The role of fatty acids in drug resistant epilepsy

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Abstract

Refractory epilepsy is a complex disorder, affecting approximately 40% of epilepsy patients. To date, no medication has been found to be effective in the treatment of this disorder, except for the ketogenic diet and ω-3 supplementation, but the mechanism(s) by which these two treatments inhibit seizures are not yet known. It is known that both these treatments increase the concentrations of individual and total ω-3 fatty acids in the plasma. DHA (an ω-3 fatty acid) is an important component of phospholipids in the cell membrane and influences the membrane integrity and fluidity.

We explored the theory that there might be a defect in the biosynthesis of these ω-3 fatty acids, particularly docosahexaenoic acid (DHA), that could lead to an alteration in membrane integrity and possibly affect the transport of AEDs (antiepileptic drugs) across the membrane causing a refractory status in these patients. Carnitine facilitates the transport of ω-3 fatty acids across the inner mitochondrial membrane, thus a defect in the carnitine transport or biosynthesis, could lead to a decrease in the ω-3 fatty acids in the plasma as well. This investigation was a pilot study defining two aims: (a) are there any differences in the concentrations and concentration ratios of the fatty acids in patients with drug-resistant epilepsy versus patients with drug-responsive epilepsy and healthy individuals? (b) Are there any differences in the acetylcarnitine concentrations in patients with drug-resisitant epilepsy versus patients with drug responsive epilepsy and healthy individuals?

We gathered urine and plasma samples from children in three groups, namely a control group (healthy individuals), a group of drug-responsive epileptic children and a group of refractory epileptic children. The urine samples were used for an organic acid analysis (for screening purposes, to determine if there were metabolic disorders in any of the children that would exclude them from this study) and an acylcarnitine analysis to determine if there were any defects. The acylcarnitine analysis was performed by use of ESI MS-MS (electrospray ionization tandem mass spectrometry). In the plasma we determined the concentrations and concentration ratios of ω-3 fatty acids by using the GC-MS (gas chromatography mass spectrometry).

The refractory epilepsy group did not reveal lower concentrations of ω-3 fatty acids, particularly DHA, in fact we found the concentrations to be higher than that of the control group and more or less the same as that of the drug-responsive group. The same tendency was evident for the long chain acylcarnitines (adipyl, suberyl, and octanoylcarnitine). Upon

merging the two epilepsy groups and dividing them into valproate and carbamazepine-treated groups, we found that the DHA-concentration was higher in both the treated groups. The valproate-group also showed increased levels of the long chain acylcarnitines. No statistically significant differences were found between the valproate drug-responsive, valproate refractory, carbamazepine drug-responsive and carbamazepine refractory groups. The mechanisms by which valproate and carbamazepine inhibit seizures, have not yet been established, but the possible mechanisms proposed for their inhibition differ vastly from each other. Thus, these elevations in the DHA and long chain acylcarnitine concentrations could possibly be attributed to the epileptogenic status of the patients, and not the AED-therarpy. Combining our results with those of Henry (2004) we propose that DHA synthesis is not affected in epileptic patients, but that the incorporation of DHA in the membranes is possibly compromised.

Uittreksel

Refraktoriese (geneesmiddel-weerstandige) epilepsie is 'n ingewikkelde sindroom wat ongeveer 40% van alle epilepsie pasiënte affekteer. Tot op hede is geen medikasie gevind, behalwe vir 'n ketogene dieet en ω-3 aanvullings, wat effektief is in die behandeling van refraktoriese epilepsie nie. Alhoewel albei hierdie behandelings effektief aangewend is om epileptiese aanvalle te verminder en selfs in sommige gevalle heeltemal te inhibeer, is die meganisme(s) waarvolgens hierdie inhibisie plaasvind nie bekend nie. Dis is bekend dat beide hierdie behandelings die konsentrasies van die individuele- en totale ω-3-vetsure in die plasma verhoog. DHA ('n ω-3-vetsuur) is 'n belangrike komponent van fosfolipiede in die selmembrane en affekteer die membraan integriteit en –vloeibaarheid.

Die teorie, dat daar moontlik 'n defek in die biosintese van hierdie ω-3-vetsure, spesifiek DHA kan wees, wat kan lei tot 'n verandering in die membraanintegriteit en moontlik 'n effek kan hê op die transport van AEMs (anti-epileptiese middels) oor die selmembraan en die BBS (bloed brein skans) het ons baie geïnteresseer. Karnitien fassiliteer die transport van ω-3-vetsure oor die binneste mitochondriale membraan, dus sal 'n defek in die karnitien transport of –biosintese ook lei tot 'n verlaging in die ω-3-vetsure in die plasma.

Ons het urien- en plasmamonsters van kinders in drie groepe versamel, naamlik 'n kontrolegroep (gesonde individue), 'n groep epileptiese kinders wat wel reageer op medikasie en 'n groep kinders wat aan refraktoriese-epilepsie ly. Die urienmonsters is aangewend in beide organiese suur (vir sifting, om te bepaal of metaboliese defekte voorkom by enige kinders wat hulle moontlik van die studie kan uitsluit) en asielkarnitienanalises om te bepaal of daar enige defekte in die karnitienbiosintese of -transport meganisme teenwoordig was. Die asielkarnitienanalise is gedoen deur middel van ESI MS-MS (elektrosprei ionisasie tandem massa-spektrometrie). In die plasma het ons die konsentrasies en konsentrasie-verhoudinge van die ω-3-vetsure bepaal met behulp van die GC-MS (gas chromatograaf massa-spektrometrie).

Ons het gevind dat die refraktoriese epilepsiegroep nie laer konsentrasies ω-3-vetsure gehad het nie, ook nie DHA nie: inteendeel, ons het gevind dat die konsentrasies hoër was as die van die kontrolegroep en min of meer dieselfde as die geneesmiddel-gekontroleerde epilepsiegroep. Hierbenewens was die konsentrasies langketting-asielkarnitiene (adipiel-, suberiel- en oktanoielkarnitien) ook hoër in die refraktoriese epilepsiegroep. Hierna het ons die twee epilepsie groepe bymekaar gevoeg en dit verdeel in 'n valproaat- en n

karbamasepienbehandelde groep. Tydens hierdie analise het ons gevind dat die konsentrasie DHA hoër was in beide behandelde groepe. Die valproaat-groep het ook verhoogde konsentrasies van die langketting asielkarnitiene gehad. Geen statisties betekenisvolle verskille is gevind tussen die valproaatgeneesmiddel gekontroleerde groep, die valproaatrefraktoriese groep, die karbamasepiengeneesmiddel gekontroleerde groep en die karbamasepienrefraktoriese groep nie. Die meganismes waarvolgens valproaat en karbamasepien epileptiese aanvalle inhibeer is nog nie vasgestel nie, maar die voorgestelde meganismes van hierdie 2 middels verskil baie van mekaar. Dus kan hierdie verhoogde konsentrasies DHA en langketting asielkarnitiene moontlik toegeskryf word aan die epileptogeniese status van die pasiënte, en nie die valproaat- of karbamasepien-behandeling nie.

In die lig van Henry (2004), en ons eie resultate, stel ons voor dat DHA-sintese nie in epileptiese pasiënte beïnvloed word nie, maar dat die inkorporering van DHA in die membrane van epileptiese pasiënte benadeel mag wees.

Chapter 1

1 Introduction

Epilepsy is the one of the oldest syndromes in the world (it was noted in biblical times) and currently affects about 50 million people, of which approximately 10.5 million are under the age of 15 (Schmidt, 2002; Kwan *et al.*,2002; Loscher, 2002, Brown *et al.*, 2001). This makes epilepsy the most common neurological disorder in the world, affecting all ages and races, though developing countries show the highest prevalence for this disfunction. Despite the fact that epilepsy is such an old disease, it is still probably one of the most misunderstood and stigmated disorders plaguing our society today (in some third world countries it is seen as a contagious disease, or a disease caused by demonic influence).

It is an extremely varying disorder, with more than 40 described types of epilepsy. This causes the diagnosis and effective treatment of epilepsy to be rather difficult. Another factor complicating the diagnosis and treatment, is that the mechanisms of action of many of the effective AEDs (Antiepileptic Drugs) have not yet been established. Treating an epilepsy patient is thus often based on trail and error. Generally, the developing or immature brain is more susceptible to seizures than the adult brain, and some idiopathic epilepsy syndromes ("genetically" acquired epilepsy syndromes) are characterized by onset in the neonatal period and others in the infantile or later childhood (Berkovic *et al.*, 2006).

Epilepsy is characterized by the spontaneous recurrence of seizures caused by a number of factors including abnormalities of potassium conductance, a defect in the voltage-sensitive calcium channels and many more. Even though there are many available treatments on the market (AEDs, vagus stimulation, dietary treatments, surgery etc), an estimated 40% of epilepsy patients have refractory seizures, that is, their seizures continue to occur despite treatment with otherwise optimized treatment (Deckers *et al*, 2003). To date, no one has been able to establish the exact mechanism of refractory epilepsy, but theories include a hyper-expression of multi-drug resistance (MDR) proteins and defects in the biosynthesis of DHA (docosahexaenoic acid), that could lead to an alteration in membrane integrity and could ultimately affect the transport of drugs across the cell membranes and BBB (blood brain barrier). With the percentage of patients suffering from refractory epilepsy being so high, and so many questions regarding this syndrome that have not been answered yet, it is vital that intensive research on this subject should be undertaken in an attempt to reduce the frequency of seizures, to avoid and reduce over-treatment and to improve the quality of life of these patients.

Chapter 2

2 Literature overview

2.1 Introduction

Proper neuronal activity is based on a tight balance between excitatory and inhibitory communication between neurons. However, excessive excitatory activity is harmful for neurons, causing neuronal death because it triggers certain molecular pathways through a process called excitoxicity. It is thought that excitotoxicity participates in the progression of many neurological disorders such as Parkinsons's disease, Alzheimer's disease and various types of epilepsy (Lutz, 2004). Epilepsy can be described as a group of chronic syndromes, and implies a spontaneous recurrence of seizures, that are generally associated with convulsions (Schwinghammer *et al.*, 2003; Trevor *et al.*, 2002; Schmidt, 2002). It is usually divided into two major groups (depending on which area of the brain it affects), namely: partial epilepsy, and generalized epilepsy.

2.2 Partial epilepsy

Partial epilepsy is a type of epilepsy categorized by both an asymmetrical seizure pattern and the fact that the seizure originates in more parts of one hemisphere. It is generally associated with changes in motor functions, as well as modifications in somatosensory and sensory symptoms. Partial epilepsy is divided into simple partial seizures, and complex partial seizures where the patient might experience memory loss as well as a loss of consciousness. Sometimes complex partial seizures are even associated with a sudden change in behaviour (Loscher, 2002; Schmidt, 2002).

2.3 Generalized epilepsy

A seizure is classified as generalized when the seizures originate in both hemispheres simultaneously. Generalized epilepsy accounts for approximately 40% of all epilepsies (Loscher, 2002; Schmidt, 2002).

2.4 Causes of epilepsy

The causes of epilepsy are about as varied as the disorder itself. It can be due to genetic factors, mutations and defects in single genes (Guerrini, 2006; Berkovic *et al*, 2006), and can also be caused by a number of external factors such as congenital infections and malformations of the central nervous system, which are classified as pre or perinatal causes. Postnatal causes include trauma, encephalopathy, meningitis and encephalitis among others. It is also important to remember that there are critical differences between adult and

childhood epilepsy. Childhood epilepsy is very dynamic, meaning that it evolves with age, most are idiopathic disorders, and there is a much higher risk when anti-epileptic drugs (AEDs) are used, therefore the consideration for using AEDs in children, is intensified (Appleton, 1995; Cavazos *et al.*, 2004).

The normal functioning of neuronal membranes depend mostly on efficient activity of inhibitory (GABA, dopamine) and excitatory neurotransmitters (acetylcholine, glutamate, peptides, aspartate, steroid hormones), as well as a sufficient supply of glucose, amino acids, oxygen, potassium, chloride, normal function of receptors and a normal pH (Schwinghammer *et al.*, 2003).

Earlier we stated that epilepsy is caused by seizures. Seizures are most commonly caused by an unstable cell membrane or its surrounding cells, causing an excess excitability to spread locally (focal seizures) or more widely (generalized seizures). The following factors may lead to an instability in neuronal membranes:

(1) an abnormality of potassium conductance,

(2) a defect in the voltage-sensitive calcium channels,

(3) or a deficiency in the membrane adenosine triphosphate *(ATP)* (Schwinghammer *et al.*, 2003).

2.5 The mechanism of epilepsy

Despite recent advances in molecular biology, the molecular mechanism of epilepsy remains a puzzle. Different theories regarding the mechanism of epilepsy exist and can be divided into the following categories:

2.5.1 Mechanism affecting the sodium channels

An action potential by an axon is achieved only through the sodium channels. The sodium channels exist in three states, which are:

(1) A resting state: during this stage the channel allows sodium to enter the cell.

(2) An active state: during this stage the channel allows an increased sodium-influx into the cell.

(3) An inactive state: no sodium is allowed to enter the cell in this phase.

When an action potential occurs, the channels are in the active state, allowing a sodium-influx into the cell. After this, a percentage of the sodium channels go into a refractory stage, which is just an inactive state of the channels.

What happens in some epileptic patients is that they experience a hyperstimulation of the axon to produce action potentials, which ultimately cause seizures. Thus by maintaining a constant stimulus of the sodium channels, many of them are being kept in the refractory period withholding the axon from propagating the action potential (Ochua & Riche, 2005).

The AEDs that focus on the sodium channels are mainly focused on maintaining this refractory stage, and preventing the channel from returning to its active stage (Trevor *et al.*, 2002). These AEDs mostly have a short-term effect that block neuronal sodium channels and are drugs like phenytoin, carbamazepine, topiramate and lamitrigine (Perucca, 2005)

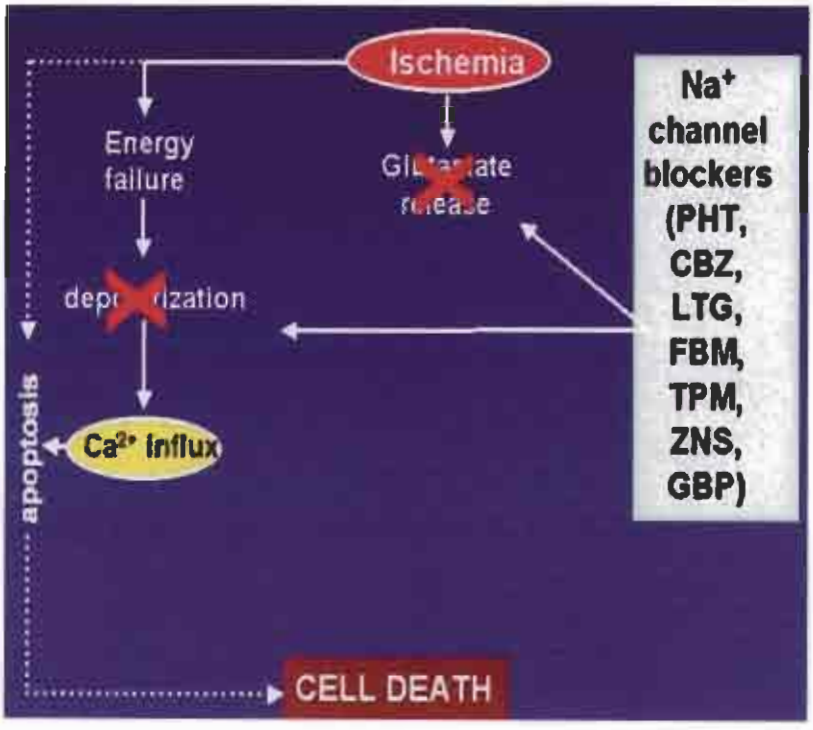


Figure 2.1. The mechanism of action of the sodium channel blockers (Moshé & Decker, 2001).

2.5.2 Mechanism affecting the calcium channels

Calcium channels are known as the "pacemakers" of the brain, meaning that they ensure normal rhythmic activity of the brain. These calcium channels are rather small and are inactivated rather quickly. An inflow of calcium during the refractory stage produces a partial depolarization of the membrane, which in turn leads to the development of an action potential after rapid depolarization of the cell. These low-threshold calcium currents often lead to cortical discharge in the thalamic region, causing seizures (Ochua & Riche, 2005; Trevor *et al.* 2002).

The calcium channels exist as three known types, namely N, L and T-types. Particularly the T-type channels have been known to play a vital role in the three per second spike-and-wave discharge associated with absence seizures (Ochua & Riche, 2005; Trevor *et al.* 2002).

AEDs that act mainly on calcium channels are, ethosuximide, gabapentine, zonisamide (Perucca, 2005).

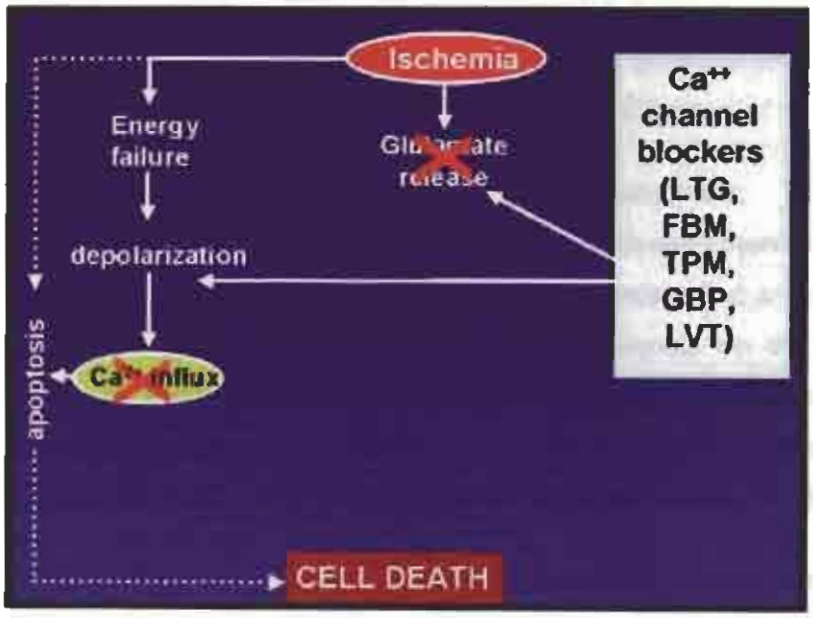


Figure 2.2. The proposed action of calcium channel blockers and GABA-related agents (Moshé & Decker, 2001).

2.5.3 Mechanism affecting the GABA receptors

GABA (gamma-amino-butyric acid) functions as one of the main inhibitory neurotransmitters in the brain, and plays a vital role in the maintenance of inhibitory tonus that opposes and

thus counterbalances excitation of neurons. This is one of the main reasons GABA plays such an important role in epilepsy (Cavazos *et al.*, 2004; Cenedella, 1999; Treiman, 2001; Perucca, 2005).

Numerous studies have stipulated the important role of GABA in the mechanism of epilepsy, indicating that there are:

(1) a dysfunctional GABAergic mechanism in genetic and acquired animal models of epilepsy,

(2) an inhibition of glutamate decarboxylase (the substrate in the synthesis of GABA), as well as a reduction in binding to GABA-A and benzodiazepine sites, GABA-mediated inhibition, and GABA in brain tissue and cerebrospinal fluid (this was reported in studies of human epileptic brain tissue),

(3) the fact that GABA-agonists suppress seizures and GABA-antagonists generate seizures (Treiman, 2001).

The major role players in epilepsy are GABA-A and GABA-B. GABA-A regulates the first part of GABA-mediated inhibitory postsynaptic potential (PSP), and GABA-B is more active in the later part of the process (Treiman, 2001; Cavazos *et al.*, 2004).

GABA-A receptors are paired with chloride channels. These chloride currents become especially significant at more depolarized membrane potentials. The chloride channels make it difficult to achieve threshold membrane potentials that are necessary to instigate an action potential. This influence of the chloride channels on the neuronal membrane potential becomes more substantial as depolarization of the neurons continue via the excitatory postsynaptic potentials (EPSPs) (Cavazos *et al.*, 2004). Many of the popular AEDs act on chloride channels, including benzodiazepines and barbiturates.

GABA-B receptors in turn are paired with potassium channels. These currents have a relatively long action duration in comparison with the chloride currents mentioned above. It is because of this long duration of action that modifications in the GABA-B receptors are thought to play a possible part in the transition between the interictal abnormality and partial onset seizure (Cavazos *et al.*, 2004).

AEDs known for their action on GABA-receptors are, valproate, felbamate, topiramate and vigabatrin (Perucca, 2005).

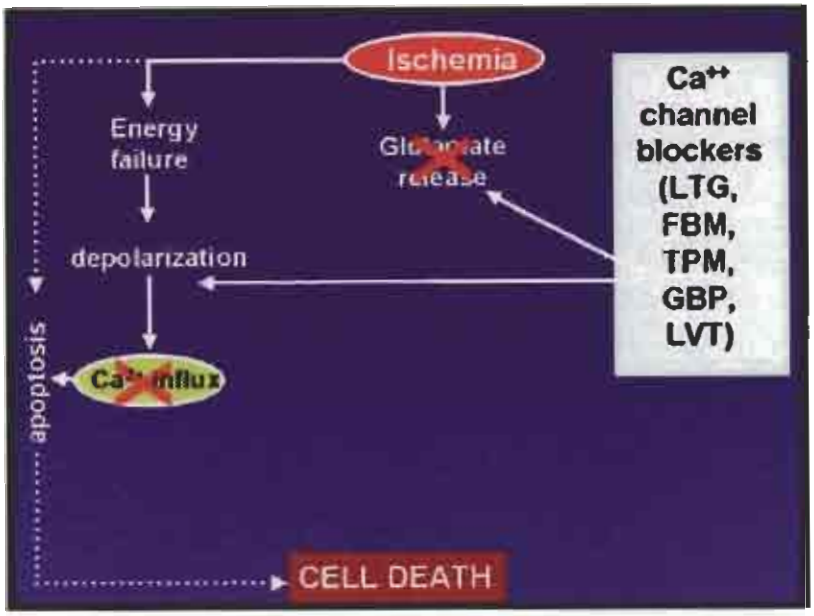


Figure 2.3. Some GABA-agents are known to inhibit calcium influx as well as glutamate release (Moshé & Decker, 2001).

2.6 Epilepsy and other neurological disorders

Epilepsy has been linked to a wide spectrum of other neurological and psychological disorders, such as:

(1) Attention deficit disorder: numerous studies have indicated that epilepsy patients have significant increase in behavioural disturbances which include attention deficiencies and hyperactivity. These attention deficiencies, associated with epilepsy, could be due to subclinical seizures, disturbed sleep (as a side-effect of the AEDs), undiagnosed learning disabilities or even attention deficit disorder (ADD) (Schubert, 2004). It is difficult to distinguish minor seizures and frequent interictal activity from ADD in patients, because attention span is decreased in epilepsy patients and attentional difficulties occur in children with complex partial, generalized tonic-clonic and absence seizures (LaJoie & Miles, 2002; Stores, 1973; Williams *et al.*, 1996).

(2) Chronic interictal psychosis: patients with epilepsy, especially those with temporal lobe epilepsy (TLE) are sometimes known to experience chronic interictal (schizophrenia-like) psychosis (Flugel *et al.*, 2006; Flor-Henry, 1969; Kanemoto & Kawasaki, 2001; Slater *et al.*, 1963).

(3) Cerebral palsy (CP): is a collective and non-specific term referring to motor disorders resulting from brain dysfunction (due to various etiologies, e.g. intracranial infection, intra or periventricular hemorrhage etc) early in life. The syndrome is non-progressive,

but often changing and affects about 1.5-2.5 in 1000 live births (LaJoie *et al.*, 2002; Kulak *et al.*, 2003). It was estimated that 15-90% of patients with CP suffer from epilepsy (LaJoie & Miles, 2002; Kulak & Sobaniec, 2003; Aicardi, 1990; Aicardi, 1994; Aksu, 1990). The frequency of epilepsy associated with CP varies by the specific kind of CP; it is seen in approximately 50-90% of the patients suffering from the quadraparetic form, 16-27% of diplegic cases, 34-60% of patients with the hemiplegic variant etc. It is also interesting that seizure control diminishes with increased severity of the CP, as does successful withdrawal from AEDs (LaJoie & Miles, 2002).

2.7 Treatment of epilepsy

The main focus of the treatment of epilepsy is to ultimately decrease the frequency of seizures and enable the patient to lead a normal life (Schwinghammer *et al.*, 2003; Sheth *et al.*, 2005). The type of treatment depends on a number of factors, but is mainly determined by the type of epilepsy, the severity of the case, the preference of the patient as well as the noted side-effects. The age of the patient is also a very important factor, because children under the age of two years and neonates often require higher dosages than adult patients because of a much higher rate of drug metabolism (Appleton, 1995). AEDs are the most common and effective treatment of epilepsy, but like most effective treatments, this does not come without a price.

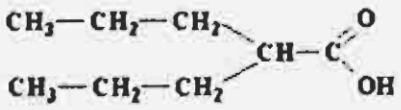
Several AEDs are associated with high toxicity, especially neuro-toxic dosages required for intractable or severe epilepsy in children. It is quite often that these high dosages cause a loss in their general function and abilities, therefore interfering with their learning abilities. It is important to note that the mechanisms by which many of the AEDs prevent seizures are mere speculations. Two of the most popular AEDs are valproate and carbamazepine. These drugs have proven to be very successful in the treatment of epilepsy, often when other AEDs have failed to succeed.

Valproate

Valproate is a short-chain fatty acid and is still one of the most effective AEDs on the market today. It has a broad spectrum of activity and is especially effective in the treatment of generalized seizures (including primarily generalized tonic-clonic, absence and myoclonic seizures). However, the precise mechanisms by which it exerts anti-epileptic properties remain to be categorically determined (Toth, 2004; Rogawski, 2006). Various studies have reported different mechanisms of action, including the blockage of voltage dependant sodium-channels, the blockage of T-type calcium channels, an elevation of whole brain GABA-levels (Kwan *et al.*, 2001) and the alteration of fatty acyl-CoA metabolism within the

mitochondrion which leads to carnitine deficiency and altered ω-3 fatty acid synthesis (Silva *et al.*, 2001; Luef *et al.*, 2003; Farkas *et al.*, 1996; Chang *et al.*, 2001).

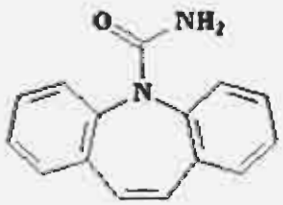
Valproic Acid
$C_8H_{16}O_2$



Carbamazepine

Carbamazepine is chemically related to tricyclic antidepressants and was first introduced in 1963 or the treatment of generalized tonic-clonic and partial seizures. As the case with valproate, carbamazepine is also highly effective in the treatment of epilepsy and certain psychiatric disorders. The exact mechanism of action by which it conquers seizures is not clear. Some say that carbamazepine influences the sodium channels, preventing the channel to return to its active state. It was also recently published that carbamezepine prevents arachidonic acid turn-over, causing an increase in certain ω-3 fatty acids. Carbamazepine has been reported to stabilize the inactive form of the sodium-channel, prolonging the return of the channel to its inactive state, to have an effect on the regulation of the monamine levels, to influence glutamatergic neurotransmission and to inhibit arachidonic acid turn-over in the brain (Kwan *et al.*, 2001; Bazinet *et al.*, 2006).

Carbamazepine
$C_{15}H_{12}N_2O$



Even though AEDs have proven to be very successful in the treatment of epilepsy, an estimated 40% of all people suffering from epilepsy do not respond to AEDs and have medically refractory seizures (Appleton, 1995; Loscher, 2002; Lutz, 2004, LaRoche *et al*, 2004).

2.8 Drug-resistant epilepsy

The prognosis for patients experiencing frequent seizures, is usually closely linked to the origin of the epilepsy (Gordon, 2004; Aicardi, 1988). If there is extensive brain damage, whether from trauma, tumors or disease, there is likely to be a poor response to any kind of treatment. Localization is also a keyfactor, because mesial temporal sclerosis that causes complex partial seizures, are very difficult to treat (Gordon, 2004).

Medically refractory seizures are seizures that do not respond to, or are not completely controlled by pharmacological treatment (AEDs). This means that the seizures continue to occur despite treatment with a maximum dose of a first line AED as monotherapy, or in at least one combination with an adjuvant medication. Generalized seizures are the most common type of refractory epilepsy in children, and complex partial seizures in adults (Loscher, 2002). In children, epilepsy is only labeled as refractory when at least 3 AEDs have been tried and found to be unsuccessful in the treatment of epilepsy (Sheth *et al.*, 2005). Refractory epilepsy can be labeled as a distinct medical condition with versatile dimensions which includes, cognitive decline, neurobiochemical plastic changes and psychosocial dysfunction which ultimately leads to dependant behaviour and a restricted lifestyle (Kwan *et al.*, 2002). The mechanisms behind refractory epilepsy are still unknown, but there a few speculations regarding this subject, including the role of very long chain fatty acids, especially docosahexaenoic acid (DHA) and compounds such as acetylcarnitne and p-glycoprotein (p-gp).

The terms intractable, refractory and drug-resistant epilepsy are interchangeable.

2.8.1 The role of very long chain fatty acids

It is only recently that the biochemical and physiological roles of essential fatty acids (particularly ω-3 fatty acids) have become more defined, emphasizing the fact that these fatty acids have the potential to prevent or diminish a variety of serious disorders that are common in industrialized nations (Kendler, 2006). The very long chain fatty acids (VCLFAs) are the main components of dietary lipids and form part of the so-called polyunsaturated fatty acids (PUFAs) (Doh *et al.*, 2005). These long chain fatty acids are very important in the development and function of the brain (Knoll *et al.*, 1999). *n*-3 PUFAs are important components of phospholipids in membranes and thereby influence the structure, functioning and fluidity of membranes. Not only are these PUFAs occupied with several physiological processes, including visual and cognitive functions and neuronal development, but they also act as substrates for eicosanoid synthesis, influence eicosanoid signaling also affecting gene expression. The fact that they play such an important part in eicosanoid synthesis and

signalling, make them all the more important in the central nervous system (CNS) because eicosanoids exert a large variety of biological actions (Youdim *et al.*, 2000; Yehuda *et al*, 2006., Kendler, B.S., 2006).

n-3 is the abbreviation representing the position of the first double bond when counting from the methyl carbon atom at the distal end of the fatty acid chain (Youdim *et al.*, 2000).

Out of all these PUFAs, docosahexaenoic acid (DHA) probably is the most important and is highly enriched in neural membranes. DHA comprises approximately 30-40% of the phospholipids in the grey matter of the cerebral cortex and photoreceptor cells in the retina, indicating its importance in the CNS (Horrocks & Farooqui., 2003).

2.8.1.1 DHA (C22:6n-3)

DHA is an essential PUFA, and large percentages of DHA are found in neural membranes. Neurons lack the enzymes necessary for the *de novo* DHA and arachidonic acid (C20:4n-6, AA) synthesis, so these fatty acids are derived either directly from the diet or synthesized from dietary linoleic acid (LA) and linolenic acid (ALA) in liver, from where they are transported to the brain tissue (Youdim *et al.*,2000; Horrocks & Farooqui., 2003). Even though cerebral endothelium and astrocytes are able to synthesize DHA, it is supplied to the brain tissue mainly from plasma. DHA is taken up by neurons from the extracellular medium after release from the glial cells or capillary endothelium.

2.8.1.1.1 DHA synthesis

Originally it was thought that the biosynthesis of DHA from dietary ALA (C18:3n-3) occurred only in the endoplasmic reticulum (microsomes) through a series of elongation and desaturation reactions (Ferdinandusse *et al.*, 2001).

However, this pathway requires that *n*-3 docosapentaenoic acid (C22:5n-3) becomes desaturated at position 4 by a microsomal acyl-CoA-dependant Δ4-desaturase to form DHA (C22:6n-3). Several studies have indicated that such a Δ4- desaturase does not exist (Ferdinandusse *et al.*, 2001; Luthria *et al.*, 1999; Qiu, 2002). Instead, Ferdinandusse found that a 24-carbon *n*-3 fatty acid is synthesized, which is desaturated at position 6 to produce tetracosahexaenoic acid (C24:6n-3), followed by a single round of β-oxidation with C22:6n-3 as the final product. Although still disputed, the peroxisome is the likely site of C24:6n-3 β-oxidation (Ferdinandusse *et al.*, 2001).

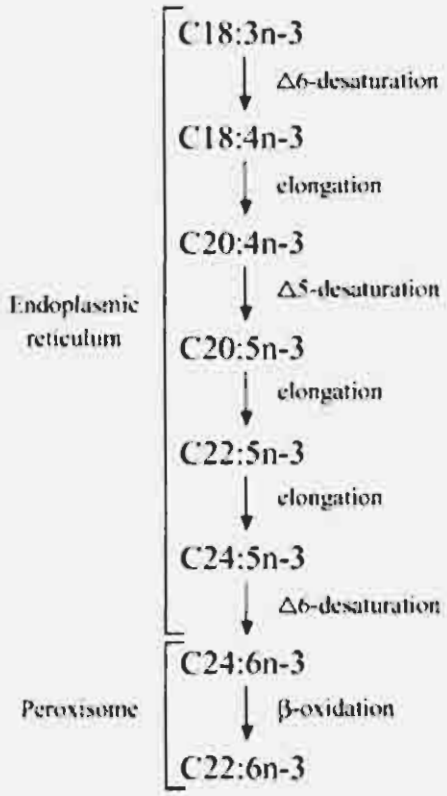


Figure 2.4. The biosynthesis pathway of DHA in the ER and the peroxisome. The direct precursor of DHA, tetracosahexaenoic acid (C24;6n-3) has to move from the ER to the peroxisome where it is β-oxidized to DHA (Ferdinandusse *et al.*, 2001).

After its synthesis, DHA is transported back to the endoplasmic reticulum, where it is esterfied into membrane lipids. The involvement of two different organelles in the biosynthesis of DHA implies that intracellular movement of fatty acids occurs between the endoplasmic reticulum (ER) and the peroxisome. The direct precursor of DHA, C24:6n-3, has to move from the ER after synthesis, to the peroxisome to be β-oxidized to form DHA. Because DHA is the most abundant *n*-3 PUFA in most tissues and is found at the sn-2 position of phospholipids, the DHA-CoA must move back to the ER from the peroxisome via thio-esterase (TE), probably as a free fatty acid to be incorporated into membrane lipids (Ferdinandusse *et al.*, 2003).

Carnitine plays a key role in the biosynthesis of DHA, because it is responsible for the transport of DHA out of the peroxisome. After β-oxidation, DHA-CoA has to move back to the ER to be incorporated into membrane lipids, but DHA-CoA cannot pass through the inner mitochondrial membrane on its own, thus carnitine replaces the CoA and in turn binds to DHA. This enables DHA to be effectively transported across the membrane and be successfully incorporated into lipids.

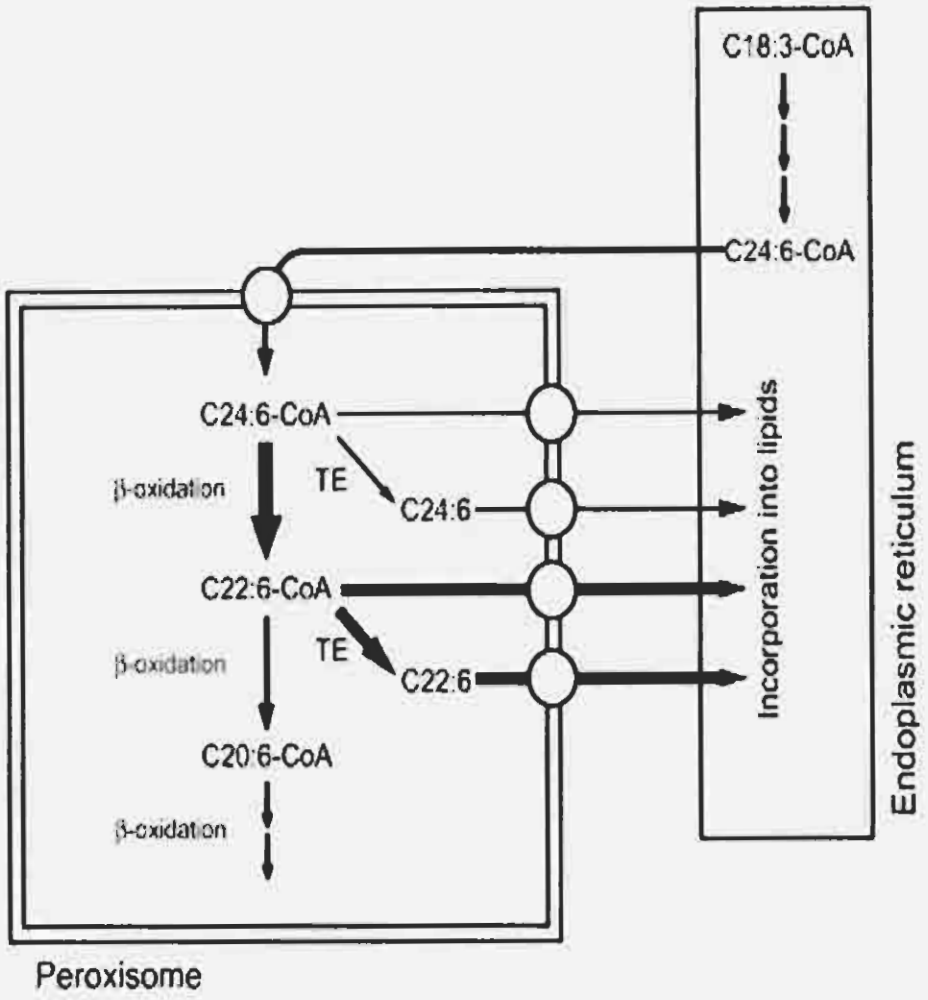


Figure 2.5. The intracellular movement of PUFAs and their metabolism. DHA is synthesized from dietary ALA (C18:3n-CoA) through a series of microsomal elongation and desaturation reactions, followed by retro-conversion of C24:6n-3 to C22:6n-3 in the peroxisome via one round of β-oxidation. Red arrows indicate the transport of DHA out of the peroxisomes and into the mitochondria via carnitine (Ferdinandusse *et al.*, 2003).

2.8.1.1.2 DHA and neural function

The CNS contains the second highest concentration of lipids after adipose tissue. Long chain fatty acids, particularly arachidonic acid and DHA are integral components of neural membrane phospholipids. Alterations in neural membrane phospholipids components cannot only influence crucial intracellular and intercellular signaling, but also alter many membrane physical properties such as fluidity, phase transition temperature, bilayer thickness, and lateral domains. A deficiency of DHA markedly affects neurotransmission, membrane-bound enzyme and ion channel activities, gene expression, intensity of

inflammation, and immunity and synaptic plasticity. DHA deficiency is associated with aging, Alzheimer's disease, hyperactivity, schizophrenia, and peroxisomal disorders (Freemantle *et al.*, 2006). Even though the molecular mechanism of DHA involvement in the disorders are unknown, the supplementation of DHA in the diet restores gene expression and modulates neurotransmission. Also, improvements are seen in signal transduction processes associated with behavioural deficits, learning activity, peroxisomal disorders, and psychotic changes in schizophrenia, depression, hyperactivity, stroke, and Alzheimer's disease (Horrocks & Farooqui, 2004).

It is known that DHA and PUFAs increase the resistance of forebrain cholinergic neurons against NMDA-induced neurotoxicity. This suggests that DHA-supplementation increases neural cell resistance against the exitotoxic damage produced by NMDA by being incorporated into neural membrane phospholipids. It is also possible that DHA may affect noradrenergic and serotonergic neurotransmission by incorporation into neural membranes, and in this way it may have positive effects on the behaviour and brain function (Youdim *et al.*, 1999).

DHA is provided by the liver and then obstinately retained during early development of the brain. However, due to free radical generation during the aging process, an unfavourable decline in DHA levels in neural membranes are detected. The deterioration of memory and ability to learn with age, may be partially due to decreased levels of DHA. This decrease in DHA with increasing age is coupled to the loss of phosphatidylserine during aging. It is interesting to note that this loss of DHA in the aged brain may contribute to cholinergic dysfunction in the hippocampus. Dietary supplementation of DHA not only restores its levels, but also increases cerebral choline and acetylcholine levels, and improves passive avoidance performance in stroke-prone, spontaneously hypertensive rats, and also in rat hippocampus during aging (Minami *et al.*, 1997; Freemantle *et al.*, 2006)).

The role of DHA in phospholipids and the role it plays in the integrity of trans cell membrane proteins are thus well known. Mechanisms of intractable epilepsy are not well understood, but may include alterations of pharmacological targets, and poor penetration of AEDs into the brain because of increased expression of multiple drug-resistance proteins, such as p-gp, and also the role that acetylcarnitine might play.

2.8.2 Acetylcarnitine

Carnitine is a water-soluble amine with vital intracellular functions. It is mainly synthesized in heart, liver, kidneys, brain and skeletal muscle (the major tissue reservoir of carnitine) and performs a wide variety of tasks in the body. It is amongst others, responsible for esterifying

potentially toxic acyl-CoA metabolites that could damage the Krebs cycle, and also performs a facilitating role in mitochondrial fatty acid oxidation, by transferring long chain fatty acids as acylcarnitine-esters across the inner mitochondrial membrane, as well as transporting acyl-CoA products of peroxisomal β-oxidation to the mitochondrial matrix in the liver (Coppola *et al.*, 2005).

Among the risk factors for carnitine deficiency are neurological disabilities (cerebral palsy, mental retardation), young age, a diet low in wheat and dairy products, hypoglycemia as well as therapy with various AEDs including valproate (Coppola *et al.*, 2005).

2.8.2.1 The role of carnitine in long chain fatty acid (LCFA) synthesis and metabolism

Carnitine plays an indispensable role in the synthesis and metabolism of LCFAs. It does however play two different parts in the peroxisome and mitochondria respectively. In the peroxisome, carnitine transfers the shortened fatty acids out of the peroxisomes back to the mitochondria. In the mitochondria, carnitine facilitates the uptake of LCFAs in the mitochondrial matrix (Wanders *et al.*, 2000).

The fatty acyl-CoA molecules cannot pass directly across the inner mitochondrial membrane and need to be transported as carnitine esters (carnitine replaces the CoA). For the β-oxidation pathway to function, acyl-CoA acts as the substrate in all microsomal elongation reactions, including the elongation and desaturation of LCFAs (see figure 2.6) (Knoll *et al.*, 1999). Short and medium-chain fatty acids can be transported quite easily without the assistance of carnitine.

Acetyl-CoA is transformed to acetyl-carnitine in the peroxisome via carnitine acyltransferase (CrAT), which enables the traffic of acetylcarnitine from the peroxisome to the mitochondria.

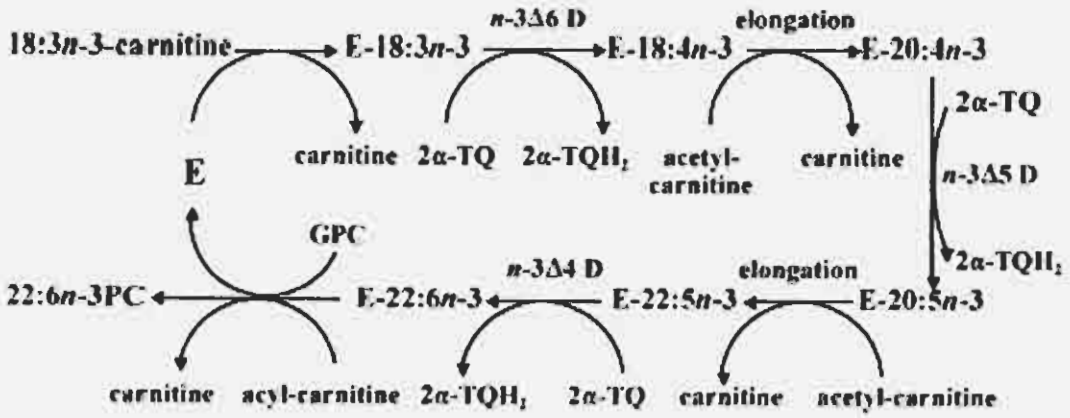


Figure 2.6. The role that acetyl-carnitine plays in the biosynthesis of DHA. Acetyl-carnitine is continuously transformed into carnitine (Infante *et al.*, 1999).

2.8.2.2 The role of carnitine in epilepsy

Carnitine-deficiency is quite common amongst patients with epilepsy, especially since some AEDs are known to inhibit carnitine. Several studies have shown that total plasma carnitine concentrations are remarkably lower in patients taking multiple AEDs, including valproate or valproate alone, and thus carnitine deficiency is mainly linked to valproate-usage (patients using phenobarbital, phenytoin and other AEDs showed no difference) (Coppola *et al.*, 2005).

Usually GC-MS analysis of the urine of valproate-treated patients mostly detect octanoyl and hexanoylcarnitine esters. Apparently, valproate and its metabolites act by inhibiting certain steps in the fatty acid oxidation process, and this causes an accumulation of medium and short-chain carnitine esters (Farkas *et al.*, 1996; Baillie *et al.*, 1989; Kossak *et al.*, 1993). Although this mechanism of action of valproate with respect to carnitine is certainly active, it alone is not sufficient to explain the development of carnitine deficiency. Another mechanism, namely impaired carnitine biosynthesis has to be involved. Farkas (1996) proposes that valproate lowers the level of α-KG, the co-enzyme for Bu-hydroxylase in the liver, in the solitary place where Bu to carnitine conversion occurs (Farkas *et al.*, 1996).

2.8.3 P-glycoprotein (p-gp)

Earlier we mentioned that one of the possible mechanisms indicated in refractory epilepsy might be poor penetration of AEDs into the brain because of an increased expression of multiple drug-resistance proteins, specifically p-gp. Several articles were published on the possible role of the cell membrane transporter protein, p-gp, in multi-drug resistant epilepsy (van Vliet *et al.*, 2005; Sills *et al.*, 2005; Summers *et al.*, 2004; Marchi *et al.*, 2004; Iannetti *et al.*, 2005).

P-gp is a cell membrane-associated protein that is mainly involved in the transportation of a large number of drug substrates (Matheny *et al.*, 2001). It is the 170-kD protein product of the MDR1 (multidrug resistance) gene. Human p-gp consists of two halves (6 hydrophobic transmembrane domains and a hydrophilic nucleotide-binding domian). These 2 halves are joined by a 60 amino acid linker region. This organization of the domains is typical of ATP-binding cassette transporters (Ramakrishnan, 2003). P-gp forms part of the transporter superfamily; ABC (ATP Binding Casette) transporters. At this stage, 49 different human ABC transporters have been identified, which are divided into 7 families, including proteins with ion channel function and other membrane-related proteins that transport endogenous products and drugs.

P-gp plays an especially significant role in cells found in the organs that influence drug delivery, such as the glial cells which comprise the blood brain barrier (BBB), drug absorption in organs like small intestine, and also plays a part in organs that have excretory function, for instance the liver and kidneys (Sills *et al.*, 2002; van Zyl, 2004; Matheny *et al.*, 2001). It functions mainly as an efflux protein, transporting a variety of drugs and harmful substances out of the cells. Changes in the secretion or activity of p-gp could have great effects on the distribution of active metabolites to sites such as the CNS, as well as drug-absorption from the gastro-intestinal tract (Matheny *et al.*, 2001).

2.8.3.1 The mechanism of action of p-gp in the CNS

The BBB plays a vital role in the protection of the brain and pharmacological action of drugs. The junctions of the glial cells that comprise the BBB are very tight, and the lipophilicity and composition of the drug are both key factors in the successful transport of the drug over the BBB (van Zyl, 2004). Immunocytochemical studies showed the existence of p-gp on brain microvessel endothelial cells as well as an expression of p-gp in the choroids plexus (an integrated complex of endothelial cells that form a barrier between the cerebrospinal fluid and blood). It is exactly this particular localization of p-gp in the brain which makes this especially important, for it is consistent with the supposed defensive role of p-gp in the CNS, by either limiting the uptake of substrates or increasing their efflux from the brain (van Zyl, 2004).

2.8.3.2 P-gp and refractory epilepsy

Regarding refractory epilepsy, there have been more and more proof that p-gp plays a facilitating role in this disorder. Firstly, the localization pattern of p-gp in the CNS is consistent with an assumed defensive role by either increasing the efflux of p-gp-substrates from the brain, or limiting their uptake (Matheny *et al.*, 2001). Recently several studies have indicated an over-expression of p-gp in the epileptic foci region (Sills *et al.*, 2002; Seegers *et al.*, 2002). There are also interesting assumptions that many of the AEDs (such as topiramate) act as substrates for p-gp. Together with an elevated level of p-gp, this would make it virtually impossible for these AEDs to reach their proposed site of action, and ultimately have an effect (Sills *et al.*, 2002; Seegers *et al.*, 2002). Before the drug enters the brain and then the epileptic foci, it is intercepted by p-gp from the inner leaflet of the cell and transported out of the capillary cells back into the blood.

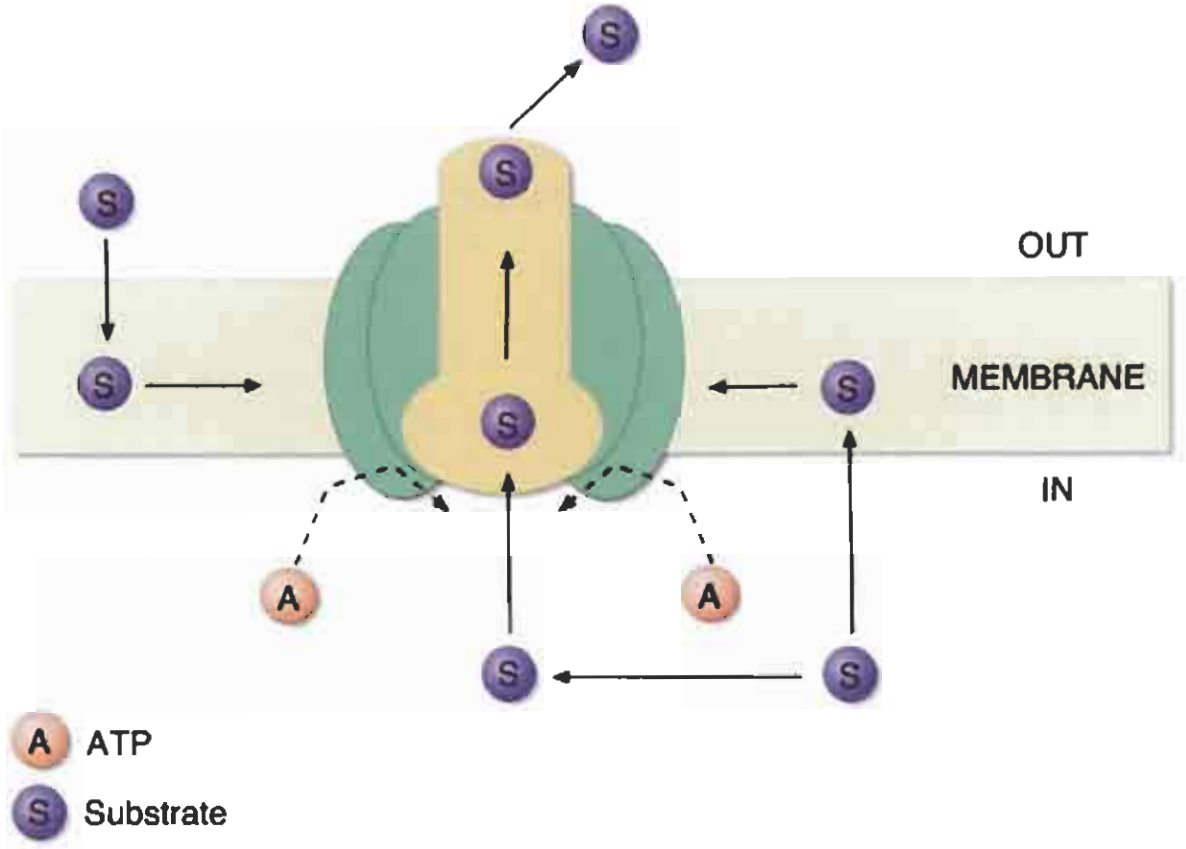


Figure 2.7. The structure and function of p-glycoprotein. The P-glycoprotein molecule spans the cell membrane and in this way is in contact not only with the membrane but also the inside and the outside of the cell. The central portion of the molecule is a channel or pore through which toxic chemicals are pumped back out into the environment. The toxic chemicals can enter the transport pore either from the interior of the cell or from its membrane as shown. Molecules of ATP power the pumping action (Edwards, 2003).

The putative role of p-gp in epilepsy is an interesting hypothesis, but there is a lack of adequate controls to test its validity, such as the difficulty to obtain brain tissue from patients with drug-responsive epilepsy. In a recent study (Volk and Loscher, 2005) a rat model of temporal lobe epilepsy to examine whether AED-responders differed from non-responders (refractory subjects) in their expression of p-gp in the brain. P-gp expression was studied by immunohistochemistry and showed striking overexpression in non-responders compared with responders in limbic brain regions. The p-gp overexpression was confined to brain capillary endothelial cells which form the BBB.

2.9 Treatment of refractory epilepsy

To date, no medication except for a ketogenic diet (KD) have been effective against refractory epilepsy. The KD has been used to treat epilepsy since the 1920s, but even today little is understood about the actual physiological and biochemical mechanisms behind its miraculous results. The KD is a high-fat, low-carbohydrate diet, working on the basis that the body utilises ketone bodies instead of glucose as an energy source (Mantis *et al.*, 2004; Cullinford *et al.*, 2004; Massieu *et al.*, 2003).

2.9.1 Energy metabolism and the KD

Energy homeostasis is achieved by the integration of lipid energy metabolism with carbohydrate and protein metabolism.

Glucose, derived from food-based carbohydrates, undergoes glycolysis to produce pyruvate. Pyruvate is then converted to acetate in the mitochondria by pyruvate dehydrogenase. Under conditions of starvation, when this glucose-derived energy source is not available, lipolysis is stimulated to mobilize free fatty acids (FFA). The process of β-oxidation of FFA yields acetyl-CoA which enters the Krebs cycle for complete oxidation to two molecules of carbon dioxide and water. The reduced co-factors that result from substrate oxidation enter the electron transport chain, where they are reconverted into their oxidized form with concomitant production of energy (Fukao *et al.*, 2004; Cunnane *et al.*, 2002; Cullingford, 2004).

The relationship between blood glucose and insulin is a fragment of a complex and circular feedback relationship among energy substrates, intermediates, and several hormones that include insulin, glucagons, epinephrine, cortisol and growth hormone (Cullingford, 2004; Pittier *et al.*, 2001). The initial period of fasting facilitates lowering the insulin to glucagons ratio, stimulating lipolysis and the production of ketone bodies. Fasting leads to lowered blood glucose, which triggers the secretion of glucagons to stimulate the release of FFAs by adipose cells (Fukao *et al.*, 2004; Veech, 2003; Beardsall, 2003).

However, disruption of the ketotic state occurs readily upon ingestion of carbohydrates by increasing blood glucose and increasing the insulin to glucagon ratio. When ketogenesis is halted, the insulin levels produced by carbohydrate ingestion tend to be higher and more sustained, hence ketosis may not be re-achieved for several hours or a day, thus vulnerability to so-called breakthrough seizures. In such a circumstance, a brief period of fasting may help reset the insulin to glucagon ratio. Another important point is that the ketone bodies themselves limit further mobilization of FFA from lipid stores, thus the need for the continuous intake of the high proportion of lipid calories in the KD (Cunnane *et al.*, 2002;

Fukao *et al.*, 2004; Cullingford., 2004). Under equilibrium conditions, the patient does not sustain a loss of body weight, even though the patient is in a continuous state of ketosis, mimicking a state of starvation (Mantis *et al.*, 2004; Sankar & Sotero de Menezes, 1999).

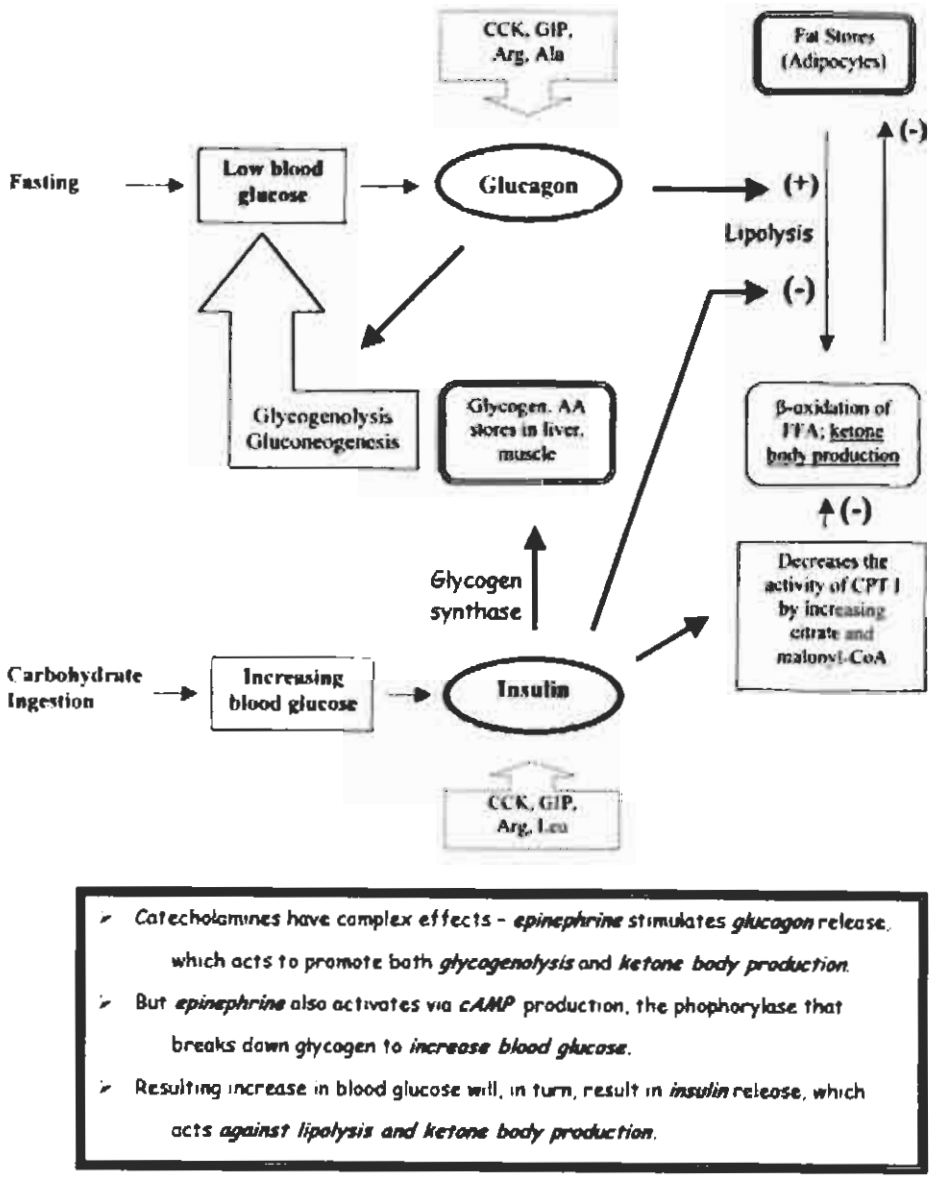


Figure 2.7. The process of fasting lowers the insulin to glucagon ratio at first, then lipolysis is stimulated, followed by the production of ketone bodies (Sankar & Sotero de Menezes, 1999).

Thus the KD is employed when other anticonvulsant drugs have failed. The exact mode of action of the KD is unknown, but it induces a complex cascade of metabolic changes, which include an increase in plasma ketones and a shift in the main substrate of energy metabolism in the brain from glucose to ketones. Although it is believed that the induction of systemic ketosis and its maintenance for the entire 2-3 year course of this therapy are the

key factors, the efficacy of the KD is inconclusive and require further research (Yudkoff *et al.*, 2003; Cullingford, 2004).

Dahlin and coworkers (2005) found that several cerebrospinal fluid (CSF) amino acids to be changed after initiation of the KD and of particular interest are the alterations of GABA levels. The altered amino acids included those that increase inhibition and also decrease excitation and some have yet unclear actions. The increases and decreases in these brain amino acid levels are a clear indication that the diet could influence excitability in the CNS.

2.10 Hypothesis

Regarding the current knowledge, the following hypothesis is viable:

In drug-resistant epilepsy, a defect of the biosynthesis of DHA (deficient enzyme) could lead to a decrease in the DHA content of phospholipids and eventually affect the transmembrane proteins in the BBB and their regulation. A KD could lead to an increase in acetyl-CoA concentrations and therefore acetylcarnitine concentrations. Increased acetylcarnitine concentrations may lead to increased productions of DHA (the remnant activity of the deficient enzyme is used optimally), leading to the correction and the role of the phospholipids in the brain.

2.11 Pilot study

This particular investigation is a pilot study posing the following questions:

(1) Are there any differences in the concentrations and concentration ratios of the fatty acids in patients with drug-resistant epilepsy versus patients with drug-responsive epilepsy and healthy individuals?

(2) Are there any differences in the acetylcarnitine concentrations in patients with drug-resistant epilepsy versus patients with drug-responsive epilepsy and healthy individuals?

The experimental procedures and results of these experiments will be discussed in the chapters 3 and 4.

The mechanism of the KD as a treatment for refractory epilepsy will be explored in future studies.

Chapter 3

3 Experimental procedures

3.1 Introduction

This project was approved by the ethics committee of the North-West university (05M13). For this study we collected blood and urine samples from three different groups of children, aged between 2 and 16 years. The first group was the control, consisting of 12 healthy individuals. These samples were obtained from the metabolic laboratory at the North-West University. The second group was a group of 14 drug-responsive epileptic patients, and the third group consisted of 8 drug-resistant epileptic patients. The range in age and gender was similar in all three groups The samples of group 2 and 3 were collected at the Red Cross Children's Hospital in Cape Town. Blood (3 ml) was collected in heparin tubes, centrifuged to obtain serum and transfered into another tube, frozen, and sent on dry ice. Urine (40ml), frozen, was also sent on dry ice with the serum. All containers was sealed to avoid leakage. The attached consent form in Appendix D was be used for our patients as well as the control subjects. The parents of both groups gave consent.

Three different analyses were performed on these samples: an organic acid analysis on urine(for screening purposes), an acylcarnitine analysis were conducted on urine samples and PUFAs were analyzed in plasma. Following is a discussion of experimental procedures used for the analysis.

3.1 The Organic Acid Analysis

3.1.1 Introduction

We performed urinary organic acid analyses to rule out any metabolic disorders or illnesses in our patients. Organic acid analysis is the standard procedure for the diagnosis of inherited disorders of amino acid and organic acid metabolism. The analysis of organic acids by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) is well established as an important and fast method of carrying out these proceedings.

Organic acids are isolated from physiological fluids with ethyl acetate and diethylether extractions. The most important steps of this method are the isolation of the organic acids from physiological fluids, formation of volatile derivatives and GC-MS analysis.

3.1.2 Experimental procedures for organic acid analysis in urine

Volume urine used according to creatinine values:

- Creatinine < 100 mg% use 1 ml urine
- Creatinine > 100 mg% use 0,5 ml urine
- Creatinine < 5 mg% use 2 ml urine
- Creatinine < 2 mg% use 3 ml urine

6 drops of a 5 M HCl solution were added to the urine to adjust the pH to 1. Internal standard was also added (5 x creatinine mg%=volume in µl). Ethyl acetate (6 ml) was added, the samples were shaken for 30 minutes (Roto-torque) and then centrifuged for approximately 3 minutes. The organic (top) phase was aspirated into a clean tube and 3ml of diethylether was added to the acqueous (lower) phase, shaken for 10 minutes and then centrifuged for about 3 minutes. Again, the organic phase was aspirated and added to the ethyl acetate phase. Two spatulas of Na_2SO_4 were added after which the samples were vortexed. **. It was centrifuged again and the organic phase poured into clean, smaller kimax tubes. The samples were dried under a flow of nitrogen at 40 °C for 1 hour. After drying, BSTFA was added (2 x creatinine mg% = volume in µl) along with TMCS (0.4 x creatinine mg% = volume in µl). The samples were then incubated at 60 °C for 1 hour and injected, 1 µl air, 1 µl external standard, 1 µl air, 0.4 µl sample.

** *Note*: the Na_2SO_4 must now be powder (not flakes). Can add more**

Procedure notes

- Hamilton syringes may be rinsed with pyridine, acetone or sonicated if dirty.
- Hamilton syringes are well rinsed with hexane between and after use (5x), and the plunger is removed when not in use.
- If silylation crystallize, derivatize again (BSTFA & TMCS)
- If water condenses with the sample during evaporation, add a few drops hexane and evaporate.

3.2 The determination of PUFAs in plasma

3.2.1 Introduction

There are a number of methods for determining the PUFAs in plasma, and after careful consideration we chose the method of Assies *et al* (2004). This method enabled us to determine the different fatty acids in the metabolic synthesis pathway of DHA.

All but three of the fatty acids noted in figure 3.1. were determined. Linolenic acid is found in the diet and was not important in the context of this study, while tetracosapentaenoic and tetracosahexaenoic acid both only exist fleetingly, making it virtually impossible to determine them in the plasma.

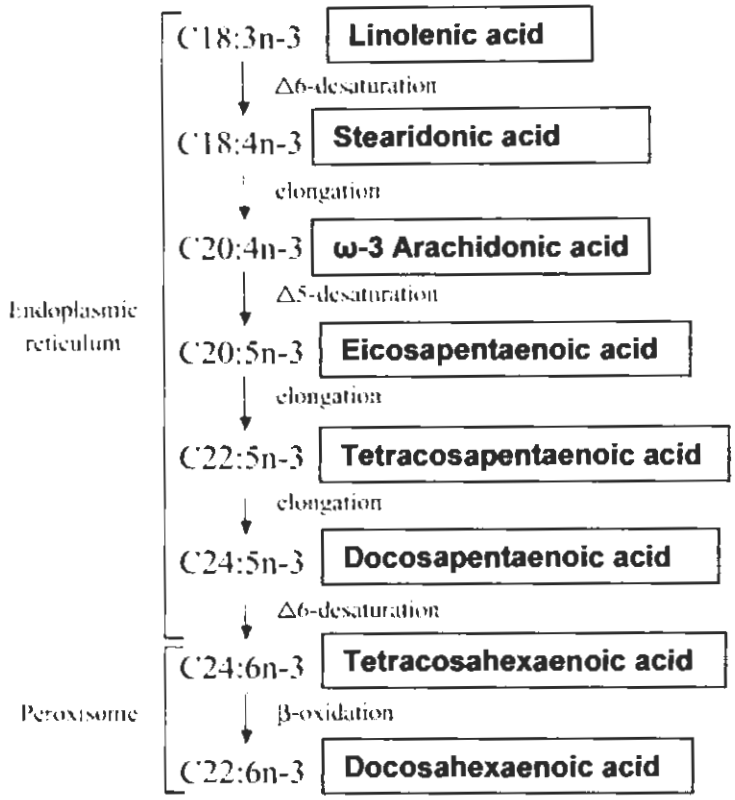


Figure 3.1. The biosynthesis pathway of DHA in the ER and the peroxisome. The direct precursor of DHA, tetracosahexaenoic acid (C24;6n-3) has to move from the ER to the peroxisome where it is β-oxidized to DHA (Ferdinandusse *et al.*, 2001).

3.2.2 Materials

Chemical or substance	Supplier
Linolenic acid (9c,12c,15c) 99% pure	Larodan chemicals, Sweden
Stearidonic acid	Larodan chemicals, Sweden
ω- 3 Arachidonic acid/Eicosatetraenoic acid (8c,11c,14c,17c) 98% pure	Larodan chemicals, Sweden
Eicosapentaenoic acid (5,8,11,14,17- all cis) 99% pure	Larodan chemicals, Sweden
Docosapentaenoic acid (7c,10c,13c,16c,19c) 99% pure	Larodan chemicals, Sweden
Tetracosapentaenoic acid	Larodan chemicals, Sweden
Tetracosahexaenoic acid	Larodan chemicals, Sweden
Docosahexaenoic acid (4,7,10,13,16,19- all cis) 99% pure	Larodan chemicals, Sweden
Heptadecaenoic acid (internal standard)	Sigma Aldrich, USA

All solutions were made up from analytical grade reagents. Methanol and *n*-hexane were GC-MS grade.

3.2.3 Preparation of Standards

In clear, screw-capped glass tubes (rinsed with ethanol) a 0,0025 g/5 ml dichloromethane solution of each standard and a 1 g/100 ml ethanol solution of butylated hydroxytoluene (BHT) was prepared. 100 µl of each standard was used and 100 µl of BHT and internal standard (0,0025 g/5 ml) was added. The standards were dried under a stream of nitrogen to rid them of ethanol. After drying, 1 ml of methanolic hydrochloric acid (HCl) was added to the standards which were tightly capped and put into the oven at 90 °C for 4 hours. The standards were left to cool at room temperature, then 2 ml of hexane was added. The standards were shaken for 10 minutes and then centrifuged for a further 2 minutes at 1500 revolutions/minute (RPM). The top layer (the methyl derivatives) was extracted and again another 2 ml of hexane was added. The standards were then shaken for a further 5 minutes and centrifuged for another 2 minutes. The top layer was extracted again and added to the other extracted part. It was then set to dry under a flow of nitrogen. After drying, 100 µl of hexane was added to each standard, then vortexed for about 5 seconds, and finally injected into the GC-MS for analysis.

3.2.4 Sample preparation

The blood samples were collected on Wednesday afternoons at the Red Cross Children's Hospital, centrifuged, and sent to us as plasma samples. The samples were stored at -20 °C until analysis. In clear, screw-capped glass tubes, 100 µl of both BHT and internal standard were added to 50 µl of plasma. The samples were the dried under a flow of nitrogen. After drying, 1ml of methanolic HCl was then added to the samples, which were then tightly capped. The samples were incubated in the oven at 90 °C for 4 hours, and then left to cool down to room temperature before 2 ml of hexane was added to each one. The samples were shaken for ten minutes and then centrifuged for 2 minutes at 1500 RPM. The top layer (the methylated derivatives) was extracted and another 2 ml of hexane was added to the samples. They were then shaken for a further 5 minutes and centrifuged for 2 minutes. Again, the top layer was extracted, and added to the other extracted part. The methylated derivatives were dried under a flow of nitrogen. After drying, 100 µl of hexane was added, the samples were then vortexed for about 5 seconds, and finally injected into the GC-MS for analysis.

3.2.5 GC-MS analysis

One microliter of sample was injected onto a GC-MS system. An Agilent 6890 GC ported to a 5973 Mass Selective detector (California, USA) was used for identification and quantification of individual fatty acids. For the aquisition of an electron ionization mass spectrum, an ion source temperature of 200 °C and electron energy of 70 eV was used. The

GC was equipped with a SE-30 capillary column (Chemetrix, USA), a split/splitless injection piece (250 °C) and a direct GC-MS coupling (260 °C). Helium (1 ml/min) was used as the carrirer gas. Initial oven temperature was 50 °C, maintained for 1.5 minutes and then increased to 190 °C (30 °C/min). The oven temperature was maintained at 190 °C for 5 minutes and then allowed to increase to 220 °C at a rate of 8 °C/min. The oven temperature was again maintained for 2 minutes and finally ramped to 230°C at a rate of 3 °C/min. The temperature was maintained at 230 °C for 24 minutes.

3.3 Acylcarnitine analysis

3.3.1 Introduction

Carnitine plays a key role in the biosynthesis of DHA, because it is responsible for the transport of DHA out of the peroxisome. After β-oxidation, DHA-CoA has to move back to the ER to be incorporated into membrane lipids, but DHA-CoA cannot pass through the inner mitochondrial membrane on its own, thus carnitine replaces the CoA and in turn binds to DHA. This enables DHA to be effectively transported across the membrane and be successfully incorporated into lipids (see figure 2.5).

Recently, a technique based on isotope-dilution tandem mass spectrometry for quantifying carntine in small volumes of plasma, whole blood and urine was 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 derivatized 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.

3.3.2 Experimental procedure

3.1.1.1 Materials and preparation of stock solutions

Preparation of butanolic hydrochloride

Caution: Acetylchloride may explode when in contact with water. Always wear a mask, goggles, and gloves to make this reagent. Procedure must be performed in a fume cupboard.

Butylation is performed to add a butaryl group to the carboxylic end of the molecules in the sample. This is done to stabilize the 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 is important for this step generates heat), while stirring frequently. The solution is covered with parafilm and left on ice for 20 minutes. The solution remains stable for one week.

Acetonitrile: Water 50 % (v/v)

Add 500 ml of acetonitrile 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/100 ml (20.83 µmol)
B) Propionylcarnitine: 5 mg/100 ml (19.69 µmol)
Isovalerylcarnitine: 5 mg/100 ml (17.73 µmol)
Octanoylcarnitine: 5 mg/100 ml (15.43 µmol)
C) Palmitoylcarnitine: 5 mg/100 ml (11.47 µmol)
D) Free carnitine: 5 mg/100 ml (30.446 µmol)
Dilutions of the different carntine 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 the volumes of each internal standard stock solution indicated in table 1 into the volumetric flask, and make up to 1 l with methanol.

Table 1. Unlabeled carnitine

Compound	(g/mol)	Stocksolution concentration (5 mg/100 ml)
Free carnitine	162	308.64 µmol/l
Acetylcarnitine	204	245.09 µmol/l
Propionylcarnitine	218	229.36 µmol/l
Isovalerylcarnitine	246	203.25 µmol/l
Octanoylcarnitine	288	173.611 µmol/l
Palmitoylcarnitine	400	125.0 µmol/l

3.3.2 Experimental procedure

(1) Creatinine determinations

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

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

(1) Protocol for urine samples

100 µl of urine was centrifuged for 30 minutes to rid the urine of crystals. 10 µl of the urine sample was added to 410 µl amino acid and carnitine isotopes mixture. The sample was centrifuged for 20 minutes and the supernatant was transferred to a new tube. The supernatant was dried under a flow of nitrogen at 55 °C for 30 minutes. After drying, the caps on the microtube (in which the sample was present) was closed, 200 µl 3 N butanolic HCl was added and incubated at 55 °C for 20 minutes. The solvent was then dried under nitrogen again (35-40 minutes). The residue was reconstituted in acetonitrile: water and 1 % formic acid (80:20). The sample was then injected into the VG Quatro tandem mass-spectrometer (injection volume: 25 µl).

Chapter 4

4 Results and discussion

4.1 Introduction

This pilot study had two main aims: to determine if there were any differences in the concentrations and concentration ratios of the ω-3 fatty acids in the plasma of patients with drug-responsive epilepsy, patients with medically refractory epilepsy and healthy individuals, and secondly, to determine if there were any differences in the urine concentrations of the acylcarnitines the same groups.

We did not administer any medication or other substances to the patients, but all of the patients in both the drug-responsive and refractory epilepsy groups were on AED-therapy. Statistical analyses were carried out at the Department of Statistical Services at the North-West University (Potchefstroom Campus) using Statistica® software.

4.2 The Organic Acids

The organic acid analysis was performed to eliminate patients with any metabolic disorders or other illnesses that could influence our fatty acid and acylcarnitine analyses. None of our patients showed any metabolic defects.

See appendix A for the raw data of the organic acid analyses.

4.3 The Fatty Acid Analysis

The unequal N HSD test (a generalization of the Tukeys multiple comparisons test) was used to compare the individual ω-3 fatty acids of the control, drug responsive and drug resistant groups. A probability of $p \leq 0.05$ was employed to declare statistical differences and each analysis was followed by multiple comparisons p-values (2-tailed) and the Kruskal-Wallis test: H (2, N = 34).

See Appendix B for the raw data of the fatty acid analyses.

4.3.1 ω-3 fatty acid concentrations

Figure 4.1 shows the differences in the concentrations of the individual ω-3 fatty acids in the control, drug-responsive and refractory epilepsy groups.

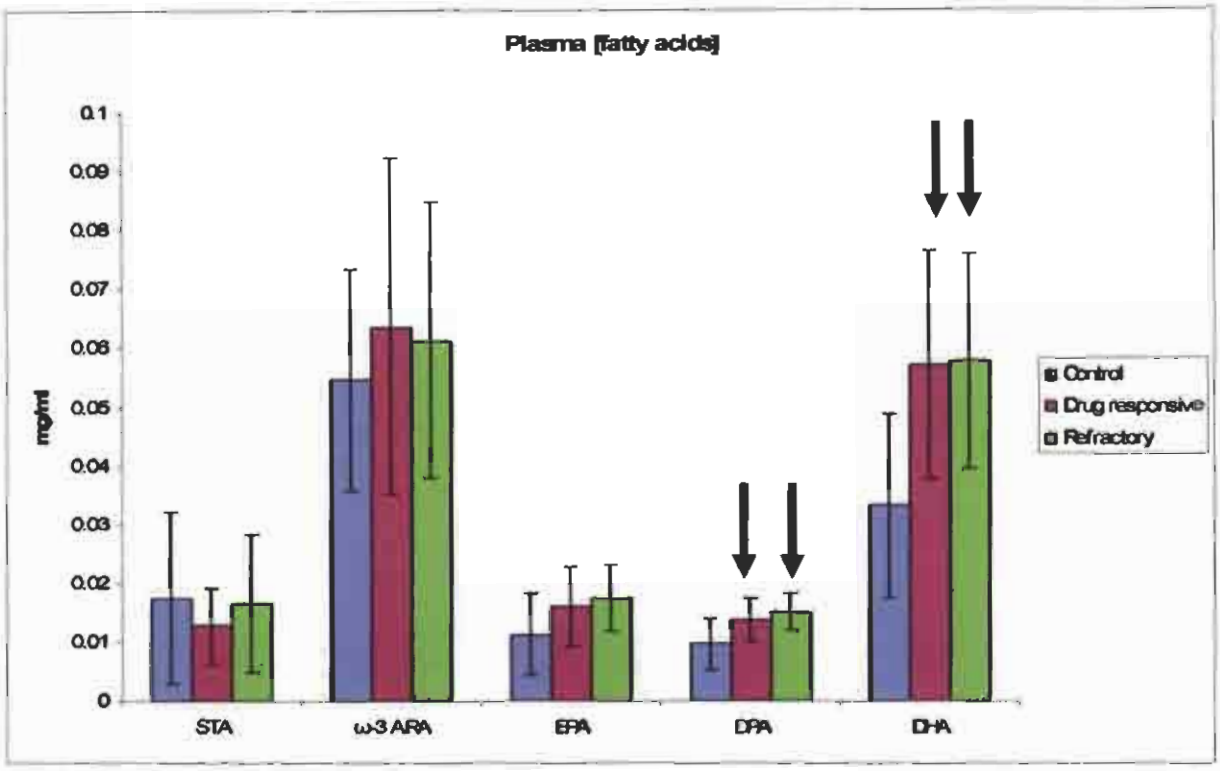


Figure 4.1. Individual ω-3 fatty acid concentrations (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

The following ω-3 fatty acids showed a statistical difference:

DPA

The analysis revealed a statistical difference in the docosapentaenoic acid between the control group and the drug-responsive epileptic group ($p = 0.045214$) and a statistical difference between the control group and the refractory epilepsy group ($p = 0.020695$). However there was no statistical difference between the drug-responsive and the refractory epilepsy groups.

DHA

A statistical difference was found in the docosahexaenoic acid, between the control and the drug-responsive epilepsy groups ($p = 0.010507$) as well as between the control and refractory epilepsy groups ($p = 0.026168$). No statistical difference was found between the drug-responsive and refractory epilepsy groups.

All of the individual ω-3 fatty acids showed a tendency of increased levels in the epileptic patients (both drug-responsive and refractory) except for stearidonic acid.

No statistical differences were found in the concentrations of the stearidonic, ω-3 arachidonic and eicosapentaenoic acid.

4.3.2 ω-3 fatty acids as a percentage of the total free fatty acids (TFFA)

Individual ω-3 fatty acids was calculated as a percentage of the total fatty acids. Figure 4.2 shows the differences in the percentages of ω-3 fatty acids between the control, drug-responsive and refractory epilepsy groups.

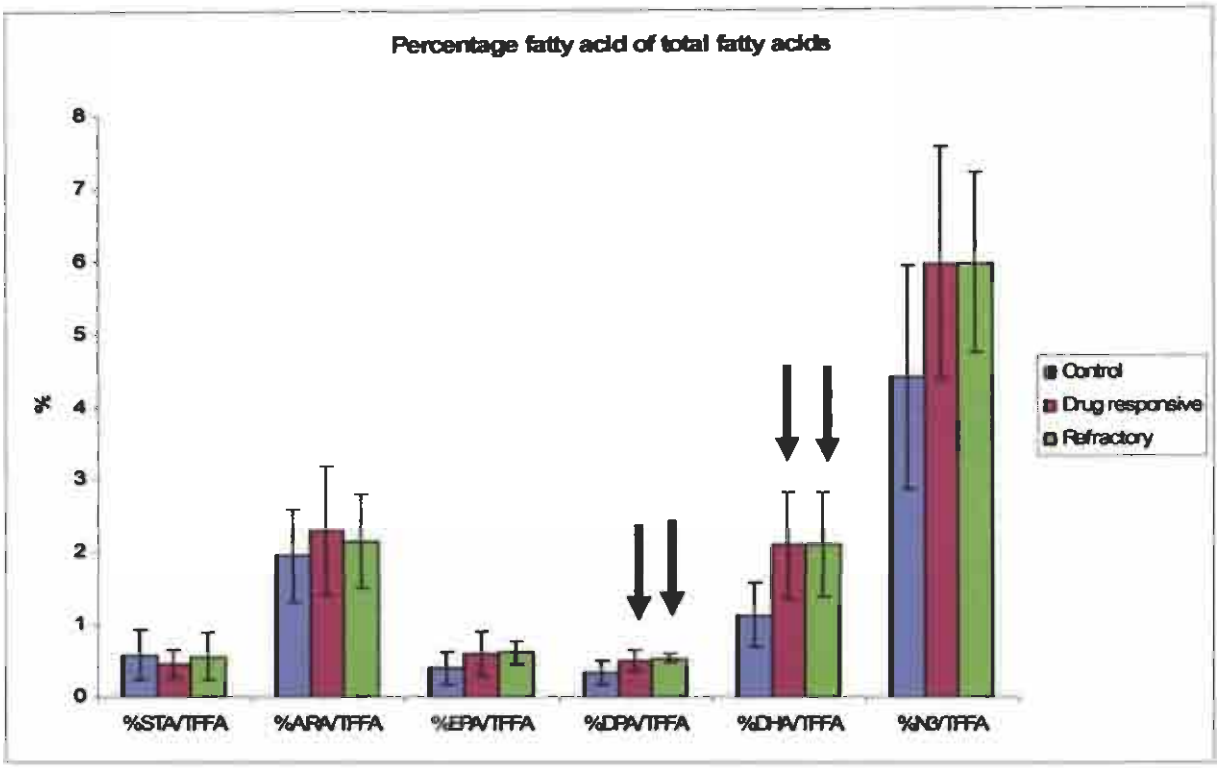


Figure 4.2. ω-3 fatty acids as a percentage of the total fatty acid concentration (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid, TFFA= total fatty acids).

The following fatty acids did show a statistical difference:

%DPA/TFFA

A statistical difference was found in the % docosapentaenoic acid/ total fatty acids, between the control and drug-responsive epilepsy groups ($p = 0.022792$) and the control and refractory epilepsy groups ($p = 0.017739$). There was no statistical difference between the drug-responsive and refractory epilepsy groups.

%DHA/TFFA

A statistical difference was found in the % docosahexaenoic acid/ total fatty acids between the control and drug-responsive epilepsy groups ($p = 0.003889$) as well as the control and the refractory epilepsy groups ($p = 0.012642$). No statistical difference was detected in the drug-responsive and the refractory epilepsy groups.

The analysis revealed that there were no statistical differences between the groups in the percentages of the STA, ω-3 ARA, EPA and total ω-3 fatty acids, but again there was a clear tendency of increased percentage levels in both epilepsy groups.

4.3.3 ω-3 fatty acid ratios

Fatty acid concentrations were determined and expressed in consequential ratios because these fatty acid ratios could give an indication of enzyme defects in the fatty acid biosynthesis pathway (figure 3.1). 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.

Figure 4.3 shows the differences in the ratios of the ω-3 fatty acids between the different groups.

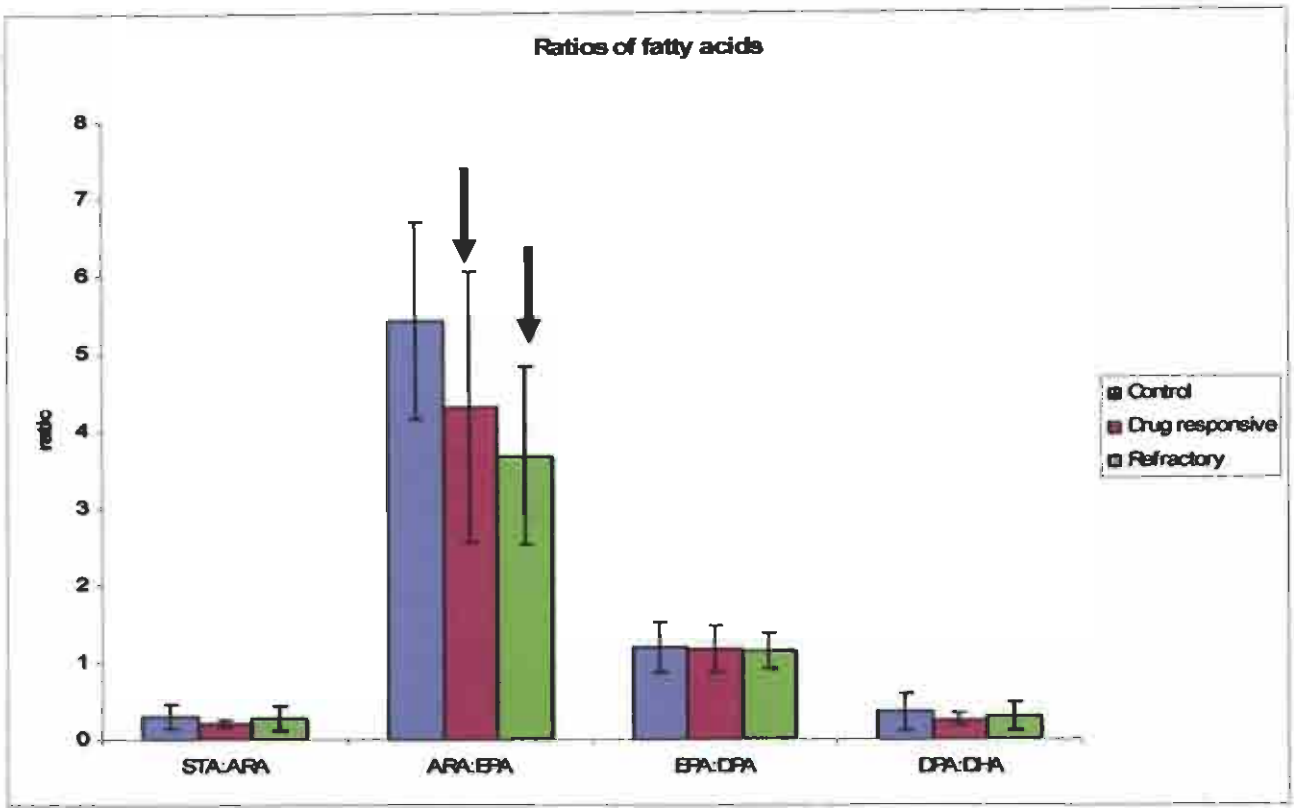


Figure 4.3. **ω-3 fatty acid ratios (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).**

4.3.4 ω-3 fatty acids as a percentage of the total ω-3 fatty acids

Individual ω-3 fatty acid was expressed as a percentage of the total ω-3 fatty acids and the data was analyzed. Figure 4.4 shows the differences in these percentages between the various groups.

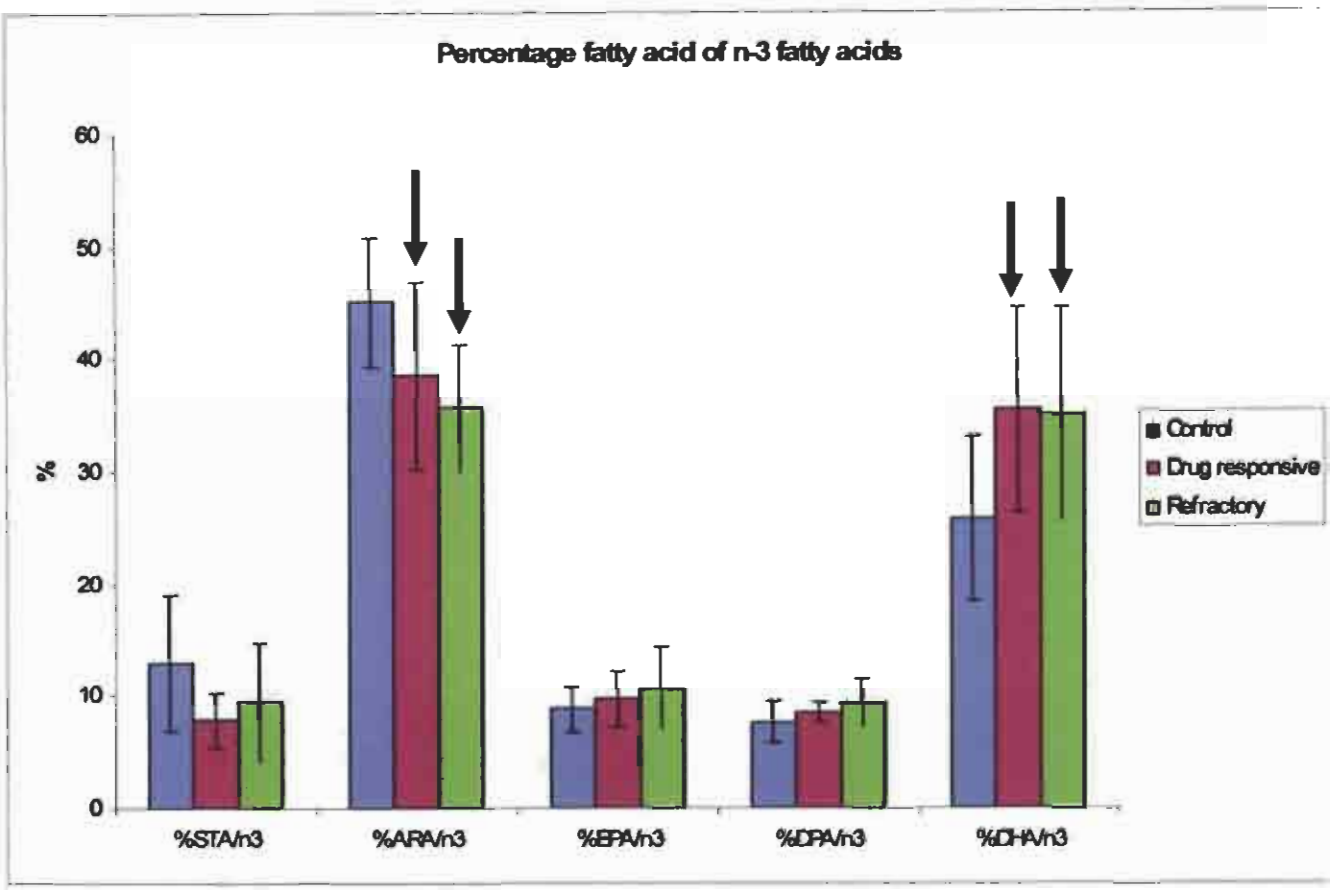


Figure 4.4. Individual ω-3 fatty acids as a percentage of the total ω-3 fatty acids (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

The following fatty acids showed a statistical difference:

% STA/ ω-3

There was a statistical difference in the stearidonic acid/ total ω-3 fatty acids between the control and drug-responsive epilepsy groups ($p = 0.042130$), showing a decrease in the levels of the drug-responsive epilepsy group, and even though there was no statistical difference between the control and refractory epilepsy group, one can see the decreasing tendency in the refractory group as well.

% ω-3- ARA/ ω-3

A statistical difference was found in the % ω-3- arachidonic acid/ total ω-3 fatty acids, between the control and refractory epilepsy groups ($p = 0.030385$), showing a clear decrease in the levels of ω-3 arachidonic acid in the refractory epilepsy group (and also a noticeable decrease in the drug-responsive epilepsy group, but this was not a statistically different

decrease). This is in contrast with our findings of elevated levels of ω-3 arachidonic acid in the drug-responsive and refractory epilepsy groups.

% DHA/ ω-3

A statistical difference was found in the % docosahexaenoic acid/ total ω-3 fatty acids, between the control and refractory epilepsy groups ($p = 0.035608$) showing an increase in the % DHA in the refractory epilepsy group (also in the drug-responsive epilepsy group, but this difference was not statistically significant). This correlates with our findings of elevated DHA levels as individual concentrations or as a percentage of the total fatty acids, leading to the speculation that there might be a mechanism in epilepsy that could possibly influence peroxisomal β-oxidation to cause higher levels of DHA in the plasma.

There were no statistical differences in the percentages of the eicosapentaenoic and docosapentaenoic acid.

4.3.5 Valproate and Carbamazepine-treated groups

There was no statistical difference between the drug responsive epilepsy group and the refractory group.

Bearing in mind that the refractory group could not be without any medication we combined our drug responsive and refractory group and identified two groups: one treated with valproate and the other with carbamazepine. This somewhat complicated the study because we could not be sure that our findings were due to the epileptogenic status of the patients, or if it was because of the medication they were taking.

Valproate is a branched short-chain fatty acid and known to undergo extensive metabolism in humans, primarily via β-oxidation in the mitochondria (Silva *et al.*, 2001). This means that valproate competes with short and medium chain fatty acids for oxidation leading to an increase in the total ω-3 fatty acids, because the short and medium chain fatty acids are used in the biosynthesis of the long chain ω-3 fatty acids. Valproate has been shown to reduce arachidonic acid turn-over in the brain (Chang *et al.*, 2001), which could also lead to an increase in certain ω-3 fatty acids.

It was recently published that carbamazepine too reduces the turn-over and incorporation rate of arachidonic acid in the brain (Bazinet *et al.*, 2006), therefore leading to an increase in several of the ω-3 fatty acids. The mechanisms by which these two drugs inhibit seizures are not yet established but the literature clearly indicates that these two medications differ in their mechanism of action.

To refine the results, the groups were renamed to control, valproate and carbamazepine-groups.

4.3.5.1 ω-3 fatty acid concentrations

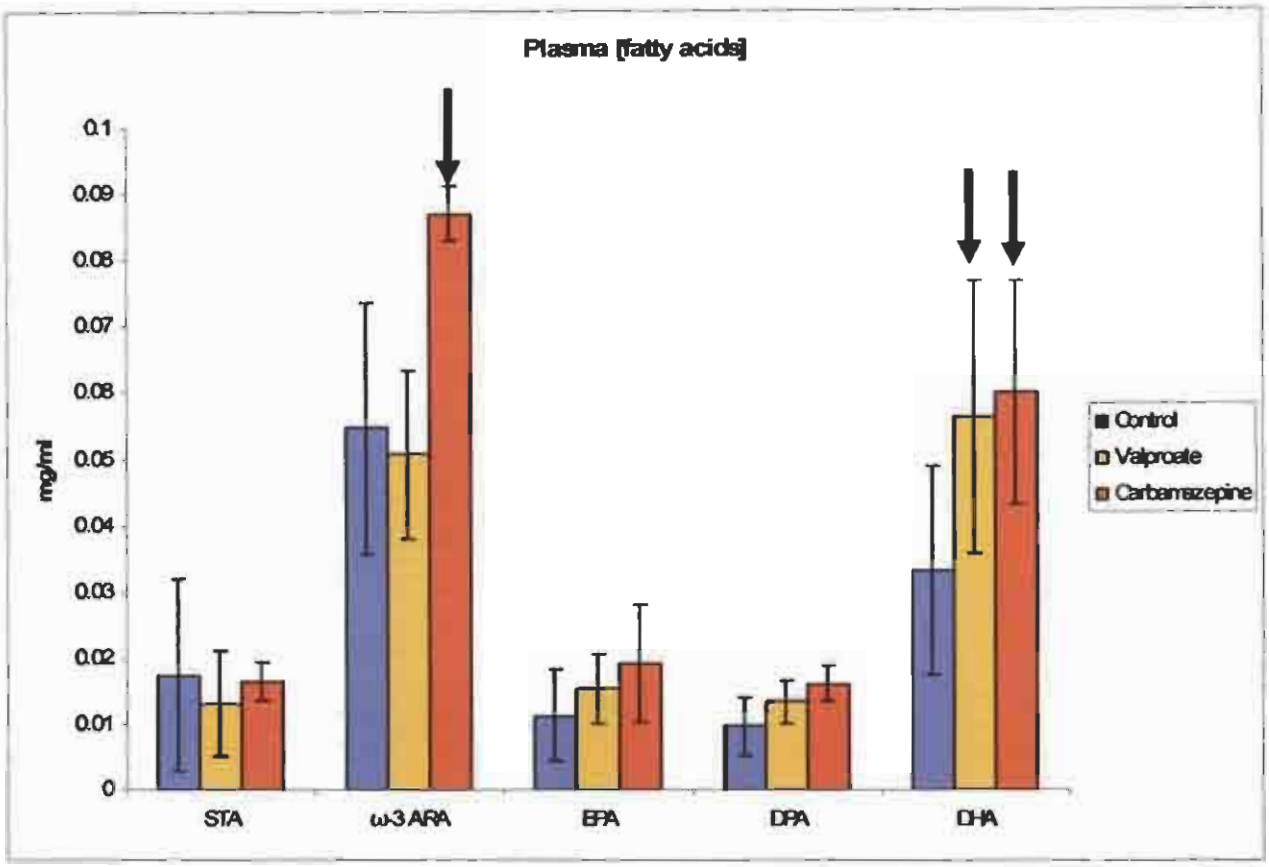


Figure 4.5: Concentration ω-3 fatty acids in the valproate and carbamazepine groups (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

Analysis of the concentration of each individual ω-3 fatty acid revealed that there were no statistical differences in the STA and EPA acid concentrations between the respective groups. The following fatty acids did show a statistical difference:

ω-3 ARA

A statistical difference was detected in ω-3 arachidonic acid between the control and carbamazepine-group ($p = 0.011713$), showing a clear increase in the levels of the ω-3 arachidonic acid in the carbamazepine-group. This correlates with the literature that carbamazepine inhibits the turn-over of arachidonic acid, thus leading to increased levels of arachidonic acid (Bazinet *et al.*, 2006). There was also a statistical difference between the

valproate and the carbamazepine-groups ($p = 0.004431$). The carbamazepine-group, having much higher levels of ω-3 ARA.

DPA

There was a statistical difference in docosapentaenoic acid between the control and carbamazepine-group ($p = 0.008372$), the carbamazepine-group showing increased levels of the docosapentaenoic acid (there was also a noticeable increase in the levels docosapentaenoic acid in the valproate group, but this was not statistically significant).

DHA

There was a statistical difference in docosahexaenoic acid between the control and valproate-group ($p = 0.013928$) and between the control and carbamazepine-group ($p = 0.023636$). No statistical difference was found between the valproate and carbamazepine group with respect to their DHA concentration. This led us to the speculation that the differences in the DHA concentration could be attributed to the epileptogenic status of the patients and not to their medication, because the suggested mechanism of action of valproate and carbamazepine differ from each other.

4.3.5.2 ω-3 fatty acids as a percentage of the total free fatty acids (TFFA)

The percentage of each ω-3 fatty acid calculated as a percentage of the total fatty acids, as well as the amount of the total ω-3 fatty acids calculated as a percentage of the total fatty acids, are shown in figure 4.6.

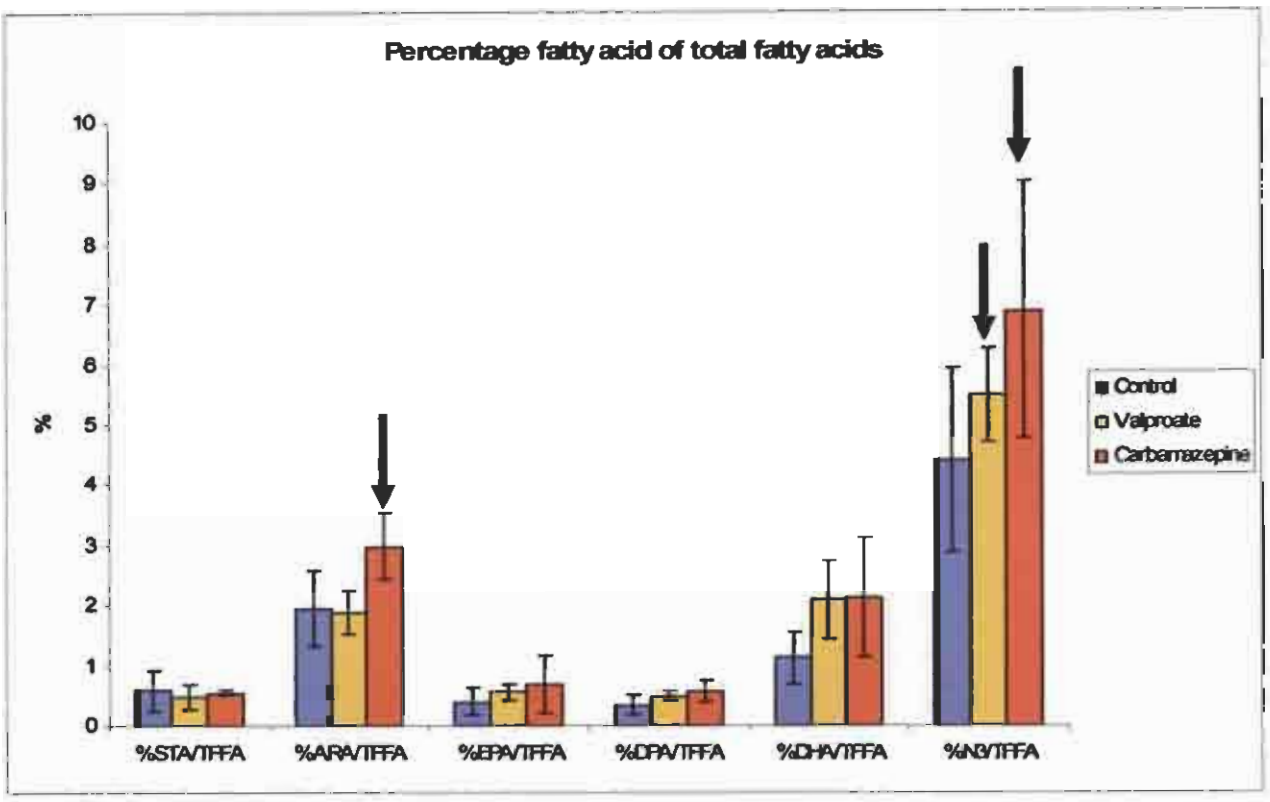


Figure 4.6: ω-3 fatty acids as a percentage of the total free fatty acids (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid, TFFA= total fatty acids).

The analysis of the percentage ω-3 fatty acids in terms of the total free fatty acids revealed that there were no statistical differences in the stearidonic and eicosapentaenoic acid percentages. The following ω-3 fatty acids did show statistical differences in their percentages:

% ω-3 ARA/TFFA

A statistical difference was found in the % ω-3 arachidonic acid/ total fatty acids, between the control and carbamazepine groups ($p = 0.009687$) and between the valproate and carbamazepine-groups ($p = 0.005690$). Once again, the increase in the levels of the % ω-3 arachidonic acid/TFFA in the carabamazepine-group correlates with the literature that carbamazepine inhibits the turn-over of ω-3 arachidonic acid (Kwan *et al.*, 2001; Bazinet *et al.*, 2006).

% DPA/TFFA

There was a statistical difference in the % docosapentaenoic acid/ total fatty acids, between the control and valproate-groups ($p = 0.029768$) and the control and carbamazepine-groups

($p = 0.011864$). Both the valproate and the carbamazepine-groups revealed an increase in their levels of % docosapentaenoic acid/T. This result, as well as the increase in the % docosapentaenoic acid/T found in the previous analysis indicate that both the drug-responsive and refractory epilepsy groups exhibited higher percentages docosapentaenoic acid than the control. This could be due to the epileptogenic status of the patients, and not the use of valproate or carbamazepine, as stated in 4.2.5.1.

% DHA /TFFA

There was a statistical difference in the docosahexaenoic acid/ total fatty acids, between the control and valproate-groups ($p = 0.004054$) and between the control and carbamazepine-groups ($p = 0.018377$). The increases in the % DHA /TFFA is in agreement with our data in 4.2.1 and supports our theory that the increased levels in DHA could possibly be attributed to the epileptogenic status of the patients.

4.3.5.3 ω-3 fatty acid ratios

The ratio of each individual ω-3 fatty acid to its precursor was determined and then analyzed. Figure 4.7 show the differences in the ratios of the various ω-3 fatty acids.

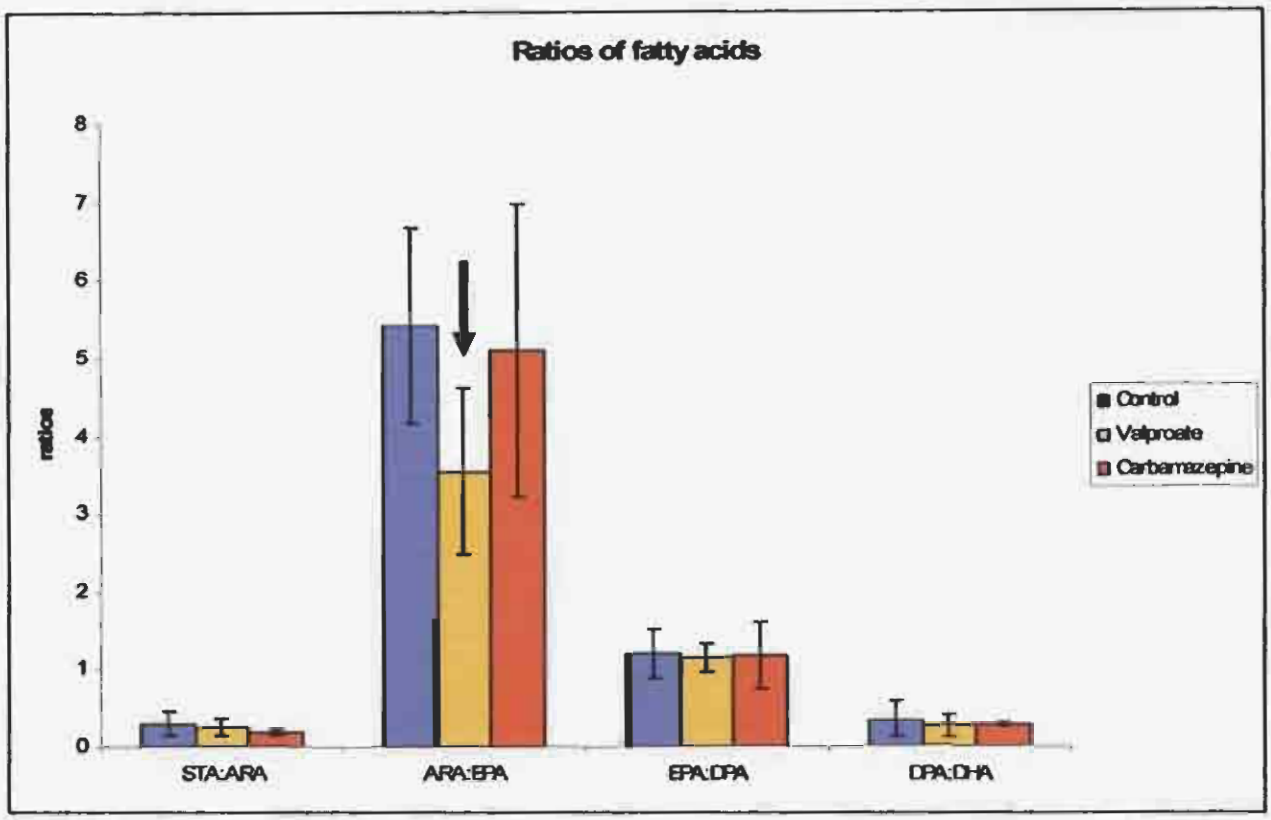


Figure 4.7. ω-3 fatty acid ratios (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

The analysis of the ratios of the ω-3 fatty acids showed only a statistical difference in the ARA: EPA ratio.

ω-3 ARA:EPA

There was a statistical difference between the control and valproate groups ($p = 0.007867$); the valproate group showing a lower ARA:EPA ratio than the control and the carbamazepine group (although not statistical significant). In section 4.2.3 we speculated that the lower ARA:EPA ratio could be due to a lower turn-over rate of the Δ5-desaturase enzyme that facilitates the turn-over of arachidonic acid to eicosapentaenoic acid in epileptic patients. These ratios, calculated for the valproate and carbamazepine-groups suggest that valproate stimulates the Δ5-desaturase enzyme, leading to a decrease in the ω-3 ARA:EPA ratio. This theory, will be explored in future research.

4.3.5.4 ω-3 fatty acids as a percentage of the total ω-3 fatty acids

We calculated each ω-3 fatty acid as a percentage of the total ω-3 fatty acids and analyzed the data to determine whether there were any differences between the various groups. Figure 4.8 show the differences between the various groups in their specific percentages.

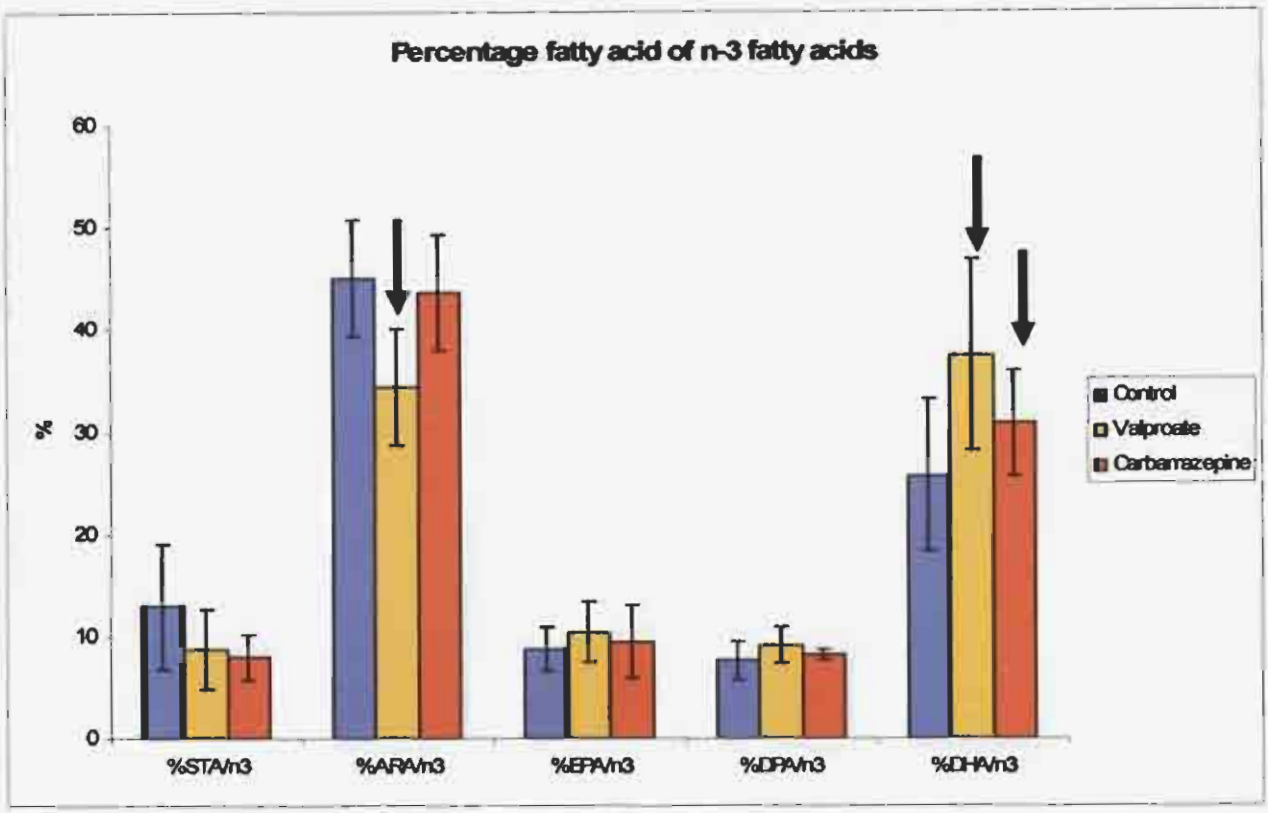


Figure 4.8. Individual ω-3 fatty acids as a percentage of the total ω-3 fatty acids (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

The analysis of the percentage individual ω-3 fatty acids in terms of the total ω -3 fatty acids showed no statistical differences in the percentages of STA/ ω-3, EPA/ ω-3, DPA/ ω-3. There were statistical differences in the following fatty acids:

% ω-3 ARA/ ω-3

A statistical difference was found in the % ω-3 arachidonic acid/ ω-3 fatty acids, between the control and valproate-group ($p = 0.000779$) and between the valproate and carbamazepine group (0.018449). The levels of % ω-3 ARA/ ω-3 in valproate was noticeably lower than both the control and carbamazepine groups. This correlates with the lowered ratio of ω-3 ARA:EPA in section 4.2.5.3, speculating that valproate might have an influence on the Δ5-desaturase enzyme.

% DHA/ ω-3

There was a statistical difference in the % docosahexaenoic acid/ ω-3 fatty acids between the control and valproate groups ($p = 0.006433$), the valproate-group showing much higher levels of % DHA/ ω-3 than the control-group, correlating with the previous findings that the

DHA concentration was increased in the valproate group. Carbamazepine DHA levels were also increased but not statistically significant. This correlates with our data in section 4.2.5.1 and 4.2.5.2.

4.3.6 Drug responsive and resistant subgroups of valproate and carbamazepine

To determine whether the increases in the ω-3 fatty acid levels were due to the epileptogenic status of the patients or if it was caused by the medication, we divided both treated groups into two subgroups, drug responsive and drug resistant.

4.3.6.1 ω-3 fatty acid concentrations

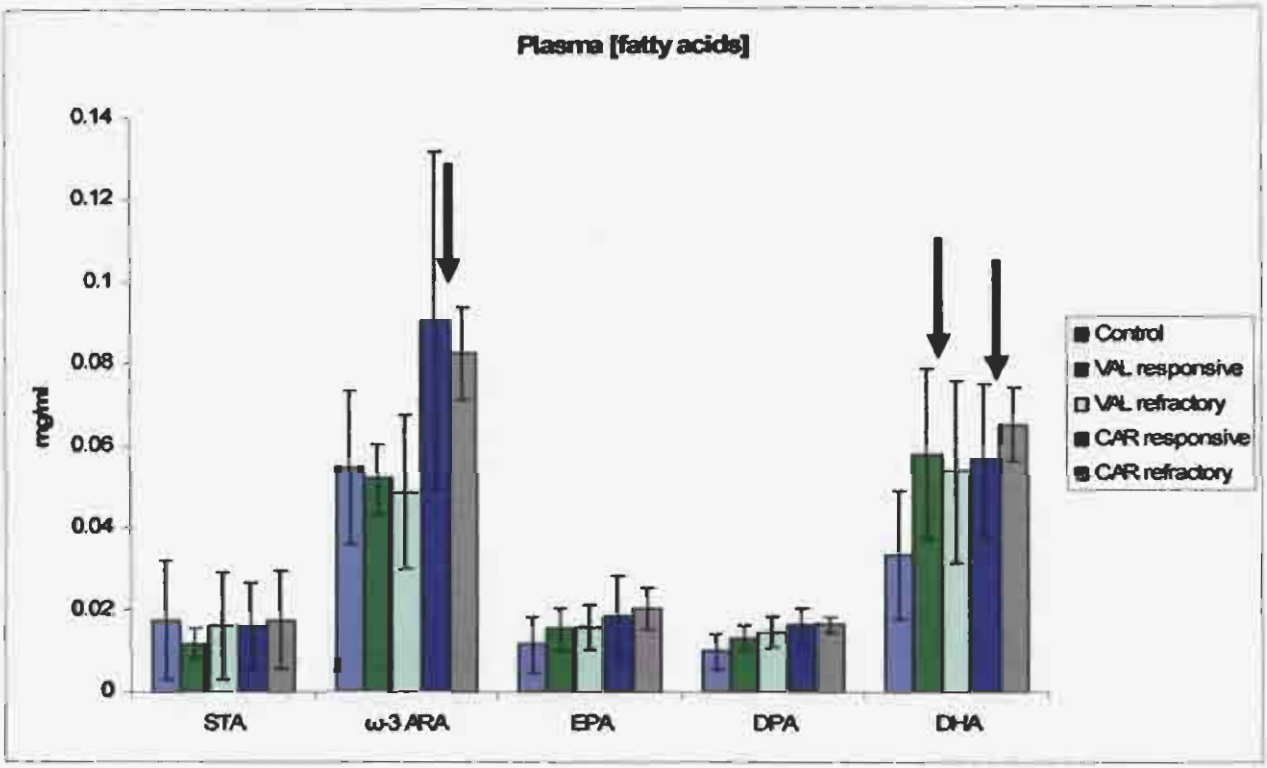


Figure 4.9. ω-3 fatty acids (the valproate and carbamazepine subgroups) (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

The results of the analysis (figure 4.9) showed that there was a notable increase in the levels of ω-3 arachidonic acid in both the drug responsive and resistant carbamazepine-groups, supporting our data in section 4.2.5.1 and 4.2.5.2. The DHA concentration levels were also elevated in both the valproate and carbamazepine drug responsive and refractory groups, supporting data in section 4.2.5.1 and 4.2.5.2. Because we could not find statistical differences between the drug-responsive and refractory epilepsy subgroups, one could

assume that the elevated levels of ω-3 fatty acids were due to the epileptogenic status of the patients, and not the use of valproate and carbamazepine respectively.

4.3.6.2 ω-3 fatty acids as a percentage of the total free fatty acids (TFFA)

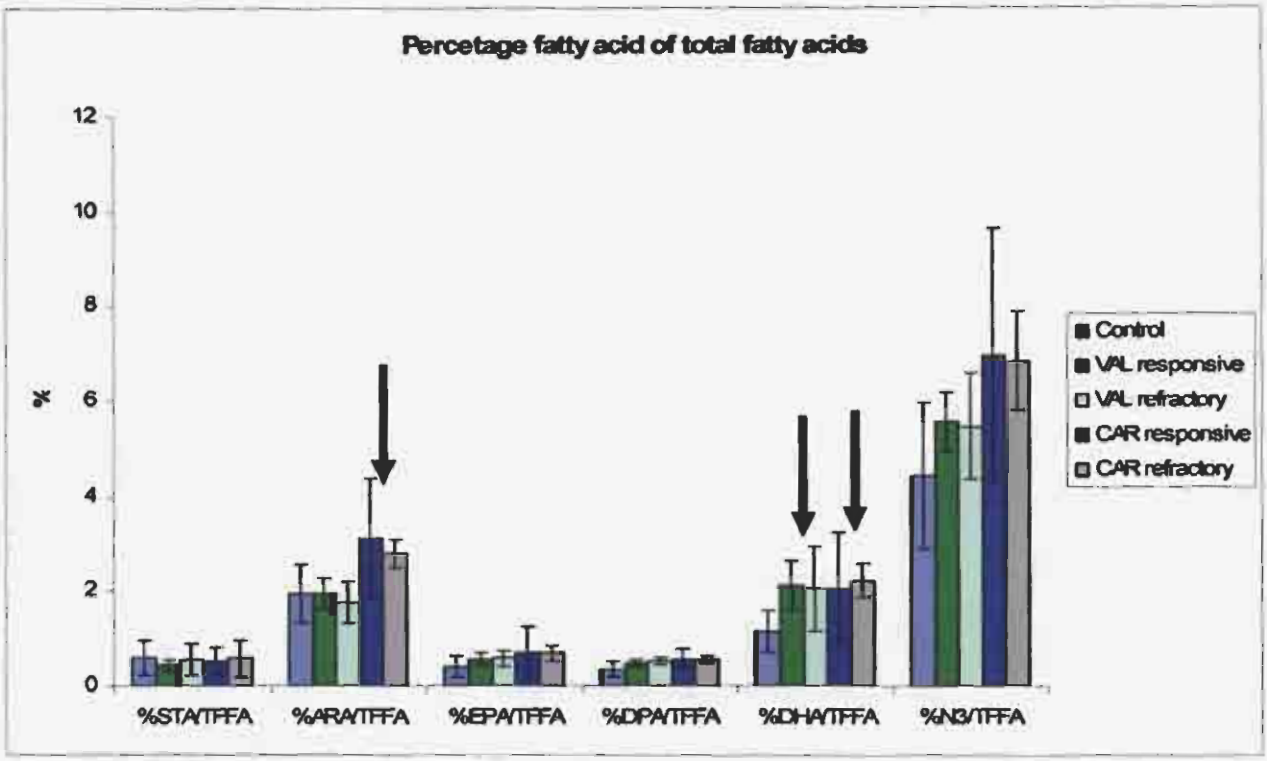


Figure 4.10. ω-3 fatty acids as a percentage of the total free fatty acids (valproate and carbamazepine treated subgroups). (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid, TFFA= total fatty acids).

Analysis of the data in figure 4.10 showed no statistical differences between the different subgroups but once again an increase in almost all of the acids (except for stearidonic) could be detected. This correlates with our previous findings that the elevated levels of ω-3 fatty acids are not statistically different between the different subgroups, and could therefore be attributed to the epileptogenic status of the patients.

4.3.6.3 ω-3 fatty acid ratios

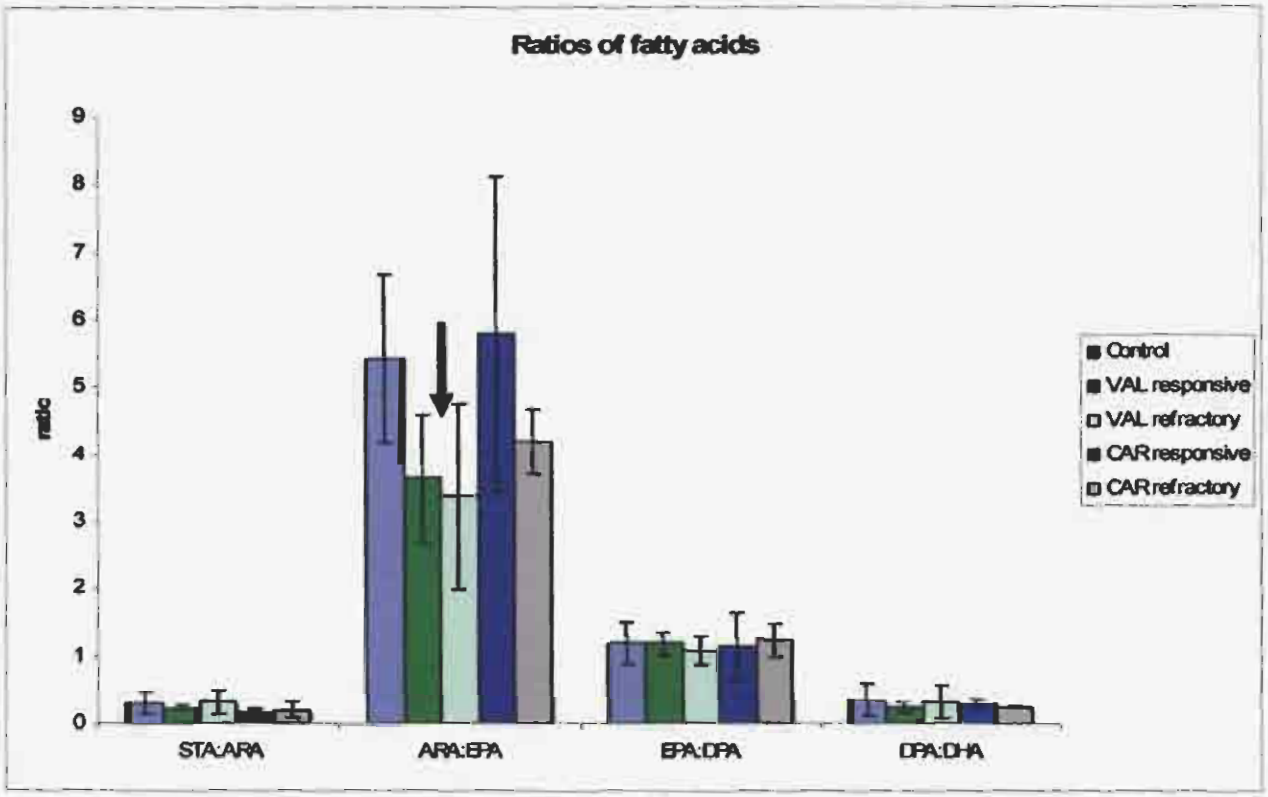


Figure 4.11. ω-3 fatty acid ratios (valproate and carbamazepine treated subgroups) (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

The data in figure 4.11 revealed that there were no statistical differences in the ratios of the ω-3 fatty acids between the different subgroups. A notably lower ratio, was detected in ω-3 ARA:EPA in both the drug responsive and drug-resistant valproate groups acid as well as in the drug-resistant carbamazepine-group. This data supports the data in section 4.2.5.3 suggesting that valproate may have an influence on the Δ5-desaturase enzyme, leading to a decrease in the ω-3 ARA:EPA ratio. This theory will be explored in future research.

4.3.6.4 ω-3 fatty acids as a percentage of the total ω-3 fatty acids

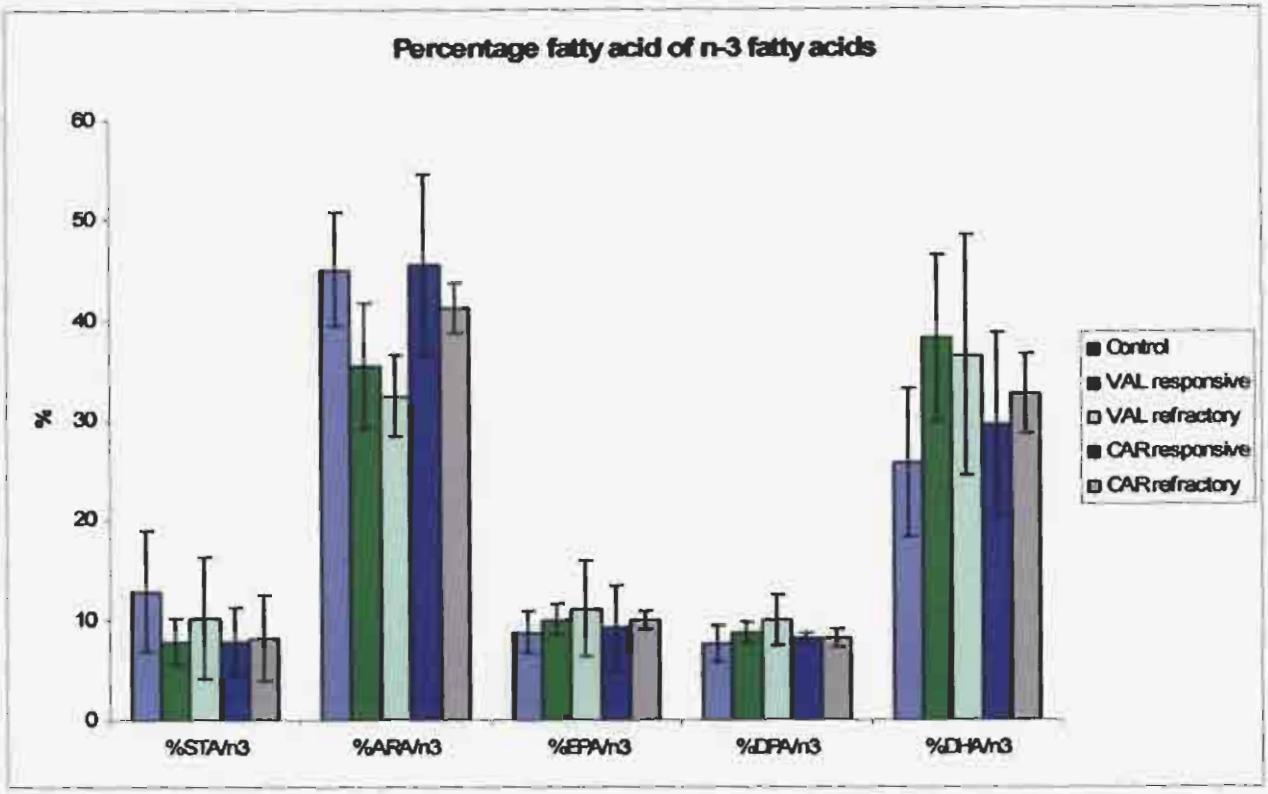


Figure 4.12. ω-3 fatty acids as a percentage of the total ω-3 fatty acids (valproate- and carbamazepine-treated subgroups) (STA= stearidonic acid, ω-3 ARA= ω-3 arachidonic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid).

Analysis of the data in figure 4.12 revealed no statistical differences between the different subgroups, but even though it was not statistically significant, the % DHA was higher in all the treated groups, especially the drug-responsive valproate group. This correlates with our previous findings that the increased concentrations of DHA in the plasma could be due to the epileptogenic status of the patients, because all the patients, refractory and drug-responsive cases showed increased concentrations of DHA, regardless of the medication they were taking.

4.4 Acylcarnitines

The data of the acylcarnitines were analyzed to determine if there were any statistical differences in the concentrations of the various acylcarnitines in the different groups. An analysis was performed using the unequal N HSD test, with a probability of $p \leq 0.05$. The test was followed by multiple comparisons p-values (2-tailed) and the Kruskal-Wallis test. The groups were the same as those used for the fatty acids.

See Appendix C for the raw data of the acylcarnitines.

4.4.1 Acylcarnitines in urine

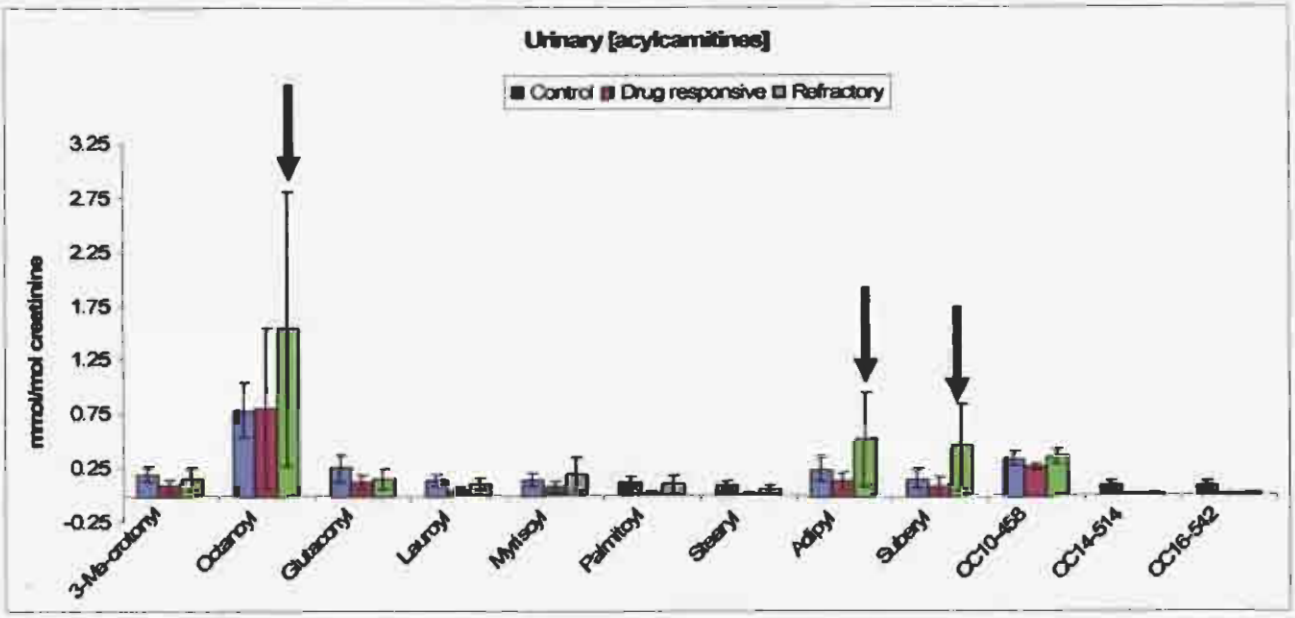


Figure 4.13. Individual acylcarnitines

The results (figure 4.13) revealed that there were no statistical differences in the concentrations of the hexanoylcarnitine, 3-hydroxyisovalerylcarnitine, carnitine, acetylcarnitine, propionylcarntine, butanoylcarnitine. However, there were statistical differences between in the concentrations of the following acylcarnitines:

3 – Methylcrotonylcarnitine

There was a statistical difference between the control and drug-responsive epilepsy group ($p = 0.002431$). The drug-responsive epilepsy group showed lower levels of 3-methylcrotonylcarnitine.

Octanoylcarnitine

No statistical differences were detected in the concentrations octanoylcarnitine, but clear elevated levels of this acylcarnitine can be seen in the refractory epilepsy group. Octanoylcarnitine is a long-chain acylcarnitine.

Glutaconylcarnitine

There was a statistical difference between the control and the drug-responsive epilepsy ($p = 0.010200$). The drug-responsive epilepsy group showed a decrease in its glutaconylcarntine concentration levels.

Lauroylcarnitine

There was a statistical difference between the control and drug-responsive epilepsy groups ($p = 0.000145$). A lowered concentration of lauroylcarntine was detected in the drug-responsive epilepsy group.

Myrisoylcarnitine

There was a statistical difference between the drug-responsive and refractory epilepsy groups ($p = 0.032539$). The refractory-group showed significantly higher levels of myrisoylcarnitine concentrations.

Palmitoylcarnitine

There was a statistical difference between the control and drug-responsive epilepsy groups ($p = 0.0003$), and between the drug-responsive and refractory epilepsy groups ($p = 0.020999$). The drug-responsive epilepsy group showed decreased levels of palmitoylcarnitine compared to both the control and refractory epilepsy groups.

Stearylcarnitine

There was a statistical difference between the control and drug-responsive epilepsy groups ($p = 0.000126$). The drug-responsive epilepsy group had significantly decreased levels in its stearylcarnitine concentration.

Adipylcarnitine

There was a statistical difference between the drug-responsive and refractory epilepsy groups ($p = 0.005481$). The refractory epilepsy group showed a considerable increase in its concentration adipylcarnitine compared to the drug-responsive epilepsy group.

Suberylcarnitine

There was a statistical difference between the control and refractory epilepsy groups ($p = 0.020677$) as well as between the drug-responsive and refractory epilepsy groups ($p = 0.003678$). The refractory epilepsy group showed an increase in its levels of suberylcarnitine compared to both the control and drug-responsive epilepsy groups and even though there was not a statistically significant difference, the levels of the drug-responsive epilepsy group showed a clear tendency to be lower than that of the control group.

CC10-458

There was a statistical difference between the control and drug-responsive epilepsy groups ($p = 0.001184$) also between the drug-responsive and refractory epilepsy groups ($p = 0.002487$). Both the control and the refractory epilepsy group showed higher levels of CC10-458 than the drug-responsive epilepsy group.

CC14-514

There was a statistical difference between the control and drug-responsive epilepsy groups ($p = 0.000123$) and the control and refractory epilepsy groups ($p = 0.000186$). Both the control and the refractory epilepsy groups had higher levels of CC14-514 compared with the drug-responsive epilepsy group.

CC16-542

There was a statistical difference between the control and drug-responsive epilepsy groups ($p = 0.000123$) and between the control and refractory epilepsy groups ($p = 0.000177$).

Of interest was that the adipylcarnitine, suberylcarnitine and octanoylcarnitine were increased in the refractory epilepsy group. Because the medication had such a dramatic effect on the fatty acids, we could not assume that these increased levels in certain acylcarnitines in the refractory group were due to the refractory status of the patients. Again we merged the drug-responsive and refractory epilepsy groups and divided them into a valproate and carbamazepine-treated group.

4.4.2 Individual acylcarnitine concentrations in the valproate and carbamazepine groups

Carnitine transports long-chain fatty acids as acylcarnitine esters across the inner mitochondrial membrane, thus acting as a cofactor for mitochondrial fatty acid oxidation (Coppola *et al.*, 2006). However, it is known that valproate undergoes extensive metabolism in the human mitochondria, primarily via β-oxidation. Valproate generates four acyl-CoA esters in this process, valproyl-CoA, Δ^2-valproyl-CoA, 3-hydroxyvalproyl-CoA and 3-ketovalproyl-CoA. The accumulation of these xenobiotic-CoA esters in the matrix leads to reduction of free CoASH and affects various CoA-dependant reactions regulated by the ratio [acyl-CoA]/[CoASH] (Silva *et al.*, 2001). This leads to accumulation of middle and shortchain carnitine esters and an increase in certain acylcarnitines in urine (Farkas *et al.*, 1996). The effect of carbamazepine on the acylcarnitines is not yet known.

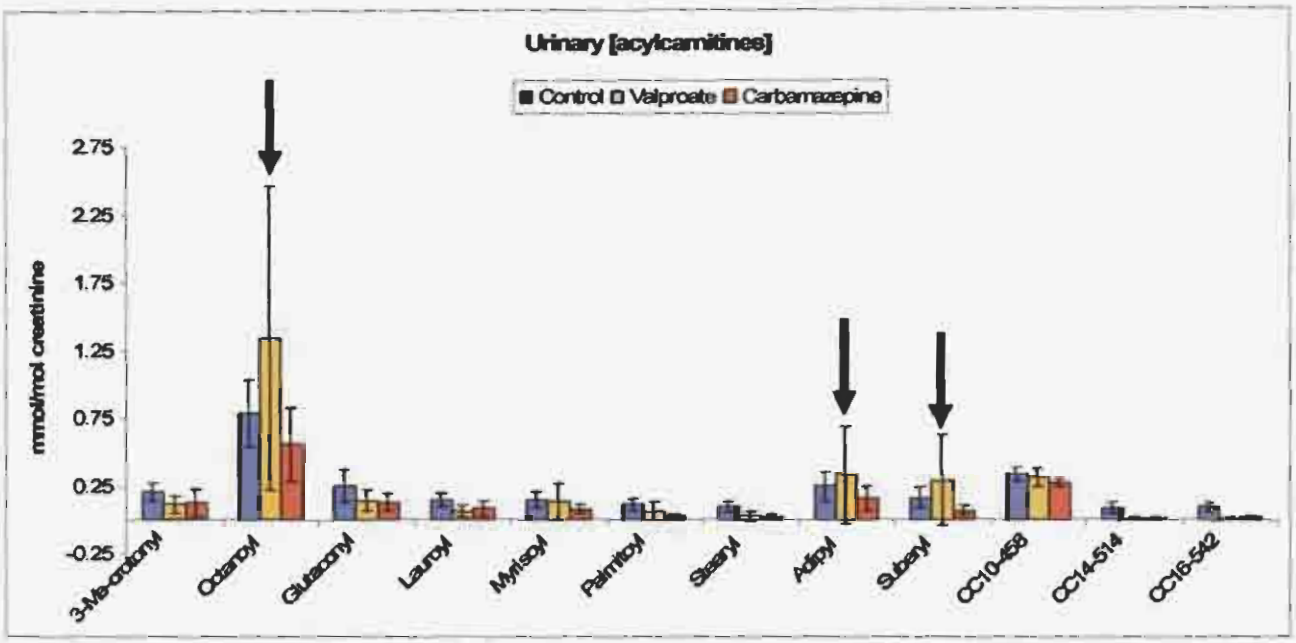


Figure 4.14: Acylcarnitine concentrations in the valproate and carbamazepine groups.

The results of the analysis of the data in figure 4.14 revealed that there were no statistical differences in the concentrations of the octanoyl, adipyl, suberylcarnitine and CC10-458. The following acylcarnitines did show a statistical difference in their concentrations between the respective groups:

3-Methylcrotonylcarnitine

There was a statistical difference between the control and valproate-groups ($p = 0.009030$). The valproate-group showed lowered levels of 3-methylcrotonoylcarnitine than the control group and even though it was not statistically significant, it had lower levels than that of the carbamazepine group as well.

Glutaconylcarnitine

There was a statistical difference between the control and valproate-groups ($p = 0.020610$). The valproate-group showed lower levels of glutaconylcarnitine than the control-group.

Lauroylcarnitine

There was a statistical difference between the control and valproate groups ($p = 0.000795$). The valproate-group showed lowered levels of lauroylcarnitine in comparison with the control-group.

Palmitoylcarnitine

There was a statistical difference between the control and carbamazepine groups ($p = 0.012655$). The levels of the concentration palmitoylcarnitine were much lower in the carbamazepine-group.

Stearylcarnitine

There was a statistical difference between the control and valproate-groups ($p = 0.000332$) and between the control and carbamazepine groups ($p = 0.004373$). Both the valproate and carbamazepine groups showed lower levels of stearylcarnitine.

CC14-514

There was a statistical difference between the control and valproate groups ($p = 0.000123$) and the control and carbamazepine groups ($p = 0.000151$).

CC16-542

There was a statistical difference between the control and valproate groups ($p = 0.000123$) and the control and carbamazepine groups ($p = 0.000212$).

Even though no statistical differences were detected in the concentrations of adipyl, suberyl and octanoylcarnitine, a definite tendency of increased concentration levels in the valproate-group can be observed. This correlates with the literature that certain short-chain acylcarnitines are lowered in patients using valproate, and certain other long-chain acylcarnitines accumulate with short-term valproate-usage, leading to increased levels of the concentrations of these acylcarnitines. Valproate affects β-oxidation in the mitochondria, leading to an increase in several long-chain acylcarnitines (see 2.8.2.2).

The results showed that there were statistical differences in several of the acylcarnitines, especially between the control and valproate group. Because both treated groups contained a mixture of drug-responsive and refractory epilepsy patients, we still could not be certain that the increased levels were due to the valproate or the refractory status of the patients. Thus we divided the treated groups in subgroups of drug-responsive and refractory patients.

4.4.3 Drug responsive and resistant subgroups of valproate and carbamazepine

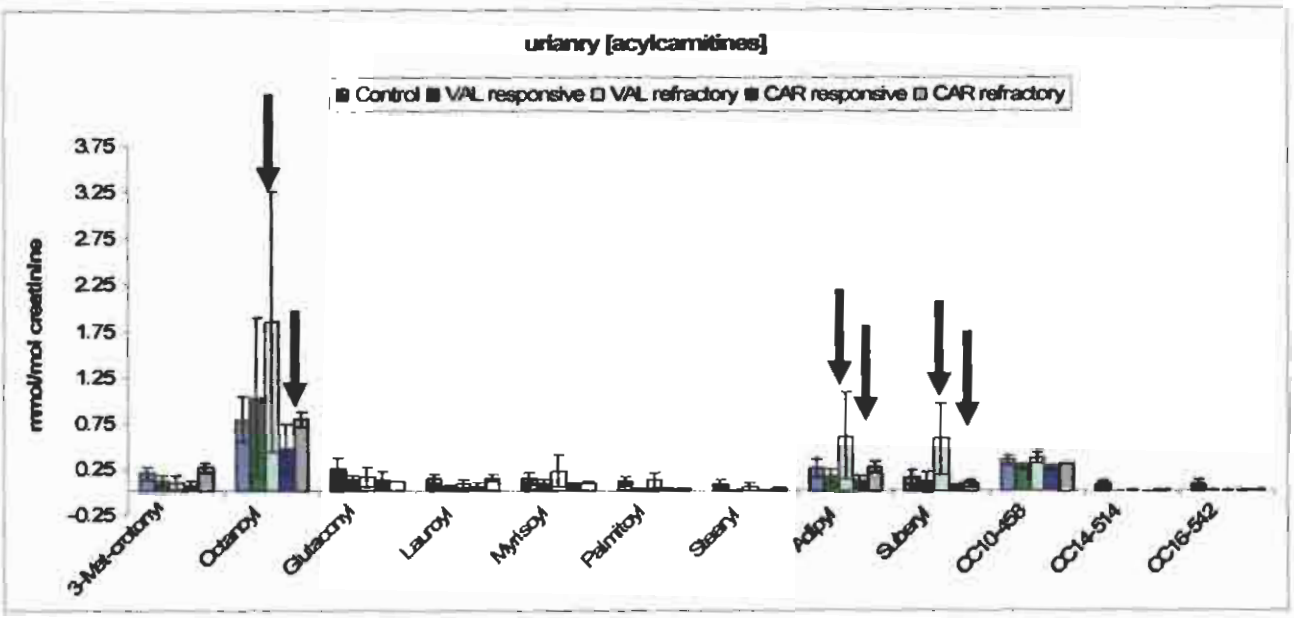


Figure 4.15: Acylcarnitines in the valproate and carbamazepine treated subgroups.

No statistical differences were evident between the various groups (fig. 4.15). However, in the concentrations adipyl, suberyl and octanoylcarnitine in the refractory valproate and carbamazepine subgroups are clearly increased. This could be attributed to the fact that these patients received higher dosages of valproate than their drug-responsive counterparts. Higher dosages of valproate means a more extensive inhibition of β-oxidation in the mitochondria which in turn leads to an accumulation of short-chain fatty acids that are metabolized via ω-oxidation to long-chain acylcarnitines such as adipyl, suberyl and octanoylcarnitine. Thus, the higher the dosage of valproate, the more extensive the inhibition of the β-oxidation and the higher is concentration of long-chain acylcarnitines produced, which could explain the elevated levels of these acylcarnitines. The increased levels of acylcarnitines in the carbamazepine drug-resistant group remains a mystery. The effect of the higher dosage valproate and carbamazepine in the refractory groups is clearly evident in the total acylcarnitines as shown in figure 4-16.

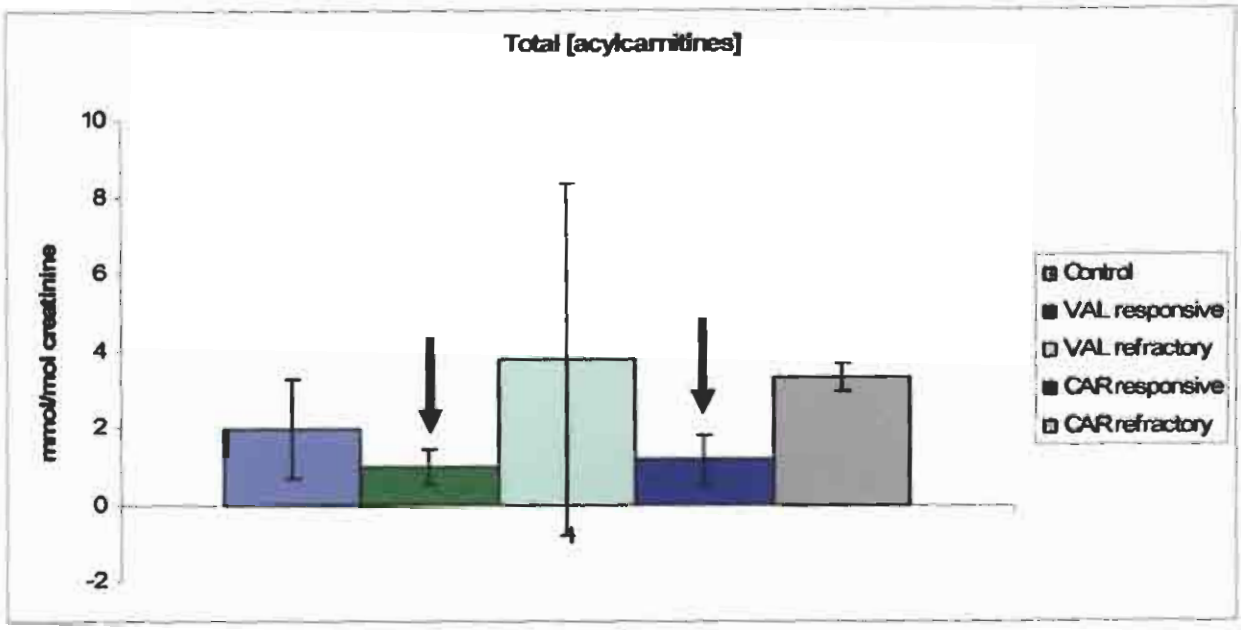


Figure 4.16. Total acylcarnitines in the valproate and carbamazepine subgroups.

4.5 Discussion

When valproate is administered to a patient, the mitochondrial β-oxidation is inhibited. This inhibition leads to an increase in the fatty acyl-CoA concentration in the mitochondria, which in turn leads to an increase in the concentrations of various fatty acids, including several ω-3 fatty acids (ω-3 arachidonic acid, DHA, etc). These free fatty acids may subsequently be transported to the mitochondria for further oxidation, be subject to ω-oxidation, or be excreted in the urine as free carboxylic acids (Silva *et al.*, 2001).

Dicarboxylic acids are formed via ω-oxidation of fatty acids in the endoplasmic reticulum and are degraded as the CoA ester via β-oxidation in peroxisomes (Westin *et al.*,2005). Thus, an increase in the concentration of fatty acids will ultimately lead to an elevation of the dicarboxylic acids (figure 4-17).

However, carbamazepine also increased the concentration of ω-3 arachidonic acid, DHA and several other ω-3 fatty acids. The chemical structures of valproate (a short chain fatty acid) and carbamazepine differ vastly from each other, and so do the suggested mechanisms by which these two drugs prevent seizures. If carbamazepine did in fact function in the same manner as valproate, *i.e.* inhibiting mitochondrial β-oxidation, it would have increased the concentration long chain acylcarnitines. In our analysis, the carbamazepine-group did not show increased concentrations of acylcarnitines, but the carbamazepine drug resistant subgroup did show increased concentrations of some acylcarnitines.

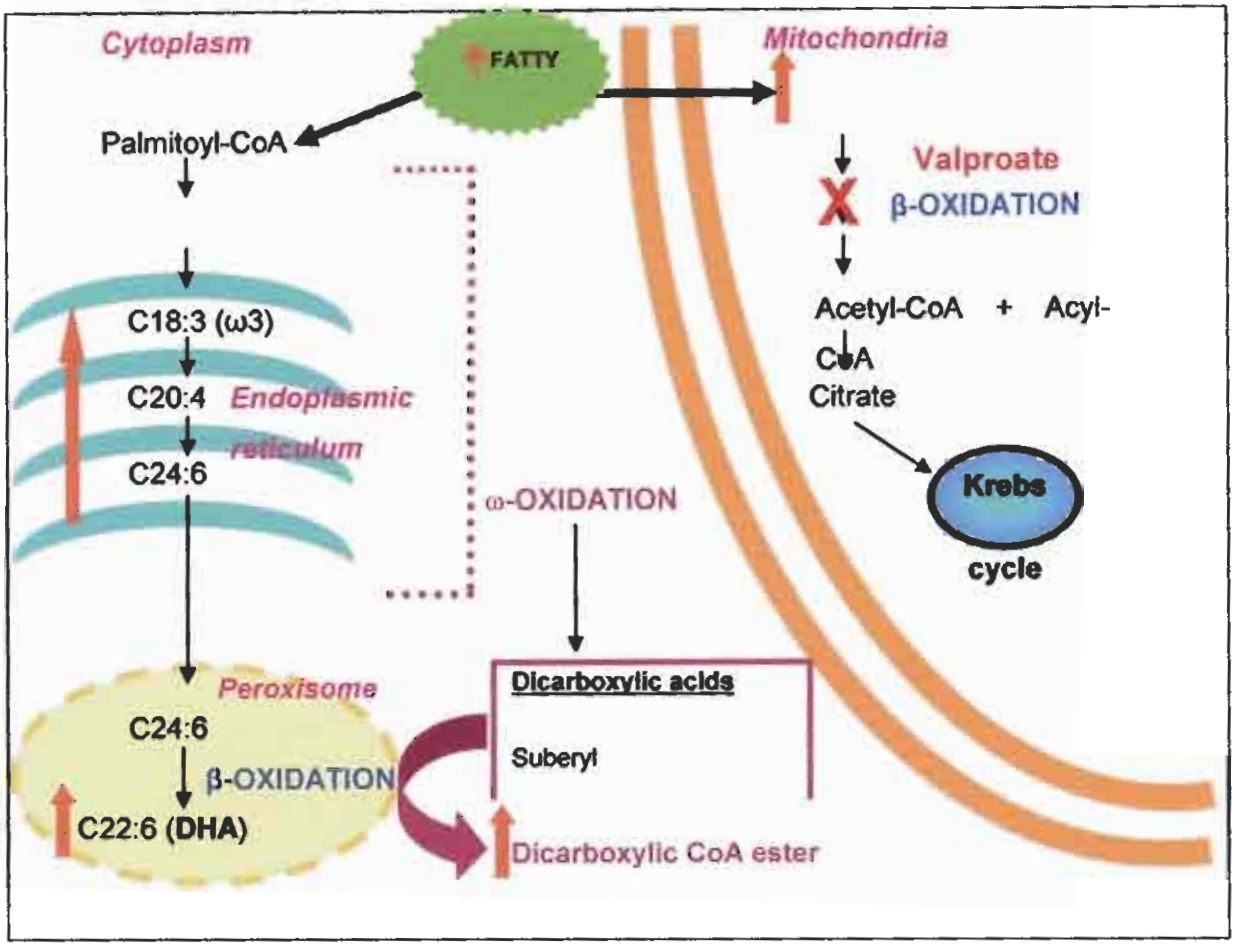


Figure 4.17. The influence of valproate on fatty acid synthesis and -β- oxidation.

The difference in the chemical structures, proposed mechanisms of action and different effects on the acylcarnitine levels of valproate and carbamazepine, suggest that the elevated levels of fatty acids in the plasma of both the drug-responsive and refractory epilepsy patients are possibly due to the epileptogenic status of the patients, and not the use of the medication.

Measurement of fatty acids in the plasma represents the fatty acid biosynthesis, whereas measurement in the red blood cells represents a combination of the biosynthesis and incorporation of DHA in the cell membrane. Thomas R. Henry (Henry, 2004) found abnormally low levels DHA in the red blood cell membranes of patients with uncontrolled epilepsy. Our results showed elevated levels of DHA in the plasma of patients with controlled and uncontrolled epilepsy. Combining our results with those of Henry we propose that DHA synthesis is not affected and propose that the incorporation of DHA in the membranes and blood brain barrier is compromised. Therefore, further trials should be performed to better understand the incorporation of DHA in target tissues.

Chapter 5

5 Conclusion

Currently there are more than 40 types of epilepsy (Schmidt, 2002), making it an extremely variable disease also complicating the effective treatment of this disorder. Despite the fact that epilepsy is the most common neurological disorder and probably one of the oldest, it still has scientists guessing today. Worldwide, an estimated 10.5 million children have active epilepsy (developing countries accounting for the highest percentage). Over the past 15 or 20 years syndrome-orientated clinical and EEG diagnosis, and better aetiological diagnosis, has helped to clarify the diversity of epilepsy (Guerrini, 2006). But even though the diagnostic tools have improved, there are still an estimated 40% of epilepsy patients that suffer from medically refractory seizures. Refractory epilepsy is poorly understood and accounts for numerous tragedies including SUDEP (sudden unexplained death in epilepsy), brain injuries causing brain damage, suicides, learning disabilities, social isolation etc. The patients suffering from refractory epilepsy often lead lives of social imbalance, isolating themselves from society because of low self-esteem and other phobias.

In the literature, we found numerous articles where refractory epilepsy was successfully treated with either the ketogenic diet, or ω-3 supplementation (Yuen *et al.*, 2005; Sheth *et al.*, 2005; Vining, 1999). With both treatments the patients became either seizure-free or had a decrease in the frequency of their seizures. Valproate was found to increase the DHA-levels, whilst carbamazepine increased the ω-3 arachidonic acid levels in the plasma. DHA (an ω-3 fatty acid) is an important component of phospholipids and plays an essential role in membrane-integrity and fluidity as well as neuronal cell communication, to name but a few.

The first aim of this study was to determine if there were any differences in the concentrations and concentration ratios of the various ω-3 fatty acids in the plasma between a healthy individuals (control-group), a group of drug-responsive epileptic children and a group of refractory epileptic children. Carnitine facilitates the transport of fatty acids over the mitochondrial membrane, thus, the second aim of our study was to determine the concentrations of the various acylcarnitines in the urine in the same groups.

The results indicated an increase in several of the ω-3 fatty acids, especially DHA, also an increase in several of the acylcarnitines, especially the long-chain acylcarnitines (adipyl, suberyl and octanoylcarnitine) in both the drug-responsive and the refractory groups. All of the epileptic children (both the drug-responsive and the refractory groups) were treated with AEDs, mostly valproate and carbamazepine. Not knowing if our findings were the result

of the AEDs or the epileptogenic status of the patients, we merged the two epileptic groups, re-dividing them into valproate and a carbamazepine-groups. DHA-levels were increased in both the valproate and carbamazepine-groups, but there was no statistical difference between the two groups. The mechanisms by which valproate and carbamazepine inhibit seizures have not been established yet, but the proposed mechanisms in the literature differ a great deal from each other. Because we could not find statistical differences between the valproate and carbamazepine group in their DHA or long chain acylcarnitine levels, we speculated that the elevated DHA-levels are possibly due to the epileptogenic status of the patients, and not the AED-therapy they are taking. Combining our results with those of Henry (2004), (Henry found there to be abnormally low levels DHA in the red blood cell membranes of patients with uncontrolled epilepsy) we propose that DHA synthesis is not affected in epileptic patients, but that the incorporation of DHA in the membranes and blood-brain barrier is compromised. We would like to explore this theory in future research using both plasma and red blood cells for our analysis.

5.1 Future Research

One aspect that complicated this study, was the lack of an epilepsy control group. All of the epilepsy patients who participated in the study, were on one or more AED. It is also quite difficult to find refractory epilepsy patients on the ketogenic diet (KD), and because this diet is rather unpleasant (the diet consists mainly of fat and protein and includes side-effects like nausea and diarrhea) it is sometimes difficult to ensure patient compliance even though it has proven to be very effective. Another aspect tha complicated our study was that there was no indication whether the samples from the different patient were taken at approximately the same time of the day. Considering the ethical implication of depriving these patients of their medication, we are planning to further our studies by:

(1) Working in association with pediatric neurologists who will be able to give us plasma- and urine samples of epilepsy patients directly after diagnosis, before the treatment is commenced. This will allow us to gather an epilepsy control group.

(2) To surmount the difficulty of assembling a group of (refractory) epilepsy patients, treated with the ketogenic diet, we contacted a dietician specializing in this particular treatment of epilepsy who will provide us of urine and plasma samples of these patients on a continuous basis (i.e. after one week of treatment, one month of treatment etc).

(3) We also plan on using animal models, specifically the GEPR (Genetic Epileptic Prone Rat). The GEPR has been used successfully in several articles as an animal model for epilepsy. We plan on administering several AEDs to these rats, especially valproate

and carbamazepine as well as the KD to study the effects of these treatments on the rats. It has not yet been determined if the mechanism by which the KD inhibits/prevents seizures, is by altering the plasma and tissue content of PUFAs, particularly an increase in brain DHA, but studies by Taha *et al* (2005) showed that there was a lower specific action of DHA in the liver, but higher activity in the brain in rats on the KD, this supports tissue redistribution rather than higher synthesis of DHA under these experimental conditions. This could mean that even if there is not a defect in the biosynthesis of DHA in refractory-epilepsy patients, there could well be a defect in the incorporation of DHA in the tissue.

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Appendix A

Organic acids

Organic acids							
Patient	Lactic acid	Glycolic acid	Oxalic acid	2-hydroxy-3-methylbutyric acid	3-hydroxyisobyturic acid	2-hydroxy-iso-valeric-acid	2-methyl-3-hydroxybutyric acid
20-9-6-05C	14.66	11.3	68.82		20.55		3.39
2-25-5-05C	7.16	20.78	21.84		10.84		3.83
1-15-9-05C	24.02	66.6	227.71	0.14	28.32	3.42	0.14
2-2-8-05C	10.75	33.14	80.44		11.19		3.09
2-4-10-05C	11.1	27.86	37		10.36	0.46	
3-26-10-05C	100.71	44.46	81.19		26.54	0.81	
4-5-9-05C	16.87	55.53	35.29		19.93	2.06	5.98
4-13-4-05C	33.08	32.51	35.69		10.4		3.33
5-18-10-05C	9.27	36.84	11.09		9.39		1.18
5-30-9-05C	61.15	23.26	104.34		21.66	5.57	4.88
6-2-8-05C	14.6	30.63	70.76		31.34		2.14
6-30-9-05C	46.55	20.19	108.38		7.65	1.13	
8-14-4-05C	16.55	45.41	19.04	1.56	12.1		1.87
16-23-5-05C	13.21	41.56	38.02		15.89	0.75	5.47
4-20-4-06E	23.32	52.35	56.54	23.72	2		22.06
3-20-4-06E	168.64	98.76	49.74		48.68	2.29	1.1
2-20-4-06E	6.15	7.96	11.75		2.85	5.58	
3-13-4-06E	3.62	6.35	13.43		2.35		
2-6-4-06E	38.33	42.41	69.99		4.93	0.64	1.17
3-6-4-06E	83.84	43.24	71.41	1.69	13.89	0.68	0.61
14-16-3-06E	23.84	33	12.9	0.71	20.05		
15-16-3-06E	13.94	35.78	19.32	0.28	10.55		5.99
16-16-3-06E	9.57	40.09	18.98		7.53		0.56
7-23-3-06E	30.83	18.84	48.66		20.85		1.77
11-23-3-06E	24.19	27.49	36.55		2.02		
10-23-2-06E	18.4	127.09	54.94	0.12	25.8		4.07
11-23-2-06E	7.02	22.38	7.79	0.09	5.97		0.98
12-23-2-06E	9.35	25.25	5.62		3.6		
3-4-5-06R	13.39	9.14	42.07		11.75	1.23	1.73
17-16-3-06R	17.76	44.7	39.32	0.51	11.03	0.62	4.27
18-16-3-06R	215.77	19.52	112.8	0.69	63.69	2.52	20.58
19-16-3-06R	22.67	9.18	12.4		17.34		2.06
9-23-3-06R	16.99	18.52	24.41		11.61	0.63	0.6
10-23-3-06R	11.97	61.47	44.61	0.27	8.22		1.91
20-16-3-06R	16.18	9.33	23.16	0.24	22.2		8.23

Organic acids								
Patient	**methylmalonic-acid**	**3-hydroxyvaleric-acid**	**methylsuccininc-acid**	**succinic-acid**	**uracil**	**glyceric-acid**	**fumaric-acid**	**glutaric-acid**
20-9-6-05C			5.61	64.63	6.1	0.36	2.99	6.37
2-25-5-05C	0.64		1.66	42.91	2.82	0.76	0.66	1.46
1-15-9-05C	61.32		5.74	58.72	4.76	1.41		1.93
2-2-8-05C	7.94		10.74	73.69	2.89	0.83	0.66	0.87
2-4-10-05C	6.85		0.5	16.12	0.79	0.69	2.82	2.17
3-26-10-05C	47.72		2.3	104.1	2.14	5.95		5.67
4-5-9-05C			1.54	13.6	3	0.26	1.04	0.87
4-13-4-05C	1.68			32.19	1.27	0.45		0.47
5-18-10-05C	4.23			11.6	2.31	0.33		
5-30-9-05C				61.8	12.02	0.69	8.88	3.99
6-2-8-05C	0.43		3.72	134.77	4.8	1.92	4.24	3.41
6-30-9-05C			2.4	4.36	2.12			2.46
8-14-4-05C			10	31.79	0.68	0.88		0.16
16-23-5-05C			3.39	27.85	2.03	0.51		2.19
4-20-4-06E	48.54	3.12	3.3	4.1	3.12	1.81	4.1	2.12
3-20-4-06E		27.5		22.54	1.64	0.79	1.6	4.64
2-20-4-06E		3.63		12.57	1.02		0.07	2.71
3-13-4-06E		4.71	0.45	2.42	0.4		0.3	0.09
2-6-4-06E		1.49	2.85	22.85	0.83	2.24		0.84
3-6-4-06E		25.17	1.81	32.14	1.87	0.56	0.85	1.29
14-16-3-06E		20.36	0.68	24.51	0.86	0.69	0.85	1.49
15-16-3-06E		9.92	1.16	5.07	3.12			0.72
16-16-3-06E		14.05	1.03	8.26	0.3		0.34	0.58
7-23-3-06E		24.91	4.63	25.94	0.56	0.53	2.88	3.01
11-23-3-06E			0.96	11.27	1.31	0.83		1.76
10-23-2-06E		25.03	0.91	19.27	0.2	0.42	1.78	1.35
11-23-2-06E		3.33	1.02	20.34	0.47	0.36		1.12
12-23-2-06E		4.55	1.24	9.29	0.47			0.83
3-4-5-06R	0.87	19.84	1.64	34.02	1.84		0.26	2.29
17-16-3-06R		18.93	1.95	30.56	2.38	0.86		1.12
18-16-3-06R	8.17	102.73	3.17	137.54	2.81	6.85	12.43	11.62
19-16-3-06R	0.36	13.55	1.41	14.78	1.7	0.57	0.3	0.62
9-23-3-06R		16.19	1.57	30.78	0.18	0.72	0.52	2.07
10-23-3-06R		11.45	2.3	18.7	0.5			1.84
20-16-3-06R	1.19	20.49	0.86	5.48	1.56	0.45		

Organic acids								
Patient	2,4-dihydroxybutyric-acid	3-methylglutaconic-acid	mandelic-acid	adipic-acid	isovalerylglycine	3-methyladipic-acid	tiglylglycine	2-hydroxyphenylacetic-acid
20-9-6-05C	0.72	7.61	5.91	6.17		1.88		
2-25-5-05C		0.32	2.8	1.69				0.77
1-15-9-05C	0.58	6.34	5.89	2.57		4.71		2.2
2-2-8-05C		4.77	1.65	4.32	0.33	1.93		1.5
2-4-10-05C		3.16	1.77	1.58		0.8		0.65
3-26-10-05C		1.32	0.1	1.65		1.89		
4-5-9-05C	0.29	3.35	3.2	0.96		0.87		1.48
4-13-4-05C	1.78	2.14	5.03	1.77	0.97	1.86	1.47	1.01
5-18-10-05C		1.62	1.85	0.64		0.35		0.25
5-30-9-05C		6.98	11.8	6.48		4.56	0.81	3.15
6-2-8-05C		3.95	3.58	9.84	0.82	5.5		0.98
6-30-9-05C	1.11	4.64	4.75	1.85		1.84		0.65
8-14-4-05C	4.81	3.65	6.08	4.45		3.67	1.49	1.24
16-23-5-05C		3.35	7.44	2.22	2.29	2.74		1.7
4-20-4-06E	2.64	3.06	0.24	7.02	0.81	0.87	0.34	
3-20-4-06E		1.2		7.89	0.64	1.75	0.1	0.26
2-20-4-06E				1.08	0.86	0.17		0.43
3-13-4-06E	0.14	0.47	0.13		0.17	0.14		
2-6-4-06E		1.27		2.73	0.81	1.82	0.54	0.84
3-6-4-06E		0.98		11.43	0.39	1.2	0.28	
14-16-3-06E		0.54			0.33	0.81	0.17	0.66
15-16-3-06E	0.65	1.72	0.02		0.35	1.92		0.57
16-16-3-06E		0.74		3	0.73	1.24	0.09	0.17
7-23-3-06E		1.71	0.3	7.77		2.11	0.15	0.61
11-23-3-06E		1.1				0.67		
10-23-2-06E	1.44	2.57		3.05	2.37	1.05	2.52	0.79
11-23-2-06E	0.34	1.38			0.95	0.52	0.27	0.67
12-23-2-06E	0.56	1.33			0.53	0.81		0.39
3-4-5-06R	0.64	1.57	0.49	18.16	0.55	1.07	0.57	0.58
17-16-3-06R	2.4	3.54			1.45	0.65	0.13	1.16
18-16-3-06R	3.43	11.22		98.38	4.32	0.43	2.24	1.41
19-16-3-06R		0.97		7.61	0.24	0.79	0.06	0.36
9-23-3-06R		0.74		6.94		0.36	0.04	0.52
10-23-3-06R	0.93	3.32	0.32		2.86	0.99	2.18	1.42
20-16-3-06R	0.45	2.49		7.75	0.26	1.53		0.45

Organic acids							
Patient	2-hydroxyglutaric-acid	3-hydroxy-3-methylglutaric-acid	4-hydroxyphenylacetic-acid	suberic acid	orotic acid	aconitic acid	azelaic-acid
20-9-6-05C	8.95	16.05				73.67	6.44
2-25-5-05C	2.64		18.13		0.55	28.77	
1-15-9-05C	1.34	15.66	426		0.61	109.36	
2-2-8-05C	2.5		72.64			56.84	
2-4-10-05C	3.78	3.03	17.11			49.11	3.45
3-26-10-05C	3.83	3.58	18.49	5.33	0.15	57.23	11.28
4-5-9-05C	2.12	4.52	19.64			32.55	2.35
4-13-4-05C	3.14	1.78				44.52	
5-18-10-05C	1.86	3.16	7.67			66.57	0.98
5-30-9-05C	15.26	15.76	167.71		1.57	84.66	
6-2-8-05C	5.92	4.45	24.61			58.37	
6-30-9-05C	9.96	4.23	36.94		1.03	92.52	
8-14-4-05C	4.02	9.95	61.86			98.64	
16-23-5-05C	2.59	1.81	62.4			46.59	
4-20-4-06E	9.2	5.14	49.6	5.29	0.94	130.32	12
3-20-4-06E	5.89	3.86	19.6	2.34	1.29	73.6	4.09
2-20-4-06E	2.77	1.5	18.47		0.36	15.53	0.18
3-13-4-06E	0.97		8.52	0.48	0.18	11.42	
2-6-4-06E	4.13	12.08	28.61	2.95	0.71	65.92	9.37
3-6-4-06E	5.18	5.76	20.5	14.03	0.55	75.3	23.13
14-16-3-06E	3.09	1.94	36.82	0.37	0.4	32.67	2.16
15-16-3-06E	2.16	2.37	45.55	4.73	0.5	29.41	8.65
16-16-3-06E	2.24	1.7	15.22	0.44	0.56	38.47	1.79
7-23-3-06E	6.97	4.88	32.48	2.66	1.4	255.58	6.88
11-23-3-06E	1.9	0.3	10.38		0.38	44.9	0.22
10-23-2-06E		1.39	22.01	9.4	0.48	51.34	37.85
11-23-2-06E	2.64		44.71	0.67	0.22	22.22	2.66
12-23-2-06E	3.64		14.85	0.61	0.44	40.32	7.35
3-4-5-06R	8.18	5.15	60.8	5.63	0.43	42.95	20.65
17-16-3-06R	8.94	3.85	57.64	1.86	1.81	70.94	4.78
18-16-3-06R	38.24	7.94	71.48	38.64		76.44	30.06
19-16-3-06R	3.46		21.74	1.74	0.26	32.69	5.58
9-23-3-06R	5.18	2.45	24.34	9.66	1.14	38.96	30.84
10-23-3-06R	4.51	2.36	66.4	3	0.66	75.15	8.59
20-16-3-06R	0.99	1.17	13.14		0.21	16.59	2.88

Organic acids								
Patient	**hippuric-acid**	**citric-acid**	**methylcitric-acid**	**indol-3-acetic-acid**	**4-hydroxyphenyllactic acid**	**acetyltyrosine**	**5-hydroxyindolacetic-acid**	
20-9-6-05C	121.07	174.46	2.11	4.68	3.47			0.64
2-25-5-05C	90.86	222.56	0.8	0.07	0.62	0.19		0.09
1-15-9-05C	224.93	416.73	2.98	5.48	2.7	0.81		0.63
2-2-8-05C	223.72	210.8	1.57	0.27	1.61			0.1
2-4-10-05C	158.86	202.61	0.61		0.9	0.34		0.44
3-26-10-05C	182.25	476	0.54	3.6	1.34	0.36		0.28
4-5-9-05C	76.75	209.38	0.62	1.73	1.82			0.85
4-13-4-05C	42.03	60.67			2.5			
5-18-10-05C	52.91	13.25	0.61	0.29	0.72			0.17
5-30-9-05C	232.74	244.74	3.58	6.25	3.97			3.54
6-2-8-05C	174.6	162.14	1.25	0.37	1.33	0.36		0.1
6-30-9-05C	64.83	223.47	1.6	2.1	4.16	0.24		0.4
8-14-4-05C	371.87	122.8	0.35		2.02			
16-23-5-05C	6.18	99.12	0.83	1.65	6.19			
4-20-4-06E	94.9	226.95	1.3	0.25	1.66	0.63		2.38
3-20-4-06E	232.15	411.74	0.76	1.22	1.74	0.6		0.97
2-20-4-06E	161.46	28.34		1.1	0.31			0.95
3-13-4-06E	48.16	33.39			0.41	0.42		0.13
2-6-4-06E	545.18	76.22	0.43	0.83	2.25			0.26
3-6-4-06E	676.3	609.76	1.15	3.24	1.82	0.52		1.29
14-16-3-06E	111.7	171.09		5.77	0.21			1.22
15-16-3-06E	72.58	94.65	0.17	0.45	0.65	0.16		0.61
16-16-3-06E	97.99	225.35	0.19	0.62	0.41			0.26
7-23-3-06E	265.1	373.12	0.49	7.55	1.01	0.54		1.17
11-23-3-06E	65.67	19.01		0.16	0.42			
10-23-2-06E	33.53	158.15	0.31	2.13	1.6	0.19		1.75
11-23-2-06E	178.18	25.35	0.07	3.04	0.49	0.25		0.2
12-23-2-06E	46.64	72.03	0.08	1.66	1.21			0.57
3-4-5-06R	94.15	111.53	0.41	20.19	3.03	0.56		1.35
17-16-3-06R	321.08	210.56	0.55	1.03	1.56	0.26		0.49
18-16-3-06R	102.06	347.26	1.61	1.64	8.75	7.92		3.52
19-16-3-06R	366.9	80.45	0.22	4.83	0.44	0.3		1.02
9-23-3-06R	12.96	246		2.59	1.61	0.24		0.92
10-23-3-06R	287.35	87.62	0.33	1.75	2.78	0.77		1.82
20-16-3-06R	28.32	40.82	0.25	3.85	1.5	0.15		1.39

Appendix B

Fatty Acids

Patient	Group	STA	ω-3 ARA	EPA	DPA	DHA	Total ω-3 FA	total other FA	Total FFA
2C4-5-9-05	1	0.006290693	0.023207827	0.004241877	0.003231155	0.014577208	0.051548761	1.07254242	1.1240912
C16-23-5-05	1	0.009559744	0.028093822	0.00470583	0.004184166	0.004184166	0.050727729	1.93134206	1.9820698
C8-14-4-05	1	0.008242224	0.060915638	0.010243751	0.008268925	0.035232548	0.122903086	2.574692207	2.6975953
C7-10-6-05	1	0.008388125	0.041134033	0.006982839	0.004578966	0.030390965	0.091474928	2.638351796	2.7298267
C6-2-8-05	1	0.005662934	0.040487992	0.008245945	0.007896025	0.022072326	0.084365221	2.341065554	2.4254308
C6-30-9-05	1	0.009559883	0.058234352	0.010813516	0.008530341	0.048765708	0.135903799	5.229974222	5.365878
C2-4-10-05	1	0.012876919	0.057693665	0.010070006	0.014068872	0.033925029	0.12863449	3.102951459	3.2315859
C20-9-6-05	1	0.049827737	0.080959632	0.010979165	0.011481582	0.039173577	0.192421694	4.370132328	4.562554
C2-2-8-05	1	0.016414408	0.070167141	0.011280413	0.014184277	0.028912819	0.140959058	1.912141083	2.0531001
C4-13-4-05	1	0.035917534	0.073517776	0.029786638	0.016067984	0.057376337	0.212666269	2.735271468	2.9479377
C5-18-10-05	1	0.02962004	0.066796027	0.016148762	0.013062857	0.048521022	0.174148708	3.270895701	3.4450444
E2-20-4-06	2	0.015011273	0.052046483	0.015215727	0.015191893	0.038928002	0.136393358	2.15969266	2.2960862
E2-6-4-06	2	0.006848832	0.072238026	0.031627274	0.017108917	0.078640944	0.206263993	1.891719403	2.0979834
2E10-23-2-06	2	0.015693102	0.060378729	0.026955725	0.018904342	0.091990538	0.213922437	3.287816789	3.5017392
2E11-23-2-06	2	0.013463926	0.069756819	0.010063744	0.014995265	0.060399803	0.168679557	3.422023407	3.590703
2E12-23-2-06	2	0.011314859	0.067229237	0.010231787	0.01046137	0.034083244	0.133320497	2.746091889	2.8794124
2E15-16-3-06	2	0.01075992	0.056201884	0.016594913	0.013851673	0.07187621	0.1692846	2.813706719	2.9829913
2E4-20-4-06	2	0.015067003	0.063447114	0.012650615	0.011583463	0.046245219	0.148993414	2.998415117	3.1474085
2E7-23-3-06	2	0.005501709	0.041474789	0.011422451	0.012093875	0.081107659	0.151600483	2.705325794	2.8569263
E14-16-3-06	2	0.031197406	0.15205125	0.020782696	0.020823322	0.050556818	0.275411493	2.932835185	3.2082467
E3-13-4-06	2	0.012233018	0.057654292	0.011396929	0.010512668	0.034670311	0.126467218	2.503861658	2.6303289
E3-6-4-06	2	0.009213613	0.041067251	0.012690986	0.011561433	0.049620072	0.124153356	2.465571238	2.5897246
E16-16-3-06	2	0.008121291	0.041398628	0.010122722	0.007936956	0.037325327	0.104904924	1.591894733	1.6967997
E3-20-4-06	2	0.012121374	0.051252885	0.019060873	0.0133906	0.066574449	0.162400181	2.4994022	2.6618024
R17-16-3-06	3	0.031055649	0.095421869	0.026265336	0.017648595	0.071431509	0.241822958	2.804443998	3.046267
R19-16-3-06	3	0.010373422	0.062438902	0.012463038	0.016075826	0.083855954	0.185207142	2.544671739	2.7298789
R3-4-5-06	3	0.008627517	0.030879798	0.01363259	0.011376658	0.055942172	0.120458735	1.964017638	2.0844764
R20-16-3-06	3	0.005012741	0.037138309	0.009021741	0.009317147	0.0450063	0.105496238	2.165537701	2.2710339
R10-23-3-06	3	0.012549471	0.077807298	0.017174672	0.014291669	0.05484977	0.17667288	2.831562304	3.0082352
R9-23-3-06	3	0.037251273	0.074533452	0.019851232	0.017771223	0.059253801	0.208660982	3.304475581	3.5131366
R8-23-3-06	3	0.008588959	0.074527954	0.016997887	0.017043186	0.068864413	0.186022398	2.612456656	2.7984791
R18-16-3-06	3	0.018784463	0.038066449	0.022894615	0.017255249	0.023146304	0.12014708	2.881216582	3.0013637

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

Patient	Group	%STA/TFFA	%ARA/TFFA	%EPA/TFFA	%DPA/TFFA	%DHA/TFFA	%N3/TFFA
2C4-5-9-05	1	0.5596248	2.0645858	0.3773606	0.287446	1.2967994	4.5858167
C16-23-5-05	1	0.4823111	1.4173982	0.23742	0.2111009	0.2111009	2.5593311
C8-14-4-05	1	0.3055397	2.2581459	0.3797364	0.3065295	1.3060724	4.5560239
C7-10-6-05	1	0.3072768	1.5068368	0.2557979	0.1677384	1.1132928	3.3509427
C6-2-8-05	1	0.2334816	1.6693114	0.3399786	0.3255515	0.9100374	3.4783603
C6-30-9-05	1	0.1781606	1.0852716	0.2015237	0.1589738	0.9088113	2.5327411
C2-4-10-05	1	0.3984706	1.785305	0.3116119	0.435355	1.049795	3.9805375
C20-9-6-05	1	1.0921019	1.7744367	0.2406364	0.2516481	0.8585888	4.2174119
C2-2-8-05	1	0.7994938	3.417619	0.5494332	0.6908712	1.4082518	6.8656689
C4-13-4-05	1	1.2183953	2.4938714	1.0104229	0.5450584	1.9463212	7.2140692
C5-18-10-05	1	0.8597869	1.9389018	0.4687534	0.3791782	1.4084295	5.0550497
E2-20-4-06	2	0.6537765	2.2667469	0.662681	0.661643	1.6954068	5.9402542
E2-6-4-06	2	0.3169154	3.4432125	1.5075083	0.8154935	3.7484064	9.831536
2E10-23-2-06	2	0.4481516	1.7242497	0.7697811	0.5398558	2.6269957	6.1090339
2E11-23-2-06	2	0.3749663	1.9427065	0.2802723	0.4176136	1.6821164	4.6976751
2E12-23-2-06	2	0.3929572	2.3348249	0.3553429	0.3633162	1.1836875	4.6301286
2E15-16-3-06	2	0.3607091	1.884078	0.5563178	0.4643551	2.4095347	5.6749947
2E4-20-4-06	2	0.4787114	2.0158525	0.4019375	0.3680317	1.469311	4.7338441
2E7-23-3-06	2	0.1925744	1.4517277	0.3998161	0.4233177	2.8389833	5.3064191
E14-16-3-06	2	0.9724129	4.7393878	0.6477898	0.6490562	1.5758395	8.5844862
E3-13-4-06	2	0.4650756	2.1919043	0.4332891	0.3996712	1.318098	4.8080382
E3-6-4-06	2	0.3557758	1.5857768	0.4900516	0.4464349	1.9160366	4.7940756
E16-16-3-06	2	0.4786241	2.4398065	0.5965773	0.4677604	2.1997486	6.1825168
E3-20-4-06	2	0.4553822	1.9254955	0.716089	0.5030651	2.5011041	6.1011359
R17-16-3-06	3	1.0194658	3.1324198	0.8622139	0.5793516	2.3448867	7.9383377
R19-16-3-06	3	0.3799957	2.2872408	0.4565418	0.5888842	3.071783	6.7844454
R3-4-5-06	3	0.4138937	1.4814175	0.6540055	0.5457801	2.6837518	5.7788487
R20-16-3-06	3	0.2207251	1.635304	0.3972526	0.4102602	1.9817537	4.6452955
R10-23-3-06	3	0.4171706	2.5864766	0.5709219	0.4750848	1.8233205	5.8729743
R9-23-3-06	3	1.0603423	2.1215643	0.5650572	0.5058506	1.6866353	5.9394498
R8-23-3-06	3	0.3069152	2.6631593	0.6073973	0.609016	2.46078	6.6472678
R18-16-3-06	3	0.6258643	1.2683051	0.7628071	0.5749136	0.7711929	4.003083

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

Patient	Group	STA:ARA	ARA:EPA	EPA:DPA	DPA:DHA	%STA/n3	%ARA/n3	%EPA/n3	%DPA/n3	%DHA/n3
2C4-5-9-05	1	0.2710591	5.471122	1.3128052	0.221658	12.203384	45.021115	8.2288634	6.2681527	28.278484
C16-23-5-05	1	0.3402792	5.9700032	1.1246757	1	18.845203	55.381589	9.2766431	8.2482824	8.2482824
C8-14-4-05	1	0.1353055	5.9466145	1.238825	0.2346956	6.7062789	49.563962	8.33482	6.7280042	28.666935
C7-10-6-05	1	0.2039218	5.8907318	1.5249815	0.1506687	9.1698622	44.967549	7.6336099	5.0057064	33.223273
C6-2-8-05	1	0.139867	4.910049	1.0443159	0.3577342	6.712403	47.99133	9.7741041	9.3593369	26.162826
C6-30-9-05	1	0.1641623	5.3853302	1.2676534	0.174925	7.0343012	42.849687	7.9567427	6.2767494	35.88252
C2-4-10-05	1	0.2231947	5.7292581	0.715765	0.4147048	10.010471	44.850852	7.8283874	10.937091	26.373198
C20-9-6-05	1	0.615464	7.3739334	0.9562415	0.2930951	25.895073	42.074067	5.7057834	5.9668855	20.358192
C2-2-8-05	1	0.233933	6.2202632	0.7952759	0.4905878	11.644805	49.778384	8.0026169	10.062693	20.511502
C4-13-4-05	1	0.4885558	2.4681462	1.8537882	0.2800455	16.889154	34.569552	14.006282	7.5554923	26.979519
C5-18-10-05	1	0.4434402	4.136294	1.236235	0.2692206	17.008475	38.355741	9.2729725	7.5009787	27.861833
E2-20-4-06	2	0.2884206	3.4205702	1.0015688	0.3902562	11.005868	38.159089	11.155768	11.138294	28.54098
E2-6-4-06	2	0.0920406	2.2840421	1.8485842	0.2175574	3.2234572	35.022121	15.333396	8.2946698	38.126356
2E10-23-2-06	2	0.2599111	2.2399223	1.4259013	0.2055031	7.3358841	28.224589	12.6007	8.8370076	43.001819
2E11-23-2-06	2	0.1930123	6.9314977	0.6711281	0.2482668	7.9819549	41.354637	5.9661907	8.8897941	35.807423
2E12-23-2-06	2	0.1683026	6.5706252	0.9780542	0.3069359	8.4869611	50.426783	7.6745791	7.8467831	25.564894
2E15-16-3-06	2	0.1914512	3.3866935	1.198044	0.1927157	6.3561129	33.199644	9.8029667	8.1824765	42.4588
2E4-20-4-06	2	0.2374734	5.0153383	1.0921272	0.2504791	10.11253	42.583838	8.490721	7.7744796	31.038432
2E7-23-3-06	2	0.1326519	3.6309886	0.9444823	0.1491089	3.829084	27.357953	7.5345741	7.977465	53.500924
E14-16-3-06	2	0.2051769	7.3162428	0.998049	0.4118796	11.327561	55.208753	7.5460525	7.5608037	18.35683
E3-13-4-06	2	0.2121788	5.0587566	1.0841139	0.3032182	9.6728765	45.588329	9.0117657	8.3125635	27.414465
E3-6-4-06	2	0.2243543	3.2359386	1.0977001	0.2329991	7.4211552	33.077842	10.222024	9.3122198	39.966758
E16-16-3-06	2	0.196173	4.0896735	1.2753909	0.2126427	7.7415732	39.462997	9.649425	7.5658569	35.580148
E3-20-4-06	2	0.2365013	2.6889055	1.4234517	0.2011372	7.4638917	31.559623	11.736978	8.2454341	40.994073
R17-16-3-06	3	0.3254563	3.6329964	1.4882394	0.2470702	12.842308	39.459392	10.86139	7.2981472	29.538762
R19-16-3-06	3	0.1661372	5.0099265	0.7752658	0.1917076	5.6009836	33.71301	6.7292425	8.6799167	45.276847
R3-4-5-06	3	0.2793903	2.2651453	1.1982949	0.2033646	7.1622175	25.635167	11.317229	9.4444439	46.440943
R20-16-3-06	3	0.134975	4.1165345	0.9682943	0.2070187	4.7515831	35.203443	8.5517182	8.8317343	42.861521
R10-23-3-06	3	0.1612891	4.5303513	1.2017262	0.2605602	7.1032245	44.040318	9.7211707	8.0893393	31.045948
R9-23-3-06	3	0.4997927	3.7546007	1.1170437	0.299917	17.852534	35.71988	9.5138294	8.5167926	28.397164
R8-23-3-06	3	0.1152448	4.3845423	0.9973421	0.247489	4.6171637	40.063968	9.1375485	9.1618997	37.01942
R18-16-3-06	3	0.4934651	1.6626813	1.3268203	0.7454862	15.634557	31.683208	19.05549	14.361771	19.264974

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

Patient	STA	ω-3 ARA	EPA	DPA	DHA	Total ω-3 FA	total other FA	Total FFA
Control								
2C4-5-9-05	0.006290693	0.023207827	0.004241877	0.003231155	0.014577208	0.051548761	1.07254242	1.124091181
C16-23-5-05	0.009559744	0.028093822	0.00470583	0.004184166	0.004184166	0.050727729	1.93134206	1.982069789
C8-14-4-05	0.008242224	0.060915638	0.010243751	0.008268925	0.035232548	0.122903086	2.574692207	2.697595293
C7-10-6-05	0.008388125	0.041134033	0.006982839	0.004578966	0.030390965	0.091474928	2.638351796	2.729826725
C6-2-8-05	0.005662934	0.040487992	0.008245945	0.007896025	0.022072326	0.084365221	2.341065554	2.425430775
C6-30-9-05	0.009559883	0.058234352	0.010813516	0.008530341	0.048765708	0.135903799	5.229974222	5.365878021
C2-4-10-05	0.012876919	0.057693665	0.010070006	0.014068872	0.033925029	0.12863449	3.102951459	3.231585949
C20-9-8-05	0.049827737	0.080959632	0.010979165	0.011481582	0.039173577	0.192421694	4.370132328	4.562554022
C2-2-8-05	0.016414408	0.070167141	0.011280413	0.014184277	0.028912819	0.140959058	1.912141083	2.053100141
C4-13-4-05	0.035917534	0.073517776	0.029786638	0.016067984	0.057376337	0.212666269	2.735271468	2.947937736
C5-18-10-05	0.02962004	0.066796027	0.016148762	0.013062857	0.048521022	0.174148708	3.270895701	3.44504441
valproate								
E2-20-4-06	0.015011273	0.052046463	0.015215727	0.015191893	0.038926002	0.136393358	2.15969286	2.296086218
2E10-23-2-06	0.015693102	0.060378729	0.026955725	0.018904342	0.091990538	0.213922437	3.287816789	3.501739226
2E15-16-3-06	0.01075992	0.056201884	0.016594913	0.013851673	0.07187621	0.1692846	2.813706719	2.982991319
2E4-20-4-06	0.015067003	0.063447114	0.012650615	0.011583463	0.046245219	0.148993414	2.998415117	3.147408532
2E7-23-3-06	0.005501709	0.041474789	0.011422451	0.012093875	0.081107659	0.151600483	2.705325794	2.856926277
E3-13-4-06	0.012233018	0.057654292	0.011396929	0.010512668	0.034670311	0.126467218	2.503861658	2.630328876
E3-6-4-06	0.009213613	0.041067251	0.012690986	0.011561433	0.049620072	0.124153356	2.465571238	2.589724594
E16-16-3-06	0.008121291	0.041398628	0.010122722	0.007936956	0.037325327	0.104904924	1.591894733	1.696799657
E3-20-4-06	0.012121374	0.051252885	0.019060873	0.0133906	0.066574449	0.162400181	2.4994022	2.66180238
R19-16-3-06	0.010373422	0.062438902	0.012463038	0.016075826	0.083855954	0.185207142	2.544671739	2.72987888
R3-4-5-06	0.008627517	0.030879798	0.01363259	0.011376658	0.055942172	0.120458735	1.964017638	2.084476373
R20-16-3-06	0.005012741	0.037138309	0.009021741	0.009317147	0.0450063	0.105496238	2.165537701	2.271033939
R9-23-3-06	0.037251273	0.074533452	0.019851232	0.017771223	0.059253801	0.208660982	3.304475581	3.513136563
R18-16-3-06	0.018784463	0.038066449	0.022894615	0.017255249	0.023146304	0.12014708	2.881216582	3.001363662
Carbamazepine								
R8-23-3-06	0.008588959	0.074527954	0.016997887	0.017043186	0.068864413	0.186022398	2.612456656	2.798479054
R10-23-3-06	0.012549471	0.077807298	0.017174672	0.014291669	0.05484977	0.17667288	2.831562304	3.008235184
E2-6-4-06	0.006648832	0.072238026	0.031627274	0.017108917	0.078640944	0.206263993	1.891719403	2.097983396
2E11-23-2-06	0.013463926	0.069756819	0.010063744	0.014995265	0.060399803	0.168679557	3.422023407	3.590702964
2E12-23-2-06	0.011314859	0.067229237	0.010231787	0.01046137	0.034083244	0.133320497	2.746091889	2.879412387
R17-16-3-06	0.031055649	0.095421869	0.026265336	0.017648595	0.071431509	0.241822958	2.804443998	3.046266955
E14-16-3-06	0.031197406	0.15205125	0.020782696	0.020823322	0.050556818	0.275411493	2.932835185	3.208246678

Patient	%STA/TFFA	%ARA/TFFA	%EPA/TFFA	%DPA/TFFA	%DHA/TFFA	%N3/TFFA
Control						
2C4-5-9-05	0.559624841	2.064585826	0.377360592	0.287445991	1.29679945	4.5858167
C16-23-5-05	0.482311146	1.417398235	0.237420012	0.211100858	0.211100858	2.559331109
C8-14-4-05	0.305539666	2.258145933	0.379736391	0.306529478	1.306072411	4.556023879
C7-10-6-05	0.307276823	1.506836779	0.255797892	0.167738352	1.113292813	3.350942659
C6-2-8-05	0.23348156	1.66931137	0.339978556	0.325551457	0.910037352	3.478360296
C6-30-9-05	0.178160639	1.085271639	0.201523694	0.158973813	0.908811335	2.532741121
C2-4-10-05	0.398470566	1.78530497	0.311611893	0.435355017	1.049795034	3.98053748
C20-9-6-05	1.092101858	1.774436685	0.240636386	0.251648137	0.858588784	4.21741185
C2-2-8-05	0.799493788	3.417619003	0.549433178	0.690871155	1.408251783	6.865668908
C4-13-4-05	1.218395278	2.493871394	1.010422897	0.545058446	1.946321198	7.214069213
C5-18-10-05	0.859786894	1.938901768	0.46875337	0.379178203	1.408429499	5.055049734
valproate						
E2-20-4-06	0.653776545	2.266746891	0.662681001	0.861842983	1.69540679	5.94025421
2E10-23-2-06	0.448151648	1.724249725	0.769781059	0.539855793	2.626995688	6.109033913
2E15-16-3-06	0.360709073	1.884078032	0.556317846	0.464355112	2.409534679	5.674994741
2E4-20-4-06	0.478711401	2.015852515	0.401937498	0.368031747	1.469310977	4.733844138
2E7-23-3-06	0.192574407	1.451727651	0.399816083	0.423317729	2.838983269	5.306419139
E3-13-4-06	0.465075599	2.191904291	0.433289141	0.39967123	1.318097967	4.808038228
E3-6-4-06	0.355775794	1.585776788	0.490051571	0.448434861	1.916036624	4.794075638
E16-16-3-06	0.478624063	2.439806452	0.596577324	0.467760373	2.199748603	6.182516814
E3-20-4-06	0.455382176	1.925495514	0.716088951	0.503065143	2.501104121	6.101135905
R19-16-3-06	0.379995675	2.287240754	0.456541781	0.588884208	3.071782959	6.784445378
R3-4-5-06	0.41389371	1.481417523	0.654005512	0.545780119	2.683751797	5.778848661
R20-16-3-06	0.220725079	1.635303989	0.397252586	0.41026016	1.981753727	4.645295541
R9-23-3-06	1.06034231	2.121564327	0.56505724	0.505850619	1.686635289	5.939449783
R18-16-3-06	0.625864293	1.268305121	0.762807094	0.574913635	0.771192905	4.003083048
Carbamazepine						
R8-23-3-06	0.306915239	2.663159264	0.607397323	0.609016013	2.460780007	6.647267847
R10-23-3-06	0.417170553	2.586476572	0.570921858	0.475084821	1.823320531	5.872974335
E2-6-4-06	0.316915354	3.443212479	1.507508301	0.815493452	3.748406414	9.831536001
2E11-23-2-06	0.374966304	1.942706463	0.28027225	0.417613638	1.682116399	4.697675054
2E12-23-2-06	0.392957217	2.334824903	0.355342884	0.363316152	1.183687479	4.630128635
R17-16-3-06	1.019465782	3.13241978	0.862213851	0.579351568	2.344886702	7.938337683
E14-16-3-06	0.972412944	4.739387756	0.647789838	0.64905615	1.575839493	8.584486181

Patient	STA:ARA	ARA:EPA	EPA:DPA	DPA:DHA	%STA/n3	%ARA/n3	%EPA/n3	%DPA/n3	%DHA/n3
Control									
2C4-5-9-05	0.271059132	5.471121973	1.3128052	0.221658015	12.20338443	45.02111535	8.228863398	6.268152655	28.27848417
C16-23-5-05	0.340279206	5.970003219	1.124675731	1	18.84520311	55.38158897	9.276643067	8.248282424	8.248282424
C8-14-4-05	0.135305545	5.946614512	1.238825033	0.234695623	6.706278853	49.56396176	8.334820031	6.728004204	28.66693516
C7-10-6-05	0.20392177	5.890731804	1.524981548	0.150668674	9.16986216	44.9675489	7.633809948	5.005706435	33.22327256
C6-2-8-05	0.139866992	4.910049001	1.044315879	0.357734171	6.712402989	47.99133006	9.774104097	9.359336862	26.16282599
C6-30-9-05	0.164162255	5.385330206	1.267653398	0.174924989	7.034301203	42.84968666	7.956742709	6.276749401	35.88251983
C2-4-10-05	0.223194677	5.729258124	0.715765022	0.414704779	10.01047141	44.8508519	7.828387365	10.93709126	26.37319806
C20-9-6-05	0.615463976	7.373933395	0.956241477	0.293095067	25.89507254	42.07406694	5.705783425	5.96688552	20.35819158
C2-2-8-05	0.233932977	6.220263242	0.795275898	0.490587808	11.64480547	49.77838356	8.00261687	10.06269257	20.51150153
C4-13-4-05	0.488555778	2.468146161	1.853788165	0.280045476	16.88915427	34.5695518	14.00628227	7.555492332	26.97951933
C5-18-10-05	0.443440152	4.136294034	1.23623501	0.269220578	17.00847548	38.35574069	9.272972465	7.500978691	27.86183268
valproate									
E2-20-4-06	0.28842062	3.420570212	1.001568848	0.390256183	11.00586813	38.15908901	11.15576838	11.13829408	28.54098041
2E10-23-2-06	0.259911103	2.239922255	1.425901267	0.205503113	7.335884109	28.22458919	12.60070037	8.837007627	43.00181871
2E15-16-3-06	0.191451238	3.386693497	1.198043979	0.192715679	6.356112895	33.19964366	9.802966725	8.182476517	42.45880021
2E4-20-4-06	0.237473425	5.015338272	1.092127245	0.250479138	10.11252984	42.58383792	8.490720987	7.774479602	31.03843165
2E7-23-3-06	0.132651883	3.630988625	0.944482256	0.14910892	3.62908398	27.35795295	7.534574126	7.977464985	53.50092396
E3-13-4-06	0.212178789	5.05875657	1.084113913	0.303218152	9.67287648	45.5883291	9.011765732	8.312563488	27.4144652
E3-6-4-06	0.22435427	3.235938586	1.097700053	0.232999127	7.421155208	33.07784248	10.22202418	9.312219812	39.96675832
E16-16-3-06	0.196172964	4.089673467	1.275390902	0.21264265	7.741573172	39.46299744	9.64942501	7.565856865	35.58014751
E3-20-4-06	0.236501291	2.688905494	1.423451734	0.201137225	7.463891688	31.55962339	11.73697754	8.24543415	40.99407323
R19-16-3-06	0.166137157	5.009926469	0.775265791	0.191707623	5.600983637	33.71301008	6.729242493	8.67991671	45.27684708
R3-4-5-06	0.279390316	2.265145317	1.198294861	0.203364603	7.162217498	25.63516731	11.31722857	9.44444389	46.44094273
R20-16-3-06	0.134974953	4.116534539	0.968294327	0.20701874	4.751583129	35.20344345	8.551718228	8.831734305	42.66152088
R9-23-3-06	0.499792675	3.754600733	1.117043686	0.299917013	17.8525343	35.71987986	9.513629383	8.516792582	28.39716388
R18-16-3-06	0.493465084	1.662681339	1.326820323	0.745486156	15.63455681	31.68320781	19.05549011	14.36177136	19.26497391
Carbamazepine									
R8-23-3-06	0.115244793	4.384542312	0.997342122	0.247489012	4.617163712	40.06396802	9.137548497	9.161899706	37.01942007
R10-23-3-06	0.161289129	4.530351282	1.201726162	0.260560232	7.10322452	44.04031798	9.721170664	8.089339294	31.04594754
E2-6-4-06	0.092040603	2.284042135	1.848584188	0.217557373	3.223457193	35.02212145	15.33339553	8.294669847	38.12635598
2E11-23-2-06	0.193012332	6.931497711	0.671128108	0.248266789	7.981954894	41.35463693	5.966190663	8.889794059	35.80742345
2E12-23-2-06	0.168302649	6.570625191	0.978054186	0.306935875	8.486961116	50.42678265	7.674579082	7.846783126	25.56489402
R17-16-3-06	0.32545631	3.632996357	1.488239437	0.247070175	12.84230809	39.45939194	10.86139045	7.298147179	29.53876234
E14-16-3-06	0.205176912	7.31624283	0.998048994	0.411879606	11.32756142	55.20875281	7.546052543	7.560803716	18.35682952

Appendix C

Acylcarnitines

Acylcarnitine concentrations	Group	µmol/L Carnitine	Acetylcarnitine	Propionylcarnitine	Butanoylcarnitine	3-Methylcrotonylcarnitine	Isovalerylcarnitine
01-15-09-05C	1	9.6516	5.6604	0.3396	1.0057	0.1868	0.28
02-02-08-05C	1	12.7599	4.4036	0.2721	1.1272	0.2355	0.4427
02-04-10-05C	1	96.4556	26.4248	3.3943	1.862	0.1764	0.6535
02-25-05-05C	1	13.5173	3.7463	0.5358	1.4532	0.2615	0.4054
03-26-10-05C	1	17.7263	5.7882	0.4012	0.9134	0.1917	0.4067
04-05-09-05C	1	38.7028	7.9706	0.5312	1.4342	0.3022	0.4004
04-13-04-05C	1	55.1803	14.1839	0.9472	3.7107	0.2678	0.6801
05-18-10-05C	1	4.3035	2.2605	0.2114	0.3109	0.156	0.2676
05-30-09-05C	1	3.3984	0.6843	0.1708	0.4355	0.0788	0.1063
06-02-08-05C	1	5.4795	4.2668	0.4356	1.3518	0.2061	0.4396
06-30-09-05C	1	8.624	3.6743	0.4436	0.5593	0.1845	0.1783
08-14-04-05C	1	6.0543	1.995	0.4814	1.1594	0.3111	0.2989
16-23-05-05C	1	13.5802	3.2311	0.6698	1.4734	0.2194	0.2866
20-09-06-05C	1	7.9929	3.2538	0.1387	0.7618	0.1367	0.1819
07-20-06-05C	1	5.8081	1.7414	0.2191	1.0186	0.0923	0.1698
02-06-04-06E	2	4.7155	1.2921	0.1004	1.1148	0.0461	0.1131
02-20-04-06E	2	4.1123	0.6332	0.1172	0.4655	0.0374	0.0543
03-13-04-06E	2	3.7229	0.3753	0.0863	0.4082	0.0236	0.0503
03-20-04-06E	2	4.5022	1.4336	0.2264	2.0674	0.1417	0.2561
04-20-04-06E	2	26.5913	9.3437	0.5391	2.8036	0.0987	0.3239
07-23-03-06E	2	5.4076	1.3166	0.0937	0.7345	0.1639	0.1562
10-23-02-06E	2	7.5942	3.3545	0.3067	1.8785	0.1089	0.264
11-23-02-06E	2	5.9334	1.4798	0.0793	2.1447	0.1115	0.2739
11-23-03-06E	2	3.1516	0.4554	0.0541	0.0766	0.0068	0.081
12-23-02-06E	2	6.3069	1.5436	0.1028	1.5512	0.1245	0.1998
14-16-03-06E	2	20.0582	8.5068	0.5919	2.8385	0.0563	0.323
15-16-03-06E	2	26.9794	9.6096	0.3301	2.6505	0.2151	0.5575
16-16-03-06E	2	32.3397	12.5692	0.6906	3.093	0.1001	0.5467
03-04-05-06R	3	5.9474	1.6927	0.3619	1.3751	0.0824	0.09
09-23-03-06R	3	23.2767	24.3194	1.2274	1.0997	0.1459	0.4051
10-23-03-06R	3	25.7346	10.0164	0.4564	2.8887	0.2137	0.5955
17-16-03-06R	3	39.8362	18.5489	0.7833	3.4294	0.2977	0.9706
18-16-03-06R	3	5.2105	2.7356	0.3417	1.0835	0.0266	0.1101
19-16-03-06R	3	4.1147	0.5651	0.1192	0.8927	0.0382	0.0515
20-16-03-06R	3	9.7541	5.4494	0.3747	3.3988	0.2069	0.4219

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

Acylcarnitine concentrations	µmol/L Group	3-Hydroxybutyrylcarnitine	Hexanoylcarnitine	3-Hydroxyisovalerylcarnitine	Octonoylcarnitine	Decanoylcarnitine
01-15-09-05C	1	0.1767	0.1995	0.253	0.5988	0.1303
02-02-08-05C	1	0.2077	0.242	0.5958	1.2893	0.2132
02-04-10-05C	1	0.1722	0.3169	0.2238	0.8213	0.1869
02-25-05-05C	1	0.1733	0.2257	0.5592	0.8719	0.1484
03-26-10-05C	1	0.1548	0.1955	0.348	0.8331	0.1337
04-05-09-05C	1	0.1821	0.2148	0.371	0.779	0.205
04-13-04-05C	1	0.1236	0.1761	0.469	0.6902	0.1737
05-18-10-05C	1	0.1485	0.1811	0.2921	1.0187	0.2199
05-30-09-05C	1	0.0758	0.0733	0.1331	0.285	0.0532
06-02-08-05C	1	0.1516	0.1962	0.2718	0.9516	0.1929
06-30-09-05C	1	0.14	0.1384	0.2997	0.846	0.1929
08-14-04-05C	1	0.2428	0.1714	0.4897	0.9316	0.2067
16-23-05-05C	1	0.1632	0.1998	0.2969	0.8822	0.1992
20-09-06-05C	1	0.1336	0.0564	0.296	0.5054	0.0537
07-20-06-05C	1	0.0607	0.1199	0.1634	0.4465	0.0742
02-06-04-06E	2	0.0458	0.0253	0.0785	0.2924	0.0589
02-20-04-06E	2	0.0165	0.012	0.0598	0.3057	0.0336
03-13-04-06E	2	0.0146	0.0235	0.0417	0.4472	0.0315
03-20-04-06E	2	0.061	0.1356	0.2457	2.9684	0.2464
04-20-04-06E	2	0.0929	0.0581	0.1478	0.9249	0.138
07-23-03-06E	2	0.0692	0.0608	0.2545	1.4351	0.1445
10-23-02-06E	2	0.0623	0.0411	0.1727	0.4512	0.067
11-23-02-06E	2	0.1408	0.0999	0.3912	0.5223	0.0941
11-23-03-06E	2	0.0191	0.0081	0.0087	0.138	0.0072
12-23-02-06E	2	0.0431	0.0387	0.2547	0.4964	0.0929
14-16-03-06E	2	0.1141	0.0576	0.1776	0.8516	0.1008
15-16-03-06E	2	0.1662	0.1029	0.3891	0.8948	0.1855
16-16-03-06E	2	0.178	0.1131	0.2441	0.7456	0.2048
03-04-05-06R	3	0.0586	0.0544	0.2254	1.923	0.2631
09-23-03-06R	3	1.4843	0.5334	1.145	1.0156	0.3474
10-23-03-06R	3	0.2305	1.7047	0.4694	0.726	0.1151
17-16-03-06R	3	0.3274	0.1216	0.5464	0.8463	0.166
18-16-03-06R	3	0.0472	0.04	0.0515	0.9258	0.0631
19-16-03-06R	3	0.0289	0.0277	0.1628	1.0654	0.0672
20-16-03-06R	3	0.1867	0.3125	0.4993	4.2587	0.3729

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

Acylcarnitine concentrations	µmol/L Group	Methylmalonylcarnitine	Glutaconylcarnitine	Glutarylcarnitine	Lauroylcarnitine	Myrisoylcarnitine	Palmitoylcarnitine
01-15-09-05C	1	0.3826	0.1967	0.4215	0.1357	0.1205	0.0984
02-02-08-05C	1	0.5499	0.3558	0.6334	0.1879	0.1976	0.1183
02-04-10-05C	1	1.0007	0.5725	0.4887	0.1192	0.1485	0.0938
02-25-05-05C	1	0.3839	0.1912	0.5023	0.1689	0.1709	0.1164
03-26-10-05C	1	0.2936	0.1464	0.2289	0.1018	0.1088	0.0782
04-05-09-05C	1	0.4341	0.2547	0.3529	0.208	0.1858	0.1909
04-13-04-05C	1	0.4318	0.1589	0.6566	0.1293	0.0991	0.0819
05-18-10-05C	1	0.8078	0.2895	1.0186	0.1624	0.135	0.0976
05-30-09-05C	1	0.0951	0.0591	0.109	0.0643	0.0397	0.0573
06-02-08-05C	1	0.2995	0.1872	0.2733	0.1032	0.1243	0.1114
06-30-09-05C	1	0.6104	0.358	0.868	0.1501	0.1768	0.1584
08-14-04-05C	1	0.6174	0.2236	0.4777	0.1959	0.2875	0.1402
16-23-05-05C	1	0.3736	0.363	0.5058	0.2338	0.1753	0.1982
20-09-06-05C	1	0.2512	0.2254	0.2913	0.0747	0.1033	0.0563
07-20-06-05C	1	0.2087	0.1466	0.2991	0.0671	0.0529	0.0503
02-06-04-06E	2	0.1805	0.0807	0.3157	0.0367	0.0498	0.0156
02-20-04-06E	2	0.2605	0.0326	0.1306	0.0403	0.0395	0.0123
03-13-04-06E	2	0.1027	0.0285	0.0737	0.0111	0.0243	0.0086
03-20-04-06E	2	0.277	0.1752	0.2989	0.0439	0.1875	0.0505
04-20-04-06E	2	0.5897	0.1483	0.5023	0.048	0.1181	0.0287
07-23-03-06E	2	0.2696	0.1209	0.1819	0.0511	0.1153	0.0378
10-23-02-06E	2	0.29	0.1654	0.2074	0.026	0.0374	0.0192
11-23-02-06E	2	0.3823	0.2206	0.5543	0.1132	0.1005	0.0206
11-23-03-06E	2	0.0337	0.012	0.0247	0.009	0.01	0.0054
12-23-02-06E	2	0.4761	0.2048	0.5733	0.0627	0.09	0.0275
14-16-03-06E	2	0.2455	0.1406	0.2017	0.0407	0.0548	0.0325
15-16-03-06E	2	0.3907	0.1494	0.5654	0.0832	0.0541	0.024
16-16-03-06E	2	0.3442	0.149	0.3607	0.0569	0.0422	0.0301
03-04-05-06R	3	0.4285	0.1738	0.2755	0.0818	0.2494	0.0853
09-23-03-06R	3	1.8906	0.1091	0.1128	0.09	0.1182	0.1147
10-23-03-06R	3	0.4712	0.1139	0.4723	0.1215	0.0963	0.0352
17-16-03-06R	3	0.4144	0.1089	0.4089	0.1704	0.1078	0.0292
18-16-03-06R	3	0.0736	0.0813	0.1005	0.0232	0.0489	0.0442
19-16-03-06R	3	0.2223	0.0958	0.2238	0.0752	0.2037	0.0957
20-16-03-06R	3	0.4687	0.3423	1.2401	0.1599	0.5057	0.2707

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

Acylcarnitine concentrations	µmol/L Group	Stearylcarnitine	Adipylcarnitine	Suberylcarnitine	CC10-458	CC12-486	CC14-514	CC16-542	Total Acylcarntine
01-15-09-05C	1	0.0812	0.265	0.1503	0.3324	0.0812	0.0751	0.073	2.8
02-02-08-05C	1	0.0916	0.3899	0.1867	0.299	0.094	0.0886	0.0866	0.86
02-04-10-05C	1	0.0878	0.2428	0.1525	0.3095	0.0809	0.0756	0.0854	2.66
02-25-05-05C	1	0.1139	0.2539	0.1653	0.3864	0.1138	0.1183	0.112	1.51
03-26-10-05C	1	0.0726	0.136	0.089	0.3065	0.0737	0.0677	0.0752	2.42
04-05-09-05C	1	0.1368	0.2877	0.1608	0.4277	0.1373	0.1271	0.1423	2.16
04-13-04-05C	1	0.0611	0.243	0.11	0.2396	0.0754	0.0683	0.0702	1.67
05-18-10-05C	1	0.0615	0.2412	0.1391	0.3555	0.0589	0.0618	0.0634	0.67
05-30-09-05C	1	0.029	0.0562	0.0368	0.3131	0.0341	0.03	0.0334	5.87
06-02-08-05C	1	0.0738	0.2041	0.1529	0.3516	0.0951	0.0661	0.0778	1.69
06-30-09-05C	1	0.1039	0.238	0.3624	0.3655	0.0943	0.082	0.088	0.7
08-14-04-05C	1	0.1415	0.4988	0.2404	0.294	0.1204	0.1257	0.1404	0.86
16-23-05-05C	1	0.1728	0.3143	0.2271	0.4449	0.1537	0.1616	0.1658	1.39
20-09-06-05C	1	0.0302	0.139	0.1043	0.3067	0.0393	0.0271	0.0326	2.16
07-20-06-05C	1	0.0342	0.1438	0.0514	0.278	0.0318	0.0322	0.0387	1.59
02-06-04-06E	2	0.0095	0.0682	0.036	0.2211	0.0082	0.0064	0.0106	1.65
02-20-04-06E	2	0.0056	0.0494	0.0246	0.2526	0.007	0.0021	0.0069	0.4
03-13-04-06E	2	0.0022	0.08	0.0329	0.2566	0.005	0.0028	0.008	0.33
03-20-04-06E	2	0.0135	0.2879	0.3705	0.3128	0.0072	0.0059	0.0119	0.95
04-20-04-06E	2	0.0104	0.2025	0.1067	0.2399	0.0088	0.0199	0.031	1.77
07-23-03-06E	2	0.0188	0.1906	0.1501	0.2863	0.0119	0.0061	0.0116	0.87
10-23-02-06E	2	0.007	0.0687	0.054	0.2354	0.0065	0.0043	0.0111	1.16
11-23-02-06E	2	0.0185	0.1664	0.0903	0.2483	0.0192	0.0055	0.0199	0.68
11-23-03-06E	2	0.004	0.0139	0.0029	0.2408	0.0027	0.0029	0.006	1.09
12-23-02-06E	2	0.0261	0.1743	0.0688	0.2942	0.0115	0.0086	0.014	0.35
14-16-03-06E	2	0.016	0.1162	0.0548	0.2482	0.009	0.0048	0.016	1.95
15-16-03-06E	2	0.0131	0.207	0.0659	0.2823	0.0052	0.0072	0.0201	0.93
16-16-03-06E	2	0.0041	0.1567	0.0683	0.2534	0.009	0.0033	0.0058	1.26
03-04-05-06R	3	0.0329	0.515	0.4466	0.4144	0.0238	0.0138	0.0197	1.02
09-23-03-06R	3	0.1185	0.4801	0.4201	0.3075	0.6075	0.0134	0.0088	11.36
10-23-03-06R	3	0.0452	0.3045	0.1212	0.2988	0.0219	0.0201	0.0239	3.55
17-16-03-06R	3	0.0329	0.218	0.0679	0.3128	0.0162	0.0133	0.0259	3.06
18-16-03-06R	3	0.0128	0.1343	0.2826	0.3372	0.0143	0.007	0.0072	4.71
19-16-03-06R	3	0.0166	0.4616	0.4856	0.3451	0.019	0.0068	0.0063	0.53
20-16-03-06R	3	0.0619	1.4111	1.2478	0.4598	0.0516	0.014	0.0074	1.14

(Group 1 = control Group 2 = drug responsive Group 3 = refractory)

	Carnitine	Acetylcarnitine	Propionylcarnitine	Butanoylcarnitine	3-Methylcrotonylcarnitine	Isovalerylcarnitine	3-Hydroxybutyrylcarnitine
Control group							
01-15-09-05C	9.6516	5.6604	0.3396	1.0057	0.1868	0.28	0.1767
02-02-08-05C	12.7599	4.4036	0.2721	1.1272	0.2355	0.4427	0.2077
02-04-10-05C	96.4556	26.4248	3.3943	1.862	0.1764	0.6535	0.1722
02-25-05-05C	13.5173	3.7463	0.5358	1.4532	0.2615	0.4054	0.1733
03-26-10-05C	17.7263	5.7882	0.4012	0.9134	0.1917	0.4067	0.1548
04-05-09-05C	38.7028	7.9706	0.5312	1.4342	0.3022	0.4004	0.1821
04-13-04-05C	55.1803	14.1839	0.9472	3.7107	0.2678	0.6801	0.1236
05-18-10-05C	4.3035	2.2605	0.2114	0.3109	0.156	0.2676	0.1485
05-30-09-05C	3.3984	0.6843	0.1708	0.4355	0.0788	0.1063	0.0758
06-02-08-05C	5.4795	4.2668	0.4356	1.3518	0.2061	0.4396	0.1516
06-30-09-05C	8.624	3.6743	0.4436	0.5593	0.1845	0.1783	0.14
08-14-04-05C	6.0543	1.995	0.4814	1.1594	0.3111	0.2989	0.2428
16-23-05-05C	13.5802	3.2311	0.6698	1.4734	0.2194	0.2866	0.1632
20-09-06-05C	7.9929	3.2538	0.1387	0.7618	0.1367	0.1819	0.1336
07-20-06-05C	5.8081	1.7414	0.2191	1.0186	0.0923	0.1698	0.0607
Valproate							
20-16-03-06R	9.7541	5.4494	0.3747	3.3988	0.2069	0.4219	0.1867
03-13-04-06E	3.7229	0.3753	0.0863	0.4082	0.0236	0.0503	0.0146
03-20-04-06E	4.5022	1.4336	0.2264	2.0674	0.1417	0.2561	0.061
04-20-04-06E	26.5913	9.3437	0.5391	2.8036	0.0987	0.3239	0.0929
07-23-03-06E	5.4076	1.3166	0.0937	0.7345	0.1639	0.1562	0.0692
15-16-03-06E	26.9794	9.6096	0.3301	2.6505	0.2151	0.5575	0.1862
10-23-02-06E	7.5942	3.3545	0.3067	1.8785	0.1089	0.264	0.0623
02-20-04-06E	4.1123	0.6332	0.1172	0.4655	0.0374	0.0543	0.0165
16-16-03-06E	32.3397	12.5692	0.6906	3.093	0.1001	0.5467	0.178
19-16-03-06R	4.1147	0.5651	0.1192	0.8927	0.0382	0.0515	0.0289
03-04-05-06R	5.9474	1.6927	0.3619	1.3751	0.0824	0.09	0.0586
09-23-03-06R	23.2767	24.3194	1.2274	1.0997	0.1459	0.4051	1.4843
18-16-03-06R	5.2105	2.7356	0.3417	1.0835	0.0266	0.1101	0.0472
Carbamazepine							
02-06-04-06E	4.7155	1.2921	0.1004	1.1148	0.0461	0.1131	0.0458
14-16-03-06E	20.0582	8.5068	0.5919	2.8385	0.0563	0.323	0.1141
11-23-02-06E	5.9334	1.4798	0.0793	2.1447	0.1115	0.2739	0.1408
10-23-03-06R	25.7346	10.0164	0.4564	2.8887	0.2137	0.5955	0.2305
17-16-03-06R	39.8362	18.5489	0.7833	3.4294	0.2977	0.9706	0.3274
12-23-02-06E	6.3069	1.5436	0.1028	1.5512	0.1245	0.1998	0.0431
11-23-03-06E	3.1516	0.4554	0.0541	0.0766	0.0068	0.081	0.0191

	Hexanoylcarnitine	3-Hydroxyisovalerylcarnitine	Octonoylcarnitine	Decanoylcarnitine	Methylmalonylcarnitine	Glutaconylcarnitine
Control group						
01-15-09-05C	0.1995	0.253	0.5988	0.1303	0.3826	0.1967
02-02-08-05C	0.242	0.5958	1.2893	0.2132	0.5499	0.3558
02-04-10-05C	0.3169	0.2238	0.8213	0.1869	1.0007	0.5725
02-25-05-05C	0.2257	0.5592	0.8719	0.1484	0.3839	0.1912
03-26-10-05C	0.1955	0.348	0.8331	0.1337	0.2936	0.1464
04-05-09-05C	0.2148	0.371	0.779	0.205	0.4341	0.2547
04-13-04-05C	0.1761	0.469	0.6902	0.1737	0.4318	0.1589
05-18-10-05C	0.1811	0.2921	1.0187	0.2199	0.8078	0.2895
05-30-09-05C	0.0733	0.1331	0.285	0.0532	0.0951	0.0591
06-02-08-05C	0.1962	0.2718	0.9516	0.1929	0.2995	0.1872
06-30-09-05C	0.1384	0.2997	0.846	0.1929	0.6104	0.358
08-14-04-05C	0.1714	0.4897	0.9316	0.2067	0.6174	0.2236
16-23-05-05C	0.1998	0.2969	0.8822	0.1992	0.3736	0.363
20-09-06-05C	0.0564	0.296	0.5054	0.0537	0.2512	0.2254
07-20-06-05C	0.1199	0.1634	0.4465	0.0742	0.2087	0.1466
Valproate						
20-16-03-06R	0.3125	0.4993	4.2587	0.3729	0.4687	0.3423
03-13-04-06E	0.0235	0.0417	0.4472	0.0315	0.1027	0.0285
03-20-04-06E	0.1356	0.2457	2.9684	0.2464	0.277	0.1752
04-20-04-06E	0.0581	0.1478	0.9249	0.138	0.5897	0.1483
07-23-03-06E	0.0608	0.2545	1.4351	0.1445	0.2696	0.1209
15-16-03-06E	0.1029	0.3891	0.8948	0.1855	0.3907	0.1494
10-23-02-06E	0.0411	0.1727	0.4512	0.067	0.29	0.1654
02-20-04-06E	0.012	0.0598	0.3057	0.0336	0.2605	0.0326
16-16-03-06E	0.1131	0.2441	0.7456	0.2048	0.3442	0.149
19-16-03-06R	0.0277	0.1628	1.0854	0.0672	0.2223	0.0958
03-04-05-06R	0.0544	0.2254	1.923	0.2631	0.4285	0.1738
09-23-03-06R	0.5334	1.145	1.0156	0.3474	1.8906	0.1091
18-16-03-06R	0.04	0.0515	0.9258	0.0631	0.0736	0.0813
Carbamazepine						
02-06-04-06E	0.0253	0.0785	0.2924	0.0589	0.1805	0.0807
14-16-03-06E	0.0576	0.1776	0.8516	0.1008	0.2455	0.1406
11-23-02-06E	0.0999	0.3912	0.5223	0.0941	0.3823	0.2206
10-23-03-06R	1.7047	0.4694	0.726	0.1151	0.4712	0.1139
17-16-03-06R	0.1216	0.5464	0.8463	0.166	0.4144	0.1089
12-23-02-06E	0.0387	0.2547	0.4964	0.0929	0.4761	0.2048
11-23-03-06E	0.0081	0.0087	0.138	0.0072	0.0337	0.012

	Glutarylcarnitine	Lauroylcarnitine	Myrisoylcarnitine	Palmitoylcarnitine	Stearylcarnitine	Adipylcarnitine	Suberylcarnitine	CC10-458	CC12-486	CC14-514	CC16-542
Control group											
01-15-09-05C	0.4215	0.1357	0.1205	0.0984	0.0812	0.265	0.1503	0.3324	0.0812	0.0751	0.073
02-02-08-05C	0.6334	0.1879	0.1976	0.1183	0.0916	0.3899	0.1867	0.299	0.094	0.0886	0.0866
02-04-10-05C	0.4887	0.1192	0.1485	0.0938	0.0878	0.2428	0.1525	0.3095	0.0809	0.0756	0.0854
02-25-05-05C	0.5023	0.1689	0.1709	0.1164	0.1139	0.2539	0.1653	0.3864	0.1138	0.1183	0.112
03-26-10-05C	0.2289	0.1018	0.1088	0.0782	0.0726	0.136	0.089	0.3065	0.0737	0.0677	0.0752
04-05-09-05C	0.3529	0.208	0.1858	0.1909	0.1368	0.2877	0.1608	0.4277	0.1373	0.1271	0.1423
04-13-04-05C	0.6566	0.1293	0.0991	0.0819	0.0611	0.243	0.11	0.2396	0.0754	0.0683	0.0702
05-18-10-05C	1.0186	0.1624	0.135	0.0976	0.0615	0.2412	0.1391	0.3555	0.0589	0.0618	0.0634
05-30-09-05C	0.109	0.0643	0.0397	0.0573	0.029	0.0562	0.0368	0.3131	0.0341	0.03	0.0334
06-02-08-05C	0.2733	0.1032	0.1243	0.1114	0.0738	0.2041	0.1529	0.3516	0.0951	0.0661	0.0778
06-30-09-05C	0.868	0.1501	0.1768	0.1584	0.1039	0.238	0.3624	0.3655	0.0943	0.082	0.088
08-14-04-05C	0.4777	0.1959	0.2875	0.1402	0.1415	0.4988	0.2404	0.294	0.1204	0.1257	0.1404
16-23-05-05C	0.5058	0.2338	0.1753	0.1982	0.1728	0.3143	0.2271	0.4449	0.1537	0.1616	0.1658
20-09-06-05C	0.2913	0.0747	0.1033	0.0563	0.0302	0.139	0.1043	0.3067	0.0393	0.0271	0.0326
07-20-06-05C	0.2991	0.0671	0.0529	0.0503	0.0342	0.1438	0.0514	0.278	0.0318	0.0322	0.0387
Valproate											
20-16-03-06R	1.2401	0.1599	0.5057	0.2707	0.0619	1.4111	1.2478	0.4598	0.0516	0.014	0.0074
03-13-04-06E	0.0737	0.0111	0.0243	0.0086	0.0022	0.08	0.0329	0.2566	0.005	0.0028	0.008
03-20-04-06E	0.2989	0.0439	0.1875	0.0505	0.0135	0.2879	0.3705	0.3128	0.0072	0.0059	0.0119
04-20-04-06E	0.5023	0.048	0.1181	0.0287	0.0104	0.2025	0.1067	0.2399	0.0088	0.0199	0.031
07-23-03-06E	0.1819	0.0511	0.1153	0.0378	0.0188	0.1906	0.1501	0.2863	0.0119	0.0061	0.0116
15-16-03-06E	0.5654	0.0832	0.0541	0.024	0.0131	0.207	0.0659	0.2823	0.0052	0.0072	0.0201
10-23-02-06E	0.2074	0.026	0.0374	0.0192	0.007	0.0687	0.054	0.2354	0.0065	0.0043	0.0111
02-20-04-06E	0.1306	0.0403	0.0395	0.0123	0.0056	0.0494	0.0246	0.2526	0.007	0.0021	0.0069
16-16-03-06E	0.3607	0.0569	0.0422	0.0301	0.0041	0.1567	0.0663	0.2534	0.009	0.0033	0.0058
19-16-03-06R	0.2238	0.0752	0.2037	0.0957	0.0166	0.4616	0.4856	0.3451	0.019	0.0068	0.0063
03-04-05-06R	0.2755	0.0818	0.2494	0.0853	0.0329	0.515	0.4466	0.4144	0.0236	0.0138	0.0197
09-23-03-06R	0.1128	0.09	0.1182	0.1147	0.1185	0.4801	0.4201	0.3075	0.6075	0.0134	0.0088
18-16-03-06R	0.1005	0.0232	0.0489	0.0442	0.0126	0.1343	0.2826	0.3372	0.0143	0.007	0.0072
Carbamazepine											
02-06-04-06E	0.3157	0.0367	0.0498	0.0156	0.0095	0.0682	0.036	0.2211	0.0082	0.0064	0.0106
14-16-03-06E	0.2017	0.0407	0.0548	0.0325	0.016	0.1162	0.0548	0.2482	0.009	0.0048	0.016
11-23-02-06E	0.5543	0.1132	0.1005	0.0206	0.0185	0.1664	0.0903	0.2483	0.0192	0.0055	0.0199
10-23-03-06R	0.4723	0.1215	0.0963	0.0352	0.0452	0.3045	0.1212	0.2988	0.0219	0.0201	0.0239
17-16-03-06R	0.4089	0.1704	0.1078	0.0292	0.0329	0.218	0.0679	0.3128	0.0162	0.0133	0.0259
12-23-02-06E	0.5733	0.0627	0.09	0.0275	0.0261	0.1743	0.0688	0.2942	0.0115	0.0086	0.014
11-23-03-06E	0.0247	0.009	0.01	0.0054	0.004	0.0139	0.0029	0.2408	0.0027	0.0029	0.006

Appendix D

Consent

Consent form

To the subject signing the consent as in part 3 of this document:

You are invited to participate in a research project as described in paragraph 2 of Part 1 of this document. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.
2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.
3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.
4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.
5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.
6. If you are a minor, we need the written approval of your parent or guardian before you may participate.
7. We require that you indemnify the University from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in Part 1. Lastly it is required to abandon any claim against the University regarding treatment of yourself or another person due to participation in this project as described in Part 1.
8. If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or death of yourself due to the project explained in Part 1.

Consent

Title of the project:

The role of fatty acids in drug resistant epilepsy .....

I, the undersigned ..... (full names)

read/listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project

.I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

(Signature of the subject)

Signed at .....on .....

Witnesses

1. .....

2. .....

Signed at ..... on .....

For non-therapeutic experimenting with subjects under the age of 21 years the written approval of a parent or guardian is required.

I, ... (full names)

parent or guardian of the subject named above, hereby give my permission that he/she may participate in this project and I also indemnify the University and any employee or student of the University, against any liability which may arise during the course of the project.

Signature: ..... Date: .....

Relationship: .....