The inhibitory effect of erucic acid on the polyunsaturated fatty acids in Sprague - Dawley rats

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Table of Contents

List of Figures

List of Tables

Opsomming

X – adrenoleukodistrofie (X-ALD) is 'n progressiewe neurodegeneratiewe sindroom wat gekenmerk word deur die opeenhoping van versadigde baie langkettingvetsure (VBLKVe), veral heksakosanoësuur (C26:0) en tetrakosanoësuur (C24:0) in die plasma en brein. Ingekorte perokisomale β-oksidasie is 'n verdere komplikasie. Dit het tot gevolg dat die serebrale witstof, rugmurg, perifere senuwees, adrenale korteks en die testis geaffekteer word (Kemp *et al.*, 2004). Die hoof oorsaak van die sindroom is 'n defek in die ABCD1 geen wat vir die peroksisomale membraan proteïen, adrenoleukodistrofie proteïen (ALDP) kodeer.

Tot hede is daar geen effektiewe behandeling vir X-ALD nie. Lorenzo se olie, wat bestaan uit oleïensuur (C18:1) en erusiese suur (C22:1), word wel gebruik. Behandeling verlaag die VBLKV vlakke in pasiënte, maar verbeter nie die neurologiese simptome nie. Behandeling van X-ALD met mono-onversadigde vetsure soos oleïensuur en erusiese suur normaliseer C26:0 vlakke omdat hierdie sure kompeteer met die mikrosomale verlengings-sisteem (Rizzo *et al.,* 1986).

Erusiese suur is 'n mono-onversadigde omega-9 vetsuur, en word aangedui as C22:1 ω-9. C22:1 is 'n potente inhibitor van VBLKVe. Die kompetisie van C22:1 met die mikrosomale verlengings-sisteem en die daaropeenvolgende inhibisie van VBLKV-sintese kan ook lei tot inhibisie van poli-onversadigde vetsuursintese wat deur dieselfde verlengings-sisteem verleng word. Kramer en mede-werkers het in 1992 eksperimente uitgevoer met doserings tussen 400 mg/kg en 1500 mg/kg erusiese suur. Spraque-Dawley rotte (10 rotte per groep) was behandel met olies met 'n 2.5 – 9% erusiese suur konsentrasie vir 'n tydperk van sewe dae. Doserings van 1500 mg/kg en hoër het noemenswaardige miokardiale lipidosis tot gevolg gehad as dit vergelyk is met laer doserings.

In hierdie studie het ons probeer om die optimale dosering waarby erusiese suur die vlakke van baie lang ketting vetsure verlaag te bepaal. Die uitwerking wat erusiese suur op die biosintese en inkorporering van poli-onversadigde vetsure in die plasma en brein fosfolipiede is ook bepaal. Sestig manlike Spraque-Dawley rotte was individueel in metaboliese hokke aangehou en het vrye toegang tot laboratorium kos en water gehad. Die rotte is in 5 groepe (n = 10) verdeel en ook 'n kontrole groep (n = 10). Doserings van 400mg/kg, 575 mg/kg, 600

mg/kg, 625 mg/kg en 800 mg/kg erusiese suur, opgelos in dimetielsulfoksied (DMSO), is respektiewelik toegedien aan die groepe rotte vir sewe dae deur 'n gastriese buis. Die kontrole groep het DMSO ontvang. Die verskillende erusiese suur dosisse was opgelos in DMSO. Die rotte is op dag agt onthoof, waarna bloed- en breinmonsters versamel is en by -20 °C gevries is. Die inhiberende uitwerking wat onderskeie dosisse erusiese suur op die totale plasma versadigde baie lang ketting versure gehad het was met behulp van 'n gestandaardiseerde metode bepaal deur gebruik te maak van die gas kromatograaf massa-spektrometrie (GC-MS). Die resultate is weergegee in µmol/L. Die versadigde baie lang ketting vetsuur en poli-onversadigde vetsuur konsentrasies in plasma en brein fosfolipiede is met behulp van 'n gestandaardiseerde metode bepaal deur gebruik te maak van GC-MS en dunlaagkromatografie. Die vetsuur konsentrasies is weergegee as 'n persentasie van die totale vetsuur samestelling. Statistiese vergelykings tussen die onderskeie groepe is gedoen deur analiese van variansie (ANOVA).

Die dosering van 600 mg/kg, alhoewel nie statisties betekenisvol nie, het die optimale inhiberende uitwerking op die C24:0 en die C26:0 sintese gehad, met 'n verlaging in die C24:0/C22:0 verhouding. Erusiese suur het die biosintese van die ω-3 vetsure inhibeer deur mededining met die verlenging van EPA na DPA in die ω-3 vetsuursintese pad. Dit was weerspiëel deur die verlaging in die DPA/EPA verhouding van die 600 mg/kg dosering. Die effek was meetbaar in beide die plasma en in die brein fosfolipiede.

Erusiese suur het die DHA (ω-3) en AA (ω-6) konsentrasies in die plasma fosfolipiede verlaag, maar het geen inhiberende effek op die DHA vlakke in die brein fosfolipiede gehad nie. Daar was 'n merkbare toename in die aragidonsuur (AA) vlakke in die brein fosfolipiede. AA is die voorganger vir 'n aantal inflammatoriese tussengangers, insluitend prostanglandiene en leukotriene. Die toename in AA kan dus 'n pseudo-inflammasie tot gevolg hê. Die verskynsel kan 'n moontlike verduideliking wees vir bevindinge dat neurologiese simptome voortduur en soms vererger in pasiënte met simptomatiese X-ALD wat met Lorenzo se olie behandel word.

Ten slotte, erusiese suurbehandeling van Spraque-Dawley rotte het tot 'n verlaging van baie langkettingvetsure in die plasma gelei. Hierdie resultate dui dus op 'n positiewe effek van behandeling. Die verhoogde AA vlakke in die brein a.g.v. die behandeling, is egter kommerwekkend. Verdere navorsing om die meganisme van erusiese suur uit te sorteer is geregverdig.

Abstract

X-adrenoleukodystrophy (X-ALD) is a progressive neurodegenerative disorder characterized by the accumulation of saturated unbranched very long chain fatty acids (VLCFA), particularly hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0) in plasma and brain. Peroxisomal β-oxidation is also impaired. It affects the cerebral white matter, spinal cord, peripheral nerves, adrenal cortex and testis (Kemp *et al.*, 2004). It is caused by a defect in the ABCD1 gene, which maps to Xq28 and codes the peroxisomal membrane protein, adrenoleukodystrophy protein (ALDP).

To date, there is no effective therapy, except for Lorenzo's oil, which consists of oleic acid (C18:1) and erucic acid (C22:1). Treatment with Lorenzo's oil elicits a good biochemical response and cause a decreased in plasma VLCFA levels in patients. However, improvement of neurological symptoms has not been reported. Treatment of X-ALD with *monounsaturated* fatty acids such as oleic acid and erucic acid lead to the normalization of C26:0 levels, possibly as a result of competition for the microsomal elongation system (Rizzo et al., 1986).

Erucic acid is a mono-unsaturated omega-9 fatty acid, denoted C22:1ω-9. It is a potent inhibitor of saturated very long chain fatty acids (SVLCFA). Because erucic acid competes for the microsomal elongation system, the inhibition of SVLCFA synthesis can lead to inhibition of polyunsaturated fatty acids that are elongated by the same system. Dosages between 400 mg/kg and 1500 mg/kg erucic acid have been tested by Kramer and co-workers (1992). Spraque-Dawley rats (10 rats per group) have been treated for one week with oils that contained 2.5 to 9% erucic acid concentration. The dosages of 1500 mg/kg and higher produced significantly increased myocardial lipidosis.

The aim in this pilot study was to determine the optimum dosage for erucic acid in Sprague-Dawley rats that will lower levels of SVLCFA. A further aim was to assess its effect on the biosynthesis and incorporation of polyunsaturated fatty acids (PUFA) into the plasma and brain phospholipids. Sixty male Sprague-Dawley rats were individually housed in metabolic cages with free access to laboratory food and water. The rats were divided into 5 groups (n = 10 in each) including a control group (n = 10). Dosages of 400mg/kg, 575 mg/kg, 600 mg/kg,

625 mg/kg and 800 mg/kg of erucic acid, dissolved in dimethylsulphoxide (DMSO), were given by gavage to the five groups respectively for 7 days. The control group received the vehicle, DMSO. The rats were decapitated on day 8 and brain and blood samples were collected and frozen at -20 °C until assayed. The inhibitory effect of different dosages of erucic acid on total plasma SVLCFA concentrations and concentration ratios was determined using a standardized method employing gas chromatography-mass spectrometry (GC-MS). These results were expressed in µmol/L. For the determination of SVLCFA and PUFA in the plasma and brain phospholipids, gas chromatography-mass spectrometry (GC-MS) and thin-layer chromatography (TLC) were used. Fatty acids were expressed as a percentage of the total lipid composition and subsequent ratios were calculated. Statistical comparisons of data between the groups were done using analysis of variance (ANOVA).

A dose of 600 mg/kg erucic acid reduced C24:0 and C26:0 levels and decreased the C24:0/C22:0 ratio the most. These reduction were, however, not significantly different. Erucic acid competed with the elongation of EPA (Eicosapentanoic acid) to DPA (Docosapentaenoic acid) in the ω-3 fatty acid pathway, which was seen as a decreased DPA/EPA ratio in the 600 mg/kg erucic acid group. This effect was seen in both the plasma and brain phospholipids.

Erucic acid slightly decreased DHA (Docosahexaenoic acid) (ω-3) and AA (Arachidonic acid) (ω-6) concentrations in the plasma phospholipids, but had no influence on DHA levels in the brain phospholipids. A result of concern was the significant increase in arachidonic acid (AA) levels in the brain phospholipids. AA is the precursor to a number of inflammatory mediators, including prostaglandins and leukotrienes that could lead to inflammation. The latter could explain why neurological symptoms persist, and sometimes even progress in patients with symptomatic ALD who use Lorenzo's oil.

Therefore, we conclude that erucic acid treatment in Sprague-Dawley rats reduced VLCFA levels in plasma and that this result is promising. Unfortunately concomitant increased levels of AA in the brain is a matter of concern. Further research into the mechanism of action of erucic acid is called for.

Abbreviations

A

AA	Arachidonic acid
ABC	ATP binding cassette
ACOX1; ACOX2	Acyl – CoA oxidases
ADHD	Attention deficit hyperactivity disorder
ALA	α – linolenic acid
ALDP; ABCD1	Adrenoleukodystrophy protein
ALDR; ABCD2	Adrenoleukodystrophy related protein
AMN	Adrenomyeloneuropathy
ANOVA	Analysis of variance
ATP	Adenine dinucleotide triphosphate

B

BBB	Blood-brain barrier
BHT	Butylated hydroxytoluene

C

CACT	Mitochondrial carnitine/acylcarnitine transporter
CAT	Asetyltransferase
CCER	Childhood cerebral Adrenoleukodystrophy
CMS	Chloroform:methanol:saline

CNS	Central nervous system
CoA	Coenzyme A
COT	Carnitine octanoyl - transferase
D	
DBP	D-bifunctional protein
ddH_2O	Double distilled water
DHA	Docosahexaenoic acid
DMSO	Dimethylsulphoxide
DPA	Docosapentaenoic acid
E	
EPA	Eicosapentanoic acid
F	
FA	Fatty acid
G	
GC-MS	gas chromatography mass spectrometry
H	
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HSD	Honest significant difference
I	
IS	Internal standard
K	
KOH	Potassium hydroxide

L

LBP	L-bifunctional protein
LCFA	Long chain fatty acid
LO	Lorenzo's oil

M

MCFA	Medium chain fatty acid
MSD	Membrane spanning domain
MUVLCFA	Monounsaturated very long chain fatty acid

N

NaOH	Sodium hydroxide
NOEL	No observed effect level

P

PUFA	Polyunsaturated fatty acid
PUVLCFA	Polyunsaturated very long chain fatty acid

R

RBC	Red blood cells
RF	Response factor
RPM	Revolutions/minute

S

SCFA	Short chain fatty acid
SIM	Single ion monitoring
SVLCFA	Saturated very long chain fatty acid

T

TFA	Total fatty acid
TLC	Thin layer chromatography
TMD	Transmembrane domain

U

USA	United States of America

V

VLCFA	Very long chain fatty acid

X

X-ALD	X-Linked Adrenoleukodystrophy

Introduction

Chapter 1

1 Introduction

X-Linked adrenoleukodystrophy (X-ALD) is an inherited neurodegenerative disease that affects the cerebral white matter, spinal cord, peripheral nerves, adrenal cortex, and testis. It is the most common peroxisomal disorder with an incidence in males estimated to be 1:21,000 and 1:14,000 in females (Moser, 2006). It appears to be the same for all ethnic groups. The biochemical signature of X-ALD is increased levels of saturated very long chain fatty acids (VLCFA) in plasma and tissue especially C26:0 and C24:0, particularly in the cholesterol esters and ganglioside fractions of the brain white matter and adrenal cortex (McGuinness *et al.*, 2001). This elevation is caused by a defect in peroxisomal VLCFA beta-oxidation activity.

X-ALD shows a wide variety of phenotypic expression with seven different phenotypes in male patients (i.e., childhood cerebral form (CCER), juvenile cerebral form, adult cerebral form, adrenomyeloneuropathy (AMN), isolated Addisons disease, olivo-ponto-cerebral and asymptomatic patients) and five in female carriers (i.e. asymptomatic, mild myelopathy, moderate to severe myeloneuropathy, cerebral involvement and clinically evident adrenal insufficiency) (Deon *et al.*, 2008). The most common variants in males are the childhood cerebral form, adrenomyeloneuropathy and Addison's disease.

The initial diagnosis of X-ALD relies on the clinical presentation and biochemical analyses of VLCFA. Analyses of the plasma VLCFA levels including lignoceric acid (C24:0), hexacosanoic acid (C26:0) and their ratios to behenic acid (C22:0) are used to confirm the diagnosis on patients suspected to suffer from the disease (Berger and Gärtner, 2006). Mutation analysis is considered the best method to establish the carrier status in women.

The current therapies available for X-ALD include Lorenzo's oil (LO), hormone replacement therapy and hematopoietic stem cell transplantation. LO is a 4:1 mixture of glyceryl trioleate and glyceryl triuricate. Oral administration of this oil with a reduction of fats in the diet lowers the levels of VLCFA in plasma of patients with X-ALD within 4 weeks. But the oil did not alter the rate of progression in individuals who were already symptomatic when therapy was

initiated; particularly those with CCER and neither improved neurological or endocrine function nor arrested progression of the disease and often induced adverse effects (Kemp *et al.*, 2005; Moser., 2006).

Erucic acid is the main component of Lorenzo's oil. This long chain monoenoic acid can normalize elevated serum levels of C26:0 and C24:0 in X-ALD by depressing their biosynthesis from shorter chain saturated fatty acids via inhibition of elongase (Sargent *et al.,* 1994). Although treatment with Lorenzo's oil seemed to be beneficial in reducing VLCFA, Lorenzo's Oil is not without side effects. Several studies showed reduced platelet counts (thromboytopaenia), increased liver enzymes, gastrointestinal complaints and gingivitis (Zinkham *et al.*, 1993; Van Geel *et al.*, 1999). Considering all of above, one can argue whether this therapy is efficacious at all, and whether a marginal therapeutic effect counterbalances the side effects.

At this time, there is no cure for X-ALD. General supportive care and symptomatic treatment for the patient and family is the cornerstone for the care and treatment of patients with X-ALD. It is therefore very important that more research must be done to develop an effective treatment for patients with X-ALD.

The symptoms and chemical pathology of X-ALD and the treatment of X-ALD will be discussed in chapter 2 and 3 respectively.

Adrenoleukodystrophy

Chapter 2

2 Introduction

X-Linked adrenoleukodystrophy (X-ALD) is a genetic disorder secondary to alterations in the ABCD1 (ATP-binding cassette, sub-family D [ALD], member 1) gene, resulting in defective peroxisomal β-oxidation and the accumulation of very long chain fatty acids (VLCFA) in all tissues (Moser *et al.*, 2007a).

It is a postnatal progressive neurodegenerative disease that primarily affects the adrenal cortex and the nervous system. The impairment of peroxisomal β-oxidation and the accumulation of saturated very long chain fatty acids in tissue and body fluids of patients are pathognomonic for X-ALD. It is characterised biochemically by the accumulation of two major VLCFA namely C26:0 (hexacosanoic acid) and C24:0 (tetracosanoic acid) (Vargas *et al.*, 2000). Analyses of the plasma VLCFA levels and their ratios to C22:0 (behemic acid) are used to confirm the diagnoses in patients suspected to suffer from the disease (Berger and Gartner., 2006).

Definitive diagnoses are achieved by demonstration of the biochemical defect and by mutation analysis. X-ALD continues to be underdiagnosed and this can have serious consequences because there is a loss of opportunity for early treatment. Magnetic resonance imaging scans of the brain are obtained as part of evaluation of clinically suggestive patients. Those with a cerebral form of the disease show characteristic white matter lesions. In the majority of cases, these lesions are symmetric and involve the corpus callosum and the periventricular parietooccipital white matter (Berger and Gartner., 2006).

Clinically, it is characterized by striking and unpredictable variations in phenotypic expression. The phenotypic manifestations of X-ALD are more varied than initially realized, ranging from rapidly progressive childhood cerebral form (CCER) to the more slowly progressive adult form adrenomeyeloneuropathy (AMN) and variants without neurological involvement (Kemp *et al.*, 2005).

2.1 Phenotypes

X-ALD is a heterogeneous disease with more than seven different phenotypes in male patients and five phenotypes in females. The phenotypes of X-ALD are subdivided based on the age of onset, the sites of most severe clinical involvement, and the rate of progression of neurological symptoms. Various phenotypes frequently occur within the same kindred although the primary defect that underlies the different phenotypes is the same.

All daughters of an affected male are carriers; none of his sons will be affected. A female who is a carrier has a 50% chance of transmitting the ABCD1 mutation with each pregnancy. Sons who inherit the mutation will be affected; daughters who inherit the mutation are carriers and will usually not be seriously affected. Many individuals with X-ALD remain asymptomatic until middle age or even later (Moser *et al.*, 1999).

Three phenotypes are seen males. The childhood cerebral form (CCER manifests between ages four and eight years. The second phenotype, adrenomyeloneuropathy (AMN), manifests in the late twenties and the third phenotype, "Addison disease only," presents with primary adrenocortical insufficiency between age two years and adulthood and most commonly by age 7.5 years. Approximately 20% of females who are carriers develop neurologic manifestations that resemble AMN but have a later onset (age ≥35 years) and milder disease than affected males.

The different X-ALD phenotypes in males as well as female carriers are summarized in Tables 2.1 and 2.2.

2.1.1 Childhood Cerebral inflammatory ALD (CCER)

The childhood cerebral inflammatory phenotype was initially described by Siemerling and Creutzfeldt, and until 1976 considered to be essentially the only phenotype (Moser., 2006). Onset is between 4 and 8 years of age with a peak at 7 years of age (Kemp and Moser., 1999a). Neurological manifestations are rarely below 3 years of age, with 21 months being the youngest known age of onset. Early development is entirely normal, with normal psychomotor development, neurological development and cognitive function. This phenotype is associated with intensely inflammatory demyelination, most severe in the parietal, occipital and posterior temporal lobes. The abnormal accumulation of VLCFA in brain white matter is thought to play a role in the myelin and axon destructive cascade that occurs in CCER (Moser., 2006).

Early behavioural changes include emotional liability, withdrawn or hyperactive behaviour, school failure, attention deficit hyperactivity disorder (ADHD) or psychological disorder.

Difficulty understanding speech, defects in auditory discrimination or visual processing, poor handwriting, impaired memory, and occasionally seizures are also seen in these children (Moser *et al.*, 2007b).

Once there are neurological manifestations, progression is rapid, with a mean interval between first neurological symptoms and an apparently vegetative state of 1.9 ± 2 years.

X-ALD phenotypes in males

Table 2.1: X-ALD phenotypes in males (Adapted from Moser *et al.*, 2007b).

Phenotype	Description	Relative frequency
Childhood cerebral (CCER)	Onset at 3 - 10 years of age. Progressive behavioural, cognitive and neurologic deficit, often leading to total disability within 3 years. Inflammatory brain demyelization.	31-35%
Adolescent	Like childhood cerebral. Onset at age 11 - 21 years. Slower progression.	4-7%
Adrenomyelo-neuropathy (AMN)	Onset at 28 ± 9 years, progressive over decades. Involves spinal cord, distal axonopathy inflammatory response mild or absent. Approximately 40 % have or develop cerebral involvement with varying degrees of inflammatory response and more rapid progression.	40-46%
Adult cerebral	Dementia, behavioural disturbances. Sometimes focal deficits, without preceding AMN. White matter inflammatory response present. Progression parallels those of CCER.	2-5%
Olivo-ponto-cerebellar	Mainly cerebellar and brain stem involvement in adolescence or adulthood.	1-2%
"Addison-only"	Primary adrenal insufficiency without neurological involvement. Onset before 7.5 years. Most develop AMN.	Varies with age. Up to 50% in childhood.
Asymptomatic	Biochemical and gene abnormality without demonstrable adrenal or neurologic deficit. Detailed studies show subtle signs of AMN.	Diminish with age. Common < 4 years. Rare > 40 years.

Phenotypes in females.

Table 2.2: X-ALD phenotypes in female carriers (Adapted from Moser *et al.*, 2007b).

Phenotype	Description	Relative frequency
Asymptomatic	No evidence of adrenal or neurologic involvement.	Diminishes with age. Most woman < 30 years neurologically uninvolved.
Mild myelopathy	Increased deep tendon reflexes and distal sensory changes in lower extremities with absent or mild disability.	Increases with age. Approximately 50% > 40 years.
Moderate to severe myeloneuropathy	Symptoms and pathology resemble AMN, but milder and later onset.	Increases with age. Approximately 15% > 40 years.
Cerebral involvement	Rarely seen in childhood and slightly more common in middle age and later.	Approximately 2%
Clinical evident adrenal insufficiency	Rare at any age.	Approximately 1%

2.1.2 Adolescent cerebral ALD

The symptoms and progression in these patients resemble those in CCER. Age of onset is between 11 and 21 years.

2.1.3 Adrenomeyloneuropathy (AMN)

AMN, characterized by myelopathy and neuropathy, was first described in 1976 in Austria by Budka and co-workers and in 1977 by Griffen and co-workers in the United States (Moser *et al.*, 2007b; Budka *et al.*, 1976; Griffen *et al.*, 1977). AMN presents in adults as a slowly progressive paraparesis, combined with sensory and sphincter disturbances. Age of onset is 28 ± 9 years. It is a non-inflammatory distal axonopathy that involves the dorsal column and corticospinal tract in the lower thoracic and lumbar regions (Moser *et al.*, 2007a).

AMN is sub-divided into two categories: 'pure AMN' and 'AMN cerebral'.

In pure AMN neurological involvement is confined to the spinal cord and peripheral nerves with no clinical evidence of brain involvement, while in the AMN cerebral phenotype there is inflammatory involvement in addition to manifestations of pure AMN. Except for mild deficits

in psychomotor speed and visual memory, neuropsychological function is normal in pure AMN. Cerebral AMN patients have normal IQ and language but impaired psychomotor speed, spatial cognition, memory, and executive functions (Edwin *et al.*, 2004).

Depression or emotional disturbances are common in AMN and impotence begins in the late twenties or thirties.

2.1.4 Adult cerebral ALD

In adulthood, the inflammatory cerebral phenotype is most commonly super-imposed on pre-existing AMN. Much less frequently, the inflammatory cerebral phenotype presents itself in adults without prior evidence of AMN (Moser *et al.*, 2007a). Adult cerebral ALD applies to patients with the biochemical defect of X-ALD who develop cerebral symptoms after 21years of age, but who do not have signs of spinal cord involvement (Moser *et al.,* 2007b).

This phenotype develops in 2 - 5% of all patients (Kemp and Moser., 1999a). Age of onset varies from early twenties to the fifties with symptoms resembling schizophrenia with dementia or a specific cerebral deficit. Psychiatric manifestations include signs of mania including disinhibition, impulsivity, loudness, hyper sexuality and perseveration (Moser *et al.,* 2007b). A white matter inflammatory response is present and visible on MRI. The prognosis is three to four years from the first neurological symptoms to the vegetative state or death.

2.1.5 Addison disease only

Seventy percent of all males with X-ALD have primary adrenocortical insufficiency (Addison disease). In most instances this is associated with CCER and AMN (Moser *et al.,* 2007b). Approximately twenty percent of male X-ALD patients have Addison disease without clinical or MRI evidence of neurological involvement (Moser., 2006). This is referred to as the Addison-only phenotype of X-ALD.

The Addison-only phenotype cannot be distinguished clinically from Addison disease, but can be set apart based upon elevated levels of plasma VLCFA. Most of the patients with the Addison-only phenotype will eventually develop neurological symptoms. The interval between adrenal insufficiency and neurological symptoms is variable and can be as long as 32 years.

2.1.6 Asymptomatic males

These patients have a biochemical abnormality and X-ALD has been demonstrated by one of the following criteria: demonstration of elevated levels of VLCFA or a mutation identified in the X-ALD gene. There is no evidence of adrenal or neurological involvement (Kemp and

Moser., 1999a). It is, however, expected that most individuals with this phenotype will develop adrenal and neurological symptoms at some point in their lives.

2.1.7 Olivopontocerebellar atrophy

This is a very rare phenotype of X-ALD. In most of the cases it presents itself in adulthood. Cerebellar ataxia is present, and in most cases it is combined with corticospinal tract involvement. The illness is progressive with neurological involvement (Moser *et al.,* 2007b).

2.1.8 Symptomatic heterozygotes

Approximately 50% of woman who are heterozygous develop AMN-like symptoms at a later stage in their lives. The mean age of onset is 37±14.6 years, ranging from 2-73 years (Moser *et al.* 2007a). The progression of this phenotype is much slower than those in males. Overt adrenal insufficiency and inflammatory cerebral phenotype occur in approximately 1% of heterozygotes (Moser., 2006; Fatemi *et al.*, 2003). The phenotypes have been described in detail and table 2.1 and 2.2 summarize their main manifestations and relative frequencies.

2.2 Gene defect in X-ALD

X-ALD is an X-linked inherited genetic disorder because it involves the X-chromosome. This disorder affects mostly only men and is transmitted by a female carrier. Women have 2 X-chromosomes while men only have one X-chromosome. In women, the affected X-chromosome (the one with the X-ALD gene) does not manifest, because of the presence of a normal copy on the other X-chromosome. In men, who have a defect on the X-chromosome, there is no protection from another normal X-chromosome like in females and therefore symptoms present in male patients (Fig 2.1 and 2.2).

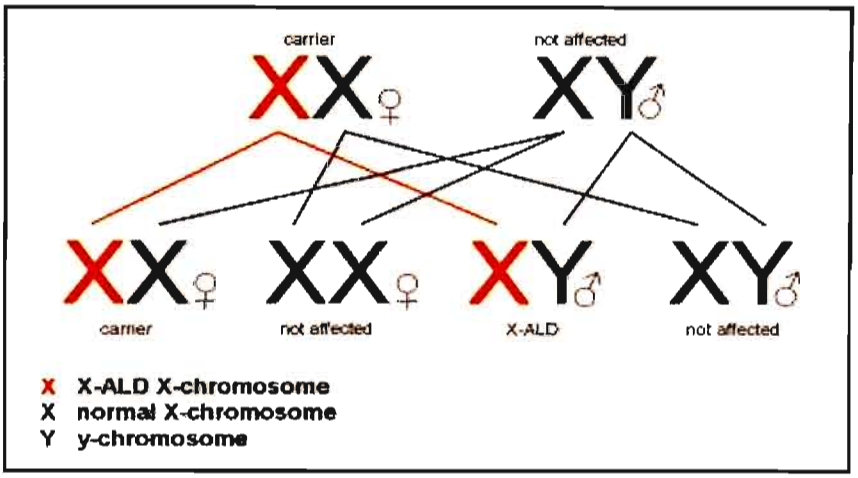

Figure 2.1: If a female is a carrier of X-ALD, her newborn daughter will have a 50% chance to be a carrier of X-ALD and a 50% chance to be normal. If the newborn is a boy there is a 50% chance that he will have X-ALD and 50% to be normal (Adapted from Kemp and Moser., 1999b)

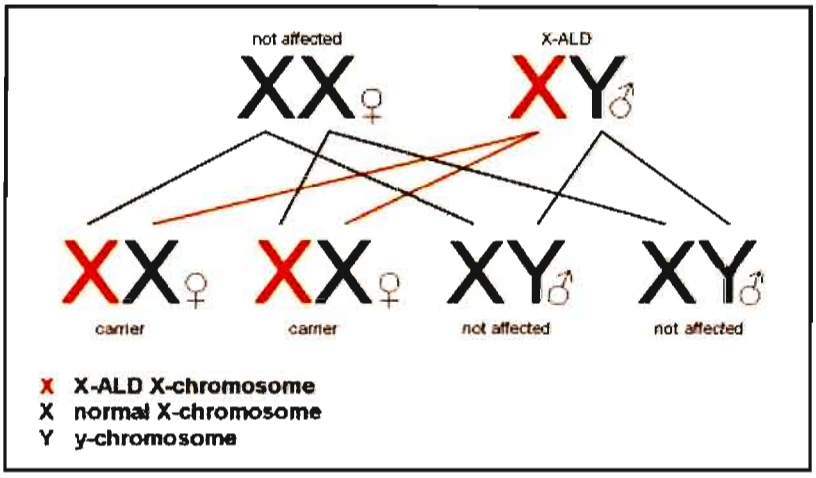

Figure 2.2: If an affected male has children, all the males will be unaffected, because the Y-chromosome is passed to his sons. His daughters will be carriers of X-ALD because the X-chromosome is passed to the daughters (Adapted from Kemp and Moser., 1999b).

2.2.1 Molecular genetics

Table 2.3: Molecular genetics of X-ALD

Affected gene	Gene locus	Enzyme/protein	Substrate
ABCD1	Xq28	ALDP	VLCFA

The defective gene in X-ALD patients was mapped to the Xq28 gene locus in 1981 and was isolated and cloned in 1993 (Moser *et al.*, 2007a; Migeon *et al.*, 1981). The gene is referred to as the ABCD1 gene and it codes for the ALDP proteien (adrenoleukodystrophy protein). Mutations in the ABCD1 gene were identified in almost all X-ALD patients, and to date there are nearly 500 different mutations known (Eichler and Van Haren, 2007). The ATP binding cassette (ABC) protein super family consists of transporters for a whole variety of organic and inorganic compounds. Their functions range from the acquisition of nutrients and excretion of waste products to the regulation of various cellular processes (Pohl *et al.*, 2005).

The 49 human ABC proteins currently known can be classified in 7 families (A-G) according to sequence similarity. Several human ABC proteins found to be mutated in lipid-linked diseases (families A, B, C, D and G) were suggested to be involved in lipid transport (Pohl *et al.*, 2005). Peroxisomal ABC transporters belong to the subclass D. All four known members of the ABCD family are involved in β-oxidation of long and very long chain fatty acids, the synthesis of bile acids, cholesterol plasmalogens and the metabolism of amino acids and purines. The four members of the ABCD subfamily are: Adrenoleukodystrophy protein (ALDP; ABCD1), adrenoleukodystrophy related protein (ALDR; ABCD2), PMP70-related protein (ABCD3) and PMP69 (P70R; ABCD4). These proteins bind ATP and use energy to drive the transport of various molecules across extra- and intra-cellular membranes. (Rottensteiner and Theodoulou., 2006).

Defects in the ABCD1 results in the inherited neurometabolic disorder X-ALD (Pohl *et al.*, 2005; Moser., 1993).

2.3 Peroxisomes

Peroxisomes are single membrane bound subcellular organelles, ubiquitous in eukaryotic cells. The organelles are usually spherical bodies in the range of 0.1 - 1 μm in diameter. Peroxisomes contain coarsely granular or fibrillar matrix, occasionally dotted with crystalline inclusions containing enzymes involved in cellular metabolism, particularly fatty acid degradation (Fig 2.3) (Nyathi and Baker., 2006).

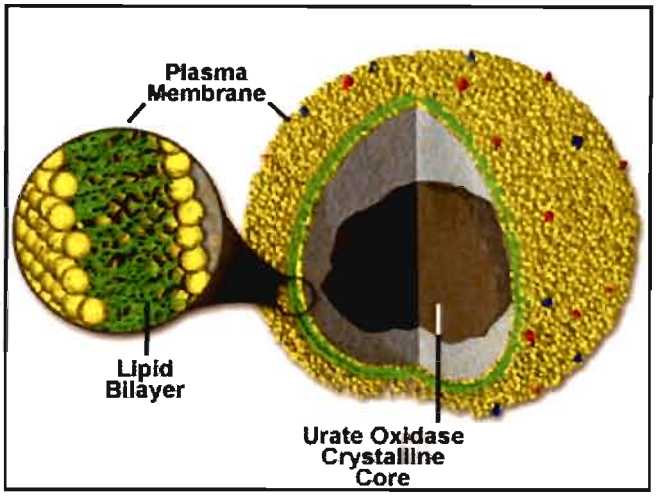

Figure 2.3: Anatomy of the peroxisome (Adapted from Davidson, 1995).

After their morphological identification in the early 1950's, peroxisomes were identified as truly distinct subcellular organelles by De Duve and Baudhuim (Wanders *et al.*, 2000; De Duve and Baudhuin., 1966). In mammals, peroxisomes are known to participate in fatty acid α- and β- oxidation, the biosynthesis of ether phospholipids and bile acids and in the degradation of purines, polyamines, L-pipecolic acid and D-amino acids (Visser *et al.*, 2007; Wanders and Waterham., 2006b).

Peroxisomes exhibit marked morphological and metabolic plasticity, depending on the organism, cell type and prevailing environmental conditions. They perform a range of functions in different taxa, some of which are organism specific and some of which are common to all eukaryotes. Although the diversity of functions is reflected in the plasticity of peroxisomes, β-oxidation of fatty acids and the generation and degradation of hydrogen peroxide are the distinctive general biochemical functions of this organelle (Theodoulou *et al.*, 2006; Gerhardt., 1986; Beevers., 2002).

The importance of peroxisomes for humans is stressed by the existence of a group of genetic diseases in humans where there is an impairment in one or more peroxisomal functions, where most of these functions have to do with lipid metabolism.

2.3.1 Fatty acid import and the role of peroxisomal ABC transporters.

In order for peroxisomes to carry out its various metabolic and developmental functions, the peroxisomal membrane must regulate import and export of metabolites and proteins.

The classical concept of a biochemical membrane is of a lipid barrier that is selectively permeable to solutes and macromolecules owing to the presence of specific transporter proteins. Whilst hydrophobic and apolar molecules can cross the lipid bilayer by diffusion, polar, hydrophilic solutes and large molecules require specific transport systems (Theodoulou *et al.*, 2006). Recent studies on isolated peroxisomes indicate that these organelles are permeable to low molecular weight and hydrophilic solutes but not to more bulky cofactors (Rottenstiener and Theodoulou., 2006; Antonenkov *et al.*, 2004).

Prior to β-oxidation, fatty acids are activated by the thioesterification to coenzyme A (CoA). This activation is catalysed by a member of the acyl-CoA synthetase enzyme family, also known as activases, which differ in substrate specificity. For example: palmitic acid (C16:0) is activated through palmitoyl-CoA ligase and lignoceric acid (C24:0) is activated through lignoceroyl-CoA ligase. The activation of fatty acids to their acyl-CoA derivatives by acyl-CoA ligases is the first step in their metabolism, and the acyl-CoA ligases in peroxisomes are localized in the peroxisomal membrane, (Singh *et al.*, 1992; Krisans *et al.*, 1980; Miyazawa *et al.*, 1985; Lazo *et al.*, 1990) whereas enzymes of β-oxidation are localized in peroxisomal matrix (Singh *et al.*, 1992; Appelkvist and Dallner., 1980). Therefore, for β-oxidation to occur in the peroxisomal matrix, the fatty acids must be activated in the peroxisomal membrane. Fatty acyl-CoA is amphipathic in nature and therefore requires a transport protein to cross lipid bilayers.

According to Singhand co-workers, the abnormality in the oxidation and accumulation of VLCFA in X-ALD may be due to the deficiency of lignoceroyl-CoA ligase. This enzyme is responsible for the activation of C24:0 (Singh *et al.*, 1992; Hashmi *et al.*, 1986). These results were confirmed by observed deficiencies in lignoceroyl-CoA ligase and lignoceroyl acid oxidation in peroxisomes isolated from X-ALD cultured skin fibroblasts (Singh *et al.*, 1992; Lazo *et al.*, 1988; Lazo *et al.*, 1989; Wanders *et al.*, 1988).

The transport of C24:0 can be through various ABC transporters. As mentioned before, there are four half-ABC transporters present in mammals: the adrenoleukodystrophy protein (ALDP; ABCD1), adrenoleukodystrophy related protein (ALDR; ABCD2), PMP70-related protein (ABCD3) and PMP69 (P70R; ABCD4). The ABCD1 subfamily encodes for the adrenoleukodystrophy protein (ALDP), a transporter in the peroxisomal membrane. Despite

the uncertainty regarding the pathology of X-ALD, it is possible that ALDP is a VLCFA or VLCFA-CoA transporter (Fig 2.4) (Rottensteiner and Theodoulou., 2006; Wanders, 2004).

ALDP selectively occurs in specific cell types of the brain (hypothalamus and basal nucleus of Meynert), kidney (distal tubules), skin (eccrine gland, hair follicles, and fibroblasts), colon (ganglion cells and epithelium), adrenal gland (zona reticularis and fasciculate), and testis (Sertoli and Leydig cells) (Hoftberger *et al.*, 2007).

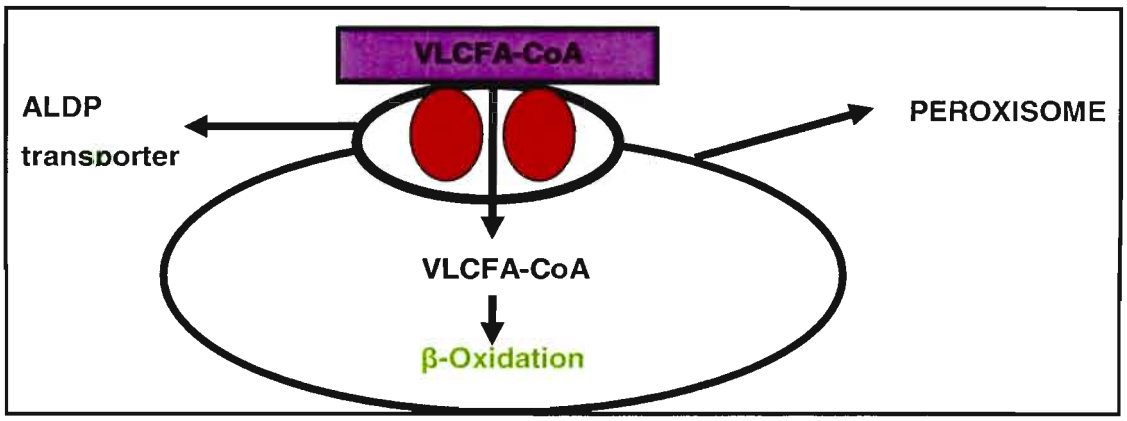

Figure 2.4: A schematic illustration of the location of the ALDP transporter protein and the transport of activated VLCFA across peroxisomal membrane for β-oxidation.

ALDP consists of 745 amino acids and is located in the peroxisomal membrane (Moser *et al.*, 2006). Structurally ALDP is a half-ABC transporter consisting of one hydrophobic transmembrane domain and a hydrophyllic nucleotide-binding domain. It has to dimerize in order to become a functional unit. A functional ABC protein contains two transmembrane domains (TMD). Each TMD has six transmembrane helices, and two ABC units (Fig 2.5) (Rottensteiner and Theodoulou., 2006; Higgens., 1992; Berger and Gartner., 2006).

The binding of two half-transporters creates a functional transporter whereby two membrane domains form a channel through which the substrate is transported against the concentration gradient into the peroxisome to undergo β-oxidation. The energy needed for this transportation is generated through the hydrolysis of ATP. This implies that ALDP might be involved in the transport of enzymes or substrates for VLCFA β-oxidation across peroxisomal membranes and because ALDP belongs to the ABC-transporter super family it may be possible that ALDP acts as a transporter for C24:0.

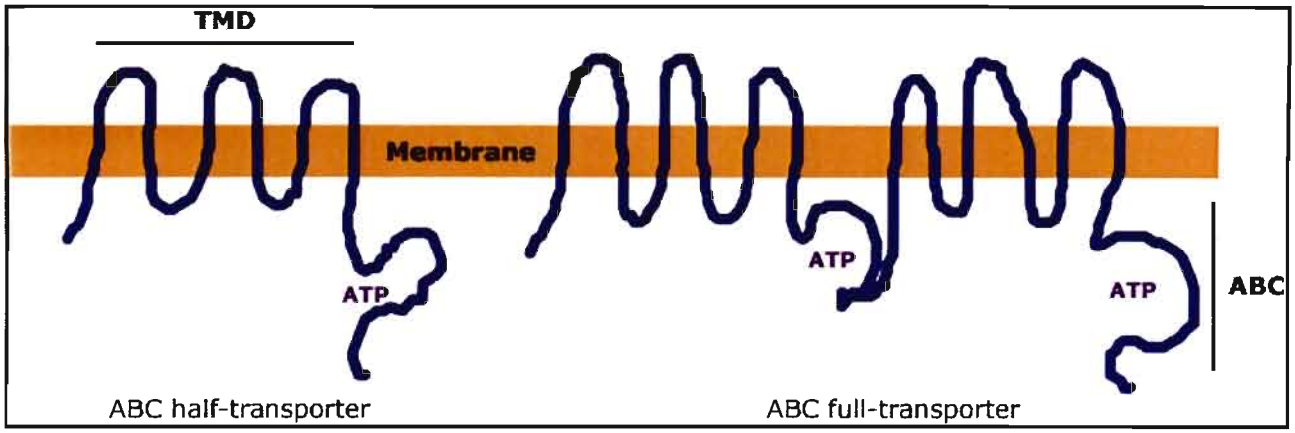

Figure 2.5: An illustration of an ABC half transporter and an ABC full-transporter (TMD = transmembrane domain)(Adapted from Kemp *et al.*, 2008).

There is no correlation between the nature of the mutation and the phenotypes of ALD. The mechanism by which ABCD1 gene deficiency leads to very long chain fatty acid accumulation and the associated phenotypic manifestations have not been defined yet (Eichler and Van Haren., 2007).

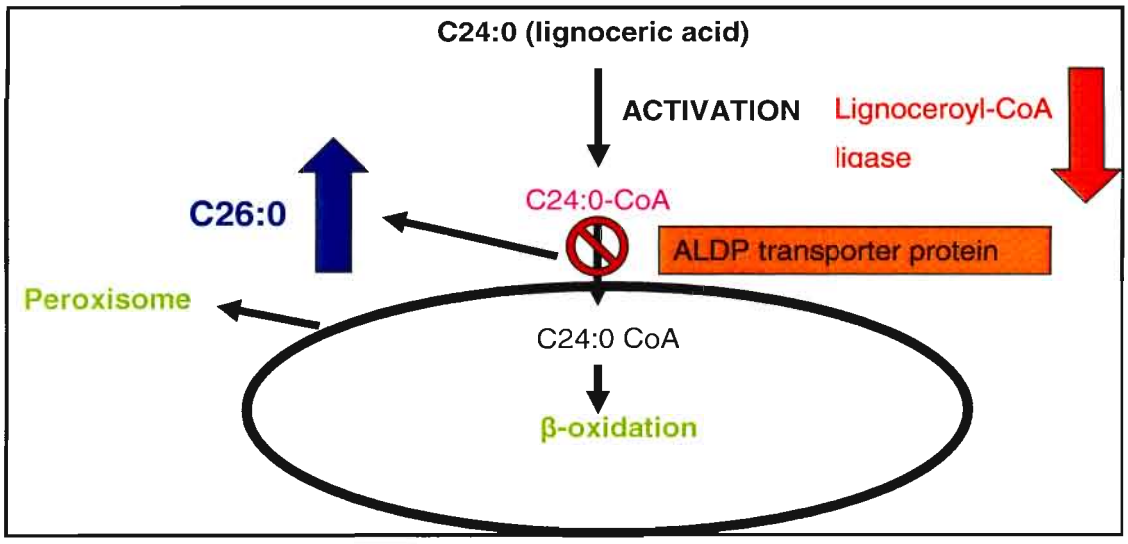

Figure 2.1: A schematic illustration of the defect in X-ALD. Because of a deficiency in the lignoceroyl CoA ligase together with a defect in the ALDP protein C24:0 CoA cannot be transported across the peroxisomal membrane for oxidation. This causes an increase in C26:0 levels.

Since the active site of lignoceroyl-CoA ligase is on the luminal surface of peroxisomal membrane, it is possible that the observed abnormality in the activation and oxidation of lignoceric acid may be due to a defect in the transport of lignoceric acid (C24:0) through the peroxisomal membrane rather than the deficiency of lignoceroyl-CoA ligase (Fig 2.6) (Singh *et al.*, 1992).

2.3.2 Peroxisomal fatty-acid β-oxidation

Although fatty acids can undergo oxidation via different mechanisms, most fatty acids are catabolised by means of β-oxidation. Peroxisomes contain a fatty acid β-oxidation machinery just like the mitochondria, although the individual reactions of the β-oxidation systems are catalyzed by distinct enzymes. The peroxisomal and mitochondrial β-oxidation systems serve different functions in human cells and catalyse the β-oxidation of different fatty acids and fatty acid derivatives (Wanders and Waterham., 2006b).

The mitochondria catalyze the β-oxidation of the excess of long-chain fatty acids derived from the diet rather than in peroxisomes. Long chain fatty acids (LCFA) are predominantly oxidized by the mitochondrion and short and medium chain fatty acids (S- and MCFA) are oxidized exclusively in the mitochondrion. But some fatty acids cannot be handled by the mitochondria and are completely dependent on peroxisomes for β-oxidation. These include the VLCFAs. VLCFAs are derived from both dietary sources, but also synthesised endogenously from shorter chain fatty acids. The mechanism of β-oxidation involves a set of four consecutive reactions: 1) dehydrogenation; 2) hydration (of the double bond); 3) dehydrogenation again, and 4) thiolytic cleavage (Fig 2.7) (Wanders and Waterham., 2006b).

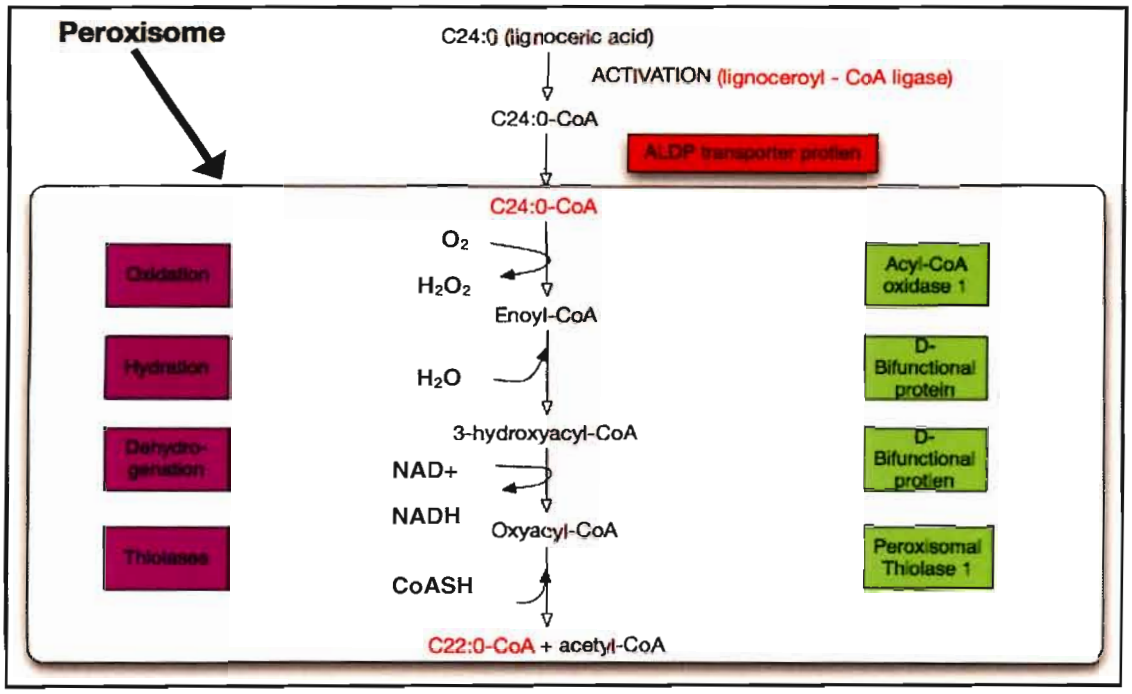

Figure 2.7: A schematic illustration of the enzymes participating in β-oxidation, the activation of VLCFA and the end product of β-oxidation.

It has been established that peroxisomes contain two acyl-CoA oxidases (ACOX1 and ACOX2) for the dehydrogenation step, two bifunctional proteins (LBP and DBP) catalyzing the second and third step and two peroxisomal thiolases (PTH1 and PTH2/SCPx) for the last step (Kemp *et al.*, 2007).

Studies in recent years have resolved the question to which of these enzymes are required for the oxidation of each particular fatty acid. The two acyl-CoA oxidases have different functions, where ACOX1 (straight-chain acyl-CoA oxidase) prefer to react with the CoA-esters of straight-chain FAs, like C26:0 and ACOX2 (branched-chain-acyl-CoA oxidase) catalyses the dehydrogenation of the CoA-esters of 2-methyl branched-chain FAs, like pristanoyl-CoA (Wanders *et al.*, 2001).

Human peroxisomes contain two bifunctional proteins, the D- and L-bifunctional proteins. The bifunctional protein harbour both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (Wanders, 2000). The first human hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D) was identified by Furuta and co-workers (Wanders, 2000; Furata *et al.*, 1980). In recent years, the L-bifunctional protein was identified by several groups (Wanders., 2000; Adamski *et al.*, 1992). The D- and L-bifunctional proteins have

different substrate specificities. The D-bifunctional protein catalyzes the formation of 3-ketoacyl-CoA intermediates from both straight-chain and 2-methyl-branched-chain fatty acids and also acts in shortening cholesterol for bile acid formation. In contrast, the L-specific bifunctional protein does not have the latter two activities (Jiang *et al.*, 1997). The enoyl-CoA esters of C26:0, are handled by D-BP catalyzing the second (hydration) and the third (dehydrogenation) steps of peroxisomal β-oxidation.

Human peroxisomes also contain two peroxisomal thiolase i.e. pTH1 and pTH2. Human pTH1 is the human equivalent of the clofibrate-inducible thiolase (Wanders and Waterham., 2006b; Miyazawa *et al.*, 1980). And pTH2 is identical to the 58 kDa sterol carrier protein domain (Wanders and Waterham., 2006b; Seedrof *et al.*, 1994). Thiolase-pTH2 plays a role in the oxidation of 2-methyl branched-chain fatty acids, and pTH1 and pTH2 are both involved in C26:0 oxidation.

The ultimate result of peroxisomal oxidation is that the first two carbon atoms of the fatty acyl-CoA ester are released as acetyl-CoA leaving a shortened acyl-CoA ester which can undergo subsequent rounds of β-oxidation (Fig 2.8).

2.3.3 Interaction between peroxisomes and mitochondria

Peroxisomes and mitochondria are capable of fatty acid β-oxidation. The mitochondria are primarily involved in the oxidation of short-, medium- and long-chain fatty acids, whereas peroxisomes are the sole site of very long-chain fatty acid oxidation. Furthermore, the first cycle of β-oxidation of the branched-chain fatty acid pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid occurs solely in peroxisomes. Since peroxisomes are incapable of oxidizing fatty acids to completion, it is clear that very long-chain fatty acids (e.g. C26 :0) and pristanic acid will only undergo a limited number of β-oxidation cycles within the peroxisomes, after which transport to the mitochondrion takes place (Wanders *et al.*, 2001).

The end products of peroxisomal β-oxidation, such as acetyl-CoA, are transported to the mitochondria in the form of the corresponding carnitine esters. For this purpose peroxisomes are equipped with different carnitine acyltransferases including acetyltransferase (CAT) and carnitine octanoyl-transferase (COT), which allows formation of carnitine esters inside peroxisomes, followed by export across the peroxisomal membrane via an unidentified carrier system (Wanders., 2004). The uptake of carnitine esters into the mitochondria occurs

via the mitochondrial carnitine/acylcarnitine transporter (CACT). In the mitochondria retroconversion takes place, where acylcarnitines are converted into the corresponding acyl-CoA esters via different acyltransferases (Fig 2.8).

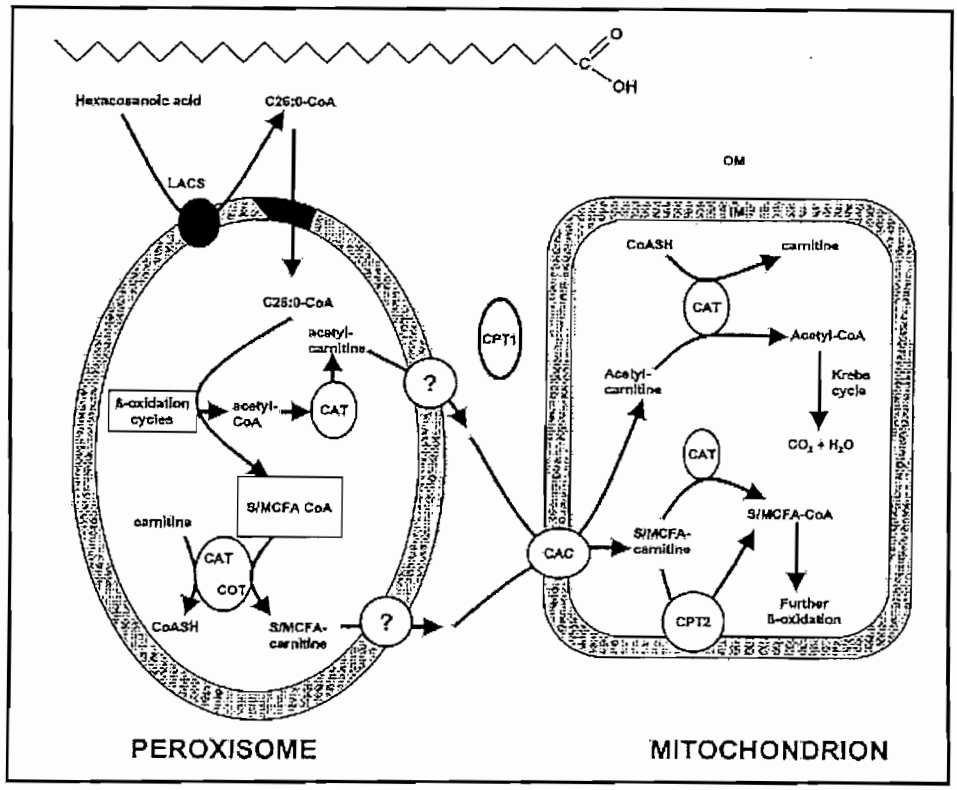

Figure 2.8: Schematic representation of the functional interaction between peroxisomes and mitochondria in the oxidation of hexacosanoic acid (C26:0) (Adapted from Wanders *et al.,* 2001).

2.4 Conclusion

X-ALD is caused by a defect in the ABCD1 gene. This gene encodes the peroxisomal transmembrane transporter, ALPD, which is known to play a role in VLCFA transportation across the peroxisomal membrane and into the peroxisomes. The defect in the ALPD protein causes increased levels of C24:0, because it is not effectively transported into the peroxisomes. This in turn leads to increased levels of C26:0. These elevated levels of VLCFA are responsible for the biochemical abnormality of X-ALD.

To date there is no effective treatment for X-ALD. Lorenzo's oil normalized plasma C26:0 and C24:0 levels within one month. However, the treatment with Lorenzo's oil does not prevent the progression of pre-existing neurological symptoms in ALD patients.

Therapies of X-linked Adrenoleukodystrophy

Chapter 3

3 Introduction

X-ALD is caused by the accumulation of saturated very long chain fatty acids in plasma and tissue especially C26:0 and C24:0. VLCFA are derived both from the diet and from endogenous synthesis by a microsomal elongation system (Fig 3.1).

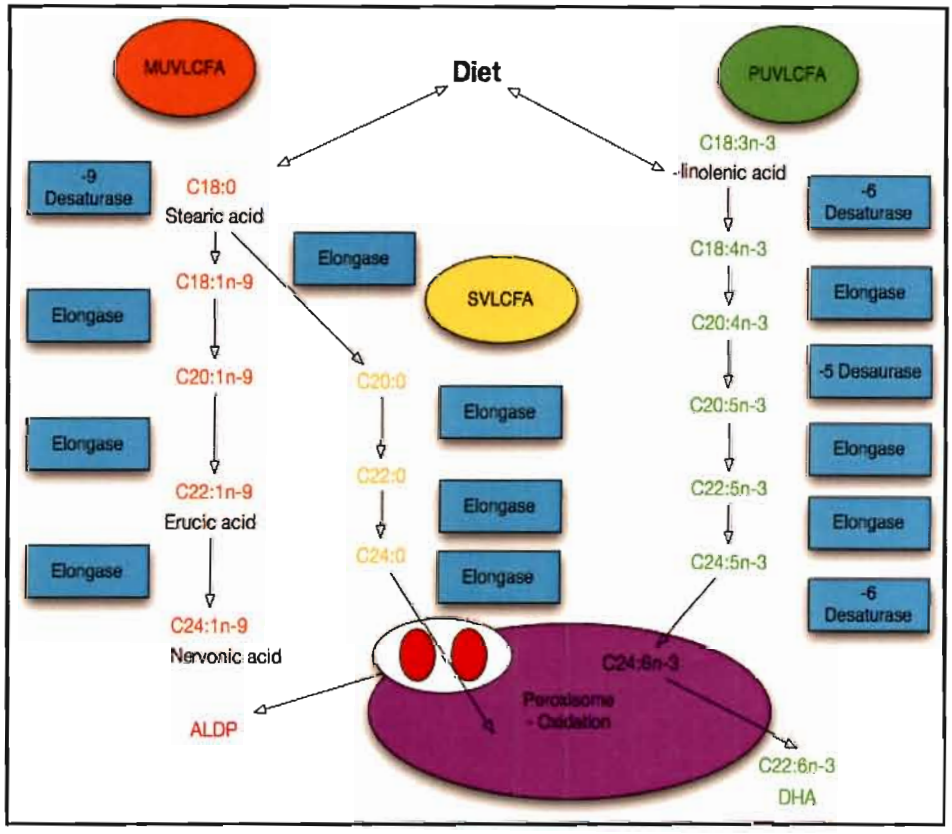

Figure 3.1: A schematic illustration of the elongation of mono-unsaturated very long chain fatty acids (MUVLCFA), saturated very-long chain fatty acids (SVLCFA) and poly-unsaturated very-long chain fatty acids (PUVLCFA) by the enzymes elongase and desaturase.

The aim of the current therapies available for the treatment of X-ALD is to lower saturated VLCFA levels and to prevent neurological progression. Prominent inflammation sets this

disorder apart from the other leukodystrophies. However, cerebral inflammation occurs in less than half of the patients with the gene defect, and there is no reliable way of predicting which patients will progress to the fatal inflammatory phase (Eichler and Van Haren., 2007). Even though the current therapies show promise, they carry a risk: their long-term efficacy is not proven and they place considerable burdens on the patients and their families. Prevention, therefore, continues to be a top priority.

3.1 Treatment of X-ALD

3.1.1 VLCFA restricted diet

The concept of dietary therapy for X-ALD was derived from the study of Kishimoto and co-workers (Moser *et al.,* 2007b; Kishimoto *et al.*, 1980). He administered deuterated C26:0 to a terminally ill patient with X-ALD and demonstrated that a substantial portion of brain C26:0 contained the label. This led to the development of a diet which restricted C26:0 to less than 15 percent of the customary U.S. intake (Van Duyn *et al.*, 1984). This diet was very limited in food choices since VLCFA are present in many plant and animal products. The simple restriction of dietary very-long chain fatty acids led to no biological or clinical improvement.

It was later shown that most of the VLCFA that accumulate in patients with X-ALD are derived from endogenous synthesis (Moser *et al.,* 2007b; Moser *et al.*, 1983; Rizzo *et al.*, 1986; Tsuji *et al.*, 1985).

3.1.2 Inhibiting the synthesis of VLCFA

The failure of dietary treatment led to the suggestion to prevent the synthesis of the toxic saturated VLCFA.

In 1986, Rizzo and co-workers observed that the addition of oleic acid (C18:1) to the medium normalizes the levels of saturated VLCFA in cultured skin fibroblasts (Moser *et al.,* 2007b; Rizzo *et al.*, 1986). This decrease in fatty acid levels are because oleic acid competes for the microsomal enzyme system that elongates saturated very long chain fatty acids. Oral administration of glyceryl trioleate reduced the levels of VLCFA in the plasma of patients with X-ALD by 50 percent. Although oleic acid is an inhibitor of the elongation of saturated very long chain fatty acids there was no improvement in the clinical manifestations.

3.1.3 Lorenzo's oil

A striking effect on plasma VLCFA levels was achieved with the administration of a 4:1 mixture of glycerol trioleate (C18:1) and glycerol trierucate (C22:1) in combination with a moderately low fat diet (Fig 3.2) (Moser *et al.*, 1993; Odone and Odone., 1989; Rizzo *et al.*, 1989). This mixture normalizes VLCFA levels within four weeks and is referred to as Lorenzo's oil. This oil is taken orally and generally well tolerated

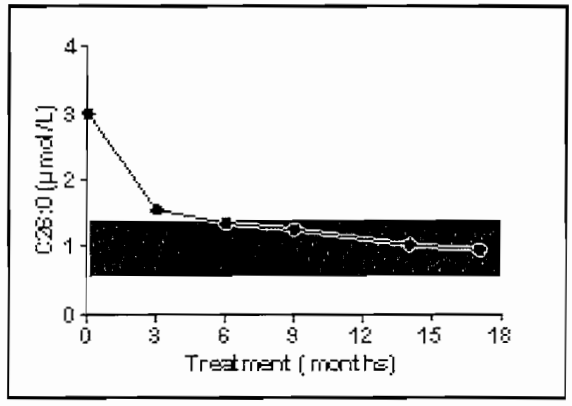

Figure 3.2: A graph showing that Lorenzo's oil lowers C26:0 levels after 6-8 weeks of treatment (Adapted from Kemp *et al.,* 1999).

From the initial studies examining the role of Lorenzo's oil in X-ALD, it was very apparent that it did not alter the progression of cerebral symptoms in affected individuals. This has been demonstrated repetitively (Raymond, 2008). Lorenzo's oil also does not alter the course of childhood or adult cerebral adrenoleukodystrophy. It is not indicated as a treatment in conditions where neurological symptoms are present. In symptomatic patients most reports indicate that the neurological disability continues to increase (Moser *et al.,* 2007b; Aubourg *et al.*, 1993; Van Geel *et al.*, 1999; Moser., 1993; Rizzo *et al.*, 1990; Rizzo., 1993). Eleven years after the introduction of Lorenzo's oil therapy, evaluation of its efficacy is still incomplete.

In the early 1990's several groups independently began studying the use of Lorenzo's oil as a preventative therapy. The use to prevent cerebral involvement in boys who were at risk of developing the disease but still asymptomatic was studied and also the use of Lorenzo's oil to slow the progression of X-ALD in men with AMN.

3.1.3.1 Adverse effects of Lorenzo's oil

Lorenzo's oil therapy has side effects, most of which can be controlled by monitoring these effects. Long-term haematological side effects are most common. Thrombocytopenia has been reported following Lorenzo's oil therapy (Konijnenberg *et al.*, 1998). Reduction in platelet count occurs in more than 30 percent of patients (Van Geel *et al.*, 1999; Stockler *et al.*, 1993; Zinkham *et al.*, 1993; Ziers *et al.*, 1993), but clinically significant abnormal bleeding has not been observed. Platelets in ALD patients also show decreased membrane anisotropy (Pai *et al.*, 2000). Slight lymphocytopenia (Unkrig *et al.*, 1994), elevation of the liver enzymes and asymptomatic neutropenia have also been reported (Van Geel *et al.*, 1999).

A reduction in the levels of very long polyunsaturated fatty acids such as docosahexanoic acid (DHA) has been reported (Moser *et al.*, 1999; Ruiz *et al.*, 1996). The levels of these polyunsaturated fatty acids can be restored by providing dietary supplements of fish oil, safflower oil or English walnut oil.

Although Lorenzo's oil was found to have led to complete normalization of plasma levels of VLCFA and showed promise as an effective therapy, there is little or no evidence that it improves or delays the progression of ALD or AMN.

3.2 Erucic acid

Erucic acid is a monounsaturated omega-9 fatty acid, denoted C22:1 ω-9. It is also known as *cis*-13-docosenoic acid and the trans-isomer is known as Brassidic acid (Fig 3.3; Table 3.1). The majority of exposure to erucic acid comes from canola oil. It is present in rape seed, wallflower seeds and mustard seeds and contributes 40 to 50% of their oils.

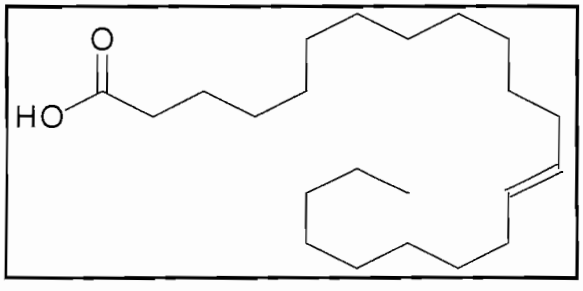

Figure 3.3: The chemical structure of erucic acid.

Table 3.1: The physical and chemical properties of erucic acid.

Physical and chemical properties	
Molecular formula	$C_{22}H_{42}O_2$
Molar mass	338.568
Physical state	Liquid > 35 °C
Odour and appearance	Pale yellow liquid with slight characteristic odour.
Specific gravity	0.8532 at 70 °C
Boiling point	281 °C 30 mm
Melting point	30 - 33 °C
Solubility in water	Insoluble. (soluble in ethanol and methanol)

3.2.1 Uses of erucic acid

It has many uses as mineral oils. It has unique chemical properties that chemists can use to make useful products. Its high tolerance to temperature makes it suitable for transmission oil. Its ability to polymerize and dry means it can be used as a binder in oil paints. High erucic oils, as exemplified by Crambe oil, can be employed as lubricants in continuous steel casting, in formulated lubricants and in the manufacture of rubber additives (Nieschlag and Wolff., 1971).

Erucic acid has also been used in the treatment of ALD in combination with oleic acid, known as Lorenzo's oil (LO). Erucic acid is the active component in LO.

3.2.2 Mechanism of erucic acid

It is a potent inhibitor of the saturated VLCFA, by competing with the elongation system. This inhibition also leads to the inhibition of polyunsaturated fatty acids that are derived from the same elongation system (Fig 3.4).

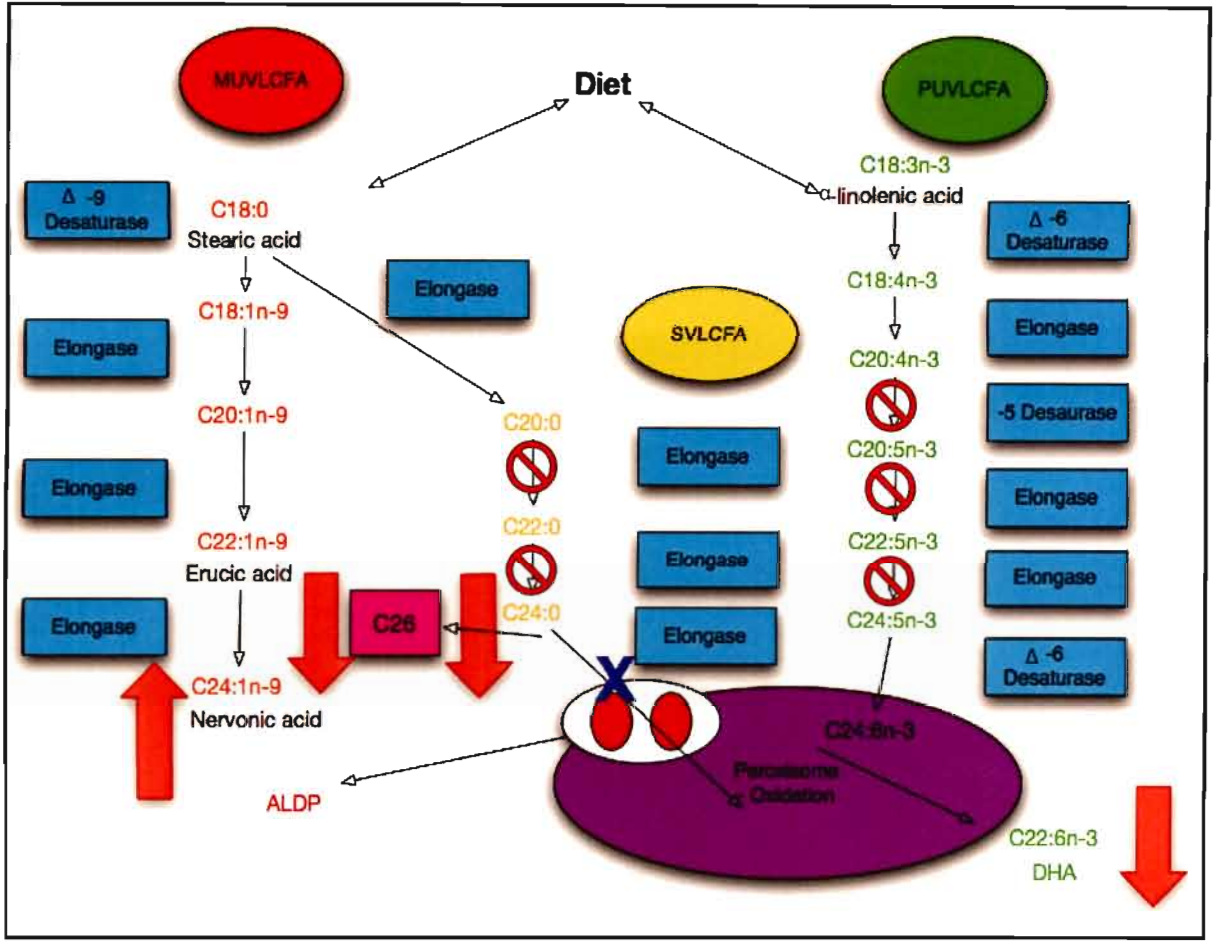

Figure 3.4: A schematic illustration showing the possible effect of erucic acid on the fatty acid elongation systems.

In X-ALD patients the levels of C24:0 is elevated because it's not taken up effectively into the peroxisomes, with a subsequent elongation to C26:0. When administering erucic acid, the inhibiting effect of erucic acid leads to a decrease in C24:0 and thus a decrease in C26:0 levels. This reduction leads to normalization of fatty acid levels that are responsible for the symptoms in X-ALD. Treatment with erucic acid also leads to increased levels of nervonic acid. Because erucic acid competes with the other elongation systems, at the PUVLCFA the levels of docosahexanoic acid (DHA) is also decreased. The levels of DHA can be restored to normal by providing dietary supplements. The specific mechanism of erucic acid is not yet fully understood and nowhere in the literature do they specify where erucic acid competes with the other elongation systems. (Fig 3.4)

3.2.3 Uptake of erucic acid into the brain

The accumulation of VLCFAs in brain lipids is associated with myelin instability and the inability of brain macrophages to remove the excess of VLCFAs. The accumulation of VLCFAs in lipids of the central white matter initiates a severe inflammatory reaction in the cerebral variants of X-ALD, resulting in demyelination (Powers *et al.,* 1992). Therefore, it was

reasoned that demyelination might be slowed down or halted if oleic and erucic acid reached the brain. Indeed, in 1989 Rizzo and co-workers al reported increased concentrations of erucic acid in the brain lipids of a patient with CCER treated with Lorenzo's oil in comparison with an untreated patient (Rizzo *et al.,* 1989), but recently researchers were unable to detect increased concentrations of erucic acid in the brain tissue of three patients with CCER and two with AMN (Rasmussen *et al.*, 1994; Poulos *et al.*, 1994). This raises the question whether erucic acid were able to cross the blood-brain barrier.

Recently, Golovko and Murphy examined the ability of erucic acid to cross the blood-brain barrier (BBB) in Sprague - Dawley rats. They demonstrated for the first time that erucic acid was taken up by the brain and esterified into brain lipid compartments. In addition, once in the brain, significant amounts of erucic acid were found to be chain-shortened to C20:1 and C18:1. They observed no elongation of erucic acid to nervonic acid (C24:1) indicating that in normal rats this process is limited. Because LO is thought to compete with the endoplasmic reticulum-localized elongases, the lack of nervonic acid in the rat brain suggested that the brain is ill-equipped to elongate this fatty acid. However, treatment of patients with LO appears to enhance the elongation of erucic acid, indicating an apparent shift in the ability of fibroblasts from these patients to elongate fatty acids (Kemp *et al.*, 2005). In conclusion they showed that erucic acid crosses the BBB in rats, and although it was significantly esterified into brain lipid pools, it was either found as C22:1 or its chain-shortened metabolites C20:1 and C18:1 (Golovko and Murphy., 2006).

3.2.4 Adverse effects

There are not many studies done on humans using erucic acid. The majority of the studies were carried out on animals. All of the available animal studies rely on short term or sub–chronic oral exposure to oils containing various proportions of erucic acid. The most common effect associated with short–term, and to a lesser extent, sub–chronic exposure to these oils is myocardial lipidosis.

Roine *et al.,* 1960 were the first to report the toxic effects of rapeseed oil. Rats were fed rapeseed oil up to 70% of the calorie content of their diet and were reported to developed myocarditis (Roine *et al.,* 1960).

In a study done by Kramer and co-workers, Sprague–Dawley rats (10 rats/group) were fed diets containing 2.5 to 9 % erucic acid for one week (calculated to be 400 and 1500 mg/kg bw/day). Dosages of 1500 mg/kg and above produced significantly increased myocardial lipidosis compared to rats fed 400 mg/kg erucic acid containing oil. An accumulation of erucic acid in heart lipids was also noted (Kramer *et al.*, 1992).

Groups of newborn Yorkshire piglets were fed milk replacer diets, containing erucic acid. The occurrence of myocardial lipidosis in the newborn piglets was attributed to the apparent low capacity of the fetus to oxidise fatty acids. Based on these studies it was concluded that it was not advisable to give un-weaned babies food containing erucic acid, because human infants that have not yet been weaned have a short supply of long-chain acyl-coenzyme A dehydrogenase (produced in the liver) to metabolize erucic acid (Kramer *et al.*, 1990; Farnworth *et al.*, 1994).

Lorenzo's oil, containing erucic acid, is not without side effects. Several studies showed reduced platelet counts (thromboytopaenia), increased liver enzymes, gastrointestinal complaints and gingivitis (Zinkham *et al.*, 1993; Van Geel *et al.*, 1999) in adrenoleukodystrophy patients using LO. In a clinical trial for the management of adrenoleukodystrophy, Kickler and co-workers analyzed the effect of Lorenzo's oil on platelet number, fatty acid composition, and function. They found that the platelet counts showed a strong inverse relationship with erucic acid levels and other omega 9 fatty acids that formed from the administration of erucic acid (Kickler *et al.*, 1996).

In the absence of adequate human data, the no observed effect level (NOEL), established for animals was extrapolated to humans in order to establish a tolerable level of human exposure. An uncertainty factor of 100 (10 for extrapolation to humans, 10 for variation within humans) was considered and therefore, a tolerable level for human exposure was found to be 7.5 mg erucic acid/kg bw/day (approximately 500 mg erucic acid/day for the average adult) (Food Standards Australia New Zealand).

The long-term effects, if any, of erucic acid is not known. Data on the effects of erucic acid on the human myocardium are lacking, and thus far an association between the consumption of erucic acid and increased myocardial lipidosis has not been established for humans.

3.3 Conclusion

While early diagnosis and application of current therapies has improved the prognosis of patients with X-ALD, there is a need to gain a better understanding of the pathogenesis of X-ALD and to develop new and more effective therapies. Lorenzo's oil reduces very long chain fatty acid levels but improvement of clinical manifestations is seldom observed.

Several studies have shown a reduction in docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) after treatment with Lorenzo's oil (Poulos *et al.*, 1994; Moser *et al.*, 2007; Aubourg *et al.*, 1993). Is it possible that erucic acid also inhibits elongation in the PUFA pathway, leading to decreased levels of DHA and thus no clinical improvement?

Regarding the adverse effects of erucic acid in rats, the human health concern and the restricted knowledge, it is important to establish the optimal dose of erucic acid with the least toxic effects.

Regarding the current knowledge discussed in chapter 2 and 3, it is very important that effective therapies for X-ALD needs to be developed.

3.4 Aim of study

The aim of this pilot study was to determine the effect of different dosages of erucic acid on the saturated VLCFA in the plasma of Sprague-Dawley rats. In addition we investigated the effect of erucic acid on the polyunsaturated fatty acid biosynthesis pathway in the plasma and brain phospholpids of Sprague-Dawley rats. Spraque-Dawley rats were used because of its availability at the Animal Research Centre.

Chapter 4 describes the experimental procedures employed to determine the VLCFAs and PUFAs. Chapter 5 contains the results obtained together with short discussions on the results. In chapter 6, conclusions are drawn from all the results obtained and suggestions for future studies.

Experimental Procedures

Chapter 4

4 Introduction

The development and undertaking of this study and the use of experimental animals was approved by the Ethics Committee for Use of Experimental Animals at the North-West University, Potchefstroom Campus (approval number – 07D02).

This study was a pilot study, designed to investigate the feasibility of the study prior to a larger study and to test logistics and gather information to improve further research.

This pilot study was conducted with the objectives to:

- Determine the inhibitory effect of different dosages of erucic acid on saturated very long chain fatty acid concentration and ratios.
- Determine the effect of erucic acid on polyunsaturated very long chain fatty acids biosynthesis and incorporation into phospholipids of the plasma and brain.

4.1 Experimental animals

Sixty Spraque-Dawley rats were provided by the Animal Research Centre, Potchefstroom Campus of the North-West University. Their weights varied from 200 – 240 g, on the first day, to 190 – 210 g at the end of the study.

The conditions at the Animal Research Centre were kept constant, with a temperature of 25 °C and humidity (55±5%) and a 12h light - dark cycle. Each rat was kept in individual metabolic cages with free access to standard laboratory food and water.

Spraque-Dawley rats were divided into 6 groups (10 rats per group) based on their weight. The experimental procedure was divided into two phases due to the availability of 30 metabolic cages. Phase 1 was conducted to establish the inhibitory effect of the different dosages of erucic acid on the SVLCFA levels, and to determine the next dosage intervals of erucic acid given in the secondary phase.

Phase 1:

Group number	Dosage	Rats per group
Group 1	Control	10
Group 2	600 mg/kg	10
Group 3	800 mg/kg	10

Phase 2:

Group number	Dosage	Rats per group
Group 4	400 mg/kg	10
Group 5	575 mg/kg	10
Group 6	625 mg/kg	10

4.2 Preparation of erucic acid

The control group received the vehicle dimethylsulphoxide (DMSO). Different dosages of erucic acid (400, 575, 600, 625 and 800 mg/kg) were dissolved in DMSO. According to Guo and co-workers DMSO has minor effects on fatty acid synthesis. They tested the inhibition of cerulenin on the endogenous fatty acid synthesis activities of oral squamous cell carcinoma and normal oral mucosa. The collected tissues were divided into three groups: cerulenin treated, DMSO treated and a control group. They concluded that the fatty acid synthetic activities remained unchanged in the DMSO group (Guo *et al.*, 2003).

The quantity of erucic acid/dosage given per group was calculated according to the average weight per group.

Example:

Group 4

- Dosage of 400 mg
- Average weight of rats for group 1: 238.02 g

400 mg erucic acid / 1000 mg

95 mg erucic acid / 238.02 g average weight rat

= 95 mg erucic acid in 0.5 ml DMSO

Table 4.1: The average weight per group and the dosage of erucic acid given to each group.

Group	Average weight	Erucic acid / 0.5 ml DMSO
Group 1	240.53 g	DMSO
Group 2	226.61 g	136 mg
Group 3	207.56 g	166 mg
Group 4	238.02 g	95 mg
Group 5	215.32 g	124 mg
Group 6	226.43 g	142 mg

See Appendix A for the weight of each rat and the average weight before and after the experimental procedures.

Each rat was given 0.5 ml erucic acid by gavage daily for 7 days and urine samples were collected daily.

4.3 Decapitation and storage

On day 8 the rats were weighed again and recorded. The rats were decapitated and trunk blood was collected. The blood was centrifuged at 4 °C for 10 minutes at 2000 revolutions/minute (RPM). The supernatant (plasma) was carefully removed and placed in a 1 ml eppendorf tube and stored at -20 °C until analysis. The brains were also removed and stored at -20 °C until analysis.

The inhibitory effect of different dosages of erucic acid on plasma SVLCFA was determined using a modified method from Vreken and co-workers (1998) employing gas chromatography mass spectrometry (GC-MS). The effect of erucic acid on plasma and brain PUFA biosynthesis was determined using a GC-MS modified method from Takemoto and colleagues (2003) and Blau and co-workers (2008) and separating the phospholipids using thin-layer chromatography (Smuts and Tichelaar.,1991).

4.4 Determination of saturated VLCFAs in total plasma lipids

4.4.1 Introduction

X-ALD in characterized by the accumulation of VLCFA in tissue and biochemical fluids. The major accumulated fatty acids is hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0). The diagnosis of X-ALD is based on the analyses of plasma VLCFA levels of C26:0 and C24:0 and their ratios to C22:0. The method used for the determination of saturated very long chain fatty acids was a modified method of Vreken and co-workers (1998). In this method the fatty acids were derivatized into methyl esters and the VLCFA concentrations were determined using gas chromatography mass spectrometry (GC-MS) as described in section 4.2.4. In order to detect possible defective enzyme activity in the VLCFA biosynthesis pathway the concentrations were expressed as the ratios: C24:0/C22:0 and C26:0/C22:0.

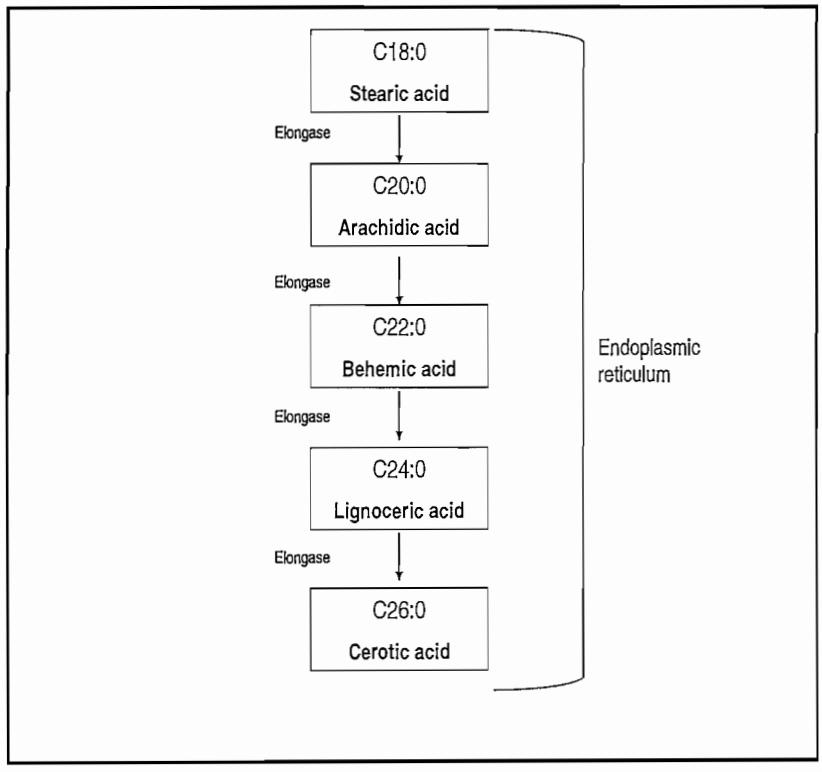

Figure 4.1: The biosynthesis pathway for saturated very long chain fatty acids through a series of elongase. C24:0 and C26:0 are transported across the peroxisomal membrane into the peroxisomes to undergo β-oxidation.

4.4.2 Materials

Table 4.2: Table of chemicals used, and their suppliers

Chemicals and substances	Suppliers
Acetonitrile	Merck, USA
Hexane	Merck, USA
Hydrochloric acid	Merck, USA
Methanol	Merck, USA
MTBSTFA	Sigma Aldrich, USA
Potassium hydroxide	Merck, USA
Pyridine	Merck, USA
Sodium Hydroxide	Merck, USA

Table 4.3: Table of standards used for GC-MS, and their suppliers

Deuterium labeled standards	Suppliers
(2-methyl-$^{2}H_{3}$,6,10,14-trimethyl) pentadecanoic acid (pristanic acid-d3)	H. ten Brink®
(3-methyl-$^{2}H_{3}$,7,11,15-trimethyl) hexadecanoic acid (phytanic acid-d3)	H. ten Brink®
(3,3,5,5, $^{2}H_{4}$) - docosanoic acid (C22:0-d4)	H. ten Brink®
(3,3,5,5, $^{2}H_{4}$) - tetracosanoic acid (C24:0-d4)	H. ten Brink®
(3,3,5,5, $^{2}H_{4}$) – hexacosanoic acid (C26:0-d4)	H. ten Brink®

Internal standard

The internal standards used to quantify the VLCFA included the fatty acid standards obtained from H. ten Brink® (see table 4.3). Preparing the internal standards entailed making a solution in toluene by adding all the standards and deuterium labeled standards to comprise the following:

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

- 1 µmolL C26:0
- 5 µmol/L pristanic acid-d3
- 8 µmol/L phytanic acid-d3
- 50 µmol/L C22:0-d4
- 50 µmol/L C22:0-d4
- 5 µmol/L C26:0-d4

This mixture was stored at 2 to 8 °C.

Acid solution for hydrolysis

HCl (37 %) and acetonitrile were mixed in a 1:23 ratio. This solution must be prepared freshly every day and stored at room temperature.

Alkaline solution for hydrolysis

8 g of NaOH was mixed with 10 ml milli-Q water and made up to 200 ml with methanol. This solution is stable for one year and was stored at room temperature.

1M KOH

56 g of KOH was made up with milli-Q water to 1 L. This solution is stable for one year and was stored at room temperature.

4.4.3 Sample preparation

In clear 10 ml screw-capped glass extraction tubes 100 µl internal standard (working solution) and 2 ml acid solution for hydrolysis were added to 100 µl of plasma. The samples were vortexed for approximately 3 seconds and left for 45 minutes at 110 °C to hydrolyze, after which the samples were left to cool down to room temperature before 2 ml of alkaline solution for hydrolysis were added to the samples. Samples were vortexed again for approximately 3 seconds, left for 45 minutes at 110 °C to hydrolyze, and left to cool down to room temperature. 0.5 ml HCl (25%) and 4 ml hexane were added to the samples to lower the pH and shaken for 5 minutes. The top layer (hexane layer) was extracted with a Pasteur pipette and transferred to clean 10 ml extraction tubes. 3.5 ml KOH (1 M) was then added to the hexane layer, shaken for five minutes and then centrifuged for 2 minutes at 1500 RPM. With a Pasteur pipette connected to a water-vacuum pump the top layer was discarded. 0.6 ml HCl (25%) and 4 ml hexane were added to the bottom phase to lower the pH and extract the fatty acids. After the samples were shaken for a further 5 minutes the top layer was extracted with a Pasteur pipette and transferred to 4 ml glass tubes. The extract was then dried under a nitrogen gas flow at 50 °C. After drying, 50 µl of pyridine were added to the samples, and vortexed. 100 µl MTBSTFA was then added and the samples vortexed again. It was then incubated for 30 minutes at 80 °C. The methylated derivatives were dried under a flow of nitrogen at 50 °C. After drying, 100 µl of hexane was added, transferred to a GC-MS vial and injected into the GC-MS for analysis.

4.4.3 GC-MS analysis

One microliter of each sample was injected into a GC-MS system. The fatty acids were analysed using a Hewlett-Packard model 6890/5973 GC-MS system with a 120-0132 DB-1ms capillary column (30 m/0.25 mm/0.25 µm). The initial oven temperature was 60 °C and maintained for 1 minute. The temperature was then increased with a rate of 30 °C/min to 240 °C and then with a rate of 10 °C/min to 270 °C. The temperature was then increased with 4 °C/min to 300 °C and maintained at 300 °C for 3 minutes. Helium was used as the carrier gas. The samples were then injected with a splitless mode at 300 °C. Electron impact ionization was applied at 0.7eV. The mass spectrum was set on single ion monitoring (SIM) mode and monitored the characteristic $[M - 57]^+$ ions. Table 4.4 represents the characteristic mass ions of the VLCFAs obtained by GC-MS analyses.

Table 4.4: Single ion monitoring (SIM)

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

4.4.4 Determination of the FA concentrations

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

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

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

For determination of C22:0 and C24:0 the following formula was used:

$$[\,] = \frac{\text{Area C22:0}}{\text{Area C22:0*}} \times Rf \times 50 = \mu g/L$$

For determination of C26:0 the following formula was used:

$$[\,] = \frac{\text{Area C26:0}}{\text{Area C22:0*}} \times Rf \times 1 = \mu g/L$$

Rf = Response factor

IS = Internal standard

[ ] = Concentration of the fatty acids

4.5 Determination of mono- and polyunsaturated fatty acids in phospholipids.

4.5.1 Introduction

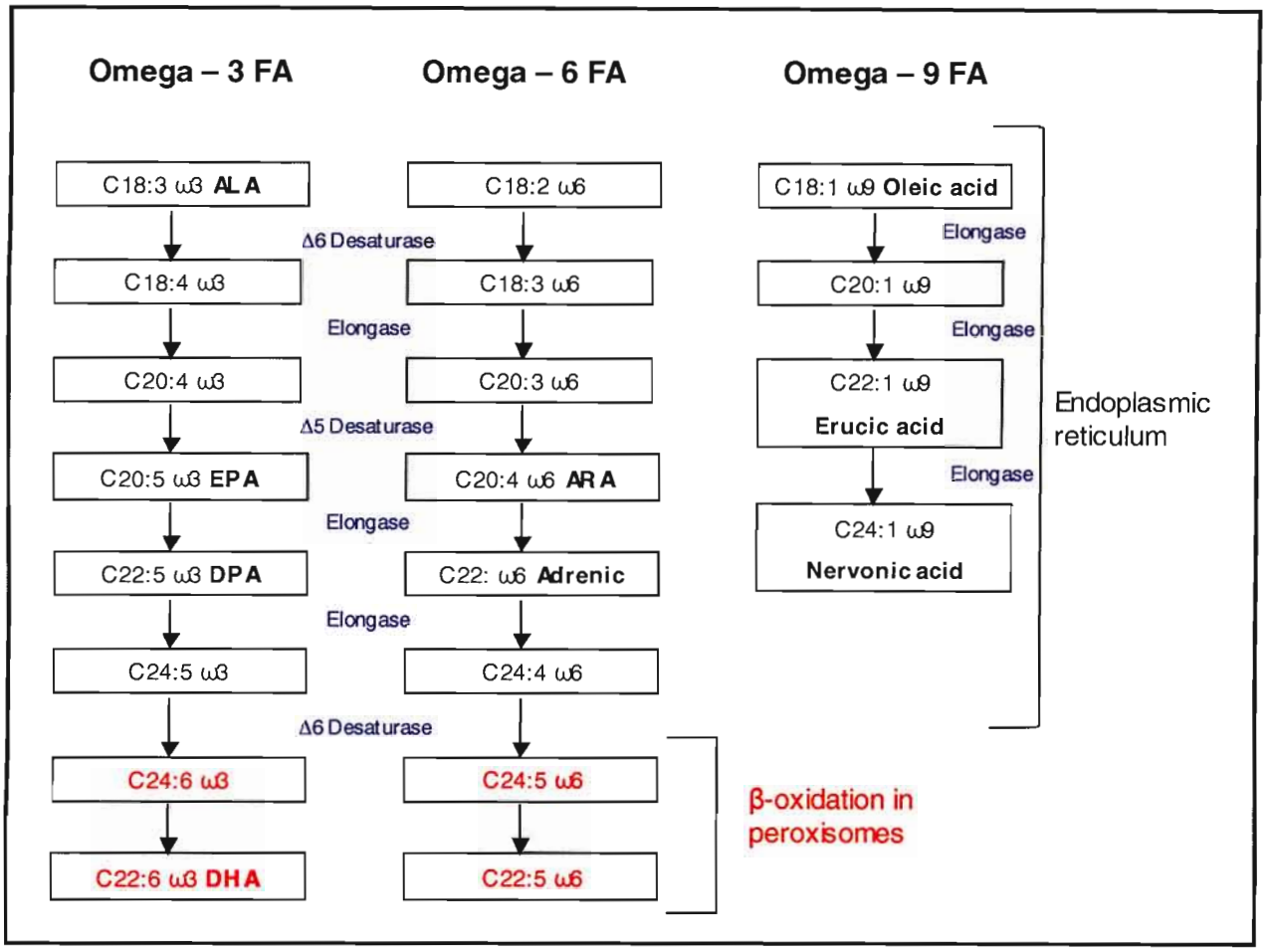

Figure 4.2: The biosynthesis pathway of polyunsaturated fatty acids. The Omega-3 and Omega-6 fatty acids (FA) are elongated and desaturated through a series of the same enzymes. C24:6 ω3 and C24:5 ω6 are transported across the peroxisomal membrane into the peroxisomes to undergo β-oxidation. C24:6 ω3 is the precursor for the production of DHA. The Omega-9 FA is elongated through a series of elongase to form erucic acid and finally nervonic acid.

Polyunsaturated fatty acids (PUFA) are derived from the same elongation system as the saturated very long chain fatty acids and it plays an integral role in numerous physiological processes. The method used for the determination of mono-and polyunsaturated fatty acids was method adapted from Smuts and Tichelaar (1991). Plasma total phospholipids were separated from neutral lipids by thin layer chromatography (TLC) on heat-activated silica gel-60 plates. The system used a diethyl ether/ petroleum ether/ acetic acid (30:90:1) solvent

system. The individual fatty acid methyl esters were determined by GC-MS. The fatty acids were expressed as a percentage of the total lipid composition.

4.5.2 Materials

Table 4.5: Table of the chemicals used, and their suppliers.

Chemicals and substances	Suppliers
Myristyc acid (C14:0)	Laroden chemicals, USA
Palmitic acid (C16:0)	Laroden chemicals, USA
Heptadecaenoic acid (C17:0) internal standard	Laroden chemicals, USA
Palmitoleate (C16:1n7)	Laroden chemicals, USA
Stearic acid (C18:0)	Laroden chemicals, USA
Vaccenic acid (C18:1n7)	Laroden chemicals, USA
Oleic acid (C18:1n9)	Laroden chemicals, USA
Linoleic acid (C18:2n6)	Laroden chemicals, USA
Alpha - Linoleic acid (C18:3n3)	Laroden chemicals, USA
Gamma - Linoleic acid (C18 3n6)	Laroden chemicals, USA
Stearidonic acid (C18:4n3)	Sigma Alderich, USA
Arachidic acid (C20:0)	Laroden chemicals, USA
Eicosenoic acid (C20:1n9)	Laroden chemicals, USA
11,14,eicosadienoic acid (C20:2n6)	Laroden chemicals, USA
11,14,17 eicosatrienoic acid (C20:3n3)	Laroden chemicals, USA
Eicosatrienoic acid (C20:3n9)	Sigma Alderich, USA
Arachidonic acid (C20:4n6)	Sigma Alderich, USA
Ecosapentaenoic acid (C20:5n3)	Sigma Alderich, USA
Behenic acid (C22:0)	Laroden chemicals, USA
Erucic acid (C22:1n9)	Laroden chemicals, USA
13,16,19 docosatrienoic acid (C22:3n3)	Laroden chemicals, USA
8,11,14 eicosatrienoic acid (C20:3n6)	Laroden chemicals, USA
Adrenic acid (C22:4n6)	Laroden chemicals, USA
Docosapentaenoic acid (C22:5n3)	Laroden chemicals, USA
Docosapentaenoic acid (C22:5n6)	Sigma Alderich, USA
Docosahexaenoic acid (C22:6n3)	Laroden chemicals, USA
Lignoceric acid (C24:0)	Laroden chemicals, USA
Nervonic acid (C24:1n9)	Laroden chemicals, USA
Cerotic acid (C26:0)	Sigma Alderich, USA

Materials and preparation of stock solutions

Methanol with 0.01% butylated hydroxytoluene (BHT)

100 mg of BHT was dissolved in 1 L of methanol (m/v). This solution is stable for one year and was stored at room temperature.

Chloroform with 0.01% butylated hydroxytoluene (BHT)

100 mg of BHT was dissolved in 1 L of chloroform (m/v). This solution is stable for one year and was stored at room temperature.

Chloroform: Methanol: Saline solution (CMS)

Chloroform, methanol and saline are mixed together in a ratio of 86:14:1 v/v/v. This solution is stable for one year and was stored at room temperature.

Saline saturated with CMS

9 g NaCl was dissolved in 1 L ddH_2O (m/v) to get a 0.9% normal saline solution. This was then saturated by mixing 100 ml CMS with 400 ml saline. This solution is stable for one year and was stored at room temperature.

Preparation of the mobile phase

Diethyl ether, petroleum ether and acetic acid were mixed in a ratio of 30:90:1 v/v/v. This solvent was prepared freshly every day and stored at room temperature.

Preparation of the trans-methylating solution

Methanol and sulphuric acid was mixed in a 95:5 ratio v/v. This solution will remain stable for one year and was stored at room temperature.

Preparation of the internal standard

1 ml of Heptadecaenoic acid (C17:0) was dissolved in 10 ml of methanol. This solution was stored in an amber coloured volumetric flask and freshly every week. This solution was stored at 2 – 8 °C.

4.5.3 Sample preparation - plasma

This is a standard procedure done by the Centre of Excellence for Nutrition at the North-West University.

In clear 10 ml screw-cap extraction tubes 3 ml methanol with 0.01% BHT was pipetted. 300 µl of serum was added to the methanol by means of a Gilson pipette and vortexed. 6 ml of chloroform with BHT were then added to the samples, which were tightly capped and vortexed well, then 1.8 ml saline saturated with CMS was added to each sample. This was vortexed again and centrifuged at 1000 RPM for 10 minutes. The top layer (water phase) was discarded with a Pasteur pipette, the middle layer perforated with a clean Pasteur pipette and the bottom phase was then transferred into a clean 10 ml extraction tube.

The extract was dried in a driblock under a nitrogen gas flow at 45 °C. The samples were concentrated twice by rinsing the inside of the extraction tube with 1 ml CMS. A filter paper lined separation tank was pre-equilibrated with the mobile phase for at least 10 minutes before use. The dried sample was dissolved in 50 µl of CMS. 30 µl of the sample was then spotted on the TLC plate (silica gel-60 plates; Merck - Germany) with a Hamilton pipette. The spotted plate was then placed in the separation tank for 15 minutes to separate the total phospholipids from the neutral lipids and then dried under a stream of nitrogen for 20 minutes. The total phospholipid fraction was marked and scraped of the plate. A funnel was used to guide the powder into clean extraction tubes. 2 ml Trans-methylating solution and 300 µl of internal standard were added to the samples. This was then tightly closed and incubated for 2 hours at 70 °C. The methyl esters were then cooled down to room temperature before 2 ml hexane and 1 ml distilled water was added. The samples were vortexed for 15-30 seconds and left until the phases has separated. The hexane phase (upper phase) was transferred to 4 ml extraction tubes with a Pasteur pipette and dried in a driblock under a nitrogen gas flow at 45 °C. After evaporation 55 µl of hexane was added and mixed well. 50 µl of the sample was then transferred into a GC-MS vial and injected into a GC-MS for analysis.

4.5.4 GC-MS analysis

One microliter of each sample was injected into a GC-MS system. The fatty acids were analysed using a Hewlett-Packard model 6890/5973 GC-MS system equipped with a 122-2361 DB-23 capillary column (60 m/0.25 mm/0.15 µm). The initial oven temperature was 160 °C and maintained for 5 minutes. The temperature was then increased to 220 °C at a rate of 3 °C/min and maintained at 220 °C for 40 minutes. Helium (1 ml/min) was used as the carrier

gas. The samples were then injected with a split mode with a split ratio of 1:5 at 220 °C. Electron impact ionization was applied at 0.7 eV.

4.5.5 Sample preparation - brain

This was a standard procedure done by the Centre of Excellence for Nutrition at the North-West University.

The left hemisphere of the brain was homogenized with a Dounce tissue homogenizer and ±100 mg of brain matter was pre-weight in a clean screw-capped extraction tube. The absolute wet weight was recorded. 300 µl of saline saturated with CMS were added to the brain samples and vortexed for 30 seconds. 3 ml methanol with 0.01% BHT was added and vortexed. 6 ml of chloroform with 0.01% BHT were then added to the samples, which were tightly capped and vortexed well. 1.8 ml saline saturated with CMS was added to each sample. This was vortexed again and centrifuged at 1000 RPM for 10 minutes. The top layer (water phase) was discarded with a Pasteur pipette, the middle layer perforated with a clean Pasteur pipette and the bottom phase was then transferred into a clean 10 ml extraction tube.

The extract was dried in a driblock under a nitrogen gas flow at 45 °C. The samples were concentrated twice by rinsing the inside of the extraction tube with 1 ml CMS. A filter paper lined separation tank was pre-equilibrate with the mobile phase for at least 10 minutes before use. The dried sample was dissolved in 100 µl of CMS. 20 µl of the sample was then spotted on the TLC plate (silica gel-60 plates) with a Hamilton pipette. The spotted plate was then placed in the separation tank for 15 minutes to separate the total phospholipids from the neutral lipids and then dried under a stream of nitrogen for 20 minutes The total phospholipid fraction was marked and scraped of the plate. A funnel was used to guide the powder into clean extraction tubes. 2 ml Trans-methylating solution and 400 µl of internal standard were added to the samples. This was tightly closed and incubated for 2 hours at 70 °C. The methyl esters were cooled down to room temperature before 2 ml hexane and 1 ml distilled water was added. The samples were vortexed for 15 - 30 seconds and left until the phases had separated. The hexane phase (upper phase) was transferred into 4 ml extraction tubes with a Pasteur pipette and dried in a driblock under a nitrogen gas flow at 45 °C. After evaporation 55 µl of hexane was added and mixed well. 50 µl of the sample was then transferred into a GC-MS vial and injected into a GC-MS for analysis.

4.5.6 GC-MS analysis

One microliter of each sample was injected into a GC-MS system. The fatty acids were analysed using a Hewlett-Packard model 6890/5973 GC-MS system equipped with a 122-2361 DB-23 capillary column (60 m/0.25 mm/0.15 µm). The initial oven temperature was 160 °C and maintained for 5 minutes. The temperature was then increased to 220 °C at a rate of 3 °C/min and maintained at 220 °C for 40 minutes. Helium (1 ml/min) was used as the carrier gas. The samples were then injected with a split mode with a split ratio of 1:5 at 220 °C. Electron impact ionization was applied at 0.7 eV.

4.5.7 Determination of the FA concentrations

The samples were quantified with Chemstation Enhanced Data analysis (D.01.XX) using unique single ions and calibration curves for each FAME. For the identification of these fatty acids the single standards obtained from Larodan Fine Chemicals were used. Fatty acids were given as a percentage of the lipid composition.

4.6 Statistics

Statistical analysis was carried out at the Department of Statistical Services at the North-West University (Potchefstroom Campus) using Statistica® software (STATSOFT, 2005).

Results and Discussion

Chapter 5

5 Introduction

This pilot study had two main aims:

- To determine the optimal dosage of erucic acid with the best inhibitory effect on SVLCFA levels and biosynthesis.
- To determine if erucic acid has any effect on PUFA biosynthesis and incorporation.

Group 1 was the control group of rats which received the vehicle, DMSO, while groups 2 - 6 received different dosages of erucic acid dissolved in DMSO. The rats were treated for 7 days. On the 8th day the rats were decapitated and brain and blood samples were collected to determine the inhibitory effect of erucic acid on the fatty acid composition.

The saturated very long chain fatty acid (SVLCFA) analysis was performed on all six groups. The mono- and polyunsaturated fatty acid analysis were performed on the optimal dosage (600 mg/kg) determined by the previously performed saturated SVLCFA analysis. For this analysis of mono- and polyunsaturated fatty acids the control group, as well as the lowest dosage (400 mg/kg) group and the highest dosage (800 mg/kg) group were included.

5.1 Saturated very long chain fatty acids in total plasma lipids

In this analysis, the focal point was to establish the effect of different dosages of erucic acid on SVLCFA concentrations and ratios in the plasma, and to determine the optimal dosage of erucic acid with the best inhibitory effect on the saturated VLCFA levels. The C24:0/C22:0 and C26:0/C22:0 ratios were determined in order to detect possible defective enzyme activity in VLCFA metabolism, focusing on the peroxisomal biosynthesis and β-oxidation pathway. These results were expressed as a concentration in µmol/L.

The unequal N HSD test (which is a generalization of the Tukey's multiple comparison test) was used to compare the control, 400 mg/kg, 575 mg/kg, 600 mg/kg, 625 mg/kg and 800 mg/kg groups. A probability of $p \leq 0.05$ was employed to declare statistical difference and

each analysis was followed by multiple comparisons p-values (2-tailed) and the Kruskal-Wallis test.

See appendix B for the data of the VLCFA analysis.

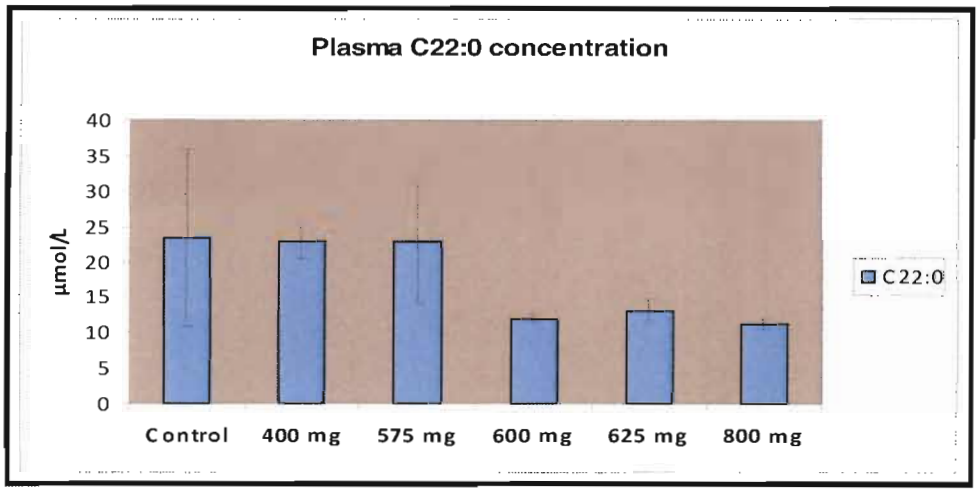

Figure 5.1: The inhibitory effect of different dosages of erucic acid on plasma C22:0 concentration.

(Control: n = 7; 400 mg: n = 10; 575 mg: n = 8; 600 mg: n = 10; 625 mg: n = 10; 800 mg: n = 8)

Figure 5.1 represents the average concentration of C22:0 obtained with different dosages of erucic acid on SVLCFA in the plasma. There was no statistical difference between the different groups and the control group.

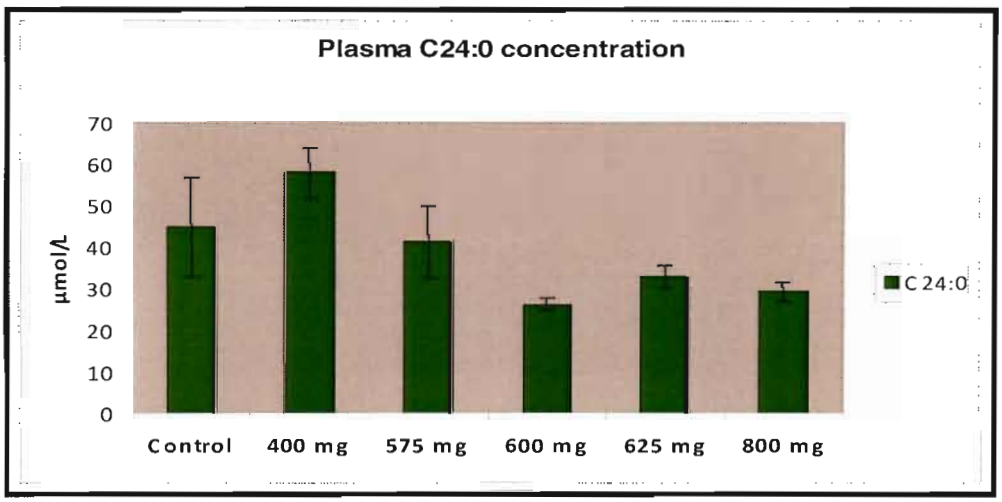

Figure 5.2: The inhibitory effect of different dosages of erucic acid on plasma C24:0 concentration.

(Control: n = 7; 400 mg: n = 10; 575 mg: n = 8; 600 mg: n = 10; 625 mg: n = 10; 800 mg: n = 8)

Figure 5.2 represents the average concentration of C24:0 obtained with different dosages of erucic acid on SVLCFA in the plasma. There was no statistical difference between the different groups and the control group.

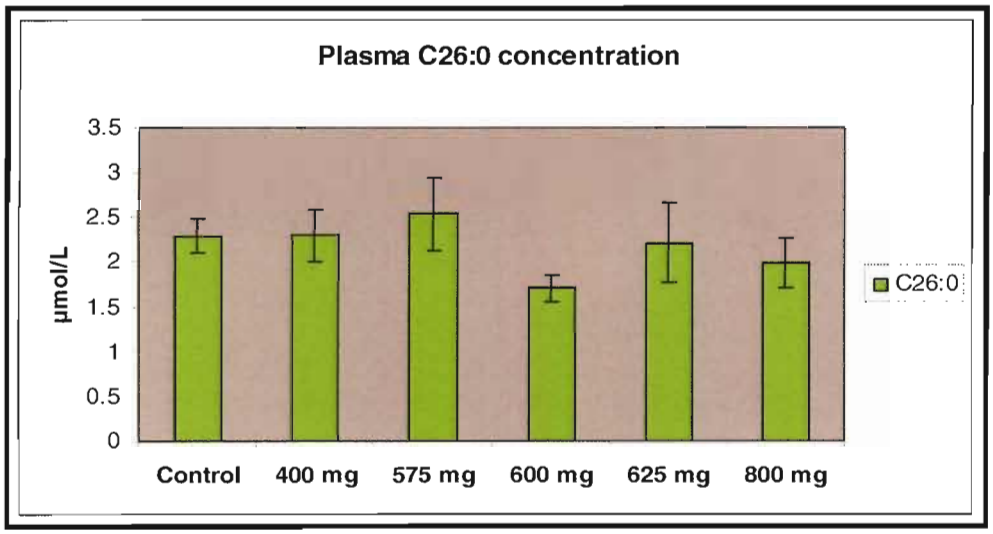

Figure 5.3: The inhibitory effect of different dosages of erucic acid on plasma C26:0 concentration.

(Control: n = 7; 400 mg: n = 10; 575 mg: n = 8; 600 mg: n = 10; 625 mg: n = 10; 800 mg: n = 8)

Figure 5.3 represents the average concentration of C26:0 obtained with different dosages of erucic acid on SVLCFA in the plasma. There was no statistical difference between the different groups and the control group.

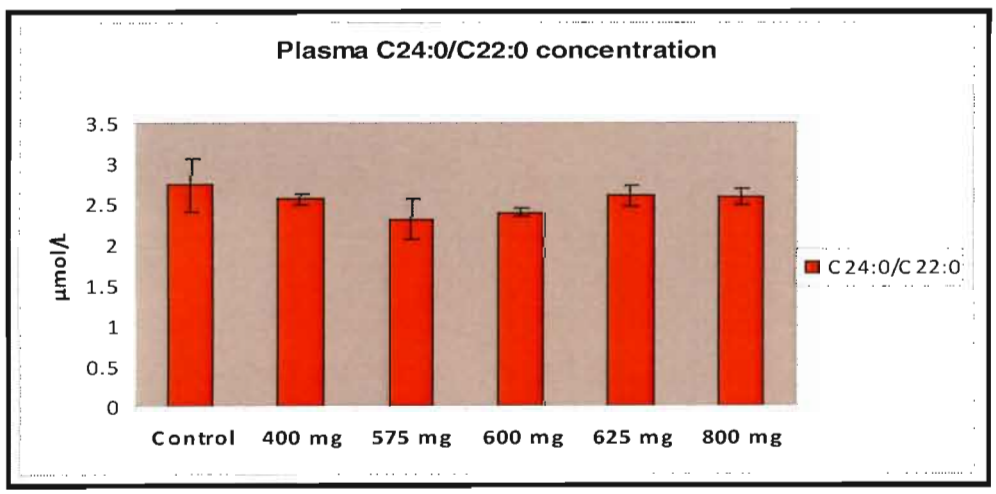

Figure 5.4: The ratios of C24:0/C22:0 of different dosages of erucic acid.

(Control: n = 7; 400 mg: n = 10; 575 mg: n = 8; 600 mg: n = 10; 625 mg: n = 10; 800 mg: n = 8)

Figure 5.4 illustrates the ratio of C24:0/C22:0 obtained with different dosages of erucic acid. There was no statistical difference between the different groups and the control group. This igure showed that the 575 mg/kg group had the best inhibitory effect on the SVLCFA levels, with a ratio of 2.31, followed by the 600 mg/kg group with a ratio of 2.39.

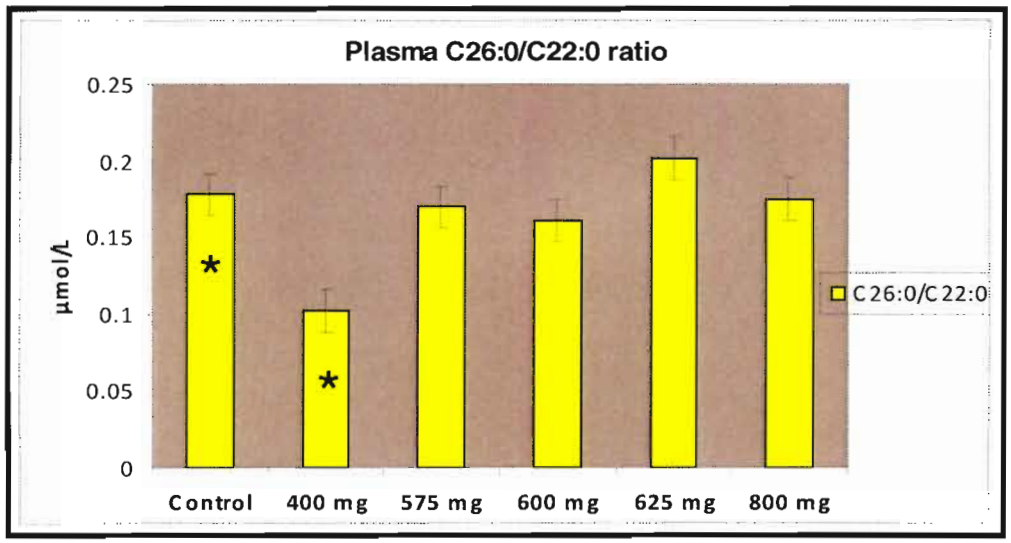

Figure 5.5: The ratio of C26:0/C22:0 of different dosages of erucic acid (*p = 0.0109).

(Control: n = 7; 400 mg: n = 10; 575 mg: n = 8; 600 mg: n = 10; 625 mg: n = 10; 800 mg: n = 8)

Figure 5.5 illustrates the ratio of C26:0/C22:0 obtained with different dosages of erucic acid. The C26:0/C22:0 ratio was significant lower in the 400 mg/kg group compared to the control group (p = 0.0109) with a ratio of 0.10, followed by the 600 mg/kg group with a ratio of 0.16.

5.1.1 Discussion

There was no statistical difference in the C22:0, C24:0, C26:0 concentrations between the different groups and the control group. Figure 5.1 – 5.3 revealed a notable decrease in the levels of C22:0, C24:0 and C26:0 in the 600 mg/kg group, although not statistically significant.

The C24:0/C22:0 ratio of the 575 mg/kg and the 600 mg/kg groups appeared to be lower compared to the other groups, although not statistically significant. The C26:0/C22:0 ratio in the 400mg/kg group was significantly lower than in the control group. The C26:0/C22:0 ratio also appeared lower in the 600 mg/kg group compared to the control group, although it was not statistically significant.

This analysis showed no statistically significant differences between the different groups, but the results did indicate that the 600 mg/kg erucic acid dosage had the best overall inhibitory effect on the SVLCFA levels.

5.2 Mono- and polyunsaturated fatty acids in plasma and brain phospholipids

In this analysis we determined the effect of different dosages of erucic acid on the biosynthesis and incorporation of the mono- and polyunsaturated fatty acids in the phospholipids of the plasma and the brain. The concentrations were expressed as a percentage of the total fatty acid (TFA) composition. An analysis was performed using the unequal N HSD (honest significant difference) test, which is a generalization of Tukey's (HSD) test used in the case of unequal sample sizes. A probability of $p \leq 0.05$ was employed to declare statistically significant differences. The test was followed by multiple comparisons p-values (2 – tailed) and the Kruskal-Wallis test. Saturated VLCFA analysis done in section 5.2 showed an overall inhibition of C24:0 and C26:0 in the 600mg/kg group. Included in the mono-and polyunsaturated fatty acid analysis was the 600 mg/kg group as well as the control group and the 400 mg/kg and 800 mg/kg groups. This was done because of cost effectiveness. Saturated VLCFAs were also measured with this method.

See Appendix C for the data on the mono- and polyunsaturated fatty acid analysis.

5.2.1 Saturated VLCFAs – plasma phospholipids

The data of the SVLCFA were analyzed to determine if there were any statistical differences in the percentages of the various erucic acid dosages.

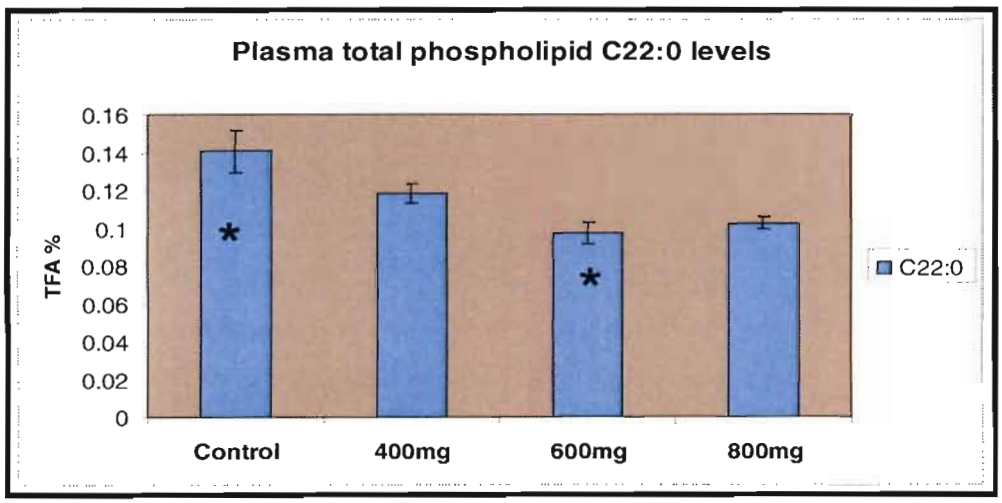

Figure 5.6: The inhibitory effect of different dosages of erucic acid on C22:0 plasma levels. TFA = Total phospholipid fatty acid (* $p = 0.0142$). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.6 illustrates the inhibitory effect of different dosages of erucic acid on plasma C22:0 levels. The C22:0 was significanty decreased in the 600 mg/kg group compared to the control group (p = 0.0142).

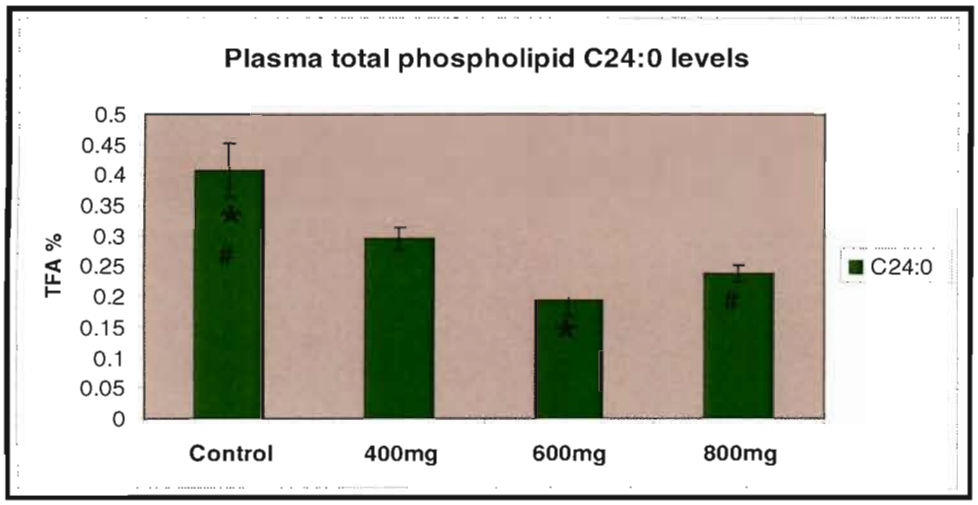

Figure 5.7: The inhibitory effect of different dosages of erucic acid on C24:0 plasma levels. TFA = Total phospholipid fatty acid (*p = 0.0009; # p = 0.0386). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.7 illustrates the inhibitory effect of different dosages of erucic acid on the C24:0 plasma levels. The C24:0 was significant decreased in the 600 mg/kg group compared to the control group (p = 0.0009), as well as in the 800 mg/kg group compared to the control group (p = 0.0386).

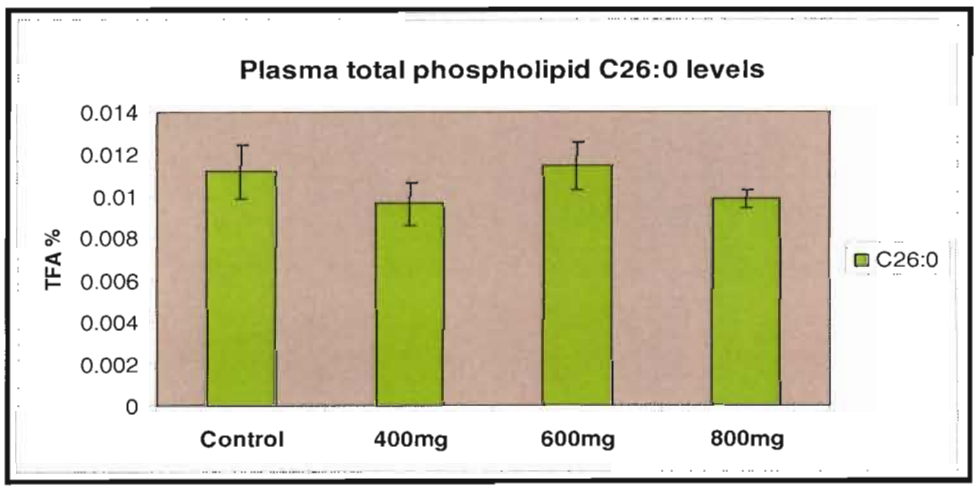

Figure 5.8: The inhibitory effect of different dosages of erucic acid on C26:0 plasma levels. TFA = Total phospholipid fatty acid. (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.8 illustrates the inhibitory effect of different dosages of erucic acid on the C26:0 plasma levels. There was no significant difference in the C26:0 levels between the experimental erucic acid groups and the control group.

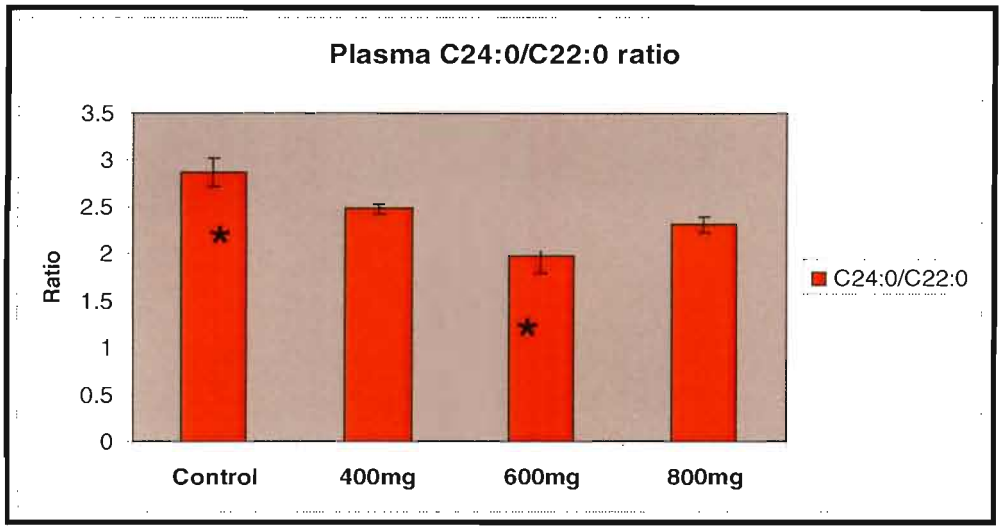

Figure 5.9: The ratios of C24:0/C22:0 of different dosages of erucic acid. TFA = Total phospholipid fatty acid (* p = 0.0009). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.9 illustrates the ratio of C24:0/C22:0 obtained by different dosages of erucic acid. The C24:0/C22:0 ratio was significant lower in the 600 mg/kg compared to the control group (p = 0.0009).

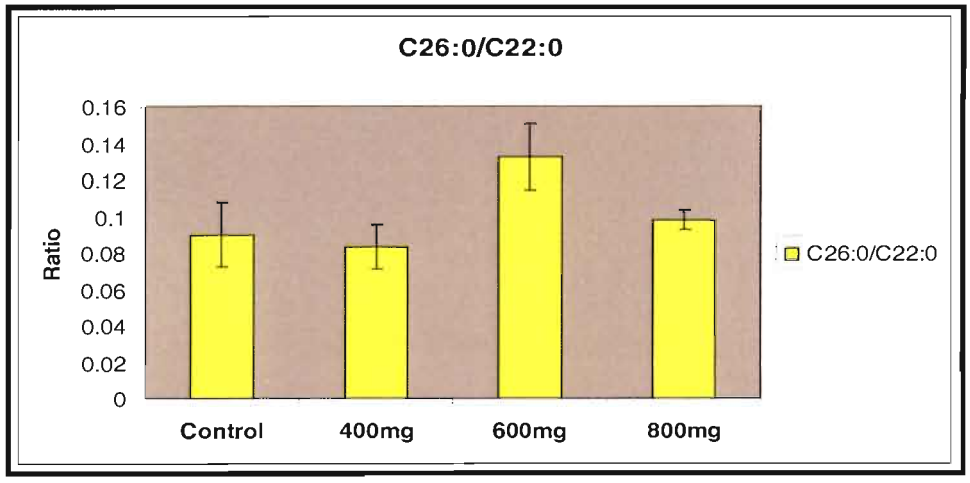

Figure 5.10: The ratio of C26:0/C22:0 of different dosages of erucic acid. TFA = Total phospholipid fatty acid. (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.10 illustrates the ratio of C26:0/C22:0 with different dosages of erucic acid. No statistical differences were found in the ratios of the experimental erucic acid groups when compared to the control group. The 400 mg/kg group had the best inhibitory effect on the SVLCFA levels with a ratio of 0.008.

5.2.1.1 Discussion

The data illustrated that the 600 mg/kg erucic acid had a statistically significant higher inhibitory effect on the C22:0 and C24:0 levels and on the C24:0/C22:0 ratio compared to the other groups. It therefore seems that the 600 mg/kg erucic acid is the optimal dosage with the best inhibitory effect on the plasma SVLCFA levels.

5.2.2 Omega-3 fatty acids – plasma phospholipids

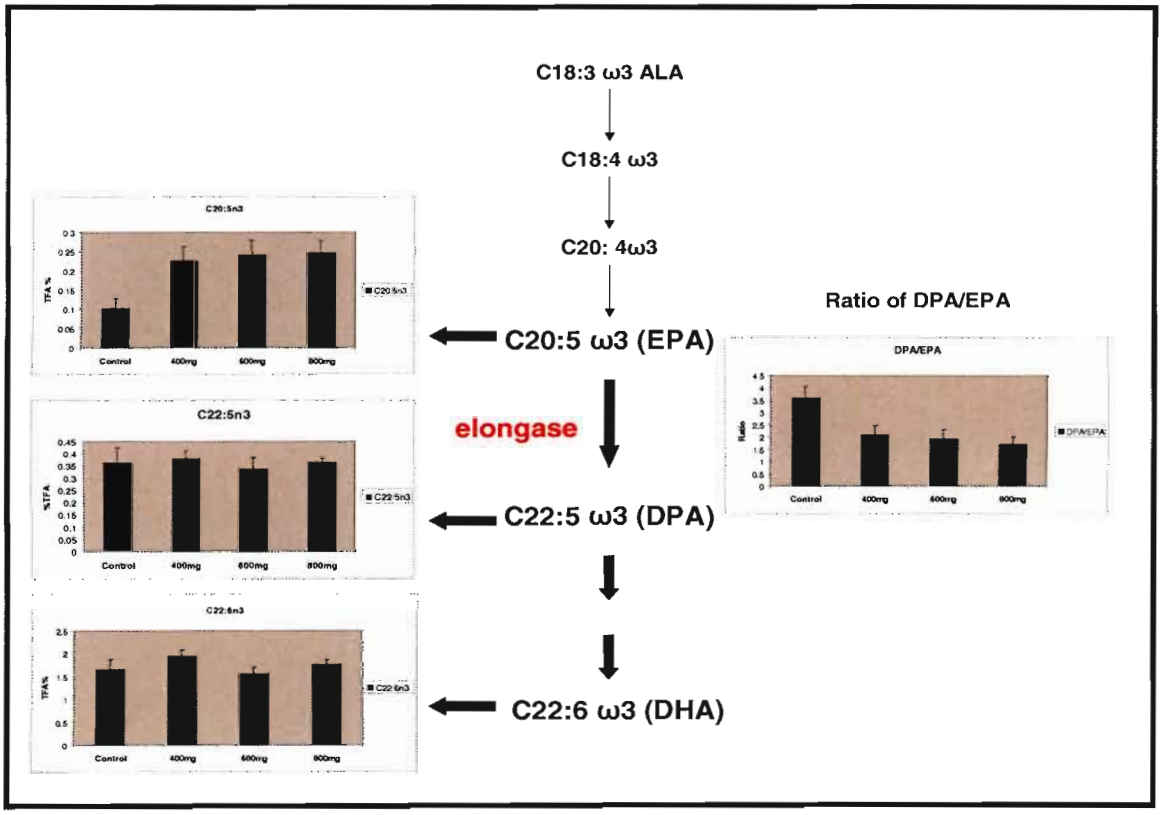

Figure 5.11: The average Omega-3 fatty acid levels and ratios in the plasma as a percentage of the total lipid composition. EPA = eicosapentaenoic acid, DPA = docosapentaenoicacid, DHA = Docosahexaenoic acid, TFA = Total phospholipid fatty acid (*p = 0.0390). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.11 represents the average ω-3 fatty acid levels and ratios. The analysis revealed no statistical difference in the EPA, DPA and DHA levels of the 400 mg/kg, 600 mg/kg and the

800 mg/kg groups compared with the control group. The DPA/EPA ratio was significant lower in the 800 mg/kg group compared to the control group ($p = 0.0390$).

Discussion

It is known that erucic acid is a potent inhibitor of VLCFA biosynthesis by competing with the other elongation systems. Figure 5.7 showed an overall increase in the EPA levels of the 400 mg/kg, 600 mg/kg and the 800 mg/kg groups compared to the control group, although not statistically significant. A notable decrease in the DPA levels was observed in the 600 mg/kg group, although it was not statistically significant.

A notable decrease in the DPA/EPA ratio was observed in the 400 mg/kg, 600 mg/kg and 800 mg/kg groups compared to the control group, with a statistical difference in the 800 mg/kg group compared to the control group. This decreased ratio is an indication of a decreased activity of the elongation at this level, thus speculating that erucic acid competes for the elongation of EPA to DPA in the ω-3 fatty acids pathway.

A slight but statistically insignificant decrease in the DHA levels was observed in the 600 mg/kg group, which could be attributed to the decreased activity of the elongation of EPA to DPA leading to a subsequent decrease in DHA.

5.2.3 Omega-6 fatty acids - plasma phospholipids

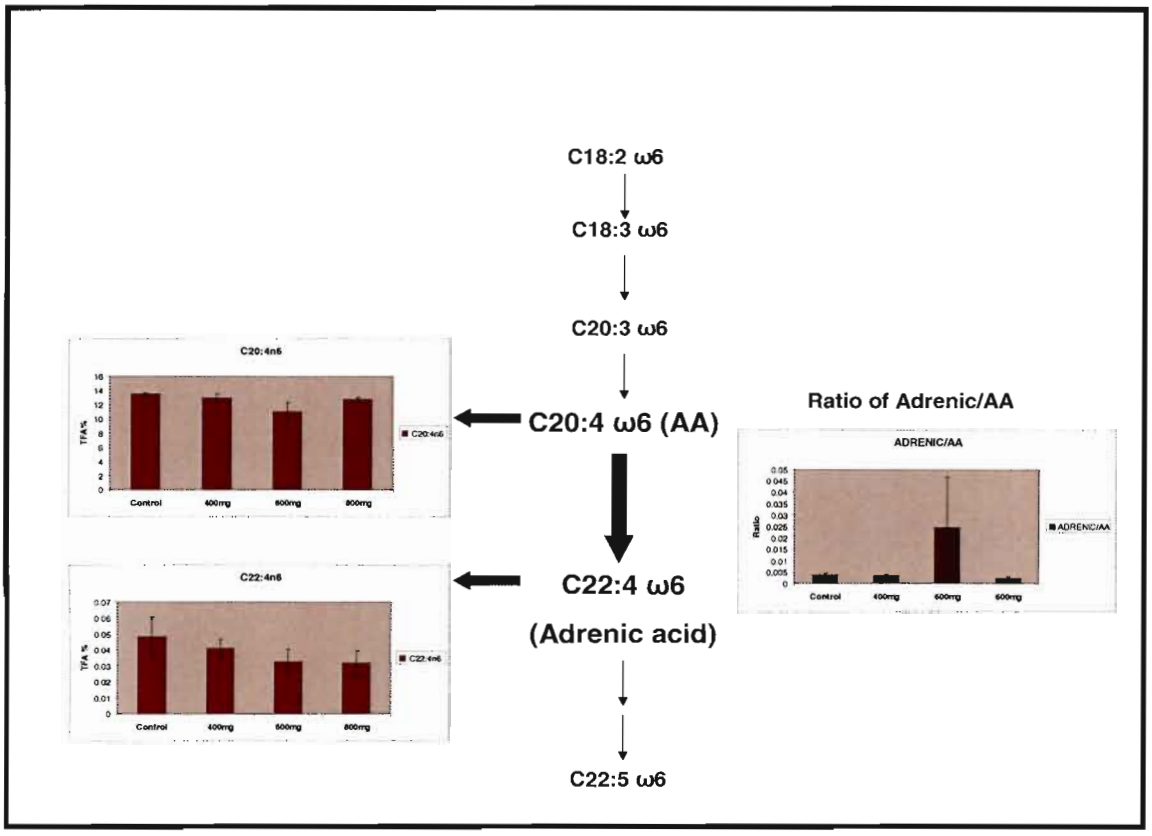

Figure 5.12: The average omega-6 fatty acid levels in the plasma as a percentage of the total lipid composition. AA = Arachidonic acid, TFA = Total phospholipid fatty acid. (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.12 represents the average ω-6 fatty acid levels. The analysis revealed no statistical differences in the levels AA and adrenic acid and in the ratio of adrenic/AA for the different dosages of erucic acid compared to the control group.

5.2.3.1 Discussion

In an experiment by Augourg *et al.* (1993), a two year trial with Lorenzo's oil as treatment for adrenomyeloneuropathy was studied. They conducted an open trail on 14 men with different phenotypes of X-ALD. The patients were on a a low-fat diet and received daily doses of glycerol trioleate and glycerol triurecate. Although all the patients received safflower and fish oil rich in ω-3 fatty acids, a 24 % decrease was found in their plasma arachidonic acid levels, but with no symptoms of essential fatty acid deficiency (Augourg *et al.*, 1993).

These results correlate with our findings which indicate a decreased level of arachidonic acid at the 600 mg/kg group, which is the optimal dosage, with the treatment of erucic acid,

although this was not statistically significant. The decrease in AA levels in turn leads to decreased levels of Adrenic acid (figure 5.12).

Although these results were established in the plasma, studies of erucic acid metabolism in tissue culture preparations of rat heart cells showed that erucic acid is rapidly taken up by the cells and that it is readily incorporated into various lipid classes. Other studies with the isolated, perfused rat heart have also produced evidence for the incorporation of erucic acid or its metabolites into neutral lipid and phospholipid components of the myocardium (Rogers., 1997; Vasdev and Kako., 1976).

5.2.4 Omega-9 fatty acids – plasma phospholipids

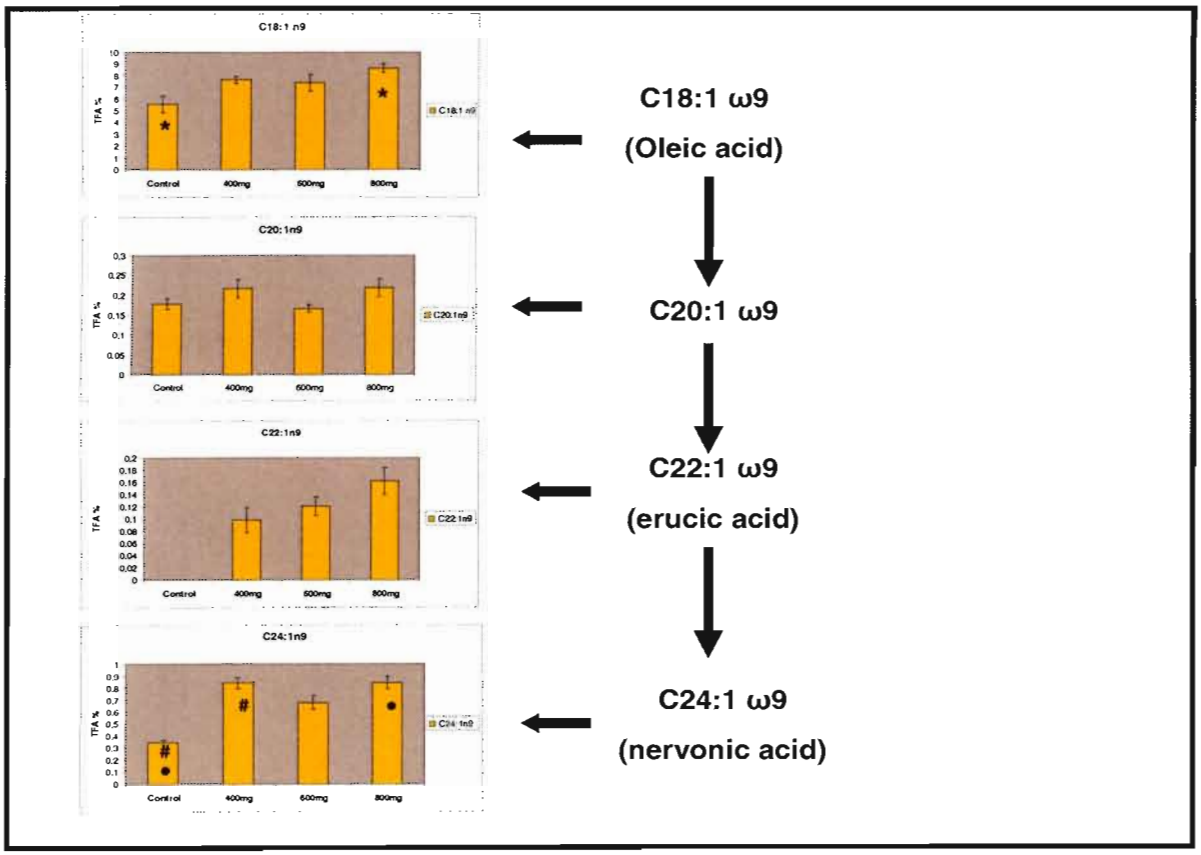

Figure 5.13: The average omega-9 fatty acid levels in the plasma as a percentage of the total lipid composition. TFA = Total phospholipid fatty acid (*p = 0.0044; # p = 0.0005; •p = 0.0007). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.13 represents the average omega-9 fatty acid levels. The oleic acid level was significant increased in the 800 mg/kg group compared to the control group ($p = 0.0044$). The nervonic acid was significant increased in the 400 mg/kg compared to the control group ($p = 0.0005$) and in the 800 mg/kg group compared to the control group ($p = 0.0007$). No statistical difference was evident in the concentration erucic acid and C20:1 between the experimental erucic acid groups and the control group, although a clear trend of increased erucic acid can be seen in all the groups.

5.2.4.1 Discussion

The analysis showed no statistical differences in the percentage erucic acid of the different groups, but although it was not statistically significant, an increase in the erucic acid levels was detected, because of an increase in the dosage of erucic acid given. The level of erucic acid in the control group was very low and not detectable. There was, however, a significant difference in the percentage nervonic acid between the 400 mg/kg and the control group and the 800 mg/kg and the control group. This increase in the nervonic acid levels was due to an increase in the erucic acid dosage given and indicates that erucic acid is subsequently elongated to nervonic acid.

In an experiment by Norseth *et al* (1978), the chain shortening of erucic acid in isolated liver cells were studied by administering ^{14}C-labelled erucic acid to male Wistar-derived rats. Isolated hepatocytes were prepared and purified from the livers of the rats. They found that erucic acid is rapidly chain shortened to C20:1 ω9, C18:1 ω9 and C16:1 ω9 in isolated liver cells. This data support our findings that the levels of oleic acid significantly increase with the administration of erucic acid (Norseth *et al.*, 1978).

Murphy and co-workers examined the uptake and metabolism of erucic acid in the rat liver and heart, following infusion of [14-^{14}C]22:1 ω9 (170 Ci/kg) under steady-state-like conditions. They stated that in most of the previous studies examining C22:1 ω-9 metabolism *in vivo,* the animals were fed high oil diets rich in C22:1 ω-9. Under these conditions, peroxisomal β-oxidation is increased, thus increasing the retro-conversion of C22:1 ω-9 to C18:1 ω-9 in cultured heart and liver cells (Murphy *et al.,* 2007). This data correlate with our findings in which the levels of oleic acid in the 800 mg/kg group increased notably.

5.2.5 Omega-3 fatty acids – brain phospholipids

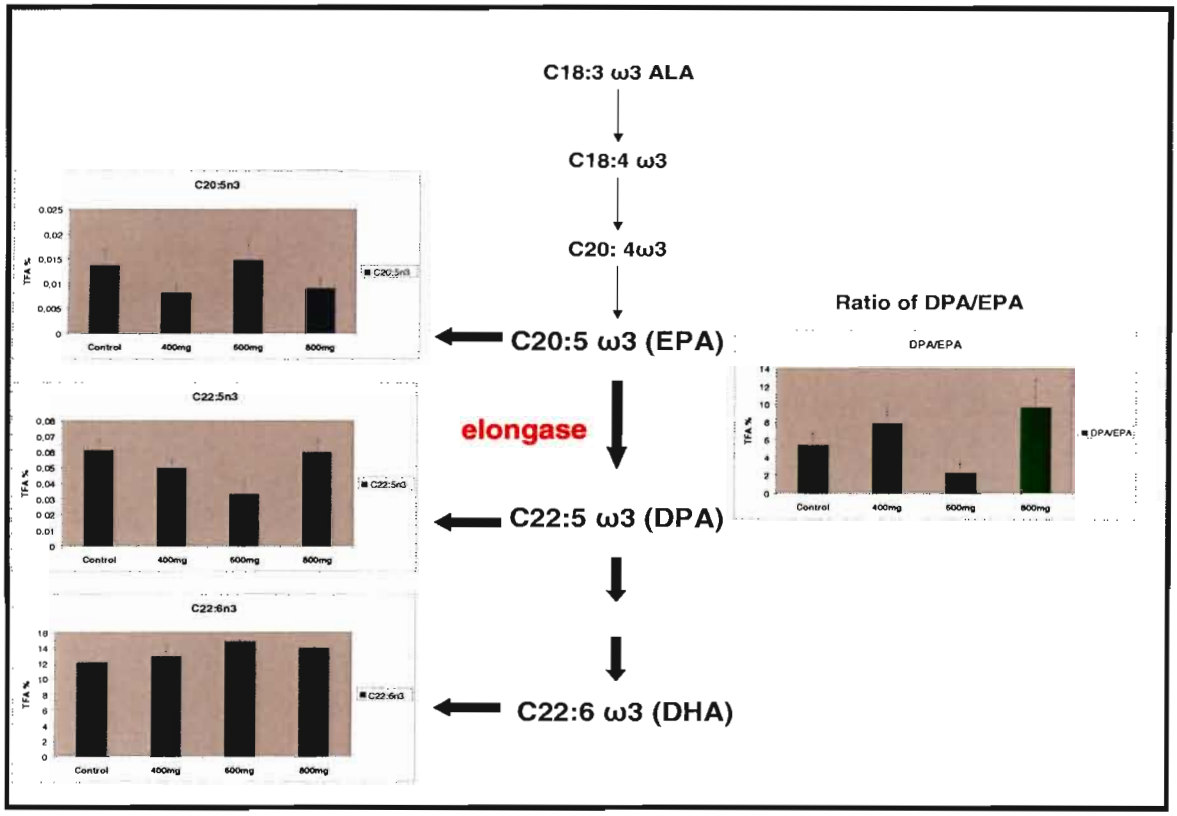

Figure 5.14: The omega-3 fatty acid levels and ratios in the brain as a percentage of the total lipid composition. EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = Docosahexaenoic acid. TFA = Total phospholipid fatty acid (*p = 0.0357, # p = 0.0001). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.14 represents the ω-3 fatty acid levels and ratios in the brain. The DHA was significant increased in the 400 mg/kg group compared to the control group (p = 0.0357) and in the 600 mg/kg group compared to the control group (p = 0.0001). No statistical differences were observed in the other concentrations and in the DPA/EPA ratio.

5.2.5.1 Discussion

There were no significant differences in the EPA, DPA and DPA/EPA levels between the erucic acid groups and the control group. A notable increase in the levels of EPA and a decrease in the levels of DPA in the 600 mg/kg group can be seen, when compared to the control group.

A notable decease is observed in the DPA/EPA ratio at the 600 mg/kg. This ratio is an indication of the activity of the enzyme elongase at the level of EPA being elongated to DPA.

Although we did not find statistical differences between the different groups, it could be assumed that this decrease in DPA/EPA ratio reflected the competition of erucic acid with the elongation enzyme in the elongation of EPA to DPA in the brain. This correlates with the findings in 5.2.2 where the DPA/EPA ratio was also notable decreased in the plasma

DHA is the major ω-3 fatty acid family member in the body, it is mainly concentrated in the brain and the retina and it is necessary for the development and function of the nervous system. Assuming that erucic acid competes at the elongation level of EPA to DPA, one would expect the levels of DHA to decrease, but no changes were seen in the DHA levels. The short time of exposure to erucic acid (7 days) may explain why DHA levels remained unchanged and the effect of erucic acid on the biosynthesis and incorporation of fatty acids in the brain could not be determined.

5.2.6 Omega-6 fatty acids – brain phospholipids

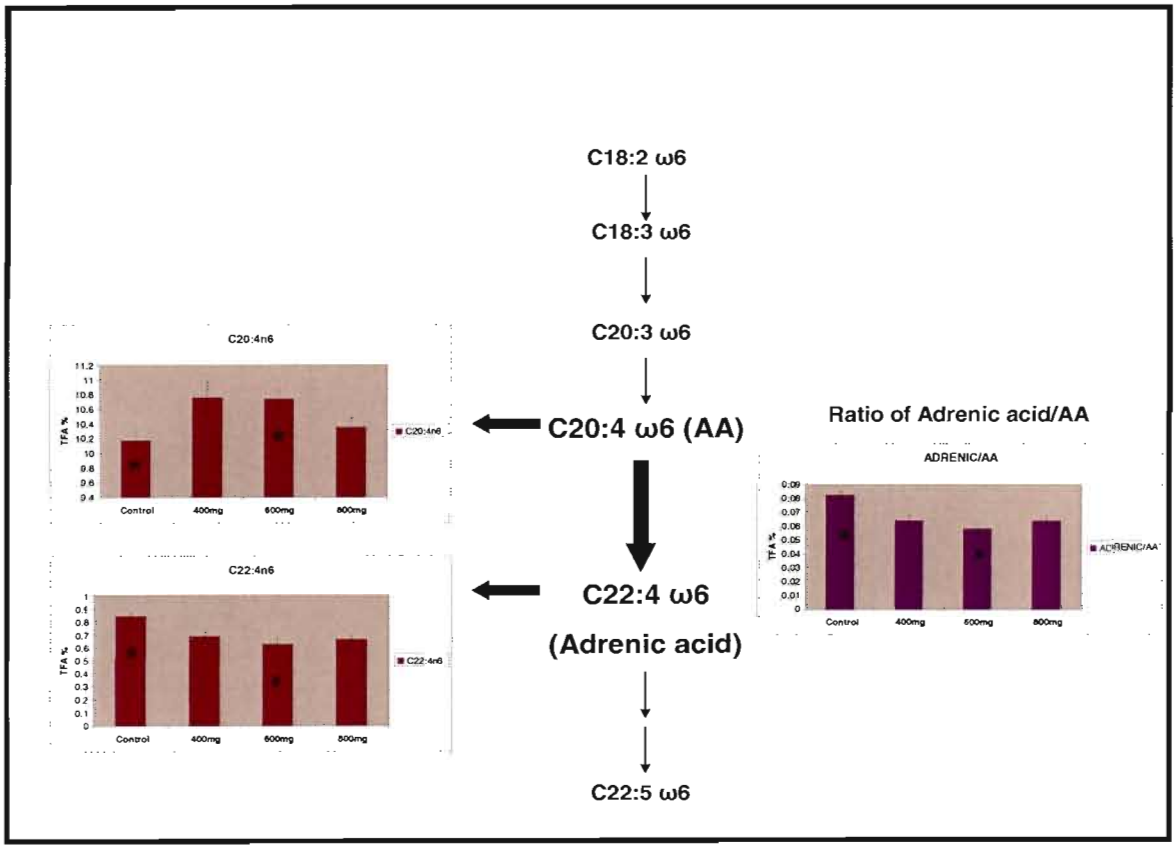

Figure 5.15: The average omega-6 fatty acid levels and ratio in the brain as a percentage of the total lipid composition. AA = Arachidonic acid. TFA = Total phospholipid fatty acid (*p = 0.0372; #p = 0.0477; •p = 0.0092). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.15 represents the average omega-6 fatty acid levels and ratios in the brain. The AA level was markedly higher in the 600 mg/kg group compared to the control group ($p = 0.0372$). The Adrenic acid level was significant lower in the 600 mg/kg group compared to the control group ($p = 0.0477$) and the adrenic acid/AA ratio was also significant lower in the 600 mg/kg group compared the control group ($p = 0.0092$).

5.2.6.1 Discussion

Long chain polyunsaturated fatty acids, mainly DHA and AA, are particularly enriched in cell membrane phospholipids, especially in neural tissues (Martin *et al.,* 2006; Uauy *et al.,* 2001; Lane and Farlow., 2005). In neurons, synapses have the highest concentration of long PUFA, especially DHA and AA.

A recent study done by Chen *et al* (2008), studied the regulation of brain polyunsaturated fatty acid uptake and turnover. They reviewed the uptake and turnover of arachidonic and docosahexaenoic acids within the brain phospholipids of unanesthetized rats, along with chronic administration of antimanic drugs to advanced their understanding of how polyunsaturated fatty acids enter the brain and the mechanism that regulate their turnover in brain phospholipids. The rate of plasma unesterified DHA, AA, palmitic acid (C16:0), linoleic acid (C18:2 ω-6), erucic acid and α-linolenic acid (C18:3 ω-3) incorporation into brain phospholipids ranged from ~ 2 – 10 pmol/g/brain/s in rats. Fatty acids with lower rates of esterification into brain phospholipids (palmitic, linoleic, erucic and α-linoleic acids) had higher rates of β-oxidation when compared to DHA and AA (Chen *et al.,* 2008).

Contradictory to the study done by Chen *et al* and the results obtained in section 5.2.3, we found elevated levels of AA in the brain of rats treated with erucic acid. AA and its metabolites are well known modulators of inflammation that are synthesized and/or released by living cells in response to various factors. The production of these derivatives and their action in the body are collectively known as the arachidonic acid cascade (Figure 5.16).

AA is generated by the action of PLA2. Leukotrienes and prostaglandins are potent mediators of inflammation and are derivatives of AA. The principal pathway of AA metabolism is:

- The 5 – lipoxygenase pathway, which produces the end metabolite leukotrienes
- The Cyclooxygenase (COX) pathway, which produces prostaglandin H_2 (PGH_2). PGH_2 serves as the substrate for two enzymatic pathways: one leading to the production of prostaglandines (PG), the other leading to the production of thromboxanes (Tx).

Arachidonic acid cascade:

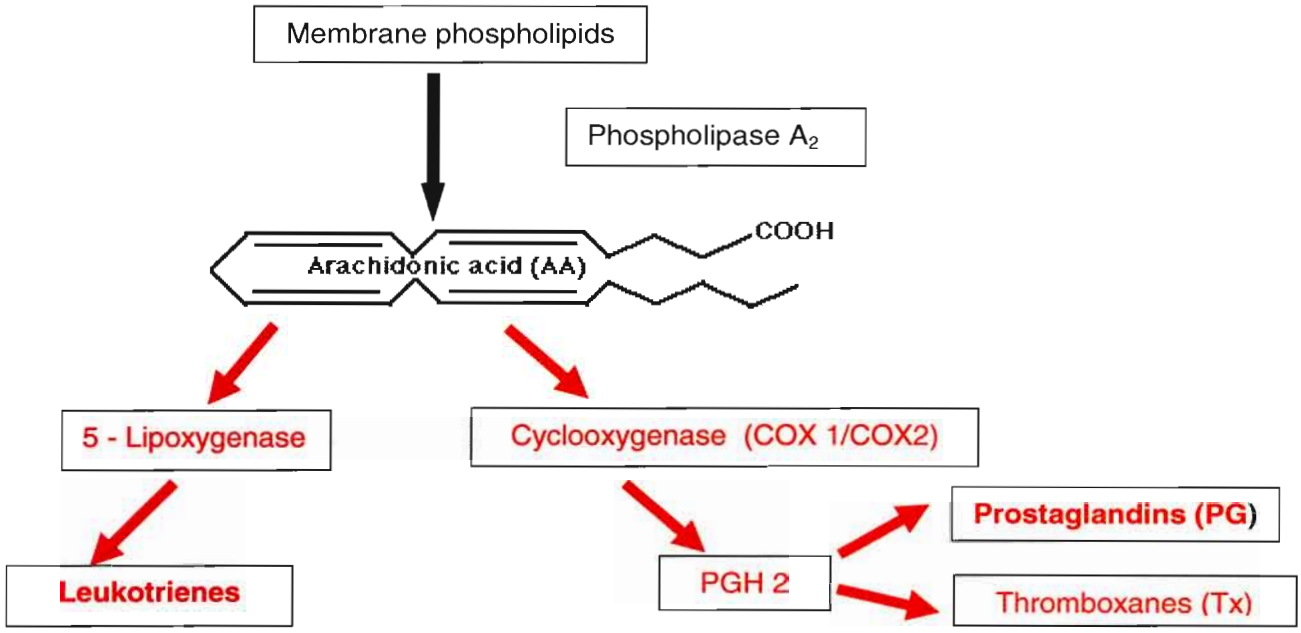

Figure 5.16: The Arachidonic acid cascade leading to the production of leukotrienes and prostaglandins.

Lorenzo's oil normalized plasma C26:0 and C24:0 levels within one month. However, the treatment with Lorenzo's oil did not prevent the progression of pre-existing neurological symptoms. A possible explanation for Lorenzo's oil's ineffectiveness in improving neurological symptoms in X-ALD patients could be the increased levels of AA in the brain due to the administration of erucic acid and this may be regarded as a possible side effect of erucic acid.

The decreased adrenic acid (22:4) and adrenic/AA ratio versus the decreased DPA (22:5) and DPA/EPA ratio confirm that erucic acid competes for the elongation system at the C20 level in the brain phospholipids.

5.2.7 Omega-9 fatty acids – brain phospholipids

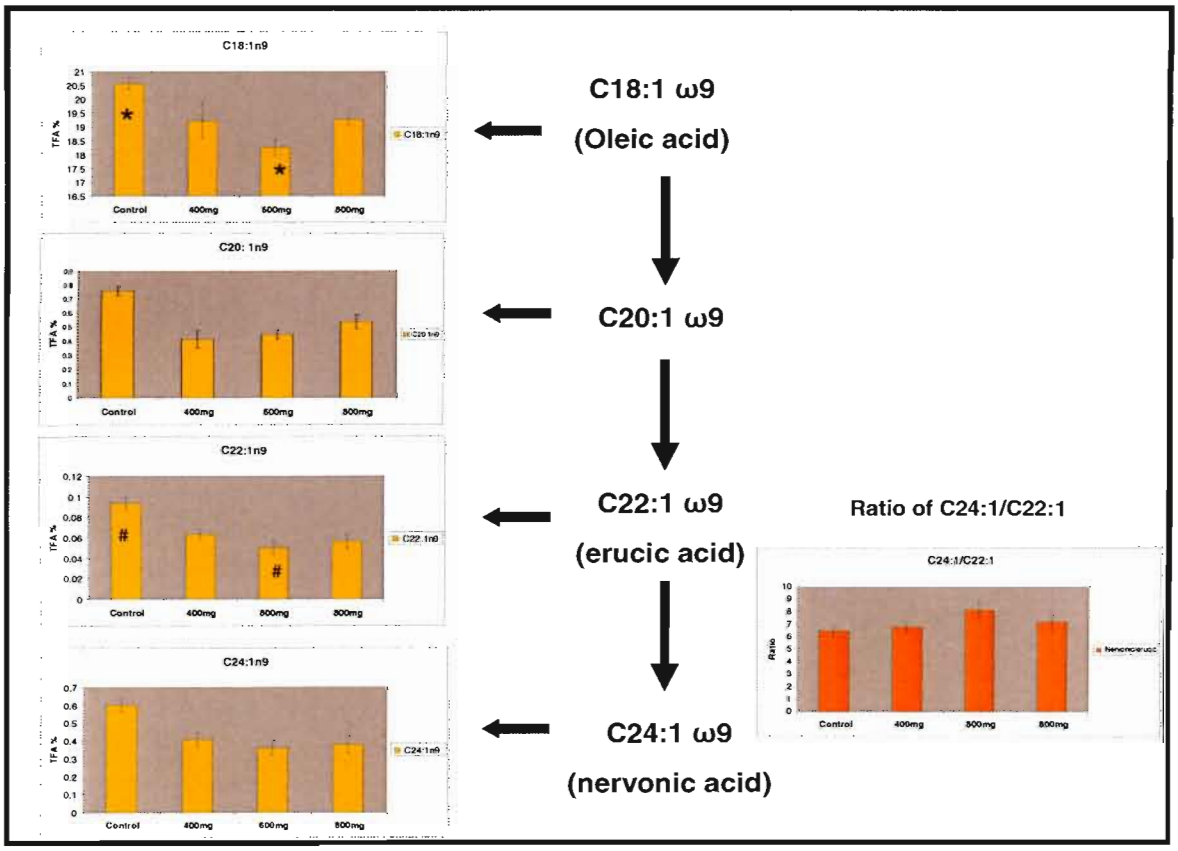

Figure 5.17: The average omega-9 fatty acid levels and ratio in the brain as a percentage of the total lipid composition. TFA = Total phospholipid fatty acid (*p = 0.0267; # p = 0.0374). (Control: n = 7; 400 mg: n = 10; 600 mg: n = 10; 800 mg: n = 8)

Figure 5.17 represents the average omega-9 fatty acid levels and ratio in the brain. The oleic acid level was significant lower in the 600 mg/kg compared to the control group (p = 0.0267). The erucic acid content was also significant lower in the 600 mg/kg group compared to the control group (p = 0.0374). No significant difference was observed in the nervonic acid content and the C24:1/C22:1 ratio between the erucic acid groups and the control group.

5.2.7.1 Discussion

Golovko and Murphy (2006) studied the uptake and metabolism of plasma-derived erucic acid by the rat brain. This study was discussed in Chapter 3, section 3.2.3. They demonstrated that erucic acid was taken up by the rat brain and crossed the BBB and found in brain metabolic compartments. In addition, once in the brain, significant amounts of erucic acid were found to be chain-shortened to C20:1 and C18:1. They observed no elongation of erucic acid to nervonic acid (C24:1), indicating that in normal rats this process is limited. Because LO is thought to compete with the endoplasmic reticulum-localized elongases, the lack of nervonic acid in the rat brain suggested that the brain is ill-equipped to elongate this fatty acid (Golovko and Murphy., 2006). This data correlated with our findings and supports the discussion in section 5.2.4.1 showing that erucic acid does cross the BBB, that retro-conversion of C22:1 to C18:1 leads to increased levels of oleic acid and explains the decrease of nervonic acid levels in the brain.

Chapter 6 Conclusion

X-Linked Adrenoleukodystrophy is an inherited disorder of peroxisomal fatty acid oxidation and is biochemically characterized by the accumulation of saturated very long chain fatty acids (SVLCFAs), particularly hexacosanoic acid (C26:0). The consequent accumulation of these fatty acids is associated with demyelination within the central nervous system (CNS), and with adrenocortical insufficiency and hypogonadism. It is a clinically heterogeneous disorder, ranging from the severe childhood cerebral form to asymptomatic persons. Males are affected most severely, but approximately 50% of woman heterozygous for X-ALD, develop some neurological deficits in middle age or later (Moser *et al.*, 2006).

As it is known that the excess of VLCFA is toxic to myelin and the adrenal cortex and testis, several attempts were made to lower the plasma concentrations of VLCFAs. Restriction of VLCFA intake alone induced neither biochemical nor clinical improvement. In 1989 Rizzo *et al* reported that the combination of oleic acid and erucic acid, in triglyceride form, normalized the plasma concentrations of C24:0 and C26:0 within one month in most X-ALD patients (Rizzo *et al.*, 1989). This combination became known as Lorenzo's oil. The aggregate of the available data on the efficacy of Lorenzo's oil in adult patients with X-ALD and neurological symptoms suggest that the oil is not efficacious.

Erucic acid, one of the components of Lorenzo's oil is a potent inhibitor of VLCFA synthesis. It reduces the concentrations of VLCFAs in patients with X-ALD, probably by competitive inhibition of the endogenous elongation system of saturated and polyunsaturated fatty acids.

In search for novel therapies for X-ALD, the potential inhibitory effect of erucic acid on VLCFA in rats was investigated. Spraque-Dawley rats were divided into 6 groups (10 rats per group), one control group and 5 groups treated with erucic acid ranging from 400 mg/kg to 800mg/kg for 7 days.

The results obtained established that the 600mg dosage of erucic acid was the optimal dosage for the best inhibitory effect on saturated VLCFA levels.

The effect of erucic acid on the biosynthesis and incorporation of FA can be summarized as follows:

- Erucic acid inhibited the biosynthesis of omega-3 FA by competing with the elongation of EPA to DPA in the brain phospholipids of Sprague-Dawley rats (Fig 5.14).

- DHA (ω-3 fatty acid) is an important component of phospholipids and plays an essential role in membrane-integrity as well as neuronal cell communication, just to name a few. Erucic acid decreased the DHA levels slightly in the plasma at the 600 mg/kg but in the brain no significant decrease was observed. It is possible that the time of exposure (7 days) to erucic acid was too short (Fig 5.11 and 5.14).

- Erucic acid significantly increased the levels of AA (ω-6 fatty acid) in the brain, which offers a possible explanation why Lorenzo's oil does not alter the clinical progression of patients with neurological symptoms. Inflammation in the brain is separate from the rest of the body, but is the foundation of many brain disorders, including Alzheimer's disease, bipolar disorder and Parkinson's disease. In these disorders, AA is rapidly converted into inflammatory prostaglandins and leukotrienes leading to inflammation (Rapoport, 2008) (Fig 5.15).

- Erucic acid inhibited the biosynthesis of omega-6 FA by competing with the elongation of AA to adrenic acid in the brain phospholipids of Sprague-Dawley rats. (Fig 5.15).

- Erucic acid crossed the BBB, but was readily chain-shortened to C20:1 and C18:1 in the brain (Fig 5.17)

In summary: erucic acid does show definite potential in lowering SVLCFA levels in the plasma and the brain of Sprague Dawley rats, but Lorenzo's Oil is not without side effects and our study showed that erucic acid markedly increased arachidonic acid (AA) levels in the brain of Sprague Dawley rats treated with erucic acid. In turn AA could be converted into inflammatory prostaglandins and leukotrienes. Erucic acid competes with the elongation of C20:0 to C22:0 in the omega-3 and omega 6 fatty acid biosynthesis pathway. However, too little is known of the mechanism and side-effects of erucic acid. There is obviously a great need for increased research.

Suggestion for future research:

- **Larger** groups and a **longer** treatment period with erucic acid might allow better results to obtain statistical significance.

- Additional groups like DHA, AA, oleic acid and combinations of these groups with erucic acid, and these groups in combination can be added to the study to determine the effect of these fatty acids on the biosynthesis and incorporation of FA.

- Analysis can be done on different tissue samples, for example the liver, heart, brain and red blood cells (RBC) and the concentration of FA in the plasma, RBC and in the phospholipids can be determined.

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Appendix A

Table A – 1: Weight of each rat and the average weight per group before experimental procedures

Weight (g)						
Rat	Control	600 mg	800 mg	400 mg	575 mg	625 mg
1	249.8	228.3	245.1	211.6	203.8	223.6
2	238.1	221.9	237.5	211.7	214.4	225.4
3	233.1	215.3	240.4	205.1	223	226.3
4	243.6	231.4	236.9	210.3	201.6	224.4
5	243.1	229	238.6	203.2	219.1	226.6
6	236.9	229.2	237.4	201.9	221.3	227
7	249	227.1	233.4	207.5	213.8	223.2
8	233.1	225.9	234.1	201.2	223	226.7
9	244	226.5	238.1	210.6	214.8	231.7
10	234.6	232.3	238.7	212.5	218.4	229.4
Average weight per group	240.53	226.69	238.02	207.56	215.32	226.43

Table A – 2: Weight of each rat and the average weight per group after experimental procedures

Weight (g)						
Rat	Control	600mg	800mg	400mg	575mg	625mg
1	203.7	215.2	212.5	216.9	197.6	206.8
2	Dead (Day 3)	206.6	191.2	230.1	Dead(Day 4)	207.1
3	219.4	209.7	201.6	204.3	218.3	222.9
4	200.3	212.1	202.6	209.8	Dead(Day 5)	203.7
5	228.3	200.3	169.2	230.1	192.9	208.4
6	196.9	239.8	217.2	161.6	217.9	210.4
7	229.2	221.4	203.7	188.7	196.4	189.6
8	Dead (Day 6)	223.5	188.7	226.3	205.4	198.2
9	Dead (Day 6)	191.2	179.7	198.3	189.2	229.4
10	219.1	215.5	132.7	214.1	176.8	208.1
Average weight per group	213.842857	213.53	189.91	208.02	199.3125	2
	Rat died on day 7. Only brain sample was collected					

Appendix B

Raw Data on the plasma saturated very long chain fatty acid analysis

Table B – 1: Plasma SVLCFA concentrations and ratios

Concentration (µg/L)						
Group	Rat	C22:0	C24:0	C26:0	C24:0/C22:0	C26:0/C22:0
Control	1	9.46	28.98	2.33	3.06	0.25
	3	12.36	37.65	2.31	3.05	0.19
	4	6.62	16.77	1.56	2.53	0.24
	5	12.68	42.97	2.65	3.39	0.21
	6	13.05	41.61	2.06	3.19	0.16
	10	85.53	101.36	2.84	1.19	0.03
600 mg	1	11.2	24.7	1.62	2.2	0.14
	2	10.8	25.6	1.29	2.4	0.12
	3	15.2	34.2	1.85	2.3	0.12
	4	9.44	21.8	2.03	2.3	0.22
	5	9.12	21.3	2.6	2.3	0.29
	6	10.2	25.6	1.29	2.5	0.13
	7	11.7	31.3	2.09	2.7	0.18
	8	10.4	23.9	1.85	2.3	0.18
	9	11.5	26.7	1.46	2.3	0.13
	10	10.1	26.4	0.96	2.6	0.1
800 mg	1	10.6	25.8	1.39	2.4	0.13
	2	14.4	33.7	1.66	2.3	0.12
	3	7.64	18.5	1.23	2.4	0.16
	4	11.9	27.5	3.5	2.3	0.29
	6	9.65	22.7	1.47	2.4	0.15
	7	11.1	32.1	1.89	2.9	0.17
	8	13	39	2.85	3	0.22
	9	11.5	33.7	1.89	2.93	0.16
400 mg	1	23.7	55.9	2.06	2.4	0.09
	2	27.9	71.8	2.98	2.6	0.11
	3	11.7	35.7	1.33	3.05	0.11
	4	27.9	65.4	2.38	2.3	0.09
	5	24.6	63.3	2.89	2.6	0.12
	6	36.3	84.2	4.13	2.3	0.11
	7	14.2	37.2	1.25	2.6	0.09
	8	23.6	61.4	2.04	2.6	0.09
	9	28.6	78.2	2.69	2.7	0.09
	10	10.5	25	1.26	2.4	0.12

Concentration (µg/L)						
Group	Rat	C22:0	C24:0	C26:0	C24:0/C22:0	C26:0/C22:0
575 mg	1	13.10	34.38	0.79	2.62	0.06
	3	12.82	25.10	4.71	1.96	0.37
	5	10.28	29.32	2.59	2.85	0.25
	6	10.72	31.62	1.76	2.95	0.16
	7	13.27	33.28	2.62	2.51	0.20
	8	29.46	38.47	3.40	1.31	0.12
	9	11.81	35.73	2.04	3.03	0.17
	10	82.09	101.93	2.40	1.24	0.03
625 mg	1	25.08	47.58	0.17	1.90	0.01
	2	12.12	34.71	1.01	2.86	0.08
	3	9.44	25.84	5.08	2.74	0.54
	5	14.34	30.98	1.79	2.16	0.13
	6	12.79	33.54	1.96	2.62	0.15
	7	13.02	41.33	2.87	3.17	0.22
	8	10.13	26.48	2.48	2.61	0.24
	9	8.18	19.27	1.89	2.35	0.23
	10	12.45	35.92	2.69	2.89	0.22

Table B – 2: Average plasma SVLCFA concentrations and ratios. (Data expressed as mean ± SEM)

Average concentration (µg/L)					
Group	C22:0	C24:0	C26:0	C24:0/C22:0	C26:0/C22:0
Control	23.28 ± 12.49	44.89 ± 11.97	2.29 ± 0.19	2.73 ± 0.33	0.18 ± 0.03
600 mg	11.92 ± 2.63	26.15 ± 6.15	1.70 ± 0.29	2.39 ± 0.07	0.16 ± 0.00
800 mg	11.22 ± 8.73	29.13 ± 8.79	1.99 ± 0.41	2.58 ± 0.25	0.18 ± 0.04
400 mg	22.90 ± 0.54	57.81 ± 1.26	2.30 ± 0.15	2.56 ± 0.05	0.10 ± 0.02
575 mg	22.95 ± 1.64	41.23 ± 2.84	2.54 ± 0.45	2.51 ± 0.13	0.17 ± 0.05
625 mg	13.06 ± 0.73	32.85 ± 2.38	2.22 ± 0.28	2.59 ± 0.11	0.20 ± 0.05

Appendix C

Raw Data on plasma mono- and polyunsaturated fatty acids analysis

Table C – 1: Plasma SVLCFA percentage and ratios

Percentage (%)						
Group	Rat	C22:0	C24:0	C26:0	C24:0/C22:0	C26:0/C22:0
Control	1	0.095370456	0.249960426	0.012210209	2.62094193	0.128029264
	3	0.147405492	0.444826385	0.006796638	3.017705644	0.046108447
	4	0.108752102	0.268681663	0.014771676	2.470588235	0.135828877
	5	0.137609655	0.462177746	0.011510421	3.358614232	0.083645443
	6	0.153737068	0.483114604		3.142473118	
	7	0.181264762	0.57119627	0.010634886	3.151171043	0.058670452
	10	0.162394252	0.374084244		2.303555941	
400 mg	1	0.12321168	0.295690746	0.011322855	2.399859698	0.09189758
	2	0.126487607	0.323270056	0.007882383	2.555744888	0.062317429
	3	0.132775561	0.344149164	0.009153279	2.591961672	0.068937982
	4	0.129425969	0.294790546		2.277676951	
	5	0.103502769	0.253932081		2.453384419	
	6	0.100091237	0.262822092	0.006739706	2.625825191	0.067335622
	7	0.098447238	0.215595281	0.015484744	2.189957653	0.157289776
	8	0.144919946	0.407513556	0.007750054	2.811990808	0.05347817
	9	0.121528509	0.308196516	0.007337365	2.536001789	0.060375671
	10	0.104891074	0.243660558	0.011483461	2.322986577	0.109479866
600 mg	1	0.083389852	0.196497144	0.009594057	2.356367583	0.115050651
	2	0.092539247	0.205590971		2.221662469	
	3	0.131377936	0.303738895	0.009740024	2.311947534	0.074137439
	4	0.080625794	0.150306366	0.01090513	1.864246643	0.135256091
	5	0.091096599	0.147857255	0.015861382	1.623082055	0.174116077
	6	0.103202136	0.230332382		2.231856739	
	7	0.112870588	0.272779289		2.416743756	
	8	0.068396218	0.147213883	0.011165836	2.152368758	0.163252241
	9	0.104145598	0.229754548		2.206089863	
	10	0.109729135	0.043020356		0.392059553	
800 mg	1	0.084911005	0.174959108	0.009492462	2.060499781	0.111793073
	2	0.103665675	0.249280447		2.404657534	
	3	0.10223569	0.188978433	0.011453238	1.848458532	0.11202779
	4	0.09965044	0.235290596		2.361159623	
	6	0.101541381	0.243611841	0.009293989	2.39913855	0.091529074
	7	0.102760944	0.272680347	0.008773105	2.653540702	0.085373925
	8	0.112516328	0.271205662	0.010328173	2.410367171	0.091792657
	9	0.114152462	0.263369084		2.307169555	

Table C – 2: The average plasma SVLCFA concentrations and ratios. (Data expressed as mean ± SEM)

Average Percentage (%)					
Group	C22:0 Behenic	C24:0 Lignoceric	C26:0 Cerotic	C24:0/C22:0	C26:0/C22:0
Control	0.140933398 ± 0.011355378	0.407720191 ± 0.044213355	0.011184766 ± 0.001295792	2.866435735 ± 0.150885419	0.090456497 ± 0.018019237
400 mg	0.118528159 ± 0.005019366	0.29496206 ± 0.017547279	0.009644231 ± 0.001043715	2.476538965 ± 0.058652958	0.083889012 ± 0.012354351
600 mg	0.09773731 ± 0.005779383	0.192709109 ± 0.023568631	0.011453286 ± 0.001144692	1.977642495 ± 0.191703925	0.1323625 ± 0.017882628
800 mg	0.102679241 ± 0.003150278	0.23742194 ± 0.013017577	0.009868193 ± 0.000468724	2.305623931 ± 0.086884829	0.098503304 ± 0.005592776

Table C – 3: Plasma Omega – 3 fatty acid percentage and ratios

Percentage (%)					
Group	Rat	C20:5 ω3 EPA	C22:5 ω3 DPA	C22:6 ω3 DHA	DPA/EPA
Control	1	0.087608251	0.189389067	1.402822215	2.161772026
	2	0.212027929	0.598811023	2.688206392	2.824208232
	3	0.02803129	0.160336655	1.124043112	
	4	0.135462188	0.537038429	2.1235008	3.964489537
	5	0.068662139	0.292124045	1.610460733	4.254514187
	7	0.077403098	0.365702837	1.431721462	4.72465374
	Info		0.382675787	1.198309015	
400 mg	1	0.059380164	0.307013601	1.979727767	5.170305677
	2	0.285028184	0.524609506	2.366100336	1.840553095
	3	0.167444931	0.33980224	1.701909148	2.029337273
	4	0.165881342	0.372587063	1.566312605	2.246105919
	5	0.279807773	0.432616459	1.756804183	1.546120231
	6	0.473867386	0.386224782	2.892760975	0.815048246
	7	0.209044043	0.230305788	1.468847077	1.101709402
	8	0.292806709	0.519768237	1.805853288	1.775124069
	9	0.120169738	0.313387023	2.129792556	2.607869742
	Info	0.20058658	0.376226332	1.727446879	1.875630621
600 mg	1	0.334283487	0.465160903	1.711664201	1.391516245
	2	0.24638283	0.371982908	1.726971927	1.509776096
	3	0.371394599	0.462913361	2.181915173	1.246419205
	4	0.314324329	0.23899073	1.227388394	0.760331633
	5	0.071406607	0.219385405	1.264535043	3.072340426
	6	0.183983826	0.369621221	1.310404497	2.008987576
	7	0.33260801	0.527651728	1.987712663	1.586407157
	8	0.236890871	0.348811955	1.400852632	1.47245841
	9	0.082914282	0.356755716	2.034687097	4.302705224
	Info		0.022054739	0.703028851	
800 mg	1	0.261545254	0.342882626	1.573142958	1.31098776
	2	0.180520282	0.41483311	2.204699094	2.297986155
	3	0.23559045	0.284288909	1.24445206	1.206708121
	4	0.369886563	0.336646545	1.901990329	0.910134563
	5	0.316578763	0.398730332	1.57035604	1.259498043
	7	0.260166771	0.43474476	1.993399089	1.671023396
	8	0.265413785	0.376998552	1.63946577	1.420418129
	9	0.089522216	0.32096895	1.966773605	3.585355286

Table C – 4: Average plasma Omega – 3 fatty acid percentages and ratios. (Data expressed as mean ± SEM)

Average Percentage (%)				
Group	**C20:5 ω3 EPA**	**C22:5 ω3 DPA**	**C22:6 ω3 DHA**	**DPA/EPA**
Control	0.101532483 ± 0.026216703	0.360868263 ± 0.062200795	1.654151961 ± 0.212295614	3.585927544 ± 0.474101776
400 mg	0.225401685 ± 0.036395346	0.380254103 ± 0.029271028	1.939555482 ± 0.135070434	2.100780428 ± 0.378428172
600 mg	0.241576538 ± 0.036613554	0.338332867 ± 0.04643478	1.554916048 ± 0.143976194	1.927882441 ± 0.36458177
800 mg	0.24740301 ± 0.029884896	0.363761723 ± 0.018108411	1.761784868 ± 0.109080743	1.707763932 ± 0.304820562

Table C – 5: Plasma Omega – 6 fatty acid percentages

Percentage (%)			
Group	**Rat**	**C20:4 ω6 AA**	**C22:4 ω6 Adrenic**
Control		13.51487007	0.04112224
		13.10457117	0.07193562
		14.22628695	
		13.55017019	0.031224163
		13.77759253	
		12.78454751	
		13.31006105	
400 mg		13.97071789	0.038895304
		12.92119557	0.046124254
		16.10379889	0.024173139
		12.77887655	
		11.43738644	0.016688665
		11.4791196	0.050693159
		10.89357693	
		12.13891487	0.050012064
		14.31495505	0.058807624
		13.26748896	
600 mg		9.909212467	0.016352134
		11.72307533	
		0.475875085	0.043230721
		11.11938351	
		13.04589591	
		12.73802269	0.021788198
		13.35015777	0.048134451
		11.62722572	
		14.44912857	
		11.53694305	
800 mg		12.03960628	
		14.13042676	0.023715298
		11.98470416	
		11.9500822	
		12.41195768	
		13.30808629	0.039478973
		12.52463067	
		13.33644411	

Table C – 6: Average plasma Omega – 6 fatty acids percentages. (Data expressed as mean ± SEM)

Average Percentage (%)		
Group	**C20:4 ω6 AA**	**C22:4 ω6 Adrenic**
Control	13.46687135 ± 13.46687135	0.048094008 ± 0.048094008
400 mg	12.93060307 ± 12.93060307	0.040770601 ± 0.040770601
600 mg	10.99749201 ± 10.99749201	0.032376376 ± 0.032376376
800 mg	12.71074227 ± 12.71074227	0.031597136 ± 0.031597136

Table C – 7: Plasma Omega – 9 fatty acid percentages

Percentage (%)				
Group	**Rat**	**C18:1 ω9 Oleic**	**C22:1 ω9 Erucic**	**C24:1 ω9 Nervonic**
Control	1	7.079522912		0.338484443
	2	7.900222087		0.400213251
	3	4.243134805		0.235590783
	4	6.977934781		0.348190223
	5	3.492500004		0.394438308
	6	5.201059372		0.365574189
	7	3.908588797		0.323309631
400 mg	1	6.776037381	0.112191344	0.87622477
	2	8.312033966	0.088214945	1.086229262
	3	7.373620249	0.101569593	0.785733042
	4	6.263841062	0.048904695	0.77467667
	5	8.537656568	0.052676697	0.687506899
	6	8.784188809	0.097448215	1.006700589
	7	6.369292085		0.600867622
	8	8.257803108	0.070053218	0.998909241
	9	8.053410584	0.253329329	0.781565277
	10	7.675879541	0.062301074	0.825709225
600 mg	1	9.473950024	0.064262078	0.540706557
	2	8.005693767	0.096968077	0.862961497
	3	9.546309692	0.106016413	1.01494794
	4	8.786166843	0.100511619	0.632738082
	5	5.704871328	0.15265821	0.590031273
	6	6.85204792	0.076355963	0.640271497
	7	7.980477878	0.121014812	0.845302558
	8	7.576172894	0.205013504	0.444662993
	9	8.019172381	0.168651672	0.701174838
	10	1.902561617		0.543880763
800 mg	1	8.680205275	0.088298512	0.649656671
	2	8.586755673	0.282254652	0.852557872
	3	8.433445587	0.137971569	0.632058946
	4	10.49831137	0.154539977	0.960236237
	5	8.97394731	0.102598344	0.826508934
	6	9.179218214	0.204841802	0.996291493
	7	7.334622667	0.135683836	0.976032564
	8	7.49733043	0.190952283	0.882189462

Table C – 8: Average plasma Omega – 9 percentages. (Data expressed as mean ± SEM)

Average Percentage (%)			
Group	**C18:1 ω9 Oleic**	**C22:1 ω9 Erucic**	**C24:1 ω9 Nervonic**
Control	5.543280394 ± 0.66652723		0.343685833 ± 0.343685833
400 mg	7.640376335 ± 0.287641009	0.098521012 ± 0.098521012	0.84241226 ± 0.84241226
600 mg	7.384742434 ± 0.709779441	0.121272483 ± 0.121272483	0.6816678 ± 0.6816678
800 mg	8.647979566 ± 0.351321707	0.162142622 ± 0.162142622	0.846941522 ± 0.846941522

Raw Data on brain mono- and polyunsaturated fatty acids analysis

Table C – 9: Brain Omega – 3 fatty acid percentages and ratios

Percentage (%)					
Group	**Rat**	**C20:5 ω3 EPA**	**C22:5 ω3 DPA**	**C22:6 ω3 DHA**	**DPA/EPA**
Control	1	0.00741965	0.042521251	13.23658208	5.7308
	2	0.014649882	0.08306697	11.02392885	5.670146138
	3	0.013123145	0.047206551	11.04529875	3.597197898
	5	0.007246726	0.079076073	12.62642539	10.91197183
	6	0.027626726	0.057953988	13.03809381	2.097750865
	7	0.012640334	0.056928844	11.8778423	4.503745318
400 mg	1	0.004867955	0.060742454	13.49167205	12.47802198
	2			12.05725054	
	3	0.003981176	0.074247672	13.60799055	18.64968341
	4	0.008515468	0.055199823	1.045443207	6.482300885
	5	0.010421608	0.062867643	16.08161076	6.032432432
	6		0.03421488	14.93648391	
	7	0.006319656	0.037248794	14.52416711	5.894117647
	8	0.006763882	0.056848817	15.46709758	8.404761905
	9	0.015358232	0.048695214	14.33878335	3.17062635
	10	0.009398064	0.018341383	14.0743589	1.951612903
600 mg	1		0.062046201	14.10761721	
	2	0.01215595	0.00982299	14.407686	0.808080808
	3	0.009962952	0.080453041	14.2002042	8.075221239
	4	0.009280904	0.008288294	15.01769327	0.893048128
	5	0.010119625	0.035587349	15.00571759	3.516666667
	6	0.016538041	0.010495295	15.07652342	0.634615385
	7	0.005347821	0.008143273	16.04897308	1.522727273
	8	0.039724689	0.037340308	15.6081589	0.93997735
	9		0.047627362	14.68156167	
	10		0.031379392	14.28281886	
800 mg	1	0.013259328	0.095553633	14.12483513	7.206521739
	2		0.067396092	13.83724368	
	3			15.09675842	
	4	0.007847636	0.088391384	13.81721885	11.26344086
	5	0.013088126	0.066880323	13.55855671	5.11
	6		0.012802485	13.46425895	
	7		0.057640849	13.98132407	
	8		0.0616489	14.42384812	
	9	0.002819818	0.058808055	13.87458264	20.85526316
	10	0.008181328	0.029873535	13.79685154	3.651428571

Table C – 10: Average brain Omega – 3 fatty acid concentrations and ratios. (Data expressed as mean ± SEM)

Average Percentage (%)				
Group	**C20:5 ω3 EPA**	**C22:5 ω3 DPA**	**C22:6 ω3 DHA**	**DPA/EPA**
Control	0.01378441 ± 0.003040594	0.061125613 ± 0.006760613	12.14136187 ± 0.398261872	5.418618177 ± 1.231806839
400 mg	0.008203255 ± 0.0012832	0.049822964 ± 0.005723153	12.9624858 ± 1.370320579	7.882944689 ± 1.908804617
600 mg	0.014732855 ± 0.004354329	0.033118351 ± 0.007894873	14.84369542 ± 0.200961581	2.341476693 ± 1.026483512
800 mg	0.009039247 ± 0.001937005	0.059888362 ± 0.00859342	13.99754781 ± 0.148966318	9.617330866 ± 3.088048111

Table C – 11: Brain Omega – 6 fatty acid concentrations and ratios

Percentage (%)				
Group	**Rat**	**C20:4 ω6 AA**	**C22:4 ω6 Adrenic**	**Adrenic/AA**
Control	1	10.51243654	0.90867298	0.086437904
	2	9.900628538	0.847949827	0.08564606
	3	9.753392885	0.866380409	0.088828618
	4	10.53934271	0.747790705	0.070952309
	5	10.28046384	0.839771205	0.08168612
	6	10.04260313	0.80443652	0.080102391
400 mg	1	10.39212183	0.823540362	0.079246604
	2	11.23470532	0.453034385	0.040324545
	3	10.89277754	0.812286773	0.074571134
	4	12.08808309	0.700566768	0.057955158
	5	10.58491695	0.662983239	0.062634713
	6	10.71144299	0.668720089	0.062430439
	7	10.26828797	0.687206787	0.066925157
	8	11.51979273	0.736715575	0.063952155
	9	9.875774131	0.736465351	0.074572924
	10	9.916372388	0.577324088	0.058219283
600 mg	1	10.20032021	0.771556752	0.075640444
	2	10.57807067	0.513312607	0.048526109
	3	10.88825006	0.891044981	0.081835463
	4	11.16800421	0.553528977	0.049563822
	5	10.28789209	0.511828155	0.049750537
	6	10.99188173	0.544674019	0.049552391
	7	11.17783733	0.607747453	0.054370755
	8	11.01925995	0.604013228	0.054814319
	9	10.73313445	0.616332247	0.057423323
	10	10.25653424	0.609223175	0.059398541
800 mg	1	10.02979265	0.836826918	0.083434119
	2	10.1821858	0.578209952	0.056786427
	3	10.80456918	0.655110049	0.060632686
	4	10.69402848	0.768477649	0.071860445
	5	10.57494392	0.614749271	0.058132627
	6	10.14675646	0.58712793	0.057863607
	7	10.31035283	0.713655726	0.069217391
	8	10.47333249	0.67924195	0.064854424
	9	10.67835556	0.673268752	0.063049853
	10	9.60688952	0.476714304	0.049622128

Table C – 12: Average brain Omega – 6 fatty acid percentages and ratios. (Data expressed as mean ± SEM)

Average Percentage (%)			
Group	**C20:4 ω6 AA**	**C22:4 ω6 Adrenic**	**Adrenic/AA**
Control	10.17147794 ± 0.132692428	0.835833608 ± 0.02246354	0.082275567 ± 0.002613516
400 mg	10.74842749 ± 0.223269029	0.685884342 ± 0.03449796	0.064083211 ± 0.00349491
600 mg	10.7301185 ± 0.119860313	0.62232616 ± 0.038019259	0.05808757 ± 0.003659365
800 mg	10.35012069 ± 0.116638282	0.65833825 ± 0.032334696	0.063545371 ± 0.002997106

Table C – 13: Brain Omega – 9 fatty acid percentages

Percentage (%)				
Group	**Rat**	**C18:1 ω9 Oleic**	**C22:1 ω9 Erucic**	**C24:1 ω9 Nervonic**
Control	1	20.10991435	0.084229053	0.705877423
	2	20.87791633	0.10239625	0.62988374
	3	21.2164949	0.112086831	0.658455546
	4	19.91431001	0.083796652	0.522019458
	5	20.36541363	0.081326771	0.485307887
	6	20.94900975	0.098092777	0.602214852
400 mg	1	21.0153913	0.083450664	0.69207882
	2	16.1086818		0.274209388
	3	20.66732631	0.0785585	0.6003969
	4	22.26078883	0.055689651	0.376451492
	5	16.45808429		0.30149429
	6	18.69753768	0.047328597	0.328439003
	7	20.38007152	0.073419528	0.440665869
	8	18.27088767	0.050020517	0.272230142
	9	20.19225985	0.054068936	0.416198125
	10	18.24684672	0.06740332	0.374457274
600 mg	1	19.84246666	0.079043687	0.631542654
	2	18.14367596	0.042054675	0.4599001
	3	19.44441995	0.066434374	0.497332045
	4	17.63634739	0.038910313	0.341755629
	5	17.466698		0.261592312
	6	17.05110191		0.257739008
	7	18.72268104	0.038812217	0.292185496
	8	17.9892109	0.052996245	0.269255115
	9	18.63463275	0.032098316	0.294457468
	10	17.71973676		0.296663856
800 mg	1	19.91330033	0.095913941	0.713985972
	2	18.65255676	0.020422065	0.244933656
	3	17.88691877	0.025788791	0.234939626
	4	19.1764792	0.050840868	0.436851739
	5	19.49882076	0.064175444	0.348580418
	6	19.10181846	0.056356455	0.303771927
	7	20.52451305	0.08476156	0.497661306
	8	19.60716367	0.065424049	0.368326355
	9	18.79609336	0.052945801	0.297082712
	10	19.54795123	0.052079997	0.362689965

Table C – 14: Average brain Omega – 9 fatty acid percentages. (Data expressed as mean ± SEM)

Average Percentage (%)			
Group	**C18:1 ω9 Oleic**	**C22:1 ω9 Erucic**	**C24:1 ω9 Nervonic**
Control	20.5721765 ± 0.211333214	0.093654722 ± 0.005078899	0.600626484 ± 0.03403
400 mg	19.2297876 ± 0.63494501	0.063742464 ± 0.004875425	0.40766213 ± 0.044093045
600 mg	18.26509713 ± 0.28126031	0.050049975 ± 0.006462954	0.360242368 ± 0.040026369
800 mg	19.27056156 ± 0.23061991	0.056870897 ± 0.00730172	0.380882368 ± 0.04497032