

Differential expression of genes involved in phase two biotransformation in an *NDUFS4* deficient mouse model

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To Morné, for being the best teammate.
To Danika, for being the newest member of our team.

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ABSTRACT

Mitochondria are involved in a plethora of cellular processes, the most prominent being the production of energy as adenosine triphosphate (ATP) through the oxidative phosphorylation (OXPHOS) system. Dysfunction of the OXPHOS system, especially complex I (CI) is one of the most common groups of inborn errors of metabolism that results in heterogeneous diseases like Leigh Syndrome, which are difficult to treat. To study mitochondrial disease *in vivo* a whole body *Ndufs4* knockout mouse model has been developed and is now commonly used in research. Although mitochondrial disorders have been extensively studied, there are still a lot of gaps in the literature, for example, its effect on biotransformation. The limited ATP of individuals with mitochondrial disorders could affect the activity and capacity of the biotransformation pathway by dysregulating any of the factors that influence metabolism including gene expression through retrograde signalling, allosteric regulation, and the availability of cofactors.

The aim of this study was to investigate differential expression of genes involved in phase two biotransformation in an *Ndufs4* knockout mouse model. This was achieved by first investigating differential gene expression of glycine conjugation as a primary pathway of phase two biotransformation between *Ndufs4* knockout (KO) and wild-type (WT) mice. This investigation was then expanded to explore if the changes observed in glycine conjugation were reflected in the gene expression of seven remaining phase two biotransformation pathways.

Significant downregulation of the glycine conjugation pathway in KO mice compared to WT mice was indicated by gene expression analysis of *Acsf2* and *Glyat*. These genes had fold changes of ~-3.6 and ~-1.6, respectively. Gene expression data was validated through enzyme activity analysis of GLYAT, which confirmed the downregulation in KO mice, by exhibiting ~50% residual GLYAT activity compared to WT mice. Significant differential expression was also revealed for 50 of the remaining phase two biotransformation pathway genes. Expression of most of these genes were significantly downregulated. As the exception, gene expression of sulfotransferases was significantly increased. Downstream effects of decreased ATP production and redox imbalance that contribute to retrograde signalling, is hypothesised to be the main contributors to the downregulation of genes investigated. Overall, the data strongly suggests that phase two biotransformation pathways are highly affected by mitochondrial dysfunction.

Keywords: *Ndufs4* knockout mouse model; transcriptomics; gene expression; mitochondrial disease; complex I deficiency; mouse liver tissue; glycine conjugation; phase two biotransformation.

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LIST OF ABBREVIATIONS

Abbreviation	Full term
2D	two dimensional
Acetyl-CoA	acetyl-coenzyme A
ACS	acyl-coA synthetase
ACSM	acyl-coenzyme A synthetases
ADP	adenosine diphosphate
AhR	aryl hydrocarbon receptor
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
AMT	amino methyltransferase
APS	ammonium persulphate
AR	androgen receptor
ARE	antioxidant response elements
ATP	adenosine triphosphate
B	blank
BAM	binary alignment map
BCA	bicinchoninic acid
BSA	bovine serum albumin
Ca	calcium
CAR	constitutive androstane receptor
cDNA	complementary DNA
CHP	chip
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CMOS	complementary metal-oxide-semiconductor
CoA	coenzyme A
COMT	catechol o-methyltransferase
COX	cytochrome c oxidase
CS	citrate synthase
CSV	comma delimited
CV	complex V
CYP450	cytochrome P450
DCIP	2,6 dichloroindophenol sodium salt hydrate

Abbreviation	Full term
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone 3-sulfate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
dsDNA	double-stranded DNA
DTNB	5,5'-dithio-bis(-2-nitrobenzoic acid)
EGTA	egtazic acid
emPCR	emulsion polymerase chain reaction
ER	estrogen receptor
EtBr	ethidium bromide
ETC	electron transport chain
FAD ⁺	oxidised flavin adenine dinucleotide
FADH	reduced flavin adenine dinucleotide
FC	fold change
FDA	food and drug administration
FDR	false discovery rate
FMN	flavin mononucleotide
FMO	flavin-dependent monooxygenase
FMO3	flavin monooxygenase 3
FXR	farnesoid x receptor
gDNA	genomic DNA
GHMT	glycine hydroxymethyltransferase
GLDC	glycine decarboxylase
GLYAT	glycine N-acyltransferase
GR	glucocorticoid receptor
GRCm38	genome reference consortium mouse reference 38
GSH	glutathione
GSSG	oxidised glutathione
GSTs	glutathione S-transferases
GTC	guanidinium thiocyanate
H ⁺	protons
H ₂ O	water molecule
HET	heterozygous
HNF-4	hepatocyte nuclear factor 4

Abbreviation	Full term
HNMT	histamine N-methyltransferase
HSP60	heat shock protein 60
IMS	intermembrane space
ISFET	ion-sensitive field-effect transistor
ISPs	Ion Sphere™ particles
kDa	kilodaltons
KEAP1	kelch-like ECH-associated protein 1
KO	knockout
L	ladder/molecular weight marker
LXR	liver X receptor
MAPK	mitogen activated protein kinase
max.	maximum
MIM	mitochondrial inner membrane
min	minutes
min.	minimum
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NAA	N-acetylaspartate
NAD ⁺	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferases
NAT1	N-acetyltransferase 1
NAT2	N-acetyltransferase 2
NATs	N-acetyltransferases
ND	nicotinamide adenine dinucleotide dehydrogenase
nDNA	nuclear DNA
NDUFS4	NADH dehydrogenase ubiquinone fe-s protein 4
<i>Ndufs4</i> ^{-/-}	<i>Ndufs4</i> knockout mice
<i>Ndufs4</i> ^{+/-}	<i>Ndufs4</i> heterozygous mice
<i>Ndufs4</i> ^{+/+}	<i>Ndufs4</i> wild-type mice
NH ₃	ammonia
NKH	nonketotic hyperglycinaemia
NMTs	N-methyltransferases
NNMT	nicotinamide N-methyltransferases
NRF-2	nuclear factor (erythroid-derived 2)-like 2

Abbreviation	Full term
NRs	nuclear receptors
NWU-AnimCareREC	North-West University Animal Care, Health and Safety Research Ethics Committee
O ₂	molecular oxygen
OAA	oxaloacetate
OXPPOS	oxidative phosphorylation
P	phosphorus
P	postnatal day
PAP	3'-phosphoadenosine-5'-phosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PARPs	poly-ADP-ribose polymerases
PCA	principal component analysis
PCDDP	Preclinical Drug Development Platform
PCR	polymerase chain reaction
PNMT	phenylethanolamine N-methyltransferase
PPAR	peroxisome proliferator activated receptor
PPi	inorganic phosphate
PR	progesterone receptor
PVDF	polyvinylidene difluoride
PXR	pregnane X receptor
Q	ubiquinone
qRT-PCR	real-time quantitative reverse transcription polymerase chain reaction
R ²	correlation coefficient
RAR	retinoic acid receptor
Rep	repeats
RIN	RNA integrity numbers
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RPM	reads per million
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptor
SAH	S-adenosyl homocysteine
SAM	S-adenosyl-L-methionine

Abbreviation	Full term
SAM	sequence alignment map
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SMTs	S-methyltransferases
SO ₃ ⁻	sulfonate
ssDNA	single-stranded DNA
SULTs	sulfotransferases
TAC	Applied Biosystems™ Transcriptome Analysis Console
TCA	tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Temp	temperature
T _m	melting temperature
TMAP	Torrent Mapping Alignment Program
TNB	5-thio-2-nitrobenzoic acid
TPMT	thiopurine S-methyltransferase
TR	thyroid hormone receptor
tRNA	transfer RNA
UCS	μmol/min/mg citrate synthase activity
UDP	uridine diphosphate
UDPG	uridine-5'-diphosphate-d-glucose
UDPGA	UDP-glucuronic acid (uridine-5'-diphospho-α-d-glucuronic acid)
UGTs	UDP-glucuronosyltransferases
VDAC1	voltage-dependent anion channel protein 1
VDR	vitamin D receptor
WT	wild-type

CHAPTER 1 INTRODUCTION

1.1 Background, research rationale and problem statement

As the main producers of energy in the form of adenosine triphosphate (ATP), mitochondria are frequently referred to as the “powerhouse of the cell”. ATP is produced by the five complexes of the mitochondrial oxidative phosphorylation (OXPHOS) system. Dysfunction of the OXPHOS system is one of the most common groups of inborn errors of metabolism (Thorburn, 2004). Of these, complex I (CI) deficiency is the most common and often presents as Leigh Syndrome (Lake *et al.*, 2016). To study mitochondrial disease *in vivo*, rodent disease models are considered to be of great value, (Wallace, 2002) and for Leigh Syndrome a whole body NADH dehydrogenase ubiquinone Fe-S protein 4 (*Ndufs4*) knockout mouse model has been developed that is now commonly used in research (Kruse *et al.*, 2008).

The impeded energy production caused by mitochondrial disease could have numerous downstream effects. One of these downstream effects that have not been thoroughly investigated is biotransformation. Biotransformation, the process of converting endogenous and xenobiotic compounds to more water-soluble compounds, is an essential process that prevents build-up of toxic compounds (Darnell & Weidolf, 2013; Sevier *et al.*, 2012). These endogenous and xenobiotic compounds are in many cases carboxylic acids, the most common type of organic acid (Darnell & Weidolf, 2013). It is well known that the metabolism of organic acids are severely affected in mitochondrial disease (Esterhuizen *et al.*, 2017). Several biotransformation enzymes, glycine-n-acyltransferase (GLYAT) for example, are located in the mitochondrion. Many of these biotransformation reactions are directly or indirectly dependent on cofactors like ATP and NAD⁺. ATP production and the redox status are severely affected in mitochondrial disease, and could therefore possibly affect biotransformation in these patients. There is currently a severe lack of investigations on the effect that a mitochondrial disease has on the capacity of patients to metabolise endogenous metabolites and drugs through their biotransformation system. Glycine conjugation is a primary pathway of phase two biotransformation which, due to its role in organic acid conjugation, can thus be used as a starting point to investigate biotransformation in a mouse model of mitochondrial disease.

This study forms part of a larger study at the Mitochondria Research Laboratory of the North-West University. The Mitochondria Research Laboratory has extensively studied the *Ndufs4* KO mouse model. Investigations of this model include metabolomics on urine, selected brain regions (Terburgh *et al.*, 2021) and muscle types (Mason *et al.*, 2018; Terburgh *et al.*, 2019), as well as the development of a method for compartment specific metabolomics (Van der Walt & Louw,

2020). These studies have shed some light on disturbances of the redox balance in these mice and how it can affect multiple metabolic pathways. During investigation of specific brain regions, Terburgh *et al.* (2021) found that *Ndufs4* KO mice with higher residual CI activity and NADH/NAD⁺ ratios that are less disturbed, had less severe metabolic disturbances. Furthermore, mice with more severe levels of CI deficiency and increased NADH/NAD⁺ redox imbalances, had preferences for reductive carboxylation, acetylation and lipid synthesis. In the same study it was also found that the increased NADH/NAD⁺ ratios resulted in increased congestion of branched chain amino acid catabolism. Investigation of muscle in these mice revealed that non-classical pathways like the glycerol-3-phosphate shuttle, electron transfer flavoprotein system, complex II, and the proline cycle can fuel the Q-cycle as an adaptive mechanism to maintain adequate ATP production (Terburgh *et al.*, 2019). Also observed were increased levels of N-acetylaspartate (NAA) in *Ndufs4* KO mice and it was hypothesised that this increase was a result of the rate of acetyl-CoA synthesis from β -oxidation and lysine catabolism exceeding the rate at which it could be used by the TCA cycle, leading to accumulation of acetyl-CoA. As a mechanism to use some of the acetyl-CoA, it is conjugated with aspartate and converted to NAA through N-acetyltransferase. In the same study they also showed how some biotransformation reactions like methylation are affected by a disturbed redox balance. It was further postulated that the decreased levels of creatine, creatinine, and 1-methylhistidine, were likely due to a disrupted one-carbon metabolism as a result of a disturbed redox balance, leading to decreased S-adenosyl methionine availability for methylation reactions (Terburgh *et al.*, 2019). Furthermore from the same group, Miller *et al.* (2021) investigated changes in gene expression in *Ndufs4* KO mice as well as *Ndufs4* KO that were cross-bred to overexpress metallothionein. The authors found that genes involved in one-carbon metabolism, but not in oxidative stress, were downregulated in mouse brain and quadriceps, further signifying the disturbed one carbon metabolism in these mice.

From the abovementioned studies that developed the background and rationale for this study, it is clear that *Ndufs4* KO mice have disrupted energy production and redox status that will likely disturb the pathways in which it is involved, including biotransformation. As in the case of humans, information about the effect of mitochondrial disease on biotransformation in these mice is currently severely limited. One tool to investigate the effect of mitochondrial disease on biotransformation is targeted transcriptomics, a novel technique that can measure the expression levels of thousands of genes (Li, Turner, *et al.*, 2015). Understanding the expression levels of genes at a specific time point and condition can provide valuable insight into the current state of the cell (Finotello & Di Camillo, 2015). Gaining knowledge on expression of genes involved in biotransformation will not only aid in understanding the mechanism and pathology of

mitochondrial diseases but could potentially assist in developing drugs for mitochondrial disease patients that are metabolised in a predictable way, thereby making treatment more effective.

1.2 Aim of the study

The aim of this study was to investigate differential expression of genes involved in phase two biotransformation in an *Ndufs4* knockout mouse model. This was achieved by first investigating differential gene expression of glycine conjugation as a primary pathway of phase two biotransformation between *Ndufs4* knockout and wild-type mice. This investigation was then expanded to explore if the changes observed in glycine conjugation were reflected in the gene expression of the remaining phase two biotransformation pathways.

1.3 Structure of the dissertation

This dissertation is arranged in five chapters. The **first** chapter provides the background and rationale of the study. The **second** chapter gives an overview of literature relevant to this study, including mitochondria, oxidative phosphorylation, mitochondrial disorders, biotransformation, and regulation of metabolic pathways. It also provides the problem statement, aims and objectives, and the experimental strategy followed. The **third** chapter lays out detailed information about the materials and methods employed during the course of this study. The **fourth** chapter provides the results obtained. These results are also discussed and interpreted in this chapter. The **fifth** and final chapter summarises this study and discusses the completion of the objectives, as well as the strengths and limitations identified. Prospects for future research are also provided. Several **annexures** are included at the end of the dissertation to provide additional information not supplied in Chapters 1-5.

CHAPTER 2 LITERATURE

2.1 Introduction

This chapter presents a detailed discussion of literature pertaining to this study. It is divided into four sections. The first section discusses mitochondria and mitochondrial disorders. The second section explains the two phases of biotransformation. This is succeeded by a focus on amino acid conjugation, specifically, glycine conjugation. The third section discusses metabolic regulation with a specific focus on gene expression¹ and allosteric regulation. The final section sets out the problem statement identified from the literature as well as the aims, research questions and objectives established to address the problem statement.

2.2 Mitochondria and associated pathology

2.2.1 The mitochondrion

The mitochondrion is considered to lie at the heart of life and death of cells because of its involvement in a multitude of cellular processes, most importantly, energy production (Duchen, 2004). Mitochondria are double membraned subcellular organelles that are found in most eukaryotic cells. The inner and outer membranes are separated by an intermembrane space. On the inside of the inner membrane lies the mitochondrial matrix. The inner membrane forms folds, called cristae, which hosts mitochondrial enzymes involved in producing energy (Duchen, 2004; McBride *et al.*, 2006). Mitochondria have a wide range of functions, the most prominent being the production of energy as adenosine triphosphate (ATP). The ATP that they produce, provide the energy required for almost all cellular processes. Beyond producing ATP through oxidative phosphorylation (OXPHOS), mitochondria are involved in several other processes. They, *inter alia*, generate reactive oxygen species (ROS); are involved in some types of apoptosis; regulate calcium levels; play key roles in cellular signalling; and synthesise and catalyse metabolites. (Brand & Nicholls, 2011; Grünewald *et al.*, 2019). Considering the vital role of mitochondria in all cell types, mitochondrial dysfunction will inevitably bring about disease that affects multiple tissues. Mitochondrial dysfunction has been implicated in many diseases including neurodegenerative diseases such as Parkinson's and Alzheimer's; diabetes and cardiomyopathy; autism; and even sepsis (Chistiakov *et al.*, 2018; Duchen, 2004; Grünewald *et al.*, 2019; Weissman *et al.*, 2008). Furthermore, mitochondria have been in the spotlight for their possible involvement in ageing. The free radical theory of ageing suggests that ROS, which are mainly

¹ Throughout this dissertation, "gene expression" specifically refers to the expression of genes coding for enzymes that are involved in biotransformation pathways.

Heteroplasmy

The condition where there are at least two different populations of mtDNA molecules in a single cell.

Homoplasmy

The condition where all the mtDNA molecules present in a cell are identical.

Adaptive homeostasis

Short-term changes in the homeostatic range of protective and repair mechanisms in response to sub-toxic signalling molecules or events.

produced in mitochondria during normal mitochondrial function, induce oxidative damage to lipids, proteins, and nucleic acids which ultimately contributes to mitochondrial dysfunction. The mitochondrial dysfunction in turn causes further increased ROS production which in turn again initiates a vicious cycle. Until recently this gradual increase in mitochondrial dysfunction and **heteroplasmy** was thought to mediate and amplify the oxidative stress that drives ageing (Bratic & Larsson, 2013), but recent research has challenged this hypothesis (Brys *et al.*, 2007; Jebb *et al.*, 2018; Lewis *et al.*, 2013; Pérez *et al.*, 2009). More recent theories have been postulated to fill the gaps in this mitochondrial free radical theory of ageing. For example, the **adaptive homeostasis** theory of ageing states that ageing involves diminished adaptive homeostasis causing deterioration of biological structures and functions which eventually results in dysfunction, disease and death (Pomatto & Davies, 2018).

2.2.2 Mitochondrial DNA

One distinctive characteristic of mitochondria is that they contain their own genome, mitochondrial DNA (mtDNA), which along with nuclear DNA (nDNA) encode the proteins of the OXPHOS system. In humans, mtDNA are circular, double stranded molecules consisting of 16 569 bp that codes for 37 genes. These genes are required for the synthesis of 13 subunits of the OXPHOS system (see Table 2.1), 22 transfer RNAs (tRNAs), and two ribosomal RNAs (rRNAs) (Bratic & Larsson, 2013; DiMauro & Schon, 2003). mtDNA is housed in the mitochondrial matrix and hundreds to thousands of copies can be present in a single cell (Taylor & Turnbull, 2005). **Homoplasmy** and heteroplasmy levels of mtDNA are directly associated with the phenotype of disease and might be able to explain the variability seen in the clinical presentation through the threshold effect (DiMauro & Schon, 2003; Taylor & Turnbull, 2005). The threshold effect describes how a minimum number of mutated mtDNA molecules must be present before oxidative dysfunction is present. This threshold is decreased in tissues with high aerobic demands, such as that of the brain, heart and skeletal muscles, making them especially vulnerable to mutations in mtDNA (DiMauro & Schon, 2003). However, mitochondrial disease is not always caused by mutations in mtDNA. It can be, and most often is, caused by mutations in nDNA that encode the remainder of the 82 structural subunits of the OXPHOS system (Shoubridge, 2001).

Table 2.1 Subunits of the OXPHOS system that are encoded by mtDNA

Complex	Subunits encoded by mtDNA
CI	ND 1, 2, 3, 4, 4L, 5, and 6
CIII	CytB
CIV	COXI, COXII, and COXIII
CV	ATP synthase subunits A6 and A8

This table shows the subunits of the OXPHOS system that are encoded by mtDNA as described by (DiMauro & Schon, 2003). Abbreviations: cytochrome b (CytB), cytochrome c oxidase (COX), *mitochondrial deoxyribonucleic acid (mtDNA)* *nicotinamide adenine dinucleotide dehydrogenase (ND)*.

2.2.3 Oxidative phosphorylation

The OXPHOS system, which is located in the inner mitochondrial membrane, consists of five multi-subunit protein complexes (see Table 2.2) and two electron carriers namely coenzyme Q10 and cytochrome C, as shown in Figure 2.1.

Table 2.2 Protein complexes of the OXPHOS system

Complex	Name	E.C. number	Approximate number of subunits ²
CI	reduced NADH dehydrogenase-ubiquinone oxidoreductase	E.C. 1.6.5.3	45 subunits
CII	succinate dehydrogenase-ubiquinone oxidoreductase	E.C. 1.3.5.1	4 subunits
CIII	ubiquinone-cytochrome c oxidoreductase	E.C. 1.10.2.2	11 subunits
CIV	cytochrome c oxidase	E.C. 1.9.3.1	13 subunits
CV	ATP synthase	E.C. 3.6.3.14	16 subunits

This table describes describes the five complexes of the OXPHOS system. Abbreviations: oxidative phosphorylation (OXPHOS), reduced nicotinamide adenine dinucleotide (NADH), complex I (CI), complex II (CII), complex III (CIII), complex IV (CIV), complex V (CV).

Electrons are transported along the electron transport chain (ETC) - which constitutes the first four complexes - to molecular oxygen (O₂), thereby forming water, while protons (H⁺) from the matrix are pumped through CI, CIII, and CIV to the intermembrane space. This forms an electrochemical proton gradient that drives ATP synthesis as H⁺ moves through CV to the matrix, which uses adenosine diphosphate (ADP) and inorganic phosphate to produce ATP (DiMauro & Schon, 2003; Hatefi, 1985; Smeitink *et al.*, 2001). CV, also known as F₀F₁-ATP synthase, is a

² Number of subunits obtained from (DiMauro & Schon, 2003; Stroud *et al.*, 2016).

super-complex consisting of two rotary motors. The first motor, F_1 , is the water-soluble part of CV and by rotating the inner subunit, hydrolyses ATP. The second and smaller motor, F_0 , is embedded in the inner mitochondrial membrane and generates rotary torque upon proton translocation driven by the proton electrochemical potential (Okuno *et al.*, 2011). The adenine nucleotide translocator has intrinsic adenine nucleotide translocase activity, thereby rendering it an important component of the mitochondrial ATP synthesis machinery (Maioli *et al.*, 2013).

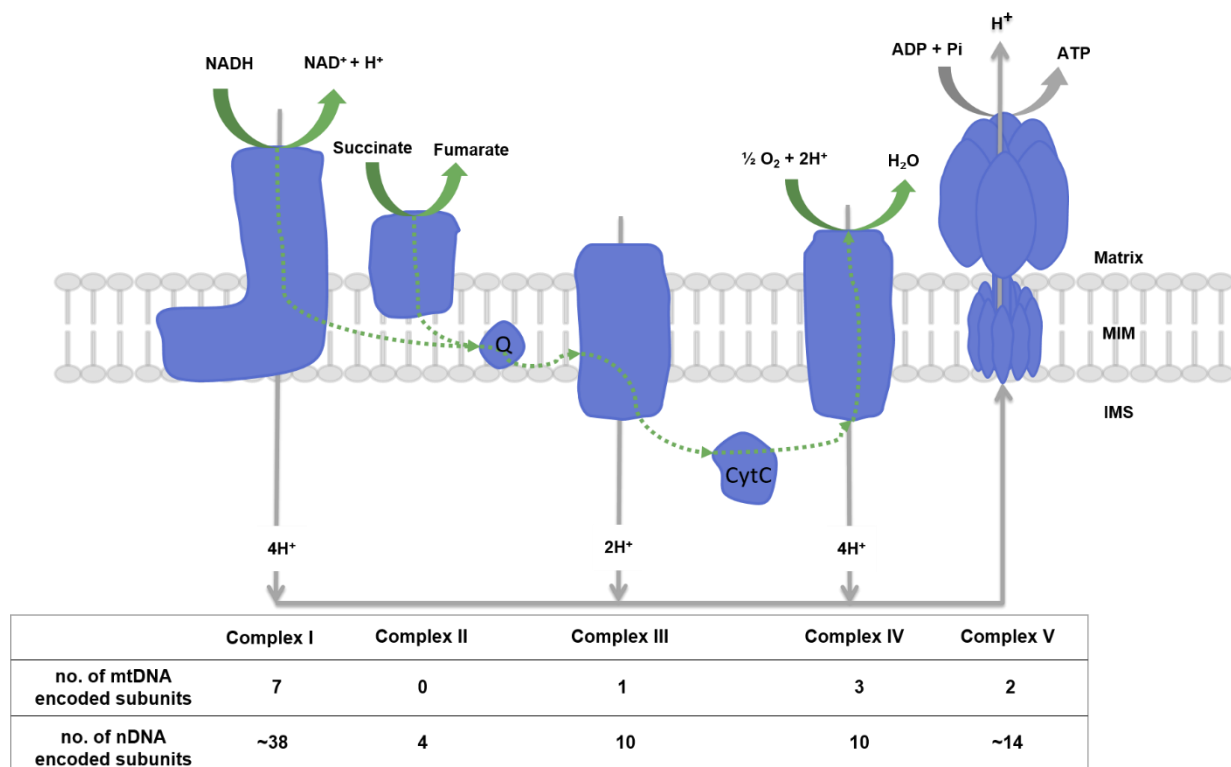


Figure 2.1 Schematic representation of the oxidative phosphorylation system

This figure shows the five subunits of the OXPHOS system as well as the two electron carriers, cytochrome C and ubiquinone. Electron flow (indicated in green) through the first four complexes is coupled to proton (H^+) translocation (grey arrows) from the matrix to the IMS. The number of subunits that are encoded by mtDNA and nDNA is shown for each complex. *Abbreviations:* adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytochrome c (CytC), oxidised flavin adenine dinucleotide (FAD^+), reduced flavin adenine dinucleotide ($FADH$), water molecule (H_2O), intermembrane space (IMS), mitochondrial inner membrane (MIM), mitochondrial DNA (mtDNA), oxidised nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), nuclear DNA (nDNA), molecular oxygen (O_2), oxidative phosphorylation (OXPHOS), ubiquinone (Q).

2.2.4 Complex I

CI is the largest of all the OXPHOS proteins and the complex that is most often involved in mitochondrial disorders. It consists of approximately 45 subunits with a size of ~1 megadaltons (Calvaruso *et al.*, 2012; DiMauro & Schon, 2003; Shoubridge, 2001). The L-shaped CI consists of a peripheral arm and a membrane arm (see Figure 2.2). The peripheral arm contains the N- and Q-module, while the membrane arm is embedded in the inner mitochondrial membrane

and contains the P-module (Hunte *et al.*, 2010). The N-module is the NADH dehydrogenase component with one flavin mononucleotide (FMN) molecule serving as the primary electron acceptor to oxidise NADH to NAD^+ and H^+ . The Q-module is a hydrogenase component that reduces ubiquinone while the P-module translocates protons across the membrane. The NADH oxidation site is connected with the ubiquinone (Q) reduction site with a chain of eight to nine iron-sulphur clusters (Brandt, 2006). Four H^+ are translocated across the inner mitochondrial membrane during the redox reaction (Brandt, 2006; Calvaruso *et al.*, 2012; Hunte *et al.*, 2010; Shoubridge, 2001). Subunits of CI is divided into central and accessory subunits. Central subunits comprise the 14 subunits necessary to perform bioenergetic functions and are divided into seven mtDNA encoded subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) and seven nuclear encoded subunits (NDUFB1, NDUFB2, NDUFB3, NDUFB4, NDUFB5, NDUFB6 and NDUFB7) (Brandt, 2006; Distelmaier *et al.*, 2009).

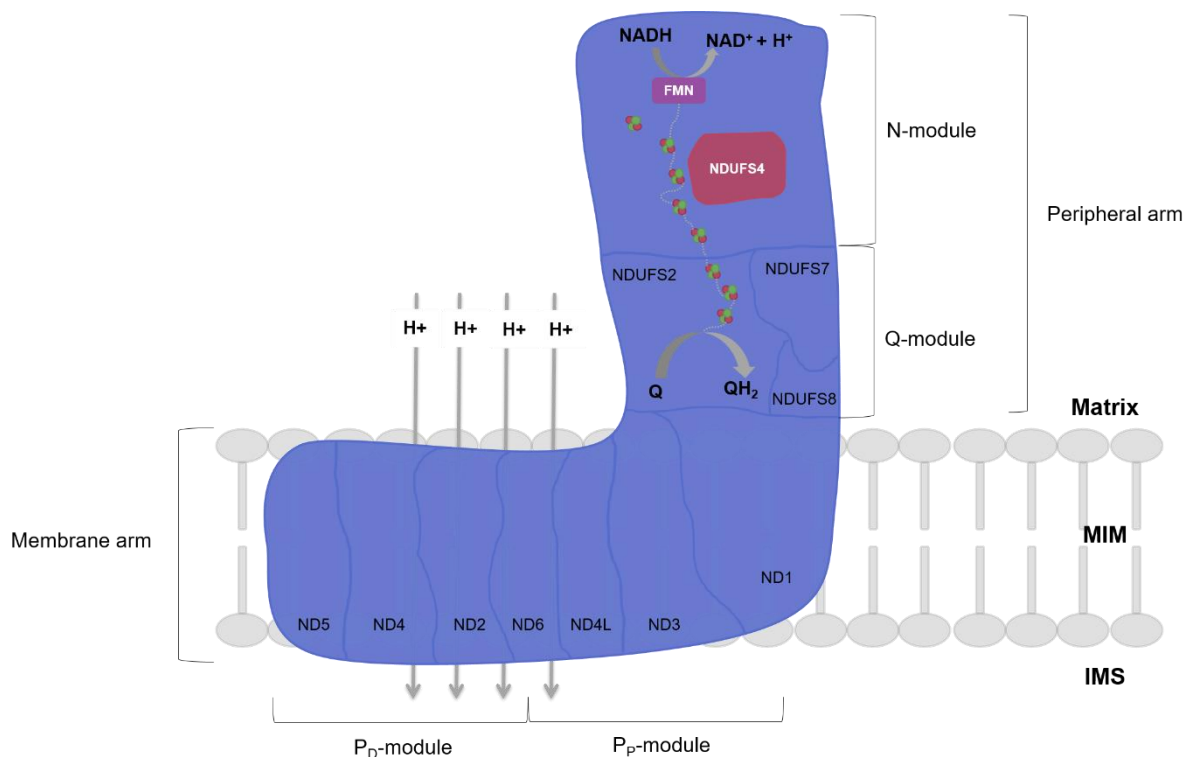


Figure 2.2 Simplified schematic representation of mammalian complex I

The peripheral arm of complex I contains the N- and Q-modules. The membrane arm consists of the two P-modules. NADH is oxidised by a FMN in the N-module. Electrons are passed along the chain of ~8 iron-sulphur clusters to the Q-binding site, where Q is reduced to QH_2 . For each oxidised NADH, four H^+ are transferred from the matrix to the IMS. Several subunits are indicated. Of particular importance for this study is the 18-kDA subunit of CI, NDUFS4 (shown in pink). *Abbreviations: flavin mononucleotide (FMN), oxidised nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide dehydrogenase (ND), NADH dehydrogenase ubiquinone Fe-S protein 4 (NDUFS4).* Adapted from several sources including Fiedorczuk and Sazanov (2018); Hirst (2013).

2.2.5 Mitochondrial disorders

Mitochondrial disorders are a group of clinically heterogeneous diseases caused by dysfunction of the OXPHOS system (Rahman & Rahman, 2018). They are the most common group of inborn errors of metabolism with an estimated prevalence of one in 5-10 000 live births (Skladal *et al.*, 2003; Smeitink *et al.*, 2001; Thorburn, 2004). So far, more than 500 pathogenic variants (mutations) have been identified in mitochondrial genes (Mitomap., 2019). These mutations can affect some of the many functions of mitochondria such as metabolism; vitamin and cofactor synthesis; calcium homeostasis; protein synthesis; and antioxidant defences (Rahman & Rahman, 2018). Mitochondrial disorders have multiple modes of transmission and can also be acquired spontaneously. Depending on whether the mutation is caused by mtDNA or nDNA mutations, they can be linked to the X-chromosome, maternally inherited, autosomal dominant or autosomal recessive (Prasun, 2019).

Of mitochondrial disorders, CI deficiency is the most common (Distelmaier *et al.*, 2009). OXPHOS is severely affected if the activity of CI is defective, especially in tissues like that of the brain, heart, and skeletal muscles that have high energy demands (Scacco *et al.*, 2003). Defects of CI where its stability is affected, as in the case of NADH dehydrogenase ubiquinone Fe-S protein 4 (*NDUFS4*)³ mutations, have been shown to be somewhat circumvented by the formation of a supercomplex between CI and CIII that aids in the stabilisation of CI (Calvaruso *et al.*, 2012).

Although complicated, several methods are used to diagnose mitochondrial disorders with the process being similar to that of diagnosis of neuromuscular disease. Tissue biopsies are used to determine mitochondrial morphology and enzyme activity. Plasma and cerebrospinal fluid are used to determine lactate and pyruvate levels through metabolomics. Next generation sequencing can detect nuclear or mitochondrial mutations (Lee *et al.*, 2009; Orsucci *et al.*, 2009). Lactate and acylcarnitines are classic markers of mitochondrial dysfunction (Legault *et al.*, 2015). The diagnostic process typically starts with patient and family history as well as a physical and neurologic examination. Patients presenting with exercise intolerance, neurosensory hearing loss, hypertrophic cardiomyopathy, ophthalmoplegia, short stature, or renal tubular acidosis are regarded as possible mitochondrial disease patients. Further diagnosis requires measurement of serum lactate; electromyography; magnetic resonance spectroscopy; enzymology and histology of muscle tissue; and genetic analysis (Orsucci *et al.*, 2009). Next generation sequencing technologies have dramatically reduced costs of sequencing in large cohorts and have thereby

³ Formatting of symbols for genes and proteins used in this dissertation: When referring to humans, gene symbols are written in uppercase and in italics (e.g., *GLYAT*). When referring to mice, the gene symbols are written in italics and only the first letter is in uppercase (*Glyat*). Protein symbols for humans and mice are fully written in uppercase and not in italics (e.g., GLYAT).

contributed to increased diagnosis of mitochondrial disorders in recent years (Rahman & Rahman, 2018).

Several obstacles are in the way for the development of successful treatment options for mitochondrial disease. A major one being the high level of clinical heterogeneity seen in patients with mitochondrial disease. Other impediments include the double membrane of the mitochondrion that hampers the penetration of drugs, and clinical trials that are difficult to conduct with sufficient numbers due to the relative scarcity of mitochondrial disorders (Rahman & Rahman, 2018). Current treatment options are mainly target symptoms and are inadequate. Some of the proposed mitochondrial therapeutics include the antioxidants vitamin C, vitamin E, N-acetylcysteine, α -lipoic acid, and coenzyme Q10 and its analogues ubiquinol and idebenone (Rahman & Rahman, 2018). Compounds that restore redox balance, including nicotinamide riboside, and metabolites such as L-carnitine and creatine, have shown some potential as therapeutics (Orsucci *et al.*, 2009; Rahman & Rahman, 2018). A more drastic approach that aims to avoid mitochondrial disease, is mitochondrial replacement therapy, in which transfer of mutated mtDNA from mother to offspring is avoided through the use of donor mitochondria (Zhang *et al.*, 2017).

2.2.6 Leigh syndrome

Leigh syndrome (also called subacute necrotizing encephalopathy) is a progressive neurodegenerative disorder, first reported in 1951 by Denis Leigh (Leigh, 1951), and although rare (incidence of one in 40 000), might be the most common disorder of the OXPHOS system in children (Lombardo *et al.*, 2014; Munaro *et al.*, 1997). Patients typically present with focal necrotizing lesions in the brainstem, basal ganglia and cerebellum. Symptoms include failure to thrive; psychomotor retardation; lactic acidosis; truncal ataxia; and death between the age of 6 months and 12 years (Budde *et al.*, 2003; Cooper *et al.*, 2006). The early-onset form typically starts to present before patients are two years old. A late-onset form is much rarer but presents with less severity than the early-onset form. Patients with late-onset Leigh Syndrome also present with intellectual decline, headache and memory loss (Hong *et al.*, 2020). Recently, 89 genes have been recognized to cause Leigh Syndrome (Rahman *et al.*, 2017). The underlying cause is commonly mutations in genes that encode subunits or assembly factors of the OXPHOS system (Finsterer, 2008). Patients with the exact same mutations don't necessarily present with the same symptoms, which accentuates this disease's clinically heterogeneous nature. This is also typical of other mitochondrial disorders (Budde *et al.*, 2003; Lachawan *et al.*, 2000; Munaro *et al.*, 1997).

At this stage, there is no cure for Leigh Syndrome. Treatment mostly involves antioxidants and OXPHOS complex cofactors, aimed at ameliorating symptoms and improving quality of life. Drugs

like riboflavin, thiamine, pyruvate, coenzyme Q10, l-carnitine, α -lipoic acid, creatine-monohydrate and vitamin B7 (biotin) are among those given to treat Leigh Syndrome. As a preventative measure, vitamins A and E can also be given for their antioxidant properties (Baertling *et al.*, 2014; Gerards *et al.*, 2016). Recently, EPI-743 has shown promising results in the treatment of not only Leigh Syndrome patients, but other mitochondrial disorders as well. EPI-743 is a synthetic derivative of coenzyme Q10 and idebenone, or a para-benzoquinone analogue. This drug is believed to be approximately 1000 – 10 000 fold more potent than coenzyme Q10 or idebenone in protecting cells that were exposed to oxidative stress (Enns *et al.*, 2012). In terms of a dietary intervention, an age and activity appropriate diet and energy intake are recommended. Drugs that impair mitochondrial function, such as valproate, barbiturates and tetracyclines, are to be avoided (Baertling *et al.*, 2014).

2.2.7 Metabolic alterations in Leigh syndrome

Metabolic pathways that are commonly disrupted in mitochondrial disease include glycolysis, the TCA cycle, mitochondrial fatty acid β -oxidation, branched chain amino acid metabolism, and one-carbon metabolism. Classic markers of mitochondrial metabolic dysfunction include lactate and acylcarnitines (Legault *et al.*, 2015). One principal repercussion of OXPHOS deficiencies is thought to be a disturbed redox balance (NADH/NAD^+ , FADH/FAD^+). NADH is oxidized to NAD^+ by CI and CI deficiency therefore results in an inability to oxidize NADH which is thought to lead to a redox imbalance and decline in NAD^+ levels (Lee *et al.*, 2019). Due to the involvement of these coenzymes in a range of cellular processes, disturbances in their ratios can affect numerous metabolic pathways. Pyruvate for example, is converted to acetyl-CoA by the pyruvate dehydrogenase complex and NAD^+ . As a result of the decreased NAD^+ levels in mitochondrial disease this reaction is inhibited, and pyruvate levels are increased. Pyruvate is then converted to lactate by lactate dehydrogenase and NADH, which aids in recycling NAD^+ which can then be used in glycolysis. The cytosolic redox state can therefore be indicated by the lactate/pyruvate ratio (Esterhuizen *et al.*, 2017). The mitochondrial redox state is reflected by the acetoacetate/3-hydroxybutyric acid ratio, with 3-hydroxybutyric acid typically being increased in mitochondrial disease (Esterhuizen *et al.*, 2017). Metabolic profiling of patients with Leigh Syndrome French Canadian variant revealed increased plasma levels of creatine, lactate, pyruvate, β -hydroxybutyrate, and decreased levels of glycocholate (Legault *et al.*, 2015). Increased lactate was also reported in children with mitochondrial disease (Shatla *et al.*, 2014). Metabolomics studies also commonly report increased TCA cycle intermediates, which is a result of the inhibition of three dehydrogenases involved in the TCA cycle as a result of increased NADH levels (Esterhuizen *et al.*, 2017). Analysis of brain regions of a mitochondrial disease mouse model, revealed perturbations in branched chain amino acid metabolism (Terburgh *et al.*, 2021).

Branched chain amino acids were increased, while by-products of their catabolism were decreased. Increased branched chain amino acids were also reported in a cohort of primary mitochondrial disease (Clarke *et al.*, 2013). Terburgh *et al.* (2021) further reported increased levels of several long chain fatty acids. Investigation of skeletal muscle of the same mouse model displayed decreased levels of fatty acid acylcarnitines, trimethylglycine, methionine, and threonine (Terburgh *et al.*, 2019). During phenylalanine and tyrosine metabolism, gut microbiota catalyse the formation of benzoic acid from cinnamic acid. Benzoic acid then undergoes glycine conjugation, a biotransformation reaction, to form hippuric acid, which is also reported to be disturbed in mitochondrial disease (Esterhuizen *et al.*, 2017).

2.3 Biotransformation

The disturbed redox balance observed in mitochondrial disease, as well as the altered ATP production could potentially affect the capacity of patients to perform biotransformation reactions, as many of these reactions are directly and indirectly dependent on cofactors like ATP and NAD⁺. Furthermore, some of these biotransformation reactions are localised in the mitochondrion, as will be discussed in the sections that follow.

Humans are exposed daily to a great deal of endogenous (e.g. bile acids) and exogenous (e.g. drugs, environmental chemicals and food preservatives) compounds through food, medicinal drugs, water, and air (Knights & Miners, 2011). These compounds are often lipophilic and would accumulate in the body which could in turn present as toxic, unless they are converted to some form that can be easily excreted (Grant, 1991). Biotransformation, or popularly also referred to as “detoxification”, is the process of converting lipophilic compounds to more water-soluble compounds that can be easily excreted in the urine and bile. This mainly takes place in the liver (Grant, 1991; Seviour *et al.*, 2012). A schematic representation of these reactions is shown in Figure 2.3 while the enzymes and cofactors involved are shown in Table 2.3. The parenchymal cells of the liver, hepatocytes, are responsible for this biotransformation. Hepatocytes contain many mitochondria, indicating that the liver plays an important role in energy metabolism (Seviour *et al.*, 2012). Drug biotransformation can also occur in mitochondria and plasma membranes (Ionescu & Caira, 2006). High levels of detoxifying enzymes are present in the small intestinal epithelium, serving as a first-defence against toxic compounds absorbed through the gastrointestinal tract (Goerres *et al.*, 2006).

There are two main biotransformation pathways which are commonly referred to as phase one and phase two biotransformation. When biotransformation renders a compound less toxic, the process is referred to as detoxification. In some cases, biotransformation renders a compound more toxic and the process is then referred to as metabolic activation (Buhler & Williams, 1988).

Metabolic activation is sometimes used in a clinical setting where an inactive drug, called a prodrug, is given to a patient and through its metabolic activation the drug is converted to an active form that has a therapeutic effect. This is employed when a prolonged or delayed response of a drug is required, when a local reaction is to be avoided, when there is poor absorption or site specificity, or even when the drug has undesirable organoleptic properties (Ionescu & Caira, 2006; Wu, 2009).

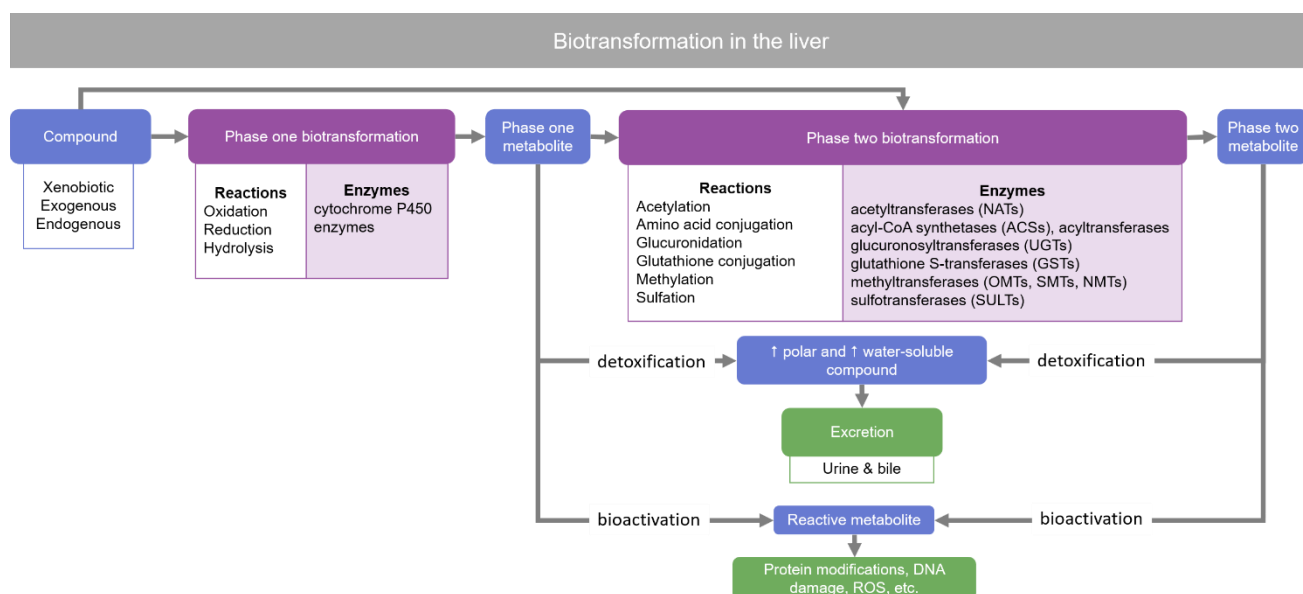


Figure 2.3 Schematic representation of typical biotransformation pathways

This scheme shows the typical pathway of biotransformation reactions. Biotransformation consists of two phases. Phase one reactions include oxidation, reduction, and hydrolysis. These reactions are mediated by cytochrome P450 enzymes. Products of phase one metabolism can undergo further metabolism through phase two biotransformation. There are several phase two reactions, each with its own set of enzymes that catalyse them. When the metabolites of phase one or two biotransformation are more polar and more water soluble, they are detoxified and can be excreted in the bile or urine. In some cases, these metabolites are bioactivated and are more reactive than the parent compound. These reactive metabolites can have several effects including protein modifications, DNA damage and ROS production.

The importance of biotransformation is highlighted by the consequences of its deficiency and its use in treatment of several diseases. Many **xenobiotics** can reach toxic and even deadly levels if they are not metabolised (Croom, 2012). Exposure to xenobiotics can disturb the microbiome (Lu *et al.*, 2015). Loots *et al.* (2007) have suggested that the clinical symptoms of isovaleric acidaemia may partially be due to the depletion of amino acids required for detoxification reactions. Patients with coeliac disease have been found to have a relatively high risk for small intestinal

Xenobiotics

A term used to describe chemicals to which organisms are exposed, that are extrinsic to their usual metabolism. This includes environmental contaminants, medicinal drugs, pesticides and foreign chemical created by other organisms (Croom, 2012).

malignancies. This could be explained by the low activity of a detoxifying enzyme, uridine diphosphate glucuronosyltransferases (UGTs) in their small intestine, resulting in decreased detoxification of potential carcinogens and toxins ingested via food and medication (Goerres *et al.*, 2006). Accumulation of several aromatic acids, like benzoic acid, 3-phelylpropionate and cinnamic acids can activate ROS production and inhibit activity of CI (Beloborodova *et al.*, 2012). Xenobiotic carboxylic acids such as benzoic acid and salicylic acid, can be activated to xenobiotic-CoAs by mitochondrial ATP-dependent acyl-coenzyme A synthetases (ACSM). Accumulation of these xenobiotic-CoAs can have toxic effects by sequestering carnitine and the cellular CoA pool, or by inhibiting important metabolic enzymes (Van der Sluis & Erasmus, 2016).

Table 2.3 Typical phase one and phase two biotransformation reactions, enzymes, cofactors, and substrates.

Type of biotransformation reactions	Enzymes	Cofactors	Examples of substrates
Phase one	cytochrome P450	Haeme	androgens, cholesterol, oestrogens, several drugs, sterols, vitamins
Acetylation	N-acetyltransferases (NATs)	acetyl-CoA	amines, amino acid, hydrazine, sulphonamide
Amino acid conjugation	acyl-CoA synthetases; acyltransferases	glycine, CoA	benzoic acid, bile acids, carboxylic acids
Glucuronidation	UDP-glucuronosyltransferases (UGTs)	UDP-glucuronic acid (UDPGA)	alcohols, carboxylic acids, hydroxylamine, phenols, sulfonamides
Glutathione S-conjugation	glutathione S-transferases (GSTs)	glutathione (GSH)	arene oxides, epoxides, hydroxylamine, nitro groups,
Methylation	methyltransferases (OMTs, SMTs, NMTs)	S-adenosylmethionine (SAM)	amines, catecholamines, phenols
Sulfoconjugation	sulfotransferases (SULTs)	phosphoadenosyl phosphosulphate (PAPS)	alcohols, aromatic amine, neurotransmitters, phenols, steroid hormones

Biotransformation is a dynamic process that can adapt to the internal milieu of hepatocytes. Exogenous drugs, endogenous steroid hormones and other xenobiotics can trigger changes in biotransformation (Sevior *et al.*, 2012). Furthermore, biotransformation is subject to large inter-individual variabilities. Factors known to affect biotransformation in individuals include genetic variation, intra-individual factors, environmental factors and disease-state factors (Ionescu & Caira, 2006). Gender and age also play a role in variations seen in enzyme activity, but is not apparent in all biotransformation enzymes (Eker *et al.*, 2020). Strassburg *et al.* (2002) for example, determined that activity of liver glucuronidation in children aged between one and two years were decreased compared to adults for a variety of drugs. Furthermore, several UGTs displayed sex-dependent expression in mouse tissue which could influence metabolism. *Ugt2b1* were predominantly expressed in male liver, while *Ugt1a1* and *Ugt1a5* showed predominant expression in females (Buckley & Klaassen, 2007). This is in contrast to expression levels of SULTs that do not show gender or age-dependent variations.

Genetic variation: The presence of genetic variants in biotransformation genes, can affect the ability of an individual to metabolise drugs by causing variations in the activity of an enzyme. Some of these genetic variants can result in a reduced ability, a complete lack of activity, or in some cases in an enhanced activity, which can result in an unexpected toxic effect or the desired therapeutic effect (Ionescu & Caira, 2006). In glycine N-acetyltransferase (GLYAT) for example,

K_{cat} (Catalytic rate constant)

The maximal number of substrate molecules that form product per active site per second when the enzyme is saturated with substrate.

characterisation of the kinetic mechanism of three haplotype variants showed that relative enzyme activity is negatively affected by a rare 156Asn>Ser, 199Arg>Cys variant which only displayed 9.8% of the **k_{cat}** of the more abundant haplotype 156Asn>Ser. Ultimately, individuals with this haplotype might have a decreased ability to metabolise benzoate (Rohwer *et al.*, 2021). Another example is 50 mutations causing the absence of or partial

deficiency of UGT1A1, resulting in Crigler-Najjar syndrome (Jancova *et al.*, 2010).

The presence of genetic variants that affect the ability of individuals to metabolise drugs has led to a field known as pharmacogenetics, which is the study of how these genetic variations affect patients' responses to drugs (Roses, 2000). **Intra-individual factors:** Physiological changes, pathological states and biological rhythms, for example hormonal balance, are thought to affect drug metabolism (Ionescu & Caira, 2006). **Environmental factors:** Environmental factors that can influence biotransformation include inter alia smoking; alcohol consumption; diet; exposure to chemicals and pollutants; and drug use (Ionescu & Caira, 2006). Consumption of vegetable-containing diets in humans for example, showed increased activity of UGT1A (Navarro

et al., 2009). Several studies showed dysregulated glucuronidation and phase one biotransformation activity in smokers, even former smokers, compared to non-smokers (Court, 2010; Lee *et al.*, 2006; O'Malley *et al.*, 2014). It is also well known that environmental chemicals like polychlorinated biphenyls and pollutants are able to induce P450 enzymes (Katzung *et al.*, 2018). Long term ethanol treatment in Wistar rats resulted in dysregulated biotransformation with some enzymes like P450E1 being induced, while others including P4503A2 and P4502C13 were decreased (Van de Wiel *et al.*, 1993). **Disease factors:** Considering that the liver is the primary biotransformation organ, any disease that affect its function can also affect the ability of the individual to perform biotransformation reactions. Liver disease, diabetes, some types of infections and thyroid dysfunction are known to affect drug metabolism (Ionescu & Caira, 2006; Maritim *et al.*, 2000). Xenobiotics are moved across membranes by several transporters. The activity levels of these transporters can be inhibited or induced by drug-drug interactions (Sevior *et al.*, 2012).

2.3.1 Phase one biotransformation

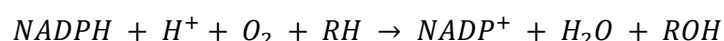
Phase one biotransformation reactions are considered a preparatory step for phase two reactions. These reactions typically add or expose a polar functional group like -NH_2 , SH or -OH by one of several reactions, including oxidation with cytochrome P450 (CYP450) enzymes, and reduction and hydrolysis reactions. The resultant metabolites are usually less lipid-soluble than the original compound, rendering them less able to penetrate tissues and less able to undergo renal tubular reabsorption. Phase one metabolites may still be active and can then be used as a substrate in phase two reactions. Compounds that already contain an -OH , -NH_2 or COOH group, can directly enter phase two biotransformation (Ionescu & Caira, 2006; Katzung *et al.*, 2018).

Oxidation reactions are the most common type of biotransformation reactions and involve the incorporation of oxygen into the substrate. They comprise the following: dehydrogenation; side-chain hydroxylation; aromatic hydroxylation; deamination; sulfoxide formation; deamination; and N-, O-, and S-dealkylation. Reduction reactions are somewhat uncommon and include reduction reactions of nitro, nitroso, and azo groups. Esters and amides commonly undergo hydrolysis reactions during biotransformation (Ionescu & Caira, 2006).

The most important enzymes involved in phase one biotransformation include monooxygenases; CYP450s; flavin-dependent monooxygenases (FMOs); hydrolases; and epoxide hydrolases (Croom, 2012). Some of these enzymes are found in mitochondria and cytosol of hepatocytes. CYP450 enzymes are regarded as the most important group of drug metabolising enzymes. They are involved in the metabolism of several endo- and exogenous compounds that include drugs, environmental chemicals and other xenobiotic compounds (Ionescu & Caira, 2006). CYP450s

contain a haeme group that is essential for their catalytic activity and become inactive when the orientation of the haeme is lost. In humans there are 14 families of CYP450 enzymes totalling 61 genes. Polymorphisms found in these genes are especially important in the metabolism of environmental xenobiotics. CYP450 reactions involve reduction of one atom of molecular oxygen to water, with the second oxygen atom being incorporated into the substrate. Electrons are transferred from nicotinamide adenine dinucleotide phosphate (NADPH) (Croom, 2012; Sevier *et al.*, 2012). A typical CYP450 reaction is shown in Equation 2.1.

Equation 2.1 A typical CYP450 catalysed reaction



where:

RH represents an oxidisable compound

ROH represents a hydroxylated metabolite

The second type of monooxygenases involved in phase one biotransformation reactions are FMOs which are mostly found in the liver. As CYP450, FMOs are microsomal enzymes that require NADPH and oxygen. They only catalyse oxygenation reactions in which electrons are directly transferred from NADPH. Currently, there are 5 types of FMOs (FMO1-FMO5) with different expressions, depending on tissue and species (Croom, 2012).

There is a range of other phase one enzymes which includes epoxide hydrolases; prostaglandin synthases; alcohol and aldehyde dehydrogenases; amine oxidases; hydrolases; and proteases (Croom, 2012). After phase one biotransformation compounds can continue biotransformation through phase two.

2.3.2 Phase two biotransformation

Phase two biotransformation reactions are conjugation reactions that result in a covalent linkage between the functional group of the parent compound to a molecule that can enhance its water solubility. Therefore, conjugated metabolites are usually more hydrophilic than the original compound and are easily excreted via the kidneys. In most cases, except for glutathione conjugation, the substrates for conjugation reactions are products of phase one reactions (Croom, 2012). Hydrophilic groups are added to the parent compound before the conjugation reaction. Reactions of phase two biotransformation include methylation; glucuronidation; acetylation; sulfation; glutathione conjugation; and amino acid conjugation. Glycine, taurine and glutamic acid

are commonly used in amino acid conjugation reactions (Grant, 1991; Ionescu & Caira, 2006; Phang-Lyn & Llerena, 2020).

Acetylation reactions

Acetylation is the transfer of acetyl-coenzyme A (acetyl-CoA) to an amine, amino acid, hydrazine, or sulphonamide, through the action of an N-acetyltransferase (see Figure 2.4). The cofactor of these reactions, acetyl-CoA can be acquired from the glycolysis pathway, fatty acid or amino acid catabolism, or synthesis from acetate and CoA (Ionescu & Caira, 2006). The two isozymes encoded in the human genome are arylamine N-acetyltransferase 1 (NAT1) and arylamine N-acetyltransferase 2 (NAT2). NAT2 is mainly expressed in the liver and intestines, while NAT1 is present in many tissues. Individuals are classified into slow, intermediate, and fast acetylators depending on their NAT2 haplotype. Fast acetylator phenotypes are encoded by four enzymatic variants including, the wild-type allele *NAT2*4*, and three allele groups – *NAT2*11*, *NAT2*12* and *NAT2*13*. Slow acetylator variants are encoded by the allele groups *NAT2*5*, *NAT*6*, *NAT2*7*, and *NAT2*14*. Intermediate acetylators possess one fast and one slow acetylator allele (Conway *et al.*, 2020). The role of NATs in endogenous metabolism is unclear, except for the recent discovery of its action on spermidine (Conway *et al.*, 2020; Jancova *et al.*, 2010). There are certain differences in NATs between species. Human NAT1 is more similar to rodent NAT2 showing 82% identity at amino acid level and both enzymes having p-aminobenzoic acid as a selective substrate (Jancova *et al.*, 2010).

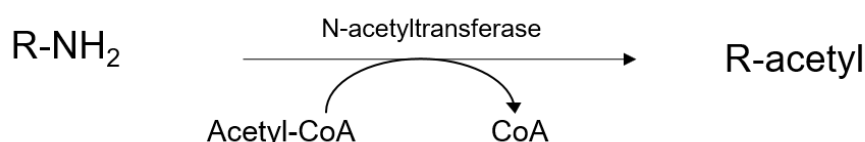


Figure 2.4 Acetylation of arylamine by NATs

With the help of cofactor acetyl-CoA, N-acetyltransferase catalyses the reaction between a substrate with NH_2 group to conjugate an acetyl group to it. *Abbreviations: coenzyme A (CoA), substrate that undergoes biotransformation (R).*

Glucuronidation reactions

Glucuronidation, which is conjugation with D-glucuronic acid, is considered the most common and most important conjugation reaction. It requires uridine-5'-diphospho- α -D-glucuronic acid (UDPGA) to provide glucuronic groups to oxygen, nitrogen, sulphur or carboxyl groups, and are catalysed by microsomal enzymes called UDP-glucuronosyltransferases (UGTs). Figure 2.5 provides a schematic representation of typical glucuronidation reactions. Human UGTs are divided into four classes, UGT1, UGT2A and UGT2B, UGT3, and UGT8 (Jancova *et al.*, 2010). UGT1 mainly binds exogenic compounds, whereas UGT2 mainly binds endogenous compounds.

Endogenous compounds conjugated through this pathway include bile acids, fatty acids, thyroid hormones, bilirubin, fat-soluble vitamins, and hydroxysteroids (Jancova *et al.*, 2010). Glucuronidation reactions also involve sulfotransferase and N-acetyltransferase enzymes (Goerres *et al.*, 2006; Ionescu & Caira, 2006). UGT3A1 is a UDP N-acetylglucosaminyltransferase, while UGT8 plays a role in the nervous system (Jancova *et al.*, 2010). The liver is the major location of most of the human UGT proteins, although they show broad tissue distribution including the gastrointestinal tract, kidneys, brain and placenta (Jancova *et al.*, 2010).

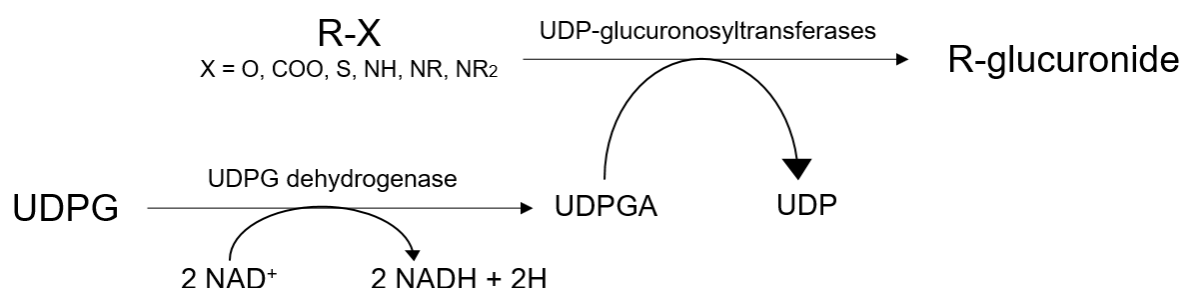


Figure 2.5 Scheme of a typical glucuronidation reactions

Synthesis of UDPGA from UDPG is mediated by UDPG dehydrogenase and NAD^+ . UDPGA is then used as a cofactor in glucuronidation reactions that are mediated by UDP-glucuronosyltransferase and a glucuronide is formed from the substrate. *Abbreviations: oxidised nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), substrate that undergoes biotransformation (R), uridine diphosphate (UDP), uridine-5'-diphosphate-d-glucose (UDPG), uridine-5'-diphospho- α -D-glucuronic acid (UDPGA),*

Glutathione S-conjugation reactions

Glutathione S-conjugation involves the conjugation of glutathione (GSH) with compounds containing electrophilic centres, by the action of a glutathione S-transferase (GST) enzyme (see Figure 2.6). GSTs are divided into two superfamilies: the soluble GSTs that are in the cytoplasm, nucleus, and mitochondria; and the membrane associated GSTs that are involved in arachidonic acid metabolism. Both superfamilies are distributed throughout the body and can be found in the liver, kidneys, heart, brain and several other tissues. In humans, GSTs are a family of proteins divided in five major classes namely, alpha, mu, pi, theta, and zeta (Jancova *et al.*, 2010; Van Bladeren, 2000). Glutathione conjugation is an important mechanism in the detoxification of drinking water as it is involved in the metabolism of halogenated compounds. Pesticides such as dichlorodiphenyltrichloroethane and organothiocyanates are also metabolites that undergo glutathione conjugation (Croom, 2012). Endogenous compounds that are metabolised via this pathway include prostaglandins and steroids (Van Bladeren, 2000). Extracts from cruciferous vegetables and grapefruit have been shown to induce human GSTs in hepG2 cell lines, while

inhibitors include glutathione derivatives or substrate analogues (Jancova *et al.*, 2010; Williamson *et al.*, 1997).

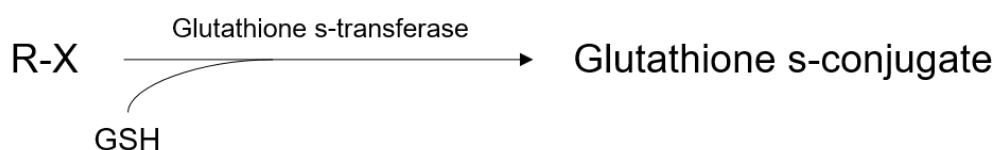


Figure 2.6 Scheme of the glutathione s-conjugation.

Glutathione s-transferase mediates the conjugation of GSH to an endogenous or xenobiotic electrophilic compound to form a glutathione S-conjugate. Abbreviations: glutathione (GSH), substrate that undergoes biotransformation (R).

Methylation reactions

Methylation reactions are mediated by three groups of enzymes namely, O-, S-, and N-methyltransferases. It requires S-adenosyl-L-methionine (SAM) to provide the methyl group (see Figure 2.7). One type of **O-methyltransferase** is catechol o-methyltransferase (COMT). COMTs play an important role in catechol-dependent functions including cognition, cardiovascular function and pain processing (Jancova *et al.*, 2010). COMTs are inhibited by several substrates. These include neurotransmitters, norepinephrine, epinephrine, dopamine as well as catechol oestrogens, catecholamine and drugs with catechol structures (Jancova *et al.*, 2010). In humans, two isoforms of COMT exist. The soluble S-COMT is expressed in the liver, blood and kidneys, while the membrane-bound MB-COMT is mostly found in the brain where it regulates extracellular dopamine levels in the prefrontal cortex (Qayyum *et al.*, 2015). The second group of methyltransferases is **S-methyltransferases (SMTs)**, a major enzyme being the cytosolic enzyme thiopurine S-methyltransferase (TPMT). TPMT catalyse S-methylation of aromatic and heterocyclic sulfhydryl compounds (Jancova *et al.*, 2010; Lennard, 2014). Substrates include thiopurine drugs like mercaptopurine and thioguanine. Currently, there are not any endogenous compounds known to be catalysed by TPMT (Lennard, 2014). High levels of TPMT are found in the liver, kidneys, and intestines, while decreased levels are found in the lungs and brain. Several benzoic acid derivatives can inhibit TPMT, including salicylic acid. Inhibition of TPMT results in elevated thioguanine nucleotide, which in turn can cause life-threatening bone marrow toxicity or myelosuppression (Lennard, 2014; Weinshilboum *et al.*, 1999). The third group of methyltransferases, **N-methyltransferases (NMTs)**, are responsible for the metabolism of endogenous neurotransmitters and hormones (Weinshilboum *et al.*, 1999). Phenylethanolamine N-methyltransferase (PNMT) for example, mediate the formation of epinephrine from norepinephrine. Other examples include histamine N-methyltransferase (HNMT) and nicotinamide N-methyltransferases (NNMT) that are highly expressed in the liver and gut. As its

name implies, HNMT catalyses the methylation of histamine. It can also catalyse methylation of other heterocyclic compounds structurally similar to histamine (Weinshilboum *et al.*, 1999). The cytosolic enzyme NNMT catalyses methylation of nicotinamide to form 1-methyl-nicotinamide (Kannt *et al.*, 2018). NNMT is expressed in several tissues, especially the liver and adipose tissue. Methyltransferases are also responsible for methylation of proteins, RNA and DNA, which will not be discussed as it goes beyond the scope of this study.

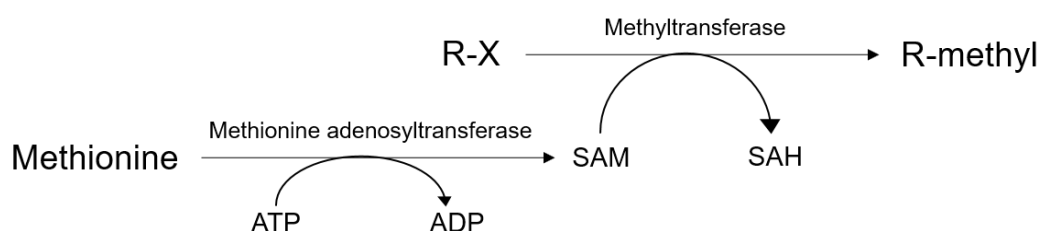


Figure 2.7 Typical methylation reactions

Methyl groups are added to substrates through mediation of methyltransferases and SAM. Synthesis of SAM from methionine is catalysed by methionine adenosyltransferase and requires ATP. *Abbreviations: adenosine triphosphate (ATP), substrate that undergoes biotransformation (R), S-adenosyl homocysteine (SAH), S-adenosyl-L-methionine (SAM).*

Sulfoconjugation reactions

Sulfoconjugation is a major route for biotransformation reactions and is catalysed by a group of enzymes known as sulfotransferases (SULTs) (Lafaye *et al.*, 2004). These enzymes typically mediate the transfer of sulfonate (SO_3^-) from the cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the hydroxyl or amino group of a wide variety of acceptor molecules (see Figure 2.8). PAPS is the universal donor of the sulfonate moieties in sulfoconjugation reactions and its depletion can lead to reduced sulfoconjugation capacity (Jančová & Šiller, 2012). Sulfotransferases are broadly divided into the cytosolic and membrane-bound classes. Cytosolic SULTs mediate sulfoconjugation of steroids, bile acids, and neurotransmitters, as well as xenobiotics. Generally, sulfoconjugation of neurotransmitters will result in loss of functional activity. Membrane-bound SULTs are located in the Golgi apparatus and mediate sulfoconjugation of peptides, proteins, lipids, and glycosaminoglycans. SULTs are also responsible for bioactivation of several drugs and xenobiotics. Sulfoconjugation of several endogenous peptides, including gastrin, fibrinogen, insulin and proenkephalin promote their activity (Dudas *et al.*, 2021; Kurogi *et al.*, 2021; Roth, 1986; Wang *et al.*, 2021). Four human SULT families are currently known and include SULT1, SULT2, SULT4 and SULT6 (Jancova *et al.*, 2010). SULT1A1, SULT1E1, and SULT2A1 are highly expressed in the liver, in contrast to SULT1A3 that is expressed in a wide variety of tissues other than the liver. SULT4A1 is found in the human brain, while SULT6B1 is expressed in the kidneys (Jancova *et al.*, 2010). There are

various differences in the SULTs of different species. SULT5A1 is found in mice and SULT3 in rabbits, while SULT1A2 has only been found in humans (Jancova *et al.*, 2010).

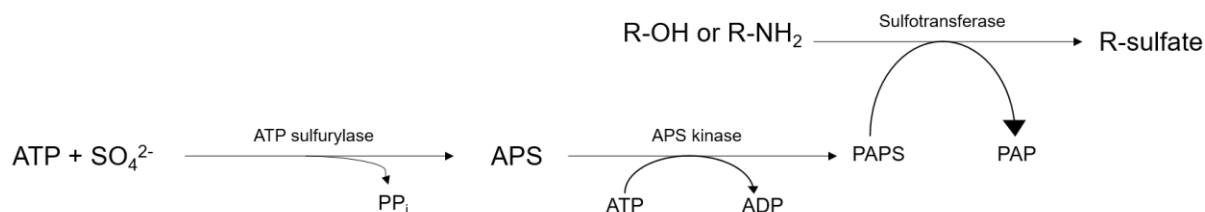


Figure 2.8 Scheme showing typical sulfoconjugation reactions

Sulfoconjugation reactions are catalysed by sulfotransferases and require the cofactor PAPS. Synthesis of PAPS is a two-step reaction that requires ATP and is mediated by ATP sulphurylase and APS kinase. Abbreviations: adenosine diphosphate (ADP), adenosine 5'-phosphosulfate (APS), adenosine triphosphate (ATP), 3'-phosphoadenosine-5'-phosphate (PAP), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), inorganic pyrophosphate (PP_i), substrate that undergoes biotransformation (R).

Amino acid conjugation reactions

Amino acid conjugation is the conjugation of an amino acid with the carboxylic group of a xenobiotic (Ionescu & Caira, 2006). One of the focus areas of this study is amino acid conjugation and will, therefore, be discussed in further detail.

2.3.3 Amino acid conjugation

Amino acid conjugation is one of several biotransformation systems that convert endogenous and xenobiotic metabolites to more hydrophilic conjugates that can be excreted in urine (Badenhorst *et al.*, 2013). Various types of aromatic acids can undergo amino acid conjugation. Contrariwise to other conjugation reactions, amino acid conjugation reactions involve a two-enzyme system. The enzymes that are involved in amino acid conjugation reside in mitochondria and conjugation consists of the following three reactions, (see Figure 2.9): 1) Activation of the carboxylic acid of the parent compound with ATP to form an acyl adenylate and pyrophosphate; 2) Formation of a high-energy CoA thioester intermediate from the bound acyl adenylate and CoA - this reaction is catalysed by an **ACSM**; and 3) Linkage of the activated acyl group to the acceptor amino acid resulting in regeneration of CoA through an acyl-CoA:amino acid N-acyltransferase (Knights & Miners, 2011; Knights *et al.*, 2007).

ACSM is one subfamily of the acyl-CoA synthetase (ACS) enzymes. The other subfamilies are **ACSS** and **ACSL**. ACSMs are the most important enzymes with regards to amino acid conjugation. They are located in the mitochondrial matrix and are involved in the activation of fatty acids and xenobiotics, suggesting that the formation of xenobiotic-CoAs have the potential to disturb mitochondrial function. In humans, several forms of ASCM enzymes have been identified (ACSM1, ACSM2A, ACSM2B, ACSM3, ACSM4, ACSM5 and ACSM6) (Knights & Miners, 2011;

Van der Sluis & Erasmus, 2016). ACSM1 is expressed in the mitochondrial matrix and can act on C4-C11 acids and corresponding 3-hydroxy- and 2,3- or 3,4-unsaturated acids. ACSM1 have been linked to schizophrenia and major depressive disorders (Van der Sluis & Erasmus, 2016). Two different loci of ACSM2 exist, namely ACSM2A and ACSM2B. ACSM2B primarily activates xenobiotics, and is expressed in the human liver. ACSM3 is expressed in human kidneys and liver and polymorphisms of its gene are associated with obesity, hypertension, and hypertriglyceridemia. In humans, ACSM4 is primarily expressed in the testis with polymorphisms associated with rapid AIDS progression in HIV-infected patients. Like ACSM3, ACSM5 is primarily expressed in the kidneys and liver. Little information is available on ACSM6 (Van der Sluis & Erasmus, 2016).

The second step of amino acid conjugation requires transfer of the acyl group to the amino group of glycine, with the action of GLYAT. GLYAT is located in the mitochondrial matrix, especially in the liver and kidneys. Substrates for GLYAT include astemizole, brompheniramine, permethrin, triflusal and m-xylene (Knights & Miners, 2011).

A limited number of substrates are conjugated with amino acids, including bile acids, herbicides, benzoates, salicylic acid and branched chain fatty acids (Knights & Miners, 2011). Several amino acids can participate in amino acid conjugation including, glycine, taurine, and glutamine, and would depend on the chemical class of the aromatic acid (Beyoğlu *et al.*, 2012). Glycine is the principle amino acid involved in human amino acid conjugation (Knights & Miners, 2011).

2.3.4 Glycine conjugation

Glycine is the simplest amino acid with only a hydrogen atom as its side chain. It functions as a neurotransmitter, a one-carbon donor in the folate one-carbon metabolism, a regulator of gene expression, and a precursor of some molecules (Alves *et al.*, 2019; Leung *et al.*, 2020). Dietary intake of glycine varies from ~1-3 g/day in adult males, depending on their dietary protein sources (Alves *et al.*, 2019; Meléndez-Hevia *et al.*, 2009). Glycine is mainly synthesised from serine through the serine hydroxymethyltransferase pathway, but can also be synthesised through the

UNDERSTANDING ENZYME NOMECLATURE

Families of acyl-CoA synthetases (ACSSs) are named according to the length of the fatty acids that they activate (Knights & Miners, 2011).

ACSS – short chain (C2-C4) fatty acids that activate acetate, propionate or butyrate (Watkins *et al.*, 2007)

ACSM – medium chain (C4-C12) fatty acids

ACSL – long chain (C12-C20) fatty acids which activate palmitate and oleate (Watkins *et al.*, 2007)

threonine dehydrogenase pathway and through the formation of sarcosine (Razak *et al.*, 2017). Although it can be synthesised in the body, several studies show that it might not be sufficient for metabolic demand and growth in humans and other mammals including rodents, and have therefore been considered as a conditionally essential amino acid (Jackson *et al.*, 1987; Li *et al.*, 2001; Persaud *et al.*, 1996; Wang *et al.*, 2013). Availability of glycine depends on its use in the glycine cleavage system, glycine conjugation pathway, and biosynthetic reactions (Leung *et al.*, 2020). The glycine cleavage system is localised in the mitochondrial matrix and is the major catabolism pathway of glycine (see Figure 2.9). It catalyses a reaction between glycine, tetrahydrofolate and NAD^+ to form 5,10-methylene-tetrahydrofolate, ammonia, carbon dioxide and NADH. This reaction is reversible (Kikuchi & Hiraga, 1982; Leung *et al.*, 2020).

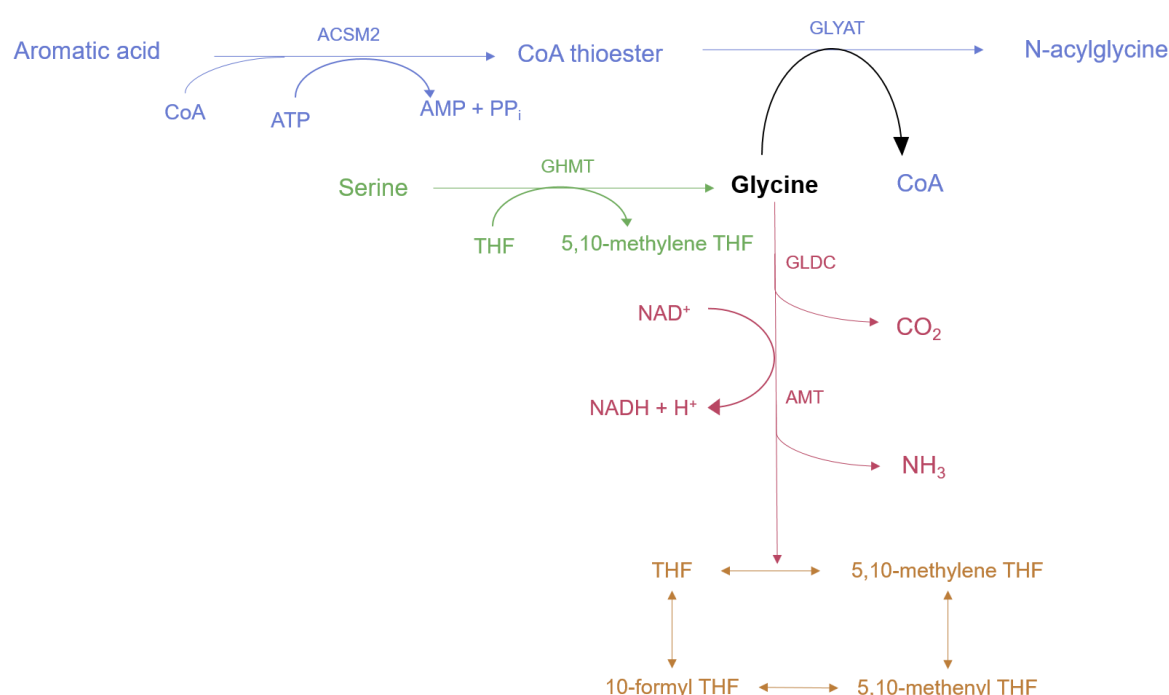


Figure 2.9 Overall metabolism of glycine

This figure shows the synthesis of glycine (green), glycine conjugation (blue), glycine cleavage system (pink), and one carbon metabolism (orange). Abbreviations: acyl-CoA synthetase medium chain family member 2 (ACSM2), adenosine diphosphate (ADP), adenosine monophosphate (AMP), amino methyltransferase (AMT), adenosine triphosphate (ATP), coenzyme A (CoA), glycine hydroxymethyltransferase (GHMT), glycine decarboxylase (GLDC), glycine N-acyltransferase (GLYAT), reduced nicotinamide adenine dinucleotide (NADH), oxidised nicotinamide adenine dinucleotide (NAD^+), ammonia (NH_3), inorganic phosphate (PP_i), tetrahydrofolate (THF).

For several years after its initial discovery there was a lack of interest in glycine conjugation, presumably because only a few drugs are conjugated to glycine (Knights *et al.*, 2007). Recently however, interest was sparked again as new theories on its main role was proposed. Beyoğlu *et al.* (2012) recently proposed the glycine deportation system. They argued that rather than a detoxification pathway, the primary purpose of amino acid conjugation is a homeostatic neuroregulatory mechanism for the amino acids that are neurotransmitters of the central nervous

system. They provide several arguments for the glycine deportation system, one main argument being that amino acid conjugation does not leave aromatic acids much more water soluble, especially in comparison to glucuronic acid conjugation. For example, benzoic acid's water solubility dramatically increases from 3.4 g/l to 263 g/l once conjugated with glucuronic acid. But, when conjugated to glycine, its solubility increased only to 3.8 g/l (Beyoğlu *et al.*, 2012). The glycine deportation system has been counter-argued by Badenhorst *et al.* (2014), as they suggested that the primary purpose of this phenomenon was rather to enable animals to limit their consumption of foods rich in aromatic compounds. They also argued that glycine conjugation is part of the phenylpropionate catabolism pathway.

Drugs such as salicylic acid and astemizole, insecticides like permethrin, and preservatives like benzoic acid are examples of xenobiotic compounds that undergo biotransformation through glycine conjugation (Knights & Miners, 2011; Knights *et al.*, 2007). Glycine conjugation is influenced by a number of factors including the availability of CoA, glycine and ATP, variations in the two enzyme-coding genes, as well as the differential expression levels of *ACSM2B* and *GLYAT* (Badenhorst *et al.*, 2014; Rohwer *et al.*, 2021). The rate of glycine conjugation can influence the toxicity of xenobiotics by influencing the clearance of xenobiotics, because this pathway can be saturated. The limiting step of glycine conjugation depends on the substrate and can either be the formation of the acyl-CoA intermediate or the transfer of the amino acid. Substrate dependence is illustrated by conjugation of benzoic acid with glycine having a 20 times higher rate than that of conjugation of salicylic acid with glycine (Knights & Miners, 2011; Van der Sluis & Erasmus, 2016). Furthermore, it seems that the capacity for glycine conjugation declines with advancing age (Knights & Miners, 2011).

The glycine conjugation pathway is sometimes exploited in the treatment of metabolic disorders of neonates and infants. Acetylated amino acids are commonly observed in urine of patients with inborn errors of metabolism, primarily as a mechanism for detoxification. Isovaleric acidemia for example, is characterised by the presence of isovaleryl-glycine in the urine. Isovaleryl-CoA is removed as nontoxic isovaleryl-glycine and isovalerylcarnitine when glycine and L-carnitine supplementation is given to these patients (Loots *et al.*, 2007). Nonketotic hyperglycinaemia (NKH) is a congenital enzymopathy where the glycine cleavage system has a reduced ability to catabolize glycine to CO₂ and NH₄⁺. Patients with NKH present with elevated plasma and cerebrospinal fluid glycine. Therapy of NKH involves administration of sodium benzoate to remove excess glycine (Beyoğlu & Idle, 2012).

Although glycine conjugation can detoxify a variety of compounds, dietary benzoates are seemingly its primary substrate (Badenhorst *et al.*, 2013; Mawal & Qureshi, 1994). Benzoic acid is an aromatic acid that comprises a benzene ring and a carboxylic acid (see Figure 2.10). It is

naturally found in apricots, snap beans, mushrooms, and honey (Nair, 2001). The salts of benzoic acid, sodium benzoate and potassium benzoate, are commonly used as preservatives in food, such as jam, soft drinks and beer. Sodium benzoate is rapidly excreted in the urine through glomerular filtration and tubular secretion (Praphanproj *et al.*, 2000). Preservatives are added to food products in order to prolong the shelf life and to delay nutritional losses as a result of microbial, enzymatic or chemical alterations. Sodium benzoate and potassium benzoate inhibit the growth of mould, yeast, and certain bacteria (Zengin *et al.*, 2011). Concerns have been raised about the use of benzoates as preservatives for their link to childhood hyperactivity, asthma, angio-oedema and urticaria (Egger *et al.*, 1985; Freedman, 1977; McCann *et al.*, 2007; Michaelsson & Juhlin, 1973). More recently, consumption of beverages rich in sodium benzoate was associated with attention deficit hyperactivity disorder-related symptoms in college students (Beezhold *et al.*, 2014). Despite these concerns, regulatory bodies still regard it safe to use in food products. Sodium benzoate is listed by the Food and Drug Administration (FDA) as a substance that is “Generally Regarded As Safe” when used as a preservative in the ranges of 0.15-9%, depending on the food product (FDA, 2020). The Joint Food and Agricultural Organization of the United Nations and World Health Organization Expert Committee on Food Additives (JECFA) established an acceptable daily intake of benzoic acid and its salts of 0 to 5 mg/kg body weight. They also have no safety concerns at current levels of intake when used as a flavouring agent (Nair, 2001). Benzoic acid and sodium benzoate are also used in cosmetic formulations and in the treatment of acute hyperammonaemia in patients with urea cycle disorders (Brusilow *et al.*, 1984; Nair, 2001; Praphanproj *et al.*, 2000).

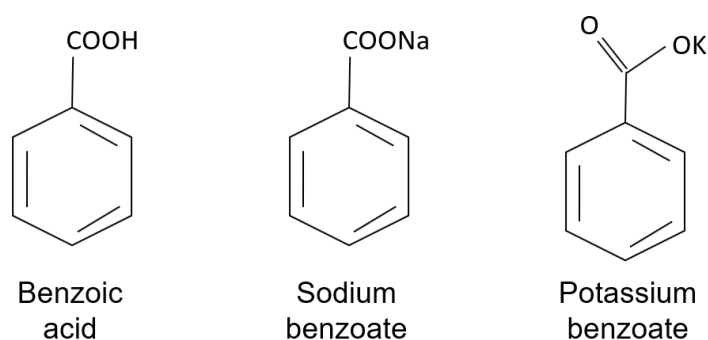


Figure 2.10 Structure of benzoic acid, sodium benzoate and potassium benzoate

This figure shows the structure of benzoic acid and two of its salt, sodium benzoate and potassium benzoate that are commonly used as preservatives.

As with other amino acid conjugations, glycine conjugation of benzoic acid is also a two-step reaction (see Figure 2.9). First, ACSM2B catalyses the reaction of benzoate and CoA to form benzoyl-CoA in the liver. This first step is ATP dependent and will release AMP and PP_i. Next, GLYAT catalyses the reaction between benzoyl-CoA and glycine to form hippurate and release

CoA (Badenhorst *et al.*, 2014; Knights, 1998). Currently, no metabolic defect in the glycine conjugation pathway has been described, indicating that it is an essential pathway (Van der Sluis *et al.*, 2015). GLYAT is a key enzyme in glycine conjugation of acyl-CoA esters and is central to maintaining CoA homeostasis in the liver (Badenhorst *et al.*, 2013). By preventing the depletion of CoA, the glycine conjugation pathway plays a vital role in homeostatic energy balance within the mitochondrion. As already mentioned, the availability of glycine can influence glycine conjugation. Low levels of glycine can result from the need to detoxify high levels of benzoate

Transcription

The process of synthesising RNA from a DNA template with DNA-dependent RNA polymerases (Garrett & Grisham, 2013).

Translation

Translation is the synthesis of amino acid sequences from mRNA (Garrett & Grisham, 2013).

Gene regulation

The biological processes that promote the tight control of expression of genes to define cellular function and identity in living organisms (Tai *et al.*, 2013).

Hormone response elements

Specific DNA sequences in target genes to which hormone receptor complexes bind. This binding can activate or repress transcription. Hormone response elements are found relatively close to the core promotor or in enhancer regions (Mason *et al.*, 2020).

which is present in natural sources like milk and berries, dietary polyphenols, and xenobiotics including pharmaceuticals. Reduced glycine levels can increase acyl-CoA intermediates like xenobiotic-CoA esters which in turn cause depletion of CoA and inhibition of ACSM2B (Rohwer *et al.*, 2021).

2.4 Regulation of metabolic pathways

Three main systems are responsible for regulation of metabolic pathways: 1) Regulation of gene expression that provides for longer term effects; 2) Posttranslational modifications that can affect protein stability or affect the balance between active and inactive enzymes relatively quickly; and 3) Allosteric regulation of enzymes to control the activity of an enzyme (Desvergne *et al.*, 2006).

2.4.1 Regulation of gene expression

The concept of the central dogma of molecular biology holds that information flows from DNA to RNA through **transcription**, then from RNA to protein through translation to ultimately determine the cellular phenotype (Shapiro, 2009). **Gene regulation** can depend on tissue type and environmental conditions (Clark & Pazdernik, 2013). Multiple transcription factors are required for activation of transcription of genes. They are divided into two categories: general transcription factors that help RNA polymerase to bind to the promotor, and specific transcription factors (also called activators) that increase the level of transcription in certain cell types or in response to signals (Clark &

Pazdernik, 2013; Mason *et al.*, 2020). Activators are proteins that bind to DNA at sites called enhancers. Enhancers are binding sites for specific transcription factors and are able to act over a large distance by DNA bending and forming a loop to position the enhancer closer to the promotor. The activator proteins can now interact with the complex to increase the transcription rate (Mason *et al.*, 2020).

One type of specific transcription factors are nuclear receptors (NRs). They are a superfamily of receptors for hormones, fatty acids, cholesterol derivatives, bile acids, benzoate derivatives, and prostaglandins that act as ligand-inducible transcription factors. They are implicated in development, detoxification and homeostasis and are divided into six classes. Selected receptors and ligands of classes I-III are shown in Table 2.4. NRs bind to **hormone response elements** to regulate transcription. NRs have the ability to repress gene expression through the action of negative hormone response elements that bind the receptors and mediate negative regulation by the ligand. Unbound receptors will increase transcription while binding of a ligand will reverse the stimulation. This is especially important for feedback mechanisms, for example with thyroid hormones (Aranda & Pascual, 2001). NRs are roughly divided into endocrine NRs and orphan NRs. A subset of orphan NRs are involved in regulation of gene transcription of several phase one and two drug metabolizing enzymes and transporters in the liver. They are known as xenobiotic receptors and include pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR) (Mackowiak & Wang, 2016).

PXR is a nuclear receptor that is mainly involved in control of expression of genes of biotransformation enzymes and drug transporters. Moreover, PXR plays a key role in endobiotic metabolism as it regulates expression of genes that are involved in glucose, lipid, and bile acid metabolism. Furthermore, the PXR pathway plays a role in regulation of energy metabolism (Pavek, 2016). Expression of PXR is mostly limited to liver, intestines and kidneys, but can be expressed in other tissues. Location is further dependent on the organism. Mouse PXR is located in the cytosol of liver cells, while human PXR is in the nucleus (Pavek, 2016). Examples of regulation by PXR in biotransformation include: Activation of PXR by rifampicin resulted in repression of *SULT1E1* by displacing HNF4 α bound to the PXR-responsive enhancer of *SULT1E1* (Kodama *et al.*, 2011). Upregulation of two CAR target genes, *Ugt1a1* and *Gsta1*, were observed in *Pxr*-null mice, indicating that ligand-free PXR acts as a CAR suppressor (Saini *et al.*, 2005).

Table 2.4 Selected nuclear hormone receptors and their ligands

Class	Receptor	Ligand⁴
Class I	Thyroid hormone receptor (TR)	Thyroid hormone (T ₃)
	Retinoic acid receptor (RAR)	Retinoic acid
	Vitamin D receptor (VDR)	1-25(OH) ₂ vitamin D ₃
	Peroxisome proliferator activated receptor (PPAR)	Benzotriene B ₄ , eicosanoids, polyunsaturated fatty acids
	Pregnane X receptor (PXR)	Pregnanes, C21 steroids, glucocorticoids, anti-glucocorticoids
	Constitutive androstane receptor (CAR)	Androstanes; 1,4-bis[2-(3,5-dichlororpyridyloxy)]benzene, dehydroepiandrosterone, dehydroepiandrosterone sulphate
	Liver X receptor (LXR)	Oxysterols
Class II	Farnesoid X receptor (FXR)	Bile acids
	Hepatocyte nuclear factor 4 (HNF-4)	Fatty acyl-CoA thioesters
Class III	Retinoid X receptor (RXR)	9-cis-retinoic acid
	Glucocorticoid receptor (GR)	Glucocorticoids
	Androgen receptor (AR)	Androgens
	Progesterone receptor (PR)	Progestins
	Estrogen receptor (ER)	Estradiol

This table shows the classes and ligands of selected nuclear hormone receptors.

CAR has a broad specificity for endogenous and exogenous ligands. It regulates expression of phase one and two enzymes and transporters of endogenous and exogenous molecules. Specifically, it can regulate CYPs, SULTs, UGTs, and GSTs. Through UGTs and membrane transporters it regulates bilirubin breakdown and haem reabsorption. Through UGTs and SULTs, it maintains hormone homeostasis (Chai *et al.*, 2016).

AhRs are inter alia, activated by xenobiotic polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons as well as the endogenous compound - tryptophan photoderivative 6-formylindolo[3,2-b]carbazole. AhRs are mostly involved in the regulation of metabolism of toxic

⁴ This table was compiled from (Aranda & Pascual, 2001; Kanda *et al.*, 2019)

compounds, immune responses and tumour promotion. When activated, AhRs increase expression of *CYP* and *UGT1A* genes (Mackowiak & Wang, 2016).

Nuclear factor (erythroid-derived 2)-like 2 (NRF-2) belongs to the basic leucine zipper nuclear transcription factor family. It is expressed in the liver, intestines, kidneys, muscles and lungs. It is thought to play a pivotal role in defence mechanisms against xenobiotic related toxicity. Several phase two enzymes are regulated by NRF-2, including GSTs, SULTs, and UGTs. In contrast to the nuclear receptors discussed above, NRF-2 regulated transcription is not activated by ligand-receptor binding. By binding to NRF-2, Kelch-like ECH-associated protein 1 (KEAP1) retains NRF-2 in the cytoplasm. In conditions of oxidative stress, NRF-2 is released from Keap1 and translocate to the nucleus to activate transcription of its target genes (Shen & Kong, 2009).

The mechanism of gene expression regulation of acyl-CoA synthetase transferase enzyme is not well understood (Badenhorst *et al.*, 2014). Regulation of human *GLYAT* is believed to occur at transcriptional level (Matsuo *et al.*, 2012). Investigation of mRNA expression in male Sprague-Dawley rats that underwent dietary restriction showed upregulation of *Glyat* and other phase two enzymes, possibly through the NRF-2 pathway (Wen *et al.*, 2013).

2.4.2 Allosteric regulation

The rate or activities of pathways are not only regulated by the expression of the genes involved, but also by the activities of the enzymes. Genetic variations of genes can alter the kinetic properties of enzymes (Van der Sluis *et al.*, 2013). Human GLYATL2 for example, a paralogue of human GLYAT, is regulated by reversible acetylation on Lys¹⁹. Substitution of lysine at position 19 with glutamine or arginine resulted in significantly decreased enzyme activity, showing the importance of lysine acetylation in regulation of the activity in this enzyme (Waluk *et al.*, 2012). Allosteric regulation is a mechanism used by proteins to regulate activity and adaptability during signal transduction, catalysis, and gene regulation (Verkhivker *et al.*, 2020). Allosteric regulation requires a regulatory trigger, for example the binding of a small molecule to a protein. These triggers can increase or decrease the activity of the protein, showing that regulation of gene expression is not the final factor that determines the activity of a pathway (Laskowski *et al.*, 2009). Numerous regulation steps occur after transcription. Any of these regulations could affect the activity of the metabolic pathway, which is why it is important to validate gene expression data at protein concentration, enzyme activity, and/or metabolite level.

2.5 The use of animal disease models

Studies investigating the effect of mitochondrial dysfunction on biotransformation in mitochondrial disease patients are scarce or lacking. Such studies would ideally need to be done in liver

samples, but biopsies are very invasive, necessitating the use of disease animal models as a first line of investigation. A well phenotyped mitochondrial disease model is the *Ndufs4* knockout mouse model, which presents with CI deficiency and a Leigh syndrome like phenotype.

2.5.1 NDUFS4

NDUFS4 is an 18-kDa subunit of CI that is located in the iron-sulphur protein fraction (Budde *et al.*, 2000; Scacco *et al.*, 2003). The NDUFS4 subunit appears to incorporate relatively late during CI assembly and is believed to affect the assembly, stability and activity of CI (Breuer *et al.*, 2013; Valsecchi *et al.*, 2010). Defects in NDUFS4 results in Leigh syndrome or a **Leigh-like** phenotype. The *NDUFS4* gene is suggested to be a mutational hotspot as it is the site of several of the mutations that results in CI deficiency (Budde *et al.*, 2003; Petruzzella & Papa, 2002). Absence of NDUFS4 results in a partially assembled sub-complex of 830 kDa and ultimately a partially assembled CI, where the NADH dehydrogenase electron influx part of CI is lost. Although CI is not fully assembled, some activity of CI still remain and activity can be tissue specific (Calvaruso *et al.*, 2012).

Leigh-like syndrome

Leigh-like syndrome is similar to Leigh syndrome, but also includes non-neurological abnormalities (Finsterer, 2008).

2.5.2 Whole body NDUFS4 knockout mouse model

As mentioned, NDUFS4 plays a role in the assembly or maintenance of integrity of CI (Distelmaier *et al.*, 2009; Kruse *et al.*, 2008; Scacco *et al.*, 2003). To study NDUFS4 mutations, Kruse *et al.* (2008) developed a whole body knockout mouse model, in which exon 2 of the *Ndufs4* gene is deleted, causing a frameshift that prevents synthesis of a mature NDUFS4 protein. Exon 2 codes for the first 17 amino acids of the mature NDUFS4 protein and the last part of a mitochondrial targeting sequence. Heterozygous (*Ndufs4*^{+/-}, HET) mice with a mixed 129/Sv x C57BL/6 genetic background were interbred to create *Ndufs4*^{+/-}, wild-type (*Ndufs4*^{+/+}, WT), and knockout (*Ndufs4*^{-/-}, KO) mice, and are born close to the expected Mendelian ratio of HET:WT:KO – 1.9:1.4:1.0. HET mice are indistinguishable from WT mice, while KO mice present with encephalomyopathy, hair-loss, retarded growth, blindness, cataracts, loss of motor skills and ataxia, which is similar to human CI deficiency (Kruse *et al.*, 2008). Furthermore, KO mice die by postnatal day 55 (P55) and are infertile – which is why HET mice are used for breeding (JAX, 2021). Interestingly, Kruse *et al.* (2008) found that even with deletion of *Ndufs4*, an intact version of CI can still form and suggested that this might be due to compensation by other proteins. This model is now widely used to investigate Leigh syndrome and mitochondrial complex I deficiency (Kayser *et al.*, 2016; Papa *et al.*, 2001; Terburgh *et al.*, 2021; Valsecchi *et al.*, 2013).

2.6 Problem statement

From the literature discussed above the following is clear: mitochondrial complex I deficiency is one of the most common groups of inborn errors of metabolism that results in heterogeneous diseases like Leigh Syndrome which are difficult to treat. These patients have severely impaired energy production resulting in numerous downstream effects (Rahman & Rahman, 2018; Smeitink *et al.*, 2001). Although mitochondrial disorders have been extensively studied, there is still a lot of gaps in the literature, for example, its effect on biotransformation. Biotransformation is an essential process used for the removal of endogenous and exogenous compounds before they accumulate to toxic levels. It is a complex system that is divided into phase one and phase two reactions. Several conjugation reactions with multiple enzymes are involved in phase two reactions (Katzung *et al.*, 2018; Sevier *et al.*, 2012). Many of these reactions are dependent on cofactors whose synthesis require other cofactors like ATP and NAD⁺. The latter are known to be decreased in patients with mitochondrial disease and could therefore possibly impair biotransformation in a mitochondrial disease model. In non-diseased individuals, there is already a lot of inter-individual variation with regards to biotransformation due to genetic, environmental, and disease factors (Ionescu & Caira, 2006). Metabolism can further be influenced by regulation of gene expression, allosteric regulation, and the availability of cofactors (Badenhorst *et al.*, 2013; Desvergne *et al.*, 2006). The limited ATP levels of individuals with mitochondrial disorders could affect the activity and capacity of the biotransformation pathway by dysregulating any of those factors. To initiate an investigation on the effect of mitochondrial disorders on biotransformation, a primary pathway like glycine conjugation can be investigated. The whole body *Ndufs4* knockout mouse model is a great model to study mitochondrial disorders like Leigh Syndrome. It is commonly used for this purpose and has been well characterised (Kruse *et al.*, 2008).

2.7 Aim, research questions and objectives

The aim of this study is to investigate differential expression of genes involved in phase two biotransformation in an *Ndufs4* knockout mouse model. The following questions were set as guidelines to fulfil this aim:

1. Is glycine conjugation differentially expressed in KO mice compared to WT mice, as an initial investigation of a primary phase two biotransformation pathway?
2. Are changes in glycine conjugation expression, if any, also reflected in the remaining phase two biotransformation enzymes genes?

To address these research questions, the following objectives were identified:

1. Characterise and select *Ndufs4* knockout and wild-type mice for samples collection.
2. Investigate the differential gene expression of glycine conjugation in KO compared to WT mouse liver samples using a targeted transcriptomics⁵ approach.
3. Validate the differential gene expression of glycine conjugation on protein level.
4. Identify the remaining enzymes and corresponding genes involved in phase two biotransformation from literature.
5. Revisit the transcriptomics dataset to investigate the differential expression of genes involved in phase two biotransformation, as identified in Objective 4.

The schematic illustration below maps the strategy followed during this study:

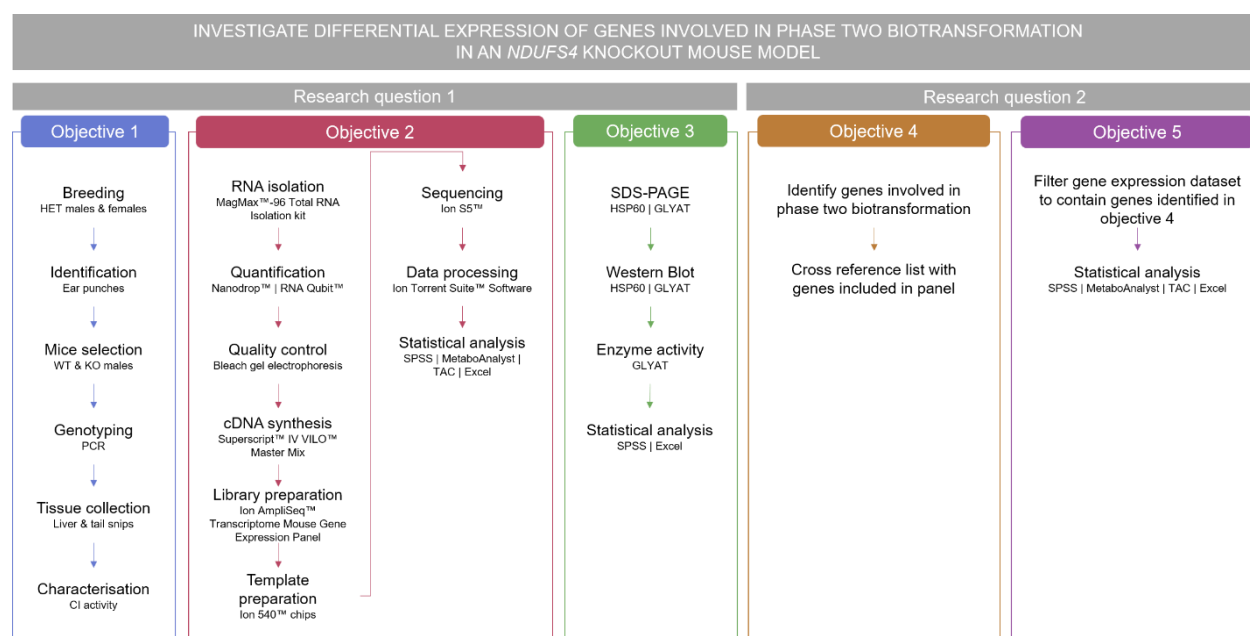


Figure 2.11 Schematic representation of the experimental strategy followed for this study

The aim of this study is to investigate differential expression of genes involved in phase two biotransformation in an *Ndufs4* knockout mouse model. Characterisation and selection of mice samples entails breeding, identification, genotyping and determination of CI activity. Investigation of differential gene expression entails RNA isolation, quantification, and quality control, as well as several steps for transcriptomics. Validation of differential gene expression will entail determination of protein expression through SDS-PAGE and western blots, as well as determination of enzyme activity. Genes involved in phase two biotransformation will be determined from literature and filtered to genes included in the transcriptome panel. The genes identified in objective 4 will be used to determine differential expression of remaining phase two biotransformation genes. *Abbreviations: complex I (CI), glycine-N-acyltransferase (GLYAT), heat shock protein 60 (HSP60), heterozygous (HET), knockout (KO), polymerase chain reaction (PCR), sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), Applied Biosystems™ Transcriptome Analysis Console (TAC), wild-type (WT).*

⁵ When using Ion Torrent™ technology, targeted transcriptomics refers to the use of an Ion AmpliSeq™ panel that aims to target all mRNA. When using this approach, mRNA data on ~24 000 genes are produced.

CHAPTER 3 MATERIALS AND METHODS

3.1 Introduction

The objective of Chapter 3 is to describe the methods that were employed during this study, and to provide details of the materials used. The chapter is divided into six sections. First, the care, breeding and identification of the mice are discussed. Secondly, aspects relating to genotyping, characterisation and mice selection are dealt with. Thirdly, methods used for isolation of ribonucleic acid (RNA) and subsequent quality evaluations are explained. Fourthly, details about the principles and methods employed for transcriptomics are discussed. Fifthly, methods used to validate the transcriptomic data are described. Lastly, the chapter ends with a discussion of the statistical analyses used.

3.2 Animal care, breeding, and identification

This project has been approved by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) with ethics approval number: NWU-00507-20-A5.

Ndufs4 heterozygous (HET, *Ndufs4*^{+/-}) mice⁶ were acquired from The Jackson Laboratory⁷ and used to breed wild-type (WT, *Ndufs4*^{+/+}) and knockout (KO, *Ndufs4*^{-/-}) mice as *Ndufs4*^{-/-} are infertile and usually die before reaching sexual maturity (JAX, 2021; Kruse *et al.*, 2008). The original whole genome SNP genotyping and detailed breeding strategy deployed for these mice upon arrival at this institution, have been described previously (Mereis, 2018).

The animals were bred and housed under controlled conditions at the specific pathogen-free unit of the Vivarium of the Preclinical Drug Development Platform (PCDDP, SAVC reg. no. FR15/13458) of the NWU. Animals were housed in individually ventilated cages under constant temperature (22 ± 1°C), humidity (55 ± 10%) and light/dark cycles (12:12 hour). Mice were fed standard laboratory chow⁸ and had *ad libitum* access to water. Nutritional information of the laboratory chow is provided in Table 3.1.

⁶ Mouse strain: B6.129S4-Ndufs4tm1.1Rpa/J; Stock nr: 027058.

⁷ Bar Harbor, Maine, USA.

⁸ Rodent Breeder, Cat. #NWU/RB2005 (LabChef, Nutritionhub, Stellenbosch, SA).

Table 3.1 Nutritional information of Rodent Breeder laboratory chow

Declaration of ingredients		
Maize, wheat bran, soybean, soybean protein concentrate, fish meal, maize protein concentrate, molasses, sucrose, calcium carbonate, sodium chloride, calcium phosphate, approved acidulants, approved antioxidants, approved vitamins & minerals.		
Nutritional value		
	As sampled (g/kg)	Dry matter (g/kg)
Crude protein (min.)	200	240
Moisture (max.)	10	-
Crude oils and fats (min.)	50	53
Linoleic acid (min.)	12	14
Crude fibre (max.)	40	45
Crude ash (max.)	70	75
Ca:P ratio	1.1-2:1	1.1-2:1
Calcium (Ca) (min.)	12	14
Phosphorus (P) (min.)	7.5	8
Vitamin A (min.)	16 000 (IU ⁹ /kg)	16 000 (IU/kg)
Vitamin D (min.)	2 000 (IU/kg)	2 000 (IU/kg)
Vitamin E (min.)	100 (mg/kg)	100 (mg/kg)

This table shows the nutritional information of Rodent Breeder laboratory chow that was fed to the animals. It contains the declaration of ingredients as well as nutritional value in an “as sampled” and “dry matter” format expressed as g/kg values. *Abbreviations: minimum (min.), maximum (max.).*

Upon weaning, postnatal day 21-23 (P21-P23), mice were marked with ear punches at specific positions on one or both ears. The site of the hole represented a specific number, and the combination of holes represented a unique identification number, ranging from 1-99 (Figure 3.1). Records on the animal's unique litter numbers (1a -1z; 2a-2z, etc.) and date of birth were also kept. Tail snips were collected after identification of the mice and used to determine their genotype as part of the routine breeding and animal selection programme. Genotyping is fully described in Section 3.3.2.

⁹ IU = international units.

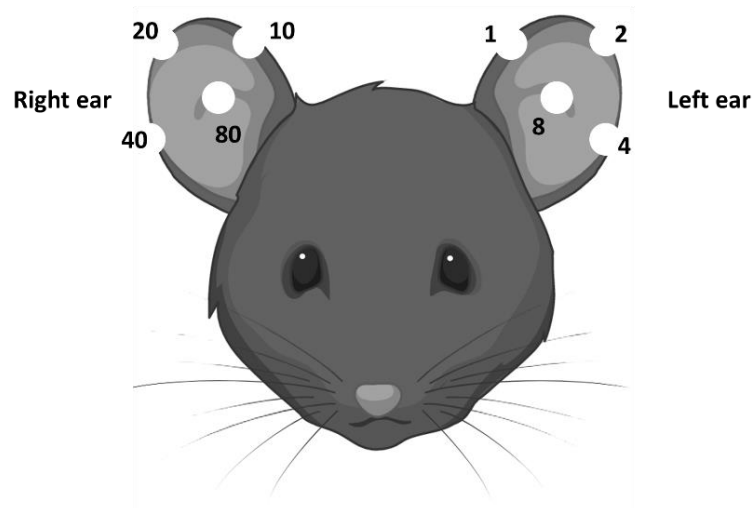


Figure 3.1 Depiction of mouse ear numbering system used to identify mice

Mice were identified by the position of the holes punched into their ears. The right ear represents numbers in tens (10-90) and the left ear numbers in ones (1-9). The sum of the values of the holes on both ears gives the identification number of the mouse. For example, mouse number 39 would have holes in position 20 and 10 in their right ear, as well as position 8 and 1 in their left ear.

3.3 Genotyping, characterisation, mice selection and tissue collection

3.3.1 Mice selection

To minimise sex-related variation during transcriptomics and protein analysis, only males were used in this study. The two transcriptomics groups consisted of eight WT and eight KO mice between the ages of 46-51 days. For enzyme analyses, females were included and analyses were performed on three WT and three KO mice that were 50 days old, an exception was one WT mouse that was 113 days old. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blots were performed on three WT and three KO male mice that were also used for transcriptomics. The genotype, age and sex of each mouse are shown in Table 3.2.

Table 3.2 Genotype and age of mice used for transcriptomics and enzymatic analyses

Animal ID	Genotype	Age (days)	Sex	Used for
1	WT	49	Male	Transcriptomics, SDS-PAGE, western blots
4	WT	49	Male	Transcriptomics
44	WT	49	Male	Transcriptomics
61	WT	49	Male	Transcriptomics
64	WT	50	Male	Transcriptomics
77	WT	46	Male	Transcriptomics, SDS-PAGE, western blots
87	WT	46	Male	Transcriptomics, SDS-PAGE,

Animal ID	Genotype	Age (days)	Sex	Used for
				western blots
88	WT	46	Male	Transcriptomics
45	KO	49	Male	Transcriptomics
31	KO	50	Male	Transcriptomics, SDS-PAGE, western blots
32	KO	51	Male	Transcriptomics, SDS-PAGE, western blots
36	KO	51	Male	Transcriptomics
40	KO	49	Male	Transcriptomics
62	KO	48	Male	Transcriptomics, SDS-PAGE, western blots
76	KO	47	Male	Transcriptomics
86	KO	47	Male	Transcriptomics
21	WT	50	Male	Enzyme activity
28	WT	50	Female	Enzyme activity
33	WT	50	Female	Enzyme activity
27	KO	50	Female	Enzyme activity
30	KO	50	Female	Enzyme activity
4.5	KO	113	Male	Enzyme activity

This table shows the ID, genotype, age and sex of each mouse used in this study. Eight WT and eight KO male mice that were between 46-51 days old, were used for transcriptomic analysis. SDS-PAGE were performed on three WT and three KO (male) mice that were 46-51 days old. Enzyme activity analyses were performed on three WT and three KO mice that were 50 or 113 days old. The sample group included both males and females. *Abbreviations: knockout (KO), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), wild-type (WT).*

3.3.2 Genotyping of mice

Principle

Tail snips were collected during identification of mice and tissue collection to be used for genotyping. The additional tail snips collected during tissue collection was to confirm that the correct mice were used. Mice could be genotyped by standard polymerase chain reaction (PCR) because the deletion of the second exon of the *Ndufs4* gene results in a size difference between the three genotypes of the amplicons spanning the *Ndufs4* gene. The primers that correspond to the introns flanking exon two, were first described by (Valsecchi *et al.*, 2012). During genotyping deoxyribonucleic acid (DNA) is first isolated from the tail snips before the purity and concentration is determined. Standard PCR is then used to amplify the DNA and the resultant products can be separated with agarose gel electrophoresis. The sizes of the DNA products are estimated by

separating a DNA ladder alongside the samples on the agarose gel. Genotypes are identified by the length of the fragments on the agarose gel. *Ndufs4*^{-/-} samples will have one band corresponding to 429 bp, *Ndufs4*^{+/+} samples will have one band corresponding to 1229 bp and the *Ndufs4*^{+/-} samples will have both bands.

Method

Genomic DNA (gDNA) was isolated from tail snips, using the Quick-DNA™ Miniprep Plus Kit¹⁰ and following its solid tissue protocol. A 200 µL master mix containing 95 µL nuclease free water, 95 µL solid tissue buffer and 1 mg/mL proteinase K for each sample was prepared. Of each tail snip, ± 3 mm were cut in a petri dish using a sterile scalpel. Each snip was then placed in its own sterile microcentrifuge tube. Master mix was added to each microcentrifuge tube containing a tail snip and vortexed for 10-15 seconds. The samples were then incubated in a heating block at 55°C for 2.5 hrs for the tissue to solubilise before vortexing again for 10-15 seconds. Of the genomic binding buffer, 400 µL was added to the solubilised sample and vortexed for 10-15 seconds. Insoluble debris was removed by centrifuging¹¹ the samples. The aqueous supernatant was transferred to a Zymo-Spin™ IIX-XL Column in a collection tube and centrifuged. The collection tube with flow through was discarded and the Zymo-Spin™ IIX-XL Column was transferred to a new collection tube. Of the DNA pre-wash buffer, 400 µL DNA was added to the spin column which was then centrifuged. The collection tube was emptied, and 700 µL gDNA wash buffer was added to the spin column before centrifuging. This was followed by adding 200 µL gDNA wash buffer to the spin column which was then centrifuged. The collection tube with flow-through was discarded and the spin column was transferred to a sterile 1.5 mL microcentrifuge tube. To elute the DNA, 25 µL DNA elution buffer (pre-heated to 65°C) was directly added on the matrix before it was incubated for five minutes at room temperature and centrifuged at ~16 000 x g for one minute. Elution was repeated once more to increase the DNA yield.

Following this, the purity and concentration (ng/µL) of isolated DNA was determined using the NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer¹². The principle behind the instrument is described in detail in Section 3.4.2.

After determining the concentration, PCR amplification and agarose gel electrophoresis of isolated DNA followed. Sample and control DNA were diluted to 25 ng/µL and primer stocks were

¹⁰ Cat. #D4069 (Inquaba Biotech™, Pretoria, SA).

¹¹ All centrifugation steps during DNA isolation were performed at 12 000 x g for one minute, unless stated otherwise.

¹² NanoDrop™ Technologies (Thermo Fisher Scientific™, USA).

diluted 10x in nuclease-free water. PCR master mixes containing the following reagents were prepared for each sample: 1x Phire Tissue Direct PCR Master Mix¹³, 0.5 µM NDUFS4 Rev¹⁴ reverse primer, 0.5 µM NDUFS4 1060¹⁵ forward primer (Table 3.3) and nuclease-free water.

Table 3.3: Details of primers used for PCR

Primer	Primer sequence (5' to 3')	T _m (°C)
NDUFS4 1060 (Forward)	AGC CTG TTC TCA TAC CTC GG	62.5
NDUFS4 Rev (Reverse)	TTG TGC TTA CAG GTT CAA AGT GA	59.2

The sequences of the forward and reverse primers used for genotyping are shown with their melting temperatures. *Abbreviations: melting temperature (T_m).*

Of the PCR master mix, 9 µL was mixed with 1 µL of the 25 ng/µL DNA. Samples were amplified in a T100™ thermal cycler¹⁶ with the protocol shown in Table 3.4. Control samples (*Ndufs4*^{+/+}, *Ndufs4*^{-/-}, and *Ndufs4*^{+/-}) were also amplified.

Table 3.4: PCR cycle conditions

Step	1	2	3	4	5	6
Temp (°C)	98	98	57.3	72	72	4
Time (min)	05:00	00:05	00:05	00:20	01:00	∞
Rep	x1	x34			x1	-

This table shows the PCR cycle conditions used in genotyping. It started with a five-minute incubation at 98°C. The second condition consisted of 34 cycles of five seconds DNA denaturation at 98°C, five seconds at 57°C for primer annealing and 20 seconds incubation at 72°C for DNA extension. Next, amplicon completion was ensured by incubation for one minute at 72°C. The amplified product could be held infinitely at 4°C. *Abbreviations: minutes (min), repeats (rep), temperature (temp).*

Agarose gel electrophoresis was used to separate the amplified product according to size. A 1% (w/v) agarose¹⁷ gel was prepared with 1x Bionic™ Buffer¹⁸ and 0.5 µg/mL ethidium bromide (EtBr). A DNA ladder was prepared by mixing GeneRuler 100 bp DNA ladder¹⁹ with GeneRuler loading dye and nuclease-free water in a 2:1:3 ratio. The prepared DNA ladder was diluted (1:3) before loading 10 µL of it along with 10 µL of diluted (1:3) PCR product onto the gel. The three control samples were also loaded onto the gel. To the gel, 40 V was applied for 10 minutes before

¹³ Cat. #F170L (Thermo Fisher Scientific™, USA).

¹⁴ Cat. #S3424 (Inqaba Biotech™, Pretoria, SA).

¹⁵ Cat. #S3C81 (Inqaba Biotech™, Pretoria, SA).

¹⁶ Cat. #1861096 (Bio-Rad, CA, USA).

¹⁷ Cat. #8100-CONDA (Thermo Fisher Scientific™, MA, USA).

¹⁸ Cat. #B6185 (Merck, Modderfontein, SA).

¹⁹ Cat. # SM0241 (Thermo Fisher Scientific™, MA, USA).

increasing the voltage to 75 V for one hour. The gel was imaged with the ChemiDoc™ MP system²⁰ and Image Lab™ software²¹.

3.3.3 Tissue collection

Method

Euthanasia via cervical dislocation and tissue collection was performed by a research animal technician from the PCDDP when the mice reached ages P45-51. Although only the liver was used in this project, various other tissues including that of the brain, heart, kidneys, muscle, pancreas and tail snips were also collected for other projects. These tissues were immediately snap-frozen in liquid nitrogen to stop metabolic activity. Thereafter, samples were stored at -80°C until required for analysis. All samples were clearly marked, and thorough records were kept of all samples.

3.3.4 Enzyme analysis for determination of complex I activity

The activities of the enzymes complex I (CI) and citrate synthase (CS) in liver tissues, harvested from three WT and three KO mice, were determined. For this, 600g supernatant samples - which is the supernatant remaining after a 10% (w/v) homogenate was centrifuged at 600g - were prepared from liver tissues, before determining protein content with a bicinchoninic acid (BCA) assay. Following this CI and CS activities were measured using kinetic spectrophotometric assays and their specific activities in each sample determined.

Reagent preparation

Method

All solutions were prepared in water which was purified with a Milli-Q® IQ 7000²² purification system, unless stated otherwise. Water purified with this system will be referred to as *Milli-Q® water*.

²⁰ Cat. #17001402 (Bio-Rad, CA, USA).

²¹ Version 5.2.1 (Bio-Rad, CA, USA).

²² Cat. #ZIQ7000T0 (Merck, Modderfontein, SA).

For preparation of the 600g supernatants, Zheng buffer was prepared from 210 mM mannitol²³, 70 mM sucrose²⁴, 5 mM HEPES²⁵ and 0.1 mM egtazic acid (EGTA)²⁶. The pH was set to 7.2 with 1 M KOH²⁷ and aliquots were stored at -80°C.

For determining CS activity, 1 M Tris.HCl was prepared from trizma base²⁸, with the pH set to 8.1 with HCl, and stored in aliquots at -80°C.

For determining CI activity, the following reagents were prepared fresh every six months and stored in aliquots at -80°C: 0.5 M KPi buffer was prepared from 0.5 M KH₂PO₄²⁹ and 0.5 M K₂HPO₄³⁰ until a pH of 7.6 was reached. A 10% (w/v) bovine serum albumin (BSA)³¹ solution was prepared in Milli-Q[®] water, and 1 mM rotenone³² was prepared in dimethyl sulfoxide (DMSO)³³, coenzyme Q1 (10 mM)³⁴ was prepared in absolute ethanol.

Preparation of 600g supernatants

Principle

Differential centrifugation at progressively higher speeds fractionates homogenates into its different components as a result of their size and density. Components typically sediment in order of high density to low density (Padh, 1992).

The use of 600g supernatants for determining CI activity is preferred in this study over crude homogenate and isolated mitochondria. Isolated mitochondria have disadvantages over 600g supernatants in that it requires a larger quantity of fresh tissue, while 600g supernatants can be prepared from frozen tissue. Furthermore, different mitochondrial subpopulations could be selected during incomplete recovery of mitochondria, leading to potential bias in the results (Spinazzi *et al.*, 2012). Crude homogenates still contain unbroken cells, nuclei and other organelles that can decrease the sensitivity and reduce accuracy to determine specific activity.

²³ Cat. #M9546 (Merck, Modderfontein, SA).

²⁴ Cat. #84100 (Merck, Modderfontein, SA).

²⁵ Cat. #H3375 (Merck, Modderfontein, SA).

²⁶ Cat. #E4378 (Merck, Modderfontein, SA).

²⁷ Cat. #P5958 (Merck, Modderfontein, SA).

²⁸ Cat. #T1503 (Merck, Modderfontein, SA).

²⁹ Cat. #P5655 (Merck, Modderfontein, SA).

³⁰ Cat. #P2222 (Merck, Modderfontein, SA).

³¹ Cat. #10775835001 (Merck, Modderfontein, SA).

³² Cat. #R8875 (Merck, Modderfontein, SA).

³³ Cat. #C6164 (Merck, Modderfontein, SA).

³⁴ Cat. #C7956 (Merck, Modderfontein, SA).

Method

The following steps were performed on ice to prevent activation of damaging phospholipases and proteases which could result in loss of activity (Spinazzi *et al.*, 2012; Wieckowski *et al.*, 2009). Frozen liver samples were thawed on ice and ± 100 mg was cut on a clean, cold metal plate with a sterile blade. Samples were then cut as fine as possible before suspending it in an ice-cold Zheng buffer to 10% (w/v). A Potter-Elvehjem homogeniser³⁵ was placed in ice water and used to homogenise the samples with a motor-driven tissue homogeniser. The homogenate was transferred to a new microcentrifuge tube and centrifuged at 600g for 10 min at 4°C to pellet the cell debris and nucleus. The supernatant was transferred to a clean, marked microcentrifuge tube and stored at -80°C. Disruption of the mitochondrial membranes is required, especially for determination of CI activity, to make the substrates accessible to the enzymes. This is performed by freeze-thawing the supernatants twice in liquid nitrogen and water.

BCA protein assay

Principle

Total protein content was determined with the BCA assay to normalise enzyme activity. BCA assays were first described by Smith *et al.* (1985) and consists of two reactions. In the first reaction, Cu^{2+} is reduced to Cu^+ when it reacts with peptide bonds in alkaline solutions. In the second reaction, Cu^+ reacts with two BCA molecules to form a purple complex that absorbs at 562 nm. The absorbance is directly proportional to the protein content of the sample.

Method

BCA assays were performed using a BCA protein assay kit³⁶ according to the manufacturer's instructions. A $\text{BCA}:\text{CuSO}_4$ solution was freshly prepared in a 50:1 ratio. Duplicate reactions of a BSA standard series (0 to 20 μg ; in 4 μg increments) were prepared in a 96-well plate. For each sample, duplicate 20 μL reactions containing 2 μL of the 600g supernatant and 18 μL Milli-Q[®] water were added to the plate. Of the BCA reagent, 200 μL was simultaneously added to samples and standards, before they incubated for 30 minutes at 37°C. Absorbance was read at 562 nm with the BioTek Synergy[™] HT Multi-Detection Microplate Reader³⁷ and Gen5[™] Data Analysis Software³⁸. A correlation coefficient (R^2) of >0.99 was deemed acceptable. Least mean squares linear regression was used to determine protein content per well (μg), before determining the

³⁵ 10 mL Glass/Teflon[®] Tight Fitting Potter-Elvehjem Homogeniser (Glas-Col, IN, USA).

³⁶ Cat. #BCA-1 (Merck, Modderfontein, SA).

³⁷ Cat. #7091000 (BioTek Instruments, VT, USA).

³⁸ Version 1.11.5 (BioTek Instruments, VT, USA).

protein concentration (see Equation 3.1) by dividing the protein content by the volume of sample added to each well (μL).

Equation 3.1 Determination of protein concentration

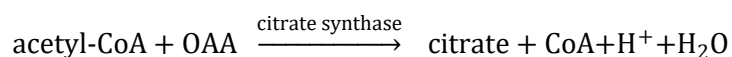
$$\text{Protein concentration } (\mu\text{g}/\mu\text{L}) = \frac{\text{Protein content } (\mu\text{g})}{\text{sample volume added } (\mu\text{L})}$$

Citrate (Si)-synthase

Principle

CS is a key regulatory mitochondrial enzyme which plays an important role in the tricarboxylic acid (TCA) cycle where it catalyses the condensation of oxaloacetate (OAA) and CoA to form citrate (Siu *et al.*, 2003). Furthermore, CS activity is a marker of abundance of mitochondria in a cell (Mogensen *et al.*, 2006; Spinazzi *et al.*, 2012) and can be measured spectrophotometrically (Siu *et al.*, 2003). The activities of enzymes of the respiratory chain is often normalised to the activity of CS, because partial respiratory enzymatic dysfunction associated with compensatory mitochondrial proliferation can be overlooked if enzyme activities are only normalised to protein content (Spinazzi *et al.*, 2012). Measurement relies on two reactions. The first being the reaction of acetyl-CoA with OAA to form citrate and free CoA (Equation 3.2), where CS is the catalyser.

Equation 3.2 The first reaction required for measurement of CS activity.



In the second reaction, the release of free CoA is coupled to 5,5'-dithio-bis(-2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB), as shown in Equation 3.3. The concentration of TNB can be measured spectrophotometrically at 412 nm.

Equation 3.3 The second reaction required for measurement of CS activity.



Method

The following reagents were prepared fresh for determination of CS activity: 1 mM DTNB³⁹, 10% (v/v) triton X-100⁴⁰, 3 mM acetyl-CoA⁴¹ and 2 mM OAA⁴². DTNB was prepared in 1 M Tris.HCl. Triton X-100 was prepared in Milli-Q[®] water and OAA was prepared in 0.1 M Tris.HCl.

Three WT and three KO samples were analysed in triplicate and were thawed on ice water (where necessary). OAA was pre-heated at 40°C in a water bath, while a master mix containing the reagents shown in Table 3.5 for each reaction, were prepared.

Table 3.5 Reagents for CS master mix

Reagent	1x (μL)	[end]
Milli-Q [®] water	106.5	
DTNB (1 mM)	20	0.1 mM
triton X-100 (10%)	0.5	0.025%
acetyl-CoA (3 mM)	20	0.3 mM
Total	147	

A 147 μL mastermix containing Milli-Q[®] water, 0.1 mM DTNB, 0.025% triton X-100 and 0.3 mM acetyl-CoA were prepared for each reaction. *Abbreviations: 5,5'-dithio-bis(-2-nitrobenzoic acid) (DTNB).*

Of the mastermix 147 μL, and 5 μL of the 600g supernatant from each sample were added to the wells of a 96-well plate. The plate was incubated for 10 minutes in the Synergy[™] HT Multi-Detection Microplate Reader. Thereafter, 0.5 mM preheated OAA was added to each reaction and a linear rate increase was recorded at 412 nm for five minutes at one-minute intervals (v_1). R^2 values > 0.99 was deemed acceptable. CS activity was calculated with Equation 3.4.

³⁹ Cat. #D8130 (Merck, Modderfontein, SA).

⁴⁰ Cat. #T9284 (Merck, Modderfontein, SA).

⁴¹ Cat. #10101907001 (Merck, Modderfontein, SA).

⁴² Cat. #O4126 (Merck, Modderfontein, SA).

Equation 3.4 Determination of CS activity

$$UCS = \frac{\overline{v1}}{\epsilon_{412}} \times \frac{V_r}{V_{Ef} \times \frac{C_P}{1000}}$$

where:

UCS is the CS activity in $\mu\text{mol}/\text{min}/\text{mg}$

$\overline{v1}$ is the average linear rate increase of the triplicate wells

ϵ_{412} is the molar extinction coefficient for TNB as determined for 200 μL reactions (7 465 Abs/mM)

V_R is the reaction volume in mL (0.2 mL)

V_{Ef} is the effective sample volume added to the reaction (0.5 μL)

C_P is the protein concentration in $\mu\text{g}/\mu\text{L}$

Complex I activity

Principle

CI (NADH-ubiquinone oxidoreductase) catalyses the transfer of electrons from NADH to coenzyme Q_{10} . The electrons produced during oxidation of NADH by CI, reduce decylubiquinine to finally deliver the electrons to an alternative terminal electron acceptor, 2,6-dichloroindophenol sodium salt hydrate (DCIP). Spectrophotometric measurements at 600 nm can follow reduction of DCIP. Reduction of DCIP is mostly as a result of CI activity, as decylubiquinine does not accept electrons produced by other NADH dehydrogenases (Janssen *et al.*, 2007). Except for CI, several other NADH oxidising dehydrogenases are present in tissue homogenates, resulting in high rotenone-insensitive NADH oxidation rates that ultimately interfere with the sensitivity of the CI assay. Specificity of the CI assay is therefore determined through rotenone sensitivity measurements (Janssen *et al.*, 2007). Specific activity of CI is calculated by subtracting activity with rotenone from total CI activity (without rotenone).

Method

On the day of analysis, 2 mM NADH⁴³ and 5 mM DCIP⁴⁴ were prepared fresh in Milli-Q® water. DCIP was heated for 20 minutes at 30°C to dissolve.

For each sample, triplicate reactions for rotenone and DMSO were prepared. The optimal volume of liver homogenate was determined in advance to be 1 μL , based on specific activity vs. protein

⁴³ Cat. #N4505 (Merck, Modderfontein, SA).

⁴⁴ Cat. #D1878 (Merck, Modderfontein, SA).

content comparisons. For each reaction, master mixes containing the reagents mentioned in Table 3.6 were prepared:

Table 3.6 Master mixes used for determining CI activity

Reagent	1x (μL)	[end]
KPi buffer (0.5 M) – pH 7.6	10	25 mM
coenzyme Q1 (10 mM)	1.4	70 μM
BSA (10%)	7	0.35 %
DCIP (5 mM)	2.4	60 μM
Milli-Q® H ₂ O	103.2	
Total	124	

Master mixes used for determining CI activity were prepared and contained KPi buffer, coenzyme Q1, BSA, DCIP and Milli-Q® H₂O. Abbreviations: *Bovine serum albumin (BSA)*, *dichloroindophenol sodium salt hydrate (DCIP)*.

To each well, 50 μL DMSO or rotenone, 1 μL homogenate and 120 μL master mix was added. Reactions incubated for 10 minutes at 37°C before 0.2 mM NADH (pre-heated to 37°C) was added to start the reactions. The linear rate decrease (v_1) was recorded at 600 nm for five minutes at one-minute intervals. R^2 values > 0.99 for v_1 and v_2 were deemed acceptable. Rotenone sensitive velocity was calculated (see Equation 3.5) and used to determine the CI activity as shown in Equation 3.6. CI activity was normalised to CS activity by using Equation 3.7.

Equation 3.5 Rotenone sensitive velocity

$$v_3 = \overline{v_1} - \overline{v_2}$$

where:

$\overline{v_1}$ is the mean velocity of replicate wells in the absence of rotenone

$\overline{v_2}$ is the mean velocity of replicate wells in the presence of rotenone

v_3 is the rotenone sensitive velocity

Equation 3.6 CI activity

$$UCI = \frac{v_3}{\epsilon_{600}} \times \frac{V_r}{V_{Ef} \times \frac{C_p}{1000}}$$

where:

UCI is the CI activity in $\mu\text{mol}/\text{min}/\text{mg}$

v_3 is the rotenone sensitive velocity as calculated in Equation 3.5

ϵ_{600} is the molar extinction coefficient for DCIP as determined for 200 μL reactions (12 712 Abs/mM)

V_{Ef} is the effective sample volume added to the reaction (0.5 μL)

C_p is the protein concentration of the sample in $\mu\text{g}/\mu\text{L}$

Equation 3.7 Normalised CI activity

$$\text{Normalised UCI} = \text{UCI} \times \frac{1000}{\text{UCS}}$$

where:

Normalised UCI is the activity of CI normalised to the activity of CS in $\text{nmol}/\text{min}/\text{UCS}$.

3.4 RNA isolation, quantification, and quality determination

RNA was isolated from eight WT and eight KO liver samples and quantified using a spectrophotometer and fluorometer. RNA quality was determined with bleach agarose gel electrophoresis.

3.4.1 Isolation of RNA

Principle

RNA was isolated with the MagMAX™-96 Total RNA Isolation Kit⁴⁵, which utilises a magnetic bead-based purification method. Samples are disrupted in a guanidinium thiocyanate (GTC), based solution (Chirgwin *et al.*, 1979) to simultaneously solubilize cellular membranes and inactivate nucleases. The magnetic beads employed in this kit are able to bind nucleic acids which are then magnetically captured while cell debris and contaminants are washed out. The magnetic beads have a nucleic acid binding surface that binds nucleic acids. The addition of salts like GTC greatly enhances adsorption of nucleic acids onto the magnetic beads' surface (Sun *et al.*, 2014).

⁴⁵ Cat. #AM1830 (Thermo Fisher Scientific™, MA, USA).

This enhancement is likely due to GTC functioning as a bridge between nucleic acids and magnetic beads. Anions are present on the surfaces of magnetic beads and nucleic acids. The water layer on the surfaces of magnetic beads and nucleic acids are dehydrated by the high concentration of saline ions. The formation of hydrated ions prompts the magnetic beads to adsorb nucleic acids (He *et al.*, 2017). The bead-nucleic acid components are then magnetically captured while cell debris and contaminants are washed out. The nucleic acids are then treated with deoxyribonuclease (DNase) to remove gDNA. Addition of a low salt elution buffer will release the purified RNA from the beads (Kovačević, 2016). The beads are then magnetically captured so that the supernatant containing the purified RNA can be transferred to a nuclease-free container. The eluted RNA is suitable for real-time quantitative reverse transcription PCR (qRT-PCR), microarray analysis, northern blotting, and ribonuclease protection assays.

Method

Special precautions were taken while working with RNA, such as preventing contamination with ribonuclease (RNase) as well as preventing tissue samples from thawing. Before isolation of RNA, all equipment including pipettes, scalpels and surfaces were cleaned with a 10% bleach solution to remove contaminating RNase. Only RNase-free pipette tips were used during RNA isolation. Tissue samples were kept at -80°C up to RNA isolation when they were transferred to a liquid nitrogen container. From the container samples were transferred to a cold vapour environment for handling. The cold vapour environment was created with an ice chest containing dry ice. The metal plate used to partition the tissue, as well as the scalpels and forceps were pre-chilled on dry ice or in liquid nitrogen. After partition, the tissue was immediately stored back in liquid nitrogen to ensure that RNA could be re-isolated from the same sample if the RNA quality was insufficient. The same samples were used for ethical reasons by keeping the number of mice that were sacrificed as low as possible.

RNA isolation was performed according to the kit manufacturer's protocol. For each sample, 100-μL lysis/binding solution was freshly prepared by adding 0.1 M β-mercaptoethanol to the lysis binding solution. The bead mix was also freshly prepared by mixing RNA binding beads and a lysis/binding enhancer in a 1:1 ratio. All mixing steps were done by an orbital shaker⁴⁶ at ~250 revolutions per minute (rpm).

The lysis/binding solution was added to ~five mg snap-frozen tissue and homogenised in a Potter-Elvehjem tissue homogeniser with a rotor-stator homogeniser⁴⁷. Homogenised tissue was transferred to a 96-well processing plate and 60 μL of 100% isopropanol was added to each

⁴⁶ Cat. #542-40010-00-3 (Heidolph Instruments, Schwabach, Germany).

⁴⁷ Cat. #501-11000-00-2 (Heidolph Instruments, Schwabach, Germany).

sample before mixing the plate for one minute. The bead mix was briefly vortexed before adding 20 µL to each sample which was then mixed for five minutes. The plate was moved to a magnetic stand to capture the RNA binding beads for two to three minutes. The supernatant was aspirated and discarded before removing the plate from the magnetic stand. Next, a 150 µL wash solution 1 containing isopropanol, was added to each sample and the plate mixed for one minute. The RNA binding beads were then captured for one to two minutes before discarding the supernatant and removing the plate from the magnetic stand. Thereafter, 150 µL wash solution 2 was added and the plate mixed for another two minutes before capturing the beads. During this time, TURBO™ DNase was prepared by mixing one-part TURBO™ DNase with 49 parts MagMAX™ TURBO™ DNase Buffer. After capturing the beads, the supernatant was discarded, and the plate removed from the magnetic stand. Immediately hereafter, 50 µL of the prepared DNase was added to each sample and the plate mixed for 10-15 minutes at room temperature. During this step, nucleic acids were released from the RNA binding beads and gDNA was degraded. To rebind the RNA to the beads, 100 µL of RNA rebinding solution was added to each sample before mixing the plate for three minutes. The RNA binding beads were magnetically captured, the supernatant discarded, and the plate removed from the magnetic stand. Next, the RNA was washed twice by adding 150 µL wash solution 2, the plate mixed for one minute, before magnetically capturing the RNA binding beads and discarding the supernatant each time. After washing the RNA, the beads were dried, and excess ethanol removed by mixing the plate for two minutes. Finally, the RNA was eluted by adding 50 µL elution buffer to each sample, mixing it for three minutes and magnetically capturing the beads. The purified RNA in the supernatant was aliquoted in nuclease-free PCR tubes and either snap-frozen in liquid nitrogen or kept on ice for downstream analyses.

3.4.2 Quantification of RNA with the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer

Principle

The Nanodrop™ Spectrophotometer is able to directly measure the quantity and quality of nucleic acid or protein samples and only requires 0.5 µL – 2 µL sample. The small sample volumes are held in place between two optical fibres with surface tension, and a xenon flash lamp is used to pass light through the top optical fibre and down through the liquid column to be detected by the internal spectrometer (Thermo Fisher Scientific., 2010).

Nucleic acids, RNA, single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA) all absorb at 260 nm while proteins, phenol and other contaminants absorb near 280 nm. The ratio of absorbance at 260 nm and 280 nm can be used to assess the purity of DNA or RNA (Nanodrop

Technologies, 2007). RNA is generally accepted as pure with a 260/280 ratio of ~2.0, a bit higher than the accepted ratio of 1.8 for DNA, due to the higher 260/280 ratio of uracil compared to thymine. Pure nucleic acids are accepted as pure with 260/230 values that are in the range of 2.0 – 2.2.

Absorbance measured by the Nanodrop™ Spectrophotometer is converted to concentrations (ng/μL) through a modified Beer-Lambert equation that is shown in Equation 3.8 (Thermo Fisher Scientific., 2010).

Equation 3.8 Beer-Lambert equation

$$c = (A * \epsilon) / b$$

where:

c is the nucleic acid concentration in ng/μL

A is the absorbance in AU

ϵ is the wavelength-dependent extinction coefficient in ng-cm/μL (dsDNA: 50 ng-cm/μL; ssDNA: 33 ng-cm/μL; RNA: 40 ng-cm/μL)

b is the pathlength in cm

Method

After elution, the RNA was directly quantified with the NanoDrop™ One spectrophotometer. The elution buffer used during RNA isolation was used as a blanking buffer. Duplicate measurements of 2 μL sample was performed to determine the RNA concentration and purity at 260 nm. Milli-Q® water was used to clean the pedestals between each sample. RNA was deemed pure with a A260/A280 ratio of ~2.0.

3.4.3 Quantification of RNA and DNA with the Qubit® Fluorometer

Principle

The Qubit® Fluorometer uses Qubit® assays containing a molecular probe dye that only emits fluorescent signals when bound to specific target molecules, making it a very accurate and sensitive method for the quantitation of DNA and RNA. Unlike ultraviolet absorbance-based methods, the Qubit® assays can differentiate between RNA and DNA in one sample, making it a much more selective method for nucleic acid quantitation. Purity of samples can also be determined with the Qubit® Fluorometer by using the Qubit® dsDNA HS Assay Kit⁴⁸ in addition to

⁴⁸ Cat. #Q32851 (Thermo Fisher Scientific™, MA, USA).

the Qubit® RNA HS Assay Kit⁴⁹. These measurements are more accurate than the A260/A280 ratio. gDNA contamination was deemed acceptable when it was lower than 10% of RNA. A similar method was employed for RNA and DNA quantification, but two different kits were used.

For RNA quantification: The Qubit® RNA HS Assay kit can accurately quantify low abundance (between 250 pg/μL and 100 ng/μL) RNA samples and requires 1-20 μL of sample. It is suitable for quantification of total RNA, ribosomal RNA, or large messenger RNA. Two standards are used during the assay, 1) Qubit® RNA HS Standard #1 (0 ng/μL) and 2) Qubit® RNA HS Standard #2 (10 ng/μL). Working solutions for RNA quantitation were prepared with Qubit® RNA HS Reagent and Qubit® RNA HS Buffer.

For DNA quantification: The Qubit® dsDNA HS Assay Kit is highly selective for dsDNA over RNA and can accurately quantify DNA from 10 pg/μL to 100 ng/μL. This assay requires 1-20 μL sample input. As with the Qubit® RNA HS Assay kit, this kit also uses two standards: 1) Qubit® dsDNA HS Standard #1 (0 ng/μL) and 2) Qubit® dsDNA HS Standard #2 (10 ng/μL). Working solutions for DNA quantitation are prepared with Qubit® dsDNA HS Reagent and Qubit® dsDNA HS Buffer.

Method

Qubit® working solution (200 μL/sample) was freshly prepared by diluting the Qubit® HS Reagent 1:200 in Qubit® HS Buffer. For each of the two standards, 190 μL working solution was mixed with 10 μL standard. For each sample, 199 μL working solution was mixed with 1 μL sample. Samples and standards were briefly vortexed and incubated for two minutes before analysing on the Qubit® Fluorometer. The concentration of the sample was calculated with Equation 3.9.

Equation 3.9 Calculation of concentration of sample from Qubit® Fluorometer

$$\text{Concentration of sample} = \text{QF value} \times \frac{200}{X}$$

where:

QF value is the value given by the Qubit® Fluorometer

X is the volume of sample added to the assay tube (μL)

⁴⁹ Cat. #Q32852 (Thermo Fisher Scientific™, MA, USA).

3.4.4 Determination of RNA integrity/purity

Principle

An essential part of RNA-based research is working with non-degraded RNA. RNA integrity is commonly affected by RNases, enzymes that quickly degrade RNA, which are found in the air, on human skin and in various human bodily fluids. RNA quality was determined with a bleach agarose gel electrophoresis, using a standard TAE-based agarose gel containing pure bleach (Aranda *et al.*, 2012). High quality RNA is suggested by two distinct bands with gel electrophoresis. The 28S rRNA, which runs at 4.7 kb, and the 18S rRNA which runs at 1.9 kb. Poor quality RNA is suggested by smeared bands or a 28S:18S band intensity ratio below 2:1 (Aranda *et al.*, 2012).

Method

A 1% (w/v) agarose gel in a TAE-buffer containing 0.06% (v/v) bleach⁵⁰ and EtBr with a final concentration of 5 µg/ml was prepared. All electrophoresis equipment was decontaminated with 10% bleach beforehand. Samples were prepared with 1x TAE-buffer, 1x DNA loading dye⁵¹ and 300-500 ng RNA and loaded on the gel. Of the GeneRuler DNA Ladder Mix⁵², 0.2 µg was also loaded on the gel. RNA was then separated according to their size by way of electrophoresis: 1 V/cm for 10 minutes, followed by 3 V/cm for 70 minutes. The gel was imaged on the ChemiDoc MP imager using Image Lab™ software.

3.5 Transcriptomics

Transcriptomics is the study of the complete set of RNA transcripts with high-throughput methods. For this study, a targeted transcriptomics approach was chosen and was performed using Ion AmpliSeq™ technology in combination with Ion Torrent sequencing. The overall workflow is shown in Figure 3.2. The first step was to synthesise complementary DNA (cDNA) from RNA. Next, libraries and templates were prepared, and sequencing chips were loaded with the template. Following chip loading, sequencing was done with an Ion S5™ Sequencer. Finally, primary and secondary data analyses were performed.

⁵⁰ Keltrade, Parys, South Africa.

⁵¹ Cat. #R0611 (Thermo Fisher Scientific™, MA, USA).

⁵² Cat. #SM0333 (Thermo Fisher Scientific™, MA, USA).

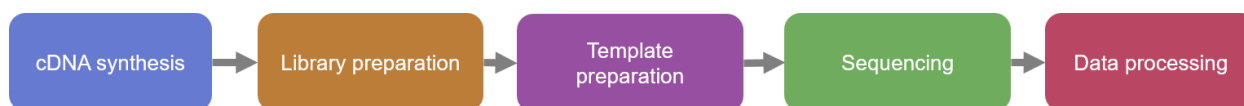


Figure 3.2 The overall sequencing workflow used during this study

The first step in the sequencing workflow was cDNA synthesis. The following steps were library and template preparation, which were performed on the Ion Chef™. After template preparation where the chips were loaded with Ion Sphere™ Particles (ISP's), the chips were sequenced. The last step was primary and secondary data processing. *Abbreviations: complementary DNA (cDNA).*

3.5.1 cDNA synthesis

Principle

Reverse transcription PCR (RT-PCR) is the process used to synthesise cDNA from an RNA template through the use of an RNA-directed DNA polymerase, reverse transcriptase (Best & Roberts, 2014). Reverse transcription reactions also require oligonucleotide primers, nucleotides, RNase inhibitors and a reaction buffer. Several different types of oligonucleotide primers can be used. Oligo(dT) primers anneal to the poly(A) tails found on most mRNA molecules. A second type of primer, random hexamer primers, is a mixture of random hexanucleotide primers that anneal throughout the RNA, ensuring that non-polyadenylated RNAs are also reverse transcribed (Best & Roberts, 2014).

cDNA was synthesised from RNA by using a SuperScript™ IV VILO™ Master Mix⁵³ kit. This kit consists of a 5x master mix that contains a reverse transcriptase, a RNase inhibitor, helper proteins, stabilizer proteins, oligo (dT)18, random hexamer primers, MgCl₂, and deoxynucleotide triphosphates (dNTPs). The kit also has a control master mix that does not contain the reverse transcriptase enzyme and is used to verify that the RNA sample does not contain gDNA.

Method

RNA was diluted to 3.3 ng/μL before preparing PCR reactions. Two PCR reactions were prepared for each sample; one to synthesise cDNA and one control reaction that did not contain reverse transcriptase. The 15 μL PCR reactions contained 40 ng RNA and either 3 μL Superscript™ IV VILO™ Master mix or SuperScript™ IV VILO™ No RT Master Mix. One cycle of the following conditions was applied to the PCR reactions: Primer annealing for 10 minutes at 25°C, reverse transcription at 50°C for 10 minutes, and reverse transcriptase inactivation at 85°C for five minutes. cDNA was stored at -20°C until needed.

⁵³ Cat. #11756050 (Invitrogen™, Thermo Fisher Scientific™, MA, USA).

3.5.2 Library preparation

Principle

Whole genome or whole exome sequencing results in overwhelmingly large data sets that are laborious to process. A novel approach, targeted transcriptome sequencing, aims to address the need for faster sequencing that results in more manageable datasets. This is done by sequencing specifically chosen areas of the transcriptome. Ion AmpliSeq™ is an example of this type of technology and uses PCR primers to amplify the specific areas of interest through a highly multiplexed amplification method (Li, Turner, *et al.*, 2015). Fundamentally, an Ion AmpliSeq™ panel consists of a pool of oligonucleotide primer pairs that are each designed to amplify a specific region of the genome (Kim, 2017). After PCR amplification of the specific regions, remaining primers are partially digested to prepare the ends of the amplicons for attachment of adapters. **Barcodes** and **adapters** are then ligated to the libraries before all libraries are pooled and ready for template preparation. Figure 3.3 shows the position of the barcodes and adapters in relation to the amplicons.

Barcodes

These are short sequences of known bases that are ligated to the template cDNA, before the samples are pooled. Each sample gets its own barcode which is used to identify the sample from which a particular read is derived after sequencing.

Adapters

These are short sequences of bases that are ligated to the ends of cDNA. They are complementary to the bases attached to the ISPs and are used to bind the cDNA template to the ISPs.

The Ion AmpliSeq™ Transcriptome Mouse Gene Expression Panel is a ready-to-use IonAmpliSeq™ panel that consists of 47 860 primers. This panel is able to measure the gene expression levels of 23 930 RefSeq genes of which 20 767 are well annotated and the remaining 3 163 are coding and non-coding genes that have provisional annotations based on the Genome Reference Consortium Mouse Reference 38 (GRCm38). The average length of the amplicons are 150 bases (Thermo Fisher Scientific., 2020). The Ion AmpliSeq™ Transcriptome Mouse Gene Expression Panel is available in a Chef-Ready kit⁵⁴, containing all the reagents and materials required for and automated library preparation on the Ion Chef™ instrument.

⁵⁴ Cat. #A36412 (Ion Torrent™, Thermo Fisher Scientific™, MA, USA).

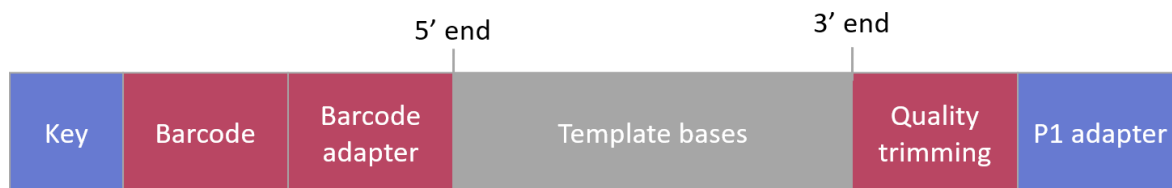


Figure 3.3 Barcodes and adapters ligated to an amplicon

This figure shows the placement of barcodes and adapters. Barcodes are ligated to the template bases on the 5' end, while P1 adapters are ligated on the 3' end. Adapted from (Thermo Fisher Scientific., 2019).

Method

Library preparation was performed using the Ion AmpliSeq™ Transcriptome Mouse Gene Expression Core Panel, Chef-Ready Kit and according to the manufacturer's protocol. Two library preparation runs were performed with eight samples each time. Briefly, a sample set was created on Torrent Suite™ Software⁵⁵. During creation of a sample set, details about the samples including a descriptive name, their position on the PCR plate and their barcode, are entered into the software. The cDNA was then diluted to 0.8 ng/μL before adding 15 μL to the wells of an Ion Code™ PCR plate. For each run, an Ion Code™ PCR plate was loaded with the cDNA of eight samples. The Ion Chef™ was loaded with the primer pools, Ion Code™ PCR plate containing the cDNA and other necessary reagents and materials contained in the Ion AmpliSeq™ Transcriptome Mouse Gene Expression Core Panel, Chef-Ready Kit, to prepare the barcoded libraries. Amplification settings were set to 13 cycles and a 16 minutes anneal/extension time, as recommended by the manufacturer for 6 145-24 576 primer pairs per pool. The barcoded libraries were equalised to 100 pM by the Ion Chef™ during the library preparation (Carino *et al.*, 2019) and stored at 4°C until required for template preparation.

3.5.3 Template preparation

Principle

To ensure a detectable signal during sequencing, the amplicons need to be amplified further through template preparation. On the Ion Torrent system, template preparation entails the preparation of template-positive Ion Sphere™ Particles (ISPs) through emulsion PCR (emPCR). ISPs are 3-micron diameter beads that are covered in specific adapter sequences complementary to adapters attached to the amplicons (Quail *et al.*, 2012). emPCR is a version of PCR where an aqueous phase PCR mixture and an oil phase containing surfactants are emulsified to form water-in-oil droplets. Millions of oil droplets each containing one amplicon attached to one ISP (except for **polyclonal ISPs**) and the reagents required for PCR, are produced and undergo PCR to replicate the amplicon sequence all over the ISP, essentially resulting in millions of isolated

⁵⁵ Version 5.12.2 (Thermo Fisher Scientific™, MA, USA).

microreactors (Siu *et al.*, 2021). ISPs containing templates are then enriched through a magnetic bead-based process before they are loaded onto a semiconductor chip. The depth of each well allows occupation of only one bead (Rothberg *et al.*, 2011). The loaded chip is then ready for sequencing.

The Ion 540™ Kit - Chef⁵⁶ enables automated template preparation and chip loading on the Ion Chef™. The Ion Chef™ allows for template preparation of two libraries and hence loading of two chips, simultaneously. In comparison to manual loading, use of the Ion Chef™ results in increased efficiency in preparing and loading templates from short tandem repeat libraries and reduced variability of loading templates from SNP libraries (Mogensen *et al.*, 2015). Ion 540™ Chips can generate 60-80 million reads from eight samples and contain ~150 million addressable wells (Thermo Fisher Scientific., 2019).

Polyclonal ISPs

These are beads that contain more than one type of amplicon. Data from these beads will be discarded as it is not possible to know which amplicon contributed to the signal during sequencing. The risk for polyclonal beads is higher when the concentration of the libraries is too high.

Method

Template preparation was performed with the Ion 540™ Kit – Chef according to the manufacturer's protocol. A planned run was first set up on the Torrent Suite™ Software by entering sample information, reference files, barcodes, and instrument details. The number of flows was set to 500 as required by the manufacturer for 200-base-read sequencing. Next, both libraries were diluted to 70 pM, as recommended by the manufacturer for Ion AmpliSeq™ Transcriptome Mouse Gene Expression libraries. Of the diluted libraries, 25 µL were added to each of the Ion Chef™ Library Sample tubes, before the necessary materials and reagents, including two Ion 540™ chips, were loaded onto the Ion Chef™. After selection of the data destination, the template preparation run was started, which took about 13 hours. When the template preparation was finished, one of the loaded chips was immediately sequenced and the second chip was stored in a chip storage container at 4°C until the sequencing of the first chip was finished. The second chip warmed to room temperature for 20 minutes before sequencing of the first chip was finished.

⁵⁶ Cat. #A30011 (Thermo Fisher Scientific™, MA, USA).

3.5.4 Sequencing

Principle

The Ion Torrent platform uses a sequencing strategy known as sequencing by synthesis, or more specifically, semiconductor sequencing (see Figure 3.4). A bead (ISP) covered in thousands of copies of the same DNA template, is added to a single reaction well of a semiconductor chip. The four nucleotides are flowed over the chip, one at a time, and a standard elongation reaction is performed. As with natural chemistry, an H^+ ion and pyrophosphate are released when a nucleotide is incorporated. For each H^+ ion that is released, the pH of the surrounding solution is changed with 0.02 pH units, which is detected by an integrated complementary metal-oxide-semiconductor (**CMOS**) and an ion-sensitive field-effect transistor (**ISFET**) sensor (Goodwin *et al.*, 2016). The change in pH is converted to a voltage and then to a digital form that can be processed by a computer. In the case of **homopolymers**, there will be a proportional increase in pH change detected as multiple bases are incorporated. Unincorporated nucleotides are then washed out of the wells to ensure that only one nucleotide is responsible for the signal before the next nucleotide is flowed over the chip.

Method

The Ion S5™ Sequencer was first initialized before a chip can be sequenced. This was typically started ~one hour before the Ion Chef™ was finished with chip loading. Initialization required installation of the reagents needed for a sequencing run. A new W2 Solution bottle, Ion S5™ Sequencing Reagents cartridge and Ion S5™ Cleaning Solution was installed and the waste reservoir was emptied. An old chip was loaded into the Ion S5™ and the initialization run was started. Once the initialization and chip loading procedures were completed, the old chip used for initialization was replaced by a newly loaded chip. The Ion S5™ automatically retrieved the information of the chips that were loaded with ISPs from the server. The second chip was loaded ~ 2.5 hours after the first chip.

CMOS

An integrated circuit design that is printed on a microchip that contains different types of semiconductor transistors (Goodwin *et al.*, 2016).

ISFET

A type of transistor that is sensitive to changes in ion concentration (Goodwin *et al.*, 2016). The change is the pH of the well during nucleotide incorporation, which induces a change in the surface potential of the metal-oxide-sensing layer, which in turn causes a change in the potential of the source terminal of the underlying field-effect transistor (Rothberg *et al.*, 2011)

Homopolymers

These are consecutive repetitions of a base. For example, in CAGGT, the GG's are the homopolymers.

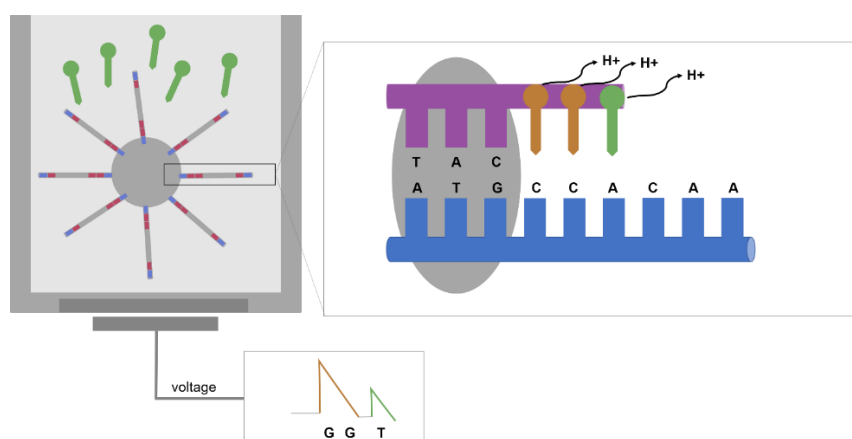


Figure 3.4 Semiconductor sequencing as performed on the Ion Torrent platform

During semiconductor sequencing, a semiconductor chip containing reaction wells loaded with one ISP per well are sequenced. Nucleotides are flowed over the chip one at a time to perform a standard elongation reaction. When a nucleotide is incorporated, a H^+ is released which changes the pH of the surrounding solution and is detected by a CMOS and ISFET sensor. The change in pH is converted to a voltage and then to a digital form that can be processed by a computer. *Abbreviations: complementary metal-oxide-semiconductor (CMOS), ion-sensitive field-effect transistor (ISFET).* Adapted from (Goodwin et al., 2016).

3.5.5 Data processing

Data obtained after sequencing needs to be processed and typically involves primary, secondary, and tertiary processing, as shown in Figure 3.5.

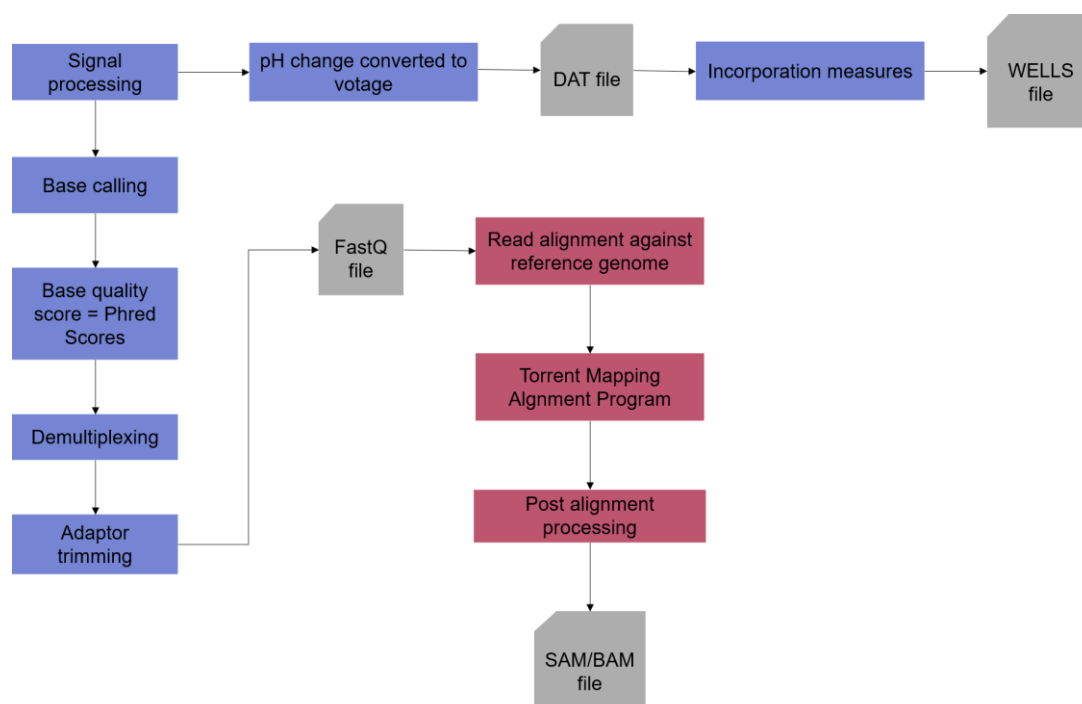


Figure 3.5 The data processing workflow for Ion Torrent transcriptomics

Primary and secondary data processing was performed with Ion Torrent Suite™ Software. Primary data analysis (blue) entailed signal processing, base calling and scoring base quality. During secondary data processing (pink), the reads were aligned to the reference genome before creation of SAM/BAM files. Adapted from (Pereira et al., 2020).

Primary data analysis entails signal processing, base calling and scoring base quality (Pereira *et al.*, 2020). During signal processing, the signal detected by the sensor due to base incorporation is converted to voltage. The raw voltage data is sent to the server as a DAT file. An acquisition file containing the raw signal measurement of each well is generated for each nucleotide flow. These raw signal measurements are then transformed into incorporation measures and stored in WELLS files. A base-caller module performs the final step, base calling. The base caller module, through a series of algorithms adjusting for signal droop and normalization, determines the most likely base sequences (Pereira *et al.*, 2020). The next step in data processing is quality control. Quality scores of the base call express errors in sequencing and is illustrated with Phred scores. A Phred score of Q10 refers to a base having a one in ten probability of being incorrect, while a Q30 score refers to a probability of an incorrect base being one in 1000 (Pereira *et al.*, 2020). Sequences are further analysed through demultiplexing, a process separates the sequencing reads from pooled libraries into separate files according to each sample's barcode (Pereira *et al.*, 2020). Demultiplexing is followed by adapter trimming where adapter sequences are removed from the ends of the reads to avoid their interference with mapping the reads to the reference genome (Kong, 2011).

Secondary analysis constitutes read alignment against the reference genome, in this case, the AmpliSeq Mouse Transcriptome v1. Read alignment is the assembly of the millions of cDNA fragments by aligning it to a known reference genome. For this, an algorithm specifically optimized for Ion Torrent, the Torrent Mapping Alignment Program (TMAP) tries to locate a position in the reference sequence that matches the read. TMAP first roughly aligns the reads against the reference genome before refining the alignment to compensate for Ion Torrent specific biases such as homopolymer alignment (Pereira *et al.*, 2020). TMAP also performs post-alignment processing to remove duplicate reads, perform intensive local realignment, and recalibrate the Phred-scaled quality score (Pereira *et al.*, 2020). The output files after alignment are the binary alignment map (BAM) and sequence alignment map (SAM) files.

Torrent Suite™ Software was used to plan the runs for library and template preparation, sequencing, as well as primary and secondary analysis of the data.

The quality of an Ion AmpliSeq™ sequencing run can be evaluated by the following metrics: Total bases, ISP loading density, total reads, read lengths, read length histogram, and key signal (Thermo Fisher Scientific., 2019). See Table 3.7 for definitions and expected values.

Table 3.7 Quality control metrics for Ion AmpliSeq™ sequencing

Metric	Definition	Expected values for good quality data
Total bases	The number of filtered and trimmed base pairs. It represents the throughput as Giga-base pairs.	For an Ion 540™ chip, with 150 base read library, 9-12 Gb is expected. Throughput = number of reads * library size.
ISP loading density	The percentage of addressable wells on the chip that contain Ion Sphere™ particles.	Generally loading of ≥80% is considered optimal
Total reads	Total number of trimmed and filtered reads, independent of length	For an Ion 540™ chip, the total number of reads is expected to be 60-80 million.
Read length and read length histogram	The length of called reads in base pairs. The histogram reflects the size distribution of the library without the Ion adapter sequences.	The histogram shape should resemble the library size (150 bp for Ion AmpliSeq™ Transcriptome Mouse Gene Expression Core Panel) distribution trace without adapter sequences closely.
Key signal	The average signal for ISPs that match the library key.	A higher starting key signal signifies a lower impact of signal droop and less 3' trimming.

This table defines the different quality control metrics of an Ion AmpliSeq™ sequencing run and gives examples of values for good quality data.

3.6 Validation

Sequencing results were validated by performing SDS-PAGE and western blots as well as determining activity of the GLYAT enzyme.

3.6.1 SDS-PAGE and western blot

Principle

SDS-PAGE is an electrophoresis technique to separate proteins according to their size. Samples are denatured by heating to unfold the proteins and thereby surrounding them with sodium dodecyl sulphate (SDS) molecules. Treatment of proteins with SDS in the presence of β-mercaptoethanol causes SDS to bind to hydrophobic regions of the protein to provide a net negative charge. The negative charge will cause the proteins to migrate to the positive pole during electrophoresis (Hnasko & Hnasko, 2015). The molecular weight of the protein determines its electrophoretic movement in the acrylamide gel, with lower molecular weight proteins moving faster. Molecular weight markers with known molecular weights are also loaded with the samples. The second step involves transfer of proteins from an SDS-PAGE gel onto a membrane, for example a polyvinylidene difluoride (PVDF) membrane, through electrophoresis (Rajput &

Sharma, 2011). The third step, identification of the protein, is performed by incubating the membrane with proteins with a primary antibody reactive to the protein of interest. Secondary antibodies which are labelled to allow detection via luminescence, are then added to interact with the first immobilized antibody, before the protein bands are detected by adding substrates that provide the luminescent signal used to detect the protein (Hnasko & Hnasko, 2015).

All imaging steps were performed using the ChemiDoc™ MP system and Image Lab™ software.

SDS-PAGE

Method

This assay was performed according to an optimised in-house protocol, based upon the method of Laemmli (1970). As described in Section 3.3.4, 600g supernatants were prepared and the protein concentrations were determined with BCA.

An SDS-PAGE gel was cast using a Mini-PROTEAN® Tetra Cell⁵⁷ and 10% TGX Stain-Free™ FastCast™ Acrylamide 1 Starter kit⁵⁸, according to the manufacturers' protocol. Briefly, 0.05% ammonium persulphate (APS)⁵⁹ and N,N,N',N'-tetramethylethylenediamine (TEMED)⁶⁰ were added to a stacking and 10% resolving solution, directly before casting to initiate polymerization of acrylamide. After polymerization of the gel, 1x running buffer consisting of 10x Tris/Glycine/SDS⁶¹ diluted with Milli-Q® water, was added to the module containing the gel. Samples for loading were prepared to contain 30 µg protein, 1x Laemmli sample buffer⁶² with 355 mM β-mercaptoethanol⁶³, and Milli-Q® water before being boiled for 5 min at 100°C. Samples and a molecular weight marker were loaded, at 20 µL into the wells of the gel before applying 200 V for approximately 25 minutes. The gel with separated proteins was imaged with a Stain-Free Gel protocol.

⁵⁷ Bio-Rad, CA, USA.

⁵⁸ Cat. #1610182 (Bio-Rad, CA, USA).

⁵⁹ Cat. #A3678 (Merck, Modderfontein, SA)

⁶⁰ Cat. #T7024 (Merck, Modderfontein, SA)

⁶¹ Cat. # 1610732 (Merck, Modderfontein, SA)

⁶² Cat. #1610737 (Bio-Rad, CA, USA).

⁶³ Cat. #M3148 (Merck, Modderfontein, SA)

Method

For western blots, a Mini TransBlot® Turbo Transfer Pack⁶⁴ with a 0.2 µm PVDF membrane and TransBlot® Turbo Transfer System⁶⁵ was used according to an optimised in-house protocol. Briefly, the blotting sandwich was assembled so that the gel was stacked on top of the PVDF membrane and between two layers of ion reservoir stacks. The blotting sandwich was placed in a cassette with cathodes at the top and anodes at the bottom. The separated proteins were electroblotted to the PVDF membrane for 30 min at a current of 1.0 A. Using the molecular marker⁶⁶ as a guide, the blot was cut between 50 and 37 kDa so that incubation of the control and test antibodies could be done separately, as a precautionary measure to avoid interference of antibodies. The blots were imaged using a Stain Free Blot protocol, before placing it in a blocking buffer (1x TBS with 1% casein)⁶⁷ for 90 min. A waving platform shaker was used to gently agitate the blots at 26 rpm during the subsequent incubation steps. The blots incubated overnight at 4°C with the primary antibodies in 1x TBS with 1% casein. The primary antibody for detection of GLYAT was a rabbit polyclonal antibody, reactive against rat GLYAT⁶⁸, in a 3:2500 dilution. The primary antibody for detection of the control protein, heat shock protein 60 (HSP60), was a rabbit polyclonal antibody⁶⁹, reactive to HSP60 in a 1:20 000 dilution.

The following day the blots were washed three times in washing buffer, consisting of 1x TBS⁷⁰ and 0.1% Tween20⁷¹, for five minutes at a time. The blots were then probed with secondary antibody for 90 min at a dilution of 1:10 000. The secondary antibody was a horse radish peroxidase-conjugated goat antibody⁷² reactive to rabbit and used for the GLYAT and HSP60 blots. Thereafter, the blots were washed again three times for five minutes at a time in washing buffer, before performing a five-minute washing step in final washing solution containing 1x TBS. The gel was incubated for one minute in Clarity™ Western ECL Substrate mix⁷³, which contained a 1:1 mix of peroxide and luminol enhancer. A Chemi Hi Resolution protocol was used to image the blot. Finally, the blot was rinsed with Milli-Q® water and air dried on Whatman Filter paper.

⁶⁴ Cat. #1704156 (Bio-Rad, CA, USA).

⁶⁵ Cat. #1704155 (Bio-Rad, CA, USA).

⁶⁶ Cat. #1610377 (Bio-Rad, CA, USA).

⁶⁷ Cat. #1610782 (Bio-Rad, CA, USA).

⁶⁸ Cat. #NBP1-54714 (Novus Biologicals, CO, USA).

⁶⁹ Cat. #ab46798 (Abcam, Cambridge, United Kingdom).

⁷⁰ Cat. #1706435 (Bio-Rad, CA, USA).

⁷¹ Cat. #822148 (Merck, Modderfontein, SA).

⁷² Cat. #ab97051 (Cambridge, United Kingdom).

⁷³ Cat. #1705060 (Bio-Rad, CA, USA).

3.6.2 GLYAT enzyme activity

Principle

The activity of GLYAT can be determined spectrophotometrically from a reaction consisting of benzoyl-CoA, glycine and DTNB. This assay is based on the principle of DTNB being reduced by the liberated thiol group of CoA to form TNB that absorbs at 412 nm, as described in Section 3.3.4.

Method

For this assay, 600g supernatants were used and protein content was determined as described in Section 3.3.4.

A 1 mM DTNB solution was prepared in 1 M Tris.HCl. In addition, 1 mM benzoyl-CoA and 1 M glycine (pH set to 8.0 with 0.5 M NaOH) was prepared in Milli-Q® water. The optimal volume of sample was determined to be 2 µL based on comparisons between specific activity and protein content comparisons.

Reaction mixtures for determining GLYAT activity are described in Table 3.8. DTNB, glycine, Milli-Q® water and homogenate were added to each well and incubated for 10 minutes, before the benzoyl-CoA was added. The linear rate increase (v_1) was recorded at 412 nm for 5 minutes at one-minute intervals. GLYAT activity was calculated with Equation 3.10 and normalised according to Equation 3.11. UCS was determined as described earlier (Section 3.3.4).

Table 3.8 Reagents for determining GLYAT activity

Reagent	1x (µL)	[end]
DTNB, Tris pH 8	20	0.1 mM
glycine	40	200 mM
Milli-Q® water	100	
Diluted homogenate (1:10)	20	2 µL
10 mM benzoyl-CoA	20	0.1 mM
Total	200	

Reaction mixtures to determine GLYAT activity contained DTNB, glycine, Milli-Q® water, sample, and benzoyl-CoA. *Abbreviations: 5,5'-dithio-bis(-2-nitrobenzoic acid) (DTNB).*

Equation 3.10 Determination of GLYAT activity

$$UGlyat = \frac{\overline{v1}}{\epsilon_{412}} \times \frac{V_r}{V_{Ef} \times \frac{C_P}{1000}}$$

where:

UGlyat is the GLYAT activity in $\mu\text{mol}/\text{min}/\text{mg}$

$\overline{v1}$ is the average linear rate increase of the triplicate wells

ϵ_{412} is the molar extinction coefficient for TNB as determined for 200 μL reactions (7 465 Abs/mM)

V_R is the reaction volume in mL (0.2 mL)

V_{Ef} is the effective sample volume added to the reaction (2 μL)

C_P is the protein concentration in $\mu\text{g}/\mu\text{L}$

Equation 3.11: Normalised GLYAT activity

$$\text{Normalised } UGlyat = UGlyat \times \frac{1000}{UCS}$$

where:

Normalised UGlyat is the activity of GLYAT normalised to the activity of CS in $\text{nmol}/\text{min}/\text{UCS}$.

3.7 Statistical analyses

The following section describes how statistical analyses were performed for enzyme activity and gene expression data. An overview of the procedure is shown in Figure 3.6.

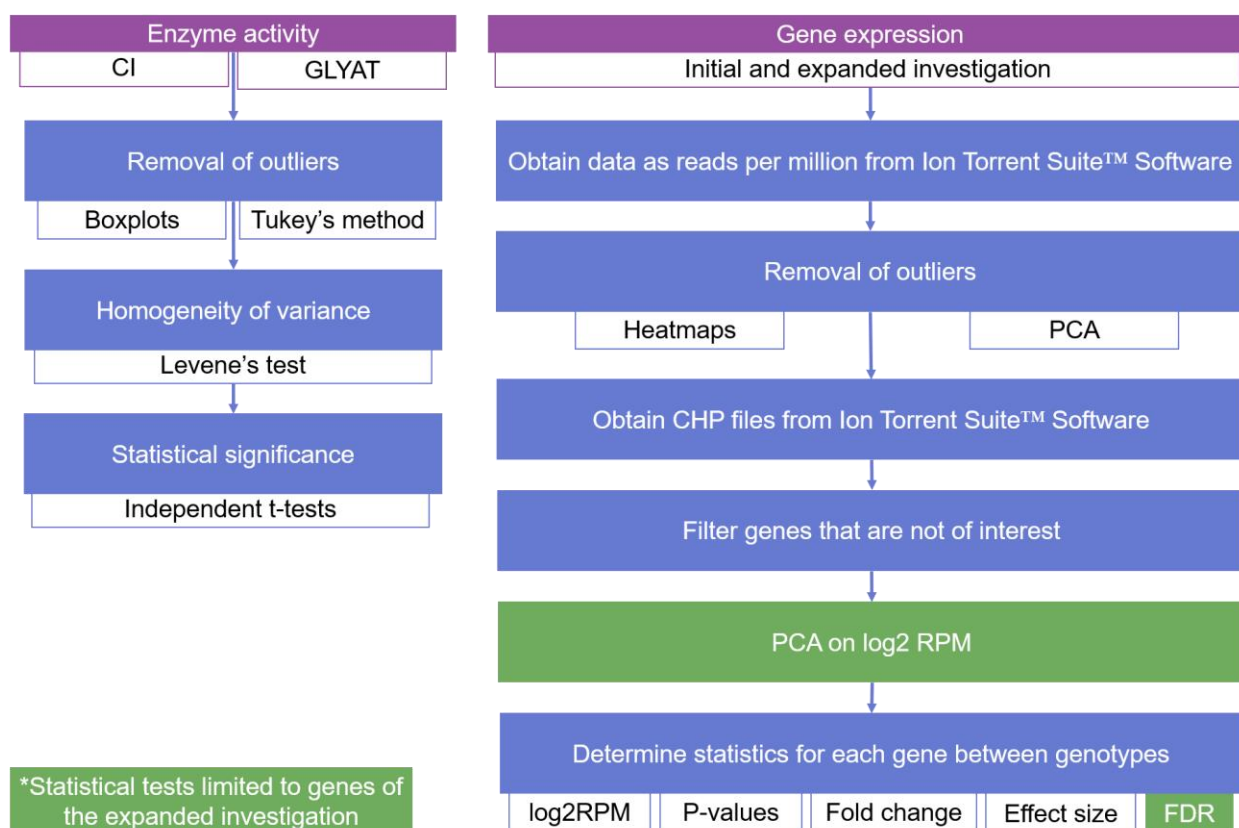


Figure 3.6 Schematic representation of statistical analyses performed

This figure gives an overview of the statistical analyses performed on enzyme activity and gene expression data. Abbreviations: chip (CHP), complex I (CI), false discovery rate (FDR), glycine N-acyltransferase (GLYAT), principal component analysis (PCA), reads per million (RPM).

3.7.1 Enzyme activity

Statistical analyses were performed using IBM SPSS^{®74} and Microsoft Excel^{®75}. Three technical and three biological repeats were analysed for each of the two genotypes. Technical and biological replicates of enzyme analysis data were visually inspected for prominent outliers via boxplots and Tukey's method and removed if detected. Enzyme activity for each biological replicate was calculated as the mean of the technical replicates. Activity of enzymes were normalised to activity of CS. (see Equation 3.1 to Equation 3.7 for CI activity and Equation 3.10 and Equation 3.11 for GLYAT activity). Levene's test was performed to test for homogeneity of variance between genotypes. Independent t-tests were performed to determine whether the changes between the two genotypes were statistically significant. P-values smaller than 0.05 were considered statistically significant.

⁷⁴ Version 27.0.0.0 (IBM Corporation, NY, USA)

⁷⁵ Microsoft Excel for Microsoft 365, Version 2008 (Microsoft Corporation, WA, USA)

3.7.2 Gene expression

Statistical analyses of gene expression data were performed using Ion Torrent Suite™ Software, MetaboAnalyst 5.0, Applied Biosystems™ Transcriptome Analysis Console (TAC)⁷⁶, and Microsoft Excel®. Chip (CHP) files containing gene-level information were downloaded from Torrent Suite™ Software after it performed primary and secondary data analysis (described in Section 3.5.5). These files provide information about the absolute normalised reads as log2 reads per million (RPM). Samples with very low reads as determined by the Torrent Suite™ Software were not included in further analyses. Heatmaps were plotted and principal component analysis (PCA) performed in MetaboAnalyst with log2RPM values to visually detect outlier samples.

CHP files for each sample, except outliers, were imported to TAC to determine the mean log2RPM per group, p-values and fold change for 23 930 genes. For the initial investigation only two genes, *Acsm2* and *Glyat* were analysed. For the expanded investigation, a list of genes involved in phase two biotransformation were determined from the literature. This list was further filtered to those present in the Ion AmpliSeq™ panel, resulting in a final list of 117 genes to be investigated. In TAC, genes that were not of interest were filtered out and a comma delimited (CSV) file containing the mean log2RPM for each sample, p-values, and fold change were exported. TAC calculated the bi-weight average for each group using Tukey's bi-weight average of the expression levels. The one step Tukey's bi-weight method gives an estimate amount of variation in the data, similar to how standard deviations estimates the amount of variation for an average (Affymetrix, 2007). Fold changes were calculated in TAC. Equation 3.12 a) shows the equation used to calculate fold change of upregulated genes, while Equation 3.12 b) was used to calculate fold change for downregulated genes. Effect sizes were calculated in Microsoft Excel® with Equation 3.13.

For the expanded investigation, other statistical tests were also performed. PCA was performed on the log2RPM data, using MetaboAnalyst. Multiple testing correction was calculated for the selected group of genes (Li *et al.*, 2008). False discovery rate (FDR) adjusted p-values were calculated in Microsoft Excel®. FDR p-values were calculated using the Benjamini Hochberg Step-Up FDR-controlling procedure (Benjamini & Hochberg, 1995). Genes with effect sizes >0.8 and p-values <0.05 were considered statistically significant. Lastly, log2 standard deviations were adjusted to proportional standard deviations according the procedure described by Livak and Schmittgen (2001), and is shown in Equation 3.14.

⁷⁶ Version 4.0.2.15 (Applied Biosystems, MA, USA)

Equation 3.12: Fold change

a)

$$\text{Fold change} = 2^{\text{Bi-weight Avg KO signal (log2)} - \text{Bi-weight Avg WT signal (log2)}}$$

b)

$$\text{Fold change} = (2^{\text{Bi-weight Avg WT signal (log2)} - \text{Bi-weight Avg KO signal (log2)}}) - 1$$

Equation 3.13: Effect size

$$\text{Effect size} = \frac{\log 2\text{RPM}_{\text{KO avg}} - \log 2\text{RPM}_{\text{WT avg}}}{\text{standard deviation}}$$

Equation 3.14: Additive formula for error propagation

$$\delta Q = \sqrt{(\delta a)^2 + (\delta b)^2 + \dots + (\delta y)^2 + (\delta z)^2}$$

where:

a, b are positive measurements

y, z are negative measurements

δ is the error associated with each measurement

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Introduction

This chapter, structured into four parts, will present and discuss the results obtained during this study. The first part will present and discuss disease model validation and sample quality evaluation data. This entails validation of the animal model and quality evaluation of the isolated RNA and sequencing data. The second part presents the gene expression, protein confirmation and enzyme activity results obtained for the genes involved in the glycine conjugation pathway. The third part continues to the gene expression results of remaining genes involved in the phase two biotransformation pathway. The chapter concludes with part four that summarises and discusses the results, in the context of the research questions posed for this study.

4.2 Part 1: Disease model validation and sample quality evaluation

4.2.1 Introduction

To ensure the quality of samples analysed and the results obtained, quality control measures were put in place during various time points of data collection. The first quality control measure was to ensure the validity of the *Ndufs4* mouse model by confirming the genotype of the mice and assessing the enzyme activity of CI in WT and KO mice (Section 4.2.2). The second quality control measure was to evaluate the quality of the RNA isolated from mouse tissue with three distinct measurements (Section 4.2.3):

- Spectrophotometric measurements to determine RNA concentrations and purity of RNA samples.
- Fluorometric measurements to more specifically determine RNA concentrations of RNA samples.
- Agarose gel electrophoresis to evaluate the integrity of RNA in samples.

The third and final data quality control measure was to assess the quality of the gene expression data (Section 4.2.4).

4.2.2 Disease model validation

To validate the genotype of mice, DNA was isolated from tail snips and amplified with standard PCR containing a forward and reverse primer pair. The sizes of the resultant products were determined by separation with agarose gel electrophoresis, and imaging with the ChemiDoc™ MP system and Image Lab™ software. *Ndufs4*^{-/-} samples were expected to have one observed

band at 429 bp, *Ndufs4*^{+/+} samples were expected to have one band observed at 1229 bp and the *Ndufs4*^{+/-} samples were expected to have both those bands. A non-specific band at 831 bp was sometimes present in *Ndufs4*^{+/-} samples. Figure 4.1 is an electrophoresis image of typical results obtained with genotyping. The genotype of all samples was confirmed before use in any of the analyses which serve to validate the model.

A table containing the age, sex and genotype of the animals used in this study is shown in Table 3.2. Eight WT and eight KO male mice between the ages of 46-51 days were used for transcriptomic analysis, six thereof were also used for SDS-PAGE and western blot analysis. Six more WT and KO mice were used for enzyme activity analysis.

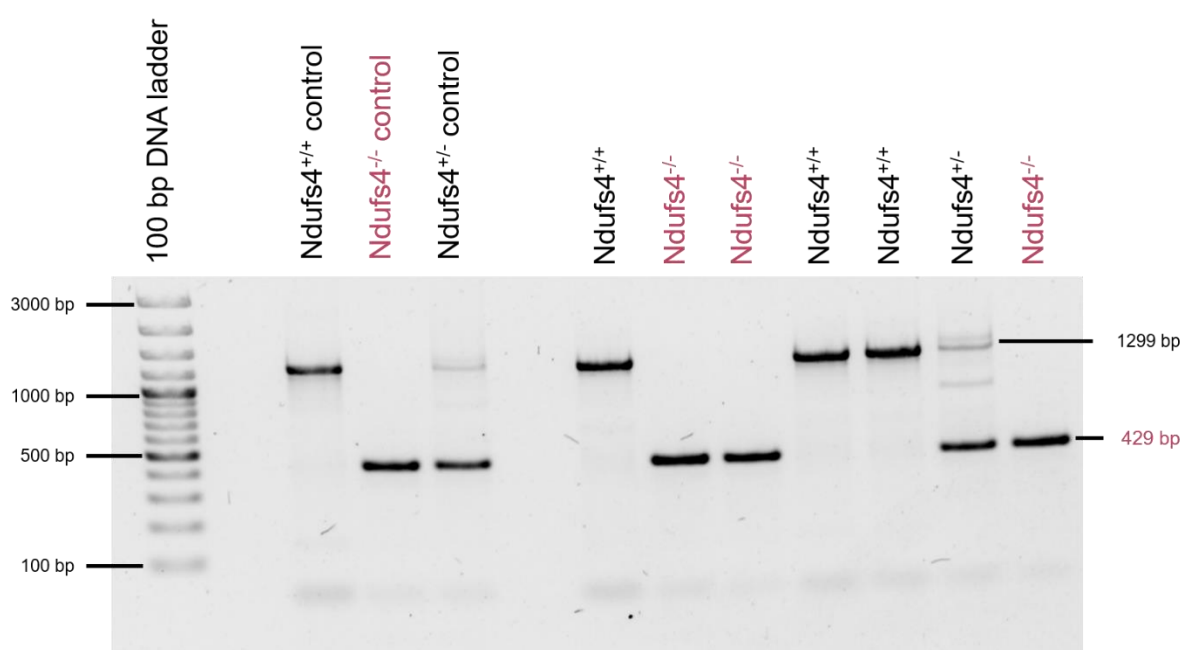


Figure 4.1 Electrophoresis image showing the DNA bands obtained during genotyping

This image shows typical results obtained after PCR amplification and EtBr-stained gel electrophoresis of isolated DNA. The DNA ladder allowed relative quantification of the size of the bands of the samples. Three controls, one for each genotype was also loaded on to a gel. *Ndufs4*^{-/-} samples were expected to have one band at 429 bp (highlighted in pink), *Ndufs4*^{+/+} samples were expected to have one band at a 1229 bp and the *Ndufs4*^{+/-} samples were expected to have both those bands. In some instances, a non-specific band at 831 bp also forms for *Ndufs4*^{+/-} samples.

To confirm a defective OXPHOS caused by the knockout of the *Ndufs4* gene, the maximal activity of CS and mitochondrial CI was determined in the liver samples of WT and KO mice. Three technical and three biological repeats were analysed for each genotype, and CI activity was normalised to activity of CS (see Section 3.3.4). For CI activity, equal variances could be assumed, and an independent samples t-test was performed. Results for the enzyme activity of CI is shown in Table 4.1 and Figure 4.2.

Table 4.1 Enzyme activity of CI in liver normalised to CS for WT and KO mice

	WT mean $\pm$ SD	KO mean $\pm$ SD	p-value
CS ($\mu\text{mol}/\text{min}/\text{mg}$)	0.16 ± 0.01	0.22 ± 0.01	<0.01
CI ($\text{nmol}/\text{min}/\text{UCS}$)	123 ± 9.20	33 ± 11.23	<0.001

Enzyme activities for CS and CI of WT (n=3) and KO (n=3) mice normalised to protein content and CS, respectively. Enzyme activities are shown as the mean activities $\pm$ SD. Levene's Test showed that equal variances could be assumed. Statistical significance between WT and KO samples was determined with an independent samples t-test. *Abbreviations: complex I (CI), citrate synthase (CS), knockout (KO), standard deviation (SD), $\mu\text{mol}/\text{min}/\text{mg}$ citrate synthase activity (UCS), wild-type (WT).*

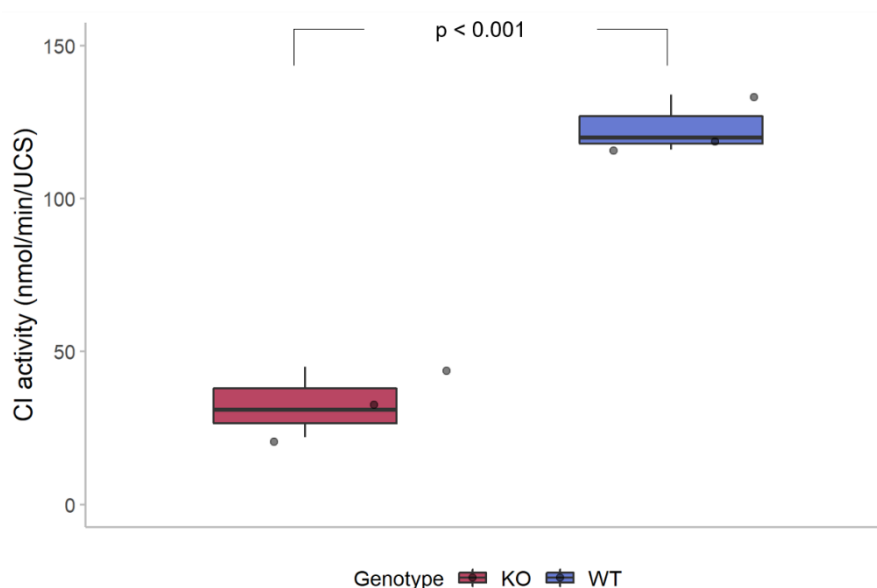


Figure 4.2 CI activity of WT and KO mouse liver samples normalised to CS activity

KO mice had significantly decreased CI activity, compared to WT mice, $p < 0.001$. *Abbreviations: complex I (CI), citrate synthase (CS), knockout (KO), $\mu\text{mol}/\text{min}/\text{mg}$ citrate synthase activity (UCS), wild-type (WT).*

As expected, CI activity was significantly (~73%) lower in the liver samples of KO mice compared to that of WT mice (33 vs 123 nmol/min/UCS, $p < 0.001$). Decreased CI activity in KO has been previously shown (Calvaruso *et al.*, 2012; Jin *et al.*, 2014; Kruse *et al.*, 2008). These results confirm a deficient CI and validates the disease model.

The genotype of all mice used in further analyses were confirmed with PCR and agarose gel electrophoresis as either WT or KO before use in any analysis.

4.2.3 RNA quality control

As a second measure to ensure good quality data, the quality of the isolated RNA was determined by measuring its concentration and purity using a NanoDrop™ One spectrophotometer. RNA concentrations ranged between 25.4 and 159.6 ng/ μL . Purity of RNA was assessed through the

A260/A280 ratio. Samples were deemed pure if they had a A260/A280 ratio between 1.8 and 2.5. NanoDrop™ values for each sample can be found in Appendix C.

RNA concentration was further evaluated using a Qubit® Fluorometer. Fluorometric measurements are more specific than spectrophotometric measurements since it can distinguish between RNA and DNA and thereby do not overestimate the concentration of RNA. For all samples, RNA concentrations measured with a Qubit® Fluorometer ranged between 19.2 - 266.7 ng/μL. Complete results are shown in Appendix C.

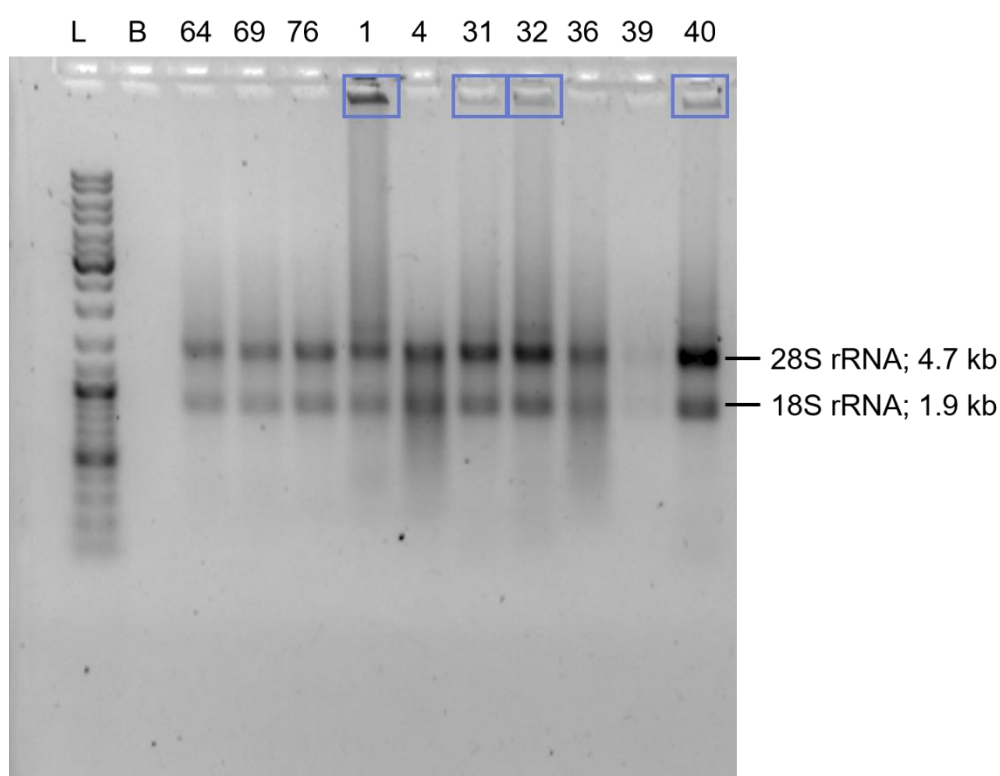


Figure 4.3 Representative image of gel from bleach agarose electrophoresis used to assess RNA integrity

This is a representation of typical results obtained for a bleach agarose gel electrophoresis. The number above each row indicates the sample number. A DNA ladder was used as a control to ensure the electrophoresis did take place in the case of no samples showing any bands and would therefore not correlate with the sizes of the RNA bands. Sample 76 shows the 28S:18S intensity ratio of 2:1 of the top and bottom band which indicated good quality RNA. Contamination is indicated in samples 1, 31, 32, and 40 by the bright bands at the top of the well (blue blocks) and the bright smears from the wells to the 28S bands. *Abbreviations: blank (B), ladder (L), ribosomal RNA (rRNA).*

Ensuring that RNA samples are not degraded and of high quality is cardinal to RNA-based research. RNA integrity can be assessed with highly sensitive technologies like the 2100 Bioanalyzer or with more cost-effective options like bleach agarose gel electrophoresis (Aranda *et al.*, 2012). Isolated RNA samples were analysed on agarose bleach gels to assess their integrity and purity. The presence of two distinct bands as described in Section 3.4.4 on the gel suggested high quality RNA. Smeared bands or a 28S:18S band intensity below ~2:1 indicated

Total bases

The number of filtered and trimmed base pairs.

ISP loading density

The percentage of addressable wells on the chip that contain Ion Sphere™ particles.

Total reads

Total number of trimmed and filtered reads, independent of length.

Read lengths and histogram

The length of called reads in base pairs. The histogram reflects the size distribution of the library without the Ion adapter sequences.

Key signal

The average signal for ISPs that match the library key. A higher starting key signal signifies a lower impact of signal droop and less 3' trimming.

poor quality RNA possibly due to RNA degradation, and the samples were then not used for downstream applications. Bright bands at the top of the wells indicated gDNA contamination or other impurities like proteins and carrier molecules (Thermo Fisher Scientific.). A representative gel is shown in Figure 4.3.

The samples with the highest quality were chosen for RNA-sequencing, resulting in all samples used having RNA concentrations >18 ng/ul, A260/A280 ratios >1.8 and 28S:18S ratios at 2:1 or above.

In conclusion, three measures were put in place to assess the quality of RNA before using it to synthesise cDNA. RNA concentration was measured with two distinct methods, RNA purity was determined with the A260/A280 ratios and integrity was assessed with bleach agarose gel electrophoresis. Samples that did not meet the acceptable ranges, were not used for further applications.

4.2.4 Gene expression data quality control

There are several bioinformatic tools that can be used to analyse RNA-sequencing data. Some of these tools are specifically designed to monitor the quality of the data and could be platform specific. Popular quality control checks for raw reads include analysis of GC content, and detection of overrepresented k-mers and duplicate reads. A good indicator of sequencing accuracy and the presence of gDNA contamination is the percentage of mapped reads, which can be monitored during read alignment. Reproducibility of sequencing data is sometimes checked among technical repeats. Determination of reproducibility among biological

repeats is more challenging, but can usually be monitored through PCA (Conesa *et al.*, 2016). These types of quality control practices can be challenging especially considering the high level of bioinformatics skill required. The use of an Ion AmpliSeq™ panel, a whole transcriptome, targeted gene quantification methodology, is another way to overcome many of the complexities of RNA-sequencing (Li, Turner, *et al.*, 2015). The Ion Torrent platform gives a report on the quality

control metrics at the end of a sequencing run. The quality of an Ion AmpliSeq™ sequencing run can be evaluated by six metrics: **Total bases**, **ISP loading density**, **total reads**, **read lengths**, **read length histogram**, and **key signal** (Thermo Fisher Scientific., 2019). An example of the output of these metrics is shown in Figure 4.4.

The 16 liver samples were analysed on two Ion 540™ chips. Total bases ranged from 8.22 - 8.7G. The percentage of wells on the chips that contained ISPs were 89% and 92%, respectively. Each chip had an average coverage of >1000x and >80 million reads (10 – 15 million reads per sample). The aligned reads accounted for 99% of the total reads. The total number of bases over all positions that align with an error rate of $\leq 2\%$ (AQ17) were 7.4 -7.8 billion bases. The mean read length for both chips was 102 bp and the read length histogram showed good distribution.

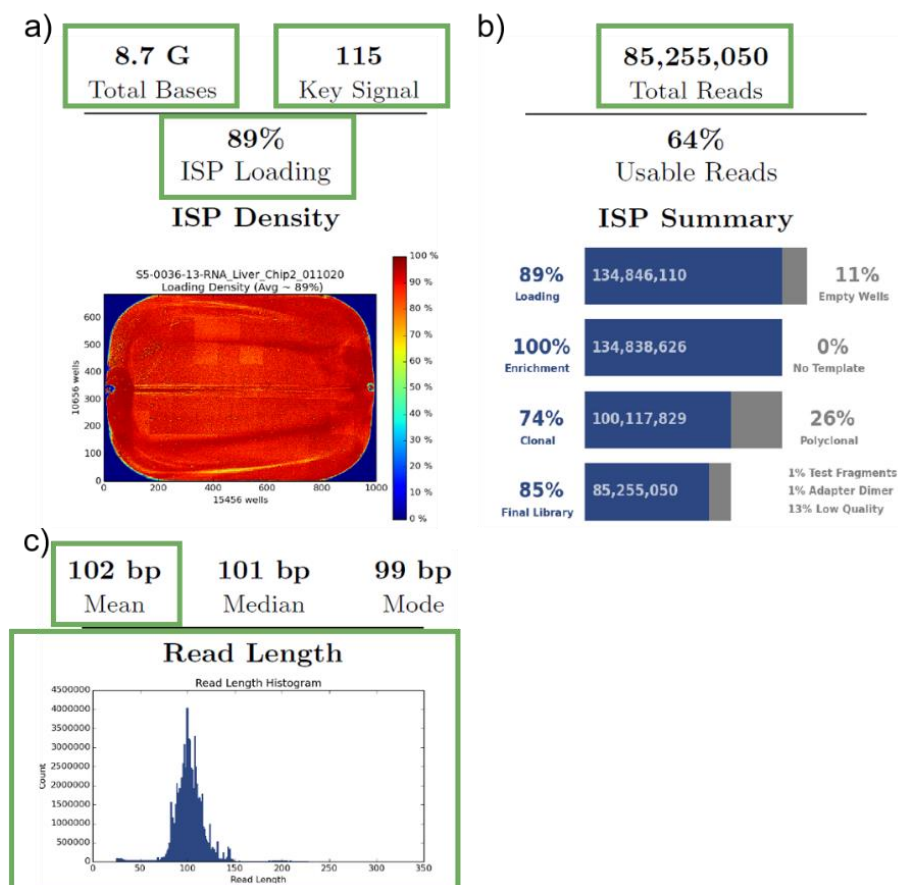


Figure 4.4 An example of some of the sequencing quality control metrics

Several sequencing quality control measures that are given in the Ion Torrent report are shown and highlighted in the green boxes. They include chip loading, total bases, key signal, read length, and total reads.

Two KO samples had very low quality data, with less than 630 000 reads per sample, and were thus removed from the group. Furthermore, visual inspection of a heatmap and PCA plot showed three additional outliers that consisted of two WT samples and one KO sample (see Figure 4.5).

The two WT samples were numbers 101 and 102, while the KO sample was number 121. WT sample 102 grouped with the KO samples, while WT sample 101 had clear differences in comparison to the other WT samples. The PCA plot also shows that sample 121 is an outlier (see Appendix D for a more complete demonstration of these differences). These three samples were removed before downstream analyses and the final two groups consisted of five KO samples and six WT samples.

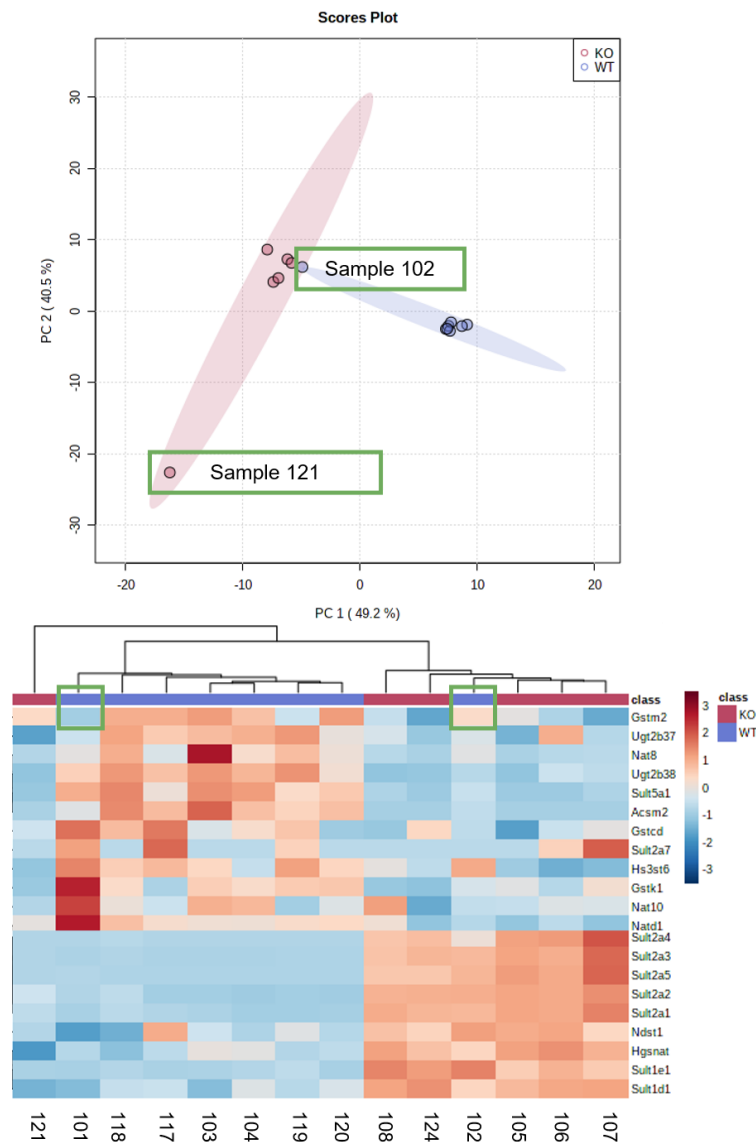


Figure 4.5 2D PCA scores plot and heatmap before outlier removal

a) The 2D PCA plot of 117 genes shows good separation between the WT and KO groups, especially on PC1 which explained ~50% of the variance between the two groups. The PCA plot also shows that samples 102 and 121 are outliers. Note that six KO and eight WT samples are shown and that some dots are overlapping. b) The heatmap and dendrogram for all samples show two obvious outliers. WT sample 102 grouped with the KO samples, while WT sample 101 had clear differences in comparison to the other WT samples. Abbreviations: knockout (KO), principal component (PC), principal component analysis (PCA), wild-type (WT).

4.2.5 Summary and conclusions

The genotypes of all samples were confirmed as either WT, HET, or KO before use in downstream applications. CI activity was significantly reduced in *Ndufs4* KO mice (33 vs 123 nmol/min/UCS, $p < 0.001$), although not completely absent. This is in line with the findings of Calvaruso *et al.* (2012) that revealed formation of respiratory supercomplexes allow formation of active CI in these mice.

The concentrations of RNA ranged between ~25-160 ng/ μ L and was considered sufficient for further analysis. The NanoDrop™ One spectrophotometer has some limitations including overestimation of concentrations due to the instrument not being able to distinguish between double-stranded and single-stranded nucleic acids. Therefore, RNA concentrations were also measured fluorometrically. These concentrations ranged between ~19-267 ng/ μ L and was considered acceptable for downstream analysis. Only samples that presented with a 28S:18S intensity ratio of 2:1, no significant smears and no gDNA contaminations when assessed with agarose gel electrophoresis, were selected for further analyses. Lastly, quality control is an important step of gene expression analyses (Li, Nair, *et al.*, 2015). Several aspects of the data were considered during quality control. These include total bases, ISP loading density, total reads, read lengths and key signal. Two KO samples were excluded because of low quality data. Another three samples were excluded as statistical outliers. Therefore, the data for five KO and six WT samples were of sufficient quality to continue with downstream data analyses.

4.3 Part 2: Phase two biotransformation: the glycine conjugation pathway

4.3.1 Introduction

Glycine conjugation is a principle amino acid conjugation pathway that converts endogenous and xenobiotic metabolites to more hydrophilic conjugates that can be excreted in the urine. The glycine conjugation pathway was chosen as initial investigation point because it specifically takes place in mitochondria and, therefore, is a simple pathway for investigation of phase two biotransformation in a model of mitochondrial disease. The glycine conjugation pathway requires two enzymes, *Acs2* and *Glyt1* (Rohwer *et al.*, 2021). The expression of both genes was analysed as a starting point to investigate the effect of a mitochondrial deficiency on biotransformation in the liver of an *Ndufs4* KO mouse model.

4.3.2 Gene expression of the glycine conjugation pathway

The RNA-sequencing data of five KO and six WT mice was statistically analysed using the Applied Biosystems™ Transcriptome Analysis Console (TAC) platform, Microsoft Excel® and

MetaboAnalyst. Both *Acsm2* and *Glyat* showed significantly decreased expression in liver samples of KO mice compared to WT mice (see Figure 4.6 and Table 4.2), suggesting downregulation of the glycine conjugation pathway in KO mice.

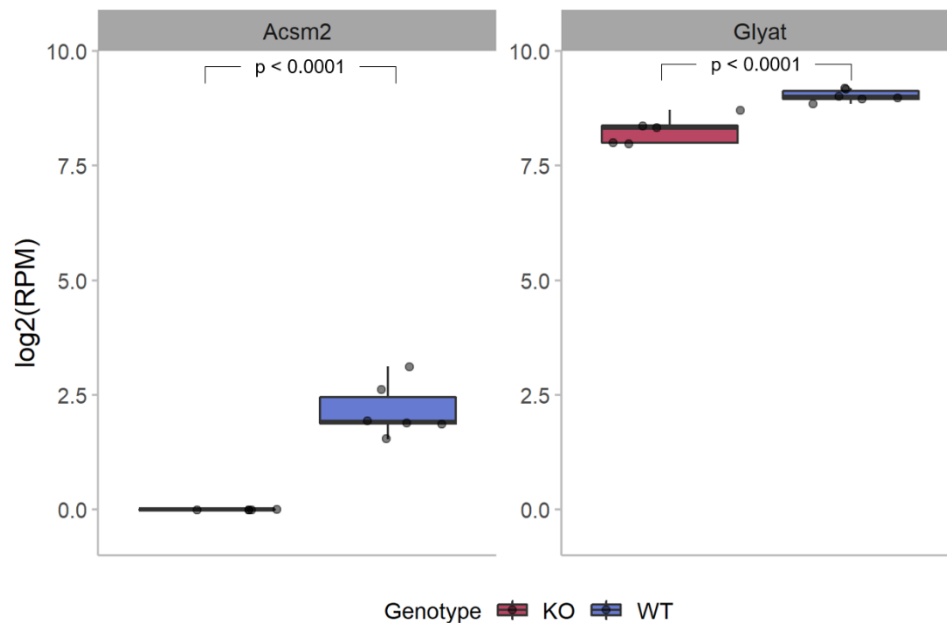


Figure 4.6 Boxplots showing log2RPM of glycine conjugation pathway genes between KO and WT samples

KO mice had no expression of *Acsm2* in contrast to WT mice (FC = -3.68, p-value <0.0001). *Glyat* had reduced expression in KO mice compared to WT mice (FC = -1.67, p-value <0.0001). Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).

The fold change analysis is useful to compare the changes between the averages of two groups. Fold change values for genes involved in glycine conjugation is shown in Table 4.2. Fold changes of -2 or 2 are generally accepted as differentially expressed, although this value is largely disputed and chosen arbitrarily (Braeuning *et al.*, 2006; Laurent *et al.*, 2013). For this study, genes that were statistically significantly expressed according to p-values and effect size, even with fold changes between -2 and 2, were kept in the list, as it was still significantly altered. *Acsm2* was substantially and significantly downregulated in *Ndufs4* KO mouse liver (FC = -3.68, p-value <0.0001), while *Glyat* showed a slighter yet statistically significant downregulation (FC = -1.67, p-value <0.0001).

Table 4.2 Mean log2RPM, fold change and statistical significance values of glycine conjugation genes in KO vs WT samples

Gene	Mean log2RPM		Fold change (FC)	p-value	Effect size
	KO	WT			
<i>Acsm2</i>	0.00	1.88	-3.68	1.88 x10 ⁻⁵	-3.19
<i>Glyat</i>	8.28	9.08	-1.67	4.00 x10 ⁻⁴	-2.39

Mean log2 (RPM), fold change, p-values, and effect size values of the glycine conjugation pathway genes. *Acsm2* and *Glyat* expression showed significant downregulation in KO samples. *Abbreviations: knockout (KO), reads per million (rpm), wild-type (WT).*

The transcriptomic data showed significant changes in the glycine conjugation pathway. Expression of both the *Acsm2* and *Glyat* genes were decreased in the KO mice, but suggestions that this pathway is downregulated in this mouse model should be made with caution at this stage. It is well known that abundance of messenger RNA (mRNA) only partially predicts abundance of protein (Vogel & Marcotte, 2012). In fact, the correlation between mRNA and protein abundance seems to lie at around 40%. This medium correlation is largely the result of several biological factors including post-transcriptional and post-translational regulation, and to a lower extent due to methodological constraints or experimental noise (Maier *et al.*, 2009; Taylor *et al.*, 2013; Vogel & Marcotte, 2012). The rate of production and degradation of mRNA and proteins largely determine the abundance of the molecules. The rate of mRNA production is much lower than proteins (two copies mRNA per hour versus dozens of copies protein per mRNA per hour). The half-life of mRNAs is also decreased compared to proteins (~3-7 hours versus ~46 hours). Furthermore, the regulation of protein abundance is dependent on the role of the protein. Housekeeping or structural proteins would have a longer lifetime than regulatory proteins that have to ramp up their production or degradation in reaction to a stimulus (Vogel & Marcotte, 2012). Considering this medium mRNA-protein correlation, it is crucial to validate gene expression results at a protein level.

4.3.3 Protein-level investigation of the glycine conjugation pathway

To verify if the changes observed in gene expression translated to protein level, SDS-PAGE and western blots were used to assess the relevant concentration of GLYAT in KO mice compared to WT mice. Ideally, ACSM2 protein levels would also be assessed, but due to time and financial constraints only GLYAT was analysed during this study. As discussed in Section 3.6.1, 600g supernatants of three mouse livers per genotype were used in SDS-PAGE and western blots. The supernatants were separated by SDS-PAGE, electroblotted to a PVDF membrane, and probed with primary antibodies reactive to GLYAT and to HSP60 (a mitochondrial housekeeping protein) that served as a control. After incubation with a secondary antibody, the bands were

detected through chemiluminescence and Image Lab™ software. Bands that corresponded to the expected molecular weight of the two proteins were identified by comparing them to a molecular weight marker that was also subjected to electrophoresis with the samples. The HSP60 protein is 60kDa and GLYAT is 34kDa.

Results are shown in Figure 4.7. Several issues hindered obtaining conclusive results. The first issue is that the control protein, HSP60, was detected only in five of the six samples. As the intensity of GLYAT is quantified relative to HSP60, it could not be done for the KO sample that did not show HSP60. This sample did show expression of GLYAT, thereby excluding the possibility that no sample was loaded (also confirmed by examining SDS-PAGE gel, see Appendix E). It might have been caused by a bubble at ~60 kDa impeding transfer of proteins to the membrane, or by poor binding of the antibodies to this sample. The second and more problematic issue was that GLYAT antibodies reactive to mice could not be acquired from distributors in South-Africa. It was therefore decided that an antibody reactive to rats would be the next best solution. GLYAT was detected in all six samples, but quantification was further hampered because the intensity of GLYAT was very low. This is likely due to the fact that the primary antibody used for detection of GLYAT is reactive to rats, and not specifically to mice. Furthermore, it seemed that although HSP60 is a control protein, it was not expressed similarly between WT and KO mice. HSP60 expression appears to be increased in KO samples. One obvious reason is inconsistent loading of samples. It is possible that the protein concentration of samples was wrongly determined and led to the amount of protein actually loaded to the SDS-PAGE gel not being 30 µg. Pipetting errors during preparation of the samples used for loading and the actual loading could also result in inconsistent loading. Inconsistent loading is further supported by differences in expression of HSP60 between the three WT samples, and between the three KO samples, and finally confirmed by analysis of the image of the SDS-PAGE gel which showed clear differences in protein levels between samples (see Appendix E).

Protein expression of GLYAT was not further investigated due to time constraints, therefore the results remain inconclusive.

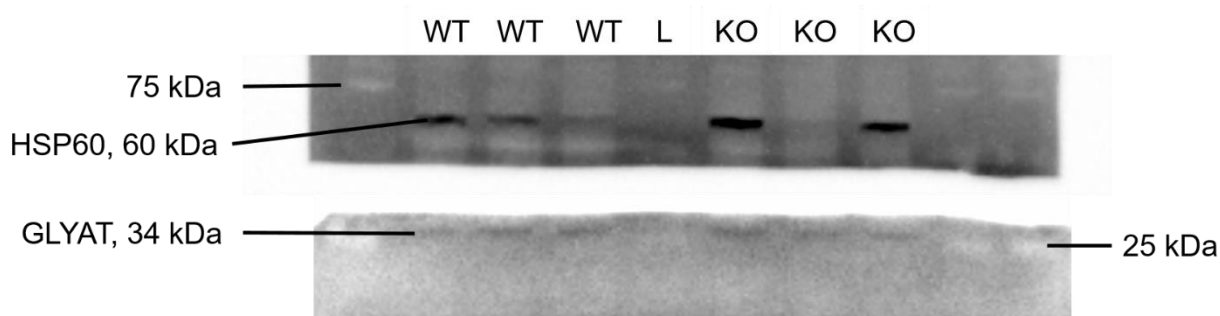


Figure 4.7 Western blot of HSP60 and GLYAT

HSP60 and GLYAT were detected in WT and KO samples. Bands of the molecular weight marker could be seen at 75 kDa and 25 kDa and were used as a reference to ensure the bands seen are HSP60 at 60 kDa and GLYAT at 34 kDa. *Abbreviations: knockout (KO), molecular weight marker (L), wild-type (WT).*

4.3.4 Enzyme activity of glycine conjugation pathway

Activity of the GLYAT enzyme was measured to see if the changes observed in gene expression would translate to an enzymatic level. The activity of GLYAT was spectrophotometrically determined in three technical and biological repeats as described in Section 3.6.2 and normalised to activity of CS. Results are shown in Table 4.3 and Figure 4.8.

Gene expression results from Section 4.3.2, showed decreased expression of *Glyat* in KO mice, suggesting that enzyme activity might also be reduced in KO samples. As anticipated, normalised activity of the GLYAT enzyme in liver samples were significantly (~50%) lower in KO mice compared to WT mice (36.2 vs 75.9 nmol/min/UCS, $p < 0.001$). This serves as a further indication that the glycine conjugation pathway is less active in *Ndufs4* KO mice than in WT mice.

Table 4.3 Enzyme activity of GLYAT in liver normalised to CS for WT and KO mice

	WT mean $\pm$ SD	KO mean $\pm$ SD	p-value
GLYAT activity (nmol/min/UCS)	75.9 $\pm$ 10.2	36.2 $\pm$ 5.2	<0.001

Enzyme activity for GLYAT of WT (n=3) and KO (n=3) mice normalised to CS. Enzyme activities are shown as the mean activities $\pm$ SD. Levene's Test showed that equal variances could be assumed. Statistical significance between WT and KO samples was determined with an independent t-test. *Abbreviations: citrate synthase (CS), knockout (KO), standard deviation (SD), μ mol/min/mg citrate synthase activity (UCS), wild-type (WT).*

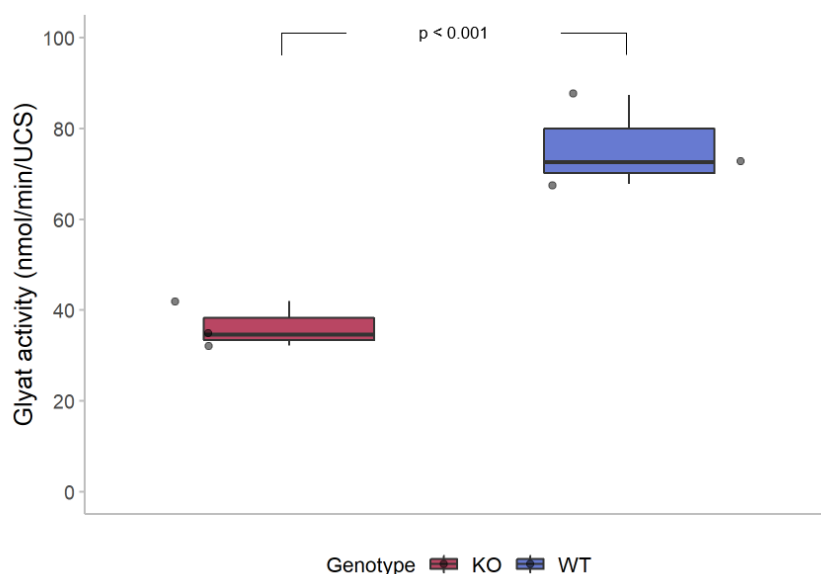


Figure 4.8 GLYAT activity of WT and KO mouse liver samples normalised to CS

The normalised activity of GLYAT was significantly lower in KO mice compared to WT mice. (p-value <0.001). Abbreviations: citrate synthase (CS), knockout (KO), $\mu\text{mol/min/mg}$ citrate synthase activity (UCS), wild-type (WT).

4.3.5 Summary and conclusions

Gene expression of enzymes involved in glycine conjugation was investigated between *Ndufs4* KO and WT mice. This served as an initial investigation of phase two biotransformation of a primary, mitochondrial contained pathway. Gene expression and enzyme activity investigations of glycine conjugation in *Ndufs4* mice liver samples indicated significantly decreased activity of this pathway in KO mice. Protein expression results were inconclusive, mainly due to issues in obtaining primary antibodies reactive to mice. This downregulation of glycine conjugation is likely due to limited ATP supply in these mice that cannot provide the ATP required in these pathways (further discussed in Section 4.5, below). Since several other phase two biotransformation pathways are also ATP-dependent (discussed in Section 2.3.2), the question remains if these pathways would also be affected by the *Ndufs4* KO. To determine if these changes are reflected in the remaining phase two biotransformation pathways, gene expression analyses was expanded to seven other phase two biotransformation pathways.

4.4 Part 3: Phase two biotransformation: remaining conjugation pathways

4.4.1 Introduction

Phase two biotransformation consists of conjugation reactions resulting in metabolites with increased water solubility that can easily be excreted. The main types of phase two reactions include acetylation, amino acid conjugation, glucuronidation, glutathione conjugation, methylation

and sulfoconjugation. The enzymes that are mainly involved in these reactions are N-acetyltransferases (NATs), acyl-CoA synthetases, amino acid acyltransferases, UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), methyltransferases, and sulfotransferases (SULTs) (Jancova *et al.*, 2010).

4.4.2 Gene expression of remaining conjugation pathways

Literature review of phase two biotransformation pathways revealed involvement of the genes in the remaining phase two pathways. Next, this list was limited to include the genes present in the Ion AmpliSeq™ panel used for gene expression analysis, resulting in a final list of 117 genes that could be further investigated. Details of the genes identified from literature and included in the panel are available in Appendix F.

PCA was performed on the log2 transformed RPM data. PCA is an unsupervised method that is used to reduce the dimension of datasets while maintaining as much of the statistical information as possible (Jolliffe & Cadima, 2016). Figure 4.9 shows the 2D PCA scores plots for selected phase two biotransformation genes of *Ndufs4* KO and WT mice. Samples naturally grouped together in accordance with their genotype and the two genotypes showed good separation. PC1 and PC2 respectively explained 82.7% and 5.7% of the variation, suggesting that the variation in gene expression data were mostly contributed by genotype-related variation. KO samples were more spread out than the WT samples, showcasing the higher levels of variation in the KO group.

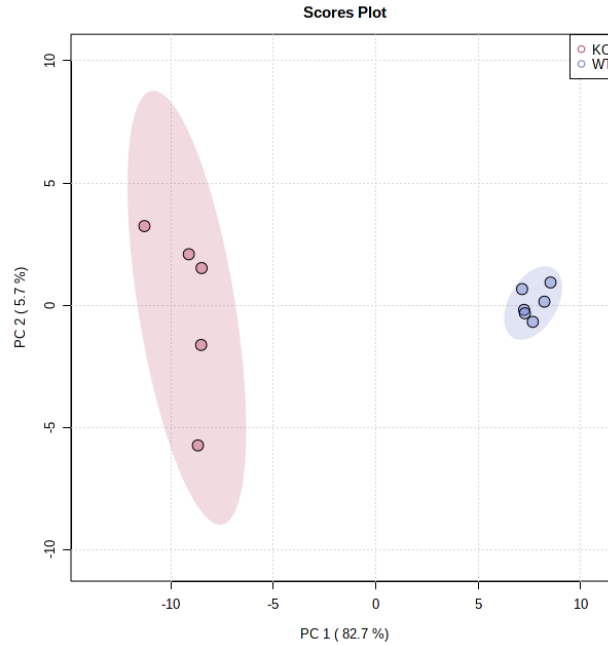


Figure 4.9 2D scores plot of WT and KO mice liver samples with phase two biotransformation gene expression

There was good separation between the two genotypes with PC1 accounting for most of the variation at 82.7%. *Abbreviations: two-dimensional (2D), knockout (KO), principal component (PC), wild-type (WT).*

The top 50 features according to effect sizes ($d > 0.8$) and p-values ($p < 0.05$) were identified. Fold changes and Benjamini-Hochberg FDR adjusted p-values were also calculated. Results are shown in Table 4.4 and Figure 4.10 to Figure 4.16. Genes that showed decreased expression in KO mice compared to WT mice, were found in all seven enzyme groups investigated. Several genes showed increased expression in KO mice compared to WT mice and included genes from the following enzyme groups: N-acetyltransferases, acyl-coenzyme A synthetases, amino acid acyltransferases, glutathione S-transferases, and sulfotransferases. The fold changes for genes that showed increased expression in KO mice ranged between 1.1 - 585.1. Fold changes for genes showing decreased expression in KO mice, ranged between -1.3 to -15.8. For the top 50 features, p-values ranged between 5.08×10^{-9} to 0.05, while Benjamini-Hochberg corrected p-values ranged between 5.94×10^{-7} and 0.11. Effect size values were mostly very far from the cut-off value of 0.8/-0.8 and ranged between -0.86 and -4.85 for genes showing decreased expression in KO mice, and ranged between 0.94 and 11.78 for genes showing increased expression in KO samples.

Table 4.4 Details of top 50 genes identified by significant p-values and effect sizes

Upregulated genes						
Gene	Name of protein	Enzyme group	Fold-change (FC)	p-value	FDR	Effect size D-value
<i>Gnpnat1</i>	glucosamine 6-phosphate n-acetyltransferase	N-acetyltransferases	1.32	0.02	0.06	1.00
<i>Hgsnat</i>	heparan-alpha-glucosaminide n-acetyltransferase	N-acetyltransferases	2.08	4.04 x10 ⁻⁵	3.38 x10 ⁻⁴	3.79
<i>AcsI3</i>	long-chain-fatty-acid-CoA ligase 3	acyl-CoA synthetases	2.64	0.03	0.08	1.77
<i>Acsm3</i>	acyl-coenzyme A synthetase acsm3, mitochondrial	acyl-CoA synthetases	1.73	0.03	0.07	1.93
<i>Dgat2</i>	diacylglycerol o-acyltransferase 2	amino acid acyltransferases	1.37	0.05	0.11	1.10
<i>Gstt2</i>	glutathione S-transferase theta-2	glutathione S-transferases	1.53	0.02	0.05	1.17
<i>Mgst3</i>	microsomal glutathione S-transferase 3	glutathione S-transferases	2.92	3.00 x10 ⁻⁴	1.95 x10 ⁻³	3.10
<i>Chst2</i>	carbohydrate sulfotransferase 2	sulfotransferases	1.82	0.05	0.11	1.26
<i>Gal3st1</i>	galactosylceramide sulfotransferase	sulfotransferases	1.75	1.90 x10 ⁻³	8.55 x10 ⁻³	1.60
<i>Ndst1</i>	bifunctional heparan sulfate n-deacetylase/n-sulfotransferase 1	sulfotransferases	1.67	0.02	0.05	1.64
<i>Ndst2</i>	bifunctional heparan sulfate n-deacetylase/n-sulfotransferase 2	sulfotransferases	1.21	0.03	0.07	1.12
<i>Sult1a1</i>	sulfotransferase 1a1	sulfotransferases	2.05	7.00 x10 ⁻⁴	4.10 x10 ⁻³	2.67
<i>Sult1c2</i>	sulfotransferase 1c2	sulfotransferases	5.10	3.22 x10 ⁻⁶	7.53 x10 ⁻⁵	5.22
<i>Sult1d1</i>	sulfotransferase 1 family member d1	sulfotransferases	2.73	1.12 x10 ⁻⁵	1.46 x10 ⁻⁴	4.03
<i>Sult1e1</i>	sulfotransferase 1e1	sulfotransferases	10.44	1.98 x10 ⁻⁶	5.79 x10 ⁻⁵	4.57
<i>Sult2a1</i>	sulfotransferase 2a1	sulfotransferases	288.90	1.66 x10 ⁻⁸	9.71 x10 ⁻⁷	8.35
<i>Sult2a2</i>	bile salt sulfotransferase 2	sulfotransferases	585.05	5.08 x10 ⁻⁹	5.94 x10 ⁻⁷	11.78

Upregulated genes						
<i>Sult2a3</i>	sulfotransferase	sulfotransferases	19.12	1.26 x10 ⁻⁶	4.91 x10 ⁻⁵	4.26
<i>Sult2a4</i>	sulfotransferase	sulfotransferases	4.06	7.87 x10 ⁻⁶	1.28 x10 ⁻⁴	3.61
<i>Sult2a5</i>	sulfotransferase	sulfotransferases	27.80	4.72 x10 ⁻⁶	9.20 x10 ⁻⁵	3.84
<i>Sult2a6</i>	sulfotransferase	sulfotransferases	1.11	0.04	0.10	0.94
<i>Sult3a1</i>	amine sulfotransferase	sulfotransferases	3.15	9.40 x10 ⁻³	0.03	1.18

Downregulated genes						
Gene	Name of protein	Enzyme group	Fold-change (FC)	p-value	FDR	Effect size
<i>Nat8</i>	n-acetyltransferase 8	N-acetyltransferases	-1.59	0.01	0.04	-1.03
<i>Nat8f2</i>	n-acetyltransferase family 8 member 2	N-acetyltransferases	-5.06	1.68 x10 ⁻⁵	1.97 x10 ⁻⁴	-3.84
<i>Natd1</i>	protein natd1	N-acetyltransferases	-1.38	1.60 x10 ⁻³	8.14 x10 ⁻³	-2.47
<i>Acs1</i>	long-chain-fatty-acid-CoA ligase 1	acyl-CoA synthetases	-1.60	0.01	0.05	-1.48
<i>Acs6</i>	long-chain-fatty-acid-CoA ligase 6	acyl-CoA synthetases	-1.45	5.90 x10 ⁻³	0.02	-1.54
<i>Acs1</i>	acyl-coenzyme A synthetase acs1, mitochondrial	acyl-CoA synthetases	-2.07	5.44 x10 ⁻⁵	4.24 x10 ⁻⁴	-3.39
<i>Acs2</i>	acyl-coenzyme A synthetase acs2, mitochondrial	acyl-CoA synthetases	-3.68	1.88 x10 ⁻⁵	2.00 x10 ⁻⁴	-3.19
<i>Acsc2</i>	acetyl-coenzyme A synthetase, cytoplasmic	acyl-CoA synthetases	-2.53	1.60 x10 ⁻³	8.14 x10 ⁻³	-2.23
<i>Glyat</i>	glycine n-acyltransferase	amino acid acyltransferases	-1.67	4.00 x10 ⁻⁴	2.46 x10 ⁻³	-2.39
<i>Mogat1</i>	2-acylglycerol o-acyltransferase 1	amino acid acyltransferases	-1.94	0.02	0.06	-2.16
<i>Mogat2</i>	2-acylglycerol o-acyltransferase 2	amino acid acyltransferases	-1.52	0.01	0.05	-1.65
<i>Ugt2b1</i>	udp-glucuronosyltransferase	glucuronosyltransferases	-3.61	2.79 x10 ⁻⁵	2.72 x10 ⁻⁴	-4.40

Downregulated genes						
<i>Ugt2b34</i>	udp-glucuronosyltransferase	glucuronosyltransferases	-1.25	0.04	0.09	-1.15
<i>Ugt2b36</i>	udp-glucuronosyltransferase	glucuronosyltransferases	-1.45	1.70 x10 ⁻³	8.29 x10 ⁻³	-2.04
<i>Ugt2b37</i>	udp-glucuronosyltransferase	glucuronosyltransferases	-4.63	0.01	0.05	-1.77
<i>Ugt2b38</i>	udp glucuronosyltransferase 2 family, polypeptide b38	glucuronosyltransferases	-15.74	3.89 x10 ⁻⁵	3.38 x10 ⁻⁴	-4.85
<i>Ugt2b5</i>	udp-glucuronosyltransferase 2b5	glucuronosyltransferases	-2.41	8.77 x10 ⁻⁶	1.28 x10 ⁻⁴	-4.23
<i>Ugt3a1</i>	udp-glucuronosyltransferase 3a1	glucuronosyltransferases	-2.05	4.80 x10 ⁻³	0.02	-1.72
<i>Gsta3</i>	glutathione S-transferase a3	glutathione s-transferases	-1.50	0.02	0.05	-1.44
<i>Gstk1</i>	glutathione S-transferase kappa 1	glutathione s-transferases	-1.82	0.04	0.09	-1.87
<i>Gstm2</i>	glutathione S-transferase mu 2	glutathione s-transferases	-2.34	1.90 x10 ⁻³	8.55 x10 ⁻³	-2.90
<i>Gstm4</i>	glutathione S-transferase mu 4	glutathione s-transferases	-2.11	2.50 x10 ⁻³	0.01	-3.00
<i>Gstm6</i>	glutathione S-transferase mu 6	glutathione s-transferases	-2.01	4.30 x10 ⁻³	0.02	-1.55
<i>Mgst1</i>	microsomal glutathione S-transferase 1	glutathione s-transferases	-1.70	1.30 x10 ⁻³	7.24 x10 ⁻³	-2.41
<i>Comt</i>	catechol o-methyltransferase	methyltransferases	-2.99	3.00 x10 ⁻⁴	1.95 x10 ⁻³	-3.43
<i>Tpmt</i>	thiopurine s-methyltransferase	methyltransferases	-1.39	0.03	0.08	-1.02
<i>Hs3st6</i>	heparan sulfate glucosamine 3-o-sulfotransferase 6	sulfotransferases	-2.07	3.60 x10 ⁻³	0.02	-2.69
<i>Sult2a8</i>	sulfotransferase	sulfotransferases	-2.50	0.01	0.04	-0.86
<i>Sult5a1</i>	sulfotransferase	sulfotransferases	-7.71	1.00 x10 ⁻⁴	7.31 x10 ⁻⁴	-3.21

This table shows the top 50 genes identified to be of statistical significance (p-val <0.05 and effect size >0.8). Fold changes, p-values, FDR values, and effect sizes are shown along with the names for the genes. *Abbreviations: False detection rate corrected p-values (FDR).*

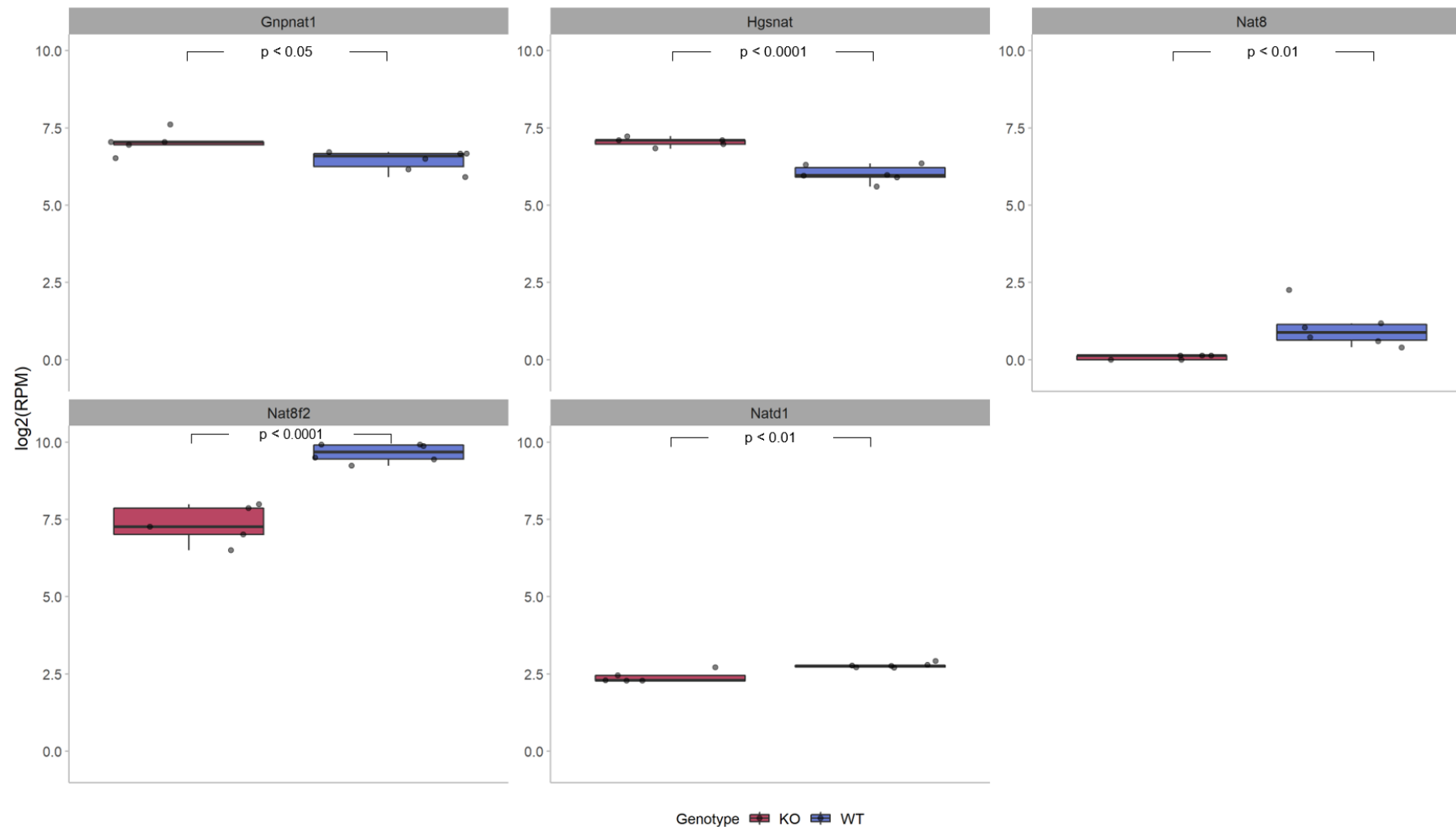


Figure 4.10 Boxplots showing log₂ reads per million values for N-acetyltransferases genes of KO and WT samples

This figure shows the log₂RPM values for five N-acetyltransferases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p-value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

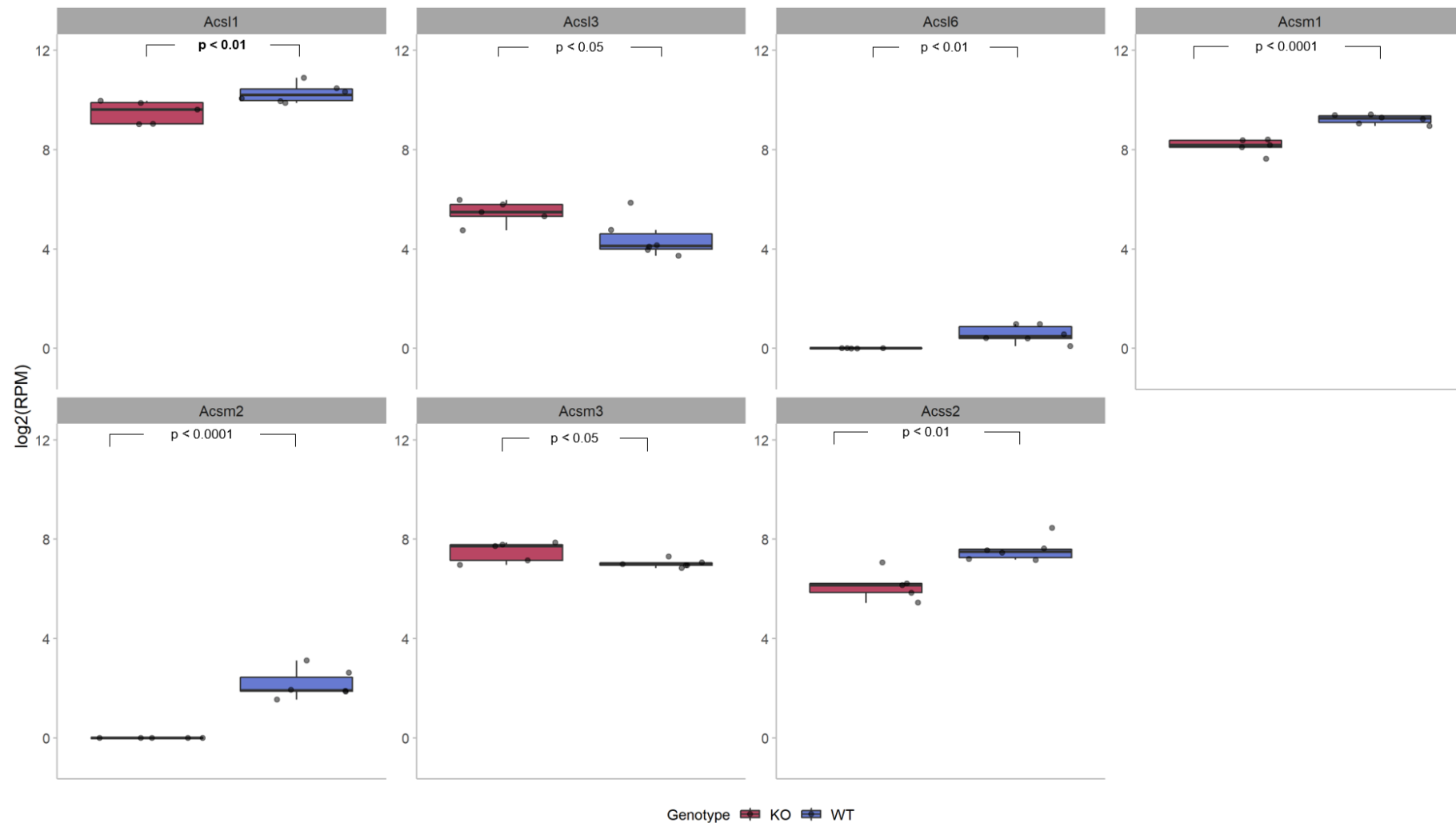


Figure 4.11 Boxplots showing log₂ reads per million values for acyl-CoA synthetase genes of KO and WT samples

This figure shows the log₂RPM values for seven acyl-CoA synthetases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p-value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

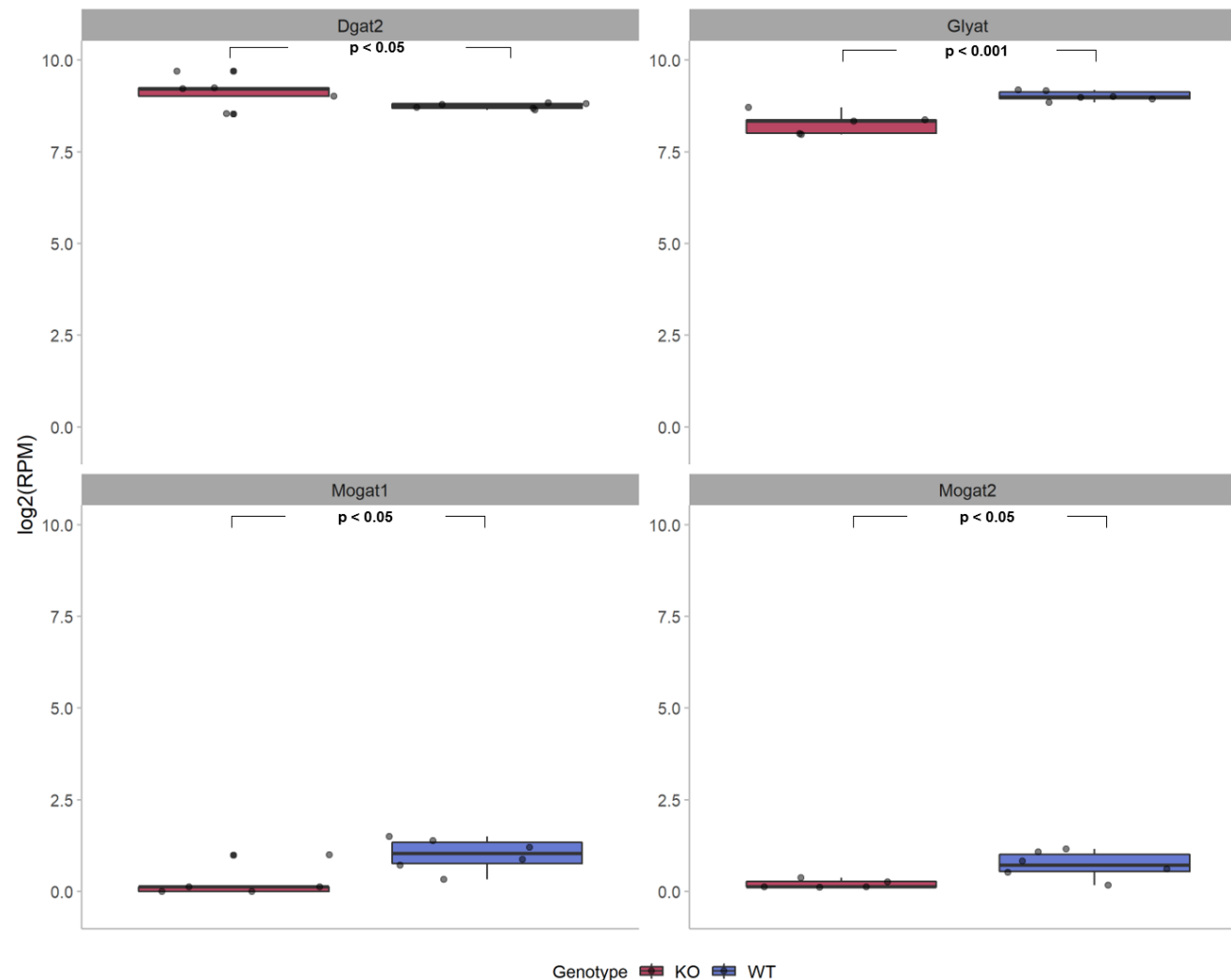


Figure 4.12 Boxplots showing log2 reads per million values for amino acid acyltransferase genes of KO and WT samples

This figure shows the log2RPM values for four amino acid acyltransferases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p -value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

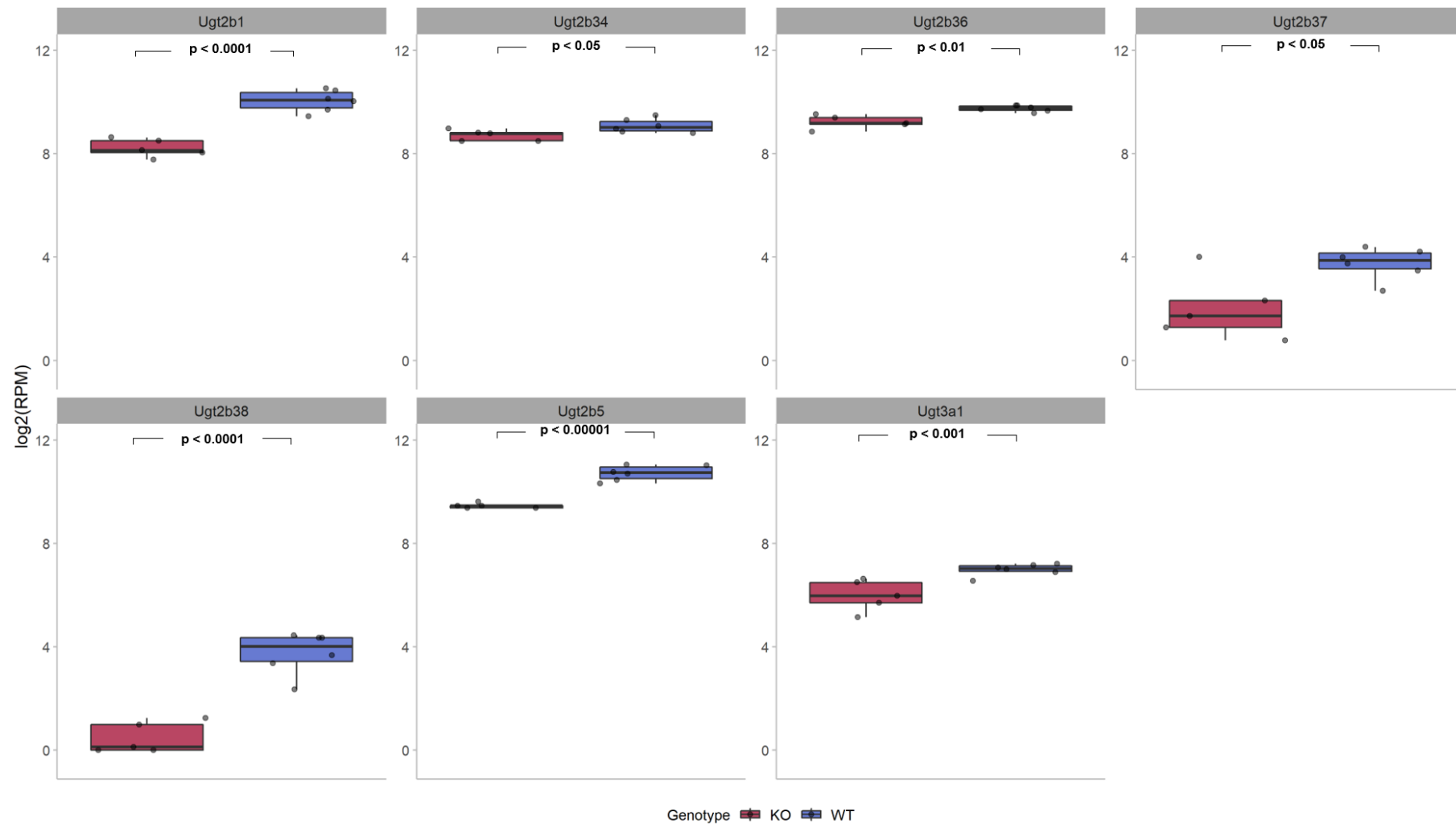


Figure 4.13 Boxplots showing log₂ reads per million values for UDP-glucuronosyltransferase genes of KO and WT samples

This figure shows the log₂RPM values for seven glucuronosyltransferases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p-value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

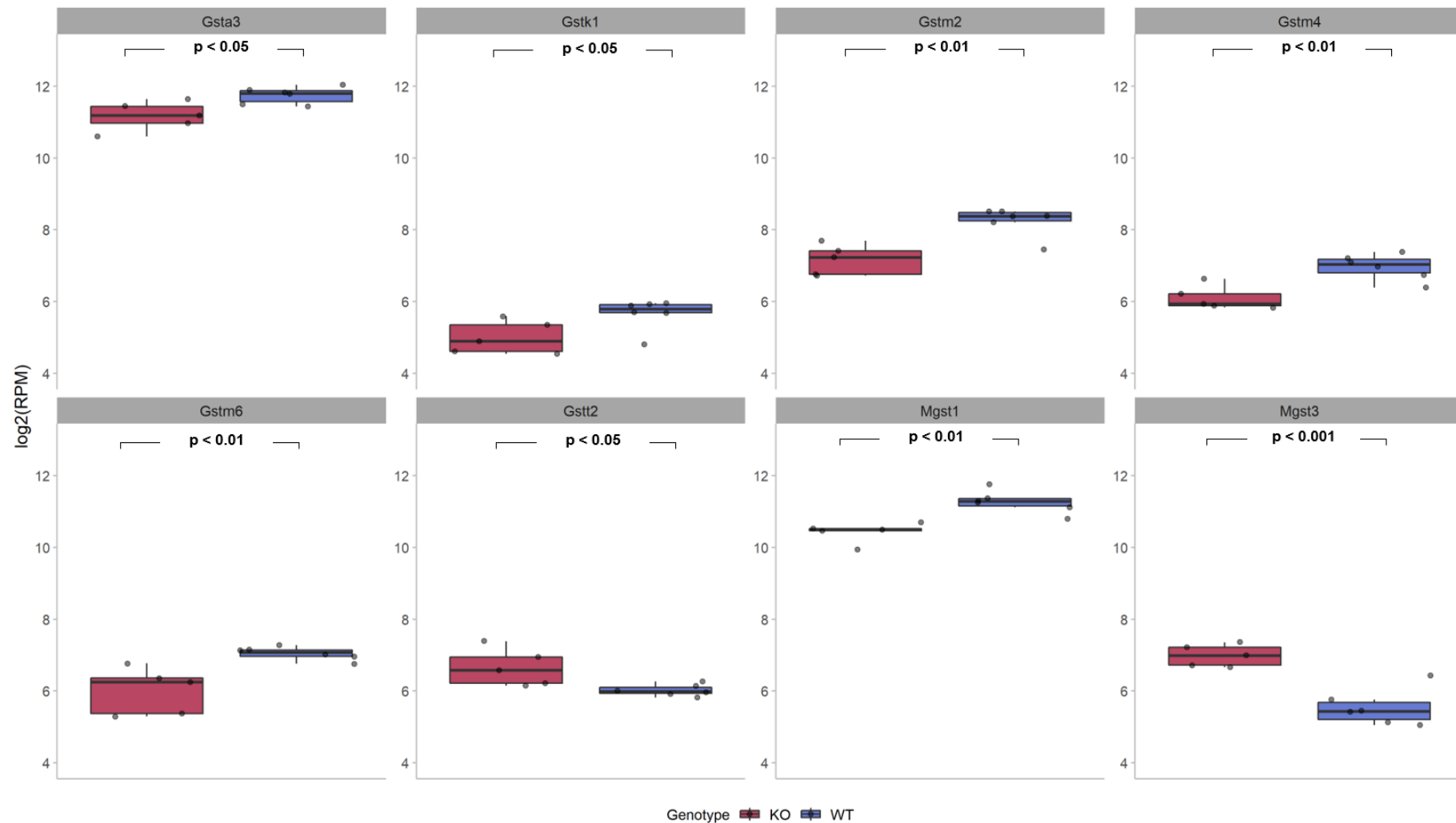


Figure 4.14 Boxplots showing log₂ reads per million values for glutathione S-transferase genes of KO and WT samples

This figure shows the log₂RPM values for eight glutathione S-transferases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p-value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

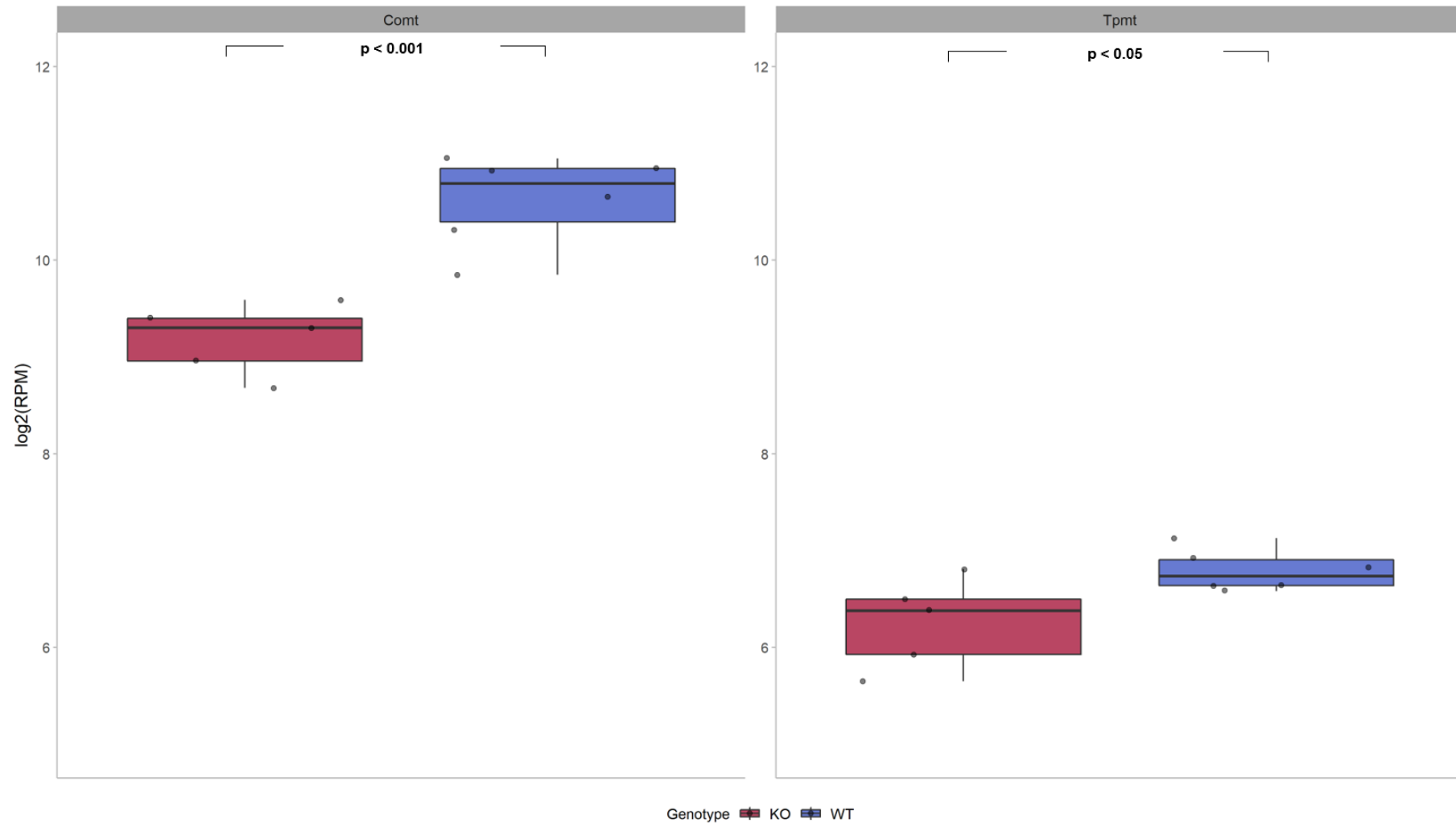


Figure 4.15 Boxplots showing log2 reads per million values for methyltransferase genes of KO and WT samples

This figure shows the log2RPM values for two methyltransferases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p-value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

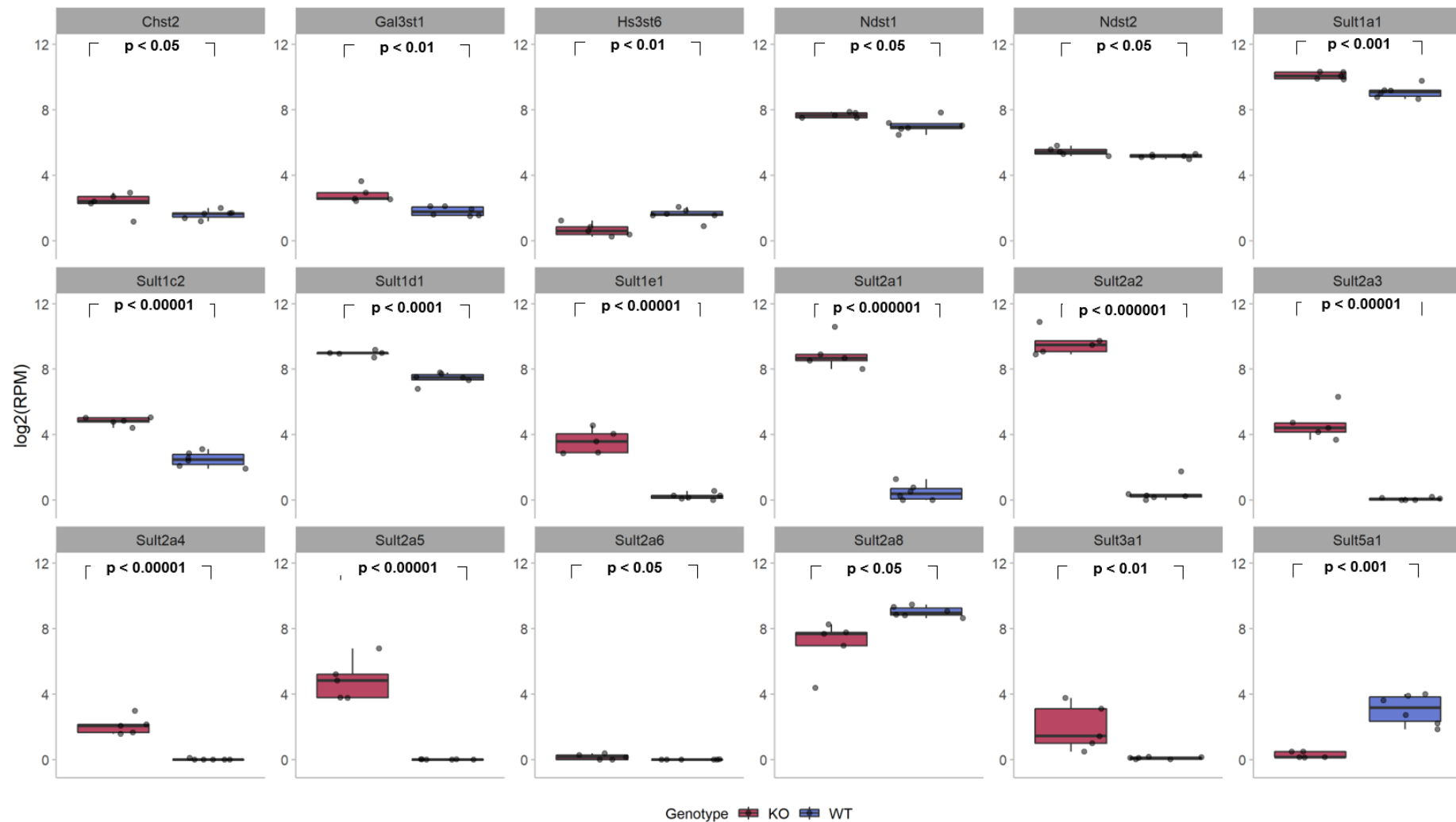


Figure 4.16 Boxplots showing log2 reads per million values for sulfotransferases genes of KO and WT samples

This figure shows the log2RPM values for 18 sulfotransferases that were statistically differentially expressed between WT and KO mice (effect size > 0.8 and p-value < 0.05). Boxes represent the interquartile range, with the whiskers representing the first quartile and third quartile respectively. The median is represented by the line in the centre of the box. Dots represent the individual samples. *Abbreviations: knockout (KO), reads per million (RPM), wild-type (WT).*

Acetylation reactions are mediated by N-acetyltransferases (NATs) and five of these genes were identified in the top 50 features. Expression of three of these genes was downregulated, and includes *Nat8f2*, *Nat8*, and *Natd1*. The two genes that showed upregulated gene expression in *Ndufs4* KO mice were *Hgsnat* and *Gnpnat1*. With only one more gene being downregulated than upregulated, a conclusive trend of gene expression of these genes in the *Ndufs4* KO mice cannot be stated. **Amino acid conjugation** reactions are catalysed by an ATP-dependent acid:CoA synthetase and an acyl-CoA:amino acid N-acyltransferase. The 11 acyl-CoA synthetases and amino acid acyltransferases genes that were included in the list of top 50 features were: *Acs11*, *Acs13*, *Acs16*, *Acsm1*, *Acsm2*, *Acsm3*, *Acss2*, *Dgat2*, *Glyat*, *Mogat1*, and *Mogat2*. Except for *Acs13*, *Acsm3*, and *Dgat*, all genes showed decreased expression in the *Ndufs4* KO mice compared to WT mice (p-values ranged from 1.88×10^{-5} to 0.05). **Glucuronidation** reactions showed significant decreased gene expression in KO mice compared to WT mice, in all seven of the genes that were included in the list of top 50 genes (p-values ranged from 3.89×10^{-5} to 0.04). These genes were: *Ugt2b1*, *Ugt2b34*, *Ugt2b36*, *Ugt2b37*, *Ugt2b38*, *Ugt2b5*, and *Ugt3a1*. **Glutathione S-conjugation** reactions are catalysed by glutathione S-transferases (GSTs). These genes predominantly showed significant downregulated gene expression in the *Ndufs4* KO mice compared to WT mice (p-values ranged from 3.00×10^{-4} to 0.04). The genes that showed downregulated differential gene expression were: *Gsta3*, *Gstk1*, *Gstm2*, *Gstm4*, *Gstm6*, and *Mgst1*. The two upregulated genes were: *Gstt2* and *Mgst3*. **Methylation** reactions are catalysed by methyltransferase enzymes. Only two methyltransferase genes were included in the top 50 features and both had decreased gene expression in *Ndufs4* KO mice. The two genes were *Comt* (p-value: 3×10^{-4}) and *Tpmt* (p-value: 0.03). **Sulfoconjugation** genes showed significant upregulated gene expression in *Ndufs4* KO mice compared to WT mice (p-values ranged from 5.08×10^{-7} to 0.05). A total of 18 sulfoconjugation genes were identified in the top 50 features of which 15 were upregulated. The upregulated sulfoconjugation genes were: *Chst2*, *Gal3st1*, *Ndst1*, *Ndst2*, *Sult1a1*, *Sult1c2*, *Sult1d1*, *Sult1e1*, *Sult2a1*, *Sult2a2*, *Sult2a3*, *Sult2a4*, *Sult2a5*, *Sult2a6*, and *Sult3a1*. The three downregulated genes were *Hs3st6*, *Sult2a8*, and *Sult5a1*.

4.4.3 Summary and conclusions

Initial investigation of phase two biotransformation through the glycine conjugation pathway, revealed significant downregulation in transcriptomic and enzymatic data. To investigate if downregulation of remaining phase two biotransformation pathways were apparent in these energy deficient mice, the transcriptomics dataset was revisited to determine gene expression of seven phase two biotransformation pathways. Gene expression of genes coding for N-acetyltransferases, acyl-CoA synthetases, amino acid acyltransferases, glucuronosyltransferases, glutathione S-transferases, methyltransferases, and sulfotransferases

were analysed. 2D score PCA plots showed that samples naturally grouped with their genotypes. The first two principal components explained 88.4% of the variation which suggested that genotype-related variation was the largest contributor to variation in gene expression data. The top 50 significantly differentially expressed ($d > 0.8$ and $p < 0.05$) genes were identified.

As expected, most of the phase two biotransformation pathways were downregulated, possibly as a mechanism for these energy deficient mice to preserve energy for more crucial processes. An unexpected finding was that sulfotransferases that were substantially upregulated. As a summary, Table 4.5 shows the trends that the different phase two enzymes underwent in the *Ndufs4* KO mice.

Table 4.5 Gene expression trends of different groups of phase two biotransformation genes in *Ndufs4* KO mice

Phase two biotransformation reactions	Group of enzymes	Cellular localisation ^{77,78}	Trend of change
Acetylation	N-acetyltransferases	Mitochondria	-
Amino acid conjugation	acyl-CoA synthetases amino acid acyltransferases	Mitochondria, cytosol, microsomes	↓
Glucuronidation	glucuronosyltransferases	Microsomes	↓
Glutathione S-conjugation	glutathione S-transferases	Cytoplasm, microsomes, mitochondria, nuclei	↓
Methylation	methyltransferases	Cytosol, microsomes	↓
Sulfoconjugation	sulfotransferases	Mitochondria, cytosol	↑

Most of the groups of phase two biotransformation genes underwent downregulation in *Ndufs4* KO mice, the exception being sulfotransferases that had an overwhelming majority of genes that were upregulated. N-acetyltransferases had a similar number of genes that were up and downregulated, so no conclusive statement can be made about the trend of change in KO mice.

⁷⁷ (Penner *et al.*, 2012)

⁷⁸ (Sevior *et al.*, 2012)

4.5 Part 4: Summative discussion in context of research questions

4.5.1 Introduction

The following section entails discussion and biological interpretation of the results obtained during this study.

Leigh syndrome is a progressive neurodegenerative disorder and the most common disorder of the OXPHOS system in children. As *NDUFS4* is a subunit of mitochondrial CI of which defects result in Leigh syndrome, a whole body *Ndufs4* knockout mouse model has been of great value to investigate the pathology of Leigh syndrome and mitochondrial CI deficiency in general. These studies cover a plethora of life-sustaining processes related to the mitochondrion, but significantly less so for biotransformation (Chen *et al.*, 2002; McBride *et al.*, 2006; Penner *et al.*, 2012; Sevier *et al.*, 2012). Biotransformation is mostly known for its role in metabolising xenobiotic compounds to water-soluble compounds that can be excreted in the urine and bile before they accumulate to toxic levels in the body (Dancygier, 2010; Sevier *et al.*, 2012). Biotransformation is divided into two phases and each phase has its own set of enzymes. Beyond its role in detoxification of xenobiotics, phase two enzymes play a crucial role in binding, transporting and inactivation of endogenous compounds like hormones and bile acids, which ultimately contribute to homeostasis (Testa, 2008). The aim of this study was to investigate differential expression of genes that are involved in phase two biotransformation in an *Ndufs4* KO mouse model. The first research question asked whether glycine conjugation, a primary pathway of phase two biotransformation, is differentially expressed in *Ndufs4* KO mice compared to WT mice.

4.5.2 Assessment of the glycine conjugation pathway as a first step toward investigation of phase two biotransformation in *Ndufs4* KO mice

Glycine conjugation is part of amino acid conjugation reactions as discussed in Section 2.3.4. It requires ACSM2 and GLYAT enzymes to conjugate glycine to endogenous and exogenous compounds (Rohwer *et al.*, 2021). Transcriptomic and enzyme activity analysis revealed downregulation of the **glycine conjugation** pathway in *Ndufs4* KO mice compared to WT mice. *Acs2* and *Glyat* genes had decreased expression and the GLYAT protein had reduced enzymatic activity in the *Ndufs4* KO mice compared to WT mice. As discussed in Section 2.3.4, several factors can influence glycine conjugation in humans including, the availability of CoA, glycine and ATP, and the differential expression of *ACSM2* (Badenhorst *et al.*, 2014; Rohwer *et al.*, 2021). *Acs2* underwent significant downregulation in the *Ndufs4* KO mice with fold changes of -3.68, a p-value of 1.88×10^{-5} , and effect size of -3.19. Furthermore, *Acs2* expression was barely detected in KO mice. The limited ATP in these mice is a possible contributing factor to the

downregulation of the glycine conjugation pathway through activating retrograde signalling pathways. Mitochondria are connected to the nucleus through retrograde signalling, which enables cells to regulate changes in gene expression in response to its physical state (Kleine & Leister, 2016; Trendelewa & Zvyagilskaya, 2018). In mammals, the exact mechanisms for these signalling pathways are not well understood but changes in metabolism can activate specialised transcription factors to elicit changes in gene expression (van der Knaap & Verrijzer, 2016). Molecules like ATP and ADP; as well as NAD^+ and NADH are known to function as molecular signals to retrograde signalling pathways (Bohovych & Khalimonchuk, 2016; Cardamone *et al.*, 2018). Increased NAD^+ levels, for example, correlate with increased SIRT1 mediated deacetylation and activation of PCG-1 α , which in turn coactivates a set of transcription factors to promote glyconeogenesis (Houtkooper *et al.*, 2010). Downregulation of *Acs2* could further potentially explain the downregulation seen in *Glyat* as well, because less CoA-thioester intermediates would form as a result of the downregulation of *Acs2*. Less CoA-thioester intermediates are then available for conjugation with glycine, which in turn could result in less GLYAT enzymes required in this pathway.

Thus, in answer to the first research question, glycine conjugation was found to be significantly downregulated in *Ndufs4* KO mice. To further address the aim, the investigation was expanded to the second research question that asked whether the changes seen in glycine conjugation were also reflected in remaining phase two biotransformation genes.

4.5.3 Expanded investigation of phase two biotransformation genes in *Ndufs4* KO mice compared to WT mice

Literature was consulted to determine the remaining phase two biotransformation enzymes and corresponding genes. The identified genes were cross-referenced with the genes analysed in the Ion AmpliSeq™ panel and resulted in a list of 117 genes to investigate. The list of genes is shown in Appendix F and consisted of genes from the following enzyme groups: N-acetyltransferases, acyl-CoA synthetases, amino acid acyltransferases, glucuronosyltransferases, glutathione S-transferases, methyltransferases and sulfotransferases. Figure 4.17 shows an overview of the differential gene expression of the top 50 genes through log2 fold changes.

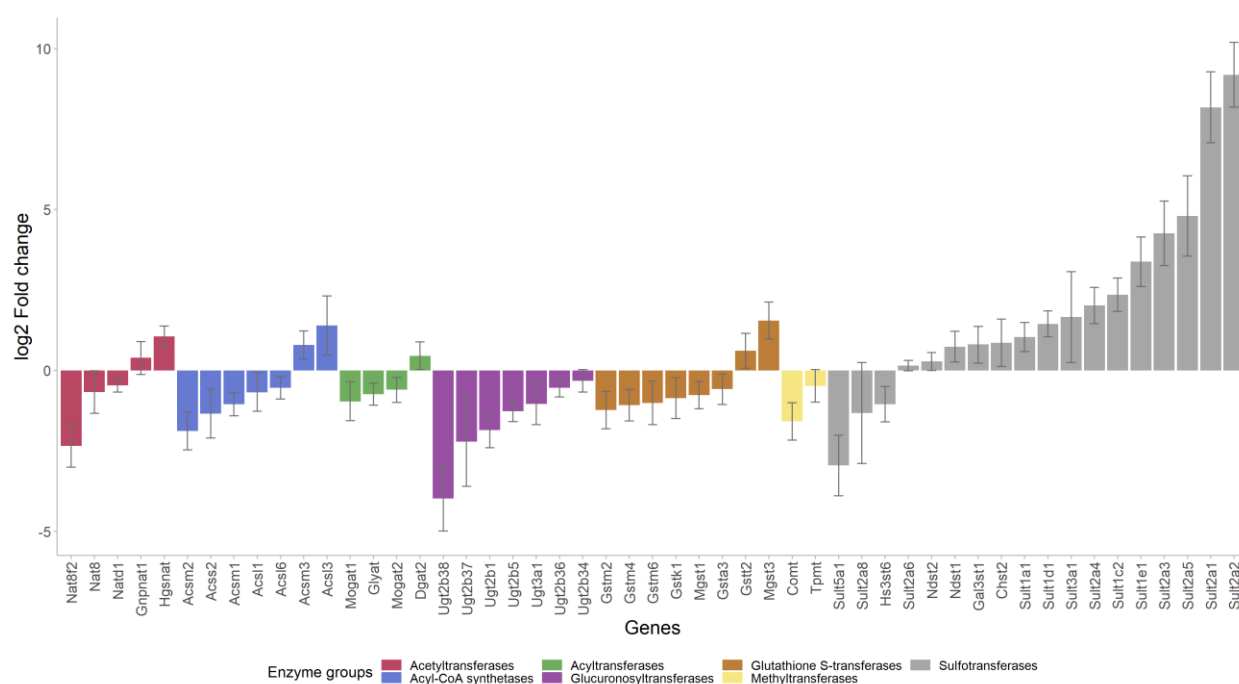


Figure 4.17 Log2 fold changes for top 50 genes in liver samples

[Log2 fold change] ≥ 1 is equivalent to a fold change of ≥ 2 . Log2 values of the fold change was calculated so that a fold change will have the same distance to the zero baseline regardless of whether it is up or downregulated. Different colours represent the seven main conjugation pathways investigated.

Downregulation of phase two biotransformation

Acetylation reactions are mediated by NATs where they catalyse the transfer of an acetyl group from acetyl-CoA to the free amino group of the substrate. The function of NATs in endogenous metabolism is still unclear (Boukouvala & Fakis, 2005; Jancova *et al.*, 2010). The two forms of NAT known in humans are NAT1 and NAT2 (Jancova *et al.*, 2010). Murine NAT2 is the functional homologue of human NAT1 and is found in a variety of tissues. Substrates for human NAT include p-aminobenzoic acid, p-aminosalicylic acid and p-aminobenzylglutamate. NAT1 on the other hand is only found in the liver and intestine. Human NAT2 is responsible for detoxification of isoniazid, hydralazine and sulphonamides (Boukouvala & Fakis, 2005). Almost equal amounts of *Nat* genes were upregulated and downregulated, making a conclusive statement on the regulation of this group of enzymes challenging. Current knowledge does not indicate a significant role for NATs in metabolism of endogenous compounds. They rather play a role in biotransformation of drugs and carcinogens (Tsirka *et al.*, 2018). *Ndufs4* KO mice used in this study were not subject to treatment of any kind and therefore did not need to metabolise any drugs, which could possibly explain the few genes that were downregulated.

As described in Section 2.3.3, **amino acid conjugation** consists of two mitochondrially located enzymatic processes which are catalysed by an ATP-dependent acid:CoA synthetase and an

acyl-CoA:amino acid N-acyltransferase (Knights *et al.*, 2007). The first reaction relies on CoA and ATP when a high-energy CoA thioester intermediate is formed. The second reaction involves transfer of the acyl group to the amino group. It can be expected that a mouse model of ATP deficiency would display downregulation of a pathway that is dependent on ATP. Acyl-CoA synthetases and amino acid acyltransferases were investigated as part of amino acid conjugation. Most genes involved in this pathway showed significant downregulation in KO mice compared to WT mice. As with the glycine conjugation pathway, limited availability of ATP can target retrograde signalling pathways, to initiate adaptive gene regulation and explain why these genes are downregulated in *Ndufs4* KO mice.

Glucuronidation is considered the most important phase two detoxification pathway in vertebrates (Jancova *et al.*, 2010; Ritter, 2000). They are catalysed by UGTs localized in the endoplasmic reticulum. These reactions require a cofactor, uridine-5'-diphospho- α -D-glucuronic acid (UDPGA), to provide the glucuronic group that is transferred to a substrate that contains oxygen, nitrogen, sulfur or carboxyl functional groups (Testa, 2008; Yang *et al.*, 2017). Enzymes of this pathway are not only crucial to the detoxification of drugs, carcinogens and environmental chemicals, but are also involved in the metabolism of endogenous compound including bile acids, fatty acids, thyroid hormones, bilirubin, fat-soluble vitamins, and hydroxysteroids (Jancova *et al.*, 2010; Jančová & Šiller, 2012; Rowland *et al.*, 2013). Metabolism of these endogenous compounds by UGTs is important to regulate their involvement in cell growth and differentiation. Some of the substances of glucuronidation can also undergo sulfoconjugation by SULTs (Ouzzine *et al.*, 2014). Metabolites that have undergone glucuronidation mostly have reduced biological activity in comparison to the parent compound (Allain *et al.*, 2020). Glucuronidation reactions require efflux transporters on the cell surface which in turn can affect the formation rate by controlling the efflux of glucuronides from the cells (Yang *et al.*, 2017). In humans, expression of UGTs are regulated by a number of factors including, epigenetic regulation of chromatin structure, transcription factors especially HNF1 α in liver and other ligand-activated transcription factors, and steroid hormones (Rowland *et al.*, 2013). Transcriptomic analysis revealed that all seven of the differentially expressed glucuronidation genes were significantly downregulated in *Ndufs4* KO mice compared to WT mice. These genes were predominantly from the UGT2b subfamily. In mice, UGT2B and UGT3A subfamilies are mainly expressed in the liver and intestine (Kurita *et al.*, 2017). Disturbed expression of UGTs can influence the response of cells to endogenous and exogenous factors, as well as progression in common malignancies (Allain *et al.*, 2020). One potential explanation of the downregulation of this pathway is that these mice are not exposed to many environmental chemicals or drugs. They live in sterile and controlled conditions in a vivarium and are therefore possibly not that reliant on biotransformation reactions to detoxify exogenous compounds. A second explanation is that during synthesis of the cofactor, UDPGA, NAD⁺ is

required but insufficient (see Figure 2.5). In this case, production of UDPGA would be hindered by the NAD^+ -dependent UDPG dehydrogenase, resulting in insufficient UDPGA for glucuronidation and ultimately causing downregulation of this pathway. CI is responsible for oxidation of NADH to NAD^+ . It is proposed that the deficient CI activity in these mice results in an $\text{NADH}:\text{NAD}^+$ ratio that is disturbed to the left, since the NADH produced in the TCA cycle cannot be oxidised to NAD^+ by CI. This redox imbalance is thought to affect several enzymes that are dependent on NAD^+ . Moreover, it is now also well documented, notably from recent metabolomics studies (Esterhuizen *et al.*, 2017), that the redox state is significantly impacted by CI deficiency. These alterations in NAD^+/NADH levels are able to target retrograde signalling pathways which can alter gene expression, as explained in Section 4.5.2.

Glutathione S-conjugation reactions (shown in Figure 2.6), which are catalysed by GSTs, protect cellular macromolecules from reactive electrophiles by conjugating GSH to endogenous and exogenous electrophilic compounds (Townsend & Tew, 2003). Some endogenous compounds like prostaglandins and steroids are metabolized via this pathway (Van Bladeren, 2000). It can provide some defence against oxidative stress and might be involved in the metabolism of arachidonic acid and steroid derivatives (Jančová & Šiller, 2012). Furthermore, it is the first step in the mercapturic acid pathway that is also involved in the elimination of toxic compounds (Townsend & Tew, 2003). Glutathione S-conjugates are more often excreted in bile than urine, due to their large size and amphipathic characteristics (Cooper & Hanigan, 2018). Transcriptomic analysis of this pathway revealed that most genes are downregulated, especially those of the mu family.

One reason to why expression of glutathione S-conjugation genes is reduced in *Ndufs4* KO mice, is possibly provided by the synthesis of the cofactor, GSH, which requires ATP. GSHs are tripeptides that bind to endogenous compounds to regulate their activity and facilitate transport over membranes. GSHs are also responsible for maintenance of the cellular redox state and transport of cysteine. They are also a cofactor in some enzymatic reactions (Cooper & Hanigan, 2018). GSHs further ensure the maintenance of protein structure. They regulate protein synthesis and degradation, and protect against oxidative damage (Wang & Ballatori, 1998). Homeostasis of GSH is maintained through two processes: recycling from oxidised glutathione (GSSG) and synthesis through the glutamyl cycle. Recycling of GSH consists of an oxidation-reduction pathway. In this pathway, GSH is oxidised to GSSH through GSH peroxidase and requires H_2O_2 . GSSG can then be reduced to GSH through glutathione reductase and NADPH as a cofactor. GSHs are synthesised in the cytosol of almost all cells through an ATP-dependent two-step reaction. The two reactions are shown in Figure 4.18. The first reaction, which is also the rate limiting step, is mediated by glutamate cysteine ligase where L-glutamate and L-cysteine

combines to form γ -glutamyl-L-cysteine. In the second step which also requires ATP, glycine combines with γ -glutamyl-L-cysteine and under mediation of GSH synthetase forms GSH (Lu, 2009). ATP supply is a limiting factor in the synthesis of GSH (Aquilano *et al.*, 2014). If the limited ATP in these mice results in decreased production of GSH, this can once again limit a phase two biotransformation pathway by limiting the available GSH for glutathione S-conjugation reactions. Although, this hypothesis is challenged by studies that measured total cellular glutathione content in patient fibroblasts with *NDUFS4* mutations (Verkaart *et al.*, 2007) and skin fibroblasts of patients with nuclear-inherited isolated CI deficiency (Iuso *et al.*, 2006), without finding any significant decrease. In contrast, perturbed glutathione metabolism was seen in neurodegeneration-prone brain regions of *Ndufs4* KO mice (Coetzer, 2020). Perturbed GSH concentrations are also seen in neurodegenerative diseases (Liu *et al.*, 2004). GSH also serves as an antioxidant and is under normal physiological conditions present in concentrations 10-100 folds higher than the oxidised species (Aquilano *et al.*, 2014). The redox ratio between GSH and GSSG serves as an indicator of the redox environment. The redox state of GSH:GSSG is disturbed to the right under conditions of oxidative stress, which could serve as another explanation of how reduced levels of GSH could contribute to downregulation of glutathione S-conjugation pathways. (Zitka *et al.*, 2012).

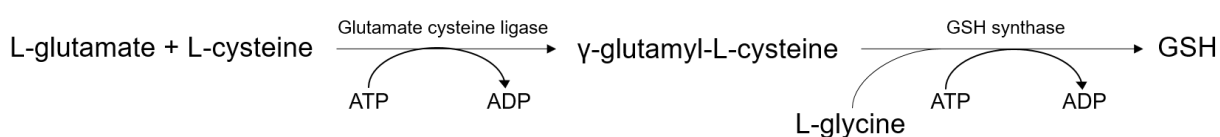


Figure 4.18 Synthesis of GSH requires ATP

Synthesis of GSH is a two-step reaction that requires two moles of ATP per mole of GSH. The first reaction is mediated by glutamate cysteine ligase and the second reaction by GSH synthase. *Abbreviations: adenosine diphosphate (ADP), adenosine triphosphate (ATP), glutathione (GSH).*

Methylation reactions are mediated by methyltransferase enzymes that are classified into three groups according to the position of the substrate: O-, S-, and N-methyltransferases. A scheme of a typical methylation reaction is shown in Figure 2.7. These enzymes rely on S-adenosyl-L-methionine (SAM) to provide a methyl group to the reaction. The two methyltransferase genes identified to be differentially expressed in *Ndufs4* KO mice were *Comt* and *Tpmt*. Both genes showed decreased expression in *Ndufs4* KO mice compared to WT mice.

The limited supply of ATP in these mice could once again result in limited production of a cofactor in a phase two biotransformation pathway, resulting in downregulation of the pathway. In this case, synthesis of the cofactor for methylation reactions, SAM, is dependent on ATP (see Figure 2.7). SAM, relies on the methionine cycle for its formation from methionine through methionine adenosyltransferase (Mentch & Locasale, 2016). This cofactor is also involved in the one-carbon

metabolism that has recently been found to be perturbed in the brain of these mice (Coetzer, 2020). Considering that there is currently no known use for TPMT for endogenous metabolism, and as discussed previously that these mice did not receive any drugs, downregulation of *Tpmt* might be because there is not any use for it in these mice.

Upregulation of phase two biotransformation

Substantial upregulation of **sulfoconjugation** pathway genes were demonstrated in *Ndufs4* KO mice. Of the 18 *Sult* genes that were included in the list of top 50 differentially expressed genes, 15 were upregulated. Of the upregulated *Sults*, *Sult2a1*, *Sult2a2*, *Sult2a3*, *Sult2a5*, and *Sult1e1* had very high fold changes, ranging from 10.44 to 585.05. Expression of mice *Sult2a* genes are dependent of age and sex. In adult mice for example, they are expressed in a strong female-specific manner (Kocarek *et al.*, 2008). This is supported in the current study where only males were used and *Sult2a1*-*Sult2a5* showed almost non-existing expression in the WT mice.

One of the factors possibly contributing to the upregulation of *Sults* is the activation of CAR, a nuclear receptor that is activated by stress. Through regulation of Ugts and *Sults*, CAR maintains hormone homeostasis (Chai *et al.*, 2016). Balyan *et al.* (2019) reported that male Sprague-Dawley rats placed under repeated restrain stress had upregulated *Sult1a1* in liver and several other tissues. CAR is known to increase *SULT1A1*, *SULT2A1* and other *SULTS*' expression, although this could be sex-specific (Alnouti & Klaassen, 2008; Mueller *et al.*, 2015; Saini *et al.*, 2004). Stress furthermore causes a release of a glucocorticoid, corticosterone, which has also been shown to enhance genetic expression of *Sult1a1* in rat hepatic cells (Fang *et al.*, 2003). Considering that dysfunctional mitochondria have been implicated in increased stress, it is possible that this stress contributes to the upregulation of *Sults* in the *Ndufs4* KO mice (Emmerzaal *et al.*, 2020; Morava & Kozicz, 2013). The mechanism by which physical stress can result in upregulation of *Sults*, more specifically *Sult2a1*, could further be illustrated by examination of one of its pathways. Dehydroepiandrosterone (DHEA), a precursor for testosterone and estrone, can undergo sulfoconjugation to form its inactive form, dehydroepiandrosterone 3-sulfate (DHEA-S) through the action of *SULT2A1* (see Figure 4.19). The reverse reaction is mediated by a steroid sulfatase. Opstad (1992) investigated regulation of androgenic steroids of male cadets undergoing continuous physical military operations for several days. It was shown that prolonged physical stress in combination with energy and sleep deprivation can alter serum levels of DHEA and DHEA-S and attributed this to an increase in sulfotransferase activity and/or reduced activity of steroid sulfatase. Furthermore, DHEA and DHEA-S act as ligands for many nuclear receptors, including CAR, pregnane X receptors and PPAR α (Prough *et al.*, 2016). Thus, physical stress and energy deprivation could potentially result in activation of CAR which in turn causes upregulation of expression of many

sulfotransferase genes, including *Sult2a1*. The increased conjugation of these androsterones might serve as a way to increase the levels of testosterone and estrone in other tissues by transporting the sulphated form (which is more soluble) from the liver to the relative tissues.

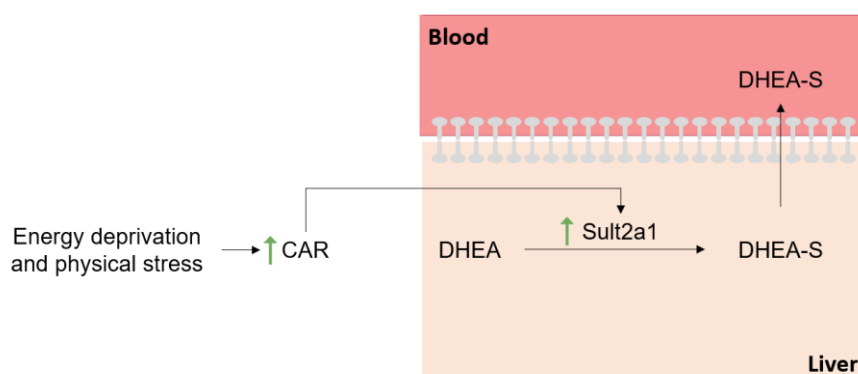


Figure 4.19 Possible mechanism for upregulation of *Sult2a1*

Energy deprivation and physical stress can activate CAR to increase expression of *Sult2a1*. Abbreviations: constitutive androstane receptor (CAR), dehydroepiandrosterone (DHEA), dehydroepiandrosterone 3-sulfate (DHEA-S).

The three downregulated sulfotransferases genes, *Hs3st6*, *Sult2a8*, and *Sult5a1*, had significant fold changes ranging from -2.07 to 7.71. The recently identified *SULT2A8* is suggested to be a major bile acid metabolising enzyme in mice (Wang *et al.*, 2021). Metabolism of bile acids is an important process as the detergent-like properties of bile acids can disrupt plasma and mitochondrial membrane lipids at high concentrations. Furthermore, accumulation of bile acids in humans can lead to a liver disease known as cholestasis where the flow of bile from the liver is blocked (Kamath *et al.*, 2020). Disturbed energy metabolism has been implicated in cholestasis. Accumulation of bile acids can affect mitochondria by hampering activities of the enzyme complexes, affecting mitochondrial membrane potential and ATP levels (Arduini *et al.*, 2012). Another notable effect of long term cholestasis is depletion of GSH in liver mitochondria due to down regulation of GSH synthetic enzymes (Serviddio *et al.*, 2004), serving as another factor that might contribute to downregulation of glutathione S-transferases as discussed in the biological interpretation of glutathione S-conjugation. Interestingly, the cofactor, PAPS, is synthesised from ATP and requires another ATP molecule during the second step of its synthesis (see Figure 2.8). Limited ATP in these mice might contribute enough to limit the expression of these three *SULT* genes, but not the majority of it.

4.5.4 Summary and conclusions

As could be expected, most of the phase two biotransformation pathways were downregulated in *Ndufs4* KO mice. In a mouse model where there is limited ATP supply, energy must be conserved for critical and life-supporting processes. Although biotransformation reactions are very important,

it seems that these reactions are not prioritised in *Ndusf4* KO mice. Downregulation of gene expression might be a mechanism to preserve energy that would otherwise be required to produce cofactors. Alternatively, it might be a consequence of the redox imbalance resulting in decreased levels of NAD⁺ which affect gene regulation, thereby inhibiting some of the biotransformation pathways in which it is involved. Several cofactor synthesis requires NAD⁺ or ATP which is known to be limited in these mice. Furthermore, these mice live in sterile, controlled conditions and do not receive any treatment for their condition. Biotransformation reactions are especially important for the metabolism of exogenous and xenobiotic compounds. It might be that these mice simply downregulate the protein expression of biotransformation enzymes because they do not require its use that often and they would rather allocate resources to more essential processes.

An unexpected result of this study was sulfoconjugation genes that were highly upregulated. SULTs are involved in the metabolism of a large range of endogenous compounds including steroids, hormones, and neurotransmitters, which upon sulfoconjugation become either activated or lose their activity. A major factor contributing to the upregulation of these genes, might be increased stress as a result of mitochondrial dysfunction.

Overall, the data strongly suggests that phase two biotransformation pathways are highly affected by mitochondrial dysfunction.

CHAPTER 5 SUMMARY AND CONCLUSIONS

5.1 Introduction

Mitochondria are at the centre of a multitude of cellular processes. Inside the inner mitochondrial membrane lies the OXPHOS system where most of the cell's energy is produced. Disruption of the OXPHOS system will inevitably have numerous downstream effects, for example patients with Leigh syndrome present with failure to thrive, psychomotor retardation and eventual death (Budde *et al.*, 2003; Cooper *et al.*, 2006). Biotransformation is an essential process responsible for metabolism of endogenous and xenobiotic compounds either by metabolising a parent compound to its active form or by excreting the compound before it accumulates to toxic levels. Several biotransformation reactions are directly or indirectly dependent on cofactors which are affected in mitochondrial disease, like ATP and NAD⁺. Some of these reactions are also localised in the mitochondrion. Biotransformation of drugs can vary greatly between individuals and knowing how a patient will metabolise a certain drug, can aid in giving them the correct dose and treatment. The downstream effects of a mitochondrial disorder on biotransformation are still unclear. Knowledge of the capacity of these patients to perform biotransformation reactions, will not only provide researchers with insight into the disease mechanism and the toxicity potential of endogenous and xenobiotic compounds, but could potentially aid in developing effective treatment. Currently there are no approved curative medications or treatment for treating inherited mitochondrial disease. Therapeutic strategies focus on improving quality of life and mitigating symptoms (Gerards *et al.*, 2016). Drug treatments mostly include compounds known for their antioxidant properties or cofactors of the OXPHOS system like coenzyme Q10, biotin, riboflavin, vitamins, and L-carnitine (Baertling *et al.*, 2014). Development of drugs for mitochondrial disease is an ongoing process with some relatively new compounds like, EPI-743, and Sonlicromanol™ (previously known as KH176) which is currently in phase two clinical trials, showing promising preliminary results (Enns *et al.*, 2012; Jiang *et al.*, 2021). This gap in knowledge prompted the current investigation which studied the effect of mitochondrial disease on biotransformation by analysing the expression of genes involved in biotransformation reactions in a mitochondrial disease mouse model. Phase two biotransformation, more specifically glycine conjugation, was selected as a starting point for this investigation before expanding to other phase two biotransformation pathways.

The aim for this study therefore was to investigate differential expression of genes involved in phase two biotransformation in an *Ndufs4* knockout mouse model.

Two research questions were set as guidelines to address this aim.

1. Is glycine conjugation differentially expressed in KO mice compared to WT mice, as an initial investigation of a simple phase two biotransformation pathway?
2. Are changes in glycine conjugation expression, if any, also reflected in the remaining phase two biotransformation enzymes genes?

Answers to these questions were sought through the completion of five objectives. The **first** objective was to characterise and select *Ndufs4* KO and WT mice. The **second** objective was to investigate the differential gene expression of glycine conjugation in KO compared to WT mouse liver samples using a targeted transcriptomics approach. The differential expression of glycine conjugation was validated on protein level for the **third** objective. For the **fourth** objective, the remaining enzymes and corresponding genes involved in phase two biotransformation were identified from literature. The **fifth** and final objective was to revisit the transcriptomics dataset to investigate the differential expression of genes involved in phase two biotransformation.

5.2 Research question one answered: The expression of genes involved in glycine conjugation is downregulated in KO mice compared to WT mice.

Characterisation and selection of samples, as well as investigation and validation of differential gene expression of glycine conjugation were successfully completed. Methods employed are described in Chapter 3 and results are fully discussed in Sections 4.2 and 4.3. As expected, *Ndufs4* KO mice displayed significantly decreased CI activity in comparison to WT mice (27% CI activity compared to WT mice). The decreased activity in KO mice confirmed the effect of deletion of exon 2 and along with confirmation of genotypes served as validation of this mouse model. High quality RNA was successfully isolated, transcribed to cDNA and then sequenced using the Ion AmpliSeq™ Transcriptome Mouse Gene Expression Panel kit as described in Sections 3.4 and 3.5, while results are presented in Sections 4.2.3, 4.2.4 and 4.3.2. Gene expression analysis of *Acsm2* and *Glyat* indicated significant downregulation of the enzymes involved in the glycine conjugation pathway in KO mice compared to WT mice. In fact, *Acsm2* showed barely detectable expression in KO mice which correlated with a fold change of ~-3.6. *Glyat* was still expressed, but significantly less and correlated to a fold change of ~-1.6. Downregulation of these genes is possibly due to the reduced production of ATP in KO mice, which targets retrograde signalling to initiate adaptive gene regulation (discussed in Section 5.4). Reduced formation of CoA thioesters could further reduce expression of *Glyat* as less GLYAT enzymes are required to catalyse reactions with CoA thioesters. Downregulation of glycine conjugation was successfully validated on enzymatic level as described in Sections 4.3.3 and 4.3.4. To verify if the changes seen in gene expression translated to protein level, gene expression data was verified with SDS-PAGE and western blots. The inconclusive results obtained are mostly attributed to the use of a GLYAT

primary antibody reactive to rat and not mouse, which could not be obtained from South-African distributors. However, enzyme activity of GLYAT in *Ndufs4* KO samples was significantly decreased by exhibiting ~50% residual activity of GLYAT compared to WT mice and served as a sufficient confirmation of gene expression data.

Completion of the first three objectives effectively answered the first research question. It can now be concluded from this investigation that glycine conjugation, as a simple and mitochondrially located pathway of phase two biotransformation, is significantly affected and decreased in a mouse model of mitochondrial deficiency.

5.3 Research question two answered: The expression of the remaining genes involved in phase two biotransformation is largely downregulated in KO mice compared to WT mice, with the exception of sulfoconjugation pathways.

The remaining phase two biotransformation pathways investigated included: N-acetylation, amino acid conjugation (enzymes are divided into acyl-CoA synthetases and amino acid acyl transferases), glucuronidation, glutathione S-conjugation, methylation, and sulfoconjugation. A list of genes to be involved in these pathways were identified after a comprehensive review of literature. This list was filtered to 117 genes that were included in the Ion AmpliSeq™ panel used for gene expression analysis. Expression of genes from these pathways was predominantly decreased in the KO mice compared to the WT mice and were contributed by all seven pathways investigated. Fold changes of expression for these genes ranged between ~-1 to -16. Genes that showed increased expression in KO mice compared to WT mice were predominantly contributed by sulfotransferases. The fold changes of expression for genes with increased expression ranged between ~1 and 580. Further interpretation of these results is discussed in Section 5.4 below.

The list of genes identified could effectively be used to determine the differential expression of the remaining phase two biotransformation genes. Therefore, objectives 4 and 5 were successfully completed.

5.4 Decreased ATP levels, redox imbalance and stress implicated in altered expression of phase two biotransformation genes

Phase two biotransformation genes were expected to show downregulation in *Ndufs4* KO mice as a means to conserve energy. For most phase two biotransformation reactions, this belief held true.

Most of the mitochondrial proteome is encoded by nuclear genes and is therefore regulated by nuclear transcription factors, which is critical to mitochondrial homeostasis (Cardamone *et al.*,

2018). As discussed in Section 2.4, transcription factors like NRs are activated by hormones, fatty acids, bile acids, and cholesterol derivatives that can act as ligand-inducible transcription factors. Binding of these compound to transcription factors can explain how they are able to alter gene expression. Mitochondrial retrograde signalling is a signalling pathway that connects mitochondria and the nucleus, through which cells can regulate changes in gene expression and metabolic reprogramming in response to their physiological state (Kleine & Leister, 2016; Trendelewa & Zvyagilskaya, 2018). However the exact mechanisms of these retrograde signalling pathways are not well understood in mammals (Cardamone *et al.*, 2018). Some of the molecular signals for retrograde signalling produced by mitochondria that are of importance in this study include, ATP and ADP; GSH and GSSG; as well as NAD⁺ and NADH (Bohovich & Khalimonchuk, 2016).

A few possible reasons for the downregulation of biotransformation in *Ndufs4* KO mice were identified. Firstly, it is believed that the limited supply of energy in these mice would be conserved for life-sustaining processes and therefore limit several biotransformation reactions. Biotransformation pathways are especially involved in the metabolism of drugs and environmental chemicals. Some of these pathways (N-acetylation for example) currently do not have any identified role in liver metabolism for endogenous compounds. These mice are bred and housed in sterile and controlled conditions and do not receive any treatment. This could mean that they are less reliant on biotransformation reactions to metabolise drugs, so it is an easy pathway to downregulate if they are trying to conserve energy. Secondly, it is believed that the limited supply of ATP would hamper the reactions that require ATP and that it may affect retrograde signalling to the nucleus and initiate adaptive gene regulation. Some of the phase two biotransformation pathways, glycine conjugation for example, are dependent on ATP. In other pathways like glutathione S-conjugation and methylation, synthesis of cofactors GSH and SAM are dependent on ATP. Depletion of intracellular ATP due to mitochondrial damage leads to increased intracellular concentrations of AMP or its derivative, adenosine. Binding of adenosine to adenosine monophosphate-activated protein kinase (AMPK) complex in conjugation with another regulator protein kinase, LKB1, promotes activation of AMPK. One of the consequences of activation of AMPK includes inhibition of ATP-dependent biosynthetic pathways as a mechanism to prevent wasteful use of cellular ATP (Bohovich & Khalimonchuk, 2016). *Ndufs4* KO mice already have difficulty to produce ATP which could deplete the intracellular pools of ATP. In turn this can activate these retrograde signalling pathways and explain the inhibition of ATP-dependent pathways like glycine conjugation and glutathione S-conjugation. Thirdly, it is thought that the redox imbalance present in *Ndufs4* KO mice, reduces the levels of cofactors that are required in some of the pathways and subsequently, via retrograde signalling, downregulates expression of the genes that code for the enzymes. Synthesis of the cofactor involved in glucuronidation, uridine-5'-diphospho- α -D-glucuronic acid, requires NAD⁺. The NADH/NAD⁺ ratio

is known to be disturbed in *Ndufs4* KO mice as a result of the CI deficiency. Seeing that CI is the primary oxidation site for NADH, CI deficiency causes its inability to oxidize NADH resulting in a redox imbalance and decline in NAD⁺ levels (Lee *et al.*, 2019). The decreased NAD⁺/NADH ratios can activate AMPK and mitogen activated protein kinase (MAPK) mediated downstream signal transduction pathways (Bohovich & Khalimonchuk, 2016). Fluctuations of NAD⁺ is also believed to affect sirtuins (SIRTs) and poly-ADP-ribose polymerases (PARPs) activity, although its poorly understood (van der Knaap & Verrijzer, 2016). Furthermore, NAD⁺ is able to cap mRNAs and subsequently mediate gene regulation (Shao *et al.*, 2020). Oxidative stress is another contributing factor to the disturbed redox balance. GSH is the cofactor required in glutathione S-conjugation reactions. Its oxidised form is known as GSSG. Under conditions of oxidative stress the redox state of GSH:GSSG is disturbed to the right, resulting in reduced levels of GSH. This reduction in GSH levels could contribute to downregulation - on transcription level - of glutathione S-conjugation pathways. Depletion of GSH/GSSG pools results in the activation of nuclear factor erythroid 2 p45-related factor 2 (NRF-2) mediated antioxidant responses (Bohovich & Khalimonchuk, 2016). NRF-2 is a master regulator of redox homeostasis and targets genes for proteins that are involved in xenobiotic detoxification. Glutathione S-transferases are among the most inducible NRF-2 dependent genes in rodents (Baird & Dinkova-Kostova, 2011). NRF-2 accumulates in the nucleus as a result of exposure to oxidants or electrophiles. In the nucleus it binds to antioxidant response elements (ARE) in the upstream regulatory regions of genes encoding for detoxification enzymes where it can ultimately alter their transcription (Holmström *et al.*, 2016; Wu *et al.*, 2012).

It is believed that a major contributor to the upregulation of SULTs in *Ndufs4* KO mice could be increased physical stress. Sulfoconjugation is involved in the detoxification of xenobiotics as well as in drug and hormone metabolism. *Sults* were significantly upregulated in *Ndufs4* KO mice compared to WT mice. Upregulation of *SULTS* could increase the metabolic rate and escalate elimination of xenobiotics. Increased sulfoconjugation of hormones and neurotransmitters could furthermore cause disturbances in homeostasis (Balyan *et al.*, 2019). Expression of *SULTS* is regulated by several nuclear receptors including among others, peroxisome proliferator-activated receptors (PPARs), pregnane X receptors, constitutive androstane receptors (CAR) and vitamin D receptors (Runge-Morris *et al.*, 2013). As discussed in Section 4.5.3, one possible explanation for the upregulation of *Sults* is increased stress as a result of dysfunctional mitochondria (Emmerzaal *et al.*, 2020; Morava & Kozicz, 2013). CAR can be activated directly or indirectly to respond to environmental or stress challenges. It participates in physiological stress adaptation responses and is known to target the expression of several *Sults* including, *Sult2a1*, *Sult2a2*, *Sult3a1*, *Sult5a1*, and *Sult1e1*. Particularly, it can induce the expression of *Sult2a1* (Alnouti & Klaassen, 2008; Oliviero *et al.*, 2020; Saini *et al.*, 2004). It is therefore possible that dysfunctional

mitochondria of *Ndufs4* KO mice result in stress which in turn activates CAR to increase the expression of genes in the sulfoconjugation pathway. Furthermore, *GSTs*, *SULTs*, and *UGTs* are regulated by NRF-2. NRF-2 regulated transcription is independent from ligand activation. By binding to NRF-2, the KEAP1 protein retains NRF-2 in the cytoplasm. In conditions of oxidative stress, NRF-2 is released from KEAP1 and translocate to the nucleus to activate transcription of its target genes (Shen & Kong, 2009).

Another explanation is that increased sulfoconjugation serves as a mechanism to decrease levels of DHEA to avoid its effect on mitochondria. DHEA was reported to inhibit CI of isolated rat kidney mitochondria in a similar fashion as rotenone (Correa *et al.*, 2003). Thereby further inhibiting ATP production and NADH oxidation, which would be detrimental in a mouse model already exhibiting these inhibitions. Increased sulfoconjugation might be the result of an altered composition of the gut microbiome. It is possible that the upregulation of *Sults* is caused by the formation of toxins through intestinal bacteria. Gastrointestinal manifestations are frequently observed in patients with mitochondrial disease. In patients with Leigh Syndrome for example, chronic diarrhoea is particularly reported and is thought to be the result of small intestinal bacterial overgrowth (Van Coster *et al.*, 1991). The importance of the composition of the gut microbiome and its involvement in health and disease have received much attention recently. Imbalance of the gut microbiome has been linked to obesity, type 2 diabetes, cardiovascular disease, autoimmunity, and atopy (Bull & Plummer, 2014; Durack & Lynch, 2019). The microbiome is crucial in several metabolic pathways including metabolism of nondigestible carbohydrates, oligosaccharides and unabsorbed sugars and it also serves as a major source of energy (Bull & Plummer, 2014). Small intestinal bacterial overgrowth is known to affect the activity of a phase I biotransformation enzyme, flavin monooxygenase 3 (FMO3). Some of the substrates of FMO like trimethylamine are derived from the gut. Although beyond the scope of this study, retrospective analysis of our gene expression data revealed that FMO3 was significantly increased in *Ndufs4* KO mice compared to WT mice (fold change: 244.1; p-value: 4.0×10^{-9}), which might suggest a disturbance in the gut microbiome of KO mice. It is therefore possible that metabolites of FMO3 could be currently unknown substrates for sulfoconjugation leading to increased expression of sulfoconjugation genes.

Whether increased expression of *Sults* is due to one, or a combination of the above mentioned reasons, is currently unknown. Investigation of these and the involvement of the other *Sults* pathway metabolites in the upregulation of *Sults* can be elucidated in future follow-up work.

5.5 Strengths, limitations, and recommendations

As with all research, this study had several strengths, limitations, and challenges which are discussed below along with some prospects for future research.

5.5.1 Strengths of the study

Use of the *Ndufs4* whole body knockout mouse model, to investigate the effect of mitochondrial dysfunction on biotransformation, was an asset to this study. This model is an excellently characterised genetic model of CI deficiency and common in investigations of Leigh Syndrome and mitochondrial CI deficiency. Apart from being well characterised, the phenotypical presentation of these mice is closely related to that of Leigh Syndrome in humans, further signifying the value of using this model to investigate human mitochondrial disorders. Furthermore, on a practical level, in the research group where this study was conducted, a lot of experience was gained, and data developed on this disease model before the onset of this study.

A second strength of this study is the sample size used for gene expression analysis. Eight KO and eight WT mice were subjected to gene expression analysis. After removal of outliers, six WT and five KO mice remained, which is in line with other gene expression studies that used at least three to four samples per group (Konieczny *et al.*, 2019; Kotlinowski *et al.*, 2021; McManus *et al.*, 2019; Watanabe *et al.*, 2021). Lamarre *et al.* (2018) demonstrated that enough statistical power can be expected to observe differential expression in ~15 000 genes, if these changes occur, when a library size of 10 million reads and five biological replicates are used. This study had 10-15 million reads per sample and, therefore, had enough statistical power. As a means to keep ethical standards of this study as high as possible, sample size was selected to use the smallest number of mice required to obtain results with sufficient statistical power.

Targeted transcriptomic analysis was an appropriate and efficient technique used to address a complex problem. Targeted transcriptome sequencing is a relatively new approach that overcomes many of the challenges posed by qPCR. The use of Ion AmpliSeq™ technology in this study produced datasets that were manageable. The Ion AmpliSeq™ Transcriptome Mouse Gene Expression Panel used, measured the expression levels of ~23 000 genes. Only a small amount of those genes was analysed during this study, providing an opportunity for future studies to investigate numerous other metabolic pathways affected in these mice without the need to generate new data. These pathways are not limited to biotransformation but include among countless others, fatty acid oxidation, oxidative stress and redox pathways, cholesterol metabolism as well as several signalling pathways. Targeted transcriptomic technology is therefore an effective technique to deeply delve into the gene expression of samples.

Another strong suit of this study is the multiple validation steps implemented during various stages of the study. Firstly, genotypes of the mice were analysed twice to account for any human error that might have occurred during the first genotyping. This was an important step especially considering the one WT sample (sample 102) that grouped with KO samples in PCA plots and heatmaps. Without double verification of the genotype, one would easily have thought that it was due to an error during genotyping. Verification that the WT sample was indeed a WT, indicated that the variation was probably due to biological variation or errors and contamination induced during RNA isolation or sequencing steps. Secondly, characterisation of the mouse model by determining CI activity served as a control to ensure that deletion of exon two of *Ndufs4* did affect CI downstream. Thirdly, by knowing the limitations of different technologies, problems could be avoided or efficiently solved when they appeared. In line with this, two technologies – namely spectrophotometry and fluorometry, were used to ensure that RNA concentrations were determined accurately. RNA-sequencing was chosen above qPCR to determine the gene expression as it provided for analysis of a large number of genes simultaneously. The use of two different methods, enzyme activity and protein expression, to verify gene expression data also proved to be useful - especially after protein expression results remained inconclusive. Determination of the enzyme activity of GLYAT was sufficient to verify *Glyat*'s gene expression data as results from these two technologies aligned.

As thoroughly discussed by Dalman *et al.* (2012), the cut-off point for statistical measures like fold change and p-values are arbitrary and changing these cut-off points can drastically alter the interpretation of data. Some genes might have highly significant p-values but miss the fold change cut off point, and vice versa. This further highlights the need to verify gene expression data on other platforms. By looking at the p-values and effect size in combination, this study aimed to provide the most accurate list of significant genes. Although fold changes of some genes were small ($<\pm 1.5$), it was argued that a small but statistically significant change was still a biological change, especially when these followed trends seen in related genes with larger fold changes. Cut-off points used in this study were effectively chosen because slight alterations in the cut-off points did not alter the biological interpretation of the data, i.e. trends of gene expression observed for the different pathways remained the same even when genes with fold changes $<\pm 1.5$ and FDR >0.05 were removed from the list of the top 50 genes (results not shown).

5.5.2 Limitations

This study had several limitations that provides potential for future follow-up investigations.

As discussed in Section 4.3.2, there is a moderate correlation between mRNA and protein abundance due to a great deal of post-transcriptional, translational and protein degradation

regulations. Also to be considered is allosteric regulation factors and interaction of these factors with the genome via retrograde signalling. The glycine conjugation pathway was investigated on a transcriptomic, proteomic and enzymatic level which provides a fair level of confidence in the interpretation of the results. Although, this could further be improved upon by performing proteomic and enzymatic analyses on ACSM2 as well. The investigation of the remaining phase two biotransformation pathways was limited to transcriptomic analyses due to cost and time restrictions. Future studies that perform protein expression and enzyme activity analysis on the remaining phase two proteins would aid in confirming the tendencies of the pathways that were observed in the gene expression data. Time and costs required to validate RNA-sequencing experiments are currently still restricting factors. However, it has been shown that validation of lists of significant results can be cost effective by confirming a small random sample of results and statistically calculating the probability that a result has been validated (Leek *et al.*, 2012). This approach is more accurate than the usual approach of confirming the most statistically significant results and should be considered for follow-up studies.

A second limitation of this study was the use of suboptimal antibodies. The GLYAT primary antibody used was specific to rat due to hindrances in sourcing antibodies reactive to mouse. This affected the results for protein expression which, ultimately, were inconclusive. Future studies to investigate protein expression of GLYAT in these mice, should aim to import GLYAT antibodies that are specific to mice. Furthermore, there were differences observed in the expression of HSP60 between WT and KO samples. A possible explanation for these differences is that HSP protein levels have been shown to increase in conditions of mitochondria-generated oxidative stress (Barrett *et al.*, 2004; Hall & Martinus, 2013). It is therefore possible that increased expression of HSP60 in KO mice is due to increased oxidative stress levels. If this is the case, future studies should consider using another loading control for *Ndufs4* samples. Cytochrome c oxidase and voltage-dependent anion channel protein 1 (VDAC1) are suitable loading controls for mitochondria, although VDAC1's size is too close to that of GLYAT (31 kDa vs 34 kDa) and is, therefore, not recommended to be used as a control when analysing expression of GLYAT. The inconsistent expression of HSP60 between genotypes impeded normalisation of GLYAT expression to the loading control and with the bands of GLYAT that were very light, it was very difficult to determine differences (if any) between expression of GLYAT in WT and KO mouse liver samples. Due to time constraints, protein expression of GLYAT could not be investigated further and results remained inconclusive.

Expression of genes is greatly depended on age and sex (Kocarek *et al.*, 2008). Furthermore, the expression of genes involved in biotransformation vary greatly during tissue development and maturation (Lu *et al.*, 2013; Rowland *et al.*, 2013). Therefore, conclusions drawn from this study

are mainly applicable to male mice that are at the ages of P45-50. Newly born animals have a low capacity to detoxify therapeutic drugs and environmental chemicals. Leigh syndrome is especially prevalent in children so future investigations that focus on the biotransformation system in *Ndufs4* KO mice during different developmental stages, could potentially elucidate how an energy deficiency impacts the biotransformation system in young mice that already have a low biotransformation capacity.

5.5.3 Analytical challenges and recommendations

A major challenge of this study was to isolate RNA of sufficient quality. RNA had to be reisolated several times from samples until sufficient quality was obtained. This resulted in time, sample and reagent waste. A prime factor contributing to this is the MagMAX™-96 Total RNA Isolation kit that was used. This kit was chosen because very low sample input is required, ≤5 mg tissue in comparison to at least 30 mg required for other kits. In a study that compared five commercial extraction kits for total RNA isolation, the MagMAX™ kit performed relatively poorly (Jeffries *et al.*, 2014). In terms of its ability to consistently extract intact total RNA it performed poorly, as RNA extracted from this kit was typically highly degraded with average RNA integrity numbers (RIN) of <5. Consensus is that RIN values ≥8 is sufficient for RNA-Sequencing. Although challenging to isolate RNA, the kit performed adequately enough for our application and ultimately provided RNA of sufficient quality for sequencing. The low sample input required was especially advantageous as livers were used in other studies as well. Therefore, this kit is recommended where low sample input is a necessity, but exploration of other total RNA extraction in conditions where sample quantity is not limited, could be preferable. The use of a dedicated lab space that is restricted to RNA work could aid in avoiding contamination and RNA degradation. Although the use of agarose bleach gel electrophoresis was sufficient to determine RNA integrity, it was sometimes challenging to distinguish between artifacts and gDNA contamination. If available, the use of a 2100 Bioanalyzer to determine RNA integrity would be useful for samples with suspected degradation.

The use of AmpliSeq technology in this study had several advantages over conventional RNA-sequencing and is recommended for future studies aiming to determine differential gene expression. Firstly, the starting material required was significantly less, which was especially useful considering the relatively small size of mice tissue and the number of times RNA had to be reisolated as discussed above. Secondly, it resulted in short turn-around times with less data processing required. Thirdly, this AmpliSeq panel is designed so that most amplicons stretch over exon-exon boundaries which enables the effect of gDNA contamination to be circumvented. None of the 117 genes investigated in this study are part of the ~2200 single exon genes in the

panel. Thus, gDNA contamination had a minimal effect of the gene expression results in this study.

Common household bleach is recommended in literature for agarose bleach gel electrophoresis (Aranda *et al.*, 2012), but it was found that pure sodium hypochlorite provided better results in that there were less artefacts in the gels.

5.5.4 Future prospects

Beyond those mentioned above, there are several analyses that could improve the current understanding of biotransformation in energy deficient mice and provide excellent opportunities for future studies to build on. The liver is considered the central organ in metabolism of endogenous, exogenous and xenobiotic compounds, but these functions are not limited to the liver (Gröger *et al.*, 2018). Other organs, including the skin, lungs and especially the kidneys and intestines, are also involved in biotransformation reactions (Brandon *et al.*, 2003; Lu *et al.*, 2013). Gene expression of phase two biotransformation genes were only investigated in the liver during this study, but future studies could benefit from investigating the kidneys and intestines to get a thorough view of the effect of an *Ndufs4* KO on biotransformation. To further complete the picture, future studies could supplement current results by investigating phase one biotransformation enzymes and the transporters of these compounds. This could easily be achieved as the dataset used already contains gene expression results for multiple phase one genes. As discussed in Section 2.3, biotransformation is an intricate process that consists of two main phases and requires several transporters to function. Another way to complement the current dataset is to perform metabolomics. The metabolome, which is the downstream product of the genome, transcriptome and proteome, can provide the closest molecular representation of the phenotype (Bathe & Farshidfar, 2014; Zhang *et al.*, 2015). The results from the phase two biotransformation pathways could therefore be further validated by performing targeted metabolic analyses on liver samples of the *Ndufs4* mice. Future studies could also benefit from expression proteomics. Expression proteomics is the quantitative comparison of proteins by analysing changes in their expression level. Most of these analyses entail extraction, isolation and separation of proteins, and protein fragmentation using mass spectrometry (Tuli & Ransom, 2009). Mass spectrometry is a useful tool to analyse proteins on a large scale, especially for shotgun proteomics. Shotgun proteomics refer to the bottom-up approach of characterising proteins by analysing the peptides released from the protein through proteolysis. During shotgun proteomics, peptides are fractionated and analysed through LC-MS/MS, before identification by comparison of the mass spectra from the peptide fragmentation with *in silico* generated tandem mass spectra (Zhang *et al.*, 2013). Using techniques like these, the expression of many proteins can be analysed relatively

easily and it would be a good approach for future studies aimed at analysing the expression of several phase two biotransformation proteins.

Mouse models to study human disease are very common in research and have proven to be very useful to provide insight into mechanisms of disease and to explore candidate drug efficacy (Justice & Dhillon, 2016; Rosenthal & Brown, 2007). Although the mice models for human disorders used to study gene expression levels by Takao and Miyakawa (2015) show significant correlations to that of human disorders, mice and humans are still very different organisms. Relevant to this study is how the functions of genes cannot always be extrapolated between animal models and humans (Coughtrie, 2016). Future studies could potentially address this problem by evaluating iPSC cell lines derived from human tissue with an *NDUFS4* KO. A study in our research group is currently underway to develop such a cell line. Although cell lines have limitations of their own, it could still serve as a good model to give researchers a better understanding of how this defect affects human cells.

5.6 Concluding remarks

This study aimed to investigate differential expression of genes involved in phase two biotransformation in an *Ndufs4* knockout mouse model, first by an initial investigation of a simple phase two pathway and secondly by an expanded investigation of the remaining phase two pathways. Differential gene expression analysis of phase two biotransformation reactions in a male mouse model of Leigh Syndrome, indicated significant downregulation of most of these pathways. Downstream effects of decreased ATP production and redox imbalance that contribute to retrograde signalling, are hypothesised to be the main contributors to the downregulation of genes investigated. Sulfoconjugation reactions genes were the exception in showing extreme upregulation which is believed to be a response to the stress endured by these mice, or to serve as a mechanism to decrease build-up of toxic metabolites, or could be the result of an altered microbiome. Overall, the data strongly suggests that phase two biotransformation pathways are highly affected by mitochondrial dysfunction. Results from this study provide a solid basis for future studies to further investigate the effect of mitochondrial disease on biotransformation in order to understand the mechanism of the disease and potentially to aid in the development of suitable therapies.

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APPENDIX A ETHICS APPROVAL LETTER OF STUDY



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Tel: 086 016 9698

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**North-West University Animal Care, Health and
Safety Research Ethics Committee (NWU-
AnimCareREC)**

Tel: 018 299-1208

Email: Ethics-AnimCare@nwu.ac.za (for animal
studies)

03 July 2020

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 03/07/2020, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Investigation of gene expression responses in an Ndufs4 knockout mouse model

Principal Investigator/Study Supervisor/Researcher: Dr M Pretorius

Student: B Fouché – 26059878

Ethics number:

N	W	U	-	0	0	5	0	7	-	2	0	-	A	5
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institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation;
A = Authorisation

Application Type: Single study

Commencement date: 03/07/2020

Expiry date: 31/07/2021

Risk:

Category 0

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of July annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:
 - annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and
 - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.
- The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- Annually a number of studies may be randomly selected for active monitoring.

- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:
 - request access to any information or data at any time during the course or after completion of the study;
 - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;
 - withdraw or postpone approval if:
 - any unethical principles or practices of the study are revealed or suspected;
 - it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;
 - submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or
 - new institutional rules, national legislation or international conventions deem it necessary.
- NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work investigating gene expression responses in previously collected tissue from Ndufs4 knockout mice), this study will be able to proceed during the current alert level, upon receipt of the following ethics approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

Special COVID-19 arrangements:

There may, in future, be certain changes that you may need to make to your study, after it has been approved, due to the impact of the COVID-19 pandemic or the lockdown e.g.:

- Changing the ages of the animals to be used in the research study
- Changing the approved methodology to use data collection methods that ensure physical distancing from farmers and owners
- The implementation of risk-mitigating strategies to prevent infection during data collection
- Changes in time schedules etc.

Please note that any 1) amendments, 2) request for extensions or 3) any other kind of modification to the proposal or other associated documentation must be submitted for review by the NWU-AnimCareREC, Faculty of Health Sciences, and must be approved, prior to these changes being implemented.

These amendment requests will be handled via the *expedited process*.

Process to be followed:

- Ensure all your amendments are indicated in **yellow highlight** in the amended documents.
- Submit your request to Ethics-AnimCare@nwu.ac.za with a *cover letter* with a specific subject title indicating, "Amendment request (COVID-19): NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted.
- Name the emails, to which you attach the documents that you send, with a *specific subject line* indicating that it is an amendment request e.g. "Amendment request (COVID-19): NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment.
- Send a short email to Prof Wayne Towers and Prof Tiaan Brink indicating to them that you have submitted an amendment request due to the COVID-19 impact.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,



Digitally signed by Christiaan B Brink
DN: cn=Christiaan B Brink, o=North-
West University,
email=Tiaan.Brink@nwu.ac.za, c=ZA
Date: 2020.07.04 05:27:31 +02'00'

Chairperson: NWU-AnimCareREC

Current details: (22654704) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
15 June 2020
File Reference: 9.1.5.4.2

APPENDIX B LANGUAGE EDITING CERTIFICATE

P Smit Language and Editing Services

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CERTIFICATE

CERTIFICATE BF/210818
DATE: AUGUST 18, 2021

CERTIFICATION OF LANGUAGE EDITING OF MSc THESIS: DIFFERENTIAL EXPRESSION OF GENES INVOLVED IN PHASE TWO BIOTRANSFORMATION IN AN NDUFS4 DEFICIENT MOUSE MODEL

Faculty of Natural and Agricultural Sciences
School of Physical and Chemical Sciences
Focus area for Human Metabolomics

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belindarenatefouche@gmail.com

This is to certify that the above MSc thesis has been edited by Textfundl for spelling, syntax, grammar and language according to UK English standards and norms. The style, layout and references were done according to prescribed and standard NWU format.

P Smit

APPENDIX C NANODROP AND QUBIT CONCENTRATIONS

Table 6.1 Nanodrop and Qubit concentrations, as well as A260/A280 ratios for all samples

Sample	Nanodrop (ng/μL)	A260/A280	Qubit (ng/μL)
IonCode_0103	25.38	1.99	19.16
IonCode_0104	56.51	1.99	43.60
IonCode_0105	53.40	2.45	37.84
IonCode_0106	64.50	1.98	46.80
IonCode_0107	156.17	2.02	129.20
IonCode_0108	121.93	2.01	98.80
IonCode_0117	39.96	1.80	40.80
IonCode_0118	149.36	2.00	176.00
IonCode_0119	49.34	1.89	52.00
IonCode_0120	148.80	1.98	74.80
IonCode_0124	71.50	1.94	72.80

This table presents the concentrations obtained from the Nanodrop and Qubit, as well as the A260/A280 values for all samples.

Figure 6.1 Heatmap for 117 genes that were analysed



APPENDIX E SDS-PAGE GEL

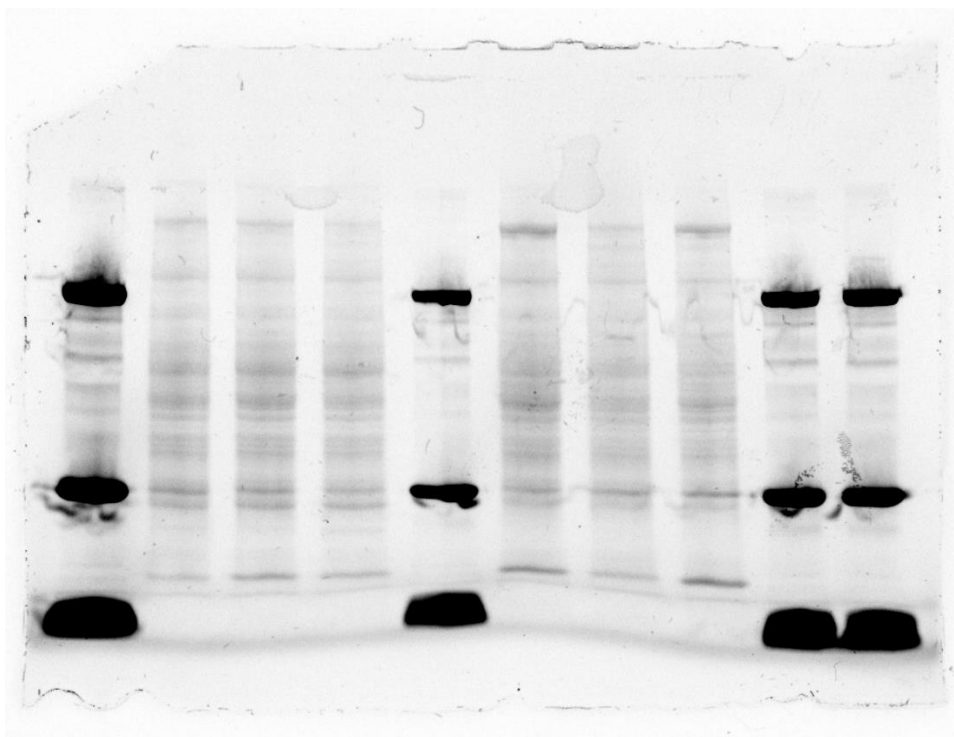


Figure 6.2 Image of SDS-PAGE gel before blotting to PVDF membrane

APPENDIX F DETAILS FOR 117 GENES ANALYSED

Table 6.2 Details of full list of genes investigated

	Gene	Name	EC number
N-acetyltransferases	<i>Aanat</i>	serotonin N-acetyltransferase	EC:2.3.1.87
	<i>Gnpnat1</i>	glucosamine 6-phosphate N-acetyltransferase	EC:2.3.1.4
	<i>Hgsnat</i>	heparan-alpha-glucosaminide N-acetyltransferase	EC:2.3.1.78
	<i>Natd1</i>	N-acetyltransferase domain containing 1	Unavailable
	<i>Nat2</i>	arylamine N-acetyltransferase 2	EC:2.3.1.5
	<i>Nat3</i>	arylamine N-acetyltransferase 3	EC:2.3.1.5
	<i>Nat8</i>	N-acetyltransferase 8	EC:2.3.1.-
	<i>Nat8f2</i>	N-acetyltransferase 8 family member 2	EC:2.3.1.-
	<i>Nat8f4</i>	N-acetyltransferase 8 family member 4	Unavailable
	<i>Nat8l</i>	N-acetyltransferase 8 like	EC:2.3.1.17
	<i>Nat9</i>	N-acetyltransferase 9	EC:2.3.1.-
	<i>Nat10</i>	N-Acetyltransferase 10	EC:2.3.1.-
	<i>Nat14</i>	N-acetyltransferase 14	EC:2.3.1.-
acyl-CoA synthetases	<i>Acss1</i>	acetyl-CoA synthetase 2-like, mitochondrial	EC:6.2.1.1
	<i>Acss2</i>	acyl-CoA synthetase short-chain family member 2, cytoplasmic	EC:6.2.1.1
	<i>Acss3</i>	acyl-CoA synthetase short-chain family member 3, mitochondrial	EC:6.2.1.1
	<i>Acs1m1</i>	acyl-CoA synthetase medium-chain family member 1, mitochondrial	EC:6.2.1.2
	<i>Acs1m2</i>	acyl-CoA synthetase medium-chain family member 2, mitochondrial	EC:6.2.1.2
	<i>Acs1m3</i>	acyl-CoA synthetase medium-chain family member 3, mitochondrial	EC:6.2.1.2

	Gene	Name	EC number
	<i>Acsm4</i>	acyl-CoA synthetase medium-chain family member 4, mitochondrial	EC:6.2.1.2
	<i>Acsm5</i>	acyl-CoA synthetase medium-chain family member 5, mitochondrial	EC:6.2.1.2
	<i>Acs1</i>	long-chain-fatty-acid-CoA ligase 1	EC:6.2.1.3
	<i>Acs3</i>	long-chain-fatty-acid-CoA ligase 3	EC:6.2.1.3
	<i>Acs4</i>	long-chain-fatty-acid-CoA ligase 4	EC:6.2.1.3
	<i>Acs5</i>	long-chain-fatty-acid-CoA ligase 5	EC:6.2.1.3
	<i>Acs6</i>	long-chain-fatty-acid-CoA ligase 6	EC:6.2.1.3
amino acyltransferases	<i>Baat</i>	bile acid-CoA:amino acid N-acyltransferase	EC:2.3.1.65
	<i>Glyat</i>	glycine N-acyltransferase	EC:2.3.1.13
	<i>Dgat1</i>	diacylglycerol O-acyltransferase 1	EC:2.3.1.20
	<i>Dgat2</i>	diacylglycerol O-acyltransferase 2	EC:2.3.1.20
	<i>Dgat2l6</i>	diacylglycerol O-acyltransferase 2-like protein 6	EC:2.3.1.-
	<i>Mogat1</i>	diacylglycerol acyltransferase 2-like protein 1	EC:2.3.1.22
	<i>Mogat2</i>	diacylglycerol acyltransferase 2-like protein 5	EC:2.3.1.22
	<i>Acat1</i>	acetyl-CoA acetyltransferase, mitochondrial	EC:2.3.1.9
glucuronosyltransferases	<i>Ugt2a3</i>	UDP-glucuronosyltransferase 2a3	EC:2.4.1.17
	<i>Ugt3a1</i>	UDP-glucuronosyltransferase 3a1	EC:2.4.1.17
	<i>Ugt3a2</i>	UDP-glucuronosyltransferase 3a2	EC:2.4.1.17
	<i>Ugt2b1</i>	UDP-glucuronosyltransferase 2b1	EC:2.4.1.17
	<i>Ugt2b5</i>	UDP-glucuronosyltransferase 2b5	EC:2.4.1.17
	<i>Ugt2b36</i>	UDP-glucuronosyltransferase 2b36	EC:2.4.1.17
	<i>Ugt2b37</i>	UDP-glucuronosyltransferase 2b37	EC:2.4.1.17
	<i>Ugt2b38</i>	UDP-glucuronosyltransferase 2b38	Unavailable

	Gene	Name	EC number
	<i>Ugt2b35</i>	UDP-glucuronosyltransferase 2b35	EC:2.4.1.17
	<i>Ugt2b34</i>	UDP-glucuronosyltransferase 2b34	EC:2.4.1.17
glutathione S-transferases	<i>Gsta3</i>	glutathione S-transferase a3	EC:2.5.1.18
	<i>Gsta4</i>	glutathione S-transferase a4	EC:2.5.1.18
	<i>Gstcd</i>	glutathione S-transferase c-terminal domain-containing protein	Unavailable
	<i>Gstk1</i>	glutathione S-transferase kappa 1	EC:2.5.1.18
	<i>Gstm1</i>	glutathione S-transferase mu 1	EC:2.5.1.18
	<i>Gstm2</i>	glutathione S-transferase mu 2	EC:2.5.1.18
	<i>Gstm3</i>	glutathione S-transferase mu 3	EC:2.5.1.18
	<i>Gstm4</i>	glutathione S-transferase mu 4	EC:2.5.1.18
	<i>Gstm6</i>	glutathione S-transferase mu 6	EC:2.5.1.18
	<i>Gstm7</i>	glutathione S-transferase mu 7	EC:2.5.1.18
	<i>Gstm5</i>	glutathione S-transferase mu 5	EC:2.5.1.18
	<i>Gsto1</i>	glutathione S-transferase omega-1	EC:2.5.1.18
	<i>Gsto2</i>	glutathione S-transferase omega-2	EC:2.5.1.18
	<i>Gstt1</i>	glutathione S-transferase theta-1	EC:2.5.1.18
	<i>Gstt2</i>	glutathione S-transferase theta-2	EC:2.5.1.18
	<i>Gstt4</i>	glutathione S-transferase theta-4	EC:2.5.1.18
	<i>Gstz1</i>	glutathione S-transferase zeta 1	EC:5.2.1.18
	<i>Mgst1</i>	microsomal glutathione S-transferase 1	EC:2.5.1.18
	<i>Mgst2</i>	microsomal glutathione S-transferase 2	EC:2.5.1.18
	<i>Mgst3</i>	microsomal glutathione S-transferase 3	Unavailable
methyltransferases	<i>Nnmt</i>	nicotinamide N-methyltransferase	EC:2.1.1.1

	Gene	Name	EC number
	<i>Hnmt</i>	histamine N-methyltransferase	EC:2.1.1.8
	<i>Pnmt</i>	phenylethanolamine N-methyltransferase	EC:2.1.1.28
	<i>Inmt</i>	indolethylamine N-methyltransferase	EC:2.1.1.49
	<i>Tpmt</i>	thiopurine S-methyltransferase	EC:2.1.1.67
	<i>Comt</i>	catechol O-methyltransferase	EC:2.1.1.6
sulfotransferases	<i>Sult1a1</i>	sulfotransferase 1a1	EC:2.8.2.1
	<i>Sult1b1</i>	sulfotransferase family cytosolic 1b member 1	EC:2.8.2.-
	<i>Sult1c1</i>	sulfotransferase 1c1	EC:2.8.2.-
	<i>Sult1c2</i>	sulfotransferase 1c2	EC:2.8.2.-
	<i>Sult1d1</i>	sulfotransferase 1 family member d1	EC:2.8.2.-
	<i>Sult1e1</i>	sulfotransferase 1e1	EC:2.8.2.4
	<i>Sult2a1</i>	sulfotransferase 2a1	EC:2.8.2.2
	<i>Sult2a2</i>	bile salt sulfotransferase 2	EC:2.8.2.14
	<i>Sult2a3</i>	sulfotransferase 2a3	EC:2.8.2.-
	<i>Sult2a4</i>	sulfotransferase 2a4	EC:2.8.2.-
	<i>Sult2a5</i>	sulfotransferase 2a5	EC:2.8.2.-
	<i>Sult2a6</i>	sulfotransferase 2a6	EC:2.8.2.-
	<i>Sult2a7</i>	sulfotransferase 2a7	EC:2.8.2.-
	<i>Sult2a8</i>	sulfotransferase 2a8	EC:2.8.2.-
	<i>Sult2b1</i>	sulfotransferase 2b1	EC:2.8.2.2
	<i>Sult3a1</i>	amine sulfotransferase 3a1	EC:2.8.2.3
	<i>Sult3a2</i>	amine sulfotransferase 3a2	EC:2.8.2.-
	<i>Sult4a1</i>	sulfotransferase 4a1	EC:2.8.2.-

Gene	Name	EC number
<i>Sult5a1</i>	sulfotransferase 5a1	EC:2.8.2.-
<i>Sult6b1</i>	sulfotransferase 6b1	Unavailable
<i>Sult6b2</i>	sulfotransferase 6b2	EC:2.8.2.-
<i>Chst1</i>	carbohydrate sulfotransferase 1	EC:2.8.2.21
<i>Chst2</i>	carbohydrate sulfotransferase 2	EC: 2.8.2.-
<i>Chst3</i>	carbohydrate sulfotransferase 3	EC:2.8.2.17
<i>Chst4</i>	carbohydrate sulfotransferase 4	EC:2.8.2.-
<i>Chst5</i>	carbohydrate sulfotransferase 5	EC:2.8.2.-
<i>Chst7</i>	carbohydrate sulfotransferase 7	EC:2.8.2.17
<i>Chst8</i>	carbohydrate sulfotransferase 8	EC:2.8.2.-
<i>Chst9</i>	carbohydrate sulfotransferase 9	EC:2.8.2.-
<i>Chst10</i>	carbohydrate sulfotransferase 10	EC:2.8.2.-
<i>Chst11</i>	carbohydrate sulfotransferase 11	EC:2.8.2.5
<i>Chst14</i>	carbohydrate sulfotransferase 14	EC:2.8.2.35
<i>Gal3st1</i>	galactosylceramide sulfotransferase	EC:2.8.2.11
<i>Gal3st3</i>	galactose-3-O-sulfotransferase 3	EC:2.8.2.-
<i>Hs2st1</i>	heparan sulphate 2-O-sulfotransferase 1	EC:2.8.2.-
<i>Hs3st1</i>	heparan sulphate glucosamine 3-O-sulfotransferase 1	EC:2.8.2.23
<i>Hs3st2</i>	heparan sulphate glucosamine 3-O-sulfotransferase 2	EC:2.8.2.29
<i>Hs3st4</i>	heparan sulphate-glucosamine 3-O-sulfotransferase 4	EC:2.8.2.-
<i>Hs3st5</i>	heparan sulphate glucosamine 3-O-sulfotransferase 5	EC:2.8.2.23
<i>Hs3st6</i>	heparan sulphate glucosamine 3-O-sulfotransferase 6	EC:2.8.2.23
<i>Hs6st1</i>	heparan sulphate 6-O-sulfotransferase 1	EC:2.8.2.-

Gene	Name	EC number
<i>Hs6st2</i>	heparan sulphate 6-O-sulfotransferase 2	EC:2.8.2.-
<i>Hs6st3</i>	heparan sulphate 6-O-sulfotransferase 3	EC:2.8.2.-
<i>Ndst1</i>	bifunctional heparan sulphate N-sulfotransferase 1	EC:2.8.2.8
<i>Ndst2</i>	bifunctional heparan sulphate N-sulfotransferase 2	EC:2.8.2.8
<i>Ndst3</i>	bifunctional heparan sulphate N-sulfotransferase 3	EC:2.8.2.8
<i>Ndst4</i>	bifunctional heparan sulphate N-sulfotransferase 4	EC:2.8.2.8

This table shows the gene symbols, names, and EC numbers for 117 genes that were analysed for their differential expression