

The effect of metallothionein overexpression on the transcription of selected genes involved in one-carbon metabolism and oxidative stress in NDUFS4-deficient mouse tissues

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This dissertation is dedicated to:

Pappa,

who always believed in me.

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ABSTRACT

Respiratory complex I (CI) defects are the most common mitochondrial disease, for which treatment is limited. Additionally, there is a lack of understanding of the impact of a CI defect upon the gene expression regulation of important metabolic pathways. In this study, the impact of a CI defect upon the gene expression of enzymes involved in one-carbon metabolism and oxidative stress was investigated. These two networks are vital for amino acid and purine production, and for the physiological antioxidant defence systems, respectively. The effect of metallothionein (MT1) overexpression was also investigated in the context of a CI defect, since metallothionein is an endogenous antioxidant with therapeutic potential. An NADH:Ubiquinone Oxidoreductase Subunit S4 (NDUFS4)-deficient, and an MT1 overexpressing mouse model and crossbred mice were used. Metallothionein (MT1), which is an endogenous antioxidant protein with therapeutic potential, was also investigated in the context of a CI defect. After the mouse models were successfully characterised on DNA, RNA and protein levels, real-time quantitative reverse transcriptase PCR (RT-qPCR) was applied to evaluate the expression of selected genes. The quadriceps and brain tissues of 24 mice (four genotypes, namely wildtype, NDUFS4 KO, MT1 overexpressing and NDUFS4 KO:MT1 overexpressing) were used. It was concluded that *Mthfd2*, *Bhmt*, *Tyms* and *Mtrr* showed the greatest downregulating change in expression in the brain tissue, whereas *Mthfd2* and *Gpx1* showed the greatest downregulating change in gene expression in quadriceps tissues. *Mt1* mitigated the downregulation only of *Tyms* in the brain tissue. It is argued that the inhibition of 1-C metabolism by the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway and alternative cellular adaptive mechanisms in the case of a CI defect, might be linked to the results. Factors that impacted the results were the differences in RNA concentration and transcription factors between the brain and quadriceps tissues, the tissue-types used and the number of genes investigated. Nevertheless, strengths of this study included the evidence-based selection of the investigated genes, the mouse models used and the in-depth evaluation of the processing of RT-qPCR data. The importance of considering the bigger gene expression regulation system of an organism, as well as the possible adverse effects of the use of antioxidants in mitochondrial myopathies during research, were also recognised. Taken together, this study has successfully applied empirical methods to investigate the expression – and its regulation – of genes involved in 1-C metabolism and oxidative stress, in the context of a CI defect and transgenic overexpression of *Mt1*.

Key Terms: complex I deficiency, NDUF54 defect, Leigh syndrome, metallothionein overexpression, oxidative phosphorylation (OXPHOS), one-carbon metabolism, mitochondrial disease, oxidative stress, mRNA, reverse transcription real-time PCR (RT-qPCR), gene expression regulation, mouse brain tissue, mouse quadriceps tissue, antioxidant therapy

OPSOMMING

Respiratoriese kompleks I (KI) -siektes is die algemeenste mitochondriale siektes en behandeling hiervoor is baie beperk. Daarmee saam is daar 'n gebrek aan begrip oor die impak van 'n KI-defek op die regulering van geenuitdrukking van belangrike metaboliese weë. In hierdie studie, is die impak van 'n KI-defek op die geenuitdrukking van ensieme, wat betrokke is by een-koolstof (1-K) -metabolisme en oksidatiewe stres, ondersoek. Hierdie twee weë is noodsaaklik vir aminosuur- en purienproduksie en vir die fisiologiese antioksidant-verdedigingsisteem, onderskeidelik. Die effek van die ooruitdrukking van metallotioneïen (MT1), 'n endogene antioksidant met die potensiaal as 'n behandeling vir KI-defekte, is ook ondersoek in die konteks van 'n KI-defek. Muis met 'n NDUFS4-defek en muis wat MT1 ooruitdruk is kruisgeteël en gebruik in hierdie studie. Nadat die muismodelle suksesvol gekarakteriseer is op DNA, RNA en proteïen-vlakke, is regstreekse kwantitatiewe omgekeerde transkriptase polimerase kettingreaksie (RT-qPCR) gebruik om die uitdrukking van die gekose gene te evalueer. Die kwadriseps- en breinweefsel van 24 muis (vier genotipes, naamlik wilde-tipe, NDUFS4 KO, MT1 oor-uitrukkend en NDUFS4 KO:MT1 ooruitrukkend) is gebruik. Die gevolgtrekking was dat die *Mthfd2*-, *Bhmt*-, *Tyms*- en *Mtrr*-gene die grootste af-regulerende verandering in die breinweefsel getoon het, waar *Mthfd2* en *Gpx1* die grootste af-regulerende verandering in geenuitdrukking in die kwadrisepsweefsels getoon het. *Mt1* het slegs die af-regulering van die geen *Tyms* in die breinweefsel reggestel. Daar word geargumenteer dat die inhibisie van 1-K-metabolisme deur die 5' adenosien monofosfaat-geaktiveerde proteïenkinase (AMPK)-weg en, in die geval van 'n KI-defek, die werking van alternatiewe sellulêre aanpassingsmeganismes moontlik aan die resultate gekoppel kan word. Faktore wat die resultate geaffekteer het, was die verskille in RNA konsentrasie en transkripsiefaktore tussen die brein- en kwadrisepsweefsels, die weefselsoorte wat gebruik is en die hoeveelheid gene wat ondersoek is. Nietemin sluit die sterkpunte van die studie die bewys-gebaseerde keuse van gene wat ondersoek is, die muismodelle wat gebruik is en die in-diepte ondersoek van die prosessering van RT-qPCR data in. Die belangrikheid om die groter geenuitdrukking-reguleringstelsel van 'n organisme, sowel as die moontlike nadelige effekte van die gebruik van antioksidante in mitochondriale miopatieë tydens navorsing in ag te neem, is ook erken. In kort, het hierdie studie empiriese metodes toegepas om die uitdrukking – en regulering van uitdrukking – van gene betrokke in die 1-K-metabolisme en oksidatiewe stres in die konteks van 'n KI-defek en transgene ooruitdrukking van *Mt1* suksesvol te ondersoek.

Sleuteltermes: kompleks I -defek, NDUF54-defek, Leigh se sindroom, metallotioneïen 1-ooruitdrukking, oksidatiewe fosforilering (OKSFOS), regstreekse kwantitatiewe omgekeerde transkriptase polimerase kettingreaksie (RTq-PCR), een-koolstof-metabolisme, mitochondriale siekte, oksidatiewe stres, mRNA, regulering van geenuitdrukking, muis-breinweefsel, muis-kwadrisepsweefsel

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LIST OF ABBREVIATIONS

#	number
%	percentage
-/-	knockout or unaltered gene
[]	concentration
+/-	heterozygous
+/+	wild type
Δ	delta (change)
ΔRn	the fluorescence of the reporter dye normalised to the fluorescence of a passive reference dye
°C	degrees celsius
[•] OH radical	hydroxyl radical
1-C	one-carbon
3'	3'-end of the polynucleotide chain
5'	5'-end of the polynucleotide chain
A	absorbance
ACTβ/ <i>Actβ</i>	β-actin PROTEIN/ <i>Gene</i>
<i>Ad libitum</i>	without restraint/available at all times
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated kinase
ANIMCARE	Animal Care, Health and Safety in Research Ethics Committee

antilog	antilogarithm
AP-1	activator protein 1
Approx.	approximately
APS	ammonium persulfate
ARE	antioxidant responsive element
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
avg	average
B	brain
BCA	bicinchoninic acid
BHMT/ <i>Bhmt</i>	betaine-homocysteine s-methyltransferase PROTEIN/ <i>Gene</i>
BN	blue native
bp	base pairs
BSA	bovine serum albumin
Ca	calcium
CA	California
Ca ²⁺	calcium ion
cAMP	cyclic adenosine 3',5'-monophosphate
Cat (in footnotes)	catalogue
CAT/ <i>Cat</i>	<i>catalase</i> PROTEIN/ <i>Gene</i>
Cd	cadmium
cDNA	complementary DNA

CI	complex I (NADH: ubiquinone oxidoreductase or NADH dehydrogenase or NADH-Coenzyme Q reductase, E.C. 1.6.5.3)
CII	complex II (succinate:ubiquinone oxidoreductase or succinate dehydrogenase (SDH), E.C. 1.3.5.1)
CIII	complex III (ubiquinol:ferricytochrome c oxidoreductase or ubiquinol cytochrome c reductase, E.C. 1.10.2.2)
CIV	complex IV (cytochrome c oxidase (COX) or ferrocytochrome-c:oxygen oxidoreductase, E.C. 1.9.3.1)
CoA	Co-enzyme A
cont.	control
COO ⁻	carboxyl group
CoQ10	coenzyme Q10
CPEO	chronic progressive external ophthalmoplegia
CpG	cytosine followed by guanine
Cre-Lox	Cre recombinase and a loxP recognition site, derived from the P1 bacteriophage
CS	citrate synthase
Ct	threshold cycle
Cu	copper
Cu ⁺	reduced copper ion
Cu ²⁺	oxidised copper ion
CuSO ₄	copper sulphate
CV	coefficient of variance

CV	complex V (ATP ¹ phosphohydrolase or F1F0-ATP synthase, E.C. 3.6.1.3)
Da	dalton
DDM	n-dodecyl-β-d-maltoside
<i>de novo</i>	anew/from the beginning
DEPC	diethyl pyrocarbonate
DF	dilution factor
DMG	dimethylglycine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOB	date of birth
dsDNA	double stranded DNA ²
dTMP	deoxythymidine monophosphate/ deoxythymidylate
DTNB	dithio-bis(-2-nitrobenzoic acid)
dUMP	deoxyuridine monophosphate/deoxyuridylate
DUQ	decylubiquinone
E	efficiency
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
eq.	equation

¹ Refer to abbreviation for ATP

² Refer to abbreviation for DNA

<i>et al.</i>	and others
EtBr	ethidium bromide
ETC	electron transport chain
ETF	electron transfer flavoprotein
ETF/ETF-QO	electron transfer flavoprotein system
ETF-QO	electron transfer flavoprotein:ubiquionone oxidoreductase
EtOH	ethanol
FAM-MGB	carboxy fluorescein minor groove binder
FLTR	from left to right
FMN	flavin mono nucleotide
Fwd	forward
g	gram
GCLC/ <i>Gclc</i>	glutamate-cysteine ligase catalytic subunit PROTEIN/ <i>Gene</i>
GEA	gene expression assay
GPX1/ <i>Gpx1</i>	glutathione peroxidase 1 (cytoplasmic isoform) PROTEIN/ <i>Gene</i>
GSH	reduced glutathione
GSR/ <i>Gsr</i>	glutathione-disulfide reductase PROTEIN/ <i>Gene</i>
GSSG	oxidised glutathione
H	hydrogen
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
Hcy	homocysteine
HEt	hydroethidine

HRP	horse radish peroxidase
i.e.	in other words
ID	identification
IMM	inner mitochondrial membrane
IMS	intermembrane space
<i>in vitro</i>	performed/taking place outside a living organism
<i>in vivo</i>	performed/taking place inside a living organism
IQR	interquartile range
JAX	Jackson Laboratories
Kb	kilobase
kDa	kilodalton
KO	knock-out
KSS	Kearns-Sayre syndrome
l	litre
LHON	Leber's hereditary optic neuropathy
LIAS/ <i>Lias</i>	lipoic acid synthase PROTEIN/ <i>Gene</i>
log	logarithm
LS	Leigh Syndrome
M	molar
MD	mitochondrial disease
MELAS	mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
MEMSA	myoclonic epilepsy myopathy sensory ataxia

MERRF	myoclonic epilepsy with ragged-red fibres
Met	methionine
mg	milligram
MGB	minor groove binder
MHC	major histocompatibility complex
min	minutes
MIRAS	mitochondrial recessive ataxia syndrome
ml	millilitre
mM	millimolar
MM	mitochondrial matrix
MP	multiplex
mPTP	mitochondrial permeability transition pore
MRE	metal responsive element
MRL	Mitochondria Research Laboratory
mRNA	messenger RNA
MT1-4/ <i>Mt1-4</i>	metallothionein isoform 1 – 4 PROTEIN/Gene
mtDNA	mitochondrial DNA
MTF-1	metal regulatory transcription factor 1
MTHFD2/ <i>Mthfd2</i>	methylenetetrahydrofolate dehydrogenase or cyclohydrolase or formyltetrahydrofolate synthetase 2 PROTEIN/ <i>Gene</i>
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
MTRR/ <i>Mtrr</i>	5-methyltetrahydrofolate-homocysteine PROTEIN/ <i>Gene</i>

mw	molecular weight
N module	NADH binding module
n	number of samples or replicates analysed
NAD ⁺	nicotine amide adenine dinucleotide (oxidized)
NADH	nicotine amide adenine dinucleotide (reduced)
NADP ⁺	nicotine amide adenine dinucleotide phosphate (oxidised)
NADPH	nicotine amide adenine dinucleotide phosphate (reduced)
NARP	neurogenic weakness with ataxia and retinitis pigmentosa
NCBI	National Centre for Biotechnology Information
nDNA	nuclear DNA
NDUFA9	NADH ³ dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9
NDUFS3	NADH ⁴ dehydrogenase [ubiquinone] iron-sulfur protein 3
NDUFS4	NADH ⁵ :ubiquinone oxidoreductase iron-sulfur protein 4
<i>Ndufs4</i> ^{-/-}	NADH:Ubiquinone Oxidoreductase Subunit S4 knock-out <i>Gene</i>
<i>Ndufs4</i> ^{FKY/fky}	Where a transposable element is inserted into the <i>Ndufs4</i> gene
<i>Ndufs4</i> -PM	Where a point mutation is introduced in the <i>Ndufs4</i> gene, resulting in the loss of the last 10-15 amino acids of the last exon of the protein
Nes <i>Ndufs4</i> ^{-/-}	Where <i>Ndufs4</i> ^{lox/lox} mice are crossed with mice expressing Cre-recombinase from the <i>Nestin</i> locus
NFE2	nuclear factor erythroid 2
NFQ	non-fluorescent quencher
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

³ Refer to abbreviation for NADH.

⁴ Refer to abbreviation for NADH.

⁵ Refer to abbreviation for NADH.

NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NH ₃ ⁺	amino group
nm	nano metre
Nm	nanometre
nM	nanomolar
Nrf1	nuclear respiratory factor 1
NRF2	NFE2 ⁶ -related factor 2
NTC	no-template control
NWU	North-West University
O ₂	oxygen
O ₂ ⁻	superoxide radical
OAA	oxaloacetate
OMIM	Online Mendelian Inheritance in Man
OMM	outer mitochondrial membrane
OVER	overexpressing
OXPHOS	oxidative phosphorylation
P module	proton pumping module
P	post-natal day
PAGE	polyacrylamide gel electrophoresis
PC <i>Ndufs4</i> ^{-/-}	A Purkinje cell specific <i>Ndufs4</i> ^{-/-} mouse
PCDDP	Preclinical Drug Development Platform

⁶ Refer to abbreviation for NFE2.

PCR	polymerase chain reaction
Pd and Pp	two proton pump modules
PGC-1 α	PPARG ⁷ coactivator 1 alpha
PHGDH/ <i>Phgdh</i>	phosphoglycerate dehydrogenase PROTEIN/ <i>Gene</i>
PPARG	peroxisome proliferator-activated receptor gamma
prop stdev	propagated standard deviation
PTFE	polytetrafluoroethylene
PVDF	polyvinylidene difluoride
Q	quadriceps
Q	ubiquinone (also abbreviated as UQ, depending on the context)
QH ₂	ubiquinol
R ²	Coefficient of determination
RC	respiratory chain
rep	repetitions
Rev	reverse
RNA	ribonucleic acid
ROS	reactive oxygen species
ROX	carboxy-X-rhodamine
rRNA	ribosomal RNA
RT	reverse transcription
RT-qPCR	reverse-transcription quantitative polymerase chain reaction in real-time

⁷ Refer to abbreviation for PPARG.

rxn	reaction
S	sulphur
S	Svedberg unit
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SANDO	sensory ataxia neuropathy, dysarthria, ophthalmoplegia
SAVC	South African Veterinary Council
SCAE	spinocerebellar ataxia with epilepsy
SDS	sodium dodecyl sulphate
Se	selenium
Seq.	sequence
Ser	serine
SHMT2/ <i>Shmt2</i>	serine hydroxymethyl transferase 2 (mitochondrial isoform) PROTEIN/ <i>Gene</i>
SOD2/ <i>Sod2</i>	superoxide dismutase 2 (mitochondrial isoform) PROTEIN/ <i>Gene</i>
stdev	standard deviation
T	thionin
TATA-box	repeating thymine and adenine sequence box
TBS	tris-buffered saline
TCA	tricarboxylic acid
TE buffer	1x Tris EDTA ⁸ buffer
temp	temperature

⁸ Refer to abbreviation for EDTA.

TF	transcription factor
TgMT1	transgenic metallothionein 1 overexpressing
THF	tetrahydrofolate
Tris.HCl	2-amino-2-(hydroxymethyl)propane-1,3-diol;hydrochloride
TYMS/ <i>Tyms</i>	thymidylate synthase PROTEIN/ <i>Gene</i>
UCS	catalytic units of citrate synthase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)
UP	ultra-pure
UQ	ubiquinone (also abbreviated as Q, depending on the context)
USA	United States of America
V	volt
VDAC1	voltage-dependent anion channel 1
vs	versus
w/v	weight (of solute) per volume (of solvent)
WB	western blot
WT	wild type
x	times
xg	centrifugal force
Zn	zinc
Zn^{2+}	zinc ion
Zn_7MT	zinc bound to metallothionein
B	beta
$\beta 2\text{M}/\beta 2m$	β -2-microglobulin PROTEIN/ <i>Gene</i>
βME	β -mercapto ethanol

ΔC_t	the difference between the average C_t value of the target gene and the average C_T value of the reference gene
$\Delta\Delta C_t$	the difference between the ΔC_t value of the target genotype and the ΔC_t value of the calibrator genotype
μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar

“Life is a relationship among molecules and not a property of any molecule.”

~Linus Pauling

CHAPTER 1: INTRODUCTION

Embedded in the inner mitochondrial membrane, are five enzyme complexes that transport electrons from NADH to oxygen for the eventual formation of water, as well as the production of energy in the form of adenosine triphosphate (ATP). Collectively, these enzymes – namely complex I (CI)¹, complex II (CII)², complex III (CIII)³, complex IV (CIV)⁴ and complex V (CV)⁵ (International Union of Biochemistry and Molecular Biology, 2019) – are known as the mitochondrial oxidative phosphorylation system (OXPHOS), the core machinery of the mitochondrial energy production from carbohydrates, proteins and fatty acids.

The first four complexes of the OXPHOS system, called the respiratory chain (RC) or electron transport chain (ETC), also play an immensely important role in the maintenance of the cellular redox balance. Under physiological circumstances, the transport of electrons from complex I to IV creates an H⁺-gradient across the inner mitochondrial membrane, which is restored by the H⁺ ion-pumping action of ATP synthase. CI, specifically, contributes to this gradient by oxidising reduced nicotinic adenine dinucleotide (NADH) from the Krebs cycle and β -oxidation, reducing ubiquinone and donating electrons to coenzyme Q10 (Hirst, 2013). It also plays the leading role in the active production of mitochondrial reactive oxygen species (ROS), which may act as cellular messengers when present in moderate concentrations.

In this study, the spotlight is aimed at a genetic disease model of a dysfunctional CI – which is the most common form of an inherited mitochondrial deficiency – and the effect it has on oxidative stress and one-carbon (1-C) metabolism.

Leigh syndrome is one of the most common phenotypes of mitochondrial CI deficiency (Lake *et al.*, 2015). Infants with this phenotype usually fail to thrive and they display progressive encephalopathy, muscle weakness and fatal respiratory failure. However, the main characteristics of Leigh syndrome are significant bilateral lesions, and abnormalities in the brain stem and basal ganglia (McKusick & Hamosh, 2018). On a molecular level, the genetic causes of Leigh syndrome most often result from an impaired CI function as a consequence of one of its 45 subunits being unstable or catalytically inactive. In addition to the resulting dysfunctional effect on overall OXPHOS function (i.e. ATP production), this leads to a dramatic surge in ROS levels inside the cell. Subsequently, the cellular reduction/oxidation

¹ NADH: ubiquinone oxidoreductase or NADH dehydrogenase or NADH-Coenzyme Q reductase, E.C. 1.6.5.3

² Succinate:ubiquinone oxidoreductase or succinate dehydrogenase (SDH), E.C. 1.3.5.1

³ Ubiquinol:ferricytochrome c oxidoreductase or ubiquinol cytochrome c reductase, E.C. 1.10.2.2

⁴ Cytochrome c oxidase (COX) or ferrocytochrome-c: oxygen oxidoreductase, E.C. 1.9.3.1

⁵ ATP phosphohydrolase or F1F0-ATP synthase, E.C. 3.6.1.3

(redox) balance shifts towards an oxidative state (i.e. increased oxidative stress) as the antioxidant defences become overburdened. This may result in a wide range of cellular consequences, including deoxyribonucleic acid (DNA), protein and lipid damage, cellular enzyme dysfunction (notably dehydrogenases, as a result of redox imbalance), affected calcium signalling and apoptosis (Jacobs & Smeitink, 2009; Ray *et al.*, 2012; Sies, 2013).

From work recently published (Terburgh *et al.*, 2019), another metabolic pathway that is affected in CI deficiency is one-carbon metabolism, which is directly involved in DNA synthesis, DNA methylation and amino acid metabolism. Yet, there is currently no effective treatment for CI defects. Treatment mainly aims to relieve the symptoms, and the fight against cellular oxidative stress depends on the exogenous intake and subsequent effect of therapeutic substances (Lukienko *et al.*, 2000; Manjeri, 2017).

Normally, the physiological antioxidant defence system works to maintain the appropriate redox balance. However, past research has shown that the expression of endogenous antioxidant, metallothionein (MT), is potently induced by ROS (Babula *et al.*, 2012; Chiaverini & De Ley, 2010). MT has four isoforms, of which MT1 is ubiquitously expressed (Thirumoorthy *et al.*, 2011). Additionally, because of the glutathione-like characteristics and immediate inducibility of MT1, it is regarded as a potential new therapy for mitochondrial CI defects (Lindeque *et al.*, 2010).

To investigate anything on a molecular level, however, a research model is necessary. In 2008, Kruse *et al.* established a mouse model in which a deletion of exon 2 of the NDUFS4 gene was induced. Physiologically, this subunit is part of the N-module of CI and is indispensable in the assembly-process of the enzyme. Today, this is a commonly used mouse model among researchers, as mice with this CI mutation display a similar phenotype to human patients with Leigh Syndrome (Distelmaier *et al.*, 2009; Exner *et al.*, 2012; Van Dyk, 2016; Winklhofer & Haass, 2010). Yet, for this study, it was decided not only to use this NDUFS4-Knockout (NDUFS4 KO) mouse model, but also to crossbreed NDUFS4 KO mice with transgenic metallothionein overexpressing mice (TgMT1 mice). Hence, the effect of metallothionein overexpression could be evaluated through all the biochemical analyses.

Therefore, in order to obtain a better understanding of the *regulation* of gene expression in 1-C metabolism in the context of a CI defect, this study aimed firstly at investigating the effect of an NDUFS4 deficiency upon the expression of selected genes involved in 1-C metabolism and oxidative stress in mouse brain and quadriceps tissues; and secondly, to evaluate whether *Mt1* overexpression would mitigate the impact of an NDUFS4 deficiency upon the expression of these genes.

CHAPTER 2: LITERATURE OVERVIEW

2.1 INTRODUCTION

The first part of this literature study will be dedicated to the significance of the mitochondrion in health and disease. Not only does it play a central role in cellular metabolism, it is also intricately involved in many diseases. Specific focus will be directed to cellular respiration, which takes place inside the mitochondrion. Hence, the oxidative phosphorylation (OXPHOS) process – and, in particular, the function and importance of complex I (CI) – will be described. Subsequently, the part that CI plays in OXPHOS dysfunction, reactive oxygen species (ROS) production and mitochondrial disease (MD), will be highlighted. Even though phenotypic and genotypic qualities, as well as the prevalence of MDs in general will be discussed, Leigh syndrome as a CI defect will be addressed specifically. A short discussion will follow on how cells respond and adapt to this defect in order to survive. Therapies that are currently available for patients suffering from Leigh syndrome will be compared in terms of their mechanism of function and effectiveness. Animal models that have been used in research on NDUFS4¹ deficiencies will then be discussed, linking it to the research conducted at the Mitochondria Research Laboratory (MRL) at the North-West University (NWU). Hereafter, a description and significance of oxidative stress metabolism will follow, together with the applicability of the endogenous antioxidant, metallothionein (MT). One-carbon (1-C) metabolism, as well as the possible effect of a CI deficiency on this metabolic cycle will be laid out. Objective 4 – the selection of the genes of interest – comprised a comprehensive study of the relevant literature. Therefore, the motivation behind the genes that were analysed in this study will be explained. This literature review will thus motivate the problem statement, aim and objectives and how it all fits into the larger study performed at the MRL. A basic hypothesis is also stated.

¹ Throughout this dissertation, proteins are written in uppercase (e.g. NDUFS4) and genes are written in italicised sentence case (e.g. *Ndufs4*) (MouseMine, 2019).

2.2 THE MITOCHONDRION

2.2.1 Mitochondrial structure and function

The mitochondrion is commonly known as the “power plant” of the cell, as it is responsible for aerobically metabolising carbohydrates, fatty acids and amino acids into the useful form of energy: adenosine triphosphate (ATP). One cell may contain hundreds of mitochondria, of which the matrix volume may occupy up to 35% of the cellular volume (Anastacio *et al.*, 2013; Winklhofer & Haass, 2010)

On a structural level, the shape of the mitochondrion might appear spherical or elongated. Mitochondria might even be found in large, interconnected networks. However, mitochondria are dynamic organelles: they constantly undergo fission and fusion and they often change their position within the cytosol. Inside the mitochondrion, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM) enclose the intermembrane space (IMS) – a space critical for bioenergetic reactions (McBride *et al.*, 2006). As opposed to the OMM, the IMM is structured in large folds protruding to the inside of the matrix (see Figure 2.1).

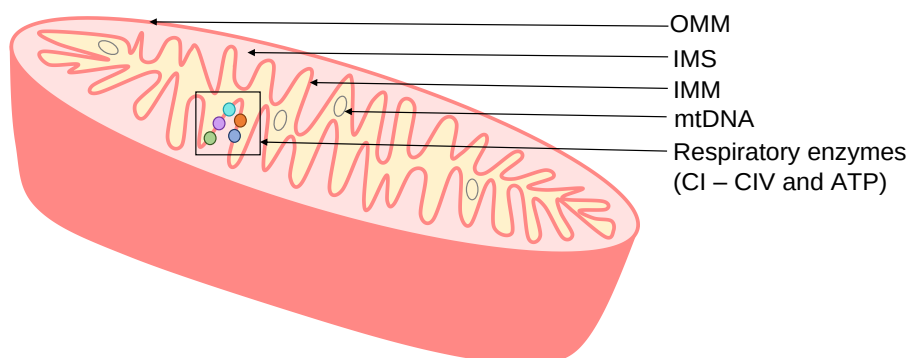


Figure 2.1 An illustration of a cross section of a mitochondrion. OMM = Outer mitochondrial membrane; IMS = Intermembrane space IMM = Inner mitochondrial membrane; CI-CIV = complex I to IV; mtDNA = mitochondrial DNA. This image is not to scale. The respiratory enzymes and the mtDNA in this illustration represent all the other molecules of their kind that are found in the mitochondrion. Adapted from CK-12 Foundation (2019) and Risha (2017).

These folds – also known as the cristae – are greatly advantageous to the metabolic reactions that take place in the mitochondrion, as they increase the surface area of the IMM (Valsecchi *et al.*, 2010). Both the OMM and the IMM are rich in proteins and lipids. The passage of proteins, such as subunits and assembly factors of the oxidative phosphorylation

(OXPHOS) system, is regulated by porins. The five enzymes that are implicated in the OXPHOS process and the regulation of the membrane potential are specifically rooted in the IMM.

In recent years, the mitochondrion has also been found to regulate apoptosis, to control cytosolic calcium concentration, to host the TCA cycle, to play a fundamental role in iron-sulphur cluster biogenesis, and to endogenously produce ROS (McBride *et al.*, 2006; Tuppen *et al.*, 2010). The fact that mitochondria also contain their own set of circular DNA, make them incomparable to other organelles. mtDNA encode the machinery for protein synthesis (2 ribosomal RNA genes as well as 22 transfer RNA genes) and it codes for 13 OXPHOS subunit genes (Anderson *et al.*, 1981).

The five complexes of the mitochondrial respiratory chain consist of an astounding 92 different proteins that require the assistance of 37 assembly factors for a cell to breathe normally. Moreover, all the complexes (except for complex II) are of a dual genetic origin (Koopman *et al.*, 2016).

Sadly, this kind of intricate design creates opportunities for a vast amount of genetic mutations. As the reader will discover, these five enzymes are directly involved in a large spectrum of MDs. Mutations of the OXPHOS enzyme subunits, as well as the assembly factors, give rise to neurodegeneration, respiratory failure and muscle atrophy. It is also associated with Leigh Syndrome, leukoencephalopathy, mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS) syndrome, Parkinsonism/MELAS, neuropathy, ataxia and retinitis pigmentosa (NARP), and Alzheimer's and Parkinson's diseases (Koopman *et al.*, 2016).

2.2.2 Oxidative phosphorylation (OXPHOS)

OXPHOS can be separated into two processes: electron transport (respiration) and adenosine triphosphate (ATP) production. As illustrated in Figure 2.2, the electron transport chain (ETC) consists of four enzyme complexes embedded in the IMM; namely NADH:ubiquinone oxidoreductase or NADH dehydrogenase or NADH-Coenzyme Q reductase/complex I (CI), succinate:ubiquinone oxidoreductase or succinate dehydrogenase (SDH)/complex II (CII), ubiquinol:ferricytochrome c oxidoreductase or ubiquinol cytochrome c reductase/complex III (CIII), and cytochrome c oxidase (COX) or ferrocytochrome-c:oxygen oxidoreductase/complex IV (CIV) (Cai & Tammineni, 2017; International Union of Biochemistry and Molecular Biology, 2019). Electrons flow from NADH (at CI) and FADH₂ (at CII), to CIII via coenzyme Q10 (CoQ10). From there the electrons are transported to CIV via a reduced cytochrome c. The fully oxidised form of CIV then receives electrons from cyto-

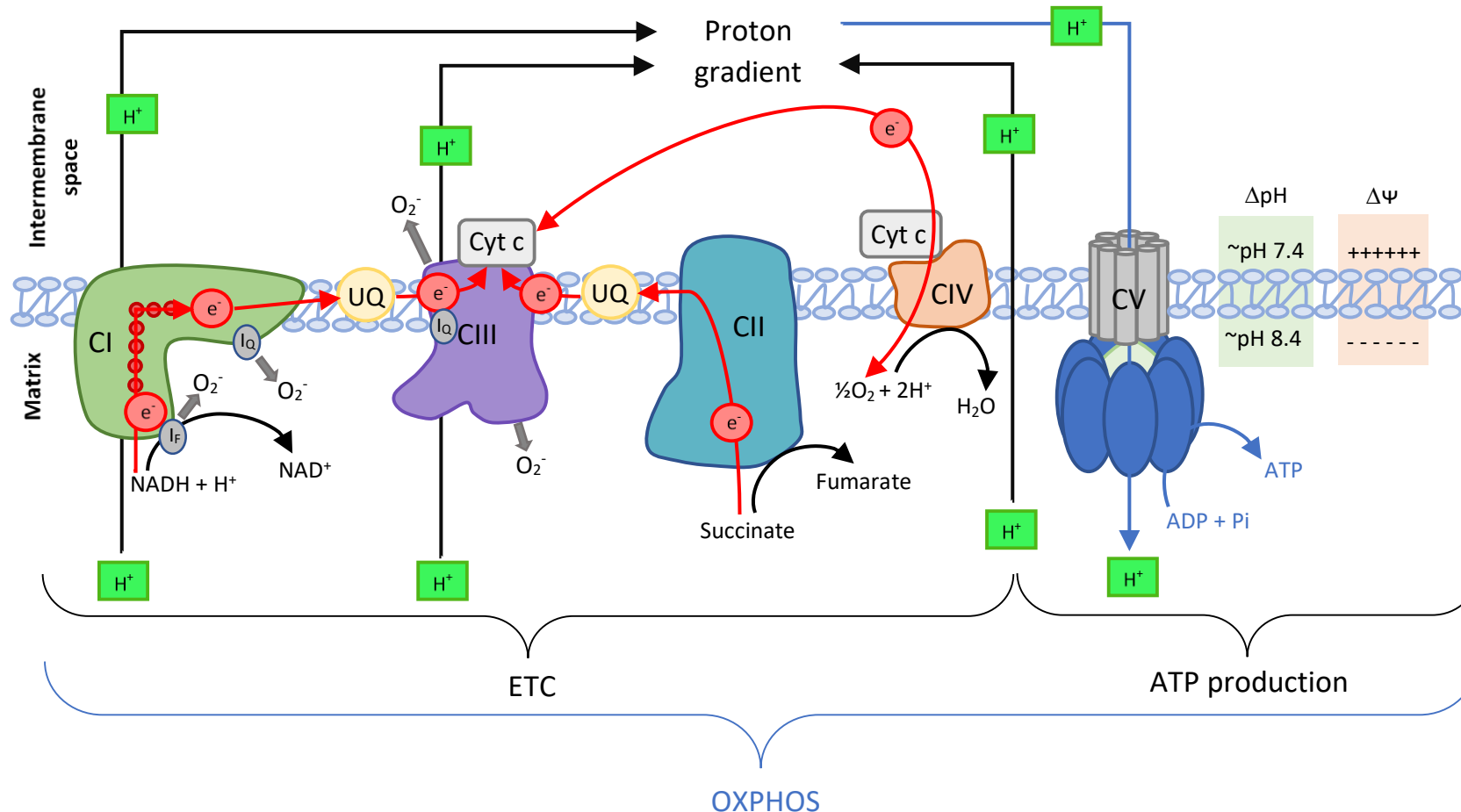


Figure 2.2 The ETC as part of the OXPHOS system. Red arrows indicate the electron path through the enzymes. Black arrows show the protons that are pumped to the intermembrane space and ETC reactions that take place inside the matrix. Blue arrows indicate the protons that are pumped back into the matrix and the formation of ATP. Grey arrows indicate electron leakage and the formation of superoxide radicals. ΔpH = difference in pH; $\Delta\psi$ = difference in membrane potential; Cyt C = cytochrome C; I_F = flavin mononucleotide site; I_Q = ubiquinone binding site; Q_O = ubiquinol binding site; Pi = inorganic phosphate; UQ = ubiquinone. Adapted from Garrett and Grisham (2010), Hasudungan (2013), Jastroch *et al.* (2010) and Koopman *et al.* (2016).

chrome c and donates electrons to O_2 , which converts the molecule to O_2^- (superoxide radical). O_2^- then rapidly takes two protons from the matrix and is firstly converted to OH^- (hydroxyl radical) and finally to the stable form of water (H_2O). In the process, a membrane potential with a proton gradient is generated over the IMM. ATP production (the phosphorylation of adenosine diphosphate) then takes place when the protons are pumped back into the mitochondrial matrix through the fifth enzyme complex, named ATP synthase (or CV). As the protons are continually pumped back and forth across the IMM, a proton motive force ($\Delta\Psi$) and an increased mitochondrial matrix pH (ΔpH) is created, which is essential for the success of all mitochondrial processes (Berg *et al.*, 2002; Koopman *et al.*, 2016; Valsecchi *et al.*, 2010).

During the process of transferring electrons among the enzymes, electrons often go astray. These stray electrons then partially reduce molecular oxygen, which leads to the formation of a spectrum of oxygen derivatives, such as the aforementioned superoxide (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) (Morgan & Liu, 2011). As the mitochondrion consumes most of the oxygen available to the cell, this organelle is responsible for the greatest amount of ROS that are formed. CIII, but especially CI of the OXPHOS system is specifically responsible for most of the mitochondrial ROS. Sites where stray electrons react with O_2 to form O_2^- , are the flavin mononucleotide site (I_F) and the ubiquinone binding site (I_Q) of CI and the ubiquinol binding site (Q_o) of CIII (indicated in Figure 2.2) (Hirst, 2013; Jastroch *et al.*, 2010).

Under normal homeostatic circumstances, ROS are vital for insulin-, cytokine-, $NF-\kappa\beta$ -, growth factor- and AP-1 signalling. Due to having an unpaired electron, however, these species are incredibly unstable. With O_2^- and OH^- having half-lives of 10^{-5} and 10^{-9} seconds, respectively, they have the ability to rapidly bind to – and oxidise – any proteins, lipids and nucleic acids in the immediate vicinity (Hanson, 2013). Indeed, by exposing human mononuclear leukocytes to H_2O_2 , Pero *et al.* (1990) already showed that prooxidants induce DNA strand breaks. This is termed 'oxidative stress'. Even though the mitochondrion is implicated in producing the greatest amount of ROS, it is also the first to be damaged by ROS as the mtDNA has no way of protecting itself. Therefore, the maintenance of a mitochondrial – indeed a cellular – redox balance is critical (Cai & Tammineni, 2017; Morrell, 2008; Sena & Chandel, 2012).

2.2.3 Complex I

CI consists of 14 core subunits and 31 accessory subunits encoded by both mitochondrial and nuclear DNA – 45 proteins in total. Approximately 16 of these subunits are directly involved with human disease (Hirst, 2013; Moreno-Lastres *et al.*, 2012; Valsecchi *et al.*, 2010). Kmita and Zickermann (2013) have established the composition of CI via X-ray crystallography and electron microscopy. As can be seen in Figure 2.3, CI has one arm embedded in the IMM, which contains two proton pump modules (Pd and Pp), and another arm – consisting of the NADH oxidising module (N) and the ubiquinone reducing module (Q) – reaching into the matrix.

The purpose of CI, according to Hirst (2013), is to oxidise nicotine amide dinucleotide (NADH), – coming from the tricarboxylic acid (TCA) cycle and β -oxidation – and to transfer electrons via iron-sulphur clusters to CoQ10. CI is also responsible for producing a large amount of ROS, as the low reduction potentials of the substrates and cofactors of CI lead to nonspecific electron transport to compounds in solution. Nevertheless, the concentrations of NADH and NAD^+ also determine the rate of H_2O_2 production.

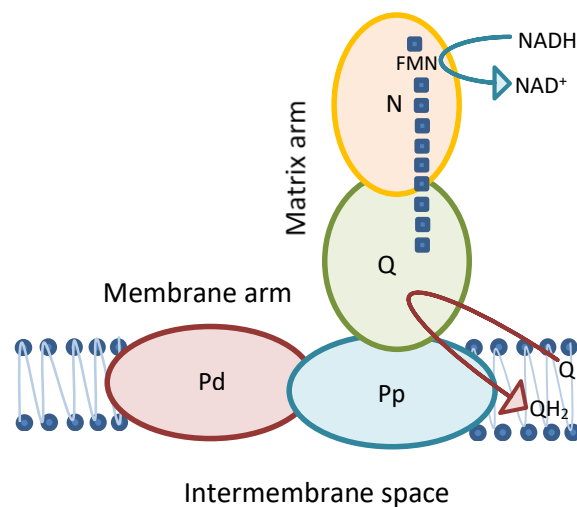


Figure 2.3 The structure of mitochondrial CI (NADH: ubiquinone oxidoreductase subunit S4). Large ovals indicate the different CI modules. Blue circles and stripes indicate the cellular membrane. Blue squares indicate iron-sulphur clusters being carried from Flavin mono nucleotide (FMN), to ubiquinone (Q). Arrows indicate the NADH oxidation and Q reduction. Figure 2.3 adapted from Kmita and Zickermann (2013) and Lazarou *et al.* (2007).

The 18 kDa nuclear NDUFS4 subunit – a subunit associated with the development of progressive neurodegenerative disease – is an accessory subunit situated on the N module of the peripheral arm of CI. This subunit is also known as the phosphorylation (activation) site of CI: when cyclic adenosine monophosphate (cAMP) phosphorylates the NDUFS4 subunit, cellular respiration and aerobic ATP production is upregulated. The NDUFS4 subunit is also important for stabilising CI (Budde *et al.*, 2001; Kahlhofer *et al.*, 2017; Lazarou *et al.*, 2007; Manjeri, 2017).

Manjeri (2017) describes the NDUFS4 subunit as a “mutational hotspot” in CI defects. To date, 9 different mutations in this gene alone have been established. A mutation in this gene renders CI unable to be activated, which leads to elevated intracellular ROS levels. This was reported by Valsecchi *et al.* (2013), who observed that the oxidation of the ROS-sensitive hydroethidine (HET) was increased in the primary muscle and skin fibroblasts of 38 to 40 day-old NDUFS4 KO mice; as well as by de Haas *et al.* (2017), who reported increased lipid peroxidation in the cerebral cortex and external capsule of NDUFS4 KO mouse brain tissue. Therefore, mutations of CI lead to rare but serious neuromuscular disorders with very limited treatment options at present. Leigh Syndrome, one of these diseases, is the focus of this study.

2.3 MITOCHONDRIAL DISEASE

2.3.1 Introduction

Luft *et al.* (1962) was the first to identify a mitochondrial defect in a patient with severe exercise intolerance and hyperthermia. An abnormal coupling between cellular respiration and phosphorylation in the mitochondria was observed. Cellular respiration was found to continue at a rapid pace, regardless of whether energy was wasted as heat or saved for metabolic reactions. Today, one in 4300 people (23 per 100 000) either have an MD or are at risk of an MD in the future, and it is suspected to be seriously underdiagnosed. (Ng & Turnbull, 2016; Schaefer *et al.*, 2019).

Essentially, MDs may be split into two categories: primary and secondary MDs. Primary MDs involve mutations in the nuclear and/or the mitochondrial DNA encoding proteins found in the mitochondrion. Secondary MDs, on the other hand are caused by factors external to the mitochondrion (Koopman *et al.*, 2016).

In this section, the phenotypes and genotypes of MDs, the cellular consequences and responses to CI deficiency – the most common primary MD (Swalwell *et al.*, 2011) – and an NDUFS4 defect will be described.

2.3.2 Phenotypes and genotypes

Over the years, the clinical profile of MDs has expanded remarkably. Initially, it was thought that the “ragged red fibres” of the muscle tissue was the hallmark of MDs, but it was later shown that neurodegeneration was also a central characteristic of this myopathy. In the cases where brain lesions is the sole determining factor in the diagnosis, the term “mitochondrial encephalopathy” is used (DiMauro, 2004).

It has been observed that paediatric MD patients tend to present a severe, progressive form of the disease as a result of a recessively inherited nuclear gene defect. This is because it is under the strict authority of Mendelian inheritance. Adult-onset cases, on the other hand, are usually mtDNA mutations, which is under the more lenient authority of mitochondrial genetics (Lightowlers *et al.*, 2015).

Yet, linking the genetic profile (genotype) and the clinical profile (phenotype) of an MD is often very difficult. As a large spectrum of seemingly unrelated clinical phenotypes is connected to MDs, the diagnostic, prognostic and treatment strategies are exceptionally challenging. In fact, these phenotypes range from exercise intolerance to blindness and MDs may ensue in any decade of life (Chinnery & Keogh, 2018; Pretorius, 2011). On genetic level, the mitochondrion is the only organelle in the cell that is under control of the nDNA as well as the mtDNA. Mutations in both the mtDNA and nuclear DNA could lead to heightened ROS production, an altered redox status and an abnormal microstructure of the mitochondrion (de Haas *et al.*, 2017; Pretorius, 2011).

Placing the focus on primary MDs, we find that diseases resulting from mutations in mtDNA are not as common as diseases resulting from nDNA. Only 13 of the 80 respiratory chain subunits are encoded by the mtDNA. Yet, mutations in mtDNA may lead to defective protein synthesis from protein coding genes, which result in severe muscle weakness, cerebellar ataxia, retinopathy and deafness, to mention only a few clinical presentations (Manjeri, 2017; Tuppen *et al.*, 2010). Nevertheless, DiMauro (2004) describes mtDNA as a “slave” of nDNA, as nDNA encodes several factors that are critical for mtDNA transcription, translation, replication and maintenance. Mutations in the 67 nuclear encoded subunits of CI-CV, as well as the subunit assembly factors, might thus lead to defective OXPHOS assembly, but also to a defective “life cycle” of mtDNA. Alterations in mitochondrial dynamics and the lipid milieu of the IMM might also occur (DiMauro, 2004; Manjeri, 2017).

Taken together, the heterogeneity of MDs complicates the development of effective therapies. Therefore, the MRL at the NWU has taken up the challenge to gain a better understanding of the mechanisms of a CI defect – using an NDUFS4 KO mouse model as a representative for the defect – with the long-term aim of developing a new, viable treatment.

2.3.3 Cellular consequences and responses to CI deficiency

The rule of nature is to adapt or die. On a cellular level, this rule is no less applicable, as a cell affected by a CI deficiency will also try to adapt in order to survive. However, the cell would first be subject to serious and direct biochemical consequences because of the functional impairment of the enzyme. These include an increase in NAD(P)H levels, increased use of succinate by CII to power the respiratory chain, redox state changes, excess ROS production, disruption of mitochondrial membrane potential, mitochondrial fragmentation, an altered calcium homeostasis, modified ATP/ADP production and an altered iron homeostasis (Manjeri, 2017; Valsecchi *et al.*, 2013; Terburgh *et al.* 2019). Additionally, Van der Westhuizen *et al.* (2003) writes that genes engaged in structural elements, mitochondrial bioenergetics and cellular stress would be differentially expressed.

The fact of the matter is that amino acid metabolism – the pathways that are responsible for the formation and breakdown of proteins and enzymes – is influenced by an OXPHOS defect. In CI-deficient mouse muscle tissue, an activated starvation response and elevated serine (Ser) levels have been observed. This means decreased formate production and severely affected thymidylate synthesis, purine synthesis and cellular methylation reactions (Tyynismaa *et al.*, 2010). Therefore, an affected amino acid metabolism would inevitably lead to a global, but tissue-specific, transcriptional response (Frazier *et al.*, 2017).

Yet, there are many possible adaptive responses that depend on the type and extent of the CI deficiency, the genetic make-up of the organism and the type of tissue that is affected. Examples of these cellular adaptive responses are cellular lipid peroxidation, thiol redox state alteration, mitochondrion filamentation and higher levels of MT expression (Manjeri, 2017; Pretorius, 2011).

The latter adaptive response is particularly important in this study. Although MT's will be discussed in detail in Section 2.4.2 of this study, it is fair to mention that the antioxidant capacities of MT open a window for investigating a possible new therapy for patients suffering from Leigh Syndrome. Thus, the next step would be to gain a better understanding of the influence of MT-overexpression on the function and regulation of the oxidative stress and 1-C metabolic pathways.

2.3.4 Leigh Syndrome: an NDUFSA4 defect

Clinical Phenotype and Prevalence

Throughout the world, Leigh Syndrome (LS) – a subacute necrotising infantile encephalopathy (OMIM #256000) – affects 1 in 40 000 live births, which makes it the most common clinical presentation of CI disease. Dr Archibald Denis Leigh was the first to report this “curious” but fatal neuropathology in a six-month-old infant.

Phenotypically, the disease distinguishes itself with developmental retardation, ataxia, hypotonia, optic atrophy, dystonia, swallowing difficulties, dysarthria, breathing abnormalities, hearing impairment and failure to thrive (Quintana *et al.*, 2012). Interestingly, Quintana *et al.* (2012) found similar symptoms being presented by *Ndufs4* knockout mice. These mice were also very small and manifested hypothermia, sensory abnormalities and lethargy.

In humans, the onset of the disease usually follows a few months of normal development. However, the progressive, episodic neurodegeneration leads to death at the early age of about 3 years. Adult-onset cases are very rare (Lake *et al.*, 2015). Nevertheless, the consistent neuropathological characteristics of Leigh disease are bilateral, symmetric lesions in the basal ganglia and brain stem. These lesions display gliosis, capillary proliferation, vacuolation and only relative neuronal preservation. Additionally, lactic acid levels are elevated in the cerebrospinal fluid and the blood. In 75% of patients, respiratory arrest is the cause of death. (Distelmaier *et al.*, 2009; Lake *et al.*, 2015; Quintana *et al.*, 2012).

On a genetic level, there are more than 35 gene mutations of nDNA as well as mtDNA origin that code for CI, that are causative of LS (Lamont *et al.*, 2017). However, Lake *et al.* (2016) mentions that mutations in the nuclear encoded *Ndufv1*, *Ndufv2*, *Ndufs1*, *Ndufs2*, *Ndufs3*, *Ndufs4*, *Ndufs7*, *Ndufs8*, *Ndufa1*, *Ndufa2*, *Ndufa9*, *Ndufa10*, *Ndufa12*, *Ndufaf2*, *Ndufaf5* and *Ndufaf6* genes, specifically, result in Leigh or Leigh-like Syndrome. However, LS is most frequently caused by a defective NDUFSA4 subunit, which leads to an unstable CI (Sterky *et al.*, 2011).

Lake *et al.* (2015) also postulates that the genetic background of an LS patient, the epigenetic changes of the DNA and environmental influences (such as infections) might contribute to the manifestation of this encephalopathy. In other words, that an LS presentation is not singularly dependent on the primary genetic mutation in CI.

The heterogeneity of LS alone emphasises the call for an effective therapy. Therefore, this study strived towards a better understanding of the mechanisms and effects of the disease

caused by the *Ndufs4* gene mutation, and the possibility of MT being a viable treatment of Leigh Syndrome.

Current Therapies

The mitochondrion has only been linked to human disease since the 1960's. Of course, research regarding pharmacological intervention in MDs started immediately. Testing substance toxicity, long term-effectiveness, manner of administration, concentration, bio-distribution, turnover and clearance was of critical importance. Substances that have been studied with positive outcome during the last 50 years include: benzothiazepine CGP37157, riboflavin, benzaifibrate, resveratrol, rapamycin, idebenone and nicotinamide riboside. During the same time, nutritional interventions (such as the ketogenic diet) and environmental interventions (such as creating hypoxic surroundings) have also shown great potential as therapies for CI-deficient patients. Exogenous antioxidants such as Vitamin E and C, N-acetyl cysteine amide, MitoQ, SkQ and the Szeto-Schiller (SS)-31 peptide have also been found to mitigate the clinical symptoms of Leigh Disease (Ferrari *et al.*, 2017; Giorgio *et al.*, 2012; Manjeri, 2017).

Yet, as stated above, most of these therapies act as small exogenous molecular effectors, aiming to relieve the symptoms, and stimulate mitochondrial respiration and ATP synthesis. Therefore, the fight against cellular oxidative stress also depends on the effective exogenous intake of antioxidants and their subsequent therapeutic effect (Giorgio *et al.*, 2012; Lukienko *et al.*, 2000).

According to research conducted at the MRL, however, MT1 opens new doors to the treatment of Leigh Syndrome (Lindeque *et al.*, 2010; Lindeque *et al.*, 2012; Lindeque *et al.*, 2015; Olivier, 2004; Pretorius, 2006; Pretorius, 2011; Reinecke *et al.*, 2006). The qualities that distinguish MT from any of the other antioxidants mentioned above, is the fact that it is an endogenous antioxidant that plays an important physiological role in neuroprotection, neuronal repair, Ca^{2+} homeostasis, and in metal detoxification and its highly inducible expression.

Models for Investigating Leigh syndrome

During the last century, rats and mice were common models in human disease research, because of simple obtainability, rapid reproduction, comparative biochemistry and the highly conserved mammalian genome (Nuffield Council on Bioethics., 2005; Perlman, 2016). Yet, in 1980 the first transgenic mice were bred and in 2007 M.R. Capecchi, M.J. Evans and O.

Smithies won the Nobel prize for the first mouse gene knock-out model (Franco, 2013). In the meantime, methods have been developed to switch gene transcription on or off *in vivo*.

Kruse *et al.* (2008) were the first to create a full body *Ndufs4* knock-out mouse in the Palmiter laboratory. They made use of the Cre-Lox gene knock-out system to delete exon 2 of the *Ndufs4* gene. This system is based on the recognition of 34 specific DNA base pairs (lox sequences) by the Cre recombinase enzyme, which then excises and rearranges the DNA according to the orientation of the lox sequences (Carter & Shieh, 2015). In this case exon 2 of the *Ndufs4* gene was excised and deleted, which caused a frameshift mutation and an inactive ~830kDa subcomplex, instead of a fully assembled CI. Native protein analyses, enzyme activity assays, electron microscopy and documentation of the phenotype and respiratory analyses via Seahorse lead them to conclude that without the NDUF54 subunit, CI fails to assemble properly and is therefore unstable and dysfunctional.

Later, mouse models that would express the human phenotype of Leigh syndrome were designed, but with different mechanisms of suppressing the function of CI. These models included the whole body *Ndufs4*^{FKY/fky} mouse (Leong *et al.*, 2012), the Nes *Ndufs4*^{-/-} and PC *Ndufs4*^{-/-} mouse (Quintana *et al.*, 2010), the conditional whole body *Ndufs4*^{-/-} mouse (Quintana *et al.*, 2010), the *Ndufs4*-PM mouse (Ingraham *et al.*, 2009), the conditional heart-specific *Ndufs4*^{-/-} mouse (Sterky *et al.*, 2012) and the conditional hematopoietic-, liver- and TLR2/4-specific *Ndufs4*^{-/-} mouse (Jin *et al.*, 2014).

The effect of MT overexpression in CI disease has been investigated at the MRL since 2003. Experimental models, including *Mt1b* and *Mt2a* overexpressing HeLa cells, plus *Mt1*, *Mt2* and *Mt3* KO mice, have been studied over the years (Lindeque *et al.*, 2010; Pretorius, 2011; Reinecke, 2004; Reinecke *et al.*, 2006). Because of the lack of a suitable NDUF54-deficient mouse model at the time, rotenone – a potent CI inhibitor – was used to externally induce a CI defect. However, *in vitro* results were inconclusive about nuclear OXPHOS gene transcription and cellular ROS levels after rotenone treatment. Furthermore, not all tissue types showed the same sensitivity towards rotenone, and the chemical induction did not imitate the full phenotypical spectrum (Pretorius, 2011; Reinecke, 2010).

Hence, the MRL sided with Jackson (JAX) Laboratories (USA) – a non-profit biomedical research institution that specialises in the breeding of genetically modified research animals – for the larger study. Heterozygous *Ndufs4*^{+/-} mice were ordered from JAX laboratories, and, with these mice, a breeding programme was launched by NWU researchers at the PCDDP Vivarium so that a series of *Ndufs4* knockout mice could be obtained (Mereis, 2018).

However, the MRL also focusses on the effect that MT1 has on a CI defect, and a new approach to external *Mt1* overexpression induction was necessary. Palmiter *et al.* (1993) was the first group of researchers to have successfully developed a transgenic MT1-overexpressing (TgMT1) mouse model by inserting a 4kb minimally marked *Mt1* gene between the original *Mt1* and *Mt2* genes. They have shown that the transgene mRNA was distributed in the tissues in a similar way to that of the endogenous MT1 mRNA, and that the transgene expression was position-independent and copy number-dependent. Therefore, a TgMT1 mouse model has also been incorporated into the breeding programme at the PCDDP Vivarium (NWU). Crossbreeding these mice with the *Ndufs4* KO opened new doors for the understanding of the impact that MT1 overexpression has on a CI deficiency.

2.4 OXIDATIVE STRESS METABOLISM

2.4.1 Definition and applicability

The term oxidative stress was first used by Helmut Sies (1985) and may be defined as a disruption of the balance between cellular ROS production and the abundance of antioxidant defences. Thus, the use of the term “oxidative stress metabolism” in this study, refers to the intracellular production of the damaging $\cdot\text{OH}$ and $\text{O}_2^{\cdot-}$ radicals, the consequent scavenging of these radicals by reduced glutathione (GSH) and MT, the conversion of $\text{O}_2^{\cdot-}$ to H_2O_2 by superoxide dismutase 2 (SOD2) and the neutralising of H_2O_2 by catalase (CAT) and glutathione peroxidase (GPX1). In short, the metabolic reactions that exist to maintain the cellular redox balance.

It is, however, important to note that GSH, MT, SOD2, GPX1 and CAT are part of a larger antioxidant defence system composed of a multitude of compounds, both dietary and endogenous, which is not mapped out in this dissertation.

As explained in Section 2.3.4, an *NDUFS4* defect not only leads to elevated ROS levels, but also to altered expression of genes involved in mitochondrial bioenergetics and cellular stress (Manjeri, 2017; Van der Westhuizen *et al.*, 2003). Additionally, it is expected that the transgenic overexpression of *Mt1* in mice would have a direct impact on the reactions in oxidative stress metabolism. Therefore, focus will be directed to the biological role and context of MT in the next sections and along the way, the metabolic functions of SOD2, CAT and GSH/GSSG will also become clear.

2.4.2 Metallothionein

Structure, function and location

Margoshes and Vallee (1957) were the first to describe MT. It was detected by way of electrophoresis while performing a study on heavy metal toxicity in a horse kidney and was described as the “cadmium protein”.

Having a molecular weight of 6 to 7kDa, MTs are small, cysteine rich proteins, found in all eukaryotes. They occur intracellularly and are distinguished by their dumbbell-like shape (see Figure 2.4). Having an abundance of sulphur atoms exposed to the outside of the molecule, an MT has the ability to bind 7-12 metal ions via a mercaptide bond – hence the name ‘**metallothionein**’ (Lindeque *et al.*, 2010; Suzuki, 1996).

Additionally, there are four isoforms of MT, namely MT1, MT2, MT3 and MT4. The isoform MT3 is primarily expressed in the central nervous system and brain, and MT4 occurs only in squamous epithelial cells of the skin, tongue and intestinal lining. Yet, MT1 and MT2 may be found in all the major organs (Park *et al.*, 2001; Lindeque *et al.*, 2010). Since it is well known which stimuli lead to the expression of the *Mt1* gene and that it is ubiquitously expressed, this study focusses on the effects of overexpression of this specific isoform in NDUFS4-deficient tissue.

Having a specific affinity to divalent essential metals, like Cu and Zn, MTs play a critical role in metal homeostasis by chaperoning metal ions (mostly Zn and Cu) to specific enzymes, metalloproteins or gene transcription factors in the mitochondrion. In fact, this is described as the main function of MTs. However, during the process of heavy metal detoxification, MTs act as metal scavengers. Because of several thiol groups that form part of the structure of MTs, the protein has the ability to bind covalently to sulfhydryl-reactive heavy metals like Cd and Hg. In the case of Cd toxicity, for example, GSH and MT form complexes with the free Cd. Consequently, it is made unavailable for the cell to metabolise and oxidative stress mechanisms are barred. In the end, the chelated MTs would be excreted by the kidneys, as MT levels have been found to be elevated in Cd-exposed patients. Only when GSH is depleted, or when Cd replaces Fenton metals from MT, is ROS production elevated (Babula *et al.*, 2012; Cuypers *et al.*, 2010; Juarez-Rebollar *et al.*, 2017; Tohyama *et al.*, 1981).

Importantly though, Lindeque *et al.* (2010) writes that these proteins have an outstanding antioxidant capacity and that they can protect the very vulnerable mtDNA against ROS. MTs have also been reported to be connected to immune system responses, protein-protein and

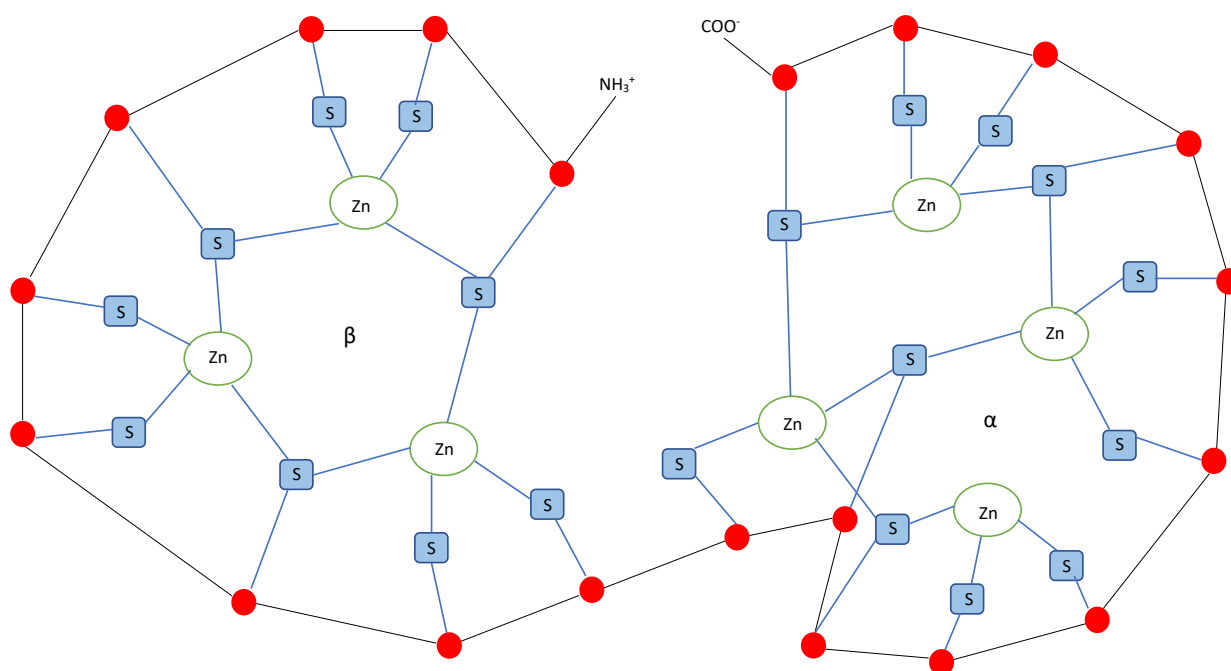


Figure 2.4 An illustration of the structure of the MT protein. The red circles indicate the twenty sulphur-bound cysteine residues, which are divalently bound to Zn: three Zn-bonds in the β-chain and four in the α-chain. Adapted from Juarez-Rebollar *et al.* (2017).

protein-nucleotide interactions, energetic metabolism regulation, cell cycle, angiogenesis, zinc fingers and Zn-based transcription factor regulation, mitochondrial function, cell differentiation and apoptosis (Babula *et al.*, 2012). Interestingly, expression of MTs is carefully regulated by the redox balance that is sustained by the mitochondrial respiratory system. In fact, MT levels are relatively low under normal physiological conditions. Expression of MTs is induced in the case of chemical and physical stress, neurodegeneration, and exposure to external substances (Babula *et al.* 2012).

Translocation of MT to the nucleus during oxidative stress is also thought to play a role in protection against oxidative damage. In a balanced system, MTs would quickly translocate through nuclear pore complexes to the inside of the nucleus, where it would be oxidised as described above. It would then be transported back to the cytosol and finally be excreted by the kidneys in its chelated form (Babula *et al.*, 2012; Tohyama *et al.*, 1981).

It is because of this wide array of functions and the inducible expression of MTs, that the MRL has decided to investigate this protein extensively in the context of CI disease.

Maintenance of the redox balance

Under normal physiological conditions, endogenous molecules such as glutathione peroxidase, superoxide dismutase, vitamins and glutathione work to maintain a stable redox balance in the cell. Of these, glutathione is the primary endogenous antioxidant. Thus, for a cell, the preservation of reduced glutathione (GSH) for the sustenance of a healthy ratio of GSH/GSSG (GSSG = oxidised glutathione) is a matter of life or death (Lindeque *et al.*, 2010). Normally, GSH and MT work together in the exchange and transport of Zn^{2+} , Cu^{2+} (oxidised) and Cu^+ (reduced) ions inside the mitochondrion, with the purpose of recycling endogenous antioxidants and maintaining the cellular metal homeostasis. This is made possible by the cluster structure of Zn^{2+} bound to MT (Zn_7MT) (Kang, 2006; Lindeque *et al.*, 2010). This not only provides thermodynamic stability with kinetic lability for Zn, but also a way for the cysteine ligand to induce oxidoreductive properties.

Even though MTs are known to be excellent scavengers of ROS – having an $\text{OH}^\bullet$ radical-scavenging ability about 340-fold greater than that of glutathione, as Abel and de Ruiter (1989) demonstrated in an *in vitro* study – they only become the primary antioxidants in the event of glutathione depletion. Under physiological conditions, MTs act as zinc chaperones for metal-dependent transcription factors, for the activity of metalloproteins and for the regulation of gene expression (Kang, 2006). However, in the event of oxidative stress, the MT molecule would release the Zn from its binding place so that all 20 of the cysteine sulphur atoms could be engaged in the quenching of radicals (Kang, 2006).

Lindeque *et al.* (2010), Kang (2006) and Ruttkay-Nedecky *et al.* (2013) explain oxidative stress and the involvement of MT1 in cellular redox homeostasis at the hand of the MT-glutathione redox and metal exchange cycle (Figure 2.5). As discussed in Section 2.2.2, molecular oxygen is converted to O_2^- when the fully oxidised form of CIV receives electrons from cytochrome c and donates electrons to O_2 . From here, the O_2^- radical may either be converted back to O_2 , or it may be catalysed by superoxide dismutase to form hydrogen peroxide. CAT is then responsible for the conversion of H_2O_2 back to water and O_2 . Yet, the H_2O_2 may follow another path; it may be broken down to $\text{OH}^\bullet$ and O_2^- inside the mitochondrial matrix, where highly reactive molecules such as these are immediately scavenged by the reduced form of glutathione (GSH).

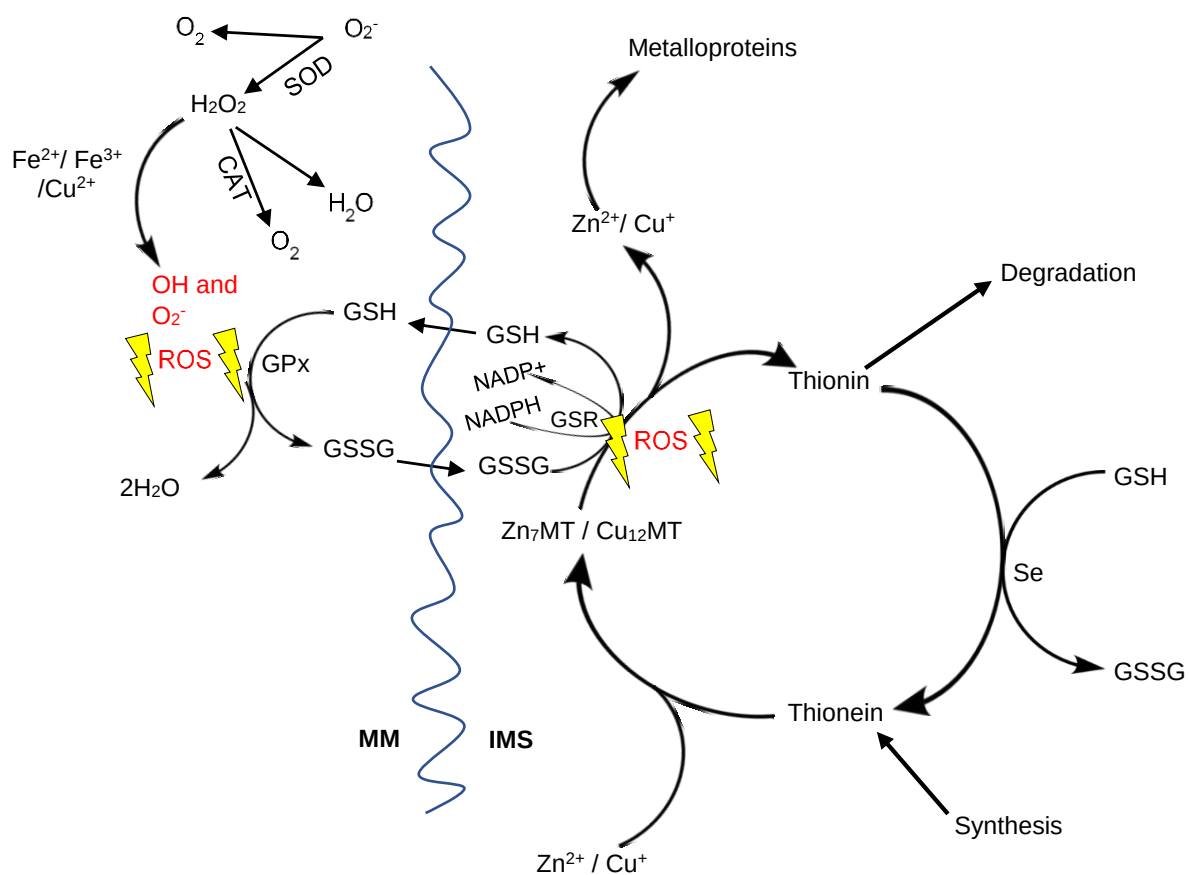


Figure 2.5 The MT-glutathione redox and metal exchange cycle, together with oxidative stress metabolism. Adapted from Kang (2006), Lindeque *et al.* (2010), Konyalioglu *et al.* (2013) and Florence (1984).

During the scavenging process of this pathway, free hydrogen peroxide is reduced to water by glutathione peroxidase and GSH is oxidized to glutathione disulfide (GSSG). GSSG is then transported to the intermembrane space (IMS), where it is reduced back to GSH by MT1. At the same time, Zn^{2+} or Cu^+ is released from the MT1 molecule and lipid peroxidation is diminished. Thionin (T), the compound that is left, is then subject to degradation. Yet, a rise in the GSH/GSSG ratio would reduce T back to a Zn-bound MT by way of a Se-catalysed reaction. In turn, Zn binds to thionine and the cycle starts again.

Metallothionein expression regulation

The expression of *Mt1* is induced by metal toxicity, ROS (oxidative stress), inflammation and increased glucocorticoid levels. It is mainly regulated by the Zn finger transcription factor MTF-1, a metalloregulatory protein that binds in a Zn-concentration-dependent fashion to the promoters with its Zn fingers. The elements in the regulatory region of *Mt1* that are responsible for expression regulation, are the TATA box (a core promoter element), MRE (metal responsive elements) and ARE (antioxidant responsive elements). The only metal that is responsible for the activation of *Mt1* is Zn, but this gene can also be activated by oxidative stress (Juarez-Rebollar *et al.*, 2017).

However, the effect of an NDUFS4 defect in mice on the expression of genes involved in oxidative stress metabolism, and whether or not *Mt1* overexpression mitigates this effect, is yet an open question. The relative expression of certain enzymes involved in this pathway was investigated in transgenic mice.

2.5 ONE-CARBON METABOLISM

2.5.1 Definition, biological importance and applicability

1-C metabolism can be defined as a set of folate-centred reactions that have the transfer of methyl groups in common. As methyl groups are too volatile to exist on their own, they travel through the pathways by binding to different intermediates at different stages of metabolism. As seen in Figure 2.6, it consists of three methylation pathways and a transsulfuration pathway. Throughout these reactions, Ser is the main 1-C contributor for all these methylation reactions (Ludueno, 2015; Nikkanen *et al.*, 2016).

This metabolism – in effect a metabolic network – is also directly involved in DNA synthesis, epigenetic DNA methylation and amino acid metabolism: Methylene-THF is used in thymidine synthesis (which is incorporated into DNA), formyl-THF is important in the synthesis of purines, and methyl-THF methylates homocysteine to form methionine (Met) (Louw, 2004).

Homocysteine (HCY) links 1-C metabolism to oxidative stress metabolism. While HCY may be remethylated to Met in a vitamin B12-dependent fashion, it may also be transsulfurated, dependent on vitamin B6, to eventually form glutathione, the main endogenous antioxidant in mammalian cells. In fact, about 50% of all cysteine residues that need to be incorporated into glutathione originate from this reaction (Dalto & Matte, 2017; Kaput & Rodriguez, 2006).

Figure 2.6 The four sections of 1-C metabolism: the folate/B12 independent remethylation pathway, the transmethylation pathway, the folate/B12 dependent remethylation pathway and the transsulfuration pathway. Enzymes are indicated in red and cofactors in green. Adapted from Skovierova *et al.* (2016), Lee *et al.* (2009), Nikkanen *et al.* (2016) and Landgraf *et al.* (2016).

Interestingly, lipoic acid synthase (LIAS) also associates with oxidative stress metabolism. Not only is it also an endogenous antioxidant that scavenges a large spectrum of ROS, but it is also a radical S-adenosyl methionine (SAM) enzyme. Taking a step back, most of the Met molecules are converted to SAM – a molecule central to all methylation reactions. LIAS, however, is part of a superfamily of enzymes that contain [4Fe-4S] cluster cofactors, with which they catalyse the reductive cleavage of SAM back to Met and a 5'-deoxyadenosyl radical.

Additionally, LIAS performs cofactor functions for mitochondrial enzymes that participate in energy production and it regenerates other antioxidants like oxidised vitamin C and E, CoQ10 and glutathione (Landgraf *et al.*, 2016; Mayr *et al.*, 2011).

As described earlier, MT is a cysteine rich endogenous antioxidant protein with a radical-scavenging ability about 340-fold greater than that of GSH, and oxidative stress induces the expression of MT when GSH is depleted (Lindeque *et al.*, 2010). Moreover, recent studies of the MRL have shown that the levels of Ser, Met and dimethylglycine (DMG) were abnormal in muscle tissue of mice with an *Ndufs4* mutation (Terburgh *et al.*, 2019). However, the effect of MT overexpression on the gene expression of enzymes involved in:

- the formation of Ser from glucose, the simultaneous interconversion between Ser and Gly, and THF and N-5,10-methylene-thf, the formation of formate and purines from N-5,10-methylene-THF, and the simultaneous formation of DHF from N-5,10-methylene-THF and dTMP from dUMP in the folate/B12-dependent remethylation pathway;
- the conversion of betaine to DMG in the folate/B12-independent remethylation pathway;
- the conversion of HCY to Met, and of Met to SAM in the transmethylation pathway and
- the formation of GSH/GSSG from HCY in the transsulfuration pathway

is investigated in this study, using an *NDUFS4*-deficient mouse model. The specific genes that were selected for the investigation, are motivated in Section 2.7.

2.6 GENE TRANSCRIPTION AND REGULATION

For cells and organisms to function correctly, the accurate synthesis of proteins is crucial. The Central Dogma of molecular biology (Figure 2.7), as described by Crick (1957), depicts the process of protein synthesis as the passing of hereditary information from nucleic acids into a protein molecule in a one-way direction.

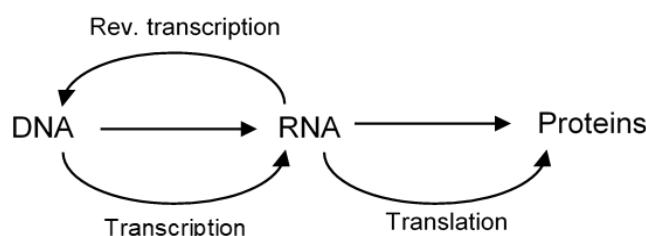


Figure 2.7 A visual depiction of the central dogma of molecular biology. Rev. = reverse. Adapted from Crick (1970).

Terburgh *et al.* (2019) used multi-platform metabolomics to investigate the metabolites produced in the skeletal muscle of NDUFS4 KO mice and currently, the phenotypes of NDUFS4 KO and TgMT1 mice are also being researched in the MRL. However, using the principle of reverse transcription, this study investigates the expression of selected genes in healthy and diseased mice.

This would provide a better understanding of the impact of an NDUFS4 defect upon the *regulation* of gene expression and of the reasons behind the production of specific metabolites. Even though the gene expression regulatory network functions on many different levels – from chromatin (or epigenetic) modification to post-translational modification (Nature Education, 2014) – transcriptional regulation is of interest in this study.

Indeed, mitochondrial ROS has been implicated in gene transcription regulation by way of retrograde mitochondria-to-nucleus signalling. When the mitochondrial membrane potential decreases as a result of a deficiency, intracellular Ca^{2+} -ions activate kinase and phosphatase enzymes that, in turn, activate transcription factors (TFs). These then bind to the *cis*-acting AREs located in the gene promoter, and enhance or repress the expression of the gene. Oxidative stress specifically triggers the binding of nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), nuclear respiratory factor 1 (Nrf1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (da Cunha *et al.*, 2015; Raghunath *et al.*, 2018; Reinecke *et al.*, 2009). These signalling pathways then defend oxidative stress, restoring redox homeostasis.

On the other hand, the mammalian target of rapamycin (mTOR) is a primary regulator of 1-C metabolism. Being sensitive to cellular Ser levels, mTOR activates the TF, termed activating transcription factor 4 (ATF4), when Ser levels are low, which subsequently binds to the promoters of genes that encode for the biosynthesis of Ser and enhances their expression. Thus, when the Ser pools have been restored, 1-C metabolism can continue to provide methyl groups for growth and cellular proliferation. However, when energy levels and nutrient levels are low, AMP-activated kinase (AMPK) is activated, so that energy may be conserved and anabolic reactions may be slowed down (Laplane & Sabatini, 2009; Rosenzweig *et al.*, 2018). Additionally, it has been reported that SAM directly regulates mammalian target of rapamycin complex 1 (mTORC1) activity; therefore, it may be said that 1-C metabolism send signals back to mTOR (Gu *et al.*, 2017).

It should, however, be taken into account that metabolism and regulation of gene expression differs greatly in brain and skeletal muscle tissue, the two respective tissue types chosen for this study, as they are both rich in mitochondria. Muscle tissue functions with oxidative metabolism, glycolysis of glycogen, anaerobic glycolysis, cleavage of phosphocreatine, phosphorylation/ rephosphorylation of ATP and ADP, as well as fatty acid catabolism. By contrast, brain tissue is not capable of anaerobic respiration and fatty acid catabolism. Instead, it directly metabolises glucose (without the use of insulin) and oxygen (Hall & Guyton, 2012). Additionally, the amount of transcription factors in mouse brain tissue is almost double than that in mouse skeletal muscle tissue (Zhou *et al.*, 2017)

Therefore, the differences in gene expression regulation among diseased, healthy and TgMT1 overexpressing tissue – both whole brain and skeletal muscle – of ten selected genes in both 1-C metabolism and oxidative stress metabolism, were of great interest.

2.7 OBJECTIVE 4: SELECTION OF THE GENES TO BE INVESTIGATED

As mentioned in Section 2.5, 1-C metabolism is directly involved in DNA synthesis, DNA methylation and amino acid metabolism. Through Ser and Met, methyl groups are transferred among folate-dependent intermediates that affect gene regulation, signal transduction, carnitine biosynthesis, polyamine biosynthesis and the transsulfuration of homocysteine to form glutathione (Ducker & Rabinowitz, 2017; Landgraf *et al.*, 2016; Louw, 2004; Luduena, 2015). Furthermore, oxidative stress metabolism is directly related to the cellular redox balance. Under steady state conditions, glutathione and other antioxidants are responsible for the scavenging of ROS. Glutathione and MT also work together in the exchange of Zn and Cu inside the mitochondrion, so that endogenous antioxidants may be recycled and reused (Lindeque *et al.*, 2010).

In the past, many studies have focussed on the expression regulation of the genes that are directly responsible for the control of the OXPHOS system and how it is influenced in the case of MD (Reinecke *et al.*, 2009). In the MRL especially, a considerable amount of research has been invested in the metabolic- and phenotypical changes that occur in the face of an OXPHOS defect (Lindeque, 2011; Pretorius, 2011; Terburgh *et al.*, 2019; Van Dyk, 2016). As these studies have brought the greatly affected 1-C metabolism and oxidative stress metabolism to light, this study aimed to dig deeper by investigating the effects of an NDUFS4 defect and *Mt1* overexpression on the expression of certain genes involved in these two metabolic pathways.

Using existing literature, 1-C metabolism and oxidative stress metabolism were studied carefully in order to identify the most important enzymes. In the end, ten genes were selected for the investigation: seven from 1-C metabolism and three from oxidative stress metabolism. Two housekeeping genes were also selected to act as reference genes in the relative quantification process.

The expression of these genes was investigated in mouse quadriceps and whole-brain tissue in the context of an NDUFS4 defect and the overexpression of *Mt1*. The motivation behind the selection of these tissue types may be seen in Section 2.6.

2.7.1 *Mthfd2*: methylenetetrahydrofolate dehydrogenase/ cyclohydrolase/ formyltetrahydrofolate synthetase 2¹

This gene encodes for the protein that acts as a gateway to the mitochondrial branch of the folate cycle. Due to being bound to both a cyclohydrolase and a formyltetrahydrofolate synthetase enzyme, this mitochondrial enzyme is a trifunctional enzyme: it is responsible for the interconversion of the methylene, methenyl and formyl derivatives of THF with the help of NAD⁺ or NADP⁺. Additionally, it contributes to purine synthesis by reversibly converting THF to N-5,10-methylene-THF, while oxidising NADPH to NADP⁺ and converting ATP to ADP (Chen *et al.*, 2002; Ju *et al.*, 2019; Nikkanen *et al.*, 2016). The mitochondrial master switch mTOR1 and the AMPK signalling pathway also have significant transcriptional effects on *Mthfd2*. When nutrients and energy are abundant, and cellular Ser pools are low, mTOR1 signalling upregulates *Mthfd2*. When nutrients and energy are low, however, AMPK acts as a brake in anabolic pathways (Ben-Sahra *et al.*, 2016; Rosenzweig *et al.*, 2018). Therefore, an energy deficiency and a redox imbalance – as observed in a CI defect – is hypothesised to impact the expression of *Mthfd2*. Because of the lower NAD⁺/NADH ratio and the lower ATP production observed in a CI defect, a downregulation of *Mthfd2* is expected.

¹ EC 6.3.4.3

2.7.2 *Gclc* : glutamate-cysteine ligase catalytic subunit²

This enzyme is also known as gamma-glutamyl synthetase. It plays an indispensable role in the transsulfuration of homocysteine and links 1-C metabolism to oxidative stress metabolism: It is rate-limiting in the synthesis of the endogenous antioxidant, glutathione. The expression of this gene is regulated by the binding of transcription factors Nuclear Factor Erythroid 2 Like 1 (Nrf1) and Nuclear Factor Erythroid 2 Like 2 (Nrf2) to the antioxidant response element (ARE) in its promoter. This means that it is sensitive to ROS levels (National Center for Biotechnology Information., 2019a; Yang *et al.*, 2005). Therefore, with oxidative stress resulting from a CI defect, upregulated *Gclc* expression levels are expected in this study.

2.7.3 *Mtrr*: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase³

This protein regenerates Met synthase to a functional state by reducing the cobalamin-dependent 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) part of the Met synthase enzyme. It is central in folate metabolism (as Met synthesis calls for 1-C group transfer by a folate donor), it is essential for the remethylation of homocysteine and it is vital for homocysteine removal during embryonic development (McKusick, 2017). Additionally, the methylation of specific CpG regions in the promoter of *Mtrr* affects the expression of this gene. This methylation of the CpG regions is, in turn, regulated by mTOR and AMPK signalling pathways (Bai *et al.*, 2019). As a disrupted Hcy metabolism (similar to that which is caused by a CI defect) leads to oxidative stress and a redox imbalance (Skovierova *et al.*, 2016), it could be reasoned that the epigenetic methylation reactions would also be affected, and thus, *Mtrr* expression as well. It is hypothesised that *Mtrr* would be downregulated in the context of a CI defect.

2.7.4 *Bhmt*: betaine-homocysteine s-methyltransferase⁴

This enzyme catalyses the conversion of betaine to DMG and of homocysteine to Met. This step is necessary for the irreversible oxidation of choline and is a key player in the folate/B12-independent remethylation pathway (see Figure 2.6) (Teng *et al.*, 2011). In a study on NDUFS4 KO mice by Terburgh *et al.* (2019), a downregulation of DMG and betaine was reported, together with perturbed SAM levels. It is postulated that the produced DMG is used in the electron transfer flavoprotein (ETF/ETF-QO) system as an adaptive mechanism. As there is a SAM-sensitive element in the promoter of *Bhmt* (Castro *et al.*, 2002), this would mean that the expression of *Bhmt* might also be affected in the case of a CI defect. In fact, it

² EC 6.3.2.2.

³ EC 2.1.1.13

⁴ EC 2.1.1.5

is predicted that *Bhmt* expression would be downregulated in the mice used in this study, as a result of the lower availability of SAM in the context of an NDUFS4 deficiency.

2.7.5 *Tyms: thymidylate synthetase*⁵

The enzyme that this gene encodes, catalyses the reductive methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP), with the folic acid coenzyme, methylene-THF, acting as a methyl donor. Therefore, it sustains the dTMP pool and purine synthesis, which is vital for DNA replication and repair (McKusick & Kniffin, 2010). Even though the *de novo* dTMP synthesis is a key player in folate-associated pathologies, 1-C metabolism is programmed to favour this pathway in the nucleus at the expense of homocysteine remethylation in the cytosol (Field *et al.*, 2016). However, Coetzer (2019) reported decreased levels of UMP in the anterior cortex of NDUFS4 KO mice, highlighting a disturbed *de novo* purine production. The cellular abundance of the TYMS enzyme is regulated at both transcriptional and posttranslational levels. In other words, the translation of *Tyms* mRNA is autoregulated by the TYMS protein – its own end product (Chu *et al.*, 1991). Therefore, if TYMS levels are low in these mouse tissues, it would lead one to expect a downregulation of *Tyms* expression in the NDUFS4 KO mice investigated in this study.

2.7.6 *Sod2: Manganese superoxide dismutase 2 (mitochondrial isoform)*⁶

The *Sod2* encodes a manganese-containing, tetrameric, mitochondrial isozyme that breaks damaging free superoxide radicals down to H₂O₂ and molecular oxygen. It plays a central role in the physiological oxidative metabolism by protecting cells against ROS (McKusick & Gross, 2009; National Center for Biotechnology Information., 2019c). The *Sod2* gene has been found to have a binding site in the proximal promoter regions where the ROS-sensitive NF-κB can bind and regulate gene expression (Miao & St Clair, 2009). Therefore, in a mouse model where ROS-levels are postulated to be elevated (Valsecchi *et al.*, 2013), it is expected that *Sod2* expression would be upregulated, in an attempt to protect the cell from damage.

2.7.7 *Cat: catalase*⁷

SOD2 and CAT work together to protect the cell against ROS. If the H₂O₂ (generated by SOD2) roams free, cellular oxidative damage (especially lipid peroxidation) would be inevitable. Hence, CAT neutralises the H₂O₂ in the peroxisome to harmless water and oxygen (Bansal & Kaushal, 2014; Glorieux *et al.*, 2015). Together with SOD and GPx, CAT

⁵ EC 2.1.1.45

⁶ EC 1.15.1.1

⁷ EC 1.11.1.6

is a cornerstone enzymatic antioxidant in the physiological oxidative stress defence mechanism. The regulation of *Cat* expression is also through the binding of ROS-sensitive NRF2 to the ARE of the promoter of the gene (Zhu *et al.*, 2005). For this reason, it is expected that *Cat* expression levels would be elevated in an NDUFS4-deficient mouse model.

***2.7.8 Gpx1: glutathione peroxidase 1 (cytoplasmic isoform)*⁸**

This Se-dependent enzyme has a function similar to that of CAT – it reductively inactivates H₂O₂ and organic hydroperoxides. This way, it maintains the thiol redox balance, protects the cell against oxidative damage and is important for mitochondrial function (Handy *et al.*, 2009; McKusick & O'Neill, 2005). Interestingly, *Gpx1* is regulated by both the presence/absence of oxygen, as well as the binding of the transcription factor NF-κβ to the gene promoter (Morgan & Liu, 2011). In the case of glutathione being overwhelmed by ROS, *Gpx1* expression might be impacted. In fact, it is hypothesised that *Gpx1* would attempt to restore the redox balance and thus, expression would be elevated in the NDUFS4 KO mouse tissues investigated in this study.

***2.7.9 Lias: lipoic acid synthase*⁹**

LIAS is one of eight Radical SAM enzymes – a super family of enzymes that contain [4Fe-4S] cluster cofactors with which they catalyse the reductive cleavage of SAM back to Met and a 5'-deoxyadenosyl radical – that are found in mammals. Lipoic acid is, however, an important physiological antioxidant. Besides scavenging ROS, it also regenerates other antioxidants like oxidated vitamin C and E, CoQ10 and glutathione. Additionally, it performs cofactor functions for mitochondrial enzymes that participate in energy production (Hartz & Kniffin, 2016; Landgraf *et al.*, 2016; Louw, 2018). *Lias* is partially regulated by glucose- and ATP levels (Padmalayam *et al.*, 2009; Rosenzweig *et al.*, 2018); however, there is also a transcription factor binding site for Nrf1: a ROS-responsive TF (Lancet *et al.*, 2019). As the NDUFS4 KO tissues used for this study are postulated to have high ROS levels and low ATP levels, it is predicted that *Lias* expression would be upregulated, in an attempt to restore SAM levels and to scavenge ROS.

⁸ EC 1.11.1.9

⁹ EC 2.8.1.8

2.7.10 *Phgdh*: phosphoglycerate dehydrogenase¹⁰

PHGDH is an enzyme responsible for the conversion of 3-phosphoglycerate to 3-phosphohydroxypyruvate. This reaction forms part of the folate/B12-dependent remethylation pathway that converts glucose to Ser, and plays a role in the synthesis of glycine and N-5,10-methylene-THF, which in turn contributes to purine synthesis (National Center for Biotechnology Information., 2019b; Ou *et al.*, 2015). *Phgdh* codes for the rate-limiting enzyme in the *de novo* biosynthesis of Ser, the main methyl donor of 1-C metabolism (Louw, 2018; Nikkanen *et al.*, 2016). However, one of the metabolomic signatures of NDUFS4 KO mouse brains is a decrease in free amino acids, which confirms perturbed mTOR activity and metabolic signalling. Additionally, a build-up of glycolytic intermediates has been reported in the brains of NDUFS4 KO mice, as energy metabolism starts to rely more on glycolysis than OXPHOS. Therefore, an upregulation of *Phgdh* expression is expected (Johnson *et al.*, 2013).

2.7.11 *Actβ*: beta actin

The ACT β protein is abundant and essential in all eukaryotic cells and it contributes to the cytoskeletal structure of a cell, muscle contraction, gene expression regulation, tissue morphogenesis, cell adhesion and developmental reprogramming (Rodriguez & Kashina, 2018). Because *Actβ*, and therefore ACT β , is essentially part of the cellular backbone, both the gene and protein are regarded as housekeeping molecules (Livak & Schmittgen, 2001; Pfaffl, 2004; Sudiwala *et al.*, 2016). Hence, it has also been chosen as one of the reference genes for this study.

2.7.12 *B2m*: β -2-microglobulin

The β 2M protein forms part of the adaptive immune molecules, the major histocompatibility complex (MHC), class I and II. It is vital for immune modulation and surveillance in all vertebrates. As it is expressed in most nucleated cells and fluids, including synovial fluid, serum and urine, it is regarded as a housekeeping gene. Also, it has been shown that *β 2m* is expressed in a stable manner in hematopoietic stem cells, even after different treatments (Matsuzaki *et al.*, 2015). This quality lead to *β 2m* also being commonly used as a reference gene for RT-qPCR assays in the MRL, and also for the assays of this study (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

¹⁰ EC 1.1.1.95

2.8 PROBLEM STATEMENT

MDs form the largest collection of congenital metabolic defects in humans, with a prevalence of one in 4300 people (23 per 100 000) (Schaefer *et al.*, 2019). The characteristic energy deficiency, oxidative damage and apoptosis are also associated with the pathogenesis of the well-known and fatal Alzheimer's and Parkinson's diseases.

Since MDs are of genetic origin, treatment approaches are extremely difficult. Clinical symptoms are often the main target of treatment; however, in most cases, it is very challenging to connect the genotypes to the phenotypes. In addition, there is no viable treatment to date, that can address severe cellular consequences like redox imbalance, excess ROS production, disruption of mitochondrial membrane potential, altered ATP/ADP ratio and, ultimately, cell death.

Metabolomics research has shown that 1-C metabolism and oxidative stress metabolism are greatly affected in CI deficiencies – the most common of MDs. This is important, as 1-C metabolism not only plays an essential role in the maintenance of the redox balance, but it also contributes to *de novo* purine production and to the formation of glutathione. The abundance of enzymes in oxidative stress metabolism are consequently impacted by possible perturbed glutathione levels and excess ROS levels. Therefore, the changed metabolite-levels point to possible changes in gene expression of enzymes in these two networks, resulting from a CI defect.

There is a lack in the complete understanding of the mechanisms behind the cellular consequences of a CI defect, which hinders the development of effective patient treatment strategies. Thus, it is a relevant problem that the changes in gene expression – and therefore the *regulation* of gene expression – of these enzymes have not yet been investigated.

Furthermore, MT1, an endogenous antioxidant, has also been shown to have therapeutic potential in the context of a CI disease, but the effect of the overexpression of Mt1 upon the expression of genes involved in 1-C metabolism and oxidative stress, has not yet been investigated.

2.9 LARGER STUDY

The impact that congenital mitochondrial energy defects have on the socio-economic status of South-Africa is unfathomable (Schoonen, 2019). Hence, the members of the MRL investigate the NDUFS4 defect – as a representative of CI deficiencies – on genetic, metabolite and phenotypical levels. The purpose is to understand the mechanisms of CI defects *in vivo* and to understand whether Mt1 overexpression might have a therapeutic effect upon subjects with a CI defect, so that a more effective, viable and affordable antioxidant therapy for paediatric patients might be developed.

Strategies to develop a breeding programme of genetically modified mice, to evaluate the phenotype of these mice, to assess the mice on a biochemical level, and to investigate their metabolome have been refined and put into practice in this laboratory (see Figure 2.8). This specific project, forms part of *Strategy 3: Biochemical evaluation of mouse strains*, as it will involve the structure-and-function analyses of RC enzymes, the investigation of the protective effect of MT against oxidative stress and the evaluation of the expression of genes involved in oxidative stress metabolism and 1-C metabolism in CI-defective mice.

2.10 AIM, OBJECTIVES AND EXPERIMENTAL STRATEGY

The aim of this study is two-fold: Firstly, to investigate the change in expression and the expression regulation of selected genes involved in 1-C metabolism and oxidative stress in a CI-deficient mouse model. Secondly, to investigate whether Mt1 overexpression mitigates these changes in a CI-deficient-TgMt1 mouse model.

As seen in Figure 2.9, a schematic representation of the experimental strategy for this study, the **first objective** is to collect tissue samples for characterisation of the mouse models, as well as for gene expression investigation. Characterisation of both the NDUFS4 and the TgMT1 mouse models via DNA, RNA and protein analyses is the **second objective**. These analyses were performed with one liver sample of each of the four genotypes. The **third objective** is the genotyping of each of the brain and quadriceps samples set aside for gene expression investigation, in order to confirm the absence/presence of the *Ndufs4* gene. **Objective four** is the selection of genes to be investigated, based on recent literature and laboratory results. The quantification of mRNA from tissue samples from each genotype and the measurement of relative expression of the selected genes collectively form **objective five**. The **sixth objective** is comprised of the comparison of gene expression in NDUFS4 KO and TgMT1 mice to that of WT:WT mice.

2.11 BASIC HYPOTHESIS

The expression of the selected genes of the 1-C and oxidative stress metabolic pathways in the KO:WT tissue samples would be up- or downregulated in correspondence with recent literature. Additionally, we expect the overexpression of *Mt1* in the KO:OVER tissue samples to affect the regulation of expression of these genes in a manner that promotes the restoration of the cellular redox balance. Taken together, we hypothesise that MT1 would prove to be a viable antioxidant treatment option for patients suffering from Leigh syndrome.

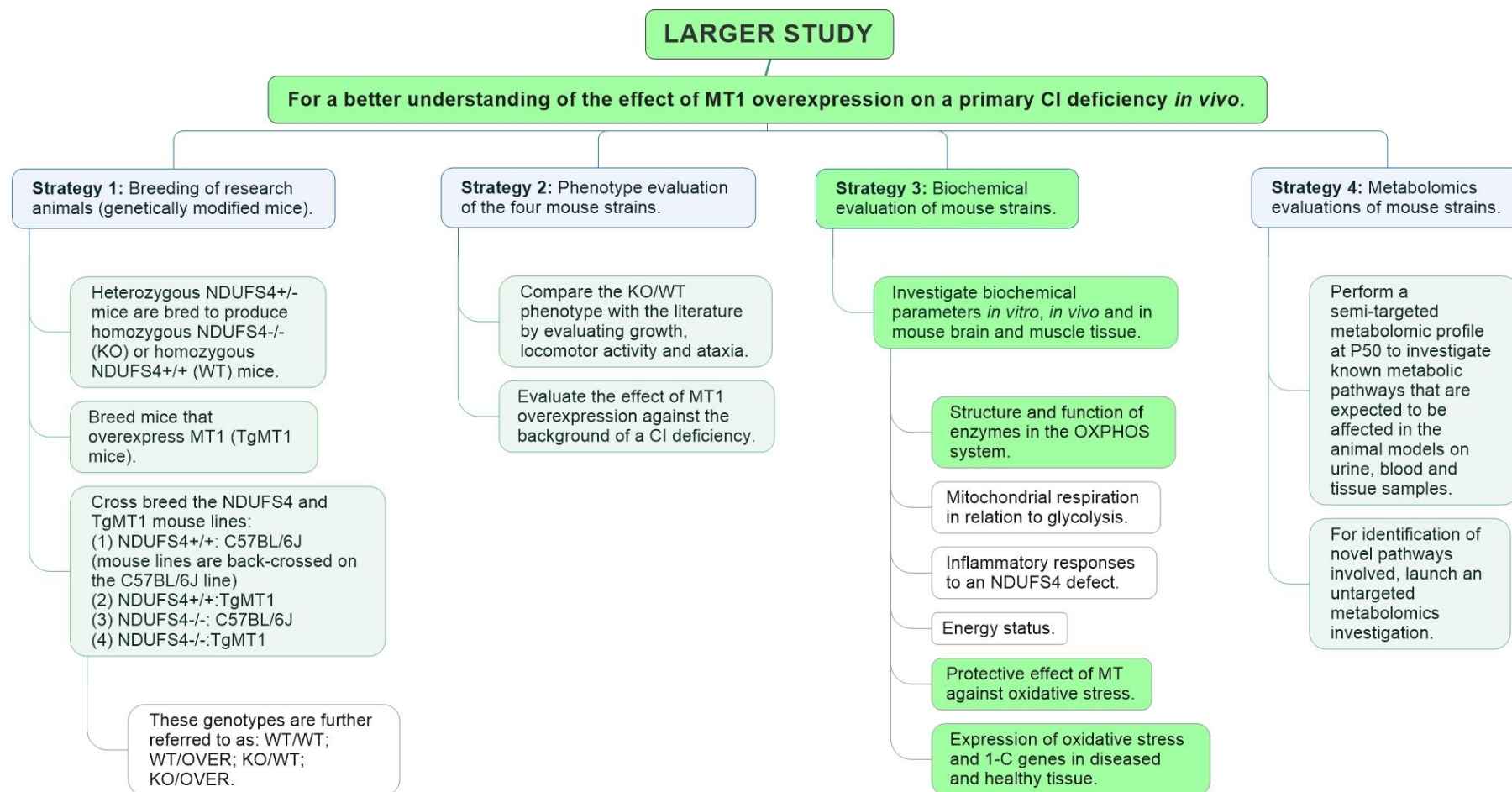


Figure 2.8 A diagram of the larger study conducted by the MRL. The study in this dissertation is indicated in bright green (Strategy 3).

EXPERIMENTAL STRATEGY

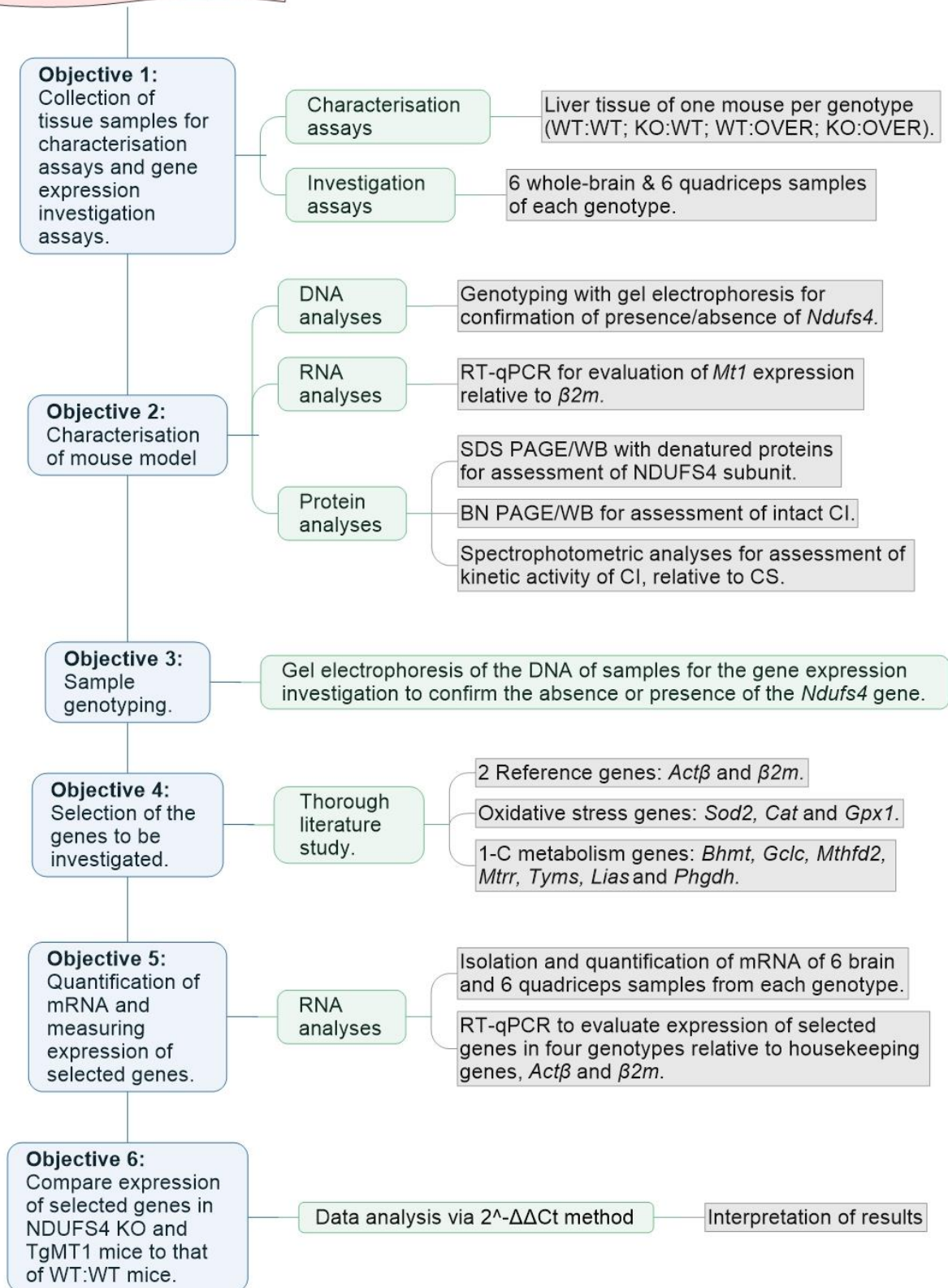


Figure 2.9 **A visual depiction of the experimental strategy of this study.** The five objectives of this study are listed vertically with descriptions protruding to the right. SDS PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; WB = western blot; BN-PAGE = blue-native polyacrylamide gel electrophoresis; CS = citrate synthase; RT-qPCR = reverse transcription quantitative real-time polymerase chain reaction.

CHAPTER 3: THE MOUSE MODELS

3.1 INTRODUCTION

As indicated in Chapter 2, this study used rodents (mice) as the experimental model to investigate the research question. As reported in this chapter, and to confirm the correct genotypes, the NDUFS4¹ mouse model used in this study was evaluated on DNA and protein levels, whereas the TgMT1 mouse model was evaluated on RNA level.

Firstly, the environment, handling and breeding of the experimental mice will be described. Methodological details, as well as the results to confirm the genetic make-up of the NDUFS4 and TgMT1 mouse models will follow thereafter. Throughout this chapter, the abbreviations listed in Table 3.1 will be used to distinguish easily among the four mouse genotypes relevant to this study.

Table 3.1 The abbreviations used for the four genotypes of importance.²

Genotype	Abbreviation
<i>Ndufs4</i> ^{+/+} / <i>TgMt1</i> ^{-/-}	WT/WT
<i>Ndufs4</i> ^{+/+} / <i>TgMt1</i> ^{+/+}	WT/OVER
<i>Ndufs4</i> ^{-/-} / <i>TgMt1</i> ^{+/+}	KO/OVER
<i>Ndufs4</i> ^{-/-} / <i>TgMt1</i> ^{-/-}	KO/WT

Under Objective 1, the sample collection and -preparation is described. The characterisation made use of liver samples, because these assays were simply for the confirmation of the molecular identity of each mouse. Objective 2 (Section 3.3) consists of a motivation of the methodology for the genotyping assays, gene expression assays, protein analyses and kinetic enzyme assays. The results for each of these investigations are also described.

¹ Throughout this dissertation, proteins are written in uppercase (e.g. NDUFS4) and genes are written in italicised sentence case (e.g. *Ndufs4*) (MouseMine, 2019).

² NDUFS4 = NADH:Ubiquinone Oxidoreductase Subunit S4; TgMT1 = transgenic metallothionein 1; WT = wild type; KO = knockout; OVER = metallothionein overexpressing.

3.2 COLLECTION OF SAMPLES FOR CHARACTERISATION ASSAYS (OBJECTIVE 1)

3.2.1 *Breeding, care and identification of mice*

This project has been approved by the Animal Care, Health and Safety in Research Ethics Committee (ANIMCARE) of the Faculty of Health Sciences of the NWU (Ethics number: NWU-00364-16-A5).

In 2015, TgMt1 overexpressing (B6.Cg-Tg(*Mt1*)174Bri/J) and CI knock-out (B6.129S4-*Ndufs4*^{tm1.1Rpa/J}) mice strains were obtained from Jackson Laboratories (Maine, USA). A six-phase strategy for the breeding of these mice (Figure 3.1) was set up by another M.Sc. student in the Mitochondrial Research Laboratory (MRL) at the Potchefstroom Campus of the NWU. The four different genotypes (WT:WT, KO:WT, WT:OVER and KO:OVER) were bred from *Ndufs4* and TgMt1 heterozygous mice (see Step 4 in Figure 3.1), as NDUFS4 KO mice do not live long enough to reach sexual maturity. The offspring were to be used by a number of students of the MRL for research purposes (Mereis, 2018).

Mouse breeding pairs and offspring were housed and cared for at the Vivarium of the Preclinical Drug Development Platform (PCDDP, SAVC reg. no. FR15/13458) of the Potchefstroom Campus of the NWU. Mice were kept in individually ventilated polysulphone cages with shredding paper and polycarbonate mouse houses or tubes. The humidity ($55 \pm 10\%$), temperature ($22 \pm 1^\circ\text{C}$) and light/dark cycles (12:12-h) were kept constant and the mice had free access to standard laboratory chow and water. The environment created for the mice complied with the code of ethics in research, training and testing of drugs in South Africa and was in accordance with national legislation.

At the age of post-natal day (P)21 to P23, mice were marked by punching holes into the ears according to this pattern: each hole in a different area of the ear represented a certain digit, therefore, combinations of digits would represent an identification number from 1-99. Furthermore, mice were categorised according to their litter number (e.g. 1a – 1z, 2a – 2z and so on) and their dates of birth (DOB). Records of the lineages were kept faithfully.

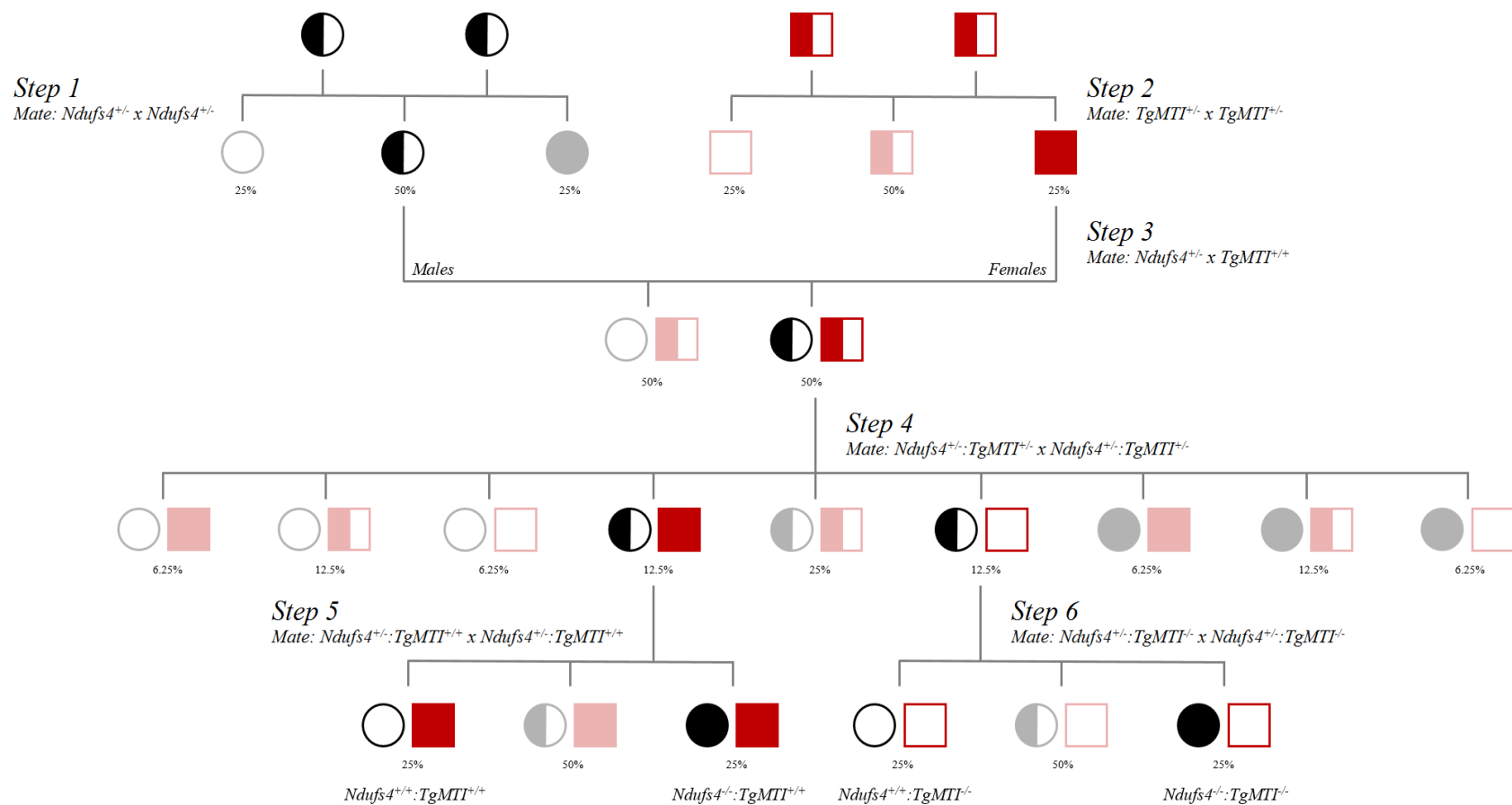


Figure 3.1 The mouse-breeding strategy. This was followed to obtain the WT:WT ($Ndufs4^{+/+}/TgMt1^{-/-}$), KO:WT ($Ndufs4^{-/-}/TgMt1^{-/-}$), WT:OVER ($Ndufs4^{+/+}/TgMt1^{+/+}$) and KO:OVER ($Ndufs4^{-/-}/TgMt1^{+/+}$) mouse genotypes.

◐ = Heterozygous $Ndufs4$ genotype; ○ and ● = Homozygous $Ndufs4$ genotypes; ◑ = Heterozygous $TgMt1$ genotype; □ and ■ = homozygous $TgMt1$ genotypes. Each step represents a new generation. Scheme used with permission from Mereis (2018).

3.2.2 Sample collection

Nine mice (see Table 3.2) were euthanised at P46-P51 by way of cervical dislocation and were dissected by a South African Veterinary Council (SAVC) authorised employee of the PCDDP in a sterile environment. Subsequently, the students from the MRL were allowed to collect tissue for their individual projects branching from the larger Mitochondrial Disease study (see Chapter 2, Section 2.9). For the characterisation of the NDUFS4 mouse model in this study, the livers were removed and transferred to 1.5ml microcentrifuge (polypropylene) tubes. Upon collection, all samples were snap frozen in liquid nitrogen, after which they were stored at -80°C. These procedures also complied with the code of ethics in research, training and testing of drugs in South Africa and were in accordance with national legislation. For the transcriptional analyses (see Chapter 4), the brain and quadriceps samples were collected in the same fashion. Before each analysis, the samples were fetched from the -80°C freezer and thawed on ice. Sample preparation differed among the characterisation analyses; therefore, the methodology of each section will contain the applicable sample preparation.

Pre-euthanasia, the wildtype and knockout mice could be distinguished based on their phenotypes, as the knockout mice were clearly smaller and weaker than the wildtype mice. However, it was difficult to distinguish the heterozygotes from the homozygotes based only on physical appearance. Therefore, the genotypes of each mouse that was bred in the larger study were genetically identified by the project manager (M. Mereis) when the mice were marked. For this project, though, each characterisation analysis used only one mouse of each genotype for the purpose of confirmation of their genotypes. Thus, Objective 2 (Section 3.3) served to confirm these genotypes in a selection of mice used in this study and to illustrate the methodology used. Each section will specify which mice were used.

Table 3.2 Mice used for the characterisation of the NDUFS4 and TgMT1 mouse models.

ID	Predicted Genotype	Sex
#12	WT:WT	Male
#75	WT:WT	Female
#65	WT:OVER	Female
#78	WT:OVER	Male
#74	KO:WT	Male
#86.1	KO:WT	Female
#2.3	KO:WT	Male
#86	KO:OVER	Female
#84	KO:OVER	Female

3.3 CHARACTERISATION OF MOUSE MODELS (OBJECTIVE 2)

3.3.1 Introduction

With the purpose of confirming the genotypes of the mice used in this study, the DNA, RNA and protein features of the mice listed in Table 3.2 were characterised. In other words, the presence, structure and function of the *Ndufs4* gene and the expression of the MT1 gene had to be confirmed on all three levels in each genotype.

DNA analyses included the isolation of DNA from the collected liver tissue, DNA amplification using polymerase chain reaction (PCR) and visualisation using gel electrophoresis. RNA analyses comprised the isolation of total RNA, which preceded the evaluation of the relative expression of metallothionein mRNA against a housekeeping gene. Protein analyses involved the assessment of the structure and function of the NDUFS4 protein via SDS-PAGE & western blot, blue native PAGE & western blot, as well as kinetic enzyme assays of CI activity relative to citrate synthase (CS) activity.

3.3.2 DNA Analyses

DNA Isolation, PCR and agarose gel electrophoresis

Principle

As explained in Section 2.3.4, the type of *Ndufs4* mutation investigated in this study is a deletion of exon two. Therefore, the sizes of the amplicons spanning this region would be different in the four genotypes of interest: the deletion of ~429 bp in the KO mice would result in a shorter PCR fragment. With primers that bind to the introns flanking exon two, the standard method of amplifying isolated DNA (first described by Mullis *et al.* in 1986), was applied. The *Ndufs4* primers that were used, were developed by Valsecchi *et al.* 2012. For the separation of PCR fragments and for visualisation and interpretation of the results, agarose gel electrophoresis (as first described by Aaij and Borst in 1972) was performed.

Methods

A ZR Genomic DNA™ – Tissue MiniPrep Kit¹ was used in the isolation of DNA from mouse liver tissue according to instructions from the manufacturer (composition of reagents was not provided). This kit was chosen for its ability to yield high quality DNA from solid tissues, the no-RNA-contamination promise from the manufacturer, the user-friendly workflow and

¹ Zymo Research Corporation (Irvine, CA, USA), Catalogue (Cat) #D3051.

affordability. The liver tissues (approx. 25mg each) from mice #74, #75, #78 and #86 (refer to Table 3.2), as well as four control samples², were used.

Before proceeding to PCR, the concentration and purity of the DNA had to be determined. This was done using a NanoDrop™ One/One^C UV-Vis Spectrophotometer³ – an instrument that uses the minimum amount of sample, requires no cuvettes and reliably yields both the concentration and purity of the DNA. The light absorbance of the 2µl DNA droplet was detected at a wavelength of 260nm. This value was then expressed as a concentration in ng/µl. A ratio of ~1.8 was deemed acceptable for DNA (Thermo Fisher Scientific, 2018b).

In preparation for the PCR, the forward and reverse primers⁴ (0.5µM each) for the *Ndufs4* gene (Table 3.3) and 1x PHIRE Tissue Direct (already containing a loading dye)⁵ was added to the diluted (25ng/ul) DNA samples and nuclease-free water. The PCR-tubes were then placed in a T100™ Thermal Cycler (BioRad), where the DNA was amplified according to the cycle conditions mentioned in Table 3.4.

Table 3.3 The sequences of the primers used in the genotyping of the *Ndufs4* gene.

Primer	Primer seq (5' to 3')	Size (bp)	Melting temp (°C)
<i>Ndufs4</i> 1060 (Fwd)	AGC CTG TTC TCA TAC CTC GG	20	62.45
<i>Ndufs4</i> Rev (Rev)	TTG TGC TTA CAG GTT CAA AGT GA	23	59.2

Fwd = Forward; Rev = Reverse; Seq = Sequence; Temp = Temperature.

Table 3.4 The applied PCR cycling conditions.

Step	1	2	3	4	5	6	7
Temp (°C)	98	98	57.3	72	-	72	4
Time (min/rep)	5:00	0:05	0:05	0:20	x 34 (step 2-3)	1:00	Infinite hold

Temp = Temperature; min = minutes; rep = repetitions.

After diluting the PCR products 3.3x, a volume of 10µl of each of the products was then loaded into a 1% (w/v) agarose gel. The gel contained 1x Bionic Buffer⁶ and ethidium bromide (EtBr) in a 1:40 ratio – the latter acting as a nucleic acid stain. The DNA alleles were then separated according to their size by way of electrophoresis: 40V for 15min and

² Genotypes of these samples were confirmed in the study of M.Mereis.

³ Thermo Fisher Scientific.

⁴ Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa

⁵ Thermo Fisher Scientific, Cat #F-170L.

⁶ Sigma Aldrich (Merck), (St. Louis, Missouri, USA), Cat #B6185.

70V for an hour. A 50bp DNA ladder⁷ was used as a molecular marker. In the end, the gel was photographed using a ChemiDoc™ MP system. The image was processed with the Image Lab (v 5.2.1) software.

Results and Discussion

The DNA concentrations of the four samples ranged from 107ng/μl to 549ng/μl, and the concentrations of the four controls ranged from 51.0ng/μl to 77.6ng/μl. The purity of the samples and controls was evaluated based on the A260/A280 ratio, and these values ranged from 1.80 to 1.86, which was deemed acceptable (Thermo Fisher Scientific, 2018b).

In the case of the WT:WT and the WT:OVER mice (#74 and #86), a large allele at ~1 229 bp could be observed. However, in the case of the KO:WT and the KO:OVER mice (#75 and #78), a much smaller allele at ~429 bp was observed. This confirmed the presence of the intact *Ndufs4* gene in the WT:WT and WT:OVER mice, as opposed to an incomplete *Ndufs4* gene in the KO:WT and KO:OVER mice. These data were similar to those of the control mice (as seen on the left of the gel image in Figure 3.2).

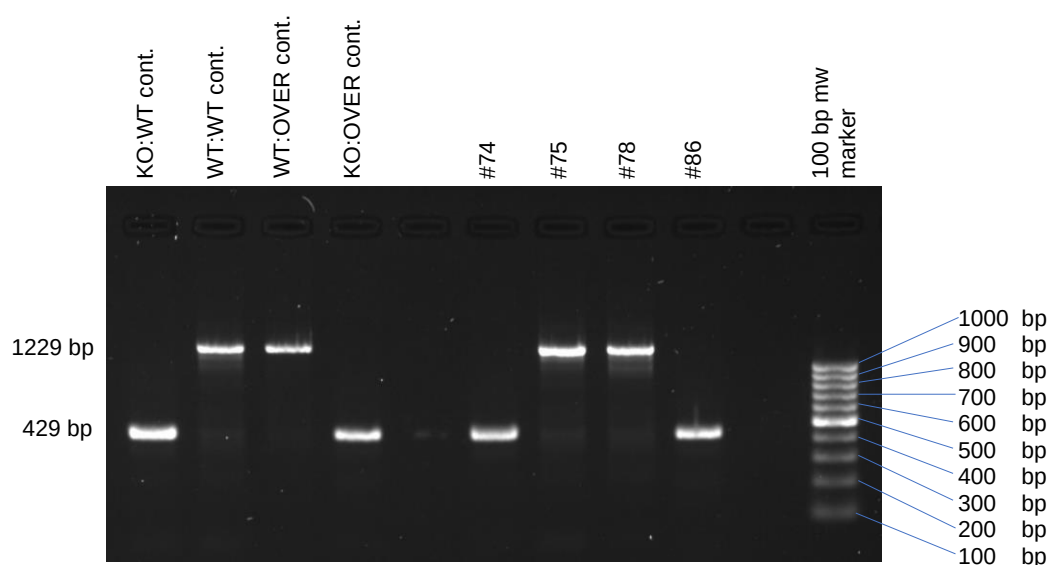


Figure 3.2 The ChemiDoc™ MP system gel image of the DNA alleles. One mouse from each of the four genotypes were used. From left to right: The DNA from four control mice, DNA from the four mice used for the characterisation assays, a 100bp molecular weight ladder. cont. = control; # = mouse number; bp = base pairs; mw = molecular weight.

⁷ Thermo Fisher Scientific, Invitrogen (Carlsbad, California, USA), Cat #10416014.

The spurious bands seen below the WT:WT and WT:OVER controls and samples #75 and #78, may be attributed to misprimed targets, leading to nonspecific amplification (Thermo Fisher Scientific, 2020). During optimisation of the *Ndufs4* primers by researchers of the MRL, it was decided to use the primers according to the method established by Valsecchi *et al.* (2012) and to use controls in all instances.

3.3.3 RNA Analyses

In order to establish the differences in expression of MT1 in the WT:WT, KO:WT, WT:OVER and KO:OVER mice, total RNA was isolated from the individual liver tissue samples of mice #2.3, #75, #78, #86 (refer to Table 3.2). Firstly, the integrity of the RNA was qualitatively examined via gel electrophoresis. This was followed by reverse transcription quantitative real-time PCR (RT-qPCR), where the complementary cDNA was synthesised and amplified and the relative expression of MT1 measured against a housekeeping gene [*β2m* (beta-2-microglobulin)].

Total RNA isolation and gel electrophoresis

Methods

Despite the manually intensive labour and the use of toxic phenol-based reagents, organic extraction methods are still considered the gold standard for total RNA isolation. This is mainly because the RNA can be stabilised, while nucleases and proteins can be rapidly denatured and biological material solubilised by these reagents. It has also been shown to yield high quality RNA with various kinds of tissues (Rio *et al.*, 2010; Thermo Fisher Scientific, 2019a). Thus, the monophasic solution of guanidinium isothiocyanate and phenol, called TRIzol® Reagent⁸, was used for all total RNA isolations in this study.

For characterisation, the total RNA of the four mouse liver tissues was isolated according to the manufacturer's protocol. Each RNA pellet was resuspended in 50µl of DEPC-treated water⁹ and divided into aliquots of 12.5µl each. While four of these aliquots were stored at -80°C, one was kept on ice. Using the NanoDrop™ One/One^C UV-Vis Spectrophotometer, the absorbance of the RNA was measured at 260nm and 280nm and the RNA concentration (ng/µl) and purity (A260/A280 ratio) values were automatically calculated. A ratio of ~2 was deemed an acceptable purity for the total RNA samples (Thermo Fisher Scientific, 2018b). In the end, the samples were diluted to 0.5µg/µl.

⁸ Thermo Fisher Scientific, Ambion, Life Technologies (Carlsbad, California, USA), Cat #15596026.

⁹ Thermo Fisher Scientific, Invitrogen, Cat #46-2224.

Consequently, the integrity of the total RNA was assessed, as one of the greatest pitfalls of RT-qPCR is working with low quality RNA samples. Indeed, degraded RNA has been found to compromise the derived gene expression results (Fleige *et al.*, 2006). Therefore, 2µl of each of the four mouse RNA samples, together with 2µl loading dye¹⁰ and 6µl DEPC-treated water were electrophoresed in an agarose gel (according to the same method explained in Section 3.3.2), so that the 18S and 28S rRNA fragments may be inspected. These fragments would indicate intact or degraded RNA (Bustin & Nolan, 2004).

Results and Discussion

A formaldehyde-based separation was not applied, as its RNA-denaturing effect would have defeated the purpose of confirming the suitability of the RNA isolation method. Referring to Figure 3.3, however, the 28S and 18S ribosomal RNA bands are well defined in all four samples. This meant that the total RNA isolation reagents and method were suitable and that the RNA was fit for further RT-qPCR analyses (Bustin & Nolan, 2004; Mansour, F.H. & Pestov, D.G. 2013). This served as a proof-of-concept analysis.

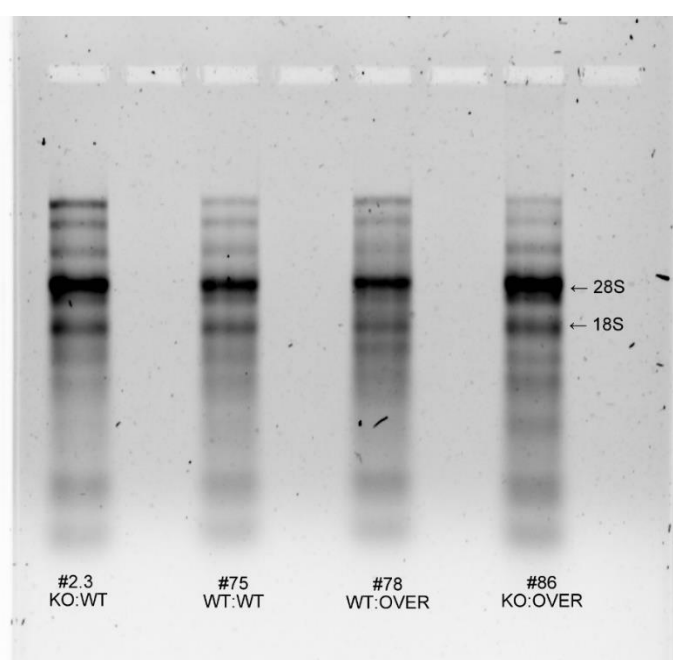


Figure 3.3 The ChemiDoc™ MP system gel image of the RNA bands in an agarose gel. Liver tissues of one mouse per genotype were used. The 28S and 18S rRNA bands are indicated with arrows, respectively.

¹⁰ Thermo Fisher Scientific, Cat #R0611.

RT-qPCR

Principle

To examine the expression of MT1 in the tissue of mice #2.3, #75, #78 and #86 (refer to Table 3.2), the mRNA that was present in the total RNA samples was subjected to RT-qPCR. It was decided that Taqman® chemistry would be used throughout this study, instead of SYBR Green-based detection, as it is more specific, reproducible and yields a high level of gene expression quantitation. The selected TaqMan® kit was based on the principle of a two-step process in one tube. The first step – catalysed by a reverse transcriptase enzyme and with gene-specific primers binding to the mRNA – was the synthesis of the cDNA from the mRNA template in a 3'→5' direction. The second step was the exponential amplification of this newly synthesised cDNA in a 5'→3' direction (Applied Biosystems, 2007; Applied Biosystems, 2016). The laser beam detection of fluorescent signals (created by the excision of the signal probe bound to the cDNA and the release of 6-carboxy-fluorescein, a.k.a. FAM) in real time, distinguished real-time PCR from conventional PCR (Life Technologies, 2013; Thermo Fisher Scientific., 2019c).

In each genotype, MT1 gene expression was measured relative to the expression of a housekeeping gene. The chosen housekeeping gene was β 2-microglobulin (*β 2m*), as it is stably expressed in most nucleated cells and fluids. Additionally, it was directly available in the MRL.

Method

The TaqMan® RNA-to-CT™ 1-Step Kit¹¹ was used according to the manufacturer's protocol¹². This kit consisted of a TaqMan® RT-PCR Mix (2x) and a TaqMan® RT Enzyme Mix (40x). The former was composed of AmpliTaq Gold® DNA Polymerase UP, dNTPs (including dUTP), a passive reference (Carboxy-X-rhodamine, or ROX), and optimized buffer components. The latter was composed of the ArrayScript™ UP Reverse Transcriptase enzyme and an RNase inhibitor. The gene-specific primer/probe mixes that were used for this characterisation assay are listed in Table 3.5.

¹¹ Thermo Fisher Scientific™, Cat #4392653.

¹² P/N 4392668.

Table 3.5 Details of the *Mt1* and $\beta 2m^{13}$ -specific primer/probe mixes.

Gene	Catalog #	Assay ID	Details
<i>Mt1</i>	4331182	Mm00496660_g1	Probe spans exons; Amplicon length: 88
$\beta 2m$	4331182	Mm00437762_m1	Probe spans exons; Amplicon length: 77

*Primer and probe sequences were not provided, as these were predesigned assays.

Each reaction had a final volume of 20 μ l and consisted of 1x TaqMan® RT-PCR Mix (2x), 1x TaqMan® RT Enzyme Mix (40x), 1x Taqman® Gene Expression Assay (20x) (i.e. either the *Mt1* or $\beta 2m$ gene-specific primer/probe mix), nuclease-free water¹⁴ and 100ng/rxn RNA template. The reactions with RNA templates of the four mice, as well as no-template controls, were loaded in triplicate into a MicroAmp™ Optical 96-Well Reaction Plate¹⁵. Before placing the plate in the heating block of the Applied Biosystems 7300 Real Time PCR System, it was sealed with a MicroAmp® Optical Adhesive Cover¹⁶. It was also centrifuged for a few seconds to free the wells of bubbles. The thermal cycling conditions (see Table 3.6) were specified in the Applied Biosystems Software.

Table 3.6 Thermal Cycling conditions for the RT-qPCR assays.

Instrument	Step	Temperature (°C)	Duration	Cycles
Applied Biosystems 7300 Real-Time PCR System	RT step	48	15 min	Hold
	Enzyme Activation	95	10 min	Hold
	Denature	95	15 sec	40
	Anneal/Extend	60	1 min	

For each reaction, the Applied Biosystems 7300 Real Time PCR System then yielded the cycle number where the fluorescence, produced by each amplification cycle of cDNA, crossed the cycle threshold. At the threshold cycle (Ct), a detectable amount of cDNA had been amplified during the exponential phase, leading to the fluorescent signal being notably greater than the background fluorescence at this point. Because of this, the Ct value of each reaction is very significant in the calculation of gene expression and in understanding the amount of gene-specific RNA present in each reaction (the higher the Ct value, the lower the amount of gene-specific RNA in the reaction). In order to compare RT-qPCR results in this

¹³ Thermo Fisher Scientific™.

¹⁴ Qiagen (Hilden, Germany), Cat #129114.

¹⁵ Thermo Fisher Scientific™, Cat #N8010560.

¹⁶ Thermo Fisher Scientific™, Life Technologies™, Cat #4360954.

study, the Applied Biosystems Software was set to consistently yield values at an arbitrary cycle threshold of 0.2 (Qiagen, 2018; Thermo Fisher Scientific, 2018a).

With the use of the $2^{-\Delta\Delta Ct}$ method (as described by Livak and Schmittgen (2001) and explained in detail in Chapter 4, Section 4.4.1), the resultant threshold cycle values (Ct values) of the reactions were used to calculate the expression of *Mt1* relative to $\beta 2m$ and normalised to the WT:WT sample. Thus, the average CT values of $\beta 2m$ was subtracted from those of *Mt1* (calculation of ΔCt). The ΔCt value of the WT:WT genotype was then subtracted from the ΔCt values of each of the other three genotypes ($\Delta\Delta Ct$) and the resultant $\Delta\Delta Ct$ values were then substituted into Equation 3.1:

$$\text{Fold change in gene expression} = 2^{(-\Delta\Delta Ct)} \dots\dots\dots \text{Equation 3.1}$$

As the control sample, the gene expression fold-change value for the WT:WT sample was set as 1.0. The *Mt1* expression of the KO:WT, WT:OVER, KO:OVER genotypes could thus be measured against that of the WT:WT genotype.

Results and Discussion

The RNA expression results of *Mt1* relative to $\beta 2m$ is summarised in Figure 3.4. The most evident feature of Figure 3.4 is the great amount of *Mt1* overexpression in the KO/OVER mouse (#86). This astounding 266.7-fold increase shows the natural cellular response because of a dysfunctional CI, in addition to the genetic stimulation of over expressing *Mt1*. Yet, it is also important to consider that upon evaluation of the validity of the reference gene, as described in Section 4.3.3, the $2^{-\Delta Ct}$ value of $\beta 2m$ was 0.65 (a stable reference gene would have a value close to 1). This might be attributed to the fact that only one sample per genotype was evaluated for *Mt1* overexpression.

The 37.4-fold overexpression of *Mt1* in the WT:OVER sample, might confirm the notion that MT1 is reserved for physiologically stressful situations: though it is described as potent, it is not abundantly available under normal circumstances (Lindeque *et al.*, 2010).

By contrast, there is practically no difference in *Mt1* expression between the KO:WT and WT:WT samples. Even though it should be taken into account that this analysis was only performed in one sample per genotype, similar findings in fibroblast cultures were reported by Mereis (2018). Moreover, in a study by Manjeri (2017), increased ROS levels, but no increased cellular lipid peroxidation or changes in thiol redox state was observed in fibroblasts from patients with an isolated CI deficiency. Though it is pointed out in Chapter 2, Section 2.4.2, that oxidative stress physiologically induces *Mt1* expression, this unchanged expression level seen in the KO:WT mouse might be attributed to other cellular adaptive

responses counterbalancing the ROS. These include mitochondrial filamentation, glycolysis upregulation and H₂O₂ scavenging by CAT (Manjeri, 2017; Tehrani & Moosavi-Movahedi, 2018). Another possible explanation might be that the results are simply mouse-specific or tissue-specific (unrelated to age).

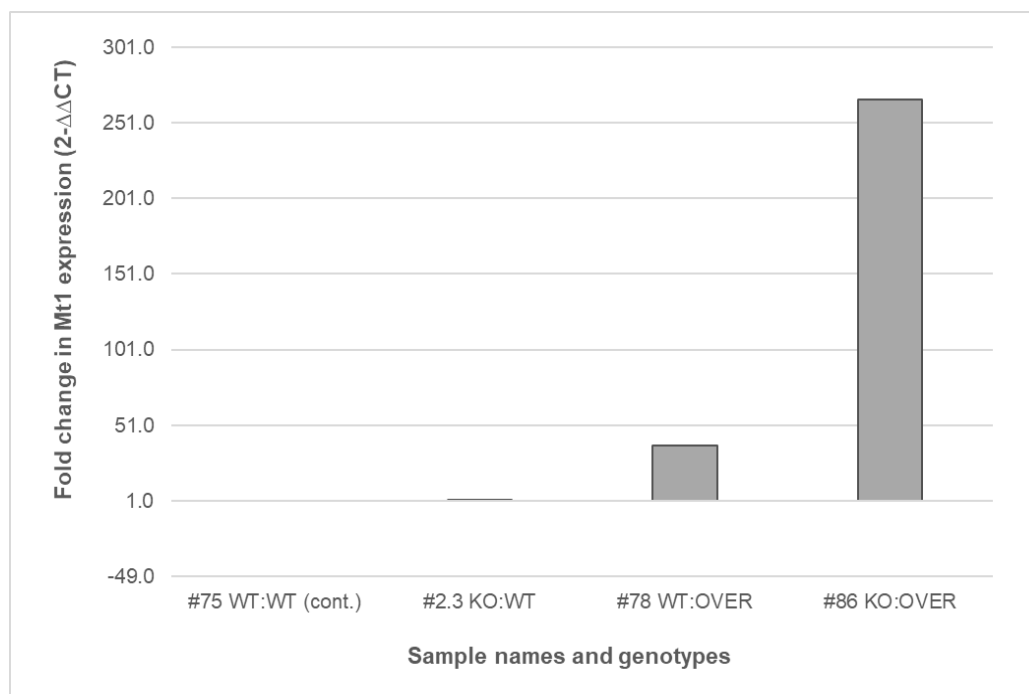


Figure 3.4 The change in expression of Mt1 in three genotypes. Results are relative to $\beta 2m$. The WT:WT genotype served as the control (cont.). Total RNA originated from liver tissue. One sample per genotype was used, thus no error bars are included.

3.3.4 Protein Analyses

Sample preparation

Method

A preparation of 600xg supernatants and enriched mitochondria was applied for the SDS-PAGE & western blot assay, BN-PAGE & western blot assay and kinetic enzyme analyses.

The liver tissues of the mice were each placed on a metal plate that rested on ice. Subsequently, they were finely minced and manually homogenised with a scalpel in order for a sample to be representative of the whole tissue. Weighed portions of the tissues were added to Zheng buffer (comprised of Mannitol¹⁷ (210 mM), Sucrose¹⁸ (70 mM), HEPES, free

¹⁷ Sigma Aldrich (Merck), Cat #M9546.

acid¹⁹ (5 mM) and EGTA²⁰ (0.1 mM), and set to a pH of 7.2 with 1M KOH²¹ at 10% (w/v). These were homogenised with a Potter-Elvehjem (polytetrafluoroethylene) PTFE pestle and glass tube²² (30 strokes, with the tube placed in ice water). When the homogenate appeared evenly opaque and no solid parts were in circulation, the homogenate was transferred to a 1.5ml microcentrifuge tube. It was then centrifuged for 10 minutes at 600xg at 4°C. The mitochondria-containing supernatant was transferred to a clean, marked tube and, if the mitochondria were not further enriched, stored at -80°C.

If the mitochondria were further enriched, as in the case of the BN-PAGE assay, these tubes were centrifuged for another 10 minutes at 600xg at 4°C. The supernatants were transferred again to clean, marked tubes and centrifuged at 20 000xg and 4°C for 20 minutes. This time, the supernatants were discarded, and the pellets were centrifuged for 5 minutes at 20 000xg and 4°C. The supernatants were removed, and the pellets were stored at -80°C. These were the enriched mitochondrial fractions.

The BCA assay for the determination of protein concentration

Method

Before any structural and functional analyses of the NDUFS4 subunit could be performed, the concentration of total protein of the 600xg supernatants had to be determined. For this, the bicinchoninic acid (BCA) method, first described by Smith *et al.* (1985), was followed. The sensitivity, accuracy and ease of performance of the BCA method had been found to be very comparable to the Kjeldahl method, which is considered the benchmark of protein quantification assays. Moreover, the BCA Protein Assay reagents from Sigma Aldrich (Merck) has been tried, tested and trusted over the years in the MRL.

The BCA working reagent was prepared in a 50:1 parts BCA:CuSO₄ ratio²³ in accordance with the amount of wells to be used in a 96-well plate. A standard series with known protein concentrations (4µg, 8µg, 12 µg, 16 µg, 20 µg) was prepared with 1µg/µl bovine serum albumin (BSA)²⁴. A constant final reaction volume of 220µl was maintained: of each 600xg sample, 2 µl was combined with 18µl MilliQ® H₂O in the respective wells of a micro-titre (96-well) plate, after which 200µl of the BCA working reagent was added to all of the wells.

¹⁸ Sigma Aldrich (Merck), Cat #S7903.

¹⁹ Sigma Aldrich (Merck), Cat #H3375.

²⁰ Sigma Aldrich (Merck), Cat #E4378.

²¹ Sigma Aldrich (Merck), Cat #P5958.

²² Sigma Aldrich (Merck), Cat #P7859-1EA.

²³ BCA (Cat #B9643-1L-KC) and CuSO₄ (Cat #C2284-25ml), Sigma Aldrich (Merck).

²⁴ Sigma Aldrich (Merck), Cat #P0914-10AMP.

The 96-well plate was then incubated for 30 min at 30°C and read at 562 nm in a Synergy™ HT Multi-detection microtiter plate reader.

The linearity of the standard curve generated by the Gene5™ software was checked before processing any data. With $R^2 > 0.98$, the protein content results were divided by the dilution ratio, after which the protein concentrations for both the standard series and the samples were computed using Equation 3.2 below.

$$\frac{\text{Avg protein content of triplicates } (\mu\text{g})}{\text{Sample volume } (2\mu\text{l})} = [\text{Protein}] \text{ in } \mu\text{g}/\mu\text{l} \dots\dots\dots \text{Equation 3.2}$$

With the known sample protein concentrations at hand, the BN-PAGE, SDS-PAGE and enzyme analyses could be performed.

Characterisation of the NDUFS4 protein

SDS-PAGE method

The process of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was first described by Laemmli (1970) as a method to denature and separate negatively charged proteins according to their differences in molecular weight in a gel-based electrical field. For the characterisation of the NDUFS4 protein, an SDS-PAGE assay was conducted to confirm the presence or absence of the NDUFS4 protein in the 600xg supernatants of liver tissue of mice #12, #65, #84 and #86 (refer to Table 3.2).

Prior to the assay, the protein concentrations of the four 600xg supernatants were determined, as described in Section 3.3.4. The assay was then conducted according to the revised and optimised protocol of the MRL, which is based upon the method of Laemmli (1970). In preparation for the samples to be loaded into the gel in the Mini-PROTEAN® Tetra Cell, a buffer mix was prepared by adding the disulphide-reducing agent β-mercapto ethanol (βME) to 2x Laemmli Sample Buffer²⁵ in a 1:19 ratio. This Laemmli Sample Buffer already contained 65.8mM Tris-HCl (pH6.8), 26.3%(w/v) glycerol, 2.1% SDS and 0.01% bromophenol blue. Each sample (diluted to 30μg) was then topped up to half of the final volume (20μl) with Milli-Q® water, then adding the buffer mix as the other half of the volume (the final concentration was therefore 1.5μg/μl). After loading the samples into the gel, the power supply was connected and the potential difference was set to 200V for 30 minutes. The proteins separated in a downwards direction. When the electrophoretic front had reached the bottom-third of the gel, the power supply was switched off and disconnected,

²⁵ Bio-Rad Laboratories (Hercules, California, USA), Cat #1610737.

the gel cassette was removed from the tank and the gel was photographed with the ChemiDoc™ MP Imaging system. A stain-free activation protocol was applied²⁶.

Western blot, immunodetection and chemiluminescent imaging method

This procedure, first described by Towbin *et al.* (1979), involved the electroblotting of the separated proteins onto a polyvinylidene difluoride (PVDF) membrane, i.e. a solid support. By way of antibody chemiluminescence, the NDUFS4 and VDAC1 protein bands could then be detected and distinguished. The housekeeping protein, VDAC, was used as a reference protein, as it is a building block of the mitochondrial permeability transition pore (mPTP) and is found in all mitochondria.

A Mini TransBlot® Turbo™ Transfer Pack²⁷, together with a TransBlot® Turbo Transfer System²⁸ was used according to the revised and optimised protocol of the MRL, which is based upon the method of Towbin *et al.* (1979), to transfer proteins from the gel onto a PVDF membrane. For the prevention of nonspecific binding of antibodies, the membrane was then incubated for 1½ hours in 1x TBS with 1% Casein²⁹ (the blocking buffer). The blot was incubated over night at 4°C with two types of primary antibodies diluted in 1x TBS with 1% Casein: mouse monoclonal anti-NDUFS4 [2C7CD4AG3]³⁰ primary antibodies and rabbit polyclonal anti-VDAC1/porin³¹ primary antibodies. Both types of antibodies were diluted in a 1:1000 ratio. However, two incubations with secondary antibodies followed, to avoid possible cross-reactions among the antibodies: the first incubation was with polyclonal goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies, and the second was with polyclonal goat anti-rabbit HRP conjugated antibodies. The secondary antibodies were diluted in a 1:10 000 ratio. The washing buffer that was used throughout the protocol consisted of 1x TBS³² and 0.001% Tween20³³, and the final wash solution was only 1x TBS. Care was taken at all times not to damage the membrane with an instrument. The moistness of the membrane was also preserved so that the final photograph could be of the best possible quality.

²⁶ Only the final photographs that were taken after the Western Blot assay have been inserted in this dissertation.

²⁷ Bio-Rad Laboratories, Cat #170-4156.

²⁸ Bio-Rad Laboratories.

²⁹ Bio-Rad Laboratories, Cat #161-0782.

³⁰ Abcam (Cambridge, United Kingdom), Cat #ab87399.

³¹ Abcam Cat #ab15895.

³² 10x concentrate purchased from Bio-Rad, Cat #170-6435.

³³ Bio-Rad, Cat #1662404.

For the chemiluminescent imaging of the blots, a Clarity™ Western enhanced chemiluminescence (ECL) Substrate³⁴ mix was prepared. The peroxide solution and the Luminol/enhancer solution was mixed in a 1:1 ratio and 0.1ml of solution was prepared per cm² of membrane. This was dispensed onto the blot before it was photographed by the ChemiDoc™ MP system. The photographs were processed using Image Lab (v 5.2.1) software.

Results and Discussion

In Figure 3.5, the SDS-PAGE result of the VDAC1 and NDUFS4 protein analysis in mouse liver tissue is shown. VDAC1 (30kDa) was the reference protein used for normalisation and the WT:WT sample (#12) served as the sample control (100%).

It is clear that the 18kDa NDUFS4 protein band was present in the WT:WT (#12) and in the WT:OVER (#65) mouse liver tissue, but absent in the KO:WT (#86) and in the KO:OVER (#84) tissue. After several repeats of the analysis, Figure 3.5 was obtained, which serves as a representative result. However, the uneven and severe background noise on the blot distorted the densitometry results to such an extent that it was inaccurate. Fortunately, for this study the SDS-PAGE & western blot analysis was purely for the confirmation of the presence/absence of the NDUFS4 protein band in the four genotypes of interest. Therefore, these results confirmed the NDUFS4 protein characteristics of the NDUFS4 mouse model (Mereis, 2018).

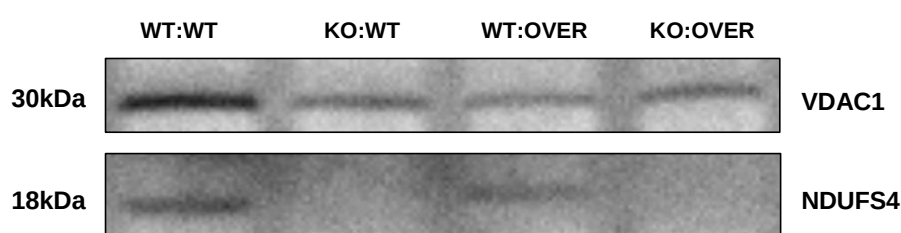


Figure 3.5 The ChemiDoc™ MP system image of the protein bands in a PAGE gel.

Top: VDAC1 protein bands at 30kDa.

Bottom: NDUFS4 proteins bands at 18kDa.

³⁴ Bio-rad, Cat #170-5060.

Characterisation of native CI

Blue Native PAGE assay method

With the direct effect of the NDUFS4 knockout confirmed using SDS-PAGE, the effect of this knockout on the stability of CI and other OXPHOS protein structures was further investigated. This was done with blue native polyacrylamide gel electrophoresis (BN-PAGE) (Wittig *et al.*, 2006).

Prior to the assay, the enriched mitochondria of liver tissues of mice #2.3, #75, #78 and #86 (refer to Table 3.2.) were prepared (refer to Section 3.3.4). These were resuspended in 1x Complete™ Mini EDTA-free Protease inhibitor Cocktail³⁵ for protection of the sample against protease enzymes. Subsequently, the protein concentration was determined via the BCA assay, as also described in Section 3.3.4. The BN-PAGE assay was performed according to the revised and optimised protocol of the MRL, which is based upon the original method first described by Schagger and von Jagow (1991).

The anode buffer used was 5% NativePAGE™ Running Buffer³⁶ and the cathode buffer was 5% NativePAGE™ Running Buffer with 5% NativePAGE™ Cathode Additive. Each sample that was loaded into the gel had a final volume of 25µl and consisted of 20µg protein, 10% n-dodecyl-β-d-maltoside (DDM)³⁷, 25% Laemmli Sample Buffer³⁸, MilliQ® H₂O and 4.5% NativePAGE™ G-250 Sample Additive³⁹. No molecular weight marker was loaded, as this assay only served to inspect the integrity of the CI protein. Samples were kept on ice throughout the procedure and care was taken to prevent the formation of bubbles.

A precast NativePAGE™ 4-16% Bis-Tris Protein Gel (1.0 mm)⁴⁰ was inserted into an XCell SureLock™ Mini-Cell and samples were loaded into the respective wells. Electrodes were connected to the Mini-Cell and the native proteins were allowed to separate according to their size and charge for approximately 2 hours. To prevent the NativePAGE™ 5% G-250 Sample Additive from dyeing the gel too intensely blue, the cathode buffer was replaced with clear, clean buffer after 30 min.

³⁵ Sigma-Aldrich (Merck), Roche, Cat #11836170001.

³⁶ Thermo Fisher Scientific, Invitrogen™, Cat #BN2001.

³⁷ Thermo Fisher Scientific, Invitrogen™, Cat #BN2005.

³⁸ Biorad Laboratories, Cat #1610747.

³⁹ Thermo Fisher Scientific™, Life Technologies™, Cat #BN2004.

⁴⁰ Thermo Fisher Scientific™, Life Technologies™, Cat #BN1002BOX.

Western blot, immunodetection and chemiluminescent imaging method

At the end of the electrophoresis process, the gel was removed from the Mini-Cell and transferred to a PVDF membrane used for western blotting. The method described after the section on SDS-PAGE was applied. In this case, however, the primary antibodies used for the first over-night incubation of the membrane was mouse monoclonal to NDUFS3⁴¹ and to NDUFA9⁴² (each diluted in a 1:1000 ratio). For the second over-night incubation, the membrane was placed in a Total OXPHOS Blue Native WB Antibody Cocktail⁴³ containing monoclonal antibodies against CI-NDUFA9⁴⁴, CII-70 kDa subunit⁴⁵, CIII-Core protein 2⁴⁶, CIV-subunit IV⁴⁷ and CV-alpha subunit⁴⁸, respectively. This cocktail was also diluted in a 1:1000 ratio. Following each over-night incubation, the membrane was treated with HRP-conjugated goat polyclonal to mouse secondary antibodies⁴⁹ (diluted in a 1:10 000 ratio) and photographed in the ChemiDoc™ Multiplex (MP) system. Photographs were processed with the Image Lab (v 5.2.1) software.

Results and Discussion

The images taken after each incubation with the secondary antibodies, were merged in a multichannel format for a comprehensible review of the results (see Figure 3.6). From these results, it was observed that CI is present and intact in the WT:WT sample and slightly more pronounced in the WT:OVER sample, but mostly absent in the KO:OVER and KO:WT samples. In both of these samples, but notably clearer in the KO:WT sample, weaker and lower sized bands were observed, which suggests a defective assembly and/or stability of the enzyme. This is similar to what was reported by (Sterky *et al.*, 2012). Notably, CIII was not detected in any of the samples. This might be due to weak binding of the primary antibody (CIII-Core protein 2) to the protein on the blot, or due to weak binding of the secondary antibody to the CIII-Core protein 2 antibody, leading to weak luminescence (Mahmood & Yang, 2012). The darker bands seen at CIV and CII of the KO:WT and KO:OVER samples, may be due to increased mitochondrial biogenesis resulting from the CI defect. Consequently, the gene expression of the other complexes would be upregulated (Reinecke *et al.*, 2009).

⁴¹ Anti-NDUFS3 antibody [3F9DD2], Abcam®, Cat #ab110246.

⁴² Anti-NDUFA9 antibody [20C11B11B11], Abcam®, Cat #ab14713.

⁴³ Abcam®, Cat #ab110412.

⁴⁴ Abcam®, Cat #ab14713 (MS111).

⁴⁵ Abcam®, Cat #ab14715 (MS204).

⁴⁶ Abcam®, Cat #ab14745 (MS304).

⁴⁷ Abcam®, Cat #ab14744 (MS407).

⁴⁸ Abcam®, Cat #ab14748 (MS507).

⁴⁹ Goat Anti-Mouse IgG H&L (HRP), Abcam®, Cat #ab205719.

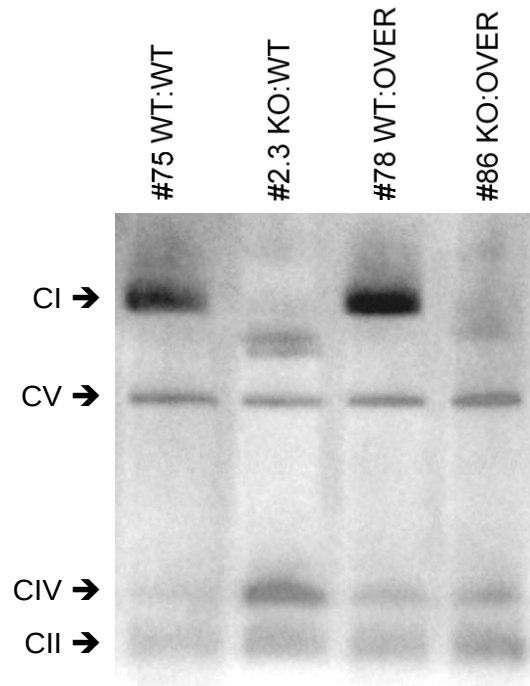


Figure 3.6 A merged/multichannel image of the two blue native PAGE images. These photographs were taken after incubation with secondary antibodies. Respiratory chain enzyme complexes are indicated on the left and mouse identities on the top.

Kinetic enzyme analyses for determination of NDUFS4 activity

To establish the amount of CI activity present in the liver tissue of each genotype, spectrophotometric enzyme analyses were conducted. The methods of both the citrate synthase (CS) and CI assays are based on the methods of Janssen *et al.* (2007) and is part of a revised and optimised protocol in the MRL. These were essentially kinetic assays, where the CI-catalysed oxidation of NADH was detected indirectly by the electron transfer to chromogen 2,6-dichloroindophenol, followed at 600 nm using a Synergy™ HT Multi-detection microtiter plate reader⁵⁰.

Before commencing the analyses, frozen portions of the 600xg supernatants of #2.3, #75, #78 and #86 (refer to Table 3.2) were thawed and the protein concentration of each sample was determined with the BCA assay (refer to Section 3.3.4).

⁵⁰ BioTek® Instruments, USA.

Citrate Synthase activity method

This assay was first described by Shepherd and Garland (1969) as well as Robinson *et al.* (1987). As CS is part of the first and rate-limiting reaction in the TCA cycle (it catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate), its maximal activity is commonly used as an indicator of mitochondrial mass. Hence, the activity of CI was measured relative to CS.

CS activity was determined with the 600xg supernatants of each of the mouse genotypes. Each sample was freeze-thawed twice with liquid nitrogen in order to break the mitochondrial membranes. The reagents involved in the reactions were prepared fresh. The reactions had a final volume of 200µl and consisted of 0.1mM Dithio-bis[2-nitrobenzoic acid] (DTNB)⁵¹, 0.04% Triton X100⁵², 60µM acetyl-CoA⁵³, MilliQ® H₂O, 0.5mM oxaloacetate (OAA)⁵⁴ and a constant volume of 3µl 600xg supernatant (diluted 3x). Each reaction was loaded in triplicate into the wells of the 96-well microtiter plate. Note that the OAA was added right before the measurement, as the reactions were immediately set in motion by OAA. All reagents were warmed to 30°C before the reading. The linear rate increase (v_1) was measured at 412nm for 5 min in 1 min intervals.

After ensuring that $R^2 > 0.98$ for v_1 , linear rate calculations were used over the first 3 minutes. For 200µl reactions, the extinction coefficient at 412nm (ϵ_{412}) for DTNB was 7465 Abs/mM and Equations 3.3 and 3.4 were used to calculate the results in catalytic units of citrate synthase (UCS):

$$\mu(\text{mol/min})/\text{mg (UCS)} = \frac{(v_1/7465) \times 0.2}{\mu\text{l protein} \times \mu\text{g}/\mu\text{l}/1000} \dots \text{Equation 3.3}$$

$$\text{nmol/min/mg} = \text{UCS} \times 1000 \dots \dots \dots \text{Equation 3.4}$$

⁵¹ Sigma Aldrich (Merck), Cat #D8130.

⁵² Sigma Aldrich (Merck), Cat #T9284.

⁵³ Roche (Basel, Switzerland), Cat #10101907001.

⁵⁴ Sigma Aldrich (Merck), Cat #17,125-5.

Complex I activity method

The method for determining CI activity is adapted from Janssen *et al.* (2007), Luo *et al.* (2008) and Rahman *et al.* (1996). The basic principle of the kinetic analysis of CI is to spectrophotometrically measure NADH oxidation in both the presence and absence of rotenone (Smet, 2016).

CI activity was also determined with the 600xg supernatants of each of the mouse genotypes. The samples were freeze-thawed twice with liquid nitrogen in order to break the mitochondrial membranes. Each sample was loaded in triplicate *with* rotenone⁵⁵ (dissolved in dimethylsulfoxide (DMSO)⁵⁶) and in triplicate *without* rotenone (only DMSO). The respective reactions had a final volume of 200µl and contained 6µl 600xg supernatant (diluted 3x). The final concentrations of the reagents in each reaction were: 50mM KPi buffer⁵⁷ (pH 7.4), 1µM Antimycin A⁵⁸ (in EtOH), 70µM decylubiquinone (DUQ)⁵⁹ (in DMSO), 60 µM 2,6-dichloroindophenol sodium salt hydrate (DCIP)⁶⁰, 0.35% BSA⁶¹ (in KPi buffer), MilliQ® H₂O, 2.5µM rotenone or DMSO and 2mM NADH⁶². Importantly, NADH was added right before the reading, as the reactions were immediately set in motion by NADH. All reagents were warmed to 30°C before the reading. The linear rate decrease of NADH in the DMSO samples (v_1) and in the rotenone-containing samples (v_2) was then spectrophotometrically measured at 600nm for 5min in 1min intervals with the Synergy™ HT multi-detection microtiter plate reader. The linear rate calculations over the first four minutes were used, after ensuring that $R^2 > 0.98$ for both v_1 and v_2 . For these 200µl reactions, $\epsilon_{600} = 12712 \text{ Abs/mM}$ for DCIP and Equations 3.5 and 3.6 were used to calculate the results. In the end, the kinetic enzyme activity of CI was normalised, relative to the citrate synthase activity of the same tissue sample.

$$\mu\text{mol/min/mg} = \frac{((v_1 - v_2)/12712) \times 0.2}{\mu\text{l protein} \times \mu\text{g}/(\mu\text{l}/1000)} \dots\dots\dots \text{Equation 3.5}$$

$$\text{nmol/min/UCS} = \mu\text{mol/min/mg} \times 1000/\text{UCS} \dots\dots\dots \text{Equation 3.6}$$

⁵⁵ Sigma Aldrich (Merck), Cat #R8875.

⁵⁶ Sigma Aldrich (Merck), Cat #D2650.

⁵⁷ KPi buffer was prepared with 19ml of 0.5M KH₂PO₄ (68g/L, product of Fluka™ (Bucharest, Romania) Cat #60218) and 81ml of 0.5M K₂HPO₄ (87.1g/L, product of Fluka™, Cat #60353).

⁵⁸ Sigma Aldrich (Merck), Cat #A8674.

⁵⁹ Sigma Aldrich (Merck) Cat #C6164.

⁶⁰ Sigma Aldrich (Merck), Cat #D1878.

⁶¹ Roche, Cat #775835.

⁶² Roche, Cat #10107735001.

Results and discussion

When considering the results, it should be taken into account that only one mouse of every genotype was analysed to confirm reduced CI activity of the NDUFS4 knockout. In comparing the CI activity of the KO:WT and the WT:WT 600xg supernatants, it was clear that the *Ndufs4* knockout had a definitive detrimental effect (reduced activity of ~40%) upon the CI activity of the KO:WT mouse (see Figure 3.11). It is, however, interesting to see the increase (~111%) of CI activity in the WT:OVER mouse compared to wild type (WT:WT) mouse and that the CI deficiency is much more pronounced in the KO:OVER mouse (~65% decrease in activity).

The data from this study therefore confirms that the CI defect is indeed present due to the *Ndufs4* knockout mouse genotypes. These results are in line with the investigation on WT:WT and KO:WT mice as published by Terburgh *et al.* (2019), who investigated the CI activity in quadriceps and soleus muscles and reported a drastic reduction in rotenone-sensitive CI activity. Upon further and more extensive investigation, however, it would be interesting to see if the more pronounced CI deficiency could be confirmed in the MT1 overexpressing mice.

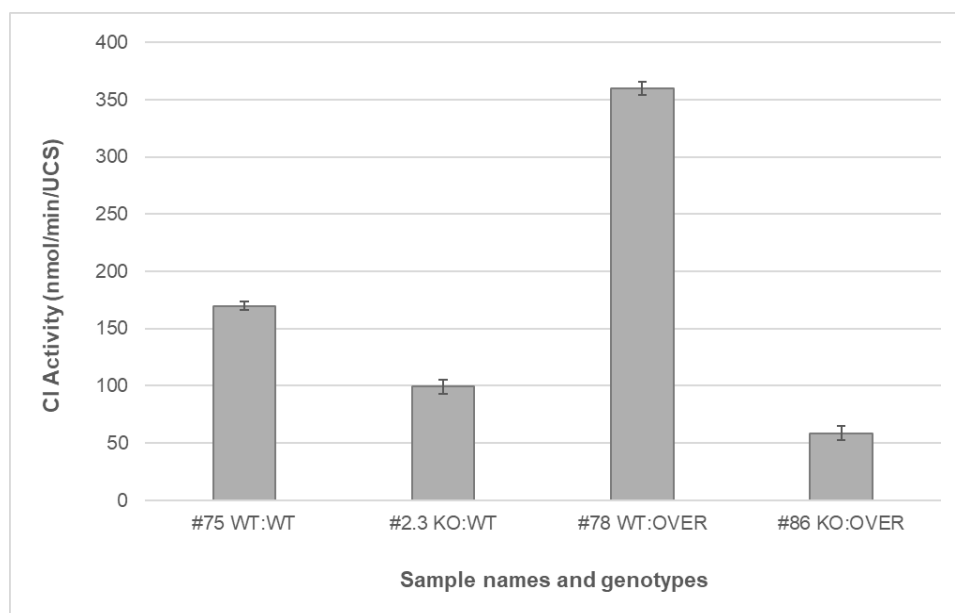


Figure 3.7 The CI enzyme activity in 600xg supernatants of the liver tissue of WT:WT, KO:WT, WT:OVER and KO:OVER mice. Error bars indicate the standard deviation among triplicates. Data is normalised to CS activity.

3.4 SUMMARY AND CONCLUSIONS

In this chapter, the methodology and results of the investigations for the characterisation of the NDUFS4 and TgMt1 mouse models for this study were described. These models were developed before the commencement of this study, but were again evaluated and characterised as part of the first two objectives.

By genotyping one sample of each of the four genotypes of interest, namely WT:WT, KO:WT, WT:OVER and KO:OVER, the presence/absence of the *Ndufs4* allele could be observed by a simple PCR-based DNA analysis. As expected, the WT:WT and the WT:OVER mice showed an intact PCR fragment at 1229bp. A shortened fragment of 429bp was observed at the KO:WT and KO:OVER samples, confirming the disruption of the *Ndufs4* gene (Mereis, 2018).

To assess the expression of the *Mt1* gene in the four genotypes, the total RNA from liver tissue of one mouse per genotype was isolated. RT-qPCR and the $2^{(-\Delta\Delta Ct)}$ method was applied, and the data was normalised against $\beta 2m$. The *Mt1* overexpression was markedly higher in the WT:OVER sample and especially in the KO:OVER sample, as opposed to that of the WT:WT tissue. In the KO:WT sample, the *Mt1* gene expression was comparable to the WT:WT sample, which might point to other cellular adaptive mechanisms (instead of physiological MT1) working to counterbalance the overwhelming amount of ROS (Manjeri, 2017; Mereis, 2018; Tehrani & Moosavi-Movahedi, 2018). These data still successfully confirmed that MT1 mRNA were overexpressed in liver samples of the TgMt1 mice.

An SDS-PAGE & western blot assay was conducted with the 600xg supernatants of the liver tissue from one mouse per genotype to characterise the NDUFS4 protein in these genotypes. With immunodetection and chemiluminescent imaging, the presence of the NDUFS4 protein was detected in the WT:WT and WT:OVER samples. In the KO:WT and KO:OVER samples, the absence of the NDUFS4 protein was observed. VDAC1 was used for normalisation purposes. Furthermore, these results confirmed the NDUFS4 protein characteristics of the NDUFS4 mouse model (Mereis, 2018).

The native structure and integrity of CI was then investigated by way of BN-PAGE & western blot, immunodetection and chemiluminescent imaging. The structure of CI appeared to be intact in the WT:WT sample and slightly more pronounced in the WT:OVER sample. In the KO:OVER and especially the KO:WT sample, a weaker and lower sized band could be observed. This is confirmed by Sterky *et al.* (2011), who have also established that an *Ndufs4* defect might cause subcomplex formation because of defective assembly and stability of the complex.

The kinetic enzyme activity of CI in one liver sample per genotype was measured with spectrophotometry, and normalised to the activity of citrate synthase. 600xg supernatants of the liver tissues were used in these assays and the WT:WT tissue sample was used as a control. The results clearly showed a weaker CI activity in the KO:WT sample and especially in the KO:OVER tissue sample.

Even though it should be taken into account that only one sample of each genotype was used and that these assays were only for confirmation purposes, the prominence of the CI structure and the activity of the CI enzyme in the WT:OVER sample was evidently greater than expected. A plausible reason for these phenomena might be related to MTs being able to transfer metals – such as Zn^{2+} and Cu^+ – to the RC enzymes by translocating themselves to the IMS. These metals act as cofactors to the RC enzymes, leading to an increase in electron flux and an increase in cellular respiration. MTs may also induce a post-translational modification that promotes the use of oxygen by the RC. The increased expression of the NDUFS4 protein might also have resulted from MT1 donating metal ions to Zn-finger proteins and transcription factors that are involved in the transcriptional pathways of *Ndufs4* (Costello *et al.*, 2004; Lindeque *et al.*, 2010; Mereis, 2018; Merten *et al.*, 2005). Additionally, the 37.4-fold overexpression of *Mt1* in the WT:OVER sample, confirms the fact that MT1 overexpression is indeed copy number-dependent (see Chapter 2, Section 2.4.2), but reserved for physiologically stressful situations; it might be described as potent, but it is not abundantly available under normal circumstances (Lindeque *et al.*, 2010).

In short, the genotyping, RT-qPCR, kinetic enzyme analyses, SDS-PAGE and BN-PAGE results all confirm the nature of the *Ndufs4* defect.

CHAPTER 4: TRANSCRIPTIONAL INVESTIGATION

4.1 INTRODUCTION

In this chapter, the methodology and results behind the investigation of the transcription of selected genes involved in one-carbon metabolism and oxidative stress in NDUFS4-deficient mouse tissues will be discussed. The effect of *Mt1* overexpression upon these genes will also be evaluated.

The first and third objectives of this study, which are the collection of tissues from the mice and DNA analysis, respectively, will be reviewed together. Here, the methodology behind the sample preparation and the confirmation of genotypical mouse identities is explained. Under Objective 5, the quantification of mRNA and measuring expression of selected genes in four genotypes: the isolation, purification, and integrity assessment of the RNA obtained from the mouse brain and quadriceps tissues, as well as the methodology for gene expression analyses, will be described. Under the sixth objective, comparison of expression of selected genes in NDUFS4 KO and TgMt1 mice to that of WT:WT mice; an explanation of the data analysis and interpretation will precede a thorough layout of the results of each gene. Finally, a discussion of the outcome of this transcriptional investigation will conclude this chapter.

Each section will contain brief background information on the methodology as well as the results and a discussion thereof.

4.2 TISSUE COLLECTION, SAMPLE PREPARATION AND GENOTYPING (OBJECTIVES 1 & 3)

4.2.1 Tissue collection and sample preparation

In total, the tissues of 24 mice were collected: one brain, one quadriceps and one liver sample from each mouse. The 24 mice consisted of 6 mice per genotype (WT:WT, WT:OVER, KO:WT and KO:OVER). All quadriceps and brain samples were saved for the gene expression assays, while the liver samples were used for genotyping. The identities of these mice may be seen in Table 4.1.

The samples were obtained from mice that were kept at the PCDDP of the NWU Potchefstroom Campus (see Section 3.2, Chapter 3, for more details about the animals). All tissues were snap-frozen in liquid nitrogen upon collection and stored in a -80°C freezer until use.

On the day of assay preparation, the tissue samples were thawed on ice. Each tissue sample was finely minced on a cold plate and mixed with the use of a scalpel blade, so that aliquots of each sample may represent the whole sample. Thus, aliquots of 10 to 30mg of the quadriceps and brain tissue samples were frozen in microcentrifuge tubes for RNA work (Samples were not stored in Trizol®. It was added right before RNA extraction.). Approximately 25mg liver tissue of each mouse was placed in its respective tube for genotyping. Each tube was marked clearly before refreezing.

Table 4.1 The identities of the mice from which tissues were collected for the transcriptional investigation.

Genotype	ID	DOB	Gender	Age (Days)	Tissues collected
WT:WT	#83	29/06/2018	F	52	B. For PCR efficiency analyses only.
	#9.1	02/10/2017	M	51	B & Q
	#10.1	07/10/2017	F	46	B & Q
	#10.5	07/10/2017	F	46	B & Q
	#10.6	07/10/2017	M	46	B & Q
	#10.7	07/10/2017	M	46	B & Q
	#13.2	30/10/2017	M	49	B & Q
WT:OVER	#1.1	07/08/2017	F	52	B & Q
	#15.2	02/08/2017	F	50	B & Q
	#15.3	02/08/2017	F	50	B & Q
	#15.5	02/08/2017	M	50	B & Q
	#4.1	03/09/2017	M	47	B & Q
	#12.1	21/10/2017	F	46	B & Q
KO:WT	#10.3	07/10/2017	M	46	B & Q
	#13.8	30/10/2017	F	49	B & Q
	#9.5	22/12/2017	M	48	B & Q
	#9.6	22/12/2017	F	48	B & Q
	#2.6	09/02/2018	M	47	B & Q
	#2.7	09/02/2018	F	47	B & Q
KO:OVER	#12.7	21/10/2017	F	46	B & Q
	#14.4	02/11/2017	M	46	B & Q
	#15.4	09/11/2017	F	49	B & Q
	#15.5	09/11/2017	M	49	B & Q
	#15.6	09/11/2017	F	49	B & Q
	#12.1	13/01/2018	F	48	B & Q

B = brain; DOB = date of birth; F = female; ID = identification number; M = male; Q = quadriceps.

4.2.2 Genotyping

Method

The aim of this procedure was to confirm the absence or presence of the *Ndufs4* gene in the 24 mice that were used for the gene expression assays (this was to confirm that the mice had been marked correctly, and was not for the characterisation of the mouse model). Thus, the DNA was isolated from the liver samples, using a similar kit and protocol as described in Section 3.3.2 of Chapter 3. On the day of genotyping, the samples involved in this investigation were genotyped together with samples from another study, thus a method suitable for high through-put had to be employed for concentration determination. The Quant-iT™ PicoGreen™ dsDNA Assay Kit¹ was therefore used for this purpose. In principle, luminescent complexes form when the PicoGreen™ fluorescent probe binds to dsDNA, therefore a signal is created that is directly proportional to the DNA concentration. This signal is then detected spectrophotometrically.

With consistent volumes, the DNA samples were mixed with 1x Tris EDTA (TE) buffer and the PicoGreen™ fluorescent probe. A DNA standard series ranging from 0 to 200ng was also set up. The samples and the standards were then dispensed in duplicate in volumes of 200µl in a 96-well microtiter plate, and measured at wavelengths of 480nm excitation and 520nm emission with a Synergy™ HT Multi-detection microtiter plate reader. With linear regression, the amount of DNA (in ng) was determined by measuring against the standard curve. The concentrations of the DNA ranged between 41.1 and 158 ng/µl.

All of the DNA samples were then diluted to have a concentration equal to that of the sample with the lowest concentration. By making use of similar primers, reagents, instrumentation and thermal cycling conditions as in Section 3.3.2 (Chapter 3), a PCR was conducted. An agarose gel electrophoresis assay with a 50bp DNA ladder² then followed with the obtained PCR products, in order to separate the DNA alleles based on their size differences.

Results and Discussion

The photographs taken with a ChemiDoc™ MP system Image Lab (v 5.2.1) software (Figure 4.1 A.-D.), shows the presence of the intact *Ndufs4* gene at 1229bp in the WT:WT and WT:OVER samples. By contrast, an incomplete *Ndufs4* subunit is seen at 429bp in the KO:WT and KO:OVER mouse samples. Similar to the genotyping results in Section 3.3.2, nonspecific bands are seen below most of the lanes, which may be attributed to misprimed

¹ Purchased from Thermo Fisher Scientific, Product of Invitrogen, Cat #P11496.

² Purchased from Thermo Fisher Scientific, Product of Invitrogen, Cat #10416014.

targets that were also extended. These results, however, established that the mice were correctly marked in the Vivarium, as their genotypes were correctly predicted.

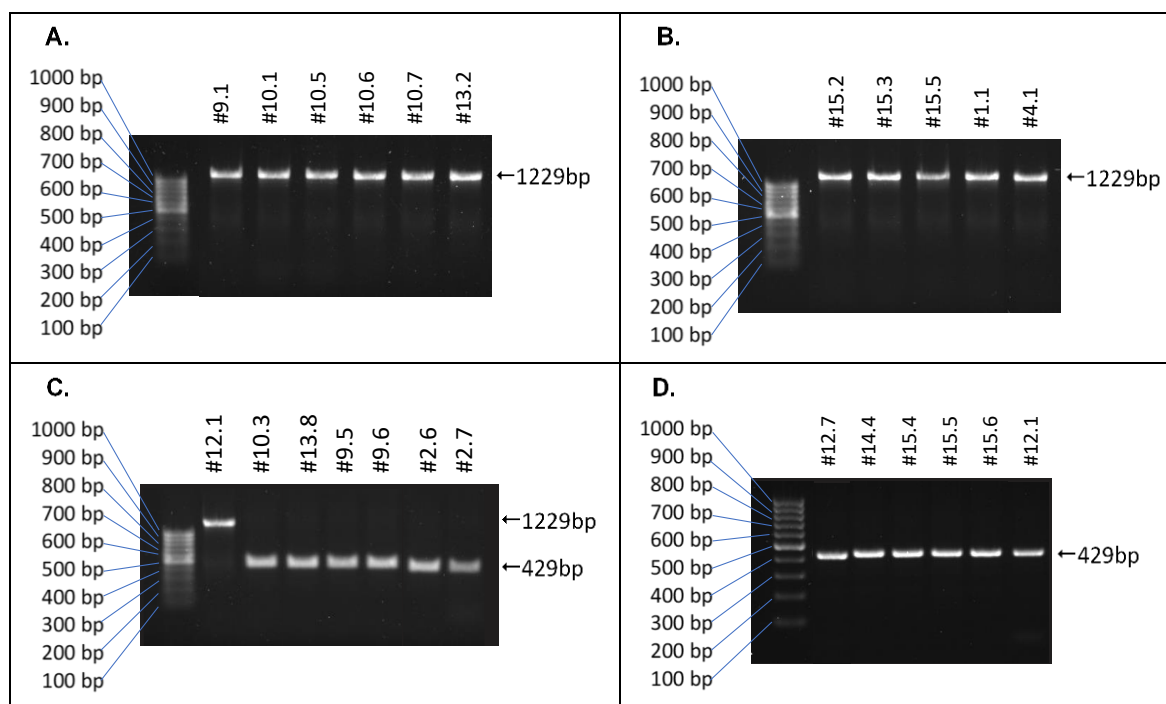


Figure 4.1 The ChemiDoc™ images of the NDUF54 alleles from the liver tissue of each mouse. Each lane represents a specific mouse and their identities are marked at the top of each image. The sizes of the alleles in the molecular weight ladder are indicated on the left and the sizes of the NDUF54 alleles are indicated on the right of each image.

4.3 QUANTIFICATION OF mRNA AND MEASURING EXPRESSION OF SELECTED GENES IN FOUR GENOTYPES (OBJECTIVE 5)

For this objective, the expression of twelve genes – seven genes involved in the 1C-metabolism, three genes involved in oxidative stress and two reference genes – were investigated in brain and quadriceps tissue via real-time quantitative polymerase chain reaction (RT-qPCR). The effect of *Mt1* overexpression upon the expression regulation of these genes was also evaluated. Details of the selected genes may be seen in Table 4.2 and the motivation behind the choice of each gene was explained in Chapter 2, Section 2.7.

Table 4.2 The TaqMan™ Gene Expression Assays (primer/probe mixes) with which the RT-qPCR analyses were conducted.

Gene	Catalog #	Assay ID	Details
<i>Mthfd2</i>	4448892	Mm00485276_m1	Probe spans exons; Amplicon length: 70
<i>Gclc</i>	4453320	Mm00802655_m1	Probe spans exons; Amplicon length: 98
<i>Mtrr</i>	4448892	Mm00549977_m1	Probe spans exons; Amplicon length: 74
<i>Bhmt</i>	4448892	Mm04210521_g1	Probe spans exons ; Amplicon length: 89
<i>Tyms</i>	4448892	Mm01702970_m1	Probe spans exons; Amplicon length: 138
<i>Sod2</i>	4453320	Mm01313000_m1	Probe spans exons; Amplicon length: 67
<i>Cat</i>	4453320	Mm00437992_m1	Probe spans exons; Amplicon length: 64
<i>Gpx1</i>	4453320	Mm00656767_g1	Probe spans exons; Amplicon length: 134
<i>Lias</i>	4448892	Mm00522477_m1	Probe spans exons; Amplicon length: 100
<i>Actβ</i>	4453320	Mm01205647_g1	Probe spans exons; Amplicon length: 72
<i>β2m</i>	4453320	Mm00437762_m1	Probe spans exons; Amplicon length: 77
<i>Phgdh</i>	4448892	Mm01623589_g1	Both primers and probe map within a single exon; Amplicon length: 73; This assay is in the 5'UTR region of the NM_016966.3 transcripts

Mus Musculus was the target species for each assay and the chosen kit size was 75rxns (XS). FAM-MGB (carboxyfluorescein minor groove binder) was the chosen fluorescent reporter dye/probe and carboxy-X-rhodamine (ROX) the specified passive reference dye for each assay. The primer and probe sequences were not provided, as these were predesigned assays.

4.3.1 RNA isolation and purification

Method

The isolation of the RNA was performed according to the method described in Chapter 3, Section 3.3.3. The total RNA of six whole brain samples and six quadriceps samples of each genotype (seven brain samples for the WT:WT genotype; refer to Table 4.1) were isolated. RNA was then dissolved in 30µl of diethylpyrocarbonate (DEPC)-treated water³, to ensure that any RNase in the solvent would be inactivated (Riche & Mabic, 2017). Determination of the concentration and purity of the RNA followed, once again using the NanoDrop™

³ Purchased from Thermo Fisher Scientific, product of Invitrogen Life Technologies, Cat #46-2224.

One/One^C UV-Vis Spectrophotometer, and concentration values ranged between 446.2 and 1413ng/μl. However, A260/A280 values <1.8 were measured for more than half of the samples, suggesting that impurities from DNA or phenol could be present. Thus, considering that the acceptable purity for RNA is ~2.0, further purification of the RNA samples was imperative (Thermo Fisher Scientific, 2018b).

For this additional purification step, the MEGAcleanTM Kit⁴ was used, as the method successfully removes phenols, proteins, salts and short oligonucleotides; the recovered RNA is thus specifically suitable for further enzyme-catalysed reactions such as RT-qPCR. Essentially, the process consisted of three steps: the binding of RNA to the membrane of the filter cartridge, the washing of RNA, and the elution of the purified RNA. The purification was conducted according to the manufacturer's instructions (Thermo Fisher Scientific, 2019b). Hereafter, another concentration and purity measurement of the purified RNA by means of the NanoDropTM One/One^C UV-Vis Spectrophotometer was performed.

Results and discussion

The purified RNA concentrations ranged between 144 and 9.84ng/μl, and this time the purity (the A260/A280 ratio) ranged between a satisfactory 1.97 and 2.19.

As the MEGAcleanTM kit had been shown to efficiently remove 5S, 16S and 23S ribosomal RNA (rRNA) and tRNAs from bacterial RNA (Thermo Fisher Scientific, 2019b), it made sense that 18S and 28S rRNA and tRNAs in the mouse RNA samples were also removed to a great extent. This was consistent with the low RNA concentrations after purification. In Chapter 3 (Figure 3.4), it has already been shown in a proof-of-concept analysis that the integrity of RNA samples is maintained after isolation with the harsh, cell-disrupting TRIzol® reagent. Therefore, based on those integrity results, it was reasoned that the RNA quality would be fit for further investigation. Furthermore, initial gene expression test assays confirmed that the samples contained intact mRNA.

⁴ Purchased from Thermo Fisher Scientific, Product of Ambion (Life TechnologiesTM), Cat #AM1908.

4.3.2 PCR Efficiency analyses

Principle

Before any gene expression assays could be performed, the amplification efficiency of each gene had to be evaluated. The purpose of these assays was to establish whether there was, in fact, a doubling of the PCR product at each thermal cycle and to determine the optimal RNA concentration for the final gene expression assays. To determine the gene expression of a target gene relative to a housekeeping gene with the $\Delta\Delta CT$ method, the PCR efficiency of the target gene and that of the housekeeping gene had to be approximately equal. This means that the PCR efficiency of both the target gene and the housekeeping genes had to average at 100% (+/-10%) (Thermo Fisher Scientific, 2018a). The exponential amplification of DNA could, therefore, be described by Equation 4.1:

$$R_n = R_0 \times (1 + E_x)^n \dots\dots\dots \text{Equation 4.1}$$

Where:

R_n = reporter fluorescence at cycle n (directly proportional to the number of target molecules at cycle n)

R_0 = initial reporter fluorescence

E_x = efficiency of target amplification

n = number of cycles

A PCR efficiency of 100% would thus be equal to an E_x value of 2.00 (Applied Biosystems, 2004).

Therefore, an RNA dilution series was set up with five data points, doubling in concentration from 10ng/rxn to 160ng/rxn. Using WT:WT mouse brain RNA and five technical replicates of each RNA concentration, an RT-qPCR assay was performed for each of the genes. The resultant C_t values of each concentration were averaged to form data points that could be plotted against their respective $\log[\text{RNA}]$ values. Substituting the slope of the trendline (ideally a value of -3.33) into the exponent, the amplification efficiency of each gene could be calculated according to Equations 4.2 or 4.3:

$$E_x = (10^{(-1/\text{slope})} - 1) \times 100 \dots\dots\dots \text{Equation 4.2}$$

$$E_x = 10^{(-1/\text{slope})} \dots\dots\dots \text{Equation 4.3}$$

Method

The brain of mouse #83 (refer to table 4.1) was collected, as described in Section 4.2.1. This mouse brain was separate from those used in the gene expression investigation. After it had been manually homogenised on a cold plate, it was divided into 8 aliquots ranging between 36.5 and 54.2mg. These aliquots were placed in microcentrifuge tubes and treated as separate samples. The total RNA was then isolated and purified as described in Section 4.3.1. Throughout the RNA isolation, the aliquots were kept on ice. During extraction of the aqueous phase from the organic phase, care was taken to avoid phenol contamination. As before, each RNA pellet was dissolved in 30µl of DEPC-treated water and the RNA concentrations (ranging between 1684 and 2316ng/µl) and purities (ranging between 1.86 and 2.03) were then determined by means of the NanoDrop™ One/One^C UV-Vis Spectrophotometer. After purification with the MEGAclear™ Transcription Clean-Up Kit, the concentrations (ranging between 335 and 370ng/µl) and purities (~2.00) were determined yet again. While six of the sample aliquots were then stowed away at -80°C, two were kept on ice for the amplification efficiency assays.

An RNA dilution series was set up with five data points, doubling in concentration from 10ng/rxn to 160ng/rxn. Using the two aliquots of total mouse brain RNA and five technical replicates of each RNA concentration, an RT-qPCR assay was performed for each of the genes. The RT-qPCR method outlined in Chapter 3, Section 3.3.3 was applied here and the gene-specific primer/probe mixes that were used for these assays are listed in Table 4.2.

Hence, each reaction had a final volume of 10µl and contained 1x TaqMan® RT-PCR Mix¹ (2x), 1x TaqMan® RT Enzyme Mix (40x)², 1x Taqman® Gene Expression Assay (20x)³ (i.e. one of the gene-specific primer/probe mixes), nuclease-free water⁴ and 10, 20, 40, 80 or 160ng/rxn RNA template. The reactions, including no-template controls, were loaded into MicroAmp™ Optical 96-Well Reaction Plates (one plate per primer/probe mix). Between each pipetting step, the sample premix was vortexed well and care was taken to place the pipet tip just under the meniscus when drawing up the fluid. The reverse transcription of the RNA and the amplification of the cDNA took place in a 96-well QuantStudio™ 5 Real-Time

¹ The concentrations of the TaqMan® RT-PCR Mix constituents (AmpliTaQ Gold® DNA Polymerase, dNTPs, ROX™ passive reference and optimised buffer components) are not provided by Thermo Fisher Scientific.

² The concentrations of the TaqMan® RT Enzyme Mix constituents (ArrayScript™ UP Reverse Transcriptase and RNase inhibitor) are not provided by Thermo Fisher Scientific.

³ Each TaqMan® Gene Expression assay contained two unlabelled primers (1x final concentration of 900nM per primer) and one 6-FAM™ dye-labelled TaqMan® MGB probe (1x final concentration of 250nM) in an optimised reaction mix.

⁴ Qiagen (Hilden, Germany), Cat #129114.

PCR System⁵. The cycle that was followed may be seen in Chapter 3, Table 3.6. In order to compare RT-qPCR results in this study, the QuantStudio™ 5 apparatus was set to consistently yield values at an arbitrary threshold value of 0.2 (Qiagen, 2018; Thermo Fisher Scientific, 2018a)

Data analysis

In a graphical representation of Real-time PCR data, ΔR_n (the fluorescence of the reporter dye normalised to the fluorescence of a passive reference dye, i.e. ROX), was plotted against the cycle number. For each dilution, the Ct values of five technical replicates were obtained. An average value, as well as the standard deviation and the coefficient of variance (CV) were calculated with these data. The data were visually inspected for outliers (where a CV value was $> 1\%$) and those were then excluded from the calculations. The resultant Ct values of each concentration were averaged to form data points that could be plotted against their respective $\log[\text{RNA}]$ values. Substituting the slope of the trendline (ideally a value of -3.33, with an R^2 value ranging between 0.990 and 1) into the exponent, the amplification efficiency of each gene was then calculated according to equation 2 or 3. See Table 4.5 for a summary of the PCR efficiency results. The graphs of the separate genes may be seen in Annexure A. Later, these results were incorporated into the gene expression calculations.

Results and Discussion

The PCR efficiency assays were performed with two total RNA samples of the brain of mouse #83. Literature suggested that RNA samples be pooled in order to have a sufficient amount of identical sample for twelve genes to be tested (Kendzioriski *et al.*, 2003; Svec *et al.*, 2015). Yet, initial PCR efficiency assays yielded unrepeatable and unreliable results, as there was no consistency even among the Ct values of the replicates. In establishing whether the fault lay with the laboratory technique, the apparatus itself, the sample, or the reagents, it was decided that two whole-brain, total RNA samples from a single WT:WT mouse be used, rather than pooled RNA. Repeatable results confirming the doubling of PCR products and pointing to the optimal RNA concentration, were of greater importance than having used identical samples. It was also decided to purify the RNA from this point forward, so that the effect of any possible impurities may be excluded, and to rather use the newer QuantStudio™ 5 Real-Time PCR System, so that any question regarding the reliability of the thermal cycler may be eliminated. However, two technique alterations finally made the assay replicates comparable: vortexing the microcentrifuge tubes between each reaction-loading step, and, ensuring that when fluid was aspirated with a micropipette, the tip would be

⁵ Purchased from Thermo Fisher Scientific, a product of Applied Biosystems™.

immersed to only 1mm below the bottom of the meniscus. This was to ensure consistency in RNA concentration among the reactions and to prevent sample from clinging to the exterior of the pipette tip, respectfully.

As seen in Table 4.5 and in Figure 4.2, the amplification efficiency of all twelve genes ranged between 90% and 106%. This corresponds exactly with the literature that states that the acceptable range is between 90% and 110% (Thermo Fisher Scientific, 2018a). The individual amplification efficiency plots for each gene may be seen in Annexure A.

Table 4.3 A summary of the PCR efficiency data for the 12 genes of interest

[RNA] range	Gene	Metabolic Pathway	% PCR Efficiency	PCR Efficiency
10ng/rxn; 20ng/rxn; 40ng/rxn; 80ng/rxn; 160ng/rxn	<i>β2m</i>	Ref gene	90.67%	1.91
	<i>Actβ</i>	Ref gene	92.20%	1.92
	<i>Gpx1</i>	Ox stress	101.72%	2.02
	<i>Lias</i>	Ox stress/1-C	96.61%	1.97
	<i>Bhmt*</i>	1-C	101.96%	2.02
	<i>Sod2</i>	Ox stress	99.03%	1.99
	<i>Cat</i>	Ox stress	99.88%	2
	<i>Gclc</i>	1-C	90.67%	1.91
	<i>Mthfd2</i>	1-C	100.68%	2.01
	<i>Tyms</i>	1-C	105.80%	2.06
	<i>Mtrr</i>	1-C	97.60%	1.98
	<i>Phgdh</i>	1-C	99.66%	2

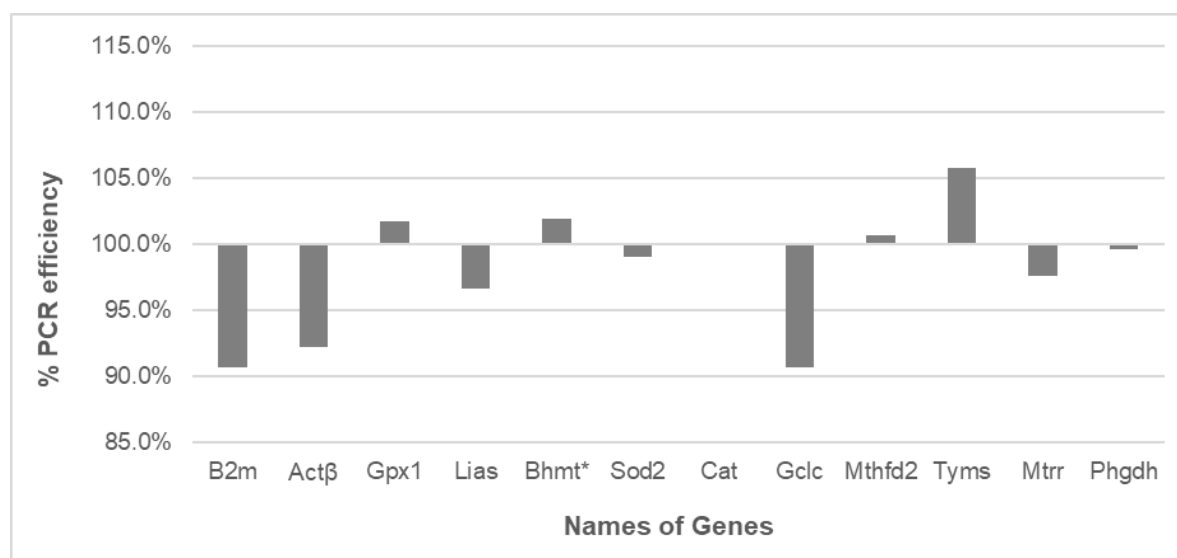


Figure 4.2 A graphical summary of the PCR efficiency results of the 12 genes of interest. Data are expressed in percentages.

*All RNA concentrations, except 160ng/rxn.

For each gene, the plot of the average Ct values against the logarithm of the corresponding RNA concentration yielded a line with an R^2 value between 0.990 and 1, meaning that the amplification efficiency was comparable for all five different RNA concentrations. This also meant that any of the five RNA concentrations would theoretically be optimal for gene expression assays. Yet, as the lowest RNA concentration of the samples purified for the gene expression investigation was close to 10 ng/μl (see Section 4.3.1), it was decided that all real-time PCR assays would be performed from RNA dilutions of 10ng/μl. Only the 160ng/rxn dilution point of *Bhmt* seemed to fall outside the linear range, therefore, this dilution point was left out of the plot as this total RNA concentration was certainly too high to yield reliable results.

4.3.3 Validation of reference genes

Method

To ensure that the data is normalised to the most reliable reference gene, it was necessary to determine whether the expression of $\beta 2m$ and *Actβ* in the brain and quadriceps tissue was affected by the NDUFS4 deficiency. For this validation, the $2^{-\Delta Ct'}$ method described by Livak and Schmittgen (2001) was applied with the final gene expression assay results (as described in Section 4.4.1). In effect, an average of the raw Ct values of each genotype of each reference gene was obtained and these values were then substituted into Equation 4.4

$$\Delta Ct' = Ct_{target} - Ct_{control}$$

$$\therefore \text{substitute } \Delta Ct' \text{ in } 2^{-\Delta Ct'} \dots\dots\dots \text{Equation 4.4}$$

where *target* = the KO:WT genotype and *control* = the WT:WT genotype.

Results and discussion

Keeping in mind that a stable reference gene would yield a fold change value close to 1 (Livak & Schmittgen, 2001), *Actβ* revealed a value of 0.78, and $\beta 2m$ a value of 0.90. From this, it was evident that $\beta 2m$ was thus the most stably expressed reference gene between the two and it was decided that *Actβ* would not be used for normalization in further analyses.

Additionally, box plots were drawn for each reference gene, so that the distribution of antilog Ct values and therefore, the consistency of expression might be visually inspected. As seen in Figure 4.3 A, the antilog Ct values of *Actβ* ranged from 9.34E-8 to 1.03E-6, which

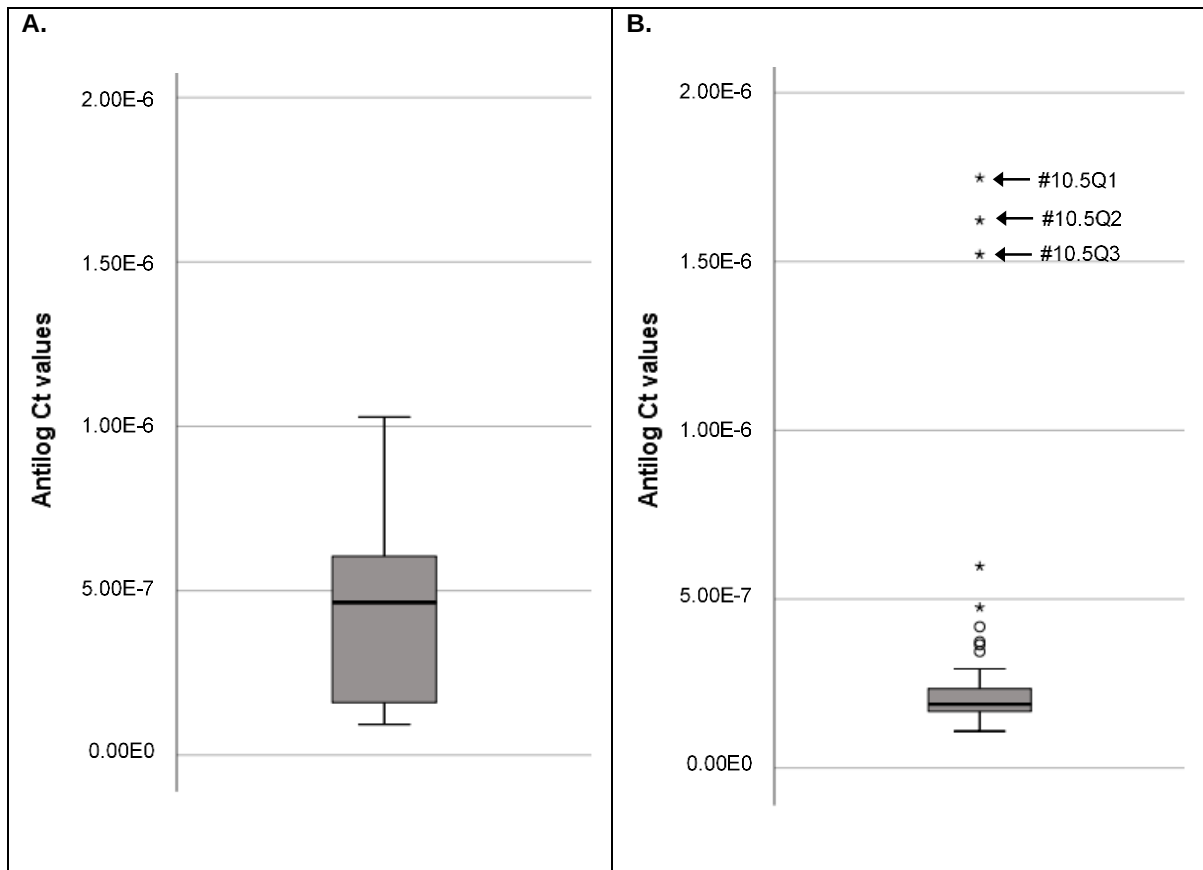


Figure 4.3 Graphical representations of the distributions of the antilog Ct values of the two reference genes. Box plots of the antilog Ct values of *Actβ* (A) and of *β2m* (B) can be seen. Quadriceps (Q) data of mouse #10.5 are indicated with arrows (refer to Section 4.4.1 for an explanation).

indicates a large distribution and an instability in gene expression in a set of exponential reactions. From Figure 4.3 B, however, it is evident that *β2m* had a much smaller distribution of data.

For any gene to qualify as a reference gene in RT-qPCR analyses, it has to maintain a stable basal level of expression – independent of the cell-cycle and across experimental and medical conditions, disease states and tissue types (Selvey *et al.*, 2001; Suzuki *et al.*, 2000). It is often assumed that all housekeeping genes (those that are essential for the maintenance of basic cellular functions) are ubiquitously expressed in all tissues and are unaffected by any treatment. This was also assumed in this study. Thus, *β2m* and *Actβ* – reference genes that are commonly used in the MRL – were selected as reference genes for this study, assuming that the effect of a CI deficiency and a transgenic Mt1 overexpression might not affect their expression (Bernard *et al.*, 1999; Janssens *et al.*, 2019; Van der Westhuizen *et al.*, 2003).

However, this assumption was tested in this study. While processing the data, the 2^{-Ct} method for assessing the suitability of a reference gene, as described by Livak and Schmittgen (2001), was applied with the Ct values obtained from *Actβ* and *β2m*. In comparison to *Actβ*, the expression of *β2m* was found to be more stable across both the brain and quadriceps tissue and across the four genotypes; therefore, it was decided to normalise the gene expression data of the other genes only to *β2m*.

In a real-time PCR study on a number of mouse models regarding the mouse uterus in the peri-implantation period, Lin *et al.* (2013) identified 10 potential reference genes and evaluated their normalisation suitability before the investigation. After a maximum amount of 10 potential reference genes had been evaluated by computer tools such as geNorm, Bestkeeper, Normfinder, RefGenes, Genevestigator and REST [also mentioned by Pfaffl (2004)], at least three of the most stable reference genes were chosen as the final reference genes for the study. In the end, the data of these reference genes were averaged to compose a reference gene index, which was used for normalisation purposes.

Interestingly, a number of publications advise against the use of *Actβ* as a reference gene, as the variability regarding its expression with certain treatments and its apparent cell-cycle dependency, have been shown (Lin & Redies, 2012; Olivier, 2004; Selvey *et al.*, 2001). It is not even expressed stably across siblings of healthy mice. It has been found that the ratios between γ-actin and β-actin genes differ from the abundance of the proteins, thus saying that the post-translational modification weighs heavier than regulation by the gene promoter. Additionally, *Actβ* expression is affected by lower levels of glutathione, albeit in the absence of oxidative stress, which in turn leads to a changed cellular morphology (Vedula & Kashina, 2018; Zepeta-Flores *et al.*, 2018). Furthermore, Pfaffl (2004) states that pseudogenes of *Actβ* interfere with RT-qPCR analyses. The findings in this study thus support those of other recent studies: *Actβ* expression is unreliable for the purpose of normalisation of target genes.

By contrast, the 2^{-Ct} method verified that *β2m* was the more stable reference gene when comparing *Actβ* and *β2m*. Independently of these calculations, however, Gabert *et al.* (2003) has shown *β2m* to be a reliable control gene in leukemic samples and Matsuzaki *et al.* (2015) states that *β2m* is appropriate for normalisation purposes in hematopoietic stem cells. Also, *β2m* has been found to be the second most reliable reference gene in HeLa cells with a rotenone-induced CI deficiency, next to 18S RNA (Olivier, 2004). Therefore, the use of *β2m* as the standard reference gene in postgraduate studies in the MRL seems justified (Mereis, 2018; Olivier, 2004; Pretorius, 2006).

In short, the unstable expression of *Actβ* disqualified it as a reference gene, whereas the expression of *β2m* was stable enough. Therefore, *β2m* was used as a reference gene for the target gene expression assays.

4.3.4 Target Gene Expression Assays

Method

For the determination of expression of ten genes relative to *β2m*, the total RNA of brain and quadriceps tissues of 24 mice was isolated, purified, and the concentrations and purities were determined as described in Section 4.3.1. Thus, the total number of samples examined per gene was 48 (i.e six mice from each of the four genotypes, and in two tissue types). Having ascertained that RNA can amplify successfully at 10ng/rxn, all gene expression assays were performed at this concentration. Dilutions of the RNA were made before the analyses and stored at -80°C.

The RT-qPCR method as outlined in Chapter 3, Section 3.3.3, was applied. However, the changes to the working method that has been described in Section 4.3.2 were also applied. Each PCR reaction had a final volume of 10μl and consisted of 1x TaqMan® RT-PCR Mix (2x), 1x TaqMan® RT Enzyme Mix (40x), 1x Taqman® Gene Expression Assay (20x) (refer to Table 4.2 for details on the gene-specific primer/probe mixes), nuclease-free water and 10ng/rxn RNA template. In Section 4.3.2, information about the final concentrations of reagent constituents is given. The plate layout is illustrated in Figure 4.4.

	1	2	3	4	5	6	7	8	9	10	11	12	
A					Blank	Blank	Blank						
B	9.1Q	9.1Q	9.1Q	9.1B	9.1B	9.1B	10.3Q	10.3Q	10.3Q	10.3B	10.3B	10.3B	GEA 1
C	10.1Q	10.1Q	10.1Q	10.1B	10.1B	10.1B	13.8Q	13.8Q	13.8Q	13.8B	13.8B	13.8B	
D	10.5Q	10.5Q	10.5Q	10.5B	10.5B	10.5B	9.5Q	9.5Q	9.5Q	9.5B	9.5B	9.5B	
E	9.1Q	9.1Q	9.1Q	9.1B	9.1B	9.1B	10.3Q	10.3Q	10.3Q	10.3B	10.3B	10.3B	
F	10.1Q	10.1Q	10.1Q	10.1B	10.1B	10.1B	13.8Q	13.8Q	13.8Q	13.8B	13.8B	13.8B	GEA 2
G	10.5Q	10.5Q	10.5Q	10.5B	10.5B	10.5B	9.5Q	9.5Q	9.5Q	9.5B	9.5B	9.5B	
H					-RT Cont	-RT Cont	-RT Cont						
	WT:WT						KO:WT						

Figure 4.4 **The plate layout for one of the RT-qPCR assays.** The quadriceps samples for Gene Expression Assay 1 (GEA 1) are marked in green and the brain samples for GEA 1 are marked in blue. The quadriceps samples for GEA 2 are marked in orange and the brain samples for GEA 2 are marked in yellow. The text in red indicates a KO:WT genotype and the text in black indicates the WT:WT genotype. The other plates were loaded in a similar fashion.

Each sample was loaded in triplicate. In order to avoid a batch effect as far as possible, the samples of each gene expression assay were distributed over two 96-well plates and it was ensured that both genotypes, as well as both tissue types could be found on one plate. With 12 samples examined for one primer/probe mix on a plate, only 36 wells were occupied. Thus, the other half of the plate was loaded with 12 samples of another primer/probe mix.

Throughout the assay, all reagents – the Taqman® RNA-to-Ct 1-Step™ kit, the RNA dilutions as well as all master mixes and premixes – were kept on ice and the sterility of the apparatus and environment was a high priority.

4.4 COMPARISON OF EXPRESSION OF SELECTED GENES IN NDUFS4 KO AND TGMT1 MICE TO THAT OF WT:WT MICE (OBJECTIVE 6)

4.4.1 Analysis of the real-time PCR data

For the reader to follow the explanation of the data analysis, the following terminology and abbreviations were used:

$\beta 2m$ = beta-2-microglobulin, the reference gene

Any other gene = target gene

WT:WT = control

Any other genotype = treated

The analysis of the RT-q-PCR data was performed by combining methods from Livak and Schmittgen (2001) and Pfaffl (2007). For each real-time PCR reaction, a Ct value was yielded at the end of the analysis. Each Ct value was obtained at a specified threshold of 0.2 from the log-linear plot of the PCR signal against the cycle number (Livak & Schmittgen, 2001). For each set of triplicates, the average, standard deviation and the coefficient of variance (CV) were calculated. Outliers in the data were removed by way of visually inspecting the CV values, and if the CV value of triplicates for a specific sample was >1%, the outlier replicate was removed.

Based upon box plots that were drawn with the antilog Ct values, all quadriceps data from mouse #10.5 were removed as well. This was the consequence of all three readings being consistently beyond 3 times the interquartile range (IQR) for most of the genes, and therefore, regarded as outliers/inaccurate data. Refer to Annexure B and Figure 4.3 for these box plots.

By subtracting the average Ct value of the reference gene of each sample from the average Ct value of the target gene of the corresponding sample, Δ Ct-values were calculated. These values were then organised according to genotype and tissue type. With the six Δ Ct values listed under each genotype (n=6 per genotype), the average and standard deviation were calculated as well. A $\Delta\Delta$ Ct value was then calculated by subtracting the average Δ Ct value of the control group from that of the treated group. (These calculations were performed in both tissues). The standard deviations that have been calculated for the Δ Ct values, had to be propagated into the $\Delta\Delta$ Ct value according to Equation 4.5, as described by Steyn *et al.* (1999) below:

$$\text{Propagated stdev} = \sqrt{\frac{(n_{\text{control}}-1) \times (\text{stdev}_{\text{control}}^2) + (n_{\text{treated}}-1) \times (\text{stdev}_{\text{treated}}^2)}{n_{\text{control}} + n_{\text{treated}} - 2}} \dots\dots \text{Equation 4.5}$$

With this information, the fold change in gene expression could be calculated. The classic approach used to calculate fold change from Ct values is given in Equation 4.6

$$\text{Fold change in gene expression} = 2^{-\Delta\Delta\text{Ct}} \dots\dots\dots \text{Equation 4.6}$$

However, this equation assumes that the PCR efficiency values of both the target gene and the reference gene are equal to 2. A more accurate approach to calculate fold change from Ct values, where PCR efficiency for each individual gene is taken into account and corrected for, was proposed by Pfaffl (2007). This method is demonstrated in Equation 4.7 below:

$$\text{Efficiency-corrected fold change} = \frac{(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{avg control}-\text{avg treated})}}{(E_{\text{ref}})^{\Delta\text{Ct}_{\text{ref}}(\text{avg control}-\text{avg treated})}} \dots\dots\dots \text{Equation 4.7}$$

In order to be able to plot error bars on a graph with these data, the propagated standard deviation was incorporated into Equation 4.7, to form Equations 4.8 and 4.9:

$$\text{Maximum} = \frac{(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{avg control}-\text{avg treated}+\text{prop stdev})}}{(E_{\text{ref}})^{\Delta\text{Ct}_{\text{ref}}(\text{avg control}-\text{avg treated})}} \dots\dots\dots \text{Equation 4.8}$$

$$\text{Minimum} = \frac{(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{avg control}-\text{avg treated}-\text{prop stdev})}}{(E_{\text{ref}})^{\Delta\text{Ct}_{\text{ref}}(\text{avg control}-\text{avg treated})}} \dots\dots\dots \text{Equation 4.9}$$

With the conventional $2^{-\Delta\Delta Ct}$ method, the gene expression of the reference gene of the control genotype would be equal to 2^0 , as $\Delta\Delta Ct$ would be equal to 0, therefore, by definition, the gene expression would be 1. When investigating target genes and treated groups, a fold change of 2 would indicate a gene expression that has doubled and a fold change of 0.5 would signify half of the original gene expression.

4.4.2 Gene expression results

Table 4.4 A and B summarise the changes in gene expression of the genes involved in 1-C metabolism and in oxidative stress metabolism, respectively. Figures 4.5, 4.6 and 4.7 are visual representations of these data. Two groups are depicted on the x-axis of each plot: the WT:WT vs KO:WT and the WT:WT vs KO:OVER. The $2^{-\Delta\Delta Ct}$ values are on the y-axis. In other words, the data is normalised with the gene expression data of $\beta 2m$ and the control in the $2^{-\Delta\Delta Ct}$ equation is the WT:WT genotype. The comparison of the WT:OVER genotype with the control is not shown here, as the effect of Mt1 overexpression on an NDUFS4 defect could clearly be observed by comparing only the KO:WT and KO:OVER genotypes to the WT:WT genotype. As cut-offs for indicating notability of real-time PCR results is population-dependent and gene-dependent, and as a relative quantification approach was followed in this study, cut-off values were selected according to this rule: Where a gene expression fold change was below 0.75 or above 1.5, it was regarded as notable (Caraguel *et al.*, 2011)⁶.

Values in brackets in the following paragraphs are indicated as:

(expression fold change; lowest value of the range to highest value of the range)⁷.

*Also, to simplify reading, the gene discussed in each paragraph is written in **bold** text.*

Data in Figures 4.5, 4.6 and 4.7 are indicated with black dots. Bottom black dot = lowest value of the range; Middle black dot = expression fold change value; Top black dot = highest value of the range. Notable data are indicated with brackets and an asterisk.

⁶ Description of fold change in gene expression:

≤ 0.75	: notable change
> 0.75 and < 1.5	: negligible change
≥ 1.5	: notable change

⁷ Description of range size (difference between lowest value and highest value):

≤ 0.2	: very narrow
0.21 – 0.40	: narrow
0.41 – 0.60	: fairly narrow
0.61 – 0.80	: fairly wide
0.81 – 1.00	: wide
1.1 – 1.2	: very wide
≥ 1.2	: exceptionally wide

Results in Brain tissue: 1-C Metabolism Genes

Figure 4.5 (A) illustrated the fold change in **Mthfd2** expression in experimental mice compared to control mice. Where the KO:WT and WT:WT genotypes are compared, a very narrow range and a notable change in gene expression in the brain tissue is observed (0.56; 0.49 to 0.64). Therefore, it would seem that *Mthfd2* expression is downregulated in KO:WT mice. Similar results were obtained where the WT:WT and KO:OVER genotypes were compared (0.58; 0.66 to 0.51).

In Figure 4.5 (B), the fold change in **Gclc** expression is depicted. Even though the range is narrow, the change in gene expression (0.90; 0.74 to 1.10) between the WT:WT and the KO:WT genotypes is negligible. The change in gene expression between the WT:WT and KO:OVER genotypes was also negligible with a narrow range (0.96; 0.79 to 1.17).

As seen in Figure 4.5 (C), the change in **Phgdh** expression is negligible and the range is narrow where the WT:WT and KO:WT genotypes are compared (0.96; 1.13 to 0.82). Similarly, where the WT:WT and KO:OVER genotypes are compared (1.07; 1.29 to 0.88), a negligible change in gene expression with a fairly narrow range is observed.

As seen in Figure 4.5 (D), the change in gene expression of **Bhmt** between the WT:WT and KO:WT genotypes, is notable according to the interpretation of this study, with a narrow range (0.40; 0.25 to 0.63). With the highest end of the range being below 0.75, it would seem that *Bhmt* expression is downregulated in the KO:WT mice. The same observation is made for the WT:WT vs KO:OVER group, though the range is fairly narrow (0.55; 0.35 to 0.87).

In Figure 4.5 (E), negligible fold changes in expression of **Lias** with narrow ranges are depicted where the WT:WT and KO:WT genotypes are compared (0.83; 0.66 to 1.05), as well as where the WT:WT and KO:OVER genotypes are compared (1.04; 0.93 to 1.18).

In Figure 4.5 (F), the expression fold change of **Tyms** is illustrated. Where the WT:WT and KO:WT genotypes were compared (0.61; 0.49 to 0.77), the range is narrow and the change in gene expression is notable. In comparing the WT:WT and KO:OVER genotypes, however (0.78; 0.60 to 1.00), the fold change is negligible with a narrow range.

The gene expression fold change of **Mtrr** is depicted in Figure 4.5 (G). The expression fold change where the WT:WT and KO:WT genotypes are compared (0.71; 0.60 to 0.84) as well as where the WT:WT and KO:OVER genotypes are compared (0.75; 0.64 to 0.88) display a notable change in gene expression. Additionally, the ranges of both groups are narrow which signifies reliable results.

Table 4.4 **A summary of the gene expression fold change data in (A.) brain tissue and (B.) quadriceps tissue.** “Max” indicates the highest value of the data range, whereas “min” indicates the lowest value of the range (calculated with equations 4.8 and 4.9, respectively). “Fold change” indicates the efficiency-corrected fold change values (calculated with equation 4.7).

A.

BRAIN TISSUE							
Gene category	Gene name	WT:WT vs KO:WT			WT:WT vs KO:OVER		
		Max	Min	Fold change	Max	Min	Fold change
1-C metabolism	<i>Mthfd2</i>	0.64	0.49	0.56	0.66	0.51	0.58
	<i>Gclc</i>	1.10	0.74	0.90	1.17	0.79	0.96
	<i>Phgdh</i>	1.13	0.82	0.96	1.29	0.88	1.07
	<i>Lias</i>	1.05	0.66	0.83	1.18	0.93	1.04
	<i>Tyms</i>	0.77	0.49	0.61	1.00	0.60	0.78
	<i>Mtrr</i>	0.84	0.60	0.71	0.88	0.64	0.75
	<i>Bhmt</i>	0.63	0.25	0.40	0.87	0.35	0.55
Oxidative stress	<i>Sod2</i>	0.94	0.73	0.83	1.00	0.74	0.86
	<i>Cat</i>	1.03	0.63	0.80	1.17	0.72	0.92
	<i>Gpx1</i>	0.93	0.72	0.82	1.14	0.69	0.88

B.

QUADRICEPS TISSUE							
Gene category	Gene name	WT:WT vs KO:WT			WT:WT vs KO:OVER		
		Max	Min	Fold change	Max	Min	Fold change
1-C metabolism	<i>Mthfd2</i>	1.50	0.69	1.01	1.02	0.50	0.71
	<i>Gclc</i>	1.78	0.94	1.30	1.24	0.62	0.88
	<i>Phgdh</i>	1.82	0.85	1.25	1.74	1.16	1.42
	<i>Lias</i>	1.67	0.73	1.10	1.56	0.71	1.05
	<i>Tyms</i>	1.43	0.48	0.83	1.01	0.39	0.63
	<i>Mtrr</i>	1.74	0.75	1.14	1.23	0.60	0.86
	<i>Bhmt</i>	3.18	0.39	1.12	1.11	0.19	0.46
Oxidative stress	<i>Sod2</i>	1.57	0.72	1.06	1.09	0.54	0.77
	<i>Cat</i>	1.56	0.77	1.09	1.52	0.75	1.07
	<i>Gpx1</i>	1.15	0.89	1.01	0.88	0.61	0.73

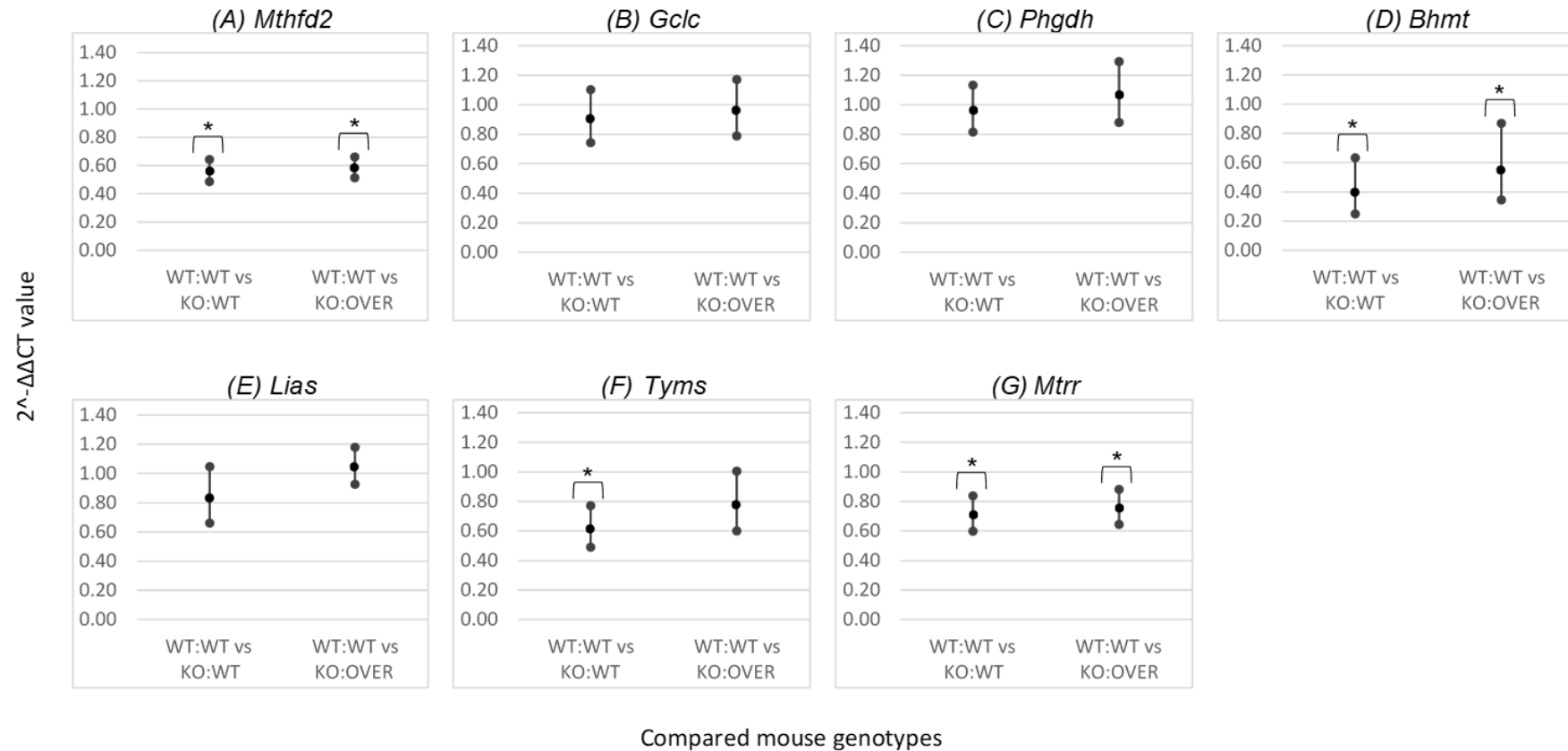


Figure 4.5 A cluster of plots of the fold change in gene expression of 1-C metabolism genes investigated in mouse brain tissue. The $2^{-\Delta\Delta CT}$ values are indicated on the Y-axes and the compared mouse genotypes are depicted on the X-axes. On the left side of each plot, the data from the WT:WT and the KO:WT brain tissue are compared and on the right side of each plot, the data from the WT:WT and the KO:OVER brain tissue are compared. Bottom black dot = lowest value of the range; Middle black dot = expression fold change value; Top black dot = highest value of the range. Notable data are indicated with brackets and an asterisk.

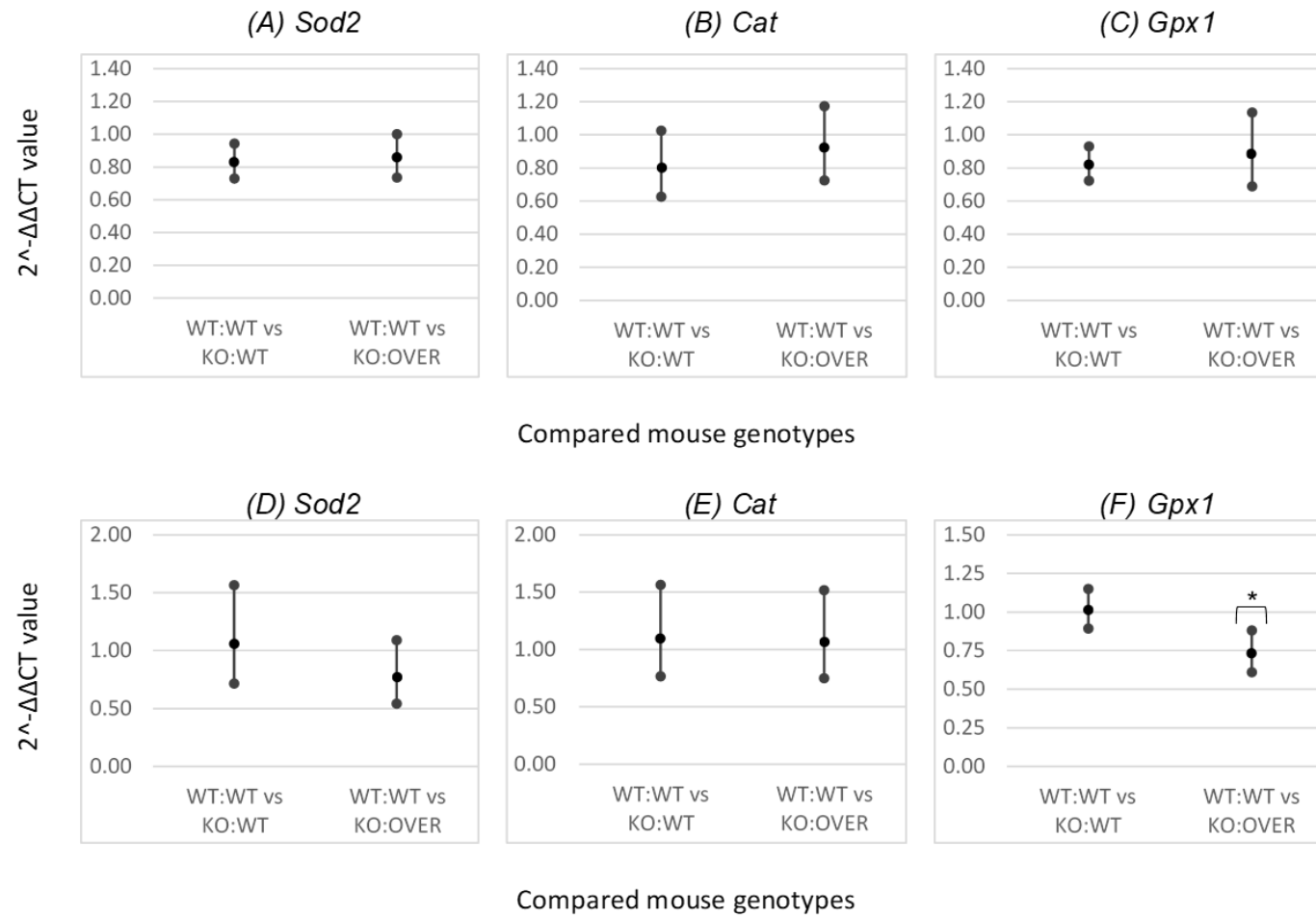


Figure 4.6 A cluster of plots of the fold change in gene expression of oxidative stress metabolism genes investigated in mouse brain tissue (A-C) and quadriceps tissue (D-F).

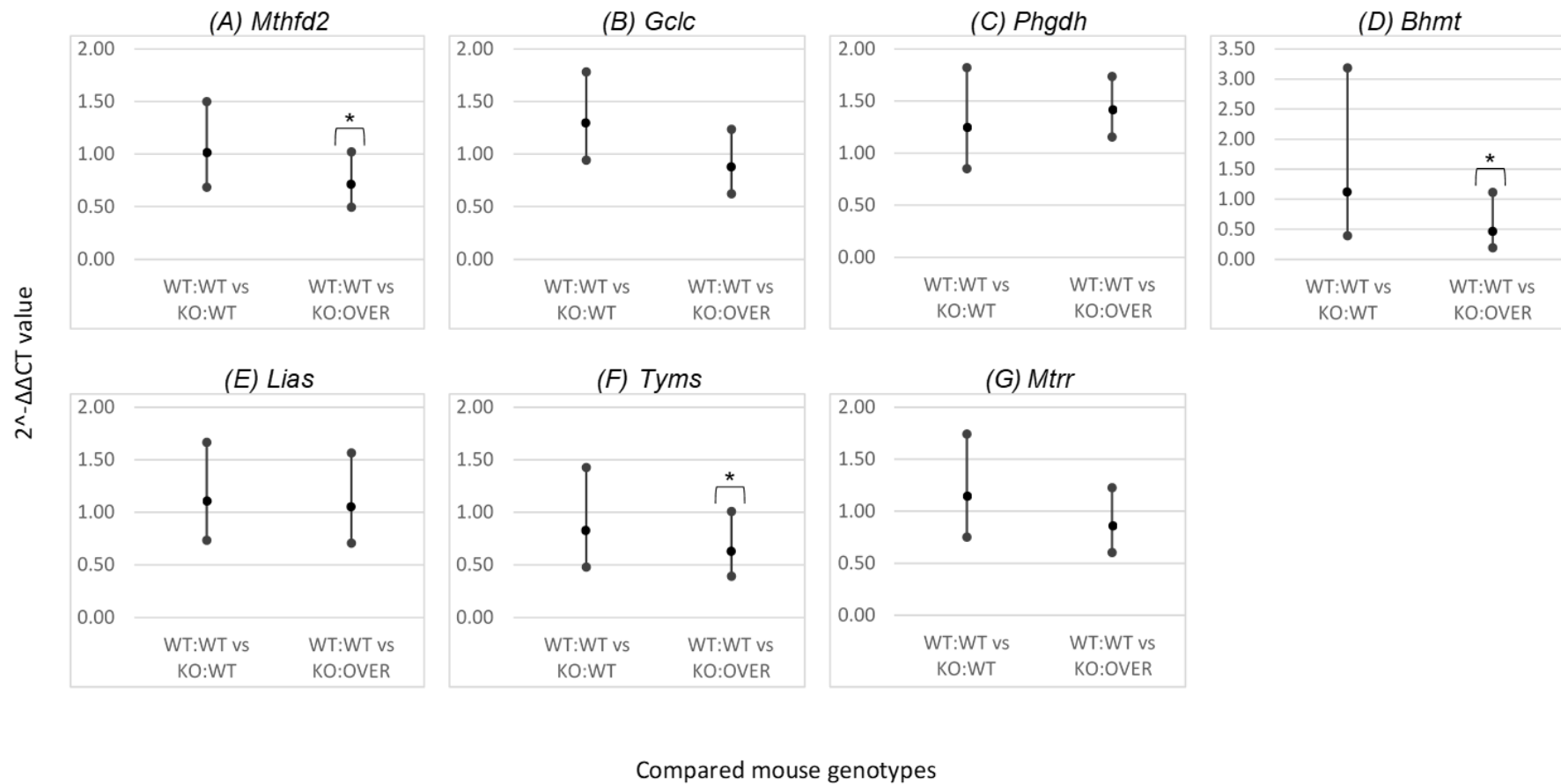


Figure 4.7 A cluster of plots of the fold change in gene expression of 1-C metabolism genes investigated in mouse quadriceps tissue.

Results in Brain tissue: Oxidative stress genes

As is evident from Figure 4.6 (A), where the fold change in **Sod2** expression is illustrated, the downregulating change is negligible where the WT:WT and KO:WT genotypes are compared (0.83; 0.73 to 0.94). A similar observation is made where the WT:WT and KO:OVER genotypes are compared (0.86; 0.74 to 1.00). Additionally, the ranges are narrow for both comparison groups.

In Figure 4.6 (B), the gene expression fold change values for **Cat** is depicted. Where the WT:WT and KO:WT genotypes are compared, the range is fairly narrow (0.80; 0.63 to 1.03) with a negligible change in gene expression. Likewise, the range is fairly narrow and the gene expression negligible where the WT:WT and KO:OVER genotypes are compared (0.92; 0.72 to 1.17).

Even though the downregulating change in **Gpx1** expression is negligible where the WT:WT and KO:WT genotypes are compared, the range is narrow (0.82; 0.72 to 0.93). As is clear from Figure 4.6 (F), the range is fairly narrow where the WT:WT and KO:OVER genotypes are compared (0.88; 0.69 to 1.14) and as the fold change value is above 0.75, the change in **Gpx1** expression is negligible.

Results in Quadriceps tissue: 1-C Metabolism Genes

The change in **Mthfd2** expression where the WT:WT and KO:WT genotypes are compared (1.01; 0.69 to 1.50) is negligible and the wide range practically extends from the lower point of notability to the higher point of notability [see Figure 4.7 (A)]. However, when comparing the WT:WT and KO:OVER genotypes, there is a notable downregulating change in gene expression (0.71; 0.50 to 1.02) with a fairly narrow range.

Though the change in **Gclc** expression, where the WT:WT and KO:WT genotypes are compared (1.30; 0.94 to 1.78), is negligible according to the interpretation of this study, it is in an upregulating direction [see Figure 4.7 (B)]. The wide range, however, negatively impacts on the reliability of the results and should be taken into consideration during the interpretation of results. The change in gene expression, where the WT:WT and KO:OVER genotypes are compared (0.88; 0.62 to 1.24), is in a downregulating direction, but negligible. The range here is fairly wide.

In addition to the negligible change in **Phgdh** expression where the WT:WT and KO:WT genotypes are compared (1.25; 0.85 to 1.82), the wide range is detrimental to the reliability of the results. The fold change where the WT:WT and KO:OVER genotypes are compared

(1.42; 1.16 to 1.74) is negligible with a fairly narrow range. However, an upregulating tendency is observed in both groups [see Figure 4.7 (C)]

In Figure 4.7 (D) the fold change in **Bhmt** expression between experimental and control mice is illustrated. Even though the fold change value where the WT:WT and KO:WT genotypes are compared (1.12; 0.39 to 3.18) presents a negligible change gene expression, the range is exceptionally wide. This makes it impossible to draw conclusions from the results. While the downregulating change in gene expression is notable where the WT:WT and KO:OVER genotypes are compared (0.46; 0.19 to 1.11), the range is again wide and the result considered unreliable.

It is clear from Figure 4.7 (E) that there is no notable change in **Lias** expression where the WT:WT and KO:WT genotypes are compared (1.10; 0.73 to 1.67), as well as where the WT:WT and KO:OVER genotypes are compared (1.05; 0.71 to 1.56). Additionally, the ranges of both comparison groups are wide.

As can be seen in Figure 4.7 (F), the change in **Tyms** expression where the WT:WT and KO:WT genotypes are compared (0.83; 0.48 to 1.43) is negligibly downregulated and it ranges from the lower point of notability to the higher point of notability. Therefore, reliability of the results is negatively impacted. The change in gene expression in the WT:WT vs KO:OVER group is, however, notable and in a downregulating direction, albeit with a fairly wide range (0.63; 0.39 to 1.01).

As seen in Figure 4.7 (G), the changes in **Mtrr** expression for both comparison groups are negligible. Where the WT:WT and KO:WT genotypes are compared (1.14; 0.75 to 1.74), the range is wide and where the WT:WT and KO:OVER genotypes are compared (0.86; 0.60 to 1.23), the range is fairly wide.

Results in Quadriceps tissue: Oxidative stress genes

Where the WT:WT and KO:WT genotypes are compared, the change in **Sod2** expression (1.06; 0.72 to 1.57) is negligible and the range is wide. However, in Figure 4.6 (D) it is clear that there is a downregulating tendency where the WT:WT and KO:OVER genotypes are compared (0.77; 0.54 to 1.09), even though the fold change value is negligible and the range is fairly narrow.

The **Cat** expression fold change results for both comparison groups are very similar, as can be seen in Figure 4.6 (E). Where the WT:WT and KO:WT genotypes are compared (1.09;

0.77 to 1.56), as well as where the WT:WT and KO:OVER genotypes are compared (1.07; 0.75 to 1.52), the change in gene expression is negligible and the range is fairly wide.

As can be seen in Figure 4.6 (F) the change in **Gpx1** expression where the WT:WT and KO:WT genotypes are compared (1.01; 0.89 to 1.15) is negligible and the narrow range confirms this. By contrast, the change in Gpx1 expression is notable with a narrow range and in a downregulating direction where the WT:WT and KO:OVER genotypes are compared (0.73; 0.88 to 0.61).

Discussion

In this section, the gene expression assay results of seven 1-C genes and three oxidative stress genes in the whole-brain and quadriceps tissues of WT:WT, KO:WT and KO:OVER mice are discussed. In Figures 4.8 and 4.9, 1-C metabolism and oxidative stress metabolism as part of the MT1-redox cycle are depicted, respectively, and the enzymes that were up-/downregulated are indicated in a visual manner.

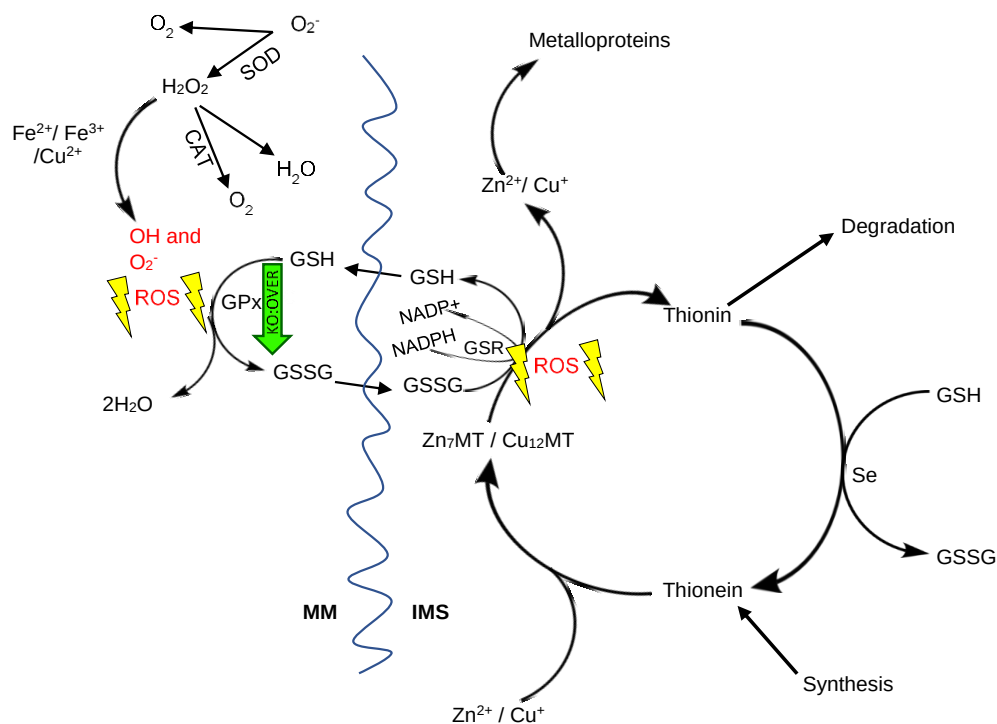


Figure 4.8 The oxidative stress metabolism and MT-redox cycle as impacted by an **NDUFS4** defect and **MT1** overexpression. Green arrows indicate downregulation in quadriceps tissue. Genotypes are indicated inside arrows.

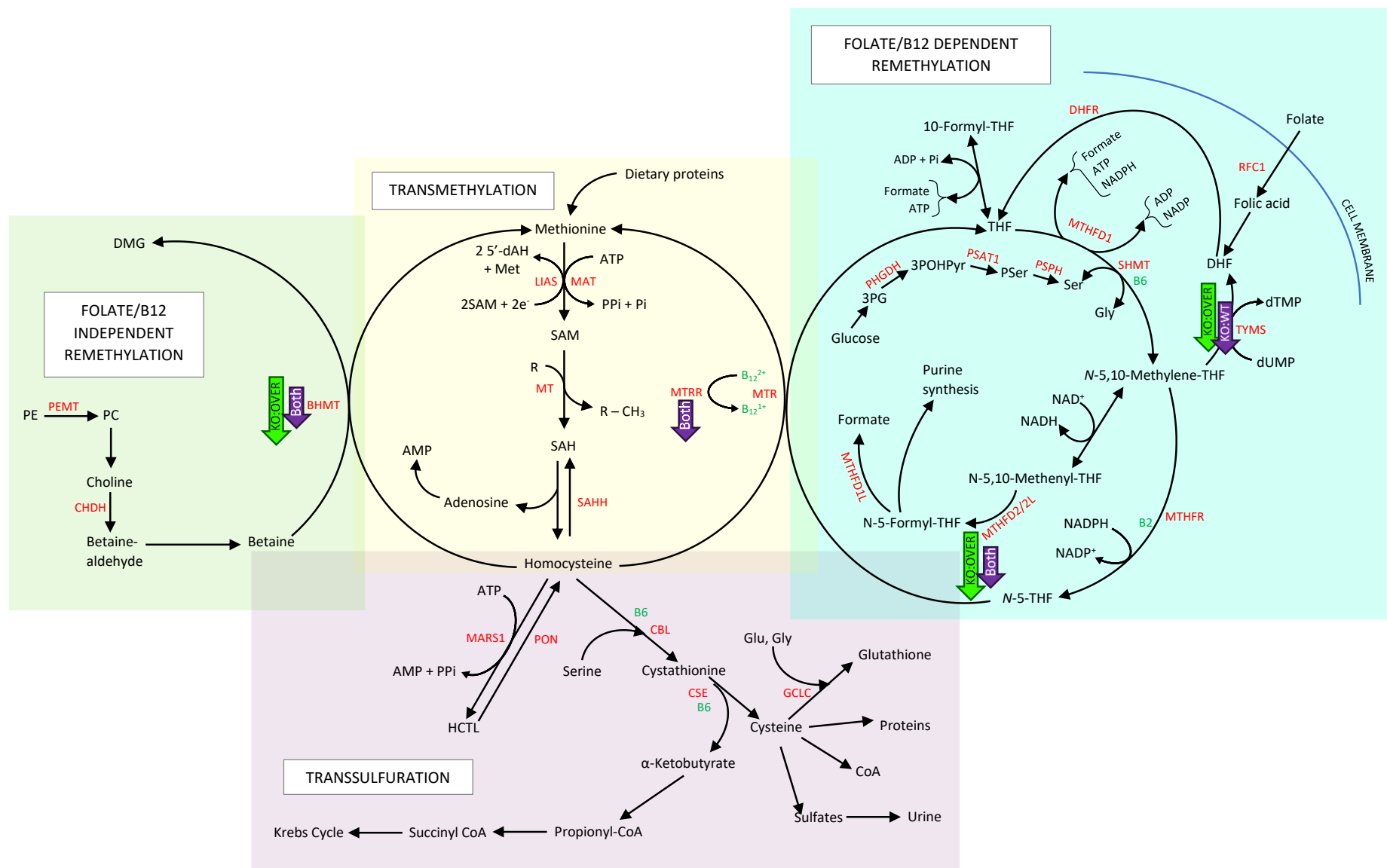


Figure 4.9 1-C metabolism as impacted by an NDUFS4 defect and MT1 overexpression. Purple arrows indicate downregulation of investigated genes in brain tissue and green arrows indicate downregulation in quadriceps tissue. Genotypes are indicated inside arrows. Both = both comparison groups.

It is evident from the results that most gene expression fold change ranges were very narrow to fairly narrow in the brain tissues and fairly wide to exceptionally wide in the quadriceps tissues. This is observed even though the assays were purposefully constructed in such a way that each 96-well plate contained samples of both tissue types and that each sample was loaded in triplicate. Possible reasons for the large ranges in the data – and therefore weak repeatability – might be inter-assay variation, the small sample size of this study and the inherent genetic variability among the mice. It might also be attributed to muscle tissue being more challenging to homogenise and extract analytes from than brain tissue, as well as the difference in mRNA content (measured concentrations) between the two tissue types. Interestingly, Zhou *et al.* (2017) has shown that the amount of DNA-binding transcription factors exposed in the skeletal muscle is 173 in total, but in the brain, it is 310. This clearly shows that the total transcriptional activity taking place in the two tissues is very different. Furthermore, this corresponds with the RNA concentrations of the brain and quadriceps tissue after purification: the average quadriceps RNA concentration being 27.59ng/μl, as opposed to an average brain RNA concentration of 91.30ng/μl. This means that the total amount of RNA per wet gram of tissue differed greatly between the brain and quadriceps.

Still, the wide ranges in the data are a great drawback, as these results cannot be interpreted with certainty. In the quadriceps tissues, the gene expression assays with fairly wide to exceptionally wide ranges, include *Gclc*, *Bhmt*, *Lias*, *Tyms*, *Mtrr*, and *Cat* in both comparison groups, and *Phgdh* and *Sod2* in the group where WT:WT and KO:WT genotypes were compared. No wide ranges were recorded in the brain tissues.

By contrast, some of the gene expression assays (*Gpx1* in both groups in the brain tissue and where WT:WT was compared to KO:WT in the quadriceps tissue; *Gclc* in both groups in the brain; *Sod2* in both groups in the brain tissue and where WT:WT and KO:OVER was compared in the quadriceps tissue; *Phgdh* in both groups in the brain tissue and where WT:WT was compared to KO:OVER in the quadriceps tissue; *Lias* in both groups in the brain tissue), yielded results with narrow ranges, even though the change in gene expression is negligible. In other words, with good repeatability, it may be said that an NDUFS4 defect had no notable effect on the expression of *Gpx1*, *Gclc*, *Lias*, *Phgdh* and *Sod2* in the mouse brain tissues and on *Gpx1* in the quadriceps tissues. On the other hand, *Mt1* had no notable impact upon the expression of *Gpx1*, *Gclc*, *Lias*, *Phgdh* and *Sod2* in the brain tissues, nor on *Sod2* and *Phgdh* in the

quadriceps tissue. The change in expression of the latter two genes did, however, show down- and upregulating tendencies, respectively.

Therefore, contrary to the predicted results in Chapter 2, Section 2.7, *Gpx1*, *Lias*, *Sod2* and *Gclc* expression was not upregulated.

Evidently, *Mt1* overexpression had a downregulating effect upon *Mthfd2* expression in the quadriceps tissue, as there is a notable difference between the gene expression in the WT:WT vs KO:WT group and the WT:WT vs KO:OVER group. In the brain, however, *Mt1* overexpression did not have any effect, but the NDUFS4 defect had a notable downregulating effect upon both comparison groups. This corresponds with the expected result that is described in Chapter 2, Section 2.7. This downregulating effect might link to AMP-activated kinase (AMPK), attempting to conserve energy, as Rosenzweig *et al.* (2018) explains. When energy levels are high and nutrients are abundant, anabolic 1-C metabolism functions to support growth and proliferation (promoted by mTOR signalling). However, when energy and nutrient levels are low, AMPK inhibits anabolic reactions and promotes catabolism. By way of the PPARG coactivator 1 alpha/oestrogen-related receptor alpha (PGC-1 α /ERR α) axis, AMPK downregulates the expression of MTHFD2. This way, the flux of methyl groups from Ser and Gly to 1-C metabolism products is limited – all in an attempt to conserve energy. *Mthfd2* is also regulated by the binding of nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) (that is stimulated by ROS), to the antioxidative response elements (AREs) in the gene promoters (Raghunath *et al.*, 2018). However, the sustained notable downregulation of *Mthfd2* in KO:OVER mouse brain samples indicate that *Mt1* overexpression does not alleviate the effect of a CI deficiency on gene expression in mice.

There is an apparent slight upregulation of the expression of *Phgdh* in both groups in the mouse quadriceps and no notable changes in gene expression in either group in the brain. It was proposed by Grant *et al.* (1996) that *Phgdh* is in fact not genetically, but allosterically regulated by Ser levels. This might be a reason why there is no observable change in *Phgdh* expression in either the NDUFS4 defect or *Mt1* overexpression model.

Though the wide ranges in both groups of *Bhmt* data in the brain makes interpretation difficult, it is implied that *Bhmt* is downregulated in the WT:WT vs KO:WT group. This corresponds with the prediction in Chapter 2, Section 2.7 and it makes sense when considering the findings of Coetzer (2019), who identified lower levels of betaine in the cerebellum of NDUFS4 KO mice. Terburgh *et al.* (2019) found elevated levels of choline and lower levels of DMG and betaine in the quadriceps tissue of NDUFS4 KO mice and stated that it might stem from the mitochondrial NAD⁺ shortage and the use of DMG by the ETF/ETF-QO system (an adaptive mechanism in

the case of a CI defect). *Bhmt* is known to have a SAM-responsive element in the promoter, therefore, perturbed SAM levels in cell would also impact *Bhmt* expression (Castro *et al.*, 2002).

Other findings by Terburgh *et al.* (2019) and Coetzer (2019) were downregulated Met in NDUFS4 KO mouse quadriceps tissue and lower levels of cysteine, Met and Ser in the anterior cortex of KO mice. This might be linked to downregulation of *Bhmt* expression, as the transmethylation of Hcy is now disturbed. Lower levels of cysteine would also impact the redox balance, as this amino acid plays a great role in the production of endogenous antioxidants like glutathione.

Additionally, when AMPK inhibits anabolic reactions, less SAM would be produced from Met, which might explain the slight downregulating tendency of *Lias* in the brain tissue of the NDUFS4 KO mice of this study. However, this negligible tendency – as opposed to the upregulation predicted in Chapter 2, Section 2.7 – might also result from lower ATP levels, which leads to lower SAM levels and consequently, lower LIAS activity (Rosenzweig *et al.*, 2018). Regarding the antioxidant function of LIAS, it would be interesting to investigate why this gene is downregulated where oxidative stress is thought to be abundant.

In the quadriceps, however, *Mt1* clearly has an effect upon the expression of *Gpx1*, as there is no change in gene expression in the WT:WT vs KO:WT group, but a notable downregulation is seen in the WT:WT vs KO:OVER group. This might be attributed to the ROS scavenging effect of Mt1 – i.e. the greater the concentration of MT1 in the cell, the fewer the ROS, and as there would be less activators of detoxifying enzymes (like NF-κB) in the environment, *Gpx1* expression might not be induced (Morgan & Liu, 2011). In the brain, the fold change is negligible but the narrow range in the WT:WT vs KO:WT group indicates good repeatability. The WT:WT vs KO:OVER group had a wide range.

With narrow ranges in the brain tissue and wide ranges in the quadriceps tissue, *Cat* showed no notable change in gene expression for any genotype in either tissue. Therefore, *Cat* was not upregulated, as predicted in Chapter 2, Section 2.7.

The gene expression of two other genes, namely *Gsr* (involved in oxidative stress) and *Shmt2* (involved in 1-C metabolism) was also evaluated in NDUFS4 KO mouse brains and quadriceps. However, after no change in gene expression was observed in either tissue or in any of the genotypes, analyses were not continued in the TgMt1 tissues.

In accordance with the prediction in Chapter 2, Section 2.7, there is notable downregulation in *Tyms* expression in the brain where the KO:WT and WT:WT genotypes were compared, which might result from AMPK promoting catabolic reactions in the mitochondrion (Rosenzweig *et al.*, 2018). Lower levels of UMP have also been observed in the anterior cortex of NDUFS4 KO

mice, which points to a disturbed *de novo* production of purine nucleotides that is catalysed by TYMS. Yet, the negligible change in gene expression where the KO:OVER and WT:WT genotypes were compared, points to a possible mitigating effect by *Mt1* overexpression in the brain. The WT:WT vs KO:WT group in the quadriceps tissue showed no notable change in *Tyms* expression, which corresponds with the findings of Field *et al.* (2016): 1-C metabolism is programmed to favour this pathway in the nucleus at the expense of homocysteine remethylation in the cytosol. On the other hand, it contradicted the prediction in Chapter 2, Section 2.7: *Tyms* expression was not downregulated in NDUFS4 KO quadriceps. The WT:WT vs KO:OVER group showed a downregulating tendency, therefore, it would appear that *Mt1* overexpression had a notable effect on *Tyms* expression in KO:OVER quadriceps tissue.

Interestingly, the results of *Mtrr* in the brain had narrow ranges in both groups and both are notably downregulated. These results were in accordance with the predicted outcome in Chapter 2, Section 2.7. This downregulation might be attributed to the role the redox imbalance plays in the regulation of the 1-C metabolism by mTOR and AMPK and, consequently, the methylation rates of specific CpG regions in the promoter of *Mtrr* (Bai *et al.*, 2019). Therefore, it can be concluded that *Mtrr* expression is influenced by an NDUFS4 defect, but this change is not mitigated by *Mt1* overexpression. In the quadriceps tissue, however, *Mtrr* is not notably up- or downregulated.

Conclusion

Considering both tissue types, however, *Mthfd2* showed the greatest change in gene expression in this study. In the brain, it is evident that the downregulation of *Mthfd2*, *Bhmt*, *Tyms* and *Mtrr* is the most significant as a result of the NDUFS4 defect. *Mthfd2* and *Gpx1* showed the greatest change in gene expression in quadriceps tissue.

Evidently, *Mt1* overexpression did not have a dramatic effect upon the expression of the investigated genes in this study, with the exception of *Tyms*. This corresponds with the work of Miller (2019), who conducted a protein oxidation investigation in NDUFS4 KO and TgMT1 mouse whole-brain tissue, and established that the parts of the brain that are greatly affected by ROS, are masked by the regions of the brain that are less affected. The switch to non-classical pathways – such as the ubiquinone (Q) cycle through complex III, in order to maintain a healthy ubiquinol (QH₂) pool – would also prevent the formation of the excessive amount of ROS that would theoretically form in an NDUFS4 defect (Terburgh *et al.*, 2019).

Therefore, it was observed that an NDUFS4 deficiency has a downregulating effect upon the expression of certain genes involved in 1-C metabolism and, possibly because of alternative cellular adaptive mechanisms, has no effect upon the expression of selected genes in the

oxidative stress metabolism in mouse brain and quadriceps tissue. Furthermore, apart from one gene, the overexpression of MT1 did not have a mitigating effect upon the expression of these genes in the absence of the NDUFS4 subunit.

Importantly, this study was interested in transcriptional regulation by proteins binding to cis-acting elements in the DNA. Yet, one must keep in mind that different ways of transcriptional regulation cannot be isolated in an intricately woven system of life. Transcription in skeletal muscle tissue is also regulated by insulin secretion, contraction and aging, and in the brain, neuronal activity has a huge influence on gene expression regulation (Hall & Guyton, 2012). All of these factors, as well as the disrupted redox balance, usage of DMG, transmethylation of Hcy and the production of *de novo* purine nucleotides would have effects on the regulation of the expression of this selection of genes. It must be remembered that they are part of an interdependent metabolic network.

CHAPTER 5: SUMMARY AND CONCLUSIONS

5.1 REVISITING THE AIM AND HYPOTHESIS

Mitochondrial disease (MD) is the most common inherited metabolic disease and affects one in 4300 people (23 per 100 000) (Schaefer *et al.*, 2019). As a group of energy deficiency disorders, they are extremely challenging to diagnose, and there are various prognoses and treatments. Of the respiratory chain deficiencies specifically, CI defects are the most common (Swalwell *et al.*, 2011). Additionally, evidence shows that the oxidative stress metabolism (the network responsible for scavenging and neutralising harmful cellular oxidants) and the one-carbon (1-C) metabolism (an anabolic network that plays an indispensable role in nucleotide and amino acid production) are frequently affected by a defective CI (Terburgh *et al.*, 2019).

Therefore, the **aim** of this project was, firstly, to investigate the effect of a CI deficiency on the gene expression regulation of selected genes in one-carbon metabolism and oxidative stress, using NDUFS4 knock-out (KO) mouse brain and quadriceps tissues. Secondly, this study aimed to investigate whether the overexpression of the endogenous antioxidant, MT1, would mitigate the effects of the CI defect in the expression of these genes, as it showed therapeutic potential in previous *in vitro* and *in vivo* studies for mitochondrial- and neurodegenerative disorders. Mice that transgenically over-express *Mt1* (TgMT1 mice) were used in this study.

The first part of the **hypothesis** for this study (refer to Chapter 2, Section 2.11) was shown to be correct: The expression of the selected genes of the 1-C and oxidative stress metabolic pathways in the KO:WT tissue samples was indeed up- or downregulated in correspondence with recent literature. The second part of the hypothesis, however, was found to be mostly incorrect; apart from one gene (*Tyms*), the overexpression of *Mt1* in the NDUFS4 KO:TgMT1 overexpressing (KO:OVER) tissue samples did not affect the expression of the selected genes in a manner that promoted the restoration of the cellular redox balance. Also, in line with other *in vitro* data, MT1 did not show the potential to be a viable antioxidant treatment for patients suffering from Leigh Syndrome (Mereis, 2018).

5.2 ACHIEVEMENT OF OBJECTIVES AND METHODOLOGY

5.2.1 Objective 1: Collection of samples for characterisation and investigation

For the characterisation of the NDUFS4 and TgMT1 mouse models, nine liver samples were collected. For the gene expression investigation, however, 24 brain samples (six of each of the four genotypes, namely WT:WT, KO:WT, WT:OVER and KO:OVER) and 24 quadriceps samples (six of each genotype) were collected. This objective was achieved successfully.

5.2.2 Objective 2: Characterisation of the mouse models

The NDUFS4 and TgMT1 mouse models were characterised successfully and are documented in Chapter 3. For each of the five characterisation assays – DNA analyses, RNA analyses, evaluation of the NDUFS4 protein, investigation of the CI structure, as well as kinetic enzyme analyses – one sample of each genotype was used.

In order to confirm the presence or absence of the *Ndufs4* gene in one sample of each genotype, the DNA of each sample was isolated, amplified and the alleles of each sample were separated by way of agarose electrophoresis. The genotypes of each sample corresponded with the expected results and the literature (see Section 3.3.2).

For the confirmation of the expression of *Mt1* in each sample, the total RNA of each liver sample was isolated and subjected to RT-qPCR, relative to beta-2-microglobulin ($\beta 2m$) and the WT:WT genotype (refer to Section 3.3.3). In the WT:OVER genotype, *Mt1* was elevated only to a small extent and in the KO:OVER genotype, the *Mt1* expression was greatly increased. This confirmed the notion that *Mt1* is reserved for physiologically stressful situations. By contrast, there was practically no elevated expression of *Mt1* in the KO:WT genotype – the genotype where the excess ROS in the mitochondria were expected to induce *Mt1* expression (see Chapter 2, Section 2.3.3 and 2.4.2). Therefore, this unchanged expression level was attributed to alternative cellular adaptive responses that counterbalanced the ROS, or simply that the results were mouse- or tissue-specific.

The absence or presence of the denatured NDUFS4 protein was investigated via SDS PAGE & western blot and measured relative to the mitochondrial membrane protein VDAC1. The photograph taken after chemiluminescent imaging (as seen in Section 3.3.4, Figure 3.5), showed that the protein was indeed present in the WT:WT and WT:OVER samples, but absent in the KO:WT and KO:OVER samples. These results corresponded with similar work done in the MRL.

In order to characterise the native structure of the CI protein in the four mouse genotypes, a blue-native PAGE (BN-PAGE) assay was performed. Indeed, CI was present and intact in the

WT:WT and WT:OVER samples. In the KO:WT and KO:OVER samples, however, weaker and lower sized bands suggested a defective assembly and/or stability of the enzyme (see Section 3.3.4, Figure 3.6). These results corresponded with the literature.

Spectrophotometric enzyme analyses were performed so that the kinetic activity of the CI enzyme could be evaluated in all four genotypes, using the WT:WT as control. As seen in Section 3.3.4 (Figure 3.7), the *Ndufs4* knockout had a definitive detrimental effect upon the CI activity of the KO:WT sample and even more so in the KO:OVER sample. There is, however, a dramatic increase in activity in the WT:OVER mouse. Even though these results confirm that the CI defect is indeed present due to the *Ndufs4* knockout (similar to what has been reported in other investigations conducted in the MRL), these observations may be spurious and require more samples to confirm a statistically significant effect.

5.2.3 Objective 3: Genotyping each sample collected for investigation

The DNA of the 48 gene expression investigation samples (24 quadriceps and 24 brain samples) was isolated, amplified and the alleles were separated by way of agarose electrophoresis. This was in order to confirm the presence or absence of the *Ndufs4* allele in each tissue sample. The genotypes matched the markings in the ears of the mice, therefore, the identities and genotypes of the mice were confirmed and correct.

5.2.4 Objective 4: Selection of the genes to be investigated

The literature was studied thoroughly in order to understand the metabolic mechanisms of 1-C metabolism and oxidative stress metabolism. In the end, ten genes (plus two reference genes) involved in these two pathways were selected, based on their metabolic importance, so that the regulation of their expression could be investigated. These genes were: *Mthfd2*, *Bhmt*, *Tyms*, *Sod*, *Cat*, *Gpx1*, *Mtrr*, *Lias*, *Acβ* (housekeeping gene), *β2m* (housekeeping gene), *Gclc* and *Phgdh*. The motivations behind the selection of these genes can be found in Chapter 2, Section 2.7. Therefore, this objective was successfully completed.

5.2.5 Objective 5: Quantification of mRNA and measuring expression of selected genes in four genotypes

The PCR efficiency of each of the ten chosen genes (seven 1-C metabolism genes and three oxidative stress genes), as well as the gene expression of each, were successfully measured via RT-qPCR. The WT:WT genotype acted as the control. The data were successfully collected and processed by combining the methods of Livak and Schmittgen (2001) and Pfaffl (2007). This way, the PCR efficiency data could be incorporated into the final gene expression data.

However, certain observations were made from the methodology of this investigation.

For the investigation of gene expression regulation of the genes subject to this study, the RNA was isolated with the Trizol reagent – a phenol chloroform extraction. Even though the isolation process was time consuming, laborious and difficult to automate, the RNA yield was of high quality. However, the need for an extra purification step was realised when the A260/280 ratios of the RNA samples were ~1.8 rather than the acceptable ~2.00. Therefore, the MEGAclear™ transcription clean-up kit was used to purify all the total RNA samples used for the gene expression investigation. This kit yielded RNA with acceptable A260/280 ratios.

Additionally, validation of the two chosen reference genes showed that *Actβ* was an unstable reference gene for this study, in comparison to *β2m*. Thus, *Actβ* was disqualified as a reference gene and the expression results of the target genes were normalised only to *β2m*. The importance of including more than one reference gene in a study was, however, realised.

5.2.6 Objective 6: Comparison of expression of selected genes in *NDUFS4* KO and *TgMT1* mice to that of *WT:WT* mice

The results were documented and interpreted in Chapter 4, Section 4.4.2. The following was observed from the results:

Genes that showed notable change in gene expression in brain tissue and in both comparison groups (KO:WT compared to WT:WT and KO:OVER compared to WT:WT) were *Mthfd2*, *Mtrr* and *Bhmt* of 1-C metabolism. No change in gene expression was observed for the oxidative stress genes. In the quadriceps tissue, it was *Mthfd2*, *Tyms* and *Bhmt* of 1-C metabolism and *Gpx1* of oxidative stress (all in the group where the KO:OVER and WT:WT genotypes were compared) that displayed notable change in gene expression. *Tyms* was the only gene of which the change in expression in brain tissue was notably impacted (in a rescuing manner) by *Mt1* overexpression.

The relative RNA quantification data was also critically evaluated. Notably, it was observed that the gene expression fold change ranges were very wide in the quadriceps tissue. This indicated weak repeatability (more likely) or large steady state variation. By contrast, the ranges were very narrow in the brain tissue. These tissue-specific variations might be due to inter-assay variation, the small sample size of this study, and the slight genetic and environmental variability among the mice. Additionally, these differences in results between the two tissue types might be attributed to differences in transcriptional activity taking place in the brain and quadriceps. Another variable to consider is the presence of a larger group of transcription factors in the brain, as opposed to the small group of transcription factors in the quadriceps. Correspondingly,

the average quadriceps RNA concentration was approximately a third of the average brain RNA concentration measured in this study – pointing to a great difference in RNA per wet gram tissue. This was also evident from the relatively higher Ct values and it impacted the quality of the data.

Nevertheless, from the gene expression fold change data reported in this study [obtained with the $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen (2001)], the downregulation of certain 1-C metabolism genes expression that was observed, may be linked to inhibition of 1-C metabolism by the AMPK pathway. The negligibility of the fold change data of the oxidative stress genes may be explained by alternative cellular adaptive mechanisms that are reverted to in the case of a knockout of the NDUFS4 subunit. Although these mechanisms have not been investigated in this study, they might include pathways that enable ATP production via CII, the use of CI (despite the deficiency) via super complexes like CI+CIII² (Calvaruso *et al.*, 2012), as well as to the use of DMG in the ETF/ETF-QO system (Terburgh *et al.*, 2019).

5.3 LIMITATIONS, STRENGTHS, RECOMMENDATIONS AND FUTURE PROSPECTS

The potential impact of this study was **limited** by using the whole-brain and only one skeletal muscle-type, instead of specific regions of the brain and an array of muscle-types. Even though Valsecchi *et al.* (2013) and de Haas *et al.* (2017) have shown that ROS levels are elevated in NDUFS4 KO mice, these studies were performed in specific cell types (fibroblasts) and brain regions (cerebral cortex and external capsule). In this study, however, it was observed that the metallothionein overexpression in the KO:OVER genotype did not notably alter the expression of the genes of interest in this study. Consistent with this, Miller (2019) – who conducted protein oxidation assays in NDUFS4 KO mouse whole-brain tissue – described an insignificant difference in the amount of protein oxidation between KO mice and those overexpressing MT1. She concluded that brain regions that might be greatly affected by ROS are masked by those that are not significantly affected. Taken together, it appears that studying the brain as a whole and studying only one type of muscle tissue is not sufficient for obtaining a better understanding of the impact of a CI defect and MT1 overexpression. The ideal would be to investigate the expression of these selected genes in specific brain regions and different muscle types, so that results may be more comparable to those of other studies.

Additionally, the small number of genes **limited** the understanding of the true effect of the NDUFS4 knockout on the function of 1-C and oxidative stress metabolism. For this study, only ten genes were selected, based on evidence, and relative expression was investigated individually. Additionally, gene expression analyses were only performed with the genes of *enzymes* involved in 1-C metabolism and oxidative stress. However, as 1-C metabolism and oxidative stress are intricate metabolic networks, it is difficult to draw wider conclusions from a

handful of isolated gene expression assays. In **future studies**, technology and techniques such as microarray analysis, QuantiGene RNA Assays¹ and Ion AmpliSeq panels² (RNA sequencing) could be used for the detection of expression of multiple genes – including genes of the *intermediates* – of these metabolic networks, so that the full effect of a CI defect and MT1 overexpression on the expression of genes of 1-C metabolism and oxidative stress metabolism might be observed.

On the other hand, the fact that the genes investigated in this study were not randomly selected is regarded a **strength** of this study. The characteristics and functions of the encoded proteins of the enzymes involved in 1-C metabolism and oxidative stress were studied carefully, after which an evidence-based selection of ten genes were made according to metabolic importance. The measured relative expression of these genes in the mouse models used in this study were then also interpreted and linked back to the literature.

Another **strength** of this study is the mouse models that were used. As opposed to the rotenone-induced CI deficiency in *in vitro* models that were previously studied in the MRL (Pretorius, 2006), the mice used in this study have been bred to be NDUFS4-deficient and to overexpress transgenic Mt1. Therefore, a larger part of the full phenotypical spectrum could be observed, and reliable conclusions could be drawn about gene transcription.

In hindsight, though, having chosen only two reference genes for the gene expression investigation was unfavourable for this study. Furthermore, one of the two reference genes (*Actβ*) was disqualified, as it was the least stably expressed across all four mouse genotypes and tissue types, according to the $2^{-\Delta Ct}$ method by Livak and Schmittgen (2001). This was in line with other studies (Lin & Redies, 2012; Olivier, 2004; Selvey *et al.*, 2001). Fortunately, *β2m* – the reference gene used for normalisation – was stably expressed across the tissue types and genotypes in this study and yielded reliable results, which corresponded with the work of Gabert *et al.* (2003), Matsuzaki *et al.* (2015), Mereis (2018) and Olivier (2004). Nevertheless, it is **recommended** that future gene expression studies should use computer tools to evaluate a set of potential reference genes and then compose a panel of reference genes with the most stably expressed genes. Thus, a reference gene index (or, in the case of a microarray, the total signal) might be a more reliable alternative for normalisation (Pfaffl, 2004; Pfaffl, 2007; Xu *et al.*, 2017).

This study did not focus on the WT:OVER genotype in the transcriptional investigation, because of the differences in gene expression effects that could be observed directly from the data where the WT:WT and KO:OVER genotypes were compared. **Future studies**, however, may

¹ Thermo Fisher Scientific, Invitrogen

² Thermo Fisher Scientific, IonTorrent

look into doing a gene expression investigation in the WT:OVER genotype where a more detailed profile of MT1 overexpression in the context of a healthy CI is desired.

Yet, the in-depth evaluation of the processing of RT-qPCR data is considered a **strength** of this study. By assessing the PCR efficiency of each gene before the gene expression assays and by applying the methods for processing exponential data, as described by Livak and Schmittgen (2001) and Pfaffl (2007), the data could be depicted accurately and precisely – within the limitations of the methodology and normalisation used.

Regarding the characterisation assays in this study, however, only one sample per genotype was used. Indeed, the purpose was only qualitative – to confirm the absence/presence of the NDUFS4 gene and protein, to confirm the expression levels of the Mt1 gene, to confirm the native structure of the CI protein and to confirm the CI activity in each of the four genotypes – and these results were obtained accordingly. Still, **future studies** may consider using a greater number of samples, as well as replicates, in order to answer characteristic-based questions of the mouse models with statistically significant findings.

Importantly, due to **ethical considerations**, the minimum number of mice necessary to observe notable changes in gene expression had to be used for both the characterisation and the transcriptional investigation. This was to ensure adherence to the ethical principles of replacement, reduction and refinement, regarding animal research (South African Bureau of Standards., 2008). Ethical accountability was a high priority in this study. Additionally, the number of mice used in this study corresponded with other transcriptional investigations where RT-qPCR was applied (Chen *et al.*, 2009; Lichter-Konecki *et al.*, 2008).

Something that must be kept in mind when discussing the regulation of the expression of the genes investigated in this study, is that gene expression regulation functions as a complex system that is controlled on many levels. These include facilitating chromatin accessibility, transcriptional regulation, post-transcriptional regulation, RNA processing control, RNA transport control, translational control and post translational modification. Isolating expression regulation on transcriptional level only, cannot be extrapolated to the final expression and function of the encoded protein. Therefore, for a broader understanding of the regulation of 1-C metabolism and oxidative stress, **future studies** may also look to investigate translational, post-translational and epigenetic regulation of gene expression.

Although it is the hallmark of most studies investigating similar aims, gene expression regulation is one mechanism of control of protein, enzyme and metabolic function. However, finer control via allosteric regulation of enzymes plays a major role in metabolic regulation, which is clearly evident from the metabolic data of mitochondrial disease (Esterhuizen *et al.*, 2017), and of

NDUFS4 KO mice investigated in the MRL (Terburgh *et al.*, 2019). The effects of allosteric regulation cannot be concluded from gene expression data, which is an important **limiting factor** to remember in gene expression studies like this one.

Importantly, Dogan *et al.* (2018) have found – via the induction of alternative oxidase in a complex IV KO model – that the use of antioxidants in mitochondrial myopathies might have detrimental effects, as it interferes with the compensatory intracellular ROS signalling. They state that antioxidants could actually block the homeostatic response to cellular bioenergetic failure, therefore worsening the clinical phenotype. It is also suggested that the AMPK enzyme is directly regulated by ROS and that the induction of mitochondrial biogenesis through mitochondriogenic pathways such as SIRT1- and/or AMPK-dependent PGC-1 α axis, might be effective approaches toward therapy for mitochondrial disease (Shao *et al.*, 2014). This finding **promotes the awareness** that antioxidants may also have negative effects in the treatment of mitochondrial disorders.

5.4 CONCLUDING REMARKS

After a thorough study of the literature, the following problem was identified: Due to shortcomings in the complete understanding of the *mechanisms* behind the cellular consequences of a CI defect, the development of effective patient treatment strategies is still severely lacking. Therefore, it was relevant that the changes in gene expression – and therefore the *regulation* of gene expression – of the enzymes involved in 1-C metabolism and oxidative stress had not yet been investigated.

Thus, a study was undertaken at the MRL, NWU Potchefstroom, as part of a larger, overall study where the phenotype of mitochondrial disease (CI defects in particular) and the protection against the pathology by MT1 overexpression is investigated. In this larger study, each researcher taking part in this larger study has a specific research focus aimed at understanding the mechanisms of CI deficiencies *in vivo*, and to understand whether *Mt1* overexpression might have a therapeutic effect upon subjects with a CI defect. The final aim is to evaluate the possibility of a more effective, viable and affordable antioxidant therapy for patients with mitochondrial disease.

Hence, an experimental strategy with aims and objectives was set up for this specific study. As stated in Section 5.1, focus was mainly placed on investigating the effect of a CI defect upon the gene expression of enzymes involved in 1-C metabolism and oxidative stress, and to establish whether *Mt1* overexpression would mitigate these effects.

A mouse model lacking the NDUFS4 subunit and another that overexpressed MT1, was crossbred and used in this study, therefore, the mice had to be characterised on molecular

level. Consequently, appropriate and advanced laboratory techniques and analytical methods were mastered to reach this objective. Similarly, for the measurement of the expression of the twelve selected genes, appropriate semi-quantitative techniques for the evaluation of gene expression in tissues were mastered and the methodology was critically evaluated. With each set of obtained results, the data was also thoroughly and critically evaluated and interpreted to establish what could be concluded with confidence. In the end, an empirical scientific approach was successfully applied.

From this study, it may finally be concluded that, firstly, the results of the characterisation of the two mouse models were in line with the literature. Secondly, that an NDUFS4 deficiency has a downregulating effect upon the expression of certain genes in the 1-C metabolism and that *Mt1* overexpression did not have a mitigating effect upon most of the genes in the absence of the NDUFS4 subunit. The importance of considering the complexity of the gene expression regulation system in an organism and the possible detrimental effects of the use of antioxidants in mitochondrial myopathies, were also recognised.

The limitations of this study (as explained in Section 5.3) prevented the visualisation of the *complete* gene expression profile of 1-C metabolism and oxidative stress in a CI-deficient mouse model, and the effect of *Mt1* overexpression upon the genes that were investigated. Yet, through the strengths of this study (refer to Section 5.3 as well), it was possible to obtain an accurate visualisation of a *partial* gene expression profile.

Therefore, all the studies (together with this one) that form part of the larger, overall study, support one another in obtaining an understanding of the protection of mitochondrial disease pathology by MT1 overexpression on both a molecular and a phenotypical level.

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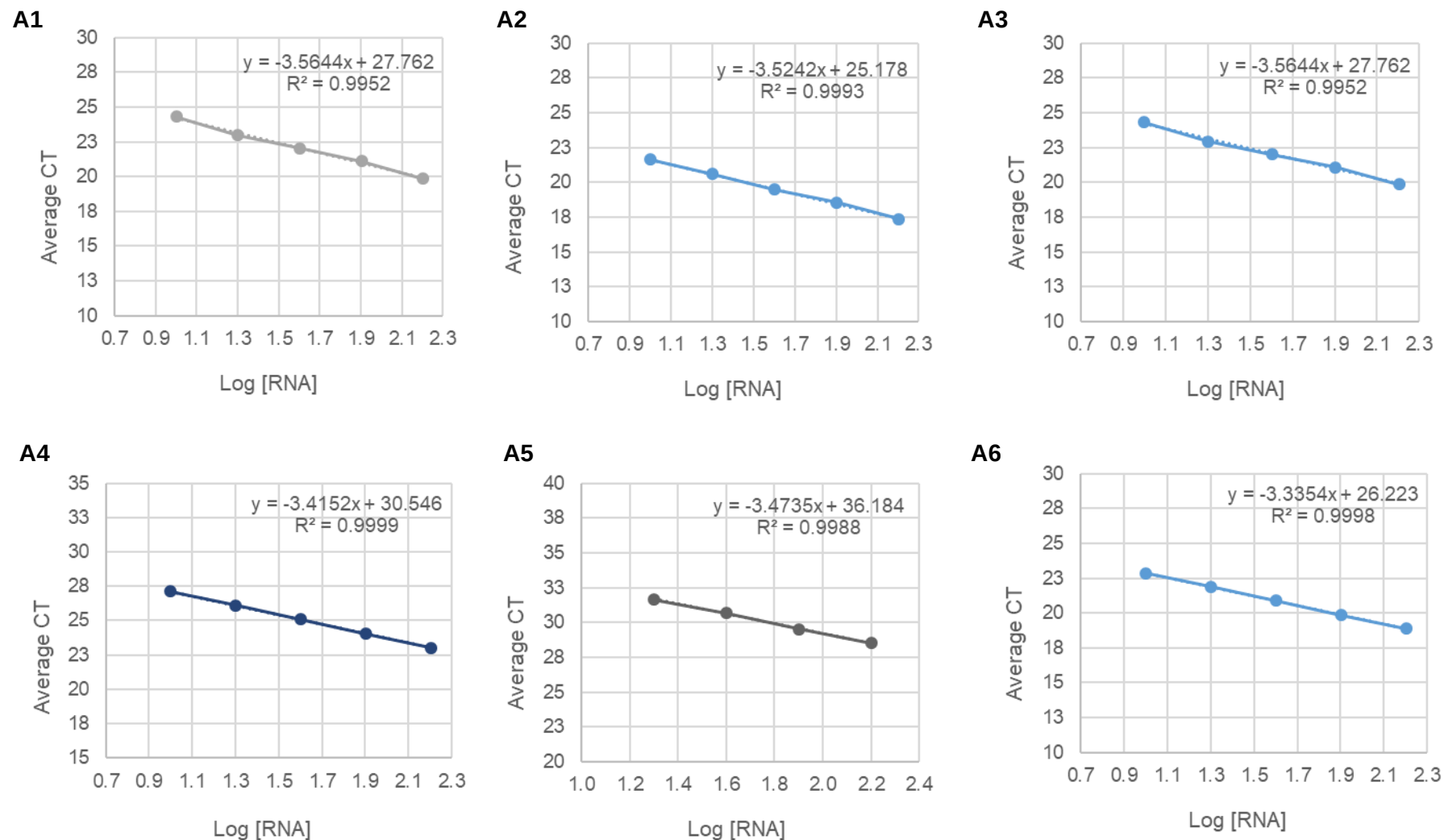
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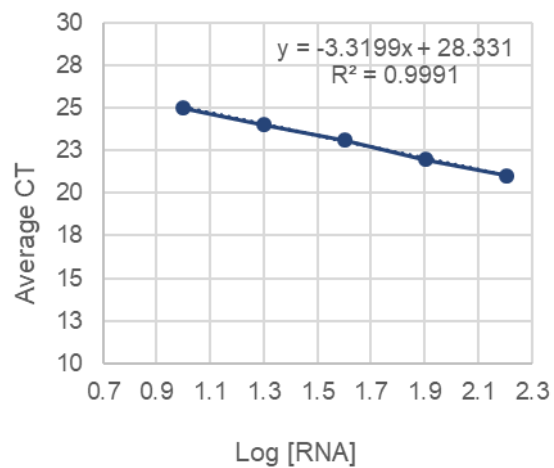
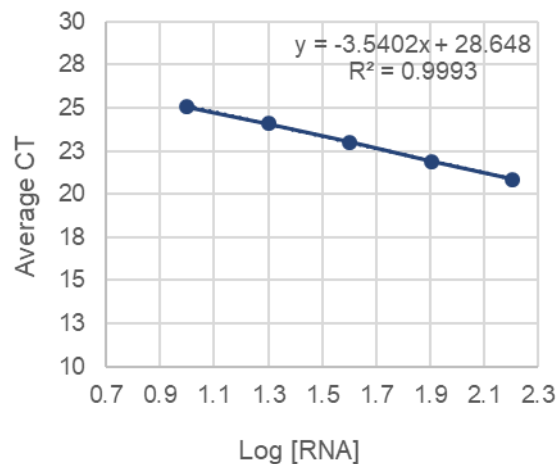
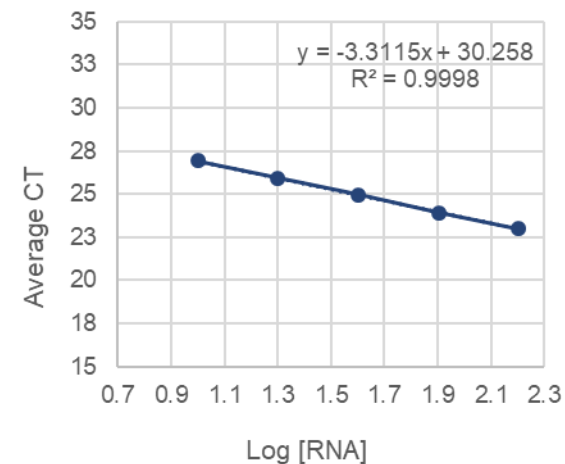
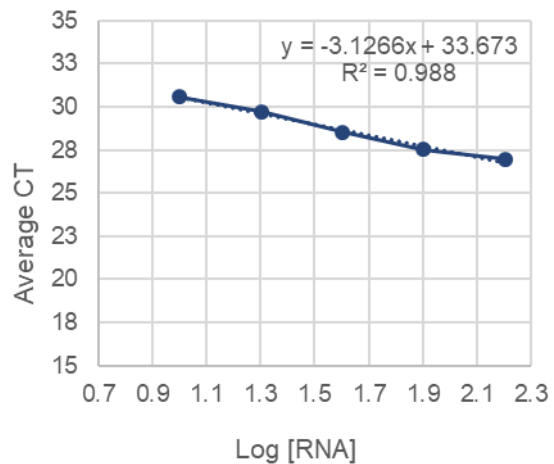
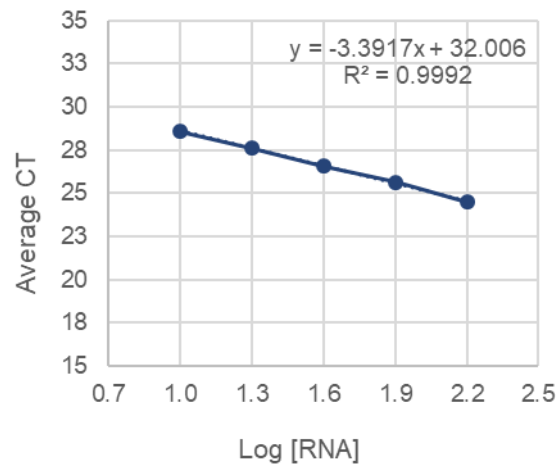
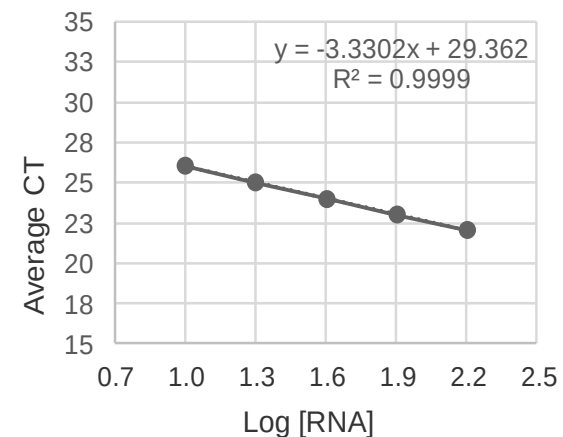
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ANNEXURE A: INDIVIDUAL PCR EFFICIENCY PLOTS



Figures A1-A6: Relative efficiency plots for (A1) $\beta 2m$, (A2) $Act\beta$, (A3) $Gpx1$, (A4) $Lias$, (A5) $Bhmt$ and (A6) $Sod2$.

A7**A8****A9****A10****A11****A12**

Figures A7-A12: Relative efficiency plots for and (A7) *Cat*, (A8) *Gclc*, (A9) *Mthfd2*, (A10) *Tyms*, (A11) *Mtrr*, and (A12) *Phgdh*.

ANNEXURE B: BOX PLOTS FOR THE IDENTIFICATION OF OUTLIERS

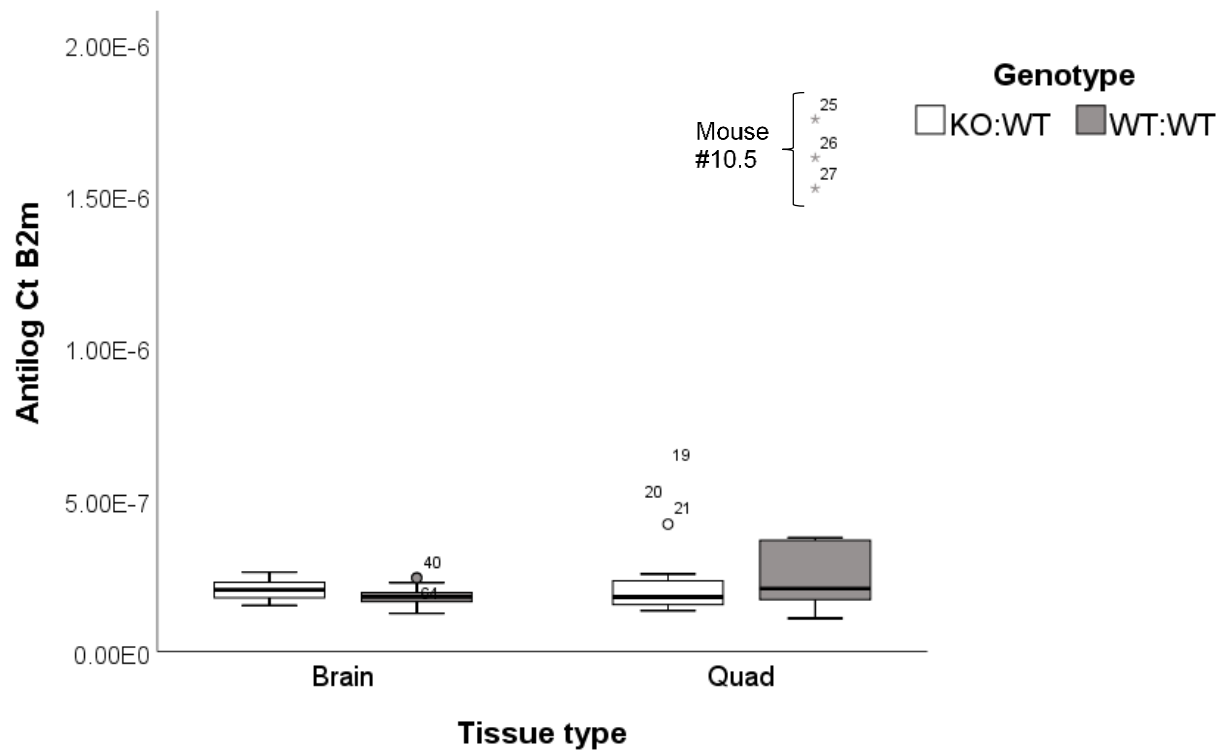


Figure B1: Box plot of the antilog Ct values of $\beta 2m$.

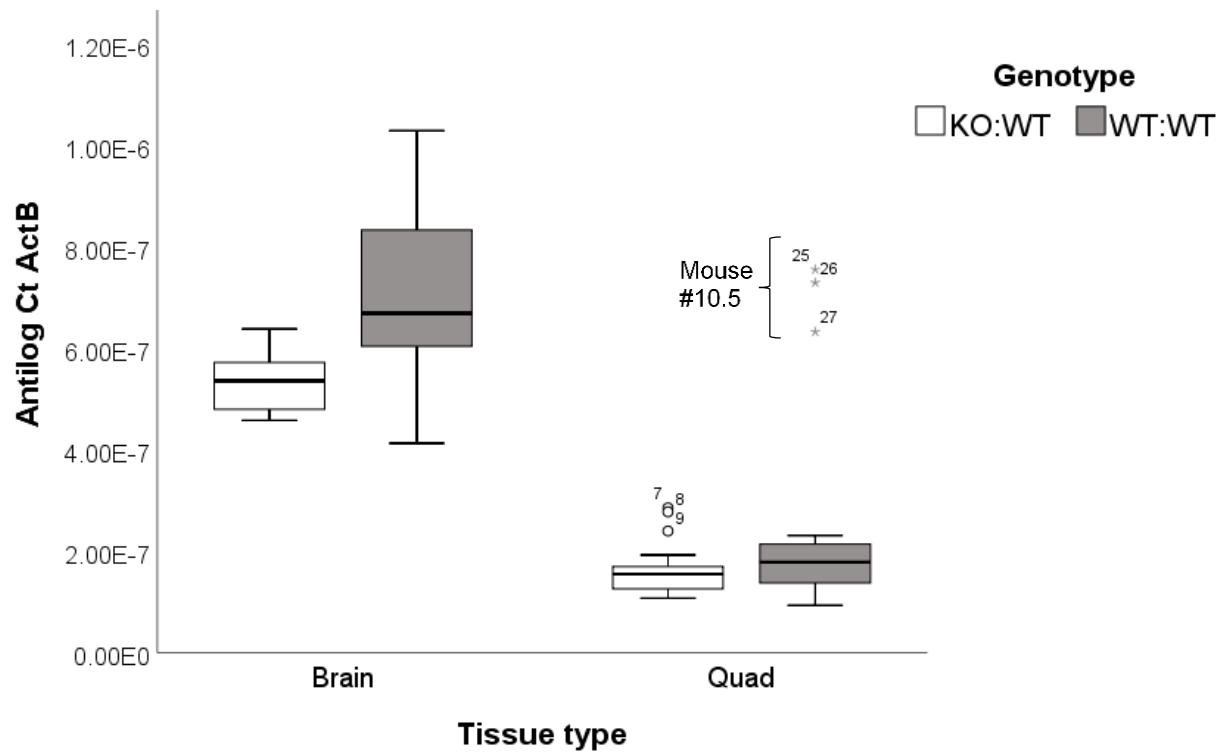


Figure B2: Box plot of the antilog Ct values of $Act\beta$.

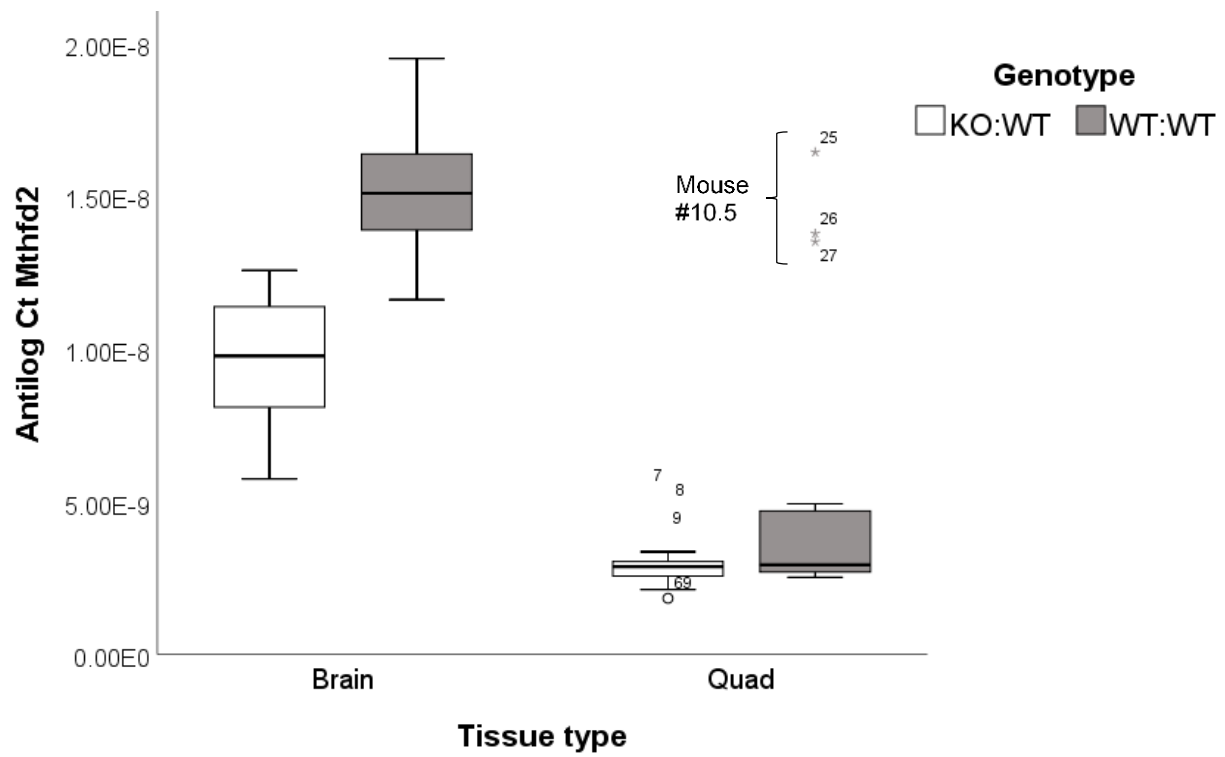


Figure B3: Box plot of the antilog Ct values of *Mthfd2*.

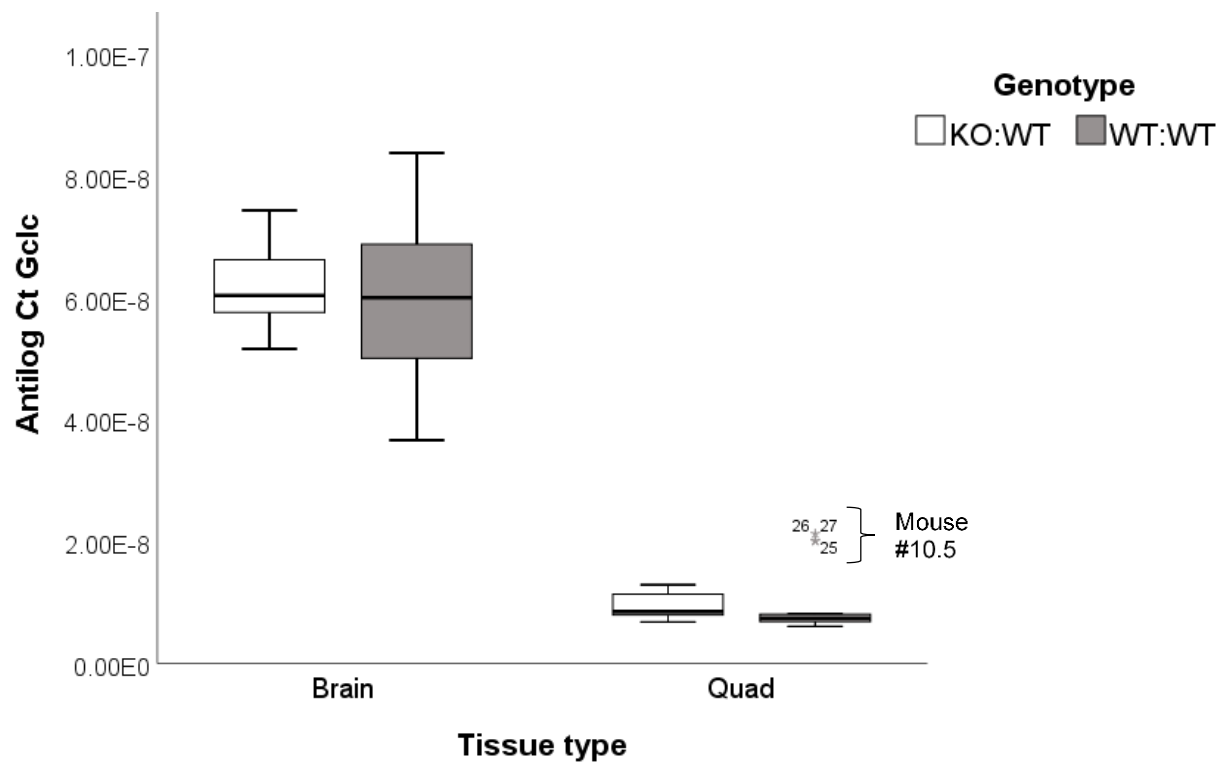


Figure B4: Box plot of the antilog Ct values of *Gclc*.

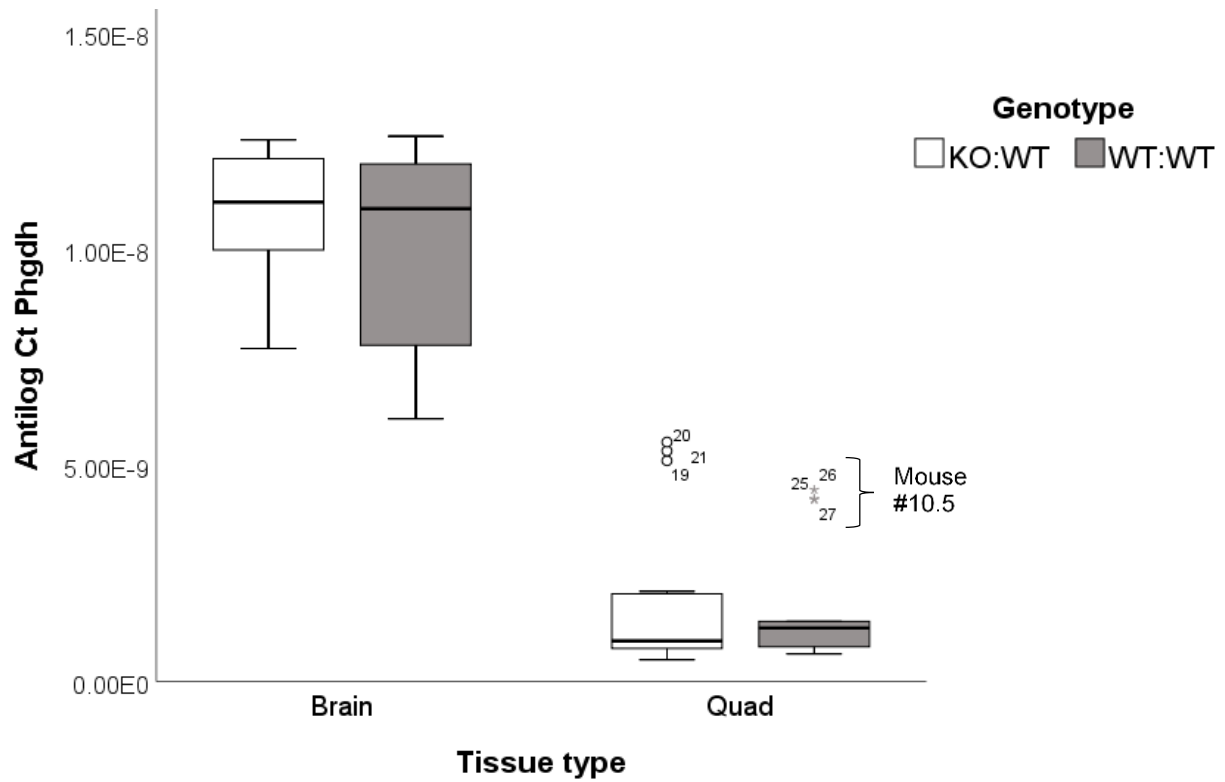


Figure B5: Box plot of the antilog Ct values of *Phgdh*.

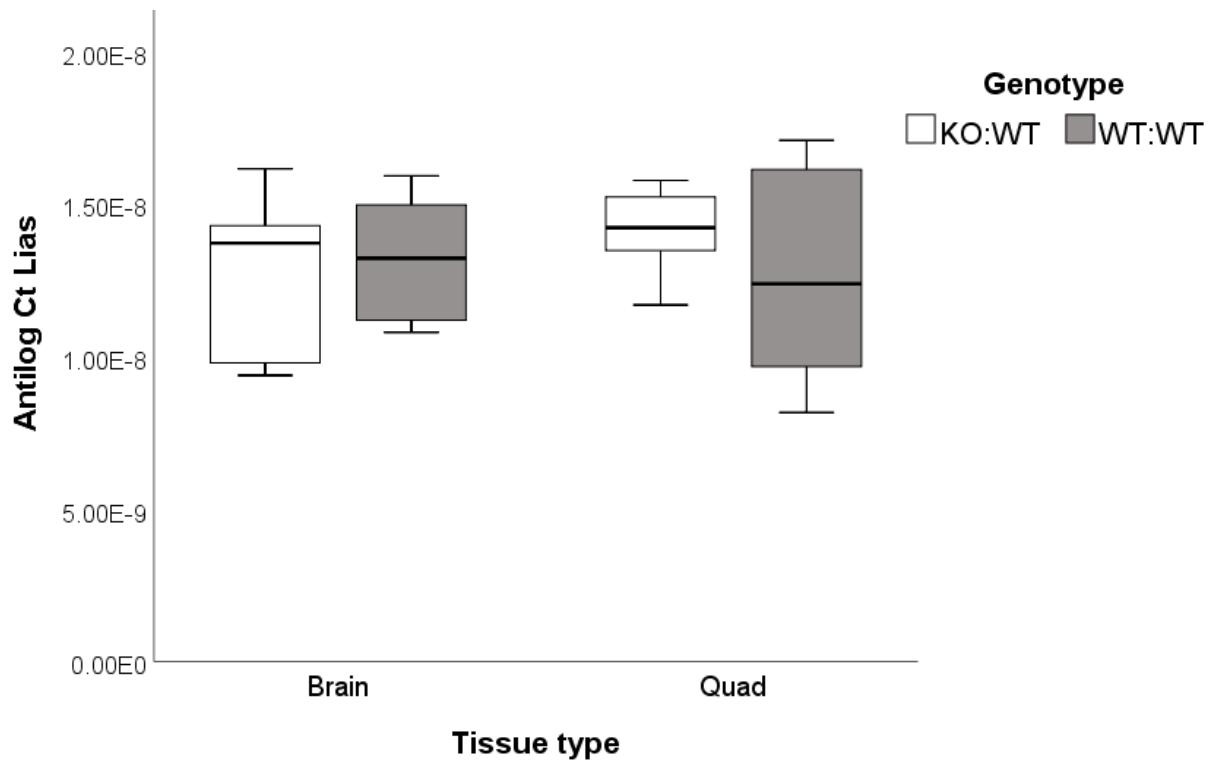


Figure B6: Box plot of the antilog Ct values of *Lias*.

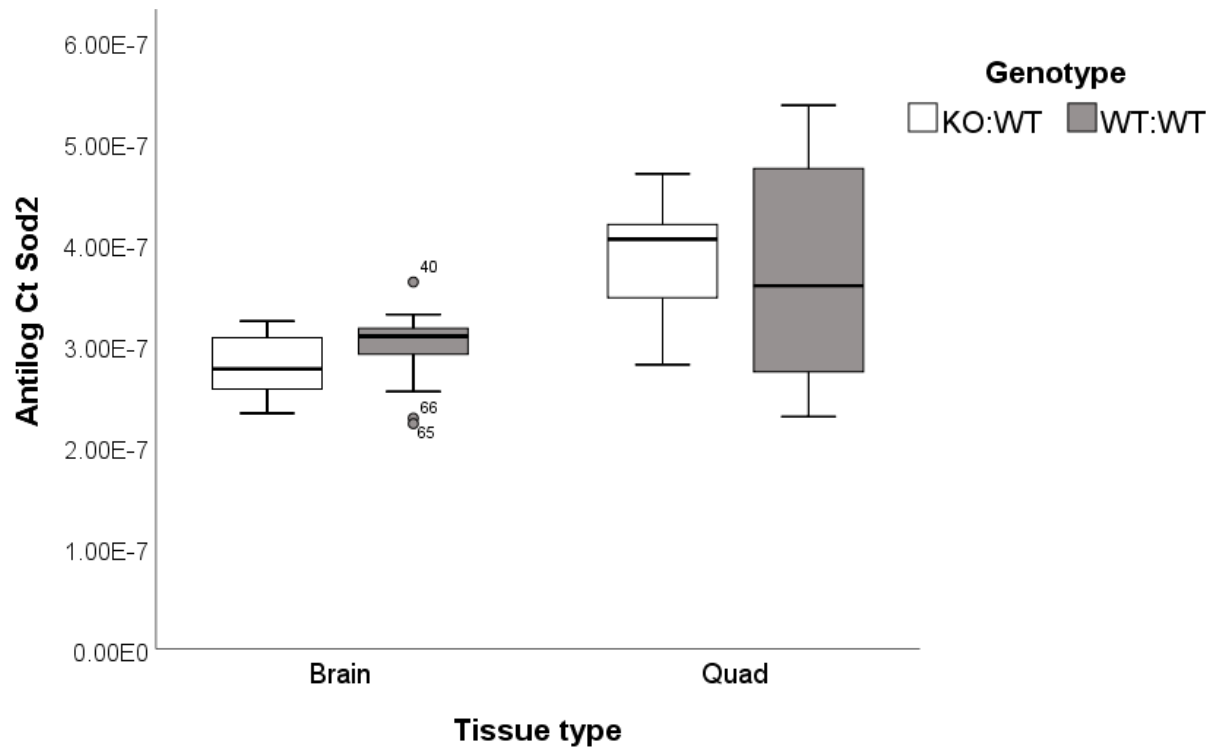


Figure B7: Box plot of the antilog Ct values of *Sod2*.

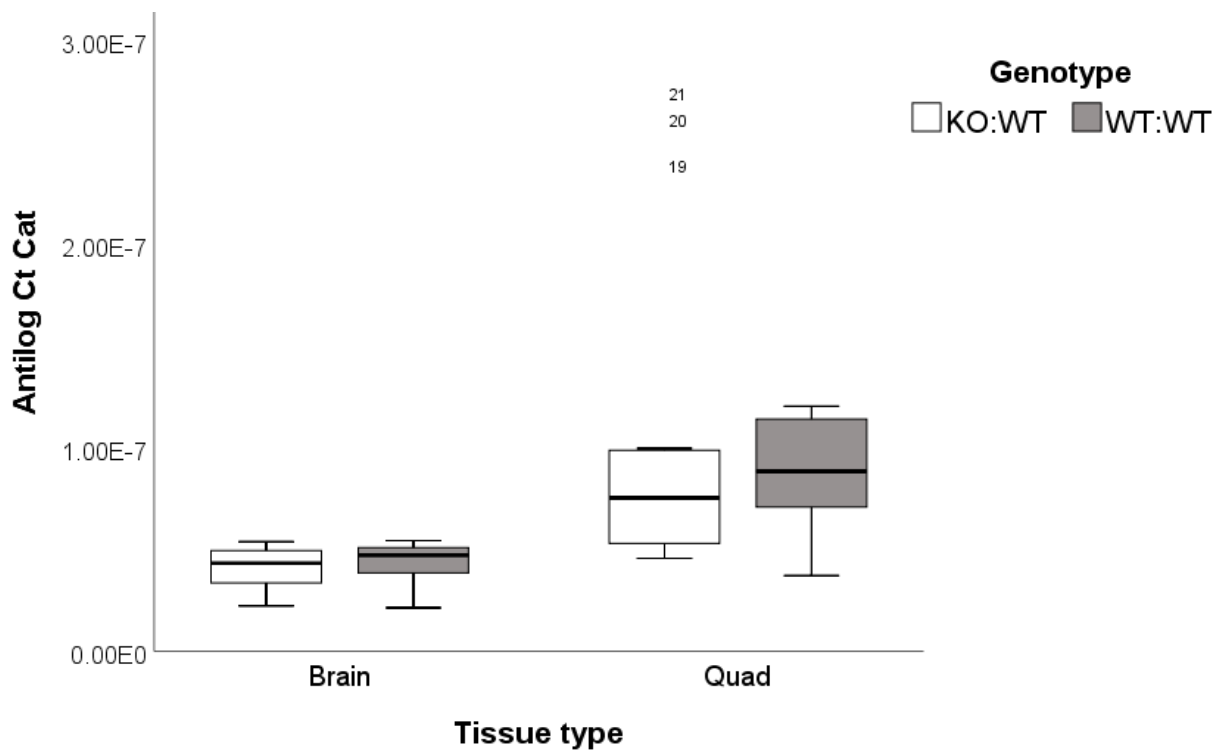


Figure B8: Box plot of the antilog Ct values of *Cat*.

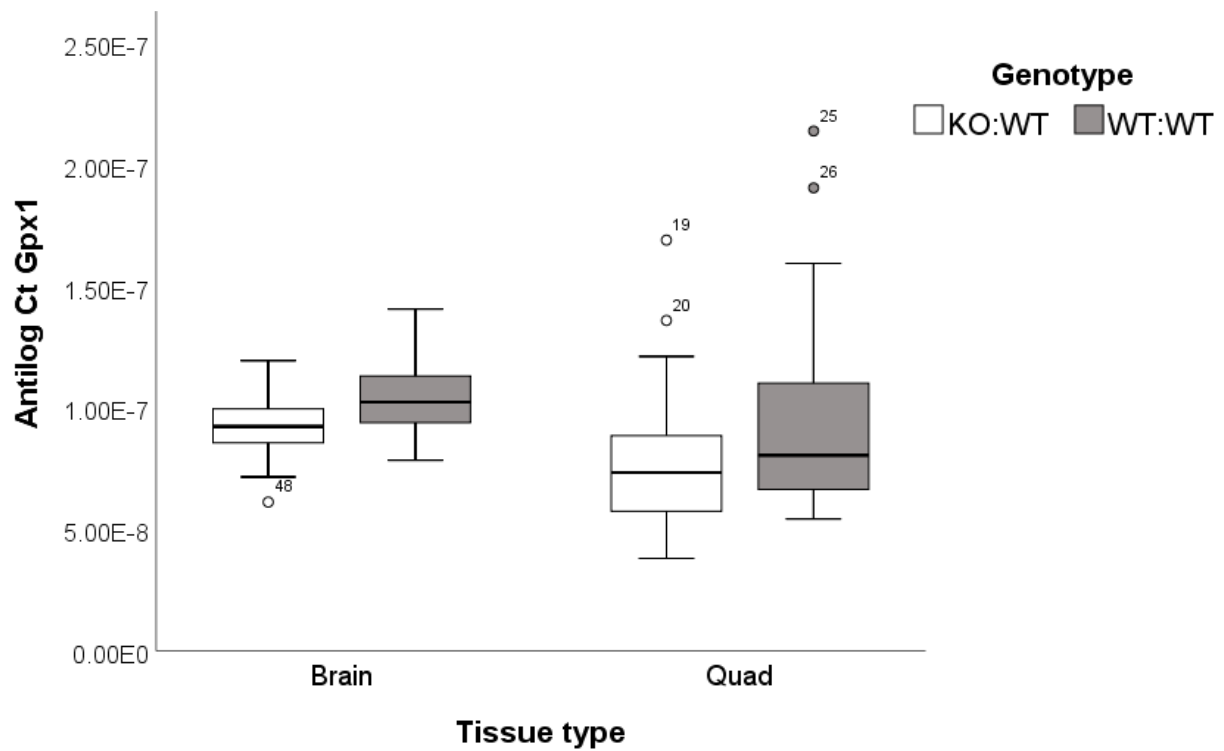


Figure B9: Box plot of the antilog Ct values of *Gpx1*.

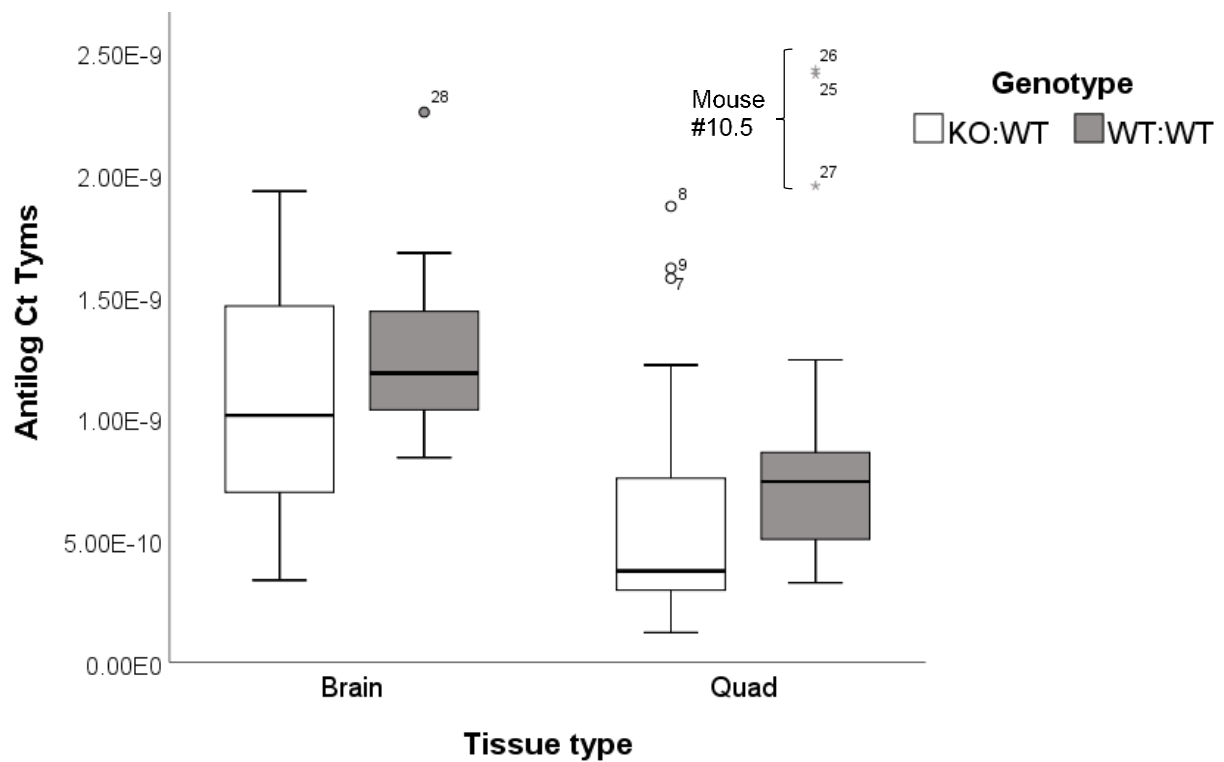


Figure B10: Box plot of the antilog Ct values of *Tyms*.

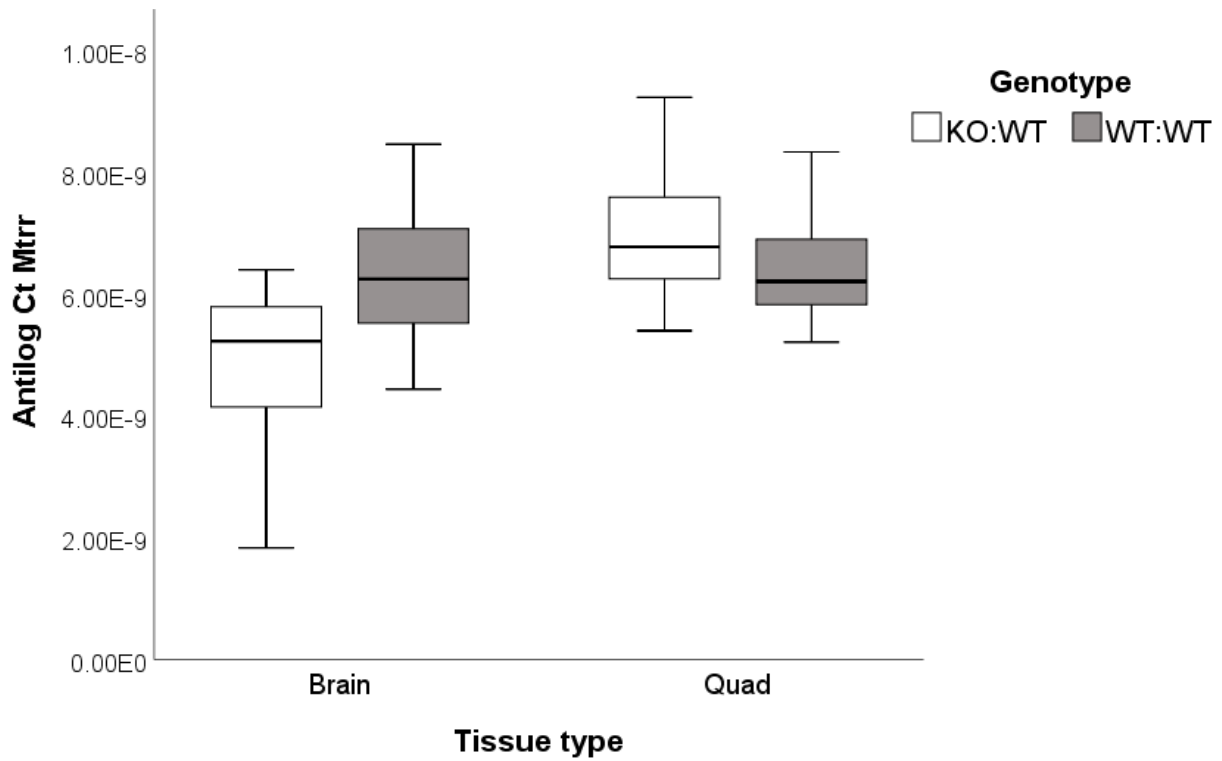


Figure B11: Box plot of the antilog Ct values of *Mtrr*.

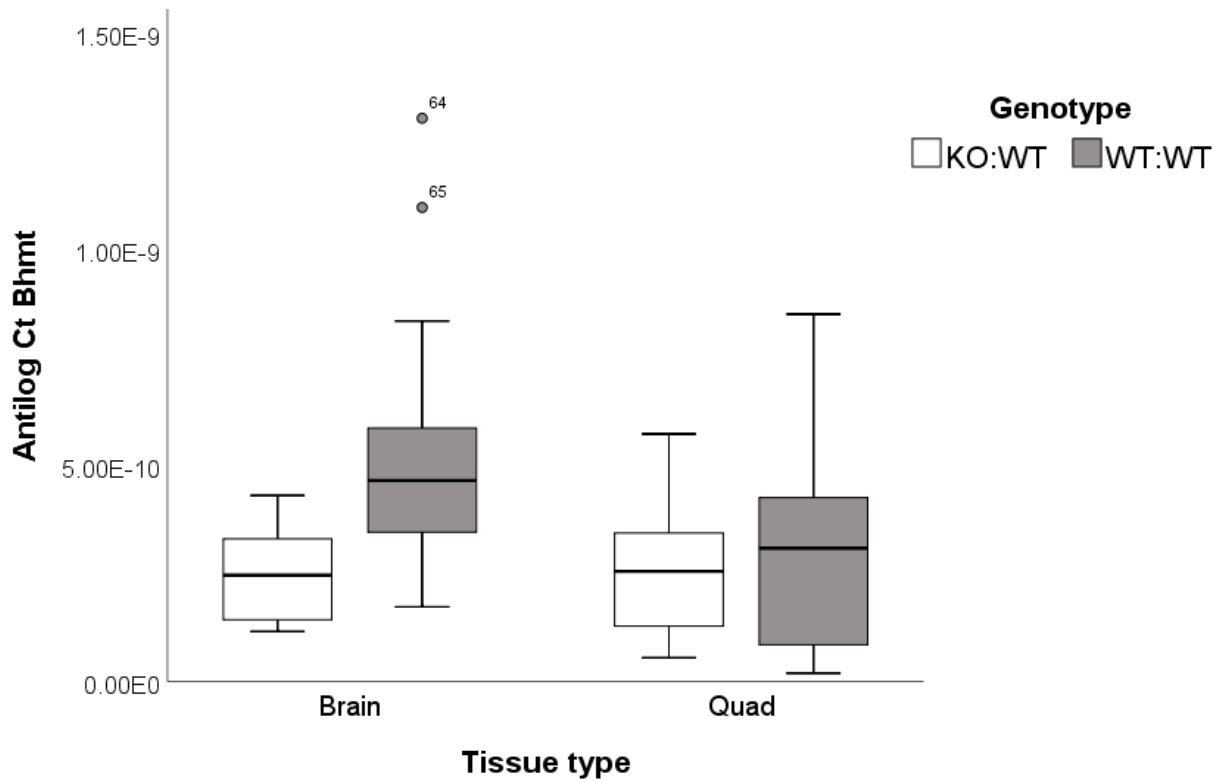


Figure B12: Box plot of the antilog Ct values of *Bhmt*.

ANNEXURE C: LANGUAGE EDITING CERTIFICATE



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

Master of Science in Biochemistry

of

L Mienie

has been edited by

Valerie Viljoen

Editing Excellence

The complete dissertation has been edited.

Date: 3 October 2019



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