

Fatty acid metabolism in HepG2 cells:
Limitations in the accumulation of
docosahexaenoic acid in cell membranes

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Abstract

The current dietary recommendations for optimal health are designed to increase our intake of two bioactive omega-3 (n-3) fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), abundant naturally in fatty fish such as salmon. Health authorities recommend that the general population consume two to three fatty fish meals per week (1) for optimal health and for the prevention of cardiovascular disease. However, some modern Western societies consume only modest amounts of fish and seafood (2;3). Land based vegetable oils may provide an alternative to meet these needs. Linseed and canola oils are rich in α -linolenic acid (ALA, 18:3n-3) (4). ALA can be converted endogenously to EPA and DHA and suggests that increasing the dietary intake of ALA may increase the conversion and accumulation of DHA in tissues and plasma. However, elevated dietary intakes of ALA in animals and humans results in an increased level of EPA in tissues yet there is little or no change in the level of DHA (5-7). The current consensus is that the synthesis of DHA from ALA in humans is limited yet the mechanisms involved in regulating the accumulation of DHA in tissues are poorly understood.

The reputed rate-limiting enzyme in the conversion of fatty acids is $\Delta 6$ desaturase (D6D). ALA is a substrate for D6D and undergoes a series of desaturation and elongation reactions to yield n-3 long chain polyunsaturated fatty acids (LCPUFA). The final step in the synthesis of DHA from ALA involves translocation of its immediate fatty acid precursor, 24:6n-3 from the endoplasmic reticulum to the peroxisome to be partially β -oxidised to yield DHA. The involvement of multiple enzymes in the desaturation-elongation pathway, and the integration of other pathways, such as phospholipid biosynthesis, suggests there are various steps that

may regulate the accumulation of DHA in cell membranes. This thesis aimed to examine the possible regulatory steps in the conversion of fatty acids to LCPUFA, particularly in the synthesis of DHA from n-3 fatty acid precursors.

The human hepatoma cell line, HepG2, was used as an *in vitro* cell system to examine the accumulation of individual fatty acids and their metabolites in isolation from other competing fatty acid substrates. The accumulation of linoleic acid (LA, 18:2n-6) and ALA in HepG2 cell phospholipids following supplementation with increasing concentrations of each respective fatty acid correlated with that described *in vivo*, as was the accumulation of their conversion products. The accumulation of DHA in cells supplemented with ALA reached a plateau at concentrations above 5 µg/ml and paralleled the accumulation of 24:6n-3 in cell phospholipids, suggesting that the $\Delta 6$ desaturation of 24:6n-3 was prevented by increasing concentrations of ALA, thereby limiting the accumulation of DHA. The accumulation of DHA in cells supplemented with eicosapentaenoic acid (EPA, 20:5n-3) or docosapentaenoic acid (DPA, 22:5n-3) was significantly greater than the level of DHA that accumulated in cells supplemented with ALA. However, regardless of substrate, the level of DHA in cell membranes reached a plateau at substrate concentrations above 5 µg/ml.

This thesis further aimed to examine the effect of fatty acid supplementation on the mRNA expression of D6D in HepG2 cells. The expression and activity of D6D mRNA is subject to nutritional and hormonal regulation. The mRNA expression of D6D in HepG2 cells following supplementation with oleic acid (OA, 18:1n-9), LA, ALA, arachidonic acid (AA, 20:4n-6) or EPA was examined by real time RT PCR. The expression of D6D mRNA was reduced by up to 50% in cells supplemented with

OA, LA, ALA , AA or EPA compared with control cells and suggests that fatty acids modulate the expression of the key enzyme involved in the conversion of fatty acids.

The effect of fatty acid co-supplementation on the fatty acid composition of HepG2 cell phospholipids was also examined in an attempt to gain insights into the role of D6D and the enzymes involved in peroxisomal β -oxidation on the accumulation of DHA from n-3 fatty acid precursors. The reduction in the accumulation of DHA in cells co-supplemented with DPA and docosatetraenoic acid (DTA, 22:4n-6) was greater than in cells co-supplemented with DPA and LA, suggesting that peroxisomal β -oxidation may have a greater role in determining the accumulation of DHA from DPA than the activity of D6D. Further investigation should be directed towards understanding the role that peroxisomal β -oxidation may play in the synthesis of DHA from precursor fatty acids.

The fatty acid composition of cell membranes *in vivo* is a result of several physiological processes including dietary intake, phospholipids biosynthesis and fatty acid conversion as well as catabolic processes. This thesis demonstrates that a greater understanding of the regulation of the conversion of fatty acids will help to define dietary approaches that enhance the synthesis of n-3 LCPUFA from n-3 fatty acid precursors to lead to improved outcomes for health.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Roxanne Portolesi

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LIST OF ABBREVIATIONS

A

1-acyl-GCP	1-acyl- <i>sn</i> -glycero-3-phosphocholine
AA	arachidonic acid
ACC	acetyl CoA carboxylase
ALA	α -linolenic acid
ALD	adrenoleukodystrophy
ANOVA	analysis of variance
ATP	adenosine triphosphate

B

bp	base pair
BSA	bovine serum albumin

C

cDNA	complementary DNA
CPT	carnitine palmitoyl transferase
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT	threshold cycle

D

d	day
D5D	Δ 5 desaturase
D6D	Δ 6 desaturase
DHA	docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
DTA	docosatetraenoic acid

E

EDTA	ethylenediaminetetraacetic acid
EFAD	essential fatty acid deficiency
en	energy
EPA	eicosapentaenoic acid

F

FABP	fatty acid binding protein
FAME	fatty acid methyl ester
FAS	fatty acyl-CoA synthetase
FAT	fatty acid transport (protein)
FBS	foetal bovine serum
FFA	free fatty acid

G

GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GLA	γ -linolenic acid
GM	genetically modified

H	
h	hour
L	
LA	linoleic acid
LCPUFA	long chain polyunsaturated fatty acid
LDH	lactate dehydrogenase
I	
INT	tetrazolium salt
M	
min	minute
mRNA	messenger ribonucleic acid
N	
NADPH	nicotinamide adenine dinucleotide phosphate
ND	not detected
NEFA	non-esterified fatty acid
NH&MRC	National Health and Medical Research Council
O	
OA	oleic acid
P	
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-activated receptor response element
PUFA	polyunsaturated fatty acid
R	
RCT	randomised control trial
RIPA	radio immuno precipitation assay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcribed PCR
S	
s	second
SC-26196	2, 2-diphenyl-5-(4-(8)piperazine-1-yl)pentanenitrile
SCD	stearoyl-CoA desaturase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
T	
TCA	tricarboxylic acid
TG	triglyceride
TLC	thin layer chromatography

V	
v	volume
VLCFA	very long chain fatty acid
vs	versus
W	
wt	weight

1 Literature Review

1.1 DIETARY FAT AND FATTY ACIDS

One of the major energy sources in the typical Western diet is fat, accounting for 26-53% total energy (9;10). Fat occurs naturally in foods such as red meat, fish, dairy foods, eggs and oils. Aside from its principal role in providing energy to the body, dietary fat also influences many physiological processes. The fatty acid composition of cell membranes modulates its structure, fluidity and function, including the production of eicosanoids and other secondary messengers (11), receptor function, and enzyme activity (12). Increased cellular fatty acid levels stimulate fatty acid oxidation (both mitochondrial and peroxisomal), inhibit glucose uptake and glycolysis and promote gluconeogenesis (13). The fatty acid composition of cell membranes therefore plays a major role in regulating cell metabolism.

Dietary fat commonly consists of three fatty acids esterified to a glycerol backbone to form a triglyceride. Generally, fatty acids are hydrocarbon chains with a carboxyl group at one end and a methyl group at the other and can be divided into three groups according to their degree of saturation (number of double bonds between carbon atoms). Saturated fatty acids are straight chain fatty acids of variable carbon chain length with no double bonds, monounsaturated fatty acids have one double bond along the fatty acid chain and polyunsaturated fatty acids (PUFA) contain more than one double bond in the fatty acid chain. Fatty acids are systematically named by the number of carbons in the fatty acid chain and the number of double bonds (14). For example, a saturated fatty acid with 16 carbon atoms is systematically named hexadecanoic acid (16:0), although its common name is palmitic acid. Linoleic acid (LA, 18:2n-6) and α -linolenic acid (ALA, 18:3n-3) belong to the omega-6 (n-6) and omega-3 (n-3) families, respectively. The 'n' nomenclature refers to the position of

the double bonds within the carbon chain from the methyl terminus. N-6 fatty acids have their first double bond on the sixth carbon atom from the methyl end, whereas n-3 fatty acids have their first double bond on the third carbon atom.

In 1929, Burr *et al.* (1929) (15) identified LA and ALA as essential for growth and reproduction and in preventing the symptoms of essential fatty acid deficiency (EFAD). These symptoms include eczema and poor growth. ALA is found in plant chloroplast membranes where it is synthesized from LA, which in turn is derived from acetate (16). In plants, ALA is found in leaves, mainly in glycolipids, and as triglycerides in certain seed oils (rapeseed, flaxseed, perilla seed, chia seed), beans (soybeans, navy beans) and nuts (walnuts) (17). LA is a major component of safflower oil. The enzymes responsible for the synthesis of LA and ALA from precursor fatty acids are the $\Delta 12$ and $\Delta 15$ desaturases. Whilst plants express $\Delta 12$ and $\Delta 15$ desaturase, animals, including humans, do not and must consume dietary LA and ALA to prevent symptoms of EFAD. As such, ALA and LA are termed essential fatty acids. The daily dietary intake of ALA in the Western diet is approximately 1.5 g/d compared with the daily intake of LA of 10.8 g (5).

In animals, including humans, ALA and LA are fatty acid precursors to the 20 carbon and 22 carbon PUFA. Arachidonic acid (AA, 20:4n-6) is derived from LA and is the main substrate for a series of enzymes that produce the 2-series eicosanoids, such as thromboxane, prostaglandins, prostacyclins, leukotrienes and lipoxins (18). ALA can be converted endogenously to eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), although EPA and DHA can be consumed directly through the diet. EPA and DHA are abundant naturally in fatty fish such as salmon. However, minimal quantities of EPA and DHA are consumed

compared with LA and ALA. The average intakes of EPA and DHA are 0.056g/d and 0.106g/d, respectively (19).

EPA and DHA are putative antiinflammatory, antiarrhythmic and antiatherogenic agents with potent triglyceride lowering effects, and some activity on plasma cholesterol, particularly in hypercholesterolemic patients (20). EPA negates the proinflammatory and atherogenic effects of lipid mediators derived from AA through its conversion to 3-series eicosanoids (21). The cardiovascular disease prevention conferred by EPA and DHA is attributed to these physiological effects and highlights the role that dietary fatty acids play in maintaining health. Mounting evidence for a positive role of n-3 fatty acids in health has resulted in health authorities recommending an increase in the dietary intake of EPA and DHA. The American Heart Association recommends that the general population consume two to three fatty fish meals per week (equivalent to 0.43-0.57 g/d EPA and DHA) (1) for optimal health.

However, some modern Western societies consume only modest amounts of fish and seafood (2;3). For instance, Australian adults consume 1/6 the quantity of fish and seafood as compared to meat, poultry and game (2;3). The recommended consumption of fish calls for major changes in dietary habits which are unlikely due to public perceptions of possible mercury contamination in seafood, dislike of the taste and/or smell of fish or the perception that seafood is more expensive than meat (22;23). As an alternative, the diet may be enriched with n-3 long chain polyunsaturated fatty acids (LCPUFA) by consuming modified foods high in n-3 LCPUFA or with fish oil supplements, rich in EPA and DHA. These practices effectively elevate the n-3 LCPUFA status of individuals but rely heavily on declining global fish stocks (24). Exploitation of this natural resource has reduced

catch size, both the number of fish and their maturity (24) and is no longer environmentally sustainable. Single-cell oils derived from the fermentation of microorganisms (microalgae or microfungi) are another source of DHA (25) but are expensive to produce in significant quantities (26). Plant oils have the potential to be developed into sustainable and affordable sources of n-3 LCPUFA through the transgenic expression of genes encoding LCPUFA biosynthetic pathways from other organisms (27;28). For the first time in seed oils, DHA has been synthesised from 18 carbon fatty acid precursors in the *Arabidopsis thaliana* plant (28). Public acceptance of the production of oils from genetically modified (GM) plants will be a major challenge for this industry as consumer attitudes towards GM food products are largely negative in many of the developed countries (29). This combination of factors clearly indicates a need for an alternative sustainable source of n-3 LCPUFA to meet the current dietary recommendations for health.

Land based vegetable oils, such as linseed and canola oils, may provide an alternative to meet the n-3 recommendations. Since ALA can be converted to EPA and DHA endogenously, it was thought that increasing the dietary intake of ALA may increase the conversion and accumulation of DHA in tissues and plasma. However, whilst elevated dietary intakes of ALA in animals and humans results in an increased level of EPA in tissues, there is little or no change in the level of DHA (5-7). Direct measurement of fatty acid conversion in humans using labelled ALA supports the conversion of ALA to EPA and docosapentaenoic acid (DPA, 22:5n-3) with limited conversion to DHA (6;30-32) suggesting a complex relationship between ingested ALA and its conversion to DHA.

1.2 HEALTH BENEFITS OF N-3 FATTY ACIDS

Among the n-9, n-6, n-3 and saturated fatty acids, the n-3 fatty acids are associated with improved health outcomes. EPA and DHA have shown particular efficacy in the prevention of cardiovascular disease (33-37) and in infant development (38-43) and are also beneficially implicated in arthritis (44) and diabetes (45-47). Whilst cell culture and animal studies support a preventive action of n-3 LCPUFA in carcinogenesis (48;49), there is divided opinion on the association between n-3 LCPUFA intake and cancer risk in humans. An extensive systematic review of 20 prospective cohort studies concluded that there was no significant association with n-3 fatty acids and the incidence of cancer (50). The role of n-3 LCPUFA in cardiovascular disease and infant development are two areas that have received considerable research.

The association between the dietary intake of n-3 LCPUFA and cardiovascular disease prevention began when Bang and Dyerberg (1980) (51) reported low mortality rates from ischemic heart disease in Inuits consuming a high proportion of fish. Several intervention studies have also shown an improvement in cardiovascular health following n-3 fatty acid supplementation (33-35;52). Bucher *et al.* (2002) (53) performed a meta-analysis of 11 randomised control trials (RCT) that compared dietary or non-dietary (supplements) intake of n-3 LCPUFA (EPA and DHA) with a control diet or placebo in patients with coronary heart disease and found significant protective effects of EPA and DHA on rates of fatal myocardial infarction, sudden death and overall mortality in treatment groups. In contrast, a Cochrane review by Hooper *et al.* (2004) (54) concluded that the evidence to support a role for dietary n-3 fatty acids in cardiovascular disease prevention was inconclusive. The Cochrane review has challenged the current opinion and sparked several responses (55-57) to refute the conclusions by Hooper *et al.* (2004) (54). One of the major criticisms of

the Cochrane review was the inclusion of the follow-up to the Diet and Reinfarction Trial (DART 2) (58), which contributed to the significant heterogeneity between studies included in the analysis. Omitting the DART 2 Study produced similar relative risks for cardiovascular disease reported by Bucher *et al.* (2002) (53). The 20% reduction in all-cause mortality reported in patients with pre-existing coronary heart disease supplemented with 850 mg EPA and DHA in the GISSI Trial (36) is striking evidence for a benefit. There are also sound biochemical theories to explain the health benefits conferred by n-3 LCPUFA in preventing cardiovascular disease including their activity as antiinflammatory, antiatherogenic and antiarrhythmic agents (33;59). A difficulty in systematic reviews is the heterogeneity between studies, including differences in population characteristics, lifestyle, dose and type of n-3 LCPUFA. Overall however, there is evidence to support a role of n-3 fatty acids in cardiovascular health.

Benefits of n-3 LCPUFA have also been described for infants. Nutritional intervention studies in infants have been spurred by reported differences in cognitive development of breast fed infants and their formula fed counter-parts (43), an observation which has been attributed to the fact that human breast milk is a rich source of DHA, whereas standard infant formulas contain no DHA (60-62). Controversy exists regarding the addition of preformed arachidonic acid (AA, 20:4n-6) and DHA, as found in human milk, to infant formulas containing only LA and ALA, for optimal brain development and to ensure the long-term integrity of functional outcomes, such as growth, in infants. A Cochrane review of n-3 and n-6 LCPUFA supplementation in preterm infants, which included 11 RCT assessing the clinical effects of feeding formula supplemented with n-3 and n-6 LCPUFA, concluded that no long-term benefits were demonstrated for infants receiving LCPUFA and there was no evidence that supplementation of formula with n-3 and n-

6 LCPUFA impaired the growth of preterm infants (63). A review of ten RCT designed to test the efficacy and safety of adding either n-3 LCPUFA or a combination of n-3 LCPUFA and AA to formulas for term infants (64) found mixed evidence for support of an effect of dietary n-3 and n-6 LCPUFA on either visual acuity or mental development. The current consensus is that the benefits of DHA supplementation for term infants are smaller than for preterm infants (65). The strongest evidence for good developmental outcomes for infants is breastfeeding for at least six months (65).

1.3 FATTY ACID METABOLISM

Fatty acids are ingested as triglycerides and phospholipids and hydrolysed in the small intestine by pancreatic lipase and other enzymes. Hydrolysis products absorbed by the intestinal mucosa are recombined into triglycerides and phospholipids, which combine with apoproteins to form lipoproteins (66). This process solubilises the fatty acids and permits their transport through the blood to other tissues where the fatty acid can enter various metabolic pathways.

1.3.1 Fatty acid transport

Fatty acids are transported between organs in the blood either as non-esterified fatty acids (NEFA) complexed to albumin or as triglycerides and phospholipids associated with chylomicrons and very low density lipoproteins. Fatty acids associated with lipoproteins can be hydrolysed by two related lipases, hepatic lipase and lipoprotein lipase (67) enabling entry of fatty acids into the cell either by passive diffusion (14;68) or facilitated membrane transport (14;67;69). Non-protein mediated, passive entry of fatty acids involves adsorption to the membrane, protonation, flip-flop within the membrane and desorption into the cytosolic space (68). Various factors can influence the rate of passive fatty acid movement across cell membranes. These include the affinity of fatty acid binding to sites on albumin (70), the transmembrane

pH gradient, the transmembrane gradient of fatty acids, modification of the fatty acid to membrane impermeable derivatives on the trans side of the membrane and the utilisation of the fatty acid for anabolic and catabolic processes (71).

Transport of fatty acids through cell membranes may also be facilitated by proteins, including fatty acid binding proteins, fatty acid translocase, (FAT, also known as CD36) and fatty acid transport proteins. All three proteins are regulated to some degree by members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (14;72-74). Inside the cell, fatty acids are bound to cytoplasmic fatty acid binding proteins (FABP) (71;75). It is believed that FABP solubilise and protect fatty acid ligands in aqueous spaces and facilitate their transport in the cytosol (76). There is evidence that FABP may influence fatty acid metabolism through facilitating uptake, transport, sequestration, and/or metabolic targeting of fatty acids (77;78). The discovery of these transport proteins indicates a potential regulatory point of entry for fatty acids into various metabolic pathways. The cellular uptake of fatty acids with their entry into these pathways may be affected by changes in the presence and/or activity of these transport proteins.

1.3.2 Activation and entry of fatty acids into several metabolic pathways

Once inside the cell, fatty acids can enter several metabolic pathways (Figure 1-1). A requisite step before entry into these pathways involves activation of fatty acids by the action of fatty acyl-CoA synthetases (FAS), abundant in microsomes and mitochondria (75), to form fatty acyl-CoA-thioesters. The FAS enzymes differ from each other with respect to their subcellular locations and their specificities for fatty acids of different chain lengths. The overlapping chain length specificities and tissue distributions mean that most saturated or unsaturated fatty acids in the range of 2-22

carbons can be activated in animal tissues, although at different rates (14). Activated fatty acids are also bound to FABP (67) and serve as a reservoir for intracellular fatty acyl-CoA that can be transported to various subcellular compartments (79). Within these compartments, the activated fatty acids can be substrates for;

- i. β -oxidation; (see Section 1.4)
- ii. elongation and desaturation reactions; or (see Section 1.5)
- iii. assimilation into complex lipids, including triglycerides, cholesterol esters or phospho- and sphingo-lipids (see Section 1.6).

Dietary fatty acid intake, fatty acid conversion and acylation into membrane lipids has a concerted influence on the fatty acid composition of cell membranes and thereby, cellular function.

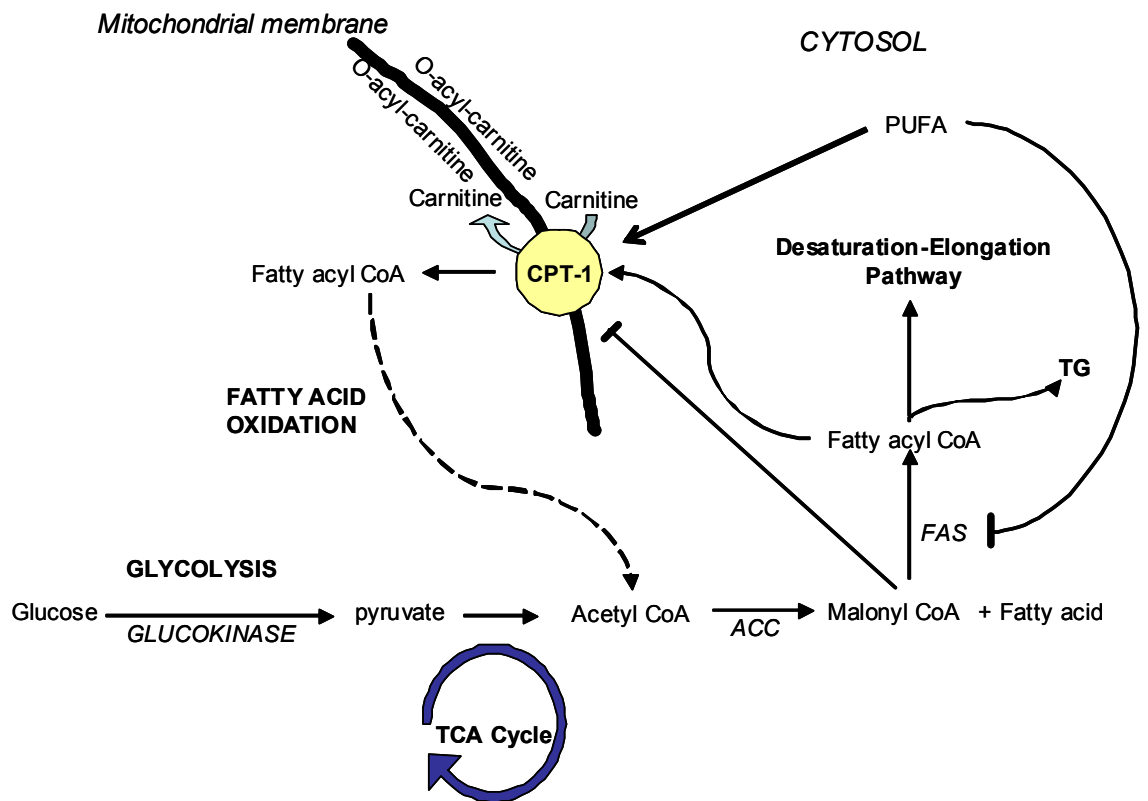


Figure 1-1. Activation of fatty acids in the cytosol and entry into the desaturation-elongation pathway or mitochondrial β -oxidation.

PUFA concomitantly increase the expression of CPT-1 and decrease the expression of FAS, directing fatty acids towards oxidation and away from the desaturation-elongation pathway. Malonyl CoA binds to the N-terminal of CPT-1, inhibiting the transport of fatty acid across the mitochondrial membrane. (ACC; Acetyl CoA carboxylase, FAS; fatty acyl-CoA synthetase, CPT-1; carnitine palmitoyltransferase-1, PUFA; polyunsaturated fatty acid, TG; triglyceride, TCA cycle; tricarboxylic acid cycle).

The expression of genes coding for the enzymes involved in the activation of fatty acids is regulated by fatty acids themselves. Nakamura *et al.* (2000) (80) reported that rats fed a fat-free diet supplemented with 4 weight (wt) % LA reduced hepatic FAS mRNA expression by approximately 50%. Ide *et al.* (2000) (81) report that hepatic FAS mRNA expression was reduced in rats fed a 15% fat diet as either perilla oil (containing ~60% total fatty acids as ALA), safflower oil (~77% total fatty acids as LA), linseed oil (~50% total fatty acids as ALA) and fish oils compared with rats fed palm oil (40-55% total fatty acids as 16:0). The reduced expression of FAS by dietary PUFA suggests that the activation of fatty acids for entry into metabolic pathways is tightly controlled.

De novo fatty acid synthesis involves acetyl CoA carboxylase (ACC) and FAS. The carboxylation of acetyl CoA to malonyl CoA is the first committed step in fatty acid synthesis. The malonyl CoA generated by the action of ACC forms the source of nearly all the carbons for the fatty acyl chain. The second step involves the activation of the fatty acyl CoA by FAS in the presence of NADPH, which results in the esterification of the fatty acid, as described above.

1.4 β -OXIDATION

The entry of fatty acids into catabolic pathways, such as β -oxidation, is also regulated by PUFA. Fatty acids enhance PUFA transport into mitochondria, the major site of fatty acid β -oxidation, via their action on carnitine palmitoyl transferase (CPT). The inner mitochondrial membrane is impermeable to CoA and its derivatives, therefore fatty acyl CoAs formed in the cytosol cannot directly enter the mitochondria for β -oxidation. Entry of activated fatty acids into the mitochondrion is governed by CPT, which transfers the long chain acyl groups from CoA to

carnitine to yield a fatty acyl carnitine. Two isoforms of CPT have been identified (82). CPT-1 is located on the outer mitochondrial membrane, and CPT-2 is on the inner membrane. Peroxisomes and the endoplasmic reticulum also contain CPTs, all of which are inhibited by malonyl CoA (75). PUFA have been shown to regulate the activity of CPT-1, thereby regulating entry into fatty acid oxidation (81;83;84). Ide *et al.* (2000) (81) reports that the activity of CPT-1 is increased by 50% in the liver of rats fed dietary perilla and fish oil, compared to those supplemented with palm or safflower oil. PUFA enhance fatty acid oxidation by co-ordinately increasing the expression of mitochondrial CPT and peroxisomal acyl CoA oxidase, by decreasing the synthesis of malonyl CoA and by decreasing the sensitivity of CPT to malonyl CoA inhibition (83;84). CPT therefore represents another site in the regulation of energy metabolism by PUFA.

Both mitochondria and peroxisomes carry out fatty acid β -oxidation. This cyclic process is similar in both organelles, with each cycle containing dehydrogenation/oxidation, hydration and thiolitic cleavage steps (Figure 1-2). In general, one cycle of β -oxidation shortens an acyl chain by two carbons, releasing one molecule of acetyl-CoA in a process mediated by a sequence of enzymes, each of which is specific for its substrate. However, there are some differences in β -oxidation between the two organelles. In mitochondrial β -oxidation, the preferred substrates are fatty acids with a chain length of less than 20 carbons. These fatty acids enter the organelle by the carnitine transport system and are usually degraded completely to acetyl-CoA via several β -oxidation cycles. Peroxisomal fatty acid β -oxidation is capable of oxidising very long chain fatty acids of 24 carbons. Entry of these substrates does not require carnitine but may involve ATP-binding cassette transporters such as the adrenoleukodystrophy (ALD) protein (85). Peroxisomal β -

oxidation does not proceed to completion but rather only through a few cycles in which the acyl chain is shortened and the shortened fatty acyl CoA returns to the endoplasmic reticulum for assimilation into cell lipids (85).

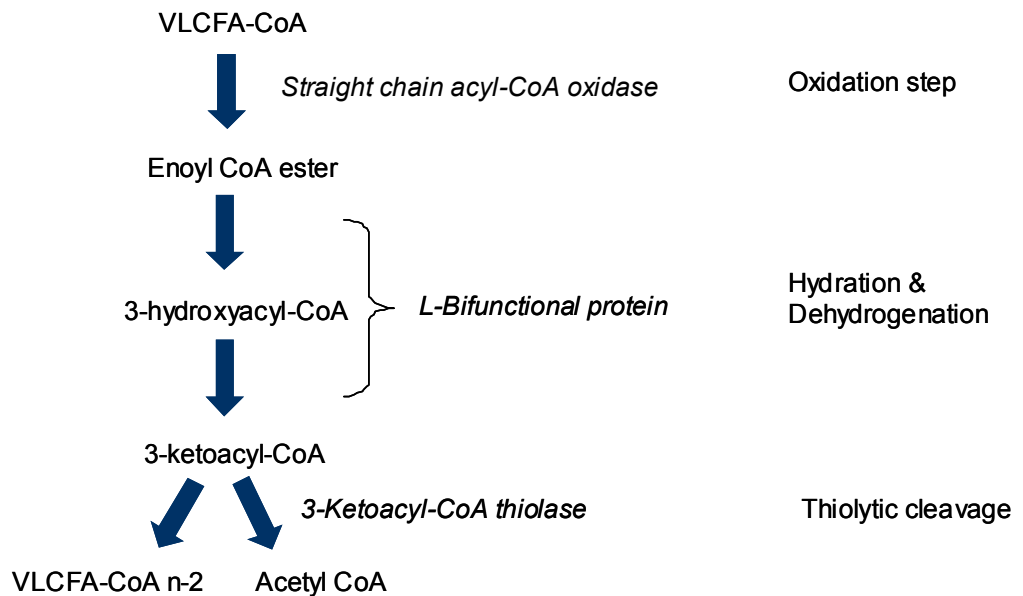


Figure 1-2. Peroxisomal β oxidation of straight chain fatty acid.

There are two complete sets of β oxidation enzymes present in the peroxisome (86). Straight chain acyl CoA oxidase is responsible for the initial oxidation of very long chain fatty acyl CoAs (VLCFA), whereas branched chain acyl CoA oxidase oxidises branched chain fatty acyl CoA. Enoyl CoA esters are then hydrated and subsequently dehydrogenated by the same enzyme. The last step is thiolytic cleavage, performed by sterol carrier protein X in the case of branched chain substrates, whereas straight chain substrates utilise 3-ketoacyl-CoA thiolase.

1.5 CONVERSION OF FATTY ACIDS

The first and rate-limiting enzyme involved in the synthesis of LCPUFAs is $\Delta 6$ desaturase (D6D) (87). D6D introduces a double bond at the $\Delta 6$ position from the

carboxyl end of a fatty acid. This reaction requires molecular oxygen, NAD(P)H and the cytochrome b5 electron transport system (88).

Figure 1-3 shows the series of desaturation and elongations that occur to n-3 and n-6 fatty acids in the endoplasmic reticulum.

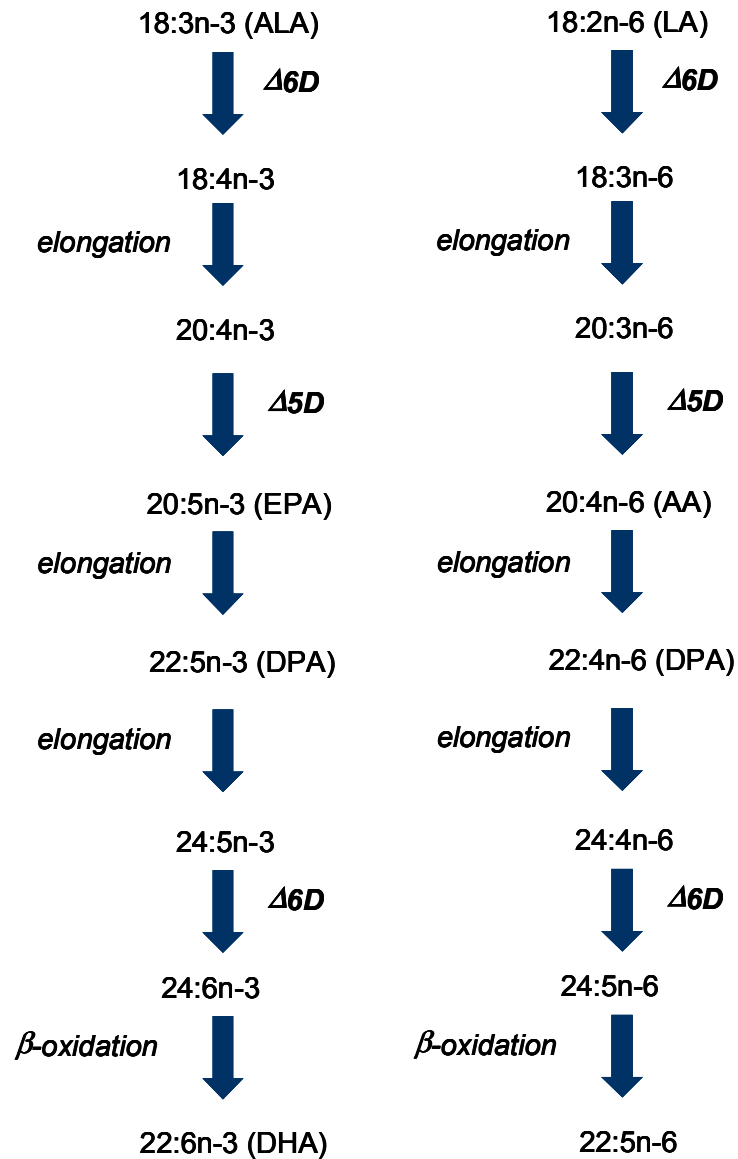


Figure 1-3. Desaturation and elongation of n-3 and n-6 fatty acids.

Elongation and desaturation steps occur in the endoplasmic reticulum. The final β -oxidation step occurs in the peroxisome. $\Delta 6$ desaturase ($\Delta 6D$) is used twice in the synthesis of 22:6n-3 and 22:5n-6 from ALA and LA, respectively.

LA and ALA are substrates for this enzyme and compete for active sites on the enzyme. The products of Δ^6 desaturation, γ -linolenic acid (GLA, 18:3n-6) and stearidonic acid (18:4n-3), are elongated to dihomo- γ -linolenic acid (20:3n-6) and 20:4n-3, respectively. Elongation is a multi-enzyme process that adds two carbon units to the carboxyl end of the fatty acid chain (79;89). Biochemical characterisation of mammalian elongation systems has indicated that the 'elongase' step in the conversion of fatty acids actually consists of four enzymatic reactions, including condensation, β -keto-reduction, dehydration and enoyl reduction. The predominant pathway for fatty acid elongation also occurs in the endoplasmic reticulum. These enzymes work in concert with the desaturases and use malonyl CoA and fatty acyl CoA as substrates for two carbon additions to the fatty acids (79). In the cell, there exist multiple microsomal elongation systems with different chain length specificity (89;90). Six distinct elongases have been described, each with overlapping substrate specificity. The chain elongation system accepts a wide variety of saturated and unsaturated fatty acids as primers for chain elongation (89).

The products of elongation, 20:3n-6 and 20:4n-3, are then further desaturated to the 20 carbon fatty acids by Δ^5 desaturase (D5D) to generate arachidonic acid (AA, 20:4n-6) and EPA. D5D accepts fatty acids with their first double bond at position eight (89) and introduces a double bond at the Δ^5 position along the carbon fatty-acyl chain. AA and EPA are further elongated to 22:4n-6 and 22:5n-3, respectively, and finally to their respective elongation products, 24:4n-6 and 24:5n-3. These final elongation products then become substrates for D6D. Several studies have indicated the utilisation of D6D by LA and ALA, and 24:4n-6 and 24:5n-3. The conversion of LA to 18:3n-6 and 24:5n-3 to 24:6n-3 has been demonstrated in COS-7 cells expressing the wild type rat D6D (91). Human skin fibroblasts isolated from a

patient with clinical evidence of an inherited abnormality in PUFA metabolism, including reduced plasma levels of AA and DHA compared with normal human plasma, showed a reduced conversion of labelled LA to AA, 24:4n-6 and 22:5n-6; and ALA to 24:5n-3 and DHA, compared with normal human fibroblasts (92). Northern blot analysis indicated that the reduced conversion of D6D substrates may be due to the 81-94% decrease in D6D mRNA content compared with normal human fibroblasts (92). de Antueno *et al.* (2001) (93) have also demonstrated that a single human D6D is active on LA, ALA, 24:4n-6 and 24:5n-3. Therefore, it is generally accepted that D6D is used twice in the conversion of ALA to DHA (and LA to 22:5n-6).

Until recently, a $\Delta 4$ desaturase was suspected in the synthesis of DHA and its n-6 homologue, 22:5n-6, from 24:6n-3 and 24:5n-6 respectively. In 1991, Sprecher's group demonstrated that DHA was synthesised by further $\Delta 6$ desaturation of 24:5n-3 to 24:6n-3 followed by peroxisomal β -oxidation to DHA (94). These results were recently confirmed by D'andrea *et al.* (2002) (95). It is now accepted that the final step in the synthesis of DHA (and 22:5n-6) from precursor fatty acids involves partial β -oxidation to remove two carbons in the peroxisome. This process relies on the movement of acyl fatty acid groups from the endoplasmic reticulum to the peroxisome, a process that must be tightly regulated (96).

Studies in patients with disorders of peroxisomal β -oxidation (Zellwegers syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease) indicate the involvement of this organelle in the synthesis of DHA. Patients with Zellwegers syndrome, who lack functional peroxisomes, have reduced levels of DHA in tissues, especially in brain and retina, but also in liver, kidney and blood (97-99). Moore *et*

al. (1995) (100) reported that normal human fibroblasts metabolised 1-¹⁴C ALA to labelled 24:5n-3, 24:6n-3 and DHA. In contrast, fibroblasts from patients with Zellwegers syndrome metabolised labelled ALA to 24:5n-3 and 24:6n-3 but not to DHA. Likewise, 3-¹⁴C 22:5n-3, 3-¹⁴C 24:5n-3 and 3-¹⁴C 24:6n-3 were all metabolised to DHA in normal fibroblast but not in fibroblasts from patients with Zellwegers syndrome. When fibroblasts from patients with Zellwegers syndrome were supplemented with 1-¹⁴C DPA, large amounts of 24:6n-3 accumulated but did not proceed further to form DHA (99). Animal models of Zellwegers syndrome, such as the PEX5 knockout mouse model, also support the role of the peroxisome in the synthesis of DHA (101). These animals have a 40% reduction in the level of DHA in brain tissue compared with the wild type (101). Fatty acid metabolic studies using peroxisomal deficiencies also demonstrate that the synthesis of DHA from ALA involves the formation of 24 carbon polyunsaturated intermediates followed by partial β -oxidation in the peroxisome to DHA, as described above. These studies highlight the importance of the peroxisome and peroxisomal β -oxidation in the synthesis of DHA from ALA. The involvement of two different organelles in the biosynthesis of DHA implies that intracellular movement of fatty acids occurs between the endoplasmic reticulum and the peroxisome, representing a further regulatory site in the synthesis and accumulation of DHA and the level of DHA in cell membranes (102).

Figure 1-4 is a circular representation of the desaturation-elongation pathway, depicting the complexities involved in fatty acid conversion and emphasises the use of D6D twice in the conversion of ALA (and LA) to DHA (and 22:5n-6). This pathway indicates that there are four competing substrates for D6D. Therefore, D6D is used twice in the synthesis of DHA from ALA. It has been reported that the 18 carbon fatty acid substrates are preferred over the 24 carbon fatty acids (87;95). The

endogenous synthesis of LCPUFA from precursor fatty acids is therefore dependent on the competition between D6D substrates. The involvement of several enzymatic steps in the conversion of PUFA to LCPUFA implicates the desaturation-elongation pathway as a potent regulator of the fatty acid composition of cell membranes, and particularly the conversion of ALA to DHA.

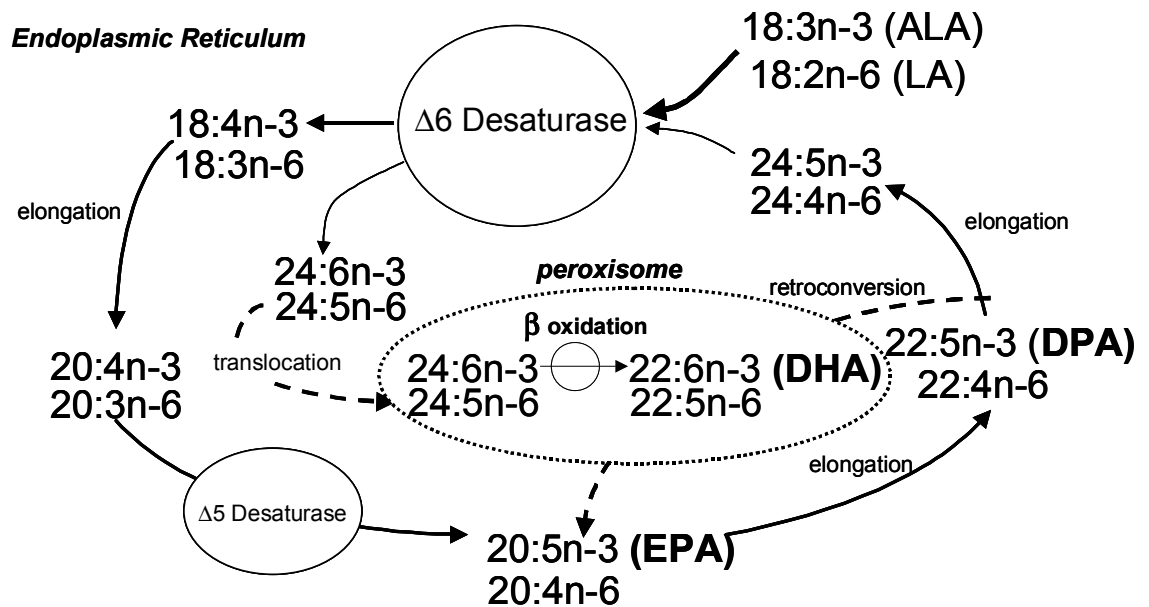


Figure 1-4. Circular representation of n-3 and n-6 fatty acid conversion.

Elongation and desaturation occurs in the endoplasmic reticulum. The final step in the synthesis of docosahexaenoic acid (DHA) and 22:5n-6 from α -linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively, occurs in the peroxisome. The diagram indicates that there are four competing substrates for $\Delta 6$ desaturase and that $\Delta 6$ desaturase is used twice in the synthesis of DHA and 22:5n-6 from ALA and LA, respectively. EPA; eicosapentaenoic acid, DPA; docosapentaenoic acid.

1.5.1 $\Delta 6$ desaturase

D6D was first cloned from the cyanobacterium; *Synechocystis*, and has since been cloned from the wild herb; *Borage officinalis* (103), the nematode; *Caenorhabditis elegans* (104), mice (105), rats (106) and humans (105). D6D is localised on human chromosome 11 (11q12-q13.1) and is expressed in nearly all human tissues, with the greatest activities found in the liver, heart and brain (107). Structurally, the enzyme

has two membrane spanning domains and three Histidine-box motifs that are characteristic of membrane-anchored desaturases and a cytochrome b5 domain (88). Research has shown that D6D enzymatic activity and mRNA expression varies with hormonal and nutritional manipulation (80;107-109).

1.5.1.1 Δ 6 desaturase activity

The activity of the microsomal enzyme, D6D, has been examined using isolated rat liver microsomes (110;111). In the presence of co-factors, such as ATP, coenzyme A and NADH, microsomal preparations have been used to demonstrate the conversion of radiolabelled substrate fatty acids to their immediate products. For example, the rate of conversion of ALA to its immediate product, 18:4n-3, was greater than the conversion of LA to GLA (110). Studies show the greatest activity of D6D when ALA was the substrate, followed by LA and OA (110;111). 24:4n-6 and 24:5n-3 are also substrates for D6D and are desaturated to yield 24:5n-6 and 24:6n-3, respectively, however, kinetic studies indicate a greater capacity for D6D to desaturate 18 carbon fatty acids over the 24 carbon fatty acids (112). One study using isolated microsomes from human fetal liver has shown a similar pattern of activity (113). The enzyme kinetics of D6D will influence the conversion of substrate fatty acids and their availability for incorporation into cellular lipids or β -oxidation.

The fatty acid composition of the background diet also modulates D6D activity. Voss *et al.* (1991) (94) has shown that the desaturation of LA and 24:5n-3 was significantly greater in animals maintained on a fat-free diet compared with those fed rat chow containing PUFA (94). The level of DHA converted from DPA in microsomes isolated from rats fed a fat free diet was seven times that observed in rats fed a chow diet (94). It has also been shown that D6D activity correlates with the

amount of D6D protein. In rats treated with clofibric acid, an increase in the activity of D6D was attributed to the induction of the protein as administration of an inhibitor of protein synthesis (cycloheximide) prevented the increased conversion of LA to GLA (114). A more direct measure of the level of protein is the use of specific antibodies to quantify the protein. Using affinity purified anti-rat D6D rabbit serum, Shoji *et al.* (2003) (115) showed that the increased conversion of [¹⁴C] LA to [¹⁴C] GLA induced by treatment with gemfibrozil correlated to an increase in the level of D6D protein. These studies indicate that the activity of the key enzyme in the conversion of fatty acids can be modulated by the diet and thereby affect the conversion of substrates and influence the fatty acid composition of cell membranes.

1.5.1.2 mRNA expression of $\Delta 6$ desaturase

Dietary studies indicate that hepatic D6D mRNA expression is induced by diets low in LA and ALA and suppressed by diets rich in vegetable or marine oils (80;105;107). D6D mRNA expression may be used as a surrogate marker for the activity of the enzyme as some studies have shown that the suppression of D6D mRNA expression by dietary PUFA is associated with reduced D6D activity in rodents (107;108). Tang *et al.* (2003) (107) demonstrated that both D6D mRNA expression and activity was reduced by 60% in rats fed a high carbohydrate diet supplemented with 10 weight (wt) % safflower oil compared with those fed a fat-free diet or those supplemented with 10 wt % triolein, a triglyceride composed of oleic acid (OA, 18:1n-9). Also, rats fed a 10 wt % fish oil supplemented diet as compared with a chow fed diet resulted in a 40% reduction in D6D activity which correlated with a decrease in hepatic mRNA D6D expression (108).

Nuclear run-on analysis suggests that modulation of D6D mRNA expression by PUFA is due to regulation at the transcriptional level (107). Fatty acids are thought

to bind to peroxisome proliferators-activated receptors (PPARs) that bind to peroxisome proliferators-activated receptor response elements (PPRE) on the D6D gene (116-118). The group of lipid lowering drugs, known as fibrates, are also agonists for PPAR and modulate fatty acid metabolism. PPREs have been identified on genes which are components of the peroxisomal long chain fatty acid β -oxidation pathway including the long chain fatty acyl CoA oxidase, the enoyl CoA hydratase and the 3-oxoacyl CoA thiolase (reviewed in (119)). However, there appears to be species differences in the control of fatty acid metabolism and their response to PPAR agonists. In rodents, PPAR agonists cause hepatic peroxisome proliferation, hepatomegaly and, with prolonged administration, hepatocarcinogenesis, in addition to the induction of the fatty acid oxidation enzymes (120). Unlike isolated hepatocytes and tumour derived liver cell lines from rats and mice, human hepatocytes and HepG2 cells do not exhibit peroxisome proliferators-dependent peroxisome proliferation or induction of peroxisome proliferator-associated genes (114). Species differences in the response to synthetic PPAR agonists may also extend to the response to natural PPAR agonists, i.e. fatty acids. Therefore, the expression and regulation of D6D and enzymes involved in peroxisomal β -oxidation in rodents may not translate to that in humans. There is considerable research into the effect of dietary fatty acids on the mRNA expression of D6D in rodents, as described below, yet research into human D6D mRNA expression and its regulation is sparse.

Diets containing corn oil, safflower oil (containing 77% of total fatty acids as LA) or fish oil (rich in EPA and DHA) reduced the expression of D6D mRNA compared with rats maintained on a diet containing either no fat or triolein (105;107). The reduced D6D mRNA expression following LA supplementation may also arise from its conversion to AA. AA has been shown to reduce the expression of D6D mRNA

dose-dependently in the human liver carcinoma cell line, HepG2 (107). Therefore, the reduced level of D6D mRNA expression following dietary LA supplementation may be due to LA, AA or both fatty acids. Similarly, the suppressive effect of fish oil on D6D mRNA expression may be attributed to either levels of EPA or DHA. Examination of D6D mRNA expression following dietary supplementation with EPA and DHA individually showed that both fatty acids decreased hepatic D6D mRNA expression and activity compared with controls (109). The effect of ALA or DPA n-3 on D6D mRNA expression has not been investigated. The regulation of D6D mRNA expression will lead to changes in the amount of the enzyme and thereby affect the rate and amount of fatty acid synthesis.

While D6D appears to be the rate-limiting step in the desaturation-elongation pathway, the overall regulation of the pathway is unclear and may contain several points of metabolic control. The importance of the fatty acid composition of cell membranes in disease states suggests that a correlation between the level of D6D mRNA expression and the accumulation of fatty acids in cell membranes needs to be examined further. A thorough description of the effect of D6D mRNA expression and tissue fatty acid composition has yet to be described. One of the aims of this thesis will be to correlate changes in D6D expression with changes in fatty acid composition of cell phospholipids.

1.5.2 $\Delta 5$ desaturase

D5D has been cloned from several animals including humans (121), rats (122) and *C. elegans* (123). The human D5D gene encodes 444 amino acids and possesses 61% amino acid identity and 75% similarity to the human D6D (88). D5D, like D6D, is a microsomal enzyme that is expressed in nearly all human tissues, with the greatest activity found in the liver, heart and brain (121). The D6D and D5D proteins possess

several other common features, including an identical polypeptide size, two membrane-spanning domains, and three histidine-rich regions. Examination of the human genome database revealed that the D5D and D6D genes are positioned in reverse sequence orientation to each other on chromosome 11 at 11q12.2 – 11q13 (121). The distance between the exon containing the translation initiation codon for D6D is approximately 11, 000 base pairs (bp) from the exon that contains the translation initiation codon for D5D. Although the specific promoters for the two desaturases have not yet been located, the proximity of the genes suggests that transcription of D5D and D6D may be co-ordinately governed by regulatory sequences within the 11,000 bp region that is common to both genes (121).

1.6 ACYLATION OF FATTY ACIDS INTO CELLULAR LIPIDS

Activated fatty acids may also be substrates for acylation into phospholipids. The cell membrane is composed of a lipid bilayer with phospholipids constituting the most abundant membrane lipid. Fatty acids are important constituents of phospholipids and confer the physical properties of cell membranes. Of the phospholipid classes, phosphatidyl choline (PC) is the most abundant in eukaryotic cell membranes, representing approximately 50% of phospholipid mass (124;125). Phosphatidyl choline (PC) is synthesised by the CDP-choline (Kennedy) pathway (126). The fatty acyl composition of the diacylglycerol molecule utilised by cholinephosphotransferase in the final step of the CDP-choline pathway determines the fatty acyl array for PC synthesised *de novo* (127). The fatty acid composition of each unique organ or cell suggests that the incorporation of fatty acids into phospholipid classes is highly selective and the conversion of fatty acid precursors to LCPUFA may be tissue-dependent (128-131). However, it must be noted that the level of n-3 LCPUFA in each tissue generally relates to the intake of n-3 LCPUFA although the absolute level is tissue specific.

The selectivity of fatty acids for acylation into membrane lipids represents another potential intracellular control in determining the fatty acid composition of cell membranes. 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GCP) is an acceptor of fatty acids that can be used to model esterification of LCPUFA into membrane lipids. In so-called 'mixing studies', 1-acyl-GCP is added to microsomes and peroxisomes isolated from the liver of rats. When labelled EPA, DPA (22:5n-3) or DHA were incubated in this mixture, 53, 5 and 30 η mol, respectively, were esterified into 1-acyl-GPC (132). Neither 24 carbon fatty acid (24:5n-3 or 24:6n-3) were readily esterified into 1-acyl-GCP in these microsomal-peroxisomal mixing studies, indicating that 24:5n-3 and 24:6n-3 are poor substrates for esterification. In cultured hepatocytes, only trace amounts of labelled 24:5n-3 and 24:6n-3 were incorporated into hepatocyte cell phospholipids (94). When these fatty acids are generated in the endoplasmic reticulum, they preferentially move to the peroxisome for further metabolism. Moreover, the rate of acylation of DHA into 1-acyl-GCP was 8.3-fold greater than for DPA (22:5n-3).

Ferdinandusse *et al.* (2003) (133) demonstrated the different rates of acylation between fatty acid substrates in cultured skin fibroblasts. They report that 24:6n-3 was β -oxidised 2.7 times faster than 22:6n-3, while 22:6n-3 was incorporated seven times faster into different lipid classes than 24:6n-3 (133) indicating that the 24 carbon fatty acids preferentially move into the peroxisome for β -oxidation rather than acylation into cellular lipids. The rate of acylation appears to be regulated by the presence of dietary PUFA. The amount of DHA esterified in hepatocyte phospholipids, derived from radiolabelled substrates was about 80-fold greater in animals fed a fat-free diet than for hepatocytes from chow-fed animals containing PUFA (94). There appears to be a preferred metabolic fate for each fatty acid and an

inverse relationship between rates of peroxisomal β -oxidation and rates of acylation. These events will particularly influence the synthesis of DHA from fatty acids precursors.

1.7 CONVERSION OF FATTY ACIDS IN ANIMALS

1.7.1 Competition between n-3 and n-6 fatty acids for $\Delta 6$ desaturase

Competition between n-3 and n-6 PUFA for enzymes in the fatty acid synthetic pathway has been demonstrated in animals (134). Mohrhaeur and Holman (1963) (134) fed rats a fat-free diet with daily supplements of either highly purified ethyl linoleate or ethyl linolenate, derivatives of LA (n-6) and ALA (n-3), or both. When dietary LA was held constant, increasing the level of dietary ALA suppressed the tissue content of n-6 products. Conversely, when ALA was held constant, increasing levels of dietary LA suppressed n-3 products (134) indicating that competition between n-3 and n-6 fatty acids exists and that this competition influenced the accumulation of LCPUFA.

1.7.2 Total PUFA affects the $\Delta 6$ desaturation of substrates

The competition between n-3 and n-6 substrates as well as the amount of each substrate appears to be important determinants of substrate desaturation and elongation in the fatty acid synthetic pathway. Lands *et al.* (1990) (135) fed rats diets with different ratios of n-3 and n-6 fatty acids and/or different total amounts of PUFA (ALA and LA). The highest level of EPA in plasma phospholipids was directly related to the energy percent intake of ALA, regardless of the presence of competing n-6 substrates. Interestingly, the highest level of DHA was observed in the liver phospholipids of rats fed a diet with 0.73 en % ALA (and 0.55 en % LA) compared with rats maintained on a diets containing up to 11.26 en % ALA. Despite

the significantly lower percent energy intake of ALA, the amount of DHA synthesised and incorporated into liver phospholipids was 12% of total fatty acids compared to 8% in those fed the highest energy percent ALA (11.26 en %). This was mirrored in plasma phospholipids where the level of DHA in rats fed 0.73 en % ALA was 7.48 % total fatty acids compared to 5.68 % total fatty acids in those fed 11.26 en %. Although the amount of ALA available for conversion to DHA was highest in the group receiving 11.26 en % ALA, its conversion and accumulation to DHA appeared to be inhibited.

Other dietary animal interventions demonstrate the paucity in the accumulation of DHA following supplementation with relatively high en % ALA (summarised in

Figure 1-5). Increasing the en % of ALA (constant en % LA) in the diet of hamsters reduced the level of DHA in plasma phospholipids in those fed the high ALA diet (136). However, the diet did contain trace amounts of DHA (0.2% total fatty acids) which may have altered the conversion of fatty acids. There was a proportional enrichment in EPA and DPA, but not in DHA, which decreased in the group fed the highest energy percent ALA. In hamsters fed the lowest en % ALA (0.37 en %) the level of DHA in plasma phospholipids was 9.88 ± 0.69 % DHA total fatty acids compared to 7.69 ± 0.19 % total fatty acids in the group receiving the highest energy percent of ALA, (14.58 en %) (136). Studies in marmosets also show that low dietary PUFA (2.15 en %) elevated the level of DHA in erythrocytes (137) and cardiac membranes (138) compared with those on a high PUFA diet (9.1 en %). In newborn piglets fed increasing amounts of ALA while keeping the level of LA constant, the accumulation of ALA, EPA and DPA was directly related to the en% of ALA in the formula (139). The accumulation of DHA was highest in piglets fed a formula containing 3.6 en% ALA. In these animals, the level of DHA in plasma

phospholipids was $6.36 \pm 1.00\%$ total fatty acids compared to $4.33 \pm 0.95\%$ total fatty acids in animals fed 16 en% ALA (139). The dose-dependent accumulation of EPA suggests that the reduced accumulation of DHA with increasing dietary ALA was not attributable to competition between LA and ALA at the first desaturation step but rather to events beyond the $\Delta 5$ desaturation of 20:4n-3 to EPA (20:5n-3). In those studies where the en % of ALA was increased; there was a counter-intuitive decrease in the level of DHA, suggesting that the enzymes utilised by ALA to synthesise DHA were saturated or inhibited. Hence, high dietary ALA reduces its conversion to DHA. Figure 1-5 shows that low dietary en % ALA results in a greater accumulation of DHA compared with higher en % ALA. The highest accumulation of tissue DHA occurs in a narrow range of en % ALA. In contrast, dietary LA does not appear to have a large influence on the accumulation of DHA in tissues (

Figure 1-5). The total en % of LA and ALA and their ratio to each other appear to be important factors in determining the optimal synthesis of DHA.

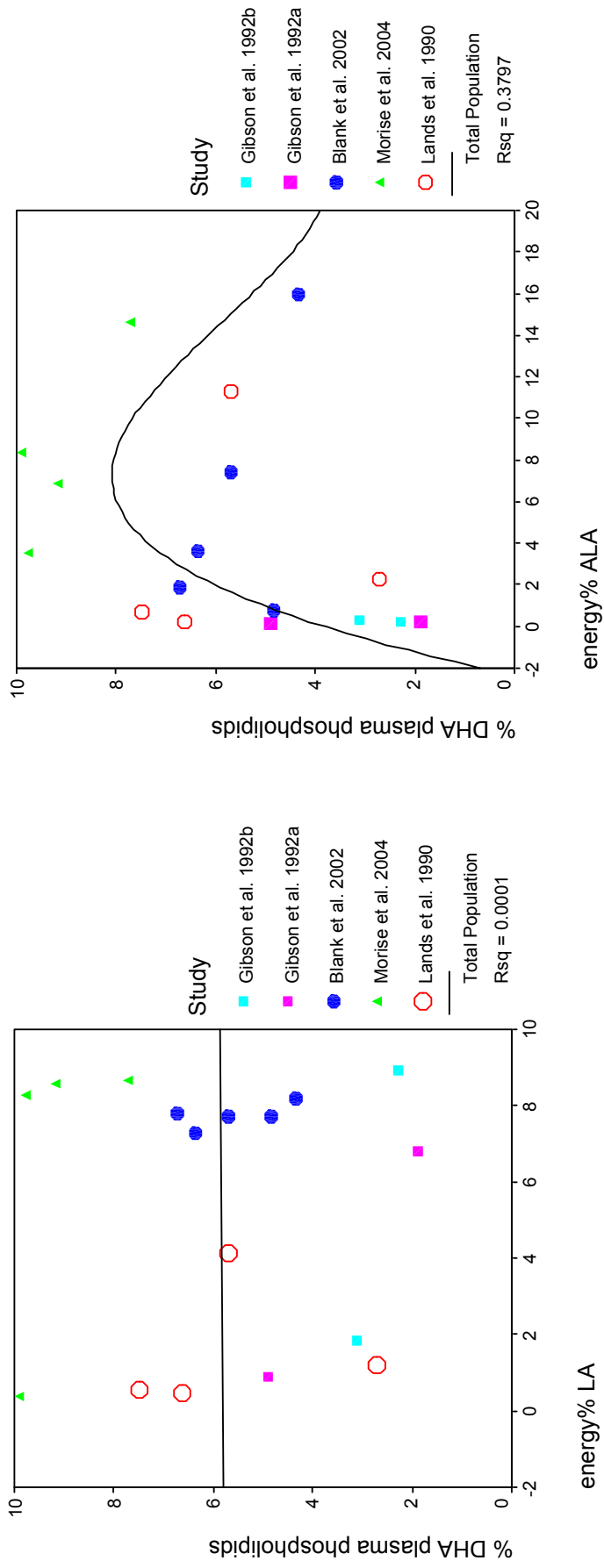


Figure 1-5. Accumulation of docosahexaenoic acid (DHA) in plasma phospholipids of animals fed different energy % of dietary PUFA (linoleic acid (LA) and α -linolenic acid (ALA)). Dietary interventions were performed in rats (Gibson *et al.* 1992a (140), Lands *et al.* 1990 (135)), marmosets (Gibson *et al.* 1992b (137)), pigs (Blank *et al.* 2002 (139)) and hamsters (Morise *et al.* 2004 (136))..

These animal studies suggest that competition exists between fatty acids of the same class for enzymes in the pathway, which may affect the fatty acid composition of cell membranes and the accumulation of LCPUFA from precursors. In the synthesis of DHA from ALA, both ALA and its downstream product, 24:5n-3, utilise D6D. In isolated liver microsomes from rats fed a fat-free diet, increasing concentrations of [1-¹⁴C] ALA inhibited the desaturation of [1-¹⁴C] 24:5n-3. Conversely, increasing amounts of [3-¹⁴C] 24:5n-3 only slightly reduced the amount of ALA that was desaturated, suggesting that ALA is the preferred substrate for D6D (112). The competition between n-3 fatty acids, particularly ALA and 24:5n-3, for enzymes in the pathway also explains the poor accumulation of DHA observed in human intervention studies, described below (see Section 1.8). It was hypothesised that high dietary intakes of ALA depress the synthesis of DHA by inhibiting the desaturation of 24:5n-3 to 24:6n-3 through its competition for D6D.

1.8 CONVERSION OF FATTY ACIDS IN HUMANS

The observation that elevated dietary intakes of ALA in humans results in an increased level of EPA but little or no change in the level of DHA in tissues or plasma (6;30-32;141) (summarised in Table 1-1) (see also (142) for a more extensive review) suggests that the endogenous synthesis and accumulation of LCPUFA from precursor fatty acids is complex. Such reports have led to the general consensus that the synthesis of DHA from ALA is limited in humans. Several studies have reported the effects of feeding high-ALA oils, typically linseed oil, containing approximately 50% ALA, on the fatty acid composition of plasma lipids. Sanders *et al.* (1983) (6) supplemented the diet of healthy human volunteers with 20 ml of linseed oil daily. This is equivalent to approximately 10 g/d ALA (~ 5 en %) and represents four and a half times the recommended dietary intake (as recommended by US National

Institute of Health (8) and Australia (NHMRC 1992, Australia, National Heart Foundation 1999, Australia)). Despite the high intake of linseed oil, only a small proportion of ALA was incorporated into platelet phosphoglycerides and suggests that ALA does not readily accumulate into cell lipids (6). EPA was significantly elevated yet there was no change in the level of DHA, suggesting a lack of conversion further down the desaturation-elongation pathway. The poor conversion of ALA to DHA in this study may result from the competition between ALA and LA for active sites on D6D as LA was not excluded in this study, providing an estimated 4 en %. Indeed, Emken *et al.* (1994) (143) have shown a 40-54% reduction in the conversion of ALA when the dietary intake of LA was doubled.

Another human intervention trial also indicated a limited conversion of ALA to DHA in humans (141). The fatty acid profile of individuals on a typical Western diet, rich in n-6 fats was compared with those maintaining a high n-3 (ALA) diet by using linseed oil and linseed spread and avoiding n-6 rich foods for four weeks. The amount of ALA consumed correlated with the level of ALA and EPA in plasma, neutrophils, mononuclear cells, erythrocytes and platelet phospholipids but inversely correlated with the level of DHA. However, participants were allowed two fish meals/week providing a source of EPA and DHA which may have affected the level of DHA in tissues due to the mechanisms mentioned previously.

De Groot *et al.* (2004) (144) supplemented pregnant women with 2.8 g/d of ALA contained in a margarine spread and measured the level of n-3 LCPUFA following supplementation. Plasma concentrations of EPA and DPA increased by 30% and 15% respectively but DHA did not differ between the ALA supplemented and control groups. In a linseed oil supplementation trial in vegetarian men, consumption of 15.4 g/d of ALA resulted in 7-, 4.5-, and 1.5-fold increases of plasma

ALA, EPA and DPA, respectively, with no change in DHA (145). The studies presented in Table 1-1 indicate that there is a limitation in the accumulation of DHA from ALA in humans. The increased accumulation of DPA in tissues following supplementation with ALA suggests that the limitation may be at a point beyond the conversion of ALA to DPA, for instance, at the second $\Delta 6$ desaturation step in the synthesis of DHA from ALA, the translocation of substrates (24:6n-3) from the endoplasmic reticulum to the peroxisome, peroxisomal β -oxidation or translocation back to the endoplasmic reticulum for membrane lipid biosynthesis.

1.8.1 Conversion of fatty acids in humans: stable isotope studies

The interpretation of human intervention studies is problematic due to the wide availability of other fatty acids in the diet and the potential for dietary fatty acids to modulate the conversion of other fatty acids to LCPUFA (146-148). By administering a short term administration of a bolus of ALA labelled with a stable isotope and monitoring the conversion of ALA to LCPUFA this problem can be briefly circumvented. Table 1-1 summarises several studies using stable isotopes to estimate the conversion of ALA to EPA and DHA in humans. Emken *et al.* (1994) (143) described the total percent conversion of deuterated ALA to deuterated EPA, DPA and DHA in a group of healthy males at 6.0%, 3.5% and 3.8%, respectively (149). As human fatty acid supplementation trials are affected by the influence of the background diet, so too are stable isotope studies (146;148). The study by Pawlosky *et al.* (2003) (147) demonstrates the effect of background diet on the conversion of labelled ALA, with reduced conversion of DPA to DHA being reported following consumption of a fish-based diet compared with a beef-based diet (147). The influence of gender on the conversion of fatty acids was also evident when the fractional conversion of DPA to DHA was calculated separately for men and women. The reduced conversion of DPA to DHA was only observed in women

consuming the fish-based diet (147). Differences in study design, background diet and the description of results contribute to the heterogeneity of the studies presented in Table 1-1. However, stable isotope studies indicate that in free-living populations the conversion of ALA to DHA is marginal.

1.8.2 Conversion of fatty acids: differences between women and men

Differences in the percent conversion of ALA to n-3 LCPUFA have been described between women and men and suggest that hormonal control may be involved in regulating the expression and activity of desaturation and elongation enzymes. The total percent conversion of deuterated ALA to deuterated EPA, DPA and DHA was reportedly 6.0%, 3.5% and 3.8%, respectively, in a group of healthy males (143). Another study reported that the conversion of [¹³C] labelled ALA to DHA was undetectable in men (31), while the conversion in women was 9% (32). A study in volunteers consuming a strictly controlled diet, containing no n-3 fatty acids from fish and a constant amount of ALA, for three weeks found that the amount of DHA derived from ALA was higher in women (14.6 ± 3.9%) than in men (150). In male-to-female gender reassignment patients the level of DHA in plasma cholesterol esters increased after administration of ethinyl estradiol plus cyproterone acetate (drugs which mimic female hormones) for four months. In female-to-male gender reassignment patients, the level of DHA was reduced after four months of testosterone administration (150). The increased accumulation of DHA in women appears to be due to the influence of oestrogen on fatty acid synthesis. The increased conversion of [¹³C] ALA to [¹³C] DHA in women compared to men suggests up-regulation of the desaturation and elongation pathway and also increased partial β-oxidation in the peroxisome, perhaps by oestrogen. This is consistent with the observation that women taking 30-35 µg ethinylloestradiol/d in a contraceptive pill,

which represents an increase in oestrogen exposure compared with the menstrual cycle, had 2.5-fold greater conversion of [¹³C] ALA to DHA than those who did not take synthetic oestrogens (31).

Ingested ALA has several metabolic fates. Apart from being a substrate for fatty acid synthesis, a major catabolic route of metabolism of ALA is complete oxidation to CO₂. In a study in humans, between 16 and 20% of ALA was expired as CO₂ in 12 h (151). In addition to the excretion of CO₂ on breath, carbon released from fatty acid β-oxidation may be recycled and used for fatty acid synthesis *de novo*. Citrate released from mitochondria into the cytosol is cleaved to form acetyl-CoA and oxaloacetate. The acetyl CoA is then available for fatty acid biosynthesis. There appears to be reciprocal relationship between partitioning of [¹³C] ALA towards β-oxidation and carbon recycling into saturated and monounsaturated fatty acids, and conversion to EPA, DPA and DHA. Women preferentially partition fatty acids towards synthesis whereas men use fatty acids as an energy source to a greater extent than women (152). It is well accepted that the presence of other fatty acids in the diet affects the conversion and accumulation of LCPUFA from precursor fatty acids. Studies which have examined the conversion of labelled fatty acids to their long chain metabolites in women and men indicate that the conversion of fatty acids is also subject to hormonal regulation.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
James <i>et al.</i> 2003 (153)	45 M + post-menopausal F	0.75 g for 3 weeks then 1.5 g for the following 3 weeks of ALA, SDA or EPA ethyl ester	6	<ul style="list-style-type: none"> Increased concentration of EPA and DPA in plasma and erythrocyte phospholipids following supplementation with SDA and EPA. No change in the concentration of DHA in plasma phospholipids or erythrocytes with any dietary group.
Mantzioris <i>et al.</i> 1995 (141)	30 M	Control (Typical Western diet, high in LA) versus High-ALA group (13.0 g/d ALA)	4	<ul style="list-style-type: none"> Intake of ALA correlated with the concentration of EPA in plasma, neutrophils, mononuclear cells, erythrocytes and platelet phospholipids but inversely correlated with DHA.
Wallace <i>et al.</i> 2003 (154)	40 M + F	Placebo (palm oil-soyabean oil (80:20 w/w), 3.5 g/d ALA, 0.44 g/d EPA+DHA, 0.94 g/d EPA+DHA, or 1.9 g/d EPA+DHA	12	<ul style="list-style-type: none"> Increased intake of ALA did not increase the concentration of ALA in plasma phospholipids but increased the concentration of EPA by 60%. No change in the concentration of DHA in plasma phospholipids following supplementation with ALA The concentration of EPA and DHA in plasma phospholipids was increase in all subjects consuming EPA+DHA.
Harper <i>et al.</i> 2006 (155)	49 F + 7 M (chronically ill)	Control (5.2 g/d olive oil) versus 5.2 g/d flaxseed oil (3.0 g/d ALA)	26	<ul style="list-style-type: none"> 70 % increase in the concentration of ALA in plasma lipids in the group receiving flaxseed oil, concomitant with a 60% increase in EPA and a 34% increase in DPA. The level of DHA in plasma lipids was unaffected in both groups
De Groot <i>et al.</i> (144)	58 F (pregnant)	From week 14 of gestation to delivery, pregnant women consumed 2.8 g/d ALA and 9.0 g/d LA or 10.9 g/d LA (control).	26	<ul style="list-style-type: none"> Plasma concentrations of EPA and DPA increased by 30% and 15% respectively but DHA did not differ between the ALA supplemented and control groups.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
Li <i>et al.</i> 1999 (145)	17 M (vegetarians)	Moderate-ALA diet (LA:ALA 3:1, 13.1 g/d LA, 3.7 g/d ALA) versus high-ALA diet (LA:ALA 1:1, 17.4 g/d LA, 15.4 g/d ALA).	4	<ul style="list-style-type: none"> • Significant increase in the level of EPA and DPA in platelet phospholipids in subjects consuming the high-ALA diet. • Significant increase in ALA and EPA in plasma phospholipids in subjects consuming the high-ALA diet. • The level of DHA in platelets and plasma phospholipids was unaffected by both diets.
Stable isotope studies				
Vermunt <i>et al.</i> 1999 (146)	15 M + F	OA-rich diet, ALA-rich diet (6.8 g/d), EPA+DHA-rich diet (1 g EPA + 0.6 g DHA). Following 6 weeks of the dietary intervention, 45 mg [¹³ C] ALA methyl ester was administered.	6	<ul style="list-style-type: none"> • [¹³C] enrichment of ALA, EPA, DPA and DHA was reduced in ALA-rich and EPA+DHA-rich diets compared with OA-rich diet. • Minimal [¹³C] enrichment of EPA, DPA and DHA in the EPA+DHA-rich diet.
Goyens <i>et al.</i> (156)	15 M + 15 F	Control (LA:ALA 19:1; 7 en% LA, 0.4 en% ALA.), Low LA (LA:ALA 7:1; 3 en% LA, 0.4 en% ALA), High ALA (LA:ALA 7:1; 7 en% LA, 1.1 en% ALA). At 19 and 61 d, subjects ingested 11.4 g [¹³ C] ALA.	61 d	<ul style="list-style-type: none"> • The level of ALA and EPA in plasma phospholipids in subjects consuming the low-LA and high-ALA diets increased significantly compared to the control group. • The level of DPA in plasma phospholipids increased significantly in subjects consuming the high-ALA diet. • The level of DHA in plasma phospholipids was not significantly different between dietary groups. • 5.2% ALA was converted to DPA in subjects consuming the low-LA diet.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
Stable isotope studies continued				
Pawlosky <i>et al.</i> 2003 (147)	5 M + 5 F	Beef-based diet (no seafood or seafood products) for 3 weeks followed by a fished-based diet for another 3 weeks, after which 1 g [² H] ALA ethyl ester was administered.	6	<ul style="list-style-type: none"> Elevated concentration of EPA and DHA in plasma lipids during the fish-based diet compared with beef-based diet. Reduced conversion of DPA to DHA following consumption of fish-based diet compared with the beef-based diet.
Pawlosky <i>et al.</i> (157)	4 M + 4 F	Beef-based diet for 21 d. During the final week of the dietary intervention, 1 g [² H] ALA ethyl ester was administered.	3	<ul style="list-style-type: none"> 0.2% [²H] ALA converted to EPA, 0.13% [²H] ALA converted to DPA, 0.05% [²H] ALA converted to DHA.
Emken <i>et al.</i> 1999 (148)	6 M	High-DHA diet (6.5 g/d DHA) versus low-DHA diet (<0.1 g/d DHA) for 90 d prior to administration of [² H] ALA triglyceride.	90 d	<ul style="list-style-type: none"> DHA supplementation reduced the conversion of ALA to EPA and DHA by 76 and 88% respectively.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
Stable isotope studies continued				
Emken <i>et al.</i> 1994 (143)	7 M	SAT diet containing 15.1 g LA versus PUFA diet containing 29.8 g LA for 12 d prior to administration of a mixture of [² H] ALA and LA triglycerides.	12 d	<ul style="list-style-type: none"> The total percent conversion of deuterated ALA to deuterated EPA, DPA and DHA was 6.0%, 3.5% and 3.8%, respectively, in all subjects. Conversion of [²H] ALA and LA was reduced by 40-54% when dietary intake of LA was increased from 15.1 g/d to 29.8 g/d.
Burdge <i>et al.</i> 2002 (31)	6 F	0.7 g [¹³ C] ALA free fatty acid	Once only administration	Conversion of ALA to EPA, DPA and DHA was 21, 6 and 9 %, respectively, in plasma lipids.
Burdge <i>et al.</i> 2002 (32)	6 M	0.7 g [¹³ C] ALA free fatty acid	Once only administration	Conversion of ALA to EPA and DPA was 8 and 8%, respectively, in plasma lipids. Conversion of ALA to DHA was not detected.

Table 1-1. Accumulation of n-3 LCPUFA in human populations: a summary of current evidence.

1.9 CONVERSION OF FATTY ACIDS *IN VITRO*

One of the major limitations of animal and human studies is the influence of other dietary fatty acids on the synthesis of DHA from ALA and the difficulty in controlling their concentration. *In vitro* models of fatty acid metabolism allow the conversion of individual fatty acids to be examined in isolation of other fatty acids. Several epithelial cell lines have been used to demonstrate the capacity of cell lines to mimic *in vivo* fatty acid metabolism. De Antueno *et al.* (2001) (158) reported the conversion of radiolabelled LA to 18:3n-6 in the ZR-75-1 (human mammary carcinoma) cell line. Following $\Delta 6$ desaturation, GLA was elongated to 20:3n-6 and further desaturated ($\Delta 5$) to 20:4n-6 and then elongated to 22:4n-6 (158), following the desaturation-elongation pathway described in Figure 1-3. Northern blot analysis of the ZR-75-1 cell line indicated the presence of both $\Delta 5$ and $\Delta 6$ desaturase transcripts (158) and indicates the usefulness of this cell line as an *in vitro* model of fatty acid conversion.

The competition between LA and ALA for common enzymes in the desaturation-elongation pathway reported *in vivo* is also demonstrated *in vitro*. When unlabeled fatty acids, ALA, EPA, DHA, LA and AA were added to cell culture media of intestinal CaCo-2 cells in the presence of radiolabelled ALA and LA, the metabolism of labelled PUFA to LCPUFA was reduced (159). The addition of unlabelled AA and EPA reduced the conversion of radiolabelled ALA to EPA by 75.8% and 90.3%, respectively. As the ratio of LA to ALA increased, conversion and incorporation of labelled ALA into CaCo-2 cell lipids decreased (159). Further elongation and desaturation of radiolabelled DPA to radiolabelled DHA was not detected (159). However, Dokko *et al.* (1998) (160) examined the metabolism of labelled LA in HepG2 cells but did not observe any conversion to AA. This was also reported by

Marra *et al.* (1992) (161) in a study using the SK-Hep1 cell line. The inability of these cell lines to convert LA to AA may have resulted from the development of essential fatty acid deficiency, which has been suggested to cause reduced conversion of n-6 fatty acids to their long chain derivatives (162). Melin and Nilsson (1997) (162) reported that an essential fatty acid deficient state occurs after five to ten days of culture in the absence of essential fatty acids. In both reports, cells were confluent and were cultured for more than five days prior to fatty acid supplementation, which may explain the inability to convert LA to AA. The conversion of LA to AA has been described in other cell lines cultured over a shorter time period (158). In the SK-Hep1 cell line, [¹⁴C] ALA was converted to 20:5n-3 and 22:5n-3 but its conversion to DHA was not detected (161). The conversion of fatty acids and the competition between fatty acid substrates reported in cell lines aligns with that described *in vivo* and suggests that *in vitro* cell systems may provide a tool in examining the potential limitations in the conversion of ALA to DHA.

1.9.1 mRNA expression of $\Delta 6$ desaturase *in vitro*

Several *in vitro* studies have utilised transformed liver cell lines to demonstrate the conversion of labelled ALA to EPA, DPA and DHA (163) and to examine the mRNA expression of the key enzymes involved in this conversion, and more specifically, D6D. Nara *et al.* (2002) (164) showed a ~50% decrease in D6D mRNA expression in HepG2 cells in the presence of AA, EPA and DHA, compared to cells without PUFA supplements or cells supplemented with oleic acid (18:1n-9), in agreement with that reported in animals (80;105;107). Tang *et al.* (2003) (107) showed a dose-dependent decrease in D6D mRNA expression in HepG2 cells supplemented with AA. These studies indicate that the expression of D6D is modulated by fatty acids. However, there is a lack of studies that thoroughly investigate the effect of D6D mRNA expression on the composition of cell

membranes and whether this is in fact the limiting step in the conversion of ALA to DHA.

HepG2 cells are a useful cell system to examine the influence of D6D mRNA expression and fatty acid supplementation on the fatty acid composition of cell membranes. The human hepatoma cell line, HepG2 is of particular interest as the liver is the primary site for the synthesis of fatty acids. The HepG2 cell line exhibits many differentiated functions of human liver cells, including expression of secretory proteins such as albumin, transferrin and fibrinogen (165;166) and retains many normal hepatic metabolic functions, including lipoprotein and apolipoprotein synthesis and cholesterol metabolism (160;167). The use of this cell line, however, is not without its limitations. Like many cell lines, HepG2 cells are derived from neoplastic tissues and as such are de-differentiated in some way from the original hepatocyte (168). Primary human hepatocytes may provide an alternative but are associated with limited supply, heterogeneity and quality (168;169), including the loss of liver-specific functions with time in culture (165). The use of the HepG2 cell line in previous studies however does suggest its use as a suitable model of fatty acid conversion and will allow direct comparison between the data produced in this thesis with other studies in the field.

1.10 AIMS

Numerous intervention studies have attempted to elevate the level of DHA in tissues by increasing dietary ALA without success. While it is generally accepted that the conversion of ALA to DHA is limited, there is little understanding as to the mechanisms governing this limitation. There are several plausible explanations for the disparity between the intake of ALA and its conversion to DHA *in vivo*. Regulation of the concentration of DHA in tissues and plasma may involve simple

substrate competition between n-3 and n-6 PUFA for common enzymes in the pathway, preferential esterification of certain fatty acids into cell lipids or peroxisomal β -oxidation or mitochondrial oxidation of fatty acids or a combination of these processes. The expression of the D6D mRNA may also influence membrane fatty acid composition by affecting the amount of D6D protein available for the conversion of fatty acids.

Much of our understanding of fatty acid conversion and accumulation into cell lipids has been gained from *in vivo* and *in vitro* studies. However, interpretation of *in vivo* studies is often limited by the multitude of other dietary fatty acids which can influence the conversion of fatty acids (147). Animal studies which examine the conversion and accumulation of a single, pure fatty acid do so by depleting the diet of all fat, thereby inducing essential fatty acid deficiency and altering the metabolic state of the animal (107;134). The altered metabolic state will affect the metabolism of fatty acids and the fatty acid composition of cell membranes. *In vitro* studies examining the conversion of labelled fatty acid substrates have demonstrated the kinetics of D6D yet are limited in their ability to show gross changes in the fatty acid composition of cell membranes as minute quantities of labelled fatty acids are administered and measured and radioactivity may not reliably be converted to mass. Moreover, it has been shown that the phospholipid distribution of incorporated labelled fatty acids may differ markedly from that of endogenous fatty acids (170). Furthermore, supplementation of animals with individual, pure fatty acids would be prohibitively expensive and more so in humans.

In vitro studies are advantageous in allowing cells to be supplemented with fatty acids individually and exclusively of other fatty acids. However, there is limited *in vitro* data which thoroughly describes the fatty acid composition of cell membranes

following supplementation with individual fatty acids. Of the limited data available, there is some discrepancy between the conversion and accumulation of fatty acids *in vitro* with that compared with *in vivo* and requires further investigation.

This thesis will use the HepG2 cell line to model the accumulation of fatty acids into cell membranes *in vivo*. This cell line reportedly retains many of the hepatic metabolic functions of the normal human liver (160;165-167), the primary site for the conversion of ALA to EPA and DHA. Changes in the fatty acid composition of HepG2 cell phospholipids following supplementation with single fatty acids will be examined in an attempt to gain insights into the potential regulatory steps in the conversion of fatty acids *in vivo*. The accumulation of fatty acids (a net effect of conversion and incorporation) into cell phospholipids following supplementation with individual fatty acids is presented in this thesis. Measuring the fatty acid composition of HepG2 cell phospholipids following fatty acid supplementation demonstrates mass changes in the fatty acid composition of cell phospholipids rather than the conversion of a fatty acid substrate to their immediate product, as in stable isotope studies. In this way, an *in vitro* system may be used to model the mass changes in the fatty acid composition of tissues *in vivo* that may occur following supplementation with fatty acids. This thesis aims to:

- (i.) examine the conversion of PUFA to LCPUFA through the accumulation of LCPUFA in HepG2 cell phospholipids following incubation with PUFA; and
- (ii.) delineate the possible regulatory steps in the conversion of ALA, and other n-3 fatty acids, to DHA.

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4 Reference List

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