Phase I and II biotransformation in patients with chronic fatigue

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ABSTRACT

This thesis, titled: '*Phase I and II biotransformation in patients with chronic fatigue*', forms part of the research and development programme of the Centre for Human Metabolomics at the NWU. There is no consensus definition of fatigue, ranging from idiopathic chronic fatigue (CF) to the more severe chronic fatigue syndrome and myalgic encephalomyelitis (CFS/ME). Against these diverse clinical presentations of fatigue, the working hypothesis formulated for the present study is: '*The wide occurrence of fatigue amongst individuals not suffering from a distinct clinical disorder may be due to an energy deprived state as a consequence of the individual's response to undefined exposure to xenobiotics, and which should be measurable in the metabolite profiles of biofluids from the fatigue cases'.*

Here we present in a thesis, structured in four parts, the first comprehensive investigation on CF, using a battery of selected indicators for phase I and II biotransformation measured in biofluids from a cohort of 576 female patients, all diagnosed by physicians to suffer from CF.

Part I includes two literature reviews. Chapter 1 deals with the mechanism of detoxification of xenobiotics by acyl-CoA synthetases, illustrated for benzoate and aspirin which highlights the complexity of multi-step detoxification. Chapter 2 presents a review on biotransformation and CF to further justify the working hypothesis. This review led to the proposal of a model for the combined influence of genome-based biotransformation and the exposome, leading to CF. A causal relationship is proposed for chronic xenobiotic exposure and the energy demand for the biotransformation responses, which may underlie idiopathic CF.

Part II covers the methodological part of the study. Chapter 3 presents detail on selected methods and on patient selection through information gained from our biotransformation test manual. Chapter 4 focus on the analytical aspects with special emphasis on method validation for phase I and II biotransformation analysis. Chapter 5 gives the statistical rationale to enable differentiation between all patients, using the complete set of data. The Chi-square Automatic Interaction Detection (CHAID) decision tree proved to be the preferred method for classification of the patient cohort.

Part III presents the results from the study. Chapter 6 is a contribution to improved understanding of the kinetic mechanism involved in human glycine-N-acyltransferase with high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS). Chapter 7 represents the culmination of the study, describing the biotransformation profiles from the cohort of patients in response to a hepatic detoxification challenge, identification of a potential biosignature for CF and the classification of all patients

through the CHAID decision tree. Chapter 8 illustrates how untargeted ^{1}H-NMR-metabolomics provided quantitative metabolite information on the detoxification challenge.

Part IV is a general discussion leading to a synthesis, proposing that the new insights could contribute to an improved detection and a future treatment strategy on CF. As predicted, no single biomarker account for CF but the use of a biosignature holds promise as a diagnostic tool for a personalized characterization of CF. Part IV is concluded with a few proposals for future research.

Keywords: Biotransformation; chronic fatigue; chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME); xenobiotics; acyl-CoA synthetases (ACSs); exposome; glycine acyl transferase (GLYAT); high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS); Chi-square Automatic Interaction Detection (CHAID); proton nuclear magnetic resonance (^{1}H-NMR); biosignature; personalized diagnostics.

Format: This thesis is presented in article format and meets the requirements set out by North-West University. Thus, the following six full papers and manuscripts form part of the thesis:

Chapter 1:
Van der Sluis, R. & Erasmus, E. 2016. Xenobiotic / medium chain fatty acid: CoA ligase – a critical review on its role in fatty acid metabolism and the detoxification of benzoic acid and aspirin. *Expert Opinion on Drug Metabolism & Toxicology*, 12(10):1169–1179. doi:10.1080/17425255.2016.1206888

Chapter 2:
Erasmus, E. Chronic fatigue, the exposome and biotransformation [Manuscript to be submitted in a revised form to *Expert Opinion on Drug Metabolism & Toxicology*].

Chapter 5:
Steffens, F.E., Erasmus, E. & Reinecke, C.J. A statistical approach for the classification of chronic fatigue cases, based on large and diverse data sets. [Manuscript to be submitted in an Afrikaans format to Suid Afrikaanse Tydskrif vir Natuurwetenskappe en Tegnologie].

Chapter 6:

van der Sluis, R., Ungerer, V., Nortje, C., van Dijk, A. & Erasmus, E. 2017. New insights into the catalytic mechanism of human glycine N-acyltransferase. *Journal of Biochemical and Molecular Toxicology*, 31(11): doi:10.1002/jbt.21963

Chapter 7:

Erasmus, E., Steffens, F.E., Van Reenen, M., Vorster, B.C. & Reinecke, C.J. 2019. Biotransformation profiles from a cohort of chronic fatigue women in response to a hepatic detoxification challenge. *PLoS ONE*, 14(5):1–22. doi:10.1371/journal.pone.0216298

Chapter 8:

Erasmus, E., Mason, S., van Reenen, M., Steffens, F.E., Vorster, B.C. & Reinecke C.J. A laboratory approach for characterizing chronic fatigue: what does metabolomics tell us? [Accepted for publication in *Metabolomics* manuscript number, MEBO-D-19-00082R1 22 Nov 2019. doi:10.1007/s11306-019-1620-4]

Reference to the following paper is included in Chapter 9.7 as an example on future approaches on biotransformation and chronic fatigue research:

Venter, M., Tomas, C., Pienaar, I.S., Strassheim, V., Erasmus, E., Ng, W.F., Howell, N., Newton, J.L., Van der Westhuizen, F.H. & Elson, J.L. 2019. MtDNA population variation in Myalgic encephalomyelitis/Chronic fatigue syndrome in two populations: a study of mildly deleterious variants. *Scientific Reports*, 9(1):1–8. doi:10.1038/s41598-019-39060-1

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LIST OF SYMBOLS, UNITS, ABBREVIATIONS & LATIN TERMS

Symbols

#	Number
$	Dollar
&	And
*	Asterisk
~	Approximately
<	Less-than
>	Greater-than
×	Times
§	Section sign
I	One (Roman numeral)
II	Two (Roman numeral)
III	Three (Roman numeral)
IV	Four (Roman numeral)
V	Five (Roman numeral)
α	Alpha
β	Beta
Δ / δ	Delta

Units

%	Percentage
°	Degrees
°C	Degrees Celsius
amu	Atomic mass unit
g	Gram
h	Hours
Hz	Hertz
L	Litres
M	Molarity; moles solute per litre of solvent
m/z	Mass-to-charge ratio
MF	Matrix Factor
mg	Milligram
MHz	Megahertz
min	Minutes
mL	Millilitre
mm	Millimetre
mM	Millimolar
mol	Mole
mV	Millivolt
N	Normality; molar concentration divided by an equivalence factor
ng	Nanogram
nm	Nanometre
nmol	Nanomole
pH	Potential of hydrogen; the negative of the base 10 logarithm of the H^+ molar concentration
ppm	Parts per million
rpm	Revolutions per minute
sec	Seconds
V	Volt

µg	Microgram
µL	Microlitre
µM	Micromolar

Abbreviations

%CV	Percentage coefficient of variance
•OH	Hydroxyl radical
^{1}H-NMR	Proton nuclear magnetic resonance
2,3-DHBA	2,3-dihydroxy benzoic acid
2,5-DHBA	2,5-dihydroxybenzoic acid
A	Acetaminophen
ACOT	Acyl-CoA thioesterases
ACS	Acyl-CoA synthetase
ACSL	Acyl-CoA synthetase long-chain.
ACSM	Acyl-CoA synthetase medium-chain.
ACSS	Acyl-CoA synthetase short-chain.
A-G	Acetaminophen glucuronide
AIDS	Acquired immune deficiency syndrome
AMP	Adenosine 5′-monophosphate
ANOVA	Analysis of variance
ANT	Adenine nucleotide transporter
A-S	Acetaminophen sulphate
ASA	Acetylsalicylic acid
ASCA	ANOVA simultaneous component analysis
ATP	Adenosine triphosphate
B	Biotransformation
BCA	Bicinchoninic acid
BOSS	Biotransformation and Oxidative Stress Status
BSTFA	O-bis(trimethylsilyl)trifluoro acetamide
CD	Compact disk
CFS	Chronic fatigue syndrome
CFS/ME	Chronic fatigue syndrome and myalgic encephalomyelitis
CHAID	Chi-square automatic interaction detection
CHE	Christian Higher Education
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase 1
CV	Coefficient of variation
CYP450	Cytochrome P450
ddH_2O	Double-distilled water
DEPPD	N,N-diethyl-paraphenylenediamine
DNA	Deoxyribonucleic acid
DOE	Diagnosis is one of exclusion
DTNB	2,2'-dinitro-5,5'-dithiobenzoic acid
E	Exposure
e.g.	*Exempli gratia* (Latin): for example
E+G	Exposome
EDTA	Ethylenediaminetetraacetic acid
ES	Effect size
ESI	Electrospray ionisation
etc.	*Etcetera* (Latin): indicates that further, similar items are included
EUFEPS	European Federation of Pharmaceutical Sciences
EWAS	Exposome-wide association studies
ExO	Exposure science ontology

FC	Fold change
FRAP	Ferri reducing ability of plasma
FS	Fibromyalgia
G	Genome
GC-MS	Gas chromatography-mass spectrometry
GLYAT	Glycine-N-acyl transferase
GP	General practitioner
GSH	Reduced glutathione
GSHt	Total red blood cell glutathione
GSSG	Oxidised glutathione; glutathione disulfide
GWAS	Genome-wide association studies
H_2O	Water
H_2O_2	Hydrogen peroxide
HA	Hippuric acid
HCl	Hydrogen chloride
HIV	Human immunodeficiency virus
HPLC	High- performance liquid chromatography
HREC	Health Research Ethics Committee
IBS	Irritable bowel syndrome
IEM	Inborn errors of metabolism
IOM	Institute of Medicine
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLOD	Lower limit of detection
LOAD	Late-onset Alzheimer disease
LOD	Limit of detection
LOQ	Limit of quantification
Max	Maximum
ME	Myalgic encephalomyelitis or Maine; relating to a state in the US
MF	Matrix factor
Min	Minimum
MPA	Metaphosphoric acid
*M*r	Molecular mass
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
MSQ	Medical symptoms questionnaire
mtCK	Mitochondrial creatine kinase
MTE	Mitochondrial thioesterase
MZ	Monozygotic
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPQI	N-acetyl-p-benzoquinone imine
NIEHS	National Institute for Environmental Health Sciences
NIH	National Institutes of Health
NINDS	Neurological disorders and stroke
NMR	Nuclear magnetic resonance
NRF	National Research Foundation of South Africa
Nrf2	Nuclear factor, erythroid 2-related factor 2
Nrf2-ARE	Nuclear factor, erythroid 2-related factor 2-antioxidant response elements
NSAID	Nonsteroidal anti-inflammatory drug
NWU	North-West University
NYD	Not yet diagnosed
OATPs	Organic anion transporting peptides
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation

PABA	P-aminobenzoic acid
PBMCs	Peripheral blood mononuclear cells
PCA	Principal component analysis
PCr	Phosphocreatine
PFS	Piper Fatigue Scale
PLS-DA	Partial least squares discriminant analysis
PU for CHE	Potchefstroom University for Christian Higher Education
p-value	Probability value; the probability of making a Type I error
QC	Quality control
QOL	Quality of life
R^2	Coefficient of determination
RF	Response factor
ROS	Reactive oxygen species
RRF	Relative response factor
RSD	Relative standard deviation
RT	Retention time
S	Salicylic acid
SAU	Salicyluric acid
SD	Standard deviation
SLC25	Solute carrier family 25
SNPs	Single nucleotide polymorphisms
SOP	Standard operating procedure
SPE	Solid phase extraction
SPECT	Single-photon emission computed tomography
SSIEM	Symposium of the Society for the Study of Inborn Errors of Metabolism
TAG	Triacylglycerol
TCA	Tricarboxylic acid
T-GSH	Total glutathione
TIA	Technology Innovation Agency
TMAO	Trimethylamine N-oxide
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TNB	5-thio-2-nitrobenzoic acid
TPTZ	2,4,6-tris-2-(pyridyl)-1,3,5-triazine
TSP	Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid
TXI	Triple-resonance inverse
UCP	Uncoupling protein
UK	United Kingdom
US	United States of America
UV	Ultraviolet
VDAC	Voltage-dependent anion carrier
VIP	Variable importance in projection
WHO	World Health Organization
X	Xenobiotica
X-OH	Oxidized intermediate

Latin terms

De novo	Anew; from the beginning
In vivo	In life; a process within a living organism

GLOSSARY OF TERMINOLOGY USED IN THIS THESIS

1. Biotransformation	According to the IUPAC vocabulary of terms used in medicinal chemistry, biotransformation is the modification of chemical compounds by living organisms or enzyme preparations. Generally this process is applicable to both xenobiotics and endogenous compounds, but medicinal chemistry usually concentrates on metabolic biotransformation of pharmaceutical agents (Borodina *et al.*, 2003).
2. Xenobiotics	Xenobiotics have been defined as chemicals to which an organism is exposed that are extrinsic to the normal metabolism of that organism and without nutritional value (Hoensch *et al.*, 1979).
3. Detoxification	The metabolic process by which toxins are changed into less toxic or more readily excreted substances.
4. Personalized medicine	The terms ‘stratified’, ‘personalised’ or ‘precision’ medicine all refer to the grouping of patients based on risk of disease, or response to therapy, using diagnostic tests or techniques (Academy of Medical Sciences, 2015).
5. Alternative medicine	The definition used by the National Center for Complementary and Alternative Medicine in the United States is “healthcare practices that are not an integral part of conventional medicine (NCCAM, 2000).
6. Translational research	Translational research is the process of applying knowledge from basic biology and clinical trials to techniques and tools that address critical medical needs. Unlike applied sciences, translational research is specifically designed to improve health outcomes. It uses an integrated team of experts who are focused on translating useful information from laboratories to doctors’ offices and hospitals. It’s a “bench to bedside” bridge (UC Davis, 2016).
7. Functional metabolism	An adaptability necessary for conserving the stability of the intracellular environment (homeostasis), which is, in turn, essential for maintaining an efficient functional state (Storey, 2004).
8. Exposome	At its most complete, the exposome encompasses life-course environmental exposures (including lifestyle factors), from the prenatal period onwards (Wild, 2005).

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PREFACE

I formally joined the inborn error of metabolism group in Biochemistry at the PU for CHE in 1986. At that stage the group had already established collaboration with South African physicians and paediatricians interested in metabolic disorders. In collaboration with Professor S.K. Wadman and his group at the clinical biochemistry division at Wilhelmina Kinderziekenhuis in Utrecht, the Netherlands, a severe case with a presumed metabolic disorder, diagnosed by a paediatrician, Dr. Celia Erasmus, turned out to be late-onset biotin-responsive multiple carboxylase deficiency (Erasmus *et al.,* 1985). This was the first diagnosis of an inborn error of metabolism from the Potchefstroom group and was related to the first case of that disorder, only reported two years earlier (Wolf *et al.,* 1983). As a young lecturer in biochemistry I clearly remember the interest in the diagnosis of a “new” metabolic disorder.

Within the group, my own focus was on the analytical aspects to identify and quantify diagnostic metabolites in inborn errors of metabolism, described in my MSc-thesis: “Die gebruik van gaschromatografie, massaspektrometrie en rekenaartegnologie in die identifisering van organiese-suururië”, PU for CHE, 1987. Even more importantly to me, was the impact of treatment and the subsequent effect on the metabolic and clinical level. These early diagnoses included cases of isovaleric aciduria, propionic academia, 3-methylglutaconic aciduria and pyroglutamic aciduria. In contrast to what we expected, treatment based on dietary intake corrections, cofactor supplementation and detoxification substrate supplementation did not always result in an improved clinical outcome. Treatment with cofactors and conjugation substrates worked well in isovaleric acidemia, but we achieved only limited success in propionic acidemia and dietary-based treatment in a patient with pyroglutamic aciduria failed to restore a normal health profile in the index case. This highlighted three very important facets in the treatment of metabolic disorders to us: (i) Early diagnosis. This point implies a close collaboration between the physicians and biochemists which specialize in the field of metabolism, including the analytical technology required for precision in diagnosis and monitoring of treatment; (ii) the success in treatment of these disorders was closely linked to the metabolic location of the defect. If the aberrant enzyme could benefit from dietary and/or coenzyme/cofactor treatment or if the conjugation moiety was a good substrate for a conjugation enzyme, then usually there was a marked improvement in the clinical features associated with the specific metabolic disorder. (iii) Even when there was an option for treatment based on improved detoxification of the accumulating metabolites, the clinical outcomes between individuals with the same defect still varied quite significantly, pointing to genetic heterogeneity and inter-individual variability in metabolic disorders.

Following our expanding competence with metabolic disorders in infants and children, we progressively experience physicians referring more and more adult patients for metabolic investigations. The reason for these referrals was often due to some idiopathic condition to which the physician could not link a specific diagnosis. In many of these cases, fatigue was one of the accompanying or even major clinical feature and mitochondrial energy abnormalities seemed to be the obvious source of fatigue. Metabolic investigations did, however, not reveal any undisputable metabolites to be biomarkers of perturbed energy metabolism in these adults. I began to suspect some sort of secondary influence on the mitochondria where accumulating endo- and exotoxins could perhaps inhibit the mitochondrial energy production system and thereby cause fatigue. The prominent role of fatigue in many of these idiopathic conditions was highlighted in a publication in the Canadian family physician journal in 2007, in which the authors stated that fatigue was amongst the top ten differential diagnoses in the family medicine practice. According to this publication fatigue was the main complaint in 45.80% of the patients under 45 years of age and in 36.60% of the patients 45 years and older (Ponka & Kirlew, 2007).

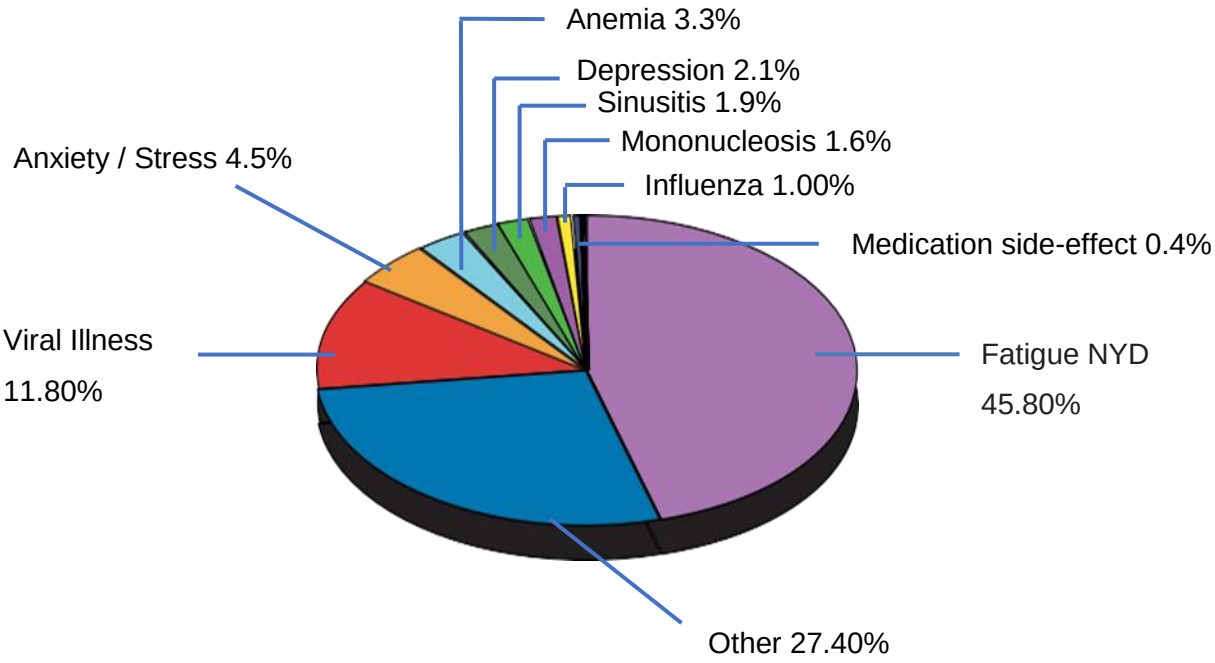


Figure I: Top ten differential diagnoses in the family practice. Adapted from David Ponka & Michael Kirlew, Canadian family physician, 2007.

I became convinced that the involvement of the liver in the metabolism of potentially harmful compounds, defined as “biotransformation” and the potential link to fatigue should be investigated. The limitation of direct metabolic investigations into liver metabolism was however also clear to us, seeing that our institution was not directly linked to a clinical set-up. In addition, investigations on biotransformation at the time were hampered by the absence of well-

established metabolites or biomarkers which indicates efficient biotransformation. In some way the functionality of the liver biotransformation had to be asserted however, and the potential that liver challenge tests held in this regard was convincingly argued for in the functional approach developed by Dr. DeAnn Liska (PhD) and clinical collaborators. In their publication "*Detoxification and biotransformational imbalances"* (Liska *et al.*, 2006), they emphasized the utility of challenge tests to monitor liver biotransformation and the effect of impaired biotransformation on oxidative stress levels (Liska, 1998). Moreover, a similar view on the value of challenge tests is shared by nutritional scientists of repute (Pellis *et al.*, 2012; Van Ommen *et al.*, 2009). I also realized that the rationale behind challenge tests on biotransformation would not be practiced by most physicians, as the general contention is that all potentially harmful compounds are metabolized and excreted naturally. This general misconception is now becoming well-acknowledged (Reviewed by Sears and Genuis (2012) in "Environmental determinants of chronic disease and medical approaches: recognition, avoidance, supportive therapy, and detoxification"). The need for pathological tests to evaluate the individual's health state with regard to exposure to endo- and exotoxins is now well-recognised, where the importance of biochemical marker testing to assess liver and kidney function is highlighted in what they referred to as a general approach to detoxification (Sears & Genius, 2012). To place the outcome of these biochemical tests into a useable clinical perspective it remains a prerequisite to obtain clinical data from these patients with regard to a wide range of clinical symptoms.

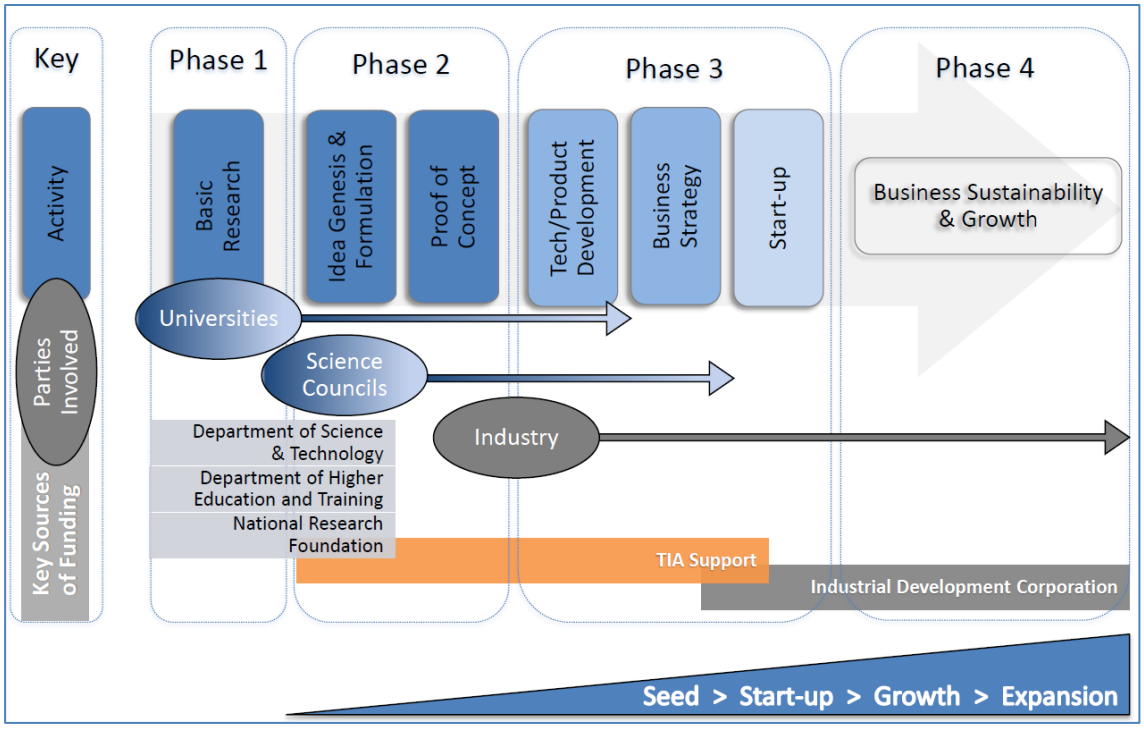


Figure II: The proposed funding model of the Technology and Innovation Agency (TIA) for the development of research activities to the commercial level which entails seed and

start-up support. The relative position of the biotransformation test development and implementation is reflected in shades of blue in the upper-half of Figure II.

Against this background, my interest shifted towards research on biotransformation and importantly included a pilot programme on biotransformation tests in fatigue adults, initiated in 2007. The pilot phase turned to the actual project with myself being the main investigator, providing outcomes which formed a major part of the present thesis. The experimental design of the comprehensive biotransformation study largely complied with the research and development requirements of the newly founded Technology and Innovation Agency (TIA) of South Africa. TIA was established as a developmental research initiative and provided seed funding and start-up support for innovative projects, as illustrated schematically in Figure II. As such, the present project was successfully registered as "The Technology Innovation Agency Biotransformation Project" (TIA-BOSS Project). The problem statement and aims of the research for the present thesis had, however, a wider scope than the TIA project.

Functional metabolism is essential to maintain homeostasis, including the key aspect of efficient energy production to sustain metabolic and bodily functions. Lessons learned from our research in inborn errors of metabolism and later from preliminary biotransformation profile results on treatment of clinically cases from our referral clinicians, demonstrated that one of the most effective treatment methods was to focus on removal of potential toxic substances. Chronic metabolite exposure at toxic levels (like what would occur in a metabolic disorder) lead to a variety of clinical symptoms, with fatigue nearly always present, although often over-shadowed by other symptoms. Similarly, fatigue is a general observation seen in individuals with persistent exposure to environmental xenobiotics, with effective removal of the affectors often resulting in marked improvement of the clinical symptoms. Against this background I formulated the following working hypothesis for the present study on fatigue and biotransformation:

> *"The wide occurrence of fatigue amongst individuals not suffering from a distinct clinical disorder may be due to an energy deprived state as a consequence of the individual's response to undefined exposure to xenobiotics, and which should be measurable in the metabolite profiles of biofluids from the fatigue cases"*

This working hypothesis complied with the aims of TIA, expressed in their Strategic Objective 1: "*to stimulate the development and commercialisation of technology-based services, processes and products".* Following application and consultation, the NWU received co-funding from TIA for the further development of the hypothesis towards practical implementation and included the co-operation of CareDoc (practice number 0346756), a private clinical trial facility in South Africa as well as co-operation from several referring physicians in private practice.

Finally, the thesis that emanated from my research is presented in article format according to the technical and academic requirements of the North-West University. The glossary provides a list of terms for the explanation of some key concepts or terms used in this thesis. Supplementary Information as well as a Google drive link with complex data are included in the final section of the thesis. The thesis itself is presented in four parts:

Part I, "Biotransformation in context", consists of two literature reviews. Chapter 1 deals with the role of medium chain fatty acid: CoA ligase in detoxification reactions, using benzoic acid and aspirin as model substances and Chapter 2 focuses on an exploration of the concept of "biotransformation", from which a refined hypothesis was formulated. **Part II** focuses on methodological aspects. This includes, firstly, the experimental design and the Biotransformation and Oxidative Stress Status (BOSS) brochure, used to generate the clinical data and collection of the biofluid samples (Chapter 3). Secondly, the analytical aspects are described for standardizing the biochemical analysis, summarized in the Standard Operational Procedure for the BOSS project (Chapter 4) and thirdly the statistical method developed to analyse three unrelated data sets (clinical, fatigue and biochemical information (Chapter 5). **Part III** presents the main contributions that emanated from the study: (1) new insights into the catalytic mechanisms of GLYAT, an important biotransformation enzyme (Chapter 6); (2) the biotransformation profiles of a cohort of fatigue patients in response to a hepatic detoxification challenge (Chapter 7); and (3) an untargeted metabolomics approach to assess a laboratory method for characterizing chronic fatigue (Chapter 8). Finally, **Part IV** is a synthesis of current results and is presented with emphasis on future developments (Chapter 9).

Based on our experience with IEM's, it was clear that studies involving human subjects requires detailed clinical information about every patient, both to compare individuals with each other and for the clinician to evaluate treatment outcome. For this purpose, I compiled a set of validated fatigue and medical symptoms questionnaires that were incorporated into the biotransformation test kit.

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PART I

INSIGHTS IN BIOTRANSFORMATION: LITERATURE REVIEWS

CHAPTER 1

On the detoxification of xenobiotics

1.1 Introduction

Human health and the environment is a key international issue. On the global level, the World Health Organization (WHO) “aims to enhance capacity in countries through regional technical networks of experts, intercountry sharing of good practices, research, policies and action on environmental and occupational hazards to health and strengthening environmental surveillance and information systems. Emphasis is placed on enabling strengthened collaboration between health and environment sectors” (WHO, s.a.). Humans are constantly and extensively exposed to numerous fabricated products, some of which are useful and others hazardous. An important question on the molecular level is how individual cells and the microbiome within the human body protect themselves against the continuously changing environment. In this regard biotransformation functions in the centre stage. Biotransformation entails metabolic processes that occur mainly in the liver and are primarily described as the enzymatic conversion of potentially harmful substances which results in inactivation, detoxification and an enhanced likelihood of excretion in urine or bile (Van der Westhuizen *et al.,* 2000; Zamek-Gliszczynski *et al.,* 2006).

Biotransformation of xenobiotics is an important biological defense mechanism against endo- and exotoxins and has been well reviewed (Arana *et al.*, 2014; Fasinu *et al.,* 2012; Park *et al.,* 2014). Many detailed aspects on the mechanism of biotransformation still remains unresolved, some of which are important for the present investigation. One of these is the biochemical link between processes within the intermediary metabolism and xenobiotic metabolism and detoxification. In this chapter a review is presented on the interphase between fatty acid metabolism and detoxification, taking benzoic acid and aspirin as model xenobiotics (Van der Sluis & Erasmus, 2016). This review paved the way towards our investigation on the catalytic mechanism of human glycine N-acyltransferase, described in Chapter 6.

1.2 A literature review and expert opinion — Xenobiotic/medium chain fatty acid: CoA ligase — a critical review on its role in fatty acid metabolism and the detoxification of benzoic acid and aspirin (Van der Sluis *et al.*, 2016 — publication I):

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REVIEW

Xenobiotic/medium chain fatty acid: CoA ligase – a critical review on its role in fatty acid metabolism and the detoxification of benzoic acid and aspirin

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ABSTRACT

Introduction: Activation of fatty acids by the acyl-CoA synthetases (ACSs) is the vital first step in fatty acid metabolism. The enzymatic and physiological characterization of the human xenobiotic/medium chain fatty acid: CoA ligases (ACSMs) has been severely neglected even though xenobiotics, such as benzoate and salicylate, are detoxified through this pathway.

Areas covered: This review will focus on the nomenclature and substrate specificity of the human ACSM ligases; the biochemical and enzymatic characterization of ACSM1 and ACSM2B; the high sequence homology of the *ACSM2* genes (*ACSM2A* and *ACSM2B*) as well as what is currently known regarding disease association studies.

Expert opinion: Several discrepancies exist in the current literature that should be taken note of. For example, the single nucleotide polymorphisms (SNPs) reported to be associated with aspirin metabolism and multiple risk factors of metabolic syndrome are incorrect. Kinetic data on the substrate specificity of the human ACSM ligases are non-existent and currently no data exist on the influence of SNPs on the enzyme activity of these ligases. One of the biggest obstacles currently in the field is that glycine conjugation is continuously studied as a one-step process, which means that key regulatory factors of the two individual steps remain unknown.

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ACSM; acyl-coenzyme A; aspirin; benzoate; glycine conjugation; medium chain fatty acid; CoA ligase; salicylate

1. Introduction

Fatty acids serve many essential functions in living organisms. They are the building blocks of lipids, including storage molecules such as triacylglycerol and cholesteryl esters, structural lipids such as phospholipids, plasmalogens, and sphingolipids, and signalling molecules such as diacylglycerol. Fatty acids can also be degraded for energy production, converted to alcohols or aldehydes, remodeled by elongation and/or insertion or removal of double bonds, or covalently bound to proteins [1].

Acyl-coenzyme A (acyl-CoA) synthetases form a large family of enzymes that catalyze the activation of fatty acids by CoA to produce an acyl-CoA, the first step in fatty acid metabolism [1]. The focus of this review will be on medium chain fatty acid and xenobiotic metabolism by the human xenobiotic/medium chain fatty acid:CoA ligases (acyl-CoA synthetase medium-chains [ACSMs]) (EC 6.2.1.2). We will review and discuss medium chain fatty acid metabolism; nomenclature and substrate specificity of the ACSM ligases; biochemical and enzymatic characterization of ACSM1 and ACSM2B; the *ACSM2* genes (*ACSM2A* and *ACSM2B*) and genetic variation as well as what is currently known regarding disease association studies. At present, in depth research on the ACSM ligases is severely lacking and we will, therefore, point out some inconsistencies in the current literature, discuss the lack of knowledge, and summarize some important future prospects regarding the pharmacogenomics of aspirin.

2. Fatty acid metabolism and roles of activated fatty acids

The diversity of fatty acids in nature is extensive. Fatty acids can range widely in their chain lengths, from the 2 carbon acid, acetate, to those containing >30 carbons in some waxes and plant lipids. Furthermore, fatty acids can be found that are totally saturated, that contain one (monounsaturated) or more (polyunsaturated) double bonds, or that have methyl branches. Thus, hundreds of naturally occurring fatty acid species exist [2]. All of these fatty acids must be activated prior to further metabolism by forming a thioester with CoA and is closely associated with oxidative phosphorylation [3,4]. In the early 1950s, the first enzyme catalyzing the activation process, named the fatty acid activating enzyme, was purified from bovine liver and characterized using butyrate as substrate [5]. Over the next three decades, various research groups elucidated the nature of fatty acid activation by members of the fatty acid ACS (fatty acid:CoA ligase) (ACSs; EC 6.2.1.x) family of enzymes [6,7]. Due to this great diversity in fatty acids, it is, therefore, not surprising that higher organisms contain multiple enzymes with ACS activity to facilitate both anabolic and catabolic reactions of fatty acids [2].

2.1. Ligase activation of fatty acids

ACSs belong to a superfamily of adenylate-forming enzymes [2]. Activation of fatty acids requires a two-step reaction

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Article highlights

- The physiological role of the individual human ACSM ligases have not been thoroughly clarified.
- CoA activation by ACSM2B is important for the detoxification of benzoate and salicylate.
- The substrate specificity of ACSM2B for salicylate is very low when compared to benzoate, which might contribute to aspirin sensitivity.
- The influence of genetic variation on the enzyme activity of the individual ACSM ligases are unknown.
- The high exposure to xenobiotics in modern times might have a detrimental effect on the CoA activation and subsequent glycine conjugation of these compounds.

This box summarizes key points contained in the article

catalyzed by these enzymes [8]. In the first step, an acyl-adenosine monophosphate intermediate is formed from adenosine triphosphate (ATP). AMP is then exchanged with CoA to produce the activated acyl-CoA. The release of AMP in this reaction defines the superfamily of AMP-forming enzymes. The length of the carbon chain of the fatty acid species defines the substrate specificity for the different ACS [9]. Figure 1 represents a schematic outline of the mobilization and activation of fatty acids.

Before the bioinformatics era, fatty acid activation activity was often characterized biochemically by chain length preference. These early investigations gave rise to the notion that there was an ACS, a butyryl-CoA synthetase, an ACSM, and a long-chain ACS (ACSL) [5,11–13]. However, there is significant overlap in the chain length specificity of, for example, ACSMs and ACSLs, and this may also vary depending on the degree of unsaturation of the fatty acid substrate. Short-chain ACSs (ACSSs) typically activate acetate, propionate, or butyrate. ACSMs are those that activate C_6–C_{10} fatty acids. ACSLs are typically thought of as those that activate palmitate (C_{16}) and oleate (C_{18}), the most common fatty acids found in nature [13,14].

Xenobiotic carboxylic acids (e.g. benzoic acid, salicylic acid, and phenylacetic acid) that undergo glycine conjugation are also activated by the mitochondrial ACSM ligases (Figure 1). The acyl-CoA species increase the reactivity of the carboxylic acid and therefore amino acid conjugation may be viewed as a mechanism of cell protection. In humans, however, formation of a xenobiotic-CoA does not necessarily result in subsequent amino acid conjugation [15]. The xenobiotic-CoA can function as an alternative substrate in pathways of intermediary metabolism, such as lipid biosynthesis [16].

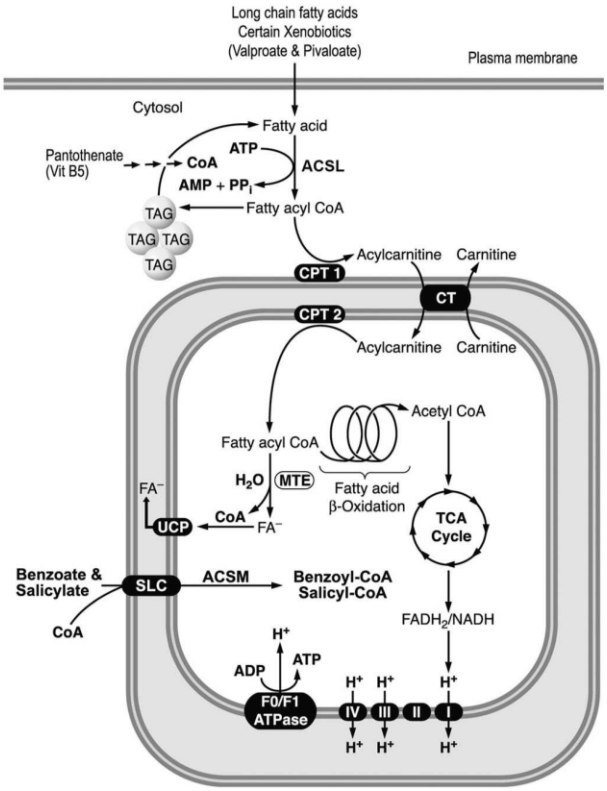


Figure 1. Illustration of the metabolism involved in the mitochondrial uptake and conjugation of fatty acids and specific xenobiotics. Long chain fatty acids and certain xenobiotics (valproate and paviloate) are activated in the cytosol by ACSL to the corresponding CoA derivative. This requires ATP and CoA. CoA is synthesized from pantothenic acid (Vitamin B_5) in the cytosol. The activated compounds are then transported into the mitochondria via the actions of carnitine palmitoyltransferase 1 (CPT1), Carnitine-acylcarnitine translocase (CACT)/CT and CPT2 in the carnitine shuttle. Mitochondrial thioesterase (MTE) can cleave long-chain acyl CoA to fatty acid anions (FA^-), which may leave the mitochondrial matrix via the uncoupling protein (UCP). The xenobiotics benzoate and salicylate are converted to CoA derivatives in the mitochondrial matrix by ASCM. The CoA pool in the mitochondria is replenished from the cytosol via the solute carrier family 25 transporter (SLC25). Benzoyl-CoA and Salicyl-CoA can then be conjugated to glycine by glycine N-acyl transferase (GLYAT). TAG – Triacylglycerol. Adapted with permission from [10].

2.2. Biosynthesis of CoA

The activation of substrates by ligases is, apart from the energy required in the form of ATP, also very dependent on the availability of CoA [17]. These molecules are truly endogenous as they need to be synthesized from a precursor from the B-vitamin group namely pantothenic acid or vitamin B_5 [18] (Figure 1). Animals and some microbes lack the capacity to synthesize pantothenate and are totally dependent on the uptake of exogenous pantothenate. The synthesis of CoA takes place in the cytosol and is accomplished in a five-step metabolic process during which the pantothenic acid is converted to CoA [19]. These reactions are conserved and absolutely essential for life. Any defects in this pathway could potentially be life-threatening as CoA is, in contrast to pantothenate, not readily available from dietary sources. Four of the enzyme reactions involved in the synthesis of CoA require ATP and the amino acid cysteine is also an essential cofactor in the second reaction, providing the thiol moiety for the CoA molecule [20]. Limited cysteine bioavailability could be detrimental to the synthesis of CoA [21]. The synthesized CoA is transported over the mitochondrial membrane by SLC25A42, a member of the solute carrier family 25 (SLC25) (Figure 1) [22].

2.3. Metabolic role of CoA and acyl-CoA

Acyl-CoA molecules have a variety of functions in the metabolism. They are crucial in the production of energy in the mitochondria via B-oxidation of activated fatty acids [23] (Figure 1). Acyl-CoA can also be instrumental in the synthesis

of triacylglycerols and phospholipids and esterification of cholesterol and retinal. A less well-known function of acyl-CoA is that of a cell signalling molecule. In this regard, it is involved in the alteration of insulin secretion, glucose transport and metabolism, and then also in apoptosis. In the case of some medium and longer chain acyl-CoAs, transport into the mitochondrial matrix cannot proceed without the help of a carrier molecule L-carnitine (Figure 1). This is accomplished with the help of three enzymes carnitine palmitoyl transferase I, a carnitine-acylcarnitine translocase, and carnitine palmitoyltransferase II. With the release of the acyl-group in the mitochondrial matrix, there is recycling of CoA and carnitine [24].

In the case of fatty acids, which is more the focal point in this review because of their association with the medium chain acyl-CoA ligases, the activated acyl-CoA is pivotal in the regulation of ion channel opening, the budding and fusion of intracellular membranes [25], and protein transport. Acyl-CoAs are also required for enzyme activation, protein acylation, cell signaling, and transcriptional control in addition to serving as substrates for oxidation and phospholipid biosynthesis [26]. CoA plays an important role in regulating cardiac energy metabolism. Accumulation of intracellular acyl-CoAs can be toxic [27], stimulate mitochondrial stress and increase the production of damaging free radicals. As already mentioned, xenobiotic carboxylic acids can also be activated to CoA-thioesters and may contribute to the compound's toxicity [28]. These xenobiotic-CoAs may be poor substrates for further metabolism, accumulation, and contribution to aberrant cellular processes. Several types of mechanisms may be involved in this, including (1) inhibition of vital metabolic enzymes by the acyl-CoA, (2) sequestration of the total cellular CoA pool as the unusual acyl-CoA, (3) direct physical–chemical effects of the acyl-CoA, and (4) sequestration and depletion of carnitine as the acyl group is transformed from the acyl-CoA to form the corresponding acylcarnitine.

Many of these toxicities are similar to sequelae observed in the inherited organic acidurias in which endogenously generated acyl-CoAs accumulate secondary to an enzymopathy [29]. In this respect, it has been suggested that dysregulated lipid metabolism, originating with the formation of acyl-CoAs, is the basis of multiple diseases including obesity and diabetes [30]. It has also been proposed that because acyl-CoA formation is a primary step in fatty acid metabolism, the activating enzyme ACS and deactivating enzyme acyl-CoA thioesterases (ACOTs) may have a direct influence on the metabolic fate of fatty acids by pointing the substrate toward or away from downstream enzymes [31]. The ACS and ACOT enzymes may also regulate fatty acid metabolism by controlling cellular concentrations and compartmentalization of free fatty acid, acyl-CoA, free CoA, and AMP/ATP.

3. Nomenclature and substrate specificity of ACSM

Mitochondria from liver contain various amounts of diverse forms of ACSMs. Xenobiotic ACSM ligases are of particular interest as these ligases have extremely broad substrate specificities. Not only do these ligases function as xenobiotic carboxylic acid:CoA ligases (benzoic acid and aspirin), but also as medium-chain fatty acid:CoA ligases (C_4–C_{12} fatty acids) [9]. Defining the substrate profile of the ACSM subgroup of enzymes has, however, been challenging as overlapping substrate and fatty acid specificity, along with the use of crude tissue and subcellular preparations have made this a complicated task [32]. Although much research has been done on several mammalian CoA-ligases [5,32–36], there is limited information in humans about the identity of individual xenobiotic CoA ligases responsible for the metabolism of a variety of drugs and chemicals [32]. Unfortunately, the current literature on the ACSM enzymes also contains many inconsistencies. This will be discussed in detail in Section 8.1.

Watkins et al. [2] used 2 conserved amino acid sequence motifs to identify 26 distinct ACS genes in the human genome [2]. Six of the 26 distinct human ACSs namely: ACSM1, ACSM2A, ACSM2B, ACSM3, ACSM4, ACSM5, and ACSM6, form part of the ACSMs (Table 1) [2,9,37,38].

The ACSM gene family consists of four members located on chromosome 16 (*ACSM1, 2, 3, 5*) while the *ACSM4* gene is located on chromosome 12 and the *ACSM6* gene on chromosome 10.

3.1. ACSM1 (HXM-B, MACS1)

The *ACSM1* (75,658 base pairs) gene is located on chromosome 16 at position 16p12.2 (ENSG00000166743 [50]). *ACSM1* encodes for a 577 amino acid protein expressed in the mitochondrial matrix that has medium-chain fatty acid:CoA ligase activity with broad substrate specificity. ACSM1 acts on acids from C_4 to C_{11} and on the corresponding 3-hydroxy- and 2,3-, or 3,4-unsaturated acids (*in vitro*) [9]. See Section 4 for a detailed discussion on the substrate specificity of ACSM1 (HXM-B).

Table 1. The six ACSMs.

ACSM	Synonyms	Nucleotide reference sequence	Protein reference sequence	Transcript ID	Chromosomal location	Strand
ACSM1	BUCS1; MACS1; LAE; HXM-B	NM_052956	NP_443188	ENST00000307493	16p12.2	Reverse
ACSM2A	LOC123876; A-923A4.1; MACS2	NM_001308172.1*	NP_001295101.1*	ENST00000573854	16p12.3	Forward
ACSM2B	HXMA; HYST1046	NM_182617	NP_872423	ENST00000567001	16p12.3	Reverse
ACSM3	SAH; SA	NM_005622	NP_005613	ENST00000289416	16p13.1	Forward
ACSM4	LOC341392	NM_001080454	NP_001073923	ENST00000399422	12p13.31	Forward
ACSM5	FLJ20581; MACS3	NM_017888	NP_060358	ENST00000331849	16p12.3	Forward
ACSM6	C10orf129	NM_207321.2	NP_997204.2	ENST00000341686	10q23.33	Forward

ACSM: Acyl-CoA synthetase medium-chain.

*It should be noted that the reference sequences given in Watkins et al. [2] (NM_001010845 and NP_001010845) has since been removed from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NM_001010845) because currently there is insufficient support for these transcripts. The correct reference sequences have been given here.

Several disease association studies have been performed for ASCM1 and link *ACSM1* to invasive apocrine carcinomas [39], plasma high-density lipoprotein cholesterol levels [40], schizophrenia [41], and major depressive disorder [42].

3.2. ACSM2

Due to a chromosomal duplication event of approximately 80 kb, two different loci of the *ACSM2* gene exist, *ACSM2A* and *ACSM2B*, located on chromosome 16 at position 16p12.3 [2,43,44]. The HXM-A form of XM-ligases was renamed by the Human Genome Organization first to *ACSM2* and, more recently, to *ACSM2B*. Although the biological function of ACSM2A has not been characterized in any species [2], the crystal structure for ACSM2A has been elucidated for a series of substrate/product/cofactor complexes [45]. *ACSM2B* is the predominant transcript identified in human liver when compared to *ACSM1, 2A, 3,* and *5* [38]. This should, however, be reinvestigated as will be explained in Section 8.1.2. The biochemical and enzymatic characteristics of ACSM2B (HXM-A) will be discussed in more detail in Section 4.

3.3. ACSM3 (SAH)

The human *SA* (*SAH, ACSM3*) gene, approximately 33 kilo bases, was mapped to human chromosome 16p13.11, consisting of 14 exons and 13 introns [46] and encodes for a 586 amino acid protein expressed in human kidneys and liver. Further studies suggested that the *SAH* protein is located in the mitochondria and has ACS activity for medium-chain fatty acids. Disease association studies have shown that polymorphisms of the *SAH* gene are associated with multiple risk factors, including hypertriglyceridemia, hypercholesterolemia, obesity [47] and hypertension [48], and non-high-density lipoprotein cholesterol levels in postmenopausal women [49].

3.4. ACSM4

ACSM4 is located on chromosome 12, spans 24,441 bases and encodes for a 580 amino acid protein [50 ENSG00000215009] that is predominantly expressed in the testis. *ACSM4* polymorphisms are associated with rapid AIDS progression in HIV-infected patients [51,52].

3.5. ACSM5 (MACS3)

ACSM5 (31,817 bases) encodes for a 579 amino acid protein that is expressed mainly in the kidney and liver [53]. Although *ACSM5* is homologous to *ACSM1* and *ACSM3*, the biological function of ASCM5 has not been characterized in any species [2].

3.6. ACSM6 (C10orf129)

ACSM6 is located on chromosome 10 at position 10q23.33 and spans 34,730 bases encoding for a 480 amino acid protein [50]. Very little research has been done on *ACSM6* with no protein functionality studies being published thus far. One disease association study was performed. To identify susceptibility genes for developing late-onset Alzheimer disease (LOAD), the rs591157 single nucleotide polymorphism (SNP) located in *ACSM6* (*C10orf129*) was one of the 1412 gene-based SNPs used to perform a chromosome-10-specific association study. No association between the *ACSM6* SNP and LOAD could be established [54].

4. Biochemical and enzymatic characterization of the ACSM ligases

ACSM2B (HXM-A) and ACSM1 (HXM-B) were isolated from human liver mitochondria and characterized. The designation of these ligases was based on their order of elution from a diethylaminoethyl-cellulose chromatography column. Kinetic analysis was conducted on each of the XM-ligases using seven different carboxylic acids, at a concentration of 100 μM in each case, to characterize the substrate specificity (Table 2). ACSM2B and ACSM1 differed significantly in their substrate specificities. ACSM2B had the highest affinity for benzoate while ACSM1 had the highest affinity for hexanoate. The two ligases were of different degrees of purity so values are only referred to as relatively apparent and cannot be compared between ACSM2B and ACSM1. There is evidence of activation of valproate by ACSM2B [9], while salicylate can be activated by both ACSM2B and ACSM1. The substrate specificity for salicylate is, however, very low when compared to benzoate and hexanoate. Neither enzyme was effective at activating short-chain fatty acids as is shown by the very poor affinity of the enzymes for butyrate [9].

A recombinant ACSM2B protein was subsequently expressed in COS-7 cells and characterized [37]. The expressed enzyme had greater activity toward benzoate than phenylacetate, which is comparable to the previously reported substrate specificity of ACSM2B isolated from liver [9]. The phenylacetate activity was, however, greater than expected, but this was attributed to the presence of the fused FLAG protein [37].

Expression of ACSM2B (HXM-A) ligase is inducible, although the mechanisms involved are not well understood. Pretreatment of rats with salicylate for 6 days increases the capacity of liver and kidney homogenates for synthesis of salicylylglycine, hippurate, and 4-aminohippurate [55]. Salicylate pretreatment also increases the rate of salicylylglycine formation in humans [56].

Table 2. A summary of the properties and substrate specificities of ACSM2B and ACSM1.

HXM-ligase		ACSM2B (HXM-A)	ACSM1 (HXM-B)
Molecular weight		48 kDa	49 kDa
Activation		Preferentially activates xenobiotics	Preferentially activates medium-chain fatty acids
Substrate specificity (V_{max}/K_m) [9]	Benzoate	7.92	0.43
	Hexanoate	1.19	3.47
	Phenylacetate	0.18	0.014
	Decanoate	0.14	0.4
	Salicylate	0.1	<0.004
	Butyrate	0.033	0.042
	Laurate	0.003	0.006

ACSM: Acyl-CoA synthetase medium-chain.

4.1. ACSM activation of benzoate and salicylate

GLYAT (EC 2.3.1.13) is responsible for the glycine conjugation of xenobiotics [57–59] such as benzoate and salicylate [60,61]. Xenobiotics that undergo glycine conjugation are activated to an acyl-CoA by the mitochondrial ACSM ligases [15,34].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most widely used drugs worldwide [62–64]. Over 30 million people receive NSAIDs daily [65], and nearly 25% of the population have experienced aspirin-related side effects that require medical care [66]. In a study involving 1225 patients with adverse drug reactions carried out in the United Kingdom, aspirin was involved in over 60% of the deaths caused by adverse drug reactions [67].

An oral dose of aspirin (also known as acetylsalicylic acid [ASA]) is first hydrolyzed to salicylate by ASA esterase [68]. Salicylate is eliminated in humans mainly by conjugation with glycine to form salicyluric acid followed by glucuronidation [69]. Roughly 10% of the dose is eliminated unchanged in urine. The rest is converted to salicylic acid, most of which is metabolized in liver. The recovery of salicylic acid in urine accounts for about 7% of the dose, with high variability (range 1–31%). The major metabolite of salicylic acid is the glycine conjugate, salicyluric acid (2-[(2-hydroxybenzoyl) amino] acetic acid), which accounts for approximately 63% (range 20–75%) of the recovery in urine, with a large inter-individual variability [70–74]. It has been proposed that the acyl glucuronides formed from NSAIDs are the cause of adverse drug reactions in humans, although convincing mechanistic evidence of this link is yet to be presented. It cannot be ruled out that CoA conjugates are responsible or partly responsible for the hepatotoxicity observed in humans [24].

The glycine conjugation pathway can be saturated and as a result the rate of glycine conjugation influences the clearance of xenobiotics and therefore also toxicity [75,76]. The half-life of salicylate elimination increases with dose and can range from 2.4 to 19 h in adults. Formation of salicyluric acid reaches a maximum rate at higher doses of aspirin (1 g or more) [77]. The formation rate of hippuric acid by the conjugation of benzoic acid with glycine also reaches a limiting value [78] but the maximum rate of conjugation of benzoic acid with glycine is about 20 times higher than the maximum rate of conjugation of salicylic acid with glycine [77]. This is because the limiting step in the pathway depends on the substrate: hippurate formation for benzoate, and salicyl-CoA formation for salicylate [79]. This is consistent with the observation that the substrate specificity of ACSM2B (HXM-A) is much lower for salicylate than benzoate [9] (Table 2). At high doses of salicylate (equivalent to 276 mg of salicylate or 360 mg of aspirin), elimination occurs mainly by salicylate excretion and salicyl glucuronide formation and increases the half-life to 22 h [77].

The appearance of salicyluric acid in the urine can be significantly inhibited by the administration of benzoic acid. This effect is not prevented by the coadministration of glycine [80]. It has also been shown that repeated administration of small doses of benzoate (0.5 g every 30 min) can inhibit salicyluric acid formation for extended periods of time [81], which means that salicylate can readily accumulate in the body so that chronic administration may result in toxicity and liver damage, especially in young children [82,83]. On average, dietary intake of benzoate together with benzoyl-CoA produced by gut microbial metabolism can result in benzoate exposure to the equivalent of 0.6–1.3 g/day, which is higher if foods preserved with benzoate are consumed frequently [84]. Aspirin doses as low as 600 mg can also inhibit glucuronidation of salicylate [69]. This can result in a situation where salicylate accumulates for long periods because it can neither be conjugated to glycine (due to inhibition by benzoate) or to glucuronic acid (due to inhibition by high levels of aspirin).

Previous studies investigating the pharmacokinetics of aspirin have measured the plasma levels of ASA and salicylate [85,86] as well as the glycine conjugate [77], namely salicyluric acid, thereby effectively treating the two-step glycine conjugation reaction as a single metabolic reaction. It is, therefore, very difficult to infer the exact role of the ACSM ligases in the elimination of salicylate. The consequence of the lack of knowledge with regard to substrate specificity of the ACSM ligases will be discussed in greater detail in Section 8.2. and 8.3.

5. The *ACSM2A* and *ACSM2B* genes and genetic variation

The *ACSM2A* and *ASCM2B* genes are almost identical and therefore difficult to distinguish, the coding sequences of these genes are 98.8% identical, and their amino acid sequences are 97.6% identical, so it would be likely to assume that experimentally determined differences were attributable to polymorphisms or sequencing errors. Although the *ACSM2A* and *ASCM2B* genes are nearly identical, there is sufficient evidence supporting the existence of both genes [2]. *ACSM2A* and *ACSM2B* both encode for 577 amino acid proteins [9].

5.1. Genetic polymorphisms of the ACSM2A gene

The *ACSM2A* gene (ENSG00000183747) is located on chromosome 16 at position 16p12 on the forward strand, spans over 36,000 base pairs and contains 14 exons, of which 13 are coding exons (Table 1). Within the *ACSM2A* gene, there are 3158 known SNPs [50 March, 2016, ENST00000573854], of which 344 are non-synonymous. Nothing is known regarding the effect of SNPs on the enzyme activity of ACSM2A.

5.2. Genetic polymorphisms of the ACSM2B gene

HXM-A is encoded by the *ACSM2B* gene [2]. *ACSM2B* is located on chromosome 16p12 on the reverse strand. It consists of just over 40,000 base pairs and contains 15 exons of which 13 are coding exons (Table 1). There are a total of 3428 SNPs located in the *ACSM2B* gene of which 314 are missense variants [50 March 2016, ENST00000567001]. No information is available on the influence of genetic variation on the enzyme activity of ACSM2B.

5.3. Distinguishing between the ACSM2A and ACSM2B genes

ACSM2A and ACSM2B both contain the two highly conserved amino acid sequence motifs found in proteins with ACS activity from a diverse group of organisms (including mammals, roundworms, fruit flies, bacteria, and yeast) (Figure 2) [2,87]. Motif I is found within the 11 amino acid putative AMP binding domain signature in the PROSITE database (PDOC00427, PS00455) (http://expasy.org/prosite). Motif II consists of 35 residues partially overlapping with a 25 amino acid signature motif (DGWLHTGDIGXW-XPXGXLK IIDRKK). This sequence appeared to be common to all ACS synthetases and signifies the ACS synthetase signature motif (FACS signature motif) [88]. A highly conserved arginine residue is found within both Motif 2 and the signature motif [2].

The *ACSM2A* and *ACSM2B* genes have 21 nucleotide differences in the coding region of which 17 results in non-synonymous substitutions. Only one of the amino acid differences lies within a conserved motif. Residue 463, found in Motif II, is asparagine in ACSM2A and aspartic acid in ACSM2B (Figure 2).

6. Disease association studies

In terms of ACSM2A and ACSM2B, very little research has been done regarding disease-association studies. It has been proposed that due to the abundant transcript level of the *ACSM2B* gene compared to transcript levels of the rest of the ACSM family, *ACSM2B* is recommended to be the more promising target for studies into the role of the ACSM family as disease risk genes [38]. As mentioned previously, this should be reinvestigated as will be discussed in Section 8.1.2.

A whole exome sequencing study on melanoma samples identified two missense SNPs (rs144594944 and rs148136861) within the *ACSM2B* gene that were not present in normal tissues. These two SNPs were, however, not recurrent mutations identified in melanoma [89].

A few disease association studies reported in the literature [53,90,91] contain several inconsistencies, which will be discussed in Section 8.1.1.

7. Summary

The existence of a wide range of ACSs suggests that each plays a unique role, directing the acyl-CoA product to a

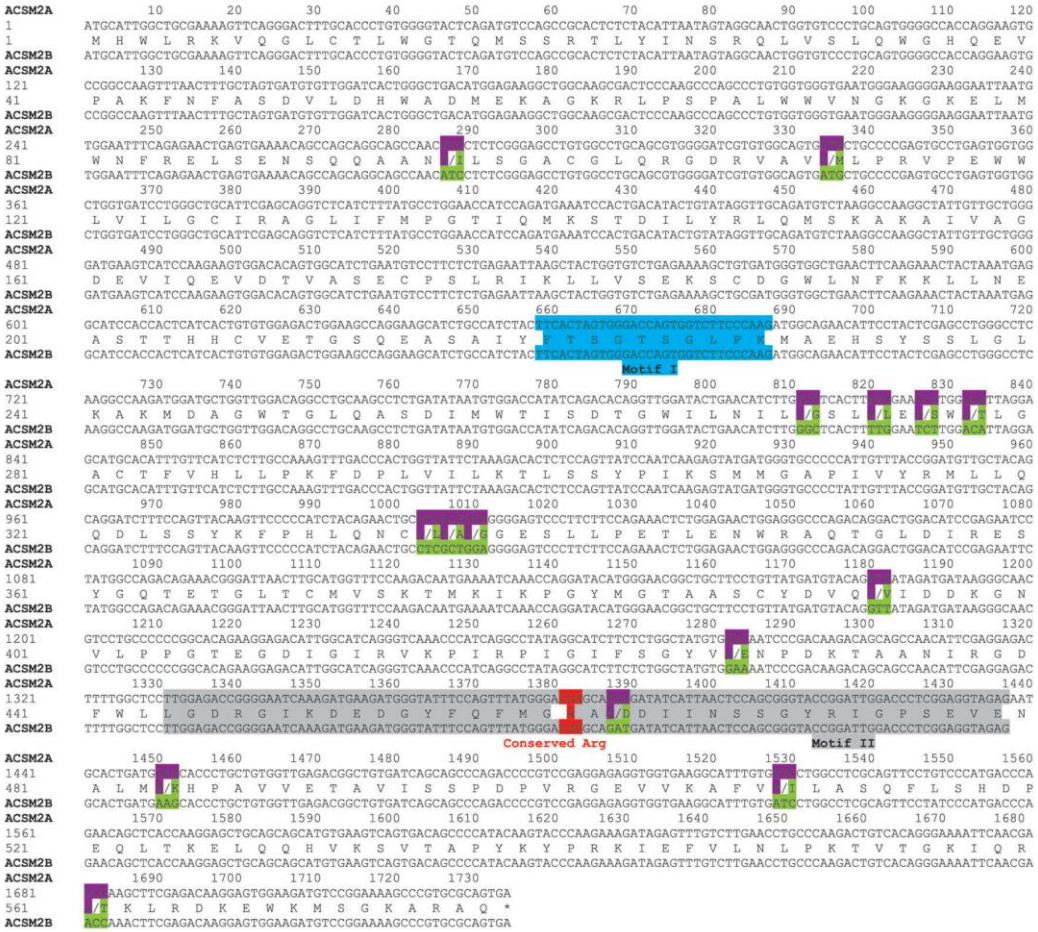


Figure 2. Alignment of the nucleic acid sequences of *ACSM2A* (ENST00000573854) and *ACSM2B* (ENST00000567001) and the corresponding amino acid sequence. The 17 non-synonymous SNPs are indicated by the purple highlight for ACSM2A and the green highlight for ACSM2B. The two conserved motifs are also indicated: Motif I in turquoise and Motif II in grey. The conserved Arginine residue found in Motif II is shown in red. Full color available online.

specific metabolic fate [2] (Figure 1). Catalytic data on the ACSM family of proteins are still limited and the xenobiotic substrate/inhibitor profiles are incomplete. There is limited information in humans concerning the identity of individual ACSM ligases responsible for the metabolism of a variety of drugs and chemicals. Previous studies have only characterized the substrate specificity of crude cell extracts of ACSM2B [37] and ACSM1 [9]. At present, factors modulating activity of human ACSM ligases *in vivo* remain unknown [32]. Characterization of the ACSM ligases in terms of substrate/inhibitor profiles and regulation will allow a greater understanding of the role of these enzymes in the metabolism of carboxylic acids [15] and xenobiotics such as salicylate.

Information on the influence of genetic variation on the enzyme activity of the human ACSM ligases does not exist. This could potentially be a key factor when investigating the causes of aspirin sensitivity, especially when taking into account the low substrate specificity of ACSM2B for salicylate (Table 2). The high degree of nucleic acid and amino acid identity between *ACSM2A* and *ACSM2B* (Figure 2) needs to be taken note of when planning future genetic variation or enzyme characterization studies. It is very important to clearly indicate which gene is studied and to give the reference sequence used for the study (Table 1). A great deal of work still needs to be done on finding significant associations between the ACSM ligases and diseases.

8. Expert opinion

8.1. ACSM nomenclature and discrepancies in the current literature

In previous studies, different synonyms for each ACSM protein are used (Table 1), which complicates literature searches and may lead to confusion for new researchers in the field. For example, the ACSM2B protein that was previously referred to as HXM-A. Some mistakes with regards to nomenclature have slipped into the literature. In a recent review, for example, it is stated that HXM-A is encoded by the *ACSM2A* gene [92]. Studies also do not clearly differentiate between the *ACSM2A* or *ASM2B* genes [90].

The high-sequence similarity between *ACSM2A* and *ACSM2B* results in the identification of SNPs that are in fact just nucleotide differences between the reference sequences of *ACSM2A* (NM_001308172.1) and *ACSM2B* (NM_182617). To prevent the incorrect reporting of SNP data on Ensembl [50], it is important to use the reference transcripts (Table 1) for future SNP identification and to always include both genes in genetic variation studies.

8.1.1. Polymorphisms in the ACSM genes and disease association

8.1.1.1. Aspirin metabolism and intolerance. A review analyzing the importance of genetic variations in enzymes involved in aspirin metabolism, namely UGT1A6, ACSM2, and CYP2C9, has some inconsistencies between the abstract and the conclusion. In the abstract, it is stated that one of the major polymorphisms related to aspirin bio-disposition is rs1133607 in the *ACSM2* gene [90]. It is not clearly stated whether the authors are referring to *ACSM2A* or *ACSM2B*. The rs1133607 SNP is found in *ACSM2A* and it results in either one of the two non-synonymous changes depending on the variant allele, that is S513W (T**C**G → T**G**G) or S513L (T**C**G → T**T**G) [50]. In the text, it is not discussed why the rs1133607 SNP is considered to be one of the major polymorphisms related to aspirin metabolism but only mentioned that this SNP has a high population frequency and should therefore be considered for future investigation. According to the 1000 genome Project [50], the wild-type allele (C) has an allele frequency of 86%. Future studies should rather investigate the effect of haplotypes on aspirin metabolism rather than individual SNPs. In a subsequent review, the rs1133607 SNP was also mistakenly reported to be associated with aspirin intolerance [92].

Variability exists in aspirin metabolism, with large differences between individuals participating in the same study and between average values observed in different studies [70–74]. In addition, gender-related differences [71,93] and interethnic variability in aspirin metabolism have been reported [70,94]. The fact that interethnic variability exists in aspirin metabolism suggests the involvement of genetic factors in variability in aspirin metabolism, because interethnic and even intraethnic variability have been described for genes coding for drug metabolizing enzymes [95,96].

What is concerning is that even though the major pathway for aspirin metabolism is the glycine conjugation pathway, no data currently exist on the putative role of either *ACSM2A, ACSM2B*, or *GLYAT* polymorphisms in aspirin metabolism or hypersensitivity. Overall information indicates that pharmacogenomic knowledge in aspirin intolerance is presently in its infancy [90].

8.1.1.2. Metabolic syndrome. Two studies found a significant association between the L513S polymorphism in *MACS2* (*ACSM2A*) and multiple risk factors of metabolic syndrome in a Japanese Suita [53] and Caucasian [91] cohort. The **S**513L SNP has been identified in both the *ACSM2A* (rs1133607) and *ACSM2B* (rs761288312) genes [50]. In the Iwai et al. study, it is difficult to ascertain if the **L**513S SNP referred to, is simply a typing error as the rs number for the SNP is not given [53]. The reference sequence (GenBank accession: AX451437) used to identify SNPs in this study has a 99% nucleic acid identity to the *ACSM2B* sequence (NM_182617). So, even though the authors use the synonym for *ACSM2A*, i.e. *MACS2*, the study in fact identified SNPs in the *ACSM2B* gene. When the reference sequence used (AX451437) is translated, a serine residue is identified at position 513. This is different from the amino acid sequence given for MACS2 in Figure 1 of the study [53]. Here, the amino acid residue is indicated as a bold letter 'L.' Is the S513L SNP in the *ACSM2B* gene, therefore, significantly associated with triglyceride level? Some follow-up studies are needed to clear up this confusion.

In the case of the Lindner et al. [91] study, the reference sequence used as well as the unique ID number of the TaqMan assay used for genotyping of the **L**513S polymorphism was not given. It is, therefore, difficult to critically evaluate the experimental protocol of this study. In Table 1 of the study, the SNP is identified as rs16970281 (base change C/T),

the record of which has been merged into the rs1133607 record. As previously mentioned, rs1133607 is a SNP located in *ACSM2A* (T**C**G → T**T**G) that results in an amino acid change **S**513L. According to the 1000 genome Project, allele frequencies [50], the C allele (serine) is found in 86% of the worldwide population. It is unlikely that an allele, which is significantly associated with risk factors of the metabolic syndrome (as stated by [91]), would be found at such a high frequency in the general population.

8.1.2. Incorrect identification of ACSM2B as the predominant ACSM transcript in human liver

A study performed a comparative analysis between disease risk genes belonging to the ACSM family in human liver and cell lines. The transcript levels of *ACSM2A* and *ACSM2B* in human liver samples were quantified using a TaqMan qPCR assay. Assays were designed to detect *ACSM2A/B* total (Hs00415261_m1) and an assay by design for *ACSM2A* (probe in 5′–3′ orientation: ACATCCTGAGAGGACTTG). It was found that *ACSM2A* is one of the least expressed transcripts with 0.24% of *ACSM2* total expression levels. The study concluded that the results suggest that *ACSM2B* is the most abundant transcript in human liver. Therefore, suggesting that genetic variations of the *ACSM2B* gene might play an important role in disease susceptibility [38].

When the probe designed to quantify *ACSM2A* (ACATCCTGAGAGGACTTG) is aligned to the *ACSM2A* (NM_001308172.1) and *ACSM2B* (NM_182617) references sequences, it aligns with 100% homology to the 5′-untranslated region of both reference sequences (Figure 3) and is not found in any of the exons of either *ACSM2A* or *ACSM2B*.

This is a fatal design flaw in the study as primers/probes must ideally span exon–exon junctions in the target mRNA to prevent amplification of the target from contaminating genomic DNA. Thus, the low level of transcript (0.24%) detected in this study for *ACSM2A* is probably due to contaminating genomic DNA. It can, therefore, not be said that the *ACSM2B* gene is the predominant transcript in human liver. It is suggested that this study needs to be repeated with correctly designed probes.

8.2. Substrate specificity of the ACSM ligases – is there an ACSM variant that preferentially activates salicylate?

Kinetic data in terms of substrate/inhibitor profiles for any of the human ACSM ligases are severely lacking. The only ACSM ligases that have been characterized in terms of substrate specificity is ACSM2B (HXM-A) [37] and ACSM1 (HXM-B) [9]. In the Vessey et al. [9] study, relative apparent kinetic parameters were determined because the ligases were of different degrees of purities. This means that, unfortunately, substrate specificities between ACSM2B and ACSM1 cannot be compared. What is very clear though, is that both enzymes have a very low substrate specificity for salicylate (Table 2). This indicates that salicylate is not the preferred substrate for either of these ligases.

In a subsequent study, a recombinant ACSM2B fusion protein was expressed and characterized in COS-7 cells [37]. Here, the recombinant ACSM2B fusion protein was only assayed for its activity toward benzoate and phenylacetate and as the protein was not purified, kinetic parameters were not determined. Although analysis of the control cells showed considerable hexanoate activity, this was not investigated further.

The amino acid sequence expressed in the Vessey et al. [37] study was aligned to the reference amino acid sequences of ACSM2A (NP_001295101.1) and ACSM2B (NP_872423). The recombinant protein has 99% amino acid identity to the ACSM2B reference sequence. There are five amino acid differences: F46L (novel), I96V (rs8056693), F133I (novel), G136E (novel), and T143A (novel). It was, therefore, not the wild-type reference sequence that was enzymatically characterized. It has previously been shown that SNPs in the *GLYAT* gene can affect the enzyme activity of GLYAT with the R199C SNP reducing enzyme activity to 5% of that of the wild-type [97]. A similar SNP might exist within the ACSM2B ligase that affects its substrate specificity toward either benzoate or salicylate.

More than a decade later, no follow-up work has been done to characterize the enzyme activity of purified human recombinant ACSM ligases. It is, therefore, imperative that the substrate specificity of purified recombinant variants of the ACSM proteins toward both benzoate and salicylate be thoroughly investigated to determine the effect of SNPs on enzyme activity and substrate specificity.

8.3. The glycine conjugation pathway is not a one-step process

One of the biggest challenges and gaps in the current literature is that studies continually focus on amino acid conjugation as a one-step process. This is especially true for pharmacogenetic studies on salicylate metabolism. The rate limiting step in salicylate metabolism is the formation of the salicyl-CoA due to the low substrate specificity of the ACSM2B ligase. Benzoate also very effectively competes with salicylate for elimination, thereby, increasing the half-life of salicylate to 24 h. With this in mind, why has research on the ACSM ligases been neglected for so many years? Why are these proteins not pharmacogenetic targets in aspirin intolerance? While this view persists, key regulatory features of the two individual steps involved that is, initial conjugation with CoA then acyltransfer to the amino acid will, remain unknown [15,32].

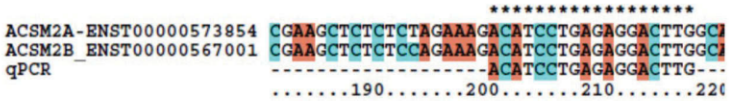


Figure 3. Alignment of the qPCR probe used for quantification of the *ACSM2A* transcript with the nucleic acid sequences of *ACSM2A* (ENST00000573854) and *ACSM2B* (ENST00000567001).

With the increase in exposure of modern humans to preservatives such as benzoate and drugs such as aspirin, it is vital to determine whether the glycine conjugation pathway is not being overloaded and whether inefficient detoxification by the glycine conjugation pathway leads to adverse health consequences.

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Declaration of interest

Any opinion, finding and conclusion or recommendation expressed in this material is that of the authors and the NRF does not accept any liability in this regard. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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CHAPTER 2

Biotransformation and chronic fatigue

2.1 Introduction

In the preface a working hypothesis for the present study was inductively formulated as:

"*The wide occurrence of fatigue amongst individuals not suffering from a distinct clinical disorder may be due to an energy deprived state as a consequence of the individual's response to undefined exposure to xenobiotics, and which should be measurable in the metabolite profiles of biofluids from the fatigue cases*"

Chapter 2 provides a review of literature related to the working hypotheses. Together with the insights from Chapter 1, these two reviews serve the basis for defining the aims and objectives of the thesis (§2.9 p31).

Chapter 2, firstly, focuses on the interrelation between fatigue, the environment and biotransformation — a metabolic response towards conserving homeostasis for maintaining an individual's efficient functional physiological state. The concept of the exposome is then introduced as an important aspect related to the working hypothesis. Secondly the review proposes an avenue for analytical measurements to assess the presumed relation between metabolic profiles of biofluids obtained from clinical cases with a defined chronic fatigue profile.

The Chapter is presented in the form of a review paper according to the instructions of the *Expert opinion* journals of the Taylor and Francis Group. The intention is that the format of the Chapter will be extended and revised by including additional information on biotransformation and fatigue, coming from the papers presented in this thesis. Thus, Chapter two has a dual purpose: (1) it provides a literature review for assessment of the working hypothesis and formulation of the aims and objectives for the study and (2) the literature review forms the basis for a manuscript to be submitted to an appropriate journal after completion of the study.

2.2 A literature review and expert opinion — Chronic fatigue, the exposome and biotransformation (Manuscript to be submitted I):

CHAPTER 2

REVIEW PAPER | MANUSCRIPT VERSION

Chronic fatigue, the exposome and biotransformation

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Abstract

Introduction: Fatigue has a complex aetiology, ranging from mild situational presentations to severe chronic fatigue syndrome and myalgic encephalomyelitis (CFS/ME). For a deeper understanding of fatigue, the US National Institutes of Health (NIH) announced strategic initiatives which significantly increased funding allocations for CFS/ME research. The present review argues for the central role of biotransformation in the understanding of chronic fatigue (CF).

Areas covered: CF is not a trivial condition but a situational presentation within the fatigue spectrum, reflected in the wide range of its effects. The relation between fatigue and the exposome is discussed through a review of factors affecting energy metabolism suspect in fatigue conditions followed by the four phases of biotransformation that are primarily involved in the enzymatic conversion of potentially harmful substances which results in their inactivation, detoxification and excretion.

Expert opinion: A causal relationship between chronic xenobiotic exposure and the energy demand for biotransformation responses may underlie idiopathic chronic fatigue. This complex relationship is illustrated here by a representative model. Based on the model a novel laboratory approach is proposed by using an intervention study that would provide a knowledge-base to explore causality, offering an avenue for prevention, diagnosis and treatment of chronic fatigue and ultimately paving the way towards personalized medicine.

2.2.1 Introduction

The US National Institutes of Health (NIH) recently announced that the agency would strengthen investigations on chronic fatigue syndrome and myalgic encephalomyelitis (CFS/ME) by increasing funding allocations to CFS/ME research from US$6 million in 2016 to $15 million in 2017 (Maxmen, 2018). A major incentive for the intensified research on CFS/ME came from the report of the US Institute of Medicine (IOM), estimating that 836,000 to 2.5 million Americans have the CFS/ME disorder (Clayton, 2015). A key finding from the survey was that CFS/ME is a "serious, chronic, complex and systemic disease." It is envisaged that the negative past perceptions of fatigue, because of the field's reputation for controversial research, will now be overcome, that the diverse presentations of severe fatigue will receive their rightful position amongst other research interests and that the millions of people who suffer from fatigue-related disorders globally will be the beneficiaries.

Fatigue presents as disproportionate levels of physical and/or cognitive/mental exhaustion, a lack of energy following simple activities, and a condition that is not ameliorated with ordinary rest, and which is common in the general population. Although there is no consensus definition of fatigue (Jason *et al.*, 2010), the current view in case definitions for CFS/ME is that it involves a multifactorial pathogenesis with varying predictions on prognosis. However, it is proposed that the strict dichotomy of an "organic" versus a "psychic" disorder, once held, should be abandoned (Brurberg *et al.*, 2014).

Until the mid-1980s, physicians often ascribed symptoms of fatigue to neuroses and depression, degraded to "yuppie flu" by the lay community. But things changed. Contemporary expertise in molecular biology and in the fields of genomics, proteomics and metabolomics provides powerful technologies that are expected to open up renewed research on associations between fatigue and several proposed underlying factors, including symptomatic viral infections (Magnus *et al.*, 2015), the immune system (Maes *et al.*, 2012), perturbed metabolism (Naviaux *et al.*, 2016) and on a proposed link between fatigue and the microbiome (Giloteaux *et al.*, 2016). Regardless of this wide coverage, the cause of CFS/ME remains uncertain. However, the NIH initiative holds the promise to expand research on fatigue in general and specifically how to target the CFS/ME field (Maxmen, 2017). In line with this expectation, this review highlights the benefits of a holistic approach towards a deeper insight into various manifestations of chronic fatigue, as explained in what follows.

First, it is progressively being realized that the risk of acquiring a chronic disease is not only influenced by a person's genetic make-up, but also by the numerous exposures expressed in the concept of the exposome (Wild, 2005), covering all external and internal exposures of an

individual's experience during an entire lifetime (Wild, 2012). Apart from genetic predisposition, it has been shown that chronic diseases often develop from a combination of a diverse range of non-genetic influences (Rappaport, 2016), supporting the call for a holistic approach to understanding an individual's ability to cope with fatigue-inducing influences.

Second, xenobiotics are a major and an increasingly important component of the exposome. Xenobiotics, and their metabolites that result from biotransformation, play a pivotal role in health and disease, but this remains a largely unexplored territory (Koppel *et al.*, 2017). Fortunately, the METLIN Exposome Database (Guijas *et al.*, 2018) was recently expanded and presently provides data on more than 950,000 unique small molecules, including analytical information on intermediates and products from the biotransformation system. The expanded database covers abundant phase I biotransformation products and a significant number of phase II conjugation products, such as 726 glucuronides (654 with associated MS/MS information) and 1989 sulphate-conjugates (366 containing MS/MS data), and hydroxylation and methylation products (Warth *et al.*, 2017).

Third, research in the 1990s proposed a link between CF and detoxification (Rigden *et al.*, 1998), but confirmation and follow-up studies are sparse or unconvincing (Dorne *et al.*, 2005). However, human metabolism is well-recognized to serve as the mainline chemical defensive network against exogenous low-molecular-weight inorganic and organic substances through a biotransformation system; several studies have demonstrated that CF is entangled in genetic effects and environmental influences (Sullivan *et al.*, 2005). In a review paper on mitochondrial dysfunction and fatigue, Filler *et al.* recently argued that, "Once the underlying mechanisms of fatigue are better understood, individualized and tailored therapies can be developed to improve quality of life of patients." (Filler *et al.*, 2014). Against this background, this review argues for a holistic view on CF, the impact of the exposome, and on the essential role of biotransformation in maintaining homeostasis.

2.2.2 Chronic fatigue

Fatigue is defined as a condition or phenomenon of decreased ability and reduced efficiency of mental and/or physical activities, caused by excessive mental or physical demands, diseases, or syndromes (Jason *et al.*, 2010). The intensity of the fatigue experience differs significantly among individuals, ranging from very mild at the lower end to CF and ultimately CFS at the upper extreme of the fatigue scale (Nicolson, 2014). Complaints of fatigue seem to be more prevalent in women than in men (Bleijenberg, 2003). In a publication from Ottawa University's Medical Faculty, it was stated that fatigue was the main complaint in 45.8% of patients under 45 years of age and in 36.6% of patients over 45 (Ponka & Kirlew, 2007). CF, however, is not

regarded to be a normal physiological condition and was originally described as neurasthenia in the Norwegian medical literature in the early 1880s. The American neurologist, George M. Beard, made the first diagnosis of neurasthenia in 1869, which he explained as occurring in people with depleted nervous energy, causing the malfunction of organs, resulting in a number of symptoms, one of which was fatigue (Schuster, 2003). Fatigue is also associated with excess mortality in the general population. This has been confirmed by results from the EPIC-Norfolk study (Basu *et al.*, 2016).

Further research about fatigue and its causes remained complex. Scientists have difficulty in understanding the aetiology and classification of fatigue within different illness groups. This is further complicated by several "fatigue syndromes" that are occasionally described in the medical literature. Epstein-Barr chronic fatigue syndrome, post-viral infection fatigue syndrome, and adrenal fatigue syndrome are among the most commonly mentioned. Of special interest, however, are patients who apparently suffer from idiopathic chronic fatigue. In a study of 375 patients with unexplained disabling fatigue, 70% showed objective signs of pathogen presence (Morris *et al.*, 2016). An early question to asked is whether CF is “a symptom or a syndrome?” (Lewis & Wessely, 1992) or whether chronic fatigue and ME/CFS are variants of sickness behaviour (Morris *et al.*, 2013). The search for a relation between ME/CFS and other conditions, like multiple sclerosis and major depression, through neuroimaging has recently shown that high-resolution SPECT imaging may have the capacity to differentiate CFS patients from those diagnosed with depression (Morris *et al.*, 2018). However, many physicians do not recognize the above aetiologies as syndromes, as the criteria to define syndromes are too diffuse and many consider the associated fatigue (sometimes chronic fatigue) as either a symptom or complication of the underlying associated diseases (Davis *et al.*, 2010). In the past this negatively affected patient–doctor interaction. This unfortunate patient–physician relationship was clearly illustrated in a recent Norwegian survey, in which the outcome of the treatment that patients who suffered from fatigue-related illnesses received was not very promising. In the 431 cases, quality of care was rated poor by 60.6% in primary care, by 47.7% in specialist care, and by 71.2% regarding coordination of care (Hansen & Lian, 2016). Fortunately, surveys like this alerted the health authorities to the seriousness of the problem. In the UK, for instance, multidisciplinary teams have now been set up, consisting of a co-ordinator, rehabilitation consultant or GP specialist, physiotherapist and occupational therapist, and a psychologist (or access to psychological services), which has led to much improved treatment of fatigued patients (Horton *et al.*, 2010).

Researchers have attempted a more systematic approach to the complexity of fatigue rating by implementing fatigue-rating scales to define the intensity of fatigue experienced by individuals.

Dittner and co-workers reported the outcome of a comprehensive literature search for the period 1966–2003, which included investigating several large databases (Dittner *et al.*, 2004). In this search they have detected no fewer than 30 different fatigue-rating scales. In an evaluation study about reliability, validity and utility of fatigue-rating scales, the authors came to the conclusion that there was no ideal measure of fatigue (Whitehead, 2009). Nevertheless, a number of these measuring instruments were rated highly in terms of reliability, amongst them the revised Piper Fatigue Scale (PFS) with a Cronbach's α value of 0.97 (Piper *et al.*, 1998). The revised PFS was developed mainly to measure subjective fatigue and influencing factors in patients with breast cancer. The multidimensionality of the Piper Fatigue Scale, however, provides an opportunity for the applicability of the scale to other fatigue-related conditions. The original Piper Fatigue Scale contains 40 items whereas the revised one retained 22 variables with an intensity rating of 0–10. The final product was designed to measure four dimensions of subjective fatigue, namely, behavioural/severity; affective meaning; sensory and cognitive/mood. The confirmation of multidimensionality of the revised Piper Fatigue Scale by Piper and co-workers was an important outcome of their study as accurate assessment and measurement is paramount to advance the science of fatigue.

Many of the scaling instruments that were developed specifically focused on diagnosing CFS, CFS/ME and ME (Jason *et al.*, 2012) while chronic fatigue in all its degrees of severity received less attention. Only one in 7 patients with chronic disabling fatigue meets criteria for CFS (David L, 2001), which leaves a large number of undiagnosed cases with chronic fatigue. While not ignoring the seriousness of CFS, it should be emphasized that CF is not trivial, and it is associated with disability comparable to that found in other chronic illnesses. Chronic fatigue also has the potential to severely limit normal functioning and health-related quality of life (Lavergne *et al.*, 2010). One prominent candidate in this respect is the effect of exposure on fatigue; fortunately promising new initiatives are under way such as the Exposure Science Ontology (ExO) proposed by Mattingly *et al.* (2012).

2.2.3 Human exposure

Exposure science focuses on understanding and characterizing receptor interactions with one or more environmental stressors of concern (Mattingly *et al.*, 2012). Monitoring exposure is no trivial task and efforts have been made to develop and apply new and better monitoring tools (Niedzwiecki & Miller, 2016), for which the term biomonitoring has been coined. The use of biomonitoring in exposure science is important, because the results can identify highly exposed or unexposed individuals, including population profiles and comparison of different population groups (Lioy, 2010).

Biomonitoring, also referred to as biological monitoring, has been defined as the periodic measurement of exposure to a biomarker, which in turn has been defined as an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism (Committee on Biological Markers of the National Research Council, 1987). In this regard, it is important to note that biomonitoring does not provide a direct measure of environmental exposure, but is an important means to assess internal exposure to specific chemical(s), which indicates the cumulative effect of environmental exposures (sources and modes of exposure), as well as to the various mechanisms of biotransformation and clearance from the body (Clewell *et al.*, 2008). The complexity of these analyses is further illustrated by the fact that information about the pharmacokinetics of the compound(s) involved in relation to the biological matrix, in which it is measured, is needed. In addition to this the exposure time (which includes variables such as geographical origin, duration, frequency and relative intensity) must also be considered (Clewell *et al.*, 2008). The practical use of biomonitoring data to estimate the degree of environmental exposure is therefore complicated by these and other confounding factors.

Against this background, various criteria have been defined to improve biomarker application. Amongst these are sensitivity, specificity and biological relevance (Silins & Högberg, 2011). The concentration of chemicals detected in human samples is normally very low, ranging from parts per billion to parts per trillion (Clewell *et al.*, 2008). This may seem extremely low but information on persistent exposures at these levels — as in industrial settings — may be clinically important. Thus, the potential impacts of chronic exposure to xenobiotics have become realized, seen in new developments in the field of epigenetic research and their implications for future generations may be far-reaching (Wei *et al.*, 2015). Adverse environmental exposures in early life and epigenetic variation and possible long-term health outcomes are just some of the emerging focal points in the field of clinical epigenetics (Vaiserman, 2015).

Great improvement in analytical capabilities, such as increased sensitivity and specificity, have made it possible to quantify many of these compounds in human physiological samples (Zitka *et al.*, 2012). This includes measurement of these toxins themselves, newly formed metabolites because of the presence of the toxins and biotransformation products of these metabolites with a combination of chromatographic separation techniques coupled to different ionization sources and mass analyzers (Roux *et al.*, 2011). It is, however, also true that in general our ability to quantify exogeneous compounds in bodily fluids is far more advanced than being able to relate any of these compounds to human health risk (Hays *et al.*, 2007). The strong point of biomonitoring tests is that they provide clear evidence of exposure, but they are of limited value regarding the source of exposure or the prediction of health outcome. This health issue is

highlighted by the following quote from De Luca *et al.*: *"The lack of satisfactory reliability and specificity of the biomarkers presently measured in the clinical setting, and the almost absolute void of epidemiological data on treatment safety, efficacy and compliance assessment by the responsible clinicians, raise undeniable ethical concerns for public health operators.*" (De Luca *et al.*, 2011). Currently, chemical legislation is limited by being based on one-by-one assessments of single substances, and so does not reflect exposure in the real world (Holmberg, 2013).

Combined exposures to chemicals are also thought to be involved in increased allergies and other environment-linked disabling conditions like multiple chemical sensitivity, chronic fatigue syndrome and fibromyalgia (De Luca *et al.*, 2011). These cumulative chemical exposures on a chronic level may overload the capacity of the liver to achieve detoxification, especially evident in cases where inherited or acquired impaired metabolism of xenobiotics triggered negative responses at exposure levels far below toxic values. This is especially relevant to lifestyle exposures of modern man, who experiences a wide array of different synthetic chemicals, many of them used on a regular basis, for example as prescription medicines (Claus *et al.*, 2016; Needham *et al.*, 2007).

The expanding contribution of the drug industry alone to adding new molecules to the number for combined chemical exposure is demonstrated in a publication in *Future medicinal chemistry*, titled "Medicinal Chemistry for 2020" (Satyanarayanajois & Hill, 2011). Herein reference is made to rational drug design in all its many manifestations, and that the "drug-discovery" process has become rather a "drug-creation" process, emphasizing the synthesis of new drug compounds (Aguzzi & Gitler, 2013). It could be argued that these chemicals are tested for their safety for human consumption, but their contribution to the overall exposure basket is largely ignored. In addition, pharmaceutically active substances are among the so-called "emerging" contaminants that have caused great concern in recent years due to the variety of drug-related hepatotoxic effects (Barceló, 2007).

This is seen in the growing trend to move away from *traditional* biomonitoring to a combination of biomonitoring with broader exposomic approaches by which the benefits of *both strategies* can be fully exploited (Dennis *et al.*, 2017).

2.2.4 The exposome

In a 2005 publication in *Cancer epidemiology, biomarkers & prevention*, the cancer researcher Christopher Wild suggested that the genome should be complemented with the "exposome" (Wild, 2005). Wild defined the exposome as the measure of all the exposures of an individual in

a lifetime and how those exposures relate to health. Later, Escher and co-workers added an elaboration on Wild's definition, to include also endogenous chemicals that are produced or altered in response to external stressors (Escher *et al.*, 2016). This expanded approach stresses the fact that including the exposome goes much further than the exposures that are quantitatively measured in daily environmental monitoring. The dramatic epidemiological difference of mainly infectious diseases in developing countries to increased chronic diseases in rich countries has led to reassessment of the health impact of environmental exposures (Bijlsma & Cohen, 2016) and to the implementation of new initiatives like the exposure biology programme funded by the National Institute for Environmental Health Sciences (NIEHS). Non-communicable diseases are the cause of high mortality worldwide and these chronic conditions are mainly the result of the combined effects of the human genome (G) and exposure (E), reflected in the exposome. The attribution of risks to G and E and their interaction is problematic due to disparities in characterising genes and exposures (Rappaport & Smith, 2010). What, however, became clear from studies of G-related factors in monozygotic (MZ) twins is the surprisingly modest influence of G on the risks for chronic diseases. This points towards a dominant role of E as well as to genetic-exposure interactions (G $\times$ E) in disease risks and that any underestimated role of E may obscure important causal factors (Rappaport, 2016).

Even though current evidence suggests that non-genetic factors may contribute as much as 90% of the risks of chronic diseases, it is not explored here how most of the human exposures might initiate disease processes (Bijlsma & Cohen, 2016). According to Rappaport and co-workers, one way of investigating and discovering E-related risks is to extend the data-driven approach embodied in Genome-Wide Association Studies (GWAS) and to conduct Exposome-Wide Association Studies (EWAS) via untargeted analysis of chemicals in blood (Rappaport *et al.*, 2014). Thus, from a focus on metabolic pathways which are under homeostatic control, systems biologists presume that clinical conditions reflect the cumulative effect of exposures or the metabolic consequences of exposures over time, including that on the sum total of the eukaryotic (human) and prokaryotic (microbiome) genomes required for human health (Nicholson *et al.*, 2012). This exposure is reflected by the number and level of chemicals in the blood, which provide for an important way to characterize these chemicals and, even more crucially, to limit the potential damage due to the exposome. From the systems-biology perspective, the most meaningful metabolites are those that participate in, or affect the pathways and products of, energy metabolism — essential nutrients provide for that requirement, which calls for attention to the important role players in energy metabolism.

2.2.5 Investigations linking chronic fatigue to metabolic abnormalities

Investigations in patients who suffered from severe chronic fatigue have revealed some relevant metabolic abnormalities. In the case of ME/CFS patients, their energy metabolism seems to be in an aberrant state with abnormalities detected in many metabolic pathways. One example of this is the high lactate levels which are often associated with ME/CFS, a condition normally associated with deficiencies in the glycolysis pathway and often also observed in mitochondrial defects. ME/CFS patients, subjected to physical exercise, displayed exercise-induced fatigue much earlier than healthy controls (Morris *et al.*, 2013). Further distinguishing features in ME/CFS patients are diminished intracellular ATP levels upon exhaustion and an increase in the rate of glycolysis in the exercised striated muscles. Repletion of ATP is also markedly reduced after strenuous exercise and this is often accompanied by prolonged elevation of lactate and slow return to normal levels. A normal response from the metabolism in a hypoglycaemic state is to turn to fatty acid oxidation for energy, during which the long-chain fatty acids require transport across the mitochondrial membrane, a carnitine-dependent process. Carnitine has been called “an essential regulator of mitochondrial metabolism”; moreover, correlated findings of deficiencies in carnitine have also been associated with CFS (Kaufman & Goodnick, 2006).

In addition, several metabolic perturbations have been shown to occur in ME/CFS. Armstrong *et al.* showed, in a group of 34 females with ME/CFS, elevated blood glucose levels whereas blood lactate, urinary lactate, pyruvate and alanine were reduced, indicating possible glycolysis inhibition. These authors proposed that in the case of ME/CFS, amino acids are increasingly used to provide intermediates for Krebs cycle metabolism. They further concluded that the dysfunctional energy metabolism appears to have influenced creatinine metabolism and that it is possibly used for anaerobic ATP production in the muscle, whilst excretion of urinary allantoin may signify elevated reactive oxygen species (ROS) in ME/CFS patients (Armstrong *et al.*, 2015). In another study seven key parameters of oxidative phosphorylation (OXPHOS) in peripheral blood mononuclear cells (PBMCs) were compared in CFS patients and healthy controls. Maximal respiration was shown to be the main mitochondrial function discriminator between the two groups ($p \leq 0.003$), which indicated that CFS PMBCs are less able to elevate respiration rate to compensate for increase in energy demand under conditions of stress (Tomas *et al.*, 2017). In further support of key metabolic processes being involved in CFS, a metabolomics-based investigation of 133 plasma samples from CFS patients by Yamano and co-workers revealed that ratios of glycolysis/tricarboxylic acid (TCA) cycle products (pyruvate/ isocitrate) and urea cycle intermediates (ornithine/citrulline) could distinguish CFS cases from healthy controls (Yamano *et al.*, 2016). In yet another study, investigation into the urinary excretion parameters in a cohort of 100 patients with CFS and 82 non-fatigued controls, revealed

decreased excretion of asparagine ($p < 0.0001$), phenylalanine ($p < 0.003$), branched-chain amino acids ($p < 0.005$) and succinic acid ($p < 0.0001$) in the CFS cases. This was accompanied by increased excretion of 3-methylhistidine ($p < 0.05$) and tyrosine ($p < 0.05$) (Niblett *et al.*, 2007).

Due to its importance in energy production, the role that impaired mitochondrial function may play in chronic fatigue and the resulting oxidative stress is a main focal point in research on this topic. Much emphasis is placed on the apparent inability of mitochondria in CFS to produce sufficient energy for cellular functions (Myhill *et al.*, 2009). Apart from its importance in energy production, mitochondria have been implicated in a number of physiological processes including production of ROS, pyrimidine and lipid biosynthesis, regulation of cellular levels of substrates, apoptosis, calcium homeostasis and flux, metal metabolism, neurotransmitter synthesis, heat production and insulin secretion (Filler *et al.*, 2014). Investigation of the metabolic features of CFS using a broad metabolomics approach, in which 612 metabolites and 63 biochemical pathways were targeted, resulted in the identification of 20 metabolic pathways which were affected in CFS, whereas 80% of the diagnostic metabolites concentrations were reduced in CFS, interpreted to be consistent with a hypometabolic syndrome (Naviaux *et al.*, 2016).

In a recent publication by Blomberg and co-workers, an explanatory model was proposed that entailed infection-elicited autoimmunity and ME/CFS (Blomberg *et al.*, 2018). The authors highlighted new dimensions about profound metabolic differences between ME/CFS patients and controls, where some of these differences were attributed to abnormal mitochondrial function. From their extensive review on ME/CFS, it is obvious that a number of important metabolic pathways are implicated in the aetiology of ME/CFS. What is also clear is that no single biomarker until now has been identified to be the golden key to unlock the causative defect in ME/CFS and will remain unlikely unless the essence of this disorder, its causes and pathophysiology becomes clearly delineated. This emphasized the fact that (1) ME/CFS is a polygenic/environmental condition and (2) research on causes of ME/CFS should take into account regulatory factors which may affect the large number of metabolic reactions and processes seen in these diseases. One possible avenue to explore here is the effect which exogenous and endogenous compounds may have on mitochondrial function and resulting energy output. Indications are that this effect may result from influences of chronic medication (Kim *et al.*, 2017), environmental chemicals (Zeise *et al.*, 2012), the food metabolome (Scalbert *et al.*, 2014) and multiple combined chemical exposures (Rosofsky *et al.*, 2017). This provides a rationale for further investigation of a possible metabolic influence into the E × G phenomenon as also indicated by the surprising analogy of acetylsalicylic acid (ASA) metabolism and that occurring in chronic fatigue: in certain individuals normal therapeutic dosages of ASA may

cause a significant inhibition of ATP production by a more pronounced uncoupling effect on the electron-transport chain (Szewczyk & Wojtczak, 2002). Even more unexpected is the urinary excretion of lactate, which correlates with the metabolite profile observed in many of the CFS/ME cases already described earlier in this section. The inhibitory effect of ASA on ATP production has also been linked to inhibition of β-oxidation (Mels *et al.*, 2011) and some of the dehydrogenase enzymes in the TCA cycle. Further analogy with the CFS/ME metabolic profile is the production of free radicals via the electron transport chain Complex I as a result of the uncoupling effect of ASA (Szewczyk & Wojtczak, 2002).

2.2.6 Biotransformation

Biotransformation is the metabolic processes that occur mainly in the liver and are primarily described as the enzymatic conversion of potentially harmful substances which results in inactivation, detoxification and an enhanced likelihood of excretion in urine or bile (Van der Westhuizen *et al.*, 2000; Zamek-Gliszczynski *et al.*, 2006). Biotransformation of xenobiotics is an important biological defense mechanism against endo- and exotoxins (Park *et al.*, 2014) and is an essential component in a holistic account on fatigue. Inefficient functioning of biotransformation may lead to secondary damage to specific tissues and organs, the effect of which may be further exacerbated by the formation of ROS (Mena *et al.*, 2009) by the mitochondria of affected tissue. The complete biotransformation process is now thought to consist of four phases (Döring & Petzinger, 2014), the first of which is denoted as phase 0, which refers to the physical uptake by an influx transporter-mediated process (Kalliokoski & Niemi, 2009); phase I refers to the activation of the xenobiotics (Omura, 1999); phase II involves a number of conjugation reactions (Taskinen *et al.*, 2003); and phase III describes the excretion of the activated and conjugated xenobiotics (Chen-Hwei, 2013). Of these four groups of reactions, phases I and II have been studied the most, especially with regard to their relevance to the pharmaceutical industry in the development of new drugs (Guo *et al.*, 2011).

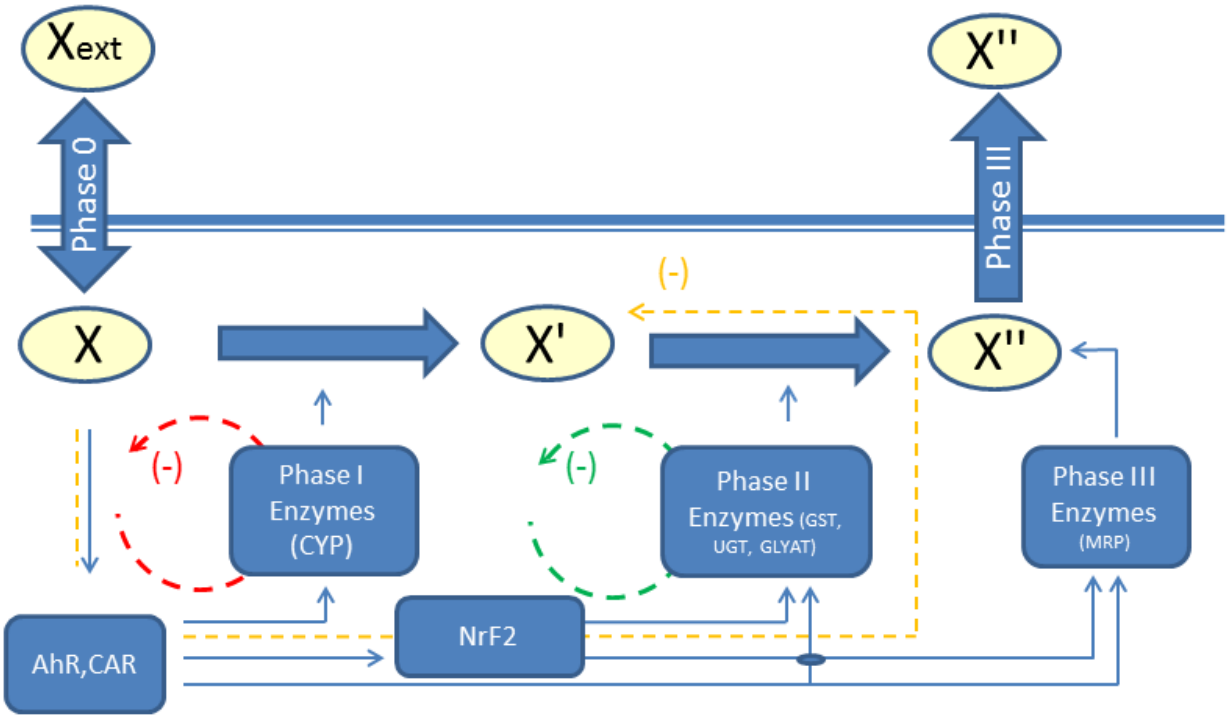


Figure 2-1: Schematic outline of the intricacies of phases 0, I, II and III xenobiotic biotransformation regulation. This demonstrates the initial role of the chemical character of the xenobiotica in phase 0 uptake (Xext–X), the role of negative feedback (red dashed line) in phase I by the xenobiotic itself, nuclear receptors such as AhR, CAR and PXR and phase I enzymes. Phase II, which also involves negative feedback (green dashed line), is influenced by the activated xenobiotica, Nrf2 and phase II enzymes like SULT, GST and UGT. Feedforward control (yellow dashed line) is achieved through phase I to phase II cross induction by X, the nuclear receptors, Nrf2 and phase II enzymes. Reproduced with permission from Zhang *et al.* (2009).

Figure 2-1 illustrates the complex regulatory mechanisms employed in the biotransformation of ~~a~~ xenobiotica, X_{ext}. It illustrates how feedback regulation by factors like the xenobiotica itself, nuclear receptors like AhR, CAR and PXR (Handschin & Meyer, 2003), and cytochrome P450 enzymes might serve to increase the metabolism of the absorbed xenobiotica X to minimize intracellular accumulation. Phase II feedback regulation by X', Nrf2 (Klaassen & Reisman, 2010) and phase II enzymes acts to speed up the conjugation of the activated xenobiotica X' and prevent its accumulation. The feed-forward control illustrated by the yellow dashed line is activated by the parent compound X to ensure sufficient capacity to prevent accumulation of reactive intermediates.

It is known that the efficiency of the biotransformation system may be affected by single nucleotide polymorphisms (SNPs). However, it has now been established that genetic factors alone are not the main causes of chronic disease (Rappaport, 2016). Instead, environmental influences seem to be much more important with more than 80–90% of deaths in the developed

world attributed to environmental exposure (Smith & Rappaport, 2009). Biotransformation is the human's main defense mechanism against chemical insults and is therefore pivotal in reducing the negative effects of the exposome. We therefore propose that there is an intricate relationship between biotransformation (B), the exposome (E + G) and chronic fatigue (CF) as discussed below and that a highly functional biotransformation process is essential and plays a crucial protective role against the insults from the individual's exposome.

The exposome functions as the encompassment of life-course environmental exposures (including lifestyle factors), emanating from the prenatal period (Wild, 2005). Environmental exposures may have a profound effect on the exposome (Rappaport *et al.*, 2014) and biotransformation has the potential to significantly limit the impact of these exposures. Xenobiotica and endogenous toxins can be biotransformed in the liver (the main detoxification organ) via a series of enzyme-catalysed reactions that alter the parent compound's polarity and render it excretable in the urine, bile and feces. Phase 0 describes the carrier-mediated absorption of the xenobiotica (X) in the liver and kidney by organic anion transporting peptides [OATPs] (Döring & Petzinger, 2014). Phase I activation, or functionalization, occurs mainly in the microsomes and may often involve oxidation by the cytochrome P450 enzyme system (Kirchheiner & Seeringer, 2007). The oxidized intermediate (X-OH) may now be conjugated in phase II biotransformation to a number of different conjugation substrates, depending on the chemical characteristics of the original compound (Wen *et al.*, 2013). Phase II reactions are highly dependent on energy and require ATP and also, for some of the reactions, coenzyme A (CoA). Imbalances between phases I and II biotransformation may potentially cause the accumulation of electrophilic compounds or reactive intermediates, which may give rise to free radicals and reduce systemic antioxidant capacity (Ferrari, 2012). It is important to note that some of these products activated in phase I may be more harmful than the original chemical, giving rise to short-lived, unstable, highly reactive chemical species that may adversely interact with biomolecules and organelles such as the mitochondria (Gu & Manautou, 2012), emphasizing the importance of a highly active and functional phase II conjugation system. The phase II conjugates are now more hydrophilic in nature, which facilitates their excretion from the liver. Phase III is the final step in this sequence of four reactions, describing the ATP-dependent excretion of metabolites (X-Con) by carrier-mediated efflux transport into the bile or sinusoidal blood. These include the so-called SLC and ABS carrier families (MPR2; MDR1 and BCRPs and other MPRs). If, however, the capacity of the biotransformation system is exceeded or imbalances occur due to cofactor and conjugation substrate depletion, accumulating endo- and exotoxins may impact on mitochondrial function (Meyer *et al.*, 2013), increase the production of free radicals (Armstrong *et al.*, 2015) from the electron transport pathway and have an adverse effect on ATP production. L-Carnitine acts as a carrier for long-chain fatty acids across the

mitochondrial membrane. This is crucial for the β-oxidation of fatty acids and energy production. Depletion of carnitine stores may have a profound effect on the carnitine shuttle, mitochondrial function and consequently energy production (Liepinsh *et al.*, 2015).

Against this background, we firstly propose a comprehensive model of fatigue in relation to the exposome and biotransformation, followed by a proposal for the experimental assessment of the environmental impact on biotransformation and fatigue.

2.2.7 Model and expert opinion

Our model illustrates the effect of a compromised biotransformation system and the relationship between the exposome (E + G), biotransformation (B) and chronic fatigue (CF) as shown in Figure 2-2. The red lines point towards possible accumulation effects in one or more of the four phases. The red arrow (c) points towards possible inhibitory effects on ATP production in the electron transport chain, red arrow (b) towards the damaging effects on mitochondrial DNA, and red arrow (a) finally contributes to increased insult to the genome (G) and ultimately to the exposome (E + G).

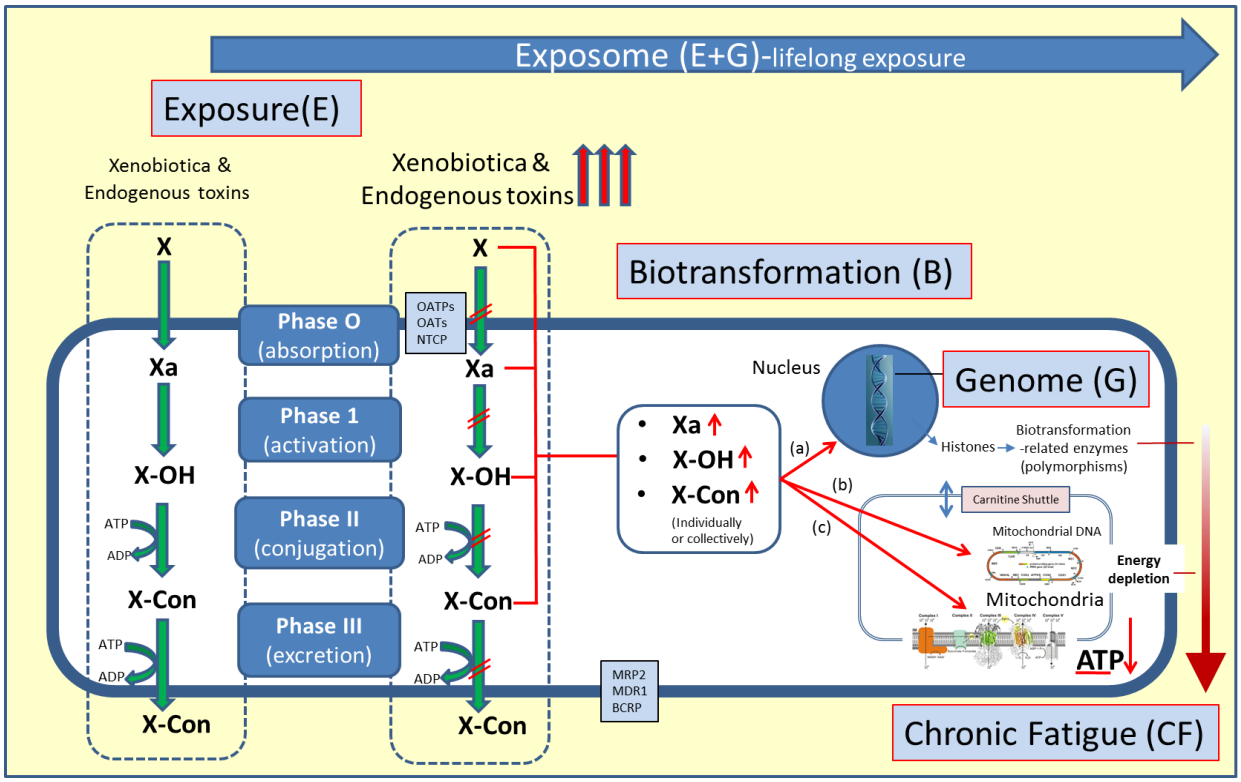


Figure 2-2: Schematic outline to illustrate the relationship between the exposome (E + G), biotransformation (B) and chronic fatigue (CF).

The significant increase in research funds allocated by the NIH for unravelling the aetiology of chronic fatigue syndrome emphasizes the realization about the scale of the effect this condition

has on the lives of many people worldwide. That lack of knowledge about the condition is still one of the principal obstacles that needs to be overcome, has recently been stressed by Walter Koroshetz, director of the NIH's National Institute of Neurological Disorders and Stroke (NINDS) and chair of the Trans-NIH ME/CFS Working Group, when he advocated the use of this increased funding to promote innovative technologies and research methods to improve understanding of this serious condition (National Institutes of Health, 2017). There are, however, several challenges which should be addressed for meaningful progress with regard to the possible metabolic involvement in the aetiology of fatigue.

First, in many of the published methods only *a limited number of compounds* are actively monitored and even less mention is made of all the possible metabolites and breakdown products which may ensue from exposure to specific chemicals.

Second, research focused on the effect of *combined chemical exposures* is even more scant and represents a serious limitation in the available knowledge about the effect of exposure (Silins & Högberg, 2011).

Third, seen against the background of increased use of prescription drugs the *total xenobiotic load on liver metabolism remains largely undefined*. To this end, it is clear that comprehensive profiling of an individual's xenobiotic load and its metabolism is required, especially when taking into account how patient-specific factors like genetics, environmental factors and physiological condition may have a direct bearing on free radical production and ultimately on the insult intensity to the exposome.

Based on these challenges, a novel approach is proposed to assess the causal relationship through experimental assessment of the biotransformation profiles of chronic fatigue patients, enabling the characterization of the detoxification phenotype in patients with chronic fatigue.

Against this background, a key opinion expressed here is that the role and importance of an effective, functional biotransformation system in the protection of the exposome is often overlooked and downplayed as something inevitably associated with "the liver can handle that". However, in reality, as demonstrated by our own research, the total xenobiotic load often exceeds the individual's biotransformation capacity, whether induced by depletion of important conjugate substrates and cofactors or by a combination of these factors with specific genetic limitations. The result is invariably an increase in circulating metabolic xenobiotica, which may directly (by inhibition of essential energy-producing processes in the mitochondria) or indirectly (by sequestration of CoA) result in increased insult to the exposome. A real-life example of this is the variety of hepatotoxic effects exerted by drugs, causing (1) inhibition of the mitochondrial

respiratory chain; (2) oxidative phosphorylation uncoupling; (3) mitochondrial DNA damage or depletion; (4) mitochondrial permeability transition pore opening; and/or (5) inhibiting β-oxidation and/or depletion of carnitine and CoA (Lauschke & Ingelman-Sundberg, 2016). Similarly, many other chemical compounds could be classified as “toxic exposome”, which represents a collection of chronically or acutely toxic compounds to which humans are exposed and are now recognised as probably the leading cause of human morbidity and mortality (Wishart *et al.*, 2015). Combining chemical/xenobiotic exposure with an aberrant biotransformation metabolism, the prospect is high for causing epidemiology conditions due to exposure, like fatigue.

In the final instance, an imbalance in biotransformation metabolism and the subsequent accumulation of xenobiotica may play a significant role in the aetiology of CF and CFS/ME. Given the multifactorial nature of chronic fatigue, it is unlikely that an objective measure, as a verified single biomarker will define fatigue conditions. However, biomarker discovery should be an essential element in characterizing health-impairing exposure and identification of a biomarker profile has been proposed for these conditions [‘biomarkers of exposure’] (Rappaport, 2012). A novel approach for the identification of fatigue related imbalances of biotransformation metabolism is offered here through an experimental intervention approach. The concept of perturbation of homeostasis through an experimental intervention to quantify health related conditions is well established for exogenous conditions affecting health (Bonassi *et al.*, 2001; De Luca *et al.*, 2011; Kitteringham *et al.*, 2009; Nakatsuji *et al.*, 2010; Pellis *et al.*, 2012). The response of the intervention, or challenge, reveals multiple aspects of metabolic health not apparent from studying biofluids obtained under homeostatic conditions (Nortje *et al.*, 2016).

A novel laboratory approach to facilitate an objective evaluation of fatigue due to imbalances of biotransformation metabolism in chronic fatigue patients require three complementary approaches: (1) use of a standardized clinical scales to score the level of fatigue in each experimental subject (Dittner *et al.*, 2004); (2) a detoxification challenge test/tests related to biotransformation (Cordts *et al.*, 2001); and (3) appropriate analytical measures, like HPLC, GC- or LC-MS to monitor the impact of the perturbation in the cohort of patients. The underlying physiological assumption for this experimental approach is that fatigue is a symptom of energy depletion. From a presumed causal relationship between exogenous stimuli, biotransformation responses and fatigue, it is anticipated that the highly energy dependent challenge test (that is, ATP required for the *in vivo* synthesis of biotransformation products) should be more pronounced in controls than in fatigue patients supposed to suffer from energy depletion (suffering from ATP depletion). The proposed intervention approach provides a knowledge-base that can link a biomarker profile related to exposure, with a potential to explore causality,

offering a novel avenue for prevention, diagnosis and treatment of chronic fatigue, ultimately paving the way towards personalized medicine.

2.3 Aims and objectives of the thesis

From the overview of the literature on biotransformation (Chapter 1 and Chapter 2: §2.2.1 to 2.2.7), we concur that the main lines of thought [*Chronic fatigue may be due to an energy deprived state as a consequence of the biochemical response(s) to exposure to xenobiotics*] are compatible with the expert opinion derived from the literature study [*A causal relationship exists between chronic xenobiotic exposure and the energy demand for biotransformation responses seen in idiopathic chronic fatigue*]. In addition, it is clear from the literature that information is scarce and unconvincing in supporting a causal link between "*measurable metabolite profiles of biofluids"* and chronic fatigue. Against this background a point of departure of the present study was to further investigate biochemical aspects of glycine N-acyltransferase (Chapter 6). Following this basic research, the focus moved to the main aim of the study as formulated in the TIA-BOSS (TIA-Biotransformation and Oxidative Stress Status) translational project (Chapter 3) and its subsequent experimental verifications (Chapters 7 to 8). The long-term strategy is the development of a practical biochemical evaluation protocol of important biofluid markers for metabolic reactions in the liver against xenobiotics, as observed in patients who suffer from idiopathic fatigue.

For the phase of translational research, the following objectives were formulated:

1. Compile a user-friendly guide for potential CF patients to give consent for participation in the project done in association with clinicians and the biochemical laboratory having the potential for further development towards a diagnostic tool.
2. Development of a battery of biochemical tests for provocation testing with certain conjugation substrates. In the process the functionality of conjugation reactions will be evaluated, and antioxidant levels and the production of reactive oxygen species monitored.
3. Obtain an objective profile on the level of fatigue for at least 750 idiopathic patients selected by the physicians, from which at least 500 cases should be selected ― based on clear exclusion criteria ― for the assessment of an experimental protocol on the proposed link between CF and biotransformation parameters.
4. Perform a correlation of the clinical features of the selected patients with the biotransformation profiles to determine a possible metabolic link to fatigue symptoms, as proposed through the model for this link (Figure 2-2).

It should be noted that feed-back interaction between the biochemists and clinicians on selective cases was frequently done. Details on the physician-biochemist interactions on individual fatigue cases as well as the outcome of a feed-back assessment are important but were not pursued as an objective for the present study. It is envisaged to form part of a future project on diagnostics of fatigue and personalized medicine.

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PART II

EXPERIMENTAL DESIGN AND METHODS

CHAPTER 3

Biotransformation and Oxidative Stress Status (BOSS) analysis



3.1 Introduction

Chapter 3 is the first of two chapters dealing in the methods used in this study (Part II). The focus of this chapter will be on the procedures followed to obtain samples for the analytical chemical methods used to obtain biochemical data on biotransformation and oxidative stress. Following the introductory background, the chapter is structured in three sections: (1) the rationale for the selection and development of investigative methods used for the measurement of the biotransformation profiles in the patient cohort; (2) the methodological approach developed for compiling a patient group, for the intervention and for collection of biofluid samples; and (3) the clinical report from the physician and the user manual which also contains the form for informed consent.

3.2 Selection and development of investigative methods for biotransformation profiling

The functions of the liver can be divided into a number of important areas of which the biotransformation and elimination of drugs and xenobiotics ranks high in terms of importance (De Luca *et al*., 2011). The use of challenge substances to monitor biotransformation, based either on the clearance of marker substances or the formation of probe substance conjugates are some of the possibilities that could be employed. The application of challenge substances to determine general and sometimes more specific liver functions is not a new concept. It is however important that selection of the challenge substance(s) is based on the focal point for the investigation with the aim of collecting as much as possible information about the reactions involved. A challenge substance can be defined as a generally safe chemical compound administered orally in specific doses to test the metabolic conversion of this compound to its product(s). According to Sear (2002), assessment of liver function requires the probe substance to display all or some of the following characteristics:

(1) Non-toxic, without serious pharmacological effects.
(2) Rapidly and completely absorbed after administration.
(3) The rate-limiting step in the liver metabolism should reflect the possible abnormality in the biotransformation of the probe substance.
(4) The parent compound, its metabolites and/or both should be readily measurable in biological fluids, saliva or breath.
(5) The probe substance should not be highly bound to proteins in plasma or tissue, unless there is an accompanying high hepatic extraction ratio.
(6) The probe substance(s) must be relatively inexpensive.
(7) There must be reliable assays available for the probe substance and/or metabolites.

(8) The probe substance(s) must be readily available and licensed for human use.

3.3 Selection of biotransformation challenge substances

The use of model test-substances for providing general information about liver function has several limitations, but there are specific methods to quantify the activity of cytochrome P450 enzymes and phase II enzymes *in vivo* (Brockmöller & Roots, 1994).

3.3.1 Phase I (CYP 1A2)

Caffeine was introduced as a model substance for determination of intrinsic hepatic clearance. It was shown that caffeine elimination was dose-dependent and therefore a specific and low dose was required. Caffeine as challenge substance has been extensively studied and has been used as an in vivo probe substance to determine CYP 1A2 activity in plasma saliva and urine (Perera *et al.*, 2010; Perera *et al.*, 2011). Saliva as a test matrix is low-cost and easy-to-collect and especially suitable for screening of large populations [Dobson *et al.*, 2016; Kaufman & Lamster 2002, reviewed in Esteban and Castaño (2009)]. Some of the guidelines proposed by Dorne and co-workers were followed. According to their review research, probe substances for each pathway of elimination should be selected on the basis that oral absorption was >95% and that the metabolic route was the primary route of elimination of the compound (60-100% of a dose). They also calculated the pathway-related uncertainty factors, and these were taken into consideration in the selection of the different probe substances in the current investigation (Dorne *et al.*, 2005). Caffeine is also a European Federation of Pharmaceutical Sciences (EUFEPS) recommended probe for the phenotyping of phase I activity (Bachmann, 2009).

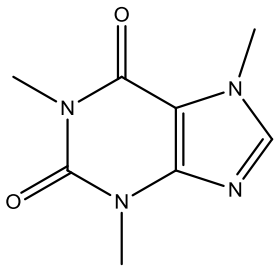


Figure 3-1: Chemical structure of caffeine. The phase I biotransformation challenge substance used in this study**.**

3.3.2 Phase II (glucuronidation, sulphation and glutathione conjugation)

Acetaminophen (paracetamol) has long been described as a good probe substance for the measurement of specific phase II conjugation reactions. Jackson and co-workers described

the metabolism of acetaminophen in a pharmacological review in which they highlighted the formation of specific metabolites from acetaminophen. According to this review acetaminophen is metabolized primarily in the liver and the proportions of excreted urinary metabolites are acetaminophen glucuronide (55%), acetaminophen sulfate (30%), the parent compound (4%) and the mercapturic acid conjugate of acetaminophen (4%) (Jackson *et al*., 1984).

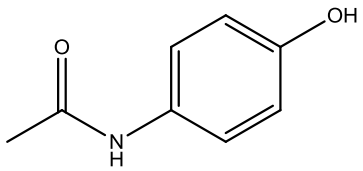


Figure 3-2: Chemical structure of acetaminophen. The first of the phase II biotransformation challenge substances used in this study.

3.3.3 Phase II (glycine conjugation)

Acetylsalicylic acid (aspirin) metabolism and pharmacokinetics also allows for this substance to be used as a probe for the amino acid glycine conjugation (reviewed by Badenhorst *et al.*, 2013). The liver is the main site for acetylsalicylic acid metabolism and it is rapidly hydrolyzed to salicylic acid (Levy, 1980). Salicyluric acid, the glycine conjugate of salicylic acid was the main excretion product and elimination of this metabolite ranged from 20 to 65% of the original dose and was related to the recovery of salicylate (Hutt *et al*., 1986).

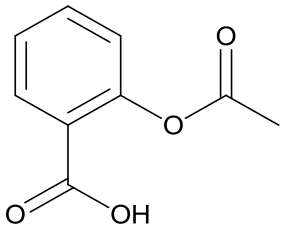


Figure 3-3: Chemical structure of acetylsalicylic acid. The second of the phase II biotransformation challenge substances used in this study.

In addition, salicylate has the potential to react with the highly reactive hydroxyl radical (•OH) to produce metabolic products like 2,3– and 2,5-dihydroxybenzoic acids (2,3-DHBA & 2,5-DHBA) and catechol (Grootveld & Halliwell, 1988; Miyakawa *et al.*, 2015). The free radical scavenging potency of the hydroxy benzoic acids have recently been characterized and confirmed by Milenković and co-workers (Milenković *et al*., 2017).

3.4 Biotransformation and Oxidative Stress Status (BOSS) analysis

The Biotransformation and Oxidative Stress Status test is based on the analysis of the specific metabolites discussed in Section 3.3 as products of the challenge tests, which served as an indication of liver detoxification. In addition to these, certain free radical markers are measured as indicators of oxidative metabolic stress (De Zwart *et al*., 1999). Detailed information about the test protocol and analytical methodology will be given in Chapter 4.

(1) All patients are clinically evaluated by a medical doctor in private practice. This included an evaluation of the complete medical history of their patients as well as a clinical investigation. Section 3.5 shows an example of the clinical questionnaire, which is completed by the physician during the evaluation. Based on the outcome of this evaluation, a decision is made by the physician whether the patient may do the Biotransformation and Oxidative Stress Status test. All the referred case samples are sent to the Biotransformation and Oxidative Stress Status (BOSS) laboratory at the Center for Human Metabolomics via the participating pathology groups (AmPath, Lancet and PathCare).

(2) In the Biotransformation section of the test, phase I activity may be affected by a number of factors and is measured with a challenge of a specific cytochrome P450 substrate, namely caffeine (Wittayalertpanya *et al*., 1996). Phase I activity measurement is crucial as this part of the biotransformation process represents activation of potential toxins before they are further metabolized in phase II. Up– or down regulated cytochrome P450 activities may contribute to biotransformation imbalances (Zhou *et al*., 2009).

(3) Phase II conjugation activity is measured for a number of conjugation pathways: these pathways all utilize different conjugation substrates to effect the conversion of reactive phase I products to more polar, conjugation products (Liska *et al.*, 2006). This is done by administering two different challenge substances, acetaminophen and acetylsalicylic acid. Urine samples are then collected over a specific period. These compounds are then metabolized and conjugated by the relevant conjugation enzymes of the detoxification pathways. Ratios of phase I to phase II biotransformation are used to determine whether there are imbalances between phase I and phase II reaction rates. Quantitatively, the conjugation ability of an individual is then expressed as percentages of the original amount of challenge substrate administered converted to specific conjugates.

(4) Urinary free carnitine and acylcarnitines are determined by analyzing the isolated and butylated carnitine derivatives with electrospray tandem mass spectrometry (Angelini *et al*., 1992). Carnitine profiles are used to determine mitochondrial metabolism abnormalities and the effect of certain exogenous compounds on the carnitine profile.

(5) The antioxidant status of the patient is determined by measuring specific marker functions which give an indication of antioxidant status (Benzie & Strain, 1996). Levels of specific endogenous antioxidants are used to determine whether a state of antioxidant depletion exists in the patient (Pasupathi *et al*., 2009).

(6) Products of oxygen metabolism are measured as an indicator of oxidative stress status (Cutler, 2005). The total pool of reactive metabolites is measured while individual markers are used to pinpoint the relative contribution of specific reactives to the pool of metabolites involved.

(7) The additional information on the report is obtained from general pathology tests as well as from the clinical questionnaires, which form part of the test. Section 3.6 shows an example of the BOSS test information booklet, which we defined as the user-friendly guideline. This booklet contains 75 questions regarding medical symptoms (Whitehead, 2009) and 24 regarding fatigue symptoms (Lukaczer, 2005). In addition to this there are also eleven lifestyle questions. The standard operating procedure (SOP) for the collection and handling of specimens for Biotransformation and Oxidative Stress Status assessments is shown in Annexure A.

The results from the BOSS analysis are then used to determine whether there is any of the phase I and/or phase II reactions which are affected. Any imbalances between phase I and II are also detected and the magnitude of imbalances are often reflected in increased free radical production. The combined use and interpretation of the biochemical information with the medical symptoms and fatigue questionnaires ultimately provides the physician with a more holistic diagnostic profile (Marschall *et al*., 2007).

3.5 BOSS clinical questionnaire



Biotransformation and Oxidative Stress Status
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Phone:+27 18 299 2042, Fax:+27 87 231 5527
e-mail: cecile.cooke@nwu.ac.za

MEDICAL REPORT: BIOTRANSFORMATION

Must be completed and signed by a Medical Practitioner

PATIENT DETAILS

Name and Surname: _____

ID nr: _____

Address: _____

Tel nr: _____

HISTORY

1. List all current problems and diseases:

_____

2. Any previous diseases and operations:

_____

3. Current problems with any of the following (Timeframe and short description)

* Chronic fatigue

_____

* Depression

_____

* Allergies like hay fever, post nasal drip, rash

_____

* Increase or decrease in weight

_____

* Poor memory

* Sleep disorders

* Increased Cholesterol

4. Any allergies for medication including aspirin, caffeine, paracetamol:

5. Previous or current heart arrhythmia or on medication for treatment of heart arrhythmia

6. Chronic medication

7. Current or previous hormone therapy, including birth control:

8. Porphyria

9. Any supplements, vitamins etc:

10. Family history of:

* Hereditary diseases: _____

* Chronic Fatigue: _____

* Cancer in the family: _____

* Obesity: _____

* Fibromyalgia/ Yuppie flu: _____

11. Smoking:_____Number of cigarettes per day: _____

12. Alcohol: _____

13. Any contra-indication for use of caffeine, paracetamol and aspirin:

Medical examination:

Weight:_____ Height: _____ BMI: _____

U-DDipstix: _____ Dex: _____ BP: _____

GENERAL EXAMINATION

Cardiovascular system:

Any signs of cardiovascular abnormalities: _____

Peripheral pulse: Speed:_____Rhythm: _____

Is peripheral pulse easily palpable: _____

Respiratory System:

Any abnormalities _____

Gastro Intestinal System:

Any abnormalities _____

Musculoskeletal System:

Any abnormalities _____

Genito Urinary System

Any abnormalities _____

Hereditary disorders, thyroid disorders, tumors: _____

Doctor's details:

Name/Surname: _____

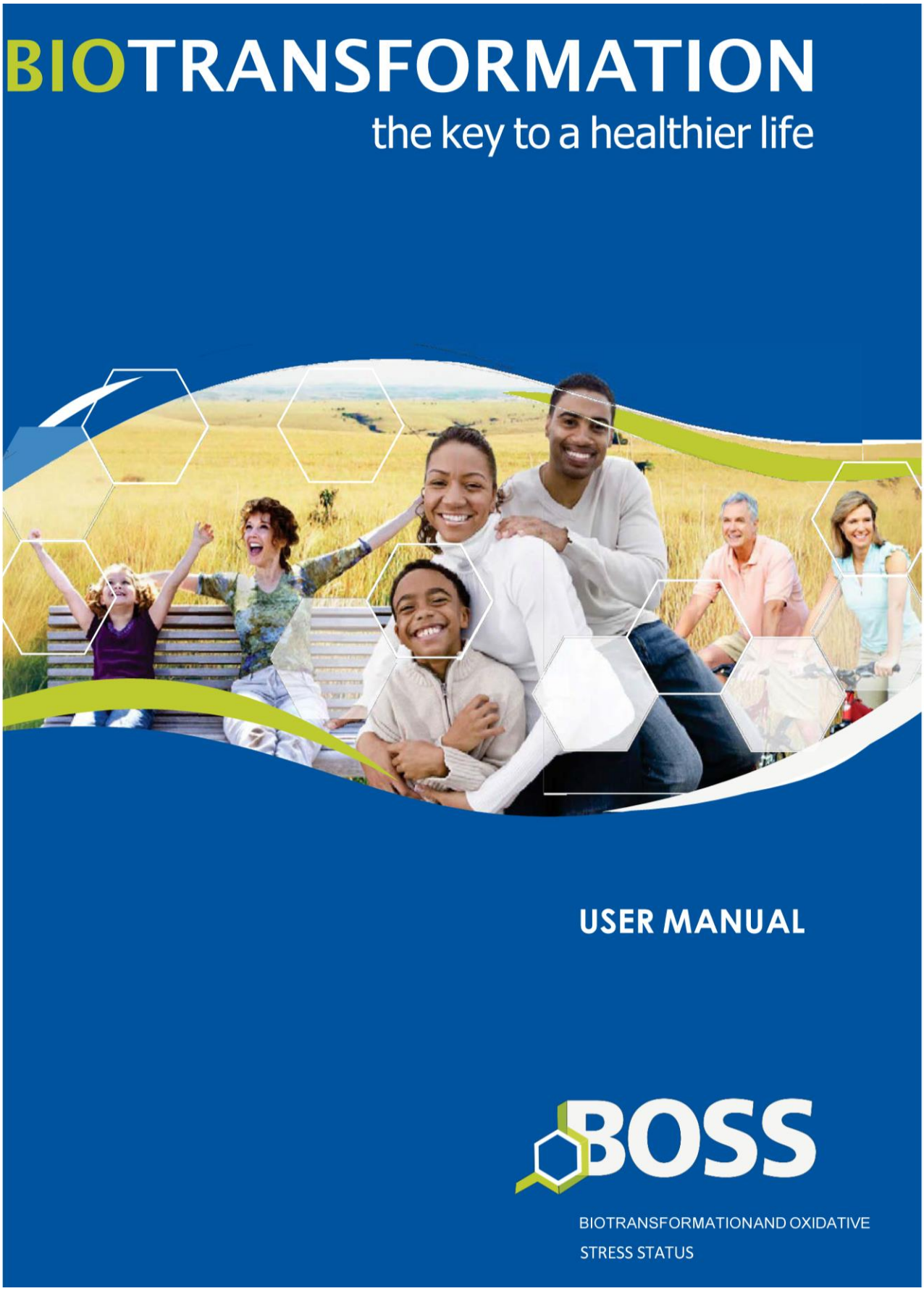
Tel nr: _____

Qualification: _____

_____

Signature: Medical Practitioner

3.6 BOSS test information booklet



A. BIOTRANSFORMATION AND OXIDATIVE STRESS STATUS PROFILE 1

Background information 1

Biotransformation and oxidative stress status test 1

B. TEST PROCEDURE 2

About the test kit and procedure 2

Instructions 3

C. IMPORTANT PAPERWORK TO BE COMPLETED 4

Patient information 4

Medical Symptoms Questionnaire 5

Lifestyle Questionnaire 5

Piper Fatigue Scale 7

Informed consent 8

IMPORTANT:

Please complete ALL the information fields on the questionnaires from p. 4 - 9 in the information booklet and submit together with all the samples taken (saliva and urine) on the day of collecting your blood sample.



A. BIOTRANSFORMATION AND OXIDATIVE STRESS STATUS PROFILE

Background information

In today's world of processed food and pollution, toxic substances exist almost everywhere. They are in the food we eat, the water we drink, and the air we breathe. It is estimated that 60 to 80 percent of all cancers are a direct result of these environmental toxins. Environmental toxins may also play a role in the development of neurological disorders such as Alzheimer's and Parkinson's disease, and in mental or behavioural disorders such as depression, anxiety, schizophrenia and attention deficit disorder. Since the nervous system is intricately connected to the immune system, toxins also affect our immune function. Thus, our very ability to think and feel normal can be drastically affected by exposure to toxins.

The liver is a key organ in your body's self-defense system. Inside the liver cells there are sophisticated mechanisms that have evolved over millions of years to break down toxic substances into forms which your body can safely eliminate. The liver uses two mechanisms called Phase I and Phase II biotransformation, designed to convert fat soluble toxins into water soluble substances so that your body can easily excrete these substances via watery fluids such as bile and urine.

In Phase I, your body's enzymes convert a toxin into a substance which can be further metabolised in Phase II. This is achieved by various chemical reactions and during this process, free radicals are produced which can damage the liver cells. Antioxidants (such as vitamin C and E) can reduce the damage caused by free radicals, but if antioxidants are low and toxin exposure high, these toxins become more dangerous and cannot be eliminated as rapidly or as completely. Slower biotransformation results in more toxic substances circulating in the body which are eventually stored for years in fatty body tissue, being released during times of exercise, stress or fasting. During the release of these toxins, symptoms such as headaches, poor memory, stomach pain, nausea, fatigue, dizziness and palpitations may occur.

Phase II is also called the conjugation pathway whereby other enzymes in liver cells add another substance to metabolites from Phase I.

This makes the toxin water soluble and less harmful, so it can then be excreted from the body via watery fluids such as bile or urine. For efficient Phase II biotransformation, the liver cells require sulphur-containing amino acids such as taurine and cysteine. The nutrients glycine, glutamine, choline and inositol are also required for efficient Phase II biotransformation. Major Phase II pathways include glutathione conjugation, sulphate conjugation, glycine conjugation and glucuronide conjugation.

A number of conditions affect how well the liver performs its detoxifying duties. Repeated exposure to toxins in food, water and the environment increases the biotransformation burden and if the Phase I and II biotransformation pathways become overloaded, there will be a build-up of toxins in the body.

Biotransformation and oxidative stress status test

One method to assess the liver's detoxifying ability is to examine the overall state of your health. Toxic substances are contributing factors in a wide range of health problems.

There are also several different liver tests (such as liver enzyme analysis) which look for clinical evidence of existing liver damage. Unfortunately, by the time these tests register abnormal, liver damage is already present.

A test to assess the liver's biotransformation function can be done. During this test the liver is challenged by common substances such as caffeine,

aspirin and paracetamol in safe doses and samples of blood, urine and saliva are collected. This test is unique because Phase I and II biotransformation pathways as well as the oxidative stress status and antioxidant capacity are assessed. Phase I activity is tested by means of the caffeine clearance test, and in Phase II the production of four conjugates are determined. The ratio between the activities of the two phases is also calculated to detect imbalances. Blood samples are used to determine the oxidative stress status and antioxidant capacity. This data can then be correlated with the results of the biotransformation capacity test.

B. TEST PROCEDURE

It is very important that the test procedures are strictly followed. The documentation and all the samples (blood, saliva and urine) should be sent together, before the laboratory can analyse any samples.

About the test kit and procedure
Three compounds, caffeine, paracetamol and aspirin, are taken orally to challenge the liver's Phase I and Phase II biotransformation capacity. Saliva and urine are analysed to determine how well the liver can convert and clear toxins from the body.

The provided kit consists of the following:

Sample	Amount	Description
A 	1	One 1 L urine container
B 	1	One small urine container
C + D 	2	Two saliva collection tubes (salivettes)
E 	1	One 150 mg caffeine tablet
F 	2	Two 300 mg aspirin tablets
	2	Two 500 mg paracetamol tablets
G 	1	Labels for all containers are supplied and should be completed with the collection time, name and date and applied to the correct containers.
H 	1	User manual containing important forms that must be completed and send back.
I	1	Extra plastic bag with document pouch attached to the outside

Pre-test instructions

During this test a normal diet can be assumed except for the following dietary restrictions.

DO NOT consume the following during the test:

- Alcohol, coffee, tea, cocoa, chocolate, cola soft drinks, rooibos tea, health tea;
- Medication containing caffeine, salicylates (aspirin) or paracetamol;
- Stone fruits (eg. sultanas, currants, raisins etc);
- Nuts and seeds, peppermint or liquorice (eg. sweets, lollies, gums, plants/tea);
- Spices (eg. curry, dill, oregano, paprika etc);
- Brassica vegetables like broccoli, cabbage, brussels sprouts, cauliflower and tomato.

INSTRUCTIONS

	Time	Use	Steps to be taken
1	Early morning		Collect an early morning baseline urine sample in the small urine sample container. All urine samples must be frozen as soon as possible.
2	08:00		Take the caffeine tablet before breakfast.
3	10:00		Collect the 1st saliva sample. [Please read detailed instructions on next page]
	PLEASE SEE DIRECTIONS ON NEXT PAGE		
4	16:00		Collect the 2nd saliva sample (see above).
5	21:00		Cease eating and drinking (except water). Empty bladder. Take the two aspirin and two paracetamol tablets.
6	Overnight 21:00 - 07:00		Collect all overnight urine until 07:00 the next morning in the 1 L container. All urine samples must be frozen as soon as possible. **Please note**: bottle has a cap and a seal.
7		Must be done by Pathologists	Collect blood sample in three tubes: two containing EDTA (purple top) and one clotted tube (yellow top). This will be done at the pathology branch when the frozen saliva and urine samples are brought back. Not necessary to fast.
8	Return all samples in the extra plastic bag. Place the blue booklet in the document pouch attached to the plastic bag. Remove strip to seal the document pouch.		

Before saliva collection make sure of the following directions:

1. Collect saliva at the specific time(s) listed by your physician.
2. Do not eat or drink anything for at least 30 minutes before collecting saliva.
3. Avoid activities that may cause your gums to bleed - do not brush or floss.
4. Remove lipstick and lip balm before collection.

How to collect saliva using the salivettes

Saliva should be collected at the precise time(s) specified by your practitioner. The provided Salivette, is a device made specifically for the purpose of collecting saliva. Please read the following directions carefully before starting your collection.

PLEASE NOTE - SALIVETTES ARE NOT INTENDED FOR USE WITH CHILDREN UNDER THE AGE OF THREE. CHILDREN UNDER THE AGE OF TEN SHOULD HAVE ADULT SUPERVISION.

INSTRUCTIONS - Follow these steps to collect your saliva:

	**STEP 1:** Remove only the top cap of the tube to expose the round swab. Do not remove the insert (smaller internal container) housing the cotton swab.
	**STEP 2:** Hold the tube to your lips and tip the tube so that the swab slides into your mouth. Do not touch the cotton swab with your fingers
	**STEP 3:** Gently roll the swab around in your mouth for 5 minutes or until you can no longer prevent swallowing excess saliva (the cotton swab should be saturated).
	**STEP 4:** Place the tube to your lips and allow the swab to slide back into the insert (small internal vessel) and firmly close with the top cap/stopper. *Do not touch the swab with your fingers. The salivette should now look exactly as it did when you started, with the cap, insert, cotton swab and centrifuge tube.*
	**STEP 5:** On the supplied labels, write your full name, date of birth, date of collection and collection time and attach it to the correct containers.
	**STEP 6:** Freeze the salivette tubes and take it to the practitioner or laboratory within 3 days.

C. IMPORTANT PAPERWORK TO BE COMPLETED

PATIENT INFORMATION			
All information from p.4 - 9 must be completed including weight, height and referring doctor.			
Tests requested	Detoxification profile:		
Monster info	Blood:	Saliva:	Urine:
Name:			
Surname:			
Date of birth:			
ID:			Sex:
Cell:			**Weight:**
Tel:			**Height:**
Postal address:			
Has anyone in the family been tested? If so, provide us with full names and family connection:			
Referring doctor:			
Medical aid information	Name of medical aid:		
	Guarantor I.D. no		
	Medical aid number:		
	Name of main member:		
ENQUIRIES			
Human Metabolomics, North-West University, Potchefstroom Campus. Enquiries: Cecile Cooke , tel 018 299 2042, fax: 087 231 5527, email: cecile.cooke@nwu.ac.za Postal address: Biochemistry, Private bag X6001, Potchefstroom, 2520			

LIFESTYLE QUESTIONNAIRE				
1	Do you use tobacco products?	No	Yes	
2	Do you live or work with someone who smokes in your presence?	No	Yes	
3	How often do you eat fast foods	Not often	Week-ly	Daily
4	How many alcoholic drinks do you have per week	< 1	1 – 5	> 5
5	Do you ever use toxic chemicals such as insect sprays or herbicides?	No	Yes	
6	Have you taken any prescription medication two or more times in the past year?	No	Yes	
7	Do you take any chronic medication? Please specify if yes. *Please specify:*	No	Yes	
8	Do you take birth-control or hormone replacement medication? Please specify if yes. *Please specify:*	No	Yes	
9	Do you suffer from gastrointestinal problems?	No	Yes	
10	Are you often tired for no reason? If yes, please complete Piper Fatigue form p. 7.	No	Yes	
11	Diagnosed with medical condition? Please specify if yes. *Please specify:*	No	Yes	

MEDICAL SYMPTOMS QUESTIONNARE													
NB!: POINT SCALE: HOW OFTEN AND SEVERELY DO YOU EXPERIENCE THE FOLLOWING SYMPTOMS - IN THE LAST 30 DAYS:		Never / Almost never	Occasionally, effect is not severe	Occasionally, effect is severe	Frequently, effect not severe	Frequently, effect is severe	NB!: POINT SCALE: HOW OFTEN AND SEVERELY DO YOU EXPERI-ENCE THE FOLLOWING SYMPTOMS - IN THE LAST 30 DAYS:		Never / Almost never	Occasionally, effect is not severe	Occasionally, effect is severe	Frequently, effect not severe	Frequently, effect is severe
		0	**1**	**2**	**3**	**4**			**0**	**1**	**2**	**3**	**4**
HEAD	Headaches	0	1	2	3	4	**EYES**	Watery or itchy eyes	0	1	2	3	4
	Faintness	0	1	2	3	4		Swollen, reddened or sticky eyelids	0	1	2	3	4
	Dizziness	0	1	2	3	4		Bags or dark circles under eyes	0	1	2	3	4
	Insomnia	0	1	2	3	4		Blurred or tunnel vision (not including near- or far-sightedness)	0	1	2	3	4
EARS	Itchy ears	0	1	2	3	4	**SKIN**	Acne	0	1	2	3	4
	Ear aches, ear infections	0	1	2	3	4		Hives, rashes, dry skin	0	1	2	3	4
	Drainage from ear	0	1	2	3	4		Hair loss	0	1	2	3	4
	Ringing in ears, hear-ing loss	0	1	2	3	4		Flushing, hot flashes	0	1	2	3	4
								Excessive sweating	0	1	2	3	4

		0	1	2	3	4			0	1	2	3	4
NOSE	Stuffy nose	0	1	2	3	4	MOUTH/ THROAT	Chronic coughing	0	1	2	3	4
	Sinus problems	0	1	2	3	4		Gagging, frequent need to clear throat	0	1	2	3	4
	Hayfever	0	1	2	3	4		Sore throat, hoarseness, loss of voice	0	1	2	3	4
	Sneezing attacks	0	1	2	3	4		Swollen or discolored tongue, gums, lips	0	1	2	3	4
	Excessive mucus formation	0	1	2	3	4		Cancer sores	0	1	2	3	4
HEART	Irregular or skipped heartbeat	0	1	2	3	4	LUNGS	Chest congestion	0	1	2	3	4
	Rapid or pounding heartbeat	0	1	2	3	4		Asthma, bronchitis	0	1	2	3	4
	Chest pain	0	1	2	3	4		Shortness of breath	0	1	2	3	4
	Excessive sweating	0	1	2	3	4		Difficulty breathing	0	1	2	3	4
EMOTIONS	Mood swings	0	1	2	3	4	ENERGY/ ACTIVITY	Fatigue, sluggishness	0	1	2	3	4
	Depression	0	1	2	3	4		Apathy, lethargy	0	1	2	3	4
	Anger, irritability, aggressiveness	0	1	2	3	4		Hyperactivity	0	1	2	3	4
	Anxiety, fear, nervousness	0	1	2	3	4		Restlessness	0	1	2	3	4
MIND	Diffculty in making decisions	0	1	2	3	4		Fatigue worsens with exertion (vigorous action or effort), plus post-exertional malaise (feeling ill after exertion)	0	1	2	3	4
	Stuttering or stammering	0	1	2	3	4							
	Slurred speech	0	1	2	3	4							
	Learning disabilities	0	1	2	3	4							
	Poor memory	0	1	2	3	4	WEIGHT	Binge eating / drinking	0	1	2	3	4
	Confusion, poor comprehension	0	1	2	3	4		Craving certain foods	0	1	2	3	4
	Poor concentration	0	1	2	3	4		Excessiveweight	0	1	2	3	4
	Poor physical coordination	0	1	2	3	4		Compulsive eating	0	1	2	3	4
								Water retention	0	1	2	3	4
								Underweight	0	1	2	3	4
DIGESTIVE TRACT	Nausea, vomiting	0	1	2	3	4	JOINT/ MUSCLE	Pain or aches in joints	0	1	2	3	4
	Diarrhea	0	1	2	3	4		Arthritis	0	1	2	3	4
	Constipation	0	1	2	3	4		Stiffness or limitation of movement	0	1	2	3	4
	Bloated feeling	0	1	2	3	4		Pain or aches in muscles	0	1	2	3	4
	Belching, passing excessive gas	0	1	2	3	4		Feeling of weakness or tiredness	0	1	2	3	4
	Heartburn	0	1	2	3	4		Migratory arthralgia - Pain in different joints at different times as if pain is travelling	0	1	2	3	4
	Intestinal / stomach pain	0	1	2	3	4							
OTHER	Frequentillness	0	1	2	3	4							
	Frequent or urgent urination	0	1	2	3	4							
	Genital itch or discharge	0	1	2	3	4							
	Painfull lymph nodes (cervical, axillary, inguinal, or supraclavicular)	0	1	2	3	4							

PIPER FATIGUE SCALE												
Please answer the questions below using the scale measurement in the next colom		Scale measurement	Please mark appropriate scale									
			1	2	3	4	5	6	7	8	9	10
1	How long have you been feeling fatigue? (Check one response only): 1 - not feeling fatigue, 2 - minutes, 3 - hours, 4- days, 5 - weeks, 6 months, 7 - other (please describe.....		1	2	3	4	5	6	7	8	9	10
2	To what degree is the fatigue you are feeling now causing you distress?	1 No distress 10 A great deal	1	2	3	4	5	6	7	8	9	10
3	To what degree is the fatigue you are feeling now interfering with your ability to complete your work or school activities?	1 Non 10 A great deal	1	2	3	4	5	6	7	8	9	10
4	To what degree is the fatigue you are feeling now interfering with your ability to socialise with your friends?	1 Non 10 A great deal	1	2	3	4	5	6	7	8	9	10
5	To what degree is the fatigue you are feeling now interfering with your ability to engage in sexual activity?	1 - Non 10 A great deal	1	2	3	4	5	6	7	8	9	10
6	Overall, how much is the fatigue which you are now experiencing interfering with your ability to engage in the kind of activities you enjoy doing?	1 Non 10 A great deal	1	2	3	4	5	6	7	8	9	10
7	How would you describe the degree of intensity or severity of the fatigue which you are experiencing now?	1 Mild 10 Severe	1	2	3	4	5	6	7	8	9	10
8	To what degree would you describe the fatigue you are experiencing now as being	1 Pleasant 10 Unpleasant	1	2	3	4	5	6	7	8	9	10
9	To what degree would you describe the fatigue you are experiencing now as being	1 Agreeable 10 Disagreeable	1	2	3	4	5	6	7	8	9	10
10	To what degree would you describe the fatigue you are experiencing now as being	1 Protective 10 Destructive	1	2	3	4	5	6	7	8	9	10
11	To what degree would you describe the fatigue you are experiencing now as being	1 Positive 10 Negative	1	2	3	4	5	6	7	8	9	10
12	To what degree would you describe the fatigue you are experiencing now as being	1 Normal 10 Abnormal	1	2	3	4	5	6	7	8	9	10
13	To what degree are you now feeling	1 Strong 10 Weak	1	2	3	4	5	6	7	8	9	10
14	To what degree are you now feeling	1 Awake 10 Sleepy	1	2	3	4	5	6	7	8	9	10
15	To what degree are you now feeling	1 Lively 10 Listless	1	2	3	4	5	6	7	8	9	10
16	To what degree are you now feeling	1 Refreshed 10 Tired	1	2	3	4	5	6	7	8	9	10
17	To what degree are you now feeling	1 Energetic 10 Unenergetic	1	2	3	4	5	6	7	8	9	10
18	To what degree are you now feeling	1 Patient 10 Impatient	1	2	3	4	5	6	7	8	9	10

PIPER FATIGUE SCALE												
Please answer the questions below using the scale measurement in the next colom		Scale measurement	Please mark appropriate scale									
			1	2	3	4	5	6	7	8	9	10
19	To what degree are you now feeling	1 Relaxed 10 Stressed	1	2	3	4	5	6	7	8	9	10
20	To what degree are you now feeling	1 Exhilarated 10 Depressed	1	2	3	4	5	6	7	8	9	10
21	To what degree are you now feeling	1 Able to concentrate 10 Unable to concentrate	1	2	3	4	5	6	7	8	9	10
22	To what degree are you now feeling	1 Able to remember 10 Unable to remember	1	2	3	4	5	6	7	8	9	10
23	To what degree are you now feeling	1 Able to think clearly 10 Unable to think clearly	1	2	3	4	5	6	7	8	9	10
24	Overall, what do you believe is most directly contributing to or causing your fatigue?											
25	Overall, the best thing you have found to relieve your fatigue is:											
26	Is there anything else you would like to add that would describe your fatigue better to us?											
27	Are you experiencing any other symptoms right now?											

INFORMED CONSENT	
Biotransformation and oxidative stress status evaluation	
1	**Background and aim of the detoxification and oxidative stress evaluation**

Detoxification of exogenous and endogenous toxins is a natural and critical biological process consisting of several biochemical pathways. The effectiveness of these pathways varies between individuals and can significantly affect the health of an individual. Similarly, the steady state between the formation of free radicals and the antioxidant defense system of the human body to cope with these free radicals, which may elevate under certain conditions mostly as a result of endogenous factors, also varies between individuals and affects health and ageing. When the antioxidant defense cannot keep the free radicals at a steady state, oxidative stress increases and incurs damage to the body. These two processes, detoxification and oxidative stress, can be measured in biological material using several tests and are useful to estimate the effectiveness of these health-related processes. It may also help decide if lifestyle habits, such as nutrition, of an individual should be adapted to support these processes. The combination of tests that is done on the material provided aims to evaluate these two processes.

2	**Procedures**

2.1 Evaluation of detoxification

2.1.1 Two saliva samples will be taken two and eight hours after caffeine loading respectively. These samples will be used to assess Phase I of the detoxification profile.

2.1.2 One 10-hour urine sample will be taken after the aspirin and paracetamol loading. The urine sample will be used to assess Phase II of the detoxification profile.

2.2 Oxidative stress evaluation

2.2.1 Two blood samples will be taken simultaneously to a total of 15 ml blood.

2.2.2 The blood samples will be processed for an array of tests that measures markers of free radical damage and antioxidant status.

2.3 Molecular genetic testing

2.3.1 DNA may be isolated from blood samples for research purposes only. This will help identify genetic and epigenetic variations that play a role in biotransformation, oxidative stress and energy metabolism deficiencies.

3 Risks and possible discomfort for the patient / individual

The usual risk and possible discomfort experienced when a blood sample is obtained.

4 Declaration of confidentiality

Information provided will be treated as highly confidential. Only individuals of the research group and the referring practitioner will have access to information. Data published in a scientific journal will include no information that could identify a patient or his/her family.

5 Withdrawal clause

I understand that I may request withdrawal of the evaluation at any time. My participation or that of my child is therefore on a voluntary basis until I request otherwise.

6 The above-mentioned project was thoroughly explained and the following additional information pointed out to me

6.1 That the blood, saliva and urine samples may be used for all relevant tests to evaluate detoxification function and oxidative stress status and that no compensation for this material will be provided.

6.2 That the biological material provided will reside only at the Centre for Human Metabolomics, North-West University, Potchefstroom, until I request (in writing) the destruction of this material at any time.

6.3 That the withdrawal clause was explained to me and that I understand its implications.

6.4 If you have any further enquiries, please refer to p.4 for our contact details.

7 Declaration of consent

I, _____

(print full name and surname) hereby consent to:

7.1 participation in the above-mentioned evaluation of detoxification and oxidative stress. I also consent that the following samples may be used for these purposes and to storage thereof until such time as it is needed for further relevant tests: (a) 15 ml venous blood sample which was taken from me as specified, (b) the urine sample which was taken as specified, and (c) the saliva samples which were taken as specified OR

7.2 participation of my child, _____

(print full name and surname) in the above-mentioned evaluation of detoxification and oxidative stress. I also consent that the following samples may be used for these purposes and to storage thereof until such time as it is needed for further relevant tests:

(a) 15 ml venous blood sample which was taken from the child named above as specified,

(b) The urine sample which was taken as specified, and

(c) the saliva samples which were taken as specified

I understand that I or my child are participating in the evaluation. I hereby acknowledge that I understand and are fully aware of the content of the forms and by signing this form I give the necessary permission.

Signed at _____ (place) on _____ (date).

Signature _____

(A parent/guardian must give consent in the case of minors.)

Signature of responsible individual: _____

Individual who explained the project and informed consent form to the participant)

CHAPTER 4

Biochemical materials and methods

4.1 Introduction

The analyses of the biotransformation variables used in this study required the application of a number of diverse methods and techniques. This included the use of fluorescence microplate readers, high pressure liquid chromatography (HPLC), gas-liquid chromatography-mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS-MS). Phase I and II biotransformation analyses were carried out using HPLC separation as these analyses were dependent on a two-time point measurement (in the case of caffeine) and the monitoring of the challenge substance induced conjugate formation (in the case of acetylsalicylic acid & acetaminophen). Both the phase I & II analytical methods were validated, and this is presented in §4.11. The methodology sections described under §4.2-§4.10 refer to that described by Erasmus *et al*. (2019) [Chapter 7, p121].

4.2 Reagents

Caffeine, allopurinol, paracetamol-glucuronide, paracetamol sulphate, high-purity trifluoroacetic acid, salicyluric acid, N,N-diethyl-paraphenylenediamine (DEPPD), sodium acetate, metaphosphoric acid (MPA) and hydrogen peroxide were supplied by Sigma Chemicals. Paracetamol-mercapturate was supplied by Toronto Research Chemicals. HPLC grade acetonitrile and water was supplied by Burdick & Jackson. Ferrous sulphate was supplied by Labchem. Acetamidophenol, phenylbutyric acid, 2,3- & 2,5-dihydroxy benzoic acid, NADPH, GSH, GSSG and catechol were all obtained from Sigma-Aldrich. Ethyl acetate and diethyl ether were purchased from Merck. N,O bis(trimethylsilyl)trifluoro acetamide (BSTFA) and trimethylchlorosilane (TMCS) were supplied by Sigma Aldrich. 2,4,6-tris-2-(pyridyl)-1,3,5-triazine (TPTZ) and Ferri Chloride ($FeCl_3.6H_2O$) were also supplied by Sigma Chemicals. Carnitine and acylcarnitine reference standards and deuterated analogues were obtained from Cambridge Isotope Laboratories.

4.3 Phase I analysis

Sample preparation: C18 solid phase extraction cartridges (HF Bond Elut C18–100 mg, Varian, Palo Alto, CA) were used for sample clean-up and extraction of caffeine; 200 µL internal standard 2-acetamidophenol (5 µg/mL) was added to 200 µL saliva sample prior to extraction. Conditioning of the SPE columns was performed with 1 mL methanol and 2 mL 18 mega ohm water (H_2O). A 200 µL sample with 200 µL internal standard was added to the conditioned SPE column and washed with 1 mL water. Samples were eluted with 1 mL methanol acetate solution (pH 3.5) (50:50% v/v), dried under nitrogen and then lyophilized. Samples were reconstituted in

200 µL mobile phase (ddH_2O:acetonitrile (95:5) solution containing 0.1% formic acid) after which 5 µL was injected into the HPLC. The Agilent 1200 HPLC system was equipped with a binary pump, inline degasser, auto sampler, heated column compartment and diode array detector. The analytical column was an Eclipse XDB C18, 5 µm, 4.6 x 150 mm reverse phase column. A flow of 1 mL/minute was used, column temperature 25°C and the UV detector set at 273 nm. The initial conditions consisted of isocratic elution at a flow rate of 1 mL/minute with 95% of mobile phase A (0.1% formic acid) and 5% of mobile phase B (acetonitrile) for 6 minutes, followed by a gradient from 95% of A and 5% of B to 100% of B over 5 minutes. The gradient program was held at 100% B for 10 minutes before returning to initial program conditions.

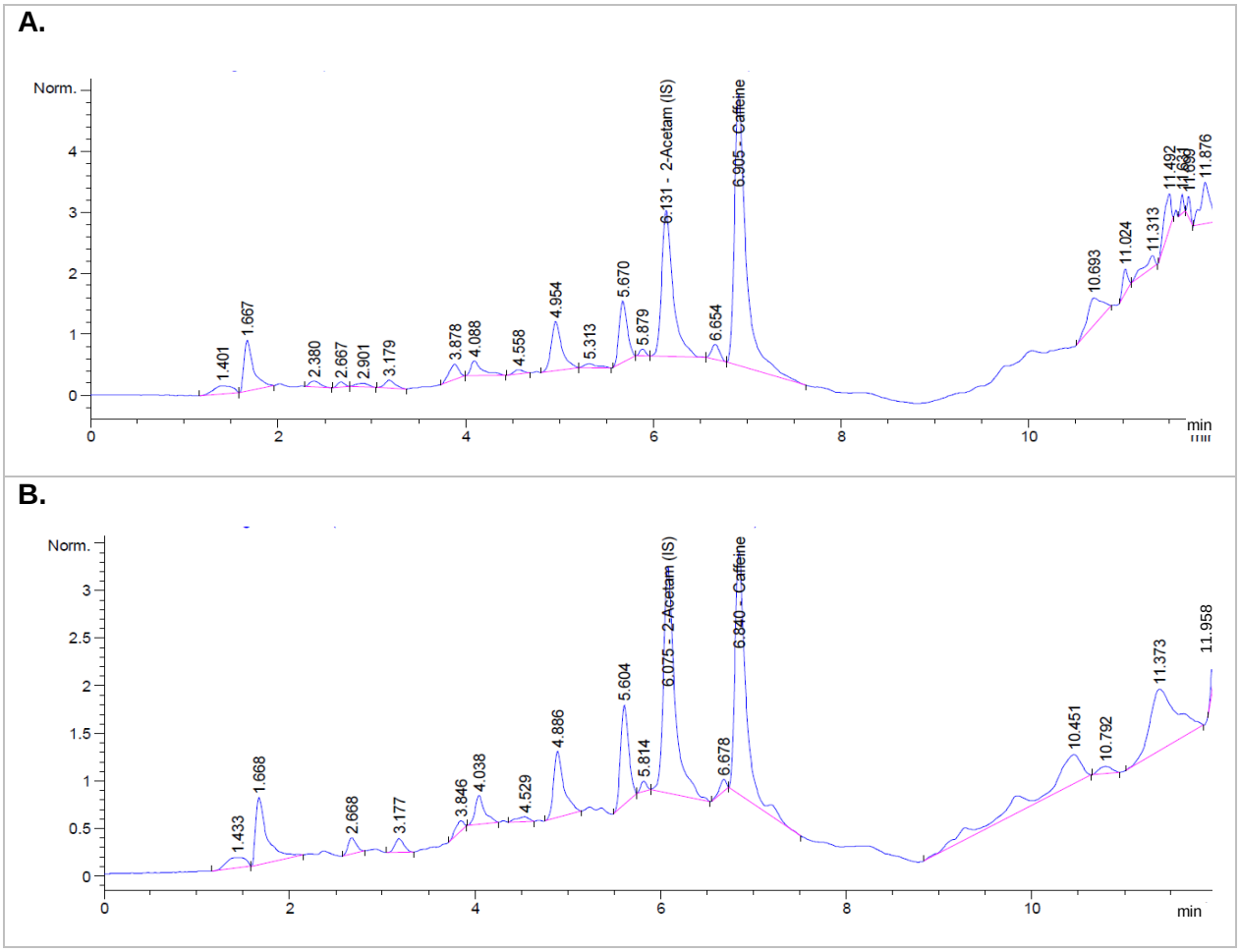


Figure 4-1 a & b: Example of a phase I caffeine challenge test analysis. (a) This profile represents the first (4 hours) of the timed saliva samples, while (b) represents the second (8 hours) timed saliva analysis. Acetamidophen (IS) eluted at retention time 6.075 while caffeine eluted at retention time 6.840 minutes.

The column was equilibrated for 10 minutes between injections. From a caffeine stock solution (1 mg/100 mL), a six-point series dilution calibration range was made up ranging from

0.156 µg/mL to 5 µg/mL for quantification and sample caffeine concentrations were determined by means of linear regression.

4.4 Phase II analysis

Aliquots of 500 µL from the 10 hour overnight collected urine samples were centrifuged and 200 µL of each sample combined with 200 µL internal standard (Allopurinol 250 µg/mL) for the HPLC analysis. The sample and internal standard mixture were then lyophilised and the dry residue reconstituted in 400 µL of mobile phase consisting of a ddH_2O:acetonitrile (95:5) solution containing 0.05% TFA. The analytical column was a Phenomenex (Torrance, CA, USA) Luna 3µ C18 (2) 100 A, 150 x 2.00 mm reverse phase column. Column temperature was held at 35°C, flow rate 0.25 mL/minute and the UV detector was set at 254 nm for analyte detection. Mobile phase A was H_2O with 0.05% TFA and mobile phase B was HPLC-grade methanol. Chromatographic separation of analytes was achieved with the following gradient mobile phase composition: 10% B from 0–2 minutes; an increase to 20% B to 16 minutes, from 16–19 minutes to 90% B and from 19–24 minutes 90% A and 10% B.

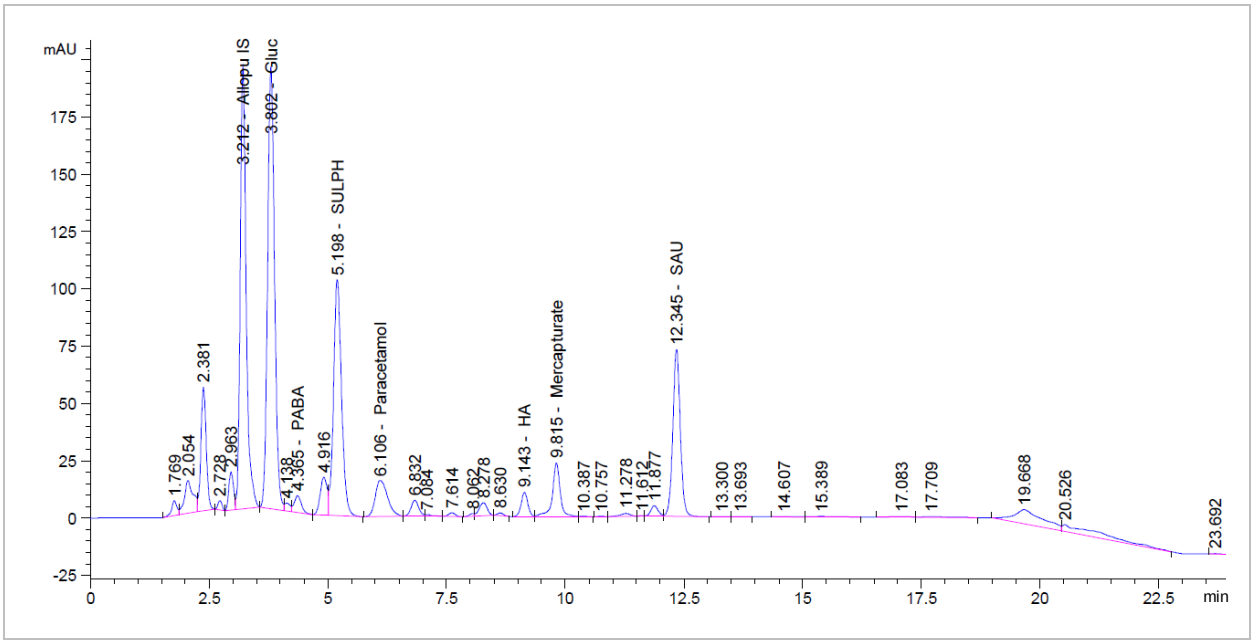


Figure 4-2: Example of a reverse phase C18 separation profile of urinary metabolites detected after an acetaminophen and acetylsalicylic acid challenge test. Allopurinol (IS) eluted at retention time 3.212 minutes; Acetaminophen glucuronide at 3.802; acetaminophen sulphate at 5.198; Acetaminophen mercapturate at 9.815 and salicyluric acid at 12.345 minutes.

To accommodate physiological concentrations for the different conjugates stock solutions a 9-point series dilution calibration range were used for each conjugate, ranging from 3.905 µg/mL to 1000 µg/mL.

4.5 Secondary products from the acetylsalicylic acid challenge

The three secondary products from the acetylsalicylic acid challenge — catechol, 2,3-DHBA and 2,5-DHBA — were analysed using GC-MS. Creatinine-corrected urine volumes were subjected to organic solvent extraction after 3-phenyl butyric acid was added as internal standard and the pH adjusted to 1 with 5N HCl. Six mL HPLC grade ethyl acetate and 3 mL diethyl ether were used as extraction solvents. After roto-torque agitation for 10 minutes, the solvent layer was removed and dried under a stream of nitrogen. Derivatization was done with BSTFA and TMCS. An Agilent 7890A gas chromatograph connected to an Agilent 5975B MSD mass spectrometer was used for analysis. The analytical column used was a 30 m x 0.25 mm x 0.25 mm DB1-MS JW Scientific column. High purity helium was used as carrier gas. A 1 µL sample was injected with a temperature programme starting at 100°C for 2 minutes, increased by 4°C to 120°C, then by 6°C to 215°C and finally by 20°C to 300°C. Post-run time was 1 minute at 295°C. Selective ion monitoring was conducted for catechol (254.1 m/z), IS (221.1 m/z), 2,3-DHBA (355.2 m/z) and 2,5-DHBA (355.2 m/z). An 8-point calibration range of the standards was used for final quantification.

4.6 Ferric reducing antioxidant assay (FRAP assay)

Measurement of the ferric reducing ability of plasma, known as the FRAP assay, provides a way for assessing 'antioxidant power' and which we used in the present study (Benzie & Strain,1996). FRAP assays were performed on a BIO-TEK FL600 fluorescence microplate reader. Amounts of 15 µL serum were added to 85 µL Milli-Q water in 96-well microplates. The sample plates were incubated for 10 minutes at 25°C. To this, 250 µL FRAP reagent (2.5 mL FRAP acetate buffer; 2.5 mL TPTZ reagent and 2.5 mL $FeCl_3$ reagent) was added. FRAP acetate buffer: 1.55 g anhydrous sodium acetate in 500 mL Milli-Q water. TPTZ reagent: 312.4 mg 2,4,6-tris-2-(pyridyl)-1,3,5-triazine in 100 mL 40mM HCl. $FeCl_3$ reagent: 4.56 mg of $FeCl_3.6H_2O$ was dissolved in 100 mL FRAP acetate buffer (300 mM). FRAP values were recorded as an end point analysis at 595 nm, 80 seconds after addition of the FRAP reagent. A 6-point calibration range containing 0, 20, 40, 60, 80 and 100 µL of a FRAP standard (0.275 $FeSO_4.7H_2O$ in 100 mL milliQ H_2O) was used as calibrator.

4.7 Reactive oxygen species (ROS assay)

The method described by Hayashi *et al.* (2007) with minor modifications was used to determine total ROS levels in serum samples. The ROS assays were also conducted on a BIO-TEK FL600 fluoresence microplate reader; 140 µL sodium acetate (0.1M; pH 4.8) was added to the wells

used in the 96-well plate; 2.5 µL samples were added in triplicate; 100 µL of a 1:25 DEPPD (100 mM) and $FeSO_4$ (43.7 µM) solution was added to the sample wells. Plates were incubated at room temperature for 1 minute; plates were read at 546 nm every minute for 10 minutes. A calibrator concentration range with 6 concentrations ranging from 0–300 mg/L hydrogen peroxide was used for quantification of samples. Serum ROS levels were calculated from the constructed calibration curve ($R^2 > 0.99$) and are expressed as equivalent to levels of hydrogen peroxide (1 unit = 1.0 mg H_2O_2/L). The calculated coefficient of variation for the ROS assay was 11%.

4.8 Total glutathione (T-GSH) assay

Total glutathione levels in blood samples were determined using the GSH/GSSG kit from Oxis International and the analyses were done with a BIO-TEK FL600 fluorescence microplate reader. Whole blood EDTA vacutainer samples were mixed and 350 µL metaphosphoric acid (MPA) mixed with 50 µL blood sample, centrifuged at 10 000 x g for 10 minutes; 12.5 µL of the MPA extract was then added to 750 µL assay buffer. A 50 µL sample was then added in triplicate to the microplate and mixed for 5 minutes; 50 µL NADPH was then added and the plates were read at 412 nm for 3 minutes at 1-minute intervals. GSH (0–3.0 µM) and GSSG (0–0.15 µM) 6-point calibration ranges were used for quantification.

4.9 Creatinine and uric acid

Both creatinine and uric acid values in urine were determined using the Konelab T series analyser with the uric acid (AOX) and creatinine (enzymatic) kits.

4.10 Carnitine and acylcarnitine analysis

Carnitine and acylcarnitines were measured on the 10-hour urine samples with an adapted method by Chace *et al.* (2003). Urine (100 µL) was transferred to an Eppendorf tube and centrifuged at 1300 rpm for 20 minutes; 10 µL of the centrifuged urine was added to 410 µL of a deuterated carnitine/acylcarnitine stable-isotope solution in a new Eppendorf tube. The sample mixture was then dried under nitrogen at 65°C for ~15 minutes; 100 µL 3N butanolic HCl was added to the dried sample and incubated at 65°C for another 15 minutes. Excess butanolic HCl was evaporated under nitrogen. The samples were then reconstituted in 100 µL acetonitrile: water (50:50) (v/v) with 0.1% formic acid. Samples were delivered to the electrospray mass spectrometer (Agilent 6410 Triple Quadrupole, Palo Alto, CA) via an Agilent 1200 series LC (Palo Alto, CA) and 96-well plate sampler. A 10 µL sample was injected per analysis at a

constant mobile phase (1% formic acid in water: acetonitrile (50:50) (v/v)), flow rate of 0.2 mL/minute. The mass spectrometry analyses were carried out in positive ionization mode with controlled collisional induced decomposition (fragmentor voltage 135 V and collision energy 20 V) and the precursor of 85 Da function used to selectively detect the different carnitine species. The deuterated analogues of the different carnitine and acylcarnitines (working solutions: free carnitine = 1.8815 µmol/L; acetylcarnitine = 0.6075 µmol/L; propionylcarnitine 0.1616 µmol/L; isovaleryl carnitine = 0.1400 µmol/L; octanoyl carnitine = 0.1233 µmol/L and palmitoyl carnitine = 0.3144 µmol/L) were used for the quantification of individual carnitine species. Linear regression calculations were used to determine the concentration of the carnitine/acylcarnitines by comparison of the signal intensity of carnitine/acylcarnitines against that of the corresponding deuterated stable isotopes. Concentrations of the analysed carnitine and acylcarnitines were expressed in mmol/mol creatinine.

4.11 Phase I and II method validation

The phase I and II biotransformation analytical methods were not part of the routine methods used in the Centre for Human Metabolomics at the North-West University and therefore it was decided to subject these methods to validation prior to implementation. Analytical method validation is a key process to demonstrate that the analytical procedures are suitable for their intended application (Tiwari & Tiwari, 2010). In their description of validation of analytical test methods Bretnall & Clarke describe validation as follows: “More specifically, analytical method validation is a matter of establishing documented evidence that provides a high degree of assurance that the specified method will consistently provide accurate test results that evaluate an analysis against its defined specification and quality attributes” (Brentnall & Clarke, 2011). The importance of effective method validation is further highlighted in the context of quality management and accreditation (Tiwari & Tiwari, 2010). Validation of the bioanalytical method for phase I & II biotransformation in this study was conducted in accordance with the guidelines established by the FDA (Anon, 2018). According to Peters & Maurer, (2002), validation should include the following validation parameters: selectivity; accuracy (which also includes biasness; precision; repeatability; intermediate precision and reproducibility); linearity; limits of detection (LOD) and limits of quantification (LOQ) and matrix effects.

4.11.1 Selectivity

The term selectivity is prefered by IUPAC over specificity (Thompson *et al*., 2002). Selectivity is one of the important parameters which plays a role in the chromatographic resolution of compounds. As a result, this parameter is high on the priority list of validation criteria (Maurer, 2005). Selectivity is normally defined as the extend to which a method can detect a specific

analyte in a complex mixture without interference from other components in the mixture (Araujo, 2009). A resolution value of at least 1.5 is acceptable for good selectivity. Kadian *et al*. (2016) describes selectivity as the ability of an bioanalytical method to produce a signal only for the analyte of interest and not for interfering compounds. The retention time of the internal standard should not interfere with the retention time of the compound of interest (Anon, 2018). In this regard it is interresting that LC-MS/MS bioanalytical methods are considered as specific while HPLC methods with other detection techniques are considered selective (Kollipara *et al*., 2011).

4.11.2 Limit of detection

The sensitivity or limit of detection (LOD) refers to "the lowest concentration of an analyte which the bioanalytical procedure can reliably differentiate from background noise" (Shah *et al*., 2000). Background noise in chromatographic separation is described as the oscillation of the baseline in the chromatogram when a blank sample was injected. The LOD is a statistical value which is indicative of the minimal concentration of a compound that will produce a signal which can be differentiated from background noise. The significance level for this differentiation should be α=5% (Peris-Vincente *et al*.,2015). The LOD value does not imply that it is possible to calculate the concentration of the relevant metabolite; it only refers to reliable detection of the compound. The LOD can be established from calculating the signal to noise ratio. A signal to noise ratio of 3:1 is acceptable for LOD or also referred to as LLOD. The calculation of the LLOD (Equation 4-1), was done for relevant phase I & II metabolites where Sa represents the standard deviation of the responses (calculated by standard deviations of y-intercepts and y-residuals of the regression line) and b represents the slope of the calibration curve (Jacobs, 2017).

Equation 4-1: The formula for the calculation of the lower limit of detection

$$LLOD = 3 \times {}^{Sa}/_{b}$$

4.11.3 Lower Limit of quantification (LLOQ)

The term LLOQ is defined as the lowest concentration at which the instrument is able to detect and quantify a compound (Shrivasta & Gupta, 2011). The LLOQ value should have a signal to noise ratio of 10:1. The LLOQ can also be determined with linear regression (Pirker *et al*., 2004) and the application of Equation 4-2. In contrast to this, the upper limit of quantification is the

Equation 4-2: The formula for the calculation of the lower limit of quantification

$$LLOQ = 10 \times {}^{Sa}/_{b}$$

highest concentration of an analyte in a biological sample that can be determined quantitatively with acceptable accuracy and precision. This is normally represented by the highest concentration of an analyte that are detected within the linear range (González *et al*., 2014).

4.11.4 Precision

Precision has been referred to as "measurement of scattering for the concentrations obtained for the replicate sampling of a homogeneous sample." It is normally calculated as coefficient of variation (%CV) (Araujo, 2009). The precision parameter is one of the important steps in achieving repeatability and reproducibility. Precision must be determined for low, medium and high QC samples from at least 3 repetitions. The accuracy and precision values for the QC samples should be determined by the calculating the mean and CV values. Precision can be determined with the mean observed concentration (should be within ± 15 % of nominal concentration) and the CV [less than 15 % at all concentrations except for LLOQ where 20 % is acceptable] (González *et al,* 2014). The use of QC samples also allows for the detection of assay biasness (Haines *et al*., 2017).

4.11.5 Accuracy

The accuracy of a method is affected by systematic (bias) as well as random (precision) error components (Peters & Maurer, 2002). Accuracy can be described as the ratio of the determined and real concentration of quality control (QC) samples (low, medium & high) on a single day and also between days (Equation 4-3). A value close to one is an indication of closeness to the true value. At least five determinations per concentration point should be done and the coefficient of variation for accuracy should not exceed 15% except for the lower QC sample where 20% is acceptable (Moein *et al*., 2017). Accuracy can be affected by factors such as sample preparation, matrix effect and calculation error.

Equation 4-3: Equation for the calculation of accuracy

$$Overall\ accuracy = \frac{\text{Response relative to metabolite calculated}}{\text{Response relative to substance in theory or in standard}}$$

4.11.6 Range

The range of concentration over which the analytical method is calculated with the linear correlation of the analyte concentration to instrument response. The usable range is affected by factors like LOD, LLOQ and method precision and accuracy (Hartmann *et al*., 1998).

4.11.7 Stability

The chemical stability limit of an analyte in a biological sample can be defined as the time required until a measured property acquires a bias higher than a defined specification (Gómez Rioja *et al.,* 2018). Time is the main variable affecting stability in medical laboratory samples and therefore it is necessary to test stability under a number of conditions. This may include stock solution stability, postpreparative stability, benchtop and freeze-thaw stability, freezer storage stability and post-validation long-term stability (Bansal & DeStefano, 2007).

4.11.8 Matrix effect

The matrix effect is notable when molecules which are present in the sample matrix coelute with the compound(s) of interest (Chambers *et al.,* 2007; Van Eeckhaut *et al*., 2009). Under these conditions the signal for the relevant sample compounds may be enhanced or suppressed. Sample preparation and optimization of chromatographic conditions can limit the matrix effect (Jacobs, 2017). The matrix effect can be measured by calculation of the matrix factor (MF), which is the ratio of the response of an analyte in a biological matrix to the response of the analyte in the absence of any matrix (Keevil, 2013). The matrix factor calculation is shown in Equation 4-4. At least 5 matrix values from different individuals are necessary for the determination of the relative matrix effect, to compensate for inter-individual variability between samples and their matrixes (Srinivas, 2009).

Equation 4-4: Equation for matrix factor calculation

$$MF = \frac{\text{Response of analyte in matrix}}{\text{Response of analyte in standard or solution}}$$

4.11.9 Preparation of standard stock solutions and calibration standards

The stock solutions of all the metabolites were prepared at concentrations of 100 µg/mL in methanol. The standards were then stored at -80˚C until used for method development. Working solutions of metabolite standards and calibration mixtures were prepared by dilution to the required concentrations. Biological matrix samples (urine and saliva) were also spiked with known amounts of the analytes. An important factor in method development and validation is that the sample composition should be as near as possible to that of the biological samples that are going to be analysed (Srinivas, 2009). QC samples for the validation of the phase I challenge test were made up at concentrations of 0.35; 2.5 and 10mg/L, representing low, medium and high QC samples respectively.

4.12 Phase I method validation

Method validation was performed on standard samples, biological matrixes and spiked matrixes. Charcoal stripping on samples were not carried out as it has been shown to limit matrix effects that might affect the results of the biological samples (Jacobs, 2017). Saliva samples was used for the liver phase I caffeine challenge test. This matrix has been evaluated for the measurement of xenobiotics because of certain distinct advantages that it offers in comparison to plasma and serum (Mullangi *et al.,* 2008). The caffeine analyses were carried out according to the phase I method described under §4.3.

4.12.1 Calibration and linearity

Calibration samples for caffeine were prepared in a concentration range of 0.02-10 µg/mL and 200 µL of a 5µg/mL of the internal standard mixture was added to each calibration sample. Calibration curves to determine linearity were constructed by using a total of 10 calibration standards. All these sample were analysed in triplicate. The calibration curves were constructed by plotting the ratio (response/IS) against the ratio of analyte/ IS concentration. The concentration ranges evaluated for caffeine is shown in Table 4-1. The linearity plot for caffeine is shown in Figure-4.3.

Table 4-1: Linear response concentrations for caffeine; R^2 values and LOD and LLOQ. The results of the matrix spikes are reported below.

Metabolite	Concentration range evaluated (µg/mL)	Concentration range linear (µg/mL)	R^2 value	LLOD (µg/mL)	LLOQ (µg/mL)
Caffeine	0.02-10.00	0.63-10.00	0.999	0.102	0.340

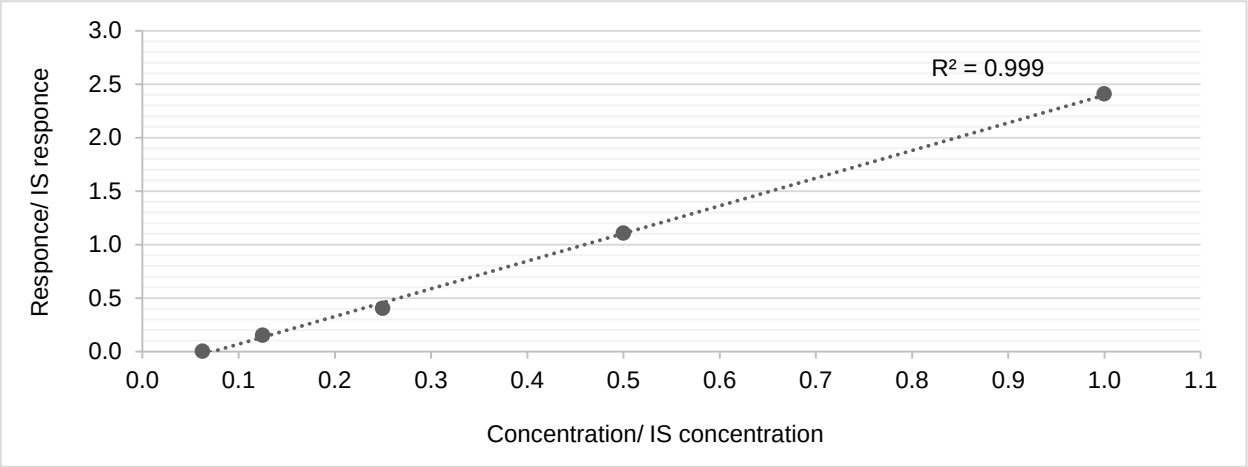


Figure 4-3: Calibration curve for caffeine. The ratio (*response/IS response*) on the y-axis was plotted against the ratio (*concentration/IS concentration*) on the x-axis.

4.12.2 Relative response factor

The relative response factor (RRF) is the ratio of the response factor (RF) [response of metabolite per unit weight] to that of the internal standard. The RRF is usually determined to improve quantification (Bartolomeo & Maisano, 2006). The RRF for a specific analyte can be calculated from the linearity curve slopes (Equation 4-5). The RRF value for caffeine is shown in Table 4-2.

Equation 4-5: Equation for the relative response factor calculation

$$Relative \text{ response factor } = \text{ Slope of metabolite of interest calibration curve*}$$

* Response/IS response vs concentration/IS concentration

Table 4-2: Relative response factor calculated for caffeine and the internal standard (IS).

Metabolite	Internal standard used for quantification	Relative response factor of IS to the metabolite of interest
Caffeine	Acetamidophenol	2.5

4.12.3 Limit of detection & quantification

The calibration data of the calibration range were used to calculate the LOD and LLOQ with linear regression, which predicts the lowest concentration at which an analyte can be detected and quantified accurately. The regression calculation is done using deviations from the data-points that best fit the linear line (Shrivastava & Gupta, 2011). The LLOD and LLOQ values for caffeine are shown in Table 4-1.

4.12.4 Accuracy

Interday and intraday accuracy of the caffeine validation were calculated for concentrations in the linear range for low, medium and high values. The accuracy analysis was also repeated on standard and matrix spiked samples, in triplicate, for five consecutive days. The accuracy can be expressed either as the percentage bias or as a percentage of the nominal concentration (Kollipara *et al*., 2011). In this study the relative accuracy was expressed as a percentage of the nominal concentration and the results are shown in Table 4-3.

4.12.5 Precision

Precision calculations were done on analyte concentrations in the linear range (low, medium, high) to determine inter- and intraday variation. The precision value was expressed as a percentage of the relative standard deviation (%RSD). The precision results for the matrix spiked samples are shown in Table 4-4.

4.12.6 Carryover

According to the new draft USFDA guidance, the carryover effect should be assessed and monitored during analysis and when detected the possible cause should be investigated and mitigated as it could seriously impact on the accuracy and precision of the validated method (Kadian *et al*., 2016). The carryover effect for caffeine was determined for all QC concentrations

with blank injections before and after the analysis of standard solutions and matrix spiked solution. No detectable carryover for caffeine could be determined.

Table 4-3: Inter and intraday accuracy values for caffeine for low, medium and high QC concentrations.

Low QC		Medium QC		High QC	
Intraday % CV	Interday % CV	Intraday % CV	Interday % CV	Intraday % CV	Interday % CV
13.890	11.631	5.572	5.787	11.480	8.604

Table 4-4: The average interday precision values for caffeine.

Low	Medium	High
RSD (%)	RSD (%)	RSD (%)
84.835	98.743	102.211

4.12.7 Stability

The stability of caffeine was determined in triplicate on QC samples (low, medium and high) and standard samples. The stability conditions and stability values (expressed as a percentage of newly prepared samples) are indicated in Table 4-5. Storage and handling methods that contributed to stability deviations larger than ±15 % were rejected.

Table 4-5: Table to illustrate the average stability of samples under certain storage conditions.

Storage method	Low QC (% of the newly prepared sample)	Medium QC (% of the newly prepared sample)	High QC (% of the newly prepared sample)
4°C for 7 days	33.77	94.29	102.40
4°C for 5 days	73.53	61.19	91.06
3 days in autosampler	101.30	100.94	114.20
5 freeze-thaw cycles	29.39	136.25	143.00
24h at room temperature	136.90	110.12	105.10

4.12.8 Selectivity

The selectivity of the caffeine analytical method was determined by analysis of six blank human saliva samples which were pooled to test for matrix interferences. The selectivity analyses were performed in triplicate on the pooled samples as well as on saliva samples spiked with caffeine concentrations representative of the LLOQ. No measurable interferences could be detected which is an indication of good selectivity.

4.12.9 Matrix effect

The matrix factor was calculated for caffeine in order to determine the matrix effect. Five different matrix samples analysed in triplicate were used for this purpose. The analyses were carried out on unspiked and spiked saliva samples which contained caffeine at high and low QC sample concentrations. Compensation calculations were done to eliminate the influence of caffeine present in the unspiked samples. The matrix effects were calculated using Equation 4-4. A matrix factor (MF) = 1 indicates no effect from the matrix. Deviations from this unit value were expressed as a coefficient of variation (CV) and values ≤15% were acceptable (Van Eeckhaut *et al.,* 2009). Calculated matrix factor values for caffeine is shown in table 4-6.

Table 4-6: The matrix factor value of caffeine at low QC and high QC values.

Metabolite	Matrix factor	
	Low QC	High QC
Caffeine	0.97066	1.0963

4.13 Phase II method validation

4.13.1 Calibration and linearity

The analytical method used for the analyses of the phase II liver conjugates in this study is described under §4.4. Calibration samples for the phase II liver conjugates, acetaminophen mercapturate; acetaminophen glucuronide; acetaminophen sulphate and salicyluric acid were prepared in a concentration range of 0.244 – 250 mg/L final concentration. 200 µL of a 250 µg/mL of the internal standard mixture was added to each calibration sample. Calibration curves to determine linearity were constructed by using a total of 10 calibration standards. All these samples were analysed in triplicate. The calibration curves were constructed by plotting the ratio (response/IS) against the ratio of analyte/IS concentration. The concentration ranges evaluated for acetaminophen mercapturate; acetaminophen glucuronide; acetaminophen

sulphate and salicyluric acid is shown in Table 4-7. The linearity plots for the conjugates are shown in Figure 4-4.

Table 4-7: Linear response concentrations for phase II conjugates; R^2 values and LOD and LLOQ. The results of the matrix spikes are reported below.

Metabolite	Concentration range evaluated (µg/mL)	Concentration range linear (µg/mL)	R^2 value	LLOD (µg/mL)	LLOQ (µg/mL)
Acetaminophen glucuronide	0.244-500	0.244-500	0.997	0.702	2.339
Acetaminophen sulphate	0.244-500	0.244-500	0.997	0.222	0.739
Acetaminophen mercapturate	0.244-500	0.98-500	0.998	2.755	9.185
Salicyluric acid	0.244-500	0.98-500	0.997	1.277	4.259

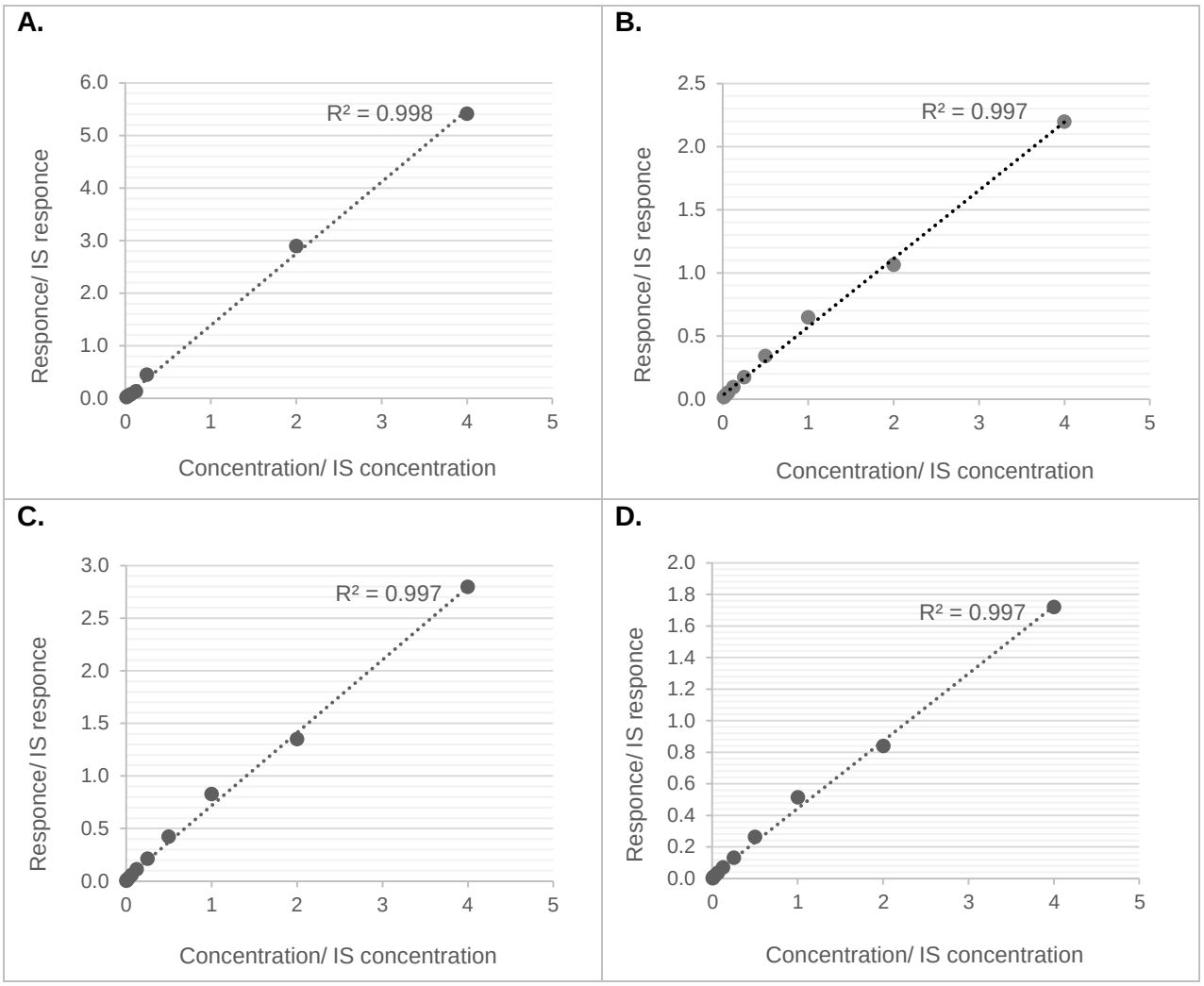


Figure 4-4: Calibration curves for (a) acetaminophen mercapturate; (b) acetaminophen glucuronide; (c) acetaminophen sulphate and salicyluric acid. The ratio (*response/IS response*) on the y-axis was plotted against the ratio (*concentration/IS concentration*) on the x-axis for the individual analytes.

4.13.2 Relative response factor

The RRF for acetaminophen mercapturate; acetaminophen glucuronide; acetaminophen sulphate and salicyluric acid was calculated from the linearity curve slopes (Equation 4-5). The RRF values for these conjugates are shown in Table 4-8.

Table 4-8: Relative response factor of the Internal standard (IS) to the phase II analytes.

Metabolite	Internal standard used for quantification	Relative response factor of IS to the analyte of interest
Acetaminophen glucuronide	Allopurinol	0.541
Acetaminophen sulphate	Allopurinol	0.693
Acetaminophen mercapturate	Allopurinol	0.683
Salicyluric acid	Allopurinol	0.427

4.13.3 Limit of detection & quantification

The calibration data of the calibration range for acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid were used to calculate the LOD and LLOQ with linear regression, which predicts the lowest concentration at which an analyte can be detected and quantified accurately. The regression calculation is done using deviations from the data-points that best fit the linear line (Shrivastava & Gupta, 2011). The LLOD and LLOQ values for the phase II liver conjugates are shown in Table 4-7.

4.13.4 Accuracy

Interday and intraday accuracy values of acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid were calculated for concentrations in the linear range for low, medium and high values. The accuracy analyses were also repeated on standard and matrix spiked samples, in triplicate, for five consecutive days. In this study the accuracy was expressed as a percentage of the nominal concentration and the results are shown in Table 4-9. The acceptance criteria of the FDA required that the mean accuracy value should be within 15 % of the actual concentration except for LLOQ where it should not deviate more than 20% (Kollipara *et al*., 2011).

4.13.5 Precision

Precision calculations were done on analyte concentrations in the linear range (low, medium, high) to determine inter- and intraday variation. The precision value was expressed as a percentage of the relative standard deviation (%RSD). The precision results for the matrix spiked samples are shown in Table 4-10.

4.13.6 Carryover

The carryover effect for acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid was determined for all QC concentrations with blank injections before and after the analysis of standard solutions and matrix spiked solution. No detectable carryover for these phase II liver conjugates were found.

Table 4-9: Intraday and interday accuracy values for acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid for low, medium and high QC concentrations.

Metabolite	Low QC		Medium QC		High QC	
	Intraday % CV	Interday % CV	Intraday % CV	Interday % CV	Intraday % CV	Interday % CV
Acetaminophen glucuronide	3.365	3.150	2.739	2.592	4.092	1.570
Acetaminophen sulphate	3.692	3.609	3.634	3.420	4.677	2.169
Acetaminophen mercapturate	6.040	7.512	3.346	2.425	1.276	4.241
Salicyluric acid	3.202	2.640	2.626	2.442	4.478	1.367

Table 4-10: Average precision values for acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid for low, medium and high QC concentrations.

Metabolite	Low	Medium	High
	RSD (%)	RSD (%)	RSD (%)
Acetaminophen glucuronide	95.3513	98.042	104.947
Acetaminophen sulphate	104.826	98.857	99.295
Acetaminophen mercapturate	100.471	115.394	141.901
Salicyluric acid	103.74	96.986	97.972

4.13.7 Stability

The stability of the four phase II liver conjugates was determined in triplicate on QC samples (low, medium and high) and standard samples. The stability conditions and stability values (expressed as a percentage of newly prepared samples) is shown in Table 4-11(a) and Table 4-11(b). Storage and handling methods that contributed to stability deviations larger than ±15 % were rejected.

Table 4-11(a): The average stability of samples under certain storage conditions. These include fridge storage of 5 or seven days, autosampler storage for three days, benchtop storage for one day and 5 freeze thaw cycles.

Storage method	Low QC (% of the freshly prepared sample)	Medium QC (% of the freshly prepared sample)	High QC (% of the freshly prepared sample)
4°C for 7 days	95.767	96.174	99.447
4°C for 5 days	102.54	102.907	107.216
3 days in autosampler	99.853	99.370	107.674
5 freeze-thaw cycles	111.06	113.538	130.557
24h at room temperature	96.492	97.998	95.132

Table 4-11(b): The average stability results of samples under certain storage conditions. These include fridge storage of 5 or seven days, autosampler storage for three days, benchtop storage for one day and 5 freeze thaw cycles for acetaminophen glucuronide, acetaminophen sulphate, and salicyluric acid for low, medium and high concentrations.

Metabolite	Concen-tration	3 days in autosampler	4°C for 7 days	5 freeze-thaw cycles	24h at room temperature	4°C for 5 days
Acetaminophen glucuronide	LOW	99.957	99.322	121.816	96.412	98.340
	MED	99.636	99.765	123.543	97.666	97.957
	HIGH	99.912	99.211	118.360	95.788	100.075
Acetaminophen sulphate	LOW	100.482	99.120	97.305	91.413	84.645
	MED	99.577	99.634	100.519	93.261	90.294
	HIGH	100.073	99.248	96.564	90.942	95.954
Salicyluric acid	LOW	100.324	98.539	109.037	94.000	91.847
	MED	99.685	99.478	117.571	96.938	99.349
	HIGH	100.347	98.977	103.902	95.331	139.160

* Stability analyses for acetaminophen mercapturate could not be carried out due to limited available quantities of the standard compound.

4.13.8 Selectivity

The selectivity of the phase II conjugate analytical method was determined by analysis of six blank human urine samples which were pooled to test for matrix interferences. The selectivity analyses were performed in triplicate on the pooled urine samples as well as on urine samples spiked with acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid concentrations representative of the LLOQ. No measurable interferences could be detected which is an indication of good selectivity.

4.13.9 Matrix effect

The matrix factor was calculated for acetaminophen glucuronide, acetaminophen sulphate, acetaminophen mercapturate and salicyluric acid in order to determine the matrix effect. Five different matrix samples analysed in triplicate were used for this purpose. The analyses were carried out on unspiked and spiked urine samples which contained the phase II conjugates at high and low QC sample concentrations. Compensation calculations were done to eliminate the influence of conjugates present in the unspiked samples. The matrix effects were again calculated using Equation 4-4. A matrix factor (MF) = 1 indicates no effect from the matrix. Deviations from this unit value were expressed as a coefficient of variation (CV) and values ≤15% were acceptable (Van Eeckhaut *et al.,* 2009). Calculated matrix factor values for the phase II conjugates are shown in table 4-12.

Table 4-12: The matrix factor value for each phase II conjugate at low QC and high QC values.

Metabolite	Matrix factor	
	Low QC	High QC
Acetaminophen glucuronide	1.091	1.075
Acetaminophen sulphate	1.119	1.076
Acetaminophen mercapturate	1.006	1.005
Salicyluric acid	1.514	1.078

CHAPTER 5

Selection and classification of a cohort of chronic fatigue patients

5.1 Introduction

In this Chapter details on the BOSS data collection and statistical analysis are presented. The data from the total analytical procedures consisted of three distinct different data sets: (1) the questionnaire for the Piper Fatigue Scale (22 items), (2) the Medical Symptoms Questionnaire (75 items) and (3) biochemical data generated from different biofluids as well as through different analytical methods (17 items). These sets provided 28 050 data points for each of the selected 576 patients, i.e. 16 156 800 data points in total. Conventional biochemical studies are mostly performed on data of limited size, obtained from reductionist studies. However, the aim of modelling complex biological interactions by integrating information from interdisciplinary fields, as in the three data sets used in the present study, requires a holistic approach for such large and diverse data sets. For this purpose, we considered various approaches in consultation with our statistical consultant (F.E Steffens, MSc DSc (PU vir CHO), Professor emeritus from the University of Pretoria and the University of South Africa), and agreed on the statistical methods described in this Chapter. After comparing various alternative classification methods, the use of the method Chi-squared Automatic Interaction Detector (CHAID) proved to be the most promising. It is a well-established method, originally proposed by Kass (1980). The advantage of CHAID is two-fold: (1) CHAID "build" non-binary trees (i.e., trees where two or more branches can attach to a single root or node), based on a relatively simple algorithm; (2) CHAID is particularly well suited for the analysis of larger datasets.

The comparative approach for the selection of the most suitable classification procedure for the present data set, as well as details on the underlying theory and mathematics of the CHAID method, are beyond the scope or the present thesis. The complete data set for the cohort of 568 patients is, however, rather unique and is provided electronically in Google drive (https://drive.google.com/open?id=1J8WuZHT6uCcj-uONpr0mm7BoKVa1bXte) format as it may be of value for statisticians interested in alternative or novel ways of analyzing complex data sets.

5.2 A statistical approach for the classification of chronic fatigue cases, based on large and diverse data sets (manuscript to be submitted II):

RESEARH PAPER | MANUSCRIPT VERSION

A statistical approach for the classification of chronic fatigue cases, based on large and diverse data sets.

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Abstract

This study involved 576 female patients, aged between 18 and 82, who complained about chronic fatigue. The patients were evaluated by means of two questionnaires, a Medical Symptoms Questionnaire and the Piper Fatigue Scale; and subsequently by analysing saliva and urine samples, after the patients took certain medications, to measure the quantities of various metabolites present. These three data sets were analysed statistically to establish the links between fatigue, metabolism and medical symptoms. The Piper Fatigue Scale was used to define five groups, and Chi-square Automatic Interaction Detection (CHAID) provided insight into the links between these measurements. The simultaneous analysis of qualitative and quantitative data sets are known in the field of statistics but seldom applied to such data sets generated through biochemical research. The value of this approach is evident from the informative classification schemes produced.

Keywords: Chronic Fatigue • CHAID • ANOVA • Factor Analysis • Principal Component Analysis • Medical Symptoms Score • Piper Fatigue Score

5.2.1 Introduction

A total of 673 women with complaints of fatigue were assessed for study eligibility. Of these subjects, 576 were eligible, following exclusion criteria for all cases which were verified as indicating diabetes, chronic high blood pressure, asthma, cancer, arthritis and some minor clinical conditions. Patients suggestive of fibromyalgia, depression or other affect syndromes remained in the final data set.

Three separate data sets were collected for each patient: a Piper Fatigue Score (PFS), from a questionnaire involving 22 items on a scale of 1–10. Second, a Medical Symptoms Questionnaire (MSQ) from the Departments of Medicine, Mercy Hospital and Maine Medical Center, Portland, ME, was completed with the assistance of a health consultant for each patient to supplement the information from the PFS and to gauge overall wellness (Jones & Quinn 2010). The MSQ contains 19 domains, most with 4 items, totaling 75 indicators to each of which the patient assigned a Likert-scale numerical ranking (0 being “never” or “seldom” and 4 being “frequently with severe effect”). Finally, biochemical measurements, of which 17 metabolite concentrations were determined from saliva, blood and urine samples collected after taking certain medications.

Traditional biochemical studies rely on reductionist data sets. However, the aim of modelling complex biological interactions by integrating information from interdisciplinary fields, as in the three data sets used in the present study, requires a holistic approach through the use of large data sets. For this purpose, we used the statistical methods as summarized below but without elaborating here on the underlying theory and mathematical details. The complete data set for the cohort of 576 patients is provided electronically in Google drive (https://drive.google.com/open?id=1J8WuZHT6uCcj-uONpr0mm7BoKVa1bXte).

The biochemical data were log-transformed because they showed high skewness and kurtosis. The PFS scores were reduced to two factors by means of factor analysis: Energy Fatigue and Mental Fatigue. From these two factors five groups were defined, depending on the combination of energy and mental fatigue. The biochemical measurements were reduced to 8 factors by means of principal components analysis, and the MSQ was divided into 15 section scores.

Using ANOVA, the means of the five fatigue groups were compared with respect to the MSQ scores and the biofactors. The five group means of the MSQ scores were highly significantly different, with p values mostly less than 0.01. In the case of the biofactors, only one of the group means differed significantly at a p value of less than 0.05 and one less than 0.10.

CHAID analysis produced models that used the overall MSQ score as a first source of differences, and additionally the metabolite information in refining the differences between the various fatigue groups.

5.2.2 Data collection

Patients suffering from low to high levels of chronic fatigue were referred by clinicians to the Centre for Human Metabolomics, North-West University (Potchefstroom campus) for evaluation. After signing an informed consent form, the patient was requested to complete two questionnaires, the Piper Fatigue Scale (PFS) Questionnaire (Piper *et al.,* 1998) and a Medical Symptoms Questionnaire [MSQ] (Jones & Quinn 2010). In addition, saliva, urine and blood samples were collected as follows: a 150-mg caffeine tablet was taken before breakfast at 08:00 in the morning, saliva samples were collected at 10:00 and 16:00; two aspirin (600 mg) and two paracetamol tablets (1000 mg) were taken at 21:00 in the evening and overnight and early morning (ending at 07:00); urine samples were collected in special containers provided in the test kit; blood samples were collected 24 hours later. The frozen blood, urine and saliva samples were handed in at a pathology depot for analysis to determine the concentrations of uric acid and creatinine.

The PFS questionnaire includes 23 questions. The first question, about how long ago the symptoms had begun, was excluded from the analysis. The 22 other questions were grouped in four dimensions: behaviour/severity, affective meaning, sensory, and cognitive/mood. The scale in each case was 0–10, with 0 indicating no to low-level symptoms and 10 indicating very severe symptoms. A total PFS score was calculated as the mean of the 22 items.

The MSQ includes 15 domains with 3 to 5 items per domain, a total of 75 items on a Likert scale of 0–4, (0 being “never” or “seldom” and 4 being “frequently with severe effect”). A total MSQ score was calculated as the mean. The domains were Head, Eyes, Ears, Skin, Nose, Heart, Emotions, Mind, Digestive tract, Mouth/Throat, Lungs, Energy/Activity, Weight and Joint/muscle, and Other (including frequent illness, frequent or urgent urination, genital itch or discharge, and painful lymph nodes).

The biochemical analysis of the body fluids yielded concentrations of 15 metabolites, filtrate volume and 5 ratios of some of the metabolite concentrations, a total of 22 variables.

Ultimately, the purpose is to find relationships or links between fatigue, as measured by the PFS on the one hand, and medical symptoms (derived from the MSQ) and the biochemical measurements on the other. The canonical correlation between the PFS variables and the

biochemical variables was 0.34, which is the maximum correlation between linear combinations of the two sets. The canonical correlation between the MSQ items PFS items was 0.798.

5.2.3 Data reduction

An attempt was made to reduce the number of variables in order to simplify the modelling. In the case of the PFS scores, factor analysis (Hair *et al.,* 2010) with principal components and principal axis factoring as extraction methods, and Varimax and Direct Oblimin rotations, were used in various combinations. The outcome was that Items 2–16 seem to measure the first factor whereas Items 17–22 measure the second factor. The first factor was termed Energy Fatigue (that is, fatigue due to lack of energy). The second factor was classified as Mental Fatigue (that is, fatigue due to stress). The Cronbach's alphas for the two groups of items were 0.974 and 0.917, respectively. A factor score was computed for each factor as the mean of the items constituting that factor. Both scores and the overall fatigue score were categorised as follows: low-level fatigue (score < 4); medium level of fatigue (4 ≤ score < 7) and high level of fatigue (score ≥ 7). The frequencies are indicated in Table 5-1.

Table 5-1: Frequencies of the Energy, Mental and Total Energy classes

	Energy Fatigue		Mental Fatigue		Total Fatigue	
Category	Frequency	Percent	Frequency	Percent	Frequency	Percent
Low Fatigue	144	25.0	176	30.6	139	24.1
Medium Fatigue	195	33.9	244	42.4	221	38.4
High Fatigue	237	41.1	156	27.1	216	37.5
Total	576	100.0	576	100.0	576	100.0

From these levels we defined five combined groupings: (1) Low Fatigue: Energy Fatigue score and Mental Fatigue score below 4; (2) Medium Fatigue: Energy Fatigue score and Mental Fatigue score below 7 and at least one of the scores equal to 4 or more; (3) Mainly Energy Fatigue: Energy Fatigue score 7 or more, Mental Fatigue score below 7; (4) Mainly Mental Fatigue: Mental Fatigue score 7 or more, Energy Fatigue score below 7; (5) Energy and Mental Fatigue: Energy Fatigue score and Mental Fatigue score both 7 or more. A scatter plot illustrates this (Figure 5-1). The frequencies of the 5 combined PFS groups are indicated in Table 5-2.

Without knowledge of the PFS, MSQ and biofactor scores, one would judge that a randomly selected patient had a 20.3% chance of falling in the Low Fatigue group and a 22.6% chance of

falling in the High Energy and Mental Fatigue group. We shall try to improve on that by using the information in the MSQ and the biochemical analyses.

Table 5-2: Frequencies of the 5 PFS groups

Category	Frequency	Percent
Low Fatigue	117	20.3
Mild Fatigue	196	34
Mainly Energy Fatigue	107	18.6
Mainly Mental Fatigue	26	4.5
High Energy and Mental Fatigue	130	22.6
Total	576	100

5.2.4 The MSQ scores

The overall MSQ score was calculated as the mean of 74 items. An attempt at a factor analysis of the 74 items failed to converge, and a PCA without rotation did not give useful results. It was therefore decided to maintain the original sections in the questionnaire. The Cronbach's alpha for the groupings varied around 0.6, but no reverse coding was necessary. The score for each section was computed as the mean of the items in the section.

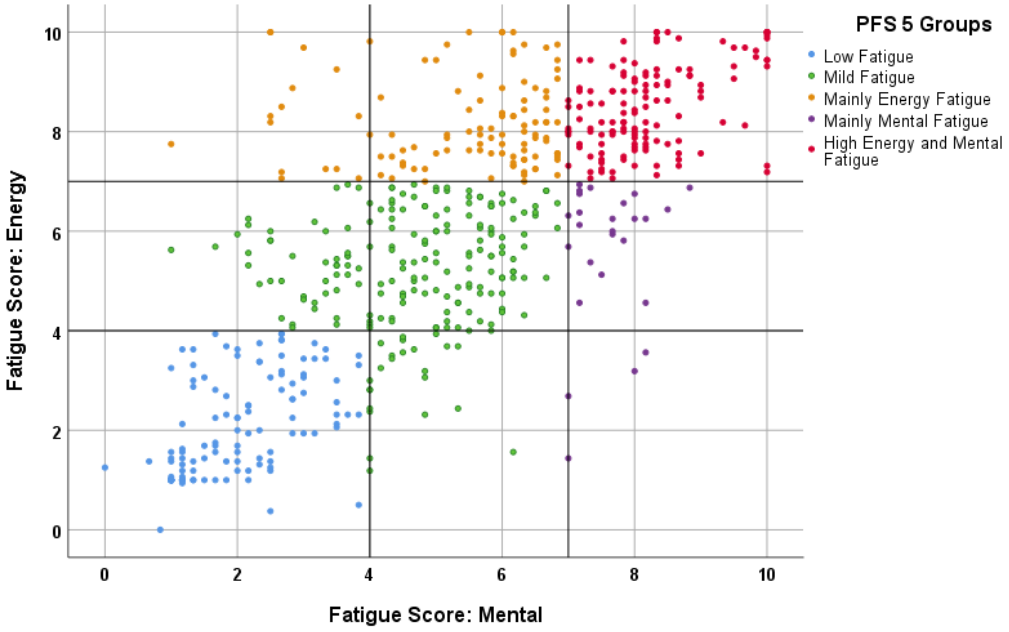


Figure 5-1: Distribution of all selected cases based on the individual's scores on energy fatigue and mental fatigue derived from the PFS. The scores are categorized as low level fatigue (0 < score < 4), medium level of fatigue (4 ≤ score < 7) and high level of fatigue (score ≥ 7). This distribution indicates the presence of five PFS groups: Low Fatigue (blue), Mild Fatigue

(green), Mainly Energy Fatigue (orange), Mainly Mental Fatigue (purple) and High Energy and Mental Fatigue (red)

5.2.5 The biochemical measurements

The biochemical variables showed relatively high skewness and kurtosis. This was improved by a log transformation. Factor analysis of the log-transformed variables was not possible because the covariance matrix is not positive definite. This is because five of the 22 variables are ratios of some of the other variables, and the log transformation turns a ratio into a difference, which means that some of the variables are linear combinations of other variables. Nevertheless, since the biochemical variables are precise measurements rather than opinions, PCA was performed, indicating the existence of 8 factors. The eight factor scores were computed and termed Biofactor1 to Biofactor8.

5.2.5.1 Comparing MSQ and biofactor scores across the fatigue groups

ANOVA tests were performed to compare the factor scores across the 5 fatigue groups. In the case of the MSQ scores, the p values were all less than 0.01 (except one of 0.014). In most cases the mean of the High Energy and Mental Fatigue group differed significantly from the means of the other groups. The differences were not sufficient to yield a successful discriminant analysis based on the MSQ subscores. In the case of the biofactors, only one (Biofactor 4) gave a p value less than 0.05 and one other less than 0.10.

5.2.5.2 CHAID analysis

In an attempt to find descriptive differences between the fatigue groups, Chi-square Automatic Interaction Detection (CHAID) analysis was selected and performed with the five PFS fatigue groups as response and the MSQ scores (total and individual sections) as well as the biofactor scores and individual measurements. CHAID is a decision tree technique that is based on adjusted significance testing. The technique was developed by Gordon V. Kass of the University of the Witwatersrand, Johannesburg, South Africa for his PhD degree (Kass, 1980). It was published in 1980 as an exploratory technique for investigating large quantities of categorical data and has since become a highly quoted paper (2902 citations). CHAID is a widely used technique within the field of consumer and medical sciences as well as in psychiatric research.

CHAID is an unsupervised technique aimed at discovering relationships between a categorical variable (the dependent variable) and several independent variables which may be categorical or measured on a continuous scale. The purpose of the analysis as applied in our study was to create a descriptive rather than a predictive model. Unlike linear model techniques, such as

ANOVA, regression and discriminant analysis, CHAID does not fit linear models. It employs a tree growing algorithm to reveal relationships between the dependent variable and the independent variables in a stepwise fashion.

The first parent node contains all the cases. If Y is the dependent variable with k categories, and X_1, X_2,……,X_m are the independent variables, then the process of building a tree representation begins by evaluating each independent variable in turn. Each X_i is evaluated by splitting its range into mutually exclusive and exhaustive categories, and then constructing a contingency table[1] with the k categories of Y in the columns and the X_i categories in the rows, with the frequencies (the number of cases) in the table. A chi-square test of independence is performed to test the independence of the rows and columns, and the significance (p value) corresponding to the chi-square test is obtained. A search algorithm is used to find the optimum split with the smallest p value (the p values are adjusted by a Bonferroni adjustment for multiple testing). The optimum split and corresponding p value is determined for each independent variable, and the independent variable corresponding to the smallest p value is selected to form child nodes. If this smallest p value is larger than 0.05, the process is stopped. If the smallest p value is 0.05 or less, the first child nodes are formed by the categories corresponding to the selected independent variable.

In the next step each child node becomes a parent node, and all the independent variables are evaluated in a similar fashion, leading to the possibility of several child nodes emanating from each parent node, provided a suitable split is found with a p value less than 0.05. The process continues with each of the new child nodes being regarded as a parent node and evaluated for the existence of independent variables that could give rise to significant splits.

Eventually, the performance of the model is evaluated in terms of the end nodes (nodes which are not split further). Each case is allocated to one end node, and the category with the highest frequency in the end node is used as a prediction of the category of all the cases in the end node. The user is able to control the process by specifying the significance level α, the minimum size for parent nodes to be split, the minimum size of child nodes and the maximum number of levels.

[1] Note: The following statistical terminology is used above: a contingency table is a cross-tabulation consisting of rows and columns with frequencies of occurrences of cases in each cell of the table. A chi-square test is performed to test the independence of the rows and columns. The null hypothesis states that the rows and columns are independent. The significance (p value) is used to judge the independence. If the p value is small (typically less than 0.05) it means that the rows in the table may be used to some extent to predict the columns or vice versa. If the p value is larger than 0.05, the interpretation is that the rows are not useful in predicting the columns.

The application of CHAID to produce classification trees using only partial data is illustrated in three examples as shown in Figures 5-2 to 5-4.

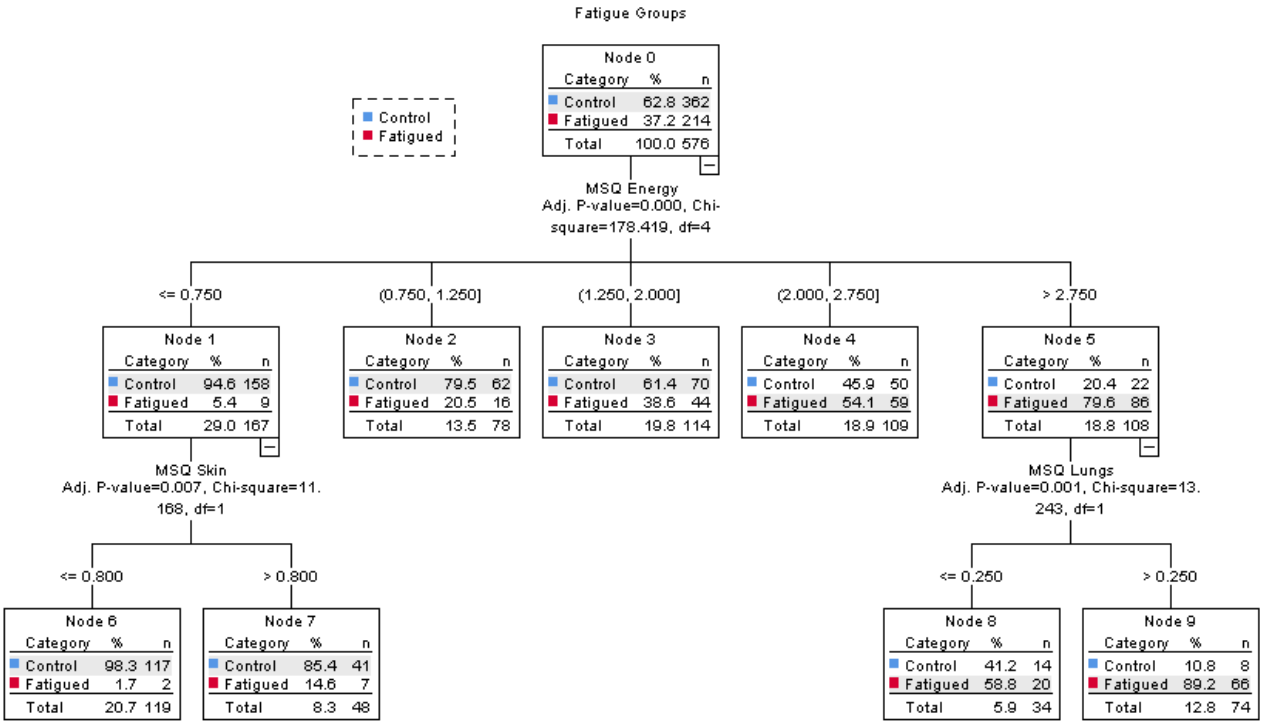


Figure 5-2: The branching of the control and High Fatigue groups based on the overall MSQ score. Total PFS and MSQ data: patient group: selected 576 cases. The "Yes" and "No" and the "Control" and "Fatigue" categories were defined as follows: PFS score ≥7; code =1 (Yes or Fatigue); PFS score < 7; code = 0 (No or Control).

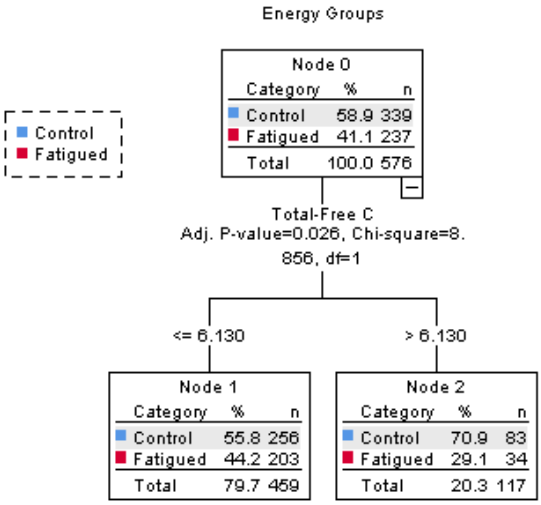


Figure 5-3: The branching of the control and High Fatigue groups based on the overall biochemical scores. Total PFS and overall biochemical data: patient group; selected 576 cases. The "Yes" and "No" and the "Control" and "Fatigue" categories were defined as above.

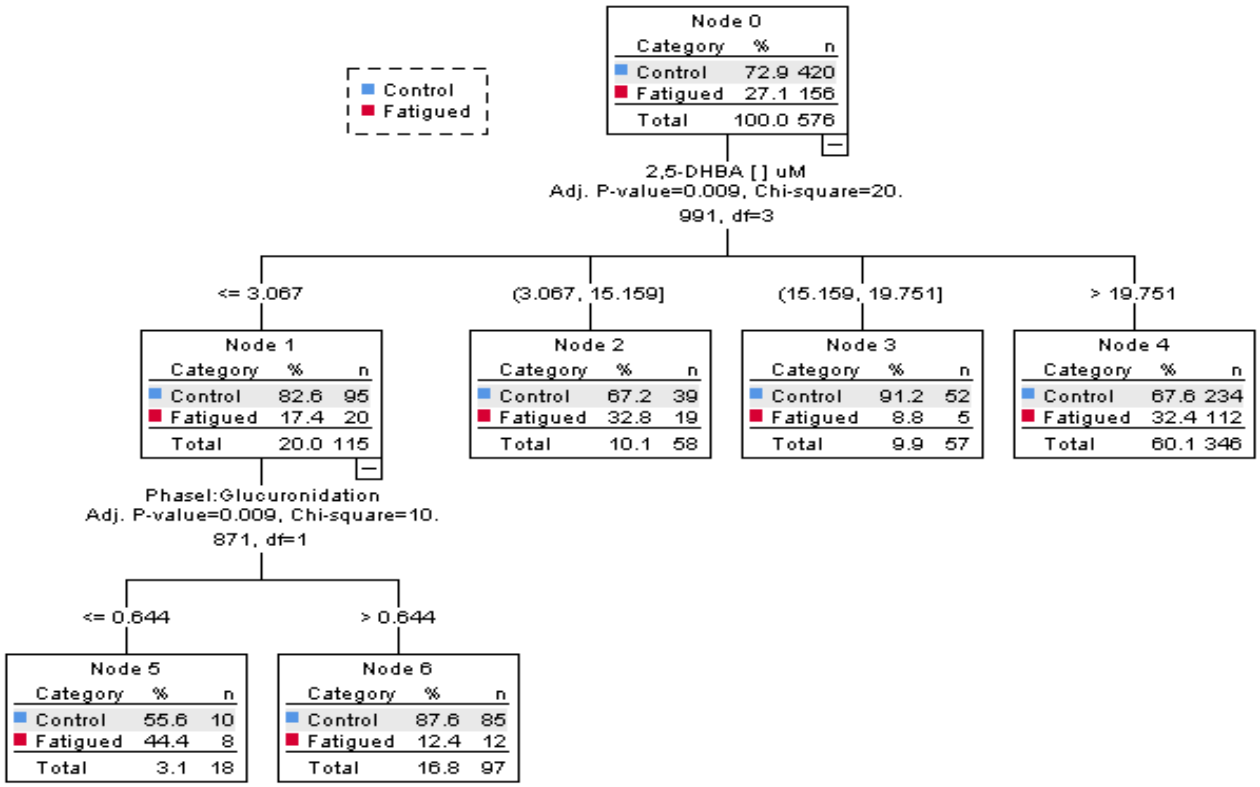


Figure 5-4: The branching of the control and High Mental groups based on the overall biochemical scores. Total PFS and overall biochemical data: patient group; selected 576 cases. The "Yes" and "No" and the "Control" and "Fatigue" categories for the Mental groups.

Finally, the CHAID analysis was applied to the total data (PFS, MSQ and biochemical) for the selected group. This outcome is presented in Chapter 7 and illustrated in Figure 4 of Erasmus *et al.* (2019)[2]. Figure 4 shows potentially important metabolites for the Low and High Fatigue groups, selected according to the CHAID analysis. At low values of MSQ, the Low Fatigue group becomes predominant, at medium levels the Mild Fatigue group becomes predominant and at high levels of MSQ the High Energy and Mental Fatigue group becomes predominant. The CHAID analysis thus provided the first results of a successful classification of a cohort of chronic fatigue patients. The outcome for this cohort of patients provided a classification tree distinguishing between five groups of patients, ranging from low to high fatigue (for further detail see Erasmus *et al.,* 2019).

5.2.6 Conclusions

The three data sets were analysed statistically with success to establish the links between fatigue, metabolism and medical symptoms for the patient groups of 576 cases under

[2] Note: This comment will be amended in the manuscript for submission to a journal.

investigation. The Piper Fatigue Scale was used to define the groups, and CHAID provided insight into the links between the MSQ and biochemical measurements. Although the model was intended as a descriptive rather than a predictive tool, prediction of low fatigue was 65.8% correct and in the case of high energy and mental fatigue it was 76.9% correct.

5.2.7 Author contributions:

All data were generated by EE through the BOSS procedure. FES did the initial data analysis: (1) it includes the conceptual view of a two-dimensional profile of patient distribution, based on the individual's scores on energy fatigue and mental fatigue derived from the PFS, and (2) the assessment of skewness and kurtosis in die biochemical data — both characteristics of the data set which may influence the outcome of the analysis if not corrected for. The conceptual design of the manuscript was agreed upon by all three authors, the first concept was drafted by FES followed by successive refinements from EE and CJR. The final version was approved by all three authors.

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PART III

INSIGHTS INTO BIOTRANSFORMATION: EXPERIMENTAL RESULTS

CHAPTER 6

New insights into the catalytic mechanism of human glycine N-acyltransferase (GLYAT)

6.1 Introduction

This chapter is a further contribution to the description of the intricacies of the human glycine-N-acyltransferase (GLYAT), a phase II conjugation enzyme system. Our research on glycine conjugation now spans the best part of two decades with some of the first contributions on amino acid substrate specificity of this enzyme system published by van der Westhuizen and co-workers at the turn of the century (Van der Westhuizen *et al.,* 2000).

Apart from the other phase II conjugation enzymes, GLYAT fulfils a very important task by removing specific acyl-compounds from the metabolism. In fact, GLYAT's evolutionary conservation points to a system which is really essential for the maintenance of life (Van der Sluis *et al.*, 2015). GLYAT is especially important for its role in the conjugation of products like benzoate which is an end-product of β-oxidation in human metabolism of flavonoids and other plant metabolites. Our research group has been active in further research about this conjugating enzyme to develop a more comprehensive understanding of the glycine conjugation pathway. More recently we have focused on the different pathways involved to ensure a functional GLYAT conjugation reaction (Badenhorst *et al*., 2014). Hepatic glycine conjugation is a two-step process that occurs in the mitochondria. The rate of glycine conjugation can be influenced by several factors, including the availability of ATP, CoASH, and glycine; genetic variation in the xenobiotic/medium chain fatty acid: CoA ligase (ACSM2B) and glycine N-acyltransferase (GLYAT) genes as well as variable expression levels of ACSM2B (E.C. 6.2.1.2) and GLYAT (E.C. 2.3.1.13) (Van der Sluis & Erasmus, 2016). Currently, the method used to study human GLYAT enzyme activity and kinetics relies on indirect colorimetric monitoring. This method however suffers from poor selectivity and absolute concentrations of the reaction product, hippuric acid cannot be accurately determined. We have therefore developed a high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) method to enable us to characterize the kinetic parameters of a recombinant GLYAT for better understanding of the kinetic mechanism involved in human GLYAT.

6.2 New insights into the catalytic mechanism of human glycine N-acyltransferase (Van der Sluis *et al.,* 2017 — publication II)

CHAPTER 6

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New insights into the catalytic mechanism of human glycine N-acyltransferase

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Abstract

Even though the glycine conjugation pathway was one of the first metabolic pathways to be discovered, this pathway remains very poorly characterized. The bi-substrate kinetic parameters of a recombinant human glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13) were determined using the traditional colorimetric method and a newly developed HPLC–ESI-MS/MS method. Previous studies analyzing the kinetic parameters of GLYAT, indicated a random Bi–Bi and/or ping-pong mechanism. In this study, the hippuric acid concentrations produced by the GLYAT enzyme reaction were analyzed using the allosteric sigmoidal enzyme kinetic module. Analyses of the initial rate (v) against substrate concentration plots, produced a sigmoidal curve (substrate activation) when the benzoyl-CoA concentrations was kept constant, whereas the plot with glycine concentrations kept constant, passed through a maximum (substrate inhibition). Thus, human GLYAT exhibits mechanistic kinetic cooperativity as described by the Ferdinand enzyme mechanism rather than the previously assumed Michaelis–Menten reaction mechanism.

KEYWORDS

glycine N-acyltransferase, human, HPLC–ESI-MS/MS, Hippuric acid, enzyme mechanism

1 | INTRODUCTION

There is a growing realization that glycine conjugation is one of the fundamentally important homeostatic mechanisms in animal physiology. The glycine conjugation pathway is important in preventing CoASH sequestration, which would result from the accumulation of acyl-CoA metabolites such as benzoyl-CoA. Glycine conjugation also decreases the toxicity of benzoate and other aromatic acids by forming less lipophilic conjugates rather than more water-soluble conjugates.[1–6] In addition to natural dietary sources of benzoate, humans are nowadays exposed to ever increasing amounts of this compound, since it is used as a preservative in food and pharmaceuticals.[7]

Hepatic glycine conjugation is a two-step process that occurs in the mitochondria and the overall rate of glycine conjugation can be influenced by several factors, including the availability of ATP, CoASH, and glycine; genetic variation in the xenobiotic/medium chain fatty acid: CoA ligase *(ACSM2B)* and glycine N-acyltransferase (*GLYAT)* genes as well as variable expression levels of ACSM2B (E.C. 6.2.1.2) and GLYAT (E.C. 2.3.1.13).[8–11] Very little is currently known about the influence of genetic variation in the human *ACSM2B* and *GLYAT* genes on the *in vivo* glycine conjugation pathway. It has, however, previously been shown that SNP variations in the human *GLYAT* gene influence the kinetic properties of a recombinantly expressed enzyme.[12] Reports on the *in vivo* interindividual variation in the glycine conjugation pathway do not discriminate between variation in acyl-CoA formation (catalyzed by ACSM2B) and variation in glycine conjugation (conjugated by GLYAT).

The catalytic mechanism of human GLYAT has not been investigated thoroughly, largely due to the scarcity of human liver samples and xenobiotic acyl-CoA substrates for research.[11] The kinetic parameters reported in the literature for human GLYAT vary substantially.[12–16] It is difficult to explain the large variation in literature values, but it has been proposed that significant differences in the reaction composition, substrate concentrations used, quality of purified protein preparations used, and data analysis may be partly responsible.[12] It is also possible that the currently used colorimetric assay introduces some variation.[17] Genetic variation might also be a contributing factor as previous studies did not specify the genetic variant that was characterized. The biggest challenge regarding the investigation of the glycine conjugation pathway remains the fact that this pathway is still very poorly characterized when compared with the cytochrome P450 (CYP450) phase I detoxification enzymes.

Currently, the method used to study human GLYAT enzyme activity and kinetics relies on indirect colorimetric monitoring of the color

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change observed by the reaction of DTNB with the free sulfhydryl group of the coenzyme A.[17] However, this method cannot determine the absolute amounts of hippuric acid (HA) formed through glycine conjugation.

An high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) method was thus developed and implemented to selectively identify and accurately quantify the amount of HA formed in the presence of varying substrate concentrations. The single biggest advantage that ESI-MS/MS offers is its very high level of selectivity. This is the first application of an HPLC–ESI-MS-MS method to characterize the bi-substrate kinetic parameters of a recombinant GLYAT to evaluate the kinetic mechanism of human GLYAT.

2 | MATERIALS AND METHODS

2.1 | Materials, reagents, standards and solutions

The S_{156} GLYAT construct was available in our laboratory.[12] Benzoyl coenzyme A, formic acid, and HA were purchased from Sigma–Aldrich (St. Louis, MO). Glycine was purchased from Melford Bio-laboratories (Suffolk, UK). Honeywell Burdick & Jackson, spectrometry-grade acetonitrile, methanol, and water were purchased from Anatech Instruments Ltd (Gauteng, SA). The HA-d5 stable isotope was purchased from Toronto Research Chemicals (Toronto, Canada). All chemicals, reagents, and solvents were of analytical grade.

2.2 | Expression and nickel-affinity purification of the recombinant human GLYAT

Bacterial expression and purification of the recombinant human GLYAT was performed as previously reported[12] with 0.5% glycine added to the expression medium in this study. For long-term storage of the purified enzyme preparations at −80°C, glycerol was added to the purified proteins to a final concentration of 10% and then snap frozen in liquid nitrogen. Protein concentrations were determined using the Qubit 2.0 Fluorometer and the Qubit Protein Assay Kit (Thermo Fisher Scientific Inc., NYSE: TMO Paisley PA4 9RF, UK).

2.3 | Bi-substrate (benzoyl-CoA and glycine) kinetic analysis of the recombinant human GLYAT using the colorimetric assay

The V_{max}, apparent K_M (benzoyl-CoA) and K_M (glycine) values were determined using a colorimetric assay that measures glycine dependent release of CoA at 412 nm, in the presence of 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB).[17] GLYAT catalyzes the reaction between benzoyl-CoA and glycine to form HA. During the colorimetric DTNB assay, the hydrolysis of the thioester of benzoyl-CoA results in the formation of CoA with a thiol group (CoA-SH). The thiol reacts with the DTNB in the reaction mixture to form 5-thio-2-nitrobenzoic acid (TNB). This yellow product (TNB) is observed spectrophotometrically by measuring absorbance at 412 nm.

To determine the bi-substrate kinetic parameters of human GLYAT, an enzyme assay was performed where both substrate concentrations were varied. The glycine concentrations were varied from 2.5 to 20 mM and the benzoyl-CoA concentrations from 20 to 80 μM. Each 200 μL reaction contained 1.2 μg of purified recombinant GLYAT and was performed in triplicate. Initial velocities were calculated over the first 6 min, where the reaction was linear. The data were processed with SigmaPlot 12.0 (Systat software, Inc., San Jose, CA) software, using the Enzyme Kinetics module, to plot the Lineweaver–Burk plots, and to calculate the kinetic parameters using nonlinear regression.

2.4 | Enzyme reactions and extractions for HPLC–ESI-MS/MS detection and quantification of HA

For quantification of HA, enzyme reactions were set up containing the purified recombinant human GLYAT, 25 mM Tris–HCl (pH 8.0), 20–80 μM benzoyl-CoA, 2.5–20 mM glycine, and water. The benzoyl-CoA concentrations were 20, 40, 60, and 80 μM. The glycine concentrations used were 2.5, 5, 10, and 20 mM. The volume of the reaction mixtures was 200 μL. The reactions were allowed to proceed for 20 min and then stopped by adding 20 μL concentrated formic acid. Before further analysis, the samples were spiked with the HA-d5 stable isotope to a final concentration of 6.44 μM. The HA and HA-d5 were then extracted using the liquid–liquid extraction method proposed in Ref. 15. The method involves the use of an organic solvent, ethyl acetate, to achieve phase separations. The extraction method used was optimized by testing its ability to extract the protein, and the high amounts of glycine present in the enzyme reactions. Approximately 2 mL ethyl acetate was added to the tubes, vortex-mixed for 1 min and then centrifuged (2800×*g*, 1 min, room temperature) to separate the phases. The top aqueous layer was then removed and transferred to a clean tube. This procedure was repeated three times, and the aqueous layer, of each individual reaction, was transferred into the same final tube. The samples were then dried in a nitrogen stream at 40°C for approximately 15 min. The residues were reconstituted with 200 μL water which served as the mobile phase. The samples were analyzed in triplicate with the HPLC–ESI-MS/MS method described in the next section and the precision/repeatability was calculated in terms of the coefficient of variation (%CV).

2.5 | Detection and quantification of HA using HPLC–ESI-MS/MS

An Agilent 6460 Triple Quad series HPLC–ESI-MS/MS (Agilent Technologies, Inc. Santa Clara, CA) coupled with the Agilent Jet Stream technology in positive ionization mode was used during this study. The Agilent ZORBAX StableBond Phenyl 2.1 × 100 mm 1.8 micron column was purchased from Agilent Technologies. The method used for HA detection and quantification was adapted from the method described by Nortje et al.[18] The source parameters of the MS were as follows: The gas temperature was 280°C, a gas flow of 6 L/min, the nebulizer set at 45 psi, sheath gas heater at 350°C, sheath gas flow of 7 L/min, a capillary voltage of 3000 V, and a nozzle voltage of 1500 V. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B

consisted of 100% methanol. The mobile phase flow rate was 0.2 mL/min with the following gradient mobile phase composition: 5% B from 0 to 1 min; an increase to 31% B over 6.5 min; another increase to 100% B from 7.5 to 9.5 min; isocratic at 100% B from 9.5 to 13.5 min and then back to 5% B over the last minute. The fragmentor voltage was set at 45 V and the collision energy at 8 V. The Masshunter optimizer software from Agilent was employed to optimize the data acquisition parameters for multiple reaction monitoring (MRM) mode for HA and HA-d5. Mass spectrometric parameters for HA (M_r = 179.17) were as follows: using a fragmentor voltage of 60 V and a collision energy of 8 V, the precursor ion was produced at 180.1 *m/z* and the most abundant product ion at 105.1 *m/z*. The mass spectrometric parameters for HA-d5 (M_r = 184.17) were: using a fragmentor voltage of 60 V and a collision energy of 8 V, the precursor ion was produced at 185.1 *m/z* and the most abundant product ion at 110.1 *m/z*.

A calibration curve was set up for HA (1–128 μM HA) to investigate the linearity of HA detection. The range was analyzed in triplicate. A second calibration curve was set up for HA with the HA-d5 isotope to investigate the linearity of HA detection in the presence of the isotope. The 1–128 μM HA sample concentrations were then diluted by the addition of 5 μL of the 650 μM HA-d5 stock solution to a final concentration of 6.44 μM. The adjusted concentration range was 0.99–126.733 μM HA, all containing 6.44 μM isotope. These samples were then also analyzed in triplicate. The coefficient of determination (R^2) was used to evaluate the linearity of the calibration curves. To test the recovery of HA after the extraction procedure, a known concentration level (16 μM) of HA (in water) was selected and placed in separate tubes. One of the samples was then put through the entire extraction procedure. Both samples were then injected into the HPLC–ESI-MS/MS system. The responses obtained from the samples were then compared to evaluate the recovery of the HA after the extraction. The %CV was calculated to assess the precision of the triplicate samples.

The raw mass spectrometric data was acquired using the on-board Agilent Masshunter B 05 software. The response data of each HA level was plotted against its concentration to construct the calibration curve. For the calibration curve including the HA-d5 stable isotope, the HA responses of each concentration level was divided by those of the HA-d5, these values were plotted on the y axis. The concentration ratios of HA and HA-d5 were plotted on the *x*-axis. HA concentrations were then calculated using the linear equation of the second calibration curve shown by Eq. (1). GraphPad Prism 7 was used for statistical analyses and data representation.

Equation 1: Linear equation of calibration curve.

$$y = 1.1176x + 0.0733$$

2.6 | Evaluation of the kinetic mechanism using the bi-substrate (benzoyl-CoA and glycine) ESI-MS/MS data

The bi-substrate (benzoyl-CoA and glycine) ESI-MS/MS data were used to re-evaluate the kinetic mechanism employed by human GLYAT. The HA concentrations determined for each endpoint enzyme reaction were analyzed using the alternative allosteric sigmoidal enzyme kinetic analyses function on GraphPad Prism 7.0 (GraphPad Software, Inc., CA La Jolla, CA 92037 USA). The sigmoidal enzyme analyses curves were fitted using the Hill equation[19] to determine V_{max}, $K_{0.5}$ as well as the Hill slope (*h*).

3 | RESULTS

The main aim of this study was to determine the bi-substrate enzyme kinetic parameters of a recombinantly expressed human GLYAT and to re-evaluate the catalytic mechanism. This was done using both the traditional colorimetric DTNB assay [17] to indirectly verify and measure the enzyme activity as well as a newly developed HPLC–ESI-MS/MS method to identify and quantify HA. The HA concentrations determined by the HPLC–ESI-MS/MS method were then used to re-evaluate the enzyme kinetic mechanism employed by human GLYAT.

3.1 | Bi-substrate (benzoyl-CoA and glycine) kinetic analysis of a recombinant human GLYAT using the colorimetric DTNB assay

It was previously shown that the human S_{156} GLYAT haplotype has the highest relative enzyme activity [12] as well as the highest allele frequency in world-wide population data analyzed.[20] For this reason, the human S_{156} GLYAT haplotype was chosen for enzymatic characterization in this study, but for the sake of simplicity will only be referred to throughout the study as a "recombinant human GLYAT."

With the exception of one study,[15] all of the previous studies[12–14,16] reported in the literature investigated GLYAT enzyme kinetics using the colorimetric DTNB assay.[17] The same method was, therefore, used in this study to enable comparison with previous studies. Lineweaver–Burk (double reciprocal) plots were generated with the SigmaPlot 12.0 software program using its enzyme kinetics module. These plots were used to determine the K_{Mapp} and V_{Max} values for benzoyl-CoA and glycine for the purified recombinant human GLYAT using nonlinear regression analysis. These values were determined individually by plotting the constant glycine values with the varying benzoyl-CoA values on a double reciprocal plot and vice versa on a second double reciprocal plot. The data were fitted using three different kinetic models, namely the random ordered mechanism, the ordered mechanism, and the ping-pong mechanism. The coefficient of determination (R^2) values, the Akaike Information Criterion (AIC), the relative likelihood as well as the kinetic parameters for each kinetic model are given in Table 1.

According to the SigmaPlot enzyme analyses, the random Bi–Bi and ping-pong Bi–Bi mechanism showed equal goodness of fit (R^2 of 0.9747 and 0.9747, respectively), whereas the ordered Bi–Bi mechanism showed convergence failure. The AIC analyses showed that the random Bi–Bi sequential mechanism has a relative likelihood of 0.84, whereas the ordered Bi–Bi sequential mechanism has a likelihood of 0.01. This indicated that the ping-pong model has the highest probability to minimize the (estimated) information loss.[21] A ping-pong kinetic

TABLE 1 The coefficient of determination (R^2) values, the Akaike Information Criterion (AIC), the relative likelihood for each model as well as the kinetic parameters for each kinetic model for human GLYAT using benzoyl-CoA and glycine as substrates

Kinetic Model	R^2	AIC	Relative Likelihood [exp (−0.5 * ΔAIC)]	K_{Mapp} Benzoyl-CoA (μM)	K_{Mapp} Glycine (mM)
Random Bi–Bi (sequential) mechanism	0.9747	1178.50	0.84	49 ± 13	20 ± 4
Ping-pong Bi–Bi (non-sequential) mechanism	0.9747	1178.15	1	53 ± 8	21 ± 2
Ordered Bi–Bi (sequential) mechanism	0.9430	1186.70	0.01	60 ± 16	5 ± 1

mechanism for human GLYAT is, however, contradictory to what was previously reported in the literature.[15] A sequential Bi–Bi reaction mechanism was determined, with the acyl-CoA substrate binding first to the enzyme, followed by the addition of glycine, before dissociation of CoA, with the release of the peptide product occurring as the last step. In this study the ordered Bi–Bi sequential mechanism was clearly eliminated as a possible model (Table 1). This prompted further investigation into the kinetic mechanism for human GLYAT.

The double reciprocal plots of both the benzoyl-CoA and glycine values are shown in Figure 1. For the random Bi–Bi kinetic mechanism, the K_{Mapp} values determined for the recombinant human GLYAT for benzoyl-CoA was 49 ± 13 μM and for glycine 20 ± 4 mM, whereas the V_{max} value was 157 ± 22 nmol/min/mg. For the ping-pong Bi–Bi kinetic mechanism, the K_{Mapp} values determined for the recombinant human GLYAT for benzoyl-CoA and glycine were 53 ± 8 μM and 21 ± 2 mM, respectively, whereas the V_{max} value was 161 ± 15 nmol/min/mg.

The bi-substrate kinetic parameters determined in this study (Table 1) were in the range of the kinetic parameters described in the literature.[12–15] Table 2 summarizes the K_{Mapp} values for benzoyl-CoA and glycine as reported in the literature and the values determined in this study (shaded in gray).

As can be seen from Table 2, the kinetic parameters reported in the literature for human GLYAT vary substantially. The K_{Mapp} (benzoyl-CoA) is reported to be between 13 μM and 57.9 mM, the K_{Mapp} (glycine) between 6.4 and 26.6 mM, and the V_{max} value between 543 nmol/min/mg and 17,100 nmol/min/mg.[12–16] The K_{Mapp} (benzoyl-CoA) of 49 ± 13 μM determined for the recombinant human GLYAT in this study is within the range reported in the literature, whereas the K_{Mapp} (glycine) value of 20 ± 4 mM is on the higher end of the range in the literature. In three previous studies, the same recombinant S_{156} variant was characterised with K_{Mapp} (benzoyl-CoA) (38 ± 4 μM[12]; 49 ± 13 μM [this study] and 209 μM[16]) and K_{Mapp} (glycine) (20 ± 4 mM [this study] and 26.6 mM[16]) determined values. Therefore, even if the same genetic variant is characterised, large variation in reported kinetic parameters is still observed.

In 1959, Ellman[22] introduced DTNB as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. The DTNB colorimetric assay used to determine the enzyme kinetics of human GLYAT is, therefore, an indirect measure of enzyme activity. The DTNB assay has a number of problems, including pH-induced disulfide disruptions, re-oxidation of the R-SH groups, and thermochromic effects.[23] The DTNB assay cannot be used to determine enzyme kinetics in crude enzyme preparations, which contain interfering substances like thiols and acytyl-CoA hydrolyse, as a high blank absorbency will be obtained. In cases where DTNB is an efficient inhibitor of the enzyme, it cannot be used for continuous enzyme kinetic analysis.[24] Although it has been shown that DTNB does not inhibit the bovine GLYAT enzyme activity,[25] similar studies have not been performed on the different human GLYAT variants.

It was subsequently proposed that the currently used DTNB colorimetric assay might contribute to the observed variation. We, therefore, investigated the suitability of an HPLC–ESI-MS/MS method to accurately detect and quantify HA formed by the recombinant glycine conjugation reaction.

3.2 | Detection and quantification of HA using HPLC–ESI-MS/MS

3.2.1 | Linearity

The linearity of HA detection was investigated both with and without the HA-d5 stable isotope. The linearity was determined using a concentration range of 1–128 μM HA as the benzoyl-CoA concentration used ranged from 20 to 80 μM. The isotope was added to a final concentration of 6.44 μM. The calibration curves were near linear within the selected calibration ranges. The calibration curves for both HA and HA with HA-d5 are illustrated in Figure 2.

3.2.2 | Organic solvent extraction

A simple extraction of HA from the enzyme reactions was performed, followed by drying under a nitrogen stream and finally resuspension in water. The recovery of HA by this method was evaluated and was found to be extremely efficient, as nearly 100% of the HA was retrieved after extraction (results not shown).

3.2.3 | Quantification of HA using the HPLC–ESI-MS/MS method

The HPLC–ESI-MS/MS method with the parameters described previously was implemented for the quantification of HA formed from the human GLYAT enzyme reactions that were set up, extracted, and spiked with the isotope. The samples were analyzed in triplicate. In Figure 3, a representative MRM chromatogram is shown. The final HA concentrations could be determined by substitution into the linear equation of the calibration curve of the HA with HA-d5 shown by Eq. (2).

Equation 2: The linear equation with the substituted values for final HA concentration determination.

$$\frac{\text{Response HA}}{\text{Response HA-d5}} = 1.1176\left(\frac{x}{\text{HA-d5 concentration}}\right) + 0.0733$$

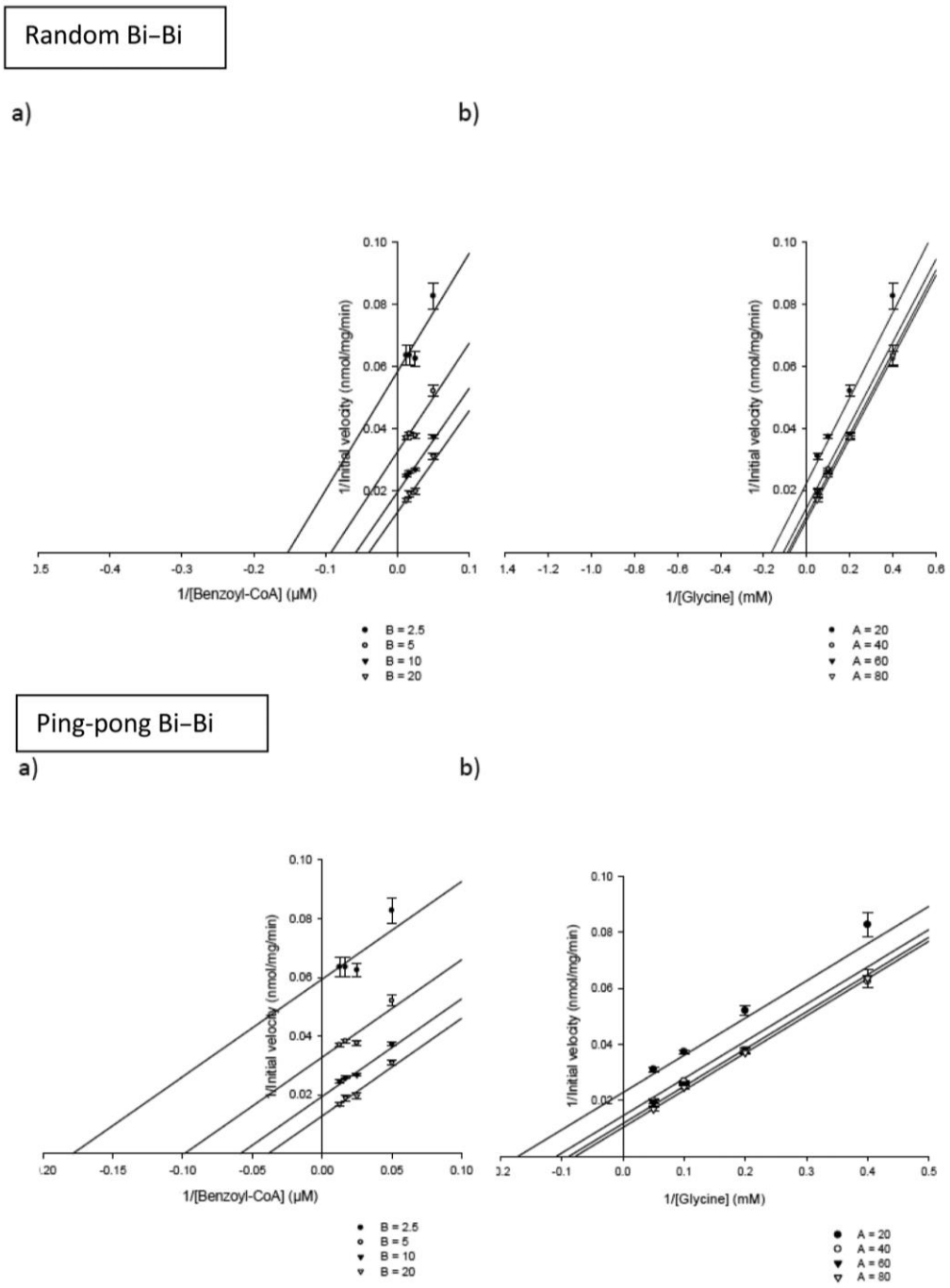


FIGURE 1 Comparison of two kinetic enzyme models for human GLYAT: The random order Bi–Bi sequential mechanism and the Ping-pong mechanism. (A) Lineweaver–Burk plots of benzoyl-CoA with varying glycine concentrations (2.5, 5, 10, and 20 mM). (B) Lineweaver–Burk plots of glycine with varying benzoyl-CoA (20, 40, 60, and 80 μM) concentrations. The data points indicate average values ± standard deviation, with $n = 3$

The final HA concentrations formed from human GLYAT activity were then calculated. The precision/repeatability was calculated in terms of the %CV using Eq. (3).

Equation 3: Calculating coefficient of variation (% CV).

$$\%\text{CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The mean and standard deviation (SD) values for each triplicate sample measurement were calculated using the concentrations calculated for each of the triplicates of each sample. The results are summarized in Table 3.

The concentrations of HA determined in the enzyme reaction samples were found to be 9.68–66.84 μM for the benzoyl-CoA range of 20–80 μM. The precision (%CV) of the triplicate measurements

TABLE 2 Kinetic parameters of benzoyl-CoA and glycine for GLYAT as reported in the literature

Parameters	Values	Recombinant Variant/Isolated from Liver	Reference
K_{Mapp} (benzoyl-CoA) (μM)	13	Purified GLYAT from human liver	[15]
	38 ± 4	Purified recombinant S_{156} variant	[12]
	49 ± 13	Purified recombinant S_{156} variant	This study (random Bi–Bi)
	53 ± 8	Purified recombinant S_{156} variant	This study (ping-pong Bi–Bi)
	67 ± 5	Partially purified GLYAT from human liver	[13]
	209	Purified recombinant S_{156} variant	[16]
	57,900	Purified GLYAT from human liver	[14]
K_{Mapp} (glycine) (mM)	6.4	Purified GLYAT from human liver	[15]
	6.5 ± 1	Partially purified GLYAT from human liver	[13]
	20 ± 4	Purified recombinant S_{156} variant	This study (random Bi–Bi)
	21 ± 2	Purified recombinant S_{156} variant	This study (ping-pong Bi–Bi)
	26.6	Purified recombinant S_{156} variant	[16]
V_{max} (nmol/min/mg)	157 ± 22	Purified recombinant S_{156} variant	This study (random Bi–Bi)
	161 ± 15	Purified recombinant S_{156} variant	This study (ping-pong Bi–Bi)
	543 ± 21	Purified GLYAT from human liver	[15]
	807	Recombinant S_{156} variant	[16]
	1230	Purified recombinant S_{156} variant	[12]
	17,100	Purified GLYAT from human liver	[14]

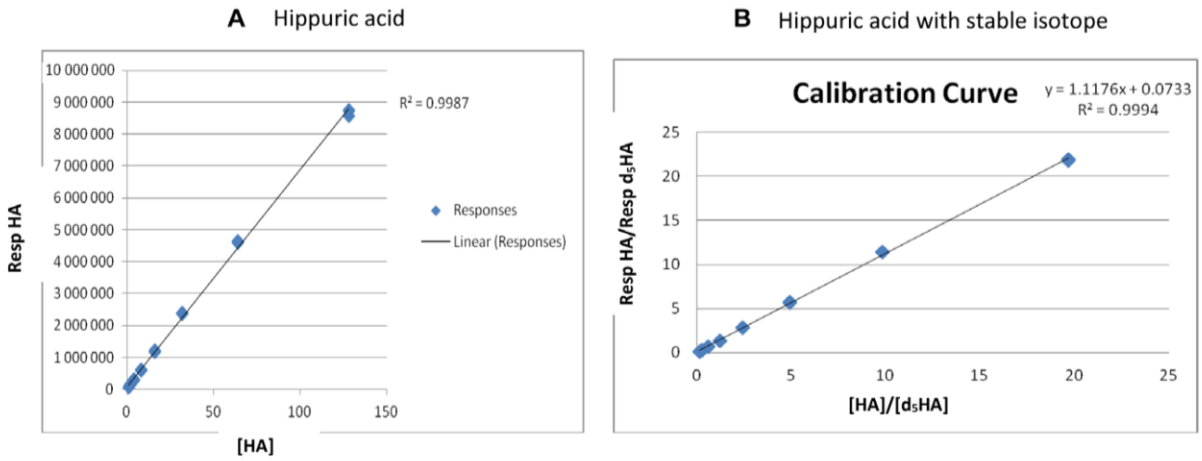


FIGURE 2 Calibration curves for HA. (A) Calibration curve of HA. The calibration curve shows the linear range for HA in water. Linearity was assessed using a concentration range of 1–128 μM of HA. (B) Calibration curve of HA with HA-d5. The calibration curve shows the linear range for HA with HA-d5 in water. Linearity was assessed using a concentration range of 0.99–126.733 μM of HA, spiked with 6.44 μM HA-d5 in each sample

were between 0.22% and 1.36% for all samples. These values showed acceptable precision, as the criterion of acceptance is generally within 15% of the %CV.[26] This demonstrated the repeatability and efficacy of this analytical method.

3.3 | Evaluation of the kinetic mechanism using the bi-substrate (benzoyl-CoA and glycine) ESI-MS/MS data

The HA concentrations determined for each endpoint enzyme reaction were converted to μM/min values. The velocity as a function of substrate concentration (glycine or benzoyl-CoA) was analyzed using the allosteric sigmoidal enzyme kinetic analyses function on GraphPad Prism 7.0 (GraphPad Software, Inc.) (Tables 4 and 5 and Figure 4).

V_{Max} is the maximum reaction rate that is observed at saturating substrate concentrations. For glycine the V_{Max} increased from 0.7825 ± 0.004079 μM/min HA formed to 3.4 ± 0.02365 μM/min with varying benzoyl-CoA concentrations (Table 4 and Figure 4A). For benzoyl-CoA, the V_{Max} increased from 1.511 ± 0.087 μM/min HA formed to 7.073 ± 1.696 μM/min with varying glycine concentrations (Table 5 and Figure 4B).

For glycine at varying benzoyl-CoA concentrations, a sigmoidal curve and a Hill slope (h) > 3 were obtained (Table 4 and Figure 4A). Thus, the binding of glycine facilitates an increased binding of

TABLE 3 Final HA concentrations, average HA concentrations, and %CV values

[Benzoyl-CoA] (μM)	[Glycine] (mM)	Final [HA] (μM)	Average [HA] (μM)	%CV
20	2.5	9.8	9.6823994	0.91
		9.7		
		9.6		
20	5	15	14.900621	0.76
		14.9		
		14.8		
20	10	15.8	15.818116	0.51
		15.7		
		15.9		
20	20	15.5	15.456734	0.29
		15.4		
		15.5		
40	2.5	18.2	18.23393	0.37
		18.2		
		18.3		
40	5	29.4	29.572761	0.39
		29.7		
		29.6		
40	10	31.8	32.311997	1.36
		32.5		
		32.7		
40	20	33	33.446127	1.17
		33.6		
		33.7		
60	2.5	24.4	24.503901	0.42
		24.6		
		24.5		
60	5	46.3	46.168682	0.69
		45.8		
		46.4		
60	10	52.4	51.957595	0.98
		52.1		
		51.4		
60	20	50.5	50.104959	1.34
		49.3		
		50.5		
80	2.5	25.5	25.34193	0.43
		25.3		
		25.3		
80	5	56.3	56.704273	1.11
		56.4		
		57.4		
80	10	67.6	67.769724	0.22
		67.8		
		67.9		
80	20	66.7	66.849286	0.23
		66.9		
		67		

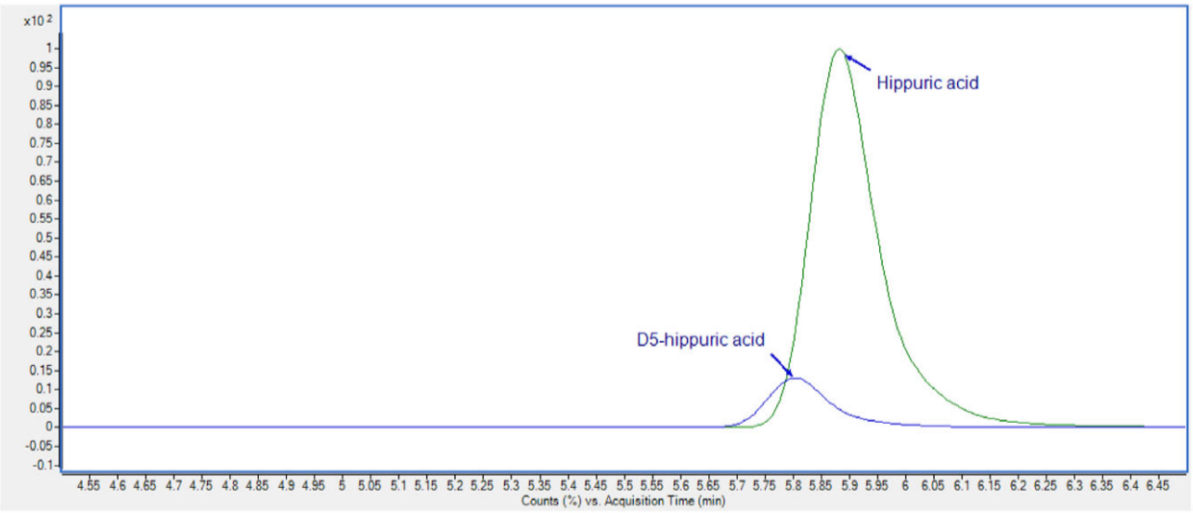


FIGURE 3 An example of the product quantification results: a representative Multiple Reaction Monitoring (MRM) chromatogram showing the transitions of Hippuric acid (180–105 amu) and D_5-Hippuric acid (185–110 amu)

TABLE 4 Hippuric acid formation (μM/min) as a function of glycine concentration (mM)

	Benzoyl-CoA (20 μM)	Benzoyl-CoA (40 μM)	Benzoyl-CoA (60 μM)	Benzoyl-CoA (80 μM)
Best-fit values				
V_{max} (μM/min)	0.7825 ± 0.004079	1.666 ± 0.0098	2.56 ± 0.02442	3.4 ± 0.02365
h	3.674 ± 0.28	2.66 ± 0.1232	3.409 ± 0.2605	3.135 ± 0.1188
$K_{0.5}$ (mM)	2.189 ± 0.02706	2.326 ± 0.02599	2.566 ± 0.03814	2.956 ± 0.03449
Goodness of fit				
R^2	0.996	0.9971	0.9931	0.9976

TABLE 5 Hippuric acid formation (μM/min) as a function of benzoyl-CoA concentration (μM)

	Glycine 2.5 mM	Glycine 5 mM	Glycine 10 mM	Glycine 20 mM
Allosteric sigmoidal			*Ambiguous*	*Ambiguous*
Best-fit values				
V_{max} (μM/min)	1.511 ± 0.087	7.073 ± 1.696	17.48 ± 8.346	~19.08 ± ~5.549
h	1.848 ± 0.203	1.289 ± 0.1157	1.199 ± 0.08332	1.157 ± 0.04344
$K_{0.5}$ (μM)	30.42 ± 2.392	108.3 ± 35.95	~261.5 ± ~149.6	~305.5 ± ~107.5
Goodness of fit				
R^2	0.9894	0.9968	0.9985	0.9996

benzoyl-CoA. The $K_{0.5}$ values (2.189 ± 0.02706 to 2.956 ± 0.03449 mM; Table 4) indicated a very high affinity of interaction between human GLYAT and glycine. Therefore, it takes a very low concentration of glycine to reach 50% of the saturating concentration. Unlike the majority of metabolic pathways that follow first-order kinetics *in vivo*, glycine conjugation is readily saturable. Previous studies have shown that the formation of HA from benzoic acid *in vivo* is limited by the availability of glycine[27] and that the administration of exogenous glycine to rats increased the amount of HA formed.[8] This phenomenon can be observed in Figure 4A where the rate of HA formation (μM/min) increased as the glycine concentration increased until a linear rate of product formation was observed.

The plot of velocity as a function of benzoyl-CoA concentration at varying glycine concentrations (Table 5 and Figure 4B) showed that as the glycine concentration increased, the $K_{0.5}$ (μM) of benzoyl-CoA also increased from 30.42 ± 2.392 to 108.3 ± 35.95 μM, which indicated that the affinity of the enzyme for benzoyl-CoA decreased.

4 | DISCUSSION

The enzyme kinetic mechanism of human GLYAT has not been thoroughly investigated. The report that GLYAT follows a sequential two-substrate mechanism,[15] contradicts our finding of equal goodness

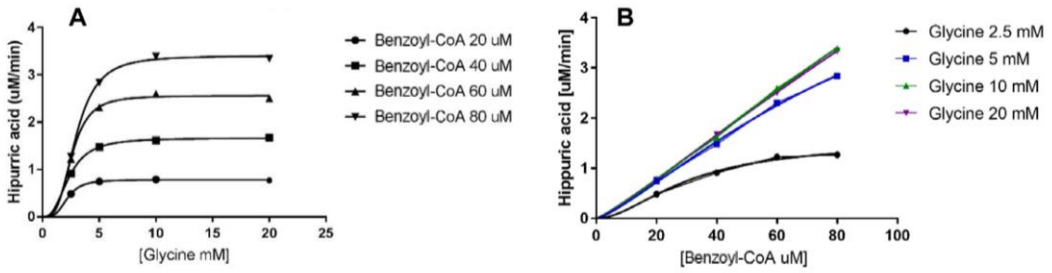


FIGURE 4 Hippuric acid formation (μM/min) as a function of substrate concentration for the human GLYAT reaction. (A) Plot of the initial rate (v) against [glycine], with [benzoyl-CoA] kept constant, produces a sigmoidal curve (substrate activation). (B) Plot of v against [benzoyl-CoA], with [glycine] constant, passes through a maximum (substrate inhibition). The data points indicate average values ± standard deviation, with $n = 3$

of fit for both the random Bi–Bi and ping-pong mechanism (Table 1), whereas the AIC relative likelihood parameter identified the ping-pong kinetic mechanism to be the best model. In previous studies, the kinetic parameters were determined using either partially purified GLYAT or mitochondrial lysate preparations from liver tissue, of which the variant analyzed was not determined. We present the first determination of the bi-substrate kinetic parameters and kinetic mechanism of a purified recombinant human GLYAT.

Previous studies[12–16] assumed that human GLYAT would also follow a sequential Bi–Bi mechanism as proposed for bovine GLYAT.[25,28] This raised the question whether the Michaelis–Menten reaction mechanism is the correct mechanism to apply to human GLYAT reaction kinetics. Over a narrow range of substrate concentrations, sulfotransferases, follow classic Michaelis–Menten kinetics, but when studied over a wide range of substrate concentrations, some sulfotransferases exhibit positive or negative cooperativity, and the kinetics fit the Hill plot (i.e., allosteric sigmoidal enzyme kinetics).[29]

We analyzed the HA concentrations produced by GLYAT using the allosteric sigmoidal enzyme kinetic module. The Hill slope (h) > 3 and sigmoidal curve determined for glycine at varying benzoyl-CoA concentrations (Table 4 and Figure 4A) clearly indicated a sigmoidal enzyme mechanism with positive cooperativity. The Hill slope (h) > 1 for benzoyl-CoA at varying glycine concentrations (Table 5 and Figure 4B) indicated a sigmoidal enzyme mechanism at glycine concentrations ≤ 5 mM. At glycine concentrations higher than 5 mM glycine, the data no longer fitted the Hill equation ($h \cong 1$) and the Hill equation was reduced to its simpler form known as the Michaelis–Menten equation.

Previous studies determined kinetic parameters using saturating glycine concentrations of 50–200 mM,[13–15] whereas the kinetic parameters of the recombinant S_{156} variant[16] was determined using very high benzoyl-CoA concentrations of 470–4700 μM.[16] This explains the variation in the K_{Mapp} values in the literature for GLYAT, as the $K_{0.5}$ value for sigmoidal enzymes varies at different substrate concentrations.[30]

We propose that human GLYAT exhibits mechanistic kinetic cooperativity.[30] Ferdinand[31] first showed that a random-order ternary-complex mechanism for a bi-substrate monomeric enzyme-catalyzed reaction can lead to sigmoidal kinetics in the absence of cooperative binding as seen in Figure 4 where the plot of the initial rate (v) against [glycine], with [benzoyl-CoA] kept constant, produces a sigmoidal curve (substrate activation), whereas the plot of v against [benzoyl-CoA], with [glycine] constant, passes through a maximum (substrate inhibition). This is supported by observations in humans that even if the amount of benzoic acid administered is increased, the hourly excretion of HA rises to a maximum and remains constant until all but a small portion is eliminated. However, when glycine is administered the hourly excretion of HA rapidly increased.[32–34]

In summary, using a highly selective and specific HPLC–ESI-MS/MS method to quantify HA indicated that human GLYAT exhibits mechanistic kinetic cooperativity. Although it is very difficult to compare our *in vitro* data to *in vivo* determined values, it is clear that the kinetic mechanism of GLYAT is far more complex than previously thought and needs to be further elucidated through the kinetic monitoring of enzymatic reactions in real time by quantitative HPLC–MS.[35,36]

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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CHAPTER 7

Biotransformation profiles from a cohort of chronic fatigue women in response to a hepatic detoxification challenge

7.1 Introduction

Chapter 7 address the fourth objective for the present study: "*Perform a correlation of the clinical features of the selected patients with the biotransformation profiles to determine a possible metabolic link to fatigue symptoms, as proposed through the model for this link.*" The outcome of this part of the study was submitted to *PLOS ONE.* The following comment was made by one of the reviewers: "*The PLOS Data policy requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception. The data should be provided as part of the manuscript or its supporting information or deposited to a public repository."* We complied to this requirement in the paper as published and include it to the thesis as well as a Google drive link (https://drive.google.com/open?id=1J8WuZHT6uCcj-uONpr0mm7BoKVa1bXte) containing all the data used for the analysis as described here.

The data used for this part of the study was generated over a period of seven years, required to compose a cohort of 500+ selected patients for the study (Chapter 2, objective 3). The final cohort of 576 chronic fatigue patients presented with a complex profile of cases distributed from low to high energy and mental fatigue (Chapter 5, Figure 5-1), but provided for a detailed analysis of the biochemical data obtained from five clusters of patients with different energy/mental characteristics. The biochemical data was obtained from a selection of phase I and phase II detoxification reactions, covering the main biotransformation pathways known to be operative in maintaining homeostasis following a perturbation due to exogenous xenobiotics (Erasmus *et al.,* 2019, Figure 1).

Polymorphisms of genes for biotransformation enzymes are known to be associated with individuals' detoxification status. This important issue was not addressed in the present study but formed part of some of other investigations done by our group. To address this issue, we included Table 2 in the discussion of the present *PLOS ONE* paper, indicating some of our observations made on polymorphisms associated with the GLYAT gene.

7.2 Biotransformation profiles from a cohort of chronic fatigue women in response to a hepatic detoxification challenge (Erasmus *et al.*, 2019 — publication III)

PLOS ONE

RESEARCH ARTICLE

Biotransformation profiles from a cohort of chronic fatigue women in response to a hepatic detoxification challenge

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Abstract

Chronic fatigue, in its various manifestations, frequently co-occur with pain, sleep disturbances and depression and is a non-communicable condition which is rapidly becoming endemic worldwide. However, it is handicapped by a lack of objective definitions and diagnostic measures. This has prompted the World Health Organization to develop an international instrument whose intended purpose is to improve quality of life (QOL), with energy and fatigue as one domain of focus. To complement this objective, the interface between detoxification, the exposome, and xenobiotic-sensing by nuclear receptors that mediate induction of biotransformation-linked genes, is stimulating renewed attention to a rational development of strategies to identify the metabolic profiles in complex multifactorial conditions like fatigue. Here we present results from a seven-year study of a cohort of 576 female patients suffering from low to high levels of chronic fatigue, in which phase I and phase II biotransformation was assessed. The biotransformation profiles used were based on hepatic detoxification challenge tests through oral caffeine, acetaminophen and acetylsalicylic acid ingestion coupled with oxidative stress analyses. The interventions indicated normal phase I but increased phase II glucuronidation and glycination conjugation. Complementarity was indicated between a fatigue scale, medical symptoms and associated energy-related parameters by application of Chi-square Automatic Interaction Detector (CHAID) analysis. The presented study provides a cluster of data from which we propose that multidisciplinary inputs from the combination of a fatigue scale, medical symptoms and biotransformation profiles provide the rationale for the development of a comprehensive laboratory instrument for improved diagnostics and personalized interventions in patients with chronic fatigue with a view to improving their QOL.

Introduction

Chronic fatigue is a condition commonly reported by patients in primary care practice, whose medical practitioners use diverse definitions and diagnostic labels to describe their symptoms

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[1]. The complaints expressed about fatigue in stressful and competitive societies range from the condition being unexplained at the one end of the fatigue spectrum [2] but commonly co-occurring with pain, sleep disturbances and depression. This cluster of symptoms associated with fatigue is recognized in functional somatic syndromes such as fibromyalgia (FS) [3] and irritable bowel syndrome (IBS) [4]; it is also common to oncology patients and is associated with a significant decline in their functional status and quality of life (QOL) [5]. Chronic fatigue syndrome (CFS) [6] and myalgic encephalomyelitis (ME) [7] occur at the other end of the fatigue spectrum, with the latter condition characterized by consistent excessive fatigue and fatigability. More broadly, fatigue appears to be one of the chronic non-communicable conditions which are rapidly becoming endemic worldwide [8][9][10][11], prompting the World Health Organization (WHO) to established a project on the development of an international quality of life (QOL) assessment instrument[12].

There is no established biological marker for CFS/ME, nor for chronic or periodic fatigue so that the diagnosis is one of exclusion (DOE) [13] after reasonable effort has been taken to eliminate at least some of the many aetiologies where fatigue is a presenting feature. The lack of objective measures of fatigability is a well-founded frustration to clinicians [14]. However, recent metabolomics studies include metabolic profiling of patients suffering from fatigue. Moreover, they have proposed that broad metabolic perturbations prevail in CFS and/or ME [15][16][17][18]. Although the compounds recorded in these studies are not all identical, the major pathways determined to be affected by ME/CFS relate to energy metabolism. These findings are in accordance with the known link between energy deprivation and fatigue [19]. As the energy demands in biotransformation reactions are key features in pharmacological and toxicological research [20][21][22][23], a link between consistent exposure to environmental toxicants and chronic conditions such as fatigue seems evident. Likewise, interest in the interface between detoxification and the impact of the exposome in health and disease is growing [24][25][26]. Moreover, xenobiotic-sensing nuclear receptors that mediate induction of biotransformation-linked cytochrome P450 (CYP450) genes are emerging as new regulators of the hepatic energy metabolism that connects sensing of the chemical environment, detoxification of xenobiotics and metabolic health [26][27]. These studies unequivocally established that the human genome harbours an extensive number of genes encoding enzymes that primarily metabolize endogenous and exogenous substances. Extensive genetic and functional variation in these genes has complex consequences. Such variability affect enzyme expression or structure and the toxicological level of induced or produced metabolites, the clinical manifestation in the patients and eventually also diagnostic and predictive strategies towards fatigue, as will be discussed below. Apart from the molecular research, these developments therefore also call for renewed attention to a rational basis for alternative strategies to identify the metabolic differences that contribute to health and disease in complex multifactorial conditions like fatigue.

A causal relationship between endogenous or exogenous stimuli, biotransformation responses and fatigue is not unequivocally established. The need for further basic research remains on the role of receptors, transcription factors and signalling cascades in the coordinated regulation of phase I and phase II metabolism, in addition to phase III transport of endogenous as well as of exogenous agents, for modelling of diseases and toxicities induced by xenobiotics. Furthermore, these developments also call for renewed attention to a rational basis for alternative strategies to identify the metabolic differences that contribute to health and disease in complex multifactorial conditions like fatigue. Against this background, this paper addresses chronic fatigue with the focus on the role of biotransformation as an essential metabolic process for maintaining energy homeostasis in patients suffering from the condition. The results presented here are the outcomes of a seven-year study and data collection

from 576 female patients suffering from low to high levels of fatigue. They were referred by clinicians to our research unit for assessment of their biochemical profiles of phase I and phase II biotransformation. From assessments of this cohort of patients, we indicate a complementarity between a fatigue scale, medical symptoms and their biotransformation profiles and associated energy-related parameters [28][29][30].

In health research, the concept of perturbation of homeostasis through an experimental challenge to quantify health related processes is well established [31][32][33][34]. The response following a challenge reveals multiple aspects of metabolic health that would not be apparent from studying homeostatic parameters. A perturbation from the homeostatic state formed the conceptual basis of our approach and the laboratory instrument used for measuring the biotransformation profile was based on hepatic detoxification challenge tests through oral caffeine, acetaminophen and acetylsalicylic acid ingestion coupled with oxidative stress analyses [35]. Taken together, the interventions with the xenobiotic substances test for the phase I and four phase II hepatic detoxification pathways and provided the scientific rationale for our proposed biotransformation assessment method. In combination with the multidisciplinary contributions from the clinicians, we propose that a novel combination of the three assessment instruments (a fatigue scale, medical symptoms, and biotransformation profiles) provides foundational knowledge for further development of a comprehensive laboratory instrument for directed personal interventions that we believe can lead to improved QOL in patients across a spectrum of chronic fatigue manifestations.

Materials and methods

Ethics statement

This study complied with all institutional guidelines of the North-West University as stipulated by the South African Guidelines for Good Clinical Practice Ethical Guidelines for Research, as well as the terms of the Declaration of Helsinki of 1975 (as revised in 2013) for investigation of human participants. Ethical approval was obtained from the Health Research Ethics Committee (HREC) of the North-West University, South Africa (NWU-00102-12-A1). All written consent was obtained based upon informed decision from all participants. An example of the informed consent form can be found in the S1 File under informed consent.

Patient procedures and selection

This study involved a prospective, observational and measurement design. Women visiting clinicians with complaints of continuous fatigue of various degrees, and the clinical label given to their fatigue condition (DOE) often reflected both the view of the patient and of the doctor about their state because of the widely varying theories about the potential underlying pathology of chronic fatigue. The clinicians informed the patients about the general intentions of the prospective study and asked whether they would consider participation. Pending informed consent, all cases were potentially eligible for the study, regardless of previous types of treatment. Prior to starting the biochemical assessment measurements, the patients underwent observations and fatigue assessment by the clinicians, and were asked to complete a self-report questionnaire that included a demographic profile and an English version of the revised Piper Fatigue Scale (PFS) [36]. The revised PFS used consisted of 22 numerical items which assessed fatigue experienced by the patients. All items were coded on a 0–10 numeric scale. The revised PFS measured four dimensions of subjective fatigue: behaviour/severity, affective meaning, sensory, and cognitive/mood. To calculate a total fatigue score the cumulative sum of all was divided by the total number of items. In addition, a generally accessible Medical Symptoms Questionnaire (MSQ, included in the S1 File) from the Departments of Medicine, Mercy

Hospital and Maine Medical Center, Portland [37] was completed with the assistance of a health consultant for each patient to supplement the information from the PFS. The MSQ contains 19 domains, most with 4 items, totaling 75 indicators to each of which the patient assigns a Likert scale numerical ranking (0 being "never" or "seldom" and 4 being "frequently with severe effect"). Finally, measurement of biochemical parameters from saliva, urine and blood was conducted on samples from all participants who agreed to the investigation.

In total, 673 women with complaints of fatigue were assessed for study eligibility. Of these, 576 were eligible; exclusion criteria for all cases were verified indications of diabetes, chronic high blood pressure, asthma, cancer, arthritis and some other minor clinical conditions, but retaining patients suggestive of fibromyalgia, depression or other affect syndromes. All eligible cases fully completed both questionnaires. The mean age of the participants was 43 years (min.: 18; max.: 82; SD: 14.3), mean body weight 72.0 kg (min.: 40; max.: 150; SD: 17.9) with a mean BMI of 26.3 (min.: 16; max.: 51; SD: 6.3).

Statistical analysis of the Piper Fatigue Scale

We analysed the data of the modified Piper Fatigue Scale (PFS) as a multidimensional model for fatigue in our patient group (data included in the S1 File). Questions 2–23 (22 questions) of the questionnaire were measured on a scale from 1 to 10. Missing values (meaning that a respondent did not answer a particular question and that was applicable to the data from the selected 576 cases) were replaced by 0. To reduce the dimensionality of the 22 items, factor analysis was performed. The sample size was sufficient to allow for factor extraction and so did not limit the factor possibilities to those outlined in the scoring instructions of the PFS. The PFS as developed for oncology patients measured four dimensions of subjective fatigue: behavioural/severity (6 items: #2–7); affective meaning (5 items: #8–12); sensory (5 items: #13–17); and cognitive/mood (6 items: #18–23) over the 22 items used to calculate the four sub-scale/dimensional scores and the total fatigue scores. Having a clinically different fatigue group, we compared various combinations of extraction methods (Principal Components and Principal Axis Factoring) and rotation methods (Varimax Rotation and Direct Oblimin rotation), all of which indicated that there were two underlying factors in our data set. The first factor was seen to load mainly on items 2–17 and is termed Energy Fatigue, whereas the second factor loads mainly on items 18–23 and is termed Mental Fatigue. The factor sores were calculated as follows: Energy Fatigue score: the mean of items 2–17 (16 items); Mental Fatigue score: the mean of items 18–23 (6 items). The reliability of the measurement for the two factors was evaluated by means of Cronbach's Alpha and proved to be high (0.974 and 0.971 for the 16 and 6 energy and mental fatigue factors, respectively).

Subsequently, the patients were categorized according to their fatigue scores. The mean Energy Fatigue score for the group was 5.88 (min.: 0; max.: 10; SD 2.59) and 5.21 (min.: 0; max.: 5.21; SD 2.37) for the Mental Fatigue score. Both scores were categorised as follows: Low level fatigue ($0 < \text{score} < 4$); Medium level of fatigue ($4 \leq \text{score} < 7$) and High level of fatigue (score ≥ 7). These thresholds are based on the severity codes included in the scoring instructions of the PFS, namely: None (0), Mild (1–3), Moderate (4–6), and Severe (7–10). From these levels we defined five combined groupings: (1) Low Fatigue: Energy Fatigue score and Mental Fatigue score below 4; (2) Medium Fatigue: Energy Fatigue score and Mental Fatigue: score below 7 and at least one of the scores equal to 4 or more; (3) Mainly Energy Fatigue: Energy Fatigue score 7 or more, Mental Fatigue: score below 7; (4) Mainly Mental Fatigue: Mental Fatigue score 7 or more, Energy Fatigue: below 7; (5) Energy and Mental Fatigue: Energy Fatigue score and Mental Fatigue score both 7 or more.

Statistical analysis of the Medical Symptoms Questionnaire

This questionnaire has 75 questions divided into 15 main sections; one question in the energy section (about hyperactivity) was omitted to enhance the reliability. The scores were calculated as the mean of the items per section and the items were also combined in an overall MSQ score. The reliability per section ranged from 0.582 for the 5 items defining MSQ related to "Skin", and 0.858 for the 6 items related to "Joint". For this data set the Factor Analysis Rotation and Extraction methods did not converge, and PCA did not provide useful outputs. We therefore decided to calculate factor scores based on the sections and total.

Statistical profile of the biochemical data

The data consist of 15 chemical measurements to define the biochemical variables and an additional variable for measurement of the filtrate volume required for some calculations. In addition the complete data set also included five ratios which we considered to be informative as well: (1) total acylcarnitine to free carnitine ratio; (2) uric acid to creatinine; (3) phase I to phase II glucuronidation; (4) phase I to phase II sulphation; and (5) phase I to phase II glycination ratio. Statistical analysis of the biochemical variables and ratios revealed extensive skewness and kurtosis of the original data that could be improved by $\log_e$-transformation of the original data. In addition to analysis of the data for the individual biochemical variables, an inherent underlying relationship among the measurements was apparent. Since the logarithm of a ratio is equal to the difference between the logarithms, this meant that some of the transformed variables are linear combinations of other variables; thus the covariance matrix was singular and factor analysis was not possible; because the variables represent precise measurements rather than opinions, principal components analysis (PCA) is considered to be a viable option. PCA suggested that 8 factors (designated as BioF1 to BioF8) showed a strong underlying relationship among the biochemical data. These factors constitute the underlying relations among the components of the factors. "Furthermore, the human urine metabolome confirmed variation in urine metabolites, based on the populations (age, gender) from which the concentrations of the metabolites were derived[38]. In our data set, confounders were confirmed if showing a practically significant correlation ($|r| \geq 0.5$). While we report on the p-values, the level of significance was not considered as this metric is known to be affected by sample size. A sample size of 576, as used here, is likely to produce statistically significant effects of little practical importance. Taken together, from the correlation analysis of our data it appeared that the age, height, weight or BMI of the present experimental group did not have a confounding effect on the biochemical data or on the classification of fatigue groups. (The correlation analysis with potential confounding effects is shown in Table A in the S1 File).

Statistical methods used

The analysis of all data generated from the investigation was performed according to well-described and validated methods[39] [40].

Biotransformation analysis

The measurement design for the biotransformation interventions is shown schematically in Fig 1.

The first intervention is a caffeine clearance test, followed after a fixed period by acetaminophen and acetylsalicylic acid interventions. The biofluids used in the study consisted of saliva, blood and urine. Samples were collected for analysis after challenge substances provided in the test kit were taken by the participants. Phase I biotransformation was challenged with a 150

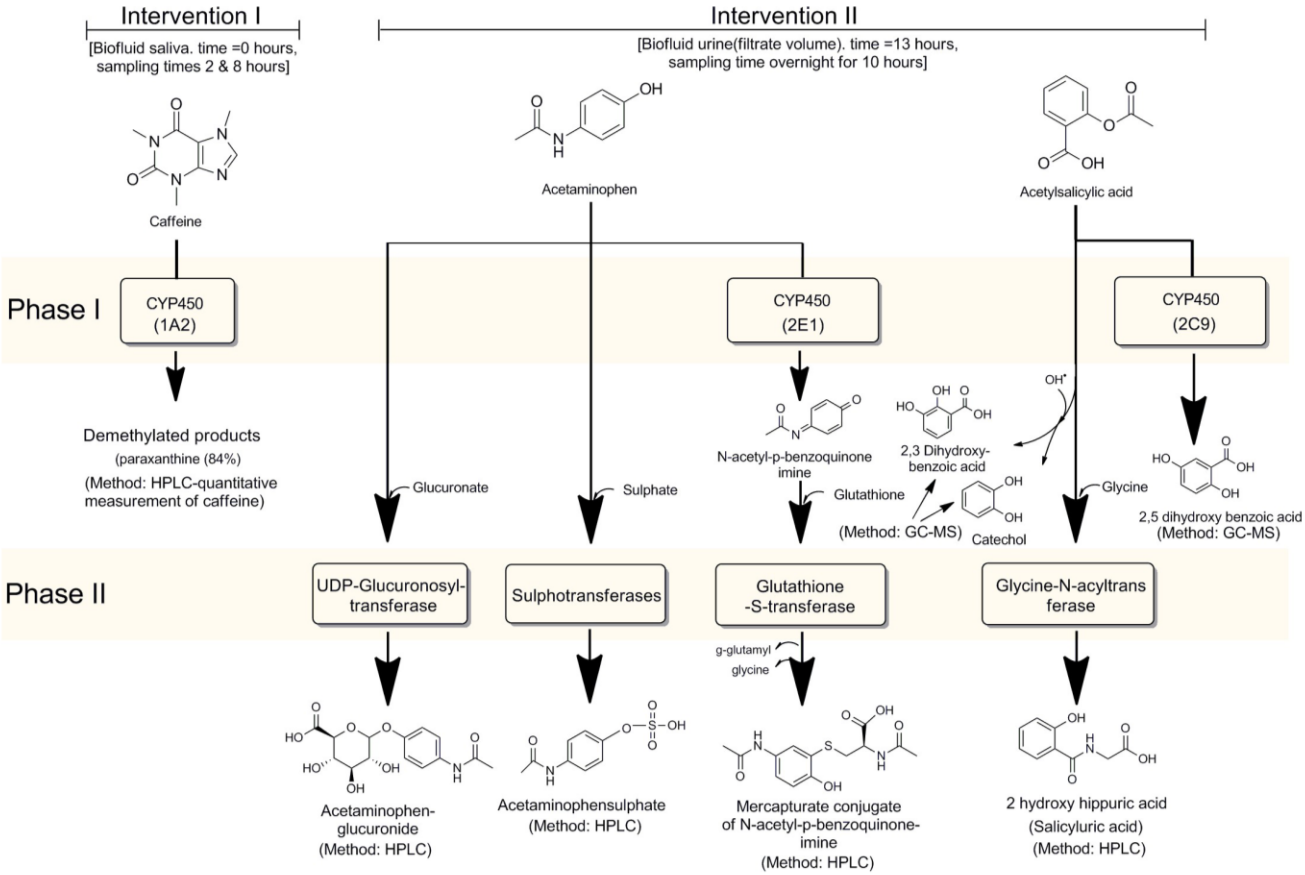


Fig 1. Measurement design for the interventions to assess the biotransformation profiles for all fatigue patients. Caffeine clearance (Intervention 1) progresses through CYP450-1A2, comprises only a phase 1 process and is measured as the residual substrate in saliva, expressed as a percentage of caffeine recovery. The acetaminophen challenge involves three phase II conjugation reactions, evaluated through the products formed following biotransformation: (1) conjugation of glucuronic acid to the hydroxyl group of acetaminophen; (2) sulphation of the phenolic hydroxyl group of acetaminophen; and (3) glutathione conjugation of N-acetyl-p-benzoquinone imine, a phase 1 activated form of acetaminophen. In the simultaneous acetylsalicylic acid challenge, glycination of the functional carboxylic group is catalysed by glycine-N-acyl transferase (GLYAT). All the substances mentioned are measured in urine samples, analysed by HPLC, as indicated. The acetaminophen and salicylate ingestion detoxification are measured through the glucuronide, sulphate and mercapturate conjugates as well as for salicyluric acid, expressed as percentage recovery. Three derivatives of salicylate may likewise be observed in this challenge (2,3-DHBA, 2,5-DHBA and catechol) and are measured by a GC-MS method. Abbreviations: CYP450-1A2, -2E1 and -2CP: mixed function CYP450 oxidase system with isozymes as indicated; GLYAT: glycine-N-acetyltransferase; HPLC: high-pressure liquid chromatography; GC-MS: gas chromatography–mass spectrometry; GSH: glutathione; 2,3-DHBA: 2,3-dihydroxy benzoic acid: 2,5-DHBA: 2,5-dihydroxybenzoic acid.

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mg caffeine tablet before breakfast at 8h00 in the morning. Saliva samples were collected in salivettes at 10h00 and 16h00. The phase II biotransformation challenge involved two aspirin (600 mg) and two paracetamol tablets (1000 mg) taken at 21h00 in the evening and overnight and early morning (ending at 7h00) urine samples collected in a special container provided in the test kit. Blood samples (2 EDTA and 1 clotted tube) for additional biochemical analyses were collected 24 hours later when the frozen urine and saliva samples were handed in at a pathology depot for selective analyses as indicated. The methods used for the measurement of phase I and II biotransformation are new and are described below with additional technical information presented in the S1 File.

Phase I analysis. Sample preparation: C18 solid phase extraction cartridges (HF Bond Elut C18–100 mg) were used for sample clean-up and extraction of caffeine; 200 μl internal

standard 2-acetamidophenol (5 μg/ml) was added to 200 μl saliva sample prior to extraction. Conditioning of the SPE columns was performed with 1 ml methanol and 2 ml water. A 200μl sample with 200μl internal standard was added to the conditioned SPE column and washed with 1 ml water. Samples were eluted with 1 ml methanol acetate solution (pH 3.5), dried under nitrogen and then lyophilized. Samples were reconstituted in 200 μl after which 5 μl was injected into the HPLC. The Agilent 1200 HPLC system was equipped with a binary pump, inline degasser, auto sampler, heated column compartment and diode array detector. The analytical column was an Eclipse XDB C18, 5 μm, 4.6 x 150 mm reverse phase column. A flow of 1 ml/min was used, column temperature 25˚C and the UV detector set at 273 nm. From a caffeine stock solution (1 mg/100 ml), a six-point series dilution calibration range was made up ranging from 0.156 μg/ml to 5 μg/ml for quantification.

Phase II analysis. Aliquots of 500 μl from the 10 hour overnight collected samples were centrifuged and 200 μl of each sample combined with 200 μl internal standard (Allopurinol 250 μg/ml) for the HPLC analysis. The analytical column was a Phenomenex Luna 3μ C18 (2) 100 A, 150 x 2.00 mm reverse phase column. Column temperature was 35˚C, flow rate 0.25 ml/min and the UV detector was set at 254 nm for analyte detection. Mobile phase A was H_2O with 0.05% TFA and mobile phase B was HPLC-grade methanol. Chromatographic separation of analytes was achieved with the following gradient mobile phase composition: 10% B from 0–2 min; an increase to 20% B to 16 min, from 16–19 min to 90% B and from 19–24 min 90% A and 10% B. To accommodate physiological concentrations for the different conjugates stock solutions a 9-point series dilution calibration range were used for each conjugate, ranging from 3.905 μg/ml to 1000 μg/ml.

Secondary products from the acetylsalicylic acid challenge. The three secondary products from the acetylsalicylic acid challenge–catechol, 2,3-DHBA and 2,5-DHBA–were analysed using GC-MS. Creatinine-corrected urine volumes were subjected to organic solvent extraction after 3-phenyl butyric acid was added as internal standard and the pH adjusted to 1 with 5N HCl. Six ml HPLC grade ethyl acetate and 3 ml diethyl ether were used as extraction solvents. After roto-torque agitation for 10 minutes, the solvent layer was removed and dried under a stream of nitrogen. Derivatization was done with BSTFA and TMCS. An Agilent 7890A gas chromatograph connected to an Agilent 5975B MSD mass spectrometer was used for analysis. The analytical column used was a 30 m x 0.25 mm x 0.25 mm DB1-MS JW Scientific column. High purity helium was used as carrier gas. A 1 μl sample was injected with a temperature programme starting at 100˚C for 2 min, increased by 4˚C to 120˚C, then by 6˚C to 215˚C and finally by 20˚C to 300˚C. Post-run time was 1 min at 295˚C. Selective ion monitoring was conducted for catechol (254.1 m/z), IS (221.1 m/z), 2,3-DHBA (355.2 m/z) and 2,5-DHBA (355.2 m/z). An 8-point calibration range of the standards was used for final quantification.

Ferric reducing antioxidant assay (FRAP assay). Measurement of the ferric reducing ability of plasma, known as the FRAP assay, provides a way for assessing 'antioxidant power' and which we used in the present study. FRAP assays were performed on a BIO-TEK FL600 fluoresence microplate reader. Amounts of 15 μl serum were added to 85 μl Milli-Q water in 96-well microplates. The sample plates were incubated for 10 min at 25˚C. To this, 250 μl FRAP reagent (2.5 ml FRAP acetate buffer; 2.5 ml TPTZ reagent and 2.5 ml $FeCl_3$ reagent) was added. FRAP acetate buffer: 1.55 g anhydrous sodium acetate in 500 ml Milli-Q water. TPTZ reagent: 312.4 mg 2,4,6-tris-2-(pyridyl)-1,3,5-triazine in 100 ml 40mM HCl. $FeCl_3$ reagent: 4.56 mg of $FeCl_3.6H_2O$ was dissolved in 100 ml FRAP acetate buffer (300mM). FRAP values were recorded 80 seconds after addition of the FRAP reagent. A 6-point calibration range containing 0, 20, 40, 60, 80 and100 μl of a FRAP standard (0.275 $FeSO_4.7H_2O$ in 100 ml milliQ H_2O) was used as calibrator.

Reactive oxygen species (ROS assay). ROS assays were also conducted on a BIO-TEK FL600 fluoresence microplate reader; 140 μl sodium acetate (0.1M; pH 4.8) was added to the wells used in the 96-well plate; 2.5 μl samples were added in triplicate; 100 μl of a 1:25 DEPPD (100 mM) and $FeSO_4$ (43.7 μM) solution was added to the sample wells. Plates were incubated at room temperature for 1 min; plates were read at 546 nm every minute for 10 min. A calibrator concentration range with 6 concentrations ranging from 0–300 mg/l hydrogen peroxide was used for quantification of samples.

Total glutathione (T-GSH) assay. Total glutathione levels in blood samples were determined using the GSH/GSSG kit from Oxis International. Whole blood EDTA vacutainer samples were mixed and 350 μl metaphosphoric acid (MPA) mixed with 50 μl blood sample, centrifuged at 10 000 x g for 10 min; 12.5 μl of the MPA extract was then added to 750 μl assay buffer. A 50 μl sample was then added in triplicate to the microplate and mixed for 5 min; 50 μl NADPH was then added and the plates were read at 412 nm for 3 min at 1-minute intervals. GSH (0–3.0 μM) and GSSG (0–0.15 μM) 6-point calibration ranges were used for quantification.

Creatinine and uric acid. Both creatinine and uric acid values in urine were determined using the Konelab T series analyser with the uric acid (AOX) and creatinine (enzymatic) kits.

Carnitine and acylcarnitine analysis. Carnitine and acylcarnitine were measured on the 10-hour urine samples. Urine (100 μl) was transferred to an Eppendorf tube and centrifuged at 1300 rpm for 20 min; 10 μl of the centrifuged urine was added to 410 μl of a deuterated carnitine/acylcarnitine stable-isotope solution in a new Eppendorf tube. The sample mixture was then dried under nitrogen at 65˚C for ~15 min; 100 μl 3N butanolic HCl was added to the dried sample and incubated at 65˚C for another 15 min. Excess butanolic HCl was evaporated under nitrogen. The samples were then reconstituted in 100 μl acetonitrile: water (50:50) (v/v) with 0.1% formic acid. Samples were delivered to the electrospray mass spectrometer (Agilent 6410 Triple Quadrupole, Palo Alto, CA) via an Agilent 1200 series LC (Palo Alto, CA) and 96-well plate sampler. A 10 μl sample was injected per analysis at a constant mobile phase (1% formic acid in water: acetonitrile (50:50) (v/v)), flow rate of 0.2 ml/min. The mass spectrometry analyses were carried out in positive ionization mode with controlled collisional induced decomposition (fragmenter voltage 135 V and collision energy 20 V) and the precursor of 85 Da function used to selectively detect the different carnitine species. The deuterated analogues of the different carnitine and acylcarnitines (working solutions: free carnitine = 1.8815 μmol/l; acetylcarnitine = 0.6075 μmol/l; propionylcarnitine 0.1616 μmol/l; isovaleryl carnitine = 0.1400 μmol/l; octanoyl carnitine = 0.1233 μmol/l and palmitoyl carnitine = 0.3144 μmol/l) were used for the quantification of individual carnitine species. Linear regression calculations were used to determine the concentration of the carnitine/acylcarnitines by comparison of the signal intensity of carnitine/acylcarnitines against that of the corresponding deuterated stable isotopes. Concentrations of the analysed carnitine and acylcarnitines were expressed in mmol/mol creatinine.

Reagents. Caffeine, allopurinol, paracetamol-glucuronide, paracetamol sulphate, high-purity trifluoroacetic acid, salicyluric acid, N,N-diethyl-paraphenylenediamine (DEPPD), sodium acetate, metaphosphoric acid (MPA) and hydrogen peroxide were supplied by Sigma Chemicals. Paracetamol-mercapturate was supplied by Toronto Research Chemicals. HPLC grade acetonitrile and water was supplied by Burdick & Jackson. Ferrous sulphate was supplied by Labchem. Acetamidophenol, phenylbutyric acid, 2,3 & 2,5 dihydroxy benzoic acid, NADPH, GSH, GSSG and catechol were all obtained from Sigma-Aldrich. Ethyl acetate and diethyl ether were purchased from Merck. N,O Bis(trimethylsilyl)fluoro acetamide (BSTFA) and trimethylchlorosilane (TMCS) were supplied by Sigma Aldrich. 2,4,6-tris-2-(pyridyl)-1,3,5-triazine (TPTZ) and Ferri Chloride ($FeCl_3.6H_2O$) were also supplied by Sigma

Chemicals. Carnitine and acylcarnitine reference standards and deuterated analogues were obtained from Cambridge Isotope Laboratories.

Results

A flow diagram of clinical and biochemical data acquisition procedures, comprehensive approaches of the statistical analyses and resulted outcomes is shown schematically in Fig 2.

The five categories of fatigue patients according to the PFS are shown in Fig 3. As reported by the physicians, the level of fatigue experienced by these subjects ranged from low (indicated by 117 blue dots in Fig 3; 20.3% of the group) to high (130 red dots in Fig 3; 22.6%). The analysis of the PFS of the mild, energy only and mental only fatigue categories were 196 (corresponding to 34.0% of the survey population), 107 (18.6%) and 26 (4.5%), respectively. These results clearly indicate that a physical experience of energy depletion, rather than a psychological mood of mental fatigue, dominates in the experimental group. The descriptive information (mean values and standard deviations) on the 15 original biochemical measurements over the five fatigue groups, as defined in Fig 3, are listed in Table 1.

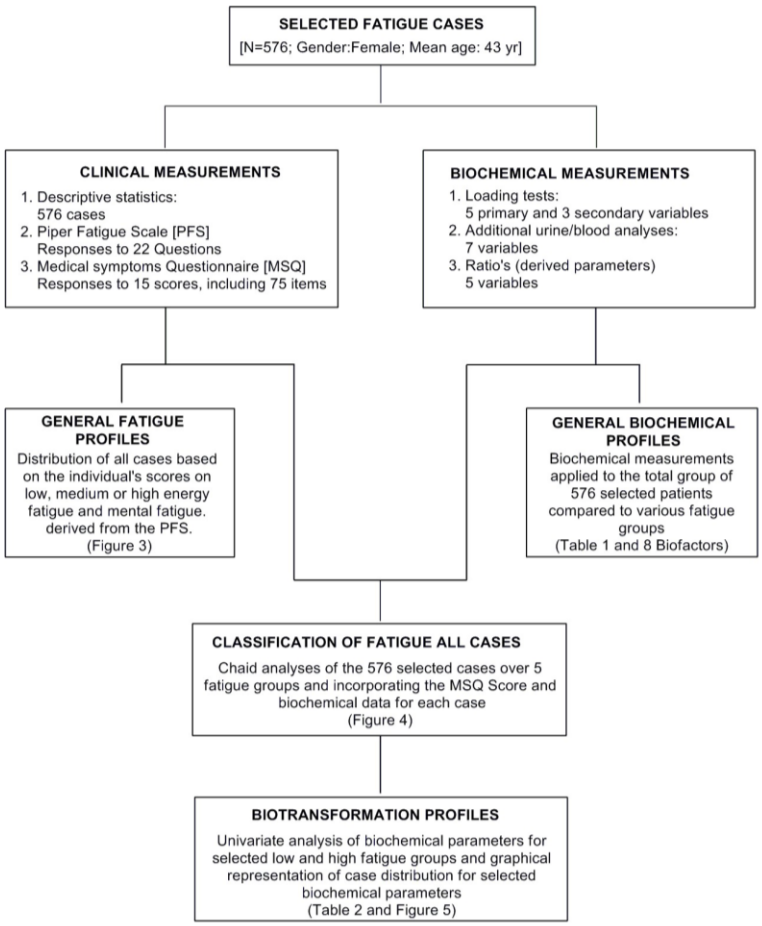


Fig 2. Schematic flow diagram for the analysis of clinical and biochemical data for exploratory biotransformation profiling of samples from all fatigue patients.

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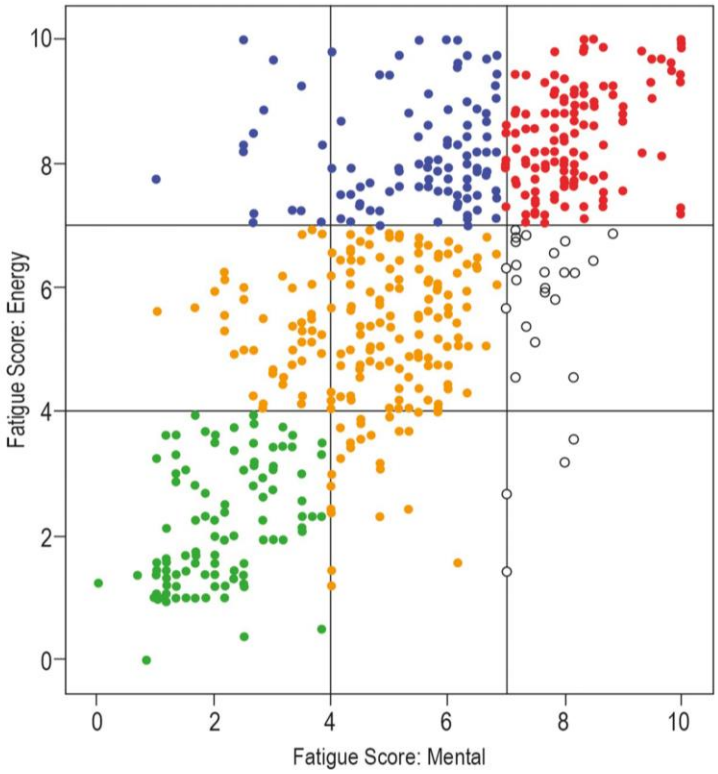


Fig 3. Distribution of all selected cases based on the individual scores in terms of energy fatigue and mental fatigue derived from the PFS. The scores are categorized as low level fatigue ($0 <$ score < 4), medium level of fatigue ($4 \leq$ score < 7) and high level of fatigue (score ≥ 7). This distribution indicates the presence of five PFS groups: Low fatigue (green), mild fatigue (orange), mainly energy fatigue (blue), mainly mental fatigue (open circles), and high energy and mental fatigue (red).

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An explorative comparison of the original data for the low fatigue group with the four other fatigue groups implies some trends based on the mean values, whereas the trends based on the untransformed data from the 576 patients do not prove to be significant ($p > 0.05$). The main trends observed are: (1) Somewhat lower phase I biotransformation (caffeine clearance) in both the Mainly Energy Fatigue [0.61% (SD = 0.53)] and Mainly Mental Fatigue [0.56% (0.45)] groups relative to the Low Fatigue [0.64% (0.59)] group. (2) This trend also seems to prevail for phase II biotransformation. (3) The antioxidant capacity in the Mainly Mental Fatigue [FRAP = 396 μmol/l (SD = 110); GSHt = 895 μmol/l (180)] group seems to be better than in the Low Fatigue [FRAP = 351 μmol/l (SD = 81); GSHt = 860 μmol/l (182)] group, which is reflected in apparent less oxidative stress in the Mainly Mental Fatigue [ROS = 103 μmol/l (SD = 38); catechol = 10 μmol/l (7)] than in the Mainly Energy Fatigue [ROS = 110 μmol/l (SD = 39; catechol = 13 μmol/l (SD = 14)] groups.

To explore these trends further, statistical clusters from the data were formed to identify potential associations between the biochemical measures and fatigue scores, for which two strategies were considered: (1) using factor analysis to identify any underlying structure in the biochemical measures observed, and (2) using a CHAID analysis to refine the 5 fatigue groups. The factor analysis of the Z-scores of the $\log_e$-transformed data suggested that 8 factors showed a strong underlying relationship among the biochemical data and some ratios between the

Table 1. Descriptive statistics of the biochemical variables and their manifestation across the original data for the five fatigue groups.

Biochemical Measure	Mean (SD)				
	Low Fatigue (N = 117)	Mild Fatigue (N = 196)	High Energy & Mental Fatigue (N = 130)	Mainly Energy Fatigue (N = 107)	Mainly Mental Fatigue (N = 26)
1. Caffeine Clearance Test					
Phase I	0.64	0.66	0.60	0.61	0.56
	(0.59)	(0.53)	(0.53)	(0.53)	(0.45)
2,5-DHBA	28.02	25.51	29.37	31.20	32.94
	(23.68)	(22.62)	(20.56)	(63.41)	(31.1)
2. Acetaminophen Challenge Test					
Glucuronidation	27.89	29.95	30.53	31.22	26.15
	(13.19)	(15.91)	(15.15)	(15.44)	(10.89)
Sulphation	17.35	17.32	17.51	15.82	15.44
	(8.38)	(8.13)	(8.93)	(7.65)	(6.45)
Glutathione Conjugation	1.56	1.73	1.74	2.50	1.48
	(0.86)	(1.02)	(1.27)	(6.09)	(0.82)
3. Acetylsalicylic Acid Challenge Test					
Salicyluric acid	10.48	12.16	11.25	11.39	10.02
	(5.37)	(7.25)	(5.51)	(5.6)	(5.06)
4. Variables Related to Oxidative Stress					
Catechol	13.41	13.75	12.99	13.34	10.31
	(14.37)	(24.14)	(12.12)	(14.43)	(7.98)
2,3-DHBA	4.27	5.17	4.31	3.61	4.24
	(4.82)	(5.59)	(4.42)	(3.02)	(5.26)
FRAP*	351.48	353.47	353.23	332.09	396.78
	(81.35)	(67.96)	(63.68)	(70.66)	(110.53)
GSHt**	860.89	876.88	873.47	846.50	895.68
	(182.95)	(181.19)	(197.39)	(174.23)	(180.09)
ROS***	107.25	109.64	106.88	110.72	103.75
	(33.37)	(39.44)	(34.89)	(39.74)	(38.93)
5. Other					
Uric acid	1.60	1.83	1.55	1.83	1.26
	(1.16)	(1.49)	(1.31)	(1.42)	(0.73)
Creatinine	8.59	10.01	8.81	9.47	9.63
	(6.1)	(6.24)	(6.14)	(6.36)	(6.75)
Total Acylcarnitine	5.36	5.15	4.76	4.55	3.88
	(3.88)	(3.65)	(3.48)	(2.96)	(2.52)
Total Free Carnitine	4.75	4.83	4.43	3.57	3.10
	(3.58)	(4.77)	(4.23)	(2.19)	(2.3)

* FRAP- Ferri reducing ability of plasma

**GSHt- total red blood cell glutathione

***ROS- Reactive Oxygen Species

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variables, with Biofactor 8 consisting of only the total acylcarnitine/total free carnitine ratio. The approach used for the factor analysis was adopted as the various metabolites were measured on different scales. This analysis indicates the underlying relation between the components of the factors and supports the selection of variables which we used for our biotransformation instrument. The 7 biofactors which consist of more than one indicator, including our broad interpretation, are:

Biofactor 1: (Phase 1; Phase 1/Phase II glucuronidation ratio); Phase 1/Phase II sulphation ratio; Phase I/Phase II glycination ratio). This biofactor refers to phase I activation in relation to the measured phase II conjugation reactions.

Biofactor 2: (Phase II glucuronidation, sulphation, mercapturation and glycination). These four variables indicate a strong underlying relationship between the four phase II conjugation reactions.

Biofactor 3: (Creatinine and uric acid). The mean urinary creatinine and uric acid excretions in the patients with gout were reported to be significantly increased as compared with those of normal controls[40], but this link is unlikely to be associated with fatigue.

Biofactor 4: (Total free carnitine and total acyl carnitine). These two variables are important indicators of fatty acid metabolism and whether enough free carnitine is available to transport long-chain fatty acids across the mitochondrial membrane. They may indicate a redox imbalance.

Biofactor 5: (Catechol, 2,3-DBHA and 2,5-DBHA). These three metabolites are formed following the acetylsalicylic acid challenge, as indicated in Fig 1, with catechol and 2,3-DBHA formed through free radical mechanisms (indications of oxidative stress) and 2,5-DHBA through phase I, catalysed by a different CYP450 isozyme from in the caffeine challenge.

Biofactor 6: (Uric acid and uric acid: creatinine). Creatine phosphate is catabolized to creatinine in ATP depletion, which may cause accelerated urate synthesis.

Biofactor 7: (ROS; FRAP; GSHt). The combination of these three variables indicates an obvious relation between antioxidant capacity (endogenous GSH and an experimental measure of antioxidant capacity through FRAP) and reactive oxygen species (ROS).

Studies on human liver microsomes indicated the formation of both 2,3- and 2,5-DHBA from salicylic acid (Biofactor 5), but apparently only 2,5-DHBA is produced by the microsomal fraction challenged through aspirin. Catechol, the third member of Biofactor 5, is a secondary product likewise resulting from a free radical reaction. A comparison between the clinical and biochemical data indicates that the canonical correlation between two sets of variables from the clinical data was high for the 22 items from the PFS and from the MSQ (0.789 for 15 items and 0.048 for the 75 sub-items), but not so for the correlations with the biochemical factor data [PFS (22 items) and biochemical factors (8 items): 0.299; MSQ (15 scores) and biochemical factors (8 items): 0.598; MSQ (75 sub-items) and biochemical factors (8 items): 0.484].

Subsequently, the data were subjected to a CHAID analysis, used as a classification instrument and for detection of interaction between the PFS grouping, on the one hand, and MSQ and the biochemical variables, on the other hand. CHAID classification is based on Bonferroni-adjusted significance testing and is a type of decision tree. Like other decision trees, CHAID's advantage is that its output is highly visual and easy to interpret. The visual model (Fig 4) clearly highlights how the medical profiles and metabolic features correspond to specific fatigue states.

Node 0 in Fig 4 shows that the highest percentage of the 576 individuals (34%, 196 women) experienced mild fatigue whereas (22.6%, 130) experienced high fatigue. Based on the MSQ overall score, the CHAID analysis next subdivided the individuals into 5 nodes (nodes 1–5). On the two extremes of the MSQ score (< = 28 and 147), 70% of the 60 cases in node 1 experienced low fatigue whereas 61.4% of the 57 cases in node 5 experienced high energy and mental fatigue. The next subdivision produced 9 nodes (nodes 6–14). The biochemical variables assigned to this subdivision were creatinine (in the mild fatigue node 2; MSQ 28–55), total free carnitine (in the mild fatigue node 3; MSQ 55–90) and 2,5-DHBA (in the energy and mental fatigue nodes 4 and 5; MSQ 90–147 and MSQ >147). 2,5-DHBA is of special interest as a discriminator between mild and high energy and mental fatigue. The high fatigued state is

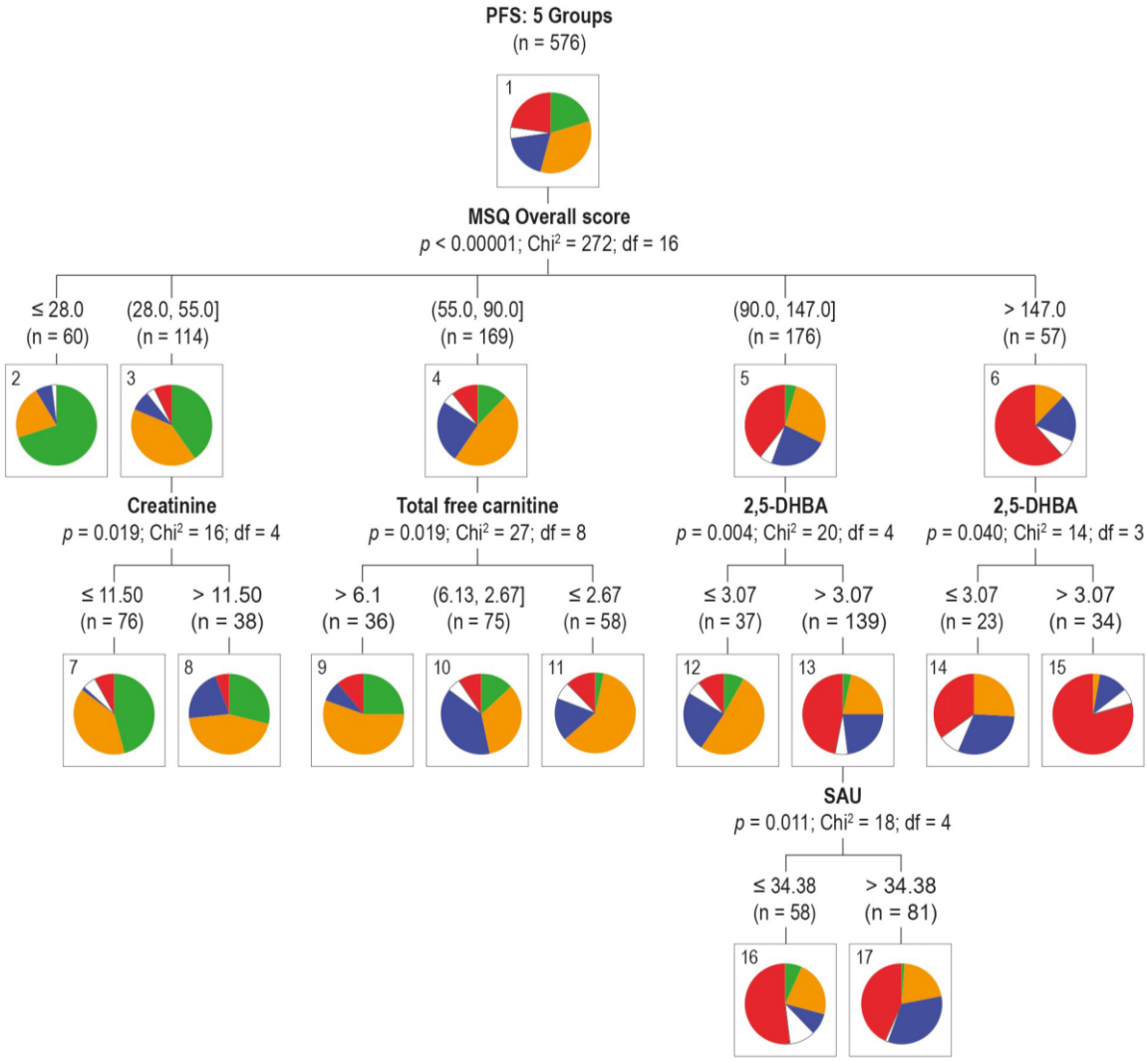


Fig 4. CHAID analysis of the 576 selected cases over 5 fatigue groups (colour code as in Fig 2), incorporating the data from the biochemical and MSQ score analysis.

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associated with levels of 2,5-DHBA >3.067 (node 12). Node 12 is further subdivided into nodes 15 and 16, where 81 of the 139 cases in node 12 displayed glycine conjugation values >34.38.

Finally, we investigated the biochemical data of a low and high fatigue group, based on the CHAID classification. The low fatigue group consisted of 37 cases, having PFS <4.0 for energy as well as mental fatigue and a score from the MSQ ≤ 28. The high fatigue group also comprised 37 subjects, having PFS >7.0 for energy as well as mental fatigue and a score from the MSQ >147. We used the original data through a parametric analysis for determination of mean group values and the fold changes (FC), and the transformed data for determination of significance, expressed as p-values and effect sizes (ES), shown graphically for important variables in Fig 5.

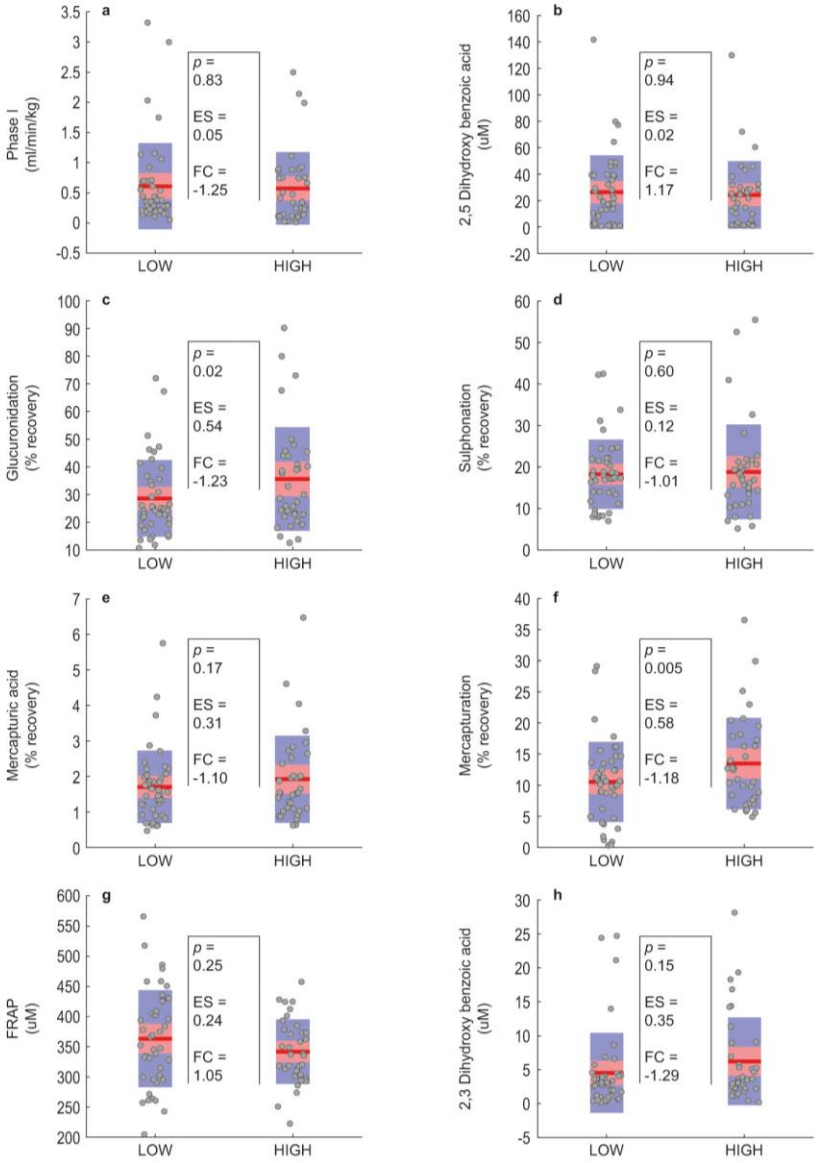


Fig 5. Graphs showing potentially important metabolites for the low and high fatigue groups, selected according to the CHAID classification. Indicated in the figure are: Patients with low fatigue (graphs to the left) relative to those with high fatigue (graphs to the right) in each panel. Values for all individual cases are shown as dots, whereas the squared area represents the 95% confidence interval (orange) and one standard deviation (blue) about the mean (red line). Fold change (FC) measures are indicated for the low relative to the high fatigue groups, and were determined from the original experimental data. The statistical and practical significance of differences (*p*-values and ES) were calculated from the transformed data. The respective p, ES and FC values shown in Fig 5 were calculated as mentioned above, but all data points in these figures represent the original experimental values.

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The graphical data indicate that phase I biotransformation was comparable between the low and high fatigue groups (Fig 5A and 5B). The mean percentage detoxification observed from the three biotransformation modes following the acetoaminophen challenge (27.89:17.53:1.53% recovery and 30.53:17.52:1.74% recovery for glucuronidation:sulphation:mercapturation for the

low and high fatigue groups as shown in Table 1), resembles the detoxification profiles reported in the literature (glucuronidation > sulphation > mercapturation) [35]. Moreover, the acetoaminophen challenge clearly indicated that phase II glucuronidation (Fig 5C) was significantly increased in the high fatigue group (FC = +1.23; p = 0.02), with practical significance (ES = 0.54). Phase II sulphation did not differ between the low and high fatigue groups (FC = +1.01), but Phase II mercapturation was somewhat increased (FC = +1.10) in the high fatigue group (Fig 5D and 5E, respectively). Salicyluric acid excretion following the salicylic acid challenge was significantly higher (FC = +1.18; p = 0.00) in the high than in the low fatigue groups (Fig 5F), which also appears to be practically significant (ES = 0.58). The statistical metrics shown for FRAP (Fig 5E) and 2,3-DHBA (Fig 5F) resemble the trends observed for these values as shown in Table 1. Taken together, these results support the factor analysis which indicated a strong underlying relationship between phase I and II biotransformation (Biofactor 1), between the four modes of phase II biotransformation (Biofactor 2) as well as on the relation between high antioxidant capacity and low oxidative stress (biofactors 5 and 7).

With regard to the remaining supplementary biochemical measurements, we observed a decrease in the total acylcarnitine within the high fatigue group (FC = −1.25; p = 0.06), which may be interpreted as being practically significant (ES = 0.41). Likewise, the free carnitine also appeared to be lower in the fatigue group, as suggested by the proposed relation between these variables by the factor analysis (Biofactor 4). Finally, the proposed relation between uric acid and creatinine (Biofactor 3) was not statistically informative (FC = +1.04; p = 0.97 and FC = −1.05; p = 0.76), although biochemically relevant.

Discussion

In this cohort study, we used two qualitative instruments (MSQ and PFS) and quantitative data (biochemical measurements) on 576 women found to suffer from periodic or chronic fatigue with no definitive tests or findings to establish a clinical diagnosis. Although the revised PFS that we used [36] was designed as a measure of fatigue in women with breast cancer, the scale was previously reported to be successfully used for different population groups [41][42][43] and the results presented here indicate that the revised PFS also provide a measure of fatigue. The state of fatigue in the patient group appeared to be highly diverse, but could be divided into five well-defined categories based on the fatigue scores from low to high. The structure of the measurements appeared to be represented by a bi-factor model (designated Energy Fatigue and Mental Fatigue), for which the reliability derived from the statistical analyses of the data proved to be high (Cronbach's Alpha of 0.974 and 0.971 for the Energy and Mental Fatigue factors, respectively). This provides evidence that the four subscales of the PFS may represent distinct aspects of the fatigue experience as reported by patients with the condition, supported by the different trends in the biotransformation profiles observed for the Mainly Energy Fatigue and the Mainly Mental Fatigue groups (Table 1). A comparison of the clinical and biochemical data indicates that the canonical correlation between two sets of clinical variables was high (Cronbach's Alpha = 0.789 for 15 main items of the MSQ) with smaller correlations with the original biochemical and factor data. Nevertheless, using CHAID analysis as the classification instrument of data derived from all three sets (MSQ, PFS and biochemical) provides a clear visual representation showing the advantage of the data from the MSQ used as a classification instrument and for detection of interaction between the three classes of variables (Fig 4). The observation that the 20 biochemical variables grouped into 8 biofactors, based on a PCA of the transformed data, emphasizes a strong underlying relationship among the biochemical variables which we developed as the biotransformation instrument and show

Table 2. Selected studies on the association between biotransformation of xenobiotics and genetic polymorphisms in the *GLYAT* gene.

PUBLICATION*	POLYMORPHIC VARIANT(S) AND RELEVANT INFORMATION ABOUT GLYCINE N-ACYLTRANSFERASE (GLYAT, EC 2.3.1.13)	OBSERVATION / INTERPRETATION
Badenhorst et al. [44]	A review paper in which: 1. It is argued that the major role of glycine conjugation is to dispose of the end products of phenylpropionate metabolism. 2. Genetic variation in the human GLYAT gene can influence the enzyme activity of a bacterially expressed recombinant human GLYAT. 3 The effects of six SNPs on enzyme indicated that the variant with the highest enzyme activity had the highest allele frequency, and that those with negative effects on enzyme activity occur very rarely. 4. The primary purpose of glycine conjugation is the detoxification of gut microbial benzoate and benzoyl-CoA, as illustrated through a schematic representation of the glycine conjugation pathway.	1. In humans the glycine N-acyltransferase enzyme (GLYAT) is important for biotransformation of xenobiotics which have a carboxylic acid group 2. Benzoate is potentially much more toxic than initially anticipated.
Van der Sluis et al. [45]	Site-directed mutagenesis was used to generate six non-synonymous SNP variants of the enzyme (K16N; S17T; R131H; N156S; F168L; R199C). The enzyme activities of the K16N, S17T and R131H variants were similar to that of the wild-type, whereas the N156S variant was more active, the F168L variant less active, and the R199C variant was inactive. An E227Q mutant, which lacks the catalytic residue proposed by Badenhorst et al. [46] was also synthesized.	1. The finding that SNP variations in the human GLYAT gene influence the kinetic properties of the enzyme may explain some of the inter-individual variation in glycine conjugation capacity.
Van der Sluis et al. [47]	With the exception of the South African Afrikaner Caucasian population (S156–86.9%; L61S156–10.7%), only two haplotypes namely S156 and T17S156, were identified in all populations as having the highest haplotype frequencies (70% and 20% respectively).	1. This supports a previous suggestion that the S156 haplotype should be considered as the wild-type sequence. 2. The results in this study indicate that the GLYAT-ORF is highly conserved.
Nortje et al.[48]	A challenge test was done in the presence of two single nucleotide polymorphisms (SNPs) of the GLYAT gene with the highest allele frequencies across available global population data, namely theN156S (rs675815) and S17T(rs10896818).	1. All individuals (n = 10) were homozygous in this polymorphic region. 2. Haplotypes which may have a negative impact on enzyme activity were present at very low frequencies.

*- All of these listed publications came from our research group which has published a number of papers on glycine N-acyltransferase

Although these polymorphisms may contribute to inter-individual variation, the challenge tests incorporated variation coming from the availability of conjugation substrate (glycine), Coenzyme A sequestration and Adenosine Triphosphate (ATP) for xenobiotic activation to the corresponding CoA-derivative.

Measuring the effect of known polymorphisms on Phase II biotransformation falls beyond the scope of this research but warrants further investigation.

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that biotransformation and associated energy-derived parameters are indicative of undefined chronic fatigue observed in the patient group.

However, some limitations by focusing primarily on the results of statistical analysis need to be taken into account. Firstly, there is compelling evidence that the genes for glucuronidase (EC 3.2.1.31), sulphotransferase (EC 2.8.2.1) and glutathione-S-transferase (EC 2.5.1.18) are highly polymorphic. Likewise single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) have been extensively reported for phase II genes. Each of these genetic aberrations affects the individuals' Phase II biotransformation status in general, and may specifically be implicated in the acetoaminophen challenge used here, but this highly complex field was taken into account in the current manuscript. By contrast, we have previously reported on the genetic variation for the gene coding for glycine-N-acyl transferase (E.C. 2.3.1.13), involved in biotransformation of acetylsalicylic acid. Our main previous observations in this regard, are summarized in Table 2.

In our review [44] we argued that the major role of glycine conjugation is disposal of the end products of the gut microbiome, like those derived from polyphenols and phenylpropionate metabolism. Natural substrates for glycine conjugation would thus include benzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, 4-aminobenzoate, and salicylate. Glucuronidation would not be ideal for the detoxification of these substances, although glucuronidated salicylate conjugates were observed in urine following aspirin dosing [49]. These substances were not detected in our analysis. However, significant inter-individual variation does exist in

glycine conjugation capacity. To investigate the influence of SNPs in the GLYAT coding sequence on enzyme activity, we expressed and characterized a recombinant human GLYAT and introduced site-directed mutagenesis to generate six non-synonymous SNP variants of the enzyme [45]. The enzyme activities of the three variants were similar to that of the wild-type, one variant was more active, one less active, and one was inactive. Although these findings suggest that SNP variations in the human GLYAT gene may influence the enzymatic kinetic properties to account for inter-individual variation, our subsequent study [47]indicated that the GLYAT open reading frame (ORF) from a cohort of 1537 South African Afrikaner Caucasians appeared to be highly conserved, supporting the hypothesis that the glycine conjugation pathway is an essential detoxification pathway. It thus seems that genetic variability in the GLYAT gene did not compromise the observed biotransformation profiles following the salicylate intervention. Finally, we quantified the urinary excretion of p-aminohippuric acid over a period of ten hours in ten human volunteers following a p-aminobenzoic acid (PABA) challenge test[48]. All of the participants were able to metabolize PABA to such an extent that the product profile showed no further excretion of PAHA after 10 h, although considerable time dependent inter-individuality occurred. It thus appears that variations in the amounts of PABA conjugated to glycine may be attributed to factors like absorption (Phase 0), metabolism (Phase II) and excretion (Phase III), each phase with its own limiting factors which were call for a study in its own right for elucidation. Not-with-standing these limitations, the present study show how the analysis and integration of a wide-range of parameters can lead to the selection of both known and new indicators of biotansformation. Three observations emerged from the fatigue and the biochemical measurements in our patient group.

First, both the original and the transformed data indicate no significant difference between low and high fatigue groups for phase I transformation (Table 1 and Fig 5A and 5B). The ability to clear caffeine following the challenge is an indicator of the detoxification capacity of the hepatic Phase I pathway and is a recognized method to assess liver function and especially Phase I biotransformation via the CYP1A2 isoenzyme [50][51]. CYP1A2 contributes to the inactivation of several common drugs and dietary constituents, including acetaminophen[52]. The administration of a combination of challenge substances as used here, exclude measurement of different Phase I cytochrome P450 enzyme reactions simultaneously. However, Phase 1 biotransformation chemically modifies exogenous substances (caffeine, acetoaminophen and salicylic acid as used here) through the inducible CYP450 mixed function oxidase system, rendering them with improved ability for excretion (caffeine derivatives) or for phase II transformation catalysed by conjugation enzymes (glucuronidase, glutathione-S-transferase and sulphotransferase). The preferred detoxification modes in our patient group operated, however, through phase II glucuronidation (Fig 5C: the acetoaminophen challenge) and phase II glycination (Fig 5F: the salicylic acid challenge). Glucuronidation is a fairly common mode of detoxification, given the abundance of required co-factors and ubiquitous nature of these transferases [53], whereas our recent kinetic study on human GLYAT indicated that glycination is observed over a range of substrate concentrations as well as over a lengthy span of time to facilitates optimal detoxification [54]. The kinetic study also supports the observation of elevated excretion of salicyluric acid, the glycine conjugate of salicylic acid, in the high fatigue group, which points to an upregulated GLYAT enzyme system and subsequent greater usage of ATP for substrate activation [41]. Taken together, we propose that these findings suggest a possible causal relationship between high exposure to some xenobiotic substance(s) or environmental condition, upregulated and chronic phase II detoxification, ATP consumption and fatigue.

Second, differences in the profiles of 2,3-DHBA and ROS appear to implicate oxidative stress in patients who experience fatigue strongly with energy factors (the Mainly Energy Fatigue group), whereas patients more inclined to mental fatigue (the Mainly Mental Fatigue

group) appeared to possess better antioxidant protection (FRAP and GSHt). Although mitochondrial dysfunction is recognized as the immediate cause of CFS symptoms like severe fatigue, a direct link to oxidative stress remains elusive [55], although increased oxidative stress remains a regularly observed condition in fatigue patients.

Third, the statistical metrics of biotransformation parameters for idiomatic fatigue, as shown here, do not comply with the requirements of diagnostic biomarkers [56] as defined for monogenetic or clinically well-defined diseases (e.g. significance [low (<0.001) p values], measures of sensitivity and specificity with high confidence intervals and validation outcomes). These biotransformation profiles underline the unique phenotypic/genetic response of individual humans towards endogenous or exogenous stressors, like toxicants. Moreover, depending on their chemical nature, some toxicants tend to bioaccumulate in the body and become stored in tissues or remain in serum [57], which most likely generates a polygenetic response in these individuals. These perturbed health profiles suggest the basis for a personalized approach to the treatment of fatigue patients, phrased as a "pragmatic clinical management" [58] in subjects with toxicant-related conditions, such as general and persistent fatigue.

The nuclear factor, erythroid 2-related factor 2-antioxidant response elements (Nrf2-ARE) as well as the androstane receptor (CAR) and pregnane receptor (PXR) are activated by a variety of endogenous and exogenous ligands[59][60] and are major determinants of phase II gene induction, so that upregulated phase II enzymes [61] may be promising targets for the treatment owing to their potent ability to detoxify harmful compounds and to combat ROS and oxidative stress. However, there is a dearth of clinical trials on inducers of Nrf2 and biotransformation through phase II involvement, which may be attributed to several concerns that preclude their translation to the clinic [62]. Nevertheless, an individualized approach implicating the application of biotransformation has been proposed by Sears and Genius [58], which we here extend, based on our observations made over several years of involvement with fatigue and biotransformation:

(a) Perform a comprehensive history to reasonably identify past and present xenobiotic exposures, while including clinical information similar to the present MSQ and PFS;

(b) apply the present broad-based biochemical instrument as a first approximation on the biotransformation status of the individual case;

(c) define a clinical approach to institute personal health-related treatment strategies directed towards the individual case.

The approach proposed here is very similar to that suggested by Beger et al. [63], in which a metabolite profile, clinical and lifestyle data were combined in a predictive patient profile, resulting in better patient stratification which facilitates personalized treatment and minimize the risk of the traditional treatment-failure approach. This precision medicine approach is fast gaining ground and has been described in a number of recent publications [64][65][66][67][68]. Incorporation of genomic data is part of our research unit's future planning involving integration of genetic and metabolic profiles. According to Contrepois et al. [69], this approach and metadata (such as nutritional background, exercise and medical records) will allow the detection of early signs of aberrant biotransformation and will result in nutrition/lifestyle recommendations and efficient drug treatments and complements. Finally, our results have a direct bearing on the recent alert by the World Health Organization (WHO) regarding chronic non-communicable diseases. We believe that our contribution could be an important step towards unraveling a very complex syndromic phenomenon.

Supporting information

S1 File.
(DOCX)

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7.3 Supplementary Information

7.3.1 Patient Selection

Female patients who were complaining about fatigue were selected during visits to clinicians. Selection was based mainly on fatigue assessment by the clinicians and the completion of a demographic profile, the revised version of the Piper Fatigue Scale and the Medical Symptoms questionnaire. The questionnaires were part of an information guide about the challenge tests.

Note: The BOSS guide (Chapter 3) was included as supplementary information in the *PLOS ONE* paper (Erasmus *et al.,* 2019).

7.3.2 Informed consent

Potential participating patients were also required to complete a section on informed consent which in essence contains information about the background and aim of the detoxification and oxidative stress evaluation, risks and possible discomforts, confidentiality, withdrawal, acknowledgement of explanation of the test procedure and finally the declaration of consent.

7.3.3 Statistics

Supplementary table 7-1 reports the correlations coefficients (r) and associated p-values, based on Spearman's rho, between suspected confounders (age, height, weight and BMI) and variables used to group cases (MSQ Overall, PFS Energy and PFS Mental scores) as well as biochemistry variables. Confounders were confirmed if showing a practically significant correlation ($|r| \geq 0.5$). While p-values are reported, the level of significance was not considered as this metric is known to be affected by sample size. A sample size of 576, as used here, is likely to produce statistically significant effects of little practical importance. Given this criteria, no confounding effects could be detected. Note the non-parametric approach was used as some concerns about normality persisted, but more powerful parametric Pearson's correlations were also performed. Pearson's results were comparable, and the same conclusions could be drawn.

Table 7-1: Correlation analysis with potential confounding effects. The table reports the Spearman's Rho non-parametric correlation coefficients (r) and associated significance levels (p-value) between variables pairs indicated as row and column headers. Correlation coefficients marked with a single asterisk (*) indicate significance at a 5% level, while correlation coefficients marked with a double asterisk (**) indicate significance at a 1% level.

	Spearman's Rho Correlation r (p-value)			
	Weight	**Height**	**BMI**	**Age**
MSQ	.087* (0.038)	-0.01194 (0.775)	.102* (0.014)	0.018704 (0.654)
PFS_Energy	.100* (0.016)	0.021687 (0.604)	.104* (0.013)	-0.02876 (0.491)
PFS_Mental	0.041353 (0.322)	-0.01059 (0.8)	0.048559 (0.245)	-0.04974 (0.233)
Creatinine	.109** (0.009)	.118** (0.005)	0.064068 (0.125)	-.391** (<0.0001)
Uric acid	.115** (0.006)	0.067687 (0.105)	.095* (0.023)	-.229** (<0.0001)
Uric acid: creatinine ratio	0.011711 (0.779)	-0.06503 (0.12)	0.046541 (0.265)	.268** (<0.0001)
Phase1	-.190** (<0.0001)	-0.05849 (0.162)	-.180** (<0.0001)	-0.03502 (0.401)
Glucuronidation	-0.04875 (0.243)	-0.02834 (0.498)	-0.03723 (0.372)	-.172** (<0.0001)
Sulphation	-.130** (0.002)	-0.02934 (0.483)	-.125** (0.003)	-0.03816 (0.361)
Mercapturation	0.012653 (0.762)	0.034901 (0.404)	-0.0063 (0.88)	-.100* (0.016)
Salicyluric acid (SAU)	0.005995 (0.886)	0.020809 (0.619)	0.008096 (0.846)	-.121** (0.004)
Catechol	-.091* (0.028)	-0.05782 (0.167)	-0.0605 (0.147)	.120** (0.004)
2,3 DHBA	-.229** (<0.0001)	-.146** (<0.0001)	-.166** (<0.0001)	0.02523 (0.546)
2,5 DHBA	-.222** (<0.0001)	-.149** (<0.0001)	-.176** (<0.0001)	-0.01479 (0.723)
ROS	.153** (<0.0001)	-0.03165 (0.449)	.172** (<0.0001)	-.124** (0.003)
FRAP	-0.02681 (0.521)	-0.02766 (0.508)	-0.02214 (0.596)	-0.03182 (0.446)

	Spearman's Rho Correlation r (p-value)			
	Weight	**Height**	**BMI**	**Age**
Total red blood cell glutathione	0.042577 (0.308)	.110** (0.008)	0.006483 (0.877)	-0.05794 (0.165)
Total free carnitine	.119** (0.004)	0.004537 (0.914)	.120** (0.004)	.156** (<0.0001)
Total acyl carnitine	0.027041 (0.517)	-0.05035 (0.228)	0.047406 (0.256)	0.061815 (0.138)
Ratio total acyl carnitine: total free carnitine	-.164** (<0.0001)	-.096* (0.022)	-.126** (0.003)	-.170** (<0.0001)
Phase1 sulfation	-.127** (0.002)	-0.05512 (0.187)	-.111** (0.008)	-0.00755 (0.857)
Phase1 glycination	-.167** (<0.0001)	-0.06549 (0.117)	-.163** (<0.0001)	0.030533 (0.465)
Phase1 glucuronidation	-.142** (0.001)	-0.06849 (0.101)	-.120** (0.004)	0.04645 (0.266)

CHAPTER 8

A laboratory approach for characterizing chronic fatigue: what does metabolomics tell us?

8.1 **Introduction** (with reference style according to *Metabolomics*)

The biochemical data generated through the BOSS protocol was based on pre-defined biochemical parameters related to the selected intervention procedures. Untargeted or semi-targeted metabolomics data opened an alternative route to gain information on metabolic perturbations. The study of the quantitative complement of metabolites in a biological system as generated through metabolomics approaches has been shown to reveal changes in metabolite concentrations or fluxes related to a wide variety of genetic or environmental determinants. Interestingly, the concept of 'metabolomics' first appeared in the journal *Xenobiotica* in 1999, there still defined as 'metabonomics': "*Metabonomics is defined as the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification'* (Nicholson *et al.* 1999).

Our researchers of the Centre for Human Metabolomics has wide experience in applying untargeted proton magnetic resonance (^{1}H NMR)-based metabolomics and chemometric methods of analysis in characterizing the metabolite profiles of biofluids from a variety of conditions (Tuberculosis meningitis: Mason *et al.* Metabolomics, 2015; HIV: Moutloatse *et al.* Metabolomics 2016; Fibromyalgia: Malatjie *et al.* BMC Neurology, 2017; Alcohol consumption: Irwin *et al.* PLOS ONE, 2018). We therefore explored the urinary metabolite profile in fatigue patients from metabolomics data, following a challenge test. The samples of the cases investigated were obtained from 39 cases with low and 31 subjects with high fatigue.

The power of untargeted metabolomics lies in its potential to broaden our understanding of the intervention and the associated biotransformation and detoxification responses. Coming from a multifactorial-multigene origin, we predicted that the metabolite profiles would deepen our insights into the interplay of several biochemical pathways and the individual's interaction with the challenge. We argued that a metabolomics approach may reveal additional differences and/or produce complementary information to the responses observed from the BOSS protocol. This study may even open a new toolbox of methods to chronic fatigue diagnostics in addition to the potential new insights into the biochemical mechanisms of xenobiotic metabolism.

8.2 A laboratory approach for characterizing chronic fatigue: what does metabolomics tell us? (Accepted manuscript I)

RESEARH PAPER | MANUSCRIPT VERSION

A laboratory approach for characterizing chronic fatigue: what does metabolomics tell us?

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Abstract

Introduction

Manifestations of fatigue range from chronic fatigue up to a severe syndrome and myalgic encephalomyelitis. Fatigue grossly affects the functional status and quality of life of affected individuals, prompting the World Health Organization to recognize it as a chronic non-communicable condition.

Objectives

Here, we explore the potential of urinary metabolite information to complement clinical criteria of fatigue, providing an avenue towards an objective measure of fatigue in patients presenting with the full spectrum of fatigue levels.

Methods

The experimental group consisted of 576 chronic fatigue female patients. The measurement design was composed of (1) existing clinical fatigue scales, (2) a hepatic detoxification challenge test, and (3) untargeted proton nuclear magnetic resonance (^{1}H-NMR) procedure to generate metabolomics data. Data analysed via an in-house Matlab script that combines functions from a Statistics and a PLS Toolbox.

Results

Multivariate analysis of the original 459 profiled ^{1}H-NMR bins for the low (control) and high (patient) fatigue groups indicated complete separation following the detoxification experimental challenge. Important bins identified from the ^{1}H-NMR spectra provided quantitative metabolite information on the detoxification challenge for the fatigue groups.

Conclusions

Untargeted ^{1}H-NMR metabolomics proved its applicability as a global profiling tool to reveal the impact of toxicological interventions in chronic fatigue patients. No clear potential biomarker emerged from this study, but the quantitative profile of the phase II biotransformation products provides a practical visible effect directing to up-regulation of crucial phase II enzyme systems in the high fatigue group in response to a high xenobiotic-load.

Keywords

Chronic fatigue; Detoxification challenge test; Piper fatigue scale; ^{1}H-NMR metabolomics; Phase II biotransformation.

Concise and informative title: Metabolomics and chronic fatigue

8.2.1 Introduction

Fatigue is one of the most common conditions reported by patients in primary care practice. Fatigue frequently co-occurs with pain, sleep disturbances and depression prompting medical practitioners to use diverse definitions and diagnostic labels to describe possible underlying causes of these manifestations. Thus, fatigue appears as a symptom of idiopathic conditions, at the one end of the fatigue spectrum, towards chronic fatigue syndrome (CFS) and myalgic encephalomyelitis (ME), at the other end. In its more persistent form, fatigue is now recognized by the World Health Organization (WHO) as one of the chronic non-communicable conditions, which are rapidly becoming endemic worldwide, recognizing that fatigue is associated with a significant decline in the functional status and quality of life (QOL) of affected individuals.

Although a diagnosis is essential for providing appropriate care, there is no established diagnostic test for any of the manifestations of fatigue. To this end, various wellness and health institutions in the United States (US) tasked the Institute of Medicine (IOM) to develop evidence-based clinical diagnostic criteria at least for ME/CFS and to recommend whether new terminology for ME/CFS should be adopted (Clayton 2015). Following extensive research involving patients and clinicians, the IOM committee proposed redefined diagnostic criteria to facilitate timely diagnosis and to improve understanding of the illness among healthcare professionals and the public (Clayton 2015). The diagnostic criteria indicative of CFS/ME specify: (1) a substantial reduction or impairment in the ability to engage in pre-illness levels of activity (occupational, educational, social or personal life); (2) symptoms that present for more than six months; (3) episodes of profound fatigue; (4) symptoms of new onset and not resulting from on-going or unusual excessive exertion; and (5) symptoms that are nor substantially alleviated by rest. From this descriptive, it is clear that clinical criteria, though relatively

subjective, remain key in the diagnosis of chronic fatigue seen in CFS/ME and that no objective measure for fatigue in general has been proposed — the issue that is addressed in this paper.

Seeing the multifactorial nature of chronic fatigue, it seems highly unlikely that an objective measure, as a verified single biomarker or biosignature comprised of a limited number of biomarkers, will be found for fatigue conditions, unless the essence of this disorder, its causes and pathophysiology becomes clearly delineated. However, it has recently been shown (Erasmus *et al*. 2019) that data from a battery of conventional tests provided some objective indicators to complement clinical and lifestyle data which enabled the classification of a cohort of patients into eleven subgroups, ranging from low to high fatigue. In addition, metabolomics research has provided insights into disorders of comparable complexity to chronic fatigue, like fibromyalgia syndrome (Hackshaw *et al*. 2018; Malatji *et al.* 2017), chronic widespread pain (Freidin *et al*. 2018; Hadrévi *et al.* 2015) and irritable bowel syndrome (Fourie *et al*. 2016; Ponnusamy *et al*. 2011). Likewise, metabolomics approaches using intervention studies proved to provide valuable insights into experimental studies on nutrition (Wittwer *et al.* 2011), detoxification (Irwin *et al*. 2016) and acute alcohol consumption (Irwin *et al*. 2018). Here we propose a laboratory approach to facilitate an objective evaluation of fatigue in a clinically selected group of chronic fatigue patients. The approach is compiled of: (1) existing clinical scales to score (Piper *et al*. 1998); (2) a known hepatic detoxification challenge test (Cordts *et al*. 2001) and (3) untargeted proton nuclear magnetic resonance (^{1}H-NMR) procedure to generate metabolomics data.

The selection of cases used for the laboratory assessment was made from 576 females identified by clinicians as suffering from chronic fatigue, supported by information from two known assessment scales. Based on exclusion criteria, the control (low fatigue) and patient (high fatigue) groups were selected from this cohort for the metabolomics study. Fatigue in our patient group was scored based on the Piper Fatigue Scale (PFS) (Piper *et al*. 1998) developed for fatigue prevailing in oncology patients. The information from the PFS was supplemented with that from a general Medical Symptoms Questionnaire (MSQ) used by the Departments of Medicine, Mercy Hospital and Maine Medical Center, Portland, for comprehensive profiling of patients with idiopathic conditions.

The hepatic detoxification test probes the gut-liver function through a hepatic detoxification challenge with acetaminophen and acetylsalicylic acid (Cordts *et al*. 2001). The laboratory instrument used for the objective measurement of fatigue is based on the biotransformation profile derived from these hepatic challenge tests. The underlying physiological assumption is that fatigue is a symptom of energy depletion (e.g. as indicated by the PFS). Based on the presumed causal relationship between exogenous stimuli, biotransformation responses and

fatigue, we speculated that the response to the highly energy dependent hepatic challenge test (that is, ATP required for the *in vivo* synthesis of biotransformation products) should be more pronounced in controls than in fatigue patients supposed to suffer from energy depletion.

In this exploratory study we followed a holistic approach to determine the response to an intervention by using metabolomics technology in the generation and analysis of untargeted ^{1}H-NMR spectral data, produced prior to and following the intervention. Important bins identified from the ^{1}H-NMR spectra provided qualitative information to compare the effect of the challenge tests on control and fatigue cases and used to identify and quantify biotransformation markers associated with fatigue.

The IOM's report on fatigue emphasized the need for increased focus on the complex phenomenon of fatigue, paving the way to learn more on conditions covering the full spectrum of fatigue conditions and to diagnose and treat patients suffering from a disease that severely affects their QOL. The outcomes of the present metabolomics study suggest that the intervention used can provide an objective measure to distinguish the response of patients with low and high fatigue, and has the potential, given further refinement, to benefit patients suffering from fatigue conditions.

8.2.2 Materials and methods

8.2.2.1 Objective, ethical approval and case selection

The primary objective of the study was to propose a laboratory-based procedure that could pave the way towards an objective indicator of fatigue in patients observed as such by physicians in general clinical practice. The point of departure for the design of the study was thus to enrol a large number of patients suffering from chronic fatigue. Next, all prospective participants were informed of the objective of the study, which complied with the requirements of the appropriate Ethics Committee of North-West University (reference no. NWU-00102-12-A1). All participants provided written consent (see Supplementary Information (SI) for **Informed consent** forms) to participate and for eventual publication of anonymized results in accordance with the ethical requirements of the university.

Patients suffering from fatigue emanating from well-defined clinical conditions such as cancer and CFS/ME or other known conditions such as diabetes or hypertension were excluded from the study. Exclusions were based on the assessment of the physician and the outcome of the MSQ (see SI on **Patient procedures and selection** for full details). Next, participants were asked to complete a self-report questionnaire that included a demographic profile and an English version of the revised Piper Fatigue Scale (PFS). The PFS used consisted of 22

numerical items that assess fatigue experienced by the patients. All items were coded on a 0–10 numeric scale and resolve into four dimensions of subjective fatigue: behavior/severity, affective meaning, sensory, and cognitive/mood. In total, 673 women with complaints of fatigue were assessed and of these 576 were found to be eligible for the study. The age, height, weight or BMI did not have a confounding effect on the biochemical data or on the classification of fatigue groups, indicated by a correlation analysis to indicate potential confounding effects (Erasmus *et al*. 2019).

Having applied the PFS to a clinically different fatigue group than the instrument was designed for, required a re-evaluation of the fatigue dimension. To this end, various combinations of factor extraction methods (Principal Components and Principal Axis Factoring) and rotation methods (Varimax Rotation and Direct Oblimin rotation) were employed. The different approaches all indicated two underlying factors in our data set. The first factor was seen to load mainly on items 2–17 and was termed Energy Fatigue, whereas the second factor loaded mainly on items 18–23 and was termed Mental Fatigue. The factor scores were calculated as based on average scores with Energy Fatigue score the mean of items 2–17 (16 items) and Mental Fatigue score the mean of items 18–23 (6 items). The reliability of the measurement for the two factors was evaluated by means of Cronbach's Alpha and proved to be high (0.974 and 0.971 for the 16 and 6 energy and mental fatigue factors, respectively).

The MSQ contains 19 domains, most with 4 items, totalling 75 indicators to each of which the patient assigns a Likert scale numerical ranking from 0 ("never" or "seldom") to 4 ("frequently with severe effect"). Note that one question, found in the energy section about hyperactivity, was omitted to enhance the reliability (see SI for further details). For this data set, factor analysis rotation and extraction methods did not converge, while PCA did not provide useful outputs. It was therefore decided to calculate factor scores based on the sections and total since the MSQ was applied in a similar context to that in which it was developed. The MSQ domain scores were calculated as the mean of the items per domain, in addition the items were combined into an overall MSQ score.

Two groups were selected for the hepatic challenge tests: (1) A low fatigue group consisted of 39 cases (control group), scoring lower than 4.0 on energy as well as mental fatigue factors and an overall score from the MSQ not exceeding 28. (2) A high fatigue group also comprised 31 subjects (patient group), scoring above 7.0 on energy as well as mental fatigue factors and an overall score from the MSQ exceeding 147. The distribution of these patients within their fatigue categories (low and high) is shown in Figure 8-1.

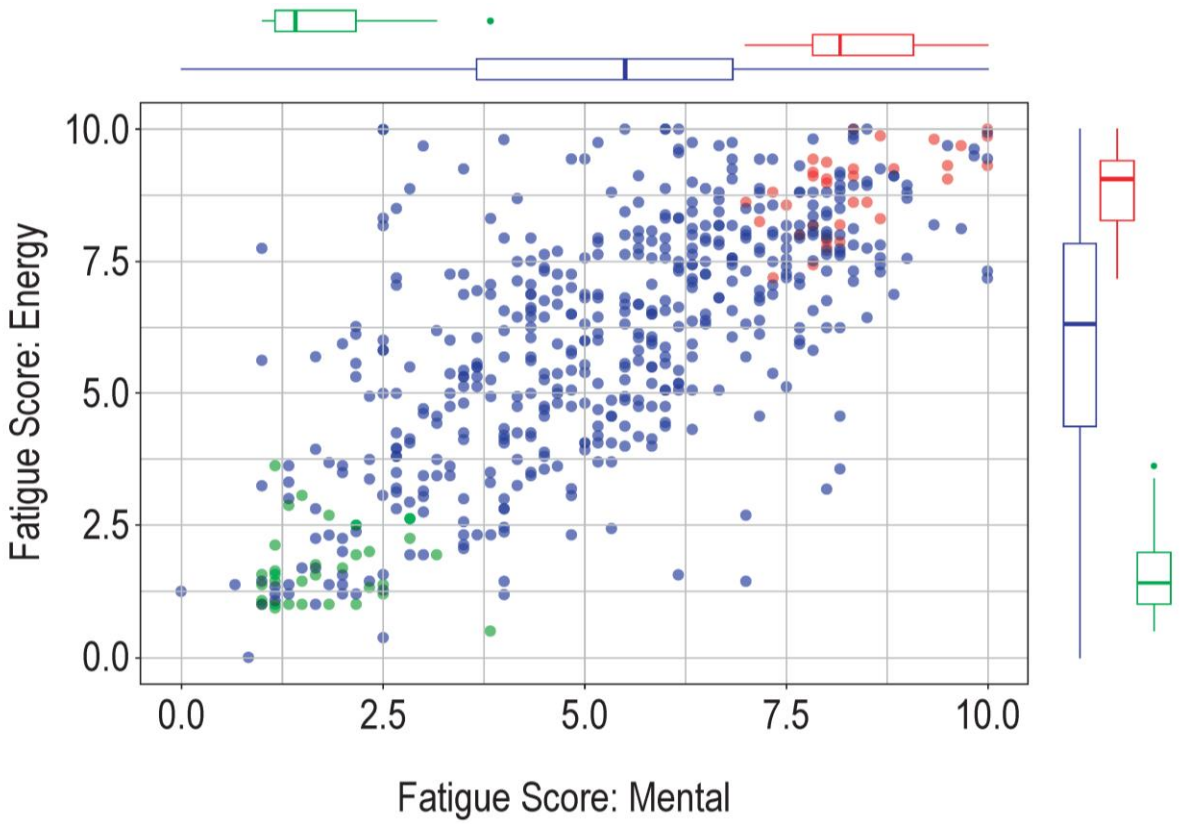


Figure 8-1: Distribution of all selected cases based on the individual's scores on energy fatigue and mental fatigue factors derived from the PFS. The scores are categorized as low level fatigue (0 < score < 4), medium level of fatigue (4 ≤ score < 7) and high level of fatigue (score ≥ 7). The distribution of all 576 cases is shown as blue, green or red dots. The green dots indicate the group of 39 cases with low fatigue (PFS Energy & Mental < 4.0 and Overall MSQ ≤ 28). The red dots indicate the high fatigue group of also 31 subjects (PFS Energy & Mental > 7.0 and Overall MSQ> 147). Box plots indicate the distribution of scores which each group as evident from the corresponding colours.

8.2.2.2 Laboratory methods: the challenge tests and metabolomics data generation

The two hepatic challenge tests were performed simultaneously. Prior to the challenge, a base urine sample was collected from each individual, and stored as -20^{o}C until analyses (Pre-sample). The first challenge involved two aspirin (600 mg) and two acetaminophen (paracetamol) tablets (1000 mg) taken at 21h00 in the evening and overnight and early morning (ending at 7h00) urine samples collected in a special container provided in the test kit and stored at -20^{o}C as well (Post-sample).

8.2.2.3 ^{1}H-NMR analysis

A volume of 600 µL of urine was centrifuged at 12 000 g for 5 minutes. Of the supernatant, 540 µL was collected in a microcentrifuge tube, with 60 µL NMR buffer solution (1.5 M potassium phosphate solution in deuterium oxide with internal standard TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid); pH 7.4). Sample was mixed under vortex to ensure completely

homogenous and centrifuged again at 12 000 g for 5 minutes. A volume of 540 µL of supernatant was transferred to a 5mm NMR tube for analysis.

Samples were measured, randomized, at 500MHz on a Bruker Avance III HD NMR spectrometer equipped with a triple-resonance inverse (TXI) ^{1}H{15N, 13C} probe head and x, y, z gradient coils. ^{1}H spectra were acquired as 128 transients in 32K data points with a spectral width of 12 000 Hz. The sample temperature was maintained at 300K and the H_2O resonance was presaturated by single-frequency irradiation during a relaxation delay of 4s, with a 90° excitation pulse of 8µs. Shimming of the sample was performed automatically on the deuterium signal. The resonance line widths for TSP and metabolites were <1 Hz. Fourier transformation and phase and baseline correction were done automatically. Software used for NMR processing was Bruker Topspin (V3.5). Bruker AMIX (V3.9.14) was used for metabolite identification and quantification. Cases were quantified across 459 equally spaced bins.

8.2.2.4 Statistical analysis

To aid interpretation two-group comparisons were performed within high and low fatigue groups as pre vs post intervention pairs. The data were pre-processed in subsets associated with each two-group comparison. First, bins with 50% or more zero-valued observation in both groups were removed. Second, the remaining zero-valued observations were replaced by random numbers generated uniformly below the lowest non-zero observation. Finally, the data were scaled by first performing a log transformation to adjust for asymmetry in distribution, since metabolomics data are known to present with a positively skewed distribution. Following this transformation, the data were auto-scaled to adjust for the effect of differences in abundance, since bins in high abundance are not necessarily of greater importance.

Univariate and multivariate methods were applied to allow for a comprehensive overview of results. The univariate method used to assess the statistical significance of differences between the dependent groups was the non-parametric Wilcoxon test. Effect sizes were used to assess the magnitude of significant differences or practical significance. Effect sizes were calculated as the respective test statistic scaled to the sample size. Scatter plots colour coded to show significance, often referred to as volcano plots, were used to summarize statistical and mean difference ratios or fold changes across bins.

Unsupervised multivariate methods were used in support of supervised methods as a form of validation. Unsupervised PCA and cluster analysis were performed to project the data onto fewer more manageable dimensions and to show purely data driven associations between cases, respectively. Euclidean distance with Ward linkage was used to form hierarchical clusters. PCA scores plots were used to explore whether a large proportion of variation in the

data can be associated with the group structure by overlaying confidence intervals for groups. These methods serve to support or caution against the interpretation of similar scores plots derived using partial least squares discriminant analysis (PLS-DA), a supervised method used to emphasize any differences between groups. Again, confidence intervals were used to assess discriminatory ability.

All pre-processing and statistical analysis were performed using an in-house Matlab script that serves to combine functions from the Statistics Toolbox [MATLAB with Statistics and Toolbox Release 2016b, The MathWorks, Inc., Natick, MA, USA)] and the PLS Toolbox as provided by EigenVector [PLS-Toolbox 8.2.1 (2016). Eigenvector Research. Software available at www.eigenvector.com.].

8.2.3 Results

8.2.3.1 Qualitative metabolomics information following the hepatic challenges.

The qualitative data was obtained from the original 459 ^{1}H-NMR profiled bins for the experimental groups before and after the intervention. Supposed changes in metabolite profiles from the two patient groups following the interventions were firstly established through two multivariate approaches. For the bin data, the group separations before and after the challenge were investigated using unsupervised PCA (Figure 8-2a and 8-2b), and supervised PLS-DA (Figure 8-2c and 8-2d). The PCA showed a virtual clear separation for the low fatigue group prior to and after the intervention. For the high fatigue group only a partial separation was detected. As expected, complete separation for both the low and high fatigue groups were detected through the PLS-DA. As this method of classification tends to overfits the data (Westerhuis *et al.,* 2008), the PCA serves as some verification, but taken with the predictive accuracy $R^2(Y)$ of the PLS-DA models and its leave-one-out cross-validated counterparts $Q^2(Y)$, some surety can be attainted. The $R^2(Y)$ and $Q^2(Y)$ values were 90.1% and 79.8% for the high fatigue group model and 93.1% and 86.3% for the low, respectively. Interestingly, both the PCA and PLS-DA models appear to have a reduced ability to discriminate pre and post intervention measures in the high fatigue group, which may support the idea that fatigue hinders the ability to respond to a metabolic challenge. Similar findings were noted in the univariate analysis below.

An incomplete group separation in the PLS-DA models was observed between low and high fatigue subjects following the hepatic detoxification test, as shown in Figure 8-2e. This observation suggests subtle differences in metabolite concentrations between low and high fatigue groups, following the intervention, which will be further investigated through a

quantitative comparison of the biotransformation products, as will be shown below. Figure 8-2f indicates the relative change before (pre) and after (post) exposure to the intervention stressors for the high and low fatigue groups. Relative change was computed in a similar manner to a fold change but based on the two observations of each participant. The relative change values were compared across fatigue groups using a PLS-DA model. The resulting scores plots indicate a difference in response of the individuals representing the low and high fatigue groups

Next, univariate analyses using Wilcoxon p-values and fold changes on the same bin data were determined and summarized in the volcano plots for the low (a) and high (b) fatigue groups (Figure 8-3). The outcome of these analyses of the bin data indicate large-magnitude changes (fold change: $|\log_2 FC| > 1.5$) that are also statistically significant (Wilcoxon test: $p \leq 0.05$), directing to several metabolites of importance that may cause the separation in both groups, following the challenge tests. Similar to the multivariate case, the number of perturbed bins was less in the high fatigue group, which may support the idea that fatigue hinders the ability to respond to a metabolic challenge, calling for a further comparison between the high and low group post-intervention bin values. For this, the perturbed bins, identified within the high and low fatigue groups when comparing profiles before and after the intervention, were identified, combined and quantified to understand if indicators of the challenge tests could also be associated with the severity of fatigue. Bins with PLS-DA VIP (variable importance in projection) scores exceeding 2, an absolute fold change exceeding 2 and p-values less than or equal to 0.05 (after correcting for multiple testing by controlling the false discovery rate) were selected to be quantified relative to creatinine.

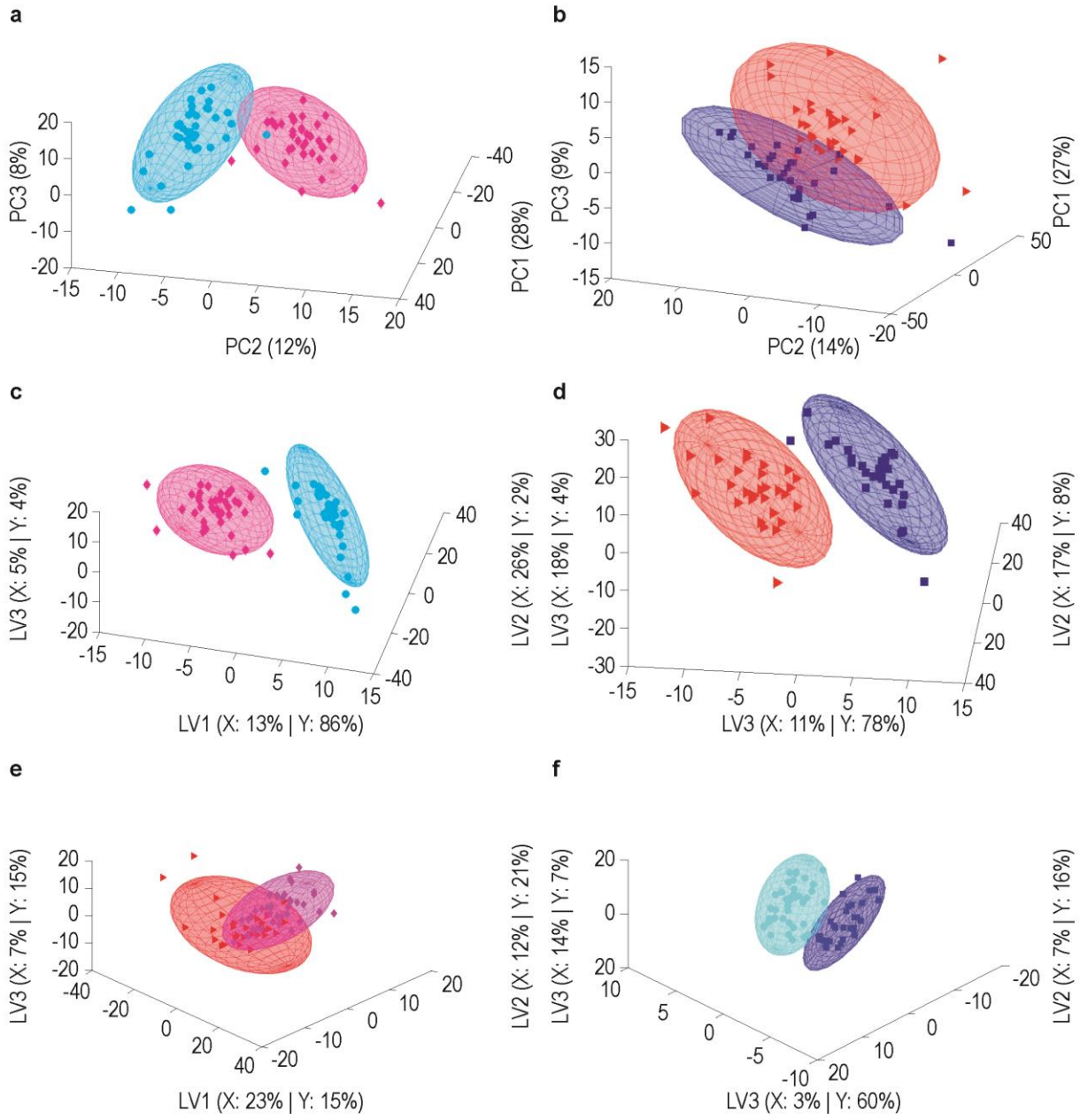


Figure 8-2: Group separation between experimental groups through multivariate analysis based on equidistant binning data. PCA indicating a clear change between pre and post intervention profiles for low (a) and high (b) fatigue cases, similarly for PLS-DA models for low (c) and high (d) fatigue cases. (e) The PLS-DA models for the low and high fatigue groups for the post intervention data. (f) The PLS-DA models from each participant's relative change before (pre) and after (post) the intervention, shown for the low and high fatigue groups. Coloured areas represent 90% confidence intervals (CI) for groups.

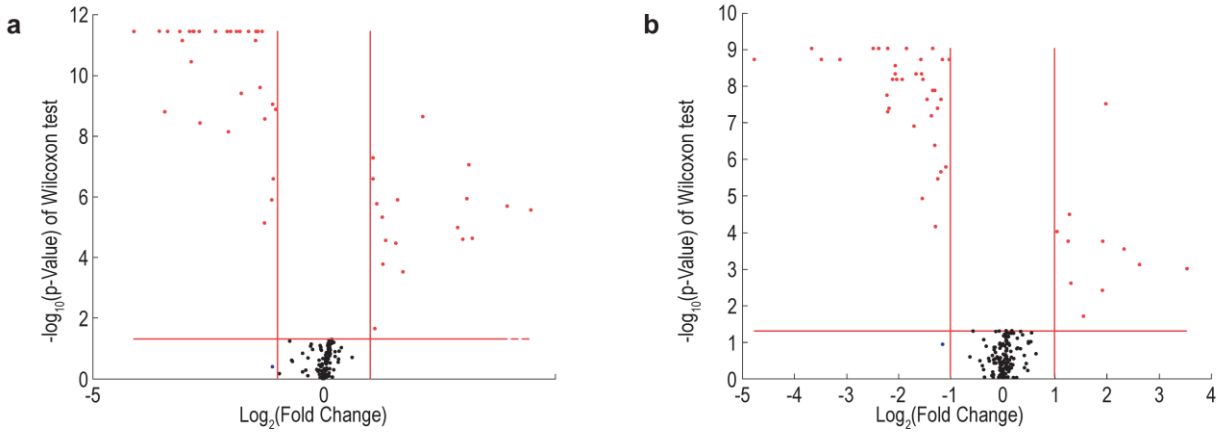


Figure 8-3: Statistical assessments of spectral bins indicative of the response following the hepatic challenges. Volcano plot mapped by the scaled fold change and *p*-values for the ^{1}H-NMR bins observed for the low (a) and the high (b) fatigue groups. Bins with high FC and significant *p*-values among cases are indicated by red dots.

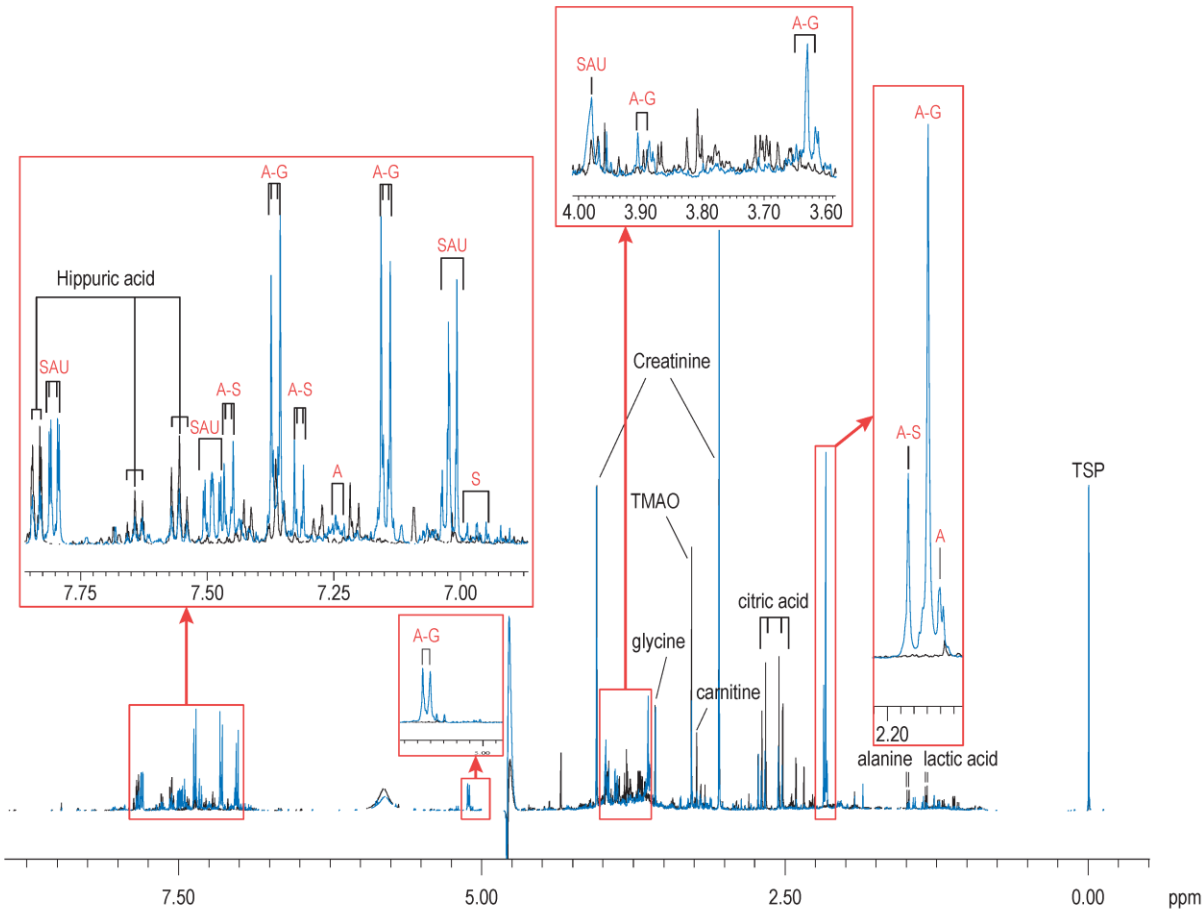


Figure 8-4: Representative ^{1}H-NMR spectra from a single fatigue patient, prior to (black) and following the challenge test (blue), scaled according to the creatinine methyl peak at 3.04 ppm. Expanded regions, framed in red in the spectra, are the bin regions where variables

important in projection (VIP) through the supervised PLS-DA are located. VIPs: A = acetaminophen; A-G = acetaminophen glucuronide; A-S = acetaminophen sulphate; S = salicylic acid; SAU = salicyluric acid

8.2.3.2 Metabolite profiles from a representative case following the hepatic challenges

Representative ^{1}H-NMR spectra from a single fatigue patient, prior to (black) and following the challenge test (blue), is shown in Figure 8-4, scaled according to the creatinine methyl peak at 3.04 ppm. Expanded regions, framed in red in the spectra, are the regions where variables associated with important bins are located. Also, indicated in Figure 8-4, are other endogenous urinary metabolites: lactic acid, alanine, citric acid, carnitine, TMAO, and hippuric acid. Regarding the acetaminophen challenge, both the challenging substance (acetaminophen) as well as two conjugation products, acetaminophen-sulphate and acetaminophen-glucuronide, were detected in the urine samples following the challenge. These results firstly indicate that two of the three major phase II conjugation reactions of acetaminophen were functional as detoxification reactions following the challenge: (1) conjugation of glucuronic acid to the hydroxyl group of acetaminophen; (2) sulphation of the phenolic hydroxyl group of acetaminophen. Apparently, the combination of phase I and phase II conjugation between acetaminophen and glutathione, producing N-acetyl-p-benzoquinone imine as final product, was not operative in the present experimental challenge. Secondly, no acetyl-salicylic acid was detected in the urine following the known high de-acylation following consumption of this challenge substance. This is related to the coupled de-acylation and glycination of the functional carboxylic group is catalysed by glycine-N-acyl transferase (GLYAT). Thus, two metabolites derived from the acetylsalicylic acid challenge could be detected: salicylic acid and salicyluric acid (2-hydroxyhippuric acid).

8.2.3.3 Quantitative comparison of the two fatigue groups following the challenges

As indicated in Figure 8-4, all substances observed following the challenge tests were detected in more than one region in the ^{1}H-NMR spectra. A summary of these areas (ppm values) and detected substances is summarized in Table 8-1. Apart for these five substances, bins associated with endogenous metabolites, tyrosine, creatine and glucose, were also selected as significantly perturbed during the challenge. We therefore quantified these eight substances for all low and high fatigue cases, followed by statistical analysis of these data.

The outcome of the univariate and multivariate analysis of the potential five diagnostic substances, derived from the challenge tests, are shown in Table 8-2. Firstly, the PLS-DA for the low and high fatigue groups when comparing substances before and after the challenge tests using the eight substances mentioned above, show complete separation, as expected

(Figure S1). An isolated peak for each identified VIP metabolite was selected, as indicated by the ‘reference bin’ in Table 8-2. Each selected peak was integrated and made relative to the creatinine methyl peak at 3.04 ppm, taking into account the number of protons representing each peak. The mean and standard deviations are given in Table 8-2 as mmol/mol creatinine concentrations.

The effect of the challenge is thus confirmed in the quantified data. The difference between the mean values of the low and high fatigue groups for these indicators of a challenge were however not statistically significant. Comparing the effect sizes (ES), based on Cohen’s d values, indicate to deliver a practical visible effect (ES ≥ 0.5)(Ellis and Steyn, 2003) for glucuronide-acetaminophen (ES = 0.7) and acetaminophen (ES = 0.5). Neither of these correlations is of practical relevance ($|r|<0.5$) and point to no more than a slight but consistent association between variables and their ranks, respectively. No correlation was observed between the biotransformation parameters and the mental fatigue scores. This leads one to conclude that an effect is visible, but greater statistical power is required to detect it.

Table 8-1: Chemical information and shifts for representative ^{1}H-NMR spectra of CFS patients. Multiplicity: s − singlet; d − doublet; dd − double doublet; m – multiplet; bs –broad singlet. ND = not determined.

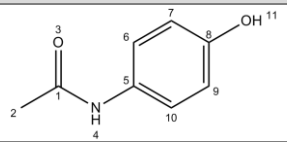
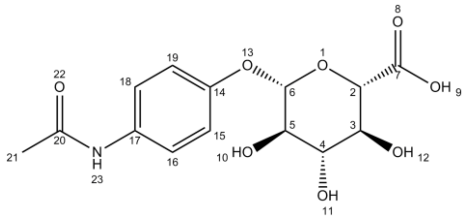
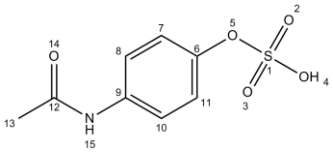
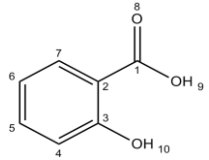
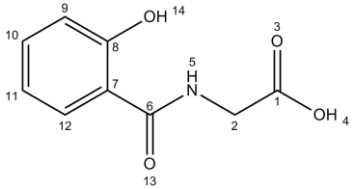
Peak assignment	Metabolite name	Chemical structure	HMDB ID	Chemical formula	δ (ppm), multiplicity (J coupling)	Chemical group	Atom(s)
A	Acetaminophen		HMDB0001859	$C_8H_9NO_2$	2.17 s	CH_3	2
					7.25 d (J = ND)	$(CH)_2$	6,10
A-G	Acetaminophen-glucuronide		HMDB0010316	$C_{14}H_{17}NO_8$	2.17 s	CH_3	21
					3.62–3.89 m	$(CH)_4$	2,3,4,5
					5.11 d (J = 7.2Hz)	CH	6
					7.15 dd (J = 6.8, 9.1Hz)	$(CH)_2$	16,18
					7.36 dd (J = 6.8, 9.1Hz)	$(CH)_2$	15,19
A-S	Acetaminophen-sulphate		HMDB0059911	$C_8H_9NO_5S$	2.18 s	CH_3	13
					7.31 dd (J = 6.7, 9.1Hz)	$(CH)_2$	8,10
					7.45 dd (J = 6.7, 9.1Hz)	$(CH)_2$	7,11
S	Salicylic acid		HMDB0001895	$C_7H_6O_3$	6.97 m	$(CH)_2$	4,6
SAU	Salicyluric acid		HMDB0000840	$C_9H_9NO_4$	3.96 bs	CH_2	2
					7.01 dd (J = Hz)	$(CH)_2$	10,12
					7.49 m	CH	11
					7.79 dd (J = Hz)	CH	9

Table 8-2: Univariate comparison between high and low fatigue groups. Table 8-2 reports the p-values obtained when comparing high and low fatigue groups across 9 compounds of interest, after exposure to the intervention. P-values were obtained using an independent samples t-test (not assuming equal variances) based on transformed data with no correction for multiple testing. Effect sizes were also calculated on untransformed data based on a variation of Cohen's d-value with the low fatigue group as a control. Also reported are means and standard deviations (SD) for each compound for each fatigue group prior to data transformation.

	Metabolite name	Reference bin (max bin VIP)	p-value (effect size)	Low Mean (SD)	High Mean (SD)
Acetaminophen challenge	Acetaminophen	2.15 (7.1)	0.08 **(0.5)**	83.02 (34.4)	100.15 (57.23)
	Acetaminophen-glucuronide	2.19 (7.4)	0.08 **(0.66)**	465.18 (163.87)	573.63 (355.05)
	Acetaminophen-sulphate	2.17 (7.7)	0.95 (≤0.001)	330.56 (104.19)	330.86 (117.66)
Acetyl-salicylic acid challenge	Salicylic acid	6.95 (4.4)	0.88 (0.12)	49.85 (40.89)	45.10 (25.44)
	SAU	7.81 (10.1)	0.06 (0.28)	408.08 (169.07)	455.17 (108.31)
Endogenous	Glucose	5.19 (2.4)	0.06 (0.24)	22.53 (12.15)	25.41 (7.32)
	Creatine	3.03 (2.8)	0.41 (0.22)	208.72 (158.99)	173.42 (142.63)
	Tyrosine	7.23 (3.2)	0.78 (0.07)	83.86 (44.59)	80.69 (39.72)

-Bold refers to compounds which were statistically significant at 10 % level (p≤0.1) and of practical relevance (ES≥0.5)

8.2.4 Discussion

Against the background of the results shown here, what did the metabolomics approach revealed on the applicability of a regimen of challenge tests to objectively assess fatigue?

Firstly, untargeted ^{1}H-NMR metabolomics proved its well-established ability as a global profiling tool to reveal the impact of toxicological interventions (Griffin 2003). However, one of the big hurdles in metabolomics studies is validation, specifically regarding sample size, as recently reviewed by Johnson *et al*. (2016). Notwithstanding the large cohort of 500+ fatigue patients from which the control (low) and patient (high) fatigue groups were selected, the exclusion criteria resulted in cases for both groups of only 30+ cases. For such a group, the model as

shown for example in Figure 8-2e and f did not validate well. Within the limitation of validation, here specifically applied on the biotransformation of challenge substances, using data from spectra (Figure 8-4) in conjunction with statistical pattern recognition technique (Figure 8-2 and 8-3) did provide a usable tool for profiling of the successive biotransformation metabolites. Biotransformation remains one of the most important defence mechanisms against xenobiotic insult (Xu *et al*. 2005). Occurring mainly in the liver, it entails initials the absorption of the xenobiotic compound, followed by phase I functionalization and phase II conjugation. The products of these reactions are often more hydrophilic and their final excretion from within the cell is facilitated by phase III (transport mediated) biotransformation.

The present results are the first of its kind on using two xenobiotics in combination in a challenge test on hepatic function in cases of fatigue. The metabolomics information on the acetaminophen challenge clearly supports the existing views on the biotransformation of both xenobiotics. Acetaminophen has a half-life of 1.5-3 hours after a therapeutic dose, while the major part of the acetaminophen probe is reportedly excreted as the acetaminophen-glucuronide (50-70%) and sulphated-acetaminophen (25-35%). A small percentage (5-15%) of the administered acetaminophen is converted by a phase I enzyme (CYP2E1) to NAPQI, a very reactive quinone, which is normally conjugated to glutathione and ultimately excreted as a mercapturate conjugate. This conjugation product was not detected in any of the present cases studied, salicylic acid and salicyluric acid, derived from each of the acetaminophen and acetyl-salicylic acid challenging substances respectively. The second xenobiotic used, acetylsalicylic acid, is rapidly deacetylated to salicylic acid. The conjugation product of salicylic acid with glycine produces salicyluric acid, the main phase II liver metabolite. The excretion of salicyluric acid normally ranges from 19.8-65% of the administered dose. The presence of the both conjugates in the urine of the patients who were subjected to the challenge tests corresponds with the expected metabolism of the administered of both challenge substances, confirming the value of metabolomics as a tool in the study of a xenobiotic challenges from more than one substance. Furthermore, the observations from the present untargeted metabolomics approach compare well to the complementary technique of targeted metabolomics that was previously described for a biotransformation, using p-aminobenzoic acid as exotoxin (Nortje *et al*. 2015).

Secondly, the functionality of the biotransformation pathways may be affected by a number of factors: (1) Genetic diversity in the enzymes coding for biotransformation enzymes may affect the ability to up-regulate the production of the enzyme under inductive conditions (Van der Sluis 2015, 2018). (2) Conjugation substrates and cofactors are needed for maintaining an effective rate of biotransformation. If depleted, it may negatively impact on biotransformation capacity of both phase I and phase II biotransformation. (3) All of the phase II reactions are heavily energy

dependent and requires ATP for activation of xenobiotica to the corresponding CoA derivative. Although we did not address these factors influencing biotransformation, we observed some qualitative differences between the pre and post-challenge profiles of the low versus high fatigue cases. Both the PCA (Figure 8-2a and b) and the PLS-DA (Figure 8-2c and d) suggest that the low fatigue group responded more to the challenge test than the high fatigue group. Likewise, the volcano plots (Figure 8-3) indicated that the number of ^{1}H-NMR-spectral bins with significant fold-changes was less in the high than in the low fatigue group. These observations will be compatible with the view that the high fatigue group suffered from energy depletion due to up-regulated biotransformation related to endogenous or exogenous conditions causative for their symptoms of high fatigue.

Thirdly, although no clear potential biomarker or biosignature for chronic fatigue emerged from this study, the quantitative profile of metabolites from the challenges hold distinct promise for follow-up studies. The phase II biotransformation of acetaminophen, as well as the urinary excretion of the parent substance proved to be a practical visible effect considering glucuronide-acetaminophen (ES = 0.7) and acetaminophen (ES = 0.5). Likewise, a somewhat related observation was seen in the excretion of salicyluric acid (VIP = 1.5 p = 0.58) in the high fatigue group. This may be further supportive of the suggestion that the high fatigue group react to a higher xenobiotic-load by up-regulating of crucial phase II enzyme systems.

These observations hold promise for further studies aimed at the development of an objective measurement of chronic fatigue. Directives for future research would be (1) the use of a control group without any signs of fatigue (PFS Energy & Mental ≤ 0.5, with no indications of any mental or clinical conditions), (2) doing a longitudinal analysis of urinary excretion of the diagnostic substances for optimizing an improved endpoint following the challenge and (3) eventually use an alternative experimental design and statistical approaches specifically directed to intervention studies, like ANOVA Simultaneous Component Analysis (or ASCA).

8.2.5 Conclusion

Our view still holds that it seems unlikely that an objective measure, as a verified single biomarker or biosignature, will be found for fatigue, given the multifactorial nature of these conditions. The renewed emphasis on fatigue conditions may reveal the causes and pathophysiology underlying fatigue. Meanwhile we believe that the outcomes of the present metabolomics study indicate that intervention studies clearly provide an avenue for an objective way for group differentiation towards a challenge provided for cases of low and high fatigue, which is a corner stone to pave the way towards an objective indicator or marker for fatigue.

8.2.6 References (according to *Metabolomics* format)

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8.2.7 Declarations

8.2.7.1 Ethics approval and consent to participate

This study complied with all institutional guidelines of the North-West University as stipulated by the South African Guidelines for Good Clinical Practice Ethical Guidelines for Research, as well as the terms of the Declaration of Helsinki of 1975 (as revised in 2013) for investigation of human participants. Ethical approval was obtained from the Health Research Ethics Committee (HREC) of the North-West University (NWU-00102-12-A1). All written consent was obtained based upon informed decision from all participants. An example of the informed consent form can be found in the SI.

8.2.7.2 Consent to publish

All authors have given their approval of the version of the manuscript as submitted, their consent for publication and agreed to the accountability requirements.

8.2.7.3 Availability of data and materials

Raw data will be made available upon acceptance of publication.

8.2.7.4 Competing interests

The authors declare that there are no competing interests regarding the publication of this paper.

8.2.7.5 Funding and acknowledgement

Research funding for the analytical and computational aspects of the project was provided by the Technological Innovation Agency (TIA) of the Department of Science and Technology of South Africa. The views and findings of this investigation are those of the authors and do not reflect or represent any policies of the TIA funder. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

8.2.7.6 Authors' contributions

EE was responsible for the experimental design on fatigue in the cohort of patients, interaction with the physicians and for the collection of the samples and the biochemical analysis, as well as data collection from the questionnaires. SM did the ^{1}H-NMR analysis and data generation and provided the quantitative metabolite information on the detoxification challenge in the selected cases. FES did the statistical analysis on the link between the medical and fatigue

scales. MvR did all pre-processing of the data and provided the univariate and multivariate analysis of all data. CV provided the input for the clinical aspects of the study. CJR participated in all areas of the research, data analysis and proposed the original outline of the manuscript, to which all authors provided their expert contributions. All authors have read and approved the final manuscript.

8.3 Supplementary Information

8.3.1 Patient procedures and selection

Female patients who were complaining about fatigue were selected during visits to clinicians. Selection was based mainly on fatigue assessment by the clinicians and the completion of a demographic profile, the revised version of the Piper Fatigue Scale and the Medical Symptoms questionnaire. The questionnaires were part of an information guide about the challenge tests.

Note: The BOSS guide (Chapter 3) was included as supplementary information in the *Metabolomics* submitted manuscript (Erasmus *et al.* 2019).

8.3.2 Informed consent

Potential participating patients were also required to complete a section on informed consent which in essence contains information about the background and aim of the detoxification and oxidative stress evaluation, risks and possible discomforts, confidentiality, withdrawal, acknowledgement of explanation of the test procedure and finally the declaration of consent.

8.3.3 PLS-DA of eight substances selected

The PLS-DA for the low and high fatigue groups when comparing substances before and after the challenge tests using the eight substances mentioned above, show complete separation, as expected The PLS-DA for the low and high fatigue groups when comparing substances before and after the challenge tests using the eight substances mentioned above, show complete separation, as expected (Figure S1). Neither PLS-DA models validated well with respect to predictive accuracy. Therefore, the findings should only be interpreted within the context of the current study and should not be generalized.

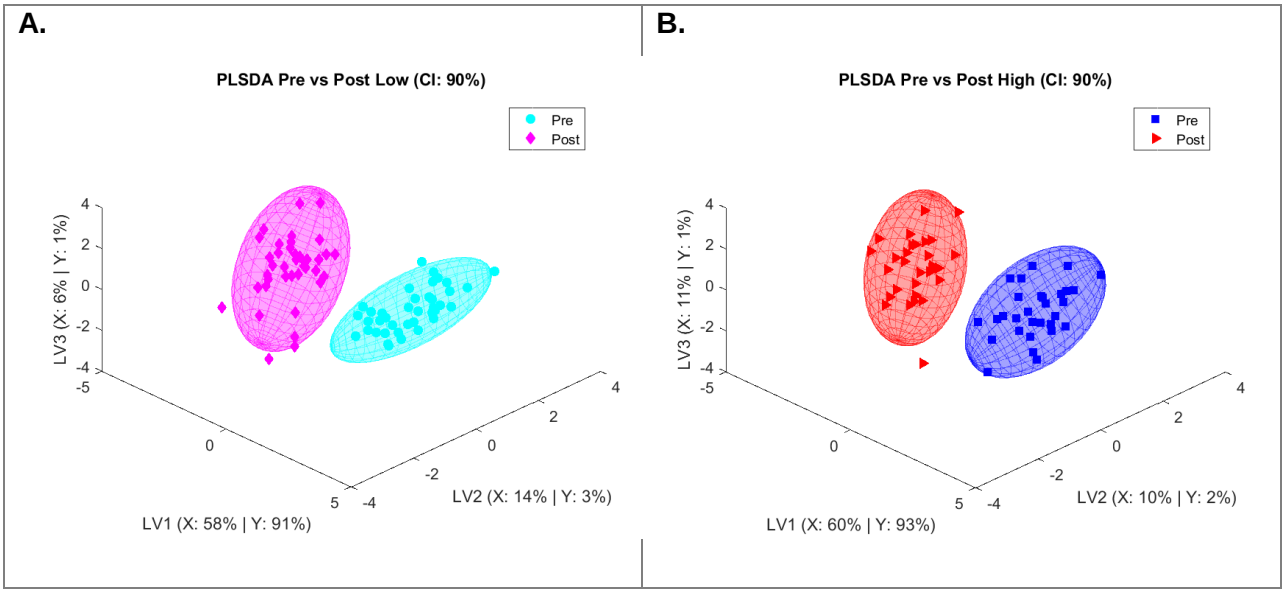


Figure S1: Group separation between experimental groups through multivariate analysis based on eight substances, identified as important from the equidistant binning data. PLS-DA, indicating the separation between the low (a) and high (b) fatigue cases before and after the hepatic challenges.

8.3.4 Summary table of the clinical diagnosis

Summary table of the clinical diagnosis information of 39 patients in low fatigue group and 31 patients in high fatigue group.

Table S1: Summary table of the clinical diagnosis information of 39 patients in low fatigue group and 31 patients in high fatigue group

		Mean	SD	ES
Energy	High	8.90	0.77	9.49
	Low	1.61	0.75	
Mental	High	8.44	0.90	7.52
	Low	1.66	0.71	

8.3.5 Table of a Pearson's and a Spearman's correlation test

Table of a Pearson's and a Spearman's correlation test between the energy and mental fatigue scores for four of the detoxification metabolites (acetaminophen glucuronide; acetaminophen sulfate; acetaminophen mercapturate and salicyluric acid).

Table S2: A Pearson's and a Spearman's correlation test between the energy and mental fatigue scores for four of the detoxification metabolites (acetaminophen glucuronide; acetaminophen sulphate; acetaminophen mercapturate and salicyluric acid). The outcome is shown in the table below:

Measure	Pearson's correlation coefficient		Spearman's rho correlation coefficient	
	PFS Energy	PFS Mental	PFS Energy	PFS Mental
Acetaminophen glucuronide	0.112**	0.060	0.090*	0.035
Acetaminophen sulphate	-0.015	0.007	-0.033	-0.012
Acetaminophen mercapturate	0.085*	0.017	0.034	0.019
Salicyluric acid	0.053	0.023	0.015	0.004

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

PART IV

SYNTHESIS AND FUTURE PROSPECTS

CHAPTER 9

Synthesis and future prospects

CHAPTER 9

9.1 Introduction

The main purpose of this chapter is to provide a general discussion of the results obtained in this thesis leading to a synthesis on how this could contribute to an improved detection and future treatment strategy on CF.

The working hypothesis as formulated in the preface was:

> *"The wide occurrence of fatigue amongst individuals not suffering from a distinct clinical disorder may be due to an energy deprived state as a consequence of the individual's response to undefined exposure to xenobiotics, and which should be measurable in the metabolite profiles of biofluids from the fatigue cases"*

Against the background of the working hypothesis, it was clear to us that our investigations should take a number of important points into account:

- The diverse set of conditions that could contribute to the symptom of fatigue.
- The biochemical complexity of biotransformation and secondary factors which could influence these reactions.
- Biochemical investigations which could produce results regarding the functioning of specific biotransformation pathways
- Analytical methods suitable to investigate specific biotransformation pathways
- The largely absence of treatment protocols for aberrant biotransformation metabolism and associated fatigue.

These points are covered in the various objectives, though not necessarily in the same order.

9.2 New insights on biochemical diagnostics of multifactorial conditions

9.2.1 Introduction

The complexity of biotransformation metabolism poses special challenges to us. Normally metabolic investigations have very limited application in the detection of biotransformation imbalances and therefore we had to revert to special approaches to monitor biotransformation and possible related fatigue on individual basis. The first of these approaches included the completion of a medical examination by a medical practitioner [Part II, Chapter 5], and next evaluation of patients in terms of fatigue degree. For this an international acceptable fatigue-measuring scale, the Piper Fatigue Scale was used. In addition to this a large number of medical symptoms were evaluated using a Medical Symptoms Questionnaire, seeing that clinical conditions may be confounding factors towards CF. All of this together with a number of

life-style questions was compiled into a user-friendly instruction guide [Part II, Chapter 5]. The relatively large number of questions in the questionnaires required self-completion by the patients which may raise queries about objective reporting. To investigate this, we have appointed a general practitioner to complete the questionnaires of 35 patients during individual consultations. These results were compared with the self-completed versions of the questionnaires and a high degree of correlation was found. This convinced us that the self-completed data sets were indeed appropriate to use. This data, together with the clinical information gathered from the clinician's medical reports, resulted in the availability of a large set of clinical information about individual patients which was stored in a computer database for use in combination with the biochemical data.

Thus, through the protocol to generate the data for the present study, including compliance with the Ethical Principles for such studies objective 1 of the study was achieved [objective 1 — Chapter 2, § 2.9 (1)]:

> *"In association with clinicians a user-friendly guide for patients complaining on CF was compiled and implemented for the purpose of their informed cooperation in a project aiming towards development of a diagnostic biochemical tool to complement the clinical information."*

The required generation of an informative biochemical data set for biotransformation as envisaged from objective 1, was characterised by unique challenges. The effectiveness of phase I & phase II biotransformation metabolism could only be assessed by implementing a functional challenge test. This was achieved by using caffeine as a phase I challenge substance while aspirin (acetyl salicylic acid) and paracetamol (acetaminophen) were the challenge substances for phase II. Phase I and II analytical methods were also validated to prove their applicability to routine laboratory analysis [Part II, Chapter 4].

The complete biochemical evaluation included also measurements of carnitine and acylcarnitines, antioxidant status and oxidative status. In total, 20 variables were produced which also contained 5 ratio calculations. The global strategy behind the combination of these specific measurements were to assess the possible impact of xenobiotics on liver biotransformation reactions and the accompanying effect on carnitine metabolism, free radical production and antioxidant status. With the development of the biotransformation and associated tests the aim of objective 2 [Chapter 2, § 2.9(2)]) was met for application in the present investigation:

"A battery of biochemical tests for provocation testing with selected conjugation substrates was compiled and validated for evaluation of the functionality of conjugation reactions, antioxidant levels and the production of reactive oxygen species."

In retrospect, three aspects of biotransformation research became evident in the course of the present investigation. These are (1) modes of transformation of xenobiotics, (2) environmental influences, contributing to the indiviual's exposome and (3) comorbidity with related affect conditions. These aspects will now be discussed (9.2.1. 9.2.2 and 9.2.3) before attention is given to the main aim of the present study — contributing to a diagnostic tool for CF (9.3).

9.2.2 Biotransformation of xenobiotics

A limitation of a biochemical profiling study as presented here, is that the battery of proposed analytical tests would cover only a subsection of the extensive phase I and II biotransformation reactions. A contribution to a perspective on this complexity is presented in the expert review presented in Part I, Chapter 1. [*Xenobiotic/medium chain fatty acid: CoA ligase — a critical review on its role in fatty acid metabolism and the detoxification of benzoic acid and aspirin.*]

This paper highlighted the important role that the human xenobiotic/medium chain fatty acid: CoA ligases play in the activation of specific substrates to generate coenzyme A derivatives, a reaction without which the formation of glycine conjugates would not proceed. Special reference is made of the fact the glycine conjugation is continuously studied as a one-step reaction and that important regulatory factors of the individual pathways (CoA synthesis, CoA activation and glycine conjugation) are ignored. Of special importance is the fact that one of the phase II challenge substances used in this study, aspirin would be activated to the corresponding salicyl-CoA by a specific ligase enzyme (ACSM2B) before conjugation with the amino acid glycine. We conclude that the *expert opinion* section in the ligase publication serves to motivate our argument about the complexity of phase II conjugation reactions with special reference to factors like substrate availability and polymorphisms which may affect the activation of xenobiotic substrates and contribute to large inter-individual variability. The following are some of the important focus points extracted from the *expert opinion* section:

- *Polymorphisms in the ACSM genes and disease association*
- *Substrate specificity of the ACSM ligases- is there an ACSM variant that preferentially activates salicylate?*
- *The glycine conjugation pathway is not a one-step process* (Van der Sluis & Erasmus, 2016).

9.2.3 Chronic fatigue, the exposome and biotransformation

A next complex aspect of CF, is the contribution towards the individual case, associated with inherited (genomic) and acquired characteristics (the exposome), derived from the environment. The proposed interplay between chronic fatigue, the exposome and biotransformation is discussed in the literature review [Part I, Chapter 2]. From the material covered in this review, I formulated an *expert opinion* (Erasmus, E. Review paper to be submitted to *Expert opinion in Drug Metabolism and Toxicology).* In this review a model was constructed which displays the interplay between the above-mentioned concepts [Chapter 2, Figure 2-2]. An extension of this model, which is essentially a combination of Figure 2-2 and Figure 4, chapter 7, will be presented in Section 9.3.

In this context, a key opinion expressed here is that the role and importance of an effective, functional biotransformation system in the protection of the exposome is often overlooked. However, in reality, results from our group demonstrates that the total xenobiotic load often exceeds the individual's biotransformation capacity, whether induced by depletion of important amino acid conjugate substrates and cofactors or by a combination of these factors with specific genetic limitations (Gómez-Lechón *et al.,* 2016). The result is invariably an increase in circulating metabolic xenobiotica, which may directly (by inhibition of essential energy-producing processes in the mitochondria) or indirectly (by sequestration of CoA) result in increased insult to the exposome (Morris *et al.,* 2019; Zolkipli-Cunningham & Falk, 2017). Combining chemical/xenobiotic exposure with an aberrant biotransformation metabolism, the prospect is high for causing epidemiology conditions due to exposure, like fatigue.

9.2.4 Comorbid clinical conditions in Chronic Fatigue:

Prolonged fatigue is a common symptom in primary care and could be ascribed to a large number of different clinical conditions. The severe form of this, Chronic Fatigue Syndrome (CFS) is less prevalent but is characterised by debilitating persistent fatigue. Ickmans and coworkers underline disease heterogeneity in CFS by indicating that a measure of endogenous pain inhibition might be a significant predictor of cognitive functioning in CFS patients with FM (Ickmans *et al.,* 2015). Some medical conditions like cancer, AIDS, multiple sclerosis, schizophrenia and bipolar disorder are exclusion factors for CFS.

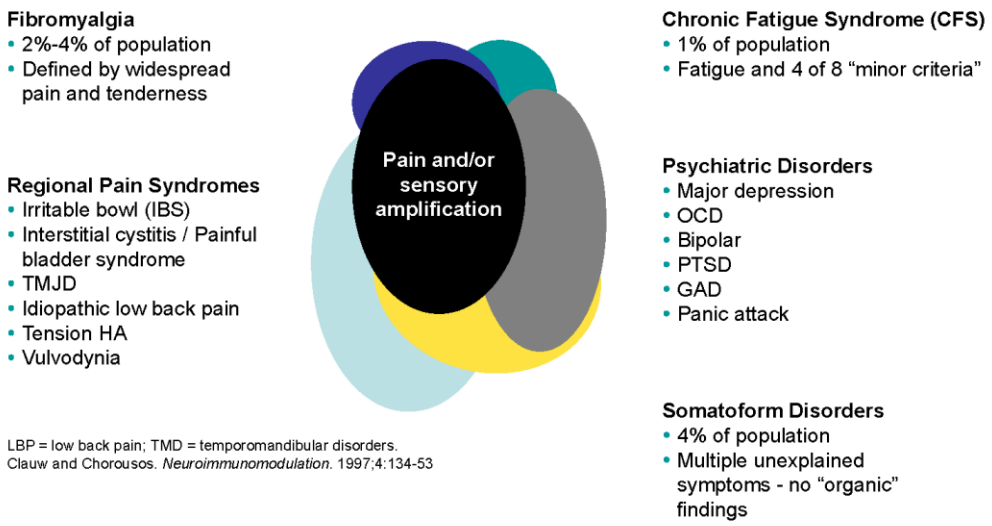


Figure 9-1: Examples of overlapping systemic syndromes characterized by otherwise unexplained chronic pain and fatigue. Adapted from Clauw (2010).

What is highly relevant to the present study is that patients with CF and CFS often present with comorbid medical and psychiatric illnesses which are normally not considered as part of the exclusion criteria (Figure 9-1) These may include exposure syndromes, somatoform disorders, multiple chemical sensitivity and fibromyalgia. In a Co-Twin control study, Aaron and co-workers reported on 127 twin pairs with some of the twins suffering from fibromyalgia, irritable bowel syndrome, multiple chemical sensitivities, temporomandibular disorder, interstitial cystitis, post-concussion syndrome, tension headache, chronic low back pain, chronic pelvic pain (women), and chronic nonbacterial prostatitis (men). They concluded that the prevalence of these comorbid clinical conditions was significantly higher in the fatigued twins compared to their non-fatigued co-twins (Aaron *et al.,* 2001). It is therefore a prerequisite that comorbid conditions should be exclusion factors for any study about fatigue.

The potential for comorbidity with CF was therefore taken into account, as formulated in objective 3. [Chapter 2, § 2.9 (3)]:

> *"Obtain an objective profile on the level of fatigue in at least 750 idiopathic patients selected by the physicians, from which at least 500 cases should be selected — based on clear exclusion criteria — for the assessment of an experimental protocol on the proposed link between CF and biotransformation parameters."*

Thus, in total, 673 women with complaints of fatigue were assessed for study eligibility. Of these, 576 were eligible, following application of the exclusion criteria; all cases with diabetes, chronic high blood pressure, asthma, cancer, arthritis and some other minor clinical conditions

were excluded, but retaining patients suggestive of fibromyalgia, depression or other affect syndromes or presumed comorbidity conditions. All eligible cases fully completed both the PFS and MSQ. Objective 3 was thus successfully accomplished within the time constrains for the study as well as limitations of the clinical guidelines and associated information.

9.3 A biosignature for chronic fatigue

Objective 4 aimed at a correlation between the levels of fatigue and the biochemical information [Chapter 2 § 9.4(4)]:

> *"Perform a correlation of the clinical features of the selected patients with the biotransformation profiles to determine a possible metabolic link to fatigue symptoms, as proposed through the model for this link*"

Such a correlation was envisaged to pave the way towards a biomarker of biosignature for CF. Two statistical procedures (Pearson's and Spearman's correlation tests) were applied on the complete data set from the 576 selected cases. The essential findings from the correlation tests are summarized in Table 9-1. (Note: the complete results from this analysis are presented in the Supplementary Material — accepted manuscript to *Metabolomics*, Erasmus *et al.,* 2019)

Table 9-1: Pearson's and Spearman's correlation tests results

Measure	Pearson's Correlation Coefficient		Spearman's rho Correlation Coefficient	
	PFS Energy	PFS Mental	PFS Energy	PFS Mental
Acetaminophen glucuronide	0.112**	0.060	0.090*	0.035
Acetaminophen sulphate	-0.015	0.007	-0.033	-0.012
Acetaminophen mercapturate	0.085*	0.017	0.034	0.019
Salicyluric acid	0.053	0.023	0.015	0.004

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

Comparing the effect sizes (ES), based on Cohen's d values, tends to deliver a practical visible effect (ES≥0.5) for acetaminophen glucuronide (ES=0.7) and acetaminophen (ES=0.5). Overall this leads one to conclude that an effect is visible, but greater statistical power is required to detect it. In addition a Pearson's correlation was significant at the 0.01 level (2-tailed) between acetaminophen glucuronide and the energy fatigue score (coeff.r=0.112**), and significant for the Spearman's correlation at the level of 0.05 (2-tailed) for the energy fatigue score as well

(coeff.r=0.09*). Thus, neither of these correlations are of practical relevance (|r|<0.5) and point to no more than a slight but consistent association between variables and their ranks in each case. No correlation was observed between the biotransformation parameters and the mental fatigue scores as well.

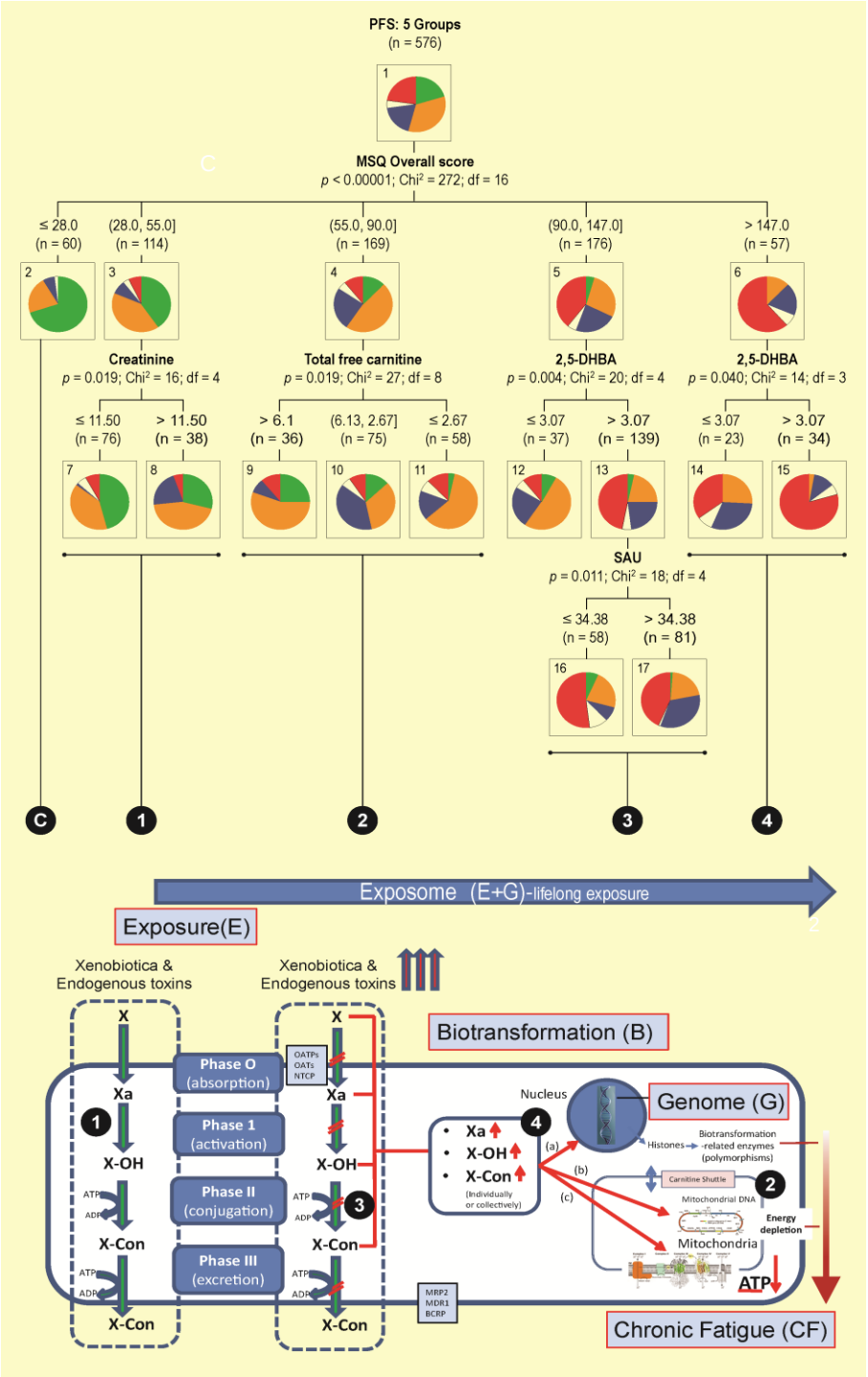
The limited information from the correlation analysis prompted the attempt to classify all cases, based on the complete data set. Using CHAID analysis as the classification instrument of data derived from all three sets (MSQ, PFS and biochemical) provides a clear visual representation showing the advantage of the data from the MSQ used as a classification instrument and for detection of interaction between the three classes of variables [Chapter 7, Figure 4]. The observation that the 20 biochemical variables grouped into 8 biofactors, based on a PCA of the transformed data, emphasizes a strong underlying relationship among the biochemical variables which we developed as the biotransformation instrument and show that biotransformation and associated energy-derived parameters are indicative of undefined chronic fatigue observed in the patient group (Erasmus *et al*., 2019). This observation clearly demonstrates the possibilities in using a biosignature as a diagnostic tool generated via these routes.

Objective 4 was, therefore, not successful through a *correlation* analysis, but was achieved through the *classification* analysis.

9.4 A synthesis of the clinical and biochemical observations

Given this observation, a link between the CHAID classification results and the model for biotransformation [Chapter 2, § 2.7 (Figure 2-2)] will now be presented in Figure 9-2. In this synthesis model, clinical classification of the 576 patients, based on the use of the Piper Fatigue scaling produced five subgroups of degrees of fatigue (see colour key in Figure 9-2). When the medical symptoms data was added to the Piper fatigue grouping, the next level CHAID classification resulted in five new nodes. The node with the lowest medical symptoms score (n=60) [MSQ ≤28.0] was marked as the control group (indicated with C in black circle). In this node the green section (low fatigue) also made up 70% of the 60 cases. It is important to revisit the definition of healthy controls in the context of this study as this was raised by a reviewer in our paper in Chapter 8:

> "*While this is an interesting approach to identify/characterise patients with chronic fatigue the authors fail to compare the data to that of control subjects who does not suffer from chronic fatigue. They only show data from low and high fatigue patients*."



Colour key:

- Green –Low fatigue
- Orange -Mild fatigue
- Blue -Mainly energy fatigue
- Open circle -Mainly mental fatigue
- Red -High energy and mental fatigue

Biochemical key

(C) "Controls"

❶ A subgroup of 114 cases in which creatinine results into further classification into two subgroups;

❷ A subgroup of 169 cases in which total free carnitine contributed to further classification into three subgroups;

❸ A subgroup of 176 cases in which 2,5 DHBA & SAU resulted in two consecutive classifications and

❹ 2,5 DHBA which on its own resulted in the further division of 57 cases into two new subgroups.

Figure 9-2: A combination of the CHAID classification results of the 576 analysed cases and the model which incorporates the contributions of exposure (E), biotransformation (B) and genome (G) on chronic fatigue (CF).

The first group of 60 cases [MSQ score<11] in the classification model may be considered as "control cases", based on the study by Winblad & Lindberg (2019). The investigators used the Fatigue Impact Scale (FIS), (which compare favourable to the PFS (Whitehead, 2009)), to distinguish between healthy controls and patients with Myotonic Dystrophy type I (DMI)

(Winblad & Lindberg, 2019). The patients classified as healthy controls showed a median score *of 18* on the FIS scale. This further supports our position on the use of "low fatigue" cases as controls in the metabolomics study, and not opting for healthy individuals as controls. [Erasmus *et al.*, *Metabolomics* — Chapter 8]. Although using different exclusion criteria, most of the "low fatigue" cases used in the metabolomics study, overlapped with the "controls" identified through the CHAID classification.

As already indicated all further subdivisions of nodes 3-6 was produced by links to the metabolism. This is indicated by branch-links ❶-❹ in Figure 9-2.

Branch-link ❶: Illustrates how creatinine divided node 3 into two new nodes, node 7 (≤11.50 mmol/L creatinine) (n=76) and node 8 (≥11.50 mmol/L creatinine) [n=38] and how these two nodes are linked to the ATP/ADP catalysed reactions in metabolism. Creatinine is produced from the breakdown of creatine and phosphocreatine (Price & Finney, 2000). The major part of creatinine is produced in the muscle. Elimination of creatinine in individuals with normal kidney function is through renal excretion, which has been shown to be fairly constant and equal to its production at steady state (Heymsfield *et al.*, 1983). In a recent study by Lidbury *et al.* it was shown that 24-hour urinary excretion of creatinine in ME/CFS was statistically significant ($p<0.05$) lower (9.9 mmol/24h) [n=68] in comparison to healthy controls (13.1 mmol/24h) [n=13] (Lidbury *et al.*, 2019). This represents total creatinine production per day, while the creatinine concentration in our study could however be influenced by fluid intake. To what extend this observation can be linked to the intricacies of the mitochondrial phosphocreatine shuttle is not clear, but the role of this system- the adenine nucleotide transporter (ANT) and the voltage-dependent anion carrier (VDAC) in intracellular energy transport - is demonstrated in Figure 9-3.

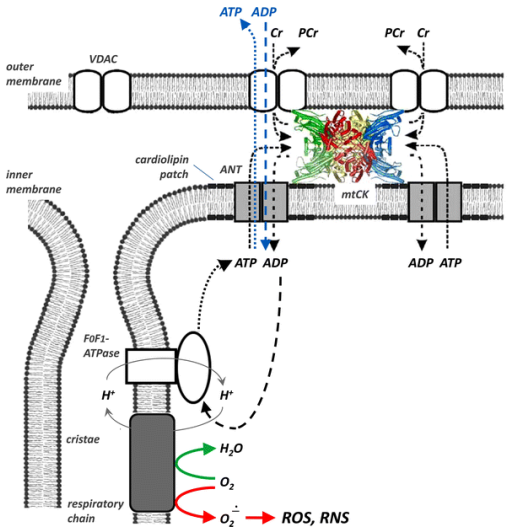


Figure 9-3: Mitochondrial mtCK functions for high-energy metabolite channelling in mitochondria. In cells with oxidative metabolism, respiration (green arrow), ATP synthesis and

ATP export through the inner mitochondrial membrane via adenine nucleotide transporter (ANT) are tightly coupled to trans-phosphorylation of ATP to PCr by mtCK and export of PCr into the cytosol by the outer membrane voltage-dependent anion channel (VDAC) as indicated by black arrows. In turn, Cr stimulates respiration by favouring constant supply of ADP to the matrix (black arrows), which also lowers ROS/RNS production in the intra-mitochondrial space (red arrows) and inhibits mitochondrial permeability transition. The tight coupling of substrate and product fluxes (black arrows) allows a so-called channelling of "high-energy" metabolites, with PCr being the one released into the cytosol, and ATP/ADP being mainly recycled within the mitochondria (reproduced with permission from Wallimann *et al*., 2011).

Branch-link ❷: Illustrates how node 4 is further divided into 3 new nodes (9-11) (total free carnitine) and how these nodes link to mitochondrial function. The role of carnitine in mitochondrial fatty acid transport is well known. It is also known that when specific acyl-groups accumulate in the mitochondria they are excreted as acylcarnitine derivatives. Over-utilization of this detoxification pathway may lead to systemic carnitine depletion which is reflected in lower concentrations of total free and acylcarnitines in the urine (Jones *et al*., 2005).

Branch-link ❸: This link displays a very important connection between free radical metabolism, glycine conjugation and increased fatigue. Node 5 was divided into two new nodes (12 and 13) by 2,5 DHBA, an induced metabolite as result of the acetylsalicylic challenge. Node 13 was again divided into two nodes (16 and 17) by the glycine conjugate of acetylsalicylic acid, salicyluric acid (SAU). It is important that both these divisions (node 5 into 12 and 13) and (node 13 into 16 and 17) generally occurs in patients suffering from significantly higher fatigue (red in pie chart). This we have linked to pathways which have to deal with higher xenobiotic load and where the availability of ATP and conjugation substrates may be a limiting factor. It is also true that accumulating xenobiotica may have a direct effect on mitochondrial energy production.

Branch-link ❹: The division of node 6 into two nodes (14 and 15) by 2,5 DHBA illustrates the effect of circulating free radicals (ROS) on the energy metabolism. Although not exclusively formed by interaction with hydroxyl radicals, 2,5 DHBA has been associated with free radical metabolism (Tai *et al*., 2004). These free radicals results in DNA damage as well as mitochondrial membrane disruption (Pizzino *et al*., 2017) Node 15 also displays the highest percentage of fatigued individuals. ROS are mainly produced by mitochondria and increased amounts of ROS are often associated with pathological conditions which impact on mitochondrial function. Therefore, we have linked these nodes to effects on energy production, impaired conjugation reactions, damage to the nucleus and DNA mutation and polymorphism formation.

A biomarker is currently defined as an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in

a compartment within an organism (National Research Council. Committee on Biological Markers of the National Research Council, 1987).

The relevance of a single biomarker for CF and ME/CFS has already been referred to in Part I, Chapter 2 §2.5: From my literature review it became clear that:

> *"No single biomarker until now has been identified to be the golden key to unlock the causative defect in ME/CFS and will remain unlikely unless the essence of this disorder, its causes and pathophysiology becomes clearly delineated. This emphasized the fact that (1) ME/CFS is a polygenic/environmental condition and (2) research on causes of ME/CFS should take into account regulatory factors which may affect the large number of metabolic reactions and processes seen in these diseases."*

The combination model presented in Figure 9-2 above shows however that a biosignature approach (a combination of metabolite indicators) may be more applicable in which detailed fatigue and medical symptoms classification combined with specific metabolic features could contribute to improved classification and treatment strategies for individuals suffering from fatigue.

9.5 Towards the development of a practical evaluation and treatment tool

The long-term strategy was to develop a practical biochemical evaluation protocol of important biofluid markers of metabolic reactions in the liver against xenobiotics, as observed in patients who suffer from idiopathic fatigue and to combine these results with that of clinical questionnaires to produce a combinatorial result which may contribute to improved diagnoses and ultimately treatment of CF. To illustrate the application of the PFS & MSQ questionnaires and the biotransformation and oxidative stress status analysis in practice, an example of the final report to the medical doctor is included in Annexure C:

9.6 Limitations of the current study

We have attempted to quantify individuals' metabolic capacity for biotransformation by implementing specific challenge tests for phase I and phase II liver detoxification reactions as well monitoring carnitine metabolism, antioxidant status and oxidative status. However, during the course of the study, certain limitations about specific facets of the study became apparent.

(1) The duration of the challenge test may allow slow metabolisers to be classified as falling within normal reference levels. This has become apparent in a study that we have done about the use of an alternative challenge substance, para amino benzoic acid, to monitor

glycine conjugation (Nortje *et al.,* 2016). A possible improvement over the current approach would be to monitor the conversion of the challenge substances over a shorter time period and thereby improve the sensitivity of the test

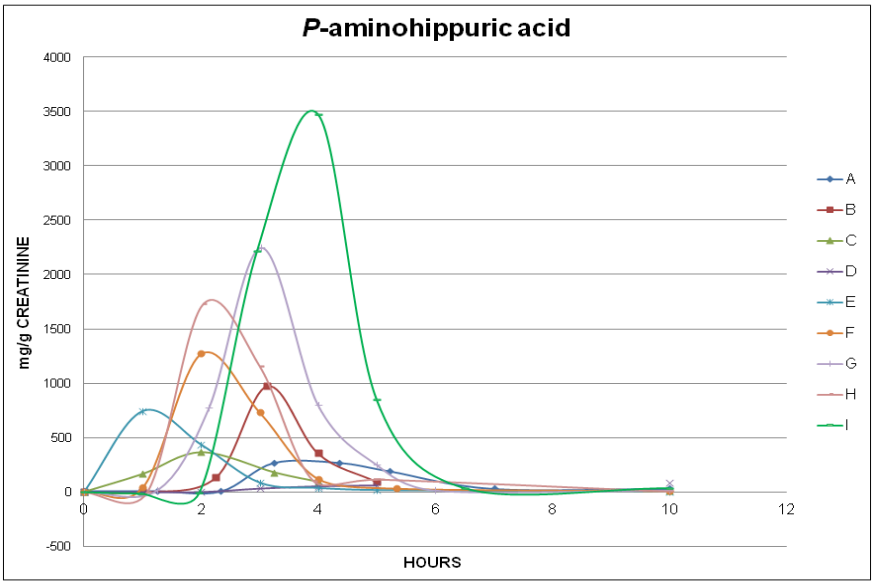


Figure 9-4: The time-dependent excretion by different individuals (A-I) of the glycine conjugate of para-amino benzoic acid. *P*-aminohippuric acid is an alternative probe to monitor glycine conjugation (Nortje *et al*., 2016).

(2) The biochemical variables monitored in the current study were compiled according to literature sources which suggest that the combination of these variables may be ideal in liver biotransformation investigations. Zamek-Gliszczynski and co-workers for example highlighted the importance of sulphate, glucuronide and glutathione as the most prevalent classes of phase II metabolism (Zamek-Gliszczynski *et al*., 2006). The scientific correctness of using these specific variables should be further investigated using metabolomics. Untargeted metabolomic investigations of patient samples could confirm whether this range of targeted metabolites should be expanded (Roux *et al*., 2011; Fang & Gonzalez, 2014; Warth *et al*., 2017).

(3) The logistics involved in the transport of the large volumes urine samples on dry ice posed problematic. In this regard we have developed a dry test sample matrix which will allow transport of dried samples from anywhere in the country by courier service. This matrix can accommodate the equivalent of 2.8 mL wet sample and has been treated with an antioxidant to prevent oxidation of labile metabolites. The tested metabolite profile produced very promising results and further tests are underway. This will however require the further development of an analytical method specific for secondary ROS marker metabolites (Breitenbach *et al*., 2014; Jin & Zangar, 2009; Wang *et al*., 2016).

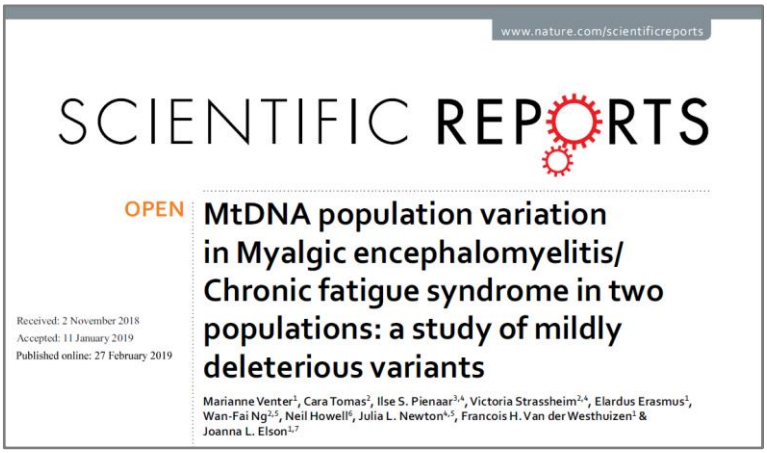
(4) The availability of single nucleotide polymorphism (SNP) information about phase I & II enzymes (De Vega *et al*., 2017). We recognise that correlation of functional enzyme data from challenge tests with information about SNP's may further improve the interpretation of aberrant biotransformation data, and with combination of our classification model, may ultimately improve the global picture about impaired biotransformation and the impact thereof on CF. In this regard we have recently applied for a South African Medical Research Council Pharmacogenomics grant (Genetic and functional profiling of biotransformation in four South African cohorts).

9.7 Future prospects

We and others (Betts & Shelton-Davenport, 2016; Romieu *et al.*, 2008) are of the opinion that biotransformation reactions in the liver will become increasingly under pressure due to the influence of exogenous factors from the environment. It is, therefore, vital that efficient methods for CF diagnosis, classification and treatment should be implemented on a practical level. The biosignature approach that we advocate as a result of this study could play an important role in this regard. There are, however, a number of factors which should be kept in mind for future expansion of this research:

1) The role of mitochondrial DNA mutations in CF

The key role that mitochondria play in energy production and possible links to CF has been highlighted in our biosignature model. A logical question to ask about mitochondrial energy production is what about the influence of mitochondrial DNA mutations on energy production? mtDNA mutations frequently cause multisystem disorders with fatigue being prevalent in these patient groups. We have been collaborating in two studies addressing this question and which have produced very interesting results. Schoeman *et al*. showed that clinically proven mtDNA mutations are not common in those with chronic fatigue syndrome (Schoeman *et al*., 2017).


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MtDNA population variation in Myalgic encephalomyelitis/ Chronic fatigue syndrome in two populations: a study of mildly deleterious variants

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In another study where two cohorts were analysed, one from the UK and the other from South Africa, we demonstrated that for both cohorts, ME/CFS patients had an excess of individuals without a mildly deleterious population variant (Venter *et al*., 2019). The role of mitochondrial DNA mutations in CF & ME/CFS should clearly be further investigated.

2) Personalized and precision medicine

Based on the outcome of our own experience with regard to biotransformation metabolism imbalances and patient specific treatment, it is one of the possibilities which should be pursued to improve the prognosis of individuals suffering from idiopathic fatigue. Although outside the scope of the current study, it is worthwhile to report on some of the diagnosed and treated cases. In a number of cases where perturbed biotransformation was indicated, a compounded treatment based on a specific biotransformation abnormality often led to notable improvement, especially with regard to fatigue ratings. In this regard it is important to realize that given the enormity of inter-individual variation in biotransformation (Betts & Shelton-Davenport, 2016), the concept of personalized and precision medicine may play an ever increasing role in the design of treatment for fatigue. Precision medicine is defined as "the emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person". In a 2011 US National Research Council report (NRC, 2011) it was stated:

> *"Precision medicine refers to the tailoring of medical treatment to the individual characteristics of each patient. It does not literally mean the creation of drugs or medical devices that are unique to a patient, but rather the ability to classify individuals into subpopulations that differ in their susceptibility to a particular disease…or in their response to a specific treatment. Although the term "personalized medicine" is also used to convey this meaning, that term is sometimes misinterpreted as implying that unique treatments can be designed for each individual. It should be emphasized that in "precision medicine," the word "precision" is being used in a colloquial sense, to mean both "accurate" and "precise.""*

However, in the context of treatment for biotransformation abnormalities, personalized medicine may be more applicable instead of patients being classified mainly on genetic information. The reason for this is that efficient biotransformation is not only affected by genetic influences but also by the availability of specific substrates, cofactors and coenzymes. This is mainly due to the fact that biotransformation reactions are unique in that they are most often multi-step reactions which produce excretion products which imply constant loss of vital conjugation factors.

Against this background, with the confidence gained from this study, we wish to advocate the use of our diagnostic model and a personalized approach to the treatment of chronic fatigue. Preliminary results obtained from the cases treated and analysed here (data not shown) strongly support the argument that personalized medicine as a potential treatment approach should be further investigated.

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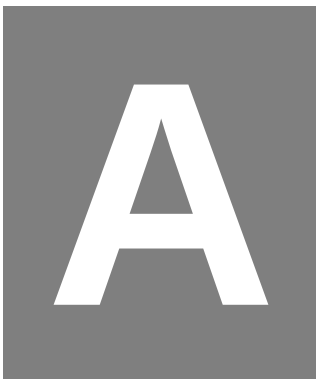
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ANNEXURE



SOP of specimen collection and handling for BOSS assessments

Biotransformation and Oxidative Stress Status laboratory Centre for Human Metabolomics	**SOP No:BOSS-001**
COLLECTION AND HANDLING OF SPECIMENS FOR BIOTRANSFORMATION AND OXIDATIVE STATUS ASSESSMENTS	**Edition 7**

CONTENTS

I. Samples required for the biotransformation and oxidative stress status assessment

II. Sample collection & handling

III. Sending of samples to the Biotransformation and Oxidative stress Status laboratory at the NWU

VI. Shipping addresses and contact details

Biotransformation and Oxidative Stress Status laboratory Centre for Human Metabolomics	SOP No:BOSS-001
COLLECTION AND HANDLING OF SPECIMENS FOR BIOTRANSFORMATION AND OXIDATIVE STATUS ASSESSMENTS	**Edition 7**

1. Samples required for the biotransformation and oxidative stress status assessment

1.1 Baseline urine collection
1.2 Two timed saliva samples
1.3 Ten-hour urine collection
1.4 Blood samples:

- 1 x EDTA tubes (purple top)
- 1 x SST tube (yellow top)

Blood samples are collected, after collection of urine and saliva samples, on the day that the samples are delivered to the lab.

2. Sample collection & handling

2.1 Baseline urine sample

The following urine collection container is needed for the collection of baseline urine samples

- 1 X 30 ml urine containers (Included in test kit)
- Collect early morning baseline urine sample. All urine samples **must be frozen as soon as possible and kept frozen for 24 hours before sending to the biotransformation and oxidative stress status laboratory. Send on dry ice.**

2.2 Saliva samples for caffeine clearance test:

Two salivettes (cotton swap tubes) is needed for the collection of saliva samples (Included in test kit).
Ensure that the patients **name,** and the **time** and **date** of saliva collection are written on the sample containers (2 saliva collection tubes/salivettes). Remove the top cap of the collection tube to expose the swab. Place swab **directly into the mouth** by tipping the container so that the swab falls into the mouth **(DO NOT TOUCH THE SWAB WITH FINGERS).** Keep the swap in the mouth for **at least**

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five minutes. Roll the swab in the mouth, **do not chew the swab**. Place the swap back into its container without touching, and replace the cap. Record the collection time, name and date on the salivette tube. During the collection it is very important that the patient **do not to cough or clear their throat** into the collection container. Remember **no consumption of coffee, tea, coke or chocolate** is allowed during the caffeine clearance test. All saliva samples **must be frozen ASAP and kept frozen for 24 hours before sending to the biotransformation and oxidative stress status laboratory. Send on dry ice with "KEEP FROZEN" sticker on cool box.**

2.3 Ten-hour urine samples:

The following urine collection container are needed for the collection of 10-hour urine samples

•1 x 1L urine container (Included in test kit)

Ensure that the patient's **name, time** and **date** of urine collection are written on the sample container. All urine samples **must be frozen ASAP and kept frozen for 24 hours before sending to the biotransformation and oxidative stress status laboratory. Send on dry ice with "KEEP FROZEN" sticker on cool box.**

- **For shipping purposes, the collecting lab <u>may</u> aliquot the urine into 2 x 50ml urine containers, <u>AFTER</u> the total volume of the 10-hour samples were measured and noted on the patients form.**

2.4 Blood samples for oxidative stress status and nucleotide analysis:

The following tubes are needed for the collection of blood samples (Not included in test kit).

- 1 x 5 ml SST tube (yellow top) • 1 x 5 ml EDTA tube (purple top)

Ensure that the patients **name,** and the **time** and **date** of sample collection are written on the sample containers.

Biotransformation and Oxidative Stress Status laboratory Centre for Human Metabolomics	**SOP No:BOSS-001**
COLLECTION AND HANDLING OF SPECIMENS FOR BIOTRANSFORMATION AND OXIDATIVE STATUS ASSESSMENTS	**Edition 7**

2.1.1 Serum for oxidative stress status analysis:

- Allow SST tube to coagulate for 30 minutes at room temperature.
- Centrifuge serum for 10 minutes.
- Freeze at -20 °C ASAP and **keep frozen for 24 hours before sending to the biotransformation and oxidative stress status laboratory. Send on dry ice with "KEEP FROZEN" sticker on cool box.**

2.1.2 EDTA blood for oxidative stress status analysis:

Freeze the 5 ml EDTA tube (purple top) at -20 °C. **Do not** transfer the blood to an eppendorf tube. **Freeze the entire 5 ml tube ASAP and keep frozen for 24 hours before sending to the biotransformation and oxidative stress status laboratory. Send on dry ice with "KEEP FROZEN" sticker on cool box.**

3. Sending of samples to the biotransformation and oxidative stress status laboratory at the NWU

IMPORTANT: Samples must be send on DRY ICE with "KEEP FROZEN" sticker on cool box.

3.1 Samples should be send to the biotransformation and oxidative stress status laboratory at the NWU for analysis as soon as possible.

3.2 **The samples should reach the biotransformation and oxidative stress status laboratory in a frozen state.** Please send the samples on **DRY ICE** with "**KEEP FROZEN" sticker on cool box** in order to keep them frozen. **The samples cannot be used for any analysis if it is thawed during transport.**

Biotransformation and Oxidative Stress Status laboratory Centre for Human Metabolomics	**SOP No:BOSS-001**
COLLECTION AND HANDLING OF SPECIMENS FOR BIOTRANSFORMATION AND OXIDATIVE STATUS ASSESSMENTS	**Edition 7**

3.3 Samples should preferably be send before Wednesday of every week, as there are no personnel available on weekends to receive samples.

4. Shipping addresses and contact details

4.1 Shipping address: ATT: Carien Jacobs
Biotransformation and oxidative stress status laboratory (BOSS) Biochemistry - Building F3
North-West University (Potchefstroom campus) Hoffman Street
Potchefstroom South-Africa

4.2 Contact details: Me. Carien Jacobs (018) 299 2042
mej.cljacobs@gmail.com

ANNEXURE B

BOSS quality control procedure

BOSS Quality Control (QC) Procedure

FREQUENCY AND SUMMARY OF INTERNAL QUALITY CONTROL (IQC) of the BOSS laboratory.

- For every test performed, there is a system of IQC (internal quality control)
- Non-conformances and corrective actions are documented, checked and stored.
- The standard protocols for all BOSS tests include the use of at least two quality control (QC) samples, validation of the test results are dependent on these control results being within a defined range.
- During transition phases from an old to a new series of IQC, duplicate assays are performed for the new QC lots. Acceptable ranges (mean 1, 2 and 3sd) are established for the IQC material on a minimum of 20 different assays.
- Internal Quality Control samples are assayed in the beginning, middle and end position of each batch depending on how many samples are analysed.

The results of both sets of controls are recorded electronically and assessed against the Westgard rules.

ANNEXURE C

Example of the BOSS report

Detoxification & Oxidative Status Profile

Refering Dr.: Dr. XXXXX

Patient name: XXXXX **D.O.B:** XXXXX

Lab number: XXXXX **Gender:** Female

Phase I Detoxification (Oxidation)

Caffeine clearance	**0.8**	0 1 2 3 4 5	Reference range: 0.5 - 1.6 ml/min/kg

Phase II Detoxification (Conjugation)

Glutathionation % Recovery	**4.2**	0 5 10 15	Reference range: 5.6 - 11.4 %
Glycination % Recovery	**33.6**	0 20 40 60 80	Reference range: 30 - 53 %
Sulfation % Recovery	**13.5**	0 10 20 30 40 50	Reference range: 16 - 36 %
Glucuronidation % Recovery	**45.7**	0 20 40 60 80	Reference range: 27 - 56 %

Carnitine Conjugation

Total free carnitine	**1.2**	0 1 2 3 4 5 6	Reference range: 0.6 - 5.1 mmol/mol creatinine
Total acylcarnitine	**5.5**	0 5 10 15	Reference range: 0.8 - 9.8 mmol/mol creatinine
Acylcarnitine : free carnitine	**4.4**	0 1 2 3 4 5 6	Reference range: <1.9

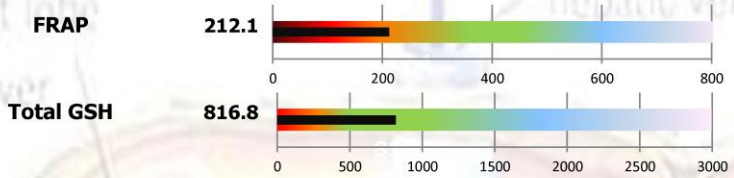
Phase I:Phase II Detoxification Ratios

Phase I : Sulfation	**5.9**	0 5 10 15	Reference range: 3.5 -13.0
Phase I : Glycination	**2.3**	0 1 2 3 4 5	Reference range: 1.3 - 3.5
Phase I : Glucuronidation	**1.7**	0 1 2 3 4 5 6	Reference range: 1.9 - 4.2

Detoxification & Oxidative Status Profile

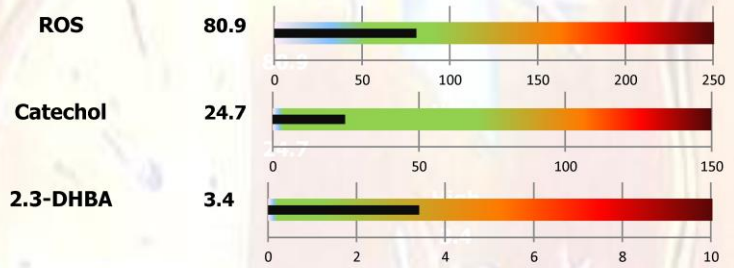
Refering Dr.: XXXXX
Patient name: XXXXX **D.O.B:** XXXXX
Lab number: XXXXX **Gender:** Female

Antioxidant Status



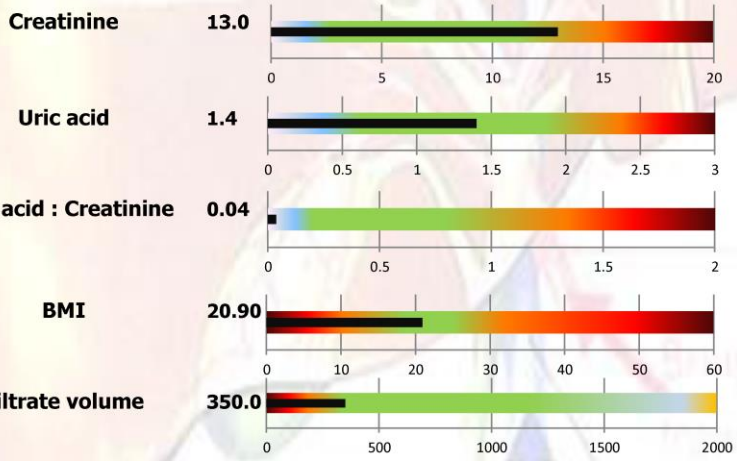
FRAP	212.1	Reference range: 355.4 - 448.0 µM
Total GSH	816.8	Reference range: 874.9 - 1025.9 µM

Oxidative Status



ROS	80.9	Reference range: 52.4 - 107.3 Units
Catechol	24.7	Reference range: 3.4 - 69.8 µM
2.3-DHBA	3.4	Reference range: 0.2 - 2.5 µM

Additional information



Creatinine	13.0	Reference range: 2.65 - 11.05 mmol/l
Uric acid	1.4	*Reference range: 0.62 - 1.86 mmol/l
Uric acid : Creatinine	0.04	*Reference range: 0.2 - 0.8
BMI	20.90	*Reference range: 18.5 - 29.9 kg/m^2
Filtrate volume	350.0	*Reference range: 333 - 833 ml/10hours

*Calculated for 10hour urine

Commentary

Detoxification metabolism is the body's method of eliminating harmful compounds that could influence normal metabolic functions. These compounds could originate from the environment or as a result of normal or affected cellular metabolism. The organ that is primarily responsible for detoxification metabolism is the liver where two distinct phases, Phase I and II are involved in detoxification. Phase I changes the chemical character of toxins to enable Phase II to transport the chemicals out of the body. Both these phases are driven by a number of enzymes and the effectiveness of these reactions are challenged in the tests that we carry out. Phase I and II must function within certain ranges under the detoxification challenge to ensure that toxins are eliminated. Phase I and II must also be in balance to prevent the accumulation of Phase I metabolites which may be harmful to the body.

Commentary (Continued)

In your case the test for Phase I detoxification displayed normal values. Glutathione conjugation tested low. This should be treated with precursor supplementation. This compound is very important in the body's own antioxidant system. Glycine conjugation tested normal. Sulphate conjugation tested low. This should be treated with precursor supplementation. Glucuronic acid conjugation tested normal. Systemic free carnitine levels tested normal. Total acylcarnitine levels tested normal. The acylcarnitine to free carnitine ratio tested high, which is an indication of high carnitine demand-carnitine should be supplemented. The Phase I:Phase II ratio for sulphate conjugation tested normal. The Phase I:Phase II ratio for glycine conjugation tested normal. The Phase I:Phase II ratio for glucuronic acid conjugation tested low. The FRAP test values were low, indicating low total reducing capacity. Total glutathione levels tested low, indicating limited antioxidant capacity from this source. The reactive oxygen species (ROS) tested within normal levels. The catechol levels were within normal range. The 2,3 DHBA levels tested increased, indicating higher than normal hydroxyl radical concentrations.

Disclaimer:

Information on this report is provided for informational purposes and is not meant to substitute for the advice provided by your own physician or other medical professional. You should read carefully all product packaging. If you have or suspect that you have a medical problem, promptly contact your health care provider.

ANNEXURE



Example of the Clinical report

Patient Name/Surname:	XXXXX
BOSS Lab.#	XXXXX
Are you often tired?	YES

Piper Fatigue Scoring

#	Numbers	Items	Grouping	Score
1	2,3,4,5,6,7	6	Behavioral/Severity	8
2	8,9,10,11,12	5	Affective Meaning	10
3	13,14,15,16,17	5	Sensory	9
4	18,19,20,21,22,23	6	Cognitive/Mood	5
5	2 up to 23	22	**PFS Overall Score**	**8**

PFS Severity Codes:	
0	**None**
1 - 3	**Mild**
4 - 6	**Moderate**
7 - 10	**Severe**

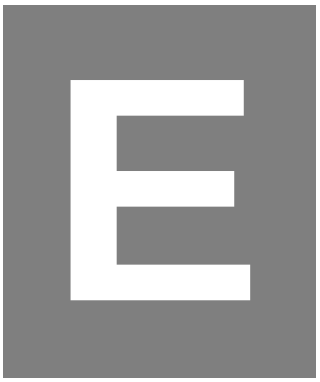
MSQ Scoring

#	Grouping	Total
1	Head	12
2	Ears	0
3	Eyes	6
4	Skin	2
5	Nose	9
6	Heart	1
7	Emotions	10
8	Mind	0
9	Digestive track	7
10	Other	1
11	Mouth/Throat	3
12	Lungs	1
13	Energy/Activity	8
14	Weight	6
15	Joints/Muscle	7
	MSQ Overall Score	**73**

MSQStage of Toxic Progression:	
0 - 36	**Maintain**
37 - 55	**Early Toxicity**
56 - 75	**Slipping**
>75	**Destructive**
10 or more in any 1 section	**Slipping**

Medication:	
Birth Control or HR:	
Medical Condition:	
Pt Comment/Symptoms:	

ANNEXURE



Ethics approval

ANNEXURE **E**

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Ethics Committee
Tel +27 18 299 4850
Fax +27 18 293 5329
Email Ethics@nwu.ac.za

19 February 2012

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title Biotransformation metabolism and oxidative status assessment

Project Leader: Mr E Erasmus

Ethics number:

N	W	U	-	0	0	1	0	2	-	1	2	-	A	1
Institution				Project Number						Year			Status	

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

Approval date: 2013/02/18 **Expiry date:** 2018/02/18

Special conditions of the approval (if any): None

General conditions:

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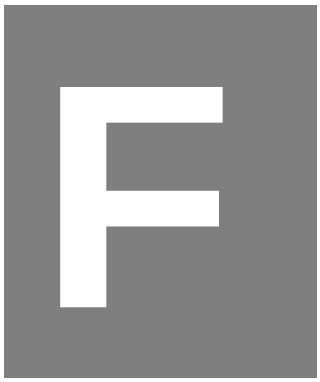
- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
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  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
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The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof Amanda Lourens
(chair NWU Ethics Committee)

ANNEXURE



Permission and contributions of co-authors

F.1 Publication I (Chapter 1)

Author permissions: The co-author (Rencia van der Sluis) of the paper titled "Xenobiotic/medium chain fatty acid: CoA ligase — a critical review on its role in fatty acid metabolism and the detoxification of benzoic acid and aspirin" had no objection to the use of the paper, as presented in Chapter 1, as part of the thesis of Elardus Erasmus submitted for the degree, *Doctor of Philosophy in Biochemistry*.

Author contributions: EE, RvdS

Conceptualization: RvdS, EE. Writing original draft: RvdS, EE. Writing — review & editing: RvdS, EE.

F.2 Publication II (Chapter 6)

Author permissions: None of the co-authors (Rencia van der Sluis, Vida Ungerer, Carla Nortje or Alberdina A. van Dijk) of the paper titled "New insights into the catalytic mechanism of human glycine N-acyltransferase" had any objection to the use of the paper, as presented in Chapter 6, as part of the thesis of Elardus Erasmus submitted for the degree, *Doctor of Philosophy in Biochemistry*.

Author contributions: EE, RvdS, VU, CN, AAvD

Conceptualization: RvdS, EE. Data curation: RvdS, VU, CN. Formal analysis: VU, CN. Funding acquisition: RvdS, EE. Investigation: RvdS, VU, EE. Methodology: EE, RvdS, VU, CN. Project administration: RvdS. Software: RvdS, VU. Supervision: EE, RvdS, AAvD. Writing — original draft: VU. Writing — review & editing: EE, RvdS, VU, CN, AAvD.

F.3 Publication III (Chapter 7)

Author permissions: None of the co-authors (Francois E. Steffens, Mari van Reenen, B. Chris Vorster or Carolus J. Reinecke) of the paper titled "Biotransformation profiles from a cohort of chronic fatigue women in response to a hepatic detoxification challenge" had any objection to the use of the paper, as presented in Chapter 7, as part of the thesis of Elardus Erasmus submitted for the degree, *Doctor of Philosophy in Biochemistry*.

Author contributions: Conceptualization: EE, BCV, CJR. Data curation: EE, FES, MvR, CR. Formal analysis: EE, CJR. Funding acquisition: CJR. Investigation: EE, BCV, CR. Methodology: EE, CJR. Project administration: EE, CJR. Software: FES, MvR. Supervision: BCV, CJR. Writing — original draft: EE, FES, MvR, BCV, CJR. Writing — review & editing: EE, FES, MvR, BCV, CJR.

F.4 Manuscript II (Chapter 5)

Author permissions: None of the co-authors (Francois E. Steffens or Carolus J. Reinecke) of the manuscript titled “A statistical approach for the classification of chronic fatigue cases, based on large and diverse data sets” had any objection to the use of the paper, as presented in Chapter 4, as part of the thesis of Elardus Erasmus submitted for the degree, *Doctor of Philosophy in Biochemistry*.

Author contributions: EE interacted with the physicians, collected the clinical information and compiled all data from the PFS and MSQ; EE collected all biofluid samples in collaboration with pathology laboratories and generated the experimental biochemical data from these samples; FES proposed the statistical methodology used, conducted all statistical analyses and wrote the original version of this paper; CJR coordinated and supervised the process. All authors contributed to the development of the paper and approved the final version.

F.5 Manuscript III (Chapter 8)

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Author contributions: EE, SM, MvR, FES, BCV, CJR

EE was responsible for the experimental design on fatigue in the cohort of patients, interaction with the physicians and for the collection of the samples and the biochemical analysis, as well as data collection from the questionnaires. SM did the ^{1}H-NMR analysis and data generation and provided the quantitative metabolite information on the detoxification challenge in the selected cases. FES did the statistical analysis on the link between the medical and fatigue scales. MvR did all pre-processing of the data and provided the univariate and multivariate analysis of all data. CV provided the input for the clinical aspects of the study. CJR participated in all areas of the research, data analysis and proposed the original outline of the manuscript, to which all authors provided their expert contributions. All authors have read and approved the final manuscript.

ANNEXURE G

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Scope of the journal: PLOS ONE is the journal of choice for authors who want their research to be available to all, immediately and without restriction. Articles are highly discoverable and accessible to researchers both in and outside of their discipline. Their active press program places articles in high profile global media outlets such as the *Huffington Post* (67 mentions in 2018), *National Geographic* (87 mentions in 2018), and *Scientific American* (46 mentions in 2018).

Impact factor: 3.167

Additional information available at Springer website:
https://journals.plos.org/plosone/s/journal-information

G.2. Metabolomics

Metabolomics is a peer reviewed scientific journal:

Scope of the journal: Metabolomics is an indispensable platform for researchers using new post-genomics approaches, to discover networks and interactions between metabolites, pharmaceuticals, SNPs, proteins and more. Its articles go beyond the genome and metabolome, by including original clinical study material together with big data from new emerging technologies.

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Additional information available at Springer website:
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G.3. Journal of biochemical and molecular toxicology

The *Journal of Biochemical and Molecular Toxicology is a peer reviewed journal:*

Scope of the journal: The *Journal of Biochemical and Molecular Toxicology* is an international journal that contains original research papers, rapid communications, mini-reviews, and book reviews, all focusing on the molecular mechanisms of action and detoxication of exogenous and endogenous chemicals and toxic agents. The scope includes effects on the organism at all stages of development, on organ systems, tissues, and cells as well as on enzymes, receptors, hormones, and genes. The biochemical and molecular aspects of uptake, transport, storage, excretion, activation and detoxication of drugs, agricultural, industrial and environmental

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Impact factor: 3.167

Additional information available at Wiley website:
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G.4. Expert opinion on drug metabolism & toxicology

Expert Opinion on Drug Metabolism & Toxicology is a MEDLINE-indexed, peer-reviewed, international journal publishing review articles on all aspects of ADME-Tox. Each article is structured to incorporate the author's own expert opinion on the scope for future development.

Scope of the journal: The Editors welcome: Reviews covering metabolic, pharmacokinetic and toxicological issues relating to specific drugs, drug-drug interactions, drug classes or their use in specific populations; issues relating to enzymes involved in the metabolism, disposition and excretion of drugs; techniques involved in the study of drug metabolism and toxicology; novel technologies for obtaining ADME-Tox data. Drug Evaluations reviewing the clinical, toxicological and pharmacokinetic data on a particular drug. The audience consists of scientists and managers in the pharmaceutical industry, pharmacologists, clinical toxicologists and related professionals.

Impact factor: 3.487

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ANNEXURE

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H.1. License agreement for Figure 2-1: Schematic outline of the intricacies of phases 0, I, II and III xenobiotic biotransformation regulation.

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