The assessment of detoxification metabolism in fatty acid oxidation deficiencies

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ABSTRACT

The concept of accumulating xenobiotics within the human body as a health risk is well known. However, these compounds can also be endogenous, as in the case of inborn errors of metabolism. Biotransformation of both exogenous and endogenous toxic compounds is an important function of the liver, and the critical balance between these systems is of fundamental importance for cellular health. Fatty acid β-oxidation deficiencies are associated with characteristic clinical symptoms as a consequence of the accumulation of specific metabolites. For these accumulated metabolites various nutrients are indispensable for optimal biotransformation and continuous accumulation of metabolites can ultimately result in the depletion of biotransformation substrates and cofactors.

In this study, a novel model (the unbalanced biotransformation metabolism model) is proposed that describes the critical balance between Phase I and Phase II biotransformation and how a disturbance in this balance will increase the oxidative stress status. The significance of this model lies within the treatment possibilities, as the assessment of biotransformation metabolism and oxidative stress status can lead to the development of nutritional treatment strategies to correct imbalances. The value of this model is illustrated by its application to a clinical case investigated.

In addition to the use of nutritional supplementation in treatment, biotransformation substrates and cofactors were also used to develop a "substrate loading cocktail". This cocktail ensured sufficient availability of biotransformation substrates and precursors to stimulate coenzyme A biosynthesis. The application of this "substrate loading cocktail" in subjects with both induced and inborn errors in fatty acid oxidation demonstrated that such a novel approach is a useful tool to give new insight into these kinds of deficiencies and open the possibility for the identification of new deficiencies.

Interesting observations made in subjects originally referred for biotransformation and oxidative stress status profiles led to the first *in vivo* evidence of an inhibitory effect of acetylsalicylic acid on short-chain fatty acid metabolism possibly at the level of isobutyryl-CoA dehydrogenase. Since not all individuals were affected to the same degree, this observation can potentially be used to detect individuals with rate-limiting polymorphisms or mutations in the isobutyryl-CoA dehydrogenase enzyme.

Key terms: biotransformation metabolism; detoxification metabolism; oxidative stress status; fatty acid β-oxidation; biotransformation substrates

UITTREKSEL

Die konsep dat akkumulering van xenobiotiese verbindings in die menslike liggaam 'n gesondheidsrisiko is, is 'n welbekende feit. Hierdie verbindings kan egter ook endogeen wees, soos in die geval van aangebore metaboliese siektetoestande. Biotransformering van beide eksogene en endogene toksiese verbindings is ‘n belangrike funksie van die lewer, en die kritiese balans tussen hierdie sisteme is van fundamentele belang vir sellulêre gesondheid. Vetsuur-β-oksidasie defekte word geassosieer met kenmerkende kliniese simptome as gevolg van die akkumulering van spesifieke metaboliete. Verskeie nutriënte speel ‘n onvervangbare rol vir optimale biotransformering van akkumulerende metaboliete en volgehoue akkumulering van metaboliete kan uiteindelik lei tot die uitputting van biotransformasiesubstrate en ko-faktore.

In hierdie studie is 'n nuwe model (die ongebalanseerde biotransformasiemetabolismemodel) voorgestel om die kritiese balans tussen Fase I en Fase II biotransformasie, en hoe 'n versteuring in hierdie balans sal lei tot verhoogde oksidatiewe stresstatus, te beskryf. Die behandelingsmoontlikhede maak hierdie model betekenisvol, omdat die ondersoek na biotransformasie metabolisme en oksidatiewe stresstatus kan lei tot die ontwikkeling van nutriëntgebaseerde behandelingstrategieë, om wanbalanse reg te stel. Die waarde van hierdie model word geïllustreer deur die toepassing daarvan op ‘n kliniese geval wat ondersoek is.

Tesame met die gebruik van nutriëntsupplementasie, is daar ook gebruik gemaak van biotransformasiesubstrate en ko-faktore, om ‘n “substraatbeladingsmengsel” te ontwikkel. Hierdie mengsel verseker voldoende beskikbaarheid van biotransformasiesubstrate en voorgangers om die biosintese van ko-ensiem A te stimuleer. Die toepassing van hierdie “substraatbeladingsmengsel” op individue met beide geïnduseerde sowel as aangebore defekte in vetsuuroksidasie, het aangetoon dat hierdie nuwe benadering 'n bruikbare instrument is wat nuwe insig kan gee in hierdie tipe defekte. Dit open verder ook die moontlikheid vir die identifisering van nuwe defekte.

Interessante waarnemings, wat in individue gemaak is, wat oorspronklik verwys is vir biotransformasie en oksidatiewe stresstatus profiele, het gelei tot die eerste *in vivo* bewyse dat asetielsalisielsuur 'n inhiberende effek het op die kortketting vetsuurmetabolisme, moontlik op die vlak van isobuteriel-KoA dehidrogenase. Alle individue word nie tot dieselfde mate

beïnvloed nie en daarom kan hierdie waarneming potensieel gebruik word om individue met tempo-beperkende polimorfismes of mutasies in die isobuteriel-KoA dehidrogenaseensiem op te spoor.

Sleutel terme: biotransformasiemetabolisme; detoksifikasiemetabolisme; oksidatiewe stresstatus; vetsuur-β-oksidasie; biotransformasiesubstrate

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2,3-DHBA	2,3-dihydroxybenzoic acid
2,5-DHBA	2,5-dihydroxybenzoic acid
4-HNE	4-hydroxynonenal
AMDIS	automated mass spectral deconvolution and identification system
ATP	adenosine triphosphate
BSTFA	bis(trimethylsilyl)trifluoroasetamide
CASTOR	coenzyme A sequestration toxicity and redistribution
CAT	carnitine acyltransferase
CoA	coenzyme A
CYP1A1	cytochrome P450 1A1
CYP1A2	cytochrome P450 1A2
CYP2B	cytochrome P450 2B
CYP3A4	cytochrome P450 3A4
CT	computed tomography
DC	dicarboxylic acid
DEPPD	N,N-diethyl-para-phenylendiamine
EDTA	ethylenediaminetetraacetic acid
ESI-MS/MS	electrospray ionisation tandem mass spectrometry
FAD	flavin adenine dinucleotide
FRAP	ferric reducing antioxidant power

GC-MS	gas chromatography mass spectrometry
GLYAT	glycine N-acyltansferase
GSH	reduced glutathione
GSSG	oxidised glutathione
GSTs	glutathione S-transferases
HCl	hydrochloric acid
H_2O	water
HPLC	high performance liquid chromatography
ICIEM	International Congress of Inborn Errors of Metabolism
IUPAC	International Union of Pure and Applied Chemistry
LCHAD	long-chain 3-hydroxyacyl-CoA dehydrogenase
LC-MS/MS	liquid chromatography tandem mass spectrometric
MCAD	medium-chain acyl-coenzyme A dehydrogenase
MDA	malondialdehyde
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTE	mitochondrial trifunctional β-oxidation enzyme
NAC	n-acetyl cysteine
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
PUFAs	polyunsaturated fatty acids

ROS	reactive oxygen species
SCAD	short-chain acyl-coenzyme A dehydrogenase
SIM	single ion monitoring
SPE	solid phase extraction
SST	serum separation tubes
TFA	trifluoroacetic acid
TMCS	trimethylchlorosylane
TPTZ	trihydrate, 2,4,6-tripyridyl-s-triazine
VLCAD	very long-chain acyl-coenzyme A dehydrogenase
VPA	valproate

Chapter 1
Introduction

1.1 Introduction

Biotransformation (or detoxification) metabolism of toxic compounds is regarded as one of the most important functions of the liver. A complex system of enzymes exists within the human body to convert highly lipophilic compounds to water soluble compounds, which can be excreted. This system includes two types of enzymatic modifications, known as Phase I and Phase II biotransformation (or detoxification) reactions. Phase I, which is also known as the oxidative phase, include oxidation, reduction and hydrolysis reactions. These reactions expose functional groups to form reactive sites, which improve water solubility itself, but also allow Phase II reactions to ensue. Phase II reactions are also known as conjugation reactions and include glucuronide conjugation, sulfate[1] conjugation, glutathione conjugation, conjugation with amino acids like glycine and carnitine conjugation. Phase II requires the presence of substrates and cofactors which can be derived from dietary sources. A great amount of inter-individual variability exists within these enzyme systems and in addition, these systems are also highly responsive to environmental conditions, lifestyle and genetic differences. Since the discovery of polymorphisms within these enzyme systems, a lot of research focused on the ability to measure biotransformation enzyme activity, as biotransformation ability plays an important role in the development of various pathological conditions (Liska, 1998; Liska *et al*., 2006).

1.2 Problem statement and substantiation

The metabolic processes that are fundamental for maintaining normal cell structure and function are highly regulated enzyme catalysed processes. Defects in these enzyme systems, whether induced or inherited, have significant consequences in humans, i.e. the accumulation of toxic substrates upstream of the enzyme defect, disturbances in metabolic intermediates downstream of the enzyme defect and the formation of intermediates by alternative biochemical pathways (Newman, 2004). On a clinical level, these biochemical abnormalities will give rise to various pathological conditions, including acute life-threatening encephalopathy, hyperammonemia, metabolic acidosis, hypoglycaemia, jaundice and liver dysfunction (Vangala and Tonelli, 2007). These clinical symptoms are a consequence of the accumulation of specific metabolites, which can ultimately result in the depletion of

[1] Sulfate was adopted as the spelling by the International Union of Pure and Applied Chemistry (IUPAC) in 1990, and is considered as the international standard.

biotransformation substrates and cofactors. Although biotransformation metabolism is a well studied discipline within the pharmaceutical industry and the concept of accumulating xenobiotics within the human body as a health risk is well known, the accumulation of endogenous compounds in the case of inborn errors of metabolism and its pathological consequences is typically not explicitly associated with unbalanced biotransformation metabolism.

The assessment of biotransformation metabolism in fatty acid oxidation deficiencies may prove that depletion of biotransformation substrates may lead to unbalanced biotransformation metabolism. This can be the first step in a vicious cycle where unbalanced biotransformation leads to increased oxidative stress status, which can then lead to further depletion of biotransformation substrates. Since fatty acid oxidation deficiencies are suspected as a result of the accumulation of certain biotransformation metabolites, depletion of these biotransformation substrates may further prove to prevent accurate diagnosis of these types of deficiencies.

1.3 Research aims and objectives

Since unbalanced biotransformation metabolism is not typically associated with the pathological consequences in inborn errors of metabolism such as fatty acid oxidation deficiencies, the aim of this study was to formulate a novel hypothetical model - the unbalanced biotransformation metabolism model, to link these two disciplines. The objectives of this study include the evaluation of biotransformation metabolism in subjects with both inborn and induced fatty acid oxidation deficiencies. A further objective was to demonstrate how substrates and cofactors involved in biotransformation metabolism can be used in a "substrate loading cocktail" to give new insight into fatty acid oxidation deficiencies. The final objective of this study was to investigate the effect of salicylic acid which is used in the phenotyping of Phase II glycine conjugation, on the acylcarnitine profile in human subjects.

1.4 Outline of thesis

This thesis consists of seven chapters. The first chapter is introductory and deals with the problem statement and substantiation. It also gives the research aims and objectives. The second chapter describes deficient fatty acid oxidation and the potential role that unbalanced

biotransformation metabolism and increased oxidative stress status plays in the pathological outcomes of deficient fatty acid oxidation. Chapter Three contains all the general materials and methods used during this study. Chapter Four involves the *in vivo* application of the unbalanced biotransformation metabolism model set in Chapter Two. Chapter Five describes how biotransformation substrates can be used in a loading cocktail to enhance fatty acid oxidation and ensure sufficient availability of biotransformation substrates. It also describes how this approach can give new insight into fatty acid oxidation deficiencies. Chapter Six illustrates the effect of the xenobiotic acetylsalicylic acid (aspirin) on the metabolism of fatty acids that originate from the branched chain amino acid metabolism and the possible use of aspirin to detect polymorphisms within this metabolic pathway. In Chapter Seven a general discussion of the results will be given, followed by concluding observations, closing remarks and recommendations. Since most of the chapters form a publishable unit some aspects will be repeated throughout the thesis and the references used in each chapter will therefore be given at the end of every chapter.

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Chapter 2
Unbalanced biotransformation metabolism and oxidative stress status: Implications for deficient fatty acid oxidation

This material has been accepted for publication in Health - **www.scirp.org/journal/health** (doi:10.4236/health.2011.31009) - Annexure A

2.1 Introduction

The indispensable role of the liver in the biotransformation or detoxification of a variety of exogenous and endogenous compounds is accomplished by two groups of enzymatic modifications known as Phase I and Phase II biotransformation metabolism. Phase I reactions expose functional groups to form reactive sites, which improve water solubility of the compound itself, or allow Phase II reactions to ensue when the products of Phase I biotransformation are conjugated with endogenous hydrophilic compounds to enhance their excretion (Grant, 1991; Liska, 1998; Liska *et al*., 2006). However, during Phase I functionalisation the resultant reactive molecule can in certain cases be more toxic than the parent compound and effective neutralisation of these noxious compounds is important in preventing covalent binding of the reactive metabolites to proteins, lipids and nucleic acids (Liska, 1998; Liska *et al*., 2006).

Maintaining the balance between Phase I and Phase II reactions is therefore of paramount importance and under normal circumstances these enzymes function adequately to minimise inefficient detoxification and potential induced intracellular damage. However, an overloaded or unbalanced system negatively affects the oxidative stress status, with serious health compromising consequences (Liska *et al*., 2006; Lampe, 2007).

The metabolic processes that are fundamental for maintaining normal cell structure and function are highly regulated enzyme catalysed processes. Defects in these enzyme systems, whether induced or inherited, have significant consequences in man, i.e. the accumulation of toxic substrates upstream of the enzyme defect, disturbances in metabolic intermediates downstream of the enzyme defect and the formation of intermediates by alternative biochemical pathways (Newman, 2004). On a clinical level, these biochemical abnormalities will give rise to various pathological conditions, including acute life-threatening encephalopathy, hyperammonemia, metabolic acidosis, hypoglycaemia, jaundice and liver dysfunction (Vangala and Tonelli, 2007). This can ultimately lead to the development of chronic diseases and eventual death.

Biotransformation metabolism is a well studied discipline within the pharmaceutical industry, and the concept of accumulating xenobiotics within the human body as a health risk is well known. However, the accumulation of endogenous compounds in the case of inborn errors of

metabolism and its pathological consequences is typically not explicitly associated with unbalanced biotransformation metabolism.

Explaining the development of the phenotypic characteristics of metabolic diseases is a formidable challenge. To this end, a model is proposed to help explain the pathological outcomes of induced and inborn errors of metabolism. This model entails that unbalanced biotransformation metabolism due to depletion of Phase II substrates and cofactors can be the first linkage in a chain of events with severe pathological outcomes. It is vital for scientific advancement and clinical applications that the phenomenon of unbalanced biotransformation metabolism be considered as a primary cause of metabolic abnormalities manifesting as increased oxidative stress status. The proposed unbalanced biotransformation metabolism model will be illustrated using defective β-oxidation of fatty acids, and its value will be demonstrated by its application in the development of individualised treatment protocols for patients suffering from induced and/or inborn errors of metabolism.

2.2 The unbalanced biotransformation metabolism model

In the unbalanced biotransformation metabolism model, a hypothesis is proposed to describe the critical balance between Phase I and Phase II biotransformation and how a disturbance in this balance will increase the oxidative stress status, with resulting pathological consequences. A defect in, or inhibition of, any one of the many enzymes involved in cellular metabolism results in the accumulation of specific metabolites that need to be removed from the body either via alternative pathways, or by Phase I and Phase II biotransformation metabolism. Phase I biotransformation of accumulating metabolites and alternative pathways, both result in additional formation of reactive oxygen species (ROS). Induced Phase I biotransformation will furthermore increase the burden on Phase II conjugation and the increased demand on the latter could lead to the depletion of conjugation substrates and cofactors. Depletion of these biomolecules will disturb the critical balance between Phase I and Phase II biotransformation, which will further increase the oxidative stress status, ultimately leading to the depletion of the endogenous antioxidant capability, further affecting Phase II conjugation. Increased circulating ROS will cause oxidative damage to macromolecules such as lipids, proteins and nucleic acids, and some of these adducts will contribute to the depletion of endogenous antioxidants. If these reactive adducts are not neutralised effectively, they can diffuse to different sites and intensify the effects of oxidative damage by decreasing respiratory chain activity. This model therefore proposes

that unbalanced biotransformation metabolism form an additional "vicious cycle" for oxidative stress which originates from inefficient biotransformation.

2.3 Regulation of the critical balance between Phase I and Phase II biotransformation metabolism

Biotransformation metabolism is under homeostatic regulation to control the detoxification of xenobiotics and their metabolites. This homeostatic system includes both negative feedback control as well as feedforward processes. In Phase I negative feedback control, xenobiotics activate a range of receptors to induce Phase I enzymes (Zhang *et al*., 2009). In most cases Phase I activity prepares the arena for Phase II conjugation to take place, because the Phase I intermediate metabolites activate transcription factors to induce synthesis of Phase II conjugation enzymes, also by means of negative feedback control (Liska, 1998; Liska *et al*., 2006).

However, many Phase II enzymes are also upregulated directly by the parent xenobiotic, which entails feedforward control by the reactive metabolites formed during Phase I. This reduces the response time for the biotransformation system to adapt and remove harmful Phase I intermediates more rapidly. However, there are also other factors involved in this process, such as nutrient concentration control (Zhang *et al*., 2009). Phase I biotransformation requires little nutritional support, whereas Phase II requires various cofactors and substrates, which must be replenished by dietary sources (Liska, 1998; Liska *et al*., 2006). Therefore, although biotransformation metabolism is under homeostatic regulation, including both negative feedback and feedforward control, depletion of Phase II substrates and cofactors will undeniably disrupt the critical balance between Phase I and Phase II biotransformation.

2.4 Consequences of disturbed balance in biotransformation metabolism

The main intracellular source of ROS is the mitochondrial respiratory chain. However, some enzymes, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (EC 1.6.3.1) and cytochrome P450-dependent oxygenases, also produce ROS during their enzymatic reactions (Turrens, 2003). ROS normally exist in all aerobic cells in balance with tightly controlled antioxidant defence and repair mechanisms. A steady state of oxidative

stress, which is always present in cells, can therefore increase (increased oxidative stress status) if the endogenous antioxidant system is not capable of coping with the continuous ROS production, or if an uncontrolled increased ROS production occurs (Cutler *et al*., 2005).

One of the most important endogenous antioxidant molecules is reduced glutathione (GSH), as it plays an important role in neutralising free radicals. A shift in the ratio between reduced glutathione (GSH) and oxidised glutathione (GSSG) could therefore further increase the oxidative stress status. In addition to its antioxidant function, GSH is also involved in Phase II conjugation, which can occur spontaneously or in an enzyme reaction catalysed by glutathione-S-transferases (GSTs) (EC 2.5.1.18) (Kidd, 2001; Townsend *et al*., 2003).

Compromised biotransformation can also have a great influence on the content and type of fatty acids and steroids involved in cellular signalling. Increased circulating ROS and free fatty acids cause lipid peroxidation and the formation of aldehyde by-products, including 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). Detoxification of these lipid peroxidation by-products enhances glutathione depletion even further. If these reactive molecules are not neutralised they can diffuse to different sites and intensify the effects of increased oxidative stress status by decreasing respiratory chain activity (Pamplona, 2008; Catala, 2009).

2.5 Verification of the unbalanced biotransformation metabolism model: Deficient fatty acid oxidation

At least 25 enzymes and transport proteins, various cofactors, coenzymes and substrates such as L-carnitine, coenzyme A, flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD) are involved in mitochondrial β-oxidation, and genetic defects in at least 22 of these proteins cause disease in humans (Sim *et al*., 2002; Vockley and Whiteman, 2002; Kompare and Rizzo, 2008). In addition to inborn errors in fatty acid oxidation, various xenobiotic compounds can also lead to inhibited enzyme activities, e.g. aspirin (acetylsalicylic acid), a widely used analgesic, and valproate (VPA), a branched-chain fatty acid, which is clinically used in the treatment of various seizure disorders. Acetylsalicylic acid is rapidly hydrolysed to salicylic acid upon ingestion and is then activated to salicyl-coenzyme A (CoA) before conjugation to glycine can take place. Valproate, on the other hand, undergoes the same metabolic reactions as natural fatty acids, including mitochondrial

β-oxidation, peroxisomal β-oxidation and cytochrome P450 dependent ω- and ω-1 hydroxylation (Fromenty and Pessayre, 1995).

Deficient mitochondrial fatty acid oxidation results in the accumulation of free fatty acids and acyl-CoA species (Fromenty and Pessayre, 1995; Sim *et al*., 2002). These metabolites need to be removed from the body either via alternative pathways, or biotransformation metabolism (Phase I and Phase II) (Figure 2.1). The alternative pathway to mitochondrial β-oxidation occurs in the peroxisomes. The first step in this pathway is catalysed by acyl-CoA oxidase (EC 1.3.3.6), which involves the reduction of oxygen to hydrogen peroxide (Cooper and Beevers, 1969; Inestrosa *et al*., 1979; Foerster *et al*., 1981). Phase I biotransformation of accumulated fatty acids involve cytochrome P450 dependent ω-oxidation of fatty acids (Johnson *et al*., 1996; Hardwick, 2008). During fatty acid ω-oxidation, the corresponding dicarboxylic acids of the metabolised fatty acids are formed (Hardwick, 2008). In addition, ROS is also formed during this reaction via flavoprotein mediated donation of electrons to molecular oxygen (Hayashi *et al*., 2005) (Figure 2.1). Both the alternative pathway and Phase I biotransformation metabolism can therefore result in enhanced production of ROS.

Phase II biotransformation of accumulated acyl-CoA and Phase I generated dicarboxylic acids involve conjugation with either glycine or L-carnitine (Sim *et al*., 2002; Vockley and Whiteman, 2002; Kompare and Rizzo, 2008). Subjects with deficient fatty acid oxidation will therefore present biochemically with elevated levels of carnitine and glycine conjugates of acyl-CoA and dicarboxylic acid species.

The increased demand on Phase II biotransformation to maintain the critical balance can result in the depletion of these Phase II conjugation substrates (Figure 2.1). If these substrates are not replenished, the critical balance between Phase I and Phase II biotransformation will become disturbed. When this balance is disturbed due to sustained induced Phase I biotransformation and reduced Phase II conjugation, it could increase the oxidative stress status (Liska *et al*., 2006) (Figure 2.1), with a consequent shift in the GSH:GSSG ratio, that could exacerbate the oxidative stress status and affect Phase II conjugation (Kidd, 2001; Townsend *et al*., 2003).

An increased amount of circulating ROS molecules, in addition to accumulated free fatty acids, especially polyunsaturated fatty acids (PUFAs), can further worsen this condition, as ROS could attack these fatty acids and initiate lipid peroxidation. Lipid peroxidation results

in the formation of aldehyde by-products, including 4-HNE and MDA (Pamplona, 2008; Catala, 2009). Increased presence and distribution of these peroxidised lipid metabolites could furthermore lead to mitochondrial instability, as phospholipids are an indispensable constituent in mitochondrial membranes for the functional assembly of the respiratory chain. The incorporation of these lipid derivatives into mitochondria could therefore lead to decreased respiratory chain activity, with resulting increased oxidative stress status (Catala, 2009).

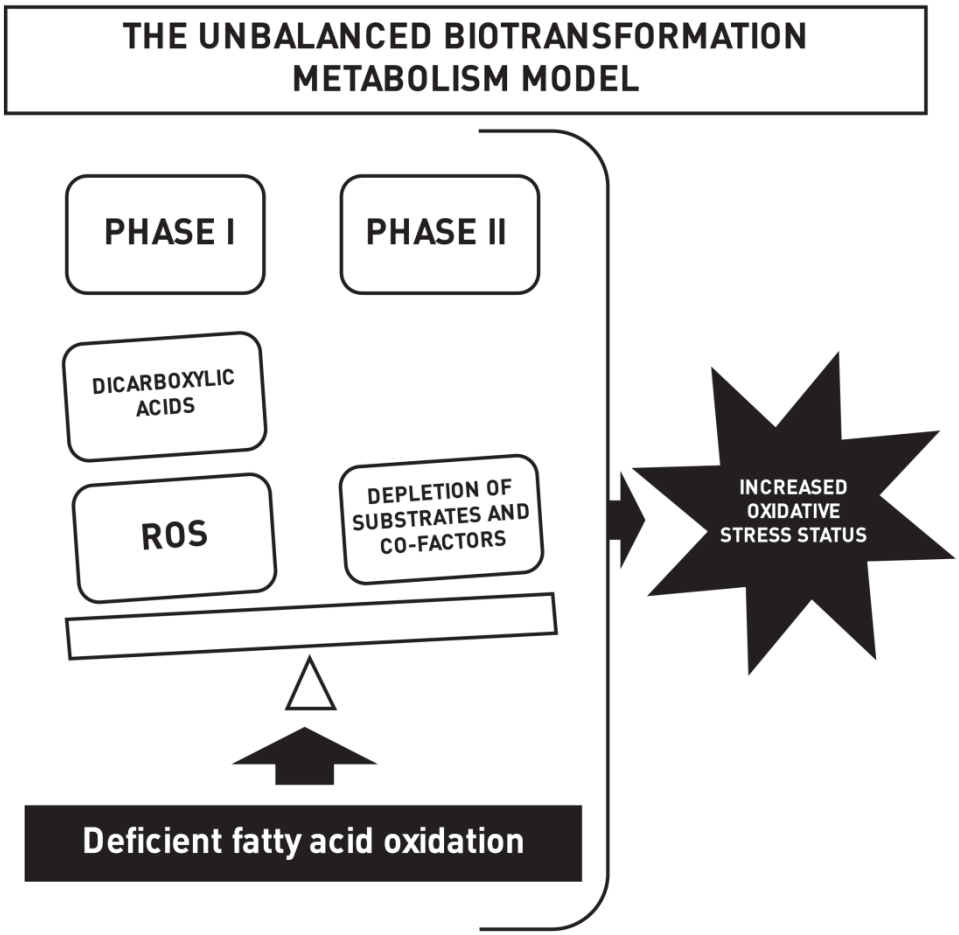

Figure 2.1 Disturbance in the critical balance between Phase I and Phase II biotransformation metabolism by deficient fatty acid oxidation can ultimately lead to an increased oxidative stress status, which is the underlying mechanism for the development of various pathologies.

Moreover, it has recently been demonstrated that two of the accumulating free fatty acids in MCAD deficiency (octanoate and decanoate) lead to increased oxidative stress status (Schuck *et al*., 2009a) and the uncoupling of oxidative phosphorylation (Schuck *et al*., 2009b) in rat brain tissue. Unbalanced biotransformation metabolism and the consequent increase in oxidative stress status are therefore a possible cause in the development of certain neurological consequences in these kinds of deficiencies.

In addition to an increased oxidative stress status, the disturbed biotransformation balance can also generate the pathological condition known as coenzyme A sequestration, toxicity and redistribution (CASTOR) (Mitchell *et al*., 2008). This phenomenon has been demonstrated in both inborn fatty acid oxidation deficiencies and xenobiotic induced fatty acid oxidation deficiencies (Fromenty and Pessayre, 1995; Mitchell *et al*., 2008). The accumulation of acyl-CoA intermediates will lead to decreased availability of free CoA and acetyl-CoA molecules, and changes in these levels can disrupt various metabolic pathways.

These metabolic pathways include the Krebs cycle, ureagenesis, biotransformation pathways as well as the mitochondrial redox state. It could also lead to further deficiencies in downstream products within these metabolic pathways (Mitchell *et al*., 2008). Taken together, defective fatty acid oxidation and its concomitant biochemical characteristics clearly verify the proposed unbalanced biotransformation metabolism model.

2.6 *In vivo* application of the unbalanced biotransformation metabolism model

The value of the proposed model is illustrated by its application to a clinical case investigated in our laboratory (Chapter 4). This case involves a non-smoking Caucasian female, 57 years of age, with metastatic small cell carcinoma of the lung. Four weeks before the end of chemotherapy, the subject suffered from severe fatigue and the first biotransformation and oxidative stress status assessments were conducted. This assessment was performed by challenging Phase I and Phase II biotransformation reactions with appropriate probe substrates. Caffeine was used as a probe substrate for cytochrome P450 1A2 (CYP1A2) (EC 1.14.14.1) activity (Phase I), and paracetamol and aspirin as probe substrates for glucuronide conjugation, sulfate conjugation, glutathione conjugation and glycine conjugation (Phase II) (Liska *et al*., 2006). In addition, the total acylcarnitine profile and oxidative stress status parameters, including the ferric reducing antioxidant power (FRAP) assay, the ROS assay,

measurement of hydroxyl radical markers like catechol and 2,3-dihydroxybenzoic acid (2,3-DHBA) as well as the determination of total glutathione, were also included in this assessment.

From the results obtained during the initial assessment, it was evident that the biotransformation metabolism and antioxidant defence systems of this subject were functioning below a healthy reference range. The results of this initial assessment were used to develop an individualised nutritional supplementation protocol in which various compounds that can be divided into different classes, including antioxidants, mitochondrial support supplementation and biotransformation substrates and cofactors were employed. After the introduction of this individualised nutritional treatment strategy, several follow-up investigations were performed over a period of seven months to monitor both biochemical and clinical characteristics.

A few weeks after the introduction of the nutritional supplementation treatment, the Phase I activity stabilised at levels well within the reference range. All the Phase II reactions also improved, with considerable improvement in glucuronide, sulfate and glutathione conjugation. In addition, the total available glutathione and the serum FRAP also increased with concomitant decreased ROS and 2,3-DHBA concentrations. The amount of free carnitine increased substantially after only eight weeks of starting the supplementation regimen. However, the ratio between acylcarnitines and free carnitine was slightly elevated. After careful investigation of the total acylcarnitine profile, it was found that the source of the elevated ratio between acylcarnitines and free carnitine in these assessments was due to increased levels of medium-chain acylcarnitines and medium-chain dicarboxylcarnitines, including hexanoylcarnitine, octanoylcarnitine, adipylcarnitine and suberylcarnitine.

It is evident in this case that the biotransformation and antioxidant defence systems were initially markedly compromised. The identification of the accumulated metabolites usually seen in fatty acid oxidation deficiencies is the most significant observation in this regard. Initial concentrations of Phase II substrates were so depleted that these metabolites were only observed after oral replenishment of the main conjugation substrate. Once the critical balance between Phase I and Phase II biotransformation was restored, the oxidative stress status decreased to levels within the reference range. In addition to the biochemical improvement, the subject also showed a significant clinical improvement, and although these

results are only preliminary and obtained from a single case, it largely supports the value of the proposed unbalanced biotransformation metabolism model.

2.7 Conclusion

The significance of testing this model, lies within the treatment possibilities, not only for inborn errors of fatty acid metabolism, but also for induced fatty acid oxidation deficiencies. If the disturbance in this critical biotransformation balance is indeed the first link in a chain of reactions to follow, which ultimately lead to pathological conditions like cancer, the assessment of these reactions is of immense importance. This kind of assessment can lead to the development of individualised treatment protocols to replenish important substrates and cofactors needed for the safe elimination of accumulated toxic compounds.

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Chapter 3
General materials and methods

3.1 Chemicals and reagents

Caffeine, allopurinol, sodium acetate, trifluoroacetic acid (TFA), acetamidophenol, acetaminophen sulfate, acetaminophen glucuronide, 3N butanolic hydrochloric acid (HCl), trimethylchlorosylane (TMCS), Bis(trimethylsilyl)trifluoroasetamide (BSTFA), 3-phenylbutyric acid, catechol, 2,3-DHBA, 2,5-dihydroxybenzoic acid (2,5-DHBA), salicylic acid, N,N-diethyl-para-phenylendiamine (DEPPD) sulfate, ferrous sulfate, hydrogen peroxide, anhydrous sodium acetate, sodium acetate trihydrate, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, ferrous sulfate heptahydrate, metaphosphoric acid, valine, leucine, isoleucine, phenylalanine, methionine, citrulline, glycine and lysine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The following reagents were purchased from Merck Chemical Co. (Darmstadt, Germany): high performance liquid chromatography (HPLC) grade acetonitrile, methanol, formic acid, salicyluric acid, HCl, ethylacetate, diethylether, pyridine, acetic acid and sodium sulfate. Acetaminophen mercapturate was obtained from Toronto Research Chemicals (Toronto, CA) and the following carnitine and acylcarnitine standards and deuterated carnitine and acylcarnitine standards were obtained from Dr. H.J. ten Brink, Free University Hospital (Amsterdam, The Netherlands): L-carnitine.HCl, acetyl-L-carnitine.HCl, propionyl-L-carnitine.HCl, isovaleryl-L-carnitine.HCl, octanoyl-L-carnitine.HCl, hexadecanoyl-L-carnitine.HCl, [methyl-d3]L-carnitine.HCl, [d_3]acetyl-L-carnitine.HCl, [3,3,3-d_3]propionyl-L-carnitine.HCl, [d_9]isovaleryl-L-carnitine.HCl, [8,8,8-d_3]octanoyl-L-carnitine.HCl and [16,16,16-d_3]hexadecanoyl-L-carnitine.HCl. The following deuterated amino acids were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA): [d_{10}]-L-isoleucine, [d_8]-L-valine, [d_2]-glycine, [d_3]-methyl-L-methionine, [d_5]-ring-L-phenylalanine, [d_5]-L-glutamine, [d_5]-indole-L-tryptophan, [d_4]-L-lysine:2HCl, [d_4]-L-citrulline. For the determination of total GSH we used the Bioxytech® GSH/GSSG-412™ kit from OxisResearch™ a division of OXIS Health Products.

3.2 Caffeine clearance

3.2.1 Solid phase extraction (SPE) of caffeine

The SPE method described by Georga *et al*. (2001) was used with minor modifications. To 200 µl of saliva, 200 µl of a 12 mg/L allopurinol solution (internal standard) was added. The SPE cartridges (HF Bond Elut C18 500 mg, 3 ml) (Varian, Palo Alto, CA) was conditioned with 3 ml methanol and 3 ml 18 megohm water (H_2O) prior to the addition of the sample and internal standard mixture. After application of the sample mixture, the SPE cartridges were washed with 2 ml ddH_2O. Caffeine and the internal standard were eluted with 3 ml methanol-acetate buffer (pH 3.5) (50:50 % v/v). The eluate was subsequently evaporated under nitrogen to remove excess methanol and the remaining eluate lyophilised. The dry eluate was then reconstituted in 200 µl mobile phase consisting of a ddH_2O:acetonitrile (95:5) solution containing 0.1 % formic acid.

3.2.2 HPLC analysis of caffeine

The samples were analysed on an Agilent 1200 HPLC system equipped with a binary pump, inline degasser, auto sampler, heated column compartment and diode array detector. The column used for this analysis was a Luna 5µm C18 (2) 100 A column from Phenomenex (Torrance, CA, USA). The column temperature was kept at 35 ºC. The initial conditions consisted of isocratic elution at a flow rate of 1 ml/min with 95 % of mobile phase A (0.1 % formic acid) and 5 % of mobile phase B (acetonitrile) for 6 min, followed by a gradient from 95 % of A and 5 % of B to 100 % of B over 5 min. The system was maintained at 100 % of B for 9 min before re-equilibration of the column with the initial mobile phase. The internal standard and caffeine were detected by diode array detection at 254 nm and 275 nm respectively, with a reference wavelength of 600 nm. Caffeine concentrations were determined by means of linear regression, with a $R^2 > 0.99$ and the calculated coefficient of variation for this method was 12.5 %.

3.3 Analysis of Phase II conjugates

3.3.1 Sample preparation

To 200 µl of the urine samples, 100 µl of a 25 mg/L acetamidophenol solution (internal standard) in methanol was added. 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.

3.3.2 High performance liquid chromatography (HPLC) analysis of Phase II conjugates

The HPLC analysis was done according to the method reported by Mutlib *et al.* (2000), with minor modifications. The samples were analysed on an Agilent 1200 HPLC system equipped with a binary pump, inline degasser, auto sampler, heated column compartment and diode array detector. The column used for this analysis was a Luna 5 µm C18(2) 100 A column from Phenomenex (Torrance, CA, USA). The column temperature was kept at 35 ºC. Two mobile phases were used, mobile phase A consisting of a ddH_2O:acetonitrile (95:5) solution with 0.05 % TFA and mobile phase B consisting of only acetonitrile. The initial conditions consisted of isocratic elution at a flow rate of 1 ml/min with 100 % of mobile phase A for 3 min. This is followed by a gradient to 75 % of A over a time period of 8 min and then to 15 % of A over the next 2 min. For the following 7 min the mobile phase constitution changed to 0 % of A, kept for 3 min before re-equilibrating the column with the initial mobile phase. The internal standard as well as acetaminophen mercapturate, acetaminophen glucuronide, acetaminophen sulfate and salicyluric acid (Phase II metabolites) were detected by diode array detection at 254 nm with a reference wavelength of 600 nm. The concentrations were determined by means of linear regression, with $R^2 > 0.99$ and calculated coefficients of variation for acetaminophen mercapturate, acetaminophen glucuronide, acetaminophen sulfate and salicyluric acid were 12.41 %, 12.68 %, 12.41 % and 11.94 % respectively.

3.4 Acylcarnitine analysis

3.4.1 Sample preparation

The electrospray ionisation tandem mass spectrometry (ESI-MS/MS) method for determination of serum acylcarnitines as described by Vreken *et al*. (1999) was adapted to determine acylcarnitines in urine. To a micro-centrifuge tube, 10 µl centrifuged urine was added to 400 µl of the deuterated acylcarnitines (internal standard solution) with the following concentrations: 30.446 µmol/L for [methyl-d_3]-L-carnitine.HCl, 20.83 µmol/L for [d_3]acetyl-L-carnitine.HCl, 19.69 µmol/L for [3,3,3-d_3]propionyl-L-carnitine.HCl, 17.73 µmol/L for [d_9]isovaleryl-L-carnitine.HCl, 15.43 µmol/L for [8,8,8-d_3]octanoyl-L-carnitine.HCl and 11.47 µmol/L for [16,16,16-d_3]hexadecanoyl-L-carnitine.HCl. The

samples were then evaporated to dryness under a gentle stream of nitrogen at 55 ºC. To the dried residue, 200 µl 3N butanolic HCl was added and the samples were incubated at 55 ºC for 20 min. The butylated samples were evaporated to dryness again under a stream of nitrogen at 55 ºC. The dried residue was reconstituted in water:acetonitrile (50:50) (v/v) containing 0.1 % formic acid.

3.4.2 ESI-MS/MS analysis of acylcarnitines

An Agilent 1200 series liquid chromatograph (Santa Clara, CA, USA) with a 96 well plate sampler was used for sample handling as well as mobile phase delivery. Samples (10 µl of each) were injected and a constant flow rate of 0.2 ml/min was maintained throughout the run. The mobile phase consisted of 0.1 % formic acid in water:acetonitrile (50:50) (v/v). The tandem mass spectrometry (MS/MS) analysis was performed on an Agilent 6410 Triple Quadrupole (Santa Clara, CA, USA) in positive ionisation. Acylcarnitines were analysed with a precursor ion scan, after controlled collision induced dissociation, with a fragmentor voltage of 135 V and collision energy of 20 V. All carnitine, acylcarnitine and other butylated species that yielded a charged mass of 85 Da after fragmentation were detected. Acylcarnitines were quantified by comparison of the signal intensity of carnitine and acylcarnitines against the signal intensity of the corresponding deuterated analogues. The concentrations of analysed carnitine and acylcarnitines were expressed as mmol/mol creatinine. Calculated coefficients of variation for free carnitine, butyrylcarnitine/isobutyrylcarnitine, hexanoylcarnitine, octanoylcarnitine, adipylcarnitine and suberylcarnitine was 13.2 %, 12.1 %, 15.2 %, 13.2 %, 17.8 % and 18.8 % respectively.

3.4.3 Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of acylcarnitine isomers

The LC-MS/MS method for the separation and identification of short-chain acylcarnitine isomers as described by Ferrer *et al*. (2007) was used, with minor modifications to separate butyrylcarnitine and isobutyrylcarnitine. A 100 µl volume of urine was prepared the same as for the ESI-MS/MS method. High-performance liquid chromatography was performed on an Agilent 1200 series liquid chromatograph equipped with a Luna C18(2) column (150 mm x 2.00 mm, particle size 5 µm) from Phenomenex (Torrance, CA, USA). Mobile phase A consisted of 10 mM ammonium acetate in water and mobile phase B of 10 mM ammonium acetate in methanol. Column temperature was maintained at 20 °C and the flow rate at 0.2 ml/min. The samples (10 µl) were injected and the mobile phase composition was changed

from 40 % of B to 60 % of B over 15 min, after which the percentage of B was further increased to 100 % over the next 5 min and kept for 5 min. The percentage of B was changed back to 40 % over 3 min and the column re-equilibrated for 7 min.

The MS/MS analysis was performed on an Agilent 6410 Triple Quadropole (Santa Clara, CA, USA) in positive ionisation after controlled collision induced dissociation, with optimised fragmentor voltages and collision energies for butyrylcarnitine, isobutyrylcarnitine and the deuterated analogues used for quantification. Mass spectrometry conditions were optimised with the MassHunter optimiser software from Agilent. Acylcarnitines were analysed in multiple reaction monitoring (MRM) mode, with the following transitions being monitored, *m/z* 288 → 85 for both butyrylcarnitine and isobutyrylcarnitine, *m/z* 277 → 85 for [3,3,3-d_3]propionyl-L-carnitine.HCl, *m/z* 311 → 85 for [d_9]isovaleryl-L-carnitine.HCl and *m/z* 347 →to 85 for [8,8,8-d_3]octanoyl-L-carnitine.HCl. The concentrations of C4-carnitine isomers were determined by comparing the signal intensity of acylcarnitines against the signal intensity of the corresponding deuterated analogues. For both butyrylcarnitine and isobutyrylcarnitine a linear relationship between concentration and intensity existed, with R^2 > 0.99. The concentrations of analysed acylcarnitine isomers were expressed as mmol/mol creatinine.

3.5 Organic acid analysis

3.5.1 Sample preparation

The amount of urine, internal standard as well as derivitisation reagents used was determined according to the creatinine value. The calculated amount of urine and a 3.197 mmol/L 3-phenylbutyric acid solution, the internal standard, were mixed in a tube after lowering of the pH to about 1.0 with 5 M HCl. To this, 6 ml of ethyl acetate was added and mixed well for 30 min. The mixture was centrifuged for 3 min, after which the organic phase was transferred to a new tube. Diethyl ether (3 ml) was added to the remaining water phase, and mixed well for 10 min, after which it was centrifuged again for 3 min. The resulting organic phase was added to the ethyl acetate organic phase and about 100 mg of sodium sulfate was added. The mixture was vortexed and the organic phase transferred to a new tube and evaporated to dryness under nitrogen. The dried residue was then derivitised with the calculated amounts of BSTFA, TMCS and pyridine (Rinaldo, 2008).

3.5.2 Gas chromatography mass spectrometry (GC-MS) analysis of organic acids

The organic acids were analysed with an Agilent 7890A gas chromatograph coupled to a - 5975B mass selective detector system equipped with a DB-1MS capillary column (30m x 0.25mm x 0.25µm) from Agilent Technologies (Santa Clara, CA, USA). The temperature programme started at 60 °C for 2 min, increasing at 4 °C/min to 120 °C and then at 6 °C/min to 285 °C, maintained for 2 min. The samples (1 µl) were injected in splitless mode at a temperature of 280 °C. The carrier gas was helium (17.73 psi) and electron impact ionisation was applied at 70 eV. Mass spectrometry acquisition was performed in scan mode. Data quantification was done using automated mass spectral deconvolution and identification system (AMDIS) software containing an in-house library with 1 100 general organic acids. The concentrations of identified organic acids were determined according to the internal standard and are reported as mmol/mol creatinine.

For the analysis of 2,3-DHBA and catechol the same procedure as for organic acid analysis was followed. However, MS acquisition was performed in single ion monitoring (SIM) mode for characteristic ions (Luo & Lehotay, 1997). Identification of peaks was done according to the mass spectra and retention times of individual compounds and the concentrations of identified compounds were determined by means of linear regression, with $R^2 > 0.99$. Calculated coefficients of variation for catechol and 2,3-DHBA was 13.1 % and 17.6 % respectively. Concentrations are reported in µM.

3.6 Amino acid analysis

3.6.1 ESI-MS/MS analysis of amino acids

Samples were prepared in the same way as for the analysis of acylcarnitines. Added internal standard solution contained deuterated amino acids with the following concentrations: 17.43 µmol/L for [d_{10}]-L-isoleucine, 32.20 µmol/L for [d_8]-L-valine, 15.99 µmol/L for [d_2]-glycine, 3.98 µmol/L for [d_3]-methyl-L-methionine, 5.77 µmol/L for [d_5]-ring-L-phenylalanine, 3.28 µmol/L for [d_5]-L-glutamine, 14.89 µmol/L for [d_5]-indole-L-tryptophan, 14.16 µmol/L for [d_4]-L-lysine:2HCl and 4.21 µmol/L for [d_4]-L-citrulline.

Amino acids were analysed in MRM mode for the following transitions: glycine *m/z* 132 → 30, [d_2]-glycine *m/z* 134 → 32, alanine *m/z* 146 → 44, serine *m/z* 162 → 60, proline and arginine *m/z* 172 → 70, valine *m/z* 174 → 72, [d_8]-L-valine, threonine *m/z* 176 → 74, leucine

and isoleucine *m/z* 188 → 86, [d_{10}]-L-isoleucine *m/z* 191 → 89, methionine *m/z* 206 → 104, [d_3]-methyl-L-methionine *m/z* 209 → 107, histidine *m/z* 212 → 110, citrulline *m/z* 215 → 113, phenylalanine *m/z* 222 → 120, [d_5]-ring-L-phenylalanine *m/z* 227 → 125, tyrosine *m/z* 238 → 136, aspartic acid *m/z* 246 → 144, glutamic acid *m/z* 260 → 158, glutamic acid-d_3 *m/z* 263 → 161, tryptophan *m/z* 261 → 159, [d_5]-indole-L-tryptophan *m/z* 266 → 164, lysine *m/z* 203 → 84 and [d_4]-L-lysine:2HCl *m/z* 207 → 88. The concentrations of the amino acids were determined by comparing the signal intensity of the amino acids against the signal intensity of the corresponding deuterated analogues. The concentrations of analysed amino acids were expressed as mmol/mol creatinine.

3.6.2 Liquid chromatography tandem mass spectrometric (LC-MS/MS) analysis of branched chain amino acids

Samples were prepared in the same manner as for the determination of acylcarnitine isomers. High-performance liquid chromatography was performed on an Agilent 1200 series liquid chromatograph equipped with a Luna C18(2) column (150 mm x 2.00 mm, particle size 5 µm) from Phenomenex (Torrance, CA, USA). Column temperature was maintained at 20°C and the flow rate at 0.2 ml/min. Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B of 0.1 % formic acid in methanol. The samples (10 µl) were injected and the mobile phase composition was changed from 40 % of B to 60 % of B over 15 min, after which the percentage of B was further increased to 100 % over the next 5 min and kept for 5 min. The percentage of B was changed back to 40 % over 3 min and the column re-equilibrated for 4 min. The MS/MS analysis was performed on an Agilent 6410 Triple Quadrupole (Santa Clara, CA, USA) in positive ionisation after controlled collision induced dissociation with optimised fragmentor voltages and collision energies for leucine, isoleucine, valine and the deuterated analogues used for quantification. Mass spectrometry conditions were optimised with the MassHunter optimiser software from Agilent. Branched chain amino acids were analysed in MRM mode, for the same transitions as described in the ESI-MS/MS analysis of amino acids. The concentrations of the branched chain amino acids were determined by comparing the signal intensity of the branched chain amino acids against the signal intensity of the corresponding deuterated analogues. The concentrations of analysed branched chain amino acids were expressed as mmol/mol creatinine.

3.7 ROS assay

The method described by Hayashi *et al.* (2007) with minor modifications was used to determine total ROS levels in serum samples. The following concentrations of hydrogen peroxide were used to construct a calibration curve: 0; 60; 120; 180; 240 and 300 mg/L. In a 96-well microtiter plate, 140 µl of a 0.1 M sodium acetate buffer (pH 4.8) was added to each well for both the calibration curve and samples to be analysed. Standards of the calibration curve were done in duplicate and samples in triplicate. A volume of 2.5 µl of both the standards and the samples were added to the buffer. To start the reaction, 100 µl of a 100 mM DEPPD and 4.37 µM ferrous sulphate, both in a 0.1 M sodium acetate buffer (pH 4.8) mixed in a 1:25 ratio, were added to each well. The 96-well microtiter plate was then incubated at room temperature for one minute after which absorbance at 546 nm was measured at 25 °C after one minute for ten consecutive minutes on a Bio-Tek®, FL600 microplate fluorescence reader. 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 10.7 %.

3.8 Ferric reducing antioxidant power (FRAP) assay

The method described by Benzie and Strain (1996) with minor modifications was used to determine the FRAP, as an indication of antioxidant capacity. The following concentrations of ferrous sulfate heptahydrate were used to construct a calibration curve: 0; 20; 40; 60; 80; 100 µM. In a 96-well microtiter plate 100; 80; 60; 40; 20; 0 µl of 18 megohm H_2O was added to each well for the standards of the calibration curve. To this, the following volumes of a 0.1 mM ferrous sulfate was added: 0; 20; 40; 60; 80; 100 µl. For all the serum samples to be analysed, 85 µl of 18 megohm H_2O was added to each well after which 15 µl of serum was added. Standards of the calibration curve were done in duplicate and samples in triplicate. To start the reaction, 250 µl of FRAP reagent (300 mM Acetate buffer pH 3.6; 10 mM TPTZ; 20 mmol/L ferric chloride hexahydrate) was added to each well. The 96-well microtiter plate was then incubated at room temperature for exactly three minutes after which absorbance at 595 nm was measured at 25 °C after exactly three minutes on a Bio-Tek®, FL600 microplate fluorecence reader. Serum FRAP levels were calculated from the constructed calibration curve ($R^2 > 0.99$) and are expressed as µM concentrations. The calculated coefficient of variation for the FRAP assay was 13.1 %.

3.9 Total glutathione (GSH and GSSG) analysis

Total glutathione levels were determined with the BIOXYTECH® GSH/GSSG-412™ kit supplied by *Oxis*Research™, a division of OXIS Health Products Inc. The kit was used according to the supplier's instruction with minor modifications. The following concentrations of GSH were used to construct a calibration curve: 0; 0.10; 0.25; 0.50; 1.50; and 3.00 µM. In a 96-well microtiter plate, 50 µl of the standards or sample, 50 µl of chromogen and 50 µl of enzyme were sequentially added to each well. The samples were incubated at room temperature for 5 minutes. 50 µL of NADPH was added to each well and within 30 seconds the change in absorbance at 412 nm was recorded for 3 minutes at 25 °C using the Bio-Tek®, FL600 microplate fluorecence reader. Standards of the calibration curve were done in duplicate and samples in triplicate. The concentrations of total GSH was calculated from the constructed calibration curve ($R2 > 0.99$) and are expressed as µM concentrations. The calculated coefficient of variation for the total glutathione assay was 8.4 %.

3.10 Ethical aspects

This study adhered to the guidelines set in the Declaration of Helsinki. Ethical approval for this study was obtained from the Ethics Committee of the North-West University, Department of Research Support (06M03). Informed consent was obtained from all participating subjects, or their legal guardians. All loading tests and treatments were requested, approved and executed under the supervision of a medical practitioner.

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Chapter 4
Biotransformation metabolism and oxidative stress status assessment, treatment and follow-up assessments in a cancer patient with impaired medium chain fatty acid oxidation

Part of this material has been accepted for publication in Health - **www.scirp.org/journal/health** (doi:10.4236/health.2011.31009) - Annexure A

Part of this material has been submitted for publication in Nutrition in Clinical Practice - **http://ncp.sagepub.com** (NCP-2011-02-016)

4.1 Introduction

The liver plays an important role in the biotransformation or detoxification of several exogenous (Liska, 1998) and endogenous compounds (Lampe, 2007). Biotransformation metabolism consists of two types of enzymatic modification reactions, known as Phase I and Phase II respectively (Grant, 1991; Liska, 1998). Phase I reactions expose functional groups to form reactive sites, which improve water solubility of the compound itself, or allow Phase II reactions to ensue. During Phase II reactions the products of Phase I biotransformation are combined with endogenous hydrophilic compounds to enhance excretion (Liska *et al*., 2006). Phase II reactions include glucuronide conjugation, sulfate conjugation, glutathione conjugation and conjugation with amino acids such as glycine and carnitine (Quistad *et al.*, 1986; Grant, 1991; Liska, 1998; Trubetskoy *et al.*, 2007; Vaz and Wanders, 2002).

The balance between Phase I and Phase II reactions is of fundamental importance because the reactive metabolites of Phase I can sometimes be more toxic than the parent compound and if reactive Phase I metabolites are not neutralised effectively by Phase II conjugation, it can lead to a state of increased oxidative stress (Liska *et al.*, 2006). Increased oxidative stress status occurs when the critical balance between the production of ROS and the proper functioning of the antioxidant defence becomes disturbed in favour of the former (Turrens, 2003; Waris and Ahsan, 2006). In addition to the formation of reactive metabolites in Phase I, ROS is also a by-product of cytochrome P450 reactions that need to be neutralised to prevent damage to proteins, lipids and nucleic acids (Liska, 1998; Nebert and Russel, 2002; Guengerich, 2006). In the case of unbalanced biotransformation and increased oxidative stress status, one of the most important treatment strategies is to meet daily nutritional needs, since various nutrients are indispensable for the optimal functioning of biotransformation and the antioxidant defence systems (Liska *et al.*, 2006). The intake of increased amounts of nutrients that function as cofactors and substrates in biotransformation metabolism can therefore enhance the functioning of these systems.

In this study, the biotransformation metabolism and oxidative stress status of a patient with metastatic small cell carcinoma of the lung were assessed. The first assessment was done at four weeks before the last chemotherapy session. The results of this initial assessment were used to develop a nutritional supplementation protocol to correct the increased oxidative stress status and improve mitochondrial function and biotransformation metabolism. In the development of a nutritional supplementation treatment strategy, various compounds were

employed that can be divided into different classes, i.e. antioxidants, mitochondrial support supplementation and biotransformation substrates and cofactors. After the introduction of such an individualised nutritional treatment strategy, several follow-up investigations were performed to monitor both biochemical and clinical improvement. Since cancer risk is determined by a combination of environmental and genetic factors, the maintenance of a proper balance between Phase I and Phase II metabolism is critical (Lampe, 2007) and therefore stresses the importance of such an approach.

4.2 Materials and methods

See Chapter 3 for a comprehensive description of all materials and methods used in this study.

4.4.1 Test subject

The subject (NWU-34588) under investigation, was a non-smoking Caucasian female, 57 years of age at the time of the study. She presented with chronic fatigue, coughing, dyspnoea, pain and anorexia and was diagnosed with metastatic small cell carcinoma of the lung, with mediastinal adenopathy of the lymphnodes measuring 15 mm in length. Full blood count results at this stage revealed anisocytosis, with normal white blood cells and sufficient platelets. The cancer also metastasised to the liver; however liver function tests was within the reference range. After the diagnosis, she commenced with a chemo combination therapy, called CAV, which consists of Cyclophosphamide, Doxorubicin and Vincristine for six repeated cycles over a period of twenty weeks. After four weeks of chemotherapy, she started to develop anaemia, which was again monitored four weeks later. At this stage she still showed lowered haemoglobin levels and in addition, the hematocrit level also dropped to just below the reference range. The full blood count revealed that the anisocytosis persisted, with normal white blood cells and sufficient platelets. Liver function tests were still within the reference range. At twelve weeks after the onset of chemotherapy, there was no difference in the pathology results when compared to the previous. However, two weeks later she developed leucopoenia and neutropoenia. At this stage, four weeks before the end of chemotherapy, the patient suffered from severe fatigue and the first biotransformation and oxidative stress status assessment was done. These results were used to develop an individualised nutritional supplementation protocol. The first follow-up assessment (second assessment) was done four weeks after completion of chemotherapy, after the introduction of

the nutritional supplementation protocol. The second follow-up assessment (third assessment) was done after twelve weeks and one more follow-up assessment were done eight weeks (fourth assessment) after the previous assessments. These time intervals were not chosen for a specific reason, but were the times when the patient was available for testing. For the whole assessment time she continued with a prescribed medication regimen consisting of sodium valproate, lamotrigine, clozapine and simvastatin (Annexure B).

4.4.2 Reference group

For the most part of this study, the test subject under investigation served as its own control, however, to place the obtained data in perspective, the results is also compared to two different reference groups. The first reference group consisted of 46 healthy individuals between the ages of 18 and 35 years, with 22 of them being male and 24 of them being female. However, due to the age of our test subject a second reference group was also used, who consisted of 30 age-matched (50 to 60 years of age) individuals, with nine of them being male and 21 of them being female. None of these individuals had cancer at the time or any history of previous diagnosed cancer of any kind.

4.4.3 Biotransformation metabolism and oxidative stress status assessment

The assessment of the biotransformation metabolic activity was done by challenging Phase I and Phase II biotransformation reactions with appropriate probe substrates. Caffeine was used as a probe substrate for CYP1A2 activity (Phase I), and paracetamol and aspirin as probe substrates for glucuronide conjugation, sulfate conjugation, glutathione conjugation and glycine conjugation (Phase II) (Liska *et al*., 2006). In addition, the total acylcarnitine profile and oxidative stress status parameters, including the FRAP assay, the ROS assay, measurement of hydroxyl radical markers like catechol and 2,3-DHBA as well as the determination of total glutathione were also included in this assessment. During this assessment a normal diet was assumed except for a few dietary restrictions, including all food and medication containing caffeine, paracetamol and salicylates, all brassica vegetables, certain fruit and spices as well as alcohol.

4.4.4 Sample collection

Blood samples were collected in serum separation tubes (SST) for the FRAP and ROS assays and ethylenediaminetetraacetic acid (EDTA) whole blood was uses for the quantification of

total glutathione before the test subject started with the biotransformation assessment procedure. Phase I activity was determined with the caffeine clearance test. After the administration of 180 mg caffeine, two saliva samples were collected at two hours and eight hours, respectively, post administration. Five hours after collection of the second saliva sample, after emptying of the bladder, the second set of probe substrates, i.e. 1000 mg paracetamol and 600 mg aspirin, were administered. The total volume of urine excreted for ten hours after administration of paracetamol and aspirin was collected and documented. This urine sample was used for the determination of paracetamol-glucuronide, paracetamol-sulfate, paracetamol-mercapturate and salicyluric acid (Phase II metabolites), the acylcarnitine profile and hydroxyl radical markers.

4.4.5 Analytical procedures

Caffeine extraction from saliva was done with SPE according to the method described by Georga *et al*. (2001) and the extracted sample analysed by HPLC with diode array detection. Phase II metabolites were analysed according to the HPLC method reported by Mutlib *et al.* (2000). The ESI-MS/MS method for determination of serum acylcarnitines by Vreken *et al*. (1999) was used to determine acylcarnitines in urine. For the analysis of catechol and 2,3-DHBA the GC-MS method published by Luo and Lehotay (1997) was used. The method described by Hayashi *et al*. (2007) was used to determine ROS levels in serum samples and the method described by Benzie and Strain (1996) was used to determine the FRAP. Total glutathione levels were determined with the BIOXYTECH® GSH/GSSG-412™ kit supplied by *Oxis*Research™, a division of OXIS Health Products Inc. The kit was used according to the supplier's instructions. All of the above mentioned analytical procedures were executed with minor modifications to suit local laboratory conditions.

4.4.6 Nutritional supplementation treatment strategy

After initial biotransformation metabolism and oxidative stress status assessment of this patient, a supplementation treatment protocol containing the following compounds were advised by a medical practitioner: 1000 mg buffered Vitamin C once a day, 1000 mg L-carnitine twice daily, 600 mg N-acetyl-cysteine twice daily, 400 mg Milk Thistle (200 mg Milk Thistle seed powder and 200 mg Milk Thistle extract containing 80% Silymarin) three times daily, 50 mg Coenzyme Q10 twice daily, 250 mg α-lipoic acid once a day, 500 mg glycine once a day and 10 ml *Rosa roxburghii* extract once a day. During the

supplementation treatment the effectiveness of the treatment was monitored three times over a seven month period. During the treatment period there were no changes made to the test subject's normal diet.

4.3 Results

The initial assessment of the biotransformation ability of this patient showed low to normal caffeine clearance (Figure 4.1 A). Two of the measured end products of Phase II conjugation reactions i.e. acetaminophen-glutathione and acetaminophen-glucuronide were also within the lower part of the reference range, while the measured end products for glycine conjugation and sulfate conjugation were found to be low (Figure 4.1 B). The amount of free carnitine and total acylcarnitines were also within the lower part of the reference range (Figure 4.1 C) with the total acylcarnitine:carnitine ratio being slightly elevated.

The second assessment was done after eight weeks of continued supplementation. The caffeine clearance in this assessment was higher than that of the first reference group and was 667% higher than at the first assessment (Figure 4.1 A). All the measured end products of the Phase II conjugation reactions showed a slight increase of 5 %, 18 %, 39 % and 9 % for glutathione, sulfate, glucuronide and glycine conjugation respectively (Figure 4.1 B). However, the ratios between Phase I oxidation and Phase II conjugation were now highly elevated when compared to the first reference group. The amount of free carnitine available increased by 128 % and that of the total acylcarnitines by 114 % (Figure 4.1 C). The ratio of free carnitine to acylcarnitines was still slightly elevated. The value for the ferric reducing ability of plasma increased by 28 % (Figure 4.2 A), however, the total glutathione available for biotransformation of xenobiotics and free radicals was 9 % lower (Figure 4.2 B). The amount of ROS (Figure 4.3 A) was 26 % lower and nearly within the range of both reference groups. The amount of 2,3-DHBA in the urine dropped marginally by 3 % (Figure 4.3 B), but that of catechol increased by 202 % (Figure 4.3 C) (Table 4.1).

At this stage a full blood count revealed normochromic normocytic anemia, with the presence of leucopoenia and neutropoenia. Both kidney and liver function tested within the reference range. Although Computed tomography (CT) scans of the lungs remained unchanged, there were still low density lesions in the liver.

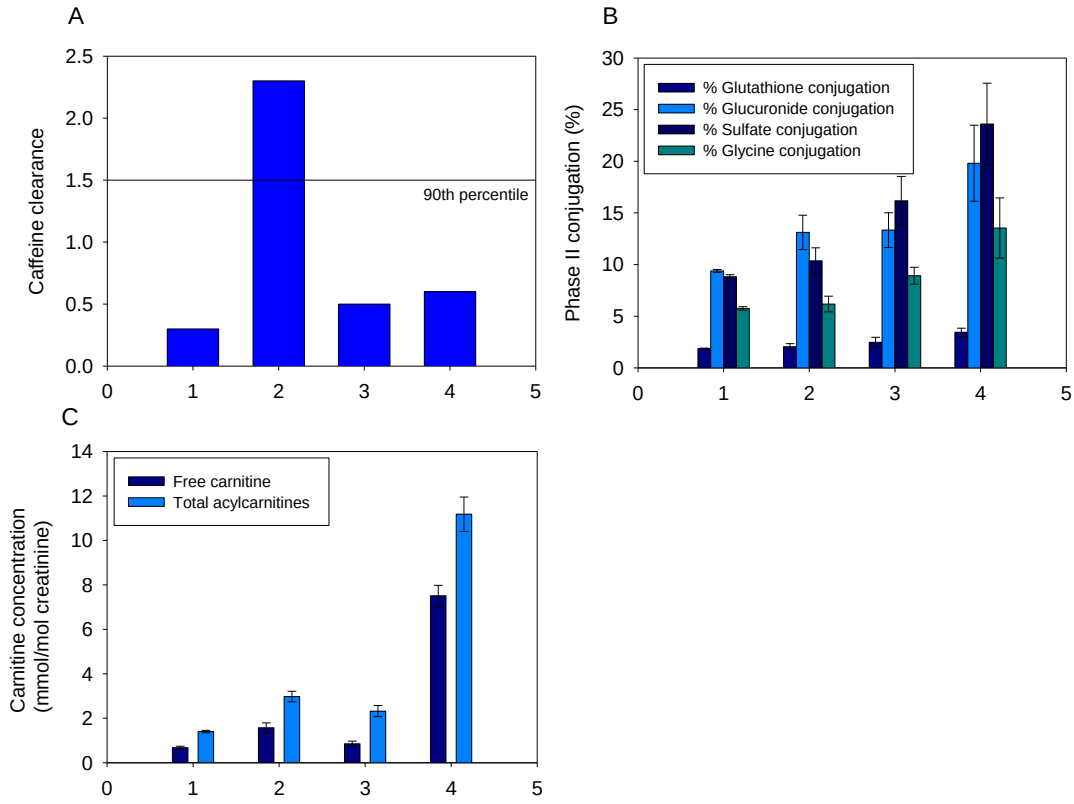

Figure 4.1 (A) Phase I (caffeine clearance), (B) Phase II (conjugation reactions), (C) free carnitine and acylcarnitines during the assessment period. The x-axis: (1) represents the initial assessment before introduction of the nutritional treatment strategy. The first follow-up assessment (2) was done after eight weeks of nutritional treatment, the second follow-up assessment (3) was done twelve weeks after the first follow-up and the third follow-up assessment (4) was done eight weeks after the second follow-up assessment. All urine samples were done in triplicate, and the average and standard deviation is reported in the above figure.

The third assessment was done twelve weeks after the previous assessment, with continued supplementation. The caffeine clearance value stabilised and was only 67 % higher than in the first assessment (Figure 4.1 A). Compared to the initial assessment, the measured end products for the different Phase II conjugation reactions improved by 31 % for glutathione conjugation, 84 % for sulfate conjugation, 41 % for glucuronide conjugation and 56 % for glycine conjugation (Figure 4.1 B). All the measured parameters for Phase II conjugation ability, with the exception of glycine conjugation, were within that of both the reference groups. The ratios between Phase I and Phase II biotransformation were also within the lower part of the first reference range. The amount of free carnitine dropped marginally in the third assessment as compared to the second assessment, but was still 28 % higher than in the initial assessment (Figure 4.1 C). The amount of total acylcarnitines followed the same pattern as free carnitine and the ratio between free carnitine and acylcarnitines was still

elevated. Values obtained with the FRAP assay (Figure 4.2 A) and the amount of total glutathione, also increased by 22 % and 5 % respectively (Figure 4.2 B), while the amount of ROS decreased by 35 % (Figure 4.3 A). The catechol concentration was still 214 % higher (Figure 4.3 C) than in the initial assessment while that of 2,3-DHBA decreased with 32 % (Figure 4.3 B) (Table 4.1). At this stage the results of a full blood count were within that of the reference range, with the exception of slight aniocytosis, and the CT scan remained unchanged.

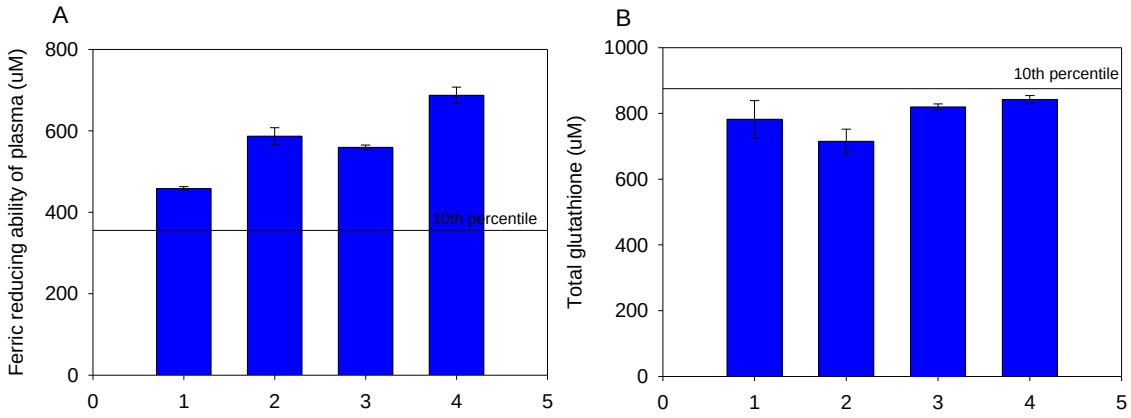

Figure 4.2 (A) Initial ferric reducing antioxidant power (FRAP) and (B) total blood glutathione (GSH) compared to the FRAP and total GSH over a seven month treatment period. Both FRAP values and GSH concentrations below the 10th percentile were considered to be low. (See Figure 4.1 for the allocation of data labels on the x-axis).

The fourth assessment was done eight weeks after the third assessment, with continued supplementation. The caffeine clearance (Figure 4.1 A) and measured end products of all the Phase II conjugation reactions have stabilised at the level of the fourth assessment, except for the glycine conjugation which remains within the lower part of the reference range. Caffeine clearance increased by 100 % and all the measured end products of the Phase II conjugation reactions also showed a further increase of 79 %, 168 %, 111 % and 137 % for glutathione, sulfate, glucuronide and glycine conjugation, respectively, when compared to the initial assessment (Figure 4.1 B). The ratios between Phase I and Phase II are also within that of both the reference groups. The concentration of free carnitine increased by 971 %, and that of the total acylcarnitines by 700 % (Figure 4.1 C).

Table 4.1 Summary of the data obtained during the initial and follow-up biotransformation metabolism and oxidative stress status assessments.

	Reference group 1 10th and 90th percentile (n = 46)	Reference group 2 10th and 90th percentile (n = 30)	1st assessment	2nd assessment	3rd assessment	4th assessment
Caffeine clearance (ml/min/kg)	0.1 – 1.5	0.4 – 5.3	0.3	2.3	0.5	0.6
Glutathione conjugation (%)	1.6 – 4.0	1.4 – 4.3	1.9	2.0	2.5	3.4
Sulfate conjugation (%)	9.5 – 26.6	9.7 – 22.8	8.8	10.4	16.2	23.6
Glucuronide conjugation (%)	9.2 – 34.4	12.5 – 33.1	9.4	13.1	13.3	19.8
Glycine conjugation (%)	9.5 – 26.6	11.0 – 27.2	5.7	6.2	8.9	13.5
Free carnitine (mmol/mol creatinine)	0.6 – 5.1	2.0 – 10.3	0.7	1.6	0.9	7.5
Total acylcarnitines (mmol/mol creatinine)	0.8 – 9.8	3.9 – 18.1	1.4	3.0	2.3	11.2
Acylcarnitine:free carnitine	<1.9	<1.8	2.0	1.9	2.6	1.5
Phase I:Sulfate conjugation	1.6 – 7.0	4.1 – 23.2	3.0	12.6	1.7	2.1
Phase I:Glucuronide conjugation	1.1 – 4.2	3.2 – 16.0	3.1	10.6	1.9	2.5
Phase I:Glycine conjugation	1.1 – 5.5	3.6 – 19.5	5.1	21.3	3.3	4.5
FRAP (µM)	355.4 – 448.0	335.7 – 500.2	458.2	586.7	559.2	687.2
Total GSH (µM)	874.9 – 1025.9	562.6 – 1141.1	782.1	715.2	819.6	842.5
ROS (Units)	52.4 – 107.3	68.3 – 114.7	144.2	107.4	93.5	89.1
Catechol (µM)	3.4 – 69.8	6.2 – 39.3	11.7	35.3	36.7	51.8
2.3-DHBA (µM)	0.2 – 1.0	0.8 – 7.8	7.7	7.5	5.2	7.2

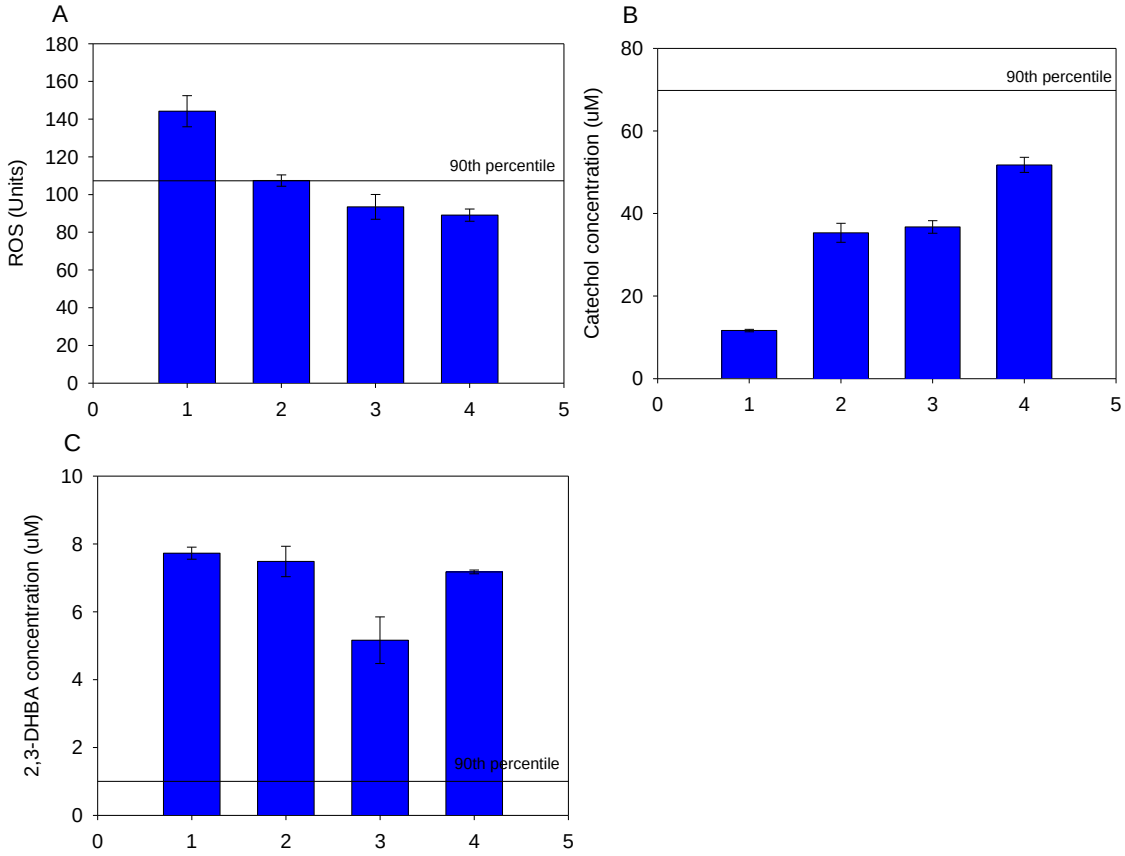

Figure 4.3 Changes in (A) serum ROS, (B) urinary catechol and (C) 2,3-DHBA levels over the seven month treatment period compared to the initial ROS, urinary catechol and 2,3-DHBA levels. (See Figure 4.1 for the allocation of data labels on the x-axis).

The ratio between free carnitine and acylcarnitines was now within that of both the reference groups. There was also an increase in the FRAP value (Figure 4.2 A) and the total available glutathione (Figure 4.2 B), with an improvement of 50 % for FRAP and 8 % for total glutathione. The ROS value decreased by 38 % (Figure 4.3 A) and the concentration of 2,3-DHBA by 6 % (Figure 4.3 B), however, this concentration was still higher than that of the first reference group. The opposite was observed for catechol, as the concentration increased by 342 % (Figure 4.3 C), but it was still within that of the first reference group (Table 4.1). Computed tomography scans of the abdomen and thorax revealed no changes at this stage. Although the CT scan of the thorax and abdomen remained unchanged at seven weeks after the fourth assessment, a fine needle aspiration of a lymph node was positive for malignant cells. Eleven weeks later a chest x-ray was done, which did not reveal any new lesions or metastatic disease. However, the abdominal sonar did reveal new liver metastases as well as

pancreatic metastases. At this stage the patient decided not to start with any active chemotherapy, but continued with the supplementation treatment protocol.

4.4 Discussion

4.4.1 Initial assessment

From the values obtained during the initial assessment of the patient, it was evident that the biotransformation metabolism and antioxidant defence systems of this subject that suffers from metastatic small cell carcinoma of the lung, were not functioning at levels associated with healthy individuals. The activity of Phase I (CYP1A2), measured as the caffeine clearance value, was within the lower part of the reference range. Lower Phase I activity can lead to slower metabolism of substrates for these enzymes (Kivisto and Kroemer, 1997; Nebert and Russel, 2002; Frye, 2004). In this case the availability of clozapine, one of the chronic medications used by the patient, will be present in the circulation for a longer period since its metabolism is catalysed by CYP1A2 and cytochrome P450 3A4 (CYP3A4) (Eiermann *et al.*, 1997).

All the measured end products for the different Phase II conjugation reactions in the initial assessment were also in the lower part of the reference range, with glycine conjugation being very low. When all the Phase II reactions are low, one has to consider the availability of sufficient amounts of energy since all these reactions are highly dependent on adenosine triphosphate (ATP) (Liska *et al.*, 2006). In addition, the chronic medication used by this patient could also be responsible for the lower Phase II activity. Glucuronide conjugation is involved in the metabolism of VPA, lamotrigine and simvastatin. It has been shown that the concomitant use of VPA and lamotrigine increases the half-life of lamotrigine, possibly due to competitive inhibition of glucuronide conjugation (Yuen *et al.*, 1992). The lower glutathione conjugation seen in this assessment may be due to the lower availability of total glutathione as glutathione is needed to quench the high amount of ROS levels observed and could also be further depleted by the use VPA. The use of VPA could also provide a partial explanation for the lower glycine conjugation observed, as glycine conjugation is also involved in valproate metabolism (Silva *et al.*, 2008).

In this assessment the concentration of free carnitine was just within the reference range. Again, the use of VPA can have a significant lowering effect on carnitine levels (Kelly, 1998a). Mitochondrial fatty acid oxidation is the primary source of energy in both heart and

skeletal muscle (Kelly, 1998a) and L-carnitine is an important cofactor in the transportation of fatty acids to the mitochondria for subsequent β-oxidation (Kelly, 1998a; Brass, 2002; Vaz and Wanders, 2002). Carnitine deficiency can therefore impair mitochondrial function, which could lead to symptoms of generalised fatigue, myalgia, muscle weakness and malaise (Werbach, 2000). The observed carnitine deficiency may therefore have contributed to the symptom of chronic fatigue experienced by this patient.

The ROS levels and the 2,3-DHBA were exceptionally high and in this case it is unlikely to be due to imbalanced biotransformation, since the ratios between Phase I and Phase II is within the reference range. After the evaluation of this initial assessment a nutritional supplementation protocol containing Vitamin C, L-carnitine, N-acetyl-cysteine, Milk Thistle, coenzyme Q10, α-lipoic acid, glycine and *Rosa Roxburghii*, was introduced. At this stage the improvements on the CT scan of the thorax and abdomen, must be the result of the chemotherapy.

4.4.2 Follow-up assessments

After a period of eight weeks of continued supplementation as described above, the patient was subjected to a second assessment of biotransformation metabolism and oxidative stress status. The Phase I activity in this assessment was markedly elevated and such high activity could lead to the formation of more free radicals (Liska *et al.*, 2006). As to the reason for this elevation in Phase I activity one can only speculate, however, CYP1A2 is subject to induction by various environmental and nutritional agents, including 3-indole-carbinol which is a constituent of cruciferous vegetables (Bland, 2007). During the assessment there are certain dietary restrictions as mentioned previously, however, it is difficult to control exposure to agents that can lead to the induction of certain enzymes. In the third (12 weeks after the second assessment) and fourth (8 weeks after the third assessment) assessment, the Phase I activity stabilised to levels within the reference ranges. This may be due to the slight inhibitory effect of silymarin on cytochrome P450 enzymes and in particular on cytochrome P450 1A1 (CYP1A1), CYP1A2 and cytochrome P450 2B (CYP2B) (Baer-Dubowska *et al.*, 1998).

During the entire assessment time of seven months, the capacity of glucuronide and sulfate conjugation improved dramatically. This may be as a result of increased ATP synthesis due to supplementation with Coenzyme Q10, as most cellular functions and especially

biotransformation reactions, are dependent on sufficient supply of ATP (Liska *et al.*, 2006). Coenzyme Q10 is an important cofactor in the electron transport chain and can therefore improve ATP synthesis (Gaby, 1996a). In addition, the supplementation with silymarin could also enhance glucuronide conjugation as it is an inhibitor of intestinal bacterial beta-glucuronidase. This enzyme catalyses the removal of glucuronic acid from the conjugated substance, which then has to undergo reconjugation with glucuronic acid (Luper, 1998). The glycine conjugation also improved after the introduction of the nutritional treatment strategy, however, glycine conjugation was still in the lower part of the reference range. This improvement may again be due to increased ATP supply (Gregus *et al.*, 1996a), and to the supplementation with glycine. Apart from the importance of both glycine and ATP in glycine conjugation, coenzyme A is an important cofactor in the first step of the reaction, which is considered to be the rate-determining step (Vessey *et al.*, 1999). It has been reported by Gregus *et al*. (1996b) that α-lipoic acid has an inhibiting effect on glycine conjugation in a dose-dependent manner in experimental animals. Supplementation with α-lipoic acid can therefore lead to depletion of Coenzyme A. This may explain why there was improvement in glycine conjugation, although it was still in the lower part of the reference ranges.

Glutathione conjugation as well as the total available glutathione also improved over the seven month period of treatment. This is very important, since glutathione has a dual purpose in the conjugation of reactive molecules and quenching of free radicals. However, after eight weeks of treatment the total available glutathione was lower than in the initial evaluation. This may be due to improved conjugation. In the treatment strategy various supplements could lead to this improvement. Silymarin (Campos *et al.*, 1988) and Vitamin C (Wang and Ballatori, 1998) can protect cells against glutathione depletion, while n-acetyl cysteine (NAC) stimulates glutathione synthesis, enhances GST activity and thereby promotes glutathione conjugation (Kelly, 1998b; Atkuri *et al.*, 2007). α-Lipoic acid could also increase the amount of reduced glutathione due to improved regeneration (Kagan *et al.*, 1990; Patrick, 2004). However, this cannot be seen in our results as the GSH/GSSG ratio was not determined. The supplementation of glycine could also have an effect on the improved availability of glutathione as it has been demonstrated *in vitro* that glycine supplementation can stimulate GSH synthesis when the concentration of L-glutamate is low (Wessner *et al.*, 2003). However, it is not known if there was in fact a glutamate deficiency and if the effect would be the same *in vivo*.

In addition to the improvement of the total glutathione levels, the serum FRAP also improved and the amount of serum ROS and 2,3-DHBA decreased. This was expected due to the supplementation with various compounds with antioxidant properties including silymarin (Pietrangelo *et al*., 1995), Vitamin C (Padayatty *et al.*, 2003), selenium (Patrick, 2004), NAC (Kelly, 1998b; Atkuri *et al.*, 2007), Coenzyme Q10 (Gaby, 1996b), α-lipoic acid (Suzuki *et al.*, 1991) and *Rosa Roxburghii* (Yong-Xing *et al*., 1997; Zhang *et al.*, 2001; Janse van Rensburg *et al*., 2005; Van der Westhuizen *et al*., 2008). The increased amount of catechol detected may have a dietary origin as it is also found in a wide range of foods and beverages (McDonald *et al*., 2001).

The amount of free carnitine increased dramatically after eight weeks of commencing the supplementation regimen. However, the ratio between acylcarnitines and free carnitine was slightly elevated. In the third assessment (12 weeks after the second assessment), the amount of free carnitine was marginally lower and the ratio between acylcarnitines and free carnitine was still elevated; however, in the fourth assessment (8 weeks after the third assessment), these values normalised. After careful investigation of the total acylcarnitine profile, the source of the elevated ratio between acylcarnitines and free carnitine in these assessments were due to increased levels of medium chain acylcarnitines and medium chain dicarboxylcarnitines, including hexanoylcarnitine, octanoylcarnitine, adipylcarnitine and suberylcarnitine. The elevation of these acylcarnitines species is typically indicative of impaired medium-chain fatty acid β-oxidation. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, is the most common inborn error of fatty acid oxidation (Kompare and Rizzo, 2008), and the possibility of MCAD deficiency in this patient is currently being investigated. However, it is not clear whether the chronic medication used by this patient could have induced this type of profile, as both VPA and simvastatin are metabolised via the β-oxidation pathway (Mclaughlin *et al.*, 2000; Wen *et al.*, 2000; Prueksaritanont *et al*., 2002) and it has been demonstrated that both VPA and the Phase I metabolite, Δ^4-valproic acid have an inhibitory effect on fatty acid β-oxidation. This effect may either be due to sequestration of available Coenzyme A or specific inhibition of one or more of the β-oxidation enzymes (Silva *et al*., 2001).

In addition to the biochemical improvements, there were also improvements in the clinical symptoms of the patient as CT scans revealed no further progression of the cancer and full blood count results stabilised. However, although the CT scan of the thorax and abdomen

remained unchanged at seven weeks after the fourth assessment, there was deterioration as malignant cells were detected in a lymph node shortly afterward. Eleven weeks later, new liver metastases as well as pancreatic metastases were detected. At this stage the patient decided not to start with any active chemotherapy, but did continue with the supplementation treatment protocol, as the patient felt that it improved her quality of life.

4.5 Conclusion

The initial assessment of this patient revealed that her biotransformation and antioxidant defence capacity were markedly compromised. After the introduction of a nutritional supplementation treatment strategy, there was an overall improvement in the biotransformation metabolism and antioxidant defence capacity of this patient. Phase I activity stabilised at a level within the reference range, the activity of Phase II biotransformation reactions also improved and it could be concluded that the Phase I and Phase II biotransformation systems were in balance. The initial systemic carnitine deficiency was replenished. The oxidative stress status was reduced and the antioxidant capacity improved since the level of ROS decreased and the FRAP and total GSH increased. In addition to the biochemical improvement, the patient also showed a significant clinical improvement. Various nutrients are critical for the optimal functioning of biotransformation and antioxidant defence systems and nutritional treatment strategies based on biotransformation and oxidative stress status assessment can therefore be of great benefit in the treatment of various pathological conditions, especially when biotransformation and antioxidant defence systems are compromised. The value of this study lies in the improvement of the clinical features of the patient, although this intervention was initiated too late in this case, since the improvement could not be maintained to reach a more satisfactory level. However, the observations made, strongly underline the value and potential of a dietary intervention aimed at enhancing the biotransformation metabolism and oxidative stress status, even under pathological conditions.

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Chapter 5
The use of a “substrate loading cocktail” to enhance fatty acid oxidation and ensure sufficient availability of detoxification substrates, gives new insight into fatty acid oxidation deficiencies

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5.1 Introduction

Fatty acid β-oxidation, which occurs in both mitochondria and peroxisomes, results in the production of acetyl-CoA and reducing equivalents which are used in the Krebs cycle and respiratory chain for the production of ATP. Fatty acid oxidation disorders individually are rare, but as a group they are quite common due to the number of different enzymes involved. In this study, detoxification substrates and cofactor precursors, which are normally used in treatment regimens, were used in a "substrate loading cocktail" to enhance aberrant metabolite profiles in fatty acid oxidation deficiencies (Sim *et al.,* 2002; Vockley and Whiteman, 2002; Kompare and Rizzo, 2008).

In the case of fatty acid oxidation deficiencies such as short-chain acyl-coenzyme A dehydrogenase (SCAD), MCAD and very long-chain acyl-coenzyme A dehydrogenase (VLCAD) the respective acyl-CoA substrates for these enzymes accumulate to abnormal levels (Figure 5.1) (Van Maldegem *et al*., 2006; Kompare and Rizzo, 2008). In addition to inborn errors in fatty acid oxidation, various xenobiotic compounds such as aspirin (acetylsalicylic acid) and VPA can also lead to inhibited fatty acid oxidation and concomitant accumulation of acyl-CoA species (Fromenty and Pessayre, 1995).

The accumulation of these substrates brings about the pathological condition known as CASTOR which includes a cascade of events that can result either in chronic illness or acute decompensation (Mitchell *et al.*, 2008). These accumulating substrates are metabolised by both Phase I and Phase II detoxification reactions. Phase I detoxification involves cytochrome P450 dependent ω-oxidation of fatty acids (Johnson *et al*., 1996; Hardwick, 2008), while Phase II detoxification of accumulated acyl-CoA and Phase I generated dicarboxylic acids involve conjugation with either glycine or L-carnitine (Figure 5.1) (Sim *et al*., 2002; Vockley and Whiteman, 2002; Kompare and Rizzo, 2008). Patients with fatty acid oxidation deficiencies, such as SCAD, MCAD and VLCAD, therefore present biochemically with elevated levels of different acylcarnitine and acylglycine species, with subsequent lower levels of free carnitine in both serum and urine (Mueller *et al*., 2003; Van Maldegem *et al*., 2006; Kompare and Rizzo, 2008).

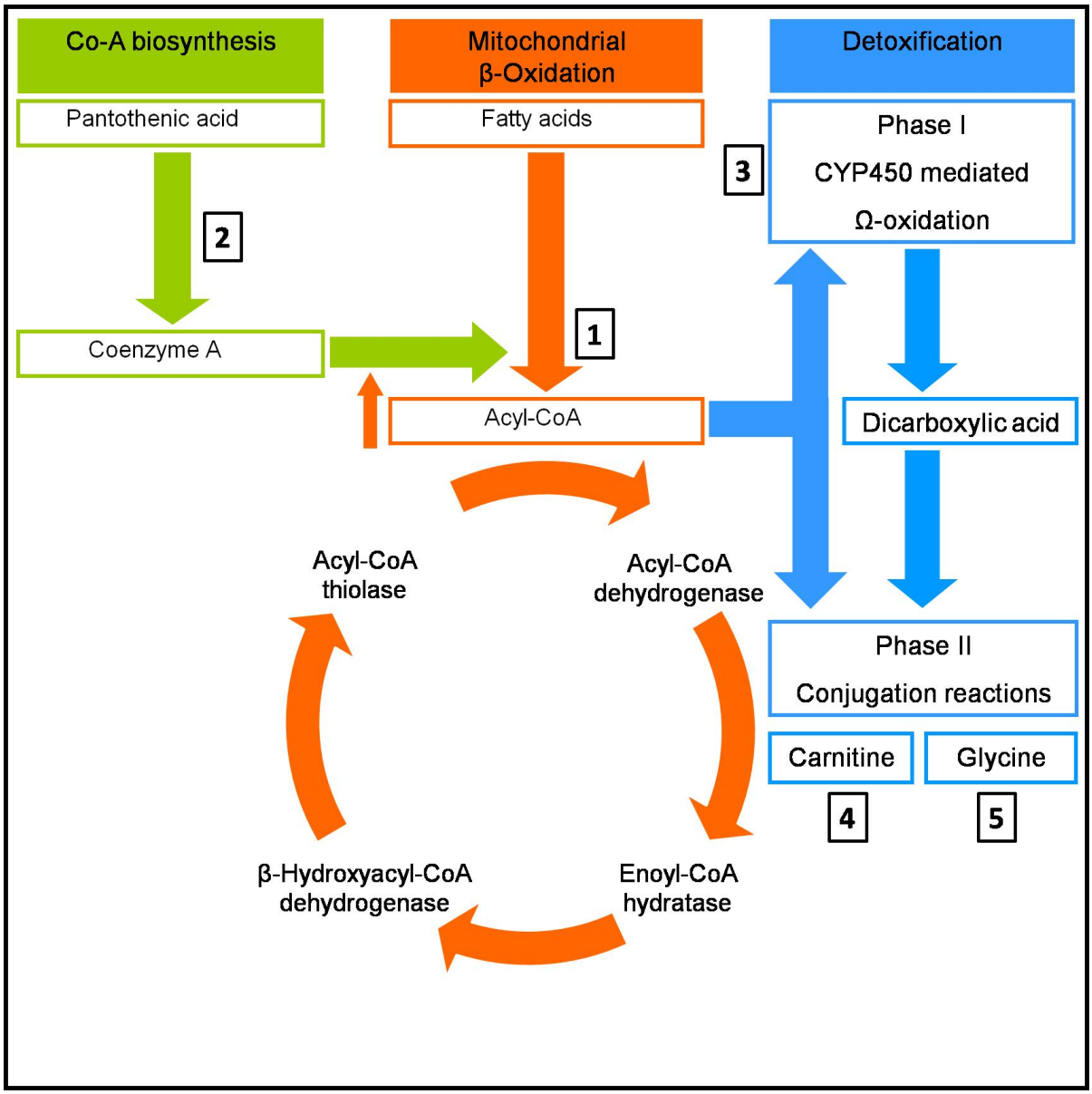

Figure 5.1 Consequences of deficient fatty acid oxidation. (1) The first step in the β-oxidation of fatty acids includes the activation of fatty acids to their corresponding acyl-CoA intermediates, catalysed by acyl-CoA synthetase enzymes. This reaction is the rate-determining step in both fatty acid β-oxidation and detoxification of accumulated fatty acids and highly dependent on the availability of coenzyme A. (2) Pantothenic acid is an important precursor in the biosynthesis of coenzyme A. In the case of deficient fatty acid β-oxidation, accumulated fatty acids undergo both Phase I and Phase II detoxification metabolism. (3) Phase I detoxification include the cytochrome P450 mediated Ω-oxidation and result in the formation of dicarboxylic acids. Both accumulated fatty acids and Phase I produced dicarboxylic acids can undergo Phase II detoxification. Phase II detoxification include (4) conjugation with carnitine and (5) conjugation with glycine.

In this study substrates were used that are frequently utilised in treatment strategies in these types of deficiencies (Vockley and Whiteman, 2002; Rinaldo *et al*., 1993), to develop a "substrate loading cocktail" to enhance fatty acid oxidation and ensure sufficient availability of detoxification substrates. However, in addition to the use of conjugation substrates, pantothenic acid (Vitamin B5), a precursor in the biosynthesis of coenzyme A (Daugherty *et al*., 2002), was also included in the "substrate loading cocktail" to stimulate coenzyme A biosynthesis. Furthermore, the developed "substrate loading cocktail" was applied to investigate the effect on subjects with both induced and inborn errors in fatty acid oxidation. These two groups of fatty acid oxidation deficiencies were not included to be compared to one another, but to indicate the multiple possible applications of the "substrate loading cocktail".

5.2 Materials and methods

See Chapter 3 for a comprehensive description of all materials and methods used in this study.

5.2.1 Subjects

The first subject (NWU-34588) under investigation was a Caucasian female, 57 years of age, who presented with clinical symptoms ranging from chronic fatigue, coughing, dyspnoea and pain to anorexia. For the entire assessment time she continued with a prescribed medication regimen consisting of sodium VPA, lamotrigine, clozapine and simvastatin. Traditional biochemical investigations (i.e. acylcarnitine and organic acid analysis) revealed a metabolite profile indicative of an MCAD deficiency. This profile was observed only after the introduction of a nutritional supplementation regimen to correct low initial free L-carnitine levels, among other abnormal metabolic features. Mutation analysis for the common A985G mutation in the *ACADM* gene was negative and, although other MCAD mutations could not be excluded, it is suspected that this patient has a medication-induced fatty acid oxidation deficiency profile, as this type of profile is typical in VPA therapy.

The second part of this study was conducted on a Caucasian MCAD family. The family consists of a father (NWU-40507) (age 36 years), mother (NWU-40525) (age 37 years) and four siblings (two boys and two girls). Both the father and mother are heterozygous for the common A985G mutation in the *ACADM* gene, while one of the boys (NWU-32842) (age eight years) is homozygous for this mutation. The other siblings did not present with any

clinical symptoms and their metabolite profiles were normal, therefore they were not tested for this mutation. One of the boys in the family did not participate, because he was too young at the time the study commenced.

A control group consisting of four (one male and three females), with ages ranging from 28 to 54 years, also participated in this study. They were considered to be control subjects since their metabolic screening profiles were normal. Since the two siblings of subject NWU-32842 tested normal on the metabolic screening, they were used as controls for subject NWU-32842.

5.2.2 L-Carnitine, glycine and pantothenic acid loading protocols

All test subjects and the control group participated in the following substrate loading protocols: on the first day at 21:00 all subjects ceased eating and drinking (except water) for the day and collected a baseline urine sample, after which they emptied their bladders. All adult subjects took 3 000 mg of L-carnitine and collected all the overnight urine until 7:00 the next morning. On the second day the same protocol as on the first day was followed, except that all the adult subjects took 3 000 mg of glycine in addition to the 3 000 mg of L-carnitine. On the third day the same protocol as on the second day was followed except that all the adult subjects took 550 mg pantothenic acid in addition to the 3 000 mg of L-carnitine and 3 000 mg of glycine. All the loading protocols were performed overnight. The three siblings in the MCAD family received 50 mg/kg body weight L-carnitine and glycine and 10 mg/kg body weight pantothenic acid, according to the same time schedule as the adults.

5.2.3 Analytical procedures

The ESI-MS/MS method for determination of serum acylcarnitines by Vreken *et al*. (1999), was used to determine acylcarnitines in urine. For the analysis of organic acids the GC-MS method of Rinaldo (2008) was used. Both these analytical procedures were executed with minor modifications to suit local laboratory conditions.

5.3 Results

5.3.1 Acylcarnitine analysis

Comparison of the data obtained for free carnitine after the different substrate loading protocols to baseline samples from all the participating subjects, revealed that free carnitine excretion was more or less the same in all the samples of all the participants. However, for subject NWU-34588 there was a 45% increase in free carnitine excretion after loading with both L-carnitine and glycine. The same kind of excretion pattern was observed for the MCAD patient in the following sample, which is the baseline sample before the next loading protocol (Figure 5.2).

Comparison of the data obtained after the different substrate loading protocols to baseline samples from the same subject, revealed differences in the excretion of various medium-chain acylcarnitine species by subject NWU-34588. Although circumstances during baseline sampling were not controlled, it is included to demonstrate that the previous loading protocol did not affect the consecutive loadings. Following L-carnitine loading, the excretion of hexanoylcarnitine increased by 55% as compared to the first base line sample of this subject and is above the 90th percentile of the reference range (Mueller *et al*., 2003). After loading with both L-carnitine and glycine, the concentration of hexanoylcarnitine (C6) decreased marginally by 2% and after loading with L-carnitine, glycine and pantothenic acid, it decreased again by 11% when compared to the loading with L-carnitine only (Figure 5.3 A).

Both these concentrations were also above the 90th percentile of the reference range (Mueller *et al*., 2003). The data obtained for octanoylcarnitine (C8) followed the same trend, with a 50% increase in concentration after L-carnitine loading, followed by a 13% decrease in concentration after loading with L-carnitine and glycine and a 20% decrease in concentration after loading with L-carnitine, glycine and pantothenic acid, when compared to the values obtained after L-carnitine loading. However, in the case of octanoylcarnitine these concentrations were within that of the reference range (Figure 5.3 B) (Mueller *et al*., 2003).

As expected in the case of a homozygous MCAD patient, the amount of hexanoylcarnitine and octanoylcarnitine excreted was found to be much higher. In addition, the excretion pattern observed for these two subjects (i.e. homozygous MCAD and subject NWU-34588) also differed.

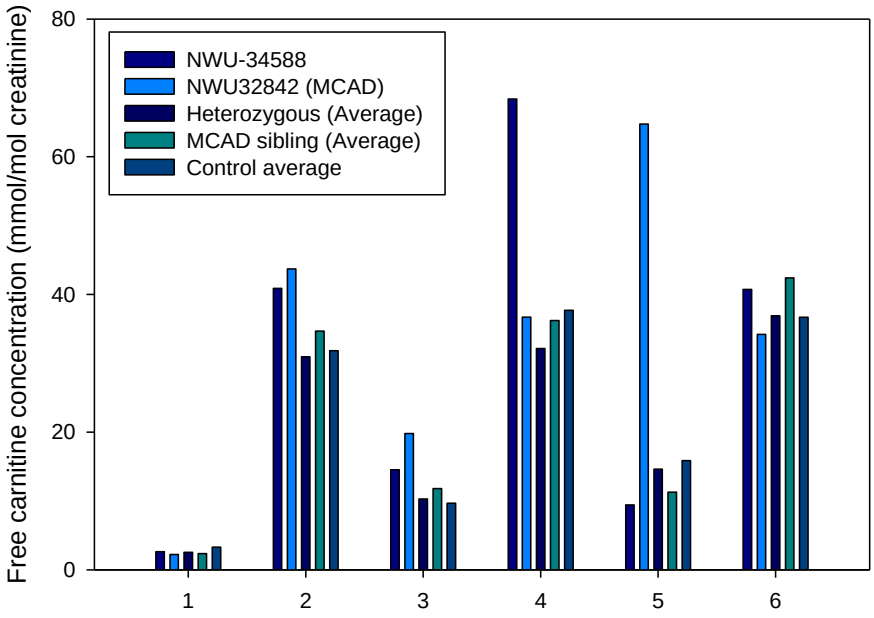

Figure 5.2 Free carnitine concentrations in urine of subject NWU-34588, subject NWU-32842 (MCAD patient homozygous for the A985G mutation), two subjects heterozygous for the same mutation, two siblings of the MCAD patient and four control subjects. The x-axis: (1) acylcarnitine concentrations obtained in the collected base line urine samples; (2) acylcarnitine concentrations after L-carnitine loading; (3) acylcarnitine concentrations obtained in the collected base line urine samples; (4) acylcarnitine concentrations after L-carnitine and glycine loading; (5) acylcarnitine concentrations obtained in the collected base line urine samples; (6) acylcarnitine concentrations after L-carnitine, glycine and pantothenic acid loading.

In the case of subject NWU-34588, the major increase in the excretion of these two acylcarnitine species occurred during the 10 hour urine collection period after the respective loading protocols were followed, while in the case of the MCAD patient the concentrations of these acylcarnitine species were the highest in the following baseline samples. These baseline samples were taken just before the next loading protocol, which is 24 hours after administration of the previous loading substrate(s) (Figure 5.3 A – B). Since this observation was rather extraordinary, the whole "substrate loading protocol" was repeated in this patient. The concentrations of these two acylcarnitine species remained more or less the same in all of the samples collected for the heterozygous MCAD subjects, the siblings of the MCAD patient and the control group.

After loading with L-carnitine, the excreted amount of C6 DC-carnitine in subject NWU-34588 increased only slightly by 7% when compared to the first baseline sample, after which it decreased by 11% and 38% during the consecutive loadings when compared to its

concentration after the L-carnitine loading (Figure 5.3 C). When compared to the first base line sample, the excretion of C8 DC-carnitine increased by 23%, after which it decreased in the consecutive loadings by 8% and 48%, respectively, in comparison to the amount after only L-carnitine loading (Figure 5.3 D). In turn, the observed excretion of these dicarboxylic acid carnitine species was much lower in the homozygous MCAD patient. C6 DC-carnitine and C8 DC-carnitine excretion in the MCAD patient followed the same basic trend as hexanoylcarnitine and octanoylcarnitine excretion, in that the observed increase in excretion occurred only 24 hours after administration of the previous loading substrate(s).

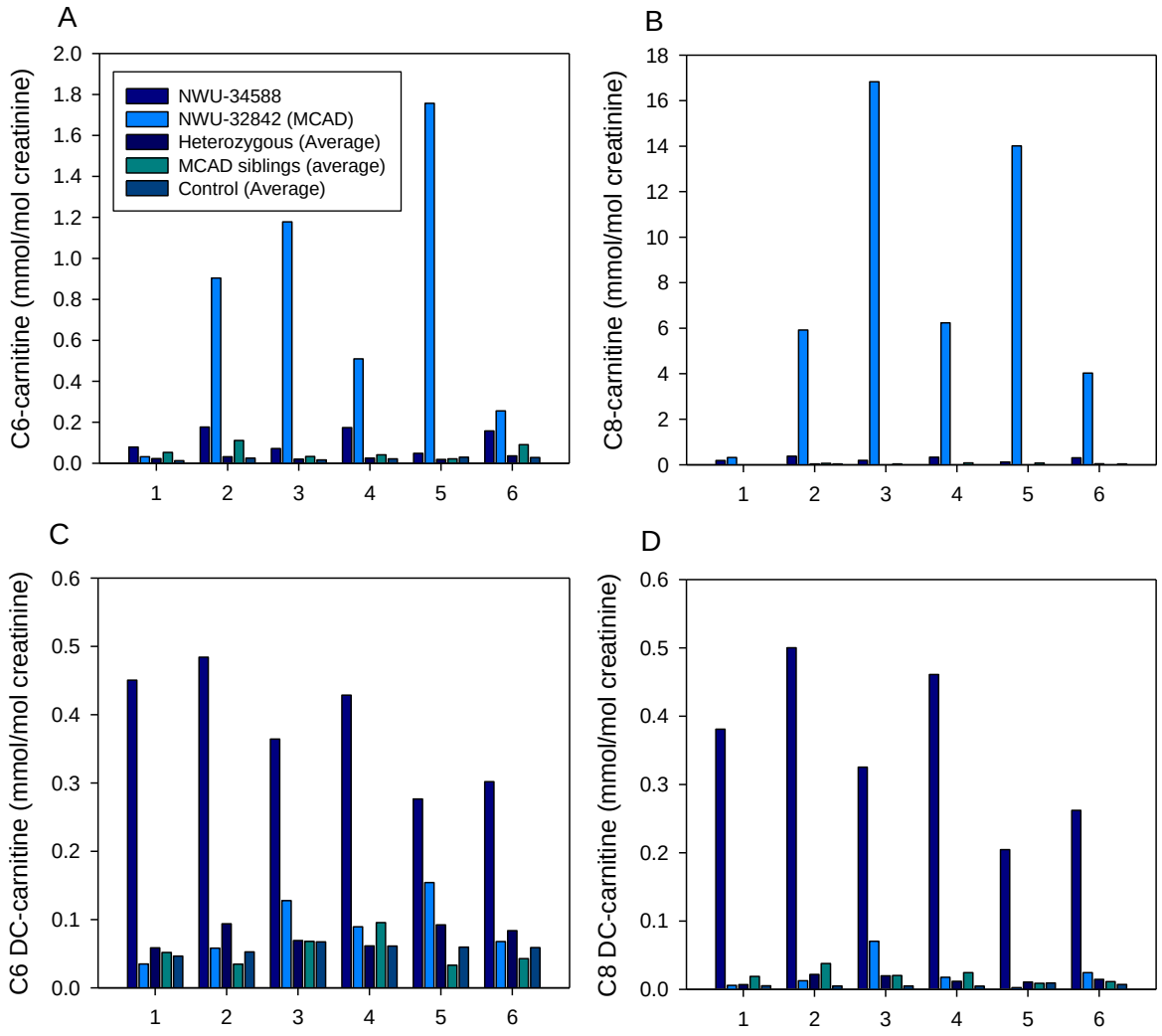

Figure 5.3 C6-carnitine (A), C8-carnitine (B), C6 DC-carnitine (C) and C8 DC-carnitine concentrations in urine of subject NWU-34588, subject NWU-32842 (MCAD patient homozygous for the A985G mutation), two subjects heterozygous for the same mutation, two siblings of the MCAD patient and four control subjects. (See Figure 5.2 for the allocation of data labels on the x-axis.)

5.3.2 Organic acid analysis

Organic acid analysis in the case of subject NWU-34588, showed increased excretion of adipic acid after the respective loading tests, although the excreted amounts remained within the reference range for adults. In the case of the MCAD patient, adipic acid levels higher than the reference range for children older than 5 years were measured in the second and third baseline samples. The average excretion of adipic acid by the siblings of this patient was above the reference range in the samples obtained after L-carnitine and glycine loading. Both the heterozygous parents and control subjects excreted adipic acid levels within the reference range for adults (Table 5.1).

Table 5.1 Urinary adipic acid concentrations in mmol/mol creatinine

Sample	NWU-34588	NWU-32842 (MCAD)	Heterozygous average	MCAD sibling average	Control average
1	5.14	0.53	0.53	4.34	1.72
2	20.72	1.92	1.87	3.33	2.16
3	12.43	24.63*	1.55	2.50	3.19
4	17.60	4.25	0.92	6.87*	2.05
5	13.16	6.70*	2.16	1.47	3.28
6	26.91	1.43	1.01	2.47	3.72

In the sample column (1) adipic acid concentration obtained in the collected base line urine samples; (2) adipic acid concentrations after L-carnitine loading; (3) adipic acid concentrations obtained in the collected base line urine samples; (4) adipic acid concentrations after L-carnitine and glycine loading; (5) adipic acid concentrations obtained in the collected base line urine samples; (6) adipic acid concentrations after L-carnitine, glycine and pantothenic acid loading.

* Reference ranges for different age groups were used. The reference range for adults is 5.1 to 35 mmol/mol creatinine and that of children above the age of 5 years, 1.1 to 5.3 mmol/mol creatinine (Hoffmann and Feyh, 2003).

Suberic acid excretion in subject NWU-34588 was above the reference range in all the samples obtained except the first baseline sample. When compared to the reference range for children older than 5 years, the MCAD patient again excreted higher levels of suberic acid in the second and third baseline samples. The average amount of suberic acid excreted by the MCAD siblings after L-carnitine and glycine loading was again above the reference range for children older than 5 years. The average suberic acid excreted by the heterozygous parents never reached values above that of the reference range, while the control average was higher than the reference range in both the third baseline sample and the samples obtained after L-carnitine, glycine and pantothenic acid loading (Table 5.2).

Table 5.2 Urinary suberic acid concentrations in mmol/mol creatinine

Sample	NWU-34588	NWU-32842 (MCAD)	Heterozygous average	MCAD sibling average	Control average
1	2.42	4.73	0.80	5.57*	1.39
2	4.17*	3.12	0.22	1.29	1.31
3	4.13*	108.93*	1.50	0.00	1.29
4	3.71*	8.24*	0.00	11.10*	0.68
5	5.38*	11.60*	0.67	1.70	3.54
6	5.79*	3.59	0.00	1.49	3.66

In the sample column (1) suberic acid concentration obtained in the collected base line urine samples; (2) suberic acid concentrations after L-carnitine loading; (3) suberic acid concentrations obtained in the collected base line urine samples; (4) suberic acid concentrations after L-carnitine and glycine loading; (5) suberic acid concentrations obtained in the collected base line urine samples; (6) suberic acid concentrations after L-carnitine, glycine and pantothenic acid loading.

* Reference ranges for different age groups were used. The reference range for adults is 0.5 to 2.9 mmol/mol creatinine and that of children above the age of 5 years, 1.1 to 5.3 mmol/mol creatinine (Hoffmann and Feyh, 2003).

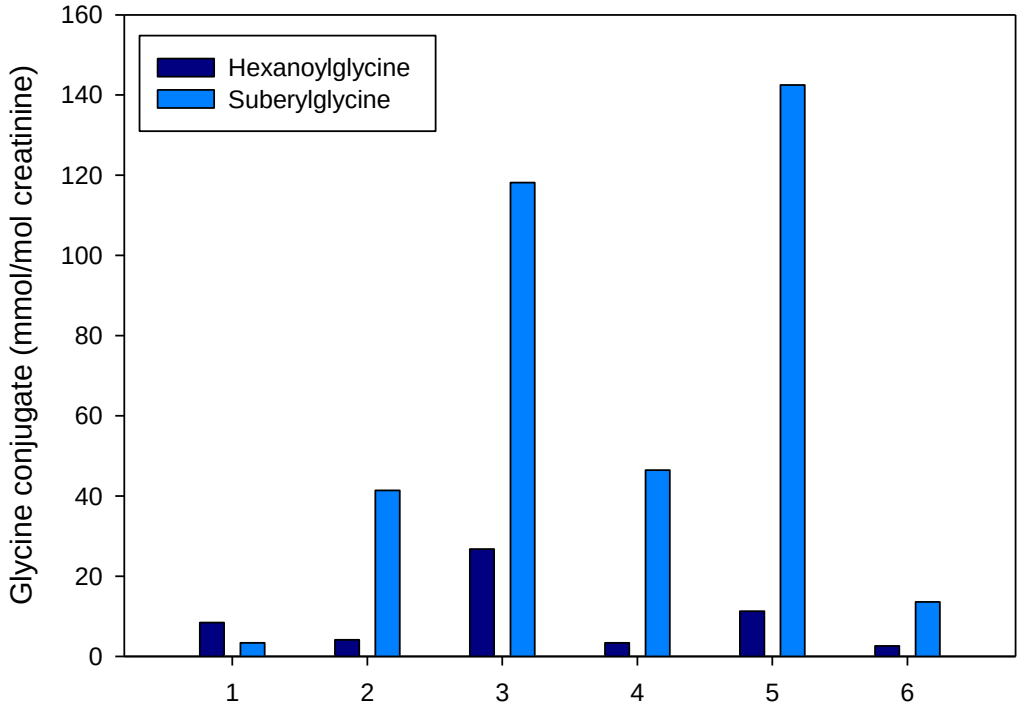

Figure 5.4 Hexanoylglycine and suberylglycine concentrations in urine of the MCAD patient (homozygous for the A985G mutation). (See Figure 5.2 for the allocation of data labels on the x-axis.)

No acylglycine conjugates were detected in any of the samples obtained from subject NWU-34588, the heterozygous subjects, the siblings of the MCAD patient or the control subjects. Both hexanoylglycine and suberylglycine were detected in all the samples obtained from the MCAD patient. A 103% decrease in the excretion of hexanoylglycine was observed after L-carnitine loading when compared to the first baseline sample and its excretion decreased further in the ensuing loading protocols by 23% and 59% respectively, when compared to loading with only L-carnitine. The excretion of suberylglycine increased with 92% after L-carnitine loading, when compared to the first baseline sample. After loading with both L-carnitine and glycine, there was a slight increase of 11% in the excretion of suberylglycine with a steep decrease in excretion after the third loading protocol by 204% when compared to loading with only L-carnitine. However, hexanoylglycine and suberylglycine excretion again followed the same perplexing trend, as there was massive increased excretion of these acylglycine species 24 hours after administration of the previous loading substrate(s) (Figure 5.4). Because the method used for the analysis of acylglycine species in this case, is not as sensitive as a stable isotope dilution method, these results should be considered with caution. However, with that in mind, the general trend observed is still of value.

5.4 Discussion

The treatment of fatty acid oxidation deficiencies often includes supplementation with substrates and cofactors to enhance detoxification metabolism (Rinaldo *et al.*, 1993; Vockley and Whiteman, 2002). This approach was applied to develop a "substrate loading cocktail" to enhance fatty acid oxidation and ensure adequate availability of detoxification substrates and precursors to stimulate coenzyme A biosynthesis. The "substrate loading cocktail" was then used to investigate detoxification patterns in two groups of fatty acid oxidation deficiencies to indicate multiple possible applications of this approach.

5.4.1 Subject NWU-34588

A comparison of the results obtained for subject NWU-34588, which was considered to have a medication-induced fatty acid oxidation deficiency profile, to the control samples and to the baseline samples of this subject, showed an overall increased excretion of acylcarnitines after the first loading. The excretion of medium-chain acylcarnitines and dicarboxylic acid carnitines were the most prominent (Figure 5.3 A-D). The hexanoylcarnitine levels increased to a value above that of the 90^{th} percentile of the reference range (Mueller *et al*., 2003).

In the second loading protocol, glycine was added to the diet supplementation because glycine conjugation to acyl groups can further favour the release of coenzyme A and therefore improve fatty acid oxidation. In this case, it was suspected that acylcarnitine excretion could drop marginally, with a consequent increase in the excretion in acylglycines by subject NWU-34588. The former was indeed the case, since the excretion of medium chain acylcarnitines and dicarboxylic acid carnitines dropped by between 2% and 13% (Figure 5.3 A-D). However, the corresponding acylglycines remained undetected. This observation can be explained by one or more of the following: Firstly, the activity of the glycine N-acyltansferase (GLYAT) enzyme is restricted towards preferred acyl-CoAs (Mitchell *et al.*, 2008), which in this case seem unlikely since the predominant mechanism for excretion of hexanoyl-CoA and octanoyl-CoA in MCAD deficient patients occurs via glycine conjugation (Figure 5.1(5)) (Rinaldo *et al*., 1993). However, in the same study it was also concluded that glycine supplementation did not increase acylglycine formation and that the physiological glycine pool is sufficient to meet conjugation demands in the case of a slight accumulation of medium chain fatty acids and intermediate metabolites (Rinaldo *et al*., 1993). The second possibility is that the co-administration of L-carnitine and glycine could

have an inhibitory effect on glycine conjugation (Rinaldo *et al*., 1993). Thirdly, glycine conjugation is dependent on the availability of both glycine and coenzyme A (Gregus *et al*., 1992). However, in the third loading, pantothenic acid was added (Figure 5.1(2)) to stimulate the biosynthesis of coenzyme A, and thereby included all the possible agents to enhance coenzyme A availability. Nevertheless, medium-chain acylglycine conjugates were still not detected, which brings us to the fourth possibility for this observation, which is a possible inhibitory effect or the presence of a polymorphism in the GLYAT enzyme, which affects the activity of this enzyme. This possibility is supported by the detoxification and oxidative status analysis of this subject, which revealed continued low glycine conjugation with salicylic acid in several follow-up investigations (Chapter 4).

A further decrease was also expected in the accumulation of medium-chain acylcarnitine and dicarboxylic acid carnitine species after the third loading regimen. It was indeed observed that the excretion of the corresponding carnitine conjugates was decreased by between 11% and 63% (Figure 5.3 A-D). However, when compared to the MCAD patient it is important to note that the excretion of the dicarboxylic acid carnitine conjugates were much higher in subject NWU-34588. This may be due to increased excretion of propylglutarylcarnitine, a C8 DC-carnitine metabolite of VPA (Silva *et al.*, 2001), or increased cytochrome P450 mediated Ω-oxidation in response to inhibited fatty acid β-oxidation or the stimulation of the cytochrome P450 system directly (Figure 5.1(3)).

5.4.2 MCAD family

The same basic trend, but more pronounced, was expected in the excretion of acylcarnitine species in the homozygous MCAD patient as was seen in subject NWU-34588. Furthermore, it was also expected that the two heterozygous parents of the MCAD patient would closely resemble the observed pattern in subject NWU-34588. However, this was not the case since the MCAD patient revealed a totally different excretion pattern. In addition, the excretion patterns of the heterozygous parents and the siblings of the MCAD patient resembled that of the control subjects involved in this study.

The excretion pattern of accumulated medium-chain acylcarnitine species in the MCAD patient differed from that expected, in that the excretion of these species reached peak concentrations in the baseline samples taken 24 hours after the different loading protocols and not during the 10 hour urine collection period in which it was expected to occur.

Although the baseline samples were analysed during this study to demonstrate that the previous loading protocol was not affecting the consecutive loadings, it led to this interesting observation. Since this observation was rather extraordinary, the "substrate loading protocol" was repeated, and the exact same results were obtained. It therefore implies a lag phase before the excretion of these species reached a maximum level. Various possible explanations can be posed for this phenomenon. The first entails slower absorption of carnitine in this patient. It has been reported that the absorption of carnitine depends on the given dosage. In the case of high dosages i.e. more than 6 g, only 5-15% of the carnitine is absorbed, while in the case of low dosages i.e. less than 1 g, more than 75% of the carnitine is absorbed (Stieber *et al*., 2004). In this study, the MCAD patient received a dosage just above 1 g, which implies that, theoretically, a large portion of the ingested carnitine should have been absorbed. Besides, if the transporters are not saturated, 98% to 99% of free carnitine can be re-absorbed and transported through the circulation to be taken up by various tissues (Stieber *et al.*, 2004). In addition, the excretion pattern of free carnitine in the MCAD patient was the same as all the other participating subjects, which also suggests that the absorption of carnitine was more or less the same in all the participating subjects (Figure 5.2)

The observed lag effect in the excretion of different acylcarnitine species in response to the different loading protocols (Figure 5.3 A-D) can also be due to slower export of these species from the mitochondrial matrix. Since L-carnitine and acylcarnitines are readily transported across mitochondrial and cell membranes by way of well defined transport systems (Forster, 2004), it can be expected that if one of these transporters is not functioning at optimal levels, it could manifest as the observed lag effect seen in this MCAD patient. However, since the same lag effect was also observed for the glycine conjugates (Figure 5.4), it seems unlikely that the export of both acylcarnitine and acylglycine species would be affected.

The third possible explanation for the observed lag effect in this MCAD patient is slower Phase II detoxification of accumulated fatty acids. This include both carnitine and glycine conjugation reactions. Slower Phase II detoxification of accumulated fatty acids seems to be a more valid explanation, as the lag effect in response to the different loading protocols were observed in both carnitine and glycine conjugation (Figure 5.3 & 5.4). Both these reactions consist of two sequential steps of which the first include the activation of fatty acids to their corresponding acyl-CoA species within mitochondria. This reaction is catalysed by the medium-chain acyl-CoA synthetase enzyme, which is also the first step in the metabolism of

medium-chain fatty acids (Figure 5.1(1)) (Kasuya *et al*., 2006). In the case of carnitine conjugation the second reaction in this biotransformation pathway is catalysed by carnitine transferase enzymes, while in the case of glycine conjugation the reaction is catalysed by GLYAT (Mitchell *et al*., 2008). The common feature, and the rate-determining step in both these processes, is the activation of accumulated fatty acids (Vessey *et al*., 1999), while the supply of carnitine and glycine (Gregus *et al*., 1993) is considered as rate-limiting. However, during this study both carnitine and glycine were used in the loading protocols to ensure that the rate-limiting aspect of these reactions was taken care of. We therefore propose that in this patient, in addition to the proven MCAD deficiency there also seems to be slower activation and therefore detoxification of accumulated fatty acids. We also propose that the slower activation is due to a possible polymorphism in the medium-chain acyl-CoA synthetase enzyme, as the third substrate loading protocol contained several agents to ensure sufficient availability of coenzyme A.

5.5 Conclusion

It became evident during this study that the application of different well defined loading protocols with appropriate substrates and cofactors involved in detoxification metabolism can be used to enhance the detection of aberrant metabolite profiles. This novel approach has the potential to contribute tremendously in describing and eventually differentiating subjects with different fatty acid oxidation deficiencies. In addition, the rates of transformation that can be observed by such a test on individuals may be of value to decide upon subsequent individualised therapy as well as the timing of the therapy. However, this approach was only applied on one MCAD deficient patient due to a lack of available mutation confirmed cases within our laboratory and one patient with medication induced fatty acid oxidation deficiency. Final conclusions can therefore not be drawn from the available data, but it provides a base for further investigation.

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Chapter 6
Increased excretion of C4-carnitine species after a therapeutic acetylsalicylic acid dose: evidence for an inhibitory effect on short-chain fatty acid metabolism

Part of this material has been accepted for publication in ISRN Pharmacology - **http://www.hindawi.com/isrn/pharmacology/** **(**doi:10.5402/2011/851870) - Annexure C

6.1 Introduction

The dehydrogenation of acyl-CoA intermediates in the catabolism of fatty acids and branched chain amino acids in humans are catalysed by the mitochondrial acyl-CoA dehydrogenase enzymes (Nguyen *et al*., 2002; Pedersen *et al*., 2006). Several inherited defects in this group of enzymes have been characterised. Defects in, or inhibition of isovaleryl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase result in the accumulation of C5-carnitine species (Gibson *et al*., 2000), whilst defects in, or inhibition of short-chain acyl-CoA dehydrogenase and isobutyryl-CoA dehydrogenase result in the accumulation of C4-carnitine species (Nguyen *et al*., 2002) (Figure 6.1).

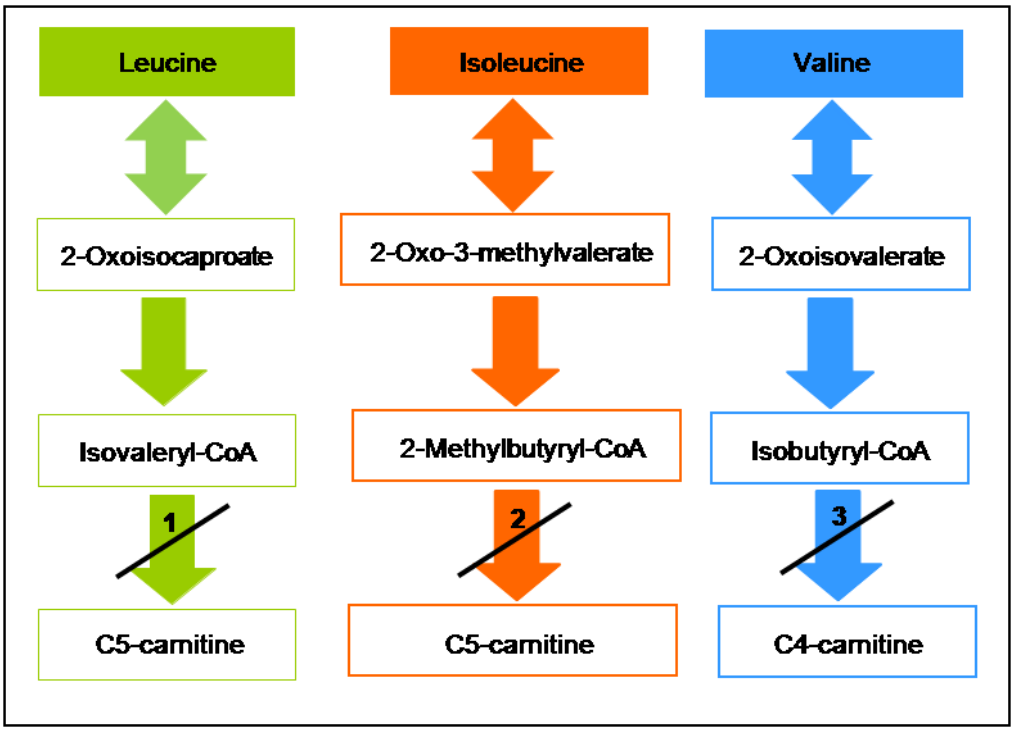

Figure 6.1 Proposed inhibition by salicylic acid of the branched chain amino acid catabolism. Inhibition (or deficiency) of (1) isovaleryl-CoA dehydrogenase, (2) 2-methylbutyryl-CoA dehydrogenase, and (3) isobutyryl-CoA dehydrogenase, will result in the accumulation of C4-carnitine and C5-carnitine species.

In the human body acetylsalicylic acid is hydrolysed to salicylic acid, which is excreted in its conjugated form with glycine and glucuronic acid, in its hydroxylated form as 2,5-dihydroxybenzoic acid or unchanged as salicylic acid (Ingelman-Sundberg *et al*., 1991). Numerous *in vitro* and *in vivo* studies have been conducted that demonstrated various effects of acetylsalicylic acid (and its metabolites) on the metabolism and especially on mitochondrial function, such as the uncoupling of oxidative phosphorylation (Brody, 1955;

Deschamps *et al*., 1991), inhibition of fatty acid oxidation (Brody, 1955; Yoshida *et al*., 1988; Deschamps *et al*., 1991; Kasuya *et al*., 2001) with the concomitant stimulation of cytochrome P450 dependent ω-oxidation (Kundu *et al*., 1991) and therefore accumulation of dicarboxylic acids (Yoshida *et al*., 1998). In addition, the inhibition of both the Krebs cycle enzymes α-ketoglutarate dehydrogenase and succinate dehydrogenase (Kaplan *et al*., 1954; Deschamps *et al*., 1991), as well as an increased flux through the Krebs cycle (Rognstad, 1991), have been reported. Other effects of salicylic acid include the stimulation of oxygen consumption, stimulation of ATP hydrolysis (Brody, 1955), the activation of pyruvate dehydrogenase and the inhibition of gluconeogenesis (Rognstad, 1991). It can also lead to decreased blood glucose concentrations, increased hepatic triglycerides (Deschamps *et al*., 1991) and a slight increase in (iso)butyryl-CoA, β-methylcrotonyl-CoA, isovaleryl-CoA and octanoyl-CoA concentrations (Kilpatrick *et al*., 1989).

The exact mechanism of inhibition that acetylsalicylic acid and/or its metabolites exert on fatty acid oxidation is not clear, since different studies indicate different sites of inhibition. These include an inhibitory effect on the activation and transportation of medium- and long-chain fatty acids into the mitochondria (Yoshida *et al*., 1988), due to the sequestration of extra-mitochondrial coenzyme A and carnitine (Deschamps *et al*., 1991), inhibition of the medium-chain acyl-CoA synthetase (Kasuya *et al*., 2001), or inhibition of the carnitine acyltransferase (CAT) enzymes (Vessey *et al*., 1991). Conversely, it was demonstrated that the target of inhibition was found to be at the level of the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) activity of the mitochondrial trifunctional β-oxidation enzyme (MTE) and not at the level of uptake or activation of fatty acids (Glasgow *et al*., 1999).

While performing biotransformation metabolism and oxidative stress status profiling studies, on individuals referred to our laboratory, by using acetylsalicylic acid and acetaminophen as probe substrates, we made the rather startling observation of the presence of elevated C3-, C4- and C5-carnitine species in their urine. These observations led to a more in depth investigation into the nature of these acylcarnitine species and whether their increased excretion was due to the separate or combined effect of the acetylsalicylic acid and acetaminophen administration. Since the accumulation of C4- and C5-carnitine species are predominantly associated with deficient branched chain amino acid metabolism at the level of different acyl-CoA dehydrogenase enzymes (Gibson *et al*., 2000; Pedersen *et al*., 2006), we hypothesised that acetylsalicylic acid administration is associated with the elevation of these

species, which may be linked to the inhibition of the metabolism of C4 and C5 fatty acids (Figure 6.1). The formulation of this hypothesis was supported by the previous observation that a slight increase in (iso)butyryl-CoA, β-methylcrotonyl-CoA, isovaleryl-CoA and octanoyl-CoA concentrations occur after acetylsalicylic acid intake (Kilpatrick *et al*., 1989).

This investigation provides the first evidence of a significant increase in isobutyrylcarnitine excretion following acetylsalicylic acid intake at therapeutic doses in humans and we propose that this accumulation may be due to the inhibitory effect of acetylsalicylic on the metabolism of C4 and C5 fatty acids.

6.2 Materials and methods

See Chapter 3 for a comprehensive description of all the materials and methods used in this study.

6.2.1 Subjects

The test subjects were divided into two groups: The first group included 30 test subjects, 19 female and 11 male between the ages of 10 and 65 years. Participants in this group were originally referred for biotransformation metabolism and oxidative stress status assessment, which include the administration of both acetaminophen and acetylsalicylic acid. The second group included seven randomly chosen participants from the first group and were used to ascertain whether the observed effects were due to acetylsalicylic acid intake, acetaminophen intake or a combination thereof.

6.2.2 Loading protocol and sample collection

Fasting baseline urine samples of all the test subjects in the first test group were collected the morning of the test day. At 21:00, on the same day, all the test subjects ceased eating and drinking (except water), emptied their bladders and took 1000 mg acetaminophen and 600 mg acetylsalicylic acid. The total volume of urine excreted for ten hours after administration of acetaminophen and acetylsalicylic acid was collected and the quantity of urine excreted was documented.

The second test group followed a protocol to determine if the observed effect on the acylcarnitine profile was due to acetylsalicylic acid intake, acetaminophen intake or a combination thereof. On the first day at 21:00 all subjects ceased eating and drinking (except

water) for the day and collected a baseline urine sample, after which they emptied their bladders. All subjects took 600 mg of acetylsalicylic acid and collected all the overnight urine until 7:00 the next morning. On the second occasion the same protocol was followed except that all the subjects took 1000 mg of acetaminophen and collected all the overnight urine until 7:00 the next morning and on the third occasion all the subjects took both 600 mg acetylsalicylic acid and 1000 mg acetaminophen and collected all the overnight urine until 7:00 the next morning. Subjects waited for a minimum of three days before going on to the next protocol.

6.2.3 Analytical procedures

The ESI-MS/MS method for determination of serum acylcarnitines by Vreken *et al*. (1999) was used to determine acylcarnitines in urine. The LC-MS/MS method for the separation and identification of short-chain acylcarnitine isomers as described by Ferrer *et al*. (2007) was used, with minor modifications to separate butyrylcarnitine and isobutyrylcarnitine. Amino acids were analysed and different branched chain amino acids separated and identified according to the ESI-MS/MS and LC-MS/MS method as described in Chapter 3.

6.2.4 Statistical analysis

A paired t-test was used to demonstrate statistically significant differences between the test samples. In all cases statistical significance was set at $p < 0.05$.

6.3 Results

6.3.1 Acylcarnitine analysis (ESI-MS/MS)

A comparison between the acylcarnitine profiles in baseline urine samples of the first test group (n=30) obtained before and after administration of a combination of acetylsalicylic acid and acetaminophen, revealed a statistically significant increase in the excretion of C3- (p=0.05), C4- (p=0.00) and C5-carnitine moieties (p=0.00). Comparison of the acylcarnitine profiles in baseline urine samples of the second test group (n=7) to the profiles obtained after the administration of a therapeutic dose of acetylsalicylic acid alone, revealed a statistically significant increase in the excretion of C4-carnitine (p=0.03) (Table 6.1).

Table 6.1 Paired t-test values (p-values) of acylcarnitine species in baseline urine samples compared to acylcarnitine species in urine samples obtained after acetylsalicylic acid administration (n=7), acetaminophen administration (n=7) and combined administration of both acetylsalicylic acid and acetaminophen (n=30).

Acylcarnitine species	Paired t-test (p-value)		
	Acetylsalicylic acid administration (n=7)	**Acetaminophen administration (n=7)**	**Combined administration (n=30)**
C0-carnitine	0.18	0.20	0.72
C2-carnitine	0.08	0.06	0.87
C3-carnitine	0.96	0.88	0.05*
C4-carnitine	0.03*	0.31	0.00*
C4-OH-carnitine	0.18	0.12	0.32
C4-DC-carnitine	0.16	0.05*	0.08
C5-carnitine	0.73	0.85	0.00*
C5-OH-carnitine	0.69	0.33	0.35
C5-DC/C10-OH-carnitine	0.17	0.01*	0.26
C5:1-carnitine	0.24	0.50	0.29
C6-carnitine	0.20	0.62	0.98
C6-DC-carnitine	0.29	0.01*	0.77
C8-carnitine	0.41	0.10	0.20
C8-DC-carnitine	0.84	0.00*	0.55
C10-carnitine	0.20	0.11	0.20
C5:1-DC/C10:1-OH-carnitine	0.82	0.10	0.90
C12-carnitine	0.20	0.11	0.15
C14-carnitine	0.41	0.20	0.70
C16-carnitine	0.21	0.07	0.95

* Differences are considered to be statistically significant compared to baseline values if $p<0.05$.

However, a comparison between the acylcarnitine profiles obtained before and after the administration of a therapeutic dose of acetaminophen (n=7), revealed a decreased excretion of various dicarboxylic acid carnitine conjugates i.e. C4-DC (p=0.05), C5-DC/C10-OH-carnitine (p=0.01), C6-DC (p=0.01) and C8-DC (p=0.00) (Table 6.1).

6.3.2 Acylcarnitine isomer analysis (LC-MS/MS)

Comparing the LC-MS/MS baseline data with that obtained after the administration of a combination of acetylsalicylic acid and acetaminophen, revealed that the increase in the excretion of C4-carnitine was due to the increased excretion of isobutyrylcarnitine (p=0.04) rather than due to the increase in butyrylcarnitine (p=0.46). The results obtained for the different C4-carnitine isomers revealed no statistically significant difference when either butyrylcarnitine or isobutyrylcarnitine concentrations in baseline urine samples were compared to samples (n=7) taken after acetylsalicylic acid administration (p=0.61 and p=0.20) and samples (n=7) taken after acetaminophen administration (p=0.62 and p=0.32).

6.3.3 Amino acid analysis (ESI-MS/MS & LC-MS/MS)

Concerning the amino acid analysis, a statistically significant decrease in the excretion of isoleucine (p=0.00), leucine (p=0.04), valine (p=0.01) and tryptophan (p=0.01) (Table 6.2) was evident in the urine samples obtained after acetylsalicylic acid and acetaminophen administration (n=30). A comparison between the amino acid profiles in baseline urine samples and samples taken after acetylsalicylic acid (n=7) and acetaminophen (n=7) administration revealed a statistically significant decrease in the excretion of alanine, glycine, the branched chain amino acids, methionine, phenylalanine, tyrosine, lysine, histidine, aspartic acid and glutamic acid. In addition, significantly less proline/arginine, tryptophan and serine were present in the urine after acetaminophen administration, but not after acetylsalicylic acid administration (Table 6.2).

Table 6.2 Paired t-test values (p-values) of amino acids in baseline urine samples compared to amino acids in urine samples obtained after acetylsalicylic acid administration (n=7), acetaminophen administration (n=7) and combined administration of both acetylsalicylic acid and acetaminophen (n=30).

Amino Acids	Paired t-test (p-value)		
	Acetylsalicylic acid administration (n=7)	**Acetaminophen administration (n=7)**	**Combined administration (n=30)**
Alanine	0.00*	0.00*	0.50
Glycine	0.03*	0.02*	0.82
Valine	0.02*	0.01*	0.01*
Leucine	0.04*	0.03*	0.04*
Isoleucine	0.02*	0.01*	0.00*
Methionine	0.01*	0.01*	0.73
Proline/Arginine	0.06	0.02*	0.21
Phenylalanine	0.02*	0.01*	0.43
Tryptophan	0.12	0.03*	0.01*
Serine	0.06	0.03*	0.32
Threonine	0.19	0.07	0.31
Tyrosine	0.01*	0.00*	0.13
Lysine	0.03*	0.03*	0.45
Histidine	0.01*	0.00*	0.20
Aspartic acid	0.05*	0.05*	1.00
Glutamic acid	0.02*	0.02*	0.39

* Differences are considered to be statistically significant compared to baseline values if $p<0.05$.

6.4 Discussion

Acetylsalicylic acid and acetaminophen are generally used as probe substrates in the evaluation of biotransformation metabolism and oxidative stress status in humans (Liska *et al*., 2006). Careful investigation of the acylcarnitine profiles of such subjects revealed the

presence of increased concentrations of C3-, C4- and C5-carnitine species in their urine. Since these acylcarnitine species reached concentrations normally associated with inborn errors of metabolism in some subjects (Mueller *et al*., 2003), it was investigated whether this phenomenon originated from the administration of acetylsalicylic acid or acetaminophen, or a combination thereof. An increased excretion of various acylcarnitines derived from inhibited fatty acid oxidation, such as octanoylcarnitine and palmitoylcarnitine (Yoshida *et al*., 1988) as well as certain dicarboxylic acid carnitines with chain lengths ranging from C6 to C12 (Yoshida *et al*., 1998), were expected after acetylsalicylic acid administration. In the case of acetaminophen administration, an increased excretion of palmitoylcarnitine was expected (Chen *et al*., 2009). However, our analyses revealed a statistically significant increase in the excretion of C4-carnitine species in the case of acetylsalicylic acid administration, a statistically significant decrease in the excretion of various dicarboxylic acid carnitine conjugates after acetaminophen administration and a statistically significant increase in the excretion of C3-, C4- and C5-carnitine in the case of the combined administration.

The most important observation in this respect is that the increased excretion in C4-carnitine was due to the intake of acetylsalicylic acid, as there was no increased excretion after acetaminophen administration. Although there were no statistically significant differences in the C3- and C5-carnitine concentrations after acetylsalicylic acid administration (n=7), as in the case of the combined administration, one cannot exclude that acetylsalicylic acid could be the cause of this increase, taking cognisance of the relatively small sample size.

It is known that butyrylcarnitine (C4-carnitine) accumulates with deficient or inhibited short-chain acyl-CoA dehydrogenase and that isobutyrylcarnitine (C4-carnitine) accumulates when isobutyryl-CoA dehydrogenase is deficient or inhibited (Pedersen *et al*., 2006). In the case of C5-carnitine, the isomers include isovalerylcarnitine and 2-methylbutyrylcarnitine, which will accumulate with deficient or inhibited isovaleryl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase (Gibson *et al*., 2000) (Figure 6.1). As three of these enzymes are dehydrogenases involved in the catabolism of branched chain amino acids, it was hypothesised that acetylsalicylic acid may have an inhibitory effect on this metabolism, possibly at the level of these enzymes. It was demonstrated by Glasgow *et al*. (1999) that the metabolites of acetylsalicylic acid can exercise mixed inhibition on β-oxidation at the level of the LCHAD activity of the MTE, as a result of structural similarities between acetylsalicylic acid and the acyl-part of fatty acids. It was therefore necessary to determine whether the

observed increased excretion of C4-carnitine was due to increased butyrylcarnitine or isobutyrylcarnitine. The chromatographic separation of these isomers from each other in both the baseline samples and the samples obtained after acetylsalicylic acid and acetaminophen administration was of utmost importance and during the chromatographic separation, when performing a precursor ion scan for a product with an *m/z* of 85, two precursor ions were detected for C4-carnitine with an *m/z* of 288.

A comparison of the amount of the C4-carnitine isomers in the baseline samples to the test samples (after acetylsalicylic acid and acetaminophen administration respectively) revealed no statistically significant difference. However, in the samples analysed to investigate a possible combined effect of acetylsalicylic acid and acetaminophen administration (n=30) a statistically significant increase in only the isobutyrylcarnitine concentration was observed. These results support the hypothesis that acetylsalicylic acid may have an inhibitory effect on short-chain fatty acid metabolism, possibly at the level of isobutyryl-CoA dehydrogenase involved in the catabolism of branched chain amino acids.

Since there was no observed effect on these metabolic pathways after acetaminophen administration alone, the possibility also exists that the increase in C5-carnitine species observed after the combined administration may be due to the same inhibitory effect on the short-chain fatty acid metabolism. In this case it can be at the level of the isovaleryl-CoA dehydrogenase and S-2-methylbutyryl-CoA dehydrogenase enzymes.

In addition to the increased excretion of the C4- and C5-carnitine species, there was also an increased excretion in C3-carnitine species after the administration of combined acetylsalicylic acid and acetaminophen administration. There are various factors that can contribute to the accumulation of C3-carnitine species, including propionyl-CoA carboxylase deficiency or inhibition, methylmalonyl-CoA mutase deficiency or inhibition and defects or inhibition of the processing of biotin and cobalamin (Chapman *et al*., 2008). However, in this case we propose that the inhibitory effect of acetylsalicylic acid on the Krebs cycle (Kaplan *et al*., 1954) may be the reason for the increased amount of C3-carnitine observed after acetylsalicylic acid and acetaminophen administration, as propionyl-CoA, which is formed during the catabolism of various amino acids, including isoleucine and valine, undergo enzymatic conversion to methylmalonyl-CoA, which is subsequently isomerised to succinyl-CoA, which can enter the Krebs cycle (Fenton *et al*., 2001). However, since it has been demonstrated that salicylic acid inhibits the Krebs cycle (Kaplan *et al*., 1954),

propionyl-CoA can no longer enter this cycle and would therefore accumulate and be excreted as propionylcarnitine. Conversely, if this was indeed the case, one would expect that methylmalonylcarnitine will also accumulate, but no statistically significant differences in the methylmalonylcarnitine excretion were detected.

To further demonstrate the possible inhibitory effect of acetylsalicylic acid and/or its metabolites on the branched chain amino acid metabolism, the total amino acid profiles were analysed. These analyses were also done on both baseline samples and samples taken after acetylsalicylic acid and acetaminophen administration, individually and combined. The comparison between the amino acid profiles in baseline urine samples and samples taken after acetylsalicylic acid (n=7) and acetaminophen (n=7) administration revealed a statistically significant decrease in the excretion of various amino acids, including the branched chain amino acids. While the combined administration of acetylsalicylic acid and acetaminophen revealed a statistically significant decrease in only the branched chain amino acids and thryptophan. However, an increase in branched chain amino acid excretion after acetylsalicylic acid administration was expected due to the proposed inhibitory effect downstream in the branched chain amino acid catabolism. In an *in vivo* study on children who ingested excessive amounts of salicylate, Andrews *et al*. (1961) reported amino aciduria with prominent excretion of cysteine, arginine, histidine, methionine, asparagine, lysine, glycine, glutamic acid, alanine, proline, tyrosine, valine and leucine. The authors suggested that this may be due to changes in carbohydrate, lipid and amino acid utilisation as a result of the uncoupling action of salicylates. However, the test subjects in this study received one therapeutic dosage of acetylsalicylic acid which could not have led to intoxication.

On the other hand, compromised energy metabolism, as a result of the uncoupling of oxidative phosphorylation, inhibition of the Krebs cycle and gluconeogenesis (Rognstad, 1991) can result in hypoglycaemia, since the decreased availability of energy affects glucose homeostasis due to impaired glucose transport and intestinal absorption (Arvanitakis *et al*., 1977). Amino acids are therefore the only other available energy source, and branched chain amino acids in particular, play an important role in the body in times of stress. During times of stress, the use of branched chain amino acids is preferred to the use of other amino acids, since it can be used as a direct source of energy in addition to glucose (Lai, 1988). It is therefore possible that, although this pathway seems to be inhibited, the body attempt to use more of these amino acids to cope with the energy demand. One has to also keep in mind

that all these reported inhibitory effects of acetylsalicylic acid and/or its metabolites are incomplete and occur to different degrees.

In only some of the subjects the amount of excreted isobutyrylcarnitine after the administration of acetylsalicylic acid, reached concentrations normally associated with isobutyryl-CoA dehydrogenase deficiency. In this regard, we would like to speculate as to the possible implications of this observation. Since not all individuals are affected to the same degree, it is possible that the inhibitory effect of acetylsalicylic acid is more pronounced in carriers of the isobutyryl-CoA dehydrogenase deficiency or in individuals with rate-limiting polymorphisms in the same enzyme system. If this is indeed the case, it opens the opportunity to investigate whether the administration of acetylsalicylic acid can be used to predict the carrier status in isobutyryl-CoA dehydrogenase deficiency. Since the first patient with isobutyryl-CoA dehydrogenase deficiency was diagnosed only over a decade ago (Roe *et al*., 1998) and since there is substantial variation in the clinical presentation of this deficiency, it also poses the opportunity to investigate other biochemical effects involved in the pathology of deficient isobutyryl-CoA dehydrogenase, as the elucidation of the development of phenotypic characteristics of metabolic diseases remains a formidable challenge.

6.5 Conclusion

From literature sources, it is clear that acetylsalicylic acid and/or its metabolites have various effects on metabolism and especially on mitochondrial function. In this study the first *in vivo* evidence in human subjects of a statistically significant increase in isobutyrylcarnitine excretion as a result of the administration of a therapeutic dose of acetylsalicylic acid was provided. Since it was previously demonstrated that the structural similarities between acetylsalicylic acid and the acyl-portion of fatty acids can result in mixed inhibition on β-oxidation (Glasgow *et al*., 1999), we propose a possible inhibitory effect on isobutyryl-CoA dehydrogenase, as elevated isobutyrylcarnitine excretion is generally a result of a deficiency in the branched chain amino acid catabolism.

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Chapter 7
General discussion and concluding remarks

7.1 General discussion of results

Biotransformation (or detoxification) metabolism of toxic compounds is regarded as one of the most important functions of the liver. The accumulation of xenobiotics within the human body is a well known health risk, and a well studied discipline within the pharmaceutical industry. However, the accumulation of endogenous compounds in inborn errors of metabolism and the concomitant pathological consequences, is not explicitly associated with unbalanced biotransformation metabolism. The aim of this study was therefore to formulate a novel hypothetical model - the unbalanced biotransformation metabolism model, to link these two disciplines. This model originated from a thorough literature study and was applied on deficient fatty acid oxidation for evaluation (Chapter Two). In the evaluation of biotransformation metabolism in subjects with both inborn and induced fatty acid oxidation deficiencies, various advanced research techniques were applied. These techniques varied from the use of specialised equipment to the use of important compounds within the human metabolism to challenge certain metabolic pathways. In addition to the formulation of this novel model, the study further aimed to demonstrate how substrates and cofactors involved in biotransformation metabolism can be used in a "substrate loading cocktail" (Chapter Five). It was indicated that this novel approach has the potential to contribute tremendously in describing and eventually differentiating subjects with different fatty acid oxidation deficiencies. Furthermore, monitoring of the rates of biotransformation may be of value to decide upon subsequent individualised therapy as well as the timing of the therapy. The final objective of this study was to investigate the effect of salicylic acid which is used in the phenotyping of Phase II glycine conjugation, on the acylcarnitine profile in human subjects. During this part of the study the first ever *in vivo* evidence in human subjects of a statistically significant increase in isobutyrylcarnitine excretion, as a result of the administration of a therapeutic dose of acetylsalicylic acid, was provided (Chapter Six). Three papers that originated from these investigations were submitted for publication. One of these papers was accepted for publication in Health (paper id: 8201301), while some of the data were presented at the 11th International Congress of Inborn Errors of Metabolism (ICIEM) (San Diego, CA, August 29 - September 2, 2009).

7.1.1 Subject NWU-34588

Subject NWU-34588, a 57 year old Caucasian female, with metastatic small cell carcinoma of the lung, presented with various clinical symptoms. After the diagnosis she commenced

with a chemo combination therapy for six repeated cycles over a period of twenty weeks. Four weeks before the end of chemotherapy she suffered from severe fatigue and the first biotransformation and oxidative stress status assessment was done. The results obtained during this initial assessment revealed that both Phase I and Phase II reactions were functioning below normal, antioxidant defence was low and the oxidative stress status high. These results lead to the development and implementation of an individualised nutritional supplementation regimen and several follow-up investigations were performed over a period of 7 months to monitor both biochemical and clinical characteristics.

Shortly after the introduction of the nutritional supplementation treatment both Phase I and Phase II reactions improved and the critical balance between Phase I and Phase II biotransformation was restored, the antioxidant defence improved and the oxidative stress status was much lower. In addition to the biochemical improvement, the subject also showed a significant clinical improvement. However, one of the most significant observations made in this case was the slower improvement of glycine conjugation as compared to the other Phase II reactions. Traditional biochemical investigations (i.e. acylcarnitine and organic acid analysis) revealed in addition to this, a metabolite profile indicative of an MCAD deficiency. This profile was observed only after the introduction of nutritional supplementation with L-carnitine as part of the individualised nutritional supplementation regimen. Mutation analysis for the common A985G mutation in the *ACADM* gene was negative, but other MCAD mutations were not excluded. However, due to the inclusion of sodium VPA as part of her prescribed medication regimen, it is suspected that this patient has a medication-induced fatty acid oxidation deficiency profile, since this profile is typical of VPA therapy (Silva *et al*, 2001).

These observations were further probed with the "substrate loading cocktail". After the first loading test (L-carnitine loading), the excreted hexanoylcarnitine levels in this patient increased to a value above that of the 90^{th} percentile of the reference range (Mueller *et al*., 2003). In the second loading test (L-carnitine and glycine) acylcarnitine excretion dropped marginally. However, although a concomitant increase in acylglycine excretion was expected, the corresponding acylglycines remained undetected. Various explanations for this phenomenon can be given, but considering the slower improvement of glycine conjugation as compared to the other Phase II reactions after the introduction of the nutritional supplementation treatment, the most feasible explanation is an inhibitory effect on, or the

presence of a rate limiting polymorphism in the GLYAT enzyme. This possibility is still under investigation. Unfortunately, since this patient is deceased, we were not able to conduct any further tests to further clarify whether this patient suffered from an MCAD deficiency or whether the observed metabolite profiles were induced by medication.

7.1.2 MCAD family

The "substrate loading cocktail" was also evaluated by its application to a Caucasian MCAD family. In this family both of the parents are heterozygous for the common A985G mutation in the *ACADM* gene, while only one of the four siblings is homozygous for this mutation. The other siblings did not present with any clinical symptoms or abnormal metabolite profiles.

Since the same basic trend as observed in subject NWU-34588 were expected, the results obtained after the application of the "substrate loading cocktail" to this family, were rather astonishing. The excretion patterns of the heterozygous parents and the siblings of the MCAD patient resembled that of the control subjects involved in this study and the MCAD patient revealed a totally different excretion pattern. The excretion pattern of accumulated medium-chain acylcarnitine species in the MCAD patient revealed a lag phase before the excretion of these metabolites reached a maximum level.

Various possible explanations can be posed for this phenomenon with the most plausible being slower Phase II detoxification of accumulated fatty acids, because the lag effect in response to the different loading protocols were observed in both carnitine and glycine conjugation. The common feature and rate-determining step in these detoxification reactions is the activation of fatty acids to their corresponding acyl-CoA species within mitochondria (Vessey *et al*., 1999). It therefore appears as if the activation and therefore detoxification of accumulated fatty acids in this MCAD deficient patient is slower. Since the third substrate loading protocol contained several agents to ensure sufficient availability of coenzyme A, we suggest that the slower activation is due to a possible polymorphism in the medium-chain acyl-CoA synthetase enzyme.

7.1.3 Isobutyryl-CoA dehydrogenase inhibition

Acylcarnitine analysis as part of the biotransformation metabolism and oxidative stress status assessment revealed increased excretion of C3-, C4- and C5-carnitine species after

administration of acetylsalicylic acid and acetaminophen as probe substrates for Phase II biotransformation reactions. Further investigation showed a statistically significant increase in the excretion of C4-carnitine in the case of acetylsalicylic acid administration, a statistically significant decrease in the excretion of various dicarboxylic acid carnitine conjugates after acetaminophen administration and a statistically significant increase in the excretion of C3-, C4- and C5-carnitine in the case of the combined administration.

As the two isomers of C4-carnitine, butyrylcarnitine and isobutyrylcarnitine accumulate as a result of the inhibition or a defect in two different enzymes, it was necessary to determine whether the observed increased concentration of C4-carnitine was due to increased butyrylcarnitine or isobutyrylcarnitine. The chromatographic separation of these isomers revealed a statistically significant increase in only the isobutyrylcarnitine concentration when baseline samples were compared to samples obtained after acetylsalicylic acid and acetaminophen administration. These results support the hypothesis that acetylsalicylic acid has an inhibitory effect on the short-chain fatty acid metabolism. We propose that this effect is at the level of isobutyryl-CoA dehydrogenase involved in the catabolism of branched chain amino acids. In addition, the increase in C5-carnitine species observed after the combined administration of acetylsalicylic acid and acetaminophen, can also be due to the same inhibitory effect of acetylsalicylic acid on isovaleryl-CoA dehydrogenase and S-2-methylbutyryl-CoA dehydrogenase as no effect on these metabolic pathways were observed after acetaminophen administration alone.

An interesting observation in this respect is the fact that in some of the subjects the isobutyrylcarnitine concentrations reached levels associated with isobutyryl-CoA dehydrogenase deficiency. It is therefore possible that the inhibitory effect could be more pronounced in carriers of the isobutyryl-CoA dehydrogenase deficiency or in individuals with rate-limiting polymorphisms in this enzyme.

7.2 Concluding observations

During this study a hypothesis was proposed to describe the critical balance between Phase I and Phase II biotransformation, and how a disturbance in this balance will increase the oxidative stress status, with resulting pathological consequences. The significance in testing the unbalanced biotransformation metabolism model lies within the treatment possibilities, as the assessment of biotransformation metabolism and oxidative stress status can lead to the

development of individualised treatment protocols to replenish important substrates and cofactors needed for the safe elimination of accumulated toxic compounds. Although this model was verified in fatty acid oxidation deficiencies (both inborn and induced) it can be significant in various other pathological conditions in which biotransformation and antioxidant defence systems are compromised. The value of the proposed model was illustrated by its application to a clinical case investigated in our laboratory and lies in the improvement of the clinical features of this patient. Although this intervention was initiated too late in this case, the observations we made strongly underline the value and potential of a dietary intervention aimed at enhancing the biotransformation metabolism and oxidative stress status.

However, the potential of dietary supplementation is not only directed towards the treatment possibilities as described above, but can also be applied as a diagnostic tool. During this study it became evident that the application of different well defined loading protocols with appropriate substrates and cofactors involved in detoxification metabolism can be used to enhance the detection of aberrant metabolite profiles. This approach can contribute significantly to the description and differentiation of subjects with different fatty acid oxidation deficiencies. Furthermore, this approach allows us to observe the rate of transformation by individuals and can be of great value in the development of individualised therapy and also in the timing of such therapy.

In addition, this study did not only indicate the importance of dietary supplementation, but also the potential importance of xenobiotic substrates, such as acetylsalicylic acid in metabolic processes. The first ever *in vivo* evidence was provided of a statistically significant increase in isobutyrylcarnitine excretion by human subjects following the administration of a therapeutic dose of acetylsalicylic acid was provided. Since not all individuals are affected to the same degree, it may be possible to use this inhibitory effect of acetylsalicylic acid as a diagnostic tool to predict the mutation carrier status or the existence of rate-limiting polymorphisms in the isobutyryl-CoA dehydrogenase enzyme. In addition, the observed inhibitory effect of acetylsalicylic acid on this enzyme also poses the opportunity to investigate other biochemical effects involved in the pathology of deficient isobutyryl-CoA dehydrogenase.

7.3 Closing remarks and recommendations

Although the proposed hypothesis was supported in fatty acid oxidation deficiencies, it is important to apply this model to other pathological conditions in which biotransformation metabolism and oxidative stress status are influenced. The value of this model was illustrated in the discussed clinical case, however since the intervention was initiated too late to maintain the observed improvement, it is recommended to further investigate the applicability of this model in systems which are more stable, such as cell cultures.

The application of the developed "substrate loading cocktail" lead to the discovery of a possible new deficiency in an MCAD patient. However, it is not known whether the observed lag effect in the activation of fatty acids in this patient is a unique feature or universal to MCAD deficiencies. It is therefore recommended to apply the "substrate loading cocktail" to other confirmed MCAD cases, and if this phenomenon is indeed unique to this specific patient, the additional deficiency in the activation of fatty acids needs to be investigated. Although one of the shortcomings of this study is the limited number of subjects investigated, it is important to bear in mind that many other metabolic deficiencies was initially identified in only one case.

In addition, the application of the "substrate loading cocktail" in both the inborn and induced fatty acid oxidation deficient subjects, revealed a similar excretion magnitude of the relevant metabolites after loading with L-carnitine, and L-carnitine and glycine. However, in the case of the third loading test, where pantothenic acid was added, there was a decrease in relevant metabolite excretion. Unfortunately, since the primary metabolites were not measured in blood samples (e.g. octanoic acid) it cannot be concluded definitively, that the decreased metabolite excretion after added pantothenic acid was due to improved octanoyl-CoA metabolism.

Since the proposed inhibitory effect of acetylsalicylic acid on isobutyryl-CoA dehydrogenase is not the same in all individuals, it is recommended that those individuals with higher isobutyrylcarnitine excretion be further investigated to prove whether acetylsalicylic acid administration can be used to detect rate-limiting polymorphisms or mutations in the isobutyryl-CoA dehydrogenase enzyme. Furthermore, it seems as if more acyl-CoA dehydrogenase enzymes involved in branched chain amino acid metabolism could be subject

to acetylsalicylic acid inhibition. However, the mechanism of salicylic acid inhibition on these enzyme systems has to be investigated and confirmed. In addition, since there is substantial variation in the clinical presentation of this deficiency, this inhibitory effect further poses the opportunity to investigate other biochemical effects involved in the pathology of deficient isobutyryl-CoA dehydrogenase. It is therefore also recommended that this phenomenon be further investigated *in vitro*, since the elucidation of the development of phenotypic characteristics of metabolic diseases remains a formidable challenge.

The development of novel hypotheses is the foundation of scientific advancement and one of the most important ways to contribute to the expansion of our current scientific knowledge. During this study the various aims and objectives set in Chapter One were achieved. However, more important in this respect is the fact that novel diagnostic and treatment ideas were developed during this study, which could prove to make an enormous contribution to the understanding of how unbalanced biotransformation metabolism and the consequences thereof can impact on various other functions in the human body. The unique contribution made in this thesis is perhaps best illustrated when it is weighed against a similar approach by Krieger & Tanaka, published in Pediatric Research in 1976. This publication brought about a total new approach in detoxification treatment. Likewise, as a result of all the investigations reported on in this thesis, we feel that this cocktail approach will contribute to the transformation of treatment regimens and improve the prognosis of conditions in which detoxification metabolism plays a crucial role.

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Annexure A

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Unbalanced biotransformation metabolism and oxidative stress status: implications for deficient fatty acid oxidation

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ABSTRACT

The concept of accumulating xenobiotics within the human body as a health risk is well known. However, these compounds can also be endogenous, as in the case of inborn errors of metabolism, and lead to some of the same symptoms as seen in xenobiotic intoxication. Biotransformation of both exogenous and endogenous toxic compounds is an important function of the liver, and the critical balance between these systems is of fundamental importance for cellular health. We propose a novel model, to describe the critical balance between Phase I and Phase II biotransformation and how a disturbance in this balance will increase the oxidative stress status, with resulting pathological consequences. We further used deficient fatty acid oxidation to verify the proposed model, as deficient fatty acid oxidation is associated with the accumulation of characteristic metabolites. These accumulating metabolites undergo both Phase I and Phase II biotransformation reactions, with resulting depletion of biotransformation substrates and co-factors. Depletion of these important biomolecules is capable of disturbing the balance between Phase I and Phase II reactions, and disturbance of this balance will increase oxidative stress status. The value of the proposed model is illustrated by its application to a clinical case investigated in our laboratory. In this case the possibility of deficient fatty acid oxidation only became evident once the critical balance between Phase I and Phase II biotransformation was restored with oral replenishment of biotransformation substrates. In addition to biochemical improvement, there was also significant clinical improvement. The significance of this model lies within the treatment possibilities, as the assessment of biotransformation metabolism and oxidative stress status can lead to the development of nutritional treatment strategies to correct imbalances. This in turn may reduce the chances of, or delay the onset of certain disease states.

Keywords: Biotransformation Metabolism; Detoxification; Fatty Acid Oxidation; Oxidative Stress Status

1. INTRODUCTION

The indispensable role of the liver in the biotransformation or detoxification of a variety of exogenous and endogenous compounds is accomplished by two groups of enzymatic modifications known as Phase I and Phase II biotransformation metabolism. Phase I reactions expose functional groups to form reactive sites, which improve water solubility of the compound itself, or allow Phase II reactions to ensue when the products of Phase I biotransformation are conjugated with endogenous hydrophilic compounds to enhance their excretion [1-3]. However, during Phase I functionalization the resultant reactive molecule can in certain cases be more toxic than the parent compound, and effective neutralization of these noxious compounds is important in preventing covalent binding of the reactive metabolites to proteins, lipids and nucleic acids [2,3].

Maintaining the balance between Phase I and Phase II reactions is therefore of paramount importance, and under normal circumstances these enzymes function adequately to minimize inefficient detoxification and potential induced intracellular damage. However, an overloaded or unbalanced system negatively affects the oxidative stress status, with serious health compromising consequences [3,4].

The metabolic processes that are fundamental for maintaining normal cell structure and function are highly regulated enzyme catalyzed processes. Defects in these enzyme systems, whether induced or inherited, have

significant consequences in man, *i.e.* the accumulation of toxic substrates upstream of the enzyme defect, disturbances in metabolic intermediates downstream of the enzyme defect, and the formation of intermediates by alternative biochemical pathways [5]. On a clinical level these biochemical aberrations will give rise to various pathological conditions including acute life-threatening encephalopathy, hyperammonemia, metabolic acidosis, hypoglycemia, jaundice and liver dysfunction [6]. This can ultimately lead to the development of chronic diseases and eventual death.

Biotransformation metabolism is a well studied discipline within the pharmaceutical industry, and the concept of accumulating xenobiotics within the human body as a health risk is well known. However, the accumulation of endogenous compounds in the case of inborn errors of metabolism and its pathological consequences is typically not explicitly associated with unbalanced biotransformation metabolism.

Explaining the development of the phenotypic characteristics of metabolic diseases is a formidable challenge. To this end we propose a model to help explain the pathological outcomes of induced and inborn errors of metabolism. This model entails that unbalanced biotransformation metabolism due to depletion of Phase II substrates and co-factors can be the first linkage in a chain of events with severe pathological outcomes. It is vital for scientific advancement and clinical applications that the phenomenon of unbalanced biotransformation metabolism be considered as a primary cause of metabolic aberrations manifesting as increased oxidative stress status. The proposed unbalanced biotransformation metabolism model will be illustrated using defective β-oxidation of fatty acids, and its value will be demonstrated by its application in the development of individualized treatment protocols for patients suffering from induced and/or inborn errors of metabolism.

2. THE UNBALANCED BIOTRANSFORMATION METABOLISM MODEL

In the unbalanced biotransformation metabolism model, a hypothesis is proposed to describe the critical balance between Phase I and Phase II biotransformation and how a disturbance in this balance will increase the oxidative stress status, with resulting pathological consequences. A defect in, or inhibition of any one of the many enzymes involved in cellular metabolism results in the accumulation of specific metabolites that need to be removed from the body either via alternative pathways or by Phase I and Phase II biotransformation metabolism. Phase I biotransformation of accumulating metabolites and alternative pathways, both result in additional formation of reactive oxygen species (ROS). Induced Phase I biotransformation will furthermore increase the burden on Phase II conjugation and the increased demand on the latter could lead to the depletion of conjugation substrates and co-factors. Depletion of these biomolecules will disturb the critical balance between Phase I and Phase II biotransformation, which will further increase the oxidative stress status, ultimately leading to the depletion of the endogenous antioxidant capability, further affecting Phase II conjugation. Increased circulating ROS will cause oxidative damage to macromolecules such as lipids, proteins, and nucleic acids, and some of these adducts will contribute to the depletion of endogenous antioxidants. If these reactive adducts are not neutralized effectively they can diffuse to different sites and intensify the effects of oxidative damage by decreasing respiratory chain activity. This model therefore proposes that unbalanced biotransformation metabolism form an additional "vicious cycle" for increased oxidative stress status which originates from inefficient biotransformation.

3. REGULATION OF THE CRITICAL BALANCE BETWEEN PHASE I AND PHASE II BIOTRANSFORMATION METABOLISM

Biotransformation metabolism is under homeostatic regulation to control the detoxification of xenobiotics and their metabolites. This homeostatic system includes both negative feedback control as well as feedforward processes. In Phase I negative feedback control, xenobiotics activate a range of receptors to induce Phase I enzymes [7]. In most cases Phase I activity prepares the arena for Phase II conjugation to take place, because the Phase I intermediate metabolites activate transcription factors to induce synthesis of Phase II conjugation enzymes, also by means of negative feedback control [2,3]. However, many Phase II enzymes are also upregulated directly by the parent xenobiotic, which entails feedforward control by the reactive metabolites formed during Phase I. This reduces the response time for the biotransformation system to adapt and remove harmful Phase I intermediates more rapidly. However, there are also other factors involved in this process, such as nutrient concentration control [7]. Phase I biotransformation requires little nutritional support, whereas Phase II requires various co-factors and substrates, which must be replenished by dietary sources [2,3]. Therefore, although biotransformation metabolism is under homeostatic regulation which includes both negative feedback and feedforward control, depletion of Phase II substrates and co-factors

will undeniably disrupt the critical balance between Phase I and Phase II biotransformation.

4. CONSEQUENCES OF DISTURBED BALANCE IN BIOTRANSFORMATION METABOLISM

The main intracellular source of ROS is the mitochondrial respiratory chain. However, some enzymes including NADPH oxidases and cytochrome P450-dependent oxygenases also produce ROS during their enzymatic reactions [8]. ROS normally exist in all aerobic cells in balance with tightly controlled antioxidant defence and repair mechanisms. A steady state of oxidative stress, which is always present in cells, can therefore increase (increased oxidative stress status) if the endogenous antioxidant system is not capable of coping with the continuous ROS production, or if an uncontrolled increased ROS production occurs [9].

One of the most important endogenous antioxidant molecules is reduced glutathione (GSH), as it plays an important role in neutralizing free radicals. A shift in the ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) could therefore further increase the oxidative stress status. In addition to its antioxidant function, GSH is also involved in Phase II conjugation, which can occur spontaneously or in an enzyme reaction catalyzed by glutathione-S-transferases (GSTs) [10,11].

Compromised biotransformation can also have a great influence on the content and type of fatty acids and steroids involved in cellular signaling. Increased circulating ROS and free fatty acids cause lipid peroxidation and the formation of aldehyde by-products, including 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). Detoxification of these lipid peroxidation by-products enhances glutathione depletion even further. If these reactive molecules are not neutralized they can diffuse to different sites and intensify the effects of oxidative stress by decreasing respiratory chain activity [12,13].

5. VERIFICATION OF THE UNBALANCED BIOTRANSFORMATION METABOLISM MODEL: DEFICIENT FATTY ACID OXIDATION

At least 25 enzymes and transport proteins, various co-factors, co-enzymes, and substrates such as L-carnitine, co-enzyme A, FAD and NAD are involved in mitochondrial β-oxidation, and genetic defects in at least 22 of these proteins cause disease in humans [14-16]. In addition to inborn errors in fatty acid oxidation, various xenobiotic compounds can also lead to inhibited enzyme activities, e.g. Aspirin (acetylsalicylic acid), a widely used analgesic, and Valproate (VPA), a branched-chain fatty acid, which is clinically used in the treatment of various seizure disorders. Acetylsalicylic acid is rapidly hydrolyzed to salicylic acid upon ingestion, and is then activated to salicyl-CoA before conjugation to glycine can take place. VPA, on the other hand, undergoes the same metabolic reactions as natural fatty acids, including mitochondrial β-oxidation, peroxisomal β-oxidation, and cytochrome P450 dependent ω- and ω-1 hydroxylation [17].

Deficient mitochondrial fatty acid oxidation results in the accumulation of free fatty acids and acyl-CoA species [14,17]. These metabolites need to be removed from the body either via alternative pathways, or biotransformation metabolism (Phase I and Phase II) (**Figure 1**). The alternative pathway to mitochondrial β-oxidation occurs in the peroxisomes. The first step in this pathway is catalyzed by acyl-CoA oxidase, which involves the reduction of oxygen to hydrogen peroxide [18-20]. Phase I biotransformation of accumulated fatty acids involve cytochrome P450 dependent ω-oxidation of fatty acids [21,22]. During fatty acid ω-oxidation the corresponding dicarboxylic acids of the metabolized fatty acids are formed [22]. In addition, ROS is also formed during this reaction via flavoprotein mediated donation of electrons to molecular oxygen [23] (**Figure 1**). Both the alternative pathway and Phase I biotransformation metabolism can therefore result in enhanced production of ROS.

Phase II biotransformation of accumulated acyl-CoA and Phase I generated dicarboxylic acids involve conjugation with either glycine or L-carnitine [14-16]. Subjects with deficient fatty acid oxidation will therefore present biochemically with elevated levels of carnitine and glycine conjugates of acyl-CoA and dicarboxylic acid species.

The increased demand on Phase II biotransformation to maintain the critical balance can result in the depletion of these Phase II conjugation substrates (**Figure 1**). If these substrates are not replenished, the critical balance between Phase I and Phase II biotransformation will become disturbed. When this balance is disturbed due to sustained induced Phase I biotransformation and reduced Phase II conjugation, it could increase the oxidative stress status [3] (**Figure 1**), with a consequent shift in the GSH:GSSG ratio, that could exacerbate the oxidative stress status and affect Phase II conjugation [10,11].

An increased amount of circulating ROS molecules, in addition to accumulated free fatty acids, especially poly-unsaturated fatty acids (PUFAs), can further worsen this condition, as ROS could attack these fatty acids and initiate lipid peroxidation. Lipid peroxidation results in the formation of aldehyde by-products, in-

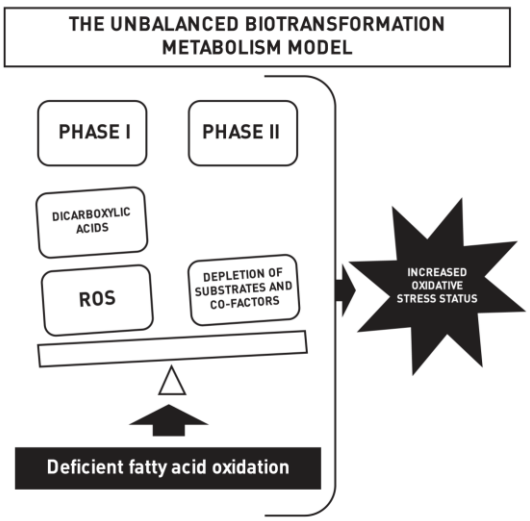

Figure 1. Disturbance in the critical balance between Phase I and Phase II biotransformation metabolism by deficient fatty acid oxidation can ultimately lead to an increased oxidative stress status, which is the underlying mechanism for the development of various pathologies.

cluding 4-hydroxynonenal (4-HNE) and malondialde hyde (MDA) [12,13]. Increased presence and distribution of these peroxidized lipid metabolites could furthermore lead to mitochondrial instability, as phospholipids are an indispensable constituent in mitochondrial membranes for the functional assembly of the respiratory chain. The incorporation of these lipid derivatives into mitochondria could therefore lead to decreased respiratory chain activity, with resulting increased oxidative stress status [13].

Moreover, it has recently been demonstrated that two of the accumulating free fatty acids in MCAD deficiency (octanoate and decanoate) lead to increased oxidative stress status [24], and the uncoupling of oxidative phosphorylation [25] in rat brain tissue. Unbalanced biotransformation metabolism and the consequent increase in oxidative stress status are therefore a possible cause in the development of certain neurological consequences in these kinds of deficiencies.

In addition to an increased oxidative stress status, the disturbed biotransformation balance can also generate the pathological condition known as co-enzyme A (CoA) sequestration, toxicity and redistribution (CASTOR) [26]. This phenomenon has been demonstrated in both inborn fatty acid oxidation deficiencies and xenobiotic induced fatty acid oxidation deficiencies [17,26]. The accumulation of acyl-CoA intermediates will lead to decreased availability of free CoA and acetyl-CoA molecules, and changes in these levels can disrupt various metabolic pathways. These metabolic pathways include the Krebs cycle, ureagenesis, biotransformation pathways as well as the mitochondrial redox state. It could also lead to further deficiencies in downstream products within these metabolic pathways [26]. Taken together, defective fatty acid oxidation and its concomitant biochemical characteristics clearly verify the proposed unbalanced biotransformation metabolism model.

6. *IN VIVO* APPLICATION OF THE UNBALANCED BIOTRANSFORMATION METABOLISM MODEL

The value of the proposed model is illustrated by its application to a clinical case investigated in our laboratory. A non-smoking female Caucasian, 57 years of age presented with chronic fatigue, coughing, dyspnoea, pain and anorexia and was diagnosed with metastatic small cell carcinoma of the lung. The cancer also metastasized to the liver although liver function tests were within the reference range. After the diagnosis she was started on a chemo combination therapy, called CAV, which consists of Cyclophosphamide, Doxorubicin and Vincristine for six repeated cycles over a period of twenty weeks. For the whole assessment time she continued with a prescribed medication regimen consisting of: Epilim (sodium valproate), Lamicton (Lamotrigine), Leponex (Clozapine) and Simvastin (Simvastatin, ascorbic acid and butylated hydroxyanisole).

Four weeks before the end of chemotherapy, the subject suffered from severe fatigue and the first biotransformation and oxidative stress status assessments were done. This assessment was performed by challenging Phase I and Phase II biotransformation reactions with appropriate probe substrates. Caffeine was used as a probe substrate for CYP1A2 activity (Phase I), and paracetamol and aspirin as probe substrates for glucuronide conjugation, sulfate conjugation, glutathione conjugation and glycine conjugation (Phase II) [3]. In addition to this, the total acylcarnitine profile and oxidative stress status parameters including the ferric reducing antioxidant power (FRAP assay), the ROS assay, measurement of hydroxyl radical markers like catechol and 2,3-dihydroxybenzoic acid (2,3-DHBA) as well as the determination of total glutathione were also included in this assessment.

From the results obtained during the initial assessment, it was evident that the biotransformation metabolism and antioxidant defense systems of this subject were functioning below normal. The activity of Phase I (CYP1A2) measured as the caffeine clearance value, as well as all the measured end products for the different Phase II conjugation reactions were also in the lower part of the reference range, with glycine conjugation being very low. The measured concentration of free carnitine was just within the reference range. The total glutathione (GSH

and GSSG) concentration was low, with ROS levels and 2,3-DHBA levels being exceptionally high.

The results of this initial assessment were used to develop an individualized nutritional supplementation protocol in which various compounds that can be divided into different classes including antioxidants, mitochondrial support supplementation and biotransformation substrates and co-factors were employed. After the introduction of this individualized nutritional treatment strategy, several follow-up investigations were performed over a period of 7 months to monitor both biochemical and clinical characteristics.

Shortly after the introduction of the nutritional supplementation treatment, the Phase I activity was markedly elevated, which could lead to the formation of more free radicals. However, after a few weeks the Phase I activity stabilized at levels well within the reference range. All the Phase II reactions also improved, with considerable improvement in glucuronide, sulfate and glutathione conjugation. Although the glycine conjugation also improved, values remained just below or just within the reference range. In addition to this the total available glutathione and the serum FRAP also increased with concomitant decreased ROS and 2,3-DHBA concentrations. The amount of free carnitine increased substantially after only eight weeks of starting the supplementation regimen. However, the ratio between acylcarnitines and free carnitine was slightly elevated. After careful investigation of the total acylcarnitine profile, the source of the elevated ratio between acylcarnitines and free carnitine in these assessments was due to increased levels of medium-chain acylcarnitines and medium- chain dicarboxylcarnitines, including hexanoylcarnitine, octanoylcarnitine, adipylcarnitine and suberylcarnitine.

It is evident in this case that the biotransformation and antioxidant defense systems were initially markedly compromised. The identification of the accumulated metabolites usually seen in fatty acid oxidation deficiencies is the most significant observation in this regard. Initial concentrations of Phase II substrates were so depleted that these metabolites were only observed after oral replenishment of the main conjugation substrate. Once the critical balance between Phase I and Phase II biotransformation was restored, the oxidative stress status decreased to levels within the reference range. In addition to the biochemical improvement, the subject also showed a significant clinical improvement, and although these results are only preliminary, it supports the value of the proposed unbalanced biotransformation metabolism model.

7. CONCLUSION

The significance in testing this model lies within the treatment possibilities, not only for inborn errors of fatty acid metabolism, but also for induced fatty acid oxidation deficiencies. It can furthermore also be significant in various metabolic aberrations manifesting as increased oxidative stress status. If the disturbance in this critical balance is indeed the first link in a chain of reactions to follow, which ultimately lead to pathological conditions like cancer, the assessment of these reactions is of immense importance. This kind of assessment can lead to the development of individualized treatment protocols to replenish important substrates and co-factors needed for the safe elimination of accumulated toxic compounds.

8. ACKNOWLEDGEMENTS

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Annexure B

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02.12.2009

TO WHOM IT MAY CONCERN

RE :

Me was known to this practice since March 2006 with the diagnosis of smallcell lung cancer. She was initially seen at Wilgers Hospital and was subsequently referred to me in February 2007.

At that stage we did a CT of the thorax and she had a pneumonitis which was reported to be less severe than before, with no visible masses. Her upper abdominal organs were all within normal limits.

In October 2007 I sent her again for a CT of the thorax, abdomen and pelvis. The scan revealed larger mediastinal glands with liver metastases. I then started treating her with a chemo combination called CAV which consists of Cyclophosphamide, Doxorubicin and Vincristine. She was treated with six cycles of this chemo combination up untill the 26.02.2008.

We then sent her again for a CT of the thorax and abdomen and it revealed a much improved CT scan in comparison to the previous one.

In July 2008 she was sent again for a CT scan and although her lungs were unchanged, she still had this low density lesions in the liver and it was decided that the scan be repeated after two to three months. In September 2008 I saw her again with the complaints of servere tiredness. She had this feeling of something pressing on her lungs. We did a chest x-ray which did not reveal any new masses. The liver ultra-sound also revealed no liver metastases.

In November 2008 another CT scan of the thorax and abdomen was done. It was reported to be unchanged. I did a fine needle aspiration of a lymph node to the left side of her neck and it was reported to be positive for malignant cells.

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In Janaury 2009 we sent her again for a chest x-ray which did not reveal any new lesions or metastatic disease. The abdominal sonar did reveal new liver metastases as well as pancreatic metastases. At that stage it was decided not to treat her with any active chemotherapy any more.

It was also the last consultation with me and in April and May 2009 she was treated with Duragesic transdermal stickers which delivers Fentinel transdermally.

Unfortunately Me passed away.

I am also including the CT scans and blood results that are relevant. Any further enquiries can be addressed towards me.

Kind regards.

DR J DUMINY
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Annexure C

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Research Article

Increased Excretion of C4-Carnitine Species after a Therapeutic Acetylsalicylic Acid Dose: Evidence for an Inhibitory Effect on Short-Chain Fatty Acid Metabolism

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Acetylsalicylic acid and/or its metabolites are implicated to have various effects on metabolism and, especially, on mitochondrial function. These effects include both inhibitory and stimulatory effects. We investigated the effect of both combined and separate oral acetylsalicylic acid and acetaminophen administration at therapeutic doses on the urinary metabolite profile of human subjects. In this paper, we provided *in vivo* evidence, in human subjects, of a statistically significant increase in isobutyrylcarnitine after the administration of a therapeutic dose of acetylsalicylic acid. We, therefore, propose an inhibitory effect of acetylsalicylic acid on the short-chain fatty acid metabolism, possibly at the level of isobutyryl-CoA dehydrogenase.

1. Introduction

The dehydrogenation of acyl-CoA intermediates in the catabolism of fatty acids and branched-chain amino acids in humans is catalysed by the mitochondrial acyl-CoA dehydrogenase enzymes [1, 2]. Several inherited defects in this group of enzymes have been characterised. Defects in, or inhibition of, isovaleryl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase result in the accumulation of C5-carnitine species [3] whilst defects in, or inhibition of, short-chain acyl-CoA dehydrogenase and isobutyryl-CoA dehydrogenase result in the accumulation of C4-carnitine species [2] (Figure 1).

In the human body, acetylsalicylic acid is hydrolysed to salicylic acid, which is excreted in its conjugated form with glycine and glucuronic acid, in its hydroxylated form as 2,5-dihydroxybenzoic acid, or unchanged as salicylic acid [4]. Numerous *in vitro* and *in vivo* studies have been conducted that demonstrated various effects of acetylsalicylic acid (and its metabolites) on the metabolism and, especially, on mitochondrial function, such as the uncoupling of oxidative phosphorylation [5, 6], inhibition of fatty acid oxidation [5–8] with the concomitant stimulation of cytochrome P450 dependent ω-oxidation [9], and, therefore, accumulation of dicarboxylic acids [10]. In addition, the inhibition of both the Krebs cycle enzymes α-ketoglutarate dehydrogenase, and succinate dehydrogenase [6, 11], as well as an increased flux through the Krebs cycle [12], have been reported. Other effects of salicylic acid include the stimulation of oxygen consumption, stimulation of ATP hydrolysis [5], the activation of pyruvate dehydrogenase, and the inhibition of gluconeogenesis [12]. It can also lead to decreased blood glucose concentrations, increased hepatic triglycerides [6], and a slight increase in (iso)butyryl-CoA, β-methylcrotonyl-CoA, isovaleryl-CoA, and octanoyl-CoA concentrations [13].

The exact mechanism of inhibition that acetylsalicylic acid and/or its metabolites exert on fatty acid oxidation is not clear, since different studies indicate different sites of inhibition. These include an inhibitory effect on the activation

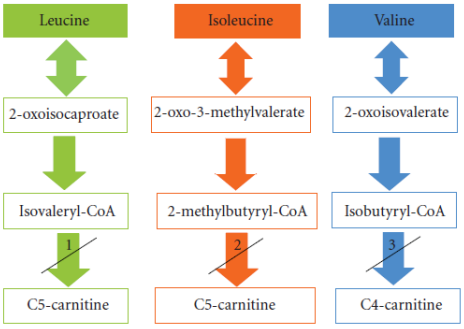


Figure 1: Proposed inhibition by salicylic acid of the branched-chain amino acid catabolism. Inhibition (or deficiency) of (1) isovaleryl-CoA dehydrogenase, (2) 2-methylbutyryl-CoA dehydrogenase, and (3) isobutyryl-CoA dehydrogenase, will result in the accumulation of C4-carnitine and C5-carnitine species.

and transportation of medium- and long-chain fatty acids into the mitochondria [7], due to the sequestration of extramitochondrial coenzyme A and carnitine [6], inhibition of the medium-chain acyl-CoA synthetase [8], or inhibition of the carnitine acyltransferase (CAT) enzymes [14]. Conversely, it was demonstrated that the target of inhibition was found to be at the level of the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) activity of the mitochondrial trifunctional β-oxidation enzyme (MTE) and not at the level of uptake or activation of fatty acids [15].

While performing biotransformation metabolism and oxidative stress status profiling studies, on individuals referred to our laboratory, by using acetylsalicylic acid and acetaminophen as probe substrates, we made the rather startling observation of the presence of elevated C3-, C4-, and C5-carnitine species in their urine. These observations led to a more in-depth investigation into the nature of these acylcarnitine species and whether their increased excretion was due to the separate or combined effect of the acetylsalicylic acid and acetaminophen administration. Since the accumulation of C4- and C5-carnitine species is predominantly associated with deficient branched-chain amino acid metabolism at the level of different acyl-CoA dehydrogenase enzymes [2, 3], we hypothesized that acetylsalicylic acid administration is associated with the elevation of these species, which may be linked to the inhibition of the metabolism of C4 and C5 fatty acids (Figure 1). The formulation of this hypothesis was supported by the previous observation that a slight increase in (iso)butyryl-CoA, β-methylcrotonyl-CoA, isovaleryl-CoA, and octanoyl-CoA concentrations occur after acetylsalicylic acid intake [13].

This investigation provides the first evidence of a significant increase in isobutyrylcarnitine excretion following acetylsalicylic acid intake at therapeutic doses in humans, and we propose that this accumulation may be due to the inhibitory effect of acetylsalicylic on the metabolism of C4 and C5 fatty acids.

2. Materials and Methods

2.1. Subjects. The test subjects were divided into two groups. The first group included 30 test subjects, 19 female and 11 male between the ages of 12 and 65 years. Participants in this group were originally referred for biotransformation metabolism and oxidative stress status assessment, which include the administration of both acetaminophen and acetylsalicylic acid. The second group included seven randomly chosen participants from the first group and were used to ascertain whether the observed effects were due to acetylsalicylic acid intake, acetaminophen intake, or a combination thereof. The study adhered to the guidelines set in the Declaration of Helsinki. Approval for this work was obtained from the Ethics Committee of the North-West University, and informed consent was obtained from all participating subjects.

2.2. Loading Protocol and Sample Collection. Fasting baseline urine samples of all the test subjects in the first test group were collected the morning of the test day. At 21:00, on the same day, all the test subjects ceased eating and drinking (except water), emptied their bladders, and took therapeutic doses (1000 mg) acetaminophen and (600 mg) acetylsalicylic acid, as recommended by the different pharmaceutical companies for the relieve of mild to moderate pain. The total volume of urine excreted for ten hours after administration of acetaminophen and acetylsalicylic acid was collected and the quantity of urine excreted was documented.

The second test group followed a protocol to determine if the observed effect on the acylcarnitine profile was due to acetylsalicylic acid intake, acetaminophen intake, or a combination thereof. On the first day at 21:00, all subjects ceased eating and drinking (except water) for the day and collected a baseline urine sample, after which they emptied their bladders. All subjects took 600 mg of acetylsalicylic acid and collected all the overnight urine until 7:00 the next morning. On the second occasion, the same protocol was followed except that all the subjects took 1000 mg of acetaminophen and collected all the overnight urine until 7:00 the next morning and on the third occasion all the subjects took both 600 mg acetylsalicylic acid and 1000 mg acetaminophen and collected all the overnight urine until 7:00 the next morning. Subjects waited for a minimum of three days before going on to the next protocol.

2.3. Reagents. The following reagents were purchased from Merck Chemical Co. (Darmstadt, Germany) acetonitrile, formic acid, and methanol. 3N butanolic HCl, valine, leucine, isoleucine, phenylalanine, methionine, citrulline, glycine, and lysine were purchased from Sigma-Aldrich Co. (St. Louis, USA). The following carnitine and acylcarnitine standards and deuterated carnitine and acylcarnitine standards were obtained from Dr. H. J. ten Brink,

Free University Hospital (Amsterdam, The Netherlands): L-carnitine·HCl, acetyl-L-carnitine·HCl, propionyl-L-carnitine·HCl, isovaleryl-L-carnitine·HCl, octanoyl-L-carnitine·HCl, hexadecanoyl-L-carnitine·HCl, [methyl-d_3]-L-carnitine·HCl, [d_3]-acetyl-L-carnitine·HCl, [3,3,3-d_3]-propionyl-L-carnitine·HCl, [d_9]-isovaleryl-L-carnitine·HCl, [8,8,8-d_3]-octanoyl-L-carnitine·HCl, and [16,16,16-d_3]-hexadecanoyl-L-carnitine·HCl. The following deuterated amino acids were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA): [d_{10}]-L-isoleucine, [d_8]-L-valine, [d_2]-glycine, [d_3]-methyl-L-methionine, [d_5]-ring-L-phenylalanine, [d_5]-L-glutamine, [d_5]-indole-L-tryptophan, [d_4]-L-lysine:2HCl, and [d_4]-L-citulline.

2.4. Electrospray Ionisation Tandem Mass Spectrometric (ESI-MS/MS) Analysis of Acylcarnitines. The electrospray ionisation tandem mass spectrometry (ESI-MS/MS) method for determination of serum acylcarnitines as described by Vreken et al. [16] was adapted to determine acylcarnitines in urine. To a microcentrifuge tube, 10 μL centrifuged urine was added to 400 μL of the deuterated acylcarnitines (internal standard solution) with the following concentrations: 30.45 μmol/L for [methyl-d_3]-L-carnitine·HCl, 20.83 μmol/L for [d_3]acetyl-L-carnitine·HCl, 19.69 μmol/L for [3,3,3-d_3]propionyl-L-carnitine·HCl, 17.73 μmol/L for [d_9]isovaleryl-L-carnitine·HCl, 15.43 μmol/L for [8,8,8-d_3]octanoyl-L-carnitine·HCl, and 11.47 μmol/L for [16,16,16-d_3]hexadecanoyl-L-carnitine·HCl. The samples were then evaporated to dryness under a gentle stream of nitrogen at 55°C. To the dried residue, 200 μL 3N butanolic HCl was added and the samples were incubated at 55°C for 20 min. The butylated samples were evaporated to dryness again under a stream of nitrogen at 55°C. The dried residue was reconstituted in water : acetonitrile (50 : 50) (v/v) containing 0.1% formic acid.

An Agilent 1200 series liquid chromatograph (Santa Clara, CA, USA) with a 96-well plate sampler was used for sample handling as well as mobile phase delivery. Samples (10 μL of each) were injected, and a constant flow rate of 0.2 mL/min was maintained throughout the run. The mobile phase consisted of 0.1% formic acid in water : acetonitrile (50 : 50) (v/v). The tandem mass spectrometry (MS/MS) analysis was performed on an Agilent 6410 Triple Quadrupole (Santa Clara, CA, USA) in positive ionisation. Acylcarnitines were analysed with a precursor ion scan, after controlled collision induced dissociation, with a fragmentor voltage of 135 V and collision energy of 20 V. All carnitine, acylcarnitine, and other butylated species that yielded a charged mass of 85 Da after fragmentation were detected. Acylcarnitines were quantified by comparison of the signal intensity of carnitine and acylcarnitines against the signal intensity of the corresponding deuterated analogues. The concentrations of analysed carnitine and acylcarnitines were expressed as mmol/mol creatinine.

2.5. Liquid Chromatography Tandem Mass Spectrometric (LC-MS/MS) Analysis of Acylcarnitine Isomers. The LC-MS/MS method for the separation and identification of short-chain acylcarnitine isomers as described by Ferrer et al. [17] was used, with minor modifications to separate butyrylcarnitine and isobutyrylcarnitine. A 100-μL volume of urine was prepared the same as for the ESI-MS/MS method. High-performance liquid chromatography was performed on an Agilent 1200 series liquid chromatograph equipped with a Luna C18(2) column (150 mm × 2.00 mm, particle size 5 μm) from Phenomenex (Torrance, CA, USA). Mobile phase A consisted of 10 mM ammonium acetate in water and mobile phase B of 10 mM ammonium acetate in methanol. Column temperature was maintained at 20°C and the flow rate at 0.2 mL/min. The samples (10 μL) were injected and the mobile phase composition was changed from 40% of B to 60% of B over 15 min, after which the percentage of B was further increased to 100% over the next 5 min and kept for 5 min. The percentage of B was changed back to 40% over 3 min, and the column re-equilibrated for 7 min.

The MS/MS analysis was performed on an Agilent 6410 Triple Quadrupole (Santa Clara, CA, USA) in positive ionisation after controlled collision induced dissociation, with optimised fragmentor voltages and collision energies for butyrylcarnitine, isobutyrylcarnitine, and the deuterated analogues used for quantification. Mass spectrometry conditions were optimised with the MassHunter optimiser software from Agilent. Acylcarnitines were analysed in multiple reaction monitoring (MRM) mode, with the following transitions being monitored, m/z 288 → 85 for both butyrylcarnitine and isobutyrylcarnitine, m/z 277 → 85 for [3,3,3-d_3]propionyl-L-carnitine·HCl, m/z 311 → 85 for [d_9]isovaleryl-L-carnitine·HCl and m/z 347 → to 85 for [8,8,8-d_3]octanoyl-L-carnitine·HCl. The concentrations of C4-carnitine isomers were determined by comparing the signal intensity of acylcarnitines against the signal intensity of the corresponding deuterated analogues. For both butyrylcarnitine and isobutyrylcarnitine, a linear relationship between concentration and intensity existed, with $R^2 > 0.99$. The concentrations of analysed acylcarnitine isomers were expressed as mmol/mol creatinine.

2.6. Electrospray Ionisation Tandem Mass Spectrometric (ESI-MS/MS) Analysis of Amino Acids. Samples were prepared in the same way as for the analysis of acylcarnitines. Added internal standard solution contained deuterated amino acids with the following concentrations: 17.43 μmol/L for [d_{10}]-L-isoleucine, 32.20 μmol/L for [d_8]-L-valine, 15.99 μmol/L for [d_2]-glycine, 3.98 μmol/L for [d_3]-methyl-L-methionine, 5.77 μmol/L for [d_5]-ring-L-phenylalanine, 3.28 μmol/L for [d_5]-L-glutamine, 14.89 μmol/L for [d_5]-indole-L-tryptophan, 14.16 μmol/L for [d_4]-L-lysine:2HCl, and 4.21 μmol/L for [d_4]-L-citrulline.

Amino acids were analysed in MRM mode for the following transitions: glycine m/z 132 → 30, [d_2]-glycine m/z 134 → 32, alanine m/z 146 → 44, serine m/z 162 → 60, proline and arginine m/z 172 → 70, valine m/z 174 → 72, [d_8]-L-valine, threonine m/z 176 → 74, leucine and isoleucine m/z 188 → 86, [d_{10}]-L-isoleucine m/z 191 → 89, methionine m/z 206 → 104, [d_3]-methyl-L-methionine m/z 209 → 107, histidine m/z 212 to 110, citrulline

m/z 215 → 113, phenylalanine *m/z* 222 → 120, [d_5]-ring-L-phenylalanine *m/z* 227 → 125, tyrosine *m/z* 238 → 136, aspartic acid *m/z* 246 → 144, glutamic acid *m/z* 260 → 158, glutamic acid-d_3 *m/z* 263 → 161, tryptophan *m/z* 261 → 159, [d_5]-indole-L-tryptophan *m/z* 266 → 164, lysine *m/z* 203 → 84, and [d_4]-L-lysine: 2 HCl *m/z* 207 → 88. The concentrations of the amino acids were determined by comparing the signal intensity of the amino acids against the signal intensity of the corresponding deuterated analogues. The concentrations of analysed amino acids were expressed as mmol/mol creatinine.

2.7. Liquid Chromatography Tandem Mass Spectrometric (LC-MS/MS) Analysis of Branched-chain Amino Acids. Samples were prepared in the same manner as for the determination of acylcarnitine isomers. High-performance liquid chromatography was performed on an Agilent 1200 series liquid chromatograph equipped with a Luna C18(2) column (150 mm × 2.00 mm, particle size 5 μm) from Phenomenex (Torrance, CA, USA). Column temperature was maintained at 20°C, and the flow rate at 0.2 mL/min. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B of 0.1% formic acid in methanol. The samples (10 μL) were injected, and the mobile phase composition was changed from 40% of B to 60% of B over 15 min, after which the percentage of B was further increased to 100% over the next 5 min and kept for 5 min. The percentage of B was changed back to 40% over 3 min, and the column re-equilibrated for 4 min. The MS/MS analysis was performed on an Agilent 6410 Triple Quadrupole (Santa Clara, CA, USA) in positive ionisation after controlled collision induced dissociation with optimised fragmentor voltages and collision energies for leucine, isoleucine, valine, and the deuterated analogues used for quantification. Mass spectrometry conditions were optimised with the MassHunter optimiser software from Agilent. Branched-chain amino acids were analysed in MRM mode, for the same transitions as described in the ESI-MS/MS analysis of amino acids. The concentrations of the branched-chain amino acids were determined by comparing the signal intensity of the branched-chain amino acids against the signal intensity of the corresponding deuterated analogues. The concentrations of analysed branched-chain amino acids were expressed as mmol/mol creatinine.

2.8. Statistical Analysis. A paired *t*-test was used to demonstrate statistically significant differences between the test samples. In all cases, statistical significance was set at $P < .05$.

3. Results

3.1. Acylcarnitine Analysis (ESI-MS/MS). A comparison between the acylcarnitine profiles in baseline urine samples of the first test group ($n = 30$) obtained before and after administration of a combination of acetylsalicylic acid and acetaminophen, revealed a statistically significant increase in the excretion of C3- ($P = .05$), C4- ($P = .00$), and C5-carnitine moieties ($P = .00$). Comparison of the acylcarnitine profiles in baseline urine samples of the second test group ($n = 7$) to the profiles obtained after the administration of a therapeutic dose of acetylsalicylic acid alone, revealed a statistically significant increase in the excretion of C4-carnitine ($P = .03$) (Table 1). However, a comparison between the acylcarnitine profiles obtained before and after the administration of a therapeutic dose of acetaminophen ($n = 7$), revealed a decreased excretion of various dicarboxylic acid carnitine conjugates, that is, C4-DC ($P = .05$), C5-DC/C10-OH-carnitine ($P = .01$), C6-DC ($P = .01$), and C8-DC ($P = .00$) (Table 1).

3.2. Acylcarnitine Isomer Analysis (LC-MS/MS). Comparing the LC-MS/MS baseline data with that obtained after the administration of a combination of acetylsalicylic acid and acetaminophen, revealed that the increase in the excretion of C4-carnitine was due to the increased excretion of isobutyrylcarnitine ($P = .04$) rather than due to the increase in butyrylcarnitine ($P = .46$) (Figure 2). The results obtained for the different C4-carnitine isomers revealed no statistically significant difference when either butyrylcarnitine or isobutyrylcarnitine concentrations in baseline urine samples were compared to samples ($n = 7$) taken after acetylsalicylic acid administration ($P = .61$ and $P = .20$) and samples ($n = 7$) taken after acetaminophen administration ($P = .62$ and $P = .32$).

3.3. Amino Acid Analysis (ESI-MS/MS & LC-MS/MS). Concerning the amino acid analysis, a statistically significant decrease in the excretion of isoleucine ($P = .00$), leucine ($P = .04$), valine ($P = .01$), and tryptophan ($P = .01$) (Table 2) was evident in the urine samples obtained after acetylsalicylic acid and acetaminophen administration ($n = 30$). A comparison between the amino acid profiles in baseline urine samples and samples taken after acetylsalicylic acid ($n = 7$) and acetaminophen ($n = 7$) administration revealed a statistically significant decrease in the excretion of alanine, glycine, the branched-chain amino acids, methionine, phenylalanine, tyrosine, lysine, histidine, aspartic acid, and glutamic acid. In addition, significantly less proline/arginine, tryptophan, and serine were present in the urine after acetaminophen administration, but not after acetylsalicylic acid administration (Table 2).

4. Discussion

Acetylsalicylic acid and acetaminophen are generally used as probe substrates in the evaluation of biotransformation metabolism and oxidative stress status in humans [18]. Careful investigation of the acylcarnitine profiles of such subjects revealed the presence of increased concentrations of C3-, C4- and, C5-carnitine species in their urine. Since these acylcarnitine species reached concentrations normally associated with inborn errors of metabolism in some subjects [19], it was investigated whether this phenomenon originated from the administration of acetylsalicylic acid or acetaminophen, or a combination thereof. An increased excretion of various acylcarnitines derived from inhibited

Table 1: Paired t-test values (P-values) of acylcarnitine species in baseline urine samples compared to acylcarnitine species in urine samples obtained after acetylsalicylic acid administration ($n = 7$), acetaminophen administration ($n = 7$), and combined administration of both acetylsalicylic acid and acetaminophen ($n = 30$).

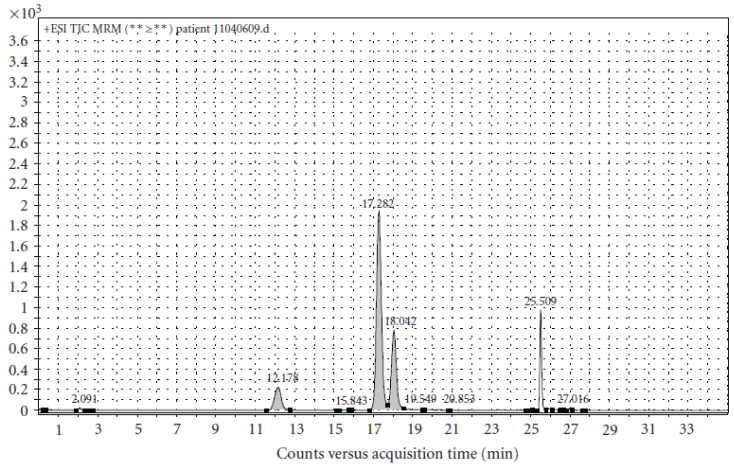
Acylcarnitine species	Paired t-test (P-value)		
	Acetylsalicylic acid administration ($n = 7$)	Acetaminophen administration ($n = 7$)	Combined administration ($n = 30$)
C0-carnitine	0.18	0.20	0.72
C2-carnitine	0.08	0.06	0.87
C3-carnitine	0.96	0.88	0.05*
C4-carnitine	0.03*	0.31	0.00*
C4-OH-carnitine	0.18	0.12	0.32
C4-DC-carnitine	0.16	0.05*	0.08
C5-carnitine	0.73	0.85	0.00*
C5-OH-carnitine	0.69	0.33	0.35
C5-DC/C10-OH-carnitine	0.17	0.01*	0.26
C5 : 1-carnitine	0.24	0.50	0.29
C6-carnitine	0.20	0.62	0.98
C6-DC-carnitine	0.29	0.01*	0.77
C8-carnitine	0.41	0.10	0.20
C8-DC-carnitine	0.84	0.00*	0.55
C10-carnitine	0.20	0.11	0.20
C5 : 1-DC/C10 : 1-OH-carnitine	0.82	0.10	0.90
C12-carnitine	0.20	0.11	0.15
C14-carnitine	0.41	0.20	0.70
C16-carnitine	0.21	0.07	0.95

* Differences are considered to be statistically significant compared to baseline values if $P < .05$.

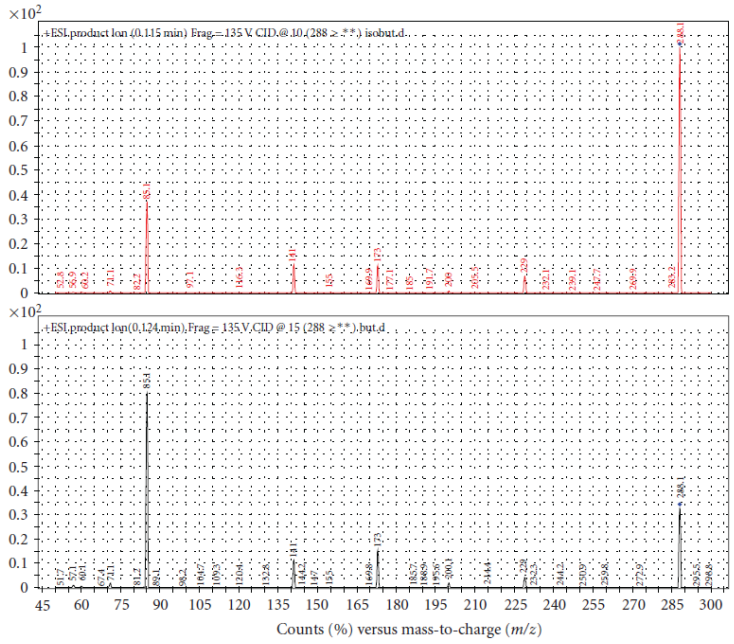
Table 2: Paired t-test values (P-values) of amino acids in baseline urine samples compared to amino acids in urine samples obtained after acetylsalicylic acid administration ($n = 7$), acetaminophen administration ($n = 7$), and combined administration of both acetylsalicylic acid and acetaminophen ($n = 30$).

Amino Acids	Paired t-test (P-value)		
	Acetylsalicylic acid administration ($n = 7$)	Acetaminophen administration ($n = 7$)	Combined administration ($n = 30$)
Alanine	0.00*	0.00*	0.50
Glycine	0.03*	0.02*	0.82
Valine	0.02*	0.01*	0.01*
Leucine	0.04*	0.03*	0.04*
Isoleucine	0.02*	0.01*	0.00*
Methionine	0.01*	0.01*	0.73
Proline/Arginine	0.06	0.02*	0.21
Phenylalanine	0.02*	0.01*	0.43
Tryptophan	0.12	0.03*	0.01*
Serine	0.06	0.03*	0.32
Threonine	0.19	0.07	0.31
Tyrosine	0.01*	0.00*	0.13
Lysine	0.03*	0.03*	0.45
Histidine	0.01*	0.00*	0.20
Aspartic acid	0.05*	0.05*	1.00
Glutamic acid	0.02*	0.02*	0.39

* Differences are considered to be statistically significant compared to baseline values if $P < .05$.



(a)



(b)

Figure 2: Separation and identification of short-chain acylcarnitine isomers. (a) In the LC-MS/MS analysis of acylcarnitine isomers, isobutyrylcarnitine is detected at 17.282 min and butyrylcarnitine at 18.042. (b) Product ion scan of *m/z* 288 for both isobutyrylcarnitine and butyrylcarnitine.

fatty acid oxidation, such as octanoylcarnitine and palmitoylcarnitine [7] as well as certain dicarboxylic acid carnitines with chain lengths ranging from C6 to C12 [10] were expected after acetylsalicylic acid administration. In the case of acetaminophen administration, an increased excretion of palmitoylcarnitine was expected [20]. However, our analyses revealed a statistically significant increase in the excretion of C4-carnitine in the case of acetylsalicylic acid administration, a statistically significant decrease in the excretion of various dicarboxylic acid carnitine conjugates after acetaminophen administration, and a statistically significant increase in the excretion of C3-, C4-, and C5-carnitine in the case of the combined administration. The most important observation in this respect is that the increased excretion in C4-carnitine was due to the intake of acetylsalicylic acid and not acetaminophen or a combined effect. Although there was no statistically significant difference in the C3- and C5-carnitine concentrations after acetylsalicylic acid administration ($n = 7$), as in the case of the combined administration, one cannot exclude that acetylsalicylic acid could be the cause of this increase, taking cognisance of the relatively small sample size.

It is known that butyrylcarnitine (C4-carnitine) accumulates with deficient or inhibited short-chain acyl-CoA dehydrogenase and that isobutyrylcarnitine (C4-carnitine) accumulates when isobutyryl-CoA dehydrogenase is deficient or inhibited [2]. In the case of C5-carnitine, the isomers include isovalerylcarnitine and 2-methylbutyrylcarnitine, which will accumulate with deficient or inhibited isovaleryl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase [3] (Figure 1). It was demonstrated by Glasgow et al. [15] that the metabolites of acetylsalicylic acid can exercise mixed inhibition on β-oxidation at the level of the LCHAD activity of the MTE, as a result of structural similarities between acetylsalicylic acid and the acyl-part of fatty acids. It was, therefore, necessary to determine whether the observed increased excretion of C4-carnitine was due to increased butyrylcarnitine or isobutyrylcarnitine. The chromatographic separation of these isomers from each other in both the baseline samples and the samples obtained after acetylsalicylic acid and acetaminophen administration was of utmost importance and during the chromatographic separation, when performing a precursor ion scan for a product with an m/z of 85, two precursor ions were detected for C4-carnitine with an m/z of 288 (Figure 2).

A comparison of the amount of the C4-carnitine isomers in the baseline samples to the test samples (after acetylsalicylic acid and acetaminophen administration, resp.) revealed no statistically significant difference. However, in the samples analysed to investigate a possible combined effect of acetylsalicylic acid and acetaminophen administration ($n = 30$) a statistically significant increase in only the isobutyrylcarnitine concentration was observed. These results support the hypothesis that acetylsalicylic acid may have an inhibitory effect on short-chain fatty acid metabolism, possibly at the level of isobutyryl-CoA dehydrogenase involved in the catabolism of branched-chain amino acids. Since there was no observed effect on these metabolic pathways after acetaminophen administration alone, the possibility also exists that the increase in C5-carnitine species observed after the combined administration may be due to the same inhibitory effect on the short-chain fatty acid metabolism. In this case, it can be at the level of the isovaleryl-CoA dehydrogenase and S-2-methylbutyryl-CoA dehydrogenase enzymes. Furthermore, it has been demonstrated that methyl-enecyclopropylacetic acid, a metabolite of hypoglycin can irreversibly inhibit all three acyl-CoA dehydrogenase enzymes in the branched-chain amino acid metabolism [21].

To further demonstrate the possible inhibitory effect of acetylsalicylic acid and/or its metabolites on the branched-chain amino acid metabolism, the total amino acid profiles were analysed. These analyses were also done on both baseline samples and samples taken after acetylsalicylic acid and acetaminophen administration, individually and combined. The comparison between the amino acid profiles in baseline urine samples and samples taken after acetylsalicylic acid ($n = 7$) and acetaminophen ($n = 7$) administration revealed a statistically significant decrease in the excretion of various amino acids, including the branched-chain amino acids. While the combined administration of acetylsalicylic acid and acetaminophen revealed a statistically significant decrease in only the branched-chain amino acids and tryptophan. However, an increase in branched-chain amino acid excretion after acetylsalicylic acid administration was expected due to the proposed inhibitory effect downstream in the branched-chain amino acid catabolism.

In only some of the subjects, the amount of excreted isobutyrylcarnitine after the administration of acetylsalicylic acid, reached concentrations normally associated with isobutyryl-CoA dehydrogenase deficiency. In this regard, we would like to speculate as to the possible implications of this observation. Since not all individuals are affected to the same degree, it is possible that the inhibitory effect of acetylsalicylic acid is more pronounced in carriers of the isobutyryl-CoA dehydrogenase deficiency or in individuals with rate-limiting polymorphisms in the same enzyme system. If this is indeed the case, it opens the opportunity to investigate whether the administration of acetylsalicylic acid can be used to predict the carrier status in isobutyryl-CoA dehydrogenase deficiency. Since the first patient with isobutyryl-CoA dehydrogenase deficiency was diagnosed only over a decade ago [21], and since there is substantial variation in the clinical presentation of this deficiency, it also poses the opportunity to investigate other biochemical effects involved in the pathology of deficient isobutyryl-CoA dehydrogenase, as the elucidation of the development of phenotypic characteristics of metabolic diseases remains a formidable challenge.

In conclusion, from the literature sources, it is clear that acetylsalicylic acid and/or its metabolites have various effects on metabolism and, especially, on mitochondrial function. In this study, the first *in vivo* evidence, in human subjects, of a statistically significant increase in isobutyrylcarnitine excretion as a result of the administration of a therapeutic dose of acetylsalicylic acid was provided. Since it was previously demonstrated that the structural similarities between acetylsalicylic acid and the acyl-portion of fatty acids can

result in mixed inhibition on β-oxidation [15], we propose a possible inhibitory effect on isobutyryl-CoA dehydrogenase, as elevated isobutyrylcarnitine excretion is generally a result of a deficiency in the branched-chain amino acid catabolism.

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