# The role of acetyl-L-carnitine on the long chain fatty acid metabolism in MPTP-treated rats.

By

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Dissertation submitted in partial fulfillment of the requirements for the degree Magister Scientiae in Pharmaceutical Chemistry of the North-West University.

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# Die rol van asetiel-L-karnitien in die langkettingvetsuurmetabolisme in MPTP-behandelde rotte.

Deur

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This Dissertation is dedicated to God, Who is my Savior, and leads me through life, holding my hand every moment of every day.

An education isn't how much you have committed to memory, or even how much you know. It's being able to differentiate between what you do know and what you don't.

*Anatole France (1844-1924)*

# ABSTRACT

Very long-chain fatty acids, especially *n*-3 polyunsaturated fatty acids (PUFAs), play an integral role in several physiological processes and are essential components of phospholipids in membranes. Fatty acid elongation in the cytoplasm (endoplasmic reticulum) and in the peroxisomes utilizes malonyl-CoA as a carbon source, whereas mitochondrial elongation uses acetylcarnitine or acetyl-CoA. Fatty acids are involved in the biosynthesis of docosahexaenoic acid (DHA, 22:6*n*-3) and the formation of acetylcarnitine. DHA's last biosynthesis step consists of one peroxisomal β-oxidation cycle of its precursor, C24:6*n*-3. After a few cycles of peroxisomal β-oxidation, the fatty acid is transported into the mitochondrion for further β-oxidation.

Previous research showed that MPTP causes a decrease of acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, or ETF-QO. Experimental animals treated with MPTP developed neurological damage which leads to clinical symptoms similar to Parkinson's disease and a metabolic profile similar to GA II, suggesting that it may be possible to induce a deficiency similar to GA II. GA II may in several ways be linked to extrapyrimidal symptoms, such as a decrease of acetyl-CoA production which is characteristic of GA II. We argued that if the neurological symptoms are the result of deceased acetyl-CoA production, which could prevent fatty acid elongation, the problem could possibly be rectified by supplementation with acetylcarnitine. Acetylcarnitine supplementation had already been shown to prevent the development of the clinical symptoms associated with MPTP treatment, but it has not been known if acetylcarnitine will increase the fatty acid elongation.

Our first objective of this study was to determine whether ALCAR plays a role in very long-chain fatty acid metabolism by influencing the fatty acid elongation process. To test this hypothesis, rats were treated with ALCAR and their serum analyzed for certain metabolites. The fatty acid concentrations were expressed in consequential ratios (C24:C22 and C26:C22), which provide a more sensitive criterion than concentrations

per se to indicate the possible defective enzymes involved in the fatty acid elongation pathway. The results showed that ALCAR had no prominent role in long-chain fatty acid metabolism and could therefore not be responsible for the preservation of peroxisomal β-oxidation or fatty acid elongation. However, concentrations of the C22:0 and C24:0 fatty acids were increased on certain days. A statistical significant higher concentration in the experimental group was observed in the C22:0 concentrations on day 1, 7 and 13 and for the C24:0 concentrations on day 7, 13 and 14.

Our second objective was to determine acetyl- and acylcarnitine concentrations in ALCAR treated rats before and after a single treatment with the GA II inducing chemical, MPTP. The acetylcarnitine concentration of the experimental group was statistically significant elevated on day 1, but thereafter the concentrations of both groups stabilized and were similar. The glutarylcarnitine concentrations of the experimental group significantly increased after MPTP treatment.

Our data suggest that the influence of ALCAR on VLCFA metabolism is not significant enough to substantiate the hypothesis that ALCAR plays an appreciable role in preserving peroxisomal β-oxidation and fatty acid elongation. ALCAR does not influence the VLCFA biosynthesis under normal circumstances, but it does activate VLCFA biosynthesis when their concentrations are decreased. ALCAR treatment can however be considered as safe in the treatment of VLCFA and neurodegenerative defects, since it has little/no effect on the VLCFA biosynthesis. ALCAR promoted the formation of some acylcarnitines (especially glutarylcarnitine), conjugates of the GA II metabolites, after MPTP treatment. Thus, it can be concluded that ALCAR possibly play an important role in the detoxification of GA II metabolites.

# UITTREKSEL

Baielangkettingvetsure, veral *n*-3 polionversadigde vetsure, speel 'n belangrike rol in verskeie fisiologiese prosesse en is 'n essensiële komponent van fosfolipiede in membrane. Vetsuurverlenging in die sitoplasma (endoplasmiese retikulum) of peroksisome gebruik maloniel-KoA as 'n koolstofbron, terwyl mitochondriale verlenging van asetielkarnitien of asetiel-KoA gebruik maak. Vetsure speel ook 'n rol in die biosintese van dokosaheksanoësuur en die vorming van asetielkarnitien. Dokosaheksanoësuur word gevorm na een siklus peroksisomale β-oksidasie vanaf sy direkte voorganger, C24:6*n*-3. Na 'n paar β-oksidasie siklusse word die verkortekettingvetsuur na die mitochondrion getransporteer waar dit verdere β-oksidasie ondergaan.

Vroeër is vasgestel dat MPTP 'n verlaging in asetiel-KoA-produksie veroorsaak as gevolg van die inhibisie van die asiel-KoA-dehidrogenase ensieme, elektron-oordragflavoproteien of elektronoordragflavoproteien-ubikinoonoksidoreduktase. MPTP-behandelde proefdiere het neurologiese skade ontwikkel wat gelei het tot kliniese simptome soortgelyk aan dié van Parkinson se siekte en 'n metaboliese profiel soortgelyk aan glutaarsuururie tipe II. Hierdie bevinding toon dat dit moontlik is om glutaarsuururie tipe II te induseer. Glutaarsuururie tipe II word op verskeie maniere verbind met ekstrapiramidale simptome – onder andere die verlaagde asetiel-KoA produksie wat 'n eienskap is van glutaarsuururie tipe II. Ons het gepostuleer dat indien die neurologiese simptome die produk is van verlaagde asetiel-KoA produksie, wat vetsuurverlenging kan verhoed, die probleem moontlik opgelos kan word deur asetielkarnitienaanvulling. Dit is reeds aangetoon dat asetielkarnitienaanvulling kliniese simptome, wat geassosieër word met MPTP-behandeling, voorkom, maar dit is nog onbekend of asetielkarnitien vetsuurverlenging sal verhoog.

Ons eerste doelwit was om the bepaal of asetiel-L-karnitien 'n rol speel in baielangkettingvetsuurmetabolisme deur die vetsuurverlengingsproses te beïnvloed. Om

hierdie hipotese te toets is rotte met asetiel-L-karnitien behandel en hulle serum vir bepaalde metaboliete geanaliseer. Die vetsuurkonsentrasies wat bepaal is, is weergegee as konsentrasie-verhoudings van opeenvolgende vetsure (C24:C22 en C26:C22), wat 'n meer sensitiewe meting as blote konsentrasiemetings daarstel om moontlike ensiemdefekte in die vetsuurverlengingsproses te kan weergee. Die resultate het egter getoon dat daar geen noemenswaardige verskil was in die verhoudings van die langkettingvetsure nie en dus kon ons nie die eerste hipotese bewys nie. Daar was wel 'n invloed op die C22:0- en C24:0-vetsuurkonsentrasies op sekere dae. 'n Verhoging van C22:0-konsentrasies is op dag 1, 7 en 13 en van C24:0-konsentrasies op dag 7, 13 en 14 by die eksperimentele groep waargeneem.

Ons tweede doel was om die asiel- en asetielkarnitiene te bepaal in asetiel-L-karnitien-behandelde en onbehandelde rotte voor en na 'n enkele dosering met MPTP (MPTP induseer 'n glutaarsuururie tipe II profiel). Die asetielkarnitienkonsentrasies van die eksperimentele groep op dag een was statisties betekenisvol verhoog, maar daarna het die konsentrasies vir die twee groepe gestabiliseer en was dit ongeveer dieselfde. Na die MPTP-behandeling het die eksperimentele groep se glutarielkarnitienkonsentrasies drasties verhoog.

Ons data toon dat asetiel-L-karnitien moontlik wel 'n rol kan speel in baie-langkettingvetsuurbiosintese, maar in dié studie was die waargenome verskille nie voldoende om te kon aflei dat die beskermingsmeganisme van asetiel-L-karnitien op die vlak van langkettingvetsuurmetabolisme lê en dus die peroksisomale β-oksidasieweg en vetsuurverlengingproses beskerm nie. Asetiel-L-karnitien het geen effek op baielangkettingvetsuurbiosintese nie, behalwe in situasies waar die konsentrasies verlaag is, in welke geval die asetiel-L-karnitien die biosintese sal stimuleer. Asetiel-L-karnitien-behandeling kan daarom as veilig beskou word by die behandeling van langkettingvetsuur- en neurodegeneratiewe defekte omdat dit 'n minimum/geen effek het op die baielangkettingvetsuurbiosintese. Asetiel-L-karnitien het die vorming van sekere asielkarnitiene (veral glutarielkarnitien) bevorder. Hierdie asielkarnitiene is die konjugate van glutaarsuururie tipe II metaboliete, wat na die behandeling met MPTP

gevorm word. Hieruit kan afgelei word dat asetiel-L-karnitien waarskynlik 'n belangrike rol speel by die detoksifikasie van glutaarsuururie tipe II metaboliete.

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**A**

| | |
|---|---|
| ACC | Acetyl-CoA carboxylase |
| ACP | Acyl-carrier protein |
| ADP | Adenine diphosphate |
| ALC | Acetyl-L-carnitine |
| ALCAR | Acetyl-L-carnitine |
| α-TOC | α-tocopherol |
| α-TQ | α-tocopherolquinone |
| α-$TQH_2$ | α-tocopherolhydroquinone |
| AMACR | 2-methyl-acyl-CoA racemase |
| AMP | Adenine monophosphate |
| ATP | Adenine triphosphate |

**B**

| | |
|---|---|
| β-oxidation | Beta oxidation |
| BBB | Blood brain barrier |
| BCAA | Branched-chain amino acid |
| BCKDH | Branched-chain α-keto acid dehydrogenase |
| BCOX | Branched-chain acyl-CoA oxidase |

**C**

| | |
|---|---|
| C2 | 2 Carbon units |
| C22:0 | 22 Carbon fatty acid (docosanoic acid) |
| C24:0 | 24 Carbon fatty acid (tetracosanoic acid) |
| C26:0 | 26 Carbon fatty acid (hexacosanoic acid) |
| C8 | 8 Carbon units |
| CACT | Carnitine-acylcarnitine translocase |

| | |
|---|---|
| CAT | Carnitine acyltransferase |
| $CO_2$ | Carbon dioxide |
| CoA | Coenzyme A |
| CoASH | Free coenzyme A |
| COX | Cytochrome oxidase |
| CPT I | Carnitine palmitoyltransferase I |
| CPT II | Carnitine palmitoyltransferase II |
| CPT | Carnitine palmitoyl transferase |

**D**

| | |
|---|---|
| Da | Dalton |
| DBP | D-bifunctional protein |
| DHA | Docosahexaenoic acid |
| D6D | Delta-6 desaturase |

**E**

| | |
|---|---|
| $E_1$ | Pyruvate dehydrogenase |
| $E_2$ | Dihydrolipoyl transacetylase |
| $E_3$ | Dihydrolipoyl dehydrogenase |
| EMA | Ethylmalonic-adipicaciduria |
| EPA | $\Delta^{5,8,11,14,17}$-eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| ESI | Electrospray ionization |
| ETC | Electron transport chain |
| ETF | Electron transfer flavoprotein |
| ETFA | Electron transfer flavoprotein alpha subunit |
| ETFB | Electron transfer flavoprotein beta subunit |
| ETFDH | Electron transfer flavoprotein dehydrogenase |
| ETF-QO | Electron transfer flavoprotein – ubiquinone oxidoreductase |

**F**

| | |
|---|---|
| FA | Fatty acid |
| FAD | Flavine adenine dinucleotide |
| $FADH_2$ | Reduced flavine adenine dinucleotide |
| FAS | Fatty acid synthase |
| Fe-S flavoprotein | Iron sulphur flavoprotein |
| FFA | Free fatty acid |

**G**

| | |
|---|---|
| GA I | Glutaric aciduria type I |
| GA II | Glutaric aciduria type II |
| GA III | Glutaric aciduria type III |
| GA IIA | Glutaric aciduria type IIA |
| GA IIB | Glutaric aciduria type IIB |
| GA IIC | Glutaric aciduria type IIC |
| GABA | γ-aminobutyric acid |
| GC-MS | Gas chromatography mass spectrometry |

**H**

| | |
|---|---|
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| HELLP | Hemolysis, elevated liver enzymes and low platelets |
| HMG-CoA | β-hydroxy-β-methylglutaryl-CoA |
| HUFA | Highly unsaturated fatty acid |

**I**

| | |
|---|---|
| IRD | Infantile Refsum disease |
| Is | Internal standard |

## K

| | |
|---|---|
| $K^+$ | Potassium ion |
| kg | Kilogram |
| KOH | Potassium hydroxide |
| kV | Kilovolt |

## L

| | |
|---|---|
| L | Litre |
| LBP | L-bifunctional protein |
| LCAD | Long-chain acyl-CoA dehydrogenase |
| LCFA | Long-chain fatty acid |
| LCHAD | Long-chain 3-hydroxyacyl-CoA dehydrogenase |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |

## M

| | |
|---|---|
| M | Molar |
| m/z | Mass to charge |
| MADD | Multiple acyl-CoA dehydrogenase deficiency |
| MADD:M | Multiple acyl-CoA dehydrogenase deficiency mild form |
| MADD:S | Multiple acyl-CoA dehydrogenase deficiency severe form |
| MCAD | Medium-chain acyl-CoA dehydrogenase |
| MCFA | Medium-chain fatty acid |
| µmol | Micromole |
| mg | Milligram |
| MIM | Mitochondrial inner membrane |
| ml | Millilitre |
| mmol | Millimole |
| MOM | Mitochondrial outer membrane |
| $MPDP^+$ | 1-methyl-4-phenyl-2,3-dihydropyridinium |
| $MPP^+$ | 1-methyl-4-phenyl-pyridinium |
| MPPP | 1-methyl-4-phenyl-propionoxypiperidine |

| | |
|---|---|
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| Mr | Molecular mass |
| MTBSTFA | N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide |

## N

| | |
|---|---|
| $Na^+$ | Sodium ion |
| NAD | Nicotinamide adenine dinucleotide |
| $NAD^+$ | Oxidised form of nicotinamide adenine dinucleotide |
| NADH | Reduced nicotinamide adenine dinucleotide |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Reduced form of nicotinamide adenine dinucleotide phosphate |
| NALD | Neonatal adrenoleukodystrophy |
| NaOH | Sodium hydroxide |

## O

| | |
|---|---|
| $O_2$ | Oxygen |
| $O_2^{\bullet -}$ | Superoxide radical anion |
| •OH | Hydroxyl free radical |
| OCTN2 | Organic cation transporter |
| OXPHOS | Oxidative phosphorylation system |

## P

| | |
|---|---|
| PC | Phosphatidylcholine |
| PP | Peroxisome proliferators |
| $PP_i$ | Inorganic pyrophosphate |
| PUFA | Polyunsaturated fatty acid |

## R

| | |
|---|---|
| Rf | Response factors |

**S**

| | |
|---|---|
| SAOX | Straight-chain acyl-CoA oxidase |
| SCAD | Short-chain acyl-CoA dehydrogenase |
| SCFA | Short-chain fatty acid |
| SCHOAD | Short-chain 3-hydroxyacyl-CoA dehydrogenase |
| SCOX | Straight-chain acyl-CoA oxidase |
| SCPx | Sterol carrier protein X |
| SD | Standard deviation |
| SIM | Single ion monitor |
| ss | Statistical significance |

**T**

| | |
|---|---|
| TE | Thioesterase |
| THC-CoA | Trihydroxycoprostanoyl-CoA |

**U**

| | |
|---|---|
| UV | Ultraviolet |

**V**

| | |
|---|---|
| v/v | Volume (of solute) per volume (of solvent) |
| VLCAD | Very long-chain acyl-CoA dehydrogenase |
| VLCFA | Very long-chain fatty acid |

**X**

| | |
|---|---|
| X-ALD | X-linked adrenoleukodystrophy |

# LIST OF SYMBOLS

| | |
|---|---|
| α | Alpha |
| β | Beta |
| [ ] | Concentration of fatty acid |
| °C | Degree Celsius |
| γ | Gamma |
| μ | Micro |
| ω | Omega |
| % | Percent |

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# Chapter One

## Introduction

Very long-chain fatty acids, especially *n*-3 polyunsaturated fatty acids (PUFAs), are involved in several physiological processes such as visual and cognitive functions, and neuronal development. *n*-3 PUFAs are important components of phospholipids in membranes and thereby influence the structure and functioning of membranes (Horrocks *et al.*, 2004). Docosahexaenoic acid (22:6*n*-3, DHA) is the most important *n*-3 PUFA (Ferdinandusse *et al.*, 2001). The results of Ferdinandusse and co-workers (2001) imply that the last step in the biosynthesis of DHA consists of one peroxisomal β-oxidation cycle of its direct precursor, C24:6*n*-3-CoA (synthesized in the endoplasmic reticulum).

Apart from the biosynthesis of DHA, fatty acids are also involved in the formation of acetyl-CoA and acetylcarnitine. The most important role of carnitine involves the transport of fatty acids. The fatty acids (acyl-CoAs) bind with carnitine to form acylcarnitine to facilitate the transport of the fatty acid into the mitochondria. It seems that among these acylcarnitines, at least acetylcarnitine may also be involved in fatty acid elongation (Wanders & Tager, 1998; Kunau *et al.*, 1995; Ramsay & Zammit, 2004). Once in the mitochondrion, β-oxidation takes place in which the fatty acids are shortened by two carbon atoms (acetyl-CoA) with each cycle of β-oxidation. Acetyl-CoA may be completely oxidized to $CO_2$ by the Krebs cycle, used in the ketogenesis pathway for the production of acetoacetate and 3-hydroxybutyric acid, or it may serve as a building block for fatty acid elongation for the production of *n*-3 PUFAs and other very long-chain fatty acids (Fromenty & Pessayre, 1995).

The first step in the mitochondrial β-oxidation process is the conversion of fatty acyl-CoA to 2-trans-enoyl-CoA, a reaction catalyzed by acyl-CoA dehydrogenase (Fromenty & Pessayre, 1995). Various acyl-CoA dehydrogenases donate electrons to ETF (electron transfer protein) or ETF-QO (electron transfer protein-ubiquinone oxidoreductase).

Therefore, a defective acyl-CoA dehydrogenase, ETF or ETF-QO may lead to an inability tot catabolize fatty acids and reduce the amount of acetyl-CoA. Known defects of acyl-CoA dehydrogenase, ETF and ETF-QO are the molecular basis of defects like sudden infant death syndrome caused by medium-chain acyl-CoA deficiency, long-chain acyl-CoA deficiency and glutaric aciduria type I and II (Olsen *et al.*, 2003; Copeland *et al.*, 2005). Clinical symptoms like hypoglycemia and reduced ketone body production are the result of decreased acetyl-CoA production, however, neurological damage often seen in patients with these abnormalities, may be attributed to an inability to produce *n*-3 PUFA and other very long-chain fatty acids due to the decreased amounts of acetyl-CoA. The lack of *n*-3 PUFA, as well as very long-chain fatty acids, will eventually effect the production of phospholipids and therefore normal cell membranes. Abnormal composition of cell membranes may affect the function of the total cell and contributes to neurological damage. Patients with GA II (ETF or ETF-QO deficiency) excrete abnormal urinary organic acids like glutaric acid, isovalerylglycine, isobuturylglycine, butyrylglycine, isovalerylcarnitine, isobuturylcarnitine, butyrylcarnitine, methylsuccinic acid, ethylsuccinic acid, sarcosine and proline (Frerman & Goodman, 1989). The metabolites thus excreted, represent the specific metabolic pathways involved in the generation of the GA II profile and include deficiencies in the metabolism of branched-chain amino acids, fatty acids, lysine, hydroxylysine, tryptophan and sarcosine (Tanaka & Rosenberg, 1983).

In previous studies it was found that experimental animals like baboons and rats treated with MPTP (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003) developed a metabolic profile similar to GA II, suggesting that it may be possible to induce a deficiency similar to GA II (Loots *et al.*, 2004). It is also known that MPTP causes neurological damage which leads to clinical symptoms similar to Parkinson's disease. MPTP causes a decrease of acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10. GA II may in several ways be linked to extrapyrimidal symptoms. Among these may be a decrease of acetyl-CoA production which is characteristic of GA II. If the neurological symptoms are the result

of deceased acetyl-CoA production, which could prevent fatty acid elongation, the problem could potentially be rectified by supplementation with acetylcarnitine.

Acetylcarnitine supplementation had already been shown to prevent the development of the clinical symptoms associated with MPTP treatment (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003), but it is not known if acetylcarnitine will increase the fatty acid elongation.

This study will investigate the hypothesis that the protective mechanism of acetyl-L-carnitine (ALCAR) may at least in part be attributed to its effect on fatty acid elongation in the cytoplasm and peroxisomes. To perform this study, rats will be treated with ALCAR, followed by a single treatment with MPTP. The concentrations of the very long-chain fatty acids, which could be an indication of increased elongation, will be determined before and after treatment with ALCAR. The ratios of a long-chain fatty acid (C22:0) and the various very long-chain fatty acids (C24:0 and C26:0) will be calculated to indicate the possibility of increased elongation.

In Chapter Two a literature overview and the motivation of this study is given and peroxisomal- and mitochondrial β-oxidation and disorders, especially GA II, that occur in these metabolic pathways are discussed. ALCAR, as a possible protective agent in several neurodegenerative diseases, is also discussed. The materials that were used, methodology and experimental design are described in Chapter Three. In Chapter Four and Chapter Five the results that were obtained are presented and interpreted. Finally, Chapter Six summarizes the project and gives some concluding annotations and recommendations.

# Chapter Two

## Literature overview

### 2.1. Background

Very long-chain fatty acids, especially docosahexaenoic acid (DHA), are involved in numerous physiological processes such as visual and cognitive functions, and neuronal development and are integral components of neural membrane phospholipids (Horrocks & Farooqui, 2004). Insufficient DHA is associated with normal ageing, Alzheimer's disease, schizophrenia, hyperactivity and peroxisomal disorders and influences neurotransmission, membrane-bound enzyme and ion-channel activities, gene expression, immunity and synaptic plasticity, and inflammation intensity (Horrocks & Farooqui, 2004). Very long-chain fatty acids and DHA, are synthesized respectively from long-chain fatty acids and essential fatty acids by fatty acid elongation and desaturation in the endoplasmic reticulum (Ferdinandusse *et al.*, 2001).

Glutaric aciduria type II (GA II), also known as multiple acyl-CoA dehydrogenase deficiency (MADD), is a syndrome which occurs due to a defect in ETF and/or ETF-QO which accepts electrons from a diverse group of acyl-CoA dehydrogenases (mitochondrial β-oxidation enzymes). The consequence of this deficiency is defective metabolism in the substrates of these dehydrogenases (Frerman & Goodman, 1989). It is known that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a GA II profile and causes parkinsonian symptoms. A recent study done by Loots and coworkers (2004) found that acetyl-L-carnitine (ALCAR) protected against MPTP/$MPP^+$ mediated oxidative stress. Although the mechanism of neuroprotection of ALCAR is yet to be determined, it is speculated that it is at a level of interference with organic cation transporters such as OCTN2 and/or carnitine-acylcarnitine translocase (CACT), however, if transport interference forms the basis of the neuroprotection, the free carnitine and not acetylcarnitine will exhibit the beneficial effect (Loots *et al.*, 2004). In previous studies,

where decreased acetyl-CoA was the possible explanation for GA II, it was observed that ALCAR prevented some of the symptoms that occurred (Loots *et al.*, 2004). It could be argued that ALCAR may lead to increased acetyl-CoA levels with subsequent improved fatty acids biosynthesis to form LCFAs. The purpose of this study is to elucidate the protective mechanism of ALCAR.

In this chapter the biosynthesis of very long-chain fatty acids will be discussed which includes elongation, desaturation and β-oxidation in the peroxisomes, the transport of these long-chain fatty acids to the mitochondria, and mitochondrial β-oxidation. GA II will be reviewed and the characteristics and proposed mechanisms of MPTP and ALCAR will be introduced. This chapter concludes with the hypothesis, aim and approach of this study.

## 2.2. Literature review

### 2.2.1. Biosynthesis of Fatty Acids

The biosynthesis of fatty acids, occurs through condensation of two carbon (C2) units which are derived from acetic acid.

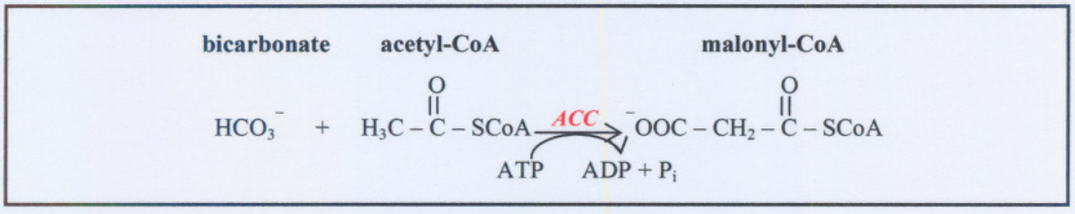



***Figure 2.1.*** ***The condensation reaction of the fatty acid biosynthesis pathway.*** *Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) (adapted from Mathews & van Holde, 2000; Voet & Voet, 1995).*

Fatty acid biosynthesis occurs in the cytosol (not mitochondria). It uses a moiety called acyl-carrier protein (ACP) instead of CoA and the reducing agent NADPH (not NAD/FAD). The synthesis of malonyl-CoA is the first committed step of fatty acid synthesis and the enzyme that catalyzes this reaction is acetyl-CoA carboxylase (ACC).

Acetyl-CoA is a precursor of the condensation reaction, bicarbonate is an absolute requirement in fatty acid biosynthesis, and malonyl-CoA an intermediate (Voet & Voet, 1995).

The synthesis of fatty acids from acetyl-CoA and malonyl-CoA is carried out by fatty acid synthase, FAS. This multifunctional enzyme catalyzes the seven different reactions whereby two carbon units from malonyl-CoA are linked together, to ultimately form palmitoyl-CoA (Mathews & van Holde, 2000; Voet & Voet, 1995).

The primary fatty acid synthesized, palmitate, is then released from the enzyme and can undergo separate elongation and/or desaturation to yield other fatty acid molecules. Palmitate or palmitoyl-CoA is the most important feedback inhibitor of fatty acid synthesis (Mathews & van Holde, 2000; Voet & Voet, 1995).

In short: fatty acid synthesis is the process of combining eight two-carbon fragments (acetyl groups from acetyl-CoA) to form a 16-carbon saturated fatty acid, palmitate. Palmitate can then be modified to give rise to the other fatty acids. These modifications may include:

- chain elongation to give longer fatty acids, such as the 18-carbon stearate,
- desaturation, giving unsaturated fatty acids.

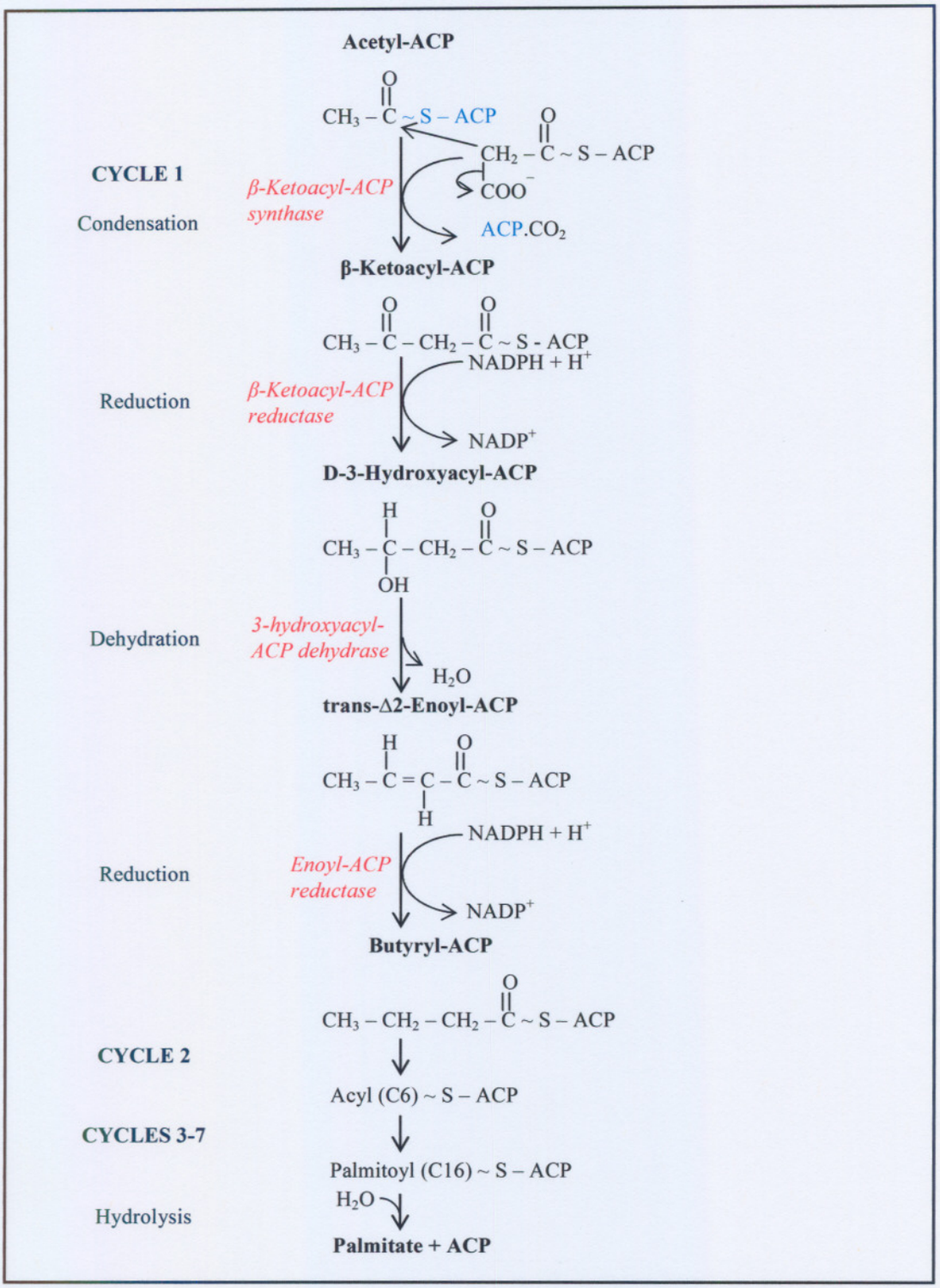



***Figure 2.2.*** ***The biosynthesis of fatty acids.*** *The pathway is repeated for seven cycles of $C_2$ elongation followed by a final hydrolysis step (adapted from Mathews & Van Holde, 2000).*

2.2.1.1. Polyunsaturated fatty acid biosynthesis

Because human cells are unable to introduce a double bond to positions beyond C9 unsaturated fatty acids like linoleic acid ($\Delta^{9,12}$-octadecadienoic acid, 18:2*n*-6), necessary for the production of the prostaglandins, α-linolenic acid ($\Delta^{9,12,15}$-octadecatrienoic acid, 18:3*n*-3), the precursor for the production of EPA ($\Delta^{5,8,11,14,17}$-eicosapentaenoic acid, 20:5*n*-3) and DHA ($\Delta^{4,7,10,13,16,19}$-docosahexaenoic acid, 22:6*n*-3), necessary for the productions of phospholipids, are all essential fatty acids. These fatty acids are also referred to as the ω-3 and ω-6 fatty acids. The ω-3 and ω-6 fatty acids are synthesized from the essential fatty acids by microsomal elongation and desaturation (Qiu, 2003; Horrocks & Farooqui, 2004).

There are two independent desaturation-elongation pathways (Infante & Huszagh, 2000):

1. a channeled carnitine and α-TOC-dependent **mitochondrial** fatty acid desaturation-elongation system which synthesizes 22:6*n*-3 (and 22:5*n*-6) by *n*-3 and *n*-6 specific multifunctional enzymes or enzyme complexes,
2. and the open microsomal system (endoplasmic reticulum, **ER**) which operates via separate desaturases and elongation enzymes and is able so synthesize only up to 22:5*n*-3 (as well as 22:5*n*-6).

In the **ER,** fatty acid elongation takes place using malonyl-CoA as a carbon source (no ACP is involved; CoA esters are used directly) and the **mitochondrial** fatty acid elongation system is known to use acetyl-CoA/acetylcarnitine as a carbon source.

Infante and Huszagh (2000) suggested that DHA is synthesized in the **mitochondria** via a carnitine and α-tocopherol (α-TOC)-dependent enzymatic pathway (figure 2.3). According to Infante and Huszagh (2000) the synthesis of fatty acids occurs in the outer mitochondrial membrane via a channeled carnitine-dependent pathway.

*Although there is not absolute experimental evidence to support the existence of the above mitochondrial pathway, a role for the mitochondrion in the biosynthesis of DHA cannot be ruled out.*

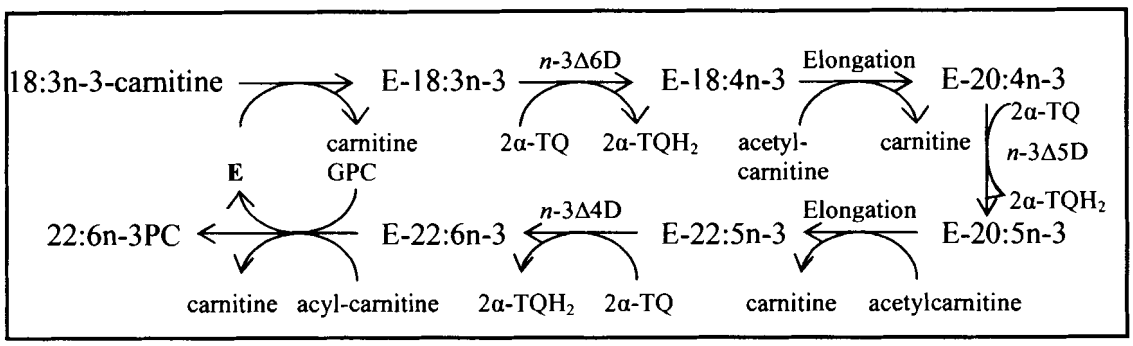



***Figure 2.3.*** ***Enzymatic mechanism for the proposed mitochondrial carnitine- and α-tocopherolquinone (α-TQ)-dependent channeled synthesis of 22:6n-3-containing phospholipids with phosphatidylcholine (PC) as an example (adapted from Infante & Huszagh, 2000).***

It was originally thought that the biosynthesis of DHA from dietary linolenic acid (C18:3*n*-3) occurred only in the endoplasmic reticulum (microsomes) via a series of elongation and desaturation reactions. The above pathway in figure 2.3 requires that *n*-3 docosapentaenoic acid (C22:5*n*-3) becomes desaturated at position 4 by a microsomal acyl-CoA-dependent $\Delta^4$-desaturase to form C22:6*n*-3 (Infante & Huszagh, 2000). Several studies, however, have indicated that such a $\Delta^4$-desaturase does not appear to exist. Instead, Ferdinandusse (2001) found that a 24-carbon *n*-3 fatty acid is synthesized, which is desaturated at position 6 to produce tetracosahexaenoic acid (C24:6*n*-3), followed by one round of β-oxidation with C22:6*n*-3 as final product.

Ferdinandusse and co-workers (2001) showed that control fibroblasts and fibroblasts from patients with Zellweger syndrome (peroxisomal biogenesis disorder) differ in their synthesis of C22:6*n*-3. When fibroblast from Zellweger patients were incubated with radiolabeled C18:3*n*-3 or C20:5*n*-3, radiolabeled C24:6*n*-3 was formed, whereas no radiolabeled C22:6*n*-3 could be detected. This showed that C24:6*n*-3 was not an elongation product from C22:6*n*-3, but that C24:6*n*-3 is an intermediate in DHA synthesis. In fibroblasts from patients with a deficiency of one of the mitochondrial

enzymes (CPT I, CACT, CPT II or VLCAD), the synthesis of radiolabeled C22:6*n*-3 from either radiolabeled C18:3*n*-3 or C20:5*n*-3 was normal. These results support the proposal that suggested that instead of a direct conversion of C22:5*n*-3 to C22:6*n*-3 by microsomal $\Delta^4$-desaturase, C22:5*n*-3 is first elongated to C24:5*n*-3, which is then desaturated by a $\Delta^6$-desaturase to C24:6*n*-3, followed by retro conversion of C24:6*n*-3 to C22:6*n*-3. This is also in agreement with deficiencies exhibited by a patient suffering from a $\Delta^6$-desaturase deficiency, a newly identified disorder. These patients hardly form any C22:6*n*-3 from C24:5*n*-3, whereas the conversion of C24:6*n*-3 to C22:6*n*-3 (peroxisomal β-oxidation) was normal. Although still disputed, the **peroxisome** is the likely site of C24:6*n*-3 β-oxidation.

The involvement of two different organelles in the biosynthesis of DHA implies that intracellular movement of fatty acids occurs between the **ER and the peroxisome** (figure 2.4). DHA is the most abundant *n*-3 PUFA in most tissues and is found at the sn-2 position of phospholipids, and must therefore move back to the endoplasmic reticulum to be acylated into phospholipids instead of being further β-oxidized in the peroxisome (Horrocks & Farooqui, 2004). The results of Ferdinandusse and co-workers (2001) imply that the last step in the biosynthesis of DHA consists of one peroxisomal β-oxidation cycle of its direct precursor, C24:6*n*-3-CoA, which is synthesized in the ER. This DHA-CoA then moves back to the ER via thioesterase (TE), probably as a free fatty acid, where it is incorporated into membrane lipids (Sprecher & Chen, 1999).

Various physiological functions, including skin integrity, eicosanoid signaling, vision and brain functions are dependent on highly unsaturated fatty acids (HUFA) (Nakamura *et al.*, 2001; Nakamura & Nara, 2003). HUFA includes arachidonic and DHA and its contents are changed in pathological conditions such as diabetes, peroxisomal disorders, growth hormone intemperance, and alcoholism (Nakamura & Nara, 2003).

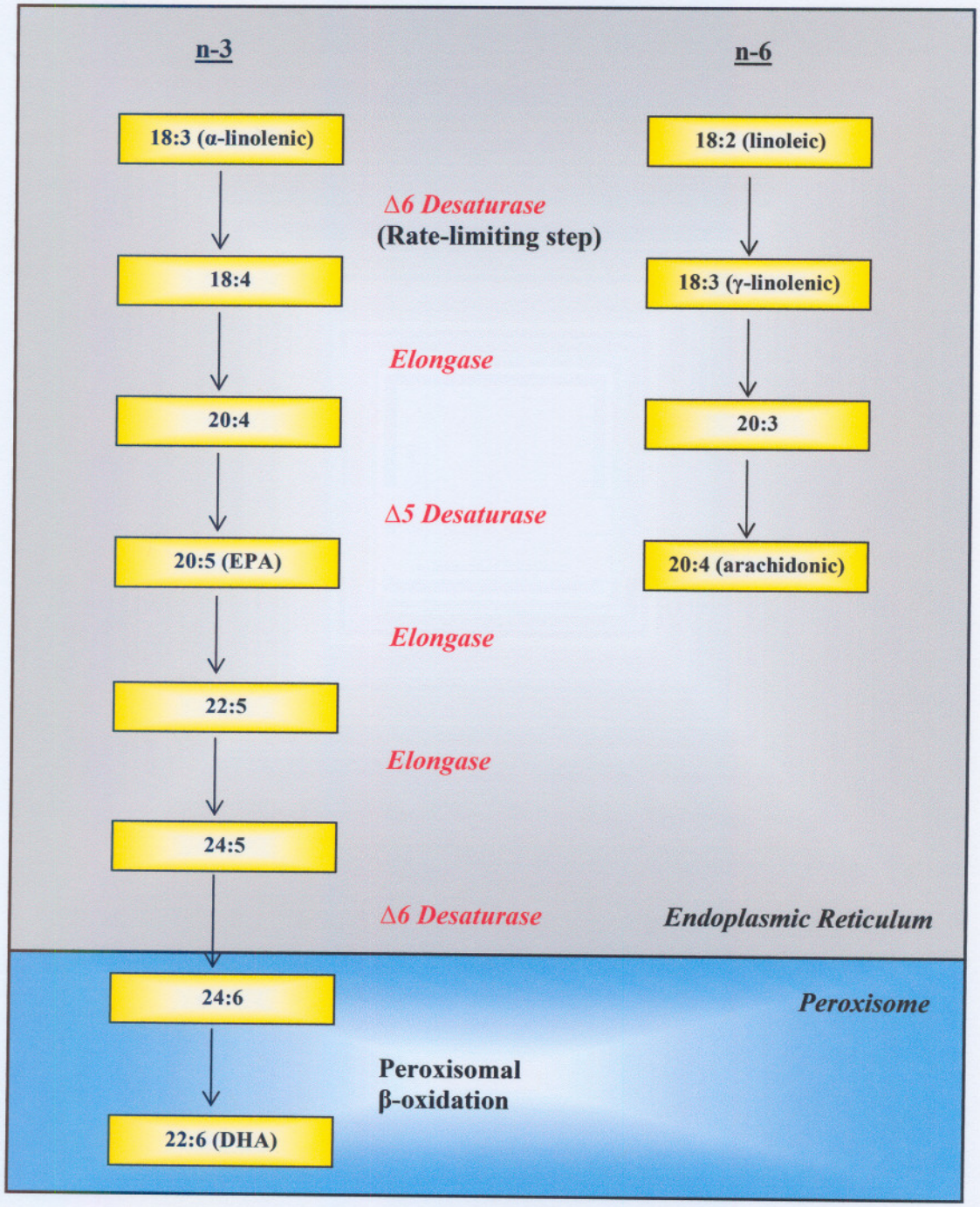



***Figure 2.4.*** ***Anabolic pathway of essential fatty acids.*** *n-3 and n-6 fatty acid synthesis in the ER is shown with the corresponding enzymes (shown in red). The final step of this pathway involves peroxisomal β-oxidation (adapted from Nakamura & Nara, 2003; Ferdinandusse et al., 2001).*

### 2.2.2. Peroxisomal β-oxidation

Peroxisomes, either spherical or oval bodies in the cytoplasm, have an osmotic behaviour (Wanders & Tager, 1998; De Duve & Baudhuin, 1966) and are freely permeable to glucose, methanol, urea, acetic acid, NAD, CoASH, ATP and carnitine (Wanders & Tager, 1998; Van Veldhoven *et al.*, 1983).

Peroxisomes and the mitochondria are able to β-oxidize fatty acids, and even though the enzymes of these peroxisomal and mitochondrial pathways are different, they result in the same chemical changes to fatty acids (Voet & Voet, 1995).

The majority of β-oxidation reactions takes place in the mitochondria, whereas peroxisomal β-oxidation most likely accounts for 10% of the β-oxidation flux of VLCFA that serves as substrates in this metabolic process, even in tissues where peroxisomes are abundant, such as the liver. Peroxisomal β-oxidation is only proficient to chain-shorten and not to degrade fatty acids to completion, whereas mitochondrial β-oxidation degrades fatty acids to completion (Wanders, 2004).

There is no carnitine requirement for the transport of fatty acyl-CoA into the peroxisomes (Wanders & Tager, 1998). A process where free VLCFA diffuse into the peroxisomes, activated by very long-chain acyl-CoA synthetases to form CoA esters and followed by direct oxidation, ultimately leads to shorter chain acyl-products. These products are then linked to carnitine for transport to mitochondria for further oxidation (Voet & Voet, 1995).

There are two complete sets of β-oxidation enzymes present in the peroxisomes, the straight- and branched-chain enzymes. β-oxidation in the peroxisomes involves 3 steps (figure 2.5) (Ferdinandusse *et al.*, 2001; Nakamura & Nara, 2003):

1. Straight-chain acyl-CoA oxidase (SCOX) is responsible for the initial oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA), whereas branched-chain acyl-CoA oxidase (BCOX) oxidizes branched-chain fatty acyl-CoAs 9 pristanoyl-CoA and THC-CoA).
2. The enoyl-CoA ester of both straight- and branched-chain fatty acids are then hydrated and dehydrogenated by the same enzyme: D-bifunctional protein (DBP). The function of the second multifunction protein, L-bifunctional protein (LBP), is still unknown.
3. The last step of the β-oxidation process, the thiolytic cleavage, is performed by sterol carrier protein X (SCPx) in the case of branched-chain substrates, whereas straight-chain substrates most likely are handled by both SCPx and the classic 3-ketoacyl-CoA thiolase.

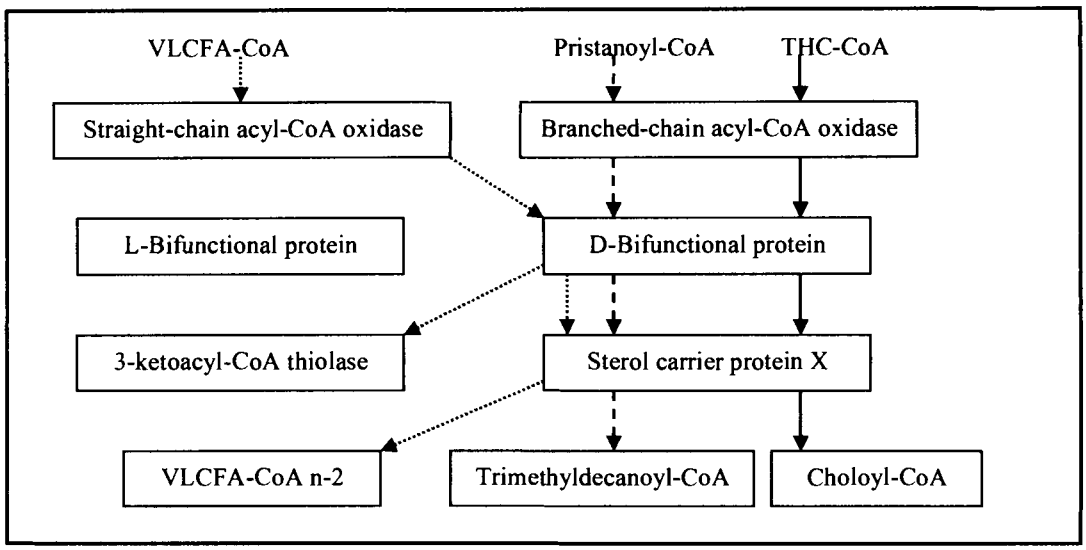



***Figure 2.5.*** ***Schematic representation of the fatty acid ß-oxidation machinery in human peroxisomes.*** *Oxidation of VLCFA-CoA (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase, D-bifunctional protein (DBP), and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), whereas oxidation of branched-chain fatty acyl-CoA involves branched-chain acyl-CoA oxidase, DBP, and SCPx, THC-CoA, trihydroxycoprostanoyl-CoA (adapted from Ferdinandusse et al., 2001).*

After peroxisomal β-oxidation, the chain shortened acyl-CoAs are converted to their carnitine esters (Voet & Voet, 1995). The carnitine esters then passively diffuse out of the peroxisome into the cytosol and are eventually transported to the mitochondrion, where they undergo further β-oxidation and are completely degraded (Ramsay & Zammit, 2004; Voet & Voet, 1995).

2.2.2.1. Peroxisomal disorders

Disorders of peroxisomal β-oxidation include X-linked adrenoleukodystrophy (X-ALD), acyl-CoA oxidase deficiency, D-bifunctional protein deficiency (3-hydroxyacyl-CoA dehydrogenase and/or enoyl-CoA hydratase deficiency) and 2-methyl-acyl-CoA racemase (AMACR) deficiency (Wanders, 2004; Bezman *et al.*, 2001; Moser *et al.*, 2000).

X-ALD is the most common single peroxisomal disorder and at least six phenotypic variants of X-ALD can be distinguished. It is characterized by the accumulation of VLCFAs, especially C24:0 and C26:0 (Wanders, 2004; Bezman *et al.*, 2001; Moser *et al.*, 2000).

A single mutation in one of many genes can alter peroxisomal biogenesis, which eventually will lead to various diseases such as Zellweger syndrome, infantile Refsum disease, and neonatal adrenoleukodystrophy (Wanders, 2004; Shimozawa *et al.*, 2004).

### 2.2.3. Fatty acid activation and import into the mitochondrion

Fatty acids must be activated (acylated) to their coenzyme A (CoA) derivatives by fatty acyl-CoA synthetase before being transported into the mithochondria. Free long-chain fatty acids (LCFA) must be activated to the CoA-derivative by long-chain acyl-CoA synthetases prior to their transport into the mitochondrion, via the carnitine transacylation pathway (figure 2.6) (Watkins, 1997).

The activation of short-chain fatty acids (SCFA, C4-C6) and medium-chain fatty acids (MCFA, C6-C14) are also acitivated by short- and medium-chain acyl-CoA synthetases, (soluble enzymes localized in the mitochondrial matrix) into acyl-CoA thioesters. These SCFAs and MCFAs can readily cross the mitochondrial outer membrane (MOM) and mitochondrial inner membrane (MIM) into the mitochondrial matrix, without the carnitine shuttle system, where it is β-oxidized (see figure 2.6) (Fromenty & Pessayre, 1995; Kunau *et al.*, 1995; Bremer, 1983; Schulz, 1991).

LCFA and VLCFA cannot freely cross the inner mitochondrial membrane and require a specific transport system as illustrated by figure 2.6:

1. LCFA and VLCFA are activated into acyl-CoA thioesters by long and very long-chain acyl-CoA synthetases respectively. These acyl-CoA synthetases are membrane-bound enzymes and are located in the MOM, peroxisomes and microsomes/ER (Fromenty & Pessayre, 1995; Kunau *et al.*, 1995; Schulz, 1991). Long-chain acyl-CoA synthetase is specific for fatty acids (saturated or unsaturated) of a chain length between ten and twenty carbon atoms and very long-chain acyl-CoA synthetase activates fatty acids containing more than 24 carbon atoms (Kunau *et al.*, 1995; Watkins, 1997).
2. Acyl-CoAs are formed by the catalytic action of long and very long-chain acyl-CoA synthetases in the MOM, followed by the conversion of acyl-CoA to acyl-carnitines, a transesterification reaction catalyzed by CPT I (Kerner & Hoppel, 2000). CPT I plays a prominent role in the transport system, where it balances the fatty acid oxidation and synthesis, and drives the LCFA and VLCFA towards oxidation instead of esterification into triglycerides (Fromenty & Pessayre, 1995).
3. Long-chain acylcarnitines are transported into the mitochondrial matrix in a transmembrane exchange reaction, where acylcarnitines are exchanged for carnitine. This reaction is mediated by CACT (Kerner & Hoppel, 2000).
4. Within the mitochondrial matrix or associated with the inner surface of the MIM, the acylcarnitines are reconverted to their respective acyl-CoAs by CPT II. These acyl-CoAs are substrates for the β-oxidation machinery (Kerner & Hoppel, 2000).

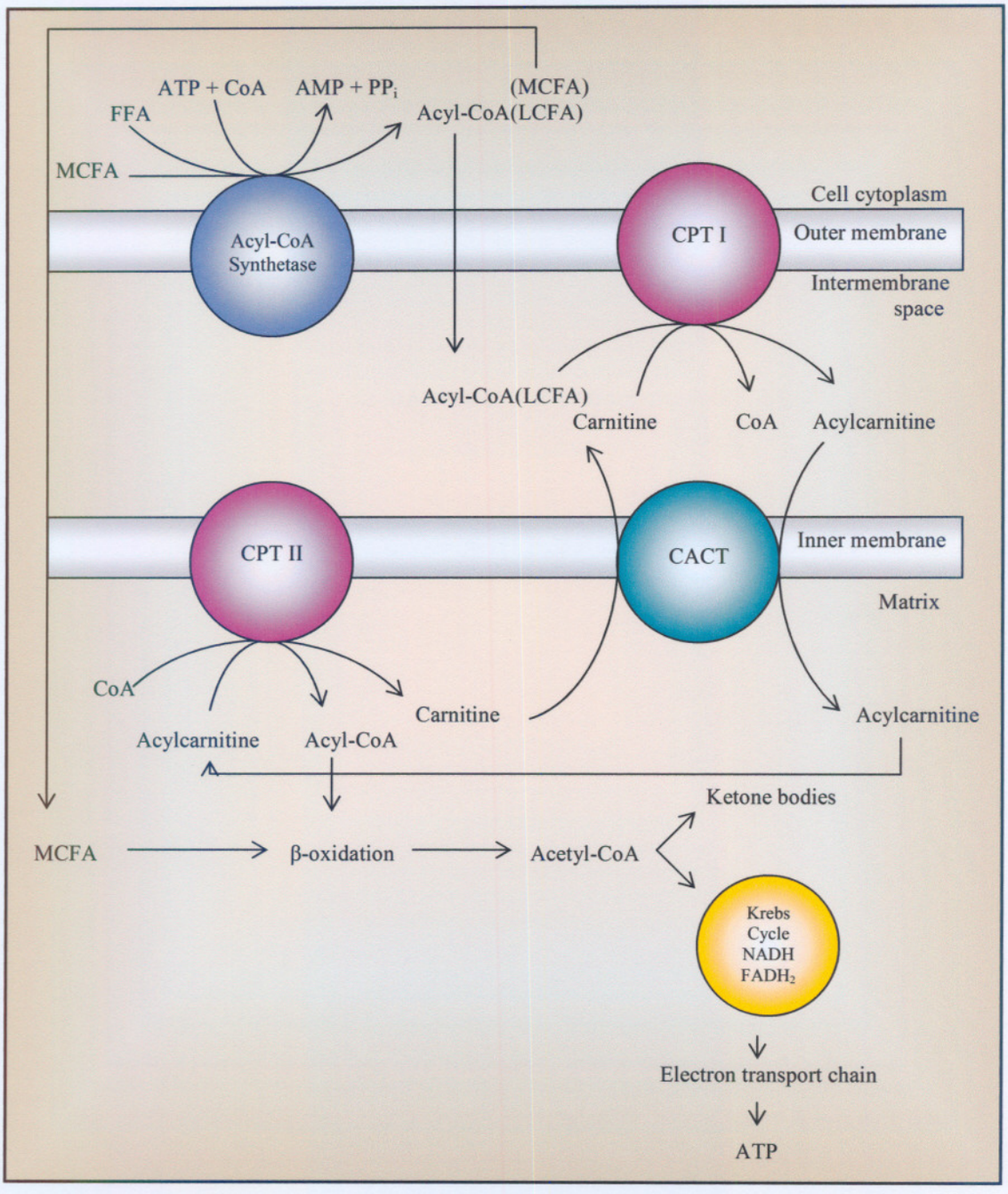



***Figure 2.6.*** ***Transport of fatty acids into the mitochondria.*** *Transport of fatty acids from the cytoplasm into the mitochondrion where the fatty-CoA is a substrate for the β-oxidation (adapted from King, 2004).*

### 2.2.4. Mitochondrial β-oxidation

Mitochondrial β-oxidation is a metabolic process which is essential for the degradation of fatty acids to acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA could then follow one of two pathways (figure 2.6), it may either condense into ketone bodies (mainly acetoacetate and β-hydroxybutyrate) or it could enter the tricarboxilic acid cycle (Krebs cycle) where it is oxidized to carbon dioxide and water (Fromenty & Pessayre, 1995).

There are four main steps in the process of β-oxidation of fatty acyl-CoAs as illustrated by figure 2.7:

1. The conversion of fatty acyl-CoA to 2-trans-enoyl-CoA catalyzed via acyl-CoA dehydrogenases. This reaction produces a trans-α,β double bond between carbon atoms 2 and 3 through dehydrogenation by the flavoenzyme acyl-CoA dehydrogenase (Fromenty & Pessayre, 1995; Schulz, 1991; Voet & Voet, 1995). There are four types of acyl-CoA dehydrogenases, short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and very long-chain acyl-CoA dehydrogenase (VLCAD) (Fromenty & Pessayre, 1995; Izai *et al.*, 1992; Bertrand *et al.*, 1993; Yamaguchi *et al.*, 1993).
2. The hydration of the double bond. Figure 2.7 illustrates the conversion reaction of 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA, catalyzed by enoyl-CoA hydratase.
3. The next step is L-3-hydroxyacyl-CoA dehydrogenation to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase. $NAD^+$ is required as a cofactor in this reaction, and the nicotinamide adenine dinucleotide (NADH, reduced form) generated by this step is reoxidized by NADH dehydrogenase (Complex I), of the respiratory chain located in the MIM.
4. The final step of this pathway, is thiolytic cleavage by 3-ketoacyl-CoA of $C_\alpha$ - $C_\beta$ to produce acetyl-CoA and an acyl-CoA chain shortened by two carbon atoms (Fromenty & Pessayre, 1995; Uchida et al., 1992; Carpenter et al., 1992).

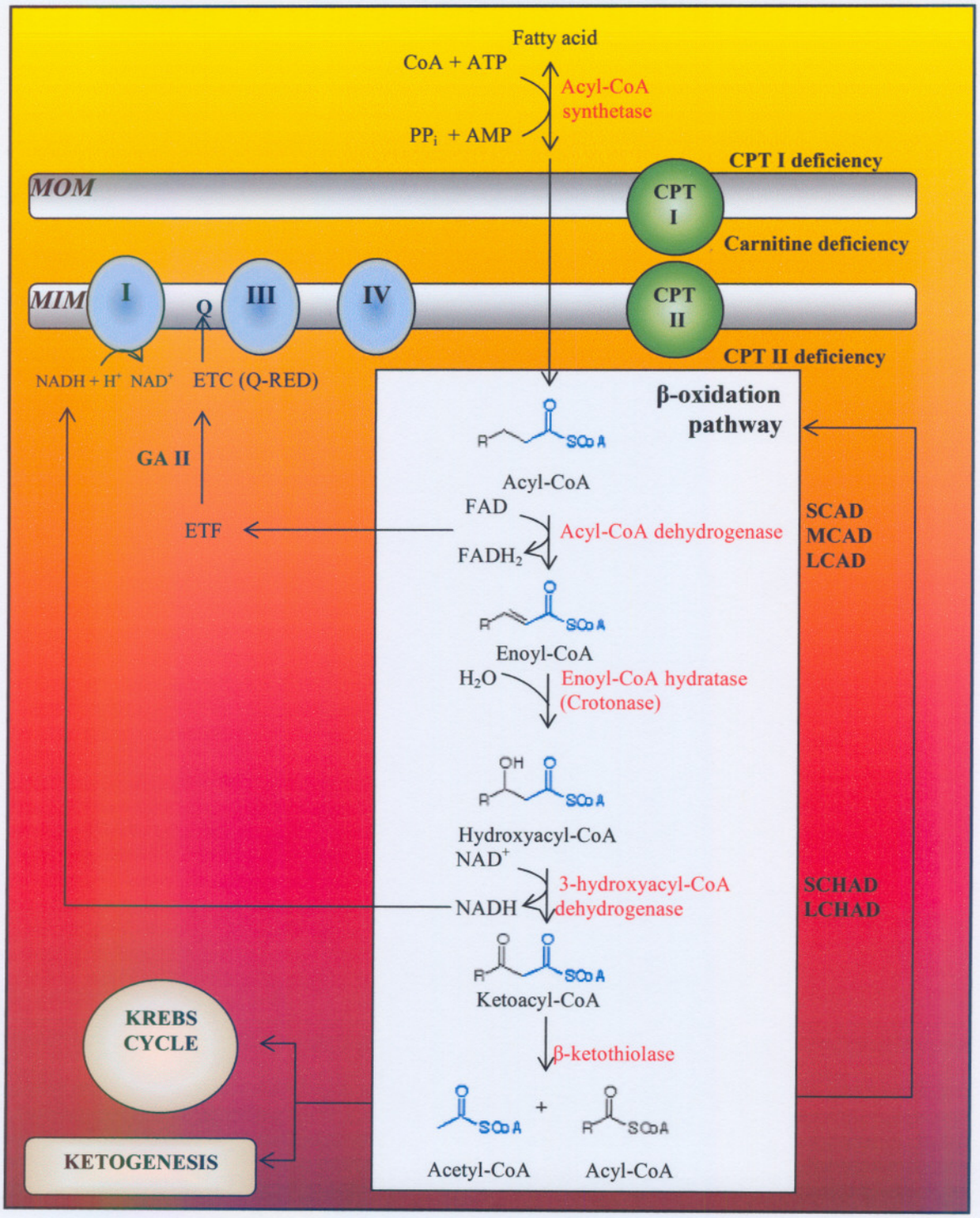



*Figure 2.7.* ***Mitochondrial β-oxidation pathway.*** *The mitochondrial β-oxidation pathway with corresponding enzymes (shown in red) and disorders indicated (adapted from various textbooks).*

2.2.4.1. Respiratory chain

The respiratory chain (figure 2.8) consists of various enzyme complexes (Complex I, II, III, IV and V) that generate energy in the form of ATP through a process called oxidative phosphorylation (Smeitink *et al.*, 2001). There are numerous metabolic pathways related to the respiratory chain such as the Krebs cycle, glycolysis, amino acid metabolism and β-oxidation of fatty acids (Mathews *et al.*, 2000).

Complex I to IV represent the electron transfer chain (ETC), while Complex V is ATP synthase, and is responsible for ATP production (Fromenty & Pessayre, 1995). Succinate dehydrogenase (an enzyme of the Krebs cycle) generates reduced FAD which translocate electrons to complex II, and reduced ETF translocate electrons directly to complex III. Electrons are shuttled from complexes I and II to complex III by ubiquinone. Cytochrome oxidase (COX) reduces cytochrome c which passes electrons from complex III to complex IV. ATP is generated by complex V from ADP and an inorganic phosphate (Leonard & Schapira, 2000; Fromenty & Pessayre, 1995).

Mitochondrial β-oxidation is related to the respiratory chain at two phases. Firstly, that of the 3-hydroxyacyl-CoA dehydrogenase to complex I via $NAD^+$/NADH and secondly the acyl-CoA dehydrogenases to ubiquinone (coenzyme Q) via electron transfer flavoprotein (ETF) (Leonard & Schapira, 2000; Fromenty & Pessayre, 1995). Inhibition at either of these phases leads to β-oxidation inhibition (Eaton *et al.*, 1996).

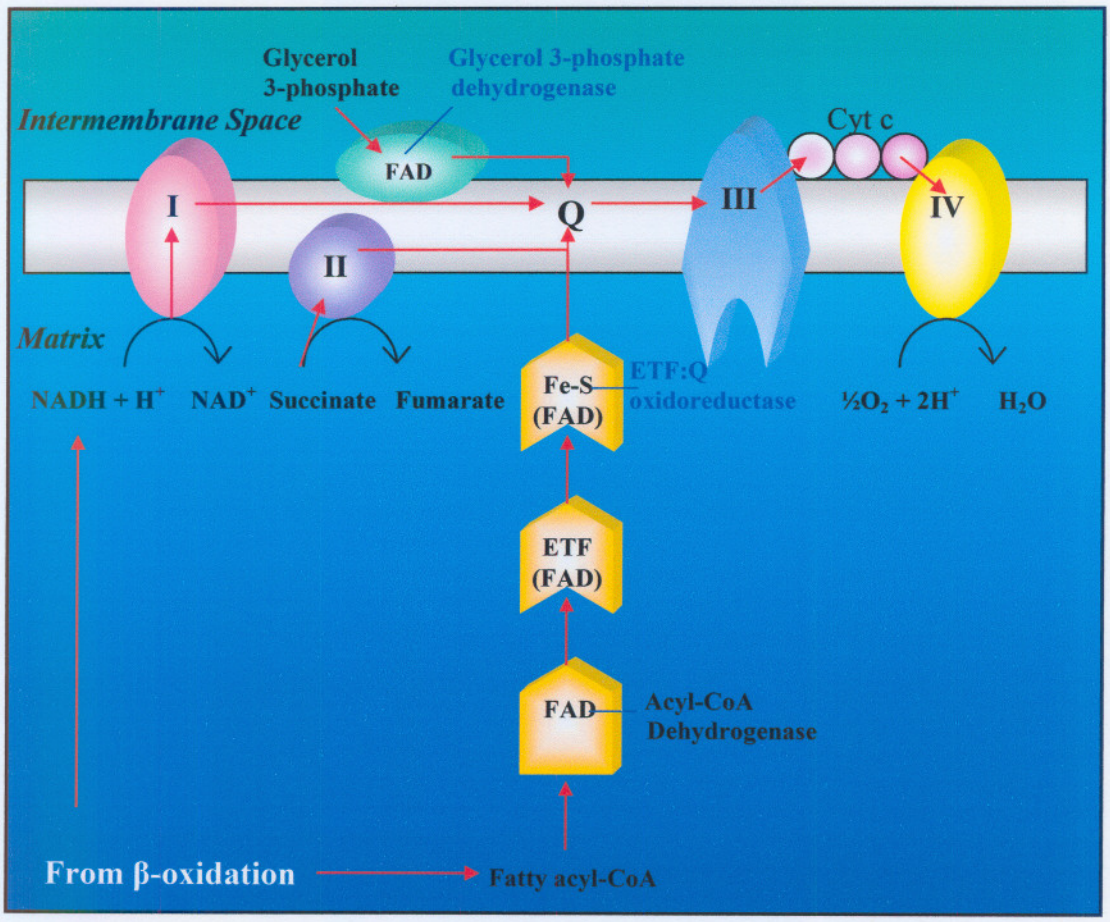



***Figure 2.8.*** ***The electron transfer chain.*** *(adapted from various textbooks).*

2.2.4.2. Mitochondrial β-oxidation disorders

Symptoms of fatty acid oxidation disorders include recurrent hypoglycemia (Reye-like), cardiomyopathy, cardiac arrhythmias, hypotonia, rhabdomyolysis, muscle weakness, unusual food preferences, retinopathy and HELLP syndrome (Hemolysis, Elevated Liver enzymes, and Low Platelets) (Roe, 2002).

Short-chain acyl-CoA dehydrogenase deficiency (SCAD) is an unusual defect. There appears to be two variants of SCAD: first and foremost, a severe infantile systemic form, which are expressed in the fibroblasts, and secondly a mild, late-onset phenotype with mainly muscle involvement (Eaton *et al.*, 1996). Medium-chain acyl-CoA

dehydrogenase (MCAD) deficiency is probably the most common mitochondrial β-oxidation disorder which presents with episodic hypoketotic hypoglycemia (Reye-like), and is provoked by fasting (Kunua *et al.*, 1995). Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD) and/or long-chain acyl-CoA dehydrogenase deficiency (LCAD) present with non-ketotic hypoglycaemia, muscle weakness and hepato- and cardiomegaly (Eaton *et al.*, 1996).

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency, a trifunctional protein deficiency, is the second most common β-oxidation disorder. LCHADs clinical presentations include episodic non-ketotic hypoglycaemia, cardiomyopathy, hepatic dysfunction, muscle weakness and sudden infant death (Eaton *et al.*, 1996).

Short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHOAD) deficiency clinically presents with recurrent Reye-like illness and dicarboxylic aciduria in patients where the defect is expressed in fibroblasts and hypoketotic hypoglycaemic encephalopathy, myoglobinuria and cardiomyopathy in patients where the defect is expressed in the muscle and presumably the liver (Eaton *et al.*, 1996).

Carnitine palmitoyl transferase (CPT) deficiency, specifically CPT II deficiency, is induced by exercise and fasting, and presents in adolescence or adulthood with recurrent myoglobinuria and rhabdomyolysis (muscle breakdown). It has however been described in infancy with the clinical presentation of hypoketonaemia, hypoglycaemia and cardiomyopathy (Eaton *et al.*, 1996).

Deficient CPT I activity is associated with the hepatic or infantile phenotypes. It clinically presents with fasting hypoglycaemia and hypoketonaemia in the presence of normal insulin levels. Insufficient CPT I activity could be observed in the liver and fibroblasts, excluding muscle (Eaton *et al.*, 1996).

Primary carnitine deficiency is rare and presents during infancy or childhood with muscle weakness and cardiomyopathy. The only well-characterized defect is that of cellular

carnitine. Just a few cases of carnitine/acylcarnitine deficiencies have been described (Eaton *et al.*, 1996).

A deficiency of either ETF or ETF-QO is known as GA II (also known as multiple acyl-CoA dehydrogenase deficiency) (Eaton *et al.*, 1996; Frerman & Goodman, 1985), in which the dehydrogenase activities of ETF and ETF-QO are impaired. Defects have been categorized in three different groups: a fatal neonatal form associated with congenital abnormalities and cardiomyopathy; an infantile or childhood form associated with metabolic acidosis, hepatomegaly and episodic hypoglycaemia; and a late onset form with conspicuous muscle involvement (Eaton *et al.*, 1996).

### 2.2.5. Glutaric Aciduria Type II

#### 2.2.5.1. Introduction

Glutaric aciduria type II (GA II) is also referred to as glutaric acidemia type II, multiple acyl-CoA dehydrogenase deficiency (MADD), ethylmalonic-adipicaciduria (EMA) (OMIM, 2005), electron transfer flavoprotein dehydrogenase deficiency, and ETF/ETF-QO deficiency (Copeland *et al.*, 2005).

Organic acids are side products in the intermediate metabolism of amino acid-, carbohydrate- and lipid metabolism. Inherited metabolic defects form a diverse group, collectively known as organic aciduria (figure 2.9)(Tang *et al.*, 2001).

Individuals who suffer from GA II, an autosomal recessively inherited disorder of fatty acid, amino acid and choline metabolism, are not capable to completely break down foods or body stores into fats and proteins, which are essential for energy use. This disorder is caused by a deficiency in either the ETF enzyme or ETF-ubiquinone oxidoreductase (figure 2.8)(Olsen *et al.*, 2003).

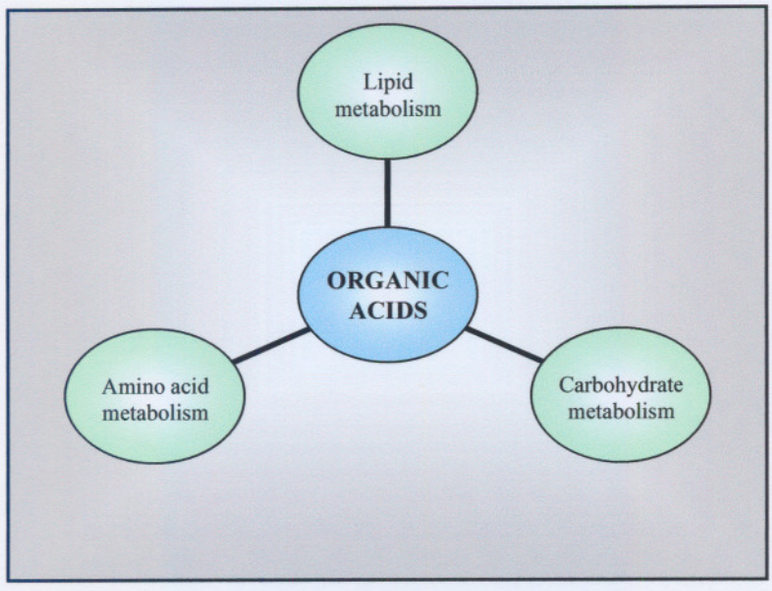



***Figure 2.9.*** ***Intermediary metabolism of organic acids.*** *Lipid-, amino acid- and carbohydrate metabolism play an essential role in the intermediary metabolism of organic acids.*

Glutaric aciduria type I (GA I) is an autosomal recessive metabolic disorder caused by a mutation in the gene encoding glutaryl-CoA dehydrogenase and is mainly characterized by gliosis and neuronal loss in the basal ganglia and progressive movement disorder (Goodman *et al.,* 1995).

GA I and II result in excessive excretion and accumulation of several metabolites, such as glutaric, lactic, ethylmalonic, isobutyric, 2-methylbutyric, and isovaleric acid (Olsen *et al.*, 2003). 2-Hydroxyglutaric acid is the diagnostic compound for GA II and distinguishes between GA I and GA II (Copeland *et al.*, 2005). The metabolites excreted derive mainly from acyl-CoA esters, normally oxidized by FAD-dependent dehydrogenases (acyl-CoA dehydrogenases) with the exception of 2-hydroxyglutaric acid (Goodman & Markey, 1981; Goodman *et al.*, 1980; Rhead & Tanaka, 1979). The only other organic aciduria with which GA II can be confused with is Jamaican vomiting sickness, which can be determined by good history taking (Goodman & Markey, 1981).

2.2.5.2. Specific catabolic pathways involved in GA II

A GA II profile is generated when fatty acid-, branched-chain amino acid (BCAA)-, lysine-, hydroxylysine-, tryptophan- and sarcosine metabolism are blocked at the acyl-CoA dehydrogenase step (Tanaka & Rosenberg, 1983).

The metabolites derived from above mentioned catabolic pathways that are indicative of GA II are given in table 2.1.

***Table 2.1. Abnormal metabolites found in the urine of GA II patients***

| Catabolic pathway | Metabolites |
|---|---|
| straight-chain fatty acids | ethylmalonic acid, adipic acid, hexanoylglycine, suberic ($C_8$) and dodecanedioic acids ($C_{10}$) |
| branched-chain amino acids<br>1. isoleucine<br>2. valine<br>3. leucine | 1. 2-methylbutyrylglycine, 2-methylbutyric acid and ethylmalonic acid<br>2. isobutyric acid and isobutyrylglycine<br>3. isovalerylglycine, isovaleric acid, 3-hydroxybutyric acid, 4-hydroxyisovaleric acid, methylsuccinic acid, methylfumaric acid, isovalerylglucuronide, isovalerylglutamic acid, isovalerylalanine, isovalerylsarcosine, isovalerylcarnitine and 3-hydroxy-isoheptanoic acid |
| lysine-, hydroxylysine | glutaric acid, 2- hydroxyglutaric acid and glutaconic acid |
| tryptophan | glutaric acid, 2-hydroxyglutaric acid and glutaconic acid |
| sarcosine | sarcosine |
| pyruvate | lactic acid |

2.2.5.3. Causes of GA II

Various metabolic pathways donate electrons that enter the respiratory chain. Electron transfer flavoprotein (ETF) and electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) facilitate the transfer of these electrons from various flavoprotein

dehydrogenases, including acyl-CoA dehydrogenases, to the mitochondrial complex III via ubiquinone (Frerman & Goodman, 1989).

ETF reduces complex III of the electron transport chain, but not complex I and II as previously thought. The subunit involved in the electron transfer between ETF and ubiquinone (coenzyme Q) is ETF-QO. Coenzyme Q rapidly reduces the flavoprotein. The reoxidation rate of the Fe-S flavoprotein by coenzyme Q is considerably higher than that of ETF. (Ruzicka & Beinert, 1977).

GA II can be caused by mutations of three different genes:

- Electron transfer flavoprotein alpha (α) subunit (ETFA) - a deficiency of the α-subunit of the electron transfer flavoprotein as seen in patients suffering from GA IIA (Indo *et al.*, 1991; Olsen *et al.*, 2003).
- Electron transfer flavoprotein beta (β) subunit (ETFB) - a deficiency of the β-subunit of the electron transfer flavoprotein as observed in patients suffering from GA IIB (Colombo *et al.*, 1994; Olsen *et al.*, 2003).
- Electron transfer flavoprotein dehydrogenase (ETFDH) – a deficiency of electron transfer flavoprotein dehydrogenase and identified in patients suffering from GA IIC (Beard *et al.*, 1993; Olsen *et al.*, 2003).

In GA II one can distinguish between MADD:S (Severe form of MADD) and MADD:M (Mild form of MADD). MADD:S provided evidence that GA II sometimes resulted from deficiency of ETF, in patients without congenital anomalies, and sometimes from a deficiency of ETF-QO, in patients with congenital anomalies. MADD:M is a result of intermediate deficiency in either ETF or ETF-QO and usually presents later in life (Frerman & Goodman, 1985; Heyer, 2002).

Experimental animals treated with MPTP (that causes neurological damage which leads to clinical symptoms similar to Parkinson's disease), developed a metabolic profile similar to GA II (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003), suggesting that it may be possible to induce a deficiency similar to GA II (Loots *et al.*, 2004). MPTP

causes a decrease of acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10. GA II may in several ways be linked to extrapyrimidal symptoms. Among these may be a decrease of acetyl-CoA production which is characteristic of GA II.

2.2.5.4. Clinical features

There are three different phenotypes in patients suffering from GA II:

- Neonatal onset with congenital anomalies (type I): Infants are often premature and usually present within the first 24-48 hours of life with hypotonia, hepatomegaly, hypoglycemia, metabolic acidosis, sweaty feet odor, palpable enlarged and cystic kidneys, facial dysmorphisms, rocker-bottom feet, muscular defects of the anterior abdominal wall and anomalies of the external genitalia such as hypospadias and chordee (Copeland *et al.*, 2005).
- Neonatal onset without anomalies (type II): Infants develop symptoms usually within the first few days of life. These symptoms include hypotonia, tachypnea, metabolic acidosis, hepatomegaly, hypoglycemia and sweaty feet odor. The few infants who survive the first week of life usually die within a few months, suffering from severe cardiomyopathy. A few others have been hypoglycemic as newborns and later developed typical episodes of Reye syndrome-like illness and survived somewhat longer (Copeland *et al.*, 2005).
- Mild or late onset (type III): Mild or late onset are extremely variable in its course and age presentation. Typical clinical features include episodes of hypoketotic, hypoglycemic and hepatic dysfunction, intermittent episodes of vomiting and lethargy (Copeland *et al.*, 2005). There is progressive lipid storage myopathy and carnitine deficiency. A few presented with progressive extrapyramidal movement disorders similar to GA I. There are reports of asymptomatic adults (Copeland *et al.*, 2005).

2.2.5.5. Treatment

Patients should avoid fasting (Copeland *et al.*, 2005), and a diet high in carbohydrates and low in fat and protein should be followed. Additional supplementation of riboflavin (reported to be therapeutic in some milder cases), glycine and carnitine may be helpful (Frerman & Goodman, 2001; Copeland *et al.*, 2005). The treatment of severe neonatal presentations is not effective (Copeland *et al.*, 2005).

## 2.2.6. MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine)

2.2.6.1. Introduction

More than two decades ago, drug users developed a rapidly progressive parkinsonian syndrome. 1-methyl-4-phenyl-propionoxypiperidine (MPPP), an analogue of the narcotic meperidine, was intravenously traced and MPTP was indicated as the responsible neurotoxic contaminant. MPTP is accountable for extensive toxicity to dopaminergic neurons and neurological injury to the substantia nigra (Langston *et al.*, 1983; Dauer & Przedborski, 2003) that causes parkinsonian symptoms and are therefore an adequate model to study Parkinson's disease (PD) and other related neurodegenerative diseases.

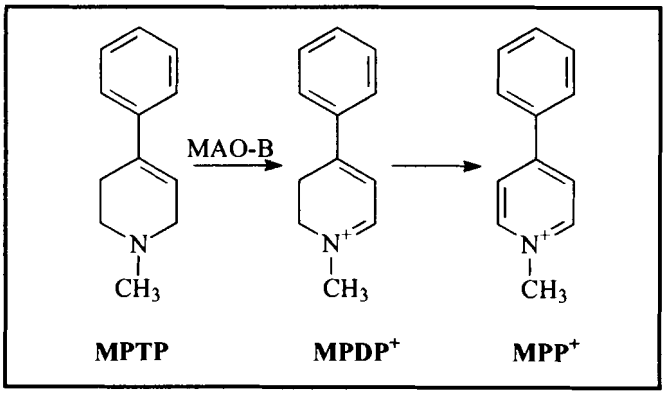



***Figure 2.10. MPTP metabolism.*** *The conversion of MPTP to $MPDP^+$ by monoamine oxidase B (MAO-B) and the oxidation to $MPP^+$ (adapted from Loots et al., 2004).*

2.2.6.2. Cellular action and mechanisms

MPTP is highly lipophilic and crosses the blood brain barrier (BBB) readily (Markey *et al.*, 1984; Dauer & Przedborski, 2003). The enzyme, monoamine oxidase B (MAO-B) in glia and serotonergic neurons, is responsible for the conversion of MPTP to the 1-methyl-4-phenyl-dihydropyridinium ($MPDP^+$). The $MPDP^+$ is then spontaneously oxidized nonenzymatically to the neurotoxin, 1-methyl-4-phenyl-pyridinium ($MPP^+$, a polar molecule), and released into the extracellular space by an unknown mechanism (Dauer & Przedborski, 2003).

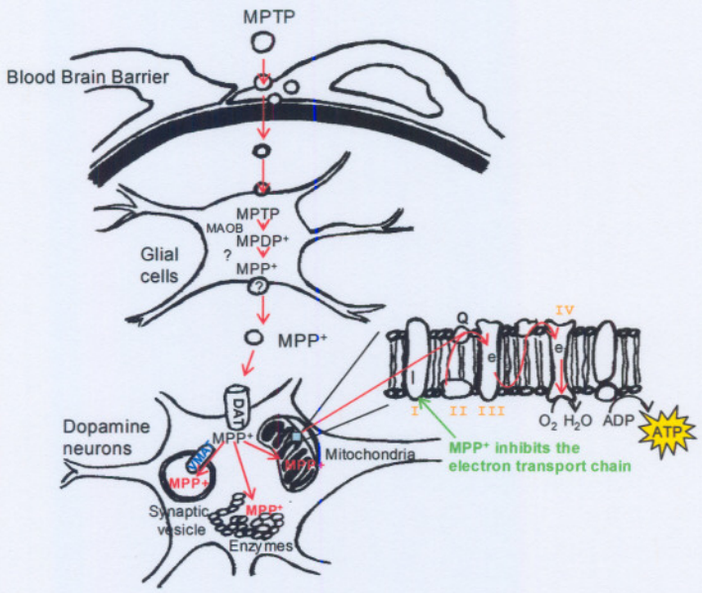



***Figure 2.11.*** ***MPTP metabolism and intracellular $MPP^+$ pathways.*** *MPTP crosses the blood-brain barrier (BBB). MPTP is converted to $MPDP^+$ by MAO-B and then to $MPP^+$ in the glail cells. $MPP^+$ is released into the extracellular space and concentrated in the dopamine neurons via the dopamine transporter (DAT). $MPP^+$ can then follow one of three routes: (1) It can be actively transported into the mitochondria where it inhibits complex I of the electron transport chain, which interrupts the transfer of electrons from complex I to ubiquinone (Q) and leads to increased production of reactive oxygen species and decreased ATP production; (2) interaction with cytosolic enzymes; (3) transported into synaptic vesicles via the vesicular monoamine transporters (VMAT) (adapted from Dauer & Przedborski, 2003).*

$MPP^+$ accumulates in the dopaminergic neurons and is actively transported into the mitochondria where it inhibits mitochondrial respiration at complex I of the electron transport chain, by blocking NADH:ubiquinone oxidoreductase. This impedes the electron transport function and alleviates the receipt of reducing equivalents utilized for OXPHOS. The rapid loss of ATP, the consequence of the former, directs to a decrease of membrane $Na^+/K^+$-ATPase activity, an increase in calcium release neuronal depolarization, and a degenerative cycle of apoptotic or excitotoxic cell death (Soliman & Mazzio, 2003; Ebadi *et al.*, 2001).

It is important to mention an additional mechanism through which $MPP^+$ causes cell death: the promotion of hydroxyl radical production ($^{\bullet}OH$), the process universally described and accepted as oxidative stress. $MPP^+$ promotes the formation of the superoxide radical anion, $O_2^{\bullet -}$ and $^{\bullet}OH$, due to its redox activity in the presence of cytochrome $P_{450}$ reductase (Loots *et al.*, 2004). Acetylcarnitine (ALCAR) supplementation had already been shown to prevent the development of the clinical symptoms in non-human primates, associated with MPTP treatment (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003)

### 2.2.7. ALCAR (Acetyl-L-Carnitine)

#### 2.2.7.1. Introduction

Acetyl-L-carnitine is known as beta-acetoxy-gamma-N,N,N-trimethylamino-butyrate, acetyl-carnitine, L-acetylcarnitine, acetylcarnitine, acetyl levocarnitine, ALC and ALCAR (White & Scates, 1990).

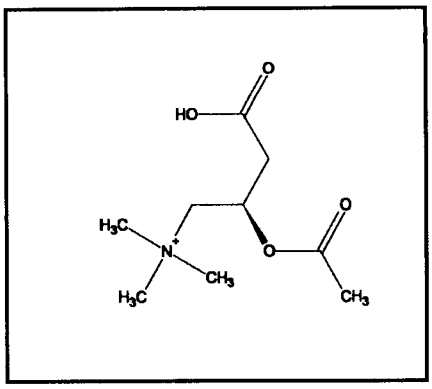



***Figure 2.12. The structure of acetyl-L-carnitine (ALCAR).***

2.2.7.2. Mechanism of action

ALCAR, a delivery form for both L-carnitine and acetyl groups, is an acetyl ester of the amino acid L-carnitine and occurs naturally in animal products (Soliman & Mazzio, 2003). The acetyl component provides for the formation of the neurotransmitter acetylcholine and acetyl-CoA, contributing to oxidative phosphorylation (OXPHOS) through enhanced energy substrate supply to the Krebs cycle (Soliman & Mazzio, 2003; Dolezal & Tucek, 1981).

L-carnitine mediates LCFA transport across the MIM into the mitochondrial matrix for oxidative phosphorylation, and SCFA and MCFA transport out of the mitochondria in order to maintain normal coenzyme A levels (Heinonen, 2005; Soliman & Mazzio, 2003). Although ALCAR functions the same as L-carnitine, it is more efficiently absorbed into the bloodstream, passes more readily through cell membranes, and is more efficiently utilized by the mitochondrion (Soliman & Mazzio, 2003).

Exogenously administered ALCAR crosses the BBB through the GABA uptake system, and attenuate neurological brain injury associated with degenerative disorders, including axotomy, human immunodeficiency virus, diabetic neuropathy, and Alzheimer's disease (Soliman & Mazzio, 2003; Pettegrew *et al.*, 2000). In rats, ALCAR is able to reduce age-associated changes in spatial and temporal memory, reduce oxidative damage to the hippocampus (Soliman& Mazzio, 2003; Liu *et al.*, 2002), and improve the cholinergic

transmission in the hippocampus through positive effects on the choline uptake acetylcholine synthesis and acetylcholine release (Soliman & Mazzio, 2003; Ando *et al.*, 2001). Extensive medical research has shown that ALCAR may have neuroprotective-, cardioprotective-, cytoprotective-, and anti-apoptotic activity and is an antioxidant (Lolic *et al.*, 1997).

The loss of mitochondrial OXPHOS ability and/or inherited mitochondrial disorders cause the effects of ageing in Parkinson's disease (Orth & Schapira, 2002; Gadaleta *et al.*, 1998). ALCAR is effective in reducing age-dependent mitochondrial functional decay, as well as the losses of mitochondrial membrane potential, cardiolipin content, metabolic oxygen ($O_2$) consumption, and β-oxidation of fatty acids (Hagen *et al.*, 2002; Gadaleta *et al.*, 1998).

ALCAR can prevent MPTP toxicity in non-human primates (Bodis-Wollner *et al.*, 1991; Soliman *et al.*, 2003). A part of ALCAR's defensive mechanisms are the interactions with organic cation transporters such as OCTN2 and/or carnitine-acylcarnitine translocase (CACT). Consequently, ALCAR may attenuate hydroxyl free radical generation in the MPTP/$MPP^+$ neurotoxic pathway (Loots *et al.*, 2004). ALCAR's defense against $MPP^+$ can be obtained by upholding cellular energy stores, by providing adequate glucose to achieve the glycolysis demands (Chalmers-Redman *et al.*, 1999; Mazzio & Soliman, 2003).

## 2.3. Hypothesis, Aim and Approach

Elongation, desaturation and peroxisomal β-oxidation are the essential steps in the biosynthesis of very long-chain fatty acids such as DHA. These polyunsaturated fatty acids (PUFAs) are involved in neural development and visual and cognitive functions. Therefore, PUFA deficiencies can lead to memory loss, learning disabilities and impaired visual acuity (Ferdinandusse *et al.*, 2001). Peroxisomal β-oxidation is necessary for the degradation of very long-chain fatty acids as well as a wide range of unusual and xenobiotic acyl groups (Eaton, 2002). This process is also very important for the

synthesis of essential very long-chain fatty acids, such as DHA. The β-oxidation pathway, therefore has an anabolic as well as a catabolic function (Sprecher & Chen, 1999).

The long-chain fatty acids are transported into the mitochondria where further β-oxidation takes place. ETF and ETF-QO are responsible for transferring electrons from various mitochondrial flavoprotein dehydrogenases, which include acyl-CoA dehydrogenases, to the mitochondrial complex III via ubiquinone. An acyl-CoA dehydrogenase deficiency could lead to a defective transport of electrons to the ETF and/or ETF-QO that may cause GA II (Frerman & Goodman, 1989).

It is known that MPTP inhibits the respiratory chain at complex I and thus causes a GA II profile. ALCAR prevents MPTP toxicity in non-human primates (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003), which is indicative of a protective mechanism of β-oxidation disorders.

Glutaric aciduria is an inborn error of glutaryl-CoA metabolism which is derived from three possible enzyme defects namely: glutaryl-CoA dehydrogenase (GA I), ETF or ETF-QO (GA II) and peroxisomal glutaryl-CoA oxidase (GA III). Some medications and other chemical substances like styrene induce glutaric aciduria. GA I and GA II, but not GA III, present with extrapyramidal symptoms, similar to those of Parkinsons disease. Since GA III patients do not develop these symptoms, one can assume that the extra-pyramidal symptoms is not caused by glutaric acid but, that they are rather the result of the metabolic processes involved that lead to the accumulation of glutaric acid. The common denominator in these metabolic defects is reduced acetyl-CoA (essential for various anabolic pathways for example ketogenesis and fatty acid biosynthesis). VLCFAs and PUFAs play an important role in membrane integrity, especially nerve cell integrity, therefore there is the possibility that a decreased production of acetyl-CoA in the above cases may lead to decreased biosynthesis of VLCFAs and the subsequent symptoms.

In previous studies, where decreased acetyl-CoA was the possible explanation for this defect, it was observed that ALCAR prevented some of the symptoms that occurred (Ricciolini *et al.*, 1998; Soliman & Mazzio, 2003). It could be argued that ALCAR may lead to increased acetyl-CoA levels with subsequent improved fatty acids biosynthesis to form LCFAs.

In this study, we hypothesize that the protective mechanism of ALCAR may be that of preserving the β-oxidation cycle, securing the transport of electrons to the ETC, and that acetyl-L-carnitine plays a role in long-chain fatty acid metabolism and subsequently in the attenuation of neurodegenerative diseases.

The aim of this study is therefore to investigate the role of acetyl-L-carnitine in the long-chain fatty acid metabolism. Rats will be pre-treated with ALCAR and the GA II will be induced with MPTP. Fatty acid concentrations in blood will be determined and expressed in consequential ratios before and after a single treatment with MPTP in rats pretreated with ALCAR. These fatty acid ratios should give an indication of enzyme defects in the fatty acid oxidation or elongation pathway and should indicate whether ALCAR protects against MPTP neurotoxicity via β-oxidation regulation. Acetyl- and acylcarnitine concentrations will be determined in the urine of treated and untreated rats. The strategy for this study will be discussed in detail in the following chapter.

# Chapter Three

## Experimental design

The strategy of this study is briefly outlined in figure 3.1. The experimental work was mainly divided into two sections namely work done in the animal research centre (discussed in this chapter) and the sample analysis (discussed in Chapter Four and Chapter Five). This project was approved by the ethics committee (05D02).

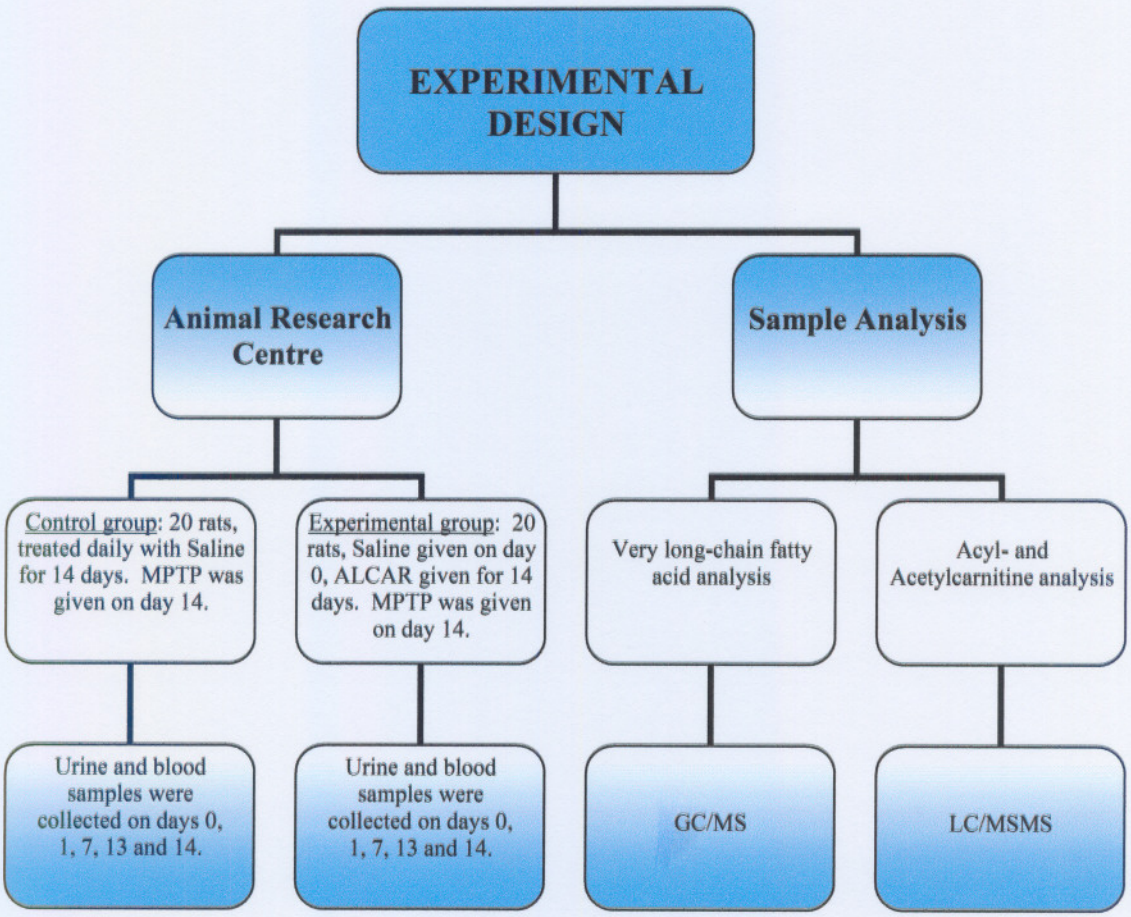



***Figure 3.1. Schematic of the experimental design.***

## 3.1. Dosing the rat and collecting urine and blood samples

*Sprague Dawley* rats (40) were chosen for this study. They are easy to handle, economical and their metabolism is comparable to that of humans (Chalmers & Lawson, 1982; Loots, 1998). The rats were individually kept in metabolic cages, which are designed in such a way that urine can be collected separately from the feces or other contaminants. Water was supplied ad.lib. and the conditions were regulated at 25°C and 40 % humidity (Chalmers & Lawson, 1982; Loots, 1998). The rats received pellets supplied by the animal research centre.

### 3.1.1. Materials

1. Acetyl-L-carnitine solution (in 0.5ml saline) was injected at a concentration of 20 mg/kg body mass. The solution was sterilized by a standard procedure (Loots, 1998).
2. An MPTP solution was made up in saline to a concentration of 35 mg MPTP/kg body mass if injected with 0.5 ml of the solution and sterilized by a standard procedure (Loots, 1998).
3. Saline was used for the control group.

***Table 3.1. Safety precautions of the chemicals used for the animal dosages.***

| Chemicals | Company | Safety precautions |
|---|---|---|
| ALCAR | Fluka | Causes eye, skin and respiratory tract irritation. Use protective eyeglasses, gloves. |
| MPTP | Sigma Aldrich | Neurotoxin, use disposable gloves and handle in a properly ventilated hood. |
| Saline | Pharmacy | None |

### 3.1.2. Procedure

40 adult male *Sprague Dawley* rats, divided into two groups, were used. The rats were fasted for four hours prior to injections because the formation of some of the metabolites is dependent on fat metabolism. On the days of blood and urine collection, the rats remained under fasting conditions until the samples were collected. 0.5 ml blood was drawn from the tail of the rat and collected in microtainer tubes. Urine was individually collected from the metabolic cages approximately four hours after the injections.

**Group A : Control group**

20 rats were injected intra-peritoneally with saline daily for 14 days and MPTP (35mg/kg) was injected 15 minutes after the saline injection on day 14. Blood and urine samples were collected on day 0, 1, 7, 13 and 14, approximately 4 hours (between 3 and 5 hours) after the injections. 2 rats receiving MPTP died, and were not included in the study.

**Group B : Experimental group**

20 rats were injected intra-peritoneally with saline on day 0. ALCAR (20 mg/kg) was injected daily for 14 days and MPTP (35 mg/kg) was injected on day 14, 15 minutes after the ALCAR injection. Blood and urine samples were collected on day 0, 1, 7, 13 and 14, approximately 4 hours (between 3 and 5 hours) after the injections. All 20 rats survived and were used to calculate the statistical significances.

# Chapter Four

## Effect of ALCAR on very long-chain fatty acid metabolism

### 4.1. Introduction

As previously mentioned in Chapter Two, very long-chain fatty acids (VLCFAs) play an integral role in numerous physiological processes, including visual and cognitive functions, and neuronal development and are essential components of neural membrane phospholipids (Horrocks & Farooqui, 2004). Insufficient VLCFAs, especially DHA, can influence neurotransmission, membrane-bound enzyme and ion-channel activities, gene expression, immunity and synaptic plasticity, and inflammation intensity (Horrocks & Farooqui, 2004).

Fatty acid elongation takes place in the endoplasmic reticulum (ER), using malonyl-CoA as a carbon source, but ACP (acyl carrier protein) is not involved. The final step of VLCFA biosynthesis (in the ER) involves one or more rounds of β-oxidation in the peroxisome (Ferdinandusse *et al.*, 2001). Therefore, a number of peroxisomal disorders can cause elevated serum concentrations of the VLCFAs. These disorders include defects of peroxisome biogenesis and β-oxidation pathway defects (Vreken *et al.*, 1998).

MPTP induces Parkinson's symptoms, which may be a result of decreased acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10 (Langston *et al.*, 1983; Dauer & Przedborski, 2003). Decreased acetyl-CoA production is a characteristic of GA II and may in several ways be linked to extrapyrimidal symptoms (Loots *et al.*, 2004). Decreased acetyl-CoA production, which prevents fatty acid elongation, is a possible explanation for neurological symptoms (caused by MPTP treatment). If this is the case, this problem could potentially be rectified by acetylcarnitine supplementation. Acetylcarnitine supplementation has already been shown to prevent the development of the clinical symptoms associated with

MPTP treatment (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003). However, it is not known if acetylcarnitine will increase the fatty acid elongation.

This study primarily investigates the hypothesis that the protective mechanism of acetyl-L-carnitine (ALCAR) may be at the level of fatty acid elongation in the cytoplasm and peroxisomes. Therefore, the VLCFA concentrations were determined using gas chromatography mass spectrometry (GC-MS) as described in section 4.2.2. These concentrations were expressed as the ratios C24:C22 and C26:C22 in order to detect possible defective enzymes in VLCFA metabolism, focusing on the peroxisomal β-oxidation and biogenesis pathways. The role of ALCAR in the metabolism of VLCFAs was considered in the presence and absence of ALCAR, before and after a single treatment on *Sprague Dawley* rats with MPTP.

## 4.2. Experimental procedure

### 4.2.1. Materials

The chemicals used including their harmful effects, are listed in table 4.1.

***Table 4.1. Harmful effects of the chemicals used for the determination of VLCFAs.***

| Chemicals | Company | Harmful effects |
|---|---|---|
| Acetonitrile | Merck | Highly flammable, harmful |
| Hexane | Merck | Highly flammable, harmful, dangerous |
| Hydrochloric acid | Merck | Corrosive |
| Methanol | Merck | Highly flammable, toxic |
| MTBSTFA | Sigma | Harmful |
| Potassium hydroxide | Merck | Corrosive |
| Pyridine | Merck | Highly flammable, harmful |
| Sodium hydroxide | Merck | Corrosive |
| Toluene | Merck | Highly flammable, harmful |

Stock solutions of standards and deuterium labeled standards

100 ml stock solutions, each for both the standards and deuterium labeled internal standards, were made up in toluene at the following concentrations:

- 0.25 mmol/L pristanic acid
- 0.25 mmol/L phytanic acid
- 1 mmol/L C22:0
- 1 mmol/L C24:0
- 0.5 mmol/L C26:0

These solutions must be stored at 2 - 8°C.

Working solutions

Stock solutions were diluted to the following concentrations:

- 1 µmol/L pristanic acid
- 4 µmol/L phytanic acid
- 50 µmol/L C22:0
- 50 µmol/L C24:0
- 1 µmol/L C26:0

These solutions must be stored at 2 – 8°C and remain stable for approximately 5 years.

Acid hydrolysis reagent: 0.5 M HCl in acetonitrile

37 % HCl and acetonitrile were mixed in a ratio of 1:23. If a different strength HCl is used, the volume should be adapted. This reagent must be stored at room temperature and prepared freshly every week.

Alkaline hydrolysis reagent: 1 M NaOH in methanol

NaOH (8g) was mixed with 10 ml milli Q water and made up to 200 ml with methanol. This solution must be stored at room temperature and will remain stable for one year.

1 M KOH

KOH (56g), made up to 1L with milli Q water. This solution remains stable for one year and must be stored at room temperature.

### 4.2.2 Procedure

This is a routine procedure used in the Biochemistry Metabolic Laboratory, of the North-West University and was adapted from Vreken and coworkers (1998).

a) Sample preparation

Serum specimens were obtained from 40 rats - 20 each in the control and experimental groups. Blood samples were collected in coagulation tubes, centrifuged at 3000 x g for 5 minutes at room temperature and the serum transferred to new tubes. The serum samples were stored at - 4°C until the day of analysis.

b) Acid hydrolysis

Serum (100 µL) was mixed with 100 µL internal standard solution and 2 ml acid hydrolysis reagent in a kimax tube and vortexed for approximately 3 seconds. The samples were hydrolyzed for 45 ± 5 minutes at 110 ± 10 °C and left to cool down to room temperature.

c) Alkaline hydrolysis

Alkaline hydrolysis reagent (2 ml) was added to the samples which were then vortexed for approximately 3 seconds. The samples were hydrolyzed for 45 ± 5 minutes at 110 ± 10 °C and left to cool down to room temperature.

d) Extraction of fatty acids

After cooling to room temperature, the pH was lowered by adding 400 µL 37% HCl. Hexane (4 ml) was added and the tube was shaken for 1 minute. The hexane layer was transferred to a new kimax tube.

e) Alkaline washing step

3.5 ml KOH was added to the hexane layer and shaken for 1 minute. The top layer was removed and the pH of the hexane layer lowered by adding 500 µL 37% HCl.

f) Extraction of fatty acids

Hexane (4 ml) was added and the tube was shaken for 1 minute. The hexane layer was transferred to a small kimax tube and evaporated to dryness under nitrogen.

g) Derivatization of fatty acids

50 µL MTBSTFA and 50 µL pyridine were added to the dried fatty acids which were incubated for 30 minutes at 80°C and evaporated to dryness under nitrogen. Hexane (200 µL) was added just before the GC-MS analysis. The reaction of MTBSTFA with fatty acids is illustrated in figure 4.1.

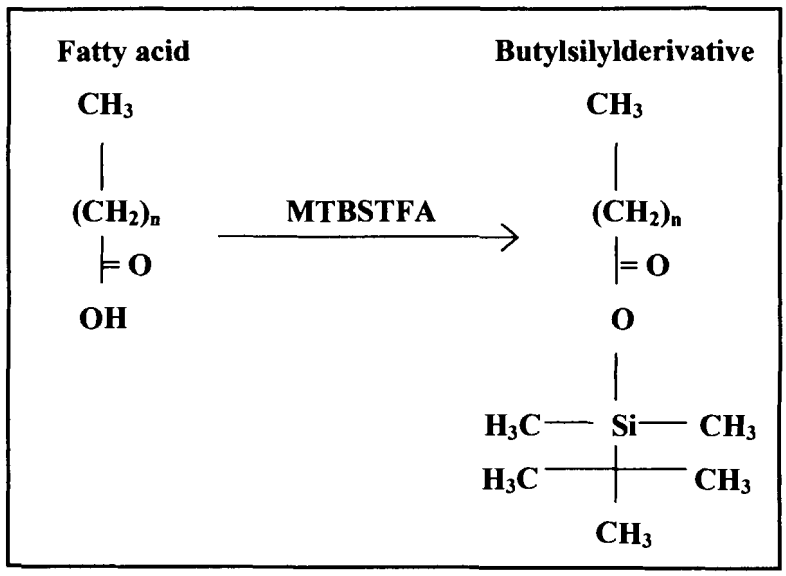



***Figure 4.1.*** ***Derivatization of fatty acids.*** *Fatty acids are derivatized by MTBSTFA to form a butylsilylderivative (Adapted from Vreken et al., 1998).*

h) GC-MS analysis

The fatty acids were analyzed on a Hewlett-Packard model 6890/5973 GC-MS system with a 120-0132 DB-1ms capillary column (30m x 0.25mm x 0.25µm) (Agilent Technologies, Chemetrix, Midrand, South Africa). The temperature program ran at 60°C

for 1 minute and increased with 30°C/min to 240°C, 10°C/min to 270°C and then with 4°C/min to 300°C. This temperature was maintained for 3 minutes. The sample was then injected (splitless mode) at 300°C. The injection volume was 1 µL and helium (14.99 psi) was the carrier gas. Electron impact ionization was applied at 0.7 eV. The mass spectrum was set on single ion monitoring (SIM) mode and monitored the characteristic $[M - 57]^+$ ions (Vreken *et al.*, 1998). Figure 4.2 represents an example of a VLCFA profile obtained by GC-MS-analyses.

***Table 4.2. Single ion monitoring (SIM).***

| Compound | $[M - 57]^+$ |
|---|---|
| C22:0 | 397.4 |
| $[^2H_4]$-C22:0 | 401.4 |
| C24:0 | 425.4 |
| $[^2H_4]$-C24:0 | 429.4 |
| C26:0 | 453.4 |
| $[^2H_4]$-C26:0 | 457.4 |

*MS acquisition was performed in the SIM (single ion monitoring) mode, monitoring the characteristic $[M - 57]^+$ ions (Vreken et al., 1998).*

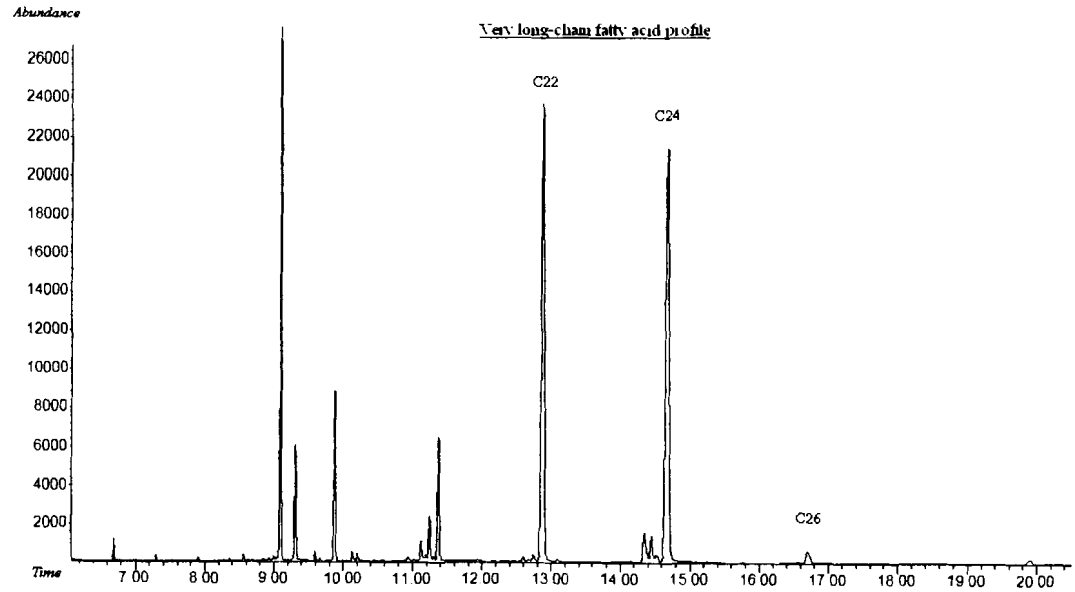



***Figure 4.2. Very long-chain fatty acid mass spectrum.*** *C22:0, C24:0 and C26:0 are indicated on the profile.*

i) Result revision

The GC-MS data was quantitatively analyzed using the HP-chemstation software. Fatty acid response factors (Rf-values) were determined as follows:

$$Rf = \frac{\text{Area under curve}_{(IS)}}{\text{Area under curve}_{(\text{fatty acid})}} \times \frac{\text{concentration}_{(\text{fatty acid})}}{\text{concentration}_{(IS)}}$$

The response factors were then used to determine the concentrations of the fatty acids:

$$[\,] = \frac{\text{Area under curve}_{(\text{Fatty acid})}}{\text{Area under curve}_{(IS)}} \times \text{concentration}_{(IS)} \times Rf$$

Rf = Response factors

Is = Internal standard

FA = Fatty acid

[ ] = Concentration of the fatty acids

j) Method and GC-MS analysis repeatability

Repeatability is the measurement of the spread around a test result from a single analyst and/or single analyzer at the same site on the same sample.

***Table 4.3.*** ***A comparison between the method repeatability obtained in this study (Marais, 2005) and those obtained from literature (Vreken et al., 1998).***

| Fatty acid | Correlation coefficient (%) (Marais, 2005) | Correlation coefficient (%) (Vreken *et al.*, 1998) |
|---|---|---|
| **C22:0** | 5.9 | 1.2 |
| **C24:0** | 5.8 | 0.9 |
| **C26:0** | 6.6 | 1.1 |

The correlation coefficient represents the standard deviation as percentage of the mean. For all the fatty acids it was smaller than 10, which is the norm in biological samples (Marais, 2005).

*Table 4.4.* ***A comparison between the GC-MS analysis repeatability obtained in this study (Marais, 2005) and those obtained from literature (Vreken et al., 1998).***

| Fatty acid | Correlation coefficient (%) (Marais, 2005) | Correlation coefficient (%) (Vreken *et al.*, 1998) |
|---|---|---|
| **C22:0** | 0.7 | 2.2 |
| **C24:0** | 0.2 | 3.1 |
| **C26:0** | 2.6 | 10.1 |

The correlation coefficient of this study (Marais, 2005) was much better than those obtained from literature (Vreken *et al.*, 1998).

### 4.3. Results and discussion

It is known that ALCAR protects against the neurotoxicity of MPTP. MPTP inhibits NADH dehydrogenase at complex I in the ETC (Soliman & Mazzio, 2003; Ebadi *et al.*, 2001) and causes a GA II profile due this inhibition (Loots *et al.*, 2004). Deficient acyl-CoA dehydrogenase, a mitochondrial β-oxidation enzyme, is one of the many causes of GA II (Frerman & Goodman, 1989).

Glutaric aciduria II (GA II) is an inborn error of glutaryl-CoA metabolism which is derived from ETF enzyme or ETF-ubiquinone oxidoreductase (Olsen *et al.*, 2003). GA II presents with extrapyramidal symptoms, similar to those of Parkinsons disease. The main characteristic in this metabolic defect is a reduced acetyl-CoA (essential for various anabolic pathways for example ketogenesis and fatty acid biosynthesis). VLCFAs and PUFAs play an important role in membrane integrity, especially nerve cell integrity, and therefore there is the possibility that a decreased production of acetyl-CoA in the above cases may lead to decreased biosynthesis of VLCFAs and the subsequent symptoms.

In previous studies, where decreased acetyl-CoA was the possible explanation for GA II, it was observed that ALCAR prevented some of the symptoms that occurred (Ricciolini *et al.*, 1998; Soliman & Mazzio, 2003). It could be argued that ALCAR may lead to increased acetyl-CoA with subsequent improved fatty acids biosynthesis to form LCFAs.

In this study, the focal point was to establish if ALCAR can participate in the protective mechanism of β-oxidation disorders. Therefore, the C24:C22 and C26:C22 ratios were determined in order to detect possible defective enzymes in VLCFA metabolism, focusing on the peroxisomal biosynthesis and β-oxidation pathway. The role of ALCAR on VLCFA metabolism was considered in the presence and absence of ALCAR, before and after a single treatment of *Sprague Dawley* rats with MPTP. Blood samples (serum) were analyzed using the GC-MS described in 4.2.2.

It is important to note that VLCFAs (C22:0, C24:0 and C26:0), and not PUFAs (DHA), were used as a marker to measure the chain lengthening process (biosynthesis) and to determine whether ALCAR activated this lengthening process. If ALCAR influenced the VLCFA biosynthesis, the same influence would occur in PUFA biosynthesis. We analyzed serum samples, therefore focusing on fatty acid elongation and oxidation in the peroxisomes and cytoplasm and not on the mitochondrial elongation of fatty acids.

Table 4.5 represents the range of values obtained for all of the rats in the control and experimental group, respectively, on that specific day. According to the results obtained (table 4.5) there were no significant differences between the two groups (control and experimental group) because their respective concentrations were within the same range.

However, the pooled concentration of C22:0 (table 4.6) gave statistical significant differences on days 1, 7 and 13 ($p < 0.05$) between the two groups. Significant differences ($p < 0.05$) were also detected on day 7, 13 and 14 for the C24:0 concentrations. The C26:0 concentrations between the groups per day did not vary ($p > 0.05$).

*Table 4.5. VLCFA concentration ranges of the control and experimental groups on days 0, 1, 7, 13 and 14.*

| Concentrations | Days | Control group [ ] Range | Experimental group [ ] Range |
|---|---|---|---|
| [C22:0] µMol/L | 0 | 16.22-55.73 | 16.26-46.76 |
| | 1 | 11.62-51.34 | 24.47-39.59 |
| | 7 | 10.47-49.97 | 19.66-32.14 |
| | 13 | 12.31-56.97 | 20.57-36.73 |
| | 14 | 22.84-42.78 | 18.00-30.4 |
| [C24:0] µMol/L | 0 | 6.86-28.29 | 6.86-24.99 |
| | 1 | 7.01-28.80 | 15.06-22.89 |
| | 7 | 7.24-34.78 | 13.86-20.88 |
| | 13 | 8.81-31.84 | 13.74-23.02 |
| | 14 | 15.96-28.79 | 12.48-17.72 |
| [C26:0] µMol/L | 0 | 1.36-8.86 | 1.36-2.75 |
| | 1 | 1.36-8.74 | 1.53-4.08 |
| | 7 | 1.09-19.39 | 1.82-3.25 |
| | 13 | 0.94-5.59 | 1.51-4.33 |
| | 14 | 1.50-3.99 | 1.67-6.16 |

The differences (table 4.6) on day 1, 7 and 13 can be attributed to up and down regulation. ALCAR was administered from day 1 to 14 in the experimental group and could have influenced their C22:0 and C24:0 concentrations by activating the VLCFA biosynthesis. According to this theory, on day 1, when ALCAR treatment was initiated in the experimental group, the VLCFA biosynthesis was activated. On the following days, down regulation took place and the concentrations decreased below the norm and were subsequently counteracted by up regulation which increased VLCFA concentrations. This was again followed by down regulation and up regulation and so forth. This pattern is normal and occurs daily in individuals. Although differences were detected, ALCAR did not have a significant effect on the VLCFA levels. The reason for

this might be that for ALCAR to stimulate VLCFA biosynthesis, the VLCFA concentrations need to be substantially lowered (for example after MPTP treatment).

MPTP, which increases the excretion of ALCAR and free carnitine (Loots, 1998) and inhibits the VLCFA biosynthesis, was administered on day 14 to both groups. MPTP was only administered once because of its toxicity to the rats. No differences in the concentration ranges detected on day 14 with MPTP can be attributed to the toxicity of MPTP, which led us to determine the VLCFA concentrations only 6 hours after MPTP administration. Because of the relatively long half-life of fatty acids it was not possible to detect the potential effect of MPTP on VLCFA concentrations.

The differences observed between the control and experimental groups were not significant enough to suggest that the protective mechanism of ALCAR is on the level of VLCFA metabolism and more specifically on the C22:0, C24:0 and C26:0 concentrations.

***Table 4.6.*** ***Average VLCFA concentrations of the control and experimental groups on days 0, 1, 7, 13 and 14. (See statistics in appendix A).***

| Concentration | Day | Average [ ] of Control group | Average [ ] of Experimental group | p value |
|---|---|---|---|---|
| C22:0 µMol/L | 0 | 41.56 | 35.74 | p > 0.05 |
| | 1 | 37.55 | 31.35 | 0.0070 |
| | 7 | 18.86 | 26.12 | 0.0009 |
| | 13 | 31.51 | 30.15 | p > 0.05 |
| | 14 | 28.74 | 22.53 | 0.0157 |
| C24:0 µMol/L | 0 | 21.69 | 18.41 | p > 0.05 |
| | 1 | 21.40 | 18.59 | p > 0.05 |
| | 7 | 12.44 | 17.98 | 0.0002 |
| | 13 | 21.72 | 18.43 | 0.0076 |
| | 14 | 20.92 | 14.46 | 0.00002 |
| C26:0 µMol/L | 0 | 3.16 | 2.18 | p > 0.05 |
| | 1 | 3.18 | 2.72 | p > 0.05 |
| | 7 | 3.73 | 2.59 | p > 0.05 |
| | 13 | 2.59 | 3.07 | p > 0.05 |
| | 14 | 2.56 | 2.84 | p > 0.05 |

VLCFAs (C24:0 and C26:0) cannot be β-oxidized by the mitochondria and therefore have to undergo a few β-oxidation cycles in peroxisomes to C22:0 before they get transported to the mitochondria. Fatty acid concentrations were determined and expressed in consequential ratios because these fatty acid ratios should give an indication of elevated C22:0, C24:0 and C26:0 concentrations and detect possible enzyme defects in the peroxisomal fatty acid oxidation pathway. The C24:C22 ratio could also be an indication of a defect in the cytoplasm (ER) or peroxisomes. If the C22:0 and C24:0 fatty acid biosyntheses are impaired in the cytoplasm or peroxisomes, this biosynthesis could still occur in the mitochondria, thus restoring the C22:0 and C24:0 levels and the defect cannot be detected. The C26:C22 ratio is indicative of peroxisomal disorders only because C26:0 fatty acids cannot be synthesized in the mitochondria. The C26:C22 ratio should therefore establish whether ALCAR has an influence on the peroxisomal biosynthesis and/or oxidation pathways. The C24:C22 and C26:C22 ratios should collectively, indicate whether ALCAR protects against MPTP neurotoxicity via the activation of the elongation process of fatty acids in the cytoplasm and peroxisomes.

Some advantages for the use of ratios are:

- It is more sensitive for the detection of abnormalities than the concentrations as such.
- Ratios eliminate the effects of other factors like kidney failure.
- Creatinine determination and internal standards (which are expensive) are unnecessary.

According to table 4.7 the C24:C22 and C26:C22 ratios between the control and experimental groups gave no indication of a default in peroxisomal oxidation. The VLCFA concentrations in table 4.6 fluctuated from day to day, but the ratios in table 4.7 stayed the same. It seemed that although VLCFA synthesis could have been stimulated and all the VLCFAs were increased, the ratios stayed the same; indicating that there was no influence on the peroxisomal biogenesis and/or oxidation pathway by ALCAR.

*Table 4.7. A comparison of the average C24:C22 and C26:C22 ratios in the control and experimental groups on day 0, 1, 7, 13 and 14.*

| Ratios | Days | Control group | Experimental group |
|---|---|---|---|
| C24:C22 | 0 | 0.423-0.589 | 0.421-0.606 |
| | 1 | 0.453-0.997 | 0.523-0.653 |
| | 7 | 0.482-0.884 | 0.645-0.724 |
| | 13 | 0.559-0.947 | 0.427-0.689 |
| | 14 | 0.664-0.809 | 0.534-0.7166 |
| C26:C22 | 0 | 0.048-0.159 | 0.047-0.083 |
| | 1 | 0.052-0.170 | 0.048-0.110 |
| | 7 | 0.080-0.388 | 0.075-0.129 |
| | 13 | 0.052-0.184 | 0.032-0.155 |
| | 14 | 0.055-0.138 | 0.087-0.203 |

The differences observed in the C24:C22 ratios were not significant enough to conclude that the protective mechanism of ALCAR is on the level of peroxisomal β-oxidation. It is important to note, that ALCAR treatment (for 14 days), used in many peroxisomal defects, could be regarded as safe, since it has little/no influence on the VLCFA metabolism.

The normal C24:C22 and C26:C22 ratios for humans as determined by Vreken and coworkers (1998) are as follows:

- C24:C22 range form 0.689 - 1.008
- C26:C22 range from 0.011 - 0.022

When one compares the results of this study to the C24:C22 values of Vreken and coworkers (1998) it is evident that it falls within the same range. The C26:C22 ratios however did vary, and could be explained by possible differences in working environment, species (rats versus humans), and treatment (none for humans and ALCAR for rats).

# Chapter Five

## Effect of ALCAR on the acetyl- and acylcarnitine concentrations

### 5.1. Introduction

ALCAR is an acetyl ester of the amino acid, L-carnitine, and a delivery form for both L-carnitine (an essential cofactor required for mitochondrial fatty acid oxidation) and acetyl groups (Soliman & Mazzio, 2003; Fontaine *et al.*, 1996). The most important role of carnitine involves the transport of fatty acids, however, it seems that acetylcarnitine may also be involved in fatty acid elongation. Activated acetate units in the cytoplasm, for which glucose is the major source, are physiologically provided by the mitochondrial conversion of acetyl-CoA to citrate. This citrate leaves the mitochondrion and serves as a cytoplasmic acetyl-CoA source by enzymatic cleavage of citrate. Ricciolini and co-workers (1998) found that ALCAR is preferentially used for elongation of PUFAs versus *de novo* synthesis in the cytoplasm. They also implicated that an oxidative pathway producing ALCAR gives rise to the acetyl units used as precursors for the elongation of PUFAs (Ricciolini *et al.*, 1998; Wanders & Tager, 1998; Kunau *et al.*, 1995; Ramsay & Zammit, 2004).

L-carnitine mediates VLCFA and LCFA transport across the MIM into the mitochondrial matrix and buffers the acyl-CoA:CoA ratio (Hoppel, 2003; Soliman & Mazzio, 2003). ALCAR is more efficiently absorbed into the bloodstream, passes more readily through cell membranes, and is more efficiently utilized by the mitochondrion than L-carnitine (Soliman & Mazzio, 2003).

Exogenously administered ALCAR crosses the BBB through the GABA uptake system (Soliman & Mazzio, 2003; Pettegrew *et al.*, 2000). Extensive medical research has shown that ALCAR may have neuroprotective, cardioprotective, cytoprotective, and anti-apoptotic activity and is an antioxidant (Lolic *et al.*, 1997). ALCAR is effective in reducing age-dependent mitochondrial functional decay, as well as the loss of

mitochondrial membrane potential, cardiolipin content, metabolic oxygen ($O_2$) consumption, and β-oxidation of fatty acids (Hagen *et al.*, 2002; Gadaleta *et al.*, 1998). ALCAR can prevent MPTP toxicity in non-human primates (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003). A part of ALCAR's defensive mechanisms is the interaction with organic cation transporters such as OCTN2 and/or carnitine-acylcarnitine translocase (CACT). ALCAR may attenuate hydroxyl free radical generation in the MPTP/$MPP^+$ neurotoxic pathway (Loots *et al.*, 2004). ALCAR's defense against $MPP^+$ can be obtained by upholding cellular energy stores, by providing adequate glucose to achieve the glycolysis demands (Chalmers-Redman *et al.*, 1999; Mazzio & Soliman, 2003).

In previous studies we found that experimental animals like baboons and rats treated with MPTP (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003) developed a metabolic profile similar to GA II, which could indicate an induced deficiency similar to GA II (Loots *et al.*, 2004). It is also known that MPTP can cause neurological damage which leads to clinical symptoms similar to Parkinson's disease. These symptoms may be the result of decreased acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10 by MPTP. If the neurological symptoms are the result of decreased acetyl-CoA production which could prevent fatty acid elongation, the problem could potentially be rectified by supplementation with acetylcarnitine. Fatty acid elongation takes place in the cytoplasm (ER) or peroxisome, and in the mitochondria. For the elongation in the ER, malonyl-CoA is used as a carbon source and for the mitochondrial elongation system, acetyl-CoA or acetylcarnitine.

This study is primarily focussed on the protective mechanism of acetyl-L-carnitine (ALCAR) and the possibility that the protection may be at the level of fatty acid elongation in the cytoplasm and peroxisomes.

A new technique, based on isotope-dilution tandem mass spectrometry, for quantifying carnitine in small volumes of plasma, whole blood and urine has been developed. This method identifies amino acids and carnitines simultaneously. Electrospray tandem mass

spectrometry analyzes the prepared and derivatised samples. Isotopically labeled acylcarnitines are added to the samples. Ions of the derivatised compounds are produced and enter the mass analyzer first. Precursor ions are fragmented by colliding them with argon gas in the collision cell which is then analyzed in a second mass analyzer according to their mass. The relation of endogenous metabolites to the internal standards is determined by measuring the virtual intensities of the mass spectra of the peaks attained. Acylcarnitines are identified by scanning precursor ions that yield a common mass fragment of 85 Da.

## 5.2. Experimental procedure

### 5.2.1. Materials

*Table 5.1.* ***Safety precautions and harmful effects of the chemicals used for this research.***

| Chemicals | Safety Precautions | Harmful effects |
|---|---|---|
| Acetonitrile (methyl cyanide) | Causes eye, skin and respiratory tract irritation. Use protectice clothing, gloves and goggles. Keep away from heat. | Highly flammable<br>Harmful |
| Acetylcarnitine | Causes eye, skin, respiratory- and digestive tract irritation. Use protective goggles and gloves. | Harmful |
| Acetylchloride | May explode when in contact with water. Wear a mask, goggles and gloves and work in a fume cupboard. | Harmful<br>Corrosive<br>Highly flammable |
| Butanolic hydrochloride | Wear protective clothing, gloves and goggles. | Toxic<br>Corrosive<br>Highly flammable |

| | | |
|---|---|---|
| Formic acid | Causes eye, skin, respiratory tract and mucous membrane irritation. Wear protective clothing, gloves and goggles. Inhalation may be fatal. | Harmful Corrosive |
| Water | None | None |

Preparation of butanolic hydrochloride

With butylation a butaryl group is added to the carboxylic end of the molecules in the sample, which leads to the stabilization of compounds and contributes towards canceling out similar molecular masses during the analysis.

Butanol (50 ml) is cooled in a beaker on ice for 5 minutes. Acetylchloride (12.5 ml) is added dropwise (this step generates heat), while stirring frequently. The solution is covered with parafilm and left on ice for 20 minutes. This solution remains stable for one week.

Acetonitrile:Water 50 % (v/v)

Add 500 ml of acetonitirile slowly to Milli Q water (500 ml) in a reagent bottle, followed by sonification for at least 20 minutes to remove any air which may be in the reagent.

Carnitine isotopes (stock solutions)

A. Acetylcarnitine: 5 mg/100ml (20.83 µmol)
B. Propionylcarnitine: 5 mg/100ml (19.69 µmol)
   Isovalerylcarnitine: 5 mg/100ml (17.73 µmol)
   Octanoylcarnitine: 5 mg/100ml (15.43 µmol)
C. Palmitoylcarnitine: 5 mg/100ml (11.47 µmol)
D. Free carnitine: 5 mg/100ml (30.446 µmol)

Dilutions of the different carnitine isotopes:

Take 1 ml of stock solution A, 2 ml of stock solution D and 1 ml of stock solution C. For a urine sample add 250 µl of B and for a blood sample, 125 µl of B.

Internal standards

Place approximately 500 ml of methanol in a 1 L volumetric flask. Measure out the volumes of each internal standard stock solution, indicated in table 5.2., into the flask and make up to 1L with methanol.

***Table 5.2. Unlabelled carnitine***

| **Compound** | **Mr (g/mol)** | **Stock solution concentration (5mg/100ml)** |
|---|---|---|
| Free carnitine | 162 g/mol | 308.64 µmol/L |
| Acetylcarnitine | 204 g/mol | 245.09 µmol/L |
| Propionylcarnitine | 218 g/mol | 229.36 µmol/L |
| Isovalerylcarnitine | 246 g/mol | 203.25 µmol/L |
| Octanoylcarnitine | 288 g/mol | 173.611 µmol/L |
| Palmitoylcarnitine | 400 g/mol | 125.0 µmol/L |

### 5.2.2. Procedure

a) Creatinine determinations

Creatinine determination must be done on urine samples before extraction to normalize results (Chalmers *et al.*, 1976). The creatinine values are expressed in µmol/L. Creatinine values are determined spectrophotometrically by a standard procedure using the UV 3.0 Spectro, Biotech, Roche.

Normal plasma or serum creatinine values are 62-132 µmol/L and 4300-9300 µmol/L in urine.

b) Urine protocol

Transfer 100 µL of urine into the first micro-centrifuge tube and centrifuge for 20 minutes at 13000 xg.

Transfer 10 µL of the above sample to the second micro-centrifuge tube, add 410 µL of the isotope mixture. Centrifuge for 20 minutes at 13000 xg.

Transfer all the liquid to the third micro-centrifuge tube, dry under nitrogen at 65°C for 60 to 90 minutes. Butalyze with 200 µL butylhydrochloride at 65°C for 15 minutes. Dry immediately under nitrogen for 60 minutes.

Dissolve the above mixture in 100 µL, 20:80 distilled water : acetonitriel and 1 % formic acid. Vortex for approximately 3 seconds and transfer 100 µL of the mixture to the LC-MS/MS inserts.

Inject the samples into the VG Quatro tandem mass-spectrometer (injection volume 25 µL).

c) Serum protocol

Transfer 10 µL serum to the first micro-centrifuge tube and add 410 µL of the isotope mixture. Centrifuge for 20 minutes at 13000 xg.

Transfer all of this solution to the second micro-centrifuge tube and centrifuge for 20 minutes at 13000 xg.

Transfer the solution to a third micro-centrifuge tube, dry under nitrogen at 65°C for 60 to 90 minutes. Butalyze with 200 µL butyl-hydrochloride at 65°C for 15 minutes. Dry immediately under nitrogen for 60 minutes.

Dissolve the above mixture in 100 µL 20:80 distilled water : acetonitriel and 1 % formic acid. Vortex for approximately 3 seconds and transfer 100 µL of the mixture to the LC-MS/MS inserts.

Inject the samples into the VG Quatro tandem mass-spectrometer (injection volume 25 µL)

d) Preset LC-MS/MS settings

This procedure is based on electrospray ionization (ESI). It is a "soft ionization" technique where little fragmentation of the ions occurs before they reach the detector. Acylcarnitines are detected using a "precursors of 85" scan.

Tuning Parameters: ES+

| | |
|---|---|
| Capillary | 3.50 kVolts |
| HV Lens | 0.50 kVolts |
| Cone | 35 Volts |
| Skimmer Offset | 5 Volts |
| Skimmer | 1.5 Volts |
| RF Lens | 0.2 Volts |
| Source Temperature | 75 °C |

Mass Spectrometer 1:

| | |
|---|---|
| Ion Energy | 1.0 Volts |
| Ion Energy Ramp | 0.0 Volts |
| LM Resolution | 15.0 |
| HM Resolution | 15.0 |
| Lens 5 | 100 Volts |
| Lens 6 | 5 Volts |
| Multiplier | 750 Volts |

Mass Spectrometer 2:

| | |
|---|---|
| Ion Energy | 1.0 Volts |
| Ion Energy Ramp | 0.0 Volts |
| LM Resolution | 13.0 |
| HM Resolution | 13.0 |
| Lens 7 | 250 Volts |
| Lens 8 | 40 Volts |
| Lens 9 | 5 Volts |
| Multiplier | 750 Volts |

Pressures:

| | |
|---|---|
| Analyser Vacuum | 2.3e-5 mBar |
| Gas Cell | 1.4e-3 mBar |

Acylcarnitine analysis functions:

| | |
|---|---|
| Scans in function | 1 |
| Cycle Time (secs) | 3.510 |
| Scan Duration (secs) | 3.47 |
| Interscan Delay (secs) | 0.04 |
| Retention Window (mins) | 0.600 to 2.000 |
| Ionization Mode | ES+ |
| Data Type | Accurate mass |
| Function Type | Parents of 85.20 |
| Mass Range | 210 to 650 |
| Cone Voltage | 35 |
| Collision Energy | 25.0 |

Figure 5.1 is an example of an MS spectrum obtained for a serum/urine sample.

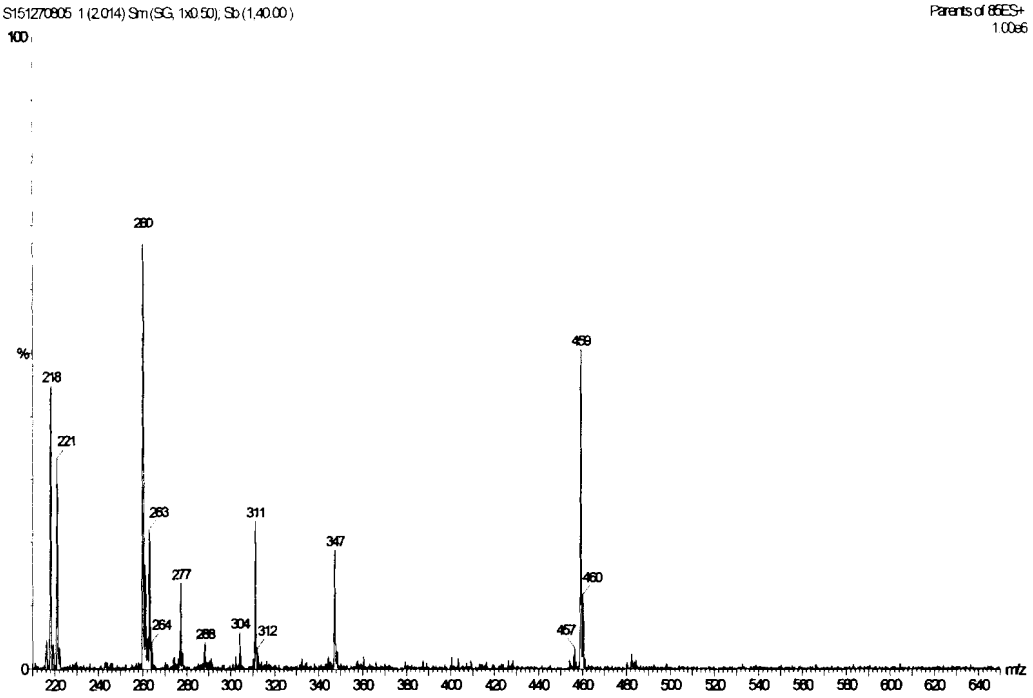



***Figure 5.1.*** ***An acyl- and acetylcarnitine mass spectrum.*** *218 = free carnitine, 260 = acetylcarnitine, 304 = 3-hydroxybutyrylcarnitine.*

e) Result revision

The acylcarnitine concentration ($C_{Acylcarnitine}$) is determined using the following formula:

$$C_{Acylcarnitine} = \frac{\dfrac{\text{Area under curve}_{(Acylcarnitine)} \times \mu l \text{ internal standard}}{\text{Area under curve}_{(Acylcarnitine\ isotope)} \times \mu l \text{ urine}} \times \text{stock solution (nmol/L)}}{\text{Creatinine (mmol/L)}}$$

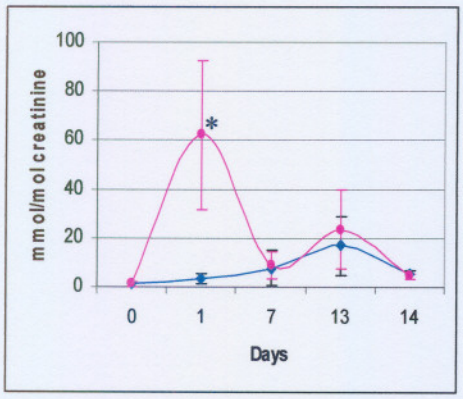
## 5.3. Results and discussion

Experimental animals treated with MPTP developed a metabolic profile similar to GA II and exhibited clinical symptoms similar to Parkinson's disease. These neurological symptoms may be the result of decreased acetyl-CoA production (due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10 by MPTP, and is characteristic of GA II), which prevents fatty acid elongation. If this is the case, this problem could potentially be rectified by acetylcarnitine supplementation. The question if acetylcarnitine will increase fatty acid elongation is the subject of this investigation.

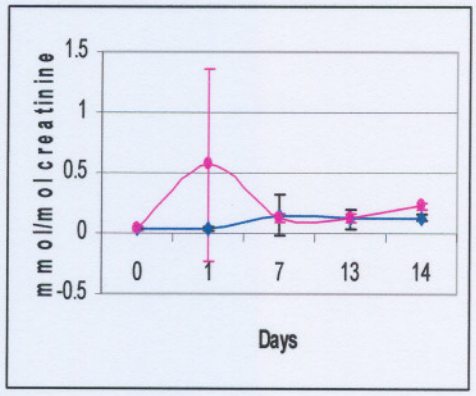
It is known that only butyryl-CoA, adipic acid, suberic acid and glutaric acid of the GA II metabolites conjugate with carnitine to form 3-hydroxybutyrylcarnitine, adipylcarnitine, suberylcarnitine and glutarylcarnitine (Loots, 1998; Chalmers & Lawson, 1982). In this study, the effects of ALCAR treatment over a period of two weeks were determined to firstly establish whether ALCAR could have an effect on the elongation of fatty acids in the cytoplasm or peroxisome over the 14 days' period and secondly how it would effect acylcarnitine concentrations. GA II metabolites conjugate with free carnitine, derived from acetylcarnitine to form acylcarnitines, which are then excreted in higher concentrations.

The results show (see figure 5.2, graph a, b, c, d and e) that the concentration versus time curves follow the same tendency: acetylcarnitine and acylcarnitine concentrations in the experimental group reached a maximum on day 1 (the first day of treatment with ALCAR), decreased and increased during the following days (probably due to up and down regulation) and increased on day 14 after the MPTP treatment in the experimental group.

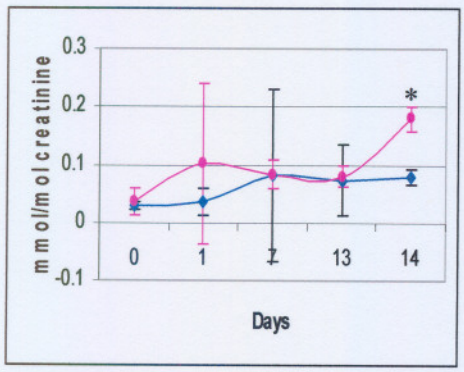
a) Acetylcarnitine



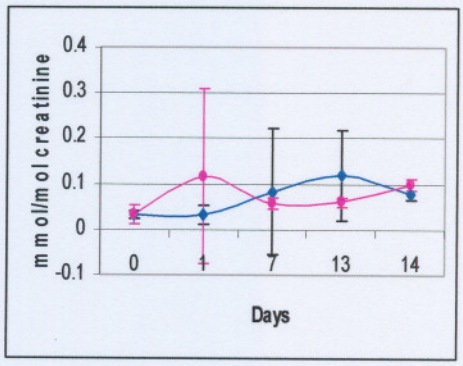

b) Butyrylcarnitine



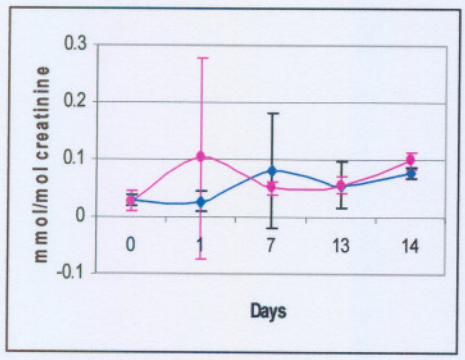

c) Glutarylcarnitine




d) Adipylcarnitine




e) Suberylcarnitine




* = *Statistical significant differences.*

***Figure 5.2.*** ***Acylcarnitine concentrations of the control and experimental groups and their corresponding standard deviations.***

*Experimental group, injected with ALCAR for 2 weeks and a single dose of MPTP on day 14.*

*Control group, injected with Saline for 2 weeks and a single dose of MPTP on day 14.*

a) Acetylcarnitine

The results obtained (see figure 5.2(a)) show that the acetylcarnitine concentrations in the experimental group reached a maximum on day 1 (the first day of treatment with ALCAR) and then stabilized in the following days. This is likely caused by the initiation of the ALCAR treatment (day 1), which might have led to the stimulation of the VLCFA biosynthesis, causing an increase in the free carnitine and ALCAR available and thus causing the acetylcarnitine concentrations to be significantly higher than the control groups ($p < 0.05$)(see statistics in appendix B).

During the following days (2 to 13) the concentrations of both groups were relatively the same, probably due to the ability of the test animals to regulate the VLCFA biosynthesis through up and down regulation. We speculate that in the days to follow, the concentrations were lowered to below the normal values through down regulation in an attempt to reach the normal values. The latter phenomenon was then counteracted by up regulation and the VLCFA concentrations increased again to above normal. This process repeated itself continually and is applicable to both groups. MPTP (administered on day 14) increased the excretion of ALCAR and free carnitine, which may be attributed to its effect on the kidneys and renal absorption through the ETC interference. Before MPTP treatment, pretreatment with ALCAR is essential to replenish the acetylcarnitine and free carnitine concentrations that will be reduced and to prevent Parkinsonian symptoms.

***Table 5.3.*** ***Acetylcarnitine concentrations on various days.*** *The red values indicate statistical significance ($p < 0.05$).*

| Day | Average [ ] Control group | Average [ ] Experimental group | p value |
|---|---|---|---|
| **0** | 1.20718 | 1.20718 | 1.000000 |
| **1** | 3.25932 | 61.9971 | **0.002189** |
| **7** | 7.57805 | 8.91606 | 0.671573 |
| **13** | 16.8066 | 23.5893 | 0.310876 |
| **14** | 5.26189 | 4.47850 | 0.227968 |

b) Butyrylcarnitine

The results did not show any statistical significance ($p > 0.05$) between the two groups although the graph (figure 5.2(b)) does indicate higher values on day 1 and 14 (see statistics in appendix B).

c) Glutarylcarnitine

Glutarylcarnitine concentrations in the experimental group tended to be higher on day 1 than those of the control group (see figure 5.2(c)), however it was not statistically significant ($p > 0.05$). MPTP is responsible for the inhibition of the VLCFA biosynthesis and therefore stimulates the production of glutaric acid. On day 14 the glutarylcarnitine concentrations of the experimental group, after the administration of MPTP, were indeed higher than those of the control group ($p < 0.05$)(see statistics in appendix B). This may be caused by the glutaric acid (a GA II metabolite) conjugating with the free carnitine to form glutarylcarnitine. The higher levels of glutarylcarnitine in the experimental group, when compared with the controls, suggest that ALCAR plays a role in the detoxification of glutaric acid.

***Table 5.4.*** ***Glutarylcarnitine concentrations on various days.*** *The red values indicate statistical significance ($p < 0.05$).*

| Day | Average [ ] Control group | Average [ ] Experimental group | p value |
|---|---|---|---|
| **0** | 0.028 | 0.035 | 0.381978 |
| **1** | 0.035 | 0.102 | 0.171724 |
| **7** | 0.083 | 0.082 | 0.991401 |
| **13** | 0.073 | 0.079 | 0.795161 |
| **14** | 0.079 | 0.180 | **0.000000** |

d) Adipylcarnitine

Although figure 5.2(d) shows slightly elevated levels of adipylcarnitine in the experimental group on days 1 and 14, no statistical significant difference ($p > 0.05$) could be established between the two groups (see statistics in appendix B).

e) Suberylcarnitine

Figure 5.2(e) shows a similar tendency for suberylcarnitine as above, where no statistical significant difference ($p > 0.05$) could be detected between the two groups (see statistics in appendix B).

All the acylcarnitine curves of the experimental group showed the same tendency: the acylcarnitine's concentrations increased on day 1 (only acetylcarnitine statistical difference – $p = 0.002189$), stabilized during the rest of the treatment and then increased again on day 14 (only glutarylcarnitine statistical difference – $p = 0.000000$), after the MPTP treatment. On day 14, when a GA II profile is developed after the MPTP treatment, the ALCAR stimulates the VLCFA biosynthesis and ensures a higher concentration of free carnitine available and leads to increased detoxification. This could be the reason for the acylcarnitine's concentrations of the experimental group to be higher than those of the control group.

Important to note is that of the conjugates measured, only adipic, suberic, glutaric acid and butyryl-CoA (a direct precursor of ethylmalonic and methylsuccinic acid) are GA II metabolites that conjugate with free carnitine. Acetylcarnitine however, is not a GA II metabolite and its concentration was determined in order to detect how administration of ALCAR influenced the acetyl- and other acylcarnitine concentrations.

# Chapter Six

## Conclusion and future studies

### 6.1. Conclusion

It is known that MPTP is accountable for neurological injury and toxicity to the dopaminergic neurons (Langston *et al.*, 1983; Dauer & Przedborski, 2003). The enzyme, MAO-B, is responsible for the conversion of MPTP to $MPP^+$, which accumulates in dopaminergic neurons and is actively transported into the mitochondria where it inhibits mitochondrial respiration at complex I of the electron transport chain, by blocking NADH:ubiquinone oxidoreductase (Soliman & Mazzio, 2003; Ebadi *et al.*, 2001). This causes a rapid loss of ATP and eventual cell death. MPTP induces parkinsonian symptoms and a GA II profile (Loots *et al.*, 2004). These neurological symptoms may be the result of decreased acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10 by MPTP. GA II is caused by a defect in ETF and/or ETF-QO, which accepts electrons from various acyl-CoA dehydrogenases (mitochondrial β-oxidation enzymes), thus leading to defective metabolism of these acyl-CoA dehydrogenase substrates (Frerman & Goodman, 1989).

ALCAR is an acetyl ester of the amino acid L-carnitine and is a delivery form for both L-carnitine and acetyl groups (Soliman & Mazzio, 2003). L-carnitine mediates both the transport of LCFAs across the MIM into the mitochondrial matrix, for oxidative phosphorylation, and the SCFA and MCFA transport out of the mitochondria, in order to maintain normal coenzyme A levels. In addition, ALCAR provides the acetyl component for the formation of acetylcholine (a neurotransmitter) and acetyl-CoA. This contributes to oxidative phosphorylation by providing increased energy substrates to the Krebs cycle (Soliman & Mazzio, 2003; Dolezal & Tucek, 1981). Therefore, through upholding cellular energy stores by providing adequate glucose to achieve the glucolysis demands, ALCAR protects against $MPP^+$ toxicity (Chalmers-Redman *et al.*, 1999; Mazzio & Soliman, 2003). In previous studies it was found that experimental animals treated with

MPTP (Bodis-Wollner *et al.*, 1991; Soliman & Mazzio, 2003) developed a metabolic profile similar to GA II, suggesting that it may be possible to induce a deficiency similar to GA II (Loots *et al.*, 2004). It is also known that MPTP causes neurological damage which leads to clinical symptoms similar to Parkinson's disease. MPTP causes a decrease of acetyl-CoA production due to the inhibition of the acyl-CoA dehydrogenase enzymes, ETF, or ETF-QO.

Glutaric aciduria is an inborn error of glutaryl-CoA metabolism which is derived from three possible enzyme defects namely: glutaryl-CoA dehydrogenase (GA I), ETF (GA II) and peroxisomal glutaryl-CoA oxidase (GA III). GA I and GA II, but not GA III, present with extra pyramidal symptoms, similar to those of Parkinsons disease. Since GA III patients do not develop these symptoms, one can assume that the extrapyramidal symptoms is not caused by glutaric acid but, that they are rather the result of the metabolic processes involved which coincidentally lead to the accumulation of glutaric acid. The common denominator in these metabolic defects is a reduced acetyl-CoA (essential for various anabolic pathways for example ketogenesis and fatty acid biosynthesis). VLCFAs and PUFAs play an important role in membrane integrity, especially nerve cell integrity.

In previous studies, where decreased acetyl-CoA was the possible explanation for these extrapyramidal symptoms, it was observed that ALCAR prevented some of the symptoms that occurred. It could be argued that ALCAR may lead to increased acetyl-CoA with subsequent improved fatty acid elongation (biosynthesis) to form LCFAs and VLCFAs.

In this study, the first objective was to elucidate the protective mechanism of ALCAR by investigating very long-chain fatty acid elongation in the cytoplasm and peroxisomes. To achieve this objective, rats were firstly treated with ALCAR, and subsequently with a single dosage of MPTP. The concentrations of the very long-chain fatty acids, which could be an indication of increased elongation, were determined before and after treatment with ALCAR. The ratios of a long-chain fatty acid (C22:0) and the various

very long-chain fatty acids (C24:0 and C26:0) were calculated to indicate the possibility of a defect in peroxisomal oxidation.

24 Carbon *n*-3 and *n*-6 fatty acids (polyunsaturated fatty acids) are synthesized in the endoplasmic reticulum and then transferred into the peroxisomes where they are partially degraded during β-oxidation, using malonyl-CoA as carbon source, to form 22:5*n*-6 and 22:6*n*-3. Peroxisomal β-oxidation is only proficient to shorten the chain but not to degrade fatty acids to completion (Wanders, 2004).

In short one can summarize peroxisomal β-oxidation as follows:

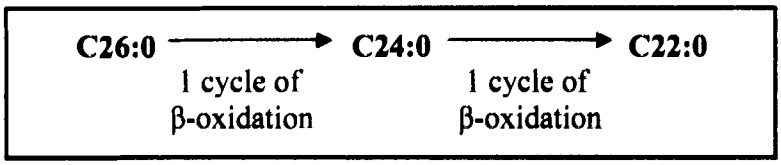



***Figure 6.1.*** ***A simplified schematic of the chain-shortening process of VLCFAs during the β-oxidation process.***

VLCFAs (C22:0, C24:0 and C26:0), not PUFAs, were used as a marker to measure the fatty acid biosynthesis, with the assumption that if ALCAR influenced the VLCFA biosynthesis, the same influence would occur in PUFA biosynthesis.

In this study, it was found that the C22:0 and C24:0 concentrations in the serum of the experimental group (receiving ALCAR for two weeks) were elevated on several days. It was assumed that this increase was possibly due to an increase in the acetylcarnitine concentrations, which could have increased the VLCFA concentrations by serving as a carbon source and activating the fatty acid elongation process. Our findings are in agreement with the concern raised by Dhopeshwarkar and Subramanian (1977). They questioned the ability of the citrate pathway to act as the sole provider of the acetyl-CoA pool for the synthesis of malonyl-CoA used for the elongation of essential PUFAs in the cytoplasm. Tuček (1993) implied that the citrate pathway may account for one-third of activated acetate units transferred from the mithochondria to cytoplasm for the synthesis of acetylcholine. The findings of Ricciolini et al. (1998) also supported the hypothesis

that an oxidative pathway producing ALCAR gives rise to the acetyl units used as precursors for the elongation of PUFAs in brain cytoplasm and mithochondria. Above research outlines the intricacy of the trafficking of acetate units between various cellular organelles.

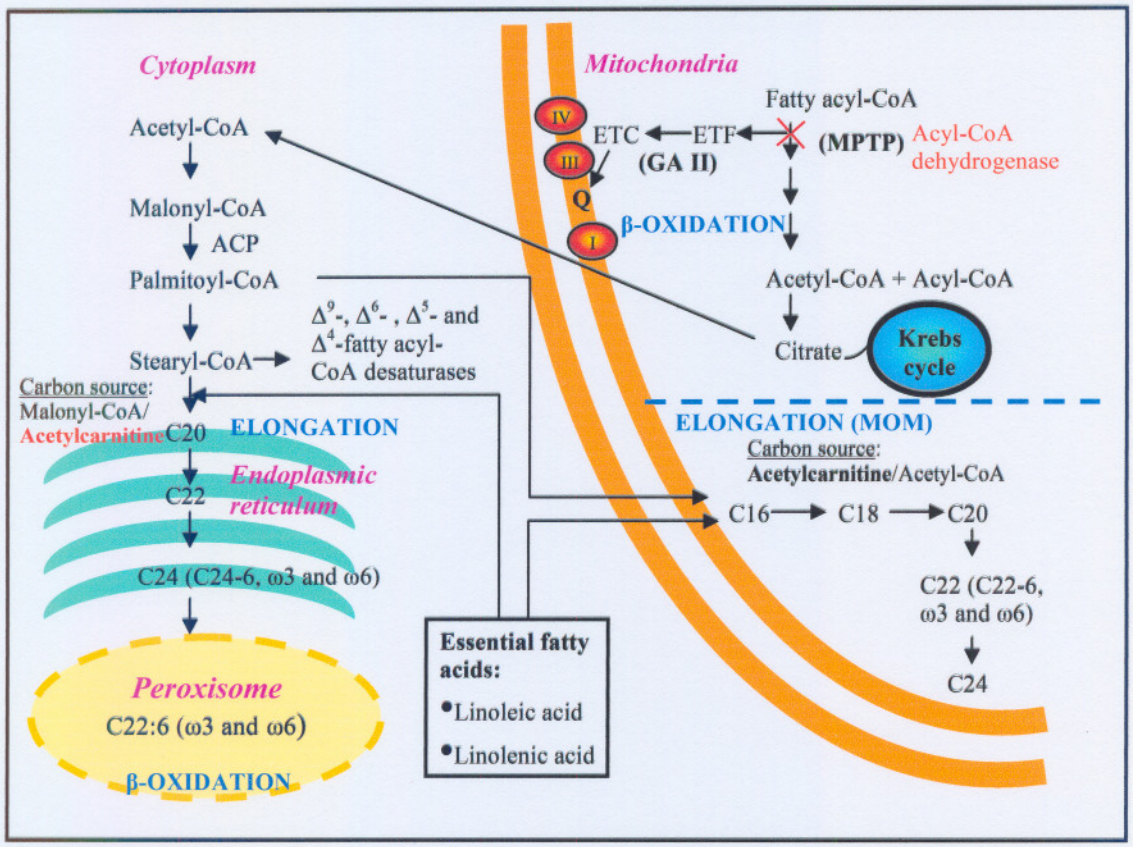



***Figure 6.2.*** ***The summary of this project.*** *There are 2 main biosynthesis or elongation pathways for fatty acids. The cytoplasm (ER, carbon source: malonyl-CoA) and peroxisomal elongation/biosynthesis pathway, and the mitochondrial elongation pathway (carbon source: acetylcarnitine or acetyl-CoA).*

The range of concentration values, obtained for all of the rats in the control and experimental group respectively, on specific days, showed no significant differences between the two groups because their respective concentrations were within the same range. However, the average pooled concentrations of C22:0 gave statistical significant differences on days 1, 7 and 13 ($p < 0.05$) and C24:0 on days 7, 13 and 14 ($p < 0.05$)

between the two groups. The average C26:0 concentrations between the groups per day did not vary ($p > 0.05$)(See chapter 4).

The VLCFA concentrations fluctuated from day to day attributed to up and down regulation, but the ratios stayed the same. It seemed that although VLCFA synthesis could have been stimulated and the VLCFAs concentrations increased, the ratios stayed the same; indicating that there was no influence on the peroxisomal biogenesis and/or oxidation pathway by ALCAR. The C24:C22 and C26:C22 ratios between the control and experimental groups gave no indication that MPTP caused a default in peroxisomal oxidation on day 14.

The second objective was to investigate the acetyl- and acylcarnitine concentrations (in urine). Mitochondrial β-oxidation is a metabolic process which is essential for the degradation of fatty acids to acetyl-CoA. The first step (and the step we are focusing on) in this pathway involves the conversion of fatty acyl-CoA to 2-trans-enoyl-CoA by the flavoenzyme, acyl-CoA dehydrogenase. There are four types of acyl-CoA dehydrogenases, short-chain- (SCAD), medium-chain- (MCAD), long-chain- (LCAD), and very long-chain acyl-CoA dehydrogenase (VLCAD) (Fromenty & Pessayre, 1995). These dehydrogenases are rather unusual in their specific requirements for ETF as electron acceptor (Heyer, 2002; Hall & Kamin, 1975). MPTP inhibits the acyl-CoA dehydrogenase enzymes, ETF, ETF-QO or even CoQ10 leading to a GA II profile. The metabolites excreted in GA II are derived mainly from acyl-CoA esters, normally oxidized by acyl-CoA dehydrogenases (Goodman & Markey, 1981; Goodman *et al.*, 1980; Rhead & Tanaka, 1979). These metabolites include glutaric acid, suberic acid, adipic acid and butyryl-CoA. When they conjugate with free carnitine they form glutarylcarnitine, suberylcarnitine, adipylcarnitine and butyrylcarnitine respectively, which are then excreted. ALCAR increases the concentration free carnitine available and is therefore involved in the detoxification of the GA II metabolites.

MPTP increases the excretion of ALCAR and free carnitine – this is thought to be due to the effect of MPTP on the kidneys and renal absorption. It is therefore important to

pretreat with ALCAR to ensure the replenishment of the lost ALCAR and carnitine and to prevent Parkinson's symptoms when the goal is to attenuate MPTP toxicity.

In this study it was observed that all the acylcarnitine graphs had the same tendency. Only acetylcarnitine concentration exhibited a significant difference on day 1 between the groups. On day 1 there was a large increase in the concentrations of the experimental group. The initial increase in the concentrations could be attributed to the initiation of the ALCAR treatment and the initial higher concentrations of free carnitine available and the stimulation of the VLCFA biosynthesis. Down regulation then lowered the concentrations to under the norm, which activated up regulation and led to concentrations which were increased to above the norm. Subsequently down regulation was then again activated and so forth with the cycle repeating itself. This is a normal process and occurred in both the control and experimental groups, although the control group did not receive ALCAR treatment.

On day 14 (MPTP treatment) the experimental group's acylcarnitine concentrations tended to be higher than those of the control group but only glutarylcarnitine exhibited a significant difference between the two groups. This was arguably caused by the inhibition of VLCFA biosynthesis by MPTP that led to decreased acylcarnitine concentrations in the control group but increased concentrations in the experimental group. This increase could be explained by ALCAR's ability to stimulate VLCFA biosynthesis when the VLCFA concentrations are inhibited (by MPTP). Free carnitine conjugates with glutaric acid, a GA II metabolite, to form glutarylcarnitine, which is then excreted

All the acylcarnitine curves of the experimental group showed the same tendency: the acylcarnitine's concentrations increased on day 1 (only acetylcarnitine statistical difference), stabilized during the rest of the treatment and then increased again on day 14 (only glutarylcarnitine statistical difference), after the MPTP treatment. On day 14, when a GA II profile is developed after the MPTP treatment, the ALCAR stimulates the VLCFA biosynthesis and ensures a higher concentration of free carnitine available and

leads to increased detoxification. This could be the reason for the acylcarnitine's concentrations of the experimental group to be higher than those of the control group (without ALCAR treatment) (see chapter 5).

In this study we hypothesized that the protective mechanism of ALCAR may be at the level of fatty acid elongation, and that ALCAR plays a role in VLCFA metabolism and subsequently in the attenuation of neurodegenerative diseases. This hypothesis could not be proven conclusively, even though some promising results were obtained. An important finding that emerged was that ALCAR treatment for 14 days can be considered safe, since it has little/no effect on the VLCFA biosynthesis. ALCAR has the unique ability to stimulate VLCFA biosynthesis when their concentrations are decreased, but it does not influence the VLCFA biosynthesis under normal circumstances.

## 6.2. Future studies

Further investigation and research in this field is necessary to fully elucidate ALCAR's effect on VLCFA metabolism and its possible protective mechanism. These studies could include:

- The determination of mitochondrial acyl-CoA dehydrogenase concentrations with and without ALCAR treatment, before and after MPTP treatment.
- To thoroughly screen for other metabolites that might have formed after MPTP treatment, with and without ALCAR treatment.
- To investigate the possibility that ALCAR has a more prominent regulatory role in the glutarylcarnitine concentrations (in GA II) than in the case of the other acylcarnitines involved in GA II.
- To examine the role of MPTP and $MPP^+$, in the presence and absence of ALCAR treatment, on acetylcarnitine and free carnitine concentrations.
- Determination of the half-life of fatty acids to establish the duration of ALCAR treatment of MPTP-treated mice. This could present a clearer picture of ALCAR's influence on the regulation of VLCFA biosynthesis following inhibition by MPTP.

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# APPENDIX A

## Statistical analysis

The data outlined in this Appendix portrays the results obtained and discussed in detail in Chapter Four.

a) C22:0, C24:0 and C26:0 concentrations independently

A two way analysis of variance, with the group as a factor and day as repeated measure, was performed on the data. No statistical significance (ss) was observed in the C22:0, C24:0 and C26:0 concentrations between the two groups, however ss did occur for the day by group interaction of the C22:0 and C24:0 concentrations when using the Greenhouse-Geisser (GG) correction.

Therefore, a parametric test was performed to determine the ss of the groups within the days for the C22:0 and C24:0 concentrations (table A.1). If $p < 0.05$, a significant difference on a 5% level occurred between the means of the two groups. Using the Student-t test for separate variances, statistical significance was observed in the C22:0 concentrations on day 1, 7 and 14, and for the C24:0 concentrations on day 7, 13 and 14.

*Table A.1.* ***T-test to determine the statistical significance between the pooled values of the groups on specific days.*** *The values indicated in red represent statistical significant values on a 5% level.*

| Concentration | Day | MEAN Control group | MEAN Experimental group | p | p 2-sided |
|---|---|---|---|---|---|
| C22:0 | 0 | 41.56 | 35.74 | 0.225118 | 0.176950 |
| | 1 | 37.55 | 31.35 | **0.039450** | **0.007025** |
| | 7 | 18.86 | 26.12 | **0.008360** | **0.000937** |
| | 13 | 31.51 | 30.15 | 0.283733 | 0.249460 |
| | 14 | 28.74 | 22.53 | **0.034074** | **0.015741** |
| C24:0 | 0 | 21.69 | 18.41 | 0.186614 | 0.196784 |
| | 1 | 21.40 | 18.59 | 0.218938 | 0.133196 |
| | 7 | 12.44 | 17.98 | **0.002710** | **0.000203** |
| | 13 | 21.72 | 18.43 | **0.021164** | **0.007602** |
| | 14 | 20.92 | 14.46 | **0.000188** | **0.000020** |

# APPENDIX B

## Statistical analysis

A two-way analysis of variance (ANOVA), with group as factor and day as repeated measure, was performed on the acetyl-, glutaryl-, butyryl-, adipyl- and suberylcarnitines.

***Table B.1.*** ***Statistical significant differences of various acylcarnitines.*** *The red values indicate statistical significance ($p < 0.05$).*

| Carnitine | p-values | |
|---|---|---|
| | Between two groups | Between two groups on certain days |
| **Acetylcarnitine** | **0.001229** | **0.000000** |
| **Butyrylcarnitine** | 0.228693 | 0.159724 |
| **Glutarylcarnitine** | 0.051053 | 0.158559 |
| **Adipylcarnitine** | 0.707930 | 0.325506 |
| **Suberylcarnitine** | 0.452503 | 0.330508 |

No statistical significant group effect ($p < 0.05$) occurred in butyryl-, glutaryl-, adipyl- and suberylcarnitine values (see figure 5.2, graph a, b, c, d and e). However, statistical significant day effect was observed in glutarylcarnitine ($p = 0.051$).

***Table B.2.*** ***Glutarylcarnitine concentrations on various days.*** *The red values indicate statistical significance ($p < 0.05$).*

| Day | Average [ ] Control group | Average [ ] Experimental group | p value | p 2-sided |
|---|---|---|---|---|
| **0** | 0.028 | 0.035 | 0.372989 | 0.381978 |
| **1** | 0.035 | 0.102 | 0.156537 | 0.171724 |
| **7** | 0.083 | 0.082 | 0.988484 | 0.991401 |
| **13** | 0.073 | 0.079 | 0.770669 | 0.795161 |
| **14** | 0.079 | 0.180 | **0.000000** | **0.000000** |

In the case of acetylcarnitine a significant day by group interaction occurred (see figure 5.2(a)) and the Student-t test (table B.3) was performed for each day to compare these two groups. Only day 1 showed a statistical significant difference between the two groups.

*Table B.3.* ***T-test to determine statistical significant differences in acetylcarnitine concentrations between the two groups.*** *The values indicated in red represent statistical significant values on a 5% level.*

| Days | MEAN Control group | MEAN Experimental group | p | p 2-sided |
|---|---|---|---|---|
| **0** | 1.20718 | 1.21000 | 1.000000 | 1.000000 |
| **1** | 3.25932 | 61.99713 | **0.000018** | **0.002189** |
| **7** | 7.57805 | 8.91606 | 0.661100 | 0.671573 |
| **13** | 16.80660 | 23.58925 | 0.318441 | 0.310876 |
| **14** | 5.26189 | 4.47850 | 0.225318 | 0.227968 |