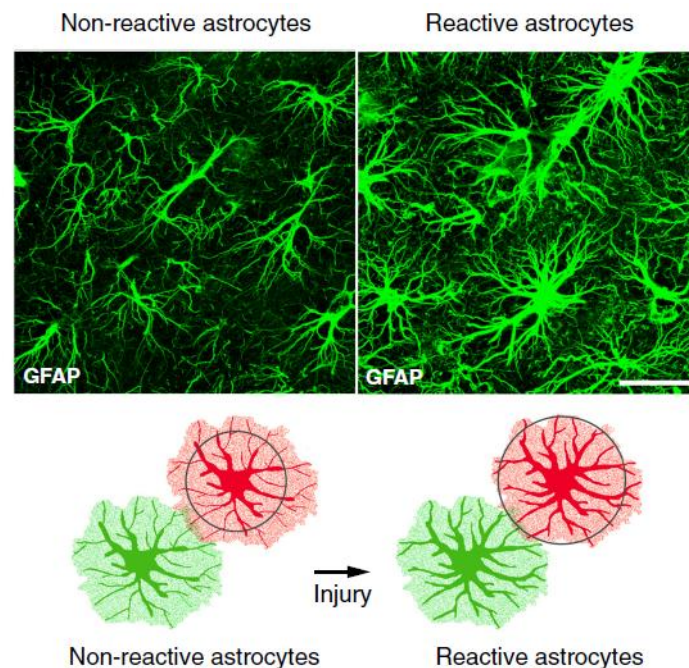


Figure 4-16: Changes in non-reactive astrocytes due to injury, where reactive astrocytes show increased GFAP and hypertrophy of the cellular processes. Image obtained with permission from Hol and Pekny (2015) (licence no. 4672980939295).

It has been observed that during pathological and experimental injury to the CNS, MT1 and 2 expression is speedily and profusely induced, primarily in the astrocytes (but the microglial cells do also contribute), and that these reactive astrocytes are advantageous for the course of recovery. The mechanism by which this occurs is not known, but astrocytes also express numerous other protective molecules, such as Cu,Zn-SOD and heat shock protein-70 (HSP-70), that may absorb or bind the surplus of extracellular ions, neurotransmitters or cytotoxic molecules that are released after CNS injury and in this way protect the neurons (West *et al.*, 2004).

4.5.2 MT, GFAP and DAPI staining

As depicted in Figure 4-17, immunohistochemistry 1 involved staining for GFAP and MT. A DAPI (4',6-diamidino-2-phenylindole) stain was also included to stain the dsDNA and thus indicate the nuclei within cells. It is important to note that the MT antibody used here was unable to distinguish between the MT1 and MT2 isoforms, and that MT2 expression, thus, cannot be ruled out in these images. In the hippocampus (Figure 4-17A-D), the MT signal in WT mice was almost absent, whilst the OVER mice showed (*TgMt1* mediated) MT overexpression in astrocytes and neurons.

Whilst MT2 expression cannot be entirely disregarded, the clear induction of MT in the OVER mice (that are known to only overexpress MT1 and not MT2) together with MT2 expression typically only being expressed at 50% of that of MT1 in the brain of non-transgenic mice (as discussed in Section 2.7.2.2), supports the assumption that the conclusions drawn from these images relate mainly to the effects of MT1. The KO mice showed an overall increase in MT signal, compared to WT mice, thus suggesting induced (i.e. non-*TgMt1* mediated) MT expression, which is in line with *in vitro* data (Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006), but contrary to the decreased *Mt1* mRNA levels observed in the whole brain samples from the KO and KO OVER mice (Section 4.3.3). Since the real-time PCR TaqMan analyses used to determine the mRNA levels of *Mt1* are able to distinguish between the MT1 and MT2 isoforms, it is thus possible that MT2 may be slightly more upregulated in response to the CI deficiency in the KO mice than MT1, and that MT2 may be responsible for the increased MT signal observed in Figure 4-17C. Other *in vivo* studies (Carrasco *et al.*, 1999; Carrasco *et al.*, 2000a; Carrasco *et al.*, 2000b; Hidalgo *et al.*, 2001) have also reported induction of MT1 in the brain as a result of injury or inflammation. KO OVER mice showed neuronal induction of MT1 together with increased astrocyte activation.

In the vestibular nucleus (Figure 4-17I-L), the MT1 signal appeared to be low with some scattered MT1 positive astrocytes, and a slightly greater MT1 signal in the OVER and KO OVER mice compared to both the WT and KO mice. MT1 and GFAP stains of the vestibular nucleus without DAPI staining is shown in Figure C11 which allows for easier visualisation of the MT1 and GFAP signal. In the vestibular nucleus of WT mice, low levels of MT1+2 expression was also previously reported, with significantly increased MT1+2 expression in astrocytes, microglia, macrophages and vascular cells in *TgMt1* mice (Penkowa *et al.*, 2004). The mRNA results thus suggest that as a whole, there was not increased *Mt1* expression in the brain of KO mice (although discrete *Mt1* increases in neurons would be difficult to detect when assessing the entire area or tissue), while the immunohistochemistry results suggest that different regions of the brain express MT1 differentially.

The lack of effect of MT1 on the vestibular nucleus is in line with the absence of improvement in the survival rate, locomotor activity or ataxia assay results of the KO OVER mice. As mentioned in Section 2.7.1, Quintana *et al.* (2012) found that by restoring *Ndufs4* in the vestibular nucleus of KO mice, breathing deficits were partially corrected and there was a moderate lifespan extension in the KO mice, thus indicating that the vestibular nucleus plays a critical role in the lethality of the disease. Greater levels of MT1 in glial cells compared to neuronal cells were previously reported (Hidalgo *et al.*, 1994), where MT1 was also shown to be induced in the hippocampus by dexamethasone.

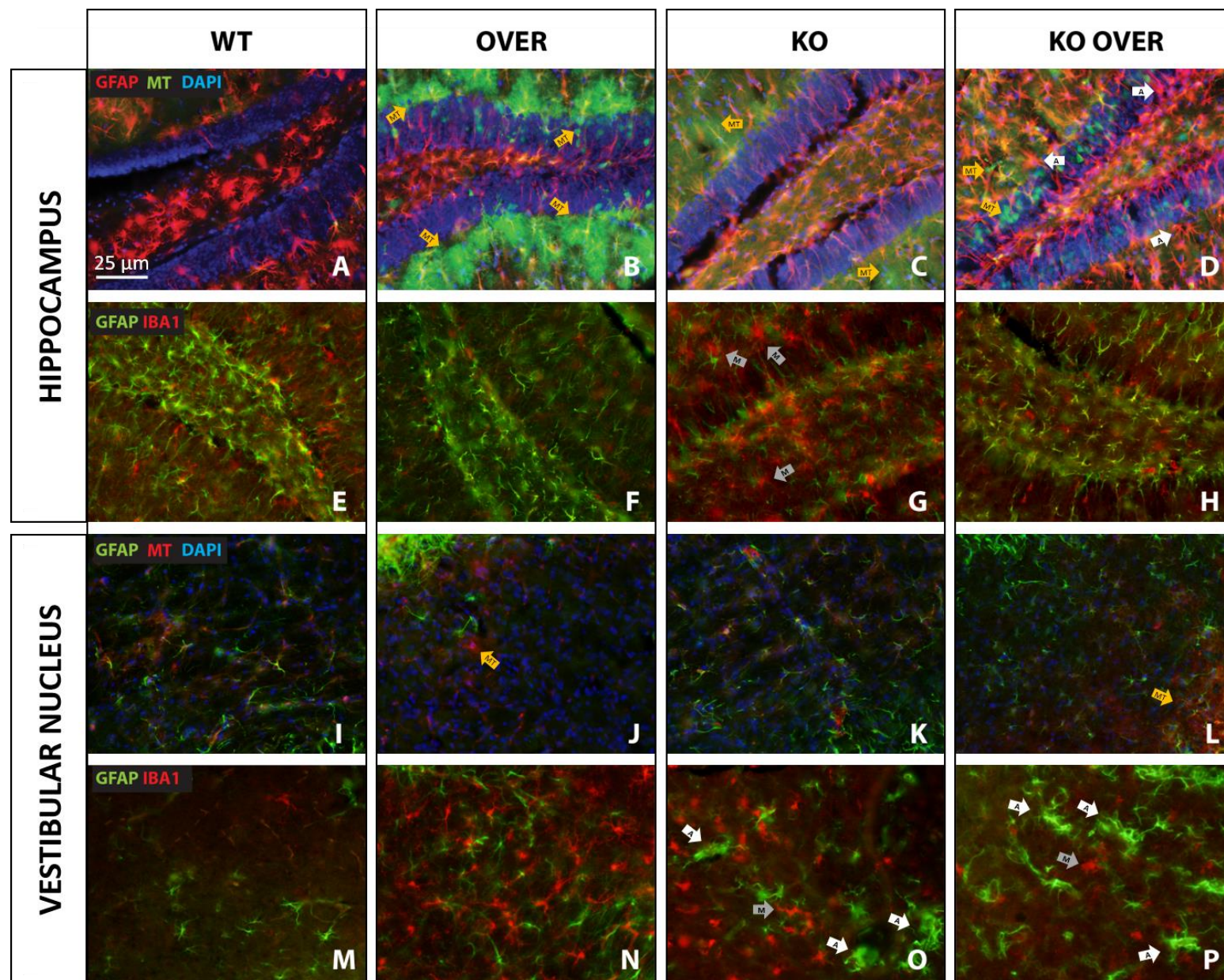


Figure 4-17: Representative immunohistochemistry stain images for MT (green in A-D, red in I-L), GFAP (red in A-D, green in E-P) and DAPI (blue) in the hippocampus (A-H) and vestibular nucleus (I-P) of WT (A, E, I, M), OVER (B, F, J, N), KO (C, G, K, O) and KO OVER (D, H, L, P) mice (n = 5/genotype).

The white horizontal line on image A is representative of the scale of 25 μm that is applicable to images A-P. The white arrows (with an A) highlight hypertrophic and reactive astrocytes, grey arrows (with an M) highlight microglial activation and yellow arrows (with MT) highlight MT expression. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA1, Ionized calcium binding adapter molecule 1; MT, metallothionein.

While no improvement was observed with MT1 overexpression in our LS model, others (Penkowa *et al.*, 2005) have shown decreased inflammation in the hippocampus, delayed neuronal degeneration and cell death in OVER mice that were administered kainic acid, which induces epileptic seizures and hippocampal excitotoxicity followed by inflammation and delayed brain damage. When crossing an Alzheimer's disease mouse model (Tg2576 mice) with *TgMt1* mice (Manoso *et al.*, 2016), increased MT1 expression was observed in the hippocampus (but not in the cortex), accompanied by an improvement in the rate of spatial learning as well as learning prompted by the eye-blink conditioning procedure. These results may thus suggest that the efficacy of MT1 overexpression depends on how severely the hippocampus is affected by a specific neurodegenerative disease, and that we may have seen improvements in the OVER and KO OVER mice had we performed spatial learning and memory tests as opposed to motor activity assays, the latter of which are more related to the functioning of the vestibular nucleus, cerebellum and motor cortex.

4.5.3 IBA1 and GFAP staining

Immunohistochemistry 2 involved staining for GFAP and IBA1. In the hippocampus (Figure 4-17E-H), there appears to be greater microglial activation (visible via IBA1 staining) in the KO mice than in any of the other three genotypes. The microglial activation thus appears to be decreased in KO OVER mice compared to the KO mice. In the vestibular nucleus (Figure 4-17M-P), there appears to be a slightly increased GFAP signal in the OVER and KO OVER mice compared to the WT and KO mice, respectively. In addition, the effect of *Ndufs4*^{-/-} seems to cause an overt morphological change (hypertrophy) in the astrocytes of the KO and KO OVER mice. Similarly, to the hippocampus, there appears to be a subtle decrease in IBA1 signal in the vestibular nucleus of KO OVER mice. To quantify or confirm the results shown here, however, western blot analyses would need to be performed.

Quintana *et al.* (2010) suggested that CI dysfunction triggers the release of cellular signals that activate glial cells and that this activation leads to oxidative stress, cytokine release by microglia, and activation of the extrinsic apoptotic pathway in neurons, which together with insufficient ATP, leads to necrotic cell death. In *Ndufs4*^{-/-} mice, they found the degree of microglial activation and lesions within the brainstem and cerebellum to correlate with ataxia, hypothermia and general morbidity. Whilst severe glial activation was observed in the vestibular nucleus of their late-stage *Ndufs4*^{-/-} animals, only mild gliosis was detected in the hippocampus, by GFAP and IBA1 staining. Similarly, microglial activation appeared to be increased in the vestibular nucleus of the KO mice (Figure 4-17O) compared to the WT mice (Figure 4-17M), and to a lesser extent in the hippocampus (Figure 4-17E and G). In the motor cortex and olfactory bulbs of *Ndufs4*^{-/-} mice at P40, Felici *et al.* (2014) observed a significant increase in GFAP positive cells, whilst at a later

stage of disease progression (P55-60) (Johnson *et al.*, 2013; Jain *et al.*, 2016), substantial neurodegeneration, glial reactivity and astrocyte activation at lesions in the cerebellum have been reported (as evidenced by an increased GFAP and IBA1 signal).

The subtle decrease in microglial activation in the vestibular nucleus and hippocampus of the KO OVER mice compared to KO mice may indicate a slight decrease in oxidative stress as a result of MT1 overexpression. Since whole brain was used for most of the other analyses, it is highly probable that subtle differences in specific brain regions would be masked and it would thus be necessary to dissect specific regions (such as the vestibular nucleus and hippocampus) to determine the cause of these subtle differences.

4.6 ELECTRON TRANSFER SYSTEM ENZYMES

As described in Section 3.7, the ETS enzyme activity was determined in the brain and quads to determine whether or not *Mt1* overexpression altered the activity of any of the enzymes. All ETS enzyme activity data was normalised according to protein and UCS and log transformed to ensure homogeneity of variance. The ETS enzyme activity data was also normalised according to protein only and CS only (see Figure C12 and Figure C13) to determine whether the normalisation method had a significant influence on the results obtained, after reports by Alam *et al.* (2015) that CS activity is affected in these mice. Line graphs depicting the interaction between genotype and tissue (using UCS normalisation) are shown in Figure C14.

4.6.1 Protein

The protein concentration was determined as described in Section 3.3.4.2. The protein concentration data is depicted in Figure 4-18A and B for the brain and quads respectively.

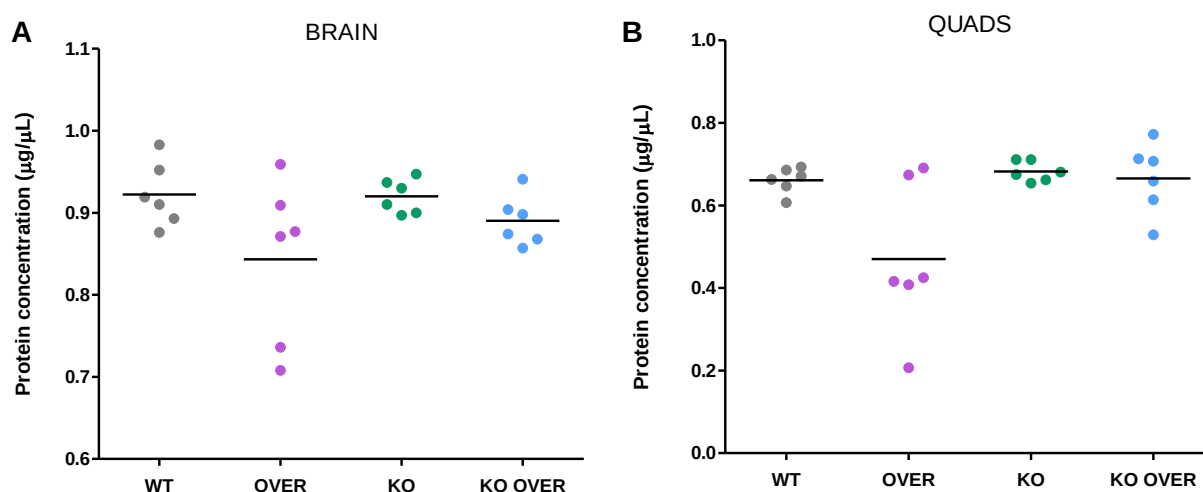


Figure 4-18: Vertical scatter plots depicting the log transformed protein concentration in the (A) brain and (B) quads (n = 6/genotype). Horizontal bar indicates the mean.

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects) on protein concentration. The results from the ANOVA were as follows:

- The interaction effect of genotype and tissue was not significant, $F(3,40) = 1.988$, $p = 0.131$, $\eta_p^2 = 0.130$.
- There was a significant main effect of genotype on the protein concentration, $F(3,40) = 7.888$, $p < 0.001$, $\eta_p^2 = 0.372$. Sidak's correction for multiple testing showed that the protein concentration in the OVER mice was significantly less than in the WT ($p = 0.002$), KO ($p = 0.001$) and KO OVER ($p = 0.006$) mice.
- There was also a significant main effect of tissue on the protein concentration, $F(1,40) = 130.468$, $p < 0.001$, $\eta_p^2 = 0.765$. The protein concentration in the brain ($M = 0.894$, $SD = 0.062$) was significantly greater than in the quads ($M = 0.620$, $SD = 0.131$).

4.6.2 Citrate synthase

The CS activity was determined as described in Section 3.7.2 and is depicted in Figure 4-19A and B for the brain and quads respectively.

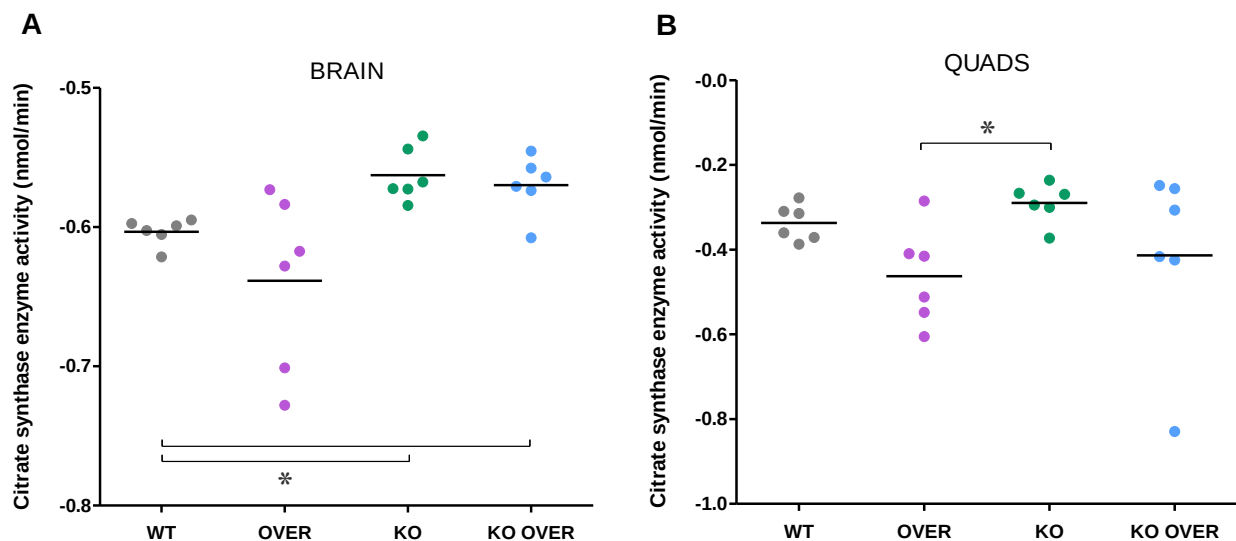


Figure 4-19: Vertical scatter plots depicting the log transformed CS activity in the (A) brain and (B) quads ($n = 6/\text{genotype}$). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects) on CS activity. The results from this ANOVA were as follows:

- The interaction effect of genotype and tissue was not significant, $F(3,40) = 1.259$, $p = 0.301$, $\eta_p^2 = 0.086$.
- There was a significant main effect of genotype on the CS activity, $F(3,40) = 3.687$, $p = 0.020$, $\eta_p^2 = 0.217$. Sidak's correction for multiple testing showed that the CS activity in the OVER mice was significantly less than in the KO mice ($p = 0.013$).
- There was also a significant main effect of tissue on the CS activity, $F(1,40) = 65.540$, $p < 0.001$, $\eta_p^2 = 0.621$. The CS activity in the brain ($M = -0.594$, $SD = 0.045$) was significantly less than in the quads ($M = -0.376$, $SD = 0.137$).

Since a significant main effect of genotype was obtained for both protein concentration and CS activity, one factor could not be chosen over the other for normalisation of complexes I-IV. It was thus decided to use UCS (equation shown in Table 3-4) as the factor against which all other enzyme complexes were normalised since UCS takes both protein concentration and CS activity into account.

4.6.3 Complex I

The CI activity was determined as described in Section 3.7.3 and is depicted in Figure 4-20A and B for the brain and quads respectively.

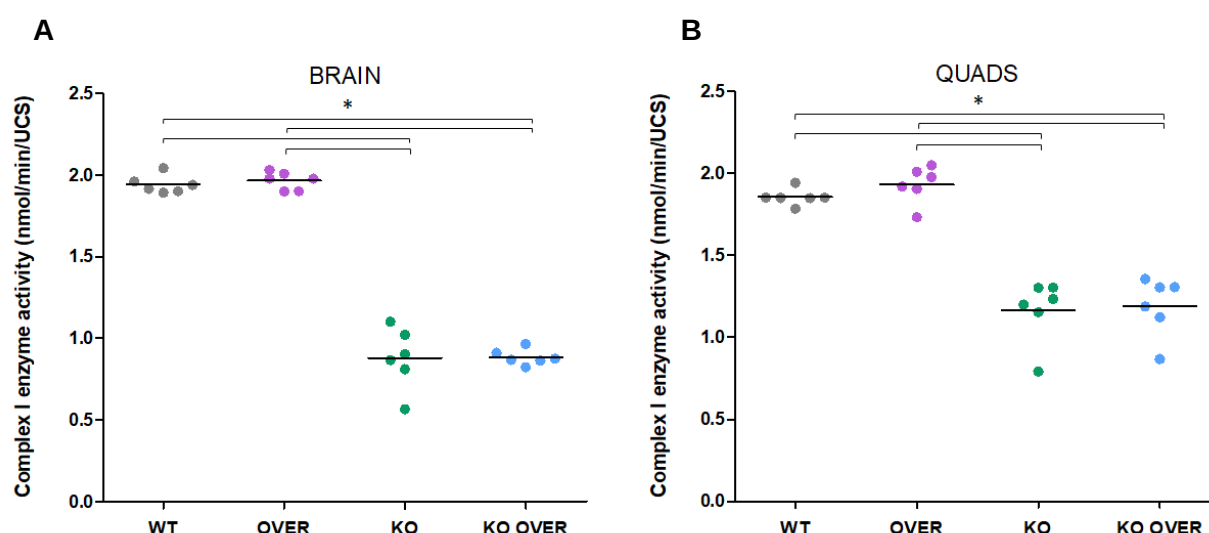


Figure 4-20: Vertical scatter plots depicting the log transformed CI enzyme activity in the (A) brain and (B) quads ($n = 6/\text{genotype}$). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects) on CI activity. The results from this ANOVA were as follows:

- The interaction effect for genotype and tissue was significant, $F(3,40) = 8.074$, $p < 0.001$, $\eta_p^2 = 0.377$, as depicted in Figure C14-B. The Games-Howell post hoc test was performed and the significant differences obtained are shown in Table 4-1.
- There was a significant main effect of genotype on the CI activity, $F(3,40) = 201.376$, $p < 0.001$, $\eta_p^2 = 0.938$. Sidak's correction for multiple testing indicated that the WT and OVER mice had significantly greater CI activity than the KO and KO OVER mice (all p-values < 0.001).
- There was a significant main effect of tissue on the CI activity, $F(1,40) = 10.643$, $p = 0.002$, $\eta_p^2 = 0.210$. The CI activity in the brain ($M = 1.416$, $SD = 0.556$) was significantly lower than in the quads ($M = 1.535$, $SD = 0.391$).

Table 4-1: Games-Howell post hoc test results for CI enzyme activity

Genotype 1	Genotype 2	Mean difference (Genotype 1 – 2)	SE	p-value
BRAIN				
WT	KO	1.063	0.079	<0.001
WT	KO OVER	1.057	0.030	<0.001
OVER	KO	1.087	0.079	<0.001
OVER	KO OVER	1.081	0.030	<0.001
QUADS				
WT	KO	0.691	0.081	0.001
WT	KO OVER	0.664	0.076	0.001
OVER	KO	0.768	0.091	<0.001
OVER	KO OVER	0.741	0.087	<0.001

To evaluate the amount of residual CI activity in the KO and KO OVER mice, Figure 4-21 was constructed where all CI activity is indicated as a percentage of the respective WT activity. It can be seen in Figure 4-21 that there was a fair increase in the CI activity of the OVER compared to WT mice in the quads, and to a lesser extent in the brain. It should be noted that the variation in the data was however high, and that these percentages merely provide a rudimentary indication of the residual CI activity. Whilst the varying *Mt1* mRNA levels displayed in Figure 4-3 suggest a biological origin for the variability in the ETS enzyme activity data, technical variation cannot be ruled out due to the fairly narrow linear range during which the mean v of each enzyme reaction can be measured. It is thus possible that some reactions may have proceeded more quickly than others and that the absorbance measurements selected may have included some of the plateau

phase, and not only the linear phase of the enzyme reaction, which may have added to the biological variation in the samples. The CI activity in the brain dropped by on average 91% in the KO and KO OVER mice, respectively, compared to the WT mice. For the quads, it dropped by on average 78% and 77% in the KO and KO OVER mice, respectively, compared to the WT mice. The CI activity in the two different tissues from the KO and KO OVER mice was very similar.

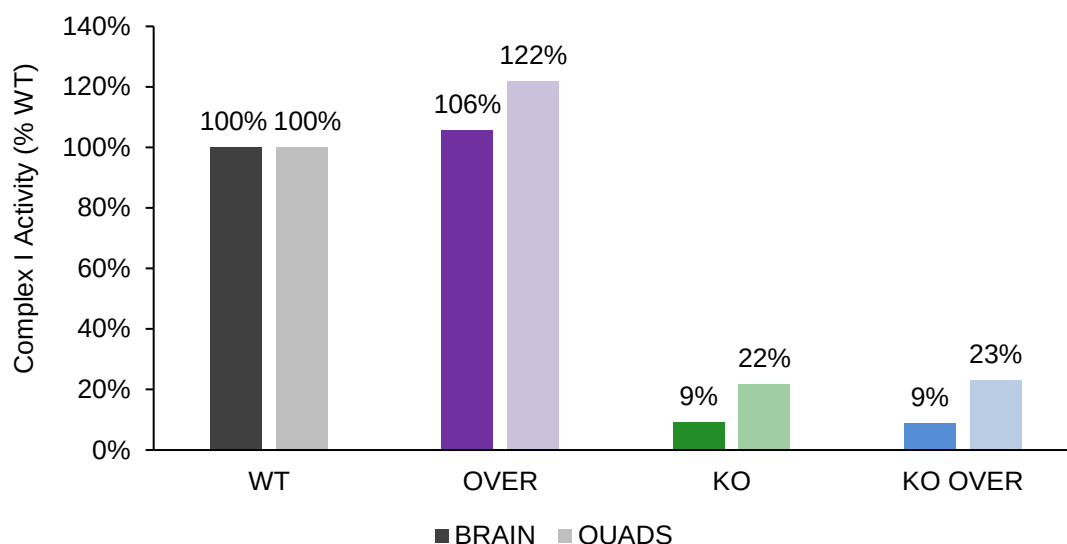


Figure 4-21: Bar graph depicting the average CI enzyme activity (not logged), as a % of the WT, in the brain (darker colour) and quads (lighter colour) (n = 6/genotype).

4.6.4 Complex II

The CII activity was determined as described in Section 3.7.4 and is depicted in Figure 4-22A and B for the brain and quads respectively.

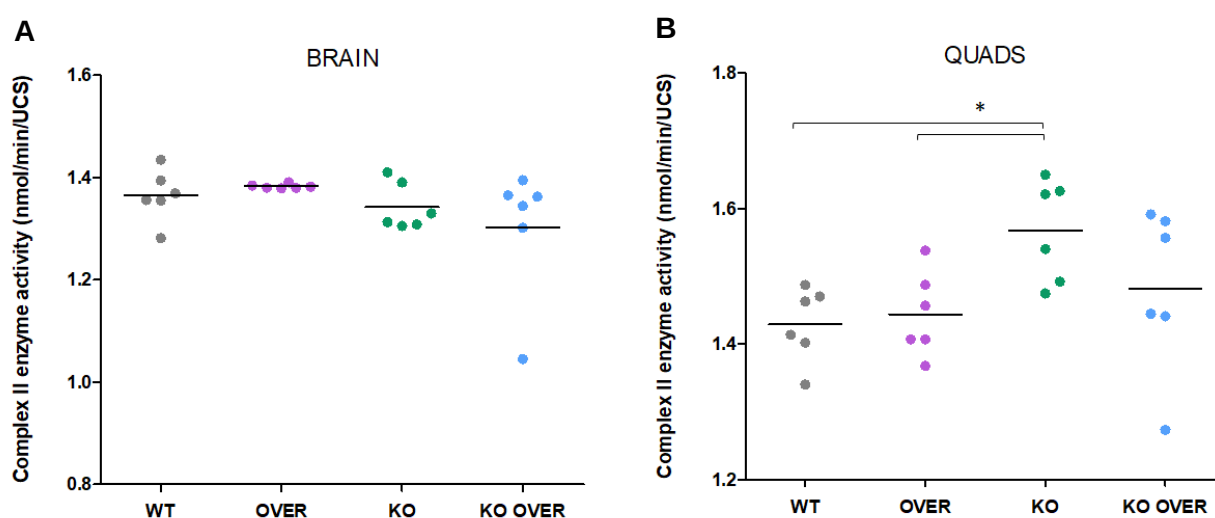


Figure 4-22: Vertical scatter plots depicting the log transformed CII enzyme activity in the (A) brain and (B) quads (n = 6/genotype). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects) on CII activity. The results from this ANOVA were as follows:

- The interaction effect for genotype and tissue was significant, $F(3,40) = 3.340$, $p = 0.029$, $\eta_p^2 = 0.200$, as depicted in Figure C14-C. The Games-Howell post hoc test was performed and showed no significant differences in the brain, but in the quads, the KO mice were observed to have 0.138 units and 0.123 units higher CII enzyme activity than WT (SE = 0.0379, $p = 0.023$) and OVER mice (SE = 0.0398, $p = 0.048$) respectively.
- There was a non-significant main effect of genotype on the CII activity, $F(3,40) = 1.606$, $p = 0.203$, $\eta_p^2 = 0.108$.
- There was a significant main effect of tissue on the CII activity, $F(1,40) = 34.352$, $p < 0.001$, $\eta_p^2 = 0.642$. The CII activity in the brain ($M = 1.348$, $SD = 0.075$) was significantly lower than in the quads ($M = 1.480$, $SD = 0.094$).

4.6.5 Complex III

The CIII activity was determined as described in Section 3.7.5 and is depicted in Figure 4-23A and B for the brain and quads respectively.

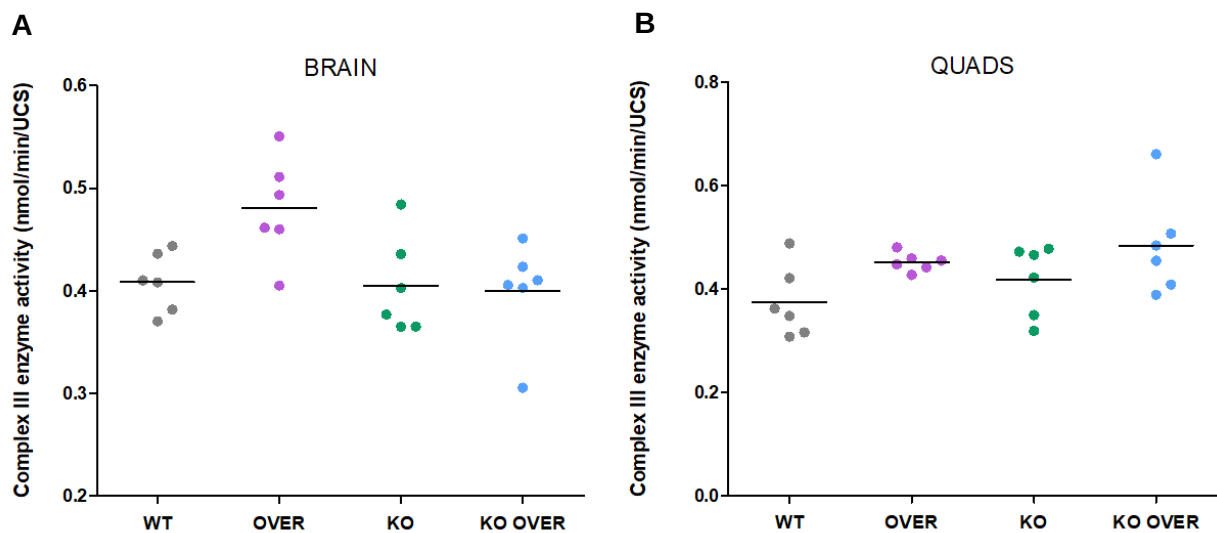


Figure 4-23: Vertical scatter plots depicting the log transformed CIII enzyme activity in the (A) brain and (B) quads ($n = 6/\text{genotype}$). Horizontal bar indicates the mean.

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects) on CIII activity. The results from this ANOVA were as follows:

- The interaction effect for genotype and tissue was not significant, $F(3,40) = 2.634$, $p = 0.063$, $\eta_p^2 = 0.165$.
- There was a significant main effect of genotype on the CIII activity, $F(3,40) = 3.851$, $p = 0.016$, $\eta_p^2 = 0.224$. Sidak's correction for multiple testing indicated that WT mice had significantly lower CIII activity than OVER mice ($p = 0.019$).
- There was a non-significant main effect of tissue on the CIII activity, $F(1,40) = 0.284$, $p = 0.597$, $\eta_p^2 = 0.007$.

4.6.6 Complex IV

The CIV activity was determined as described in Section 3.7.6 and is depicted in Figure 4-24A and B for the brain and quads respectively.

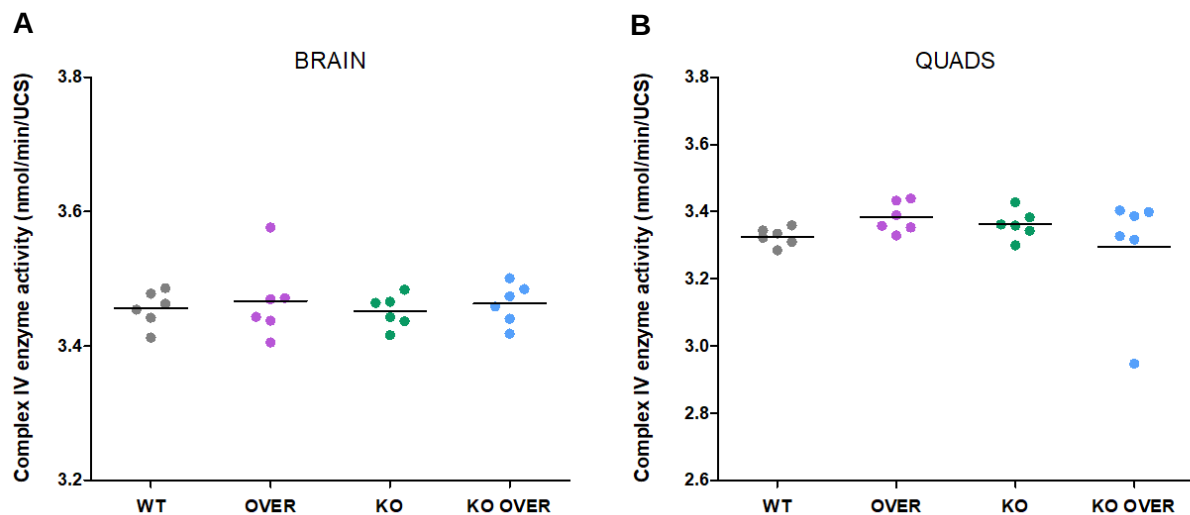


Figure 4-24: Vertical scatter plots depicting the log transformed CIV enzyme activity in the (A) brain and (B) quads ($n = 6/\text{genotype}$). Horizontal bar indicates the mean.

As discussed in Section 3.9, a two-way ANOVA was performed to determine the effect of genotype and tissue (the between-factor subjects) on CIV activity. The results from this ANOVA were as follows:

- The interaction effect for genotype and tissue was not significant, $F(3,40) = 0.874$, $p = 0.463$, $\eta_p^2 = 0.062$.
- There was a non-significant main effect of genotype on the CIV activity, $F(3,40) = 0.925$, $p = 0.437$, $\eta_p^2 = 0.065$.

- There was a significant main effect of tissue on the CIV activity, $F(1,40) = 32.242$, $p < 0.001$, $\eta_p^2 = 0.446$. The CIV activity in the brain ($M = 3.460$, $SD = 0.0360$) was significantly greater than in the quads ($M = 3.342$, $SD = 0.0940$).

4.6.7 Discussion

Despite the absence of NDUF54, residual CI activity is still present in *Ndufs4*^{-/-} mice due to the formation of respiratory supercomplexes with CIII, which allow the formation of active CI (Calvaruso *et al.*, 2012). As expected, CI enzyme activity was significantly decreased in the KO and KO OVER mice, with a decrease of ~91% in the brain and ~78% in the quads. This decrease was similar to that reported by others using the *Ndufs4*^{-/-} mice as well as similar methods (Rodenburg, 2011) and sample sizes. Jain *et al.* (2016) reported a decrease in CI activity of ~85% in the brain, and Calvaruso *et al.* (2012) a decrease of 74% in the brain and 71% in the muscle of *Ndufs4*^{-/-} mice compared to WT mice. Using skeletal muscle from the entire hind limb of 5 week old mice, Alam *et al.* (2015) reported a reduction of 79% in CI activity in *Ndufs4*^{-/-} compared to WT mice. Furthermore, they mathematically predicted that the *Ndufs4*^{-/-} mice skeletal muscle mitochondria have a significant spare CI capacity and that major metabolic consequences only occur when CI activity drops more than 90% below ordinary levels. A related study on selected brain regions in *Ndufs4*^{-/-} mice (using slightly altered methods) demonstrated varied ETS enzyme activities in different brain regions where those areas known to be more vulnerable in *Ndufs4*^{-/-} mice (cerebellum, brainstem and olfactory bulbs) showed lower residual CI activities (~15-28%) than the anterior cortex (~38%), an area believed to be less affected by CI dysfunction (Coetzer, 2020). Similarly, Terburgh (2019) also saw differences in the residual CI activity of oxidative compared to glycolytic muscle fibres. In addition, Kayser *et al.* (2016) also reported decreased OXPHOS capacities in brain regions more prone to neurodegeneration (cerebellum, brainstem and olfactory bulbs) than other more resilient areas in *Ndufs4*^{-/-} mice, thus supporting the notion that there are region-specific biochemical consequences in these mice, which may not be detected in whole tissues.

For the other complexes, Calvaruso *et al.* (2012) found no significant differences in complexes II, III or IV and Alam *et al.* (2015) found a 45-72% increase in complexes II, III, IV and CS activity of *Ndufs4*^{-/-} mice compared to WT mice. In the quads, Terburgh (2019) observed no significant differences in the activity of complexes II-IV or CS of KO compared to WT mice, whilst Coetzer (2020) observed increased CII activity in the cerebellum only and increased CIV activity in the anterior cortex, cerebellum and olfactory bulbs, but not in the brainstem. Similarly to Alam *et al.* (2015), we also saw significantly increased CS and CII activity in the quads of the KO mice. For CIII and CIV, however, our results were more comparable to Terburgh (2019) and Calvaruso *et al.* (2012) with no significant changes. The overexpression of *Mt1* did not seem to have any effect

on any of the enzyme complexes between KO and KO OVER mice, but the OVER mice appeared to have significantly increased CIII activity in the brain compared to WT mice and significantly decreased CS activity compared to KO mice. Besides the expected decrease in CI activity, the increased CII activity in the quads of the KO mice appears to be the most noteworthy OXPHOS alteration and corresponds to the metabolomics results reported below in Section 5.5.4.1.

4.6.8 Enzyme, *Mt1* mRNA and phenotyping data correlations

To determine whether there was any correlation between the *Mt1* mRNA levels or the enzyme activities of the two different tissues and the phenotyping data, Spearman's rank correlation coefficient was determined (see Appendix C3), where green blocks indicated significant positive correlations and red blocks indicated significant negative correlations ($p < 0.05$). The correlations between the enzyme activity and mRNA data is shown in Table C3, while the correlations between the mRNA data, together with enzyme activity data, and the phenotyping results is shown in Table C4, Table C5, Table C6 and Table C7 for WT, OVER, KO and KO OVER mice, respectively. The *Mt1* mRNA levels did not seem to be an influencing factor on the ETS enzyme activity (Table C3). For the phenotyping data, *Mt1* mRNA levels only seem to show some degree of correlation in the KO OVER mice, with some positive correlations in the brain and some negative correlations in the quads (Table C7). When correlating the ETS enzyme data with the phenotyping data, the KO OVER mice were again the group that seemed to show a few correlations, including a negative correlation between CI activity and the rotarod and wire grid hang test at P30 (Table C7). Overall, there did not appear to be noteworthy correlations between the *Mt1* mRNA, ETS enzyme activity and phenotyping data. More specifically, the mRNA levels of CI and the CI enzyme activity did not appear to correlate repeatedly with one another or with the phenotyping data, thus indicating that other factors may be contributing to the variability seen in mice from the same genotypes and to the differences in disease severity of mice of the same age. This also agrees with Alam *et al.* (2015) and that major metabolic consequences may only occur when CI activity drops by more than 90%, which was higher than that seen in the KO and KO OVER mice. In future, classifying the mice according to disease progression scores (such as weight, motor skills, body temperature and other behavioural traits) as was done in Quintana *et al.* (2010), rather than age, could greatly assist in reducing the variability in the data. Identifying other biological markers that correlate with the phenotypic disease severity in *Ndufs4*^{-/-} mice would also be useful.

4.7 PROTEIN OXIDATION

To investigate one of the downstream damaging effects of oxidative stress within the cell, protein oxidation was determined. As described in Section 3.8, the relative volume of oxidised proteins was measured in one mouse per genotype. Multiple oxidised protein bands were formed, but

these were all quantified together as one unit to give the total amount of oxidised protein. The same mouse was used for both the brain and the quads and the blot images are shown in Figure 4-25 and Figure 4-26, respectively. While other techniques for measuring ROS were attempted, they were however not successful, and new methods required different sample preparation to that already performed here.

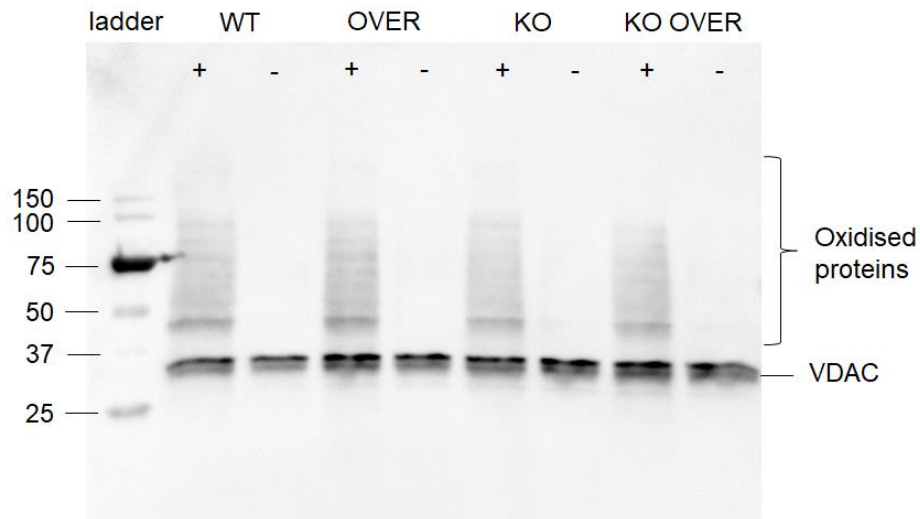


Figure 4-25: Western blot of four brain samples from one mouse per genotype that were stained for oxidised proteins and VDAC using the OxyBlot kit. The first row represents the protein ladder (sizes in kDa) and the following rows are indicated with a "+" for the sample derivatised with DNPH and a "-" for the negative control.

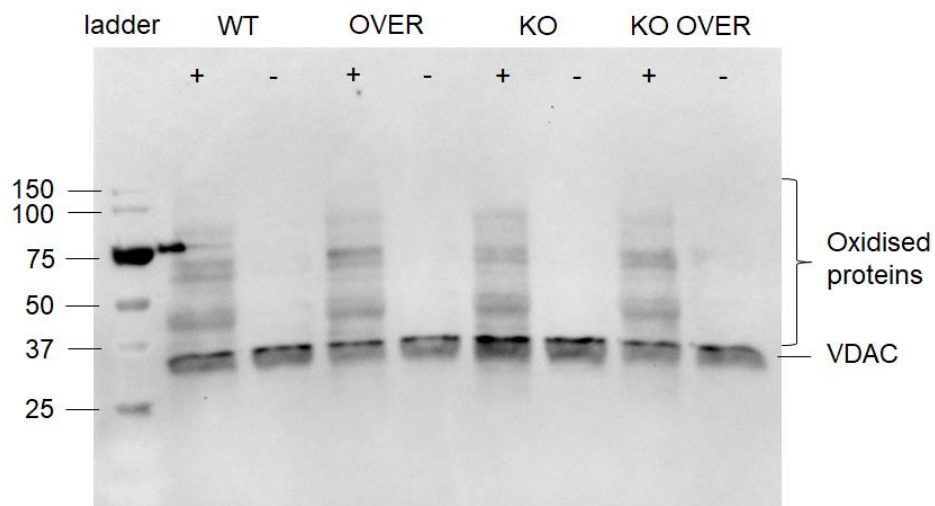


Figure 4-26: Western blot of four quad samples from one mouse per genotype that were stained for oxidised proteins and VDAC using the OxyBlot kit. The first row represents the protein ladder (sizes in kDa) and the following rows are indicated with a "+" for the sample derivatised with DNPH and a "-" for the negative control.

The intensity of oxidised proteins was normalised according to VDAC, the negative control intensity subtracted, and then expressed as a % of the WT for both the brain and the quads, as shown in Figure 4-27. The dark 75 kDa band from the ladder that overlapped into the WT lane for both the quads and the brain was corrected for by measuring the volume of that dark band area in the WT lane, subtracting it from the WT intensity and then adding an equivalent adjacent area's intensity. In addition, the relative intensities were also quantified without normalising according to VDAC, in the event that VDAC expression may be altered in the different genotypes, as shown in Figure C15.

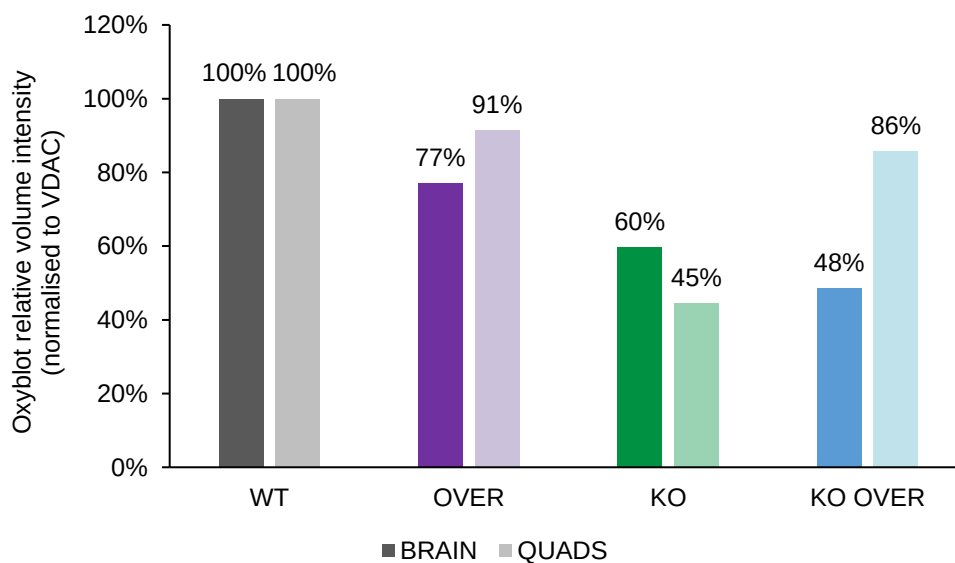


Figure 4-27: Bar graph depicting the percentage of oxidised proteins (normalised to VDAC) in the brain and quads of one mouse per genotype, relative to the WT. The darker bar indicates the brain tissue and the lighter bars indicate the quads samples.

Since only one mouse per genotype was used, no statistical tests could be performed to assess significant differences. These results indicated that the KO and KO OVER mice had fewer oxidised proteins than WT mice. In the brain, the overexpression of *Mt1* seemed to slightly decrease the amount of oxidised proteins when comparing OVER mice with WT mice, and KO OVER mice with KO mice. In the quads, however, the overexpression of *Mt1* appeared not to affect the oxidised protein levels since OVER and KO OVER mice showed similar oxidised protein levels when compared to WT mice. Contrary to Quintana *et al.* (2010) who showed significantly increased oxidised proteins in the olfactory bulb of *Ndufs4*^{-/-} mice compared to WT mice, in both the brain and quads of our KO mice, the number of oxidised proteins appeared to be decreased in the KO mice compared to WT mice. In the brain, the oxidised protein levels were similar between KO and KO OVER mice, while in the quads, the oxidised protein levels were increased in the KO OVER mice compared to KO mice. The difference seen between Quintana *et al.* (2010) and the results shown here could be attributed to the fact that they made use of the olfactory

bulbs as opposed to whole brain and muscle. Piroli *et al.* (2016) also did not find an increase in oxidative stress markers in the brainstem of *Ndufs4*^{-/-} mice and proposed that this explained why antioxidant therapies in isolation were not sufficient to treat MDs and suggested that strategies to improve mitochondrial function were required.

4.8 CONCLUSION

This study aimed to determine whether or not overexpression of *Mt1* in the *Ndufs4*^{-/-} mice would increase the age of survival, as well as whether any improvements could be observed in hallmark phenotypic motor activity deficiencies of these mice. Other than mild reductions in inflammation in the vestibular nucleus and hippocampus, no significant differences were found in the growth curves, lifespan, motor activity assays, *Mt1* mRNA gene expression analysis or protein oxidation in the brain and quads of the KO vs KO OVER mice. While the expected worsened phenotype was seen in the KO and KO OVER compared to WT and OVER mice as a result of the CI deficiency, a protective effect from MT1 in our OVER or KO OVER groups was glaringly absent, contrary to the multiple reports of their protective role in mitochondrial dysfunction and neurodegenerative diseases.

It has been shown that excitatory glutamatergic neurons in *Ndufs4*^{-/-} mice are a major contributor to the volatile anaesthetic hypersensitivity observed in these mice, whilst inhibitory GABAergic neurons and cholinergic neurons (that transfer information from the brain to the tissues) were not found to play a role (Zimin *et al.*, 2016). This was further evidenced by Bolea *et al.* (2019) who showed that selective inactivation of *Ndufs4* in glutamatergic neurons resulted in motor and respiratory activity impairment, lessened neuronal firing, inflammation in the brainstem and premature death. This inactivation in the GABAergic neurons also resulted in premature death but did not, however, lead to respiratory or motor defects. In contrast, the GABAergic neurons displayed signs of basal ganglia inflammation, hypothermia and severe epileptic seizures. Selective inactivation of *Ndufs4* in the cholinergic neurons had no impact on motor activity, survival or growth. This suggests that, in addition to the brain region specific differences that are present in these mice (Kayser *et al.*, 2016), different cellular populations also play a role and that when studying whole brain or combined cellular populations, important differences may be masked or compensated for by other cell types.

CHAPTER 5: METABOLOMICS RESULTS AND DISCUSSION

5.1 INTRODUCTION

As described in Section 3.10, three platforms were used for metabolomics analysis of the brain and quad samples, namely NMR, LC-MS/MS and GC-TOF-MS. Quad samples were analysed on all three platforms in two batches, while brain samples were analysed using NMR and GC-TOF-MS only, in a single batch. Other researchers within our laboratory, upon which the methods used here were based, have studied the metabolomics profiles within *specific* brain and muscle regions of the WT and KO mice (Terburgh, 2019; Coetzer, 2020) in parallel to this study, while here *whole* brain and muscle were studied with the addition of the OVER and KO OVER mice. Here, the quality of the metabolomics data will first be discussed, followed by the list of significant metabolites identified, and lastly, the metabolic pathways associated with these metabolites in the brain and quads will be explored in detail.

5.2 DATA QUALITY

As described in Section 3.10.6.3, the quality of the data was checked by examining CV%, relative intensity plots and sample and QC clustering.

5.2.1 *Relative intensity plots*

The relative intensity plots of the samples in run order showed that the total signal obtained for the NMR data (Figure 5-1A and B), in both the brain and quads, had good horizontal alignment and no evident batch effects, with excellent alignment of the QCs both intra- and inter-batch. The LC-MS/MS plot (Figure 5-1E) showed greater deviations from horizontal alignment in the QCs, but the scatter pattern of the samples and QCs still appeared to be random with no clear trend that could be attributed to any technical variations and no visible inter-batch effects could be seen.

For the GC-TOF-MS data (Figure 5-1C and D), the quad data showed good horizontal alignment of QCs and samples with even scatter distribution, with the exception of two samples in batch 2 which lay much higher than all the other samples and could clearly be identified as outliers and were thus removed. For the brain GC-TOF-MS relative intensities, a clear drift could be seen in the first 3-4 samples that were injected (including the first QC). This was attributed to instrument drifts caused by not injecting enough blank samples prior to the first QC, to fully equilibrate the instrument. The first QC was thus excluded from all quality checks thereafter and was not used when applying the QC filter. Those first few samples were, however, still included in the data processing thereafter due to the already low number of brain samples available.

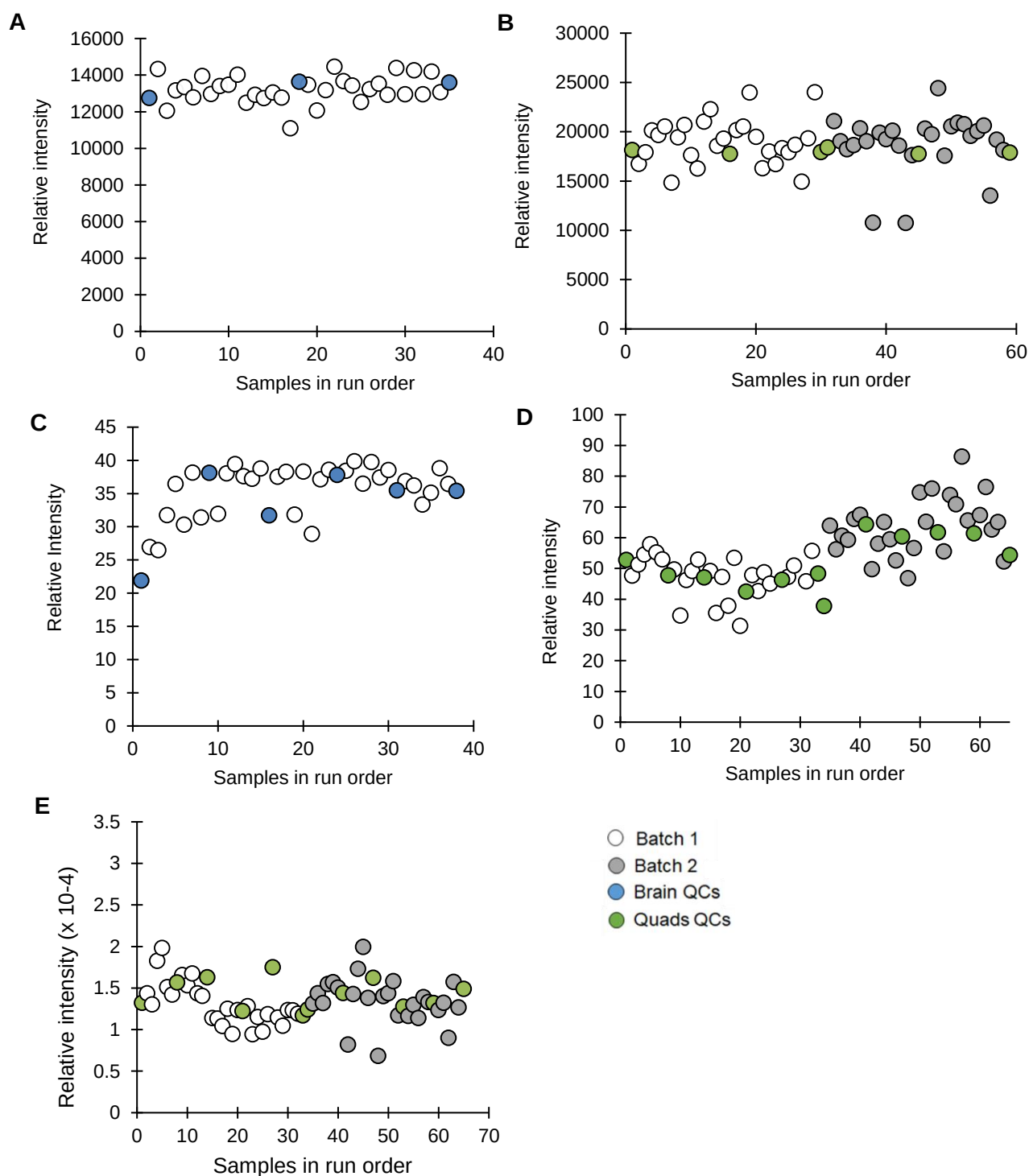


Figure 5-1: Sequential total signal scatter plot of various metabolic profiling methods. Scatter plots depicting the total signal obtained for NMR analysis in (A) brain and (B) quads; for GC-TOF-MS analysis in (C) brain and (D) quads; and for (E) LC-MS/MS analysis in quads. Brain samples were analysed in one batch whilst quad samples were analysed in two batches over two days. The number of samples per genotype were as follows (n = brain/quads): WT (n = 9/14), OVER (n = 6/13), KO (n = 8/12) and KO OVER (n = 9/12). For NMR, three QCs per batch was analysed, whilst for GC-TOF-MS and LC-MS/MS, six QCs per batch were analysed. The x-axis specifies the sample run order on each analytical platform, while the y-axis specifies the sum of the relative peak intensities of all features/metabolites measured in the sample. White and grey symbols respectively indicate samples of batch one and batch two, while blue or green symbols indicate brain or quad QC samples, respectively, analysed throughout the batches.

5.2.2 CV%

The CV was examined in Table 5-1 and Figure 5-2 in order to evaluate repeatability. A CV cut-off of >50% was used in data processing (Dunn *et al.*, 2012). The untargeted NMR had the highest precision, with a CV of less than 10%, for 100% of the metabolites in the brain (Figure 5-2A), 79% of the metabolites intra-batch and 74% inter-batch in the quads (Figure 5-2B). For the targeted LC analysis, 90-95% of the metabolites in the quads had a CV of <20% both intra- and inter-batch (Figure 5-2E). The untargeted GC-TOF-MS analyses had the lowest precision with 72-77% of metabolites having a CV of <50% in the quads (both intra- and inter-batch) (Figure 5-2D), but improved precision in the brain of 81% of metabolites with a CV of <30% (Figure 5-2C). This high CV distribution is due to the untargeted nature of the GC-TOF-MS analyses as well as to sample derivatisation reactions and the length of time that samples stand before being analysed, during which time further compound derivatisation or decomposition may occur (Erban *et al.*, 2007; Fiehn, 2008).

Table 5-1: NMR, GC-TOF-MS and LC-MS/MS intra- and inter-batch CV values for brain and quads

CV range (%)	BRAIN	QUADS		
NMR	<i>Intra-batch</i>	<i>Intra-batch 1</i>	<i>Intra-batch 2</i>	<i>Inter-batch</i>
0-10	100	79	74	79
10-20	0	16	21	11
20-30	0	0	5	11
30-40	0	5	0	0
40-50	0	0	0	0
GC-TOF-MS	<i>Intra-batch</i>	<i>Intra-batch 1</i>	<i>Intra-batch 2</i>	<i>Inter-batch</i>
0-10	38	7	7	0
10-20	28	33	28	10
20-30	15	23	23	26
30-40	7	8	15	30
40-50	6	7	2	7
> 50	6	23	26	28
LC-MS/MS		<i>Intra-batch 1</i>	<i>Intra-batch 2</i>	<i>Inter-batch</i>
0-10		0	53	0
10-20		91	42	93
20-30		7	5	7
30-40		2	0	0
40-50		0	0	0
> 50		0	0	0

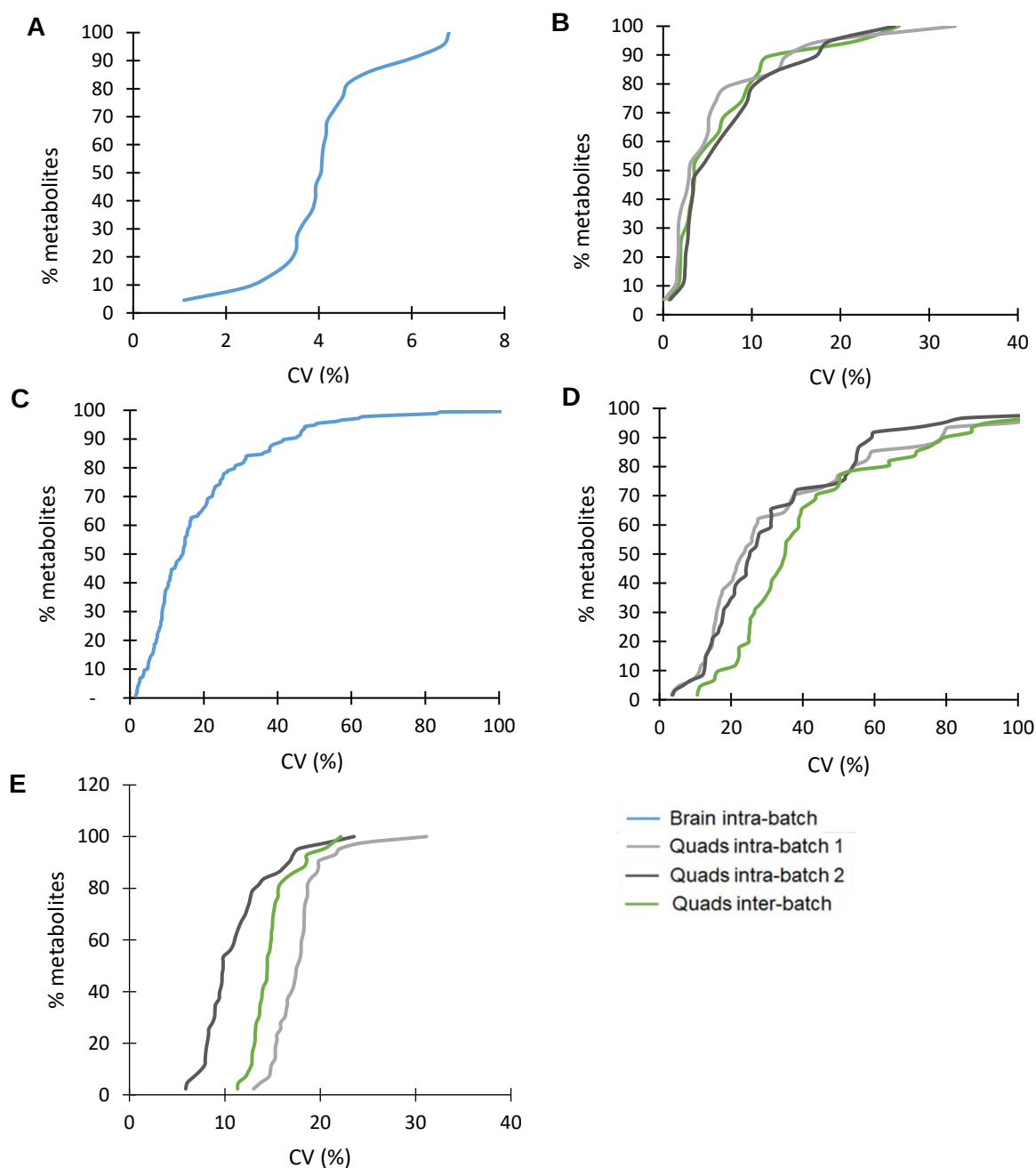


Figure 5-2: Performance of the various metabolic profiling methods. The intra-batch (grey) and inter-batch (brain = blue; quads = green) relative coefficient of variance (CV%) of sample replicates from NMR analysis of (A) brain and (B) quad samples; GC-TOF-MS analysis of (C) brain and (D) quads samples; and (E) LC-MS/MS analysis of quad samples.

5.2.3 QC clustering

Lastly, PCAs were constructed of the samples vs QCs for each platform, as shown in Figure 5-3. A close clustering of QCs within the sample cluster was expected for all samples (since the QCs represented identical replicates). This was clearly seen for all the NMR and LC data and to a less extent in the GC data. For the brain GC data, both the first and last QCs had to be removed.

Overall, it was however clear that the data was repeatable, and that the variability of each metabolomics technique was sufficiently low and that good quality data had been obtained which could be used for downstream processing.

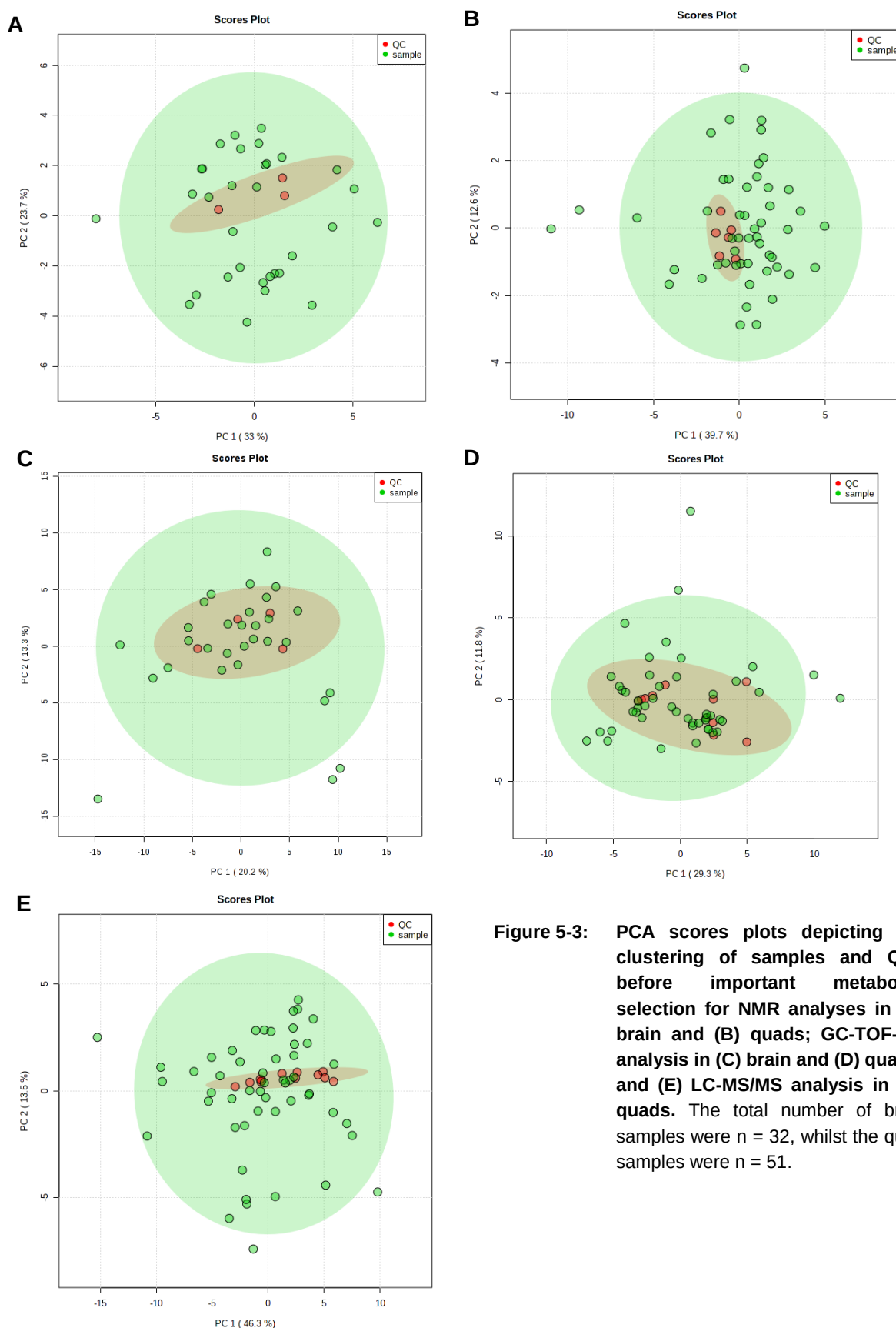


Figure 5-3: PCA scores plots depicting the clustering of samples and QCs before important metabolite selection for NMR analyses in (A) brain and (B) quads; GC-TOF-MS analysis in (C) brain and (D) quads; and (E) LC-MS/MS analysis in the quads. The total number of brain samples were $n = 32$, whilst the quad samples were $n = 51$.

5.3 SIGNIFICANT METABOLITES

By using our multi-platform metabolomics approach, we relatively quantified 187 metabolites in the brain and 109 metabolites in the quads (following data pre-processing). Discriminatory metabolites were identified in the brain and quads (see Table 5-2 and Table 5-3 respectively), as described in Section 3.10.6.4, using independent t-tests. Each of these metabolites were categorised according to a class, based on which part of the metabolism they belong to. The mean (prior to auto-scaling or glog transformation) was given together with the p-value (<0.05) and effect size (Cohen's d). All metabolites were ranked in alphabetical order for each genotype comparison and Venn diagrams (Figure 5-5) were constructed to display metabolites that differ consistently across the different genotype comparisons, as well as the up- or down-regulation of these metabolite levels compared to WT or OVER mice. The discriminatory metabolites obtained from the different platforms were all grouped according to the genotype comparisons that were used in the t-tests, namely:

- WT vs OVER
- WT vs KO
- WT vs KO OVER
- OVER vs KO
- OVER vs KO OVER
- KO vs KO OVER

The combined data matrices from the different platforms were also used to construct PCAs as shown in Figure 5-4. The data from each platform was auto-scaled separately and the auto-scaled data was then combined for the different platforms and used to construct the PCA (without glog transformation). A distinct separation could be seen between the ellipses of the WT/OVER and KO/KO OVER genotypes in the brain (Figure 5-4A), indicating that the final selected discriminatory metabolites did indeed provide good separation between the genotypes. For the quads (Figure 5-4B), no distinct separation could be seen in the WT data, but the KO and KO OVER data did, however, align very closely. The lack of separation observed for the quads can be ascribed partly to the NMR data, where the WT mice showed greater variability, as opposed to the LC data, where a greater degree of separation was seen between WT/OVER and KO/KO OVER mice. Furthermore, as these mice predominantly show a neurological phenotype, it is understandable that there are less significant differences in the quads.

It is important to note that both male and female mice were used in this study due to the limited number of mouse samples available for each of the genotypes. It is known that sex is one of the most relevant biological variables to play a role in metabolomics (Audano *et al.*, 2018) and as

such, by analysing mice from both sexes, the biological variation within the samples was increased and may make it more difficult to identify specific metabolite markers that are altered in different ways from mice of different sexes. However, it is thus believed that only metabolites that are greatly altered across the genotypes, regardless of sex, will be identified here. Furthermore, in both LS in humans (Sofou *et al.*, 2014) and the *Ndufs4*^{-/-} mouse model (Lee *et al.*, 2019), gender has been shown to have minimal to no differences on phenotype. Strict criteria were also used during statistical analysis with a BH-FDR adjusted p-value of <0.05. Many of the significant metabolites identified when using a BH-FDR adjusted p-value of <0.1 were supportive of the metabolic pathways identified here but will not be discussed.

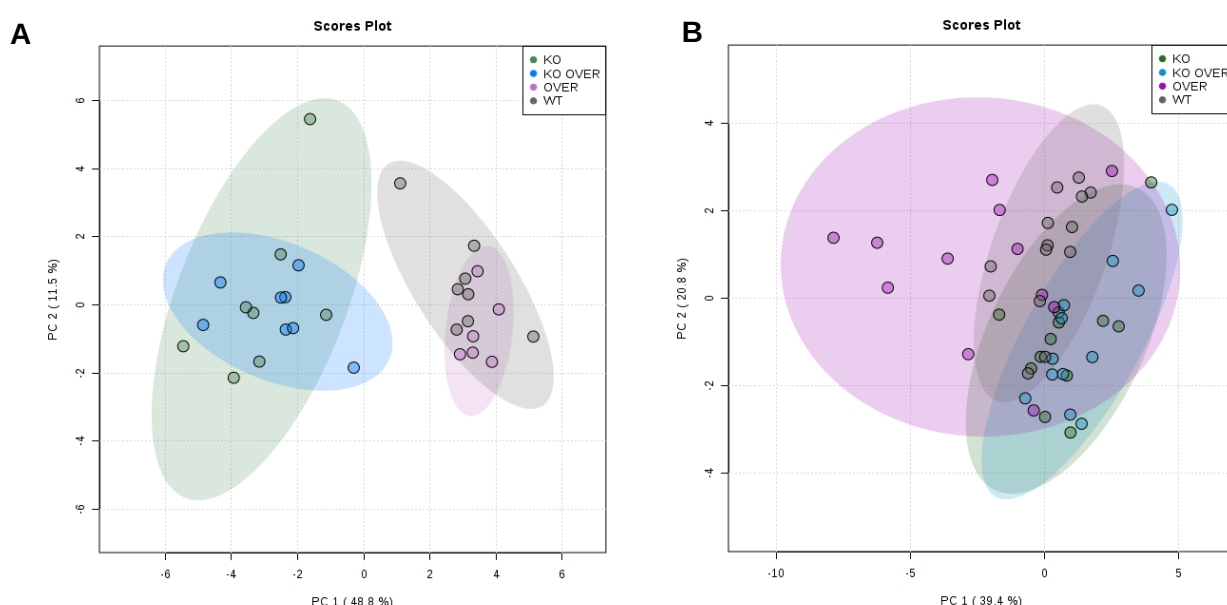


Figure 5-4: PCAs of combined NMR and GC-TOF-MS discriminatory auto-scaled metabolite data matrices of the (A) brain and combined NMR and LC-MS/MS discriminatory auto-scaled metabolite data matrices of the (B) quads where ellipses indicate 95% confidence intervals.

No discriminatory metabolites were identified in the KO vs KO OVER mice in either the brain or the quads. For WT vs OVER mice, only two significant metabolites were identified in the quads. For the GC-TOF-MS data, there was also no discriminatory metabolites identified in the quads of any of the genotype comparisons. The Venn diagrams (Figure 5-5) show that many metabolic perturbations were found in the different groups, but these could all essentially be attributed to the CI deficiency, which appeared to have a dominating effect on the metabolome and thus masked any possible MT effects. As mentioned previously, one of the main metabolic consequences of CI dysfunction is the prevention of electrons being pumped into the respiratory chain by oxidation of NADH to NAD⁺, thus leading to a disturbance of the NADH/NAD⁺ ratio with a build-up of NADH. This redox state alteration is, in fact, a key hallmark of many of the metabolic perturbations associated with MD (Esterhuizen *et al.*, 2017). Due to the altered redox state, the

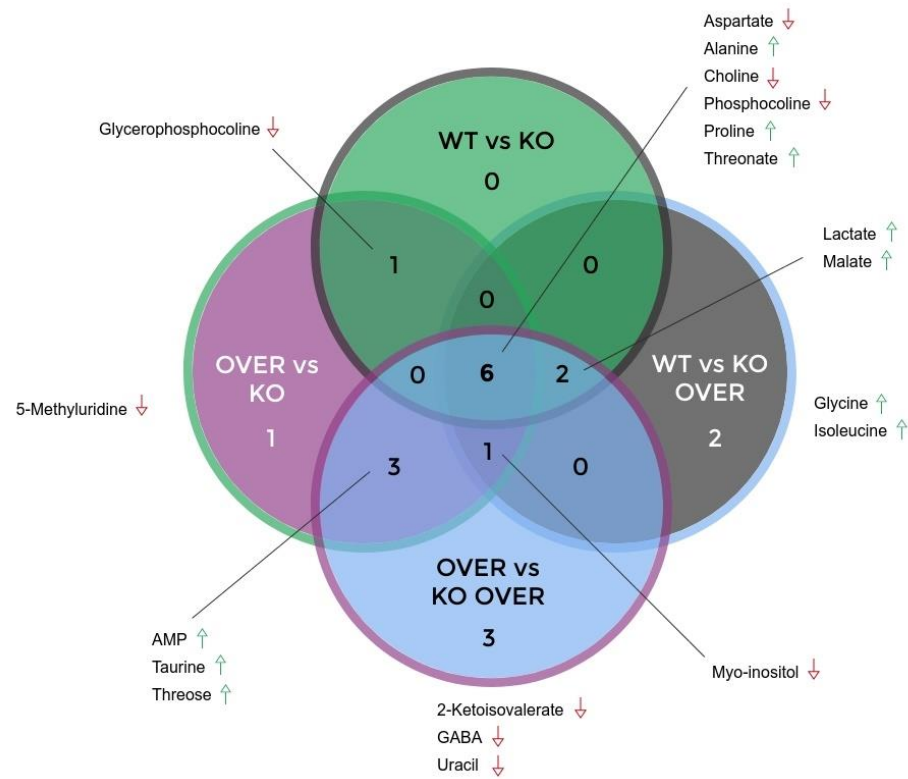
cellular metabolism is affected in a very broad manner rather than just in a single metabolic pathway. Some of the CI linked perturbed metabolic pathways detected included altered Krebs cycle intermediates and central carbon metabolism, disturbed 1C metabolism, purine and pyrimidine metabolism, BCAA metabolism, lysine metabolism and altered urea cycle intermediates. While there were differences between the discriminatory metabolites identified in the KO and KO OVER mice compared to WT and OVER mice, collectively, all of these metabolites are however indicative of differences in the metabolism as a result of CI deficiency. Thus, despite the different overlaps across the genotypes, the role of the metabolites will be discussed as a whole as they represent different ways in which the KO genotype affected the metabolism in different mice.

5.4 ALTERED BRAIN METABOLISM

A summary figure of the significantly altered metabolites in the brain is presented in Figure 5-6 where the interaction between the different metabolic pathways can be seen. Each specific metabolite, its biosynthesis and catabolism are discussed in more detail below. It is important to note that since no LC-MS/MS was performed on the brain tissue, one less platform was used than on the quads tissue.

Johnson *et al.* (2013) also performed metabolomic analyses on whole brain samples (of mice at P30) and reported that the metabolic signature of the *Ndufs4*^{-/-} mouse brain includes an accumulation of pyruvate, lactate and glycolytic intermediates and increased oxidative stress markers together with decreased free amino acids, free fatty acids, nucleotides, products of nucleotide metabolism and GABA. Similarly, we also found decreased GABA, increased lactate and increased AMP. In Esterhuizen *et al.* (2017) altered metabolite concentrations in MDs in various models were reviewed and many similarities were reported for the metabolites shown here. These included elevated lactate, malate, alanine, proline, AMP, taurine, glycine and isoleucine. On the contrary, we also found the following metabolites but they were altered in the opposite direction to the studies reported in Esterhuizen *et al.* (2017): choline, aspartate, 2-ketoisovalerate, GABA and uracil. In parallel to this study, Coetzer (2020) performed metabolomics analyses on the anterior cortex (less affected region), brainstem, olfactory bulbs and cerebellum (more vulnerable areas) in *Ndufs4*^{-/-} mice using the same LC-MS/MS and GC-TOF-MS methods applied here. Across all the brain regions studied, the only common discriminatory metabolites to those identified here included decreased aspartate and increased proline in the KO compared to WT mice. They showed a correlation between the residual CI activity and the severity of the metabolic disturbances in brain regions more vulnerable to CI dysfunction than those less affected. They also showed that the olfactory bulbs were the most severely affected in *Ndufs4*^{-/-} mice, exhibiting potentially neurotoxic features.

A



B

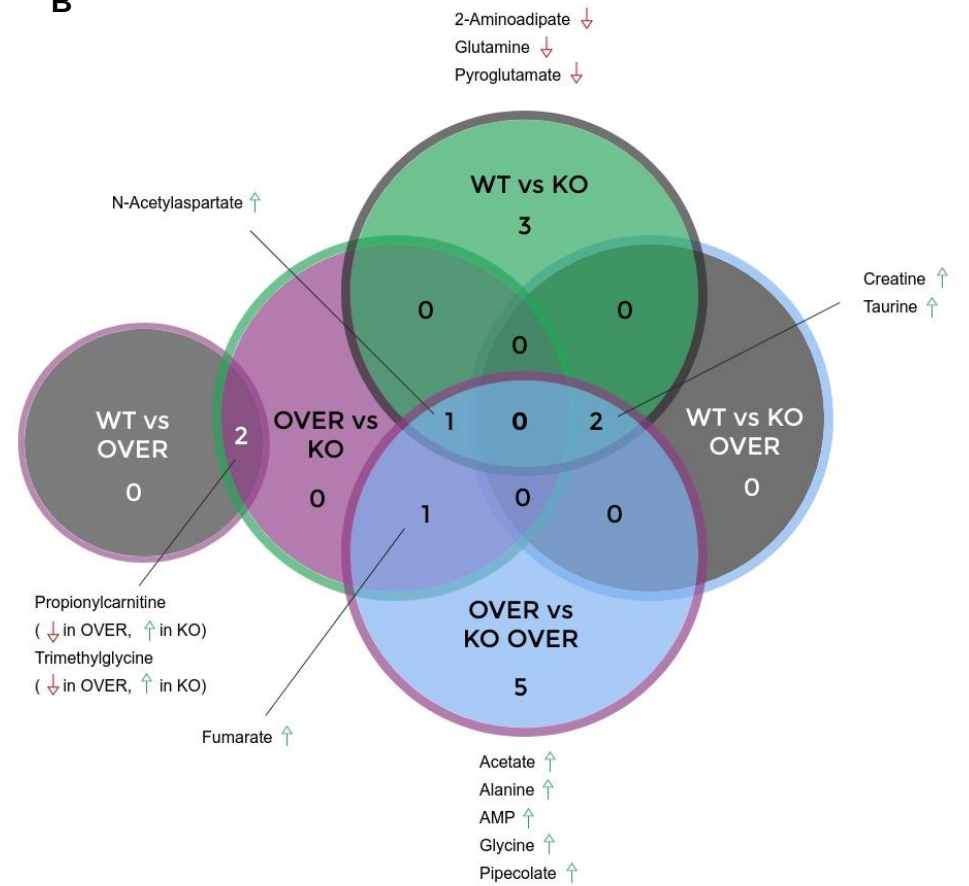


Figure 5-5: Venn diagram depicting important metabolites in the (A) brain and (B) quads which overlap between the genotype groups (WT vs OVER, WT vs KO, WT vs KO OVER, OVER vs KO and OVER vs KO OVER). The direction of metabolite alteration is indicated as KO or KO OVER relative to WT or OVER. For the WT vs OVER group, the direction indicates OVER relative to WT. *Abbreviations:* AMP, adenosine monophosphate; GABA, γ -aminobutyrate.

Table 5-2: List of significant metabolites identified in the brain using NMR and GC-TOF-MS

METABOLITES	↑/↓	ID	CLASS	<u>NMR</u>				<u>GC-TOF-MS</u>			
				MEAN (AU)		T-TEST		MEAN (x10 ⁻³) (AU)		T-TEST	
WT vs KO				WT	KO	p	ES	WT	KO	p	ES
Alanine	↑	1	Amino acid	133.0	192.5	0.003	2.283	411.8	702.4	0.010	2.244
Aspartate	↓	1	Amino acid	557.4	414.4	0.003	2.099	1060.4	785.4	0.001	3.446
Choline	↓	1	Organic acid	44.1	29.6	0.004	1.768				
Glycerophosphocholine	↓	1	Organic acid	134.5	110.8	0.020	1.456				
Lactate	↑	1	Organic acid	2092.8	2595.1	0.041	1.344				
Malate	↑	1	Organic acid					40.4	51.7	0.045	1.796
Phosphocholine	↓	1	Organic acid	108.3	86.7	0.020	1.417				
Proline	↑	1	Amino acid					45.7	68.5	0.001	3.553
Threonate	↑	3	Sugar acid					2.4	3.8	0.020	1.995
WT vs KO OVER				WT	KO OVER	p	ES	WT	KO OVER	p	ES
Alanine	↑	1	Amino acid	133.0	185.9	0.000	2.459	411.8	700.3	0.005	2.763
Aspartate	↓	1	Amino acid	557.4	423.8	0.005	1.956	1083.1	831.5	0.014	2.074
Choline	↓	1	Organic acid	44.1	33.4	0.047	1.282				
Glycine	↑	1	Amino acid	194.3	242.0	0.014	1.512				
Isoleucine	↑	1	Amino acid					13.1	17.5	0.027	1.855
Lactate	↑	1	Organic acid	2092.8	2491.8	0.022	1.283				
Malate	↑	1	Organic acid					40.4	53.1	0.007	2.547
Myo-Inositol	↓	1	Sugar alcohol	1016.5	912.5	0.024	1.353				
Phosphocholine	↓	1	Organic acid	108.3	83.8	0.000	2.861				
Proline	↑	1	Amino acid					45.7	69.1	0.036	1.718
Threonate	↑	3	Sugar acid					2.4	3.4	0.007	2.336

METABOLITES	↑/↓	ID	CLASS	NMR				GC-TOF-MS			
				MEAN (AU)		T-TEST		MEAN (x10 ⁻³) (AU)		T-TEST	
OVER vs KO				OVER	KO	p	ES	OVER	KO	p	ES
5-Methyluridine	↓	2	Nucleic acid	138.3	192.5	0.003	1.995	24.3	16.9	0.028	2.612
Alanine	↑	1	Amino acid					453.7	702.4	0.031	1.883
AMP	↑	1	Nucleic acid					140.6	183.2	0.004	2.026
Aspartate	↓	1	Amino acid					537.4	414.4	0.002	2.218
Choline	↓	1	Organic acid					47.5	29.6	0.000	3.611
Glycerophosphocholine	↓	1	Organic acid					135.4	110.8	0.015	1.498
Myo-Inositol	↓	1	Sugar alcohol					1016.9	908.1	0.048	1.136
Phosphocholine	↓	1	Organic acid					103.6	86.7	0.045	1.147
Proline	↑	1	Amino acid					42.4	68.5	0.003	3.830
Taurine	↑	1	Amino acid					1386.5	1724.4	0.002	2.189
Threonate	↑	3	Sugar acid	2.3	3.8	0.021	2.222				
Threose	↑	2	Pentose	142.3	170.7	0.038	2.217				
OVER vs KO OVER				OVER	KO OVER	p	ES	OVER	KO OVER	p	ES
2-Ketoisovalerate (enol)	↓	3	Amino acid	138.3	185.9	0.000	2.745	9.4	7.2	0.032	2.321
Alanine	↑	1	Amino acid					453.7	700.3	0.014	2.319
AMP	↑	1	Nucleic acid					140.6	185.5	0.031	1.161
Aspartate	↓	1	Amino acid					537.4	423.8	0.004	1.742
Choline	↓	1	Organic acid					47.5	33.4	0.004	1.723
GABA	↓	1	Amino acid					535.2	471.7	0.032	1.343
Lactate	↑	1	Organic acid					2275.7	2491.8	0.032	1.336
Malate	↑	1	Organic acid					43.2	53.1	0.032	1.972
Myo-Inositol	↓	1	Sugar alcohol					1016.9	912.5	0.010	1.676

METABOLITES	↑/↓	ID	CLASS	<u>NMR</u>				<u>GC-TOF-MS</u>			
				MEAN (AU)		T-TEST		MEAN (x10 ⁻³) (AU)		T-TEST	
Phosphocholine	↓	1	Organic acid	103.6	83.8	0.000	2.371				
Proline	↑	1	Amino acid					42.4	69.1	0.032	1.970
Taurine	↑	1	Amino acid	1386.5	1652.1	0.000	2.813				
Threonate	↑	3	Sugar acid					2.3	3.4	0.007	2.712
Threose	↑	2	Pentose					142.3	168.5	0.032	2.229
Uracil	↓	1	Nucleic acid					3.1	1.5	0.032	1.987

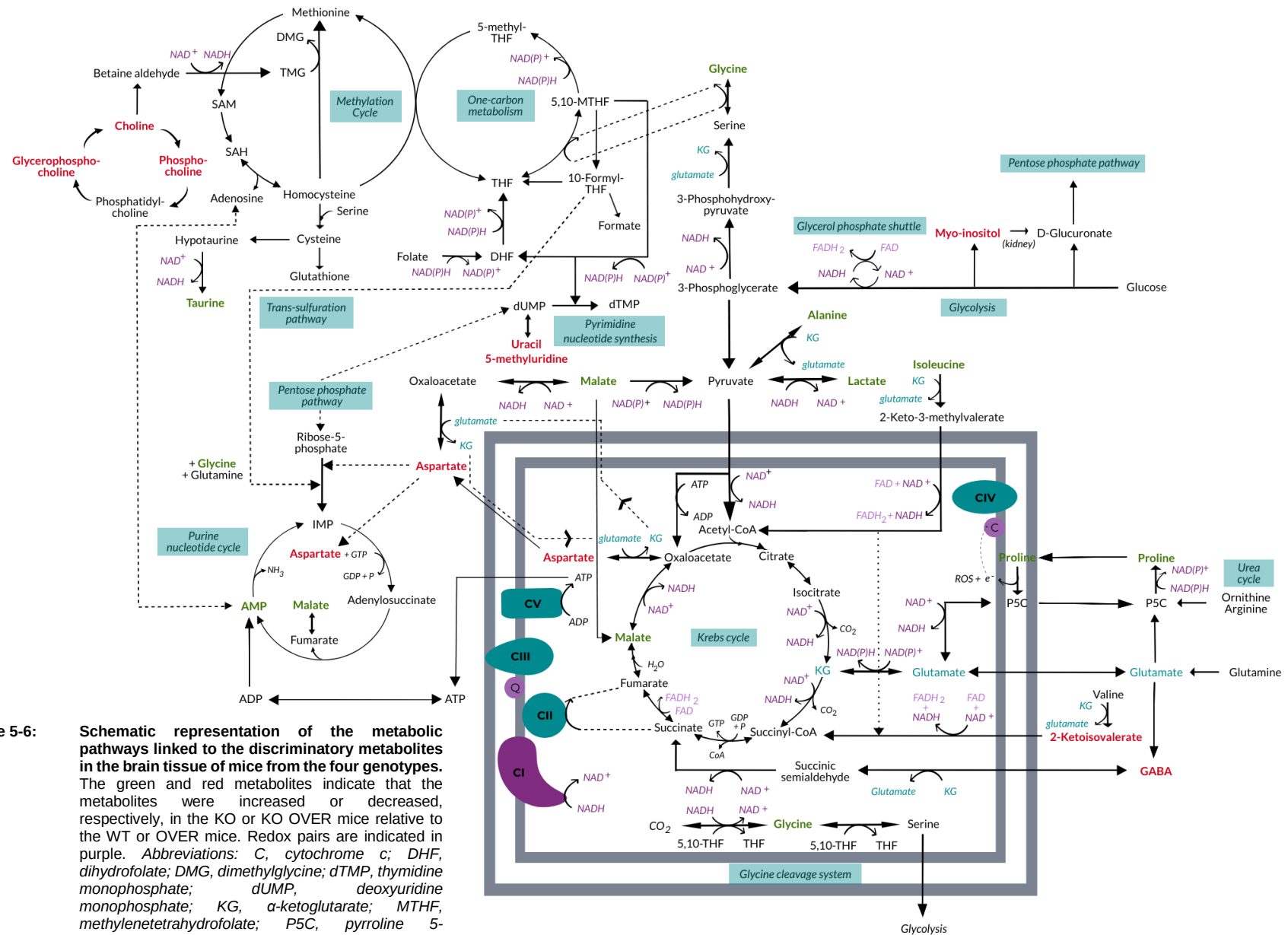
* Means (and not concentrations) are given for the untransformed raw data. ID indicates the level of identification obtained. Independent t-test results: P < 0.05 = BH-FDR 5% adjusted p-value. ES = Effect size (Cohen's d-value; where 0.8 is significant). *Abbreviations: AU, Arbitrary units; AMP, adenosine monophosphate; GABA, γ-aminobutyrate.*

Table 5-3: List of significant metabolites identified in the quads using NMR and LC-MS-MS

METABOLITES	↑/↓	ID	CLASS	NMR				LC-MS/MS			
				MEAN (AU)		T-TEST		MEAN (x10 ⁻⁶) (AU)		T-TEST	
WT vs OVER				WT	OVER	p	ES	WT	OVER	p	ES
Propionylcarnitine (C3)	↓	1	Acylcarnitine					0.025	0.017	0.049	1.369
Trimethylglycine	↓	1	Amino acid					0.064	0.045	0.049	1.259
WT vs KO				WT	KO	p	ES	WT	KO	p	ES
2-aminoadipate	↓	1	Amino acid					0.063	0.042	0.032	1.321
Creatine	↑	1	Organic acid	4177.6	4542.9	0.043	1.209				
Glutamine	↓	1	Amino acid					14.776	11.046	0.032	1.266
N-acetylaspartate (NAA)	↑	1	Amino acid					0.019	0.031	0.032	1.371
Pyroglutamate	↓	1	Amino acid					2.123	1.634	0.049	1.054
Taurine	↑	1	Amino acid	5236.4	5806.7	0.025	1.358				

METABOLITES	↑/↓	ID	CLASS	NMR				LC-MS/MS			
				MEAN (AU)		T-TEST		MEAN (x10 ⁻⁶) (AU)		T-TEST	
WT vs KO OVER				WT	KO OVER	p	ES	WT	KO OVER	p	ES
Creatine	↑	1	Organic acid	4177.6	4561.2	0.025	1.313				
Taurine	↑	1	Amino acid	5236.4	5833.3	0.003	1.664				
OVER vs KO				OVER	KO	p	ES	OVER	KO	p	ES
Fumarate	↑	1	Organic acid	15.0	24.8	0.010	1.424				
N-acetylaspartate (NAA)	↑	1	Amino acid					0.013	0.031	0.000	2.101
Propionylcarnitine (C3)	↑	1	Acylcarnitine					0.017	0.026	0.031	1.323
Trimethylglycine	↑	1	Amino acid					0.045	0.068	0.033	1.279
OVER vs KO OVER				OVER	KO OVER	p	ES	OVER	KO OVER	p	ES
Acetate	↑	1	Organic acid	115.0	129.3	0.035	1.034				
Alanine	↑	1	Amino acid	349.2	469.8	0.034	1.086				
AMP	↑	1	Nucleic acid	858.0	1036.3	0.034	0.927				
Creatine	↑	1	Organic acid	3726.6	4561.2	0.035	0.881				
Fumarate	↑	1	Organic acid	15.0	21.9	0.034	1.082				
Glycine	↑	1	Amino acid	420.2	589.2	0.034	1.256				
N-acetylaspartate (NAA)	↑	1	Amino acid					0.013	0.028	0.000	1.924
Pipecolate	↑	1	Organic acid					0.028	0.042	0.031	1.281
Taurine	↑	1	Amino acid	4613.4	5833.3	0.034	0.950				

* Means (and not concentrations) are given for the untransformed raw data. ID indicates the level of identification obtained. Independent t-test results: P < 0.05 = BH FDR 5% adjusted p-value. ES = Effect size (Cohen's d-value; where 0.8 is significant). *Abbreviations: AU, Arbitrary units; AMP, adenosine monophosphate.*



5.4.1 Central carbon metabolism

As depicted in Figure 5-7, the organic acids lactate and malate were observed to be increased in KO OVER mice compared to WT mice. Of the non-essential amino acids, alanine was found to be increased, and aspartate decreased, in the KO and KO OVER mice compared to the WT and OVER mice. Increased lactate and alanine are known diagnostic markers of MDs and more specifically of LS, but they are not exclusive to these diseases (Mitochondrial Medicine Society's Committee on Diagnosis *et al.*, 2008; Esterhuizen *et al.*, 2017).

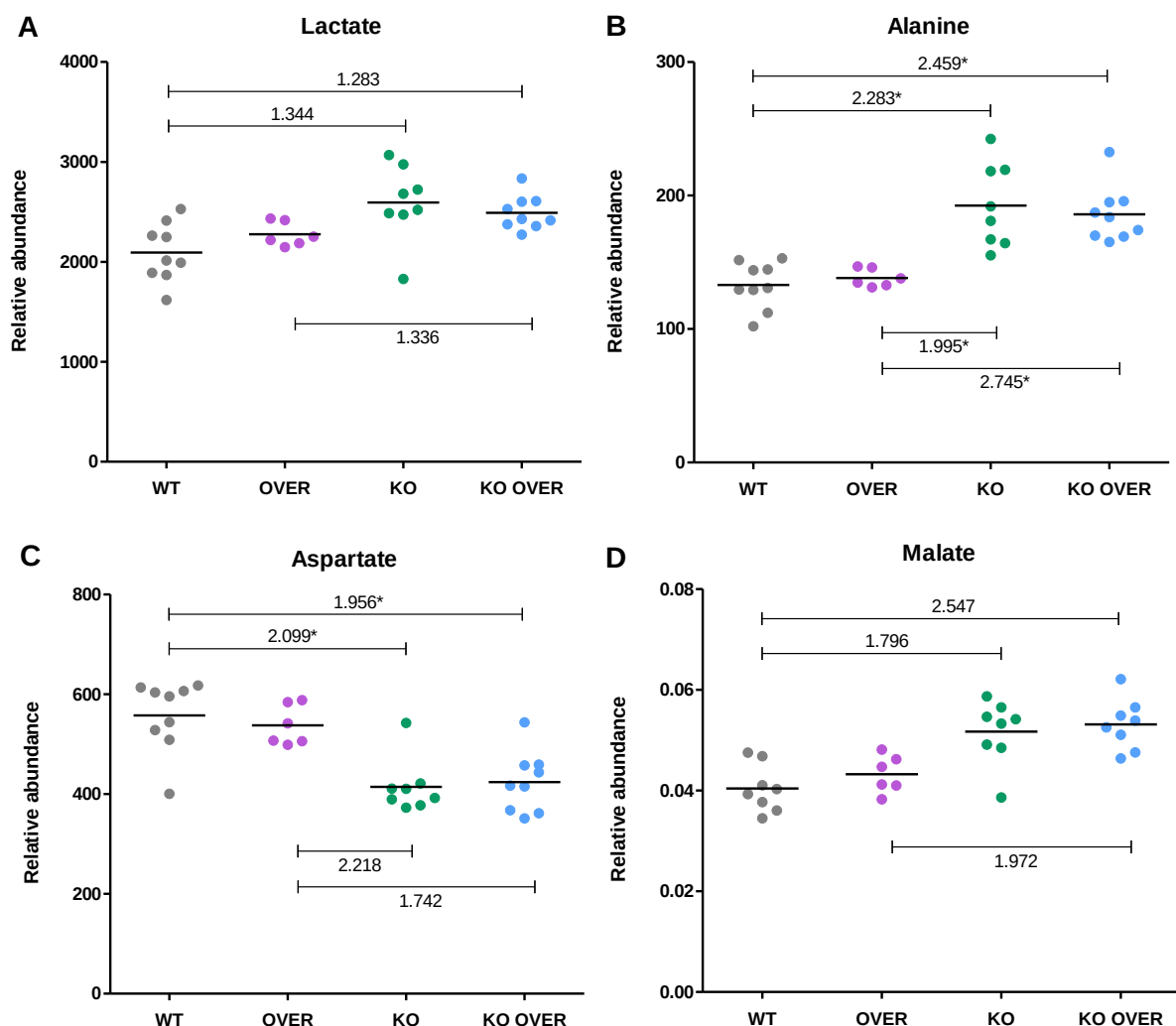


Figure 5-7: Vertical scatter plots depicting the relative abundance of (A) lactate; (B) alanine; (C) aspartate and (D) malate in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant). An asterisk (*) indicates that a significant difference was seen in both NMR and GC-TOF-MS, where only the NMR data is shown.

5.4.1.1 Lactate

Pyruvate, an important metabolite at the centre of many metabolic pathways, is converted to acetyl-CoA (via pyruvate dehydrogenase with reduction of NAD^+ to NADH) which is then oxidised in the Krebs cycle to produce energy via the OXPHOS system. As described above, CI dysfunction leads to a state of disturbed redox balance where there is a build-up of NADH. In an attempt to restore this balance, pyruvate is reduced to lactate by lactate dehydrogenase, with the simultaneous oxidation of NADH to NAD^+ (thus allowing glycolysis to proceed by regenerating the NAD^+ pool) (Esterhuizen *et al.*, 2017; Kanehisa *et al.*, 2019). This leads to increased lactate levels, as seen in our KO OVER mice, and has previously been reported in the brain tissue of both humans (Mitochondrial Medicine Society's Committee on Diagnosis *et al.*, 2008) and *Ndufs4*^{-/-} mice (Johnson *et al.*, 2013). Increased lactate was also reported in the blood (Kruse *et al.*, 2008) and fibroblasts (Valsecchi *et al.*, 2012) of *Ndufs4*^{-/-} mice, however not at levels high enough to necessarily suggest toxic acidosis.

5.4.1.2 Alanine

Similarly to lactate, alanine can also be found in equilibrium with pyruvate and forms part of the glucose-alanine cycle. The BCAAs (discussed in Section 5.4.3), aspartate and glycolysis all produce pyruvate, which can then be transaminated to form alanine via alanine transaminase (and conversion of glutamate to KG). Alanine formed in the muscle is then transported to the liver where it is used for gluconeogenesis and ultimately for energy production in the brain (Salway, 2004; Kanehisa *et al.*, 2019).

Falk *et al.* (2008) studied the metabolic pathways of CI mutants in *C. elegans*. Their data suggested that oxidation of ketoacids (such as pyruvate and KG) is impaired during mitochondrial dysfunction making them more accessible for transamination with glutamate to produce their related amino acids. This accumulation of ketoacids led to increased alanine, aspartate, leucine, valine and glycine, together with decreased glutamate, as the transaminases in the equilibrium reactions regulating these amino acids were tipped away from the ketoacids. This correlates with the KO and KO OVER mice as well as that seen in patients with OXPHOS disorders, as discussed in Section 2.5.3.4, where alanine levels are consistently reported as elevated since this increase in available pyruvate disturbs the equilibrium of the reversible alanine aminotransferase reaction and leads to conversion of pyruvate to alanine (with the concurrent conversion of glutamate to KG) (Smeitink *et al.*, 2006; Esterhuizen *et al.*, 2017). While decreased glutamate was not detected here, a related study (Coetzer, 2020) using various brain regions from the same KO model did indeed see decreased glutamate in all four brain regions investigated, thus supporting this hypothesis.

5.4.1.3 Aspartate

Aspartate is synthesised by transamination of oxaloacetate in the reversible reaction catalysed by aspartate aminotransferase (AST), in the presence of glutamate (to produce KG). Aspartate is used as an amino donor in urea synthesis, where it provides the carbon backbone, and is also essential for *de novo* pyrimidine and purine synthesis, with converts inosine monophosphate (IMP) to AMP, respectively. Aspartate also forms part of the malate/aspartate shuttle where cytosolic malate dehydrogenase reduces oxaloacetate to malate using NADH. Malate is transported into the mitochondrial matrix in exchange for KG, where it is then oxidised back to oxaloacetate with release of NADH to be used by the OXPHOS system. Oxaloacetate is then transported back to the cytosol by transforming it to aspartate (by AST) which is transported across the membrane by the glutamate/aspartate carrier. Aspartate is finally transaminated back to oxaloacetate in the cytosol (Salway, 2004; Kanehisa *et al.*, 2019).

Biosynthesis of aspartate by the ETS has been identified as an essential role during cell proliferation. When the ETS is inhibited, cytosolic AST reverses to generate aspartate in the cytosol, to compensate for the loss of mitochondrial aspartate synthesis. Aspartate can be synthesised from KG via both oxidative and reductive pathways as shown in Figure 5-8. The reductive carboxylation pathway converts glutamine to glutamate which enters the Krebs cycle via glutamate dehydrogenase (using NAD^+ as co-substrate) to produce KG. Oxidative synthesis utilises the Krebs cycle functioning in the opposite direction via succinate and malate but requires the net transfer of two electron pairs. A pathway that does not require electron acceptors to synthesise aspartate includes carboxylation of pyruvate to oxaloacetate, which can then be transaminated to form aspartate. However, in all of these mechanisms, high levels of pyruvate and KG are required, and the synthesis thereof requires electron acceptors. This explains the decreased aspartate seen in the KO and KO OVER mice due to the limited availability of electron acceptors, regardless of the biosynthesis pathway used. Without sufficient aspartate cells are unable to proliferate and cell death eventually occurs (Birsoy *et al.*, 2015; Sullivan *et al.*, 2015), a possible contributing factor to the mortality of CI dysfunction.

Depleted KG was also evidenced when measuring ADP-stimulated respiration in isolated mitochondria from whole brain samples of *Ndufs4*^{-/-} mice. Utilisation of pyruvate/malate or glutamate/malate as substrates lowered the CI dependent OXPHOS capacity in the *Ndufs4*^{-/-} samples compared to controls, while the use of KG/malate as substrate resulted in normalisation of the CI dependent OXPHOS capacity of *Ndufs4*^{-/-} samples (to levels similar to that of controls) (Kayser *et al.*, 2016).

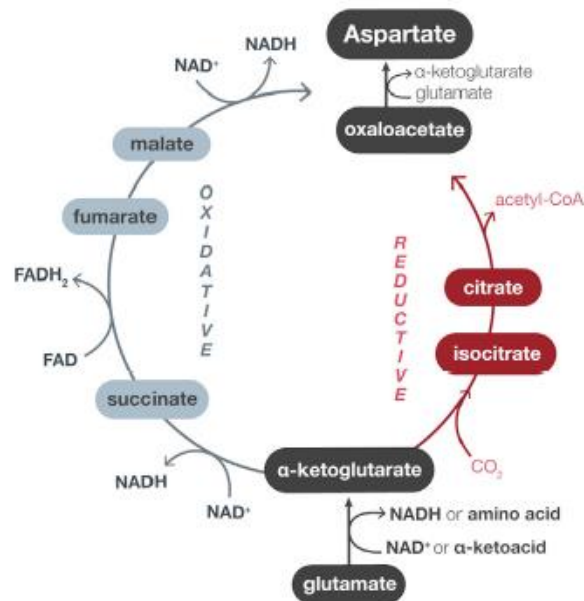


Figure 5-8: Schematic representation of the reductive and oxidative pathways in the Krebs cycle for synthesising aspartate. Image obtained with permission from Sullivan *et al.* (2015) (licence no. 4673101226721).

5.4.1.4 Malate and the Krebs cycle

Malate forms part of the Krebs cycle and is also intertwined with the malate/aspartate shuttle mentioned above, as well as the purine nucleotide cycle (discussed in Section 5.4.7.1). Malate dehydrogenase oxidises oxaloacetate and NADH to form malate and NAD⁺. The pyruvate/malate cycle functions to transport acetyl-CoA from the mitochondria to the cytosol via conversion of oxaloacetate to malate in the cytosol (following citrate cleavage by ATP citrate lyase), as well as to generate NADPH (which is required for fatty acid synthesis) in the reaction catalysed by malate dehydrogenase (Salway, 2004; Kanehisa *et al.*, 2019).

Reduced turnover of NADH in the mitochondria during MD has been shown to impair the malate/aspartate cycle and thus increase cytosolic NADH. As seen by the increased malate in our KO OVER mice, this increased cytosolic NADH stimulates reductive carboxylation (described above) to form malate which in turn provides carbon atoms for NADH-coupled malate dehydrogenase and stimulates glyceraldehyde-3-phosphate dehydrogenase (a critical enzyme in the glycolytic pathway dependent on reduction of NAD⁺ for glycolysis to proceed) thus regulating redox balance and increasing glycolytic flux to support ATP production in the cytosol (Gaude *et al.*, 2018). Zielinski *et al.* (2016) also proposed a mechanism for increased malate where the Krebs cycle was split so that both the forward and reverse reactions of the Krebs cycle were used to produce malate from pyruvate (described in Section 2.5.3.4).

5.4.2 GABA and proline

The non-essential amino acid, proline, was increased in the KO and KO OVER mice compared to the WT and OVER mice, as shown in Figure 5-9, while GABA was found to be decreased in KO OVER mice compared to OVER mice, in agreement with reports that a deficiency of GABA is typically associated with neurodegeneration (Ravasz *et al.*, 2017).

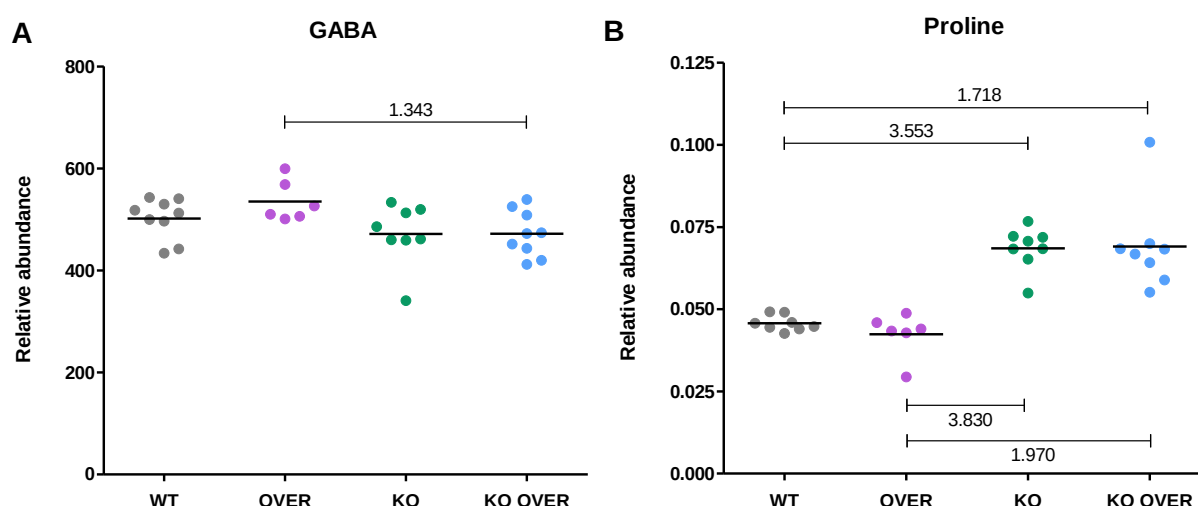


Figure 5-9: Vertical scatter plots depicting the relative abundance of (A) GABA and (B) proline in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

5.4.2.1 GABA

GABA is principally known as a neurotransmitter for rapid inhibitory or excitatory synaptic transmission in the CNS. GABA is synthesised via the GABA shunt whereby glutamate (obtained from KG) is irreversibly converted to GABA by the cytosolic enzyme glutamate decarboxylase. GABA is then converted to succinic semialdehyde by GABA transferase (with the simultaneous conversion of KG to glutamate) which is in turn converted into succinate by succinate semialdehyde dehydrogenase (with reduction of NAD^+). Succinate then enters the Krebs cycle. Proline can also contribute to the biosynthesis of GABA via the polyamine pathway. GABA synthesis occurs only in neurons, whilst catabolism occurs in glia too (Ravasz *et al.*, 2017).

Falk *et al.* (2008) also found decreased GABA in their *C. elegans* model of CI dysfunction. They hypothesised that this may be due to decreased glutamate levels (as discussed in Section 5.4.3) or due to increased transamination of GABA to succinate (since this requires KG dehydrogenase, which oxidises NADH and KG to form glutamate). The production of succinate would also be favourable during CI dysfunction as it can be oxidised via CII.

5.4.2.2 Proline cycle

Proline can be synthesised by glutamine, arginine and ornithine (from the urea cycle). In addition, proline is also synthesised via the proline cycle where glutamate (formed from the Krebs cycle intermediate KG) is converted to pyrroline-5-carboxylate (P5C) by delta-P5C synthetase, followed by NADH-dependent P5C conversion to proline by pyrroline-5-carboxylate reductase. Proline is catabolised to glutamate by the FAD-dependent enzyme proline dehydrogenase, which is located in the mitochondrial inner membrane and donates electrons directly to cyt c in the ETS.

Proline plays an important role in protein synthesis and structure, metabolism (especially synthesis of arginine and polyamines in addition to glutamate), as well antioxidant reactions (scavenging of free radicals in response to oxidative stress) and immune responses (Wu *et al.*, 2011; Kanehisa *et al.*, 2019). Both Zielinski *et al.* (2016) and Terburgh *et al.* (2019) identified the proline cycle as an essential compensatory mechanism in CI deficiency. Synthesis of proline in the mitochondrion from pyrroline 5-carboxylate restores redox balance with its concurrent oxidation of NADH and catabolism of proline by proline dehydrogenase assists energy production by direct transfer of electrons to cyt c. Elevated proline levels are a diagnostic marker in mitochondrial disease with the former reaction considered to play a larger mechanistic role due to inhibition of the latter by elevated lactate levels (Smeitink *et al.*, 2006; Mitochondrial Medicine Society's Committee on Diagnosis *et al.*, 2008).

5.4.3 Branched-chain amino acids

Of the BCAAs, isoleucine was increased in KO OVER mice compared to WT mice, and 2-ketoisovalerate (a derivative of valine) was decreased in KO OVER mice compared to OVER mice, as shown in Figure 5-10. BCAAs (leucine, isoleucine and valine), which are major donors of amino groups, are reversibly catabolised by BCAA aminotransferase to the respective α -keto acids, with concurrent conversion of KG to glutamate. During further BCAA catabolism, valine utilises three NAD⁺ molecules and one FAD molecule to form propionyl-CoA, isoleucine utilises two NAD⁺ molecules and one FAD molecule to form propionyl-CoA and acetyl-CoA, and leucine utilises one NAD⁺ and one FAD molecule to form acetyl-CoA and acetoacetate. Propionyl-CoA leads to succinyl-CoA production, which then enters the Krebs cycle and can be used for pyruvate formation and can thus also lead to transamination of pyruvate to alanine via the glucose alanine cycle (Salway, 2004; Kanehisa *et al.*, 2019). The succinate that is formed from succinyl-CoA is also used to drive ATP production via CII, an important fuel during CI dysfunction.

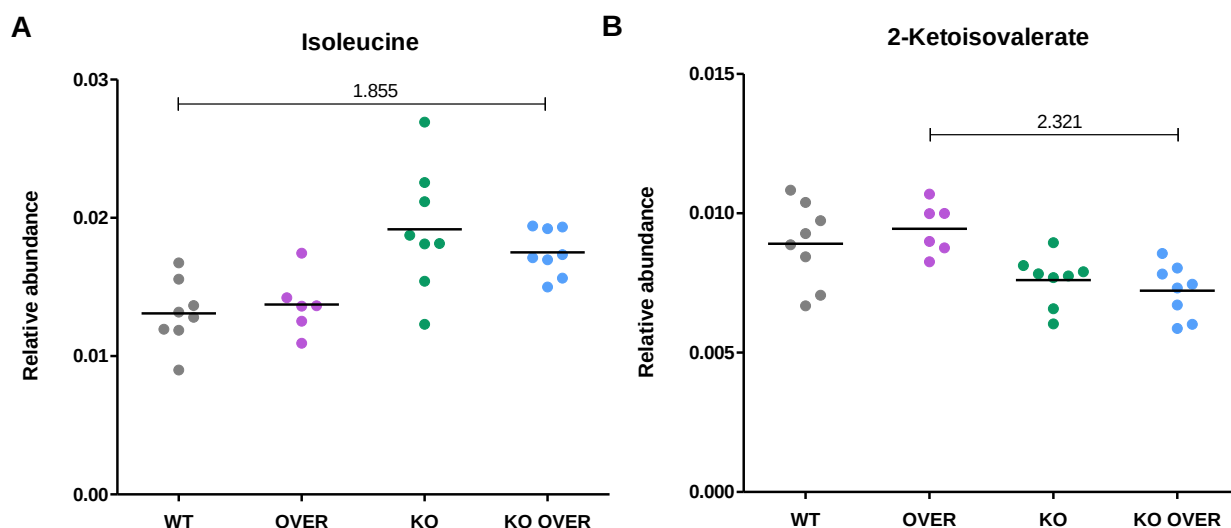


Figure 5-10: Vertical scatter plots depicting the relative abundance of (A) isoleucine and (B) 2-ketoisovalerate in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

Due to the increased NADH/NAD⁺ ratio in CI dysfunction, the activity of the branched-chain α -ketoacid dehydrogenase complex (responsible for the oxidative decarboxylation of the α -keto acids and fuelled by NAD⁺) is inhibited thus decreasing BCAA catabolism (Legault *et al.*, 2015; Esterhuizen *et al.*, 2017). The catabolism of BCAAs has also been reported to be dysregulated by an increased FADH₂/FAD redox state due to deficient electron delivery to CII during ETS dysfunction (Reinecke *et al.*, 2012; Smuts *et al.*, 2013). Falk *et al.* (2008) hypothesised that increased BCAAs are formed due to transamination of the relative ketoacid with glutamate (following on from Section 5.4.1.2). Taken together, these shifts in equilibrium lead to accumulation of the BCAAs (such as isoleucine seen in the KO OVER mice), and depletion of their respective α -keto acids (such as 2-ketoisovalerate seen in the KO OVER mice) and glutamate. Some glutamate may be restored by reversing the glutamate dehydrogenase reaction to utilise KG and the increased NADH, but this reaction is not believed to be robust enough to prevent total reduction of glutamate, thus indicating that BCAAs may be sensitive indicators of redox state (Falk *et al.*, 2008).

5.4.4 Threonate and threose

Threonate was found to be increased in KO and KO OVER mice compared to WT and OVER mice, together with threose, which was also increased in KO and KO OVER mice compared to OVER mice. Threonate is a sugar acid derived from threose. However, threose was identified at a level 2 ID and threonate at a level 3 ID, and while both features are most likely sugars, there is uncertainty as to whether or not the exact identification is correct. Threonate may be produced by the breakdown of the sugar components in glycated proteins (Harding *et al.*, 1999), and has also

been shown to be linked to species in the gut microbiome (Visconti *et al.*, 2019), but the exact source and ID warrants further investigation.

5.4.5 Myo-Inositol and osmolytes

Myo-inositol was found to be decreased in KO and KO OVER compared to OVER mice as well as in KO OVER compared to WT mice, as shown in Figure 5-11. In WT vs KO mice, myo-inositol was also found as a significant metabolite with BH-FDR adjusted $p < 0.1$.

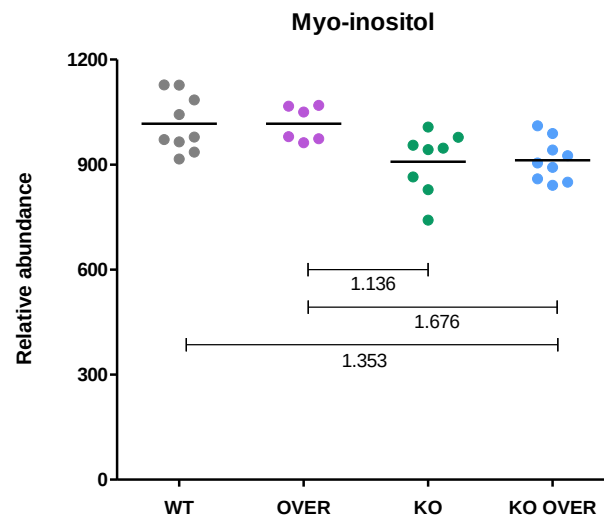


Figure 5-11: Vertical scatter plot depicting the relative abundance of myo-inositol in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

Myo-inositol is one of the most abundant metabolites in the brain that is located mainly in glial cells, acts as an organic osmolyte and has been shown to increase under hypertonic- and decrease under hypotonic-conditions (Kwon *et al.*, 1992; Lee *et al.*, 1994; Videen *et al.*, 1995). It is a cyclitol that provides the structural basis for numerous secondary messengers and is thus important for cell growth and survival, development and functioning of peripheral nerves, osteogenesis, glucose homeostasis and reproduction. It is synthesised endogenously from glucose whereby glucose is phosphorylated to glucose-6-phosphate, which is in turn converted to myo-inositol-1-phosphate, and finally dephosphorylated to form myo-inositol. By phosphorylation or methylation, myo-inositol can then be converted into one of many derivatives, which are thereafter bound to phospholipids. Catabolism of myo-inositol occurs in the kidneys where it is broken down to D-glucuronate and enters the pentose phosphate shuttle (Croze & Soulage, 2013).

In mitochondrial recessive ataxia syndrome, mitochondrial myopathy, encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and maternally inherited diabetes and deafness (MIDD) patients, Buzkova *et al.* (2018) saw significantly increased carbohydrate derivatives, including glucuronate and myo-inositol, and identified myo-inositol (together with alanine, sorbitol and cystathionine) as one of the critical blood biomarkers for distinguishing primary MDs from secondary or non-MDs. This was contrary to the decrease in myo-inositol seen in our CI dysfunctional mice and may be attributed to the fact that Buzkova *et al.* (2018) did not specifically look at patients with CI dysfunction.

Taken together, myo-inositol, choline, glycerophosphocholine (GPC), taurine and betaine (other discriminatory metabolites discussed below) are all organic osmolytes that play an important osmoregulatory role in the brain, and whether the altered levels caused by the CI defect in our model attempt to exert a protective effect or exasperate the pathological symptoms in the disease phenotype, remains to be elucidated (Burg & Ferraris, 2008). Under significant chemical and physical stress, proteins can lose their functionally active conformation leading to misfolding or aggregation, which can lead to complete loss of their functional activity. Osmolytes typically accumulate intracellularly and maintain the stability and folding of proteins without disturbing other cellular processes (Atamna & Kumar, 2010). Astrocytes have been identified as an essential component of the osmotic regulatory system in cells and tissues and have been shown to express GFAP during osmotic challenges to encourage cell repair and survival (Wang & Parpura, 2018). Astrocyte swelling has been proposed as a possible central cause of seizures in epilepsy sufferers by decreasing the extracellular space and increasing glutamate concentrations, which leads to pathological neuronal firing and synchrony (Murphy *et al.*, 2017). Furthermore, taurine storage in astrocytes *in vitro* has been shown to have an important effect on ion homeostasis (specifically of calcium and potassium) in the CNS and may act indirectly on neuronal excitability (Walz & Allen, 1987). As seizures and glial activation are known to occur in *Ndufs4*^{-/-} mice (Quintana *et al.*, 2010), as seen in the KO and KO OVER mice too (Figure 4-17O and P), in conjunction with the known disturbances of calcium homeostasis in CI deficiency (see Section 2.5.3.3), it is possible that the disturbance of these organic osmolytes in the KO and KO OVER mice may play a direct or indirect pathophysiological role for the phenotype seen in *Ndufs4*^{-/-} mice.

5.4.6 1C metabolism, the methylation cycle and transsulfuration pathway

Reports of remodelling of the 1C metabolism in mammalian cells as a result of OXPHOS dysfunction have implicated the central involvement of the 1C folate cycle, methyl cycle, transsulfuration pathway and purine synthesis in MD pathology (Bao *et al.*, 2016; Nikkanen *et al.*, 2016), as also seen by the altered metabolites in these pathways in our KO mice. To understand

the mechanism by which these pathways are affected in MDs, a brief overview of these metabolic pathways and how they are linked is given below (and can also be seen in Figure 5-6).

An intermediate in the glycolytic pathway, 3-phosphoglycerate, is metabolised via 3-phosphoglycerate dehydrogenase (and reduction of NAD^+) to 3-phosphohydroxypyruvate and eventually to serine (via conversion of glutamate to KG). Serine and glycine are integral to the folate cycle and can be found in a reversible reaction where conversion of serine to glycine results in the simultaneous conversion of tetrahydrofolate (THF) to 5,10-methylenetetrahydrofolate (MTHF) by serine hydroxymethyltransferase (this occurs in both the mitochondrion and the cytosol). In the mitochondrion, 5,10-MTHF is oxidised to 10-formyl-THF and then to formate. Mitochondrial formate is then released into the cytosol where it is incorporated into cytosolic 10-formyl-THF. In the cytosol, conversion of 5,10-MTHF to 10-formyl-THF is required (together with glycine, glutamine and aspartate) to convert ribose-5-phosphate to IMP which is used for purine synthesis. The conversion of 5,10-MTHF to dihydrofolate (DHF) by thymidylate synthetase results in concurrent conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) which is used for pyrimidine DNA synthesis.

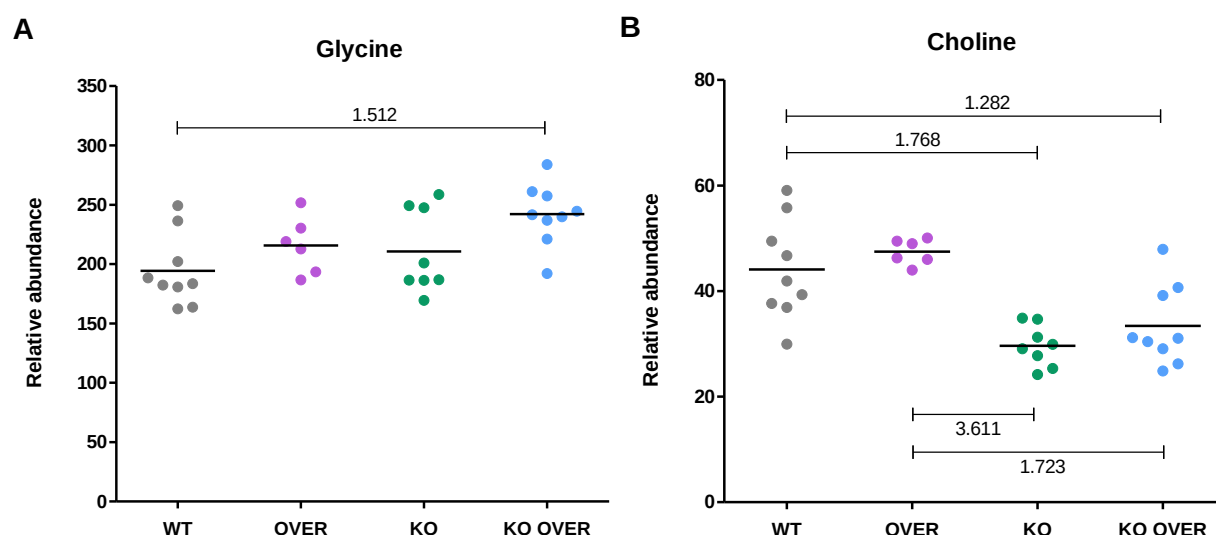
The folate cycle links to the methylation cycle when 5,10-MTHF is converted to 5-methyl-THF (with oxidation of NADPH to NADP^+) and when 5-methyl-THF is converted back to THF by methionine synthase, there is simultaneous conversion of homocysteine to methionine. Methionine is used to produce S-adenosylmethionine (SAM), which is an important methyl group donor for methylation reactions, polyamine biosynthesis and carnitine biosynthesis. Following conversion of SAM to S-adenosyl homocysteine (SAH) and its conversion to homocysteine, homocysteine can also be converted to methionine via a different pathway utilising betaine-homocysteine methyltransferase and conversion of trimethylglycine (TMG, also known as betaine) to DMG. TMG is synthesised from choline (obtained from diet and phospholipids) by choline oxidase and betaine aldehyde dehydrogenase (which reduces NAD^+) (Morrow *et al.*, 2015; Bao *et al.*, 2016; Nikkanen *et al.*, 2016; Esterhuizen *et al.*, 2017; Konno *et al.*, 2017; Kanehisa *et al.*, 2019).

Bao *et al.* (2016) offered evidence that during mtDNA depletion, cells activate serine biosynthesis and transsulfuration. They furthermore showed that inhibition of OXPHOS in cells impaired the mitochondrial production of formate (a precursor of DNA building blocks) from serine. Besides its function as a major source of 1C units in the folate metabolism (to support methylation reactions) and in purine and pyrimidine synthesis, serine also plays an important role in protein synthesis, regulation of the exit of carbons from glycolysis, and acts as a precursor for phospholipid synthesis as well as cysteine and glutathione biosynthesis via transsulfuration. This crucial role of serine explained why Bao *et al.* saw a serine dependence of certain cell lines on cell growth

(related to the NAD⁺/NADH redox status rather than to energy deficiency), which could be rescued by supplementation with formate (bypassing the need for mitochondrial synthesis thereof) or hypoxanthine (an alternative source of purines). Breakdown of choline and glycine can also provide 1C units to support growth but were shown to be insufficient to completely replace serine-derived 1C units.

Nikkanen *et al.* (2016) provided further *in vivo* evidence that in MDs, this 1C remodelling leads to cellular nucleotide imbalance associated with glutathione synthesis and deficient metabolite methylation. They hypothesised that MD may induce a stress pathway in the affected tissues which promotes glucose-driven *de novo* serine biosynthesis and uses the transsulfuration pathway to synthesise glutathione (an antioxidant and reductant that also promotes dNTP synthesis via the glutaredoxin system). Glutathione may be used to avert damage or to offer repair to the cellular metabolic and/or DNA stress caused by MDs. The disturbances in 1C metabolism and the methyl cycle may also explain some of the phenotypic symptoms seen in *Ndufs4*^{-/-} mice since ataxia and severe epilepsy have been reported in childhood diseases where there are disturbances in creatine synthesis and folate supply to the brain (Stöckler *et al.*, 1996).

In relation to these metabolic pathways, as shown in Figure 5-12 and further discussed below, glycine was increased in our KO OVER compared to WT mice, choline and phosphocholine (PC) were found to be decreased in KO and KO OVER mice vs WT and OVER mice and GPC was decreased in KO mice compared to WT and OVER mice.



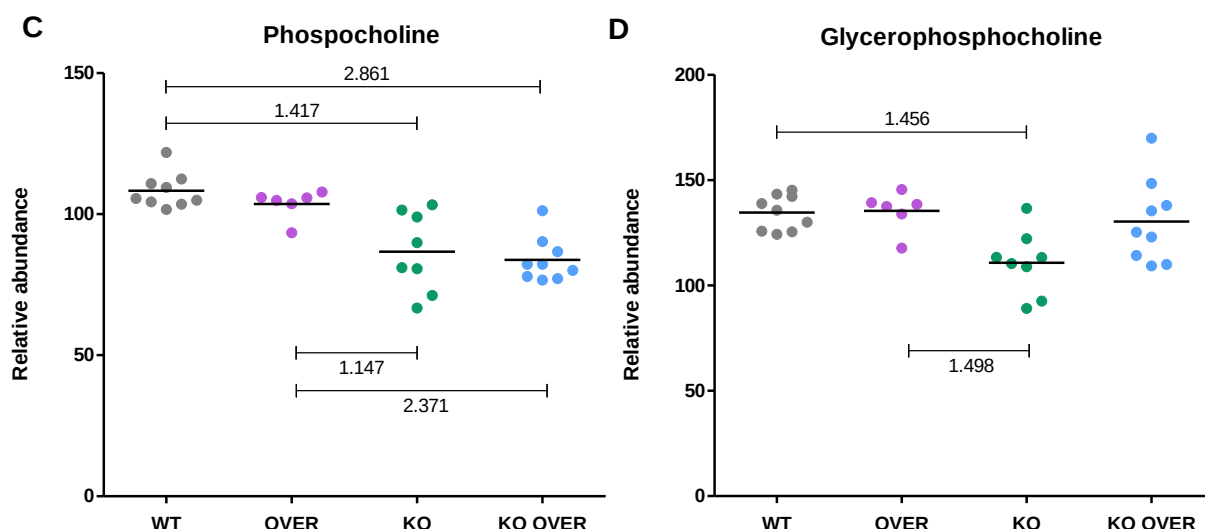


Figure 5-12: Vertical scatter plots depicting the relative abundance of (A) glycine; (B) choline; (C) phosphocholine and (D) glycerophosphocholine in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

5.4.6.1 Glycine

Glycine can be biosynthesised by either glycine synthase or serine hydroxymethyltransferase, both of which use serine. Glycine contributes to cellular proteins, synthesis of purines, collagen, porphyrins, creatine, glutathione and conjugation with bile salts. The glycine cleavage system is one of the most important glycine catabolism routes and is located in the mitochondrial inner membrane where glycine is cleaved to form 5,10-MTHF, as discussed above (Kikuchi, 1973; Kanehisa *et al.*, 2019).

In correlation with the increase seen in our KO OVER mice, increased glycine in the plasma or cerebrospinal fluid in humans is a known diagnostic marker of MDs (Mitochondrial Medicine Society's Committee on Diagnosis *et al.*, 2008) and has also previously been found to be increased in the blood of *Ndufs4*^{-/-} mice (Leong *et al.*, 2012). Nikkanen *et al.* (2016) also reported increased glycine in the heart and skeletal muscle of mice with an mtDNA replication disorder. This correlates with the hypotheses of Nikkanen *et al.* (2016) and Bao *et al.* (2016) that there is an increase in serine biosynthesis, where glycine is produced as a result of the increased flux towards serine biosynthesis and the transsulfuration pathway.

Reversal of the glycine cleavage system may be favoured during CI deficiency due to the increased NADH/NAD⁺ ratio, thus leading to the conversion of 5,10-MTHF to THF by glycine synthase with the concurrent oxidation of NADH and resulting in increased glycine, which can also be used to replenish the serine levels (Salway, 2004; Nikkanen *et al.*, 2016).

5.4.6.2 Choline, phosphocoline and glycerophosphocholine

Choline and its metabolites are responsible for the structural integrity of cell membranes, signalling across cell membranes, lipid-cholesterol transport, cholinergic neurotransmission (choline acetyltransferase is used to form acetylcholine from choline and acetyl-CoA) and methyl metabolism (essential for the methylation of DNA and protein molecules). PC (synthesised from choline by choline kinase) acts as an intracellular storage pool of choline while GPC acts as a renal osmolyte (previously discussed in Section 5.4.5) to control the properties of biological fluids. As described above, the choline, folate and methionine metabolic pathways are closely related with choline and 1C metabolism intersecting at the formation of methionine from homocysteine. PC is used to form phosphatidylcholine, which is a key source of eicosanoids, important cell signalling molecules that play a role in immune and inflammatory responses. Phosphatidylcholine can then be converted to GPC, which can in turn be converted back to choline (Zeisel & Blusztajn, 1994; Baykal *et al.*, 2008).

Abnormal choline metabolism has previously been reported in numerous diseases where mitochondrial dysfunction was involved and is increasingly being used as a biomarker for MDs. Inhibition of CI using MPTP in SH-SY5Y cells resulted in elevated GPC and PC, but decreased choline levels. The decreased choline levels were attributed to activated choline kinase, inactivation of phospholipase D and GPC phosphodiesterase (Baykal *et al.*, 2008). Contrary to Baykal *et al.* (2008), we found decreased levels of choline, PC and GPC.

Approximately 80% of the methylation capacity of cells is used for metabolite methylation of creatine and phosphatidylcholine (Mudd *et al.*, 2007; Nikkanen *et al.*, 2016). In skeletal muscle of mice, Nikkanen *et al.* (2016) found an increase in the unmethylated precursors, indicating insufficient methylation capacity to synthesise these two metabolites during mitochondrial dysfunction. As discussed above, this may be due to depletion of the 1C pool by increased glutathione and purine synthesis, which in turn increases the homocysteine requirement and disturbs the methyl cycle. The depleted choline, PC and GPC levels in our KO and KO OVER mice may thus indicate an attempt at replenishing homocysteine levels.

5.4.6.3 Taurine

Taurine, an amino sulfonic acid and organic osmolyte (discussed in Section 5.4.5) involved in cell volume regulation and a substrate for the formation of bile salts, was increased in KO and KO OVER mice compared to OVER mice, as shown in Figure 5-13.

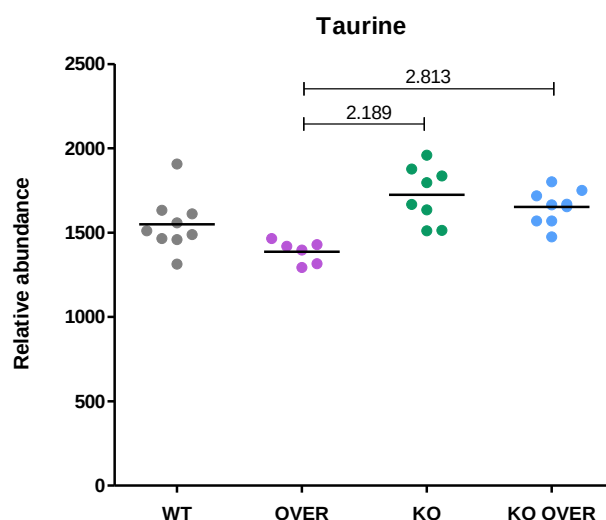


Figure 5-13: Vertical scatter plot depicting the relative abundance of taurine in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

Taurine forms part of the transsulfuration pathway, plays a role in the modulation of intracellular free calcium concentration and is abundant in the brain and muscle. It also has a wide variety of functions in the CNS, including development and cryoprotection. The main route for biosynthesis of taurine is via conversion of homocysteine to cysteine, conversion of cysteine to hypotaurine by cysteinesulfinic acid decarboxylase, and finally conversion of hypotaurine to taurine by hypotaurine dehydrogenase (with reduction of NAD^+). Taurine has been shown to improve certain forms of neuropathy and plays a critical role in brain and neural development. Taurine has also been shown to exhibit antioxidant properties in improving mitochondrial function by stabilising the ETS and inhibiting the generation of ROS. Taurine resembles the structure of GABA and it has been suggested that taurine is also able to act as a neurotransmitter (Ripps & Shen, 2012). Taurine has been linked to inhibition of pyruvate dehydrogenase phosphorylation in the rat heart, the mechanism of which is not yet known, but calcium signalling between taurine and pyruvate dehydrogenase have been suggested to possibly play a role (Militante *et al.*, 2000).

Due to the NAD^+ requirement of hypotaurine dehydrogenase, the elevated taurine levels in our KO and KO OVER mice were surprising due to the disturbed redox status of the CI deficient mice, however, taurine has also previously been reported as increased in mitochondrial dysfunction in other studies (Bao *et al.*, 2016; Nikkanen *et al.*, 2016). This increase may be linked to the activated transsulfuration pathway and increased glutathione synthesis reported above, which would lead to elevated cysteine levels and may thus lead to the elevated taurine seen here.

5.4.7 Purine and pyrimidine metabolism

5.4.7.1 AMP and the purine nucleotide cycle

AMP levels were found to be increased in KO and KO OVER mice compared to OVER mice, as shown in Figure 5-14. Purine nucleotides are essential for the synthesis of DNA and RNA, as well as for biomolecules like AMP. Their ribose base comes from the pentose phosphate pathway in the cytosol whilst the mitochondrial 1C metabolism contributes to purine ring biosynthesis via 5-methyl-THF as described in Section 5.4.6 (Zhou *et al.*, 2018).

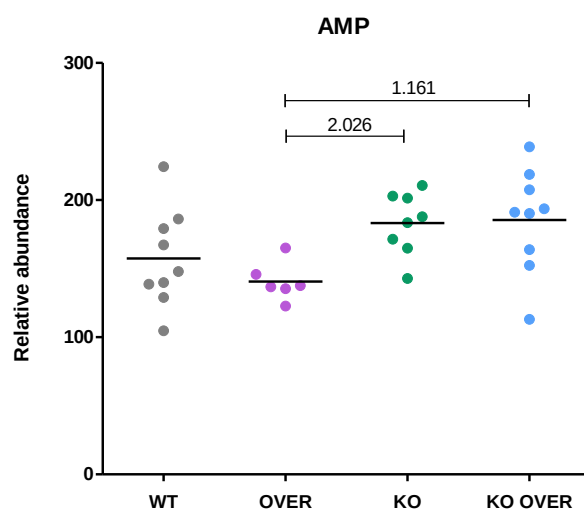


Figure 5-14: Vertical scatter plot depicting the relative abundance of AMP in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where >0.8 is significant).

Adenosine that is released when SAH is converted to homocysteine is used to form AMP by AMP phosphatase (Nikkanen *et al.*, 2016; Kanehisa *et al.*, 2019). In the purine nucleotide cycle, AMP is deaminated to form IMP with release of ammonia. Aspartate and IMP combine, together with GTP hydrolysis, to form adenylosuccinate which is then finally cleaved to again form AMP in addition to fumarate (which can then enter the Krebs cycle) (Swain *et al.*, 1984).

Increased AMP levels in the brain of *Ndufs4*^{-/-} mice has previously been reported (Johnson *et al.*, 2013). In an *in vitro* metabolomics study on fibroblast mammalian cells (Balcke *et al.*, 2011), they saw greatly increased IMP levels when inhibiting OXPHOS. They hypothesised that the energy deficiency in these cells leads to a greater rate of ATP usage than synthesis resulting in depletion of storage products. The purine cycle attempts to replenish the deficient ATP supply by favouring the adenylate kinase reaction ($2\text{ADP} = \text{AMP} + \text{ATP}$) in the direction of ATP formation (Van den Berghe *et al.*, 1992) (and consequently leads to increased AMP as also seen in our KO and KO OVER mice). As discussed above in Section 5.4.1.3, aspartate also becomes depleted during

OXPHOS dysfunction which can also cause the purine nucleotide cycle to be limited, thus leading to the increased IMP levels seen in Balcke *et al.* (2011) (and possibly to the increased AMP levels in our KO and KO OVER mice). Nikkanen *et al.* (2016) reported increased purine degradation products in the heart and greatly increased and imbalanced dNTPs cells in the skeletal muscle of mice with an mtDNA replication dysfunction, thus also indicating increased purine synthesis during mitochondrial dysfunction (as well as an increased demand of 1C units for purine and methionine synthesis as discussed in Section 5.4.6). Increased flux through the methyl cycle in our KO mice (as indicated by the depleted choline and its intermediates, as well as by increased glycine) to replenish 1C units would lead to an increased supply of adenosine with SAH conversion to homocysteine, which could in turn also be used to form AMP by AMP phosphatase.

Imbalanced dNTPs are known to be genotoxic in dividing cells (Garcia-Diaz *et al.*, 2014) and may possibly be an exasperating factor in the progression of the disease in the *Ndufs4*^{-/-} mice. Furthermore, Swain *et al.* (1984) has demonstrated that inhibition of the purine nucleotide cycle in mice led to dysfunctional energy production and muscle dysfunction, thus implicating disruptions in the purine nucleotide cycle as a possible cause or contributing factor to the ataxia and muscle dysfunction seen in the KO and KO OVER mice.

5.4.7.2 Uracil, 5-methyluridine and pyrimidine synthesis

Uracil was decreased in KO OVER mice compared to OVER mice and 5-methyluridine was decreased in KO mice compared to OVER mice, as shown in Figure 5-15.

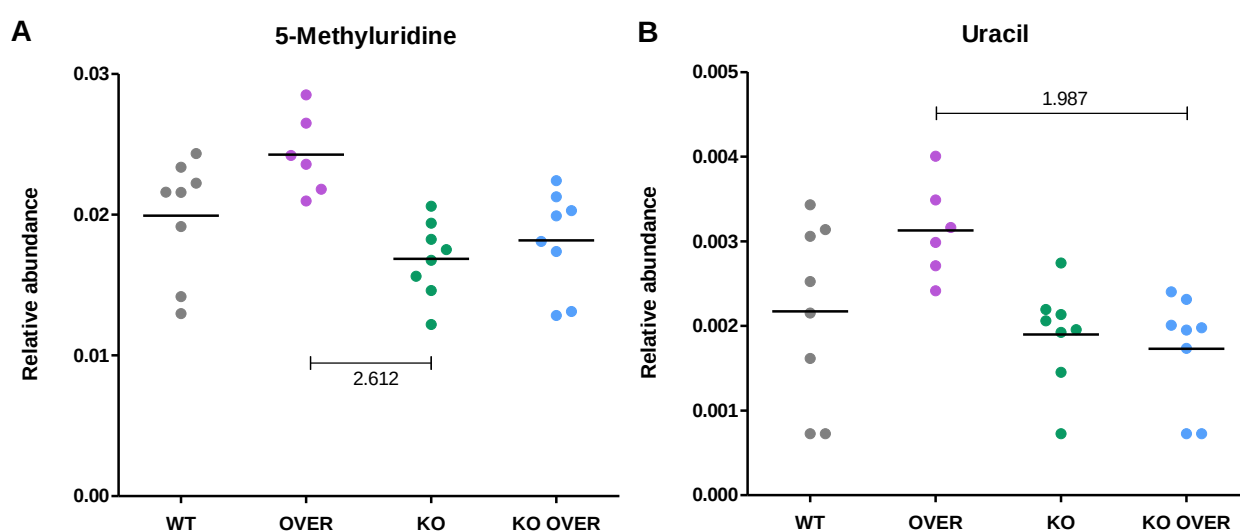


Figure 5-15: Vertical scatter plot depicting the relative abundance of (A) 5-methyluridine and (B) uracil in the brains of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

During pyrimidine biosynthesis, the ribose base comes from the pentose phosphate pathway, whilst the rest of the carbons required for the pyrimidine ring are obtained from oxaloacetate and aspartate in the Krebs cycle. After ring closure and oxidation, orotate is formed, which is then converted to orotidine monophosphate (OMP), by addition of phosphoribosyl pyrophosphate (PRPP), and finally decarboxylated to uridine monophosphate (UMP). UMP is a precursor of pyrimidine-containing nucleotides such as uracil and 5-methyluridine (whereby RNA polymerase is involved). Salvage pathways for recycling purines and pyrimidines also exist for when nucleotides and nucleic acids are degraded. The addition of PRPP to adenine or uracil results in AMP or UMP formation, respectively (Salway, 2004; Zhou *et al.*, 2018). The increased AMP and decreased uracil and 5-methyluridine thus suggests that the salvage pathway may be activated in the KO mice since the salvage pathway requires much less ATP than the *de novo* pathways (Salway, 2004).

Dihydro-orotate dehydrogenase forms part of the OXPHOS system (as mentioned in Section 2.2.2.2) but it is also essential for pyrimidine synthesis since it catalyses the conversion of dihydro-orotate to orotate while donating electrons directly to UQ (Desler *et al.*, 2010). Gattermann *et al.* (2004) displayed *in vitro* that OXPHOS inhibition (more specifically CIII inhibition and to a lesser extent CI inhibition) impaired *de novo* pyrimidine synthesis. While inhibition of CI alone did not appear to have an inhibitory effect on dihydro-orotate dehydrogenase (Desler *et al.*, 2010), the disturbed OXPHOS system in the KO and KO OVER mice may still affect the activity of the enzyme thus leading to the decreased levels of uracil and 5-methyluridine seen here.

5.5 QUADS METABOLISM

A summary figure of the significantly altered metabolites in the quads is shown in Figure 5-17. Similarly to the brain, there were also no significantly altered metabolites between the KO and KO OVER mice, while contrary to the brain, there were however two metabolites altered in the OVER compared to WT mice. Metabolites that were also reported in Esterhuizen *et al.* (2017) and were changed in the same direction included fumarate, alanine, AMP, TMG, creatine, taurine and glycine, while those that were altered in the opposite direction included glutamine, pyroglutamate and 2-aminoadipate. Increased alanine, glycine, taurine and AMP were also seen in the brain and were discussed in Sections 5.4.1.2, 5.4.6.1, 5.4.6.3 and 5.4.7.1 respectively. In isolated skeletal muscle of *Ndufs4*^{-/-} mice, Alam *et al.* (2015) studied the energy metabolism using computational and experimental analyses, and found changes in intermediate metabolite levels, a modestly increased mitochondrial NADH/NAD⁺ ratio and stimulation of the oxidative pathway of the Krebs cycle.

A very comparable study also performed in the quads by Terburgh *et al.* (2019), upon which the methods used here were based, found a much larger number of significantly altered metabolites in KO mice compared to WT mice (OVER and KO OVER mice were not studied). Terburgh *et al.* (2019) had greater statistical power since 19-20 animals were used per group (as opposed to the 12-14 animals used here) in addition to the fact that they reduced variance by only using males (while both males and females were used here), factors which most likely contributed to the greater number of discriminatory metabolites obtained. Similarly identified metabolites included 2-aminoadipate, N-acetylaspartate (NAA), pyroglutamate, glutamine, pipecolate, propionylcarnitine and TMG, in addition to alanine, but alanine was altered in the opposite direction. Contrary to Terburgh *et al.* (2019), we found significantly altered fumarate and taurine levels in the quads while they found these metabolites to be altered in the soleus only, as well as in the opposite direction. They hypothesised that the G3P shuttle, the ETF system, CII and the proline cycle provided alternative sources of electrons to the UQ pool and restored NAD⁺ levels, thus allowing electron flux through the rest of the ETS during CI dysfunction. Some of these reactions may also have potentiated the redox imbalance.

5.5.1 Central carbon metabolism

5.5.1.1 Acetate and N-acetylaspartate

Acetate was increased in KO OVER compared to OVER mice, whilst NAA was increased in KO mice compared to WT and OVER mice, as shown in Figure 5-16.

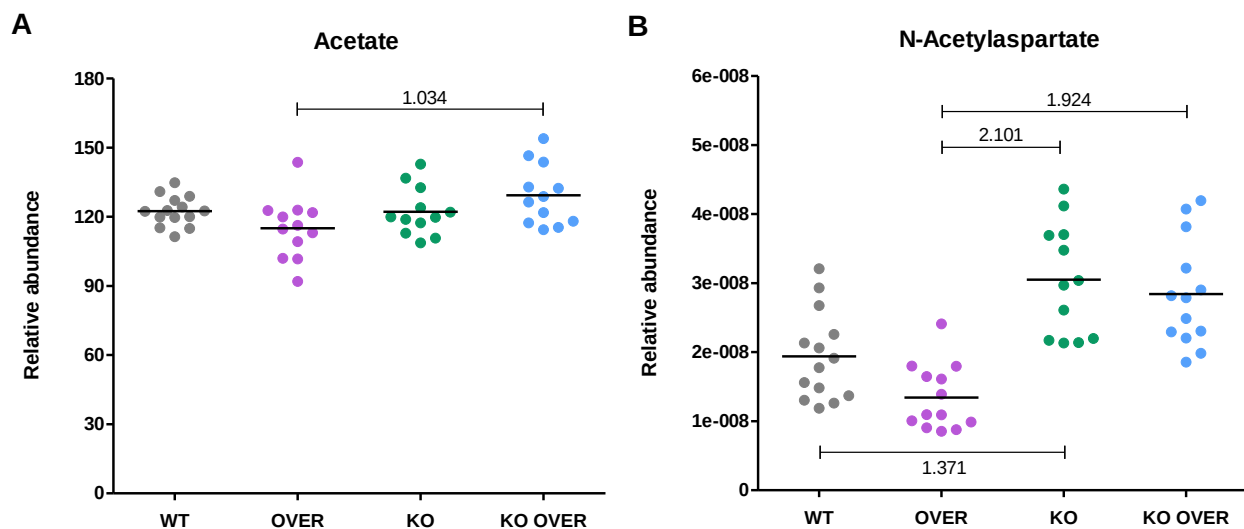


Figure 5-16: Vertical scatter plots depicting the relative abundance of (A) acetate and (B) N-acetylaspartate in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted p < 0.05. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

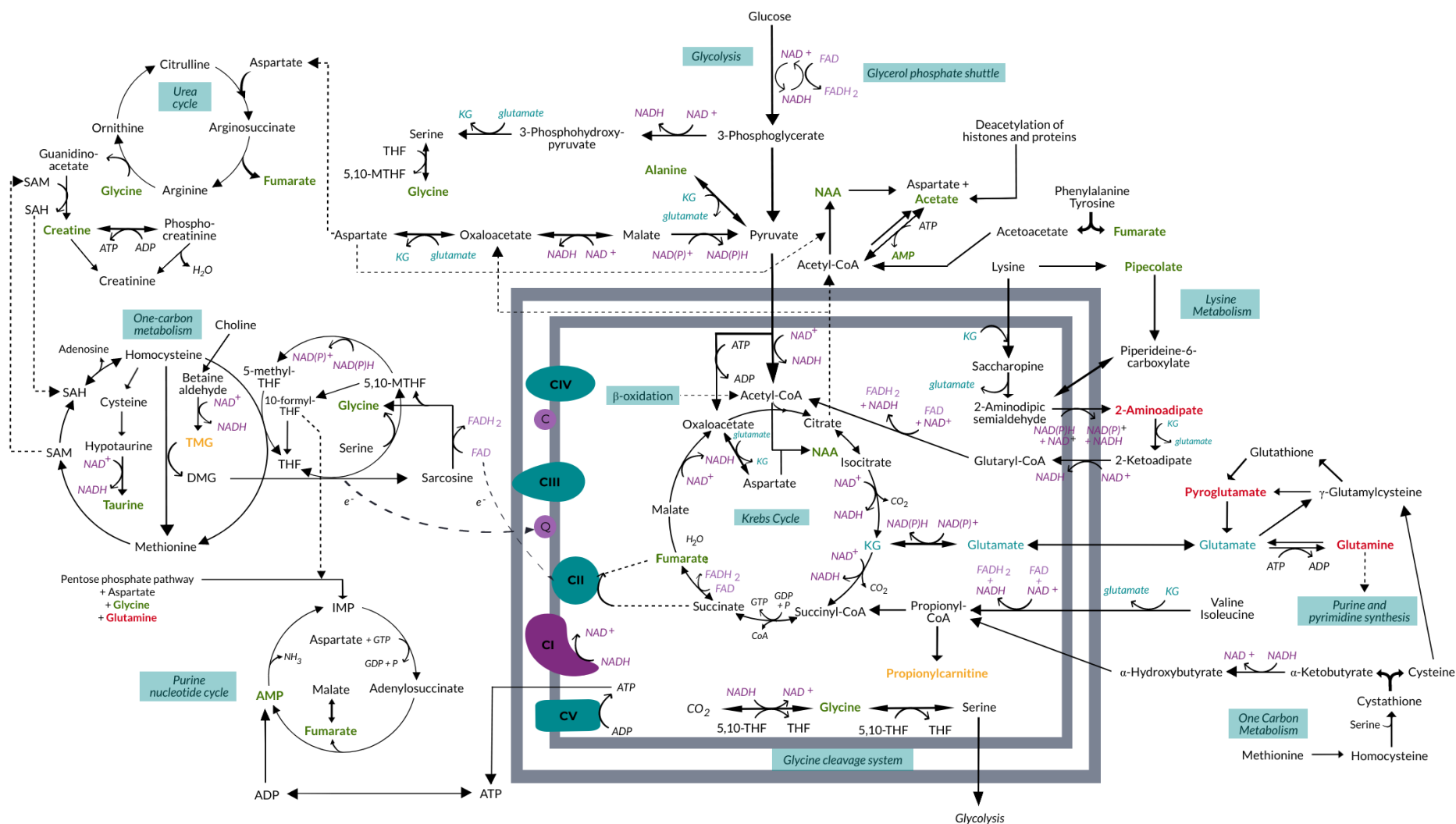


Figure 5-17: Schematic representation of the metabolic pathways involved in the significant altered metabolites in the quads tissue of mice from the four genotypes.

The green and red metabolites indicate that the metabolites were increased or decreased, respectively, in the KO or KO OVER mice relative to the WT or OVER mice. The orange metabolites were decreased in OVER mice but increased in KO mice. Abbreviations: C, cytochrome c; DHF, dihydrofolate; DMG, dimethylglycine; KG, α -ketoglutarate; MTHF, methylenetetrahydrofolate; NAA = N-acetylaspartate; Q, ubiquinone; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TMG, trimethylglycine.

Acetate is in reversible equilibrium with acetyl-CoA where acetate is converted to acetyl-CoA by acetyl-CoA synthetase (with the conversion of ATP to AMP). Acetate can be formed by hydrolysis of acetyl-CoA by acetyl-CoA hydrolase as well as by histone deacetylases which remove acetyl groups from histones. Acetyl-CoA plays a very key role in central carbon metabolism via the Krebs cycle, protein acetylation as well as in the generation of lipids (in the nucleus, mitochondria and cytosol). Due to the elevated lactate (in the brain) and alanine levels in our KO OVER mice, it is expected that the acetyl-CoA levels in these mice would also be elevated and would thus be converted to acetate, resulting in the increased acetate levels observed. Acetate can also be generated directly from pyruvate by non-enzymatic ROS-coupled reactions as well as by alternative activities of ketoacid dehydrogenases. Increased ROS and altered availability of cofactors due to mitochondrial dysfunction can lead to upregulated pyruvate-derived *de novo* acetate metabolism (Liu *et al.*, 2018; Bose *et al.*, 2019), and could thus also explain the increased acetate observed.

NAA, a derivative of aspartate, predominantly localises to neurons, axons and dendrites and is one of the best biomarkers for neuronal integrity. It is synthesised from acetyl-CoA and aspartate by aspartate N-acetyltransferase and it is catabolised by aspartoacylase to produce acetate and aspartate. Acetate can in turn be used to synthesise acetyl-CoA which can then be metabolised via the Krebs cycle for energy production or used as a source for myelin lipid synthesis (in oligodendrocytes) during brain development. NAA has also been described as a precursor to N-acetylasparylglutamate (NAG) synthesis, and to function in osmoregulation and axon-glial signalling. Decreases in NAA, contrary to that seen here, may indicate MD as it is synthesised in the mitochondria in an energy-dependent manner and thus represents a functional mitochondrial marker in neuronal populations (Mitochondrial Medicine Society's Committee on Diagnosis *et al.*, 2008; Bogner-Strauss, 2017).

Other reports of increased NAA include Legault *et al.* (2015), who found increased NAA levels in the urine of LS patients, and Terburgh *et al.* (2019), who reported increased NAA in *Ndufs4*^{-/-} mice skeletal muscle and hypothesised that the Krebs cycle was not able to use acetyl-CoA at the same rate that it was synthesised (via fatty acid oxidation and ketogenic amino acids), resulting in an accumulation of acetyl-CoA and thus its conversion to NAA. This hypothesis is further supported by the increased acetate seen here as the accumulation of acetyl-CoA may shift the equilibrium of the acetyl-CoA synthetase reaction towards acetate production, a reaction that is furthermore favoured due to the production of ATP. The increased acetate may also be a result of the elevated NAA as it is catabolised to form acetate and aspartate, with aspartate being an amino acid that has been shown to be essential for cell proliferation during mitochondrial dysfunction and a rate-limiting factor (see Section 5.4.1.3).

5.5.1.2 Glutamine and alanine

Glutamine was decreased in KO mice compared to WT mice, whilst alanine (discussed in detail in Section 5.4.1.2) was increased in KO OVER compared to OVER mice, as shown in Figure 5-18. Glutamine is synthesised by glutamine synthetase, which combines glutamate and NH_4^+ in an ATP-requiring reaction. The opposite can also occur where glutaminase catalyses the conversion of glutamine to glutamate. Glutamate is synthesised via glutamate dehydrogenase by amination and reduction of KG (many of the amino groups of which are obtained from BCAAs). Glutamine provides a vital source of nitrogen for purine and pyrimidine synthesis, fuel for the intestines and is also used to regulate the pH in acidotic conditions in the kidneys. During *de novo* purine biosynthesis, glutamine, aspartate and glycine are required for AMP synthesis. In cells that are swiftly proliferating, glutamine serves an anaplerotic purpose in restoring the Krebs cycle intermediates. In addition, glutamine also plays a role in synthesis of glutathione and neurotransmitters (Salway, 2004; Kanehisa *et al.*, 2019).

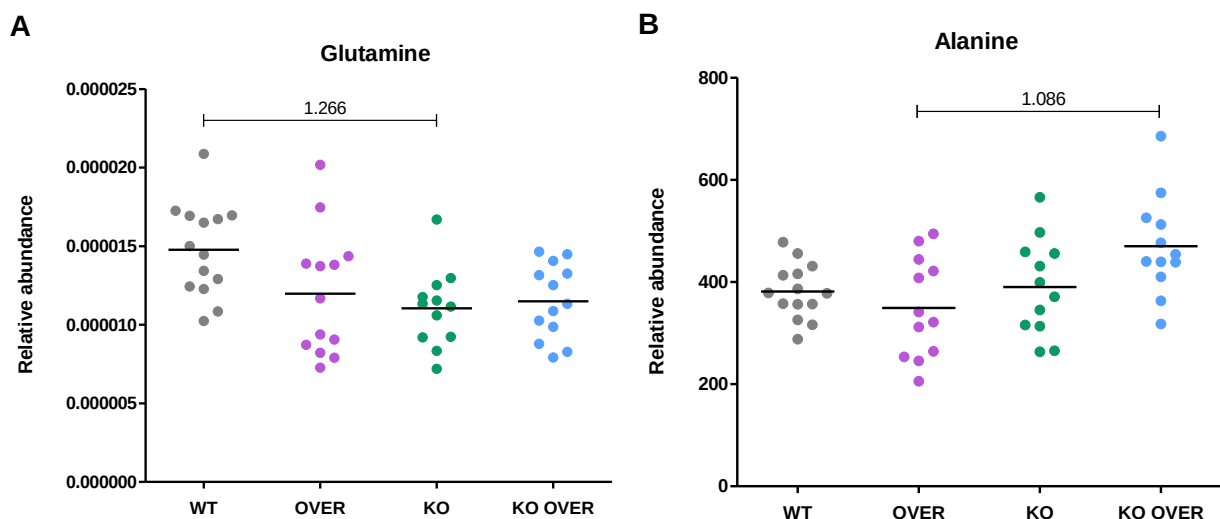


Figure 5-18: Vertical scatter plots depicting the relative abundance of (A) glutamine and (B) alanine in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

As a source of glutamate, glutamine has also demonstrated an important role in the reductive pathway of the Krebs cycle, as shown in Figure 5-8. Chen *et al.* (2018) demonstrated that the severity of an OXPHOS impairment determines whether or not KG is metabolised via the oxidative or the reductive pathway of the Krebs cycle (as explained in Section 5.4.1.3). Reductive carboxylation becomes the primary pathway only when respiration is severely compromised ($>55\%$) (due to limited NAD^+), whilst in cells with only moderate OXPHOS defects, increased KG (derived from glutamine) is used by the Krebs cycle in both directions but is predominantly oxidative. The role of aspartate is thus different in cells with severe OXPHOS defects, where

aspartate is generated from oxaloacetate through reversal of glutamate-oxaloacetate transaminase, and since part of oxaloacetate is also converted to malate, limited aspartate availability impairs nucleotide synthesis and cell proliferation. This is contrary to cells with modest OXPHOS deficiency, where aspartate supplementation would lead to KG inhibition, and rather require metabolites that stimulate glutamate generation. The depletion of glutamine here thus suggests a modest OXPHOS deficiency in the muscle of the KO mice. Chen *et al.* also demonstrated that in the muscle of mice with severe OXPHOS deficiency (CIV knockouts), glutamate-derived KG and amino acid derived ketoacids were used to drive energy production and muscle protein breakdown. This also led to increased alanine (as also seen in our KO OVER mice), which is believed to act as a by-product to safely discard of excess amino groups and to prevent toxic ammonia accumulation.

5.5.1.3 Pyroglutamate

Pyroglutamate was decreased in KO mice compared to WT mice, as shown in Figure 5-19. Pyroglutamate is produced and used by the γ -glutamyl cycle, where it acts as an intermediary metabolite in glutathione degradation. Glutathione is degraded by γ -glutamyl transpeptidase to produce γ -glutamyl amino acids, which are in turn acted upon by γ -glutamyl cyclotransferase to produce pyroglutamic acid and the corresponding amino acids. Pyroglutamate is also produced by glutamate where cysteine and ammonia are used by γ -glutamylcysteine synthetase and glutamine synthetase, respectively. Pyroglutamate can also be converted to glutamate by 5-oxoprolinase (an ATP-dependent enzyme) by degrading proteins containing pyroglutamate at the N-terminal. Glutamate and glutamine on the N-terminal of proteins are also able to spontaneously convert to pyroglutamate, thus indicating that pyroglutamate contributes to the structural and activity-related properties of proteins. In its free form, pyroglutamate is also believed to play a role as an analogue or reservoir of glutamate as well as an osmoprotector (Kumar & Bachhawat, 2012). As a reservoir of glutamate, the diminished pyroglutamate in our KO mice further support the idea of a modest OXPHOS deficiency in these mice (discussed in Section 5.5.1.2) and the requirement of glutamate-synthesising metabolites to fuel the oxidative pathway of the Krebs cycle, resulting in glutamate conversion to KG and subsequently succinate production for CII, as also evidenced by the increased fumarate seen in our KO mice (see Section 5.5.4.1 below).

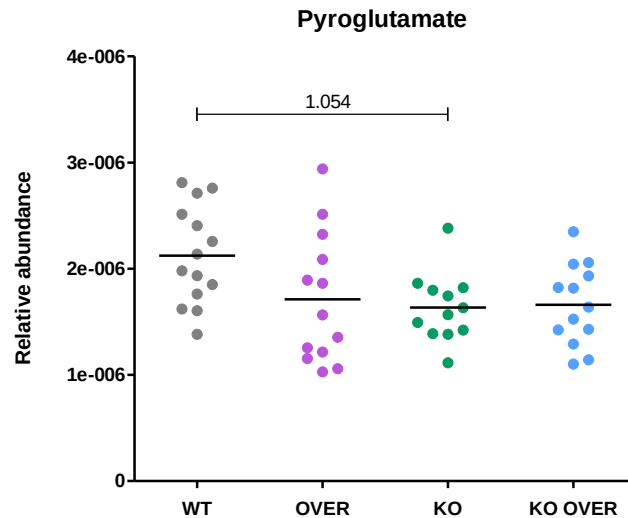


Figure 5-19: Vertical scatter plot depicting the relative abundance of pyroglutamate in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

5.5.2 Lysine metabolism

5.5.2.1 Pipecolate and 2-aminoadipate

Pipecolate was increased in KO OVER compared to OVER mice, whilst 2-aminoadipate was decreased in KO compared to WT mice, in correlation with that seen in Terburgh *et al.* (2019), as shown in Figure 5-20.

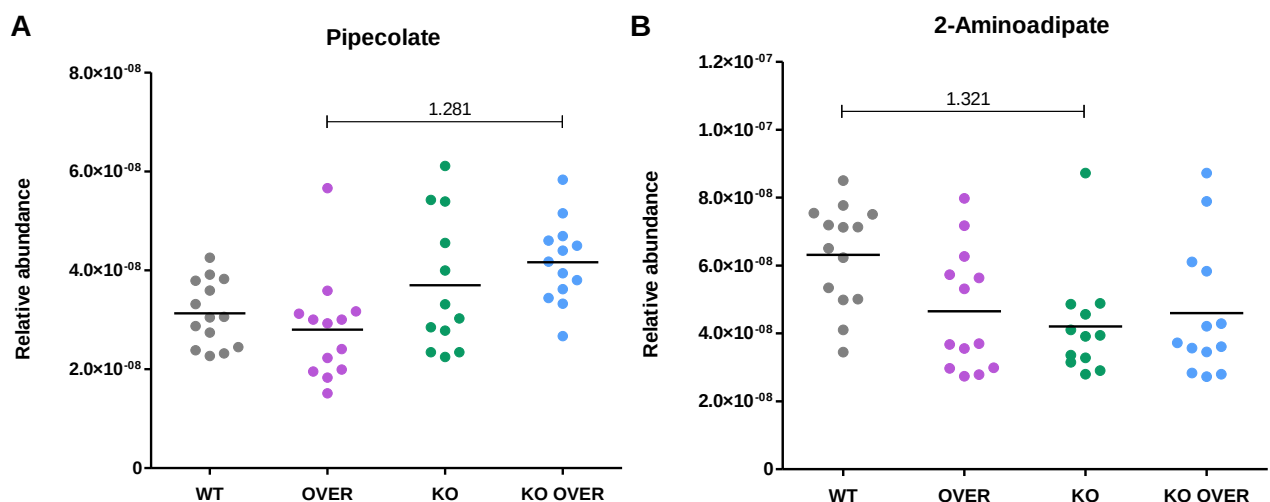


Figure 5-20: Vertical scatter plots depicting the relative abundance of (A) pipecolate and (B) 2-aminoadipate in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

Lysine is an essential amino acid, which is catabolised via two pathways, namely, the saccharopine pathway (predominantly mitochondrial) and the pipecolate pathway (predominantly cytosolic and peroxisomal). In the former pathway, lysine is converted to saccharopine, in the presence of KG, which is in turn converted to 2-aminoadipic semialdehyde (with subsequent release of glutamate), and finally to 2-aminoadipate (requiring one NADPH and two NAD⁺ molecules). In the latter pathway, there is non-enzymatic conversion of lysine, via intermediates, to pipecolate, which is converted to piperideine-6-carboxylate. The two pathways then intersect where piperideine-6-carboxylate reversibly forms 2-aminoadipic semialdehyde, which is then converted to 2-aminoadipate. Subsequently, 2-aminoadipate is transaminated to form 2-ketoadipate (with concurrent conversion of KG to glutamate) which is then converted to glutaryl-CoA (together with reduction of another NAD⁺ molecule). Glutaryl-CoA conversion is then catalysed by glutaryl-CoA dehydrogenase (a reaction dependent on FAD which transfers electrons to the ETF) and ultimately produces acetyl-CoA (with the use of another NAD⁺ molecule) in the mitochondria to be used for the Krebs cycle (Salway, 2004; Sauer *et al.*, 2011; Hallen *et al.*, 2013; Kanehisa *et al.*, 2019).

Terburgh *et al.* (2019) hypothesised that there is an increased flux through mitochondrial flavoprotein dehydrogenases (such as glutaryl-CoA dehydrogenase) to supply the ETF system with electrons. A limiting factor in the FADH₂ producing pathways is however their requirement of NAD⁺, a factor that is limited in the *Ndufs4*^{-/-} mice due to build-up of NADH as a result of CI inactivity as well as due to these compensatory reactions themselves. The pipecolate pathway thus appears to be preferred in the lysine metabolism during CI dysfunction, since it does not require NAD⁺ or KG prior to 2-aminoadipic semialdehyde formation. However, conversion of 2-aminoadipic semialdehyde to 2-aminoadipate by 2-aminoadipate semialdehyde dehydrogenase is limited during CI dysfunction since it requires two NAD⁺ molecules (as mentioned above). Furthermore, conversion of 2-aminoadipate to glutaryl-CoA requires both KG (which may be decreased to fuel the bifurcated Krebs cycle as discussed in Sections 5.4.1.3 and 5.5.1.2) and another NAD⁺ molecule, thus demonstrating further limitations in the KO mice. This limited NAD⁺ and KG may thus prevent conversion of 2-aminoadipic semialdehyde to 2-aminoadipate, leading to decreased 2-aminoadipate levels, limited flux through the pipecolate pathway and thus to the accumulation of the pipecolate seen in the KO mice.

5.5.3 One-carbon metabolism, the methylation cycle and transsulfuration pathway

5.5.3.1 Trimethylglycine

TMG was increased in KO and WT mice compared to OVER mice (one of only two metabolites to differ between WT and OVER mice), as shown in Figure 5-21. TMG is a methyl group donor,

an important osmoprotectant (also mentioned in Section 5.4.5) and has been shown to demonstrate anti-inflammatory and antioxidant properties in many diseases. It is biosynthesised from choline (as described in Section 5.4.6), and during transmethylation (primarily in liver and kidneys), betaine homocysteine methyltransferase catalyses the conversion of homocysteine to methionine, where TMG donates a methyl group so that it becomes DMG. Methionine is metabolised in the transsulfuration pathway to form cystathionine, cysteine, taurine and glutathione (Zhao *et al.*, 2018). DMG is then converted to sarcosine by DMG dehydrogenase (with concurrent conversion of THF to 5,10-MTHF and reduction of UQ to ubiquinol), and sarcosine is converted to glycine by sarcosine dehydrogenase (with release of 5,10-MTHF), an FAD-linked dehydrogenase (Mailloux *et al.*, 2016).

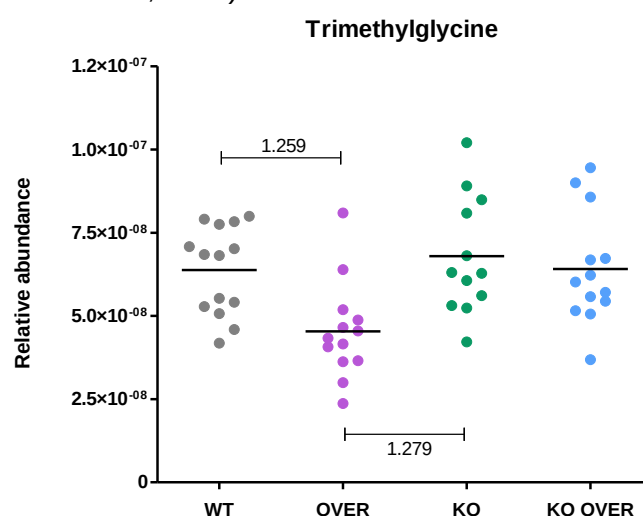


Figure 5-21: Vertical scatter plot depicting the relative abundance of trimethylglycine in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

In line with the hypothesis of Terburgh *et al.* (2019), choline supply of TMG may be increased (despite the NAD^+ molecule required) due to the ability of TMG to supply electrons directly to the UQ pool together with fuelling of CII by conversion of FAD to FADH_2 . This furthermore agrees with Nikkanen *et al.* (2016) (as discussed in Section 5.4.6) who suggested that increased TMG (which indirectly connects the folate and methyl cycles) indicates induction of alternative 1C sources to replenish the 1C pool.

For WT vs OVER mice, the decreased TMG in OVER compared to WT mice may be linked to the accelerated methionine metabolism (discussed below in Section 5.5.4.2) and homocysteinylolation of MT, which was shown to target intracellular MT and disrupt zinc, ROS and redox homeostasis, as well as impair the zinc-binding capacity of MT (Barbato *et al.*, 2007). However, since only two metabolites were identified in this group, further research is warranted to substantiate this hypothesis.

5.5.3.2 Taurine and glycine

Glycine was increased in KO OVER compared to OVER mice, whilst taurine was increased in KO OVER compared to WT and OVER mice and KO compared to WT mice, as shown in Figure 5-22. As mentioned above, the increased glycine supports the hypothesis that the catabolism of TMG is favoured due to its function as an alternative electron source to the ETS, where glycine is produced by sarcosine dehydrogenase, and increased taurine supports the theory of increased flux through the transsulfuration pathway, as discussed in detail in Sections 5.4.6.1 and 5.4.6.3.

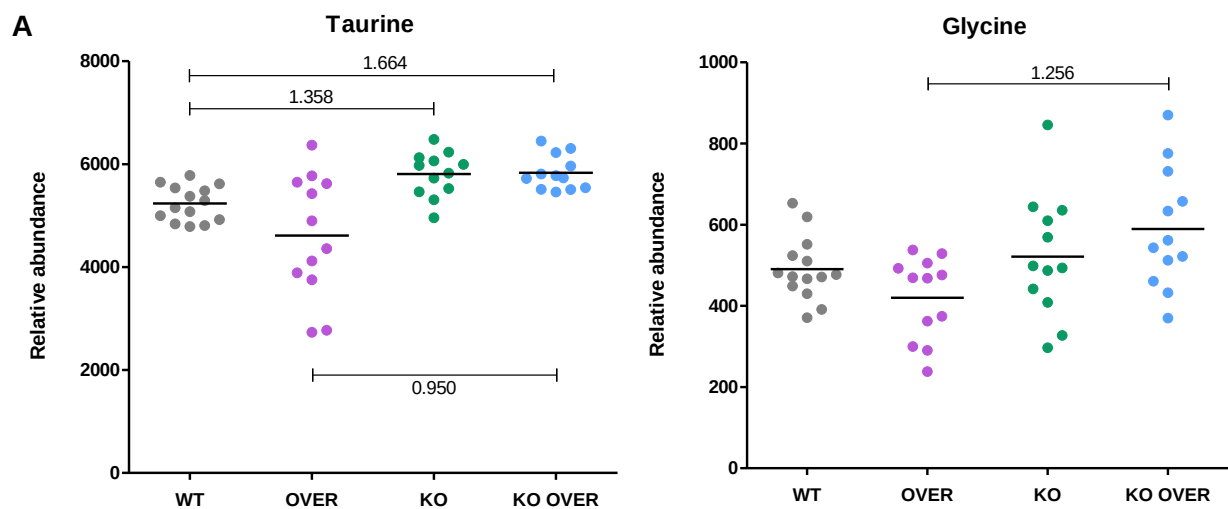


Figure 5-22: Vertical scatter plots depicting the relative abundance of (A) taurine and (B) glycine in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

5.5.4 Urea cycle

Fumarate was increased in KO and KO OVER mice compared to OVER mice and creatine was increased in KO OVER compared to WT and OVER mice as well as in KO compared to WT mice, as shown in Figure 5-23.

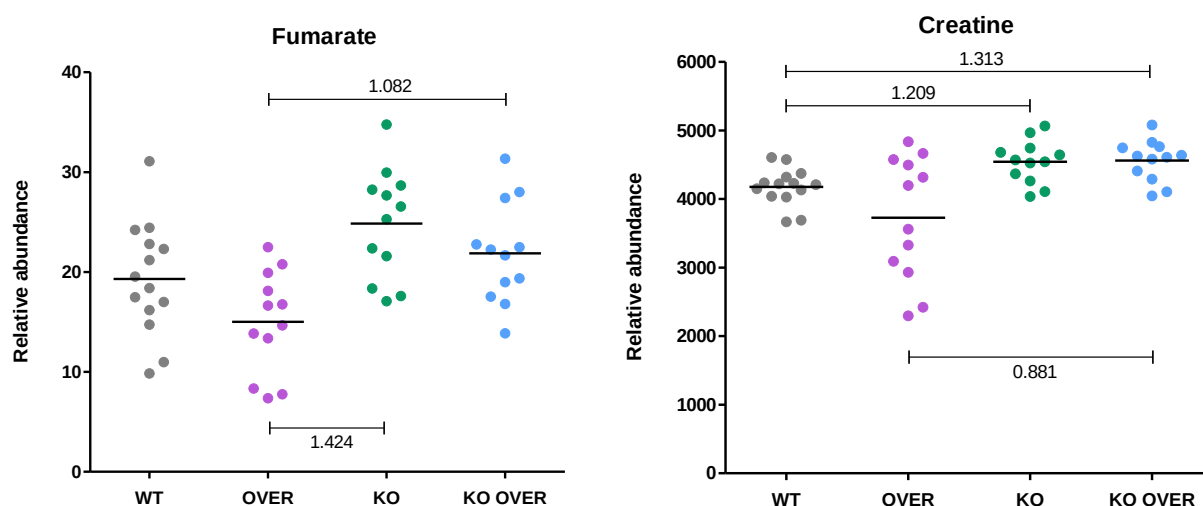


Figure 5-23: Vertical scatter plots depicting the relative abundance of (A) fumarate and (B) creatine in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH-FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

5.5.4.1 Fumarate

Fumarate can be synthesised via the purine nucleotide cycle (as discussed in Section 5.4.7.1), from the amino acids tyrosine and phenylalanine as well as from the urea cycle via the transamination route. In this route, amino acids enter the urea cycle and KG accepts their amino groups to form glutamate. Oxaloacetate then accepts the amino group from glutamate to form aspartate (as catalysed by AST). Aspartate then carries the second amino group into the urea cycle by condensing with citrulline to form arginosuccinate, which is then cleaved to form fumarate and arginine. Arginine is then ultimately hydrolysed to ornithine and urea. Succinate dehydrogenase (located in the inner mitochondrial membrane) forms part of both the Krebs cycle and CII of the ETS, where it catalyses the reversible oxidation of succinate to form fumarate together with the reduction of UQ to ubiquinol and FAD to FADH₂. Fumarate conversion to malate is catalysed by fumarase, with addition of a water molecule, and malate can then be used in the Krebs cycle (Salway, 2004; Kanehisa *et al.*, 2019).

In the model developed by Alam *et al.* (2015), as discussed in Section 5.5, they saw upregulated activity of the oxidative pathway in the Krebs cycle in *Ndufs4*^{-/-} mice as well as activation of CII. This would explain the increased fumarate seen in our KO mice, as CII is used as an alternative mechanism to fuel OXPHOS, and also correlates with the ETS enzyme data (Figure 4-22) where we saw significantly increased CII activity in the quads (but not in the brain) of our KO compared to WT and OVER mice. The decreased glutamine and pyroglutamate in the KO mice further support an activated oxidative pathway in the quads. Piroli *et al.* (2016) identified fumarate as a possible novel biochemical contributor to the progression of the neuropathology of CI dysfunction

in *Ndufs4*^{-/-} mice, but contrary to that seen here, they only saw protein succination in the brain (more specifically in the brainstem and particularly the vestibular nucleus) and not in skeletal muscle, which they correlated to the lack of muscle pathology in this model. However, Piroli *et al.* (2016) only studied cell-specific differences in protein succination and did not directly measure the fumarate levels; it is thus possible that high fumarate is necessary but not sufficient to have protein succination. It thus appears that in our KO and KO OVER mice, the fumarate is merely increased as a result of the increased flux through CII in an attempt to restore electron flow through the ETS and to allow ATP production to continue. The increased fumarate may also be as a result of increased flow through the purine nucleotide cycle (as evidenced by increased AMP) due to the perturbed 1C metabolism and its effect on purine synthesis (as discussed above).

5.5.4.2 Creatine

Creatine is primarily synthesised in the liver and kidneys by combining glycine and arginine to produce guanidinoacetate and ornithine (which goes to the urea cycle), which is then converted to creatine by methylation using SAM as the methyl donor. The majority of the body's creatine is found in the muscle, after which it is transported via the blood to tissues, like the brain and skeletal muscle, which require large amounts of energy. Creatine is in reversible equilibrium with phosphocreatine where the conversion of phosphocreatine to creatine by creatine kinase results in conversion of ADP to ATP. Creatinine in the muscle is formed by the non-enzymatic and irreversible removal of water from phosphocreatine and acts as waste product in the excretion of creatine (Wyss & Kaddurah-Daouk, 2000). It has been shown in adults that disturbances of the creatine metabolism affect the skeletal muscle much more than the brain (Balsom *et al.*, 1994).

A disturbance in the 1C metabolism, as described in Section 5.4.6, may lead to the increased creatine levels seen here. As mentioned in Section 5.4.6.2, 80% of the methylation cycle is used by creatine and phosphatidylcholine, and during mitochondrial dysfunction, the 1C pool becomes depleted due to purine and glutathione synthesis, thus increasing the need for homocysteine. Nikkanen *et al.* (2016) however reported increased unmethylated precursors of creatine rather than creatine itself, due to the insufficient methylation capacity. A possible reason for this difference is that Nikkanen *et al.* (2016) made use of mice with mtDNA replication disorders rather than a CI deficient model and the residual CI activity in our mice may allow for a greater methylation capacity. A possible hypothesis is that the methionine metabolism through SAM is accelerated in an attempt to restore homocysteine levels (Legault *et al.*, 2015) in our KO and KO OVER mice. The increased levels of 5,10-MTHF produced as a by-product of TMG catabolism (during its donation of electrons to the ETS) may lead to THF production (and oxidation of NADPH), with the concurrent production of methionine, which could in turn increase SAM availability. The increased SAM and increased glycine may thus favour the production of creatine.

Another factor may be that conversion of phosphocreatine to creatine is favoured due to the production of ATP.

5.5.5 AMP and the purine nucleotide cycle

AMP (as discussed in detail in Section 5.4.7.1) was increased in KO OVER compared to OVER mice, as shown in Figure 5-24. The purine cycle is especially important in skeletal muscle where it is involved in the myokinase reaction for muscle energy production and plays an important role in regulating ATP production under aerobic and anaerobic conditions during exercise. Accumulating IMP acts as a storage mechanism within the cell for purines that may be needed later for ATP synthesis. Flux through the purine nucleotide cycle also generates fumarate and other Krebs cycle intermediates which can be used to fuel mitochondrial respiration (Swain *et al.*, 1984).

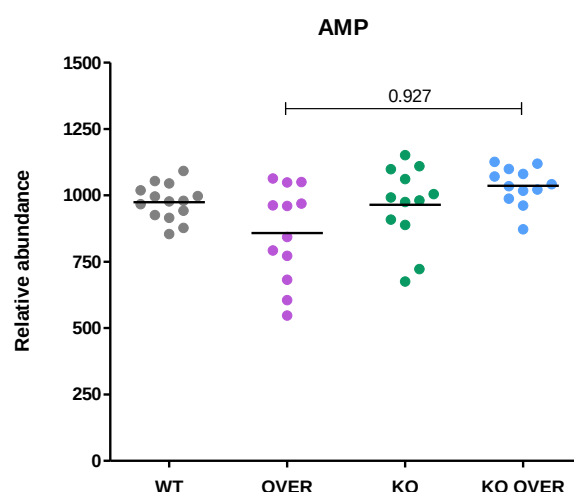


Figure 5-24: Vertical scatter plot depicting the relative abundance of AMP in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

5.5.6 Propionylcarnitine

Propionylcarnitine (also known as C3) was increased in KO and WT mice compared to OVER mice (the only other metabolite to differ between WT and OVER mice), as shown in Figure 5-25. Propionylcarnitine, an odd numbered fatty acid, is reversibly converted to propionyl-CoA in the mitochondria. Propionyl-CoA is also formed during isoleucine and valine catabolism. This, in turn, is converted to methylmalonyl-CoA by propionyl-CoA carboxylase in the presence of biotin. Methylmalonyl-CoA is then converted to succinyl-CoA (which enters the Krebs cycle) by methylmalonyl-CoA mutase, in the presence of vitamin B12 (Wikoff *et al.*, 2007).

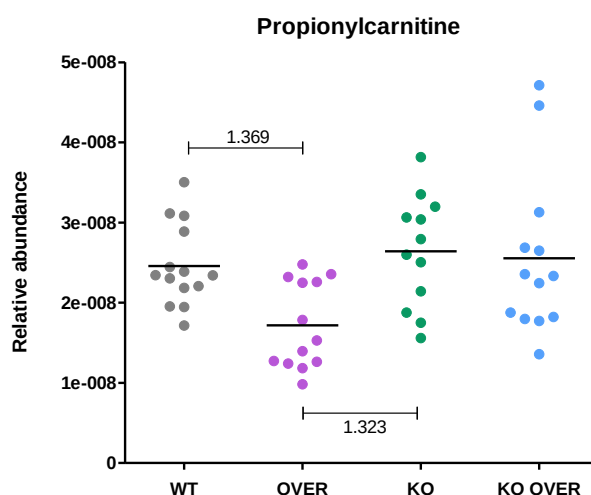


Figure 5-25: Vertical scatter plot depicting the relative abundance of propionylcarnitine in the quads of mice from the four genotypes, where the horizontal bar indicates the mean. The brackets indicate a significant difference between two genotypes with BH=FDR adjusted $p < 0.05$. The number above/below the bracket indicates the effect size (Cohen's d-value where > 0.8 is significant).

Legault *et al.* (2015) found increased α -hydroxybutyrate in the plasma of LS patients, a marker also previously identified in *Ndufs4*^{-/-} mice (Jain *et al.*, 2016), which can be transported to the mitochondria and undergo oxidative decarboxylation to form propionyl-CoA. It is synthesised by reducing α -ketobutyrate (and thus utilizes NADH), a metabolite formed from methionine and/or threonine. This appears to support the hypothesis that methionine metabolism is accelerated in the KO mice leading to increased α -hydroxybutyrate (and creatine), which is favoured due to the oxidation of NADH, and results in increased propionyl-CoA and thus increased propionylcarnitines.

5.6 CONCLUSION

Metabolomics analyses using brain and skeletal muscle tissue were performed to enhance the phenotype investigations, since metabolomics allows for a comprehensive evaluation of the metabolites and their underlying interactions, that are sensitive to small changes in the redox status of the cells and theoretically should relate closely to the phenotype of the animals (Nicholson & Lindon, 2008).

The key difference between the brain and the quads of the *Ndufs4*^{-/-} mice, as discussed in Section 5.5.1.2, may be linked to the oxidative and reductive pathways of the Krebs cycle, the latter of which is more predominant in severe OXPHOS deficiencies. Due to the predominantly neurological phenotype in the *Ndufs4*^{-/-} mice, it is probable that the brain tissue exhibits severe OXPHOS deficiency preferring the reductive carboxylation pathway (as supported by decreased aspartate and increased malate), whilst the muscle tissue may only exhibit moderate OXPHOS

deficiency leading to predominantly oxidative utilisation of KG (as evidenced by decreased glutamine and pyroglutamate). Alam *et al.* (2015) showed near normal pyruvate oxidation and ATP production in muscle from *Ndufs4*^{-/-} mice thus indicating the presence of a compensatory mechanism and CI over-capacity not seen in the brain. Furthermore, Benard *et al.* (2006) showed that muscle has a greater quantity of CI units than the brain (as discussed in Section 2.2.2.4), also evidenced by the CI activity in our KO and KO OVER mice (see Figure 4-21) where the average residual CI activity in the brain was ~9% as opposed to ~22% in the quads.

The metabolomics data presented here clearly demonstrate the significant effect of the altered NADH/NAD⁺ ratio on the metabolome of the *Ndufs4*^{-/-} mice. The discriminatory metabolites identified indicate an attempt to fuel the ETS by making use of alternative electron sources and by favouring reactions that lead to oxidation of NADH. In both the brain and the quads, bifurcation of the Krebs cycle and remodelling of the 1C metabolism were shown to play key roles in the metabolism of CI dysfunction. In addition, aspartate depletion and disturbances of the purine nucleotide cycle also appeared to be significant pathophysiological consequences of CI dysfunction. The most surprising metabolic finding, considering a putative redox-modulating role of MTs, was the lack of any discriminatory metabolites in the KO OVER vs KO mice. All discriminatory metabolites identified could be attributed to the *Ndufs4*^{-/-} genotype, but none could be distinctly accredited to the *TgMt1* genotype. However, these metabolic investigations supported the phenotypic and biochemical findings described in the previous chapter, and was in line with the conclusion reached there, that *Mt1* overexpression does not protect against *Ndufs4* knockout pathology.

CHAPTER 6: SUMMARY AND CONCLUSIONS

6.1 RATIONALE

The aim of this study was to determine whether *Mt1* overexpression could protect against the disease progression and damaging cellular consequences of complex I (CI) dysfunction in an *Ndufs4*^{-/-} mouse model. As discussed in Chapter 2, CI deficiency is the most common mitochondrial disease (MD), most frequently presenting as Leigh Syndrome (LS), a severe neurodegenerative disease with a poor prognosis, no known cure and very limited treatment options. Despite contradicting reports, increased oxidative stress and ROS have been widely reported as a key cellular consequence of CI deficiency, which led to the rationale of investigating metallothioneins (MTs), ROS scavengers and potent antioxidants, as a potential therapy for CI dysfunction. While some evidence in support of this has been shown *in vitro* (Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006), no *in vivo* studies using *bona fide* genetic disease models for MD had previously been reported. The commercial availability of the whole-body NDUFS4 knockout mouse model at the start of this study allowed investigation of this hypothesis by crossing it with the *TgMt1* mouse, a widely used and well-studied transgenic mouse model that overexpresses MT1.

The methodology used to achieve this aim was set out in Chapter 3, and all biochemical and metabolomic results were presented and discussed in detail in Chapters 4 and 5 (and Appendices A-D) respectively. In this chapter, a summary of the most noteworthy results for each objective will be presented and a final conclusion will then be given by holistically evaluating all the results obtained here as well as in other related studies performed by others from the MRL on these mice. Lastly, the strengths, limitations and future prospects of this study will be elucidated.

6.2 SUMMARY OF FINDINGS

6.2.1 Objective 1 – Breeding of WT, OVER, KO and KO OVER mice

To obtain the 68 mice required for the phenotyping section of this study (in addition to the extra three mice per genotype that were used for other biochemical and metabolic investigations) was a time-consuming process. The prolonged time frame was mainly as a result of the KO and KO OVER mice, since *Ndufs4*^{-/-} mice do not reach sexual maturity and heterozygotes must thus be used in the breeding process, with a low probability for obtaining mice homozygous for *Ndufs4* (Mereis, 2018). The WT, OVER, KO and KO OVER mice were nevertheless successfully bred and upon completion of the phenotyping analyses, they were humanely euthanised and multiple

organs harvested, including the brain and both quads, and frozen at -80°C (with the exception of selected whole brains that were fixed in PFA for immunohistochemistry analyses).

6.2.2 Objective 2 – Genetic characterisation of the mouse model

To select the correct mice from each litter to be included in the study, genotyping was performed (using tail snips) to determine the presence of the *Ndufs4* gene. It was not necessary to quantify the *Mt1* levels at this stage since the breeding pairs were set-up in such a manner that the *TgMt1* genotype was known. After euthanising the animals, liver samples were used to again determine *Ndufs4*, but to also confirm the *Mt1* relative copy number. This ensured that all mice included in the results were correctly genotyped. To further characterise the model, absence of NDUFS4 was confirmed at the protein level in both the brain and quads of KO and KO OVER mice, and the mRNA levels of *Mt1* (relative to $\beta 2m$) were also determined to confirm *Mt1* overexpression in both the OVER and KO OVER mice.

Surprisingly, mRNA results showed significantly decreased *Mt1* levels in KO and KO OVER mice compared to WT and OVER mice (Section 4.3.3), respectively. This was contrary to other reports (Van Der Westhuizen *et al.*, 2003; Reinecke *et al.*, 2006) of inherently increased MT expression in CI deficient cell lines, supposedly in response to increased ROS. OxyBlot results in the brain and quads demonstrated decreased protein oxidation in the KO and KO OVER mice compared to WT and OVER mice (Section 4.7), contrary to that reported by others (Quintana *et al.*, 2010). However, this apparent lack of protein oxidation in the KO and KO OVER mice could indicate that the ROS levels were not increased to the levels expected for protein damage and may explain the lack of non-*TgMt1* induced *Mt1* expression in the whole brain and quads of these mice. Immunohistochemistry imaging (Section 4.5) did however highlight brain region specific non-*TgMt1* mediated *Mt1* induction in KO mice.

6.2.3 Objective 3 – Phenotype investigation

The key investigation of this study was the survival analysis where ten KO and ten KO OVER mice were allowed die naturally and the age at which they died recorded. The survival curve obtained showed that there was no statistically significant difference between the age of survival of KO and KO OVER mice and that *Mt1* overexpression thus does not increase the lifespan of *Ndufs4*^{-/-} mice. All other phenotyping analyses also supported this conclusion since no statistically significant differences could be seen in the growth curves, wire grid hang test, rotarod or balance beam test of KO compared to KO OVER mice at either P30 or P50. As expected, KO and KO OVER mice performed statistically significantly worse in all phenotyping assays compared to WT and OVER mice, at P50, as a result of the CI deficiency. We thus showed that in addition to no

improvement in lifespan, *Mt1* overexpression in *Ndufs4*^{-/-} mice also did not rescue the impaired motor activity or failure to thrive reported in these mice.

6.2.4 Objective 4 – Investigation of key biochemical parameters

Despite the lack of improvement observed phenotypically in the KO OVER mice, we investigated whether changes could be seen at the biochemical level. The brain and quads were used due to the neurological and muscular involvement in this disease model. Gender did not appear to play a role in any of the biochemical or phenotypic investigations performed, and more specifically, did not show different *Mt1* mRNA expression in male compared to female mice in the brain or the quads. Alam *et al.* (2015) had also previously reported no gender differences for *Ndufs4*^{-/-} mice, while mixed reports exist for *Mt1* expression in the brain tissue of *TgMt1* mice (Iszard *et al.*, 1995; Ono *et al.*, 1997).

As mentioned above, protein oxidation results were surprising in that they showed decreased protein oxidation in both tissues of the KO and KO OVER mice, contrary to the increased protein oxidation (Quintana *et al.*, 2010) and protein nitrotyrosine (Lee *et al.*, 2019) levels previously reported in the brains of these mice. Measurements of the ETS enzyme activity showed the expected significantly decreased CI activity in KO and KO OVER mice compared to WT and OVER mice, with a residual CI activity of ~9% in the brain and ~22% in the quads. This residual activity was similar to that reported by others, but most of these other studies were performed on younger mice. In the skeletal muscle of *Ndufs4*^{-/-} mice, Alam *et al.* (2015) predicted that severe metabolic consequences only occur once CI activity drops by more than 90% due to a significant spare CI capacity in muscle mitochondria. While in a related study from the MRL using these *Ndufs4*^{-/-} mice (Coetzer, 2020), they demonstrated varied ETS enzyme activity in different brain regions, with the vulnerable regions (olfactory bulb, cerebellum and brainstem) demonstrating lower residual CI activity than other regions (anterior cortex) that are believed to be less affected by the knockout of *Ndufs4*. In these same vulnerable brain regions in *Ndufs4*^{-/-} mice, Kayser *et al.* (2016) had demonstrated decreased OXPHOS capacities, which could be distinguished even before the neurological symptoms presented, and hypothesised that the increasing energy demands as the *Ndufs4*^{-/-} mice age may bring about the region-specific neurodegeneration and contribute to progression of the disease. The KO and KO OVER mice in this study showed longer average lifespans and lack of protein oxidation compared to that reported by others, in addition, the residual CI activities were similar to that reported by others in younger *Ndufs4*^{-/-} mice. Thus, it is possible that disease progression was slightly slower in the KO and KO OVER mice compared to other *Ndufs4*^{-/-} mice, perhaps due to the genetic background against which the mice were back-crossed.

Immunohistochemistry involving glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule 1 (IBA1), markers of astrocyte reactivity and microglial activation (which is associated with inflammation) respectively, was performed on coronal sections of the vestibular nucleus and hippocampus from fixed frozen brain samples. These regions were selected based on sagittal section results and since vacuolation and activation of astrocytes and microglial cells have been demonstrated in the vestibular nucleus of *Ndufs4*^{-/-} mice, resulting in fatal breathing deficits (Quintana *et al.*, 2010; Quintana *et al.*, 2012), and there have also been multiple reports of the importance of MT expression in the hippocampus (Carrasco *et al.*, 2000b; Mocchegiani *et al.*, 2004; Manso *et al.*, 2016). As mentioned above, non-*TgMt1* mediated MT1 expression was induced in the hippocampus of KO mice, while increased *TgMt1* mediated MT1 expression was seen in the OVER and KO OVER mice (as expected). The same was not seen in the vestibular nucleus, where the MT1 signal was much lower and only slightly increased in OVER and KO OVER mice compared to WT and KO mice. The lack of MT1 expression in the vestibular nucleus, which has been associated with the morbidity of this model, correlated with the lack of improvement in the lifespan, locomotor activity, balance or motor coordination in KO OVER mice. Furthermore, despite evidence of *TgMt1*-mediated MT1 expression in the hippocampus of *Ndufs4*^{-/-} mice, it is explainable that no improvements were seen in the motor activity assays (which are regulated by the vestibular nucleus or brainstem, motor cortex and cerebellum), since the hippocampus is mainly involved in memory and learning. For the IBA1 stains, the hippocampus of KO mice had greater microglial activation than any of the other genotypes and was actually greater than that in the KO OVER mice (one of the only differences noted between KO and KO OVER mice in this study). The decreased microglial activation of the KO OVER compared to KO mice was also seen to a greater extent in the vestibular nucleus. GFAP stains in the hippocampus were slightly increased in the MT1 overexpressing mice while astrocytes from the *Ndufs4*^{-/-} mice displayed hypertrophy (a sign of glial reactivity). Reactive gliosis can have both positive or negative consequences by creating a glial scar to protect against cellular stress and release protective molecules (such as Cu,Zn-SOD and HSP-70) (West *et al.*, 2004) or by inhibiting neuronal regeneration, respectively, and in combination with the subtle decrease in microglial activation in the KO OVER mice, could be an indication of slightly decreased oxidative stress as a result of MT1 overexpression.

The biochemical data collected appears to point to significant differences in brain-specific regions, especially when looking at the immunohistochemistry results obtained together with the results seen in other studies. However, when looking at the brain as a whole, the known significant differences within brain-specific regions appear to be masked by other regions of the brain which are not affected in the same way. In order to clearly elucidate the biochemical differences between these genotypes, it appears that the most affected brain areas should be studied in isolation.

When investigating the damage to mitochondrial proteins (as a result of excessive ROS) from the more vulnerable brain regions in *Ndufs4*^{-/-} mice, however, Kayser *et al.* (2016) did not find excessive accumulated damage in the brain stem compared to controls. They concluded that the focal neurodegeneration observed in *Ndufs4*^{-/-} mice is not likely as a result of region-specific imbalances in ROS production or clearance. This suggests that ROS may not play such a significant role in the pathological consequences observed in the vestibular nucleus (located in the brain stem) of *Ndufs4*^{-/-} mice and may partially support the lack of protein oxidation observed in the KO and KO OVER mice. This may also provide an explanation as to why the antioxidant and free radical scavenging abilities of MT1 did not appear to elicit a significant effect on any of the phenotypic or biochemical consequences of CI deficiency.

6.2.5 Objective 5 – Metabolomics profiling

By using the multi-platform metabolomics approach, including NMR, GC-TOF-MS and LC-MS/MS, 187 metabolites in the brain and 109 metabolites in the quads were relatively quantified. Due to the lack of improvement observed when directly measuring the hallmark phenotypic and biochemical responses of CI deficiency to MT1 overexpression, it was reasoned that the sensitivity offered by metabolic investigations – being sensitive to finer biochemical control/allosteric mechanisms on a cellular level – may reveal finer insights into a potential effect of MT1 on the background of a CI deficiency.

For WT vs OVER mice, only two significant metabolites, propionylcarnitine and TMG, were identified in the quads. No discriminatory metabolites were identified in the KO vs KO OVER mice in either the brain or the quads. Many metabolic perturbations were found in the different groups, but these could all essentially be attributed to the CI deficiency, which appeared to have a dominating effect on the metabolome and thus masked any possible MT1 effects. One of the hallmark metabolic consequences of CI dysfunction is the prevention of electrons being pumped into the respiratory chain by oxidation of NADH to NAD⁺, thus leading to a disturbance of the NADH/NAD⁺ ratio with a build-up of NADH. Due to the altered redox state, the cellular metabolism is affected in a very broad manner rather than just in a single metabolic pathway. Some of the CI linked perturbed metabolic pathways detected included: altered Krebs cycle intermediates and central carbon metabolism, disturbed 1C metabolism, purine and pyrimidine metabolism, BCAA metabolism, lysine metabolism and altered urea cycle intermediates. Two main metabolic consequences were highlighted, the bifurcation of the Krebs cycle and the alterations in 1C and purine metabolism, however, as already stated, these altered metabolites could be attributed to the *Ndufs4*^{-/-} genotype and the metabolic investigation thus supports the phenotypic and biochemical findings that *Mt1* overexpression does not protect against *Ndufs4* knockout pathology.

6.2.5.1 Bifurcation of the Krebs cycle

The Krebs cycle can be bifurcated via the reductive carboxylation pathway (conversion of glutamine to glutamate, which then enters the Krebs cycle via KG to produce oxaloacetate via citrate) and oxidative pathway (the Krebs cycle functioning in the opposite direction to produce oxaloacetate via succinate and malate), both of which require high levels of pyruvate, KG and electron acceptors and are essential for biosynthesis of aspartate. The KO and KO OVER mice showed decreased aspartate in the brain, possibly due to the limited availability of electron acceptors during CI dysfunction, and increased malate in the KO OVER mice supports the notion that increased cytosolic NADH stimulates reductive carboxylation. On the other hand, the increased fumarate in the quads of our KO and KO OVER mice correlates with Alam *et al.* (2015) (who saw upregulated activity of the oxidative pathway in the Krebs cycle in skeletal muscle of *Ndufs4*^{-/-} mice as well as activation of CII) and indicates increased flux through CII in an attempt to restore electron flow through the ETS and to allow ATP production to continue. This also correlated with the enzyme activity data (Figure 4-22) where significantly increased CII activity in the quads (but not in the brain) of our KO compared to WT and OVER mice was observed. In further support of an activated oxidative pathway in the quads, KO mice showed decreased glutamine and pyroglutamate (a reservoir of glutamate).

Chen *et al.* (2018) demonstrated that the severity of an OXPHOS impairment determines which of the two pathways are used to metabolise KG, where reductive carboxylation is the predominant pathway during severe OXPHOS defects (as a result of limited NAD⁺), whilst in cells with only moderate OXPHOS defects, increased KG is used by the Krebs cycle in both directions, but is mainly oxidative. In support of a KG shortage in *Ndufs4*^{-/-} mice, Kayser *et al.* (2016) showed near normal OXPHOS capacity when KG and malate were used as substrates, presumably due to the ability of KG to maximise the contribution of CII to respiration, while also placing the lowest demand on residual CI activity. Due to the predominantly neurological phenotype in the *Ndufs4*^{-/-} mice, it is probable that the brain tissue exhibits severe OXPHOS deficiency preferring the reductive carboxylation pathway (as supported by decreased aspartate and increased malate), whilst the muscle tissue may only exhibit moderate OXPHOS deficiency leading to predominantly oxidative utilisation of KG (as evidenced by decreased glutamine and pyroglutamate).

6.2.5.2 Changes in 1C metabolism and purine metabolism

Reports of remodelling of the 1C metabolism in mammalian cells as a result of mitochondrial respiratory chain dysfunction have implicated the central involvement of the 1C folate cycle, methyl cycle, transsulfuration pathway and purine synthesis in mitochondrial disease pathology (Bao *et al.*, 2016; Nikkanen *et al.*, 2016), as also seen by the altered metabolites in these

pathways in our KO mice. Nikkanen *et al.* (2016) and Bao *et al.* (2016) hypothesised that the 1C pool becomes depleted in MDs due to increased serine, glutathione and purine synthesis, which in turn increases the homocysteine requirement and disturbs the methyl cycle. This correlated with the increased glycine (produced as a result of increased flux towards serine biosynthesis) and taurine seen in both the brain and quads of the KO and KO OVER mice. The depleted choline, phosphocholine and glycerophosphocholine levels in the brain of the KO and KO OVER mice also indicated an attempt at replenishing homocysteine levels. TMG, which indirectly connects the folate and methyl cycles, was also increased in the KO and WT mice compared to OVER mice in the quads and thus further indicates the induction of alternative 1C sources to replenish the 1C pool. Furthermore, in alignment with Terburgh *et al.* (2019), increased TMG indicates an increased choline supply (despite the NAD⁺ molecule required) due to the ability of TMG to supply electrons directly to the UQ pool (as also evidenced by the increased glycine in the KO OVER mice, which can be produced by sarcosine dehydrogenase) together with fuelling of CII by conversion of FAD to FADH₂. In further support of the hypothesis that methionine metabolism is accelerated, KO mice showed increased propionylcarnitine in the quads. The homocysteine produced by increased methionine metabolism may be converted to α -hydroxybutyrate (Legault *et al.*, 2015; Jain *et al.*, 2016), which is favoured due to the oxidation of NADH, and can lead to increased propionyl-CoA and consequently increased propionylcarnitine. As displayed by increased AMP levels in the brain and quads of the KO and KO OVER mice, another consequence of increased flux through the methyl cycle (to meet the demand of 1C units for purine and methionine synthesis) may be the resulting increased supply of adenosine during SAH conversion to homocysteine, which could in turn be used to form AMP.

6.2.6 Relevant results from other linked studies

Gene expression responses to alterations in the 1C metabolism and oxidative stress metabolism, have been shown to be altered in MDs and with MT expression (Lindeque *et al.*, 2013; Bao *et al.*, 2016; Nikkanen *et al.*, 2016; Terburgh *et al.*, 2019). An investigation of the fold change in gene expression of selected 1C metabolism genes and oxidative stress metabolism genes, using the same mice as this study, also did not reveal any significant differences between KO and KO OVER mice (with the exception of one gene – *Tyms*) (Mienie, 2020).

ROS levels were measured in both untreated and H₂O₂-treated bone-marrow derived macrophages (BMDMs), from the same mice used in this study, since mitochondrial function and cellular redox state have been shown to regulate the activity of macrophages in *Ndufs4*^{-/-} mice (Jin *et al.*, 2014). Surprisingly, no significant differences were detected in the relative ROS levels of untreated BMDMs from the different mice. In the H₂O₂-treated BMDMs, there was a significant increase in the relative ROS levels in all four genotypes compared to the untreated BMDMs, and

H₂O₂-induced ROS levels were significantly lower in OVER mice compared to KO mice. Although this supports the ROS scavenging abilities of MT1, no inhibition of H₂O₂-induced ROS formation was observed in the KO OVER compared to KO mice, thus indicating that *Mt1* overexpression did not provide additional antioxidant capacity to protect KO BMDMs against excess ROS production. Furthermore, no significant difference was seen in the expression of pro-inflammatory biomarkers between BMDMs from KO and KO OVER mice (Boshoff, 2020).

These results correlated with the lack of protein oxidation shown in the brain and quads of the KO and KO OVER mice. The absence of significant differences in the ROS levels, inflammation or expression of oxidative stress metabolism genes in the KO OVER compared to KO mice provided further support for the protein oxidation results, unaltered CI activity, lack of improvement in phenotype and lack of metabolic differences in the KO OVER mice. These results all culminate to form the premise that MT1 overexpression does not protect against CI dysfunction at a phenotypic, metabolic or biochemical level, in the tissues investigated here.

6.3 STRENGTHS, LIMITATIONS AND FUTURE PROSPECTS

The strengths of this study included the well-powered sample sizes compared to other studies (Johnson *et al.*, 2013; Civileto *et al.*, 2015; Jain *et al.*, 2016) as well as the holistic approach that was followed to investigate the hypothesis at a phenotypic, biochemical and metabolic level using many different analytical techniques. A further asset to this study was the confidence that could be achieved in the conclusions drawn from the data since the same result was observed consistently across every analytical platform used – the lack of any difference between KO and KO OVER mice. In addition, the availability of the results from the related studies performed on these mice by others at the MRL allowed for a broader perspective on the study and also provided some external/independent validation of the results obtained here. Obtaining the ethics required for conducting a survival analysis was also a major advantage, as this is not awarded easily, and allowed the most telling aspect of this study to be answered with a fair amount of confidence.

One of the main shortcomings of this study was the lack of more extensive and tissue-specific ROS analyses. Measurement of ROS is however challenging due to its short half-life, high reactivity and complex signaling, and despite the analytical techniques that are available for measuring ROS, most have limitations relating to selectivity, kinetics and absolute quantification, which in turn place uncertainty on the results that are obtained (Wang *et al.*, 2013). The sampling methods used here, as were required by the types of analyses performed, did not allow for direct ROS measurements. Attempts to measure the secondary effects of ROS also proved challenging with NAD⁺/NADH ratio and GSH/GSSG ratio assessments proving unsuccessful/inaccurate. The only the successful measurement was that of protein oxidation. To overcome the difficulties *in*

vivo, *in vitro* measurements of ROS and inflammation in the BMDMs by Boshoff (2020) allowed for some insight into the oxidative status in these mice, while ROS, NADH/NAD⁺ and ADP/ATP ratio measurements in fibroblasts from these mice (Mereis, 2018) did not provide any conclusive information. The lack of improvement in oxidative stress markers of the OVER and KO OVER mice was surprising due to the known antioxidant properties of MT1 and warrants further investigation where an MD model, which demonstrates more drastic oxidative stress, such as superoxide dismutase or glutathione knockout models, crossed with *TgMt1* mice would provide more information.

For the genetic characterisation of the model, the measurement of *Mt1* at the mRNA and DNA levels only was a limitation in this study due to the unsuccessful attempts at measuring MT1 at the protein level using enzyme-linked immunosorbent assays (ELISAs) and SDS-PAGE with western blot analyses (Mereis, 2018). The only successful measurement of MT was in the immunohistochemistry images – using MT antibodies (sensitive to MT1+2 but not MT3) that were generously supplied by Prof. Juan Hidalgo at the *Institut de Neurosciències* at the Autonomous University of Barcelona. While techniques like high-performance liquid chromatography coupled to inductively coupled plasma – mass spectrometry (HPLC-ICP-MS), HPLC-ESI-MS and reversed phase-HPLC coupled to fluorescent detection have shown some promise, they require expensive equipment and specialist operators (Nischwitz *et al.*, 2003). The type of metal that is bound by MT also adds to the complexity of MT quantification, since different metals require varying conditions during MT isolation (Isani & Carpenè, 2014). This correlated with other reports of difficulties in measuring this small protein due to its low detection, sensitivity and selectivity, and currently, no simple and accurate methods for the quantification of MT exist (Dabrio *et al.*, 2002; Lindeque, 2007; Lindeque, 2011; Mehus *et al.*, 2014).

MT2 and MT3 were not specifically measured in this study, and as such, it cannot be assumed that all MT isoforms are ineffective in protecting against mitochondrial dysfunction (as was seen for MT1). The closely related function, structure and localisation of MT1 and MT2 (as discussed in Section 2.6.1) suggests that MT2 would yield similar results to MT1. However, the differences in the metal binding capacities of MT1 and MT2 (Artells *et al.*, 2013; Comes *et al.*, 2019) together with the differential expression and localisation of MT3, and the general lack of knowledge regarding the exact mechanisms by which MTs are able to interact with mitochondria, does not allow one to rule out the potential therapeutic effects of the MT2 and MT3 isoforms.

A question that was not answered in this study, due to the obstacles when trying to measure MT1 at the protein level, is whether or not the MT1 expression levels in the different tissues correlated with that reported by others (Iszard *et al.*, 1995; Ono *et al.*, 1997) who characterized the *TgMt1* model. It was assumed that this was the case and that brain MT levels would have been increased

at least 1.4 times, but no data was available regarding the skeletal muscle or the vestibular nucleus, specifically. While mRNA results do show greatly increased *Mt* levels in both OVER and KO OVER mice, it cannot be certain whether the protein levels do indeed show the corresponding increase reported in literature. The immunohistochemistry results do, however, clearly show a striking increase in the MT levels of OVER and KO OVER mice in the hippocampus. The differential expression of MT1 in the hippocampus and vestibular nucleus supports other reports in literature that certain brain-specific regions better exhibit *TgMt1*-mediated MT1 induction than others. As a predominantly neurological disease, the brainstem (which includes the vestibular nucleus) and olfactory bulbs of *Ndufs4*^{-/-} mice have been reported to be crucial targets in this model, and for MT1, or any other treatment, to rescue many of the metabolic and biochemical consequences observed, it would appear that they would need to target these areas (and others that have been shown to be vulnerable).

Due to the inherent behaviour of mice, high variability was observed in the phenotyping data. To minimise this in future analyses it is recommended to score *Ndufs4*^{-/-} animals according to disease severity (by assessing weight, posture, touch response and other general responses such as hind limb claspings, tremors, etc.), as was done in Quintana *et al.* (2010), in order to classify animals as early stage, middle stage or late stage as opposed to classifying them by a postnatal day age. By then performing the phenotypic analyses, once mice have been classified to be at one of these three stages, variation should be minimised and all mice should be much closer to the same phase of disease severity or progression than when only using the age of the mice. In addition, this study would have greatly benefitted from the inclusion of not only motor activity assays, but also spatial learning and memory tests to assess whether or not the increased expression of MT1 in the hippocampus of KO OVER mice showed improvements in memory and learning compared to KO mice, as the immunohistochemistry results suggest.

The severity and impact of the tissue- and region-specific differences, observed in the biochemical and metabolic alterations in response to CI dysfunction, became apparent in the *Ndufs4*^{-/-} mice and highlighted the importance of not only investigating whole tissues, but also those specific areas that have been shown to be affected. Furthermore, the contribution of specific cell types (such as glutamatergic and GABAergic neurons) have also been shown to play a significant role in the pathology of the *Ndufs4*^{-/-} mice (Bolea *et al.*, 2019), and their involvement should thus also not be underestimated.

6.4 FINAL CONCLUSION

This study has shown for the first time, using a multifaceted approach, that *Mt1* overexpression does not protect against the devastating phenotypic and cellular consequences of CI dysfunction in an *Ndufs4*^{-/-} mouse model. Other than mild reductions in inflammation in the vestibular nucleus and hippocampus, no significant differences were found in the phenotype tests, RNA gene expression assays, oxidative stress or metabolomics analyses in the brain and quads of the KO vs KO OVER mice. While the expected worsened phenotype and altered redox reactions were seen in the KO and KO OVER compared to WT and OVER mice as a result of the CI deficiency, a protective effect from MT1 in our OVER or KO OVER groups was glaringly absent, contrary to the multiple reports of their protective role in mitochondrial dysfunction and neurodegenerative diseases.

The lack of measurable elevated oxidative stress and low-grade inflammation in the KO and KO OVER mice leads one to question whether or not *Ndufs4*^{-/-} does indeed result in systemic oxidative stress, as shown by others, or whether the oxidative stress is more localised to specific tissues and cell types, as this model seems to suggest. A Trolox derivative, KH176, developed by de Haas *et al.* (2017), was used to treat *Ndufs4*^{-/-} mice due to its potency in ROS and redox assays. Similarly, treatment with this KH176 antioxidant also did not improve the disease severity nor increase the lifespan of *Ndufs4*^{-/-} mice, which they attributed to the unrestored vestibular nuclei lesions that they observed. However, contrary to that shown here, they did demonstrate improved rotarod and gait performance, which they hypothesised was due to the remedial effect of KH176 on ROS and microstructural coherence in the brain. Likewise, treating *Ndufs4*^{-/-} mice with a poly[adenine diphosphate (ADP)-ribose] polymerase (PARP)-1 inhibitor, PJ34, which increases NAD⁺ availability, Felici *et al.* (2014) reported improvements in motor skills and coordination, but also saw no improvement in survival or body weight. In contrast, rapamycin, an inhibitor of mTOR, was shown to increase the lifespan of *Ndufs4* mice (up to 114 days) in addition to demonstrating improved motor coordination, without evidence of any improvement in oxidative stress markers nor rescue of OXPHOS function (Johnson *et al.*, 2013). Lee *et al.* (2019) demonstrated decreased NAD⁺ levels in the brain and muscle of *Ndufs4*^{-/-} mice, which were restored in the muscle only upon treatment with nicotinamide mononucleotide, an NAD⁺ precursor, and extended the lifespan (median of 110 days) of the mice despite the lack of metabolic benefits in the brain. Furthermore, treatment with dimethyl-ketoglutarate, a KG precursor that also inhibits mTOR, resulted in a similar extension of lifespan in addition to an improved neurological phenotype. Lee *et al.* (2019) suggested that the greater energy deprivation in the brain as opposed to the muscle explained the organ-specific effectivity of increased intracellular NAD⁺ levels. This leads one to question what proportion of the *Ndufs4*^{-/-} disease phenotype, and its biological consequences, are truly

dependent on oxidative stress, especially in relation to the major devastating cellular consequences that occur during CI dysfunction. The apparent absence thereof in our *Ndufs4*^{-/-} model could explain the inability of *Mt1* overexpression to improve motor activity or survival.

The more distinct overexpression of MT1 in the hippocampus than in the vestibular nucleus of the OVER and KO OVER mice appears to link to the key role that the vestibular nucleus has been shown to play in the morbidity of the *Ndufs4*^{-/-} mice (Quintana *et al.*, 2010; Quintana *et al.*, 2012). Taken together with the improved neurological phenotype observed with *Mt1* overexpression in diseases (such as Alzheimer's disease and epileptic seizures) that have a greater hippocampal involvement (Penkowa *et al.*, 2005; Manso *et al.*, 2016), and an improvement in lifespan with alleviation of vestibular nuclei lesions irrespective of an improvement in oxidative stress markers (Johnson *et al.*, 2013; de Haas *et al.*, 2017), it appears that ameliorating the pathological changes observed in the vestibular nucleus of *Ndufs4* mice is one of the key features in an effective treatment strategy for CI dysfunction. A recent report on the cell type-specific contribution of glutamatergic and GABAergic neurons to the cellular mechanisms underlying the pathology of *Ndufs4* deficiency (Bolea *et al.*, 2019) also provides an exciting new insight into CI deficiency and a promising target for new treatment options. Whether or not the *Ndufs4*^{-/-} model exhibits the extraordinary ROS levels (especially at a tissue-specific level) that would be required to see an improvement in primary MD pathology, due to the ROS scavenging abilities of MT1, remains unclear. Despite this, the tissue- and cell-specific neuroprotective role of MT1 and the potential of other MT isoforms, such as MT2 and MT3, cannot yet be ruled out in CI deficiency.

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APPENDIX A: ANIMAL INFORMATION

A1. Animals and samples

A detailed list of the animals included in this study is shown in Table A1 and Table A2, as discussed in Section 3.2.3, where the age and gender of the mice is indicated together with which analyses each mouse was used for.

Table A1: Mice included in phenotyping, biochemical analysis and metabolomics

Mouse ID	Sample ID	Date of birth	Gender	Age euthanised	Analysis
WT					
9.1	1B/1Q	2017/10/02	M	51	P, B, M, O
10.1	2B/2Q	2017/10/07	F	46	P, B, M
10.5	3B/3Q	2017/10/07	F	46	P, B, M
10.6	4B/4Q	2017/10/07	M	46	P, B, M
10.7	5B/5Q	2017/10/07	M	46	P, B, M
13.2	6B/6Q	2017/10/30	M	49	P, B, M
14.1		2017/07/30	F	51	P
14.3		2017/07/30	F	51	P
14.4		2017/07/30	M	51	P
2.2	25Q	2017/08/25	F	47	P, I, M
3.3	26Q	2017/08/25	F	47	P, I, M
7.4	27Q	2017/09/15	M	48	P, I, M
75	28Q	2017/09/18	F	49	I
76	29Q	2017/09/18	F	49	I
4.7	25B/30Q	2018/02/24	F	47	M
4.2	29B/49Q	2018/02/24	M	47	M
4.8	30B/50Q	2018/02/24	M	47	M, N
OVER					
12.5	7B	2017/07/08	F	52	P, B
1.1	7Q	2017/08/07	M	46	P, B, I, M
15.2	8B/8Q	2017/08/02	F	50	P, B, M
15.3	9B/9Q	2017/08/02	F	50	P, B, M
15.5	10B/10Q	2017/08/02	M	50	P, B, M
4.1	11B/11Q	2017/09/03	M	47	P, B, M, O
12.1	12B/12Q	2017/10/21	F	46	P, B, M
12.4		2017/07/08	F	52	P
1.3	31Q	2017/08/07	M	46	P, I, M
1.6	32Q	2017/08/07	M	46	P, I, M
1.7	33Q	2017/08/07	F	46	P, I, M
4.3	34Q	2017/09/03	F	47	P, I, M
78	35Q	2017/09/18	M	49	M
82	36Q	2017/09/18	F	49	I
80	31B/51Q	2017/09/18	M	49	M, N
KO					
10.3	13B/13Q	2017/10/07	M	46	P, B, M
13.8	14B/14Q	2017/10/30	F	49	P, B, M, O
9.5	15B/15Q	2017/12/22	M	48	P, B, M
9.6	16B/16Q	2017/12/22	F	48	P, B, M
2.6	17B/17Q	2018/02/09	M	47	P, B, M
2.7	18B/18Q	2018/02/09	F	47	P, B, M
2.3	37Q	2017/08/25	M	47	P, I, M

Mouse ID	Sample ID	Date of birth	Gender	Age euthanised	Analysis
13.7	38Q	2017/10/30	F	49	P, I, M
15.8	26B/39Q	2018/01/20	M	46	P, M
1.4	40Q	2018/01/27	F	47	P, I, M
1.6	41Q	2018/01/27	M	47	P, I, M
74	42Q	2017/09/18	M	49	I
14.2	32B/52Q	2017/07/30	M	51	P, M, N
KO OVER					
12.7	19B/19Q	2017/10/21	F	46	P, B, M
14.4	20B/20Q	2017/11/02	M	46	P, B, M
15.4	21B/21Q	2017/11/09	F	49	P, B, M, O
15.5	22B/22Q	2017/11/09	M	49	P, B, M
15.6	23B/23Q	2017/11/09	F	49	P, B, M
12.1	24B/24Q	2018/01/13	F	48	P, B, M
5.5	43Q	2017/09/04	M	46	P, I, M
5.8	44Q	2017/09/04	F	46	P, I, M
12.2	45Q	2017/10/21	F	46	P, I, M
12.5	46Q	2017/10/21	M	46	P, I, M
14.2	47Q	2017/11/02	M	46	P, I, M
15.8	27B/48Q	2017/11/09	F	49	P, M
3.6	28B	2018/02/10	M	46	M
3.4	33B/53Q	2018/02/10	M	46	M, N

* Key for analyses: P = Phenotyping; B = Biochemical analyses (RNA and enzyme analyses); I = Immunohistochemistry; M = Metabolomics; N = NDUFS4 blot; O = OxyBlot.

Table A2: Mice included in survival analysis

Mouse ID	Date of birth	Gender	Age euthanised
KO			
4.6	2017/04/03	M	67
5.3	2017/04/18	F	73
5.6	2017/04/18	F	79
5.7	2017/04/18	M	86
8.4	2017/05/24	M	75
8.6	2017/05/24	M	64
11.2	2017/07/06	M	85
11.4	2017/07/06	F	71
1.5	2017/11/19	M	65
9.1	2017/12/22	F	81
KO OVER			
9.3	2017/06/18	F	87
10.1	2017/06/23	M	69
12.3	2017/07/08	F	70
12.7	2017/07/08	M	78
1.4	2017/08/07	M	88
1.5	2017/08/07	M	69
1.8	2017/08/07	F	76
3.2	2017/11/26	F	66
7.4	2017/12/14	F	95
8.9	2017/12/24	M	77

A2. Genotype characterisation

An example of the gel electrophoresis results for the *Ndufs4* gene is shown in Figure A1, as discussed in Section 3.3.2.

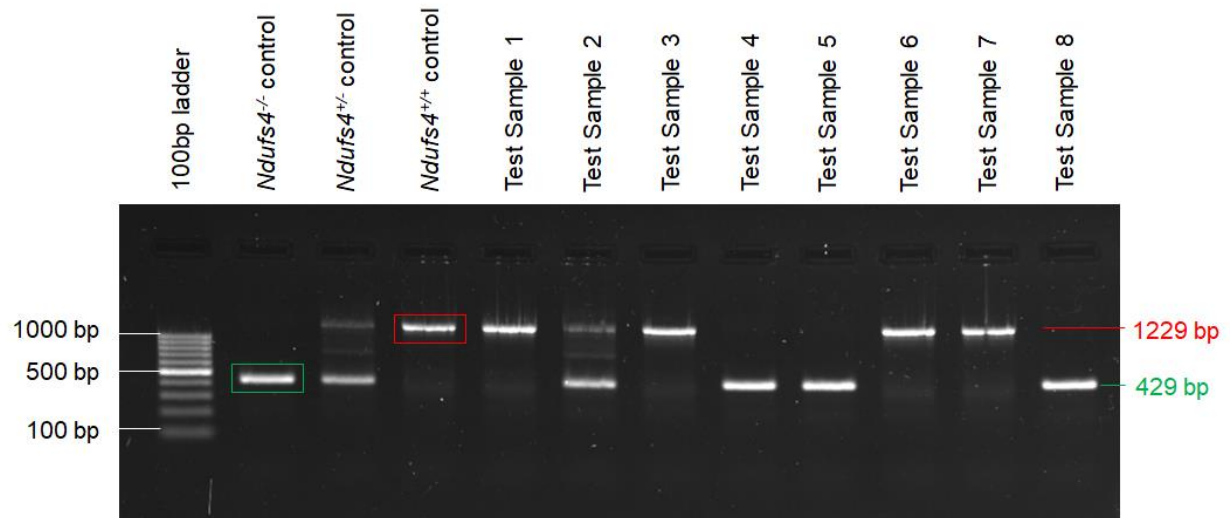


Figure A1: Example gel electrophoresis photo indicating the DNA bands obtained for wild-type *Ndufs4*^{+/+} (red - 1229 bp), knockout *Ndufs4*^{-/-} (green – 429 bp) and heterozygote *Ndufs4*^{+/-} (both 429 bp and 1229 bp) mice. A DNA ladder is depicted in the first column which allows for relative quantification of the size of the bp in the samples.

APPENDIX B: SUPPLEMENTARY PHENOTYPING DATA

B1. Growth curves

As described in Section 3.5.1, separate growth curves were constructed using: 1) the mice that were used for survival analysis, and 2) the mice that underwent all locomotor activity and ataxia assays. Furthermore, all growth curves were separated according to gender.

B1.1. Growth curves of mice included in survival analysis

The growth curves of all mice used for survival analysis (that were not euthanised) are shown in Figure B2. The body weight of the KO mice was statistically greater than the KO OVER mice at P20-25 and P32-34. Thereafter the KO and KO OVER mice appeared to plateau at similar weights. Similarly, to the KO and KO OVER mice, the WT mice showed significantly greater body weight than the OVER mice during the initial growth phase, P20-28, followed by similar body weight thereafter. The WT mice, from P35 onwards, and the OVER mice, from P50 onwards, showed significantly greater body weight than both the KO and KO OVER mice. One of the contributing factors to the larger variation seen in the WT and OVER mice was the smaller sample sizes compared to KO and KO OVER mice. Also note that the sample sizes for the KO and KO OVER mice gradually decreased with age as the mice naturally died due to CI deficiency.

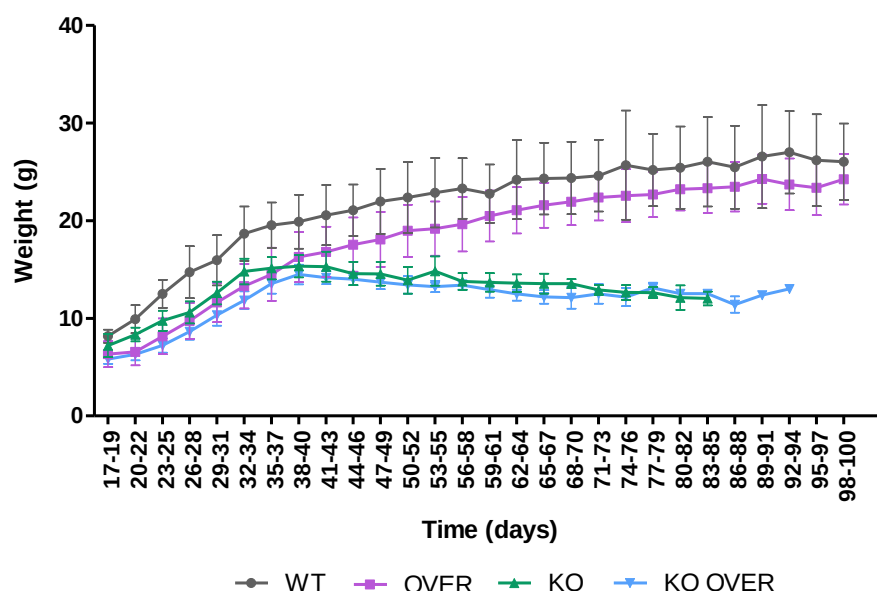


Figure B2: Growth curves of WT (n = 5), OVER (n = 6), KO (n = 10) and KO OVER (n = 10) mice, included in the survival analysis, indicating their masses over time. Error bars represent 95% confidence interval of the mean at each time intervals.

B1.2. Growth curves of mice included in locomotor and ataxia assays

Figure B3 demonstrates the growth curves constructed from the mice that were included in the locomotor and ataxia assays and were euthanised at ~P50. In Figure B4, this data was split according to gender. Due to the larger and more even sample sizes, as well as the euthanasia of all mice at the same age, the variation was much smaller than that seen in Figure B2. The OVER mice, from P26 onwards, and the WT mice, from P38 onwards, showed significantly greater body weight than the KO OVER mice. For the KO mice, the body weight of the OVER and WT mice only became significantly greater at a later age, from P38 and P41 respectively. Unlike the growth curve obtained from the mice included in the survival analysis, no significant differences were seen between the KO and KO OVER mice or between the WT and OVER mice.

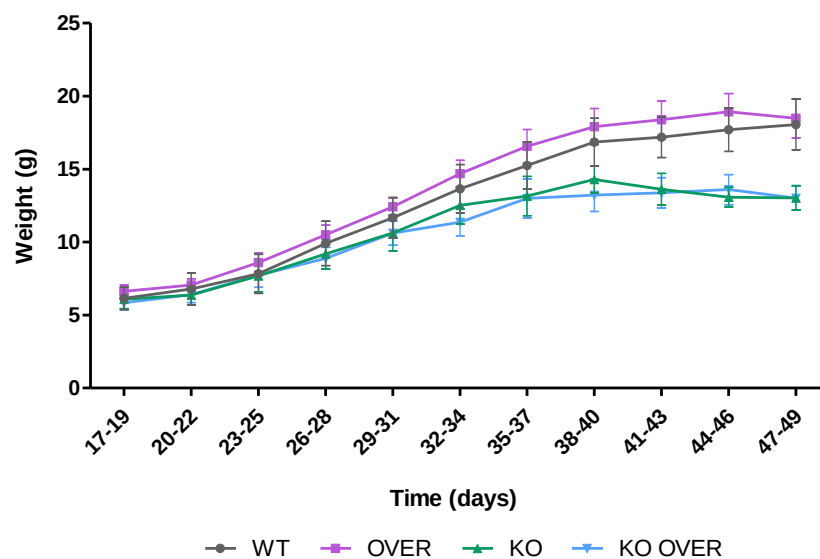
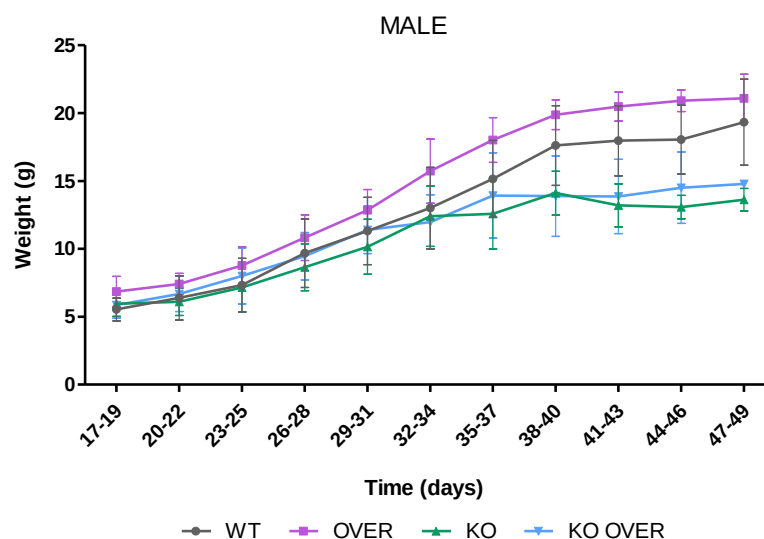


Figure B3: Growth curves of WT, OVER, KO and KO OVER (n = 12/genotype), included in the phenotyping analyses, indicating their masses over time. Error bars represent 95% confidence interval of the mean at each time intervals.



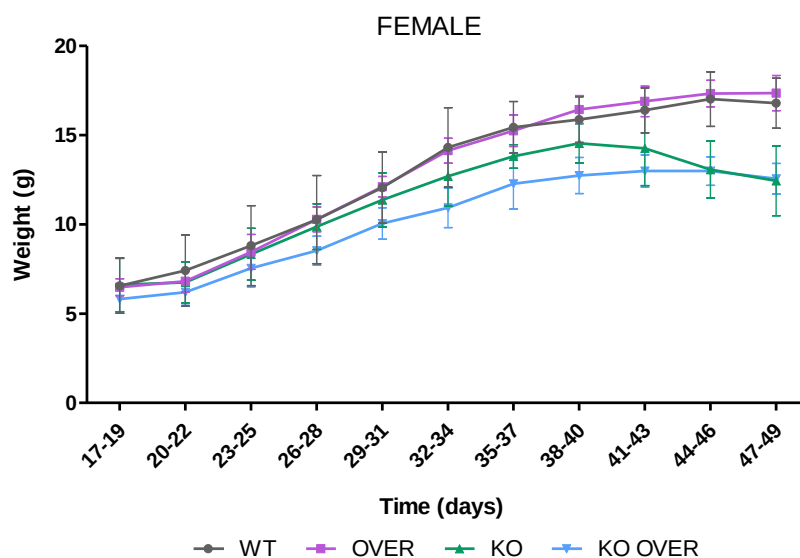


Figure B4: Growth curves of (A) male and (B) female (n = male/female) WT (n = 7/5), OVER (n = 5/7), KO (n = 7/5) and KO OVER (n = 5/7) mice that were included in the phenotyping analyses, indicating their masses over time. Error bars represent 95% confidence intervals.

B2. Survival analysis

As discussed in Section 4.4.2, survival curves were split according to gender and are shown in Figure B5. Using the Log Rank (Mantel-Cox) test, the survival distributions for the males and females were however not statistically significant, $\chi^2(1) = 0.604$, $p = 0.437$ and $\chi^2(1) = 0.331$, $p = 0.565$ respectively.

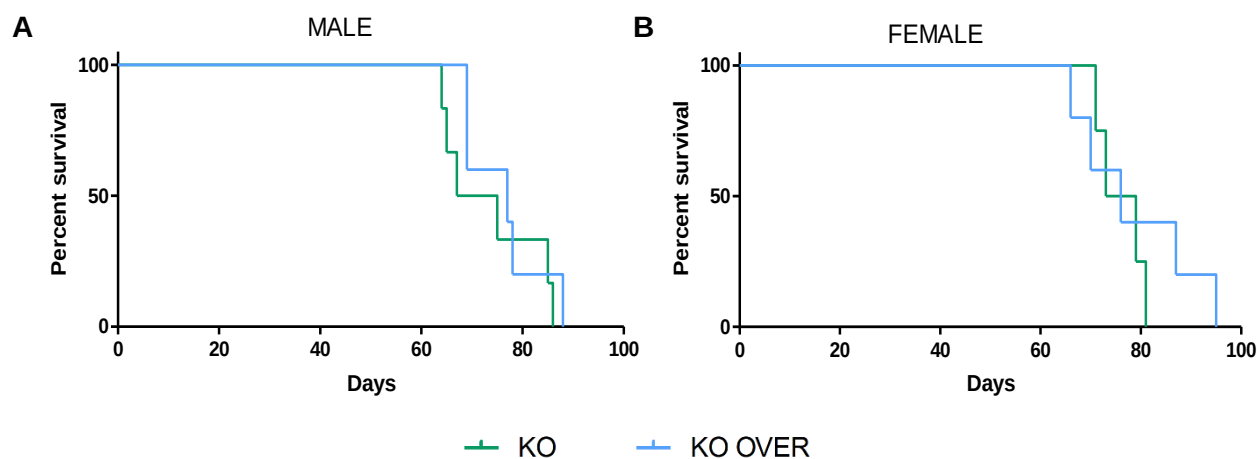


Figure B5: Survival rate of (A) male and (B) female KO OVER mice (n = 5) compared to KO mice (n = 6 males/4 females) over time.

B3. Locomotor activity

As discussed in Section 4.4.4, the total distance travelled and rest time of the mice was determined over 5 min in an open field test and is shown for both males and females at P30 (Figure B6) and P50 (Figure B7). One-way ANOVAs were performed with Games-Howell post hoc tests to determine the statistically significant differences indicated on the vertical scatter plots.

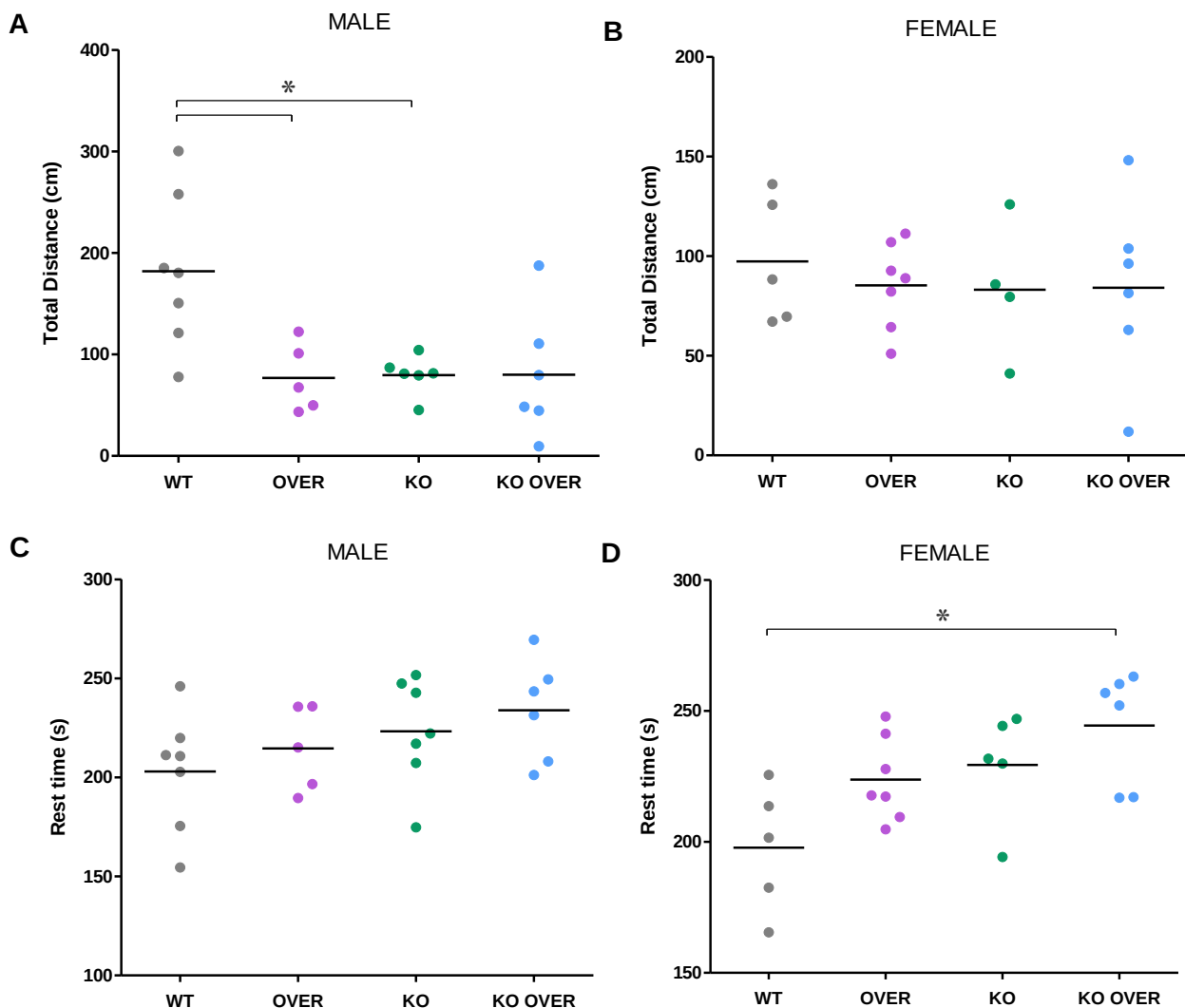


Figure B6: Vertical scatter plots of mice of the four genotypes at P30 depicting the (A-B) total distance travelled (in cm) over 5 min and the (C-D) rest time (in seconds) of males (A, C) and females (B, D). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

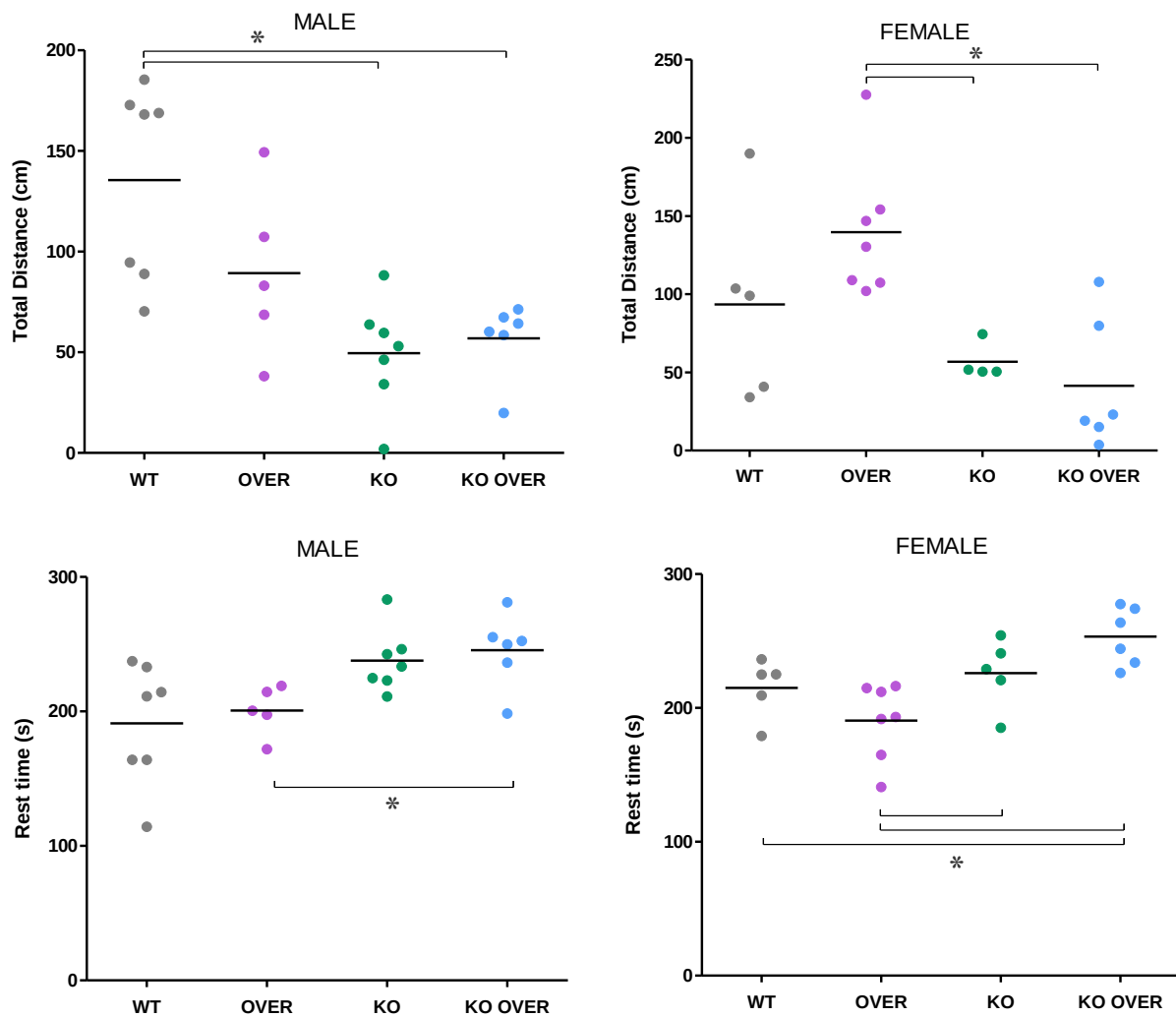


Figure B7: Vertical scatter plots of mice of the four genotypes at P50 depicting the (A-B) total distance travelled (in cm) over 5 min and the (C-D) rest time (in seconds) of males (A, C) and females (B, D). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

B4. Wire grid hang test

As discussed in Section 4.4.5.1, the mean physical impulse of the mice was determined using the wire grid hang test and is shown for both males and females at P30 and P50 in Figure B8. One-way ANOVAs were performed with Games-Howell post hoc tests to determine the statistically significant differences indicated on the vertical scatter plots.

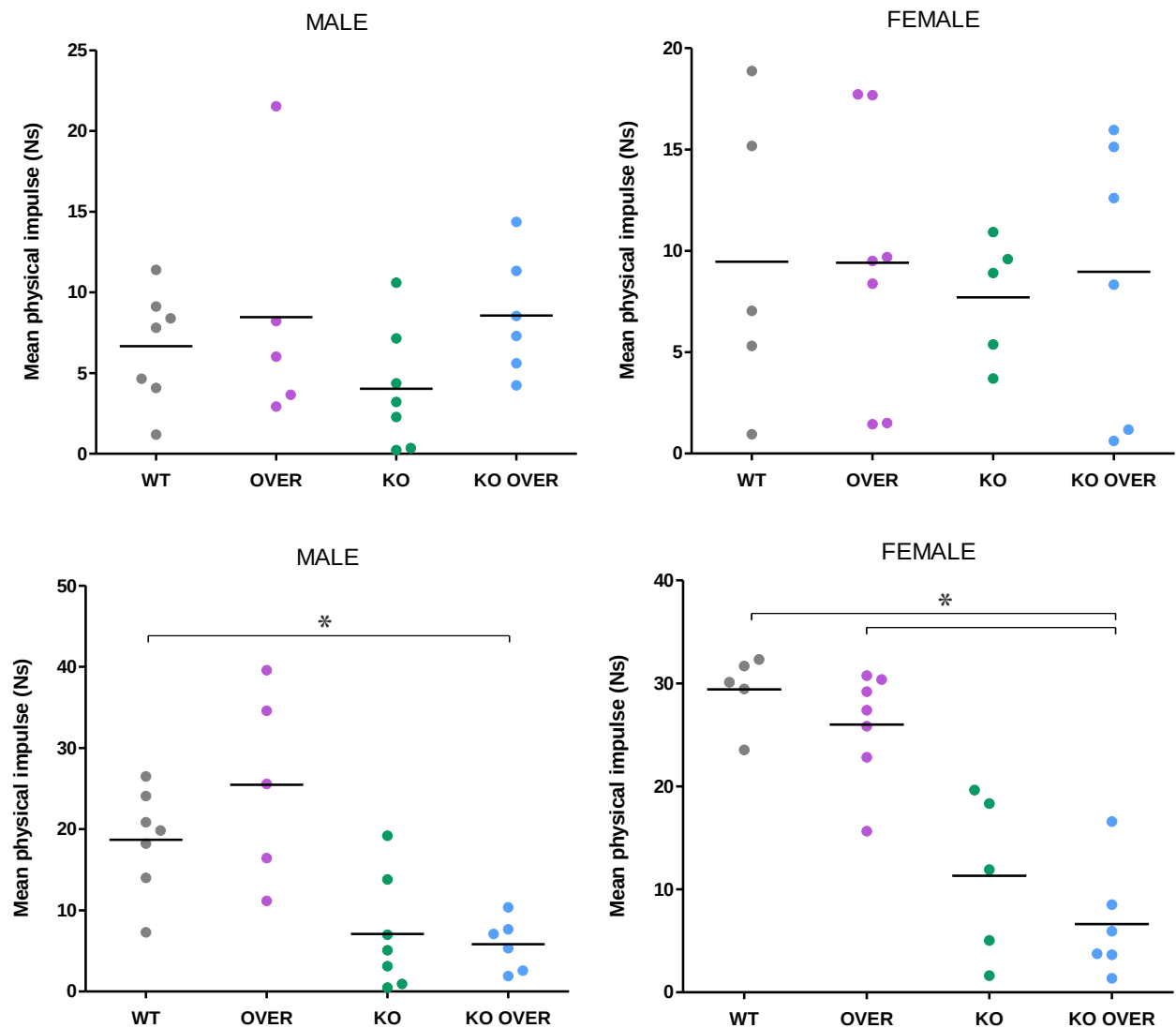


Figure B8: Vertical scatter plots depicting the mean physical impulse of mice of the four genotypes at (A-B) P30 and (C-D) P50 for males (A, C) and females (B, D). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

B5. Balance beam test

As discussed in Section 4.4.5.2, the balance of the mice was assessed using the balance beam test and is shown for both males and females at P30 and P50 in Figure B9. One-way ANOVAs were performed with Games-Howell post hoc tests to determine the statistically significant differences indicated on the vertical scatter plots.

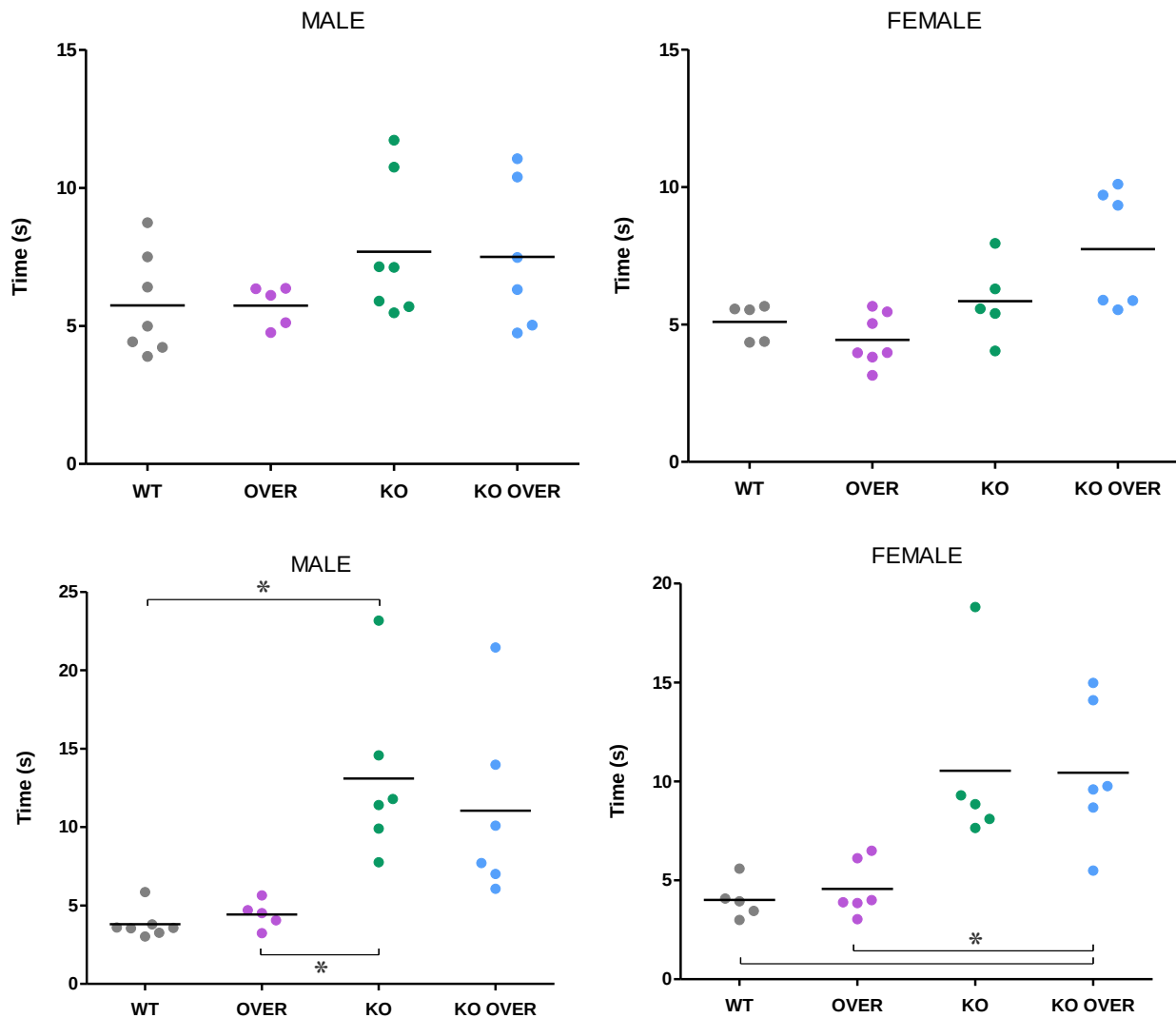


Figure B9: Vertical scatter plots depicting the time taken (in seconds) for mice of the four genotypes at (A-B) P30 and (C-D) P50 to cross a 0.8 m balance beam for males (A, C) and females (B, D). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

B6. Rotarod

As discussed in Section 4.4.5.3, the motor coordination of the mice was assessed using the rotarod and is shown for both males and females at P30 and P50 in Figure B10. One-way ANOVAs were performed with Games-Howell post hoc tests to determine the statistically significant differences indicated on the vertical scatter plots

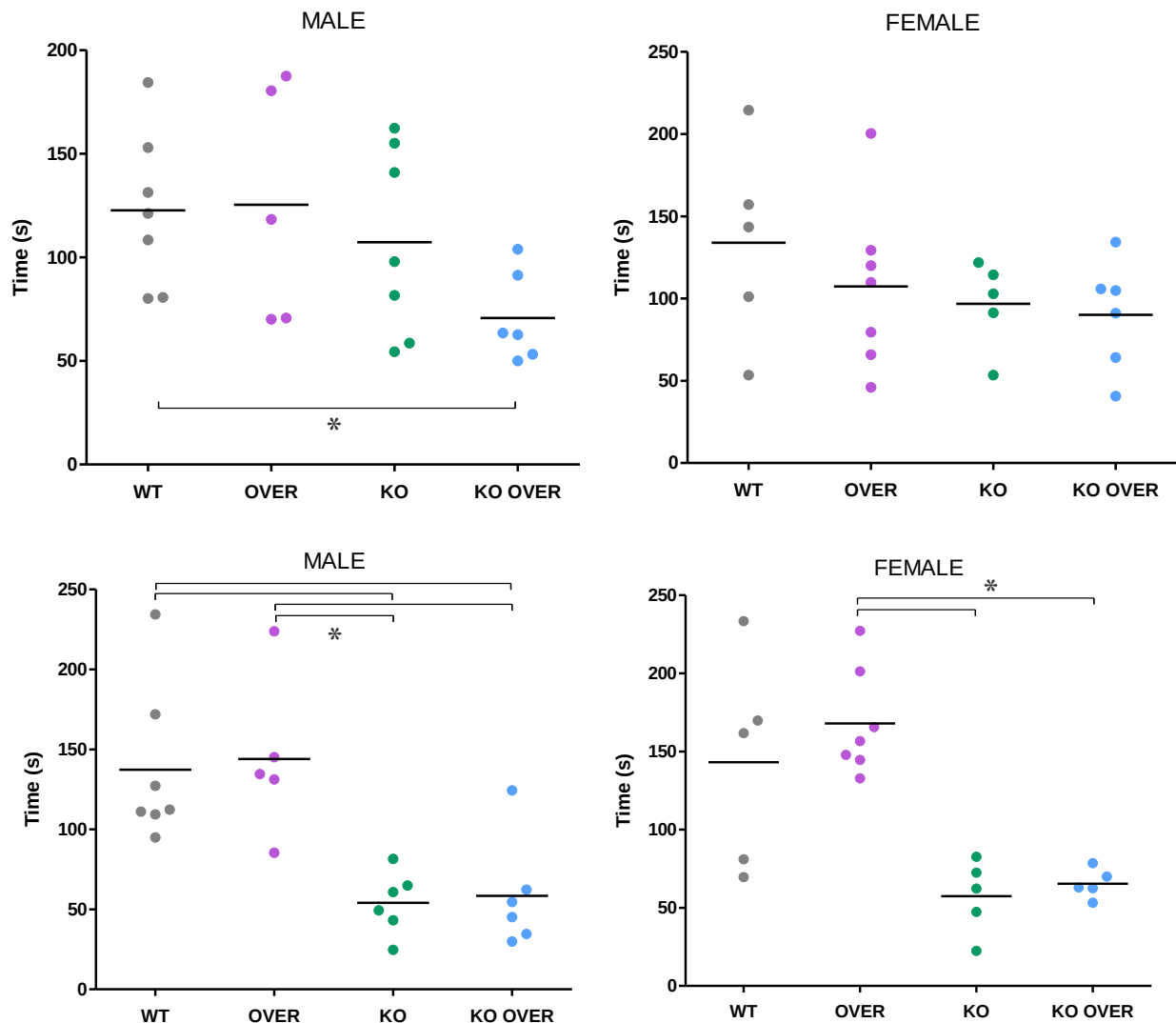


Figure B10: Vertical scatter plots depicting the latency to fall (in seconds) for mice of the four genotypes at (A-B) P30 and (C-D) P50 for males (A, C) and females (B, D). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

APPENDIX C: SUPPLEMENTARY BIOCHEMICAL DATA

C1. Immunohistochemistry

As discussed in Section 4.5.2, Figure C11 depicts the MT1 and GFAP stains of the vestibular nucleus (without DAPI).

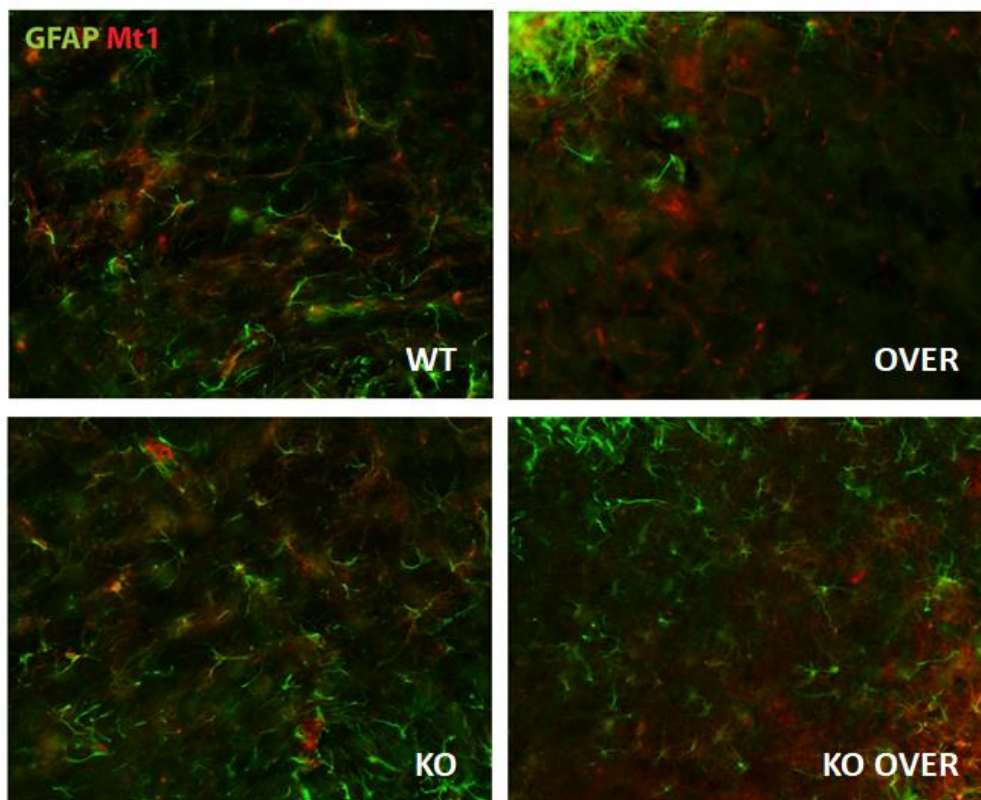


Figure C11: Immunohistochemistry stains for MT1 (red) and GFAP (green) in the vestibular nucleus of mice from the four genotypes (n = 5/genotype).

C2. Electron transport system enzyme analyses

As discussed in Section 4.6, in addition to normalising to UCS, the ETS enzyme activity was also normalised according to protein only and CS activity only, to determine the effect of the normalisation method, as shown in Figure C12 and Figure C13 respectively. Similarly to the phenotyping data, line graphs were used as opposed to bar graphs (as shown in Figure C14), even though the genotypes are independent entities, to depict the interaction between genotype and tissue type. The error bars on these line graphs also represented 95% confidence intervals, and as such, genotypes where error bars did not overlap indicated statistically significant differences between the brain and quads.

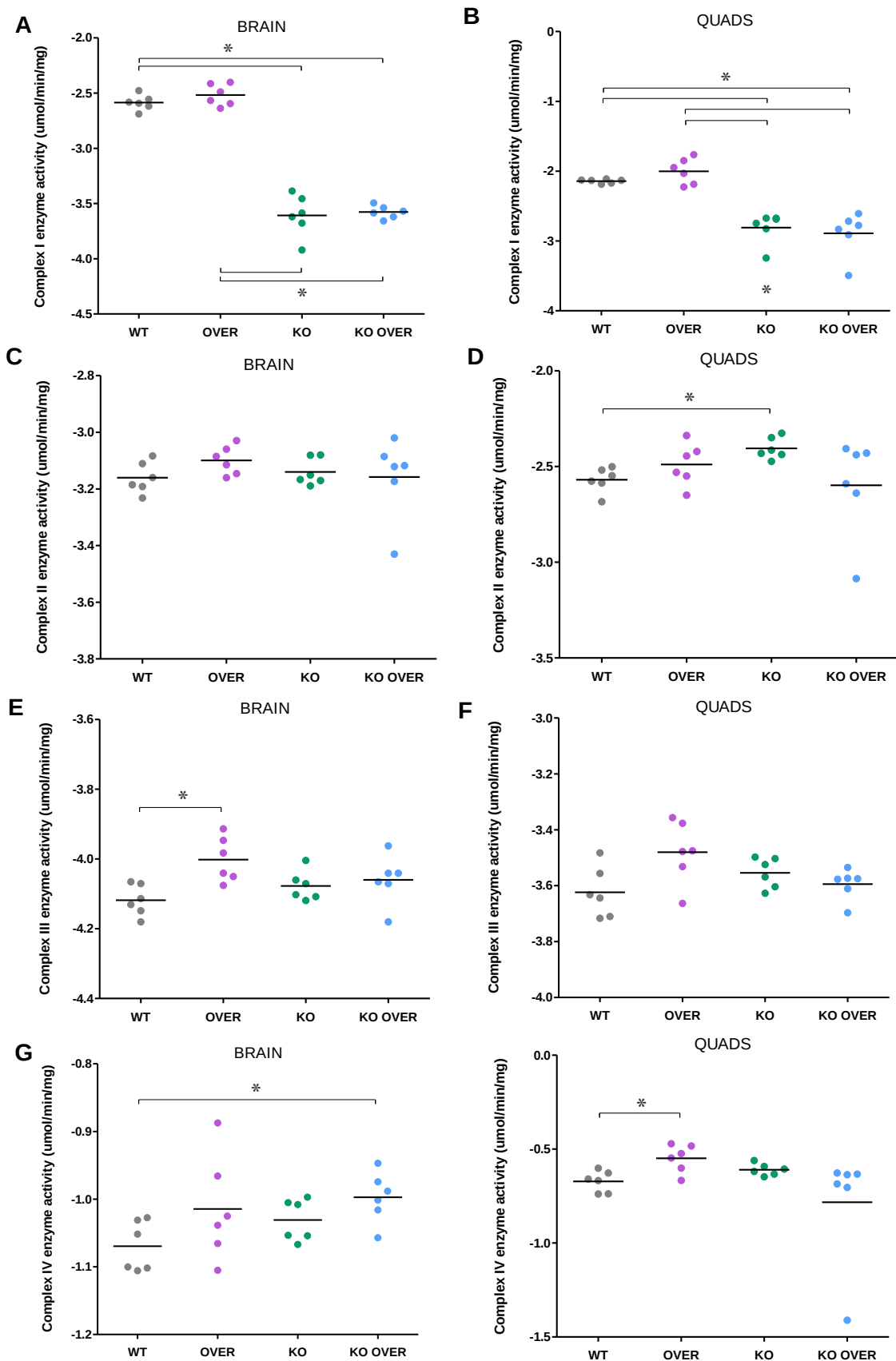


Figure C12: Vertical scatter plots depicting the log transformed (A-B) CI, (C-D) CII, (E-F) CIII and (G-H) CIV enzyme activity, normalised to protein only, in the (A, C, E, G) brain and (B, D, F, H) quads ($n = 6/\text{genotype}$). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

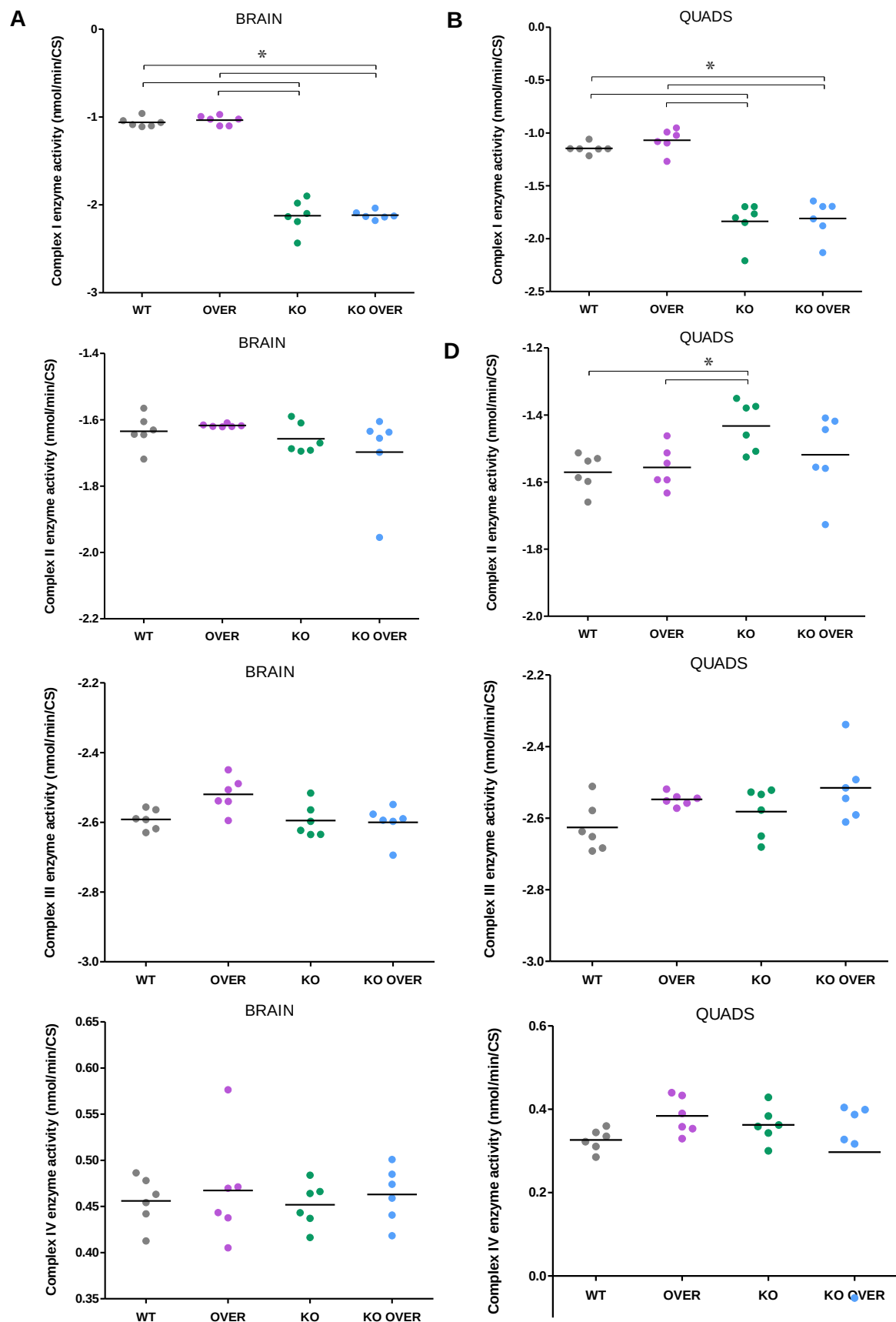


Figure C13: Vertical scatter plots depicting the log transformed (A-B) CI, (C-D) CII, (E-F) CIII and (G-H) CIV enzyme activity, normalised to CS activity only, in the (A, C, E, G) brain and (B, D, F, H) quads ($n = 6/\text{genotype}$). Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*).

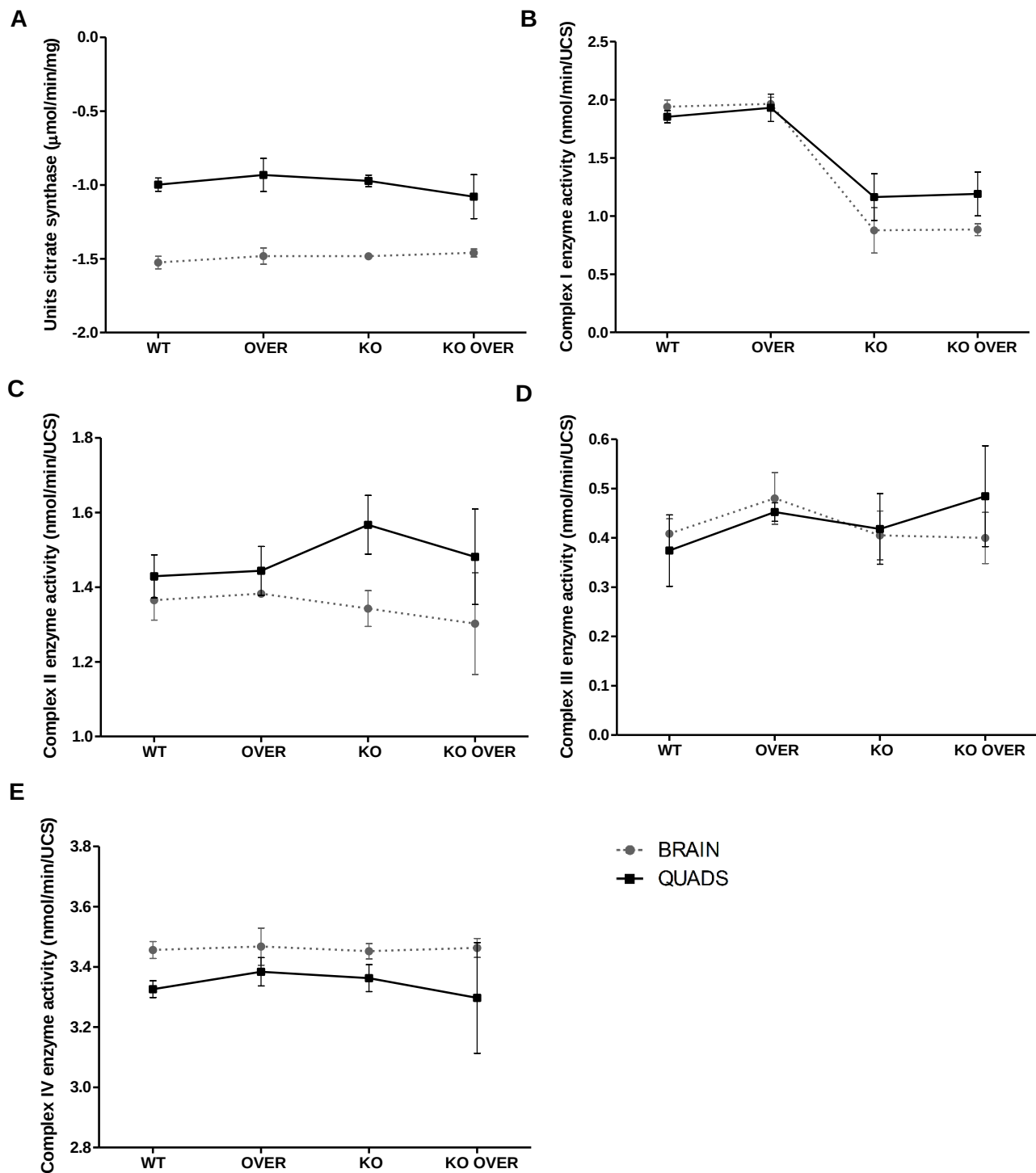


Figure C14: Line graphs depicting the log transformed (A) UCS, (B) CI, (C) CII, (D) CIII and (E) CIV activity (normalised to UCS) in the brain and quads ($n = 6/\text{genotype}$). Error bars represent 95% confidence interval of the mean.

C3. Enzyme, RNA and phenotyping correlations

Table C3: Spearman's rho correlation between *Mt1* mRNA and ETS enzyme activity data

Genotype	Parameter	Statistic	BRAIN						QUADS					
			Protein	CS	CI	CII	CIII	CIV	Protein	CS	CI	CII	CIII	CIV
WT	<i>Mt1</i> mRNA (BRAIN)	Spearman	0.486	-0.543	0.029	-0.029	-0.086	0.886	-0.371	-0.257	0.086	-0.600	0.086	-0.886
		<i>p-value</i>	0.329	0.266	0.957	0.957	0.872	0.019*	0.468	0.623	0.872	0.208	0.872	0.019*
	<i>Mt1</i> mRNA (QUADS)	Spearman	-0.600	0.371	-0.086	-0.657	-0.543	-0.486	-0.429	0.086	0.543	-0.371	-0.600	0.143
		<i>p-value</i>	0.208	0.468	0.872	0.156	0.266	0.329	0.397	0.872	0.266	0.468	0.208	0.787
OVER	<i>Mt1</i> mRNA (BRAIN)	Spearman	-0.429	0.143	-0.143	-0.600	0.486	0.714	-0.500	0.300	0.300	-0.400	0.100	-0.400
		<i>p-value</i>	0.397	0.787	0.787	0.208	0.329	0.111	0.391	0.624	0.624	0.505	0.873	0.505
	<i>Mt1</i> mRNA (QUADS)	Spearman	0.500	-0.500	0.000	0.500	-0.300	0.300	-0.371	0.086	0.029	0.086	-0.029	-0.371
		<i>p-value</i>	0.391	0.391	1.000	0.391	0.624	0.624	0.468	0.872	0.957	0.872	0.957	0.468
KO	<i>Mt1</i> mRNA (BRAIN)	Spearman	-0.086	0.371	-0.200	-0.200	-0.600	-0.371	-0.600	-0.486	-0.600	0.714	0.543	0.829
		<i>p-value</i>	0.872	0.468	0.704	0.704	0.208	0.468	0.208	0.329	0.208	0.111	0.266	0.042*
	<i>Mt1</i> mRNA (QUADS)	Spearman	0.086	0.314	-0.257	-0.257	-0.543	-0.543	-0.543	0.029	-0.771	0.429	0.371	0.771
		<i>p-value</i>	0.872	0.544	0.623	0.623	0.266	0.266	0.266	0.957	0.072	0.397	0.468	0.072
KO OVER	<i>Mt1</i> mRNA (BRAIN)	Spearman	0.429	0.086	0.429	0.486	-0.029	0.714	-0.600	-0.771	-0.486	-0.600	0.486	0.486
		<i>p-value</i>	0.397	0.872	0.397	0.329	0.957	0.111	0.208	0.072	0.329	0.208	0.329	0.329
	<i>Mt1</i> mRNA (QUADS)	Spearman	0.771	-0.886	-0.429	-0.314	-0.943	0.200	-0.086	0.029	0.371	-0.086	0.371	0.314
		<i>p-value</i>	0.072	0.019*	0.397	0.544	0.005*	0.704	0.872	0.957	0.468	0.872	0.468	0.544

* Green blocks indicate significant positive correlations while red blocks indicate significant negative correlations ($p < 0.05$).

Table C4: Spearman's rho correlation between *Mt1* mRNA, ETS enzyme activity and phenotyping data of WT mice

Tissue	Parameter	Statistic	POSTNATAL DAY 30					POSTNATAL DAY 50				
			Distance	Rest	Balance	Rotarod	FDT	Distance	Rest	Balance	Rotarod	FDT
BRAIN	<i>Mt1</i> mRNA	Spearman	0.829	-0.486	0.143	0.029	-0.086	-0.143	0.086	0.086	-0.371	0.371
		<i>p-value</i>	0.042*	0.329	0.787	0.957	0.872	0.787	0.872	0.872	0.468	0.468
	CS	Spearman	-0.600	0.143	0.314	0.429	0.200	0.829	-0.771	0.714	0.143	-0.143
		<i>p-value</i>	0.208	0.787	0.544	0.397	0.704	0.042*	0.072	0.111	0.787	0.787
	CI	Spearman	0.314	-0.200	0.543	0.543	-0.429	0.086	0.714	0.086	0.029	-0.029
		<i>p-value</i>	0.544	0.704	0.266	0.266	0.397	0.872	0.111	0.872	0.957	0.957
	CII	Spearman	0.143	0.314	0.143	0.086	-0.943	-0.029	0.657	-0.257	-0.029	0.029
		<i>p-value</i>	0.787	0.544	0.787	0.872	0.005*	0.957	0.156	0.623	0.957	0.957
	CIII	Spearman	-0.600	0.829	0.086	-0.714	-0.143	0.029	-0.314	-0.314	-0.771	0.771
		<i>p-value</i>	0.208	0.042*	0.872	0.111	0.787	0.957	0.544	0.544	0.072	0.072
	CIV	Spearman	0.543	-0.200	0.029	-0.200	-0.086	-0.029	-0.257	0.086	-0.486	0.486
		<i>p-value</i>	0.266	0.704	0.957	0.704	0.872	0.957	0.623	0.872	0.329	0.329
QUADS	<i>Mt1</i> mRNA	Spearman	-0.086	-0.543	-0.200	0.371	0.829	0.029	-0.257	0.314	0.657	-0.657
		<i>p-value</i>	0.872	0.266	0.704	0.468	0.042*	0.957	0.623	0.544	0.156	0.156
	CS	Spearman	-0.600	0.486	-0.429	-0.771	0.486	-0.257	-0.486	-0.371	-0.257	0.257
		<i>p-value</i>	0.208	0.329	0.397	0.072	0.329	0.623	0.329	0.468	0.623	0.623
	CI	Spearman	0.600	-0.829	-0.086	0.714	0.143	-0.029	0.314	0.314	0.771	-0.771
		<i>p-value</i>	0.208	0.042*	0.872	0.111	0.787	0.957	0.544	0.544	0.072	0.072
	CII	Spearman	-0.429	0.657	-0.086	-0.200	-0.657	-0.143	0.543	-0.486	0.086	-0.086
		<i>p-value</i>	0.397	0.156	0.872	0.704	0.156	0.787	0.266	0.329	0.872	0.872
	CIII	Spearman	0.029	0.486	-0.429	-0.771	-0.429	-0.771	0.657	-0.943	-0.257	0.257
		<i>p-value</i>	0.957	0.329	0.397	0.072	0.397	0.072	0.156	0.005*	0.623	0.623
	CIV	Spearman	-0.886	0.714	-0.371	-0.486	0.086	-0.143	-0.086	-0.429	0.143	-0.143
		<i>p-value</i>	0.019*	0.111	0.468	0.329	0.872	0.787	0.872	0.397	0.787	0.787

* Cases excluded pairwise. Green blocks indicate significant positive correlations while red blocks indicate significant negative correlations ($p < 0.05$).

Table C5: Spearman's rho correlation between *Mt1* mRNA, ETS enzyme activity and phenotyping data of OVER mice

Tissue	Parameter	Statistic	POSTNATAL DAY 30					POSTNATAL DAY 50				
			Distance	Rest	Balance	Rotarod	FDT	Distance	Rest	Balance	Rotarod	FDT
BRAIN	<i>Mt1</i> mRNA	Spearman	0.086	0.429	-0.600	0.486	-0.486	0.371	-0.486	0.086	0.886	-0.600
		<i>p-value</i>	0.872	0.397	0.208	0.329	0.329	0.468	0.329	0.872	0.019*	0.208
	CS	Spearman	-0.086	0.429	-0.600	-0.200	-0.314	0.371	0.371	0.600	0.029	-0.771
		<i>p-value</i>	0.872	0.397	0.208	0.704	0.544	0.468	0.468	0.208	0.957	0.072
	CI	Spearman	0.200	-0.257	-0.600	-0.543	-0.657	0.371	-0.086	0.086	-0.029	-0.600
		<i>p-value</i>	0.704	0.623	0.208	0.266	0.156	0.468	0.872	0.872	0.957	0.208
	CII	Spearman	-0.486	-0.143	0.600	-0.771	0.429	-0.086	0.086	-0.486	-0.257	0.429
		<i>p-value</i>	0.329	0.787	0.208	0.072	0.397	0.872	0.872	0.329	0.623	0.397
	CIII	Spearman	0.543	-0.086	-0.143	1.000	-0.029	-0.143	-0.257	0.257	0.200	0.143
		<i>p-value</i>	0.266	0.872	0.787		0.957	0.787	0.623	0.623	0.704	0.787
	CIV	Spearman	-0.029	0.429	-0.086	0.600	0.257	0.257	-0.314	0.257	0.657	-0.086
		<i>p-value</i>	0.957	0.397	0.872	0.208	0.623	0.623	0.544	0.623	0.156	0.872
QUADS	<i>Mt1</i> mRNA	Spearman	0.086	0.143	-0.257	-0.314	-0.314	0.600	-0.714	-0.771	0.371	-0.086
		<i>p-value</i>	0.872	0.787	0.623	0.544	0.544	0.208	0.111	0.072	0.468	0.872
	CS	Spearman	-0.029	0.257	-0.943	-0.429	-0.771	0.486	0.086	0.143	0.257	-0.886
		<i>p-value</i>	0.957	0.623	0.005*	0.397	0.072	0.329	0.872	0.787	0.623	0.019*
	CI	Spearman	0.600	-0.143	-0.771	0.314	-0.714	0.086	0.200	-0.086	-0.371	-0.257
		<i>p-value</i>	0.208	0.787	0.072	0.544	0.111	0.872	0.704	0.872	0.468	0.623
	CII	Spearman	-0.143	-0.086	0.543	-0.543	0.829	0.371	-0.086	0.314	-0.086	0.371
		<i>p-value</i>	0.787	0.872	0.266	0.266	0.042*	0.468	0.872	0.544	0.872	0.468
	CIII	Spearman	0.257	-0.371	-0.486	0.257	-0.771	-0.257	-0.371	-0.314	0.200	-0.257
		<i>p-value</i>	0.623	0.468	0.329	0.623	0.072	0.623	0.468	0.544	0.704	0.623
	CIV	Spearman	0.200	-0.543	0.771	0.657	0.486	-0.771	-0.086	-0.086	-0.314	0.829
		<i>p-value</i>	0.704	0.266	0.072	0.156	0.329	0.072	0.872	0.872	0.544	0.042*

* Cases excluded pairwise. Green blocks indicate significant positive correlations while red blocks indicate significant negative correlations ($p < 0.05$).

Table C6: Spearman's rho correlation between *Mt1* mRNA, ETS enzyme activity and phenotyping data of KO mice

Tissue	Parameter	Statistic	POSTNATAL DAY 30					POSTNATAL DAY 50				
			Distance	Rest	Balance	Rotarod	FDT	Distance	Rest	Balance	Rotarod	FDT
BRAIN	<i>Mt1</i> mRNA	Spearman	-1.000	0.771	0.086	-0.486	0.714	-0.600	-0.429	0.771	-0.314	0.486
		<i>p-value</i>		0.072	0.872	0.329	0.111	0.208	0.397	0.072	0.544	0.329
	CS	Spearman	0.000	0.600	-0.086	-0.086	-0.086	-0.371	-0.086	0.600	-0.771	0.257
		<i>p-value</i>	1.000	0.208	0.872	0.872	0.872	0.468	0.872	0.208	0.072	0.623
	CI	Spearman	0.800	-0.429	-0.429	0.314	0.314	0.143	-0.600	-0.257	0.486	0.371
		<i>p-value</i>	0.200	0.397	0.397	0.544	0.544	0.787	0.208	0.623	0.329	0.468
	CII	Spearman	0.800	-0.429	-0.429	0.314	0.314	0.143	-0.600	-0.257	0.486	0.371
		<i>p-value</i>	0.200	0.397	0.397	0.544	0.544	0.787	0.208	0.623	0.329	0.468
	CIII	Spearman	0.800	-0.943	0.086	0.486	-0.086	0.543	-0.371	-0.486	0.829	0.029
		<i>p-value</i>	0.200	0.005*	0.872	0.329	0.872	0.266	0.468	0.329	0.042*	0.957
	CIV	Spearman	0.800	0.086	0.086	-0.143	-0.543	-0.257	0.486	-0.143	-0.657	0.143
		<i>p-value</i>	0.200	0.872	0.872	0.787	0.266	0.623	0.329	0.787	0.156	0.787
QUADS	<i>Mt1</i> mRNA	Spearman	-0.800	0.600	-0.086	0.029	0.429	-0.086	-0.486	0.829	-0.143	-0.029
		<i>p-value</i>	0.200	0.208	0.872	0.957	0.397	0.872	0.329	0.042*	0.787	0.957
	CS	Spearman	0.400	-0.600	-0.257	1.000	-0.486	0.943	-0.314	-0.029	0.486	-0.657
		<i>p-value</i>	0.600	0.208	0.623		0.329	0.005*	0.544	0.957	0.329	0.156
	CI	Spearman	1.000	-0.486	-0.371	0.086	-0.029	0.029	0.029	-0.714	0.257	0.257
		<i>p-value</i>		0.329	0.468	0.872	0.957	0.957	0.957	0.111	0.623	0.623
	CII	Spearman	-0.600	0.371	-0.200	-0.486	1.000	-0.543	-0.543	0.200	0.200	0.657
		<i>p-value</i>	0.400	0.468	0.704	0.329		0.266	0.266	0.704	0.704	0.156
	CIII	Spearman	-0.600	0.086	0.086	-0.371	0.829	-0.257	-0.429	0.086	0.486	0.371
		<i>p-value</i>	0.400	0.872	0.872	0.468	0.042*	0.623	0.397	0.872	0.329	0.468
	CIV	Spearman	-1.000	0.943	-0.086	-0.371	0.314	-0.543	-0.086	0.771	-0.714	0.200
		<i>p-value</i>		0.005*	0.872	0.468	0.544	0.266	0.872	0.072	0.111	0.704

* Cases excluded pairwise. Green blocks indicate significant positive correlations while red blocks indicate significant negative correlations ($p < 0.05$).

Table C7: Spearman's rho correlation between *Mt1* mRNA, ETS enzyme activity and phenotyping data of KO OVER mice

Tissue	Parameter	Statistic	POSTNATAL DAY 30					POSTNATAL DAY 50				
			Distance	Rest	Balance	Rotarod	FDT	Distance	Rest	Balance	Rotarod	FDT
BRAIN	<i>Mt1</i> mRNA	Spearman	-0.257	0.371	0.886	0.657	0.771	-0.200	0.371	0.829	-0.200	0.257
		<i>p-value</i>	0.623	0.468	0.019*	0.156	0.072	0.704	0.468	0.042*	0.747	0.623
	CS	Spearman	-0.486	0.829	-0.143	0.714	0.200	-0.600	0.086	-0.029	1.000	0.143
		<i>p-value</i>	0.329	0.042*	0.787	0.111	0.704	0.208	0.872	0.957		0.787
	CI	Spearman	-0.886	0.771	-0.029	0.771	0.371	-0.086	-0.086	0.029	0.200	0.314
		<i>p-value</i>	0.019*	0.072	0.957	0.072	0.468	0.872	0.872	0.957	0.747	0.544
	CII	Spearman	0.257	0.200	0.543	0.314	0.257	-0.886	0.886	0.657	0.400	-0.429
		<i>p-value</i>	0.623	0.704	0.266	0.544	0.623	0.019*	0.019*	0.156	0.505	0.397
	CIII	Spearman	-0.314	0.771	-0.143	0.429	-0.257	-0.086	-0.314	-0.371	0.900	-0.143
		<i>p-value</i>	0.544	0.072	0.787	0.397	0.623	0.872	0.544	0.468	0.037*	0.787
	CIV	Spearman	-0.143	0.029	0.657	0.486	0.943	-0.371	0.486	0.886	-0.300	0.543
		<i>p-value</i>	0.787	0.957	0.156	0.329	0.005*	0.468	0.329	0.019*	0.624	0.266
QUADS	<i>Mt1</i> mRNA	Spearman	0.429	-0.886	0.029	-0.657	-0.029	0.314	0.143	0.143	-0.900	-0.029
		<i>p-value</i>	0.397	0.019*	0.957	0.156	0.957	0.544	0.787	0.787	0.037*	0.957
	CS	Spearman	0.029	-0.143	-0.771	-0.543	-0.886	0.543	-0.600	-0.943	0.000	-0.371
		<i>p-value</i>	0.957	0.787	0.072	0.266	0.019*	0.266	0.208	0.005*	1.000	0.468
	CI	Spearman	0.771	-0.657	-0.143	-0.886	-0.829	0.314	-0.029	-0.371	-0.400	-0.771
		<i>p-value</i>	0.072	0.156	0.787	0.019*	0.042*	0.544	0.957	0.468	0.505	0.072
	CII	Spearman	0.200	-0.086	-0.600	-0.429	-0.829	-0.143	0.086	-0.600	0.100	-0.771
		<i>p-value</i>	0.704	0.872	0.208	0.397	0.042*	0.787	0.872	0.208	0.873	0.072
	CIII	Spearman	-0.086	-0.143	0.429	0.314	0.829	-0.429	0.543	0.771	-0.400	0.486
		<i>p-value</i>	0.872	0.787	0.397	0.544	0.042*	0.397	0.266	0.072	0.505	0.329
	CIV	Spearman	-0.200	-0.029	0.371	0.029	0.086	0.543	-0.143	0.143	-0.600	-0.029
		<i>p-value</i>	0.704	0.957	0.468	0.957	0.872	0.266	0.787	0.787	0.285	0.957

* Cases excluded pairwise. Green blocks indicate significant positive correlations while red blocks indicate significant negative correlations ($p < 0.05$).

C4. Protein oxidation

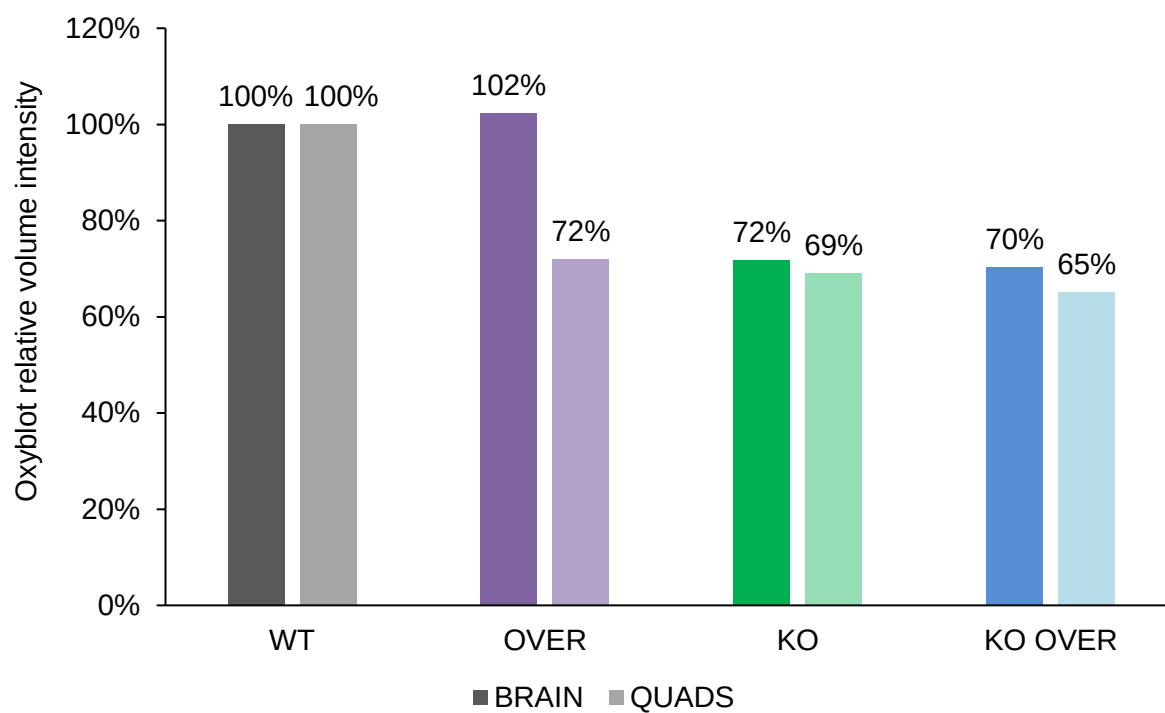


Figure C15: Bar graph depicting the percentage of oxidised proteins in the brain and quads of one mouse per genotype, relative to the WT (not normalised to VDAC). The darker bar indicates the brain tissue and the lighter bars indicate the quads samples.

APPENDIX D: METABOLOMICS

Table D8: MRM parameters for metabolites and internal standard isotopes monitored

Metabolite name	~ RT (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Fragmentor voltage (V)	Collision energy (eV)
AMINO ACIDS AND DERIVATIVES						
1-Methylhistidine	1.8	226.4	124.2	45	120	15
2-Aminoadipic acid	12.7	274.2	98.1	45	113	24
3-Methyl Histidine	1.7	226.4	96.2	45	130	25
Alanine	4.0	146.1	44.2	45	80	15
Arginine	1.9	231.2	70.1	45	103	40
Arginine__4,4,5,5__d ₄	2.0	235.2	74.2	45	108	32
Asparagine	2.6	189.1	144.1	45	89	8
Asparagine__C ¹³ ₄ __N ¹⁵ ₂	2.6	195.2	148.1	45	89	8
Aspartic acid	12.3	246.2	144.1	45	98	12
Beta-Alanine	3.7	146.2	72.1	45	90	10
Beta-Aminoisobutyric acid	6.6	160.1	86.1	45	100	10
Citrulline	4.6	232.2	70.1	45	89	32
Citrulline__4,4,5,5__d ₄	4.6	236.2	74.1	45	108	28
Creatine	6.6	188.1	90.1	45	104	16
Cystathionine	8.5	335.2	190.1	45	127	16
Cystine	8.4	353	130	45	118	16
Dimethylglycine	3.8	160.3	58.2	45	90	20
Gamma-Aminobutyric acid	6.1	160.1	87.1	45	79	8
Glutamic acid	12.5	260.2	84.1	45	89	24
Glutamine	3.4	203.1	84.1	45	89	20
Glutamine__C ¹³ ₅ __N ¹⁵ ₂	3.2	210.2	89.1	45	89	20
Glycine	2.6	132.1	76.1	45	65	4
Histidine	1.6	212.1	110.1	45	89	16
Homocysteine	10.6	192	90	45	161	12
4-Hydroxyproline	4.0	188.1	68.1	45	120	35
Isoleucine__d ₁₀	10.6	198.2	96.2	45	108	12
Leucine_Isoleucine	10.8	188.2	86.2	45	89	8
Lysine	1.7	203.2	84.1	45	89	20
Lysine__4,4,5,5__d ₄	1.6	207.2	88.2	45	118	16
Methionine	9.1	206.1	104.1	45	94	8
<i>N,N</i> -Dimethylphenylalanine	12.5	250.1	148.1	45	103	20
<i>N</i> -Acetylaspartate	14.1	288.2	144.1	45	98	16
<i>N</i> -Acetylglutamate	14.2	302.2	186.1	45	113	12
Ornithine	1.5	189.2	70.2	45	74	20
Phenylalanine	11.9	222.2	120.1	45	108	16
Phenylalanine__ring__d ₅	11.8	227.2	125.1	45	108	16
Pipecolic acid	7.9	186.1	84.1	45	108	16
Proline	6.5	172.1	70.2	45	94	20

Metabolite name	~ RT (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Fragmentor voltage (V)	Collision energy (eV)
Pyroglutamic acid	11.5	186.1	84.1	45	108	16
Sarcosine	2.8	146.1	44.2	45	80	15
Serine	2.7	162.1	60.1	45	94	12
Taurine	1.4	126	107.9	45	93	10
Threonine	3.7	176.1	74.1	45	94	12
Trimethylglycine	5.4	174.2	58.2	45	130	45
Tryptophan	12.4	261.2	244.1	45	94	8
Tyrosine	9.5	238.2	136.1	45	94	12
Valine	8.7	174.2	72.2	45	89	12
Valine__d ₈	8.5	182.2	80.2	45	103	12
ACYLCARNITINES						
Acetyl-carnitine (C2)	9.1	260.2	85.1	45	122	24
Butyryl-carnitine (C4)	12.4	288.2	85.1	45	110	20
Carnitine (C0)	6.3	218.2	103	45	132	16
Carnitine__d ₃	6.3	221.2	103.1	45	127	16
Decanoyl-carnitine (C10)	14.4	372.3	85.1	45	125	28
Dodecanoyl-carnitine (C12)	14.6	400.3	85.1	45	155	28
Dodecanoyl-carnitine__d ₃	14.6	403.4	85.1	45	155	28
Hexanoyl-carnitine (C6)	13.3	316.2	85.1	45	137	24
Isovaleryl-carnitine (C5)	12.8	302.2	85.1	45	105	24
Myristoyl-carnitine (C14)	15.0	428.4	85.1	45	150	28
Octanoyl-carnitine (C8)	13.8	344.3	85.1	45	115	28
Octanoyl-carnitine__d ₃	13.8	347.3	85.1	45	136	24
Palmitoyl-carnitine (C16)	15.3	456.4	85.1	45	160	36
Propionyl-carnitine (C3)	11.2	274.2	85.1	45	127	20
Stearoyl-carnitine (C18)	15.7	484	85.1	45	155	36
Stearoyl-carnitine__d ₃	15.7	487.5	85.1	45	160	36

Abbreviations: ~ RT, approximate retention time.

APPENDIX E: SUBMITTED PEER-REVIEWED ARTICLE

TITLE:

Metallothionein 1 overexpression does not protect against mitochondrial disease pathology in *Ndufs4* knockout mice

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ABSTRACT

Mitochondrial diseases (MD), such as Leigh Syndrome (LS), present with severe neurological and muscular phenotypes in patients, but have no known cure and limited treatment options. Based on their neuroprotective effects against other neurodegenerative diseases *in vivo* and their positive impact as an antioxidant against complex I deficiency *in vitro*, we investigated the potential protective effect of metallothioneins (MT) in an *Ndufs4* knockout mouse model (with a very similar phenotype to LS) crossed with an *Mt1* overexpressing mouse model (*TgMt1*). Despite subtle reductions in the expression of neuroinflammatory markers GFAP and IBA1 in the vestibular nucleus and hippocampus, we found no improvement in survival, growth, locomotor activity, balance or motor coordination in the *Mt1* overexpressing *Ndufs4*^{-/-} mice. Furthermore, at a cellular level, no differences were detected in the metabolomics profile or gene expression of selected one-carbon metabolism and oxidative stress genes, performed in the brain and quadriceps, nor in the ROS levels of macrophages derived from these mice. Considering these outcomes, we conclude that MT1, in general, does not protect against the impaired motor activity or improve survival in these complex I deficient mice. The unexpected absence of increased oxidative stress and metabolic redox imbalance in this MD model may explain these observations. However, tissue-specific observations such as the mildly reduced inflammation in the hippocampus and vestibular nucleus, as well as differential MT1 expression in these tissues, may yet reveal a tissue- or cell-specific role for MTs in these mice.

KEYWORDS

Ndufs4 knockout mice; Metallothionein; Mitochondrial disease; Leigh Syndrome; Phenotyping; Oxidative stress

DECLARATIONS

Funding

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Conflicts of interest

All authors declare that they have no conflict of interest.

Ethics approval

The AnimCare animal research ethics committee of the NWU approved (approval numbers NWU-00364-16-A5 and NWU-00001-15-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.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and material

The data that support the findings of this study are available from the corresponding author, FHvdW, upon reasonable request.

Code availability

Not applicable.

Authors' contributions

All authors reviewed, edited and approved the final manuscript version for submission and all authors were involved with the design of the methodology. **Hayley Christy Miller:** Conceptualization, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Roan Louw:** Conceptualization, Writing - Original Draft. **Michelle Mereis:** Investigation, Visualization. **Gerda Venter:** Conceptualization. **John-Drew Boshoff:** Formal analysis, Investigation, Visualization. **Liesel Mienie:** Formal analysis, Investigation, Visualization. **Mari van Reenen:** Conceptualization, Formal analysis. **Marianne Venter:** Formal analysis. **Jeremie Zander Lindeque:** Formal analysis. **Adán Domínguez-Martínez:** Formal analysis, Visualisation. **Albert Quintana:** Conceptualization, Investigation, Resources, Visualization, Supervision. **Francois Hendrikus van der Westhuizen:** Conceptualization, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

ABBREVIATIONS

1C, One-carbon; ADP, Adenosine diphosphate; BMDM, Bone marrow-derived macrophage; BH-FDR, Benjamini-Hochberg adjustment to control the rate of false discovery; CI, Complex I; CII, Complex II; CIII, Complex III; CIV, Complex IV; DAPI, 4',6-Diamidino-2-phenylindole; DNA, Deoxyribonucleic acid; dNTP, Deoxynucleotide triphosphate; GC-TOF-MS, Gas chromatography time-of-flight mass spectrometry; GFAP, Glial fibrillary acidic protein; GSH, Reduced glutathione; GSSG, Reduced glutathione; H₂DCF-DA, 2',7'-Dichlorodihydrofluorescein diacetate; H₂O₂, Hydrogen peroxide; IBA1, Ionised calcium binding adapter molecule 1; IL6, Interleukin 6; IL12, Interleukin 12; KO, *Ndufs4* knockout; KO OVER, *Ndufs4* knockout *Mt1* overexpressing; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LS, Leigh Syndrome; MCP1, Monocyte chemoattractant protein 1; MCSF, Macrophage colony stimulating factor; mRNA, Messenger RNA; MD, Mitochondrial disease; MT, Metallothionein; NAD⁺, Oxidised nicotinamide adenine dinucleotide; NADH, Reduced nicotinamide adenine dinucleotide; NDUFS4, NADH:ubiquinone oxidoreductase subunit S4; NMR, Nuclear magnetic resonance spectroscopy; NWU, North-West University; OVER, *Mt1* overexpressing; P, Postnatal day; PARP, Poly[adenine diphosphate (ADP)-ribose] polymerase; PBS, Physiological buffered saline; PCR, Polymerase chain reaction; RC, Respiratory chain; RNA, Ribonucleic acid; ROS, Reactive oxygen species; RT-PCR, Reverse transcription polymerase chain reaction; SI, Supporting information; TNF, Tumor necrosis factor; UCS, Units citrate synthase; WT, Wild type.

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1. Introduction

Leigh Syndrome (LS, OMIM 256000), a mitochondrial disease (MD) occurring in ~80% of children with complex I (CI) deficiency, is characterised by a muscular and neurological phenotype and is associated with increased oxidative stress and inflammation, decreased energy production, altered redox status, disturbance of the mitochondrial membrane potential and altered intracellular Ca^{2+} homeostasis [1-4]. With no known cure and a severe prognosis of death at ~3 years of age, LS treatment options are urgently sought after, yet remain lacking. Current treatment strategies focus on promoting sirtuin activity, stabilising cellular Ca^{2+} homeostasis and increased reactive oxygen species (ROS) scavenging, with potent antioxidants like Trolox (a vitamin E analogue) showing great promise for treatment of CI dysfunction [5,1,6,7].

Metallothioneins (MTs) and their protective effects against the damaging consequences of ROS have been widely reported in both mitochondrial and neurodegenerative diseases. MTs are low molecular weight intracellular proteins that have many functions, the most important of which is the detoxification of heavy metals, maintenance of zinc and copper homeostasis and transport of essential metals. Other functions include free radical scavenging, apoptosis, regulation of intracellular redox balance and anti-inflammatory processes [8,9]. They consist of four isoforms, two of which are tissue-specific, namely MT3 and MT4, which have been reported in the central nervous system (mainly the neurons) and squamous epithelial cells, respectively [10]. MT1 and MT2 are highly similar, ubiquitous isoforms that can be easily induced by many similar factors (including glucocorticoids, cytokines, ROS and metals) [11-14], are both expressed in all major organs, and display closely related functions. They have, however, been shown to differ in their metal binding capacities for Zn^{2+} , Cd^{2+} and Cu^{+} [15,16]. Contrary to MT1 and MT2, it appears that MT3 is not controlled by glucocorticoids or metals, and is thus not regarded to be inducible [17].

While MTs are typically viewed as cytosolic proteins, they have also been shown to localise within the intermembrane space of the mitochondria, the exact mechanism of which is not yet known (especially since MTs lack any mitochondrial targeting sequences) [18,19]. While various mechanisms have been proposed [20-22], a tissue-specific controlled mechanism appears to be present that is independent of the cytosolic MT concentration and that involves distant means by which MTs are able to protect mitochondria. One mechanism by which MTs can indirectly protect mitochondrial structures is via their interaction with glutathione [18]. Oxidised glutathione (GSSG), which is transported from the mitochondrial matrix to the intermembrane space, can be reduced by MTs, which may result in structural changes that allow the transfer of metal ions between glutathione and MTs [23-25]. Reduced glutathione (GSH) can then be transported back into the mitochondrial matrix where it acts as a ROS scavenger. This shift towards MT as the primary antioxidant enables GSH to be conserved and less GSSG to be formed, thus allowing glutathione to protect against ROS in the areas within the mitochondrion that MTs cannot reach [26-29]. MTs have also been shown to not only scavenge ROS but to also prevent the formation thereof by modifying a nuclear-encoded subunit of complex IV (CIV) during oxidative stress, which in turn, increases CIV ability to use molecular oxygen [30].

While Lindeque *et al.* [18] extensively reviewed the involvement of MTs in mitochondrial function and disease, others [12] have reviewed the important neuroprotective role of MT1 and 2 *in vivo* in brain disorders, such as focal and traumatic brain injury, epilepsy, ischemia and neurodegenerative diseases like Parkinson's disease and Alzheimer's disease [31-37]. Overexpression of MTs and exogenous treatment with MT1 and 2 in these mouse models have shown improved tissue repair and neurological outcomes with decreased inflammatory responses and reduced proinflammatory cytokines, decreased ROS, neuroregeneration and reduced apoptotic cell death [38]. The role of MTs as free radical scavengers, their mechanisms for preventing additional ROS formation [39] and their cooperation with the glutathione redox cycle [40,41], make these highly inducible small endogenous peptides greatly appealing as potential therapeutic effectors for patients with LS and other MD phenotypes. With limited, but promising *in vitro* data

in support of this potential [19,42], the lack of *in vivo* MD models have been limiting to effectively explore this potential [18].

To investigate the potential protective effect of MT1 on MD pathology *in vivo*, we cross-bred the *Ndufs4* whole-body knockout mouse developed by Kruse *et al.* [43] – the first and best characterised genetic model of CI deficiency, which presents very similarly to LS in humans – with the MT1 overexpressing transgenic mouse (*TgMt1*) developed by Palmiter *et al.* [44]. We aimed to determine whether or not overexpression of *Mt1* in the *Ndufs4*^{-/-} mice would increase the age of survival (typically only 2-3 months of age), as well as whether any improvements could be seen in hallmark phenotypic motor activity deficiencies of these mice, which typically present with weight loss, ataxia and loss of balance beginning at postnatal day (P) 35 [43]. These phenotype investigations were enhanced by metabolomics analyses using brain and skeletal muscle tissue, since metabolomics allows for a comprehensive evaluation of the metabolites and their underlying interactions, which are sensitive to small changes in the redox status of the cells and should theoretically relate closely to the phenotype of the animals [45]. Along with a selected set of biochemical and histochemical analyses in relevant tissues and cell lines, this study provides the first comprehensive *in vivo* data to better evaluate the potential of *Mt1* overexpression as a protective adaptive response in a primary MD phenotype.

2. Materials and methods

Comprehensive details regarding the materials and methods used can be found in the supporting information (SI methods).

2.1. Animals and sampling

Male and female whole body *Ndufs4* knockout mice (B6.129S4-*Ndufs4*^{tm1.1Rpa/J}) and *Mt1* overexpressing mice (B6.Cg-Tg(Mt1)174Bri/J), obtained from the Jackson Laboratory (ME, USA), were used together with age-matched controls. The confounding effect of gender was considered as part of all analyses performed, but no adjustments were necessary (data not shown). The breeding strategy is shown in Fig. S1, but briefly, heterozygous *Ndufs4* mice were crossbred with *TgMt1*^{+/+} mice (all backcrossed on a C57BL6/J genetic background) to obtain the four genotypes used in this study (as presented in Fig. 1), namely: WT (wild type mice); OVER (*Mt1* overexpressing mice); KO (*Ndufs4* knockout mice) and KO OVER (*Mt1* overexpressing, *Ndufs4* knockout mice). Genetic characterisation of the mouse model was performed, as further described in SI methods. This included confirmation of the *Ndufs4* and *TgMt1* genotypes by PCR [46] and real-time PCR analyses, respectively, at both the beginning (using tail snips) and end (using liver tissue) of the study. Furthermore, mRNA levels of *Mt1* and protein levels of NDUFS4 were determined in the brain and quadriceps.

All mice used were housed and bred at the Vivarium (SAVC reg. no. FR15/13458) of the Pre-Clinical Drug Development Platform (PCDDP) with the approval of the Animcare ethics committee (approval numbers NWU-00364-16-A5 and NWU-0001-15-A5) of the North-West University (NWU). The mice were housed in individually ventilated cages at constant temperature (22 ± 1°C), humidity (55 ± 10%) and a 12:12-h light/dark cycle. Food and water were provided *ad libitum*. Mice were euthanised at P46-51 by cervical dislocation, after which whole brain and quadricep (from both limbs) tissue were immediately snap frozen in liquid nitrogen (unless otherwise stated) and stored at -80°C until further use. Prior to euthanasia, whole blood samples were obtained from the ventral artery of the animals and serum was prepared by centrifugation of the blood samples at 2000 x g for 10 min, after which the supernatant was stored at -20°C.

2.2. Respiratory chain enzyme analyses

To determine the respiratory chain (RC) enzyme activities of complex I (CI), complex II (CII), complex III (CIII) and complex IV (CIV), 600 x g supernatants of the whole brain and quadriceps (n = 6/genotype) were used. The methods utilised were as described previously [47] and in SI methods. For normalisation, the

protein concentration was determined using the Bicinchoninic Acid kit (Sigma Aldrich, #BCA1), and citrate synthase activity was determined as described elsewhere [48,49] and in SI methods.

2.3. Primary culture of bone marrow-derived macrophages

Bone marrow cells were isolated from the femurs and tibiae of mice from the four genotypes by centrifugation, as previously described [50]. Mouse bone marrow cells were cultured and differentiated into bone marrow-derived macrophages (BMDMs) using 20 ng/ml MCSF, as described elsewhere [51-53] and in SI methods.

2.4. Growth and survival analysis

For construction of the growth curves, all mice included in this study (n = 68) were weighed three times a week and the masses recorded. For survival analysis, ten KO and ten KO OVER mice were allowed to age and die naturally. While the mice included in the survival analysis were also weighed, they did not undergo any of the other phenotyping assays, and pellets were placed on the floor of the cage from P50 onwards to ensure that KO and KO OVER mice had free access to food and water.

2.5. Phenotype investigation

The chronological order in which all phenotyping tests were carried out is shown in Fig. S2. Prior to training or testing for each phenotyping analysis, mice were left undisturbed in their housing cage in the room of the analysis for at least 15 min to habituate them to the environment. For all phenotyping analyses, 12 mice per genotype (n = 48) were used.

2.5.1. Open field test

Locomotor activity was evaluated using an open field test. Horizontal and vertical locomotor activity was determined using a Digiscan Animal Activity Monitor (DAAM, AccuScan Instruments), as described in SI methods.

2.5.2. Wire grid hang test

The wire grid hang test was conducted, using the cage lid, as described before [54] and detailed in SI methods. The length of time that the animal was able to hold onto the grid was recorded (up to a maximum of 180 s) and the mean physical impulse was calculated.

2.5.3. Balance beam

The 12 mm wide balance beam was constructed according to specifications described elsewhere [55] and in SI methods. The amount of time that it took each mouse to cross the centre 0.8 m of the balance beam was measured using two infrared beams (one to start and one to stop the timer when the beam was broken).

2.5.4. Rotarod

The rotarod was constructed and utilised as described before [56] and in SI methods. The rod was set to accelerate from 5 rpm to 40 rpm over a period of 300 s and the latency to fall time was recorded.

2.6. Immunohistochemistry of whole brains

Immediately following euthanasia, whole brains (n = 5/genotype) were fixed using 4% (w/v) paraformaldehyde and 30% (w/v) sucrose (in 1x PBS) as described elsewhere [57] and in SI methods. The primary antibodies (1:500 anti-MT1, #M0639 from Dako; 1:5000 anti-GFAP, #PA1-10019 from Thermo Fisher Scientific; 1:1000 anti-Iba1, #01919741 from Wako; and 1:2000 anti-GFAP, #Ab4674 from Abcam) and Alexa Fluor 555 or 488 secondary antibodies (1:500, #A31752 and #A21202 from Thermo Fisher Scientific; #Ab150173 from Abcam) used were prepared in 1% (w/v) Normal Donkey Serum – PBS 0.2% (v/v) Triton-X.

2.7. Oxidative stress, inflammation and redox metabolism investigations

2.7.1. RNA analyses of selected one-carbon (1C) metabolism and oxidative stress genes

Selected genes, with expression known to be responsive to variation in oxidative stress and 1C metabolism expression, were selected in this part of the investigation. RNA was isolated from whole brain and quadriceps (n = 6/genotype), using TRIzol Reagent and purified using the MEGAclear™ Transcription Clean-Up Kit (#AM1908, Thermo Fisher Scientific), according to manufacturer instructions, as described in SI methods. For each assay, the TaqMan® RNA-to-C_T™ 1-Step Kit (Applied Biosystems, #4392938) was used with TaqMan Gene Expression Assays (Applied Biosystems, #4332078) in triplicate, according to manufacturer instructions. The genes (and assay numbers) investigated were: *Mthfd2* (Mm00485276_m1), *Gclc* (Mm00802655_m1), *Mtrr* (Mm00549977_m1), *Bhmt* (Mm04210521_g1), *Tyms* (Mm01702970_m1), *Sod2* (Mm01313000_m1), *Cat* (Mm00437992_m1), *Gpx1* (Mm00656767_g1), *Lias* (Mm00522477_m1), *Phgdh* (Mm01623589_g1), *Actβ* (Mm01205647_g1) and *β2m* (Mm00437762_m1).

2.7.2. Protein oxidation in tissue

The OxyBlot™ Protein Oxidation Kit (Merck, #S7150) was used, according to manufacturer instructions and as described in SI methods, on 600 x g protein lysates from the brain and quadriceps of the mice (n = 1/genotype).

2.7.3. ROS determination BMDMs

The basal intracellular generation of ROS was fluorometrically measured in BMDMs, based on the methods employed by previous studies [58-60] and as described in SI methods, by staining cells with the molecular probe H₂DCF-DA (Molecular Probes, #D399). Furthermore, oxidative stress in the cells was chemically induced by treatment with H₂O₂ (Sigma-Aldrich, #H-1009), to serve as the positive controls, and to determine the antioxidant capacity of the cells.

2.7.4. Inflammatory status in serum

The BD Cytometric bead array mouse inflammation kit (BD Biosciences, #552364) and Accuri C6 flow cytometer (BD Biosciences) were used, according to manufacturer instructions and as described in SI methods, to determine the levels of selected pro-inflammatory mediators, namely, interleukin 6 (IL6), monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor (TNF) and interleukin 12 (IL12).

2.7.5. Metabolomics

Metabolic profiles were obtained from the quadriceps (n = 13-14/genotype) and whole brain (n = 6-9/genotype) samples of mice from the four genotypes, using a multi-platform metabolomics approach, as previously described [61] and in SI methods. Due to the amount of tissue required for all the biochemical assays performed, especially the immunohistochemistry, which made use of whole brain samples, fewer brain samples were available for metabolic profiling than quadricep samples, and in some specific samples, not enough tissue remained for the mass required by the metabolic profiling methods. Platforms included proton nuclear magnetic resonance (NMR) spectroscopy [62], untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) [63] and targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) [64]. Brain samples were not analysed using the LC-MS/MS due to their high apolar metabolite content. As already described [61], a modified monophasic Bligh-Dyer extraction method [65] was used for ~70 mg brain and ~80 mg quadriceps.

2.8. Statistical analysis

GraphPad Prism version 5 and Excel 365 were used to construct all graphs. The survival analysis was performed using the Log Rank (Mantel-Cox) test in GraphPad Prism version 5. IBM SPSS Statistics 25 was used to identify outliers (via box plots of the unlogged data) in the phenotyping and RNA data, as well as to perform one-way ANOVAs with Games-Howell post hoc tests on the phenotyping and enzyme data. The C_T values from the RNA data were used to determine efficiency-corrected fold change as described

elsewhere [66]. For the ROS and inflammation assays, independent sample *t*-tests and the Mann-Whitney *U* test were used. A p-value of less than 0.05 was used to indicate statistical significance.

For the metabolic data, Excel 365 and Metaboanalyst 4.0 [67] were used for all data processing, and discriminatory metabolites were identified in the brain and quadriceps using the independent sample *t*-tests (with p-value after BH-FDR correction <0.05 and Cohen's d-value effect size >0.8).

3. Results and discussion

3.1. Characterisation of the model

The *Ndufs4* genotype was characterised by determining the knockout of *Ndufs4* in DNA samples that were obtained using tail snips and liver samples from all mice included in the study, as described in Section A.2.1 (results not shown). Furthermore, the absence of NDUFS4 in the KO and KO OVER mice, compared to WT and OVER mice, was confirmed by SDS-PAGE and western blot, as discussed in Section A.2.4 and shown in Fig. S3. To confirm the *TgMt1* genotype, the mRNA levels of *Mt1* relative to β -2-microglobulin were determined in the brain and quadriceps of six mice per genotype, as shown in Fig. 2 and described in Section A.2.5. As desired, the OVER and KO OVER mice showed significantly increased *Mt1* expression at the mRNA level, thus confirming that which was seen at the DNA level (DNA data not shown). Characterisation of the *TgMt1* mouse model by Iszard *et al.* [68] established that the MT1 protein concentration is also increased in all major organs of the *Mt1* overexpressing mice, with brain MT1 levels at least 1.4 times that of controls. Similar to Iszard *et al.* [68], we did not detect any differences in the mRNA expression levels in the brain of male and female mice (data not shown). This was contrary to Ono *et al.* [69], who found that whole brain samples of *TgMt1* female mice had significantly increased MT levels, compared to male mice. Furthermore, Ono *et al.* [69] not only reported significantly increased MT in whole brains from *TgMt1* mice, but also brain region-specific differences with the greatest increase in the cerebellum (3.5-fold) and the smallest increase in the cortex (two-fold).

Contrary to the multiple reports of increased expression of MTs in MDs as a response to increased ROS, which was reviewed by Lindeque *et al.* [18] and reported by others [19,42], the KO mice did not show an increase in mRNA *Mt1* expression compared to WT mice. Rather, a decrease in the *Mt1* expression levels could be observed in the KO compared to WT mice (which was statistically significant in the brain but not in the quadriceps) and KO OVER compared to OVER mice (which was statistically significant in the quadriceps but not in the brain). These results thus suggest *TgMt1*-induced *Mt1* overexpression in the two tissues investigated, with an apparent downregulated *Mt1* expression in the *Ndufs4*^{-/-} mice.

The respiratory chain (RC) enzyme activity was determined in the brain and quadriceps, as described in Section A.3 and shown in Fig. 3, to confirm that the *Ndufs4* knockout resulted in the expected decrease in CI enzyme activity as well as to determine the effect of the model on the other RC enzyme activities. All RC data was normalised according to protein and units citrate synthase (UCS), and was thereafter log transformed to ensure homogeneity of variance. To evaluate the amount of residual CI activity in the KO and KO OVER mice, Fig. 4 was constructed where all CI activity is indicated as a percentage of the WT.

Despite the absence of NDUFS4, residual CI activity is still present in *Ndufs4*^{-/-} mice due to the formation of respiratory supercomplexes with CIII, which allow the formation of active CI [70]. As expected, CI enzyme activity was significantly decreased in the KO and KO OVER mice, with a decrease of ~91% in the brain and ~78% in the quadriceps. This decrease was comparable to that reported by others using the *Ndufs4*^{-/-} mice [71,70,72,61], similar methods [47] and similar sample sizes. The increase in CII activity in the quadriceps of KO compared to WT and OVER mice was the only other significant difference observed in the RC enzyme activities. This increase correlated with the increased fumarate observed in the metabolomics data of our KO mice (see Table S6 and Fig. 12), as well as that reported by Alam *et al.* [71], presumably due to the use of CII as an alternative mechanism to fuel the RC during CI dysfunction.

3.2. *Mt1* overexpression does not improve the weight or survival of *Ndufs4*^{-/-} mice

Due to the failure to thrive phenotype reported in *Ndufs4*^{-/-} mice [43], the weights of all mice included in the phenotyping and survival curve analyses were recorded and are shown in Fig. 5. The error bars depicted on the growth curves are at 95% confidence intervals so that ages where the error bars did not overlap, could be considered as statistically significant differences. From P38 onwards, the WT and OVER mice weighed significantly more on average than the KO and KO OVER mice, but no distinction could be made between the KO and KO OVER mice or between the WT and OVER mice. The weights obtained for the OVER and KO mice corresponded with that observed by others [73,74,43,75]. It thus appears that the overexpression of *Mt1* did not have a significant effect on the body weight of *Ndufs4*^{-/-} mice.

A Log Rank (Mantel-Cox) test was performed to determine differences in the survival rate of KO and KO OVER mice; the survival curve is displayed in Fig. 6. The survival distributions were, however, not statistically significant, $\chi^2(1) = 1.153$, $p = 0.283$. Survival analyses thus indicate that the overexpression of *Mt1* does not lead to a statistically significant increase in longevity in the *Ndufs4*^{-/-} mice. While MTs have increasingly been shown to provide a survival advantage in neurological diseases and injury [76], this protective effect does not appear to be evident in the case of MT1 overexpression in *Ndufs4* deficiency. The median number of days that these KO and KO OVER mice (74.0 and 76.5 days, respectively) survived was slightly higher than the range reported in other studies for *Ndufs4*^{-/-} mice (~P50 to P75) [74,72,77,78]. The genetic background against which the mice were backcrossed may have played a role in the differences seen in the survival age and phenotyping results that we obtained compared to other studies. Different genetic backgrounds bring about different phenotypic responses and may thus result in the disease phenotype presenting earlier or more severely than other strains [79].

3.3. *Mt1* overexpression does not improve ataxia or locomotor activity in *Ndufs4*^{-/-} mice

Phenotyping analyses were performed on mice from the four genotypes at both P30 and P50, due to the known poor motor activity and ataxia reported in *Ndufs4*^{-/-} mice. These analyses included open field testing to examine locomotor activity (Fig. 7a), the wire grid hang test to test muscle strength (Fig. 7b), a balance beam test (Fig. 7c) and the rotarod to evaluate motor coordination (Fig. 7d). At P30 (Fig. S4), significantly worse performance was only observed for the balance beam and rotarod in the KO OVER mice, while at P50, the KO and KO OVER mice performed significantly worse than the WT and OVER mice in all phenotyping tests utilised. However, no significant differences were observed between the KO and KO OVER mice (or between the WT and OVER mice), thus indicating that *Mt1* overexpression did not rescue the balance, motor coordination, locomotor activity or muscle strength in *Ndufs4*^{-/-} mice.

Kruse *et al.* [43] reported similar open field test, balance beam and rotarod results as well as worsening of the ataxia in the *Ndufs4*^{-/-} mice, confirming that the phenotype of our KO mouse model correlated with that reported. While open field testing typically takes place over much longer periods of time (such as 24-72 hours) when observing animal behaviour, the severe muscle phenotype observed in the *Ndufs4*^{-/-} mice allowed for a much shorter observation period (5 min) to obtain statistically significant differences between the WT/OVER and KO/KO OVER mice (Fig. 7a). Absolute values of mean physical impulse could not be found in other studies on KO or OVER mice, but the relative differences between the WT and KO mice in other grip and strength tests [73,74] were similar to those observed for our wire grid hang test (Fig. 7b), where the KO mice showed poor muscle strength and the inability to hold on for as long as WT mice. The relative differences for the balance beam test, where KO mice took significantly longer to cross the balance beam than the WT mice (Fig. 7c), was comparable to that reported in similar studies [74,57], but the variation in our KO and KO OVER mice was high, which was attributed to inherent spontaneous animal behaviour [80] and differences in the disease severity of mice of the same age. Rotarod results of our WT versus KO mice (Fig. 7d) were similar to those reported in Jain *et al.* [72] and Johnson *et al.* [77] at P40 and P50, where KO mice fell off the rotarod much earlier than WT mice. No differences were observed

between WT and OVER mice, corresponding with the results reported by Manso *et al.* [75]. Absolute values, however, differed slightly (while relative comparisons remained similar) from those rotarod results reported by others [73,74,43,75]. In these other studies, KO mice at P40-P50 were mostly unable to remain on an immobile rod long enough for the rotarod experiment to be conducted and the latency to fall lay closer to zero than to the average of approximately 50 s seen here. Whilst the diameter of the rod used in all these studies was the same as that used here, the width of the rod differed (depending on the rotarod supplier) and the varying widths may thus have played a role in the differences observed in the latency to fall times.

3.4. Differences in the expression of MT1 and neuroinflammatory markers were observed between the vestibular nucleus and hippocampus of *Mt1* overexpressing *Ndufs4*^{-/-} mice

Immunohistochemistry involving glial fibrillary acidic protein (GFAP) and ionised calcium binding adapter molecule 1 (IBA1), markers of astrocyte reactivity and microglial activation (which is associated with inflammation), respectively, was performed on fixed frozen brain samples from mice of the four genotypes, as vacuolation and activation of astrocytes and microglial cells have been demonstrated in the vestibular nucleus of *Ndufs4*^{-/-} mice, resulting in fatal breathing deficits [57,78]. Furthermore, there have also been multiple reports of the importance of MT expression in the hippocampus [31,75,81]. Initial sagittal sections of our samples also demonstrated glial cell reactivity in these two brain regions. We performed two different immunohistochemistries in the hippocampus and vestibular nucleus with MT1, GFAP and DAPI staining, as shown in Fig. 8a-d and Fig. 8i-l, respectively, and GFAP and IBA1 staining, as shown in Fig. 8e-h and Fig. 8m-p, respectively.

3.4.1. MT1, GFAP and DAPI staining

It is important to note that the MT antibody used here was unable to distinguish between the MT1 and MT2 isoforms, and that MT2 expression can thus not be ruled out in these images. In the hippocampus (Fig. 8a-d), the MT signal in WT mice was almost absent, whilst the OVER mice showed (*TgMt1* mediated) MT overexpression in astrocytes and neurons. Whilst MT2 expression cannot be entirely disregarded, there is a clear induction of MT in the OVER mice (that are known to only overexpress MT1 and not MT2) and MT2 expression was reported to be 50% that of MT1 in the brain of non-transgenic mice [82]. This supports the assumption that the conclusions drawn from these images relate mainly to the effects of MT1. The KO mice showed an overall increase in MT signal, compared to WT mice, thus suggesting induced (i.e. non-*TgMt1* mediated) MT expression, which is in line with *in vitro* data [19,42], but contrary to the decreased *Mt1* mRNA levels observed in the whole brain samples from the KO and KO OVER mice (Fig. 2a). The RT-PCR TaqMan assays used to determine the mRNA levels of *Mt1* were able to distinguish between the MT1 and MT2 isoforms, making it possible that elevated MT2 (and not MT1) levels may have resulted in the increased MT signal observed in Fig. 8c. However, since the *Mt1* mRNA levels were studied in whole brain as opposed to specific brain regions, this remains uncertain. Other *in vivo* studies [83,84,31,76] have also reported induction of MT1 in the brain as a result of injury or inflammation. KO OVER mice showed neuronal induction of MT1 together with increased astrocyte activation. In the vestibular nucleus (Fig. 8i-l), the MT signal appeared to be low with some scattered MT positive astrocytes, and a slightly greater MT signal in the OVER and KO OVER mice, compared to both the WT and KO mice. In the vestibular nucleus of WT mice, low levels of MT1+2 expression was also previously reported, with significantly increased MT1+2 expression in astrocytes, microglia, macrophages and vascular cells in *TgMt1* mice [85].

3.4.2. IBA1 and GFAP staining

In the hippocampus (Fig. 8e-h), there appears to be greater microglial activation (visible via IBA1 staining) in the KO mice than in any of the other three genotypes. The microglial activation thus appears to be decreased in KO OVER mice compared to the KO mice. In the vestibular nucleus (Fig. 8m-p), there appears to be a slightly increased GFAP signal in the OVER and KO OVER mice, compared to the WT and KO mice. In addition, the effect of *Ndufs4*^{-/-} seems to cause an overt morphological change (hypertrophy) in the

astrocytes of the KO and KO OVER mice. Similarly to the hippocampus, there appears to be a subtle decrease in IBA1 signal in the vestibular nucleus of KO OVER mice.

Quintana *et al.* [57] suggested that CI dysfunction triggers the release of cellular signals that activate glial cells and that this activation leads to oxidative stress, cytokine release by microglia, and activation of the extrinsic apoptotic pathway in neurons, which together with insufficient ATP, leads to necrotic cell death. In *Ndufs4*^{-/-} mice, they found the degree of microglial activation and lesions within the brainstem and cerebellum to correlate with ataxia, hypothermia and general morbidity. Whilst severe glial activation was observed in the vestibular nucleus of their late-stage *Ndufs4*^{-/-} animals, only mild gliosis was detected in the hippocampus, by GFAP and IBA1 staining. Similarly, microglial activation appeared to be increased in the vestibular nucleus of the KO mice (Fig. 8o) compared to the WT mice (Fig. 8m), and to a lesser extent in the hippocampus (Fig. 8e and g). In the motor cortex and olfactory bulbs of *Ndufs4*^{-/-} mice at P40, Felici *et al.* [74] observed a significant increase in GFAP positive cells, whilst at a later stage of disease progression (P55-60) [72,77], substantial neurodegeneration, glial reactivity and astrocyte activation at lesions in the cerebellum have been reported (as evidenced by an increased GFAP and IBA1 signal).

The subtle decrease in microglial activation in the vestibular nucleus and hippocampus of the KO OVER mice compared to KO mice may indicate a slight decrease in oxidative stress as a result of MT1 overexpression. Since whole brain was used for most of the other analyses, it is highly probable that subtle differences in specific brain regions would be masked and it would thus be necessary to dissect specific regions (such as the vestibular nucleus and hippocampus) to determine the cause of these subtle differences.

3.5. *Mt1* overexpression did not demonstrate remarkable improvements in oxidative stress, inflammation or redox status imbalances in *Ndufs4*^{-/-} mice

3.5.1. Protein oxidation in the tissue

To investigate one of the downstream damaging effects of oxidative stress within the cell, protein oxidation was determined in the brain and quadriceps tissue of mice from the four genotypes, as described in Section A.6. The relative volume of oxidised proteins was measured in one mouse per genotype and is shown in Fig. 9 and quantified in Fig. 10. Multiple oxidised protein bands were formed, but these were all quantified as one to give the total amount of oxidised protein. The results from Fig. 10 indicated that the KO and KO OVER mice had decreased oxidised proteins compared to WT mice. The apparent lack of increased oxidative stress may partially explain the lack of *Mt1* mRNA induction observed in the KO mice (Fig. 2). In the brain (Fig. 9a), the overexpression of *Mt1* seemed to slightly decrease the amount of oxidised proteins when comparing OVER mice with WT mice, and KO OVER mice with KO mice. In the quadriceps (Fig. 9b), however, the overexpression of *Mt1* appeared to not affect the oxidised protein levels since OVER and KO OVER mice showed similar oxidised protein levels when compared to WT mice. Contrary to Quintana *et al.* [57], who showed significantly increased oxidised proteins in the olfactory bulb of *Ndufs4*^{-/-} mice compared to WT mice, in both the brain and quadriceps of our KO mice, the number of oxidised proteins appeared to be decreased in the KO mice compared to WT mice. In the brain, the oxidised protein levels were similar between KO and KO OVER mice, while in the quadriceps, the oxidised protein levels were increased in the KO OVER mice compared to KO mice. The difference seen between Quintana *et al.* [57] and the results shown here could be attributed to the fact that they made use of the olfactory bulbs, as opposed to whole brain and muscle. Piroli *et al.* [86] also did not find an increase in oxidative stress markers in the brainstem of KO mice and proposed that this explained why antioxidant therapies in isolation were not sufficient to treat MDs and suggested that strategies to improve mitochondrial function were required.

3.5.2. Selective gene expression in one-carbon (1C) metabolism and oxidative stress metabolism

Gene expression responses to alterations in the 1C metabolism and oxidative stress metabolism have been shown to be altered in MDs and with MT expression [87,63,88,61]. To investigate this, the fold change in the gene expression of selected 1C metabolism genes (Fig. S5) and oxidative stress metabolism genes (Fig. S6) is shown in the SI results. For the KO and KO OVER mice compared to WT mice, there was downregulation of *Mthfd2*, *Bhmt*, and *Mtrr* in the brain. In the quadriceps, *Mthfd2*, *Bhmt*, *Tyms* and *Gpx1* downregulation was observed only for KO OVER mice. Thus, *Tyms* was the only gene where MT1 appeared to have a remedial effect in the brain, as it was downregulated in the KO mice and restored to normal levels in the KO OVER mice. Its protein product, TYMS, catalyses the *de novo* production of purine nucleotides, which may indicate a correlation with the disturbances in dNTP pools that have been reported in mitochondrial DNA replication defects [88]. This data thus indicated a tendency towards downregulation of 1C metabolism genes in *Ndufs4* deficiency and no effect on selected genes of the oxidative stress metabolism in mouse brain and quadriceps tissues. Furthermore, apart from one gene in the selection, the overexpression of *Mt1* did not have a mitigating effect on the expression of these genes in the absence of the NDUFS4 subunit.

3.5.3. ROS levels in BMDMs

Since mitochondrial function and cellular redox state have been shown to regulate the activity of macrophages [89], intracellular ROS was detected in both untreated and H₂O₂-treated mouse bone marrow-derived macrophages (BMDMs), using the molecular probe H2DCF-DA (as shown in Fig. 11). No significant differences were detected in the relative ROS levels of untreated BMDMs (Fig. 11a) from the different mice and, therefore, the genotype did not affect the basal oxidative status of these cells. In primary skin fibroblasts obtained from these mice, similar relative ROS levels were measured (results not shown). The fact that KO BMDMs did not present with oxidative stress was indeed unexpected, since this is one of the main consequences associated with a CI defect. In addition, this finding did not correspond to that of previous studies, where oxidative stress was indeed detected in KO mouse BMDMs [89], as well as other KO mouse cell lines, i.e. primary muscle and skin fibroblasts [90]. In the H₂O₂-treated BMDMs (Fig. 11b), there was a significant increase in the relative ROS levels in all four genotypes, compared to the untreated BMDMs in Fig. 11a. OVER mice presented with the lowest H₂O₂-induced ROS levels, suggesting that MT1 overexpression provided an increased ROS scavenging capacity in BMDMs. However, no inhibition of H₂O₂-induced ROS formation was observed in the KO OVER, compared to KO mice, thus indicating that *Mt1* overexpression did not provide additional antioxidant capacity to protect KO BMDMs against the excess ROS production. This corresponds with the lack of improvement in the phenotyping results from the KO OVER mice.

3.5.4. Inflammation in serum and BMDMs

Increased inflammation is one of the symptoms associated with MDs such as LS. For this reason, pro-inflammatory biomarkers were measured in the serum of mice from the four genotypes (Fig. S7), and it was found that KO and KO OVER mice presented with increased inflammation, compared to WT mice, as evidenced by significantly elevated TNF and IL12 concentrations in both the genotypes, as well as elevated IL6 in the KO mice. This increase in pro-inflammatory biomarkers was, however, not as profound as that found by Jin *et al.* [89]. Furthermore, the relative expression of inflammatory biomarker genes was not significantly altered on mRNA level in BMDMs from KO and KO OVER mice, compared to WT mice (results not shown), suggesting only minimal inflammation. Moreover, no significant difference was seen between KO and KO OVER mice either. *Mt1* overexpression, therefore, does not seem to provide effective protection against ROS-mediated inflammation associated with *Ndufs4* deficiency. This is corroborated by the fact that *Mt1* overexpression also did not protect KO BMDMs from excess ROS production (as discussed in Section 3.5.3).

In the serum, OVER mice presented with some degree of systemic inflammation, compared to WT mice, as evidenced by significantly increased IL6 and IL12 concentrations. This was unexpected, since BMDMs from OVER mice had shown increased antioxidant capacity (Fig. 11b) and an inflammatory biomarker gene expression profile (RT-PCR results not shown) similar to WT controls. We speculate that *Mt1* overexpression may, therefore, induce pro-inflammatory cytokine secretion through other mechanisms that may involve other immune cells [91].

3.5.5. Metabolomics

By using our multi-platform metabolomics approach, we relatively quantified 187 metabolites in the brain and 109 metabolites in the quadriceps (following data pre-processing). Discriminatory metabolites were identified in the brain and quadriceps (Tables S5 and S6); and Venn diagrams (Fig. 12a and Fig. 12b) displaying metabolites that differ consistently across the different genotype comparisons, as well as the up- or down-regulation of these metabolite levels compared to WT or OVER mice, were constructed.

For OVER compared to WT mice, only two significant metabolites, propionylcarnitine and trimethylglycine, were found to be decreased in the quadriceps. Trimethylglycine, which is concurrently converted to dimethylglycine (with homocysteine conversion to methionine), and propionylcarnitine (a downstream product of homocysteine) may be linked to the homocysteinylation of MT. This has been shown to target intracellular MT and disrupt zinc, ROS and redox homeostasis, as well as impair the zinc-binding capacity of MT [92]. However, since only two metabolites were identified, further research is warranted to substantiate this hypothesis.

No discriminatory metabolites were identified in the KO versus KO OVER mice in either the brain or the quadriceps. The Venn diagrams in Fig. 12 show that many metabolic perturbations were found in the different groups, but these could all essentially be attributed to the CI deficiency, which appeared to have a dominating effect on the metabolome and thus masked any possible MT1 effects. One of the main metabolic consequences of CI dysfunction is the prevention of electrons being pumped into the respiratory chain by oxidation of NADH to NAD⁺, thus leading to a disturbance of the NADH/NAD⁺ ratio with a build-up of NADH. This redox state alteration is, in fact, a key hallmark of many of the metabolic perturbations associated with MD [93]. Due to the altered redox state, the cellular metabolism is affected in a very broad manner rather than just in a single metabolic pathway. Some of the CI linked perturbed metabolic pathways detected, included altered Krebs cycle intermediates and central carbon metabolism, disturbed 1C metabolism, purine and pyrimidine metabolism, branched-chain amino acid metabolism, lysine metabolism and altered urea cycle intermediates. However, as already stated, these altered metabolites can be attributed to the *Ndufs4*^{-/-} genotype and the metabolic investigation thus supports the phenotypic and biochemical findings that *Mt1* overexpression does not protect against *Ndufs4* knockout pathology.

4. Conclusions

For the first time, this study has shown that *Mt1* overexpression does not protect against the devastating phenotypic and cellular consequences of CI dysfunction in an *Ndufs4*^{-/-} mouse model, in using a multifaceted approach and well powered sample sizes compared to other studies [94,72,77]. Other than mild reductions in inflammation in the vestibular nucleus and hippocampus, no significant differences were found in the phenotype tests, RNA gene expression assays, oxidative stress or metabolomics analyses in the brain and quadriceps of the KO versus KO OVER mice. While the expected worsened phenotype and altered redox reactions were seen in the KO and KO OVER, compared to WT and OVER mice as a result of the CI deficiency, a protective effect from MT1 in the OVER or KO OVER groups was glaringly absent, contrary to the multiple reports of their protective role in mitochondrial dysfunction and neurodegenerative diseases.

As observed in the immunohistochemistry results, the lack of effect of MT on the vestibular nucleus is in line with the absence of improvement in the survival rate, locomotor activity or ataxia assay results of the KO OVER mice. Quintana *et al.* [78] found that by restoring *Ndufs4* in the vestibular nucleus of KO mice, breathing deficits were partially corrected and there was a moderate lifespan extension in the KO mice, thus indicating that the vestibular nucleus plays a critical role in the lethality of the disease. Greater levels of MT1 in glial cells compared to neuronal cells were previously reported [95], where MT1 was also shown to be induced in the hippocampus by dexamethasone. While no improvement was observed with MT1 overexpression in our LS model, others [96] have shown decreased inflammation in the hippocampus, delayed neuronal degeneration and cell death in OVER mice that were administered kainic acid, which induces epileptic seizures and hippocampal excitotoxicity, followed by inflammation and delayed brain damage. When crossing an Alzheimer's disease mouse model (Tg2576 mice) with *TgMt1* mice [75], increased MT1 expression was observed in the hippocampus (but not in the cortex), accompanied by an improvement in the rate of spatial learning as well as learning prompted by the eye-blink conditioning procedure. These results may thus suggest that the efficacy of MT1 overexpression depends on how severely the hippocampus is affected by a specific neurodegenerative disease, and that we may have seen improvements in the OVER and KO OVER mice had we performed spatial learning and memory tests, as opposed to motor activity assays, the latter of which are more related to the functioning of the vestibular nucleus, cerebellum and motor cortex. The measurement of MT in this study (~6-7 kDa), using protein gels, was a challenge due to its low detection, sensitivity and selectivity, and currently, no simple and accurate methods for the quantification of MT exist [97-99]. The type of metal that is bound by MT also adds to the complexity of MT quantification, since different metals require varying conditions during MT isolation [100].

The lack of oxidative stress and low-grade inflammation, together with the lack of redox imbalance in the KO and KO OVER mice leads one to question whether or not *Ndufs4*^{-/-} does indeed result in systemic oxidative stress, as shown by others, or whether the oxidative stress is more localised to specific tissues, as this model seems to suggest. A Trolox derivative, KH176, developed by de Haas *et al.* [5], was used to treat *Ndufs4*^{-/-} mice due to its potency in ROS and redox assays. Similarly, treatment with this KH176 antioxidant also did not improve the disease severity nor increase the lifespan of *Ndufs4*^{-/-} mice, which they attributed to the unrestored vestibular nuclei lesions that they observed. However, contrary to that shown here, they did demonstrate improved rotarod and gait performance, which they hypothesised was due to the remedial effect of KH176 on ROS and microstructural coherence in the brain. Likewise, treating *Ndufs4*^{-/-} mice with a poly[adenine diphosphate (ADP)-ribose] polymerase (PARP)-1 inhibitor, PJ34, which increases NAD⁺ availability, Felici *et al.* [74] reported improvements in motor skills and coordination, but also saw no improvement in survival or body weight. In contrast, rapamycin, an inhibitor of mTOR, was shown to increase the lifespan of *Ndufs4* mice (up to 114 days), in addition to demonstrating improved motor coordination, without evidence of any improvement in oxidative stress markers nor rescue of RC function [77]. Lee *et al.* [101] demonstrated decreased NAD⁺ levels in the brain and muscle of *Ndufs4*^{-/-} mice, which were restored in the muscle only upon treatment with nicotinamide mononucleotide, an NAD⁺ precursor, and extended the lifespan (median of 110 days) of the mice despite the lack of metabolic benefits in the brain. Furthermore, treatment with dimethyl-ketoglutarate, a KG precursor that also inhibits mTOR, resulted in a similar extension of lifespan in addition to an improved neurological phenotype. Lee *et al.* [101] suggested that the greater energy deprivation in the brain, as opposed to the muscle, explained the organ-specific effectivity of increased intracellular NAD⁺ levels. This leads one to question what proportion of the *Ndufs4*^{-/-} disease phenotype, and its biological consequences, are truly dependent on oxidative stress, especially in relation to the major devastating cellular consequences that occur during CI dysfunction. The apparent absence thereof in this *Ndufs4*^{-/-} model could explain the inability of *Mt1* overexpression to improve motor activity or survival. Indeed, this study could have benefitted from more extensive and tissue-specific ROS analyses.

It has been shown by others [102] that excitatory glutamatergic neurons in *Ndufs4*^{-/-} mice are a major contributor to the volatile anaesthetic hypersensitivity observed in these mice, whilst inhibitory GABAergic neurons and cholinergic neurons were not found to play a role. This was further evidenced by Bolea *et al.* [103], who showed that selective inactivation of *Ndufs4* in glutamatergic neurons resulted in motor and respiratory activity impairment, lessened neuronal firing, inflammation in the brainstem and premature death. This inactivation in the GABAergic neurons also resulted in premature death but did not, however, lead to respiratory or motor defects. In contrast, the GABAergic neurons displayed signs of basal ganglia inflammation, hypothermia and severe epileptic seizures. Selective inactivation of *Ndufs4* in the cholinergic neurons had no impact on motor activity, survival or growth. This suggests that, in addition to the brain region specific differences that are present in these mice [104], different cellular populations also play a role, and that when studying whole brain or combined cellular populations, important differences may be masked or compensated for by other cell types.

When taking all of this together, it thus appears that ameliorating the pathological changes observed in the vestibular nucleus of *Ndufs4* mice is one of the key features in an effective treatment strategy for CI dysfunction. The cell type-specific contribution of glutamatergic and GABAergic neurons to the cellular mechanisms underlying the pathology of *Ndufs4* deficiency [103] also provide an exciting new insight into CI deficiency and a promising target for new treatment options. Whether or not the *Ndufs4*^{-/-} model exhibits the extraordinary ROS levels (especially at a tissue-specific level) that would be required to see an improvement in primary MD pathology, due to the ROS scavenging abilities of MT1, remains unclear. Despite the lack of phenotypic or biochemical improvement observed in this study, however, the tissue- and cell-specific neuroprotective role of MT1 and the potential of other MT isoforms, such as MT2 and MT3, can thus not yet be ruled out in CI deficiency.

FIGURES

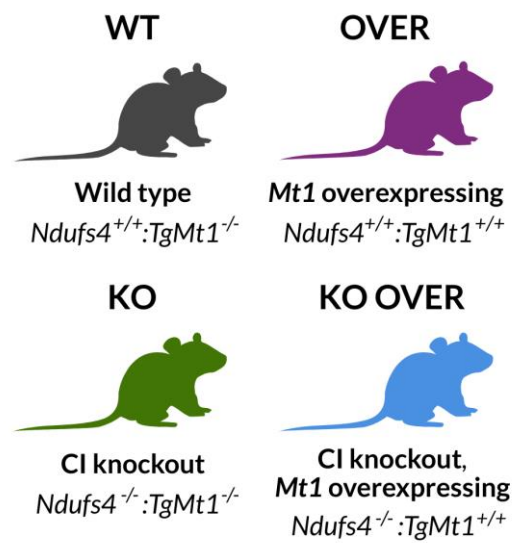


Fig. 1 The four genotypes investigated in this study, WT, OVER, KO and KO OVER. The colours depicted here correspond to the colours used in the graphs below for each respective genotype

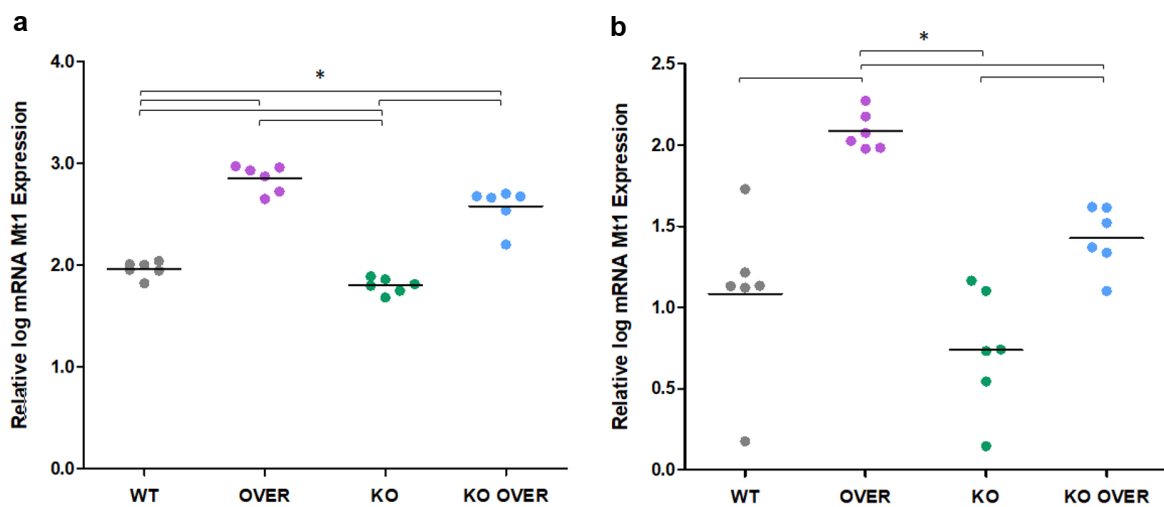


Fig. 2 mRNA *Mt1* expression relative to β -2-microglobulin in the (a) brain and (b) quadriceps ($n = 6$ /genotype). Horizontal bar indicates the mean and brackets indicate statistically significant differences, where $p < 0.05$ (*)

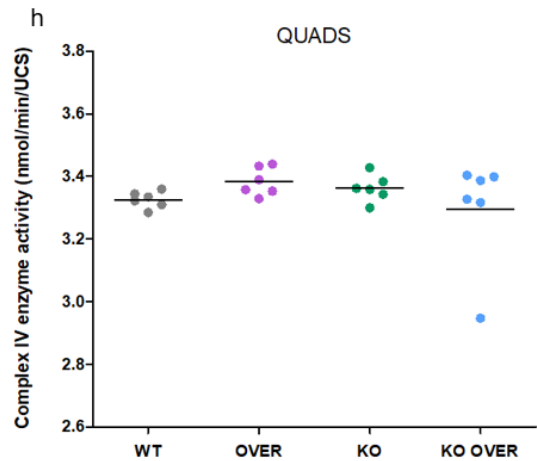
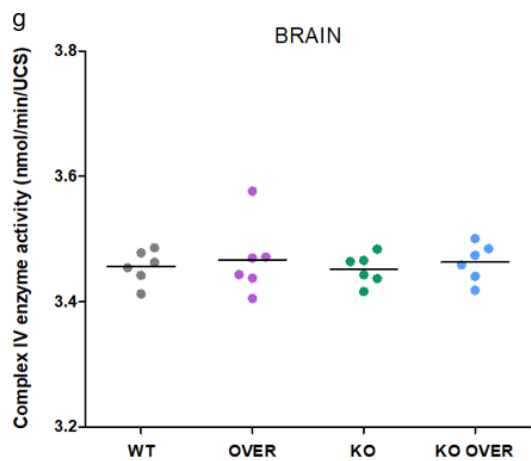
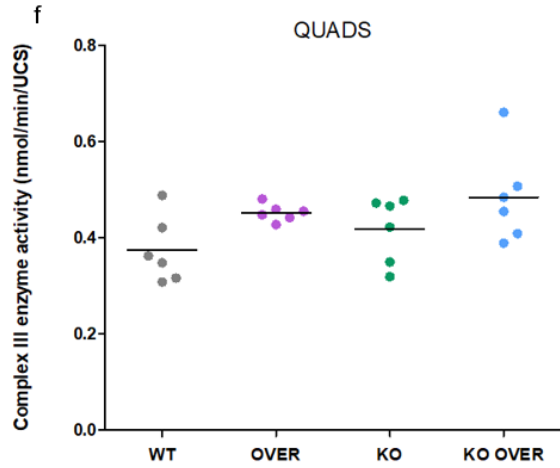
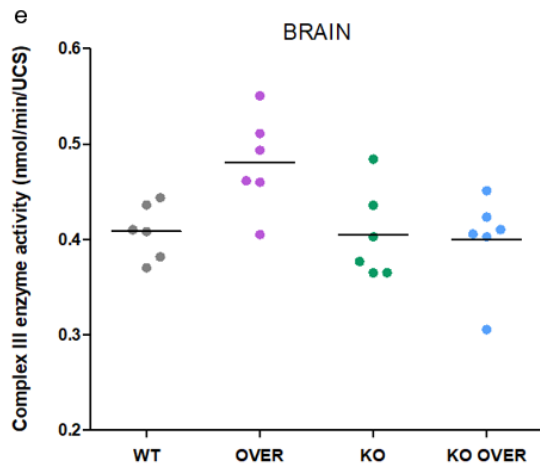
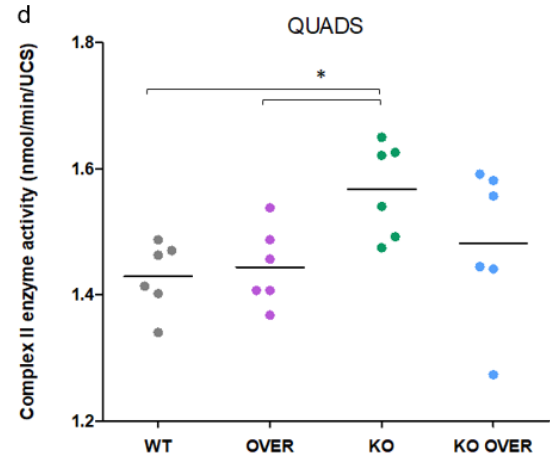
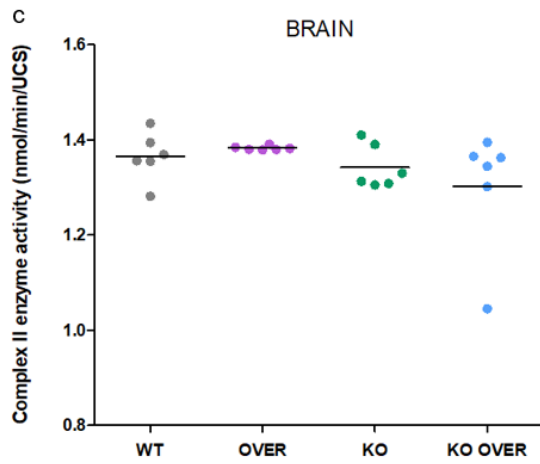
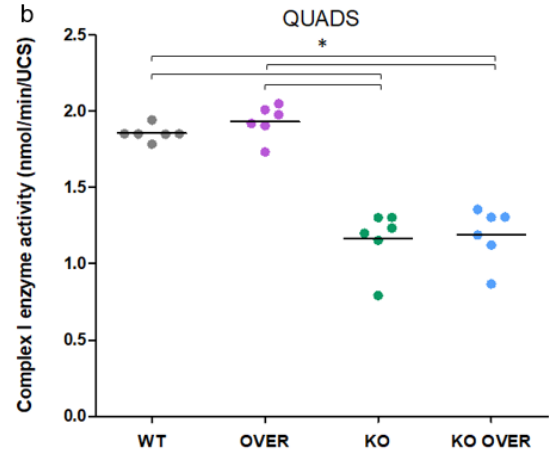
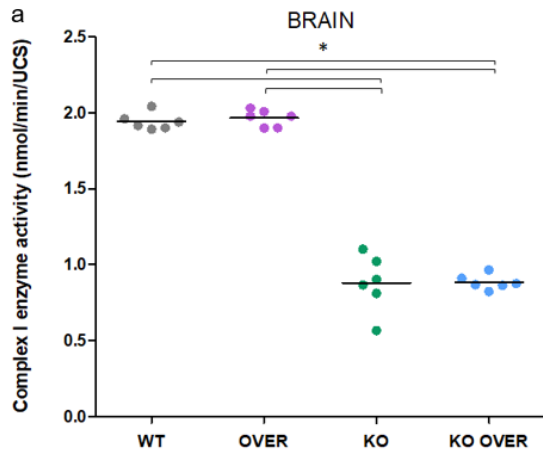


Fig. 3 Vertical scatter plots depicting the log transformed (a-b) CI, (c-d) CII, (e-f) CIII and (g-h) CIV enzyme activity in the (a, c, e, g) brain and (b, d, f, h) quadriceps (n = 6/genotype) from mice of the four genotypes. Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*)

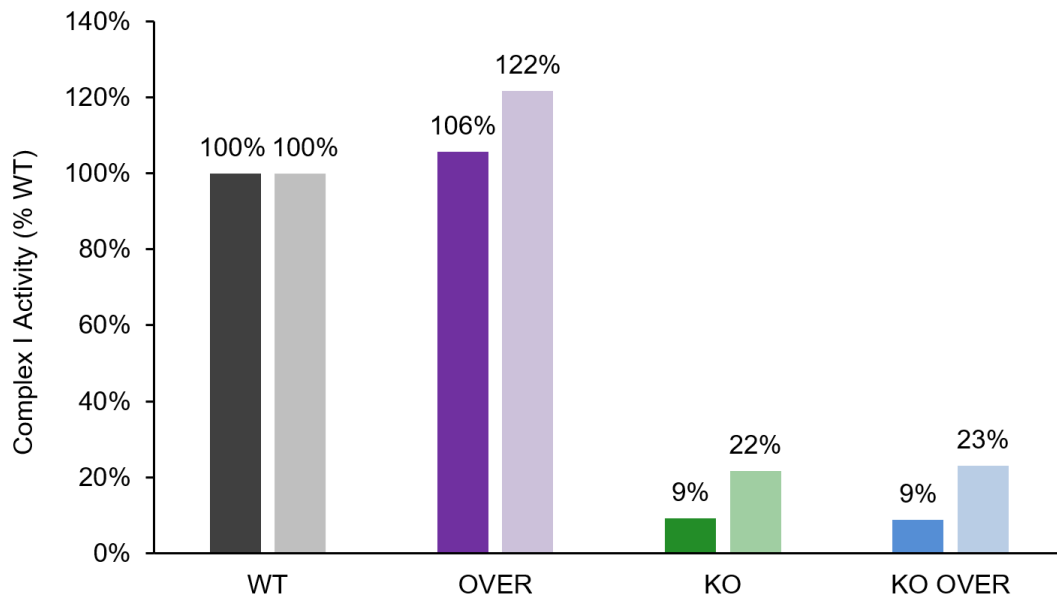


Fig. 4 Bar graph depicting the average untransformed CI enzyme activity, as a % of the WT, in the brain (darker colour) and quadriceps (lighter colour) (n = 6/genotype)

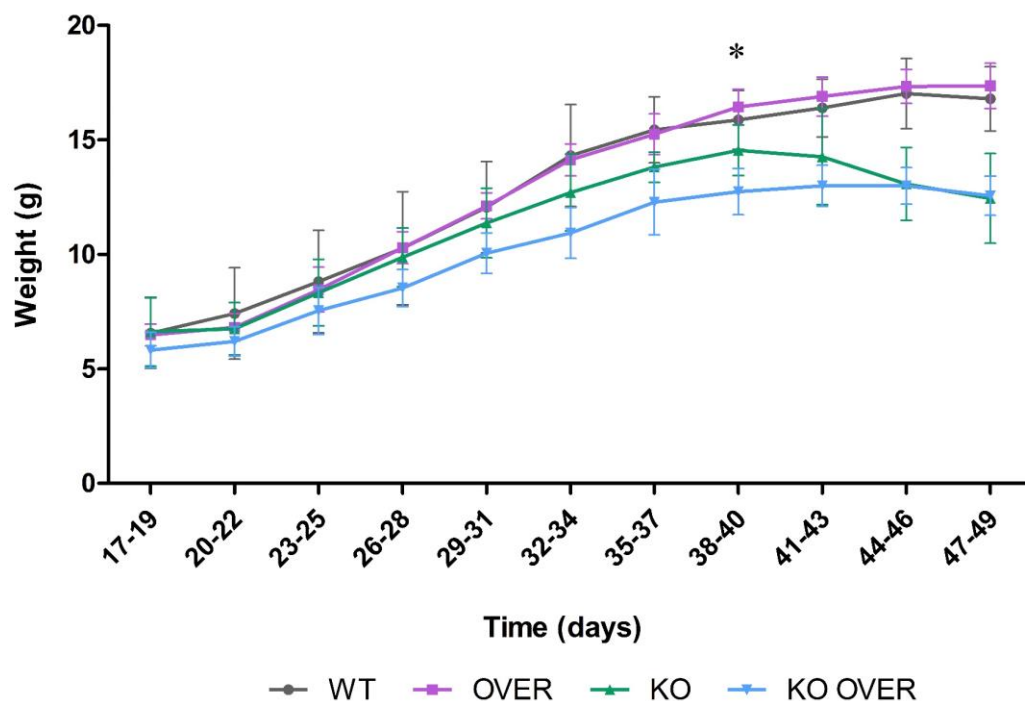


Fig. 5 Growth curves of WT (n = 12), OVER (n = 12), KO (n = 22) and KO OVER (n = 22) mice, included in phenotyping and survival analyses, indicating their weight over time. Error bars represent the 95% confidence interval of the mean at each time interval

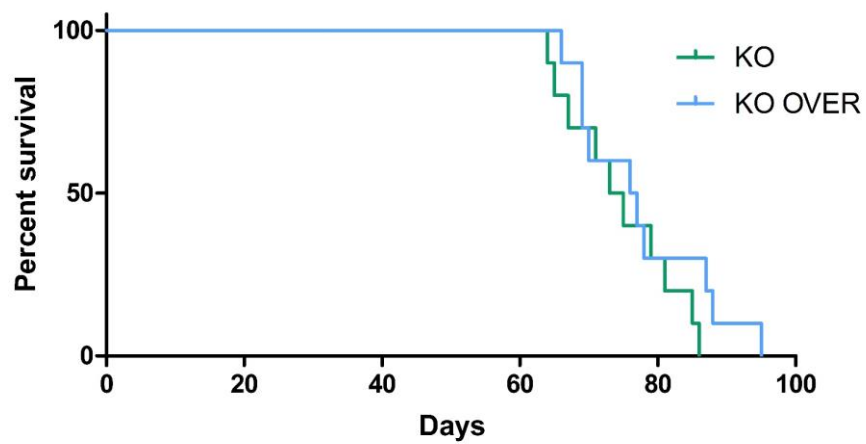


Fig. 6 Mantel-Cox survival curve of KO OVER mice compared to KO mice over time (n = 10/genotype)

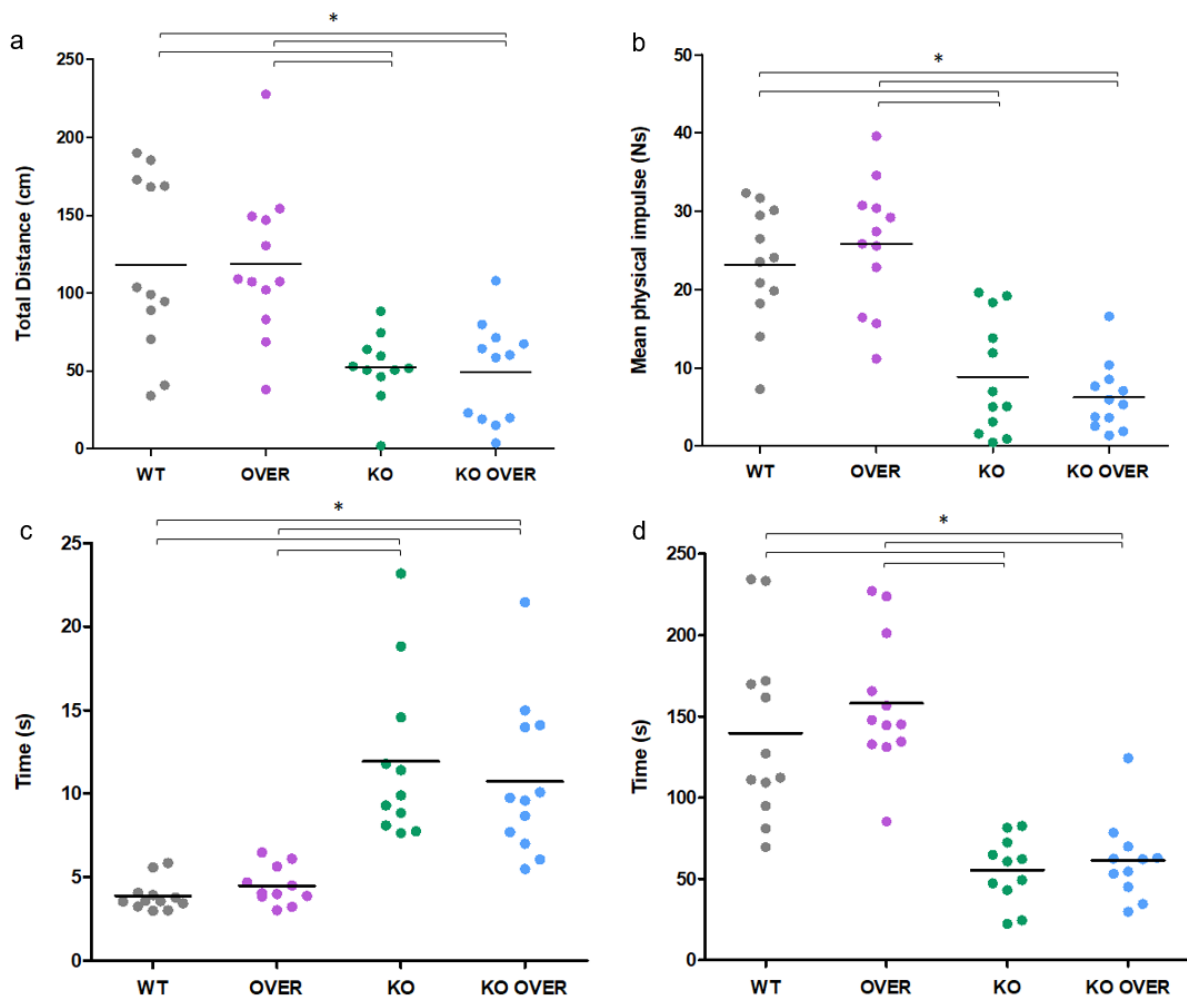


Fig. 7 Vertical scatter plots depicting the (a) total distance travelled in an open field test over 5 min, (b) mean physical impulse in a wire grid hang test, (c) time taken to cross a 0.8 m balance beam and (d) latency to fall from a rotarod, by mice of the 4 genotypes (n = 12/genotype) at P50. Horizontal bar indicates the mean and brackets indicate statistically significant differences where $p < 0.05$ (*)

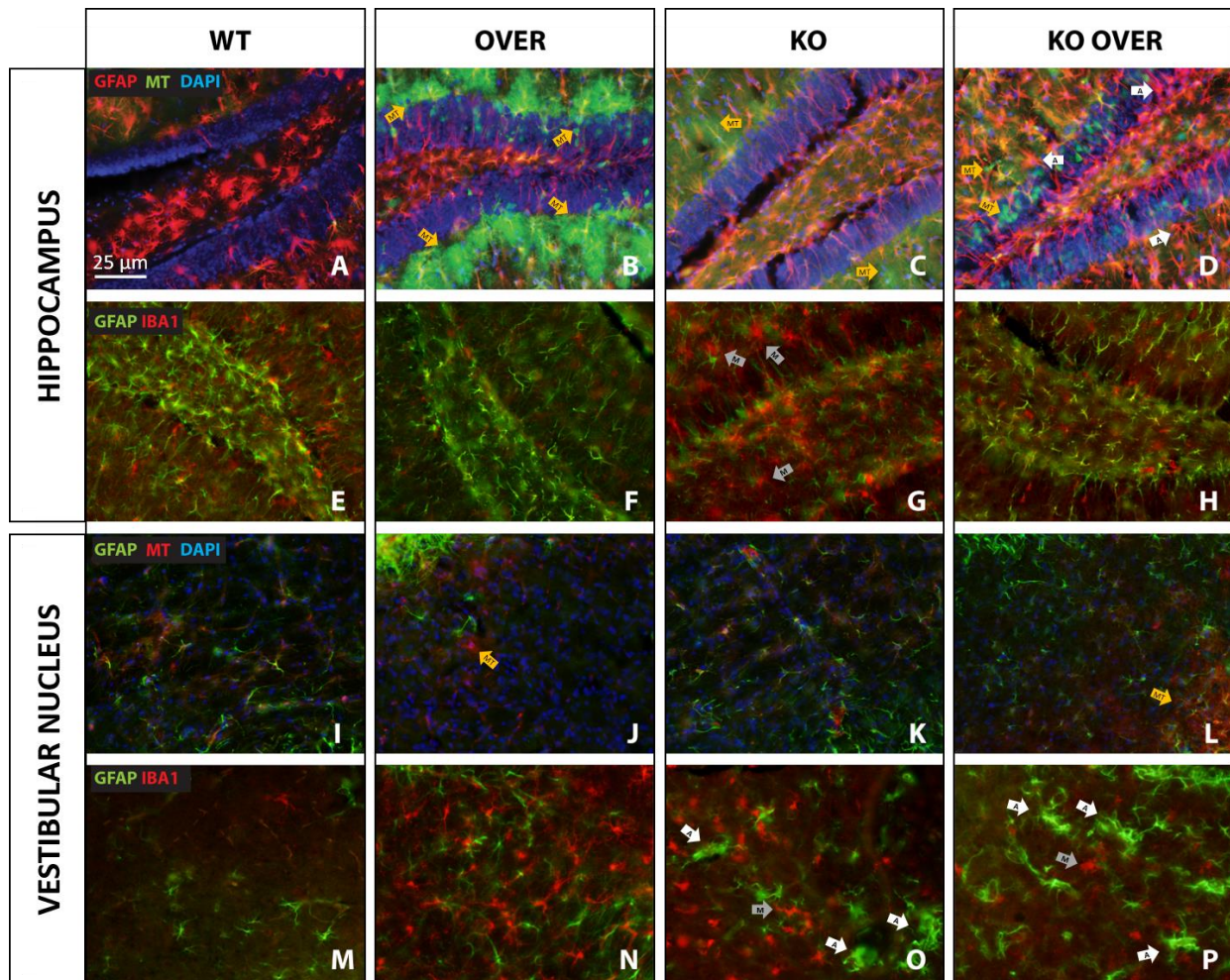
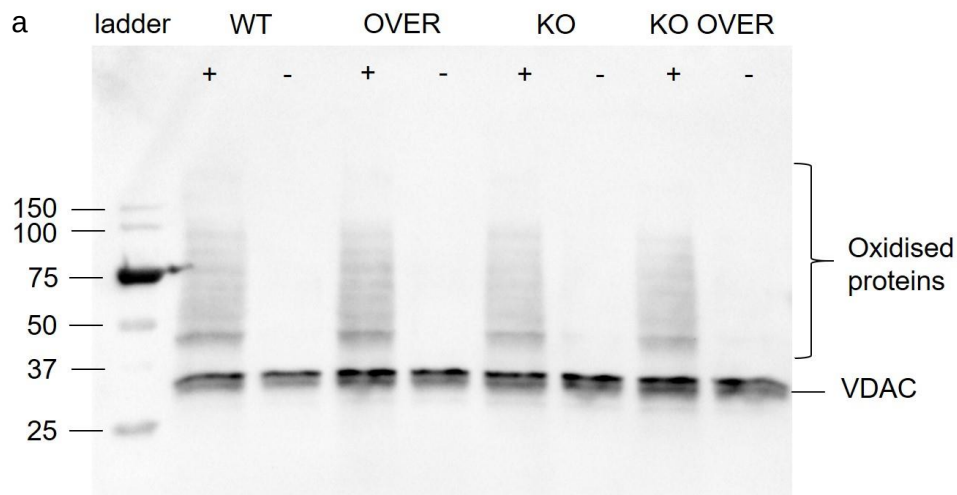


Fig. 8 Representative immunohistochemistry stain images for MT (green in a-d, red in i-l), GFAP (red in a-d, green in e-p) and DAPI (blue) in the hippocampus (a-h) and vestibular nucleus (i-p) of WT (a, e, i, m), OVER (b, f, j, n), KO (c, g, k, o) and KO OVER (d, h, l, p) mice (n = 5/genotype). The white horizontal line on image a is representative of the scale of 25 μm that is applicable to images a-p. The white arrows (with an A) highlight hypertrophic and reactive astrocytes, grey arrows (with an M) highlight microglial activation and yellow arrows (with MT) highlight MT expression. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IBA1, Ionised calcium binding adapter molecule 1; MT, metallothionein.



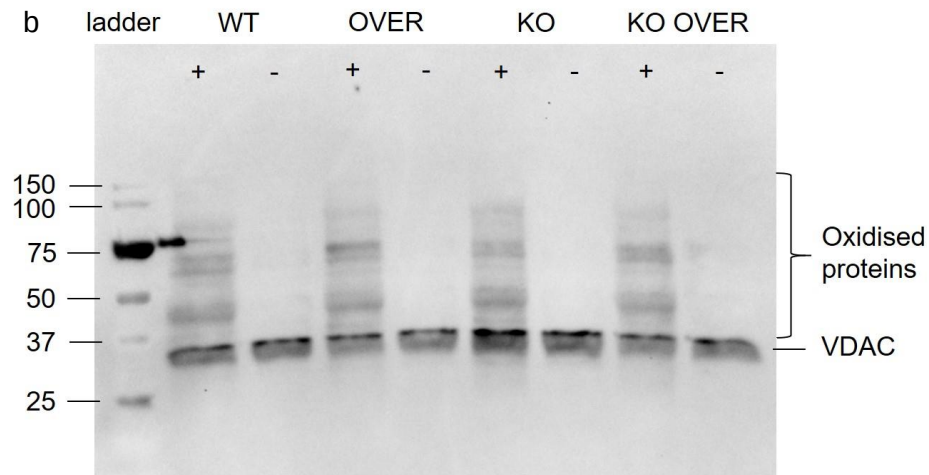


Fig. 9 Western blot of (a) brain and (b) quadriceps samples ($n = 1/\text{genotype}$) that were stained for oxidised proteins and VDAC, using the OxyBlot kit. The first row represents the protein ladder (sizes in kDa) and the following rows are indicated with a “+” for the sample derivatised with DNPH and a “-” for the negative control

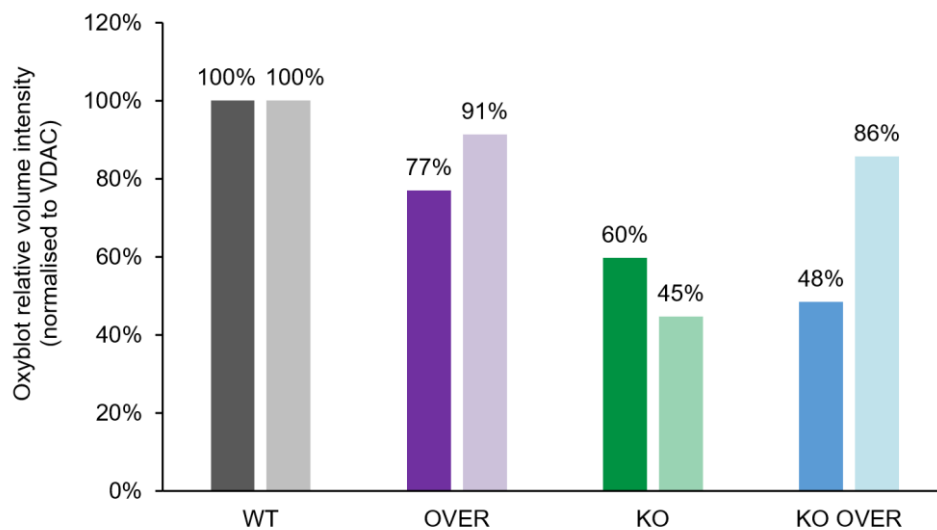


Fig. 10 Bar graph depicting the percentage of oxidised proteins (normalised to VDAC) in the brain and quadriceps ($n = 1/\text{genotype}$), relative to the WT. The darker bars indicate the brain samples and the lighter bars indicate the quadriceps samples.

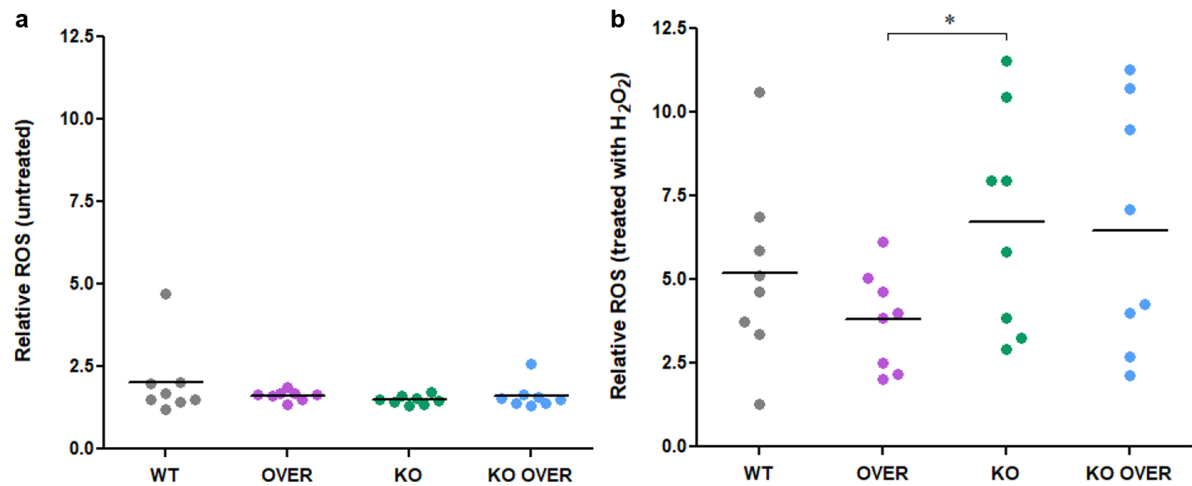
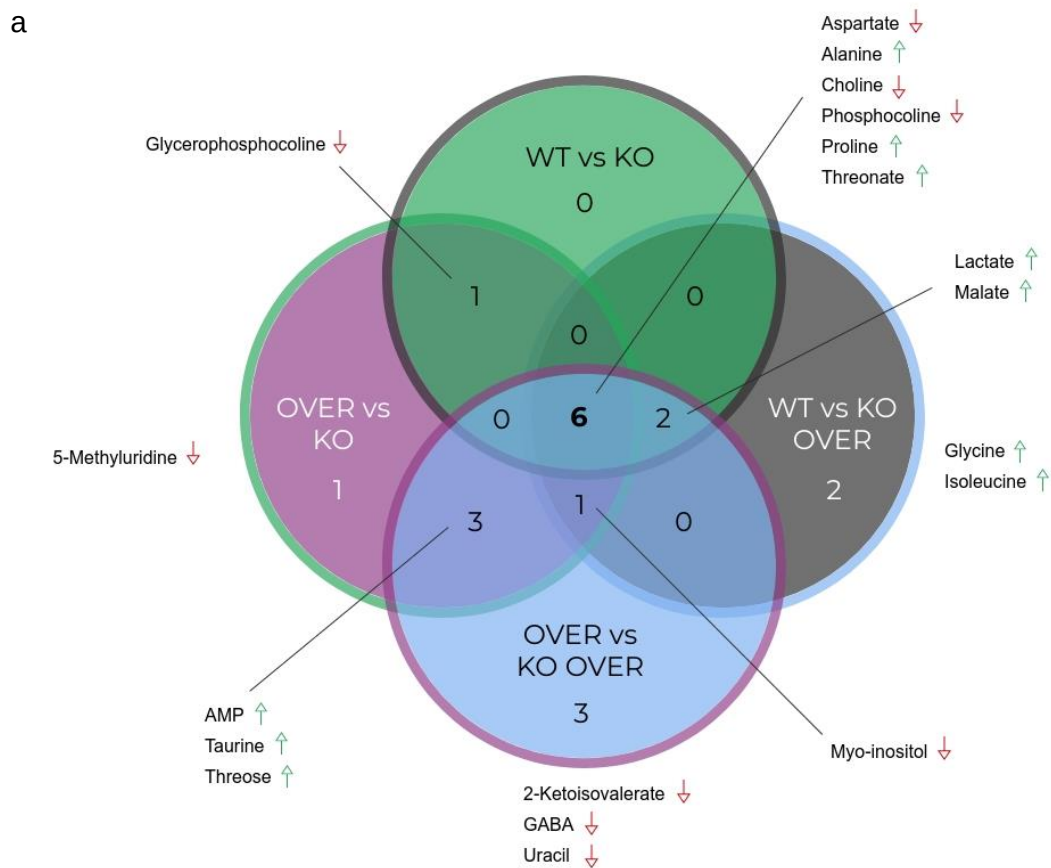


Fig. 11 Vertical scatter plots depicting the relative intracellular ROS levels (normalised to the negative controls) of both (a) untreated and (b) H₂O₂ treated cultured mouse BMDMs of mice from the four genotypes (n = 8/genotype). Significant differences are depicted by a bracket and asterisk, where p < 0.05. All untreated genotypes were significantly lower than their treated counterpart



b

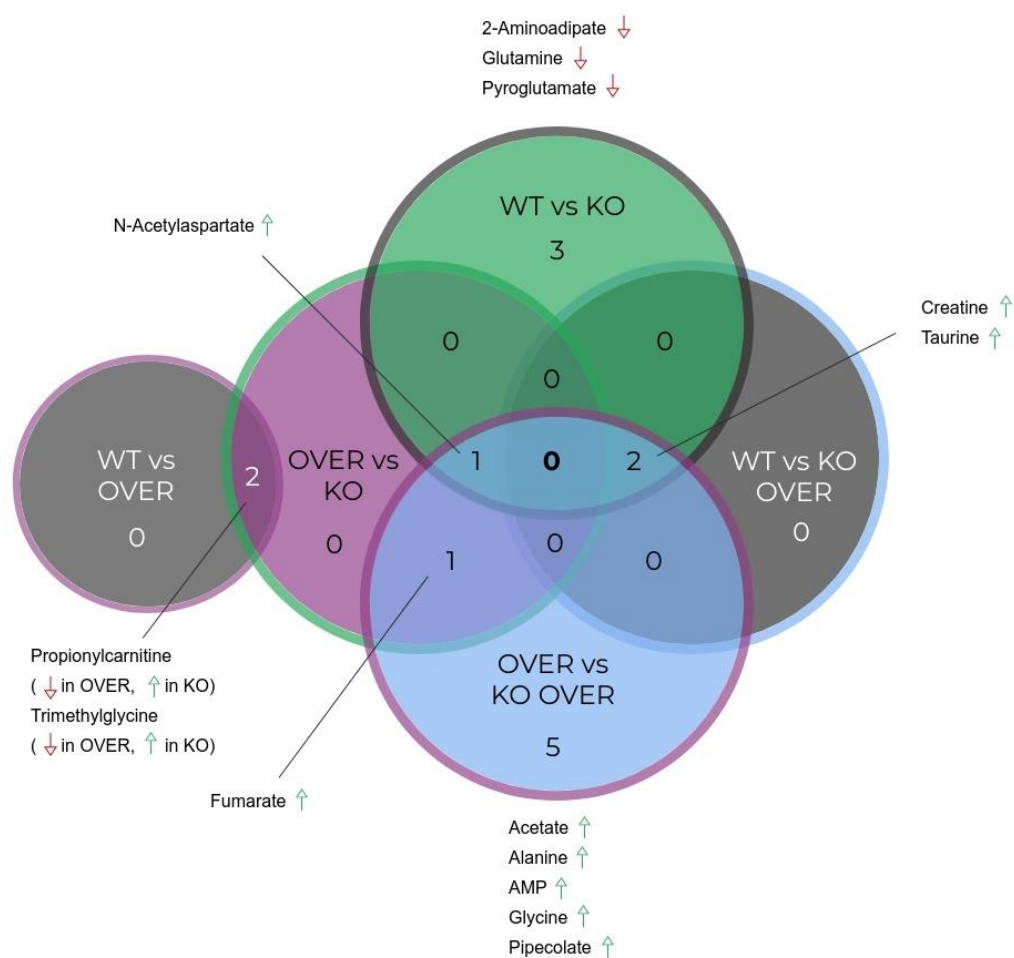


Fig. 12 Venn diagram depicting important metabolites in the (a) brain (n = 6-9/genotype) and (b) quadriceps (n = 13-14/genotype) which overlap between the genotype groups (WT vs OVER, WT vs KO, WT vs KO OVER, OVER vs KO and OVER vs KO OVER). The direction of metabolite alteration is indicated as KO or KO OVER relative to WT or OVER. For the WT vs OVER group, the direction indicates OVER relative to WT. Abbreviations: AMP = adenosine monophosphate; GABA = γ -aminobutyrate

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APPENDIX F: LANGUAGE CERTIFICATE



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*This is to certify that the dissertation in fulfilment of the
requirements for the degree*

Philosophiae Doctor in Biochemistry

of

HC Miller

has been perused by

Valerie Viljoen

of

Editing Excellence

Date: 19 January 2020



SATI INDIVIDUAL MEMBER