

Omega-3 Fatty Acids: The Good Oil?

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Omega-3 Fatty Acids: The Good Oil?

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Contents

<i>Avant-propos</i>	<i>IX</i>
<i>Foreword</i>	<i>XI</i>
<i>Acknowledgements</i>	<i>XIII</i>
<i>Summary</i>	<i>XV</i>
1 – Fatty acids: Structure, nomenclature, biosynthesis, sources, intakes	1
1-1 Fatty acids – structure and nomenclature	1
1-2 Fatty acid biosynthesis	5
1-3 Dietary sources of fatty acids	8
1-4 Intakes of fatty acids by humans	10
References	13
2 – Polyunsaturated fatty acids and cell membranes	15
2-1 Fatty acids as membrane components	15
2-2 Fatty acylation of proteins	19
2-3 Fatty acids as eicosanoid precursors	20
2-4 Fatty acids and cell signalling	25
2-5 Fatty acid composition changes as a result of increased intake of long chain <i>n</i> -3 PUFAs	30
References	35
3 – Long chain <i>n</i>-3 fatty acids and cardiovascular disease	41
3-1 Overview – atherosclerosis, cardiovascular disease, and risk factors	41
3-2 Long chain <i>n</i> -3 PUFAs and selected CVD risk factors	46
3-2-1 Hypercholesterolemia	46
3-2-2 Hypertriacylglycerolemia in combination with a low HDL cholesterol concentration	48
3-2-3 Hypertension and endothelial responsiveness	49
3-2-4 Propensity towards thrombosis	51
• Platelet aggregation	51
• Coagulation and fibrinolysis	52
• Long chain <i>n</i> -3 PUFAs and platelet aggregation	53
• Long chain <i>n</i> -3 PUFAs and coagulation factors	53
• Long chain <i>n</i> -3 PUFAs and fibrinolytic factors	55
3-2-5 Inflammation	55

3-3	Long chain <i>n</i> -3 PUFAs and cardiovascular risk: evidence from ecological, epidemiological and case-control studies	57
3-4	Secondary prevention studies in post-MI patients	59
3-5	Conclusions	64
	References	65

4 – Long chain *n*-3 fatty acids and the brain 75

4-1	The relationship between maternal and fetal docosahexaenoic acid status	75
4-1-1	Fetal and infant brain growth creates a demand for DHA	75
4-1-2	Maternal supply of DHA to the fetus during pregnancy	76
4-1-3	Maternal supply of DHA to the infant after birth	78
4-2	DHA plays special roles in brain and eye development and function	78
4-3	DHA and visual function	81
4-3-1	Pre-term infants	81
4-3-2	Term infants	81
4-4	DHA and cognitive development	82
4-4-1	Pre-term infants	82
4-4-2	Term infants	82
4-5	Dietary strategies to increase maternal DHA status	83
4-6	Long chain <i>n</i> -3 PUFAs and childhood developmental disorders	84
4-7	Long chain <i>n</i> -3 PUFAs and psychiatric and psychological disorders in adults	85
4-8	Long chain <i>n</i> -3 PUFAs and neurodegenerative diseases of ageing	86
4-9	Conclusions	87
	References	88

5 – Long chain *n*-3 fatty acids and inflammation 97

5-1	Inflammation in health and disease	97
5-2	Arachidonic acid-derived eicosanoids and inflammation	99
5-3	Long chain <i>n</i> -3 PUFAs and inflammatory eicosanoid production	100
5-4	Anti-inflammatory effects of long chain <i>n</i> -3 PUFAs other than altered eicosanoid production	104
5-4-1	<i>n</i> -3 PUFAs and inflammatory cytokine production	104
5-4-2	<i>n</i> -3 PUFAs and adhesion molecule expression	106
5-5	<i>n</i> -3 PUFAs and inflammatory gene expression	107
5-6	Clinical applications of the anti-inflammatory effects of <i>n</i> -3 PUFAs	111
5-6-1	Introductory comments	111
5-6-2	Rheumatoid arthritis	112

5-6-3 Inflammatory bowel diseases	115
5-6-4 Asthma	116
5-7 Conclusions	118
References	118
6 – Strategies to increase long chain <i>n</i>-3 PUFA status in humans	129
6-1 Recommendations for long chain <i>n</i> -3 PUFA intake and possible strategies to achieve these	129
6-2 Is α -linolenic acid a suitable substitute for long chain <i>n</i> -3 PUFAs?	132
6-2-1 Introductory comments	132
6-2-2 Stable isotope studies	133
6-2-3 Effects of chronically increased α -linolenic acid consumption	134
6-2-4 α -Linolenic acid and human health-related outcomes	138
• α -Linolenic acid and cardiovascular disease	138
• α -Linolenic acid and cardiovascular risk factors	139
6-2-5 To summarise	143
6-3 Conclusions	144
References	146
7 – Long chain <i>n</i>-3 fatty acids in artificial nutrition with an emphasis on immuno-inflammatory outcomes	153
7-1 Septic syndromes	153
7-2 Potential relevance of long chain <i>n</i> -3 fatty acids to immune-inflammatory responses in post-surgical and critically ill patients	157
7-3 Studies of long chain <i>n</i> -3 fatty acids in surgical patients	160
7-3-1 Introductory comments	160
7-3-2 Parenteral <i>n</i> -3 fatty acids	161
7-3-3 Enteral <i>n</i> -3 fatty acids	163
7-4 Studies of long chain <i>n</i> -3 fatty acids in critically ill patients	163
7-5 Conclusions	165
References	166

Avant-propos

The DANONE INSTITUTE is an association of scientists, who are specialists in the field of nutrition.

Its objectives are as follows:

- to encourage research in the field of nutrition,
- and to inform professionals in the areas of health and education about all matters related to foodstuffs.

The DANONE CHAIR contributes to this second objective, as it aims to highlight recent developments in the area of human nutrition. The chair was created in 1994 and every year it is awarded to both a Flemish-language university and a French-language university. Under their patronage, they organise instruction by Belgian or non-Belgian scholars. This course of instruction, which is aimed at nutrition specialists from a multidisciplinary background, comprises an introductory lecture followed by fifteen hours of coursework. All conferences are contained in an integrated publication in a series of monographs, which are edited by the DANONE INSTITUTE.

IX

Professor Philip CALDER of the Institute of Human Nutrition, School of Medicine of the University of Southampton (UK) was the holder of the Danone Chair awarded to the Université Libre de Bruxelles for the academic year 2003-2004. Inspired by his teaching, the work “Omega-3 Fatty Acids: The Good Oil?” complements the Danone Chair’s collection of monographs.

The DANONE INSTITUTE wishes to express its sincere gratitude to Professor Philip CALDER. The quality of the series of conferences was confirmed by the attendance of an attentive and enthusiastic audience. The monograph which was produced as a result of this will certainly answer many readers’ questions. The Institute would also like to express its gratitude to Ms Micheline POPULER for her valuable contribution to the compilation of this publication. Lastly, the DANONE INSTITUTE expresses its deep gratitude to the staff of the Université Libre de Bruxelles, and especially to Professor Yvon CARPENTIER.

Prof. Dr. Eng. Greet VANSANT
President of the
Scientific Council

Dr. Daniel BRASSEUR
President of the
Board of Directors

Foreword

The Belgian Danone Institute has established since more than a decade what has already become an annual tradition, namely the organisation of two Chairs dispensed in Universities of the country. The purpose of the lessons given by a well recognized authority in the field of nutrition is to facilitate contacts between researchers, to educate students and update or brief future and active health care professionals. The Chair has indeed as main objective to contribute and facilitate the large diffusion of scientific knowledge amongst interested parties. Since nutrition covers a large domain of activities, the different Chairs have been focusing in the recent past on both fundamental aspects and practical approaches in a number of complementary fields such as microbiology, human physiology, clinical feeding techniques, food technology, human behaviour, consumer habits, malnutrition and other food related disorders...

During the academic year 2003-2004, the 'Université Libre de Bruxelles' was in charge of organising the Chair and setting up lessons proposed to potential participants.

Professor Philip Calder was nominated by the Faculty of Medicine for his impressive contributions and publications in nutrition. The work of PC Calder raised huge interest more particularly for his original approach in the domain of lipid disorders and immunomodulation.

PC Calder was able to convincingly demonstrate that the impacts of different types of fatty acids influencing the immune responses in the body and several inflammatory reactions have important implications for the prevention and treatment of disease.

Omega 3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found in high proportions in oily fish and fish oils. Typically, human inflammatory cells contain high proportions of the omega 6 PUFA arachidonic acid and low proportions of omega 3 PUFA. Feeding on fish oil results in the partial replacement of arachidonic acid in inflammatory cell membranes by EPA. This change leads to a decreased production of arachidonic acid-derived mediators. This response alone is a potentially beneficial anti-inflammatory effect of omega 3 PUFA. However, PC Calder was able to show in his work that omega 3 PUFA have a number of other effects which might occur downstream of altered eicosanoid production or

might be independent of this activity. Fish oil feeding improves the symptoms in some animal models of chronic inflammatory disease and protects against the effects of endotoxin and similar inflammatory challenges. Clinical studies have reported that oral fish oil supplementation has interesting effects in rheumatoid arthritis and among some patients with asthma, supporting the idea that the omega 3 PUFA in fish oil are anti-inflammatory. Further there are indications that the inclusion of omega 3 PUFA in enteral formulas and parenteral solutions might be beneficial to patients in intensive care or post-surgery.

This monograph offers a comprehensive summary of a number of studies conducted by PC Calder including core work performed by other authors allowing a larger understanding of human diseases and their potential relationships with food and feeding habits.

The author of this monograph has really been successful in summing up in a very understandable text what appears to be a complex field of ongoing investigations. The Danone Institute would like to express a special thank to PC Calder for accepting to draft and write this overview.

Professor Yvon Carpentier as the local organizer of the Chair in Brussels is also warmly thanked for his kind collaboration and continuous support: he contributed to making a success of this Chair and to consolidating the recognition of the Institute in a field of excellence.

Last but not least the input of the team from the Institute Danone and the particular tribute of Mrs Fabienne Trignon should be acknowledged.

Many thanks to all and congratulation for the performance.

I am convinced that the readers will appreciate reading this monograph!

Daniel Brasseur
Chair, Management Board
Danone Institute Belgium

Acknowledgements

This monograph is based upon the course of lectures I gave at the Campus Erasme of Université Libre de Bruxelles during February and March of 2004 as holder of the Chaire Danone for 2004. I am indebted to the Belgian Institut Danone for appointing me to the chair, and so for providing me with a wonderful opportunity to spend time discussing the importance of omega-3 fatty acids to human physiology and health with members of the Belgian academic and student communities and with interested parties from industry and the public. Naturally, the lectures and this monograph reflect my own interests and my own scientific activities. However, I have attempted to provide a broad overview of the topic to promote its accessibility to interested persons, whilst also attempting to maintain suitable depth for those more familiar with the topics addressed. The monograph is written to reflect the state of knowledge in early 2004, the timing of the lecture course.

XIII

It is important for me on this occasion to acknowledge those who have made major contributions to the development of my career as a university-based research scientist. First, I would like to thank Eric NEWSHOLME of the Department of Biochemistry, University of Oxford, a truly inspirational figure, who is responsible for sending me along the path of fatty acid research. This has proved to be such an interesting and exciting path that I have not felt any need to deviate from it! Secondly, I would like to thank Bob GRIMBLE, my mentor at the Institute of Human Nutrition, University of Southampton, who has proven to be such a helpful colleague and wonderful collaborator over the last ten years. Thirdly, Parveen YAQOOB who worked so tirelessly to enable the transfer of my early research activities from purely *in vitro* studies on to animal and then human-based research. Fourthly, I would like to acknowledge my colleague Graham BURDGE for his help in assembling material for my lecture on omega-3 fatty acids and the brain and for allowing me to use some of his original figures in Chapter 4. Finally, I would like to thank all those other researchers who have worked under my supervision over the years and whose efforts have contributed so much. Among those who have made significant contributions in my research in the area of omega-3 fatty acids are Chris DONNELLAN, Jennifer GARRY, Nicola JEFFERY, Sam KEW, Liz MILES, Glen POWER, Peter SANDERSON, Frank THIES, Tim TREBBLE, and Fiona WALLACE. Hopefully they will find something of theirs in here.

Finally, I would like to thank my local host Yvon CARPENTIER for stimulating discussions, for the very convivial atmosphere he created during my visits to Brussels and for the introduction he provided to fine Belgian cuisine.

Pr. Philip CALDER

Summary

Fatty acids are the major component of dietary fat. Although dietary fat has long been considered as deleterious to human health, fatty acids play vital biochemical and physiological roles and some of the most important fatty acids must be obtained from the diet. Amongst these are the simplest polyunsaturated fatty acids (PUFAs), linoleic and α -linolenic, which cannot be synthesised by mammalian cells. Linoleic acid is an omega-6 (or *n*-6) PUFA and α -linolenic acid is an omega-3 (or *n*-3) PUFA. These two fatty acids and their derivatives play vital roles in cell membrane structure, integrity and function. In the absence of sufficient dietary intake of linoleic and α -linolenic acids deficiency symptoms occur, and so these two fatty acids are classed as essential fatty acids. However, it appears that at least some of the essential functions of linoleic acid and perhaps all of the essential functions of α -linolenic acid are due to their derivatives. These are synthesized by a series of fatty acid chain elongations and insertions of further double bonds (desaturations) that occur mainly in the liver. The major biologically important derivative of linoleic acid is arachidonic acid. The major biologically important derivatives of α -linolenic acid are eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively). There is competition between linoleic and α -linolenic acids for metabolism to their longer chain, more unsaturated derivatives. Since most Western diets contain more linoleic acid than α -linolenic acid, the metabolism of the former predominates. Thus cell membranes typically contain a fairly high proportion of arachidonic acid (10 to 25% of fatty acids). One of its principal roles is to serve as a precursor of a family of bioactive mediators termed eicosanoids. Examples of these are prostaglandins, thromboxanes and leukotrienes. This family of mediators serves to regulate gastric acid secretion, smooth muscle contraction, body temperature, renal function, platelet aggregation, and inflammation amongst other responses. The main dietary source of the long chain *n*-3 PUFAs is oily fish and few other food sources contain substantial amounts of these fatty acids. Thus, in the absence of oily fish consumption, the amounts of EPA and DHA in cell membranes are relatively low and this is compounded by the limited conversion of α -linolenic acid. One functional effect of long chain *n*-3 PUFAs in cell membranes is that they limit metabolism of arachidonic acid to eicosanoids. It seems that having a relative imbalance of arachidonic acid and long chain *n*-3 PUFAs in cell membranes is not an ideal situation and may

result in a prothrombotic and proinflammatory state. Consumption of EPA and DHA from fish or from dietary supplements such as fish oils results in the appearance of those fatty acids in cell membranes in a time- and dose-dependent manner. This results in modulation of those cell and tissue responses regulated by arachidonic acid-derived eicosanoids. Long chain *n*-3 PUFAs have other effects on cellular function such as altering the activity of membrane receptors and transporters and altering gene expression. Through these effects these fatty acids alter the activity of cells, tissues and organs in a way that appears to result in improvement in human health. In accordance with this, low intake or low status of long chain *n*-3 PUFAs is associated with increased risk of cardiovascular disease and of some cancers, and perhaps of childhood developmental disorders, adult psychiatric and psychological disorders, and neurodegenerative diseases of ageing. It is clear that, *via* effects on a range of risk factors, consumption of long chain *n*-3 PUFAs lowers the likelihood of developing cardiovascular disease and protects against mortality from myocardial infarction. These fatty acids also exert anti-inflammatory actions that make them useful as therapeutic agents in diseases with an inflammatory component. They seem to be very efficacious in rheumatoid arthritis. An emerging role of long chain *n*-3 PUFAs is in patients receiving artificial nutrition because of surgery or critical illness, where they appear to modulate the metabolic, inflammatory and immune responses that are associated with poor outcome in some patients. Long chain *n*-3 fatty acids have special roles in the brain and visual systems. There is a very high content of DHA in grey matter of the brain and in the outer segment of the retinal rods. DHA is accumulated into these regions early in life and a supply from the mother *in utero* and during the suckling period appears to be very important in determining optimal DHA accumulation and optimal brain and visual function. Inclusion of DHA in formula for pre-term infants has been shown to result in better visual function. The same may be true for term infants but the evidence is less clear. There is some evidence that early supply of DHA improves cognitive and intellectual development in children, although the effects may not be long lasting. Emerging data suggests that long chain *n*-3 PUFAs may be beneficial in childhood developmental disorders, adult psychiatric and psychological disorders and neurodegenerative diseases of ageing. Thus, long chain *n*-3 PUFAs appear to be associated with improved health and well-being right across the life cycle. Although it is evident that increased intake of these fatty acids should be encouraged in various population groups, it is clear that greater scientific evidence of protective effects is required in a number of conditions.

1 – Fatty acids: Structure, nomenclature, biosynthesis, sources, intakes

1-1 Fatty acids - structure and nomenclature

The general structure of a fatty acid is a hydrocarbon chain with a carboxyl group at one end and a methyl group at the other (**Figure 1.1**). The carboxyl group is reactive and readily forms ester links with alcohol groups for example those on glycerol or cholesterol, in turn forming acylglycerols (e.g. triacylglycerols,

1

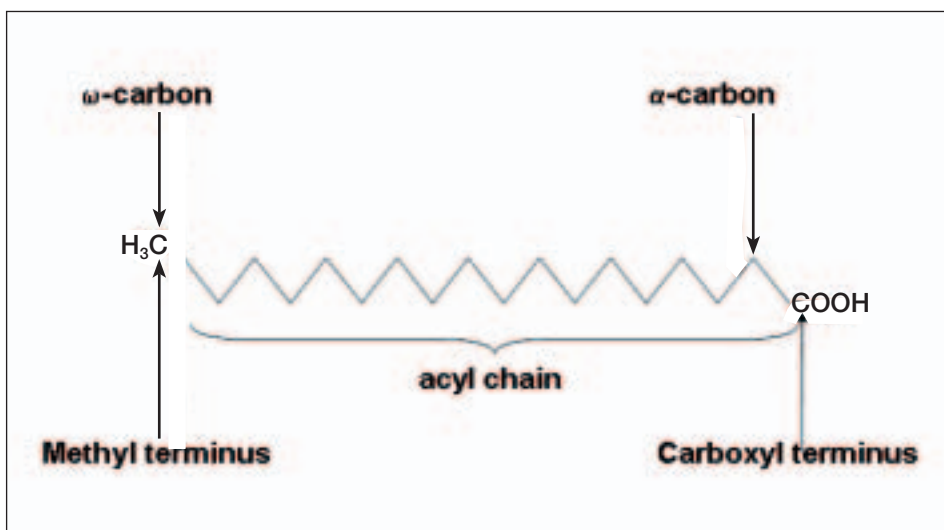


Figure 1.1 — General features of fatty acid structure.

phospholipids), and cholesteryl esters (**Figure 1.2**). Traditionally, the carbon of the carboxyl group is called carbon 1 with carbon 2 being referred to as the α carbon and the methyl terminal carbon as the ω (sometimes called n) carbon (**Figure 1.1**). The most abundant fatty acids have straight chains of an even number of carbon atoms, although branched chain, odd-numbered and substituted fatty acids do exist. Fatty acid chain lengths vary from 2 to 30 carbons or more and the chain may contain double bonds. Fatty acids containing double bonds in the acyl chain are referred to as unsaturated fatty acids; a fatty acid containing two or more double bonds is called a polyunsaturated fatty acid (PUFA). Saturated fatty acids do not contain double bonds in the acyl chain.

The systematic name for a fatty acid is determined simply by the number of carbons in the acyl chain. For example an eight carbon fatty acid (with no double bonds in the acyl chain) is termed octanoic acid while an eighteen carbon fatty acid (with no double bonds in the acyl chain) is termed octadecanoic acid. However, complications arise for the naming of unsaturated fatty acids. This is because there are multiple possibilities for the position of double bonds within the hydrocarbon chain and because each double bond may be in the *cis* or *trans* configuration. Therefore, when naming an unsaturated fatty acid it is important that the exact

2

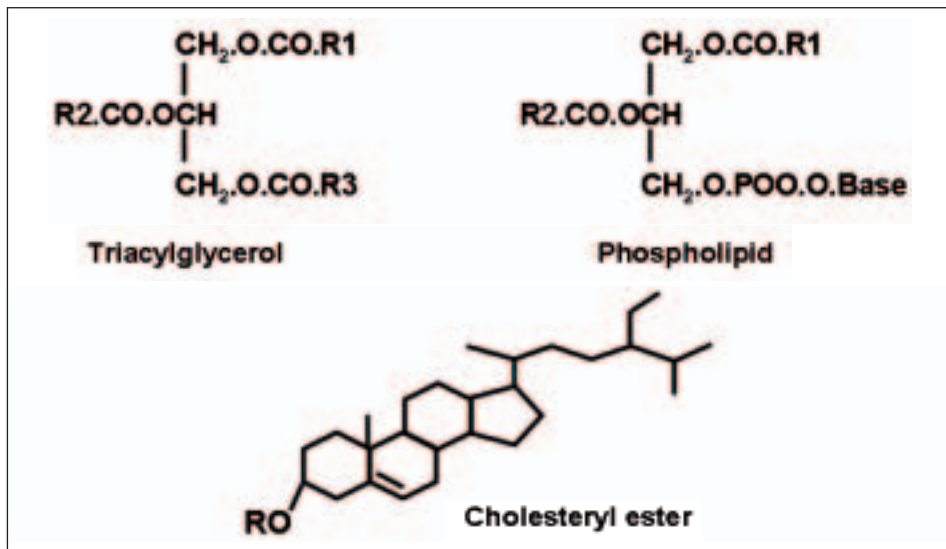


Figure 1.2 — Structures of triacylglycerols, phospholipids and cholesteryl esters. R, R1, R2 and R3 signify fatty acyl chains.

positions of double bonds and their configurations be clearly identified. Traditionally, the position of double bonds was identified by naming the carbon number (from carbon 1 (the carboxyl carbon)) on which each double bond occurs. Thus, octadecadienoic acid, an 18-carbon fatty acid with *cis* double bonds between carbons 9 and 10 and carbons 12 and 13 is correctly denoted as *cis* 9, *cis* 12-octadecadienoic acid or as *cis, cis, 9,12*-octadecadienoic acid. More recently, an alternative shorthand notation for fatty acids has come into frequent use. This relies upon identifying the number of carbon atoms in the chain, the number of double bonds and their position. Thus, octadecanoic acid is notated as 18:0, indicating that it has an acyl chain of 18 carbons and does not contain any double bonds. Unsaturated fatty acids are named simply by identifying the number of double bonds and the position of the first double bond counted from the methyl terminus (with the methyl, or ω , carbon as number 1) of the acyl chain. The way the first double bond is identified is as ω -x, where x is the carbon number on which the double bond occurs. Therefore *cis, cis, 9,12*-octadecadienoic acid is also known as 18:2 ω -6. The ω -x nomenclature is sometimes referred to as omega x (e.g. 18:2 omega 6) or *n*-x (e.g. 18:2*n*-6). In addition to these nomenclatures, fatty acids are often described by their common names. **Table 1.1** lists a range of fatty acids and shows the different ways in which they are named. In most PUFAs the double bonds are separated by a methylene (-CH₂-) group. **Figure 1.3** shows the structure of several

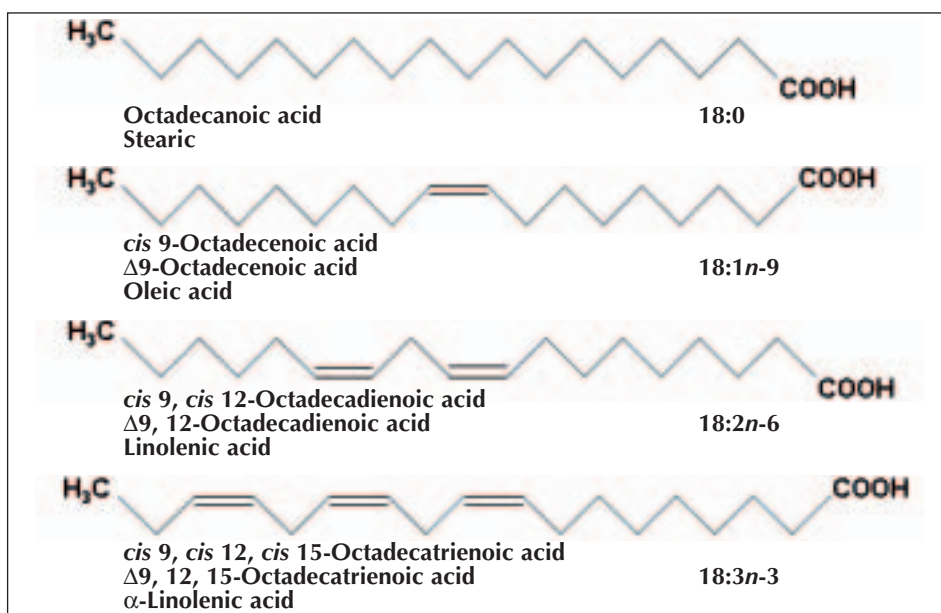


Figure 1.3 — Structures and naming of a selection of 18 carbon fatty acids.

OMEGA-3 FATTY ACIDS: THE GOOD OIL?

18-carbon fatty acids indicating the position of the double bonds in the chain and how this is reflected in their naming. Note that most common unsaturated fatty acids contain *cis* rather than *trans* double bonds. *Cis*, but not *trans*, double bonds produce a kink in the molecule so that the molecular shape of unsaturated fatty acids containing *cis* double bonds is distinct from that of saturated fatty acids.

Table 1.1 — Fatty acid nomenclature.

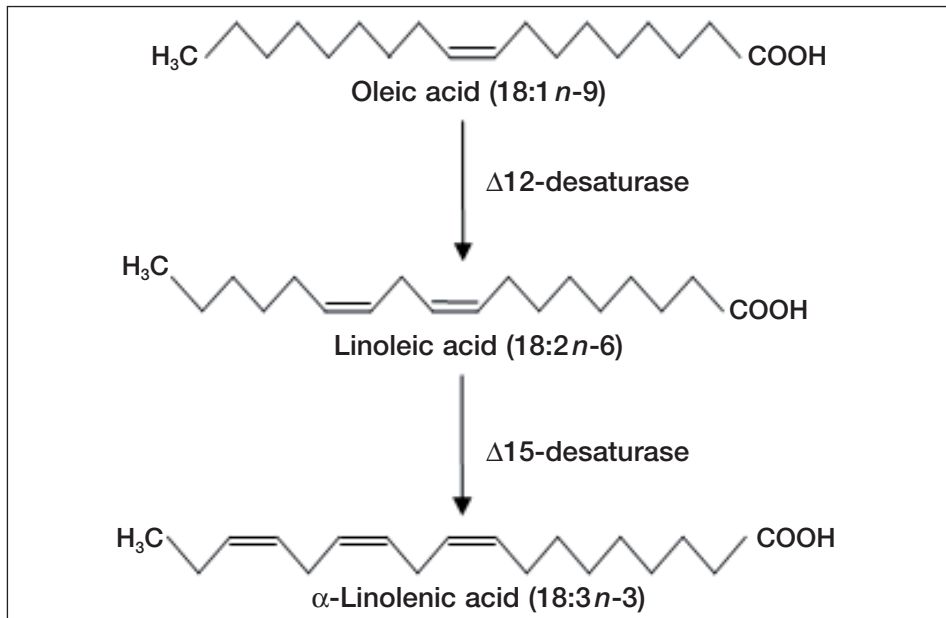
Systematic name	Trivial name	Shorthand notation	Melting point (°C)
Ethanoic	Acetic	2:0	
Propanoic	Propionic	3:0	
Butanoic	Butyric	4:0	
Hexanoic	Caproic	6:0	-8
Octanoic	Caprylic	8:0	13
Decanoic	Capric	10:0	30
Dodecanoic	Lauric	12:0	42
Tetradecanoic	Myrsitic	14:0	52
Hexadecanoic	Palmitic	16:0	61
Octadecanoic	Stearic	18:0	70
<i>cis</i> 9-Hexadecenoic	Palmitoleic	16:1 n -7	
<i>cis</i> 9-Octadecenoic	Oleic	18:1 n -9	16
<i>cis</i> 9, <i>cis</i> 12 -Octadecadienoic	Linoleic	18:2 n -6	-5
All <i>cis</i> 9, 12, 15 -Octadecatrienoic	α -Linolenic	18:3 n -3	-11
All <i>cis</i> 6, 9, 12 -Octadecatrienoic	γ -Linolenic	18:3 n -6	
All <i>cis</i> 11, 14, 17 -Eicosatrienoic	Mead	20:3 n -9	
All <i>cis</i> 8, 11, 14 -Eicosatrienoic	Dihomo- γ -linolenic	20:3 n -6	
All <i>cis</i> 5, 8, 11, 14 -Eicosatetraenoic	Arachidonic	20:4 n -6	-50
All <i>cis</i> 5, 8, 11, 14, 17-Eicosapentaenoic	Eicosapentaenoic	20:5 n -3	-54
All <i>cis</i> 7, 10, 13, 16, 19-Docosapentaenoic	Docosapentaenoic	22:5 n -3	
All <i>cis</i> 4, 7, 10, 13, 16, 19 -Docosahexaenoic	Docosahexaenoic	22:6 n -3	-44

The chain length and degree of unsaturation of a fatty acid are what determines its melting point. Increasing chain length tends to increase melting point while increasing unsaturation decreases it (**Table 1.1**). It is the nature of the constituent fatty acids that gives fat its physical properties. Thus, fats that are rich in saturated fatty acids (e.g. butter, lard) are solid at room temperature, whereas fats that are rich in unsaturated fatty acids (e.g. olive oil, sunflower oil) are liquid at room temperature.

1-2 Fatty acid biosynthesis

Saturated fatty acids are built up by successive addition of 2-carbon units to a growing acyl chain [GURR *et al.*, 2002]. The principal product of fatty acid synthesis (i.e. the fatty acid that is finally released from the growing acyl chain) is generally considered to be palmitic acid (16:0). Longer chain fatty acids are formed from palmitic acid by elongation reactions (the enzymes catalysing these are termed elongases). Elongation converts palmitic acid to stearic acid (18:0). The endoplasmic reticulum is a site for introduction of double bonds («desaturation») into fatty acids. Insertion of a *cis* double bond between carbons 9 and 10 of stearic acid (18:0) results in oleic acid (18:1 n -9). Because the double bond is inserted between carbons 9 and 10 counting from the carboxyl end of the acyl chain, the desaturase enzyme is known as delta-9 desaturase (Δ 9-desaturase), although sometimes this enzyme is referred to as stearyl CoA desaturase because the substrate is the coenzyme A ester of stearic acid.

Further desaturation of oleic acid produces PUFAs. Plant enzymes normally introduce a new double bond between an existing double bond and the terminal methyl group, whereas animal enzymes normally introduce a new double bond between an existing double bond and the carboxyl group. Insertion of a double bond between carbons 12 and 13 (counted from the carboxyl carbon) of oleic acid yields linoleic acid (18:2 n -6). The enzyme that catalyses this reaction is called Δ 12-desaturase (**Figure 1.4**). Linoleic acid can be further desaturated by insertion of a double bond between carbons 15 and 16 (counted from the carboxyl carbon) by Δ 15-desaturase to yield α -linolenic acid (18:3 n -3) (**Figure 1.4**). Linoleic and α -linolenic acids are the simplest members of the n -6 and n -3 families of fatty acids, respectively. As indicated above, mammals lack the enzymes that introduce double bonds at carbon atoms beyond carbon 9 in the acyl chain (counting from the carboxyl carbon). Because these include the Δ 12- and Δ 15-desaturases, this means that mammals cannot synthesise linoleic and α -linolenic acids. Since these fatty acids are



6

Figure 1.4 — Pathway of conversion of oleic acid to linoleic and α -linolenic acids.

required by mammalian cells, they are termed essential fatty acids, and there is a need for their consumption in the diet.

Although mammalian cells cannot synthesise linoleic and α -linolenic acids, they can metabolise them by further desaturation and elongation; desaturation occurs at carbon atoms below carbon number 9 (counting from the carboxyl carbon). Linoleic acid can be converted to γ -linolenic (18:3 n-6) by $\Delta 6$ -desaturase and then γ -linolenic can be elongated to dihomo- γ -linolenic (20:3 n-6) acid (Figure 1.5). Dihomo- γ -linolenic can be further desaturated by $\Delta 5$ -desaturase to yield arachidonic acid (20:4 n-6). Using the same series of enzymes as used to metabolise n-6 PUFA, α -linolenic acid is converted to eicosapentaenoic acid (20:5 n-3; EPA). In mammals the pathway of desaturation and elongation occurs mainly in the liver.

It is evident from the pathway shown in Figure 1.5 that there is competition between the n-6 and n-3 fatty acid families for metabolism. The $\Delta 6$ -desaturase reaction is rate limiting in this pathway [SPRECHER, 2000]. The preferred substrate for $\Delta 6$ -desaturase is α -linolenic acid [SPRECHER, 2000]. However, because linoleic acid is much more prevalent in most human diets than α -linolenic acid (see section 1-4), metabolism of n-6 fatty acids is quantitatively the more important. In the absence of

intake of linoleic and α -linolenic acids, metabolism of oleic acid by this pathway is enhanced resulting in accumulation of mead acid (20:3n-9), which is normally only found in tissues in trace amounts. Appearance and accumulation of mead acid is taken to indicate dietary essential fatty acid deficiency. The activities of Δ 6- and Δ 5-desaturases are regulated by nutritional status, hormones and by feedback inhibition by end products [British Nutrition Foundation, 1992].

A pathway for further conversion of EPA to docosahexaenoic acid (22:6n-3; DHA) exists and this involves addition of 2 carbons to form docosapentaenoic acid (22:5n-3), 2 further carbons to produce 24:5n-3 and desaturation at the Δ 6 position to form 24:6n-3 [SPRECHER, 2000, 2002] (Figure 1.5). It is unclear whether the same enzyme is responsible for the initial, rate-limiting desaturation at the Δ 6 position and for the synthesis of 24:6n-3 [HARMON

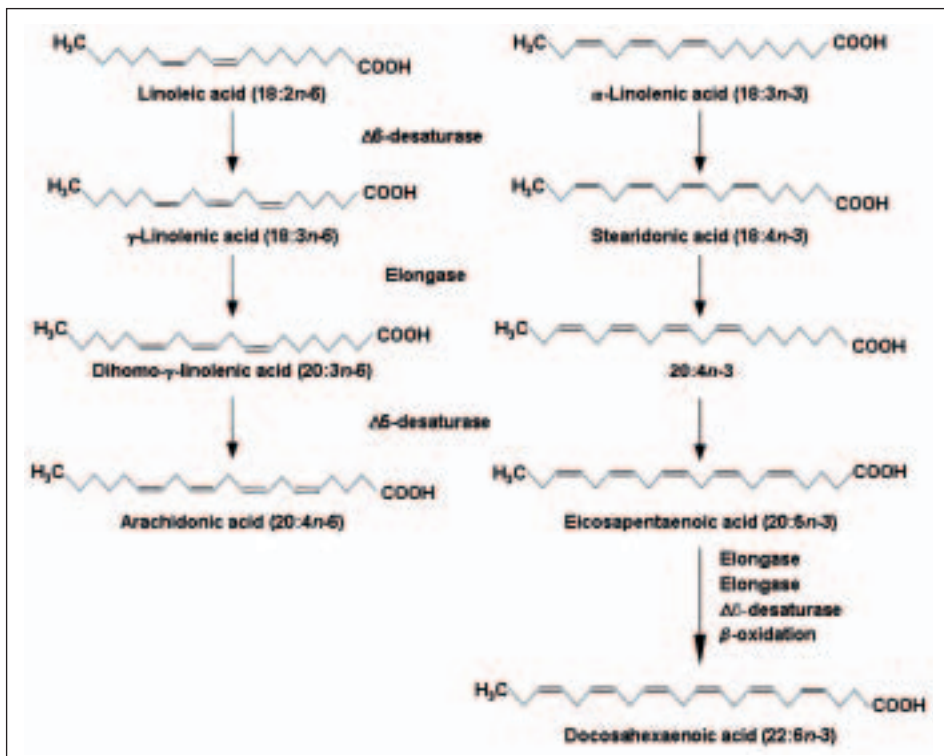


Figure 1.5 – Pathway of conversion of linoleic and α -linolenic acids to longer chain, more unsaturated fatty acids.

et al., 2003], although enzyme preparations with both activities have been reported [DE ANTUENO *et al.*, 2001; D'ANDREA *et al.*, 2002]. 24:6n-3 is translocated from the endoplasmic reticulum to peroxisomes where 2 carbons are removed by limited β -oxidation to yield DHA. Subsequent incorporation of DHA into complex lipids such as triacylglycerols and phospholipids requires transfer of DHA back to the endoplasmic reticulum. It has been suggested that the complex series of fatty acid translocation and β -oxidation steps may act as loci of metabolic control facilitating regulation of DHA synthesis independent from the up-stream activity of the pathway [SPRECHER, 2000, 2002]. EPA and DPA can also be synthesised from DHA by retro-conversion due to limited peroxisomal β -oxidation. Arachidonic acid can be metabolised by the same series of enzymes to yield, in turn, 22:4n-6, 24:4n-6, 24:5n-6 and 22:5n-6.

1-3 Dietary sources of fatty acids

8

Fatty acids in fats, oils and foodstuffs are mainly esterified to glycerol, as triacylglycerols, although some are present as esterified components of phospholipids, glycolipids, and other lipids. The fatty acid composition of cow's, sheep's, and goat's milks is typically characterised by relatively high proportions of short and medium chain saturated and monounsaturated fatty acids and low proportions of PUFAs. Ruminant milks also contain small quantities of a variety of *trans*, branched, or odd-numbered fatty acids. The proportions of different fatty acids in milks can be affected by the nature of the feed. While eggs are rich in palmitic and oleic acids, the phospholipid fraction of yolk provides linoleic acid and other PUFAs. Again different feeding regimens alter the fatty acid composition of eggs. Animal and poultry storage fat tend to be rich in saturated and monounsaturated fatty acids, but the muscle (i.e. meat) contains significant proportions of PUFAs.

The fatty acid composition of lipids in plant membranes varies little between different types of leaves. Five fatty acids generally account for > 90% of total fatty acids: palmitic (ca. 13%), palmitoleic (ca. 3%), oleic (ca. 7%), linoleic (ca. 16%) and α -linolenic (ca. 56%). Thus, green leaves are an important source of the essential fatty acid, α -linolenic acid. In contrast to the uniformity of the fatty acid composition of plant leaves, seed oils exhibit a wide range of fatty acid compositions (**Table 1.2**). Interestingly, in seed oils, one fatty acid often predominates, and seed oils are important sources of essential fatty acids. Some seed oils contain moderate to high proportions of relatively unusual fatty acids. For example, borage (starflower) and evening primrose oil contain γ -linolenic acid, and echium oil contains stearidonic

Table 1.2 — Typical fatty acid compositions of some seed oils.

	Fatty acid (g/100 g total fatty acids)										
	8:0	10:0	12:0	14:0	16:0	18:0	16:1n-7	18:1n-9	18:2n-6	18:3n-3	
Coconut oil	8	7	48	16	9	2	trace	7	2	0	
Corn oil	0	0	0	1	14	2	trace	30	50	2	
Olive oil	0	0	0	trace	12	2	1	72	11	1	
Palm oil	0	0	trace	1	42	4	trace	43	8	trace	
Palm kernel oil	4	4	45	18	9	3	0	15	2	0	
Rapeseed oil*	0	0	0	trace	4	1	2	54	29	1	
Soybean oil	0	0	trace	trace	10	4	trace	25	52	7	
Sunflower oil	0	0	trace	trace	6	6	trace	33	52	trace	
Safflower oil	0	0	trace	trace	6	2	trace	18	70	trace	
Linseed oil	0	0	0	0	6	5	trace	20	15	50	

*low erucic acid.

Data are from British Nutrition Foundation [1992]

acid (18:4 n -3). Older varieties of rapeseed oil contained nearly 60% erucic acid (22:1 n -9) which was reported to be toxic. New varieties of rapeseed oil have had the erucic acid replaced by oleic acid through breeding.

Fish can be classified into lean fish that store lipid as triacylglycerols in the liver (e.g. cod) or «fatty» («oily») fish that store lipid as triacylglycerols in the flesh (e.g. mackerel, herring, salmon, tuna). Compared with other foodstuffs, fish and other seafood are good sources of long chain n -3 PUFAs. However different types of fish contain different amounts of these fatty acids. This is partly dependent upon the metabolic characteristics of the fish and also upon their diet, water temperature, season etc. Recent studies have investigated the effect of changing the type of dietary oil fed to farmed salmon [BELL *et al.*, 2001, 2002, 2003] and identify significant effects of this on long chain n -3 PUFA content of the flesh.

The oil obtained from fatty fish flesh or lean fish livers is termed «fish oil» and it has the distinctive characteristic of being rich in long chain n -3 PUFAs. Since different oily fish contain different amounts of n -3 PUFAs then so do fish oils. Note too that it is not only the amount of n -3 PUFAs that can vary between fish and fish oils, but also the relative proportions of the individual long chain n -3 PUFAs (EPA, DPA and DHA). EPA and DHA comprise 20% to 30% of the fatty acids in a typical preparation of fish oil, which means that a one gram fish oil capsule can provide 200 to 300 mg of EPA plus DHA. It should also be mentioned that fish oils contain fairly high proportions of palmitic and palmitoleic acids, and contain some arachidonic acid.

1-4 Intakes of fatty acids by humans

Most fat consumed in the human diet (90 to 95%) is in the form of triacylglycerols, although the diet also contains phospholipids, glycolipids, other complex lipids, and cholesterol. Fatty acids are components of each of these structures apart from cholesterol, and so fatty acids form quantitatively the bulk of dietary fat. There are large differences in fat intake between countries with average intakes among adults varying from < 20 g/day in some developing countries to > 150 g/day in some developed countries. The mix of fatty acids consumed also varies in accordance with the fatty acid compositions of the fats and oils used in food preparation and of the foodstuffs eaten. Average fat consumption has changed over time and continues to do so. These time trends differ between countries. In many developing countries fat intake is increasing, while in developed countries fat intake

has tended to decline over the last 40 years or so. For example, the average consumption of fat in United Kingdom fell from 110 to 86 g per person per day between 1959 and 1990 [British Nutrition Foundation, 1992]. The type of fat consumed has also changed over time, meaning that the fatty acid composition of the human diet has changed. For example, in the United Kingdom the consumption of saturated fatty acids as a percentage of food energy decreased from 20.3% in 1975 to 16.6% in 1990 and to 13% in 2000 [Brit. Nutr. Found., 1992; HENDERSON *et al.*, 2003]. In contrast the intake of PUFAs, especially linoleic acid, increased over this period [Brit. Nutr. Found., 1992; HENDERSON *et al.*, 2003]. Much of this change has been brought about by a change in consumption from butter to margarine and from animal fats to vegetable oils. As a result the ratio of PUFAs to saturated fatty acids in the average United Kingdom diet has increased substantially over the last 40 years, more than doubling from 1970 (ratio = 0.2) and 2000 (ratio = 0.44). The main PUFA in the diet is linoleic acid followed by α -linolenic acid. On average, adult men in the United Kingdom consume about 13 and 2 g linoleic and α -linolenic acids, respectively, per day [HENDERSON *et al.*, 2003]. Adult women in the United Kingdom consume about 9.5

Table 1.3 — Consumption of linoleic and α -linolenic acids among adults in selected Western countries.

	Fatty acid intake (g per day)			
	Linoleic acid		α -Linolenic acid	
	Men	Women	Men	Women
UK	13.0	9.5	2.0	1.5
Belgium	16.6	12.8	1.7	1.4
Denmark	12.0	9.0	2.2	2.1
France	8.3	6.8	0.6	0.5
Germany	9.3	8.0	0.9	0.7
Netherlands	19.0	13.2	1.7	1.2
Italy*		14.5		0.8
Spain*		21.6		0.8
Australia*		9.9		1.2
USA	16.0	11.0	2.0	1.0
Canada	-	11.2**	-	1.6**

* Separate data are not available for men and women.

** Pregnant women.

Data for UK from HENDERSON *et al.*, 2003; data for Australia from OLLIS *et al.*, 1999; data for USA from KRIS-ETHERTON *et al.*, 2000; data for Canada from INNIS, ELIAS, 2003; other data from HULSOFF *et al.*, 1999.

and 1.5 g linoleic and α -linolenic acids, respectively, per day [HENDERSON *et al.*, 2003]. **Table 1.3** provides details of intakes of linoleic and α -linolenic acids among seventeen Western countries and demonstrates that the ratio of linoleic to α -linolenic acids varies from between 5 to greater than 20 amongst these countries.

Longer chain PUFAs are consumed in much lower amounts than linoleic and α -linolenic acids. However, intakes of longer chain PUFAs are difficult to identify precisely because these fatty acids are found in low amounts in many foods and because food composition databases frequently contain inadequate information about these fatty acids. Estimates of the intake of arachidonic acid intakes in Western populations vary between 50 and 300 mg/day for adults [SINCLAIR, O'DEA, 1993; JONNALAGADDA *et al.*, 1995; MANN *et al.*, 1995]. In the

Table 1.4 — Intakes of long chain n-3 PUFAs from a single portion of seafood or meat.

Seafood or meat	20:5n-3	22:5n-3	22:6n-3	Average portion size in UK	Total long chain n-3 PUFAs per portion
	g/100 g food			g	g
Cod	0.08	0.01	0.16	120	0.30
Haddock	0.05	0.01	0.10	120	0.19
Plaice	0.16	0.04	0.10	130	0.39
Herring	0.51	0.11	0.69	120	1.56
Mackerel	0.71	0.12	1.10	160	3.09
Kippers	1.15	0.10	1.34	130	3.37
Salmon	0.50	0.40	1.30	100	2.20
Trout	0.23	0.09	0.83	230	2.65
Canned crab	0.47	0.08	0.45	85	0.85
Prawns	0.06	trace	0.04	60	0.06
Mussels	0.41	0.02	0.16	40	0.24
Roast beef	0.02	0.02	0	90	0.04
Roast lamb	0.03	0.04	0.02	90	0.08
Roast pork	0.01	0.02	0.01	90	0.04
Roast chicken (light meat)	0.01	0.02	0.03	100	0.06
Venison	0.04	0.09	0	120	0.16

Data are from British Nutrition Foundation [1999].

absence of fatty fish or fish oil consumption, α -linolenic acid is by far the principal dietary *n*-3 PUFA. **Table 1.4** gives data on the approximate intakes of long chain *n*-3 PUFAs from fish and some other foods available in the United Kingdom. The latest estimate for fish consumption among adults in the United Kingdom is approximately 100 g lean fish and approximately 50 g oily fish per week [Sci. Adv. Com. Nutr., 2004]. The most recent estimate for average intake of long chain *n*-3 PUFAs by adults in the United Kingdom is about 200 mg/day [Sci. Adv. Comm. Nutr., 2004].

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2 – Polyunsaturated fatty acids and cell membranes

2-1 Fatty acids as membrane components

Fatty acids have important roles in membrane structure and there are several ways by which fatty acids within cell membranes can potentially influence the functions of membrane proteins (and indeed some intracellular proteins). The fluid mosaic model of membrane structure describes biological membranes as dynamic bilayer structures involving lipids and proteins [SINGER, NICHOLSON, 1972] (**Figure 2.1**).

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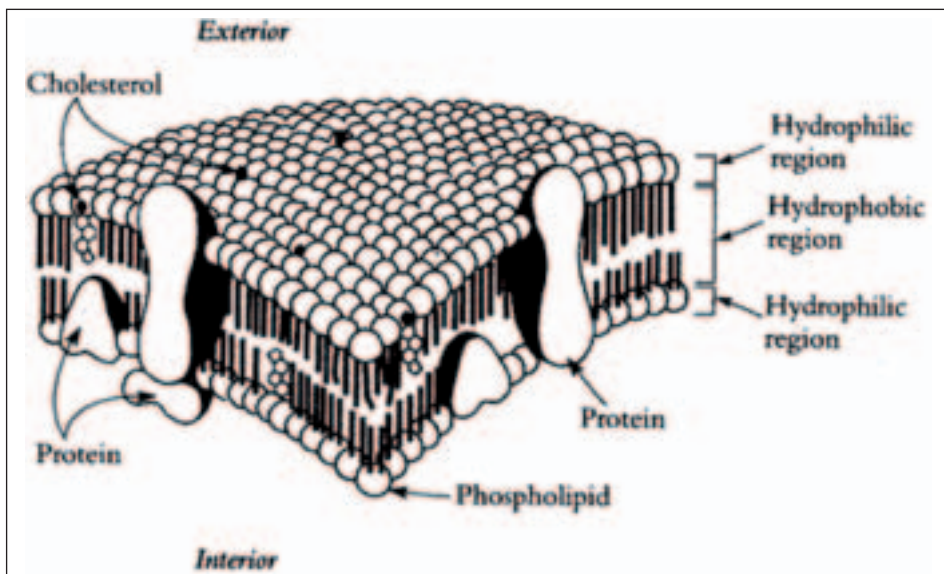


Figure 2.1 — Cell membrane structure according to the “fluid mosaic model”.

It is now also recognized that domains exist in membranes, where lipid-protein and lipid-lipid interactions may be highly specific (see Section 2-2). There is great variety in the lipid content and constituents of membranes (Table 2.1). This is not surprising since different membranes perform different functions and so will require different structural characteristics. Glycerolipids are the most widespread of membrane lipids. In higher animals phosphoglycerides (these are usually called phospholipids) predominate. Phospholipids and glycosylglycerides both contain fatty acids esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone. In some membrane lipids fatty acids may be attached by ether, rather than ester, links. These so-called plasmalogens are especially abundant in nervous tissue, white blood cells and platelets. Membranes also contain sphingolipids, which are based on sphingosine rather than glycerol, and these also contain fatty acids or fatty acid derivatives as side chains. Examples of such structures are sphingomyelin and ceramide. Membranes are asymmetric i.e. the two layers of the bilayer differ from one another structurally. For example, in erythrocyte and hepatocyte membranes 80% of the phosphatidylcholine but < 15% of the phosphatidylethanolamine is in the outer leaflet [MATHEWS, VAN HOLDE, 1990].

16

The fatty acid compositions of membrane lipids are usually characteristic for the cell [GIBNEY, HUNTER, 1993], membrane type [STUBBS, SMITH, 1984] and also for the type of phospholipid [SPERLING *et al.*, 1993]. However, the fatty acid

Table 2.1 — Typical compositions of selected membranes.

Composition	Erythrocyte	Myelin	Mitochondria
<i>Component (% by weight)</i>			
Protein	49	18	52
Lipid	43	79	48
<i>Component (% by weight of lipids)</i>			
Phosphatidylcholine	19	10	39
Phosphatidylethanolamine	18	20	27
Phosphatidylserine	8	8	< 1
Phosphatidylinositol	1	1	7
Sphingomyelin	18	9	0
Glycolipids	10	26	0
Cholesterol	25	26	3

Data are from MATHEWS, VAN HOLDE, 1990.

composition may change with the cell cycle, with age, or in response or to stimuli, to changes in the environment [STUBBS, SMITH, 1984] or diet (see Section 2-5). It is thought that these changes may have functional consequences (**Figure 2.2**). As many as 40 different fatty acids can be incorporated into the *sn*-1 or *sn*-2 positions of a phospholipid, but measurement of specific functional changes relating to such a diverse array of structural constituents is difficult and many measurements instead rely on bulk changes in membrane lipid structure and/or membrane function. Under normal conditions the membrane bilayer is in a «fluid» state, meaning that membrane proteins and lipids can migrate within the plane of the membrane [STUBBS, SMITH, 1984; BRENNER, 1984]. The fluidity of a membrane is strongly influenced by the fatty acid composition of its constituent lipids [STUBBS, SMITH, 1984; BRENNER, 1984; CALDER *et al.* 1994] (**Figure 2.3**). In turn, membrane fluidity may affect cell function [STUBBS, SMITH, 1984; BRENNER, 1984; CALDER *et al.* 1990, 1994]. This may be because fluidity, and hence protein movement, is required to establish appropriate interactions between proteins or between proteins and lipids. Often functional proteins require a specific lipid to provide a specific

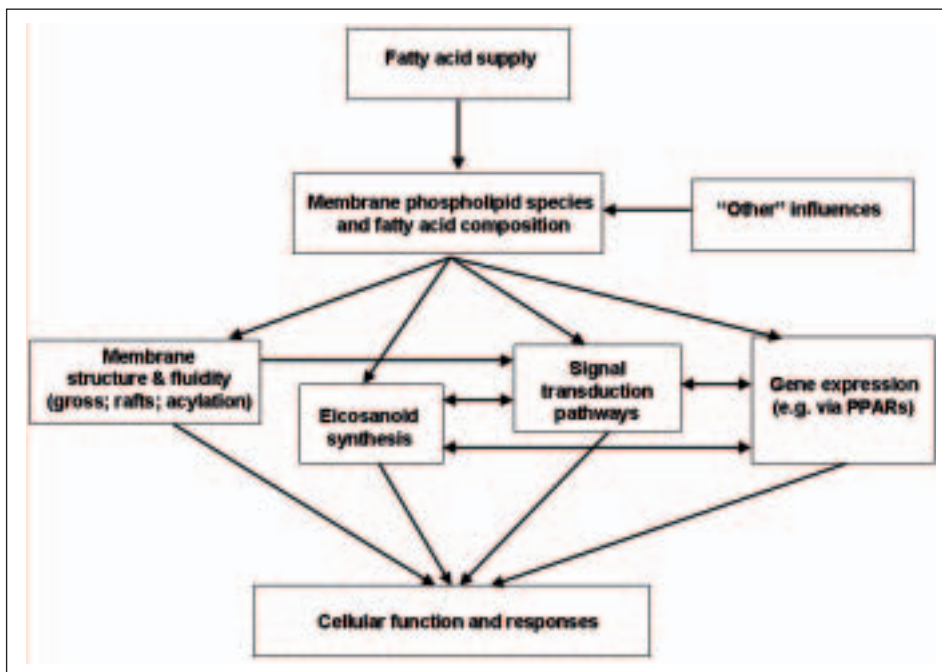


Figure 2.2 — General scheme of the interacting mechanisms whereby fatty acids might influence cell function.

microenvironment around hydrophobic regions. A number of membrane-bound enzymes, transporters and receptors have been shown to be particularly sensitive to their fatty acid environments; these include adenylate cyclase, 5' nucleotidase, the Na⁺/K⁺ ATPase, and the insulin receptor [see STUBBS, SMITH, 1984; BRENNER, 1984; MURPHY, 1990 for reviews]. These observations suggest that changing the fatty acid composition of cell membranes may alter the function of membrane proteins and so may affect cell functions (Figure 2.2).

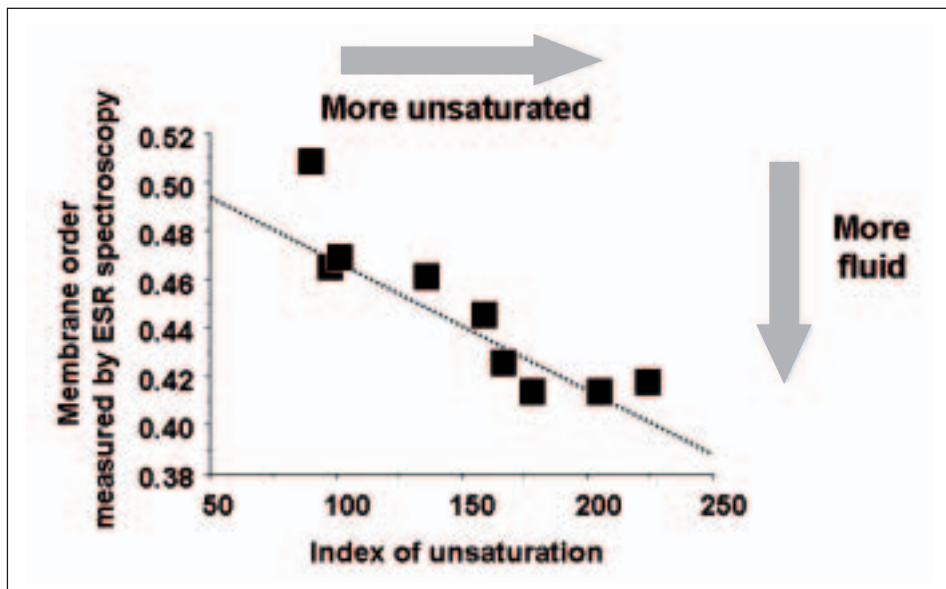


Figure 2.3 — The relationship between fatty acid composition and membrane fluidity. Rat T lymphocytes were cultured in the presence of various fatty acids. The fatty acid composition of the cell phospholipids was determined by gas chromatography and is summarised as “index of unsaturation”. The fluidity of the cells was determined by electron spin resonance (ESR) spectroscopy using a suitable spin-labelled probe and is expressed as the order parameter. Data are from CALDER *et al.* [1994].

2-2 Fatty acylation of proteins

In addition to the non-covalent interactions between membrane lipids and proteins (see Section 2-1), proteins may be anchored into membranes *via* covalent attachment to lipids. If the lipids involved are fatty acids, this process is termed protein acylation (there are other types of lipid anchoring including prenylation and anchoring *via* glycosyl phosphatidylinositol). Myristic and palmitic acids are the predominant fatty acids found attached to proteins in eukaryotic cells [see SCHMIDT, 1989; MCLHINNEY, 1990 for reviews]. Myristic acid is normally found attached to proteins by an amide bond to an N-terminal glycine and this attachment takes place as the protein is being synthesized. Generally, the attached myristic acid is stable and has a half-life similar to that of the protein to which it is bound. This contrasts with palmitic acid, which is attached to the protein post-translationally *via* an ester (usually thioester) linkage; palmitic acid can turnover much faster than the protein to which it is attached. The addition of myristic acid to a protein is catalyzed by a transferase enzyme (myristoyl-CoA:protein N-myristoyl transferase) which exhibits a high level of specificity for myristoyl-CoA; in contrast the enzyme(s) which attaches palmitic acid to proteins is less specific and may also add myristic, stearic and oleic acids. Myristic and palmitic acids are attached to very distinct classes of protein, including protein kinases, receptors, G proteins and a number of oncogene products. These proteins appear targeted or anchored to specific regions of the plasma membrane, termed rafts. Rafts have a composition that is different from other regions of the membrane: they contain more lipid, more phospholipid and more cholesterol than the bulk plasma membrane [PIKE *et al.*, 2002]. Rafts seem to function as foci for the generation of intracellular signals in response to extracellular stimuli. Lipid rafts are rich in unsaturated fatty acids, especially arachidonic acid and DHA [PIKE *et al.*, 2002], and they are considered to be more fluid than other regions of the membrane. Many proteins involved in cell signalling are located in lipid rafts [see BOWN, LONDON, 1998; SIMONS, TOMRE, 2000; PIKE, 2003 for reviews]. In simple terms, rafts can be viewed as signalling platforms that serve to co-localize the requisite components facilitating their interaction. Thus, receptors, coupling factors, effector enzymes and substrates are co-localized within a single raft. Signal transduction would occur rapidly and efficiently because of the spatial proximity of the various components involved. PIKE [2003] has recently reviewed current views of the structure, organization and function of rafts.

2-3 Fatty acids as eicosanoid precursors

One of the key functional roles of PUFAs is as precursors to eicosanoids. Eicosanoids are a family of bioactive mediators that are oxygenated derivatives of the 20-carbon PUFAs dihomo- γ -linolenic, arachidonic and eicosapentaenoic acids. Eicosanoids include prostaglandins (PG) and thromboxanes (TX), which together are termed prostanoids, and leukotrienes (LT), lipoxins (LX), hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE). In most conditions the principal precursor for these compounds is arachidonic acid and the eicosanoids produced from arachidonic acid frequently have more potent biological functions than those produced from dihomo- γ -linolenic or eicosapentaenoic acids. The precursor PUFA is released from membrane phosphatidylcholine (PC) by the action of phospholipase A₂ or from membrane phosphatidylinositol-4,5-bisphosphate (PIP₂) by the actions of phospholipase C and a diacylglycerol (DAG) lipase (**Figure 2.4**).

20

The pathways of eicosanoid synthesis begin with prostaglandin endoperoxide synthase, commonly known as cyclooxygenase (COX), which ultimately yields the PGs and TXs, or with the 5-, 12- or 15-lipoxygenases (LOX), which yield the LTs, HPETEs, HETEs and LXs (**Figure 2.5**). The amount and type of eicosanoids synthesized are determined by the availability of arachidonic acid and other precursors, by the activities of phospholipase A₂ and phospholipase C, and by the activities of COX and LOX enzymes.

COX enzymes have been found in every animal tissue and cell type examined with the exception of mature erythrocytes. Together with the hydroperoxidase which catalyses the next step in the pathway of prostanoid synthesis (**Figure 2.5**), COX forms part of the enzyme known as PGG/PGH synthase. There are two isoforms of COX: COX-1 is a constitutive enzyme and COX-2 is induced in cells as a result of stimulation (for example with inflammatory cytokines and growth factors) and is responsible for the markedly elevated production of prostanoids that occurs upon cellular activation. COX enzymes are localized in both the endoplasmic reticulum and the nuclear envelope. The major biologically active products of COX metabolism of arachidonic acid are considered to be PGD₂, PGE₂, PGI₂ (prostacyclin), PGF_{2 α} and TXA₂ (**Table 2.2**), although these are produced in a cell-specific manner. For example, platelets form mainly TXA₂ while endothelial cells form mainly PGI₂. These compounds usually have a short half-life (of the order of seconds) and act locally to the cell from which they are

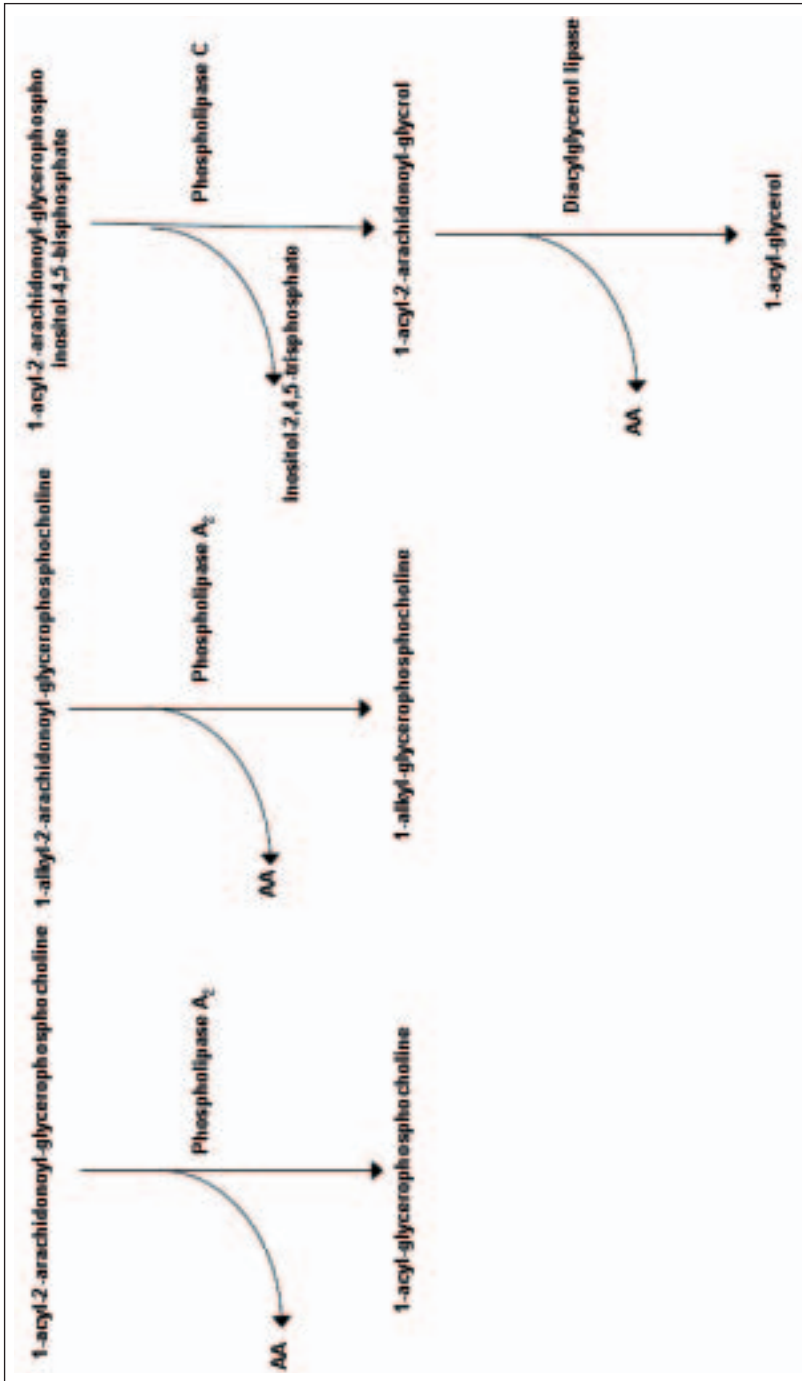


Figure 2.4 — Mechanisms of arachidonic acid release from cell membrane phospholipids. AA, arachidonic acid.

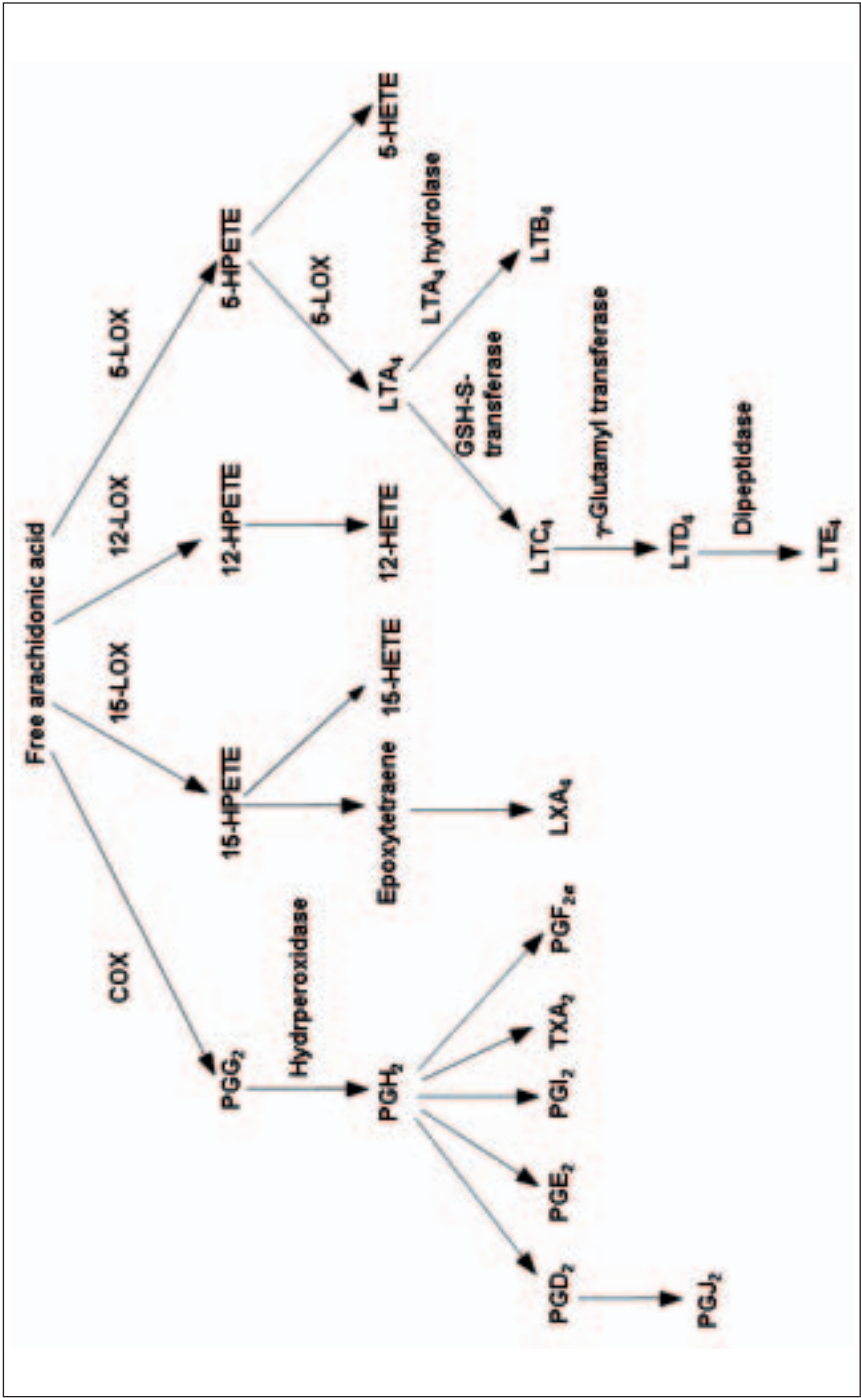


Figure 2.5 — Pathway of synthesis of eicosanoids from arachidonic acid.
 AA, arachidonic acid; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid;
 HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; PG, prostaglandin.

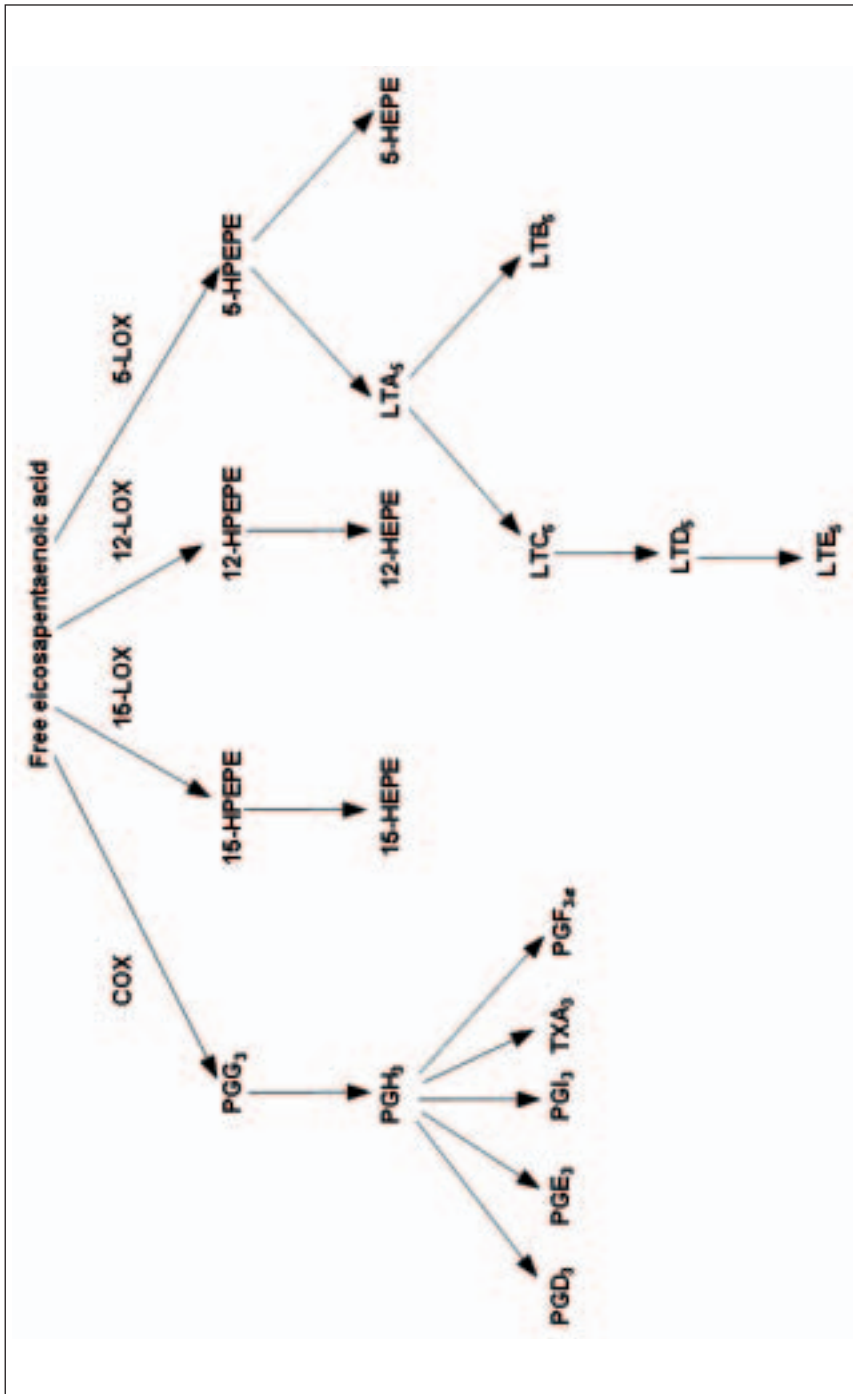


Figure 2.6 — Pathway of synthesis of eicosanoids from eicosapentaenoic acid.

COX, cyclooxygenase; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid;

HPEPE, hydroperoxyeicosapentaenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin.

Table 2.2 — Actions of arachidonic acid-derived prostanoids.

	Regulates	Receptor involved
PGD ₂	Sleep-wake cycle; Pain responses; Vasodilation; Vasoconstriction	DP
PGE ₂	Gastric acid secretion; Vasoconstriction; Vasodilation; Bronchiole relaxation; Uterine implantation; Uterine contraction; Pain responses; Temperature regulation; Inflammatory cytokine production; Immune function	EP1, EP2, EP3, EP4
PGI ₂	Platelet aggregation; Vasodilation	IP
PGF _{2α}		FP
TXA ₂	Platelet aggregation; Smooth muscle contraction; Smooth muscle proliferation	TP

produced. Their production is initiated by a stimulus and, once produced, they themselves are able to modify the response to the stimulus. Different prostanoids have different, sometimes opposite, effects; for example, TXA₂ increases platelet aggregation whereas PGI₂ inhibits platelet aggregation (see Section 3-2-4). PGs are associated with the inflammatory response (see Section 5-2) and COX inhibitors include aspirin and related non-steroidal anti-inflammatory drugs. For a recent review of prostanoids see NICOLAOU [2004].

It appears that each of the LOX enzymes has a particular cellular distribution: 5-LOX is found in mast cells, monocytes, macrophages and granulocytes, 12-LOX is found in platelets and some epithelial cells and 15-LOX is found in young myeloid cells and some epithelial cells. LTA₄ is formed by 5-LOX activity on 5-HPETE (**Figure 2.5**). Further metabolism (hydrolysis) of LTA₄ yields LTB₄. LTA₄ is also metabolized to LTC₄ via addition of a glutathione moiety. LTD₄ and LTE₄ are products of further metabolism of this glutathione moiety. Thus LTC₄, D₄ and E₄ are known as the cysteinyl LTs. They are also collectively referred to as slow-reacting substance, the mediator of the slow but sustained contractile response in airways and gastrointestinal smooth muscle; this and other effects of LTs are listed in **table 2.3**. For a recent review of lipoxygenase metabolites see FIORE [2004].

Dihomo-γ-linolenic acid competes with arachidonic acid for COX and therefore decreases the production of COX products from arachidonic acid, whilst favoring the production of the 1-series PGs and TXs for which it is the precursor (e.g. PGE₁ and TXA₁; there is no PGI₂ analogue formed from diho-

γ -linolenic acid). The prostanoids generated from dihomo- γ -linolenic acid have different (less potent) functional properties from those generated from arachidonic acid [HORROBIN, MANKU, 1990]. Dihomo- γ -linolenic acid is converted by 15-LOX to 15-hydroxy-dihomo- γ -linolenic acid, which can inhibit the 5-LOX activity.

Table 2.3 — Actions of arachidonic acid-derived leukotrienes.

	LTB₄	LTC₄, D₄ and E₄
Produced by	Neutrophils, macrophages	Mast cells, basophils, eosinophils
Increases	Leukocyte chemotaxis Leukocyte adhesion Leukocyte degranulation Leukocyte oxidative burst Inflammatory cytokine production	Vascular permeability Mucus secretion Smooth muscle proliferation Vasodilatation Vasoconstriction Smooth muscle contraction (bronchospasm)

EPA competes with arachidonic acid for COX and LOX and therefore decreases the production of COX products from arachidonic acid, whilst favoring the production of the 3-series PGs and TXs and 5-series LTs for which it is the precursor (**Figure 2.6**). The prostanoids generated from EPA frequently have different (less potent) functional properties from those generated from arachidonic acid (see Sections 3-2-4 and 5-3).

2-4 Fatty acids and cell signalling

In addition to their roles as precursors of regulatory eicosanoids, in membrane structure/fluidity/function and in the acylation of proteins involved in intracellular signalling, fatty acids may have other roles in signal transduction. Many lipids are involved directly in signalling pathways; for example hydrolysis of membrane phospholipids such as PIP₂ and PC by phospholipases C and D generates second messengers such as DAG and phosphatidic acid (**Figures 2.7 and 2.8**). Other phospholipids have roles in activating or stabilizing enzymes

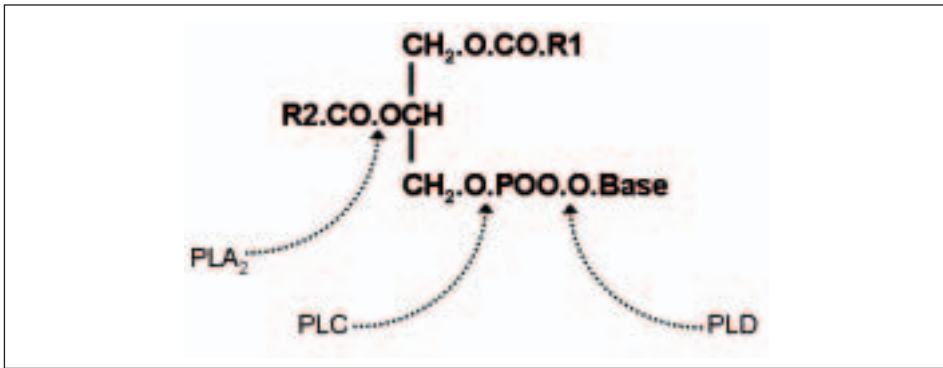


Figure 2.7 — Sites of action of phospholipase enzymes.
 PL, phospholipase.

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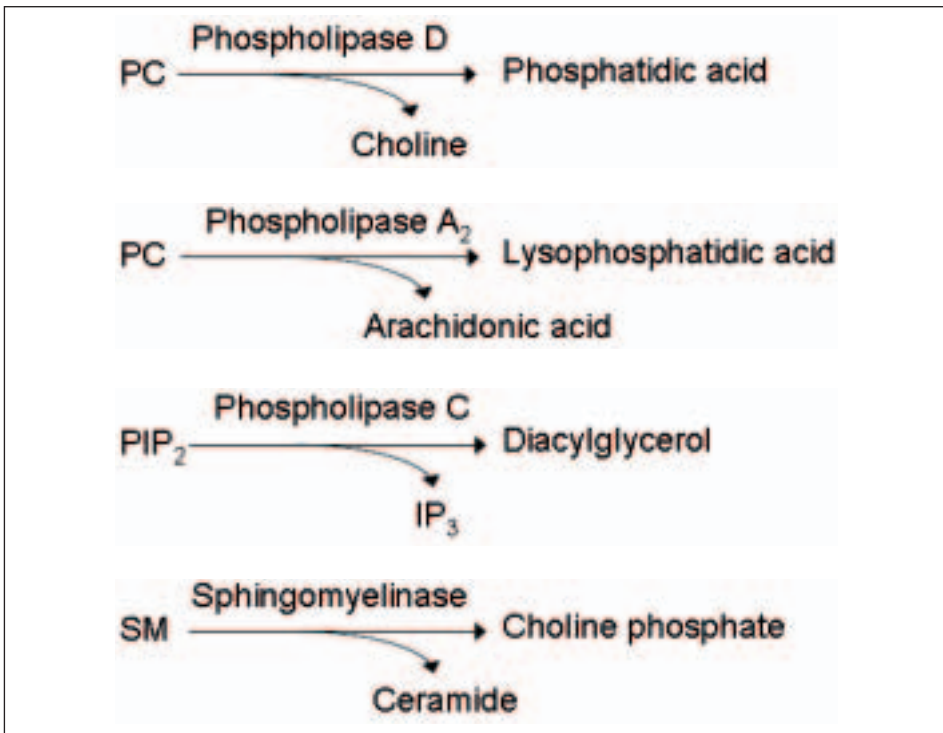


Figure 2.8 — Generation of signalling molecules from membrane phospholipids.
 IP_3 , inositol-2,4,5-trisphosphate; PC, phosphatidylcholine;
 PIP_2 , phosphatidylinositol-4,5-bisphosphate; SM, sphingomyelin.

involved in intracellular signalling; for example phosphatidylserine (PS) is required for protein kinase C (PKC) activation. Since PIP₂, PC, PS, phosphatidic acid and DAG all contain fatty acyl chains attached to the *sn*-1 and *sn*-2 positions of the glycerol moiety, it is conceivable that changing the type of fatty acid present may alter the precise properties of these compounds with regard to their functions in signal transduction. Indeed, KISHIMOTO *et al.* [1980] reported that PKC was more active with dioleoylglycerol and diarachidonoylglycerol than with diacylglycerols containing two saturated fatty acids or one saturated and one unsaturated fatty acid. BELL and SARGENT [1987] showed that the activity of rat spleen PKC differed in the presence of different combinations of PS and DAG each with differing fatty acid compositions. These observations suggest that the fatty acid composition of PS and DAG may affect the activity of PKC. In addition to the effects of fatty acids on intracellular signalling mechanisms due to changes in the fatty acid composition of the phospholipids which are involved, it has been proposed that fatty acids themselves may have a direct effect. This direct modulatory effect of fatty acids has been fairly extensively documented in relation to PKC, which has been shown to be regulated by oleic, linoleic, γ -linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acids in different ways according to the precise environment [e.g. SPEIZER *et al.*, 1991]. These observations are indicative that changes in fatty acid availability can affect the activity of cell signalling enzymes. More recently studies have shown that *n*-6 and *n*-3 PUFAs can differentially alter the expression and/or activities of other protein kinases involved in cell signalling pathways [e.g. LO *et al.*, 2000; DENYS *et al.*, 2001, 2002], and that this influences the activity of transcription factors such as nuclear factor κ B (see Section 5-5). This is one mechanism by which these fatty acids can alter gene expression in various cell types.

In addition to influencing signalling pathways that converge on transcription factor activation, fatty acids appear to more directly affect the activity of a family of transcription factors called the peroxisomal proliferator-activated receptors (PPARs). PPARs belong to the steroid hormone receptor family. There are several isoforms of PPARs (**Table 2.4**). PPARs act by forming a heterodimer with the retinoic-X-receptor (RXR), the ligand for which is *cis*-9-retinoic acid (**Figure 2.9**). PPAR- α is expressed mainly in the liver, and is involved in regulating hepatic responses to the availability of certain fatty acids, fatty acid metabolites and other peroxisome proliferators. Several key enzymes of β -oxidation and of lipoprotein metabolism have been shown to have PPAR- α -response elements in the 5'-upstream regulatory regions of their genes (**Table 2.4**) [SCHOONJANS *et al.*, 1996]. Thus, a role for activation of PPAR- α may be to partition fatty acids towards hepatic oxidation. PPAR- γ is expressed mainly in adipose tissue and is involved in regulating adipocyte differentiation and in the regulating metabolic

Table 2.4 — Distribution and targets of PPAR isoforms.

Receptor	Tissue distribution	Target genes
PPAR- α	Liver; Also kidney, heart, muscle, brown adipose tissue	↑ Apo AI, Apo AII, Enzymes of peroxisomal fatty acid oxidation, Carnitine palmitoyl transferase, Enzymes of mitochondrial fatty acid oxidation, Liver FABP ↓ Apo CII
PPAR- δ (also known as PPAR- β and FAAR)	Widespread	?
PPAR- γ 1	Widespread	?
PPAR- γ 2	Adipose tissue	↑ Factors involved in adipocyte differentiation, adipose tissue FABP (also known as aP2), lipoprotein lipase, FAT, acyl CoA synthase, GLUT4, phosphoenolpyruvate carboxykinase ↓ Leptin

Apo, apolipoprotein; FAAR, fatty acid activated receptor; FABP, fatty acid binding protein; FAT, fatty acid transporter; GLUT4, glucose transporter 4; PPAR, peroxisome proliferator-activated receptor.

responses of adipocytes. PPAR- γ is also expressed in inflammatory cells, and appears to be involved in regulation of production of inflammatory mediators (see Section 5-5). As with other steroid hormone receptors, PPARs are activated by non-covalent binding of ligands which promote alterations in transcription of genes containing PPAR response elements (**Figure 2.9**). An arachidonic acid derivative, 15-deoxy- Δ 12,14-PGJ₂, is a ligand for PPAR- γ [FORMAN *et al.*, 1995; KLEIWER *et al.*, 1995]. A number of years ago relatively high concentrations of several fatty acids including lauric, oleic, elaidic, linoleic, α -linolenic and arachidonic acids were shown to activate PPAR- α [GOTTLICHER *et al.*, 1992], although it was not clear whether they did so by binding directly to PPAR or by being metabolized to one or more common ligands. More recent studies have demonstrated that certain fatty acids are ligands for PPARs. For example,

FORMAN *et al.* [1997] demonstrated that linoleic, α -linolenic, γ -linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acids were ligands (and activators) of PPAR- α and - δ . In contrast, saturated fatty acids did not bind or

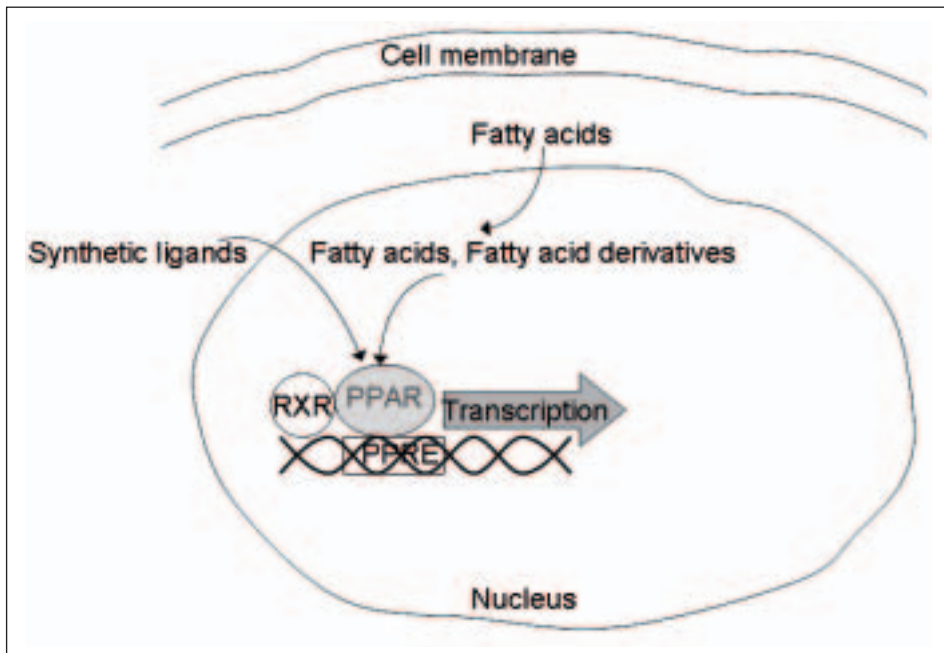


Figure 2.9 — Generalised scheme for the mechanism of action of peroxisome proliferator activated receptors.

PPAR, peroxisome proliferator activated receptor; PPRE, PPAR response element; RXR, retinoic-X-receptor.

activate PPAR- α or - δ . Although FORMAN *et al.* [1997] reported that PUFAs were poor activators of PPAR- γ , another study found that oleic, linoleic, α -linolenic and arachidonic acids were effective ligands and activators for both PPAR- α and - γ [KLEIWER *et al.*, 1997]. Thus, through activation of PPARs, fatty acids are able to regulate metabolism and other cell and tissue responses. This mechanism of action might explain some of physiological actions of certain fatty acids. For example, activation of PPAR- α by long chain *n*-3 PUFAs might explain the ability of these fatty acids to lower fasting plasma triacylglycerol concentrations (see Section 3-2-2), since this would partition fatty acids towards oxidation and away from incorporation into triacylglycerols.

2-5 Fatty acid composition changes as a result of increased intake of long chain *n*-3 PUFAs

Changes in the fatty acid composition of cultured cells according to the fatty acids to which they are exposed are readily demonstrated [ROSENTHAL, 1987]. Such studies show that EPA and DHA are fairly easily incorporated into mammalian cells, at least in culture, and that their incorporation is partly at the expense of arachidonic acid [e.g. CALDER *et al.*, 1990, 1994]. Feeding studies in laboratory animals confirm that long chain *n*-3 PUFAs are readily incorporated from the diet (e.g. from fish oil) into various cells and tissues and that this incorporation is associated with a reduction in arachidonic acid levels [e.g. YAQOUB *et al.*, 1995a, 1995b]. Increased consumption of long chain *n*-3 PUFAs by humans results in appearance of those fatty acids in plasma lipids [1], platelets [2], erythrocytes [3], leukocytes [4], cardiac cells [HARRIS *et al.*, 2004] and most likely in many other cell types [HOFFMAN *et al.*, 1999; LUND *et al.*, 1999; CONNOR *et al.*, 2001]. Some studies report that EPA is preferentially incorporated into phosphatidylethanolamine and phosphatidylcholine in platelets and erythrocytes, with little incorporation into phosphatidylserine or phosphatidylinositol [GALLOWAY *et al.*, 1985; VON SCHACKY *et al.*, 1985; POPP-SNIJDERS *et al.*, 1986]. The incorporation of long chain *n*-3 PUFAs occurs in a dose-response fashion. For example, studies using a range of EPA+DHA intakes from 1 to 5 g/day reported near linear relationships between EPA intake and the EPA content of plasma phospholipids [HARRIS *et al.*, 1991; MARSEN *et al.*, 1992], while BLONK *et al.* [1990] reported linear relationships between intakes of EPA and DHA at 1.5, 3 and 6 g/day and the proportions of those fatty acids in plasma phospholipids in male volunteers. SANDERS and ROSHANAI [1983] demonstrated dose-dependent incorporation of EPA and DHA into platelet phospholipids in men consuming between 1.6 and 6.5 g EPA+DHA/day for 3 weeks. In another study incorporation of EPA and DHA into neutrophils in

-
- [1] VON SCHACKY *et al.*, 1985; BLONK *et al.*, 1990; HARRIS *et al.*, 1991; MARSEN *et al.*, 1992; KATAN *et al.*, 1997; YAQOUB *et al.*, 2000; OTTO *et al.*, 2000.
- [2] GOODNIGHT *et al.*, 1981; SANDERS *et al.*, 1981; GALLOWAY *et al.*, 1985; VON SCHACKY *et al.*, 1985.
- [3] VON SCHACKY *et al.*, 1985; POPP-SNIJDERS *et al.*, 1986; KATAN *et al.*, 1997; OTTO *et al.*, 2000; YAQOUB *et al.*, 2000; TREBBLE *et al.*, 2003.
- [4] LEE *et al.*, 1985; ENDRES *et al.*, 1989; GIBNEY, HUNTER, 1993; SPERLING *et al.*, 1993; CAUGHEY *et al.*, 1996; HEALY *et al.*, 2000; YAQOUB *et al.*, 2000.

healthy young males occurred in a linear dose response manner (**Figure 2.10**). In an elegant study combining dose-response and time-course over 12 months in older male subjects KATAN *et al.* [1997] reported the fatty acid compositions of serum cholesteryl esters, erythrocytes and adipose tissue. The results for EPA incorporation into serum cholesteryl esters and erythrocytes are shown in **figure 2.11**. This study confirmed that EPA and DHA are incorporated into circulating lipid pools and into erythrocytes when their intakes are increased. It also demonstrated EPA and DHA incorporation into adipose tissue, a storage pool, when their intake is increased. However it also clearly shows that incorporation into different pools occurs at different rates and to differing extents (i.e. with different efficiencies) and may not be related to intake in a strictly linear fashion, at least over the intakes studied. The study of KATAN *et al.* [1997] shows that near-maximal incorporation of EPA and DHA into serum cholesteryl esters occurs within 30 days of beginning supplementation, whereas maximal incorporation into erythrocytes does not occur until sometime between 56 and 182 days. YAQOOB *et al.* [2000] reported the time dependent incorporation of EPA and DHA into peripheral blood mononuclear cells, a mixture of lymphocytes and monocytes; incorporation of both fatty

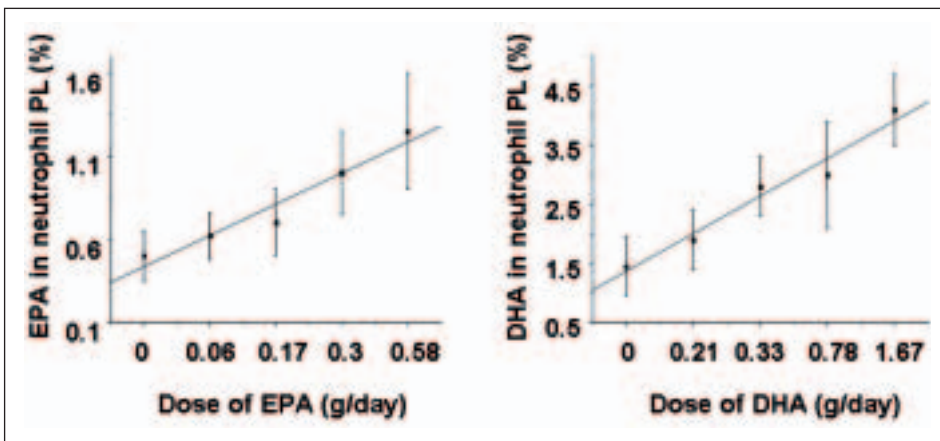


Figure 2.10 — Dose-dependent incorporation of eicosapentaenoic and docosahexaenoic acids into human neutrophils.

Healthy young males supplemented their diet with differing amounts of EPA and DHA from tuna oil (in capsules) for a period of 12 weeks. Blood neutrophil phospholipids (PL) were isolated and their fatty acid composition determined by gas chromatography. Data are mean \pm SEM from 8 subjects per dose, and are from HEALY *et al.* [2000].

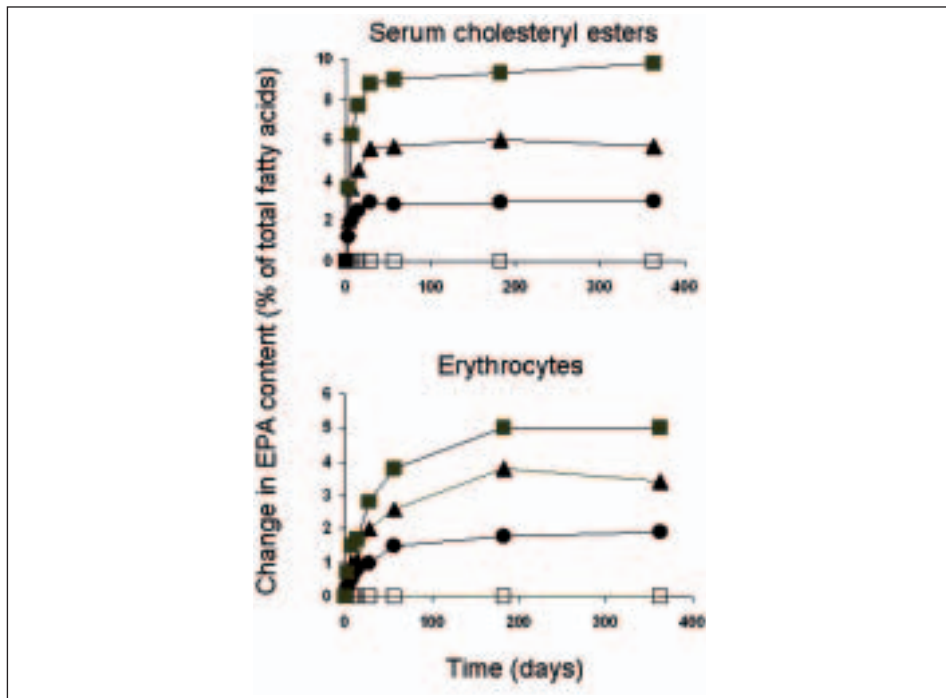


Figure 2.11 — Dose- and time-dependent incorporation of eicosapentaenoic acid into human serum cholesteryl esters and erythrocytes.

Healthy male subjects supplemented their diet with control capsules (non-3 fatty acids) or with fish oil capsules providing different amounts of EPA+DHA (1.35, 2.25 and 3.2 g/day) for a period of 12 months. Serum cholesteryl esters and erythrocytes were isolated and their fatty acid composition determined by gas chromatography. Data are mean values from 14 or 15 subjects per dose and are from KATAN *et al.* [1997]. ■ indicates highest dose (2.5 g EPA + 0.7 g DHA/day), ▲ indicates medium dose (1.75 g EPA + 0.5 g DHA/day); ● indicates lowest dose (1 g EPA + 0.35 g DHA/day); □ indicates control.

acids was near-maximal after 4 weeks of supplementation (Figure 2.12). Upon cessation of supplementation EPA in mononuclear cells returned to starting levels within 8 weeks, while the cells appeared to retain DHA (Figure 2.12). This is similar to the findings of POPP-SNIJDERS *et al.* [1986], who observed that erythrocyte EPA content returned to basal values eight weeks after stopping fish oil supplementation, whereas DHA remained elevated. The same observations of loss of EPA and selective retention of DHA upon cessation of

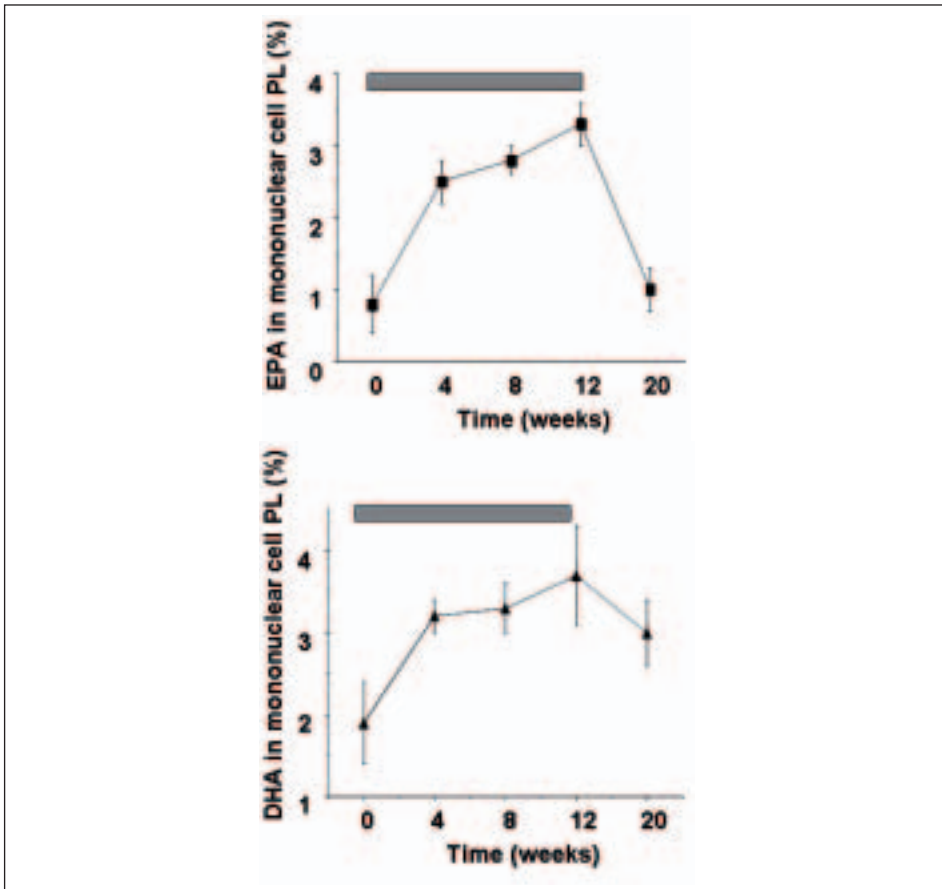


Figure 2.12 — Time course of incorporation of eicosapentaenoic and docosahexaenoic acids into human mononuclear cells.

Healthy subjects supplemented their diet with fish oil capsules providing 2.1 g EPA plus 1.1 g DHA per day for a period of 12 weeks (indicated by bar). Blood mononuclear cell phospholipids (PL) were isolated and their fatty acid composition determined by gas chromatography. Data are mean \pm SEM from 8 subjects and are from YAQOOB *et al.* [2000].

fish oil supplementation have been made for platelets [VON SCHACKY *et al.*, 1985]. Thus, a significant body of literature reports that EPA and DHA are incorporated into blood, cell and tissue lipids when their intake is increased. Given the roles of PUFAs in determining membrane structure and in regulating membrane protein-mediated responses, eicosanoid generation, cell signalling and transcription factor activity (**Figure 2.2**), it seems likely that increased

incorporation of long chain *n*-3 PUFAs into cell membranes will impact on cellular responses (Figure 2.13) and thus on cell, tissue and organ responses in a way that influences human health (Figure 2.14).

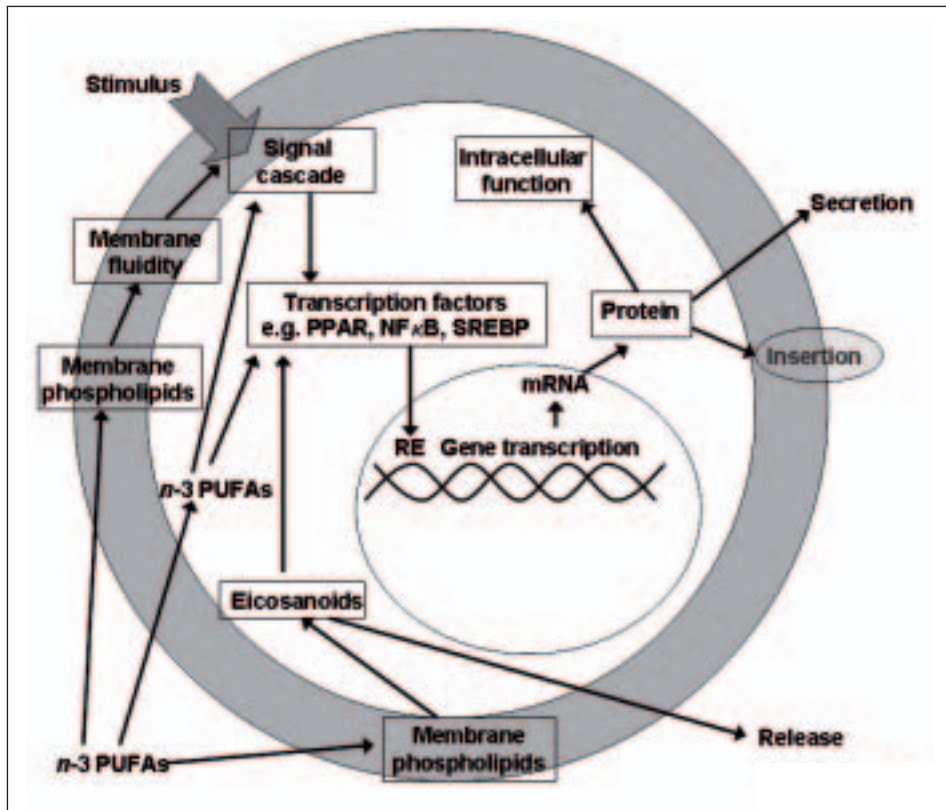


Figure 2.13 — Representation of the mechanisms by which an increased supply of *n*-3 polyunsaturated fatty acids could affect cell responses.

NFκB, nuclear factor κB; PPAR, peroxisome proliferator activated receptors; PUFAs, polyunsaturated fatty acids; SREBP, sterol receptor element binding protein.

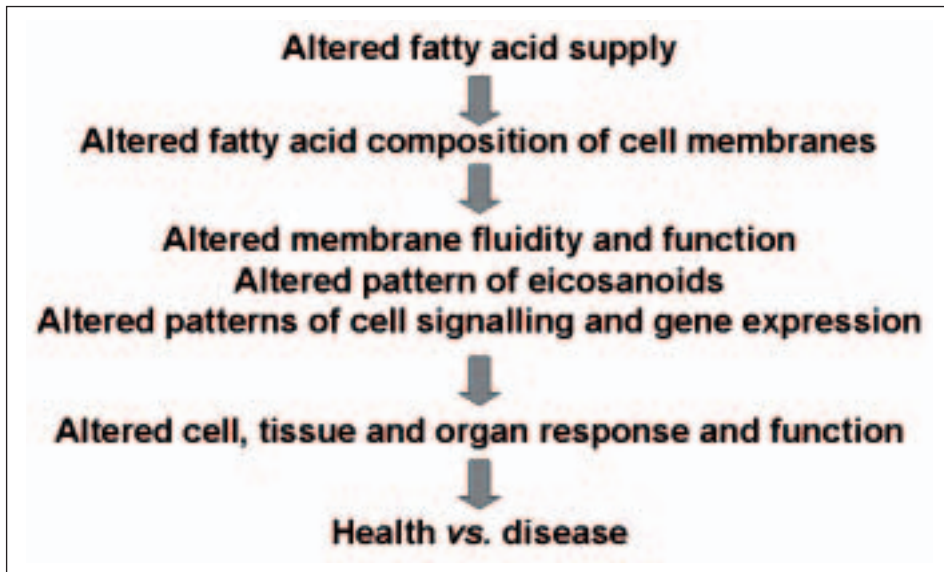


Figure 2.14 — General scheme relating fatty acid supply to human health via changes in cell, tissue and organ fatty acid composition.

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OMEGA-3 FATTY ACIDS: THE GOOD OIL?

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2 – POLYUNSATURATED FATTY ACIDS AND CELL MEMBRANES

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OMEGA-3 FATTY ACIDS: THE GOOD OIL?

3 – Long chain *n*-3 fatty acids and cardiovascular disease

3-1 Overview - atherosclerosis, cardiovascular disease, and risk factors

Cardiovascular disease (CVD) includes:

- coronary heart disease (CHD), which may manifest itself clinically as stable angina, unstable angina, myocardial infarction (“heart attack”) or sudden death;
- stroke, which may be thrombotic or haemorrhagic;
- peripheral vascular disease, which occurs in the aorta, and the iliac and leg, but rarely the arm, arteries, and which may manifest itself as leg pain, especially, but not exclusively on effort.

CVD is the major cause of death in Western countries. In the United Kingdom in 1990, CHD accounted for 30% of male deaths and 23% of female deaths. Stroke accounted for a further 15% of female deaths and 9% of male deaths. **Table 3.1** shows the age standardised mortality from CHD and stroke in European countries, the United States and Australia in 1989. In addition to being the major cause of death, morbidity from CVD (incapacitation due to myocardial infarction, stroke or peripheral vascular disease, amputation of the lower limbs due to peripheral vascular disease etc.) and the need for surgical interventions (coronary artery bypass grafts, aortic aneurysm repair, carotid endarterectomy) and pharmacological interventions (aspirin, ACE inhibitors, β -blockers, statins) place an enormous burden on sufferers, their families, health services and society at large.

Table 3.1 — Age-standardised mortality from coronary heart disease and stroke in various countries in 1989.

Country	Mortality from CHD*	Mortality from stroke*
Belgium	115	85
Denmark	250	80
France	70	65
Greece	100	140
Ireland	270	105
Italy	105	100
Luxembourg	130	120
Netherlands	140	80
Portugal	80	210
Spain	75	115
United Kingdom	225	100
Germany (West)	160	100
Australia	200	70
United States	180	50

* standardized death rate per 100,000 population. Data are from Department of Health [1994].

The underlying basis of CVD is the combination of atherosclerosis and thrombosis. Atherosclerosis is the thickening of the arterial wall as the result of the accumulation of lipid, calcium, connective tissue and cellular material in the form of plaques. The plaques, or lesions as they are sometimes called, may occur throughout the vasculature, their presence being determined by blood flow characteristics and by stress to the vascular wall. They are commonly found at bends or branches in the vasculature. The series of events leading to atherosclerosis is still not fully known. Fatty streaks, containing lipid-filled macrophages within the artery wall, have been identified in children and are likely to be the precursors of mature atherosclerotic plaques. A key event leading to formation of fatty streaks and plaques is the entry of low density lipoprotein (LDL) into the arterial intima and its subsequent modification, usually oxidation, and recognition by scavenger receptors on macrophages [STEINBERG *et al.*, 1989; WITZTUM, STEINBERG, 1991]. The modified LDL is then taken up by the macrophages in an apparently poorly regulated process that results in the macrophages becoming lipid-laden [STEINBERG *et al.*, 1989; WITZTUM, STEINBERG, 1991]. These lipid-laden macrophages are termed foam cells and their formation represents the sequestration of LDL lipids within the arterial wall. Oxidised LDL also acts as a chemoattractant for monocytes [BERLINER *et al.*, 1990], which are not usually found in high numbers within the normal intima. These monocytes are the precursors of the differentiated macrophages involved in LDL modification and uptake (**Figure 3.1**). The

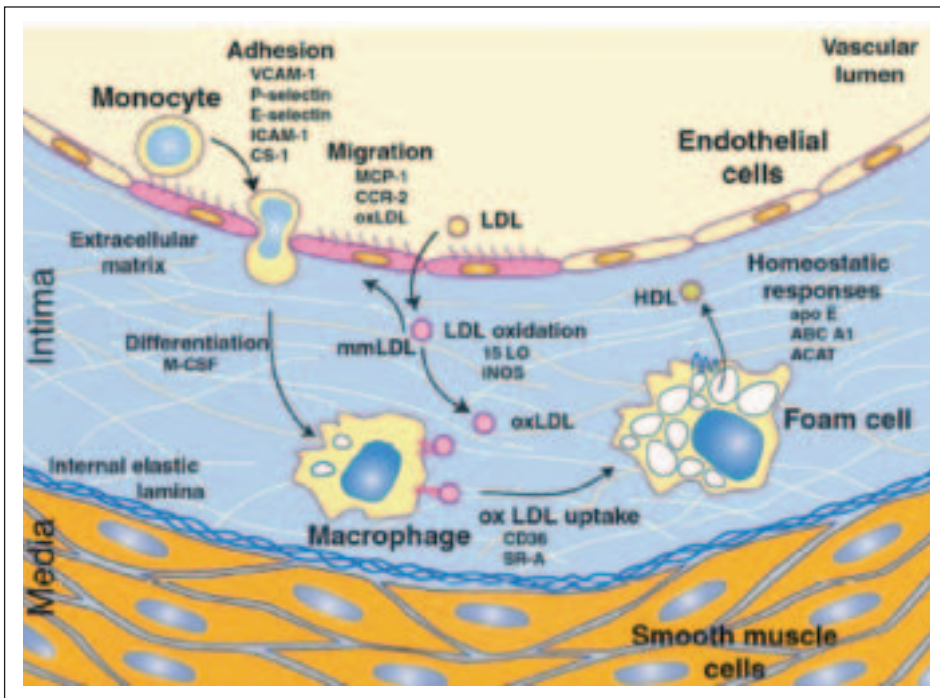


Figure 3.1 — Initiating events in atherosclerosis.

Low density lipoproteins (LDL) are subject to oxidative modifications in the subendothelial space, progressing from minimally modified LDL (mmLDL) to extensively oxidised LDL (oxLDL). Monocytes attach to endothelial cells that have been induced to express adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation.

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development of the atherosclerotic plaque is characterised by the migration of smooth muscle cells from the media layer of the artery into the intima, or subendothelial space (**Figure 3.2**). Intimal smooth muscle cells may proliferate and can take up modified LDL. They also synthesise the extracellular matrix proteins that lead to development of the fibrous cap. Interactions between monocyte/macrophages and T-cells are also important in plaque development (**Figure 3.2**). Both necrosis and rupture occur within regions of the plaque as it grows, and the latter may serve as foci for thrombus formation, suggesting that

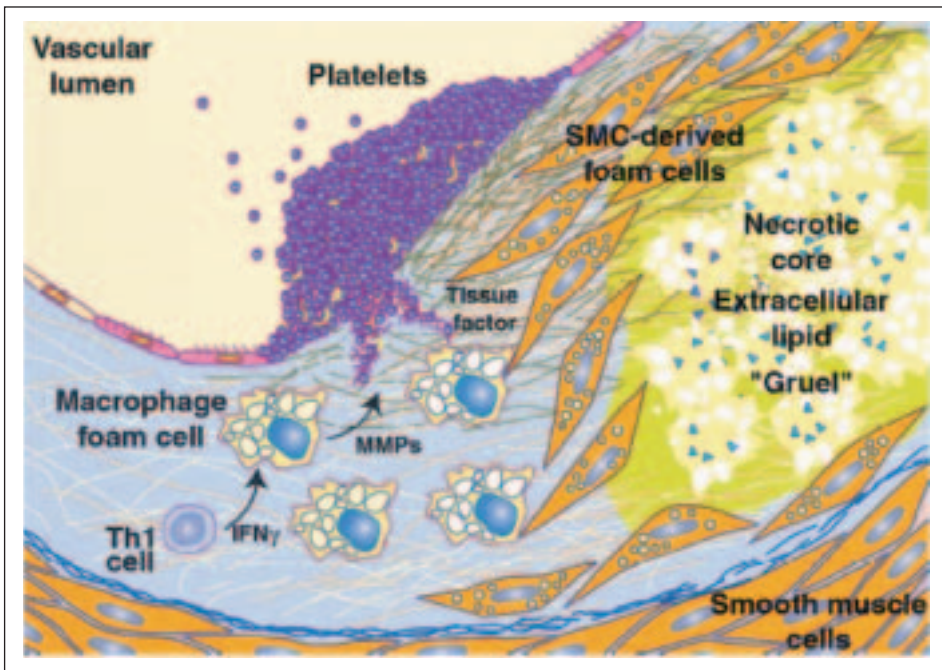


Figure 3.3 — Plaque rupture and thrombosis.

Necrosis of macrophages and smooth muscle cell (SMC) derived foam cells leads to formation of a necrotic core and accumulation of extracellular cholesterol. Macrophage secretion of matrix metalloproteinases (MMPs) weakens the fibrous cap. Plaque rupture exposes blood components to tissue factor initiating coagulation, recruitment of platelets and thrombus formation. Reprinted from *Cell*, 2001; 104:503-516, CKGLASS, JLWITZTUM, *Atherosclerosis: The road ahead*. Copyright, with permission from Elsevier.

A number of modifiable and non-modifiable risk factors for CVD have been identified (**Table 3.2**). Some of these (e.g. hypercholesterolemia, smoking) have been known for many years, but in recent years new risk factors, such as inflammation [PLUTZKY, 1999; GLASS, WITZTUM, 2001], have been identified.

Table 3.2 — Risk factors for cardiovascular disease.

<p>Non-modifiable risk factors:</p> <ul style="list-style-type: none"> Age Male gender Genetics
<p>Modifiable risk factors:</p> <ul style="list-style-type: none"> Low socioeconomic status Smoking Lack of physical activity Alcohol consumption Hypercholesterolaemia* Hypertriglyceridaemia (Fasting or post-prandial)* Hypertension* Vascular dysfunction* Diabetes* Obesity*? Propensity towards thrombosis* Inflammation* Hyperhomocysteinaemia

*indicates may be influenced by the fatty acid composition of the diet.

3-2 Long chain *n*-3 PUFAs and selected CVD risk factors

3-2-1 Hypercholesterolemia

Between country, within country, and migrant studies indicate a strong positive relationship between serum or plasma cholesterol concentration and incidence of coronary heart disease [see Department of Health, 2004 for references], with much of the risk lying with LDL cholesterol. Since movement of LDL cholesterol into the intima and its subsequent modification are necessary for foam cell formation and lipid deposition within the vessel wall,

it is clear why an elevated LDL cholesterol concentration is a risk factor for CVD. Thus, cholesterol, especially LDL-cholesterol, lowering is related to reduced risk of CVD. It is estimated that a 1% reduction in serum/plasma cholesterol concentration is associated with a 2.5% reduction in risk of an acute coronary event [see Dept. of Health, 2004]. LDL cholesterol lowering is associated with a lower rate of progression of atherosclerosis. High density lipoprotein (HDL)-cholesterol concentration has been inversely associated with CVD risk.

Numerous studies have shown that serum/plasma cholesterol concentrations are influenced by the types of fatty acids in the human diet [see KATAN *et al.*, 1995 for a review, and British Nutrition Found., 1992; Department of Health, 1994 for further references]. Detailed discussion of these studies is not necessary here but they can be summarised as follows:

- in general, saturated fatty acids increase and “polyunsaturated fatty acids (PUFAs)” (mainly linoleic acid) decrease serum cholesterol concentration, with equations being developed to predict the relationship between the change in serum cholesterol and the change in intake of saturated and polyunsaturated fatty acids;
- not all saturated fatty acids are equally potent in raising serum cholesterol concentrations. Lauric, myristic and palmitic acids are all cholesterol raising, while stearic acid and fatty acids with 10 or less carbon atoms do not appear to affect serum cholesterol concentrations. The cholesterol raising potency appears to be lauric < palmitic < myristic;
- replacement of either saturated fatty acids or carbohydrate with oleic acid decreases total and LDL cholesterol with a small HDL raising effect;
- substituting saturated fatty acids or carbohydrate with linoleic acid decreases total and LDL cholesterol concentrations;
- elaidic acid (trans-18:1 n -9) increases total and LDL cholesterol and decreases HDL cholesterol concentrations.

Numerous studies have examined the effect of long chain n -3 PUFAs on blood cholesterol, LDL and HDL concentrations; these have been reviewed by HARRIS [1996]. He identified 72 placebo-controlled studies of fish oil of more than 2 weeks duration in humans looking at circulating cholesterol concentrations and concluded that long chain n -3 PUFAs have a small cholesterol RAISING effect; overall there was a 3% increase in total cholesterol, a 4.5% increase in LDL cholesterol and a 4% increase in HDL concentration.

3-2-2 Hypertriacylglycerolemia in combination with a low HDL cholesterol concentration

Epidemiological data suggest that fasting triacylglycerol (TAG) concentrations are associated with increased CVD risk, probably in combination with decreased HDL cholesterol and the presence of small-dense LDL. Meta-analysis revealed a 30% increase in risk for each 1 mM increase in fasting TAG concentration in men (75% in women) [HOKANSON, AUSTIN, 1996]. When HDL cholesterol concentration was adjusted for, the risk was decreased (to 14% and 37%, respectively), but remained significant [HOKANSON, AUSTIN, 1996]. Thus, fasting TAG concentration is a risk factor independent of HDL cholesterol. Small-dense LDL is a risk factor because it is pro-atherogenic since it can cross the endothelium easily, and has great susceptibility to oxidation. TAG and small-dense LDL are related to one-another since TAG is a major determinant of small-dense LDL [GRIFFIN, 1997].

Long chain *n*-3 PUFAs exert a hypotriacylglycerolemic effect. A review of 72 placebo-controlled human studies which provided 1 to 7 g eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)/day for at least two weeks, demonstrated a 25 to 30% lowering in plasma TAG concentration [HARRIS, 1996]. The effect is dose-dependent [e.g. SANDERS *et al.*, 1981; BLONK *et al.*, 1990; SCHMIDT *et al.*, 1990]. Using a selection of studies providing 1 to 9 g EPA+DHA/day for 4 to 12 weeks, ROCHE [1999] derived a predictive equation for the relationship between altered fasting TAG concentration and altered intake of long chain *n*-3 PUFAs:

$$[\Delta\text{TAG (\% change)} = -7.67 - 3.05(\Delta n\text{-3 PUFA in g/day})]$$

Data from HARRIS [1996] and from ROCHE [1999] are indicative that a 20 to 30% lowering of fasting triglyceride concentrations could be expected from an intake of more than 2 g EPA+DHA per day. However, low doses of long chain *n*-3 PUFAs provided for an extended duration can induce the same TAG-lowering effect as higher doses given for a short time [ROCHE, GIBNEY, 1996].

Although there has been some focus on fasting plasma TAG concentrations, it is now recognised that the post-prandial TAG response may also be important as a risk factor, probably because of the relationship between TAG and cholesterol-rich lipoproteins [see WILLIAMS, 1997 for references]. In brief this can be explained as follows: an exaggerated or prolonged post-prandial response to fat provides greater opportunity for formation of cholesterol-rich chylomicron remnants and small-dense LDL particles [see WILLIAMS, 1997 for a detailed explanation]. The post-prandial response is examined by providing a meal

containing a known amount of fat to a fasting subject and taking a series of blood samples at times thereafter; typically both concentration and area-under-the-concentration-curve (AUC) are used as outcomes. ZAMPELAS *et al.* [1994] showed that including fish oil in the test meal resulted in lower plasma chylomicron and total TAG AUC than if an oil mix not including *n*-3 PUFAs was used. Compared with controls of olive oil or mixed oils, consumption of long chain *n*-3 PUFAs (1.6 to 9 g/day) for a period of time (4 to 42 days) resulted in lower peak TAG concentrations and lower chylomicron or TAG AUC when a saturated fatty acid rich test meal was consumed [see WILLIAMS, 1997 for references]; the reductions were between 28 and 46%. Typically a 30% reduction occurs at an intake of 1.6 to 2.8 g EPA+DHA/day. However some lower dose studies (< 1.7 g EPA+DHA/day) have failed to show an effect of long chain *n*-3 PUFAs on post-prandial lipemia [LOVEGROVE *et al.*, 1997; FINNEGAN *et al.*, 2003].

The lowering of plasma TAG concentrations either in the fasting or post-prandial state may relate to decreased entry of TAG-rich lipoproteins into the circulation or to their increased clearance from it. TAG clearance from the circulation is the result of the activity of lipoprotein lipase. Lipoprotein lipase mRNA levels were higher in adipose tissue of rats fed fish oil compared with other oils [MURPHY *et al.*, 1993]. However, human studies do not show enhanced lipoprotein lipase activity after chronic *n*-3 PUFA consumption. Fasting TAG and TAG appearing in the circulation in the late post-prandial state originate in the liver. Thus, hepatic actions of long chain *n*-3 PUFAs might explain the TAG lowering effect. These actions may relate to enhanced activation of PPAR- α , which increases the expression of enzymes involved in peroxisomal and mitochondrial fatty acid oxidation and decreases expression of some apolipoprotein genes [e.g. BERTHOUE *et al.*, 1995; see SCHOONJANS *et al.*, 1996 for a review]. These actions would have the effect of partitioning fatty acids towards β -oxidation and away from TAG synthesis and of decreasing VLDL assembly, respectively.

3-2-3 Hypertension and endothelial responsiveness

Systolic and diastolic blood pressure are directly related to risk of CHD and stroke [STAMLER *et al.*, 1989], possibly because of the damaging effects of pressure on the vessel wall that can create foci for plaque formation. A 7.5 mm Hg difference in diastolic pressure within the range 70 to 110 mm Hg is accompanied by a 29% difference in CHD risk and a 46% difference in risk of stroke irrespective of age, gender or ethnicity [MACMAHON *et al.*, 1990]. A mean

reduction of diastolic blood pressure of 4 mm Hg reduces risk of stroke by at least 34% [MACMAHON *et al.*, 1990; COLLINS *et al.*, 1990] and of CHD by 14% [COLLINS *et al.*, 1990].

A large number of studies have examined the effect of long chain *n*-3 PUFAs in the form of fish oil on blood pressure in both normotensive and hypertensive individuals. A number of reviews and meta-analyses of these studies exist [BONAA, 1989; RADACK, DACK, 1989; APPEL *et al.*, 1993; MORRIS *et al.*, 1993]. MORRIS *et al.* [1993] examined 31 placebo controlled trials using an average dose of 4.8 g EPA+DHA per day. The overall effects observed were reductions of 3 mm Hg in systolic and of 1.5 mm Hg in diastolic blood pressure. Effects were seen in hypertensive but not normotensive populations. A further meta-analysis of 36 trials, including 22 double blind, placebo controlled trials, involving over 2100 subjects was recently published [GELEIJNSE *et al.*, 2002]. The doses of fish oil used ranged from 0.2 to 15 g/day with an average dose of 3.7 g/day. Duration ranged from 3 to 52 weeks with a mean of about 12 weeks. The overall effects observed were reductions of 2.3 mm Hg in systolic and of 1.5 mm Hg in diastolic blood pressure. Effects were greater in older compared with younger populations and in hypertensive compared with normotensive populations. The data also suggested a greater effect in women than men.

The arachidonic acid derivative TXA₂ is a potent vasoconstrictor *via* its action on smooth muscle cells while PGI₂ is a vasodilator (**Table 2.2**). One of the characteristic features of increased availability of long chain *n*-3 fatty acids, especially EPA, is a reduction in the content of arachidonic acid in cell membrane phospholipids, thus decreasing the amount of substrate available for eicosanoid synthesis. Therefore, *n*-3 fatty acids are associated with a decrease in production of TXA₂ and PGI₂ (see below). As a result of incorporation of EPA into cell membrane phospholipids there is increased capacity to produce TXA₃ and PGI₃. TXA₃ is only weakly vasoconstrictive, while PGI₃ is vasodilatory. Thus, long chain *n*-3 PUFAs from fish oil might lower blood pressure by inducing vasodilation due to decreased TXA₂ and increased PGI₃ production. Alternatively they may act at the level of the kidney. Renal prostaglandins (PGE₂ and PGF₂) stimulate renin release, and renin promotes angiotensin formation causing the kidneys to retain salt and water, thus increasing extracellular fluid volume and increasing blood pressure. EPA-derived prostaglandins do not stimulate renin production [WEBER *et al.*, 1976; GERKENS *et al.*, 1981] and so fish oil may decrease blood pressure by decreasing renin activity.

There is a close relationship between blood pressure and endothelial responsiveness to vasodilators. A healthy endothelium modulates the activity of

vascular smooth muscle cells while damage to the endothelium decreases the vasodilatory response to acetylcholine and other substances. The effect of acetylcholine is mediated by nitric oxide produced by endothelial nitric oxide synthase. Damage to the endothelium results in decreased nitric oxide production and so an impaired response to acetylcholine. The impaired endothelial response may be secondary to atherosclerotic damage or a consequence of raised blood pressure and will act to increase blood pressure further. SHIMOKAWA *et al.* [1987, 1988] demonstrated that feeding cod liver oil to pigs increased nitric oxide release from coronary arteries in response to bradykinin, serotonin and thrombin, and that this was associated with an improvement in endothelium-dependent vascular relaxation. HARRIS *et al.* [1997] reported that fish oil providing 2 g EPA + 1 g DHA/day for 3 weeks resulted in a 43% increase in the concentration of nitric oxide metabolites in human urine, reflecting increased whole body nitric oxide production in humans. Interestingly 2.73 g EPA/day had no effect, suggesting that DHA may be the *n*-3 PUFA involved in regulating endothelial nitric oxide production. Several studies in humans now report improved nitric oxide-dependent and -independent endothelial relaxation and improved arterial compliance after chronic fish oil supplementation [CHIN *et al.*, 1993; McVEIGH *et al.*, 1994; GOODE *et al.*, 1997; TAGAWA *et al.*, 1999; GOODFELLOW *et al.*, 2000; NESTEL *et al.*, 2002].

3-2-4 Propensity towards thrombosis

As indicated above thrombosis is involved in the cycle of events that build up an atherosclerotic plaque and also is the ultimate cause of vessel occlusion during an acute cardiovascular event. The local responses at the vessel wall that play a role in thrombosis involve three main components: platelets, coagulation factors and fibrinolytic factors.

- **Platelet aggregation**

Cellular adhesive reactions lead to attachment of platelets to a damaged endothelial surface and their subsequent activation and aggregation. Arachidonic acid typically comprises 20 to 30% of the fatty acids in human platelet phospholipids. Platelet adhesion activates phospholipase enzymes, particularly PLA₂, releasing arachidonic acid. The released arachidonic acid is metabolised via COX (**Figure 2.5**) and then to TXA₂ a potent inducer of platelet degranulation (with the release of many factors including adenosine diphosphate (ADP), platelet-derived growth factor, fibrinogen, von Willebrand's factor, tissue plasminogen activator (tPA), plasminogen activator inhibitor (PAI)-1 and plasminogen) and aggregation (triggered in part *via* ADP release). Inhibitors of

platelet aggregation include adenosine, PGD_2 and PGI_2 . PGI_2 is synthesised by activated endothelial cells and inhibits both platelet TXA_2 synthesis and aggregation. Nitric oxide generated from endothelial cells is also an inhibitor of platelet aggregation. Thus, TXA_2 and PGI_2 /nitric oxide act as opposing factors controlling platelet aggregation (and smooth muscle contraction) (**Figure 3.4**).

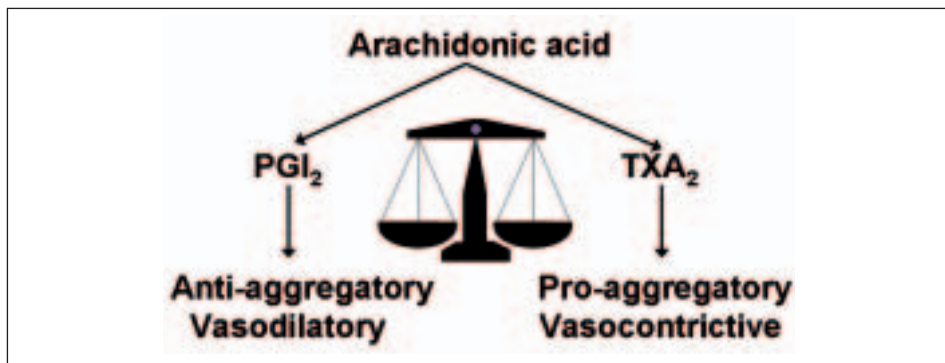


Figure 3.4 — The balance in production of cyclooxygenase products from arachidonic acid controls platelet aggregation and smooth muscle contraction. PG, prostaglandin; TX, thromboxane.

- **Coagulation and fibrinolysis**

Injury to the vascular endothelium can expose a cell membrane protein called tissue factor, which combines with circulating coagulant factor VII. The tissue factor/factor VII complex initiates a cascade of reactions (the coagulation pathway) that culminates in the generation of thrombin from prothrombin and the deposition of an insoluble fibrin network at the site of injury. The rate of coagulation is regulated by an anticoagulant pathway but this may be overwhelmed after injury. High levels of factor VII activity and of fibrinogen may predispose the blood to clotting (a hypercoagulable state) and are predictive of CHD [see British Nutrition Foundation, 1992 for references]. Disruption of the vessel wall can trigger massive deposition of platelets and fibrin (thrombosis), which may be sufficiently large to occlude the vessel lumen. There is a fibrinolytic system to protect against this [see British Nutrition Foundation, 1992]. It involves the generation of plasmin, which can degrade fibrin in a thrombus, from plasminogen. Plasminogen is converted to plasmin *via* the action of tPA. tPA is inhibited by plasminogen activator inhibitor type 1 (PAI-1).

- **Long chain *n*-3 PUFAs and platelet aggregation**

Comparison between Inuits and Danes and between inhabitants of Japanese fishing and farming villages showed that populations consuming high amounts of long chain *n*-3 PUFAs had decreased platelet aggregation and increased bleeding time [DYERBERG, BANG, 1979; HIRAI *et al.*, 1982]. One of the characteristic features of increased availability of long chain *n*-3 fatty acids, especially EPA, is a reduction in the content of arachidonic acid in membrane phospholipids in platelets [SIESS *et al.*, 1980; SANDERS *et al.*, 1981; GOODKNIGHT *et al.*, 1981; THORNGREN *et al.*, 1984; VON SCHACKY *et al.*, 1985] and presumably also endothelial cells, thus decreasing the amount of substrate available for eicosanoid synthesis. Therefore, *n*-3 fatty acids are associated with a decrease in production of TXA₂ and PGI₂ [GOODKNIGHT *et al.*, 1981; THORNGREN *et al.*, 1984; VON SCHACKY *et al.*, 1985; KNAPP *et al.*, 1986; KNAPP, FITZGERALD, 1989] (**Figure 3.5**). EPA is readily incorporated in a dose-dependent manner into platelet (and presumably endothelial) membrane phospholipids [SIESS *et al.*, 1980; GOODKNIGHT *et al.*, 1981, SANDERS *et al.*, 1981; THORNGREN *et al.*, 1984; KNAPP, FITZGERALD, 1989]. EPA is released upon platelet and endothelial activation by the action of phospholipase A₂ and acts as a substrate for COX (**Figure 2.6**). The products produced (e.g. TXA₃ and PGI₃) have a different potency from those produced from arachidonic acid: TXA₃ has a weaker pro-aggregatory effect than does TXA₂, while PGI₂ and PGI₃ have similar anti-aggregatory potencies [NEEDLEMAN *et al.*, 1979; 1980; FISHER, WEBER, 1984]. Therefore, the effect of long chain *n*-3 fatty acids is to decrease platelet aggregation in response to collagen [1] or ADP [2] and increase the time taken for blood to clot (i.e. bleeding time) [e.g. 3]. Increases of bleeding time of 20 to 100% are reported, the extent of the effect being largely related to dose of long chain *n*-3 PUFAs. It is unlikely that low-to-moderate doses of long chain *n*-3 PUFAs will dramatically affect bleeding time.

- **Long chain *n*-3 PUFAs and coagulation factors**

Incubation of a monocytic cell line with EPA or DHA did not affect basal expression of tissue factor but decreased by more than 50% the induction of tissue factor by endotoxin [CHU *et al.*, 1999]. Supplementing the diet of dogs with fish oil did not affect basal monocyte procoagulant activity, which is largely due to tissue factor expression, but significantly decreased the activity

[1] THORNGREN, GUSTAFSON, 1981; HIRAI *et al.*, 1982; SANDERS, HOCHLAND, 1983; VAN SCAHCKY *et al.*, 1985.

[2] THORNGREN, GUSTAFSON, 1981; THORNGREN *et al.*, 1984; FREESE, MUTANEN, 1997.

[3] GOODKNIGHT *et al.*, 1981; THORNGREN, GUSTAFSON, 1981; SANDERS, ROSHANAI, 1983; STEINBERG *et al.*, 1989.

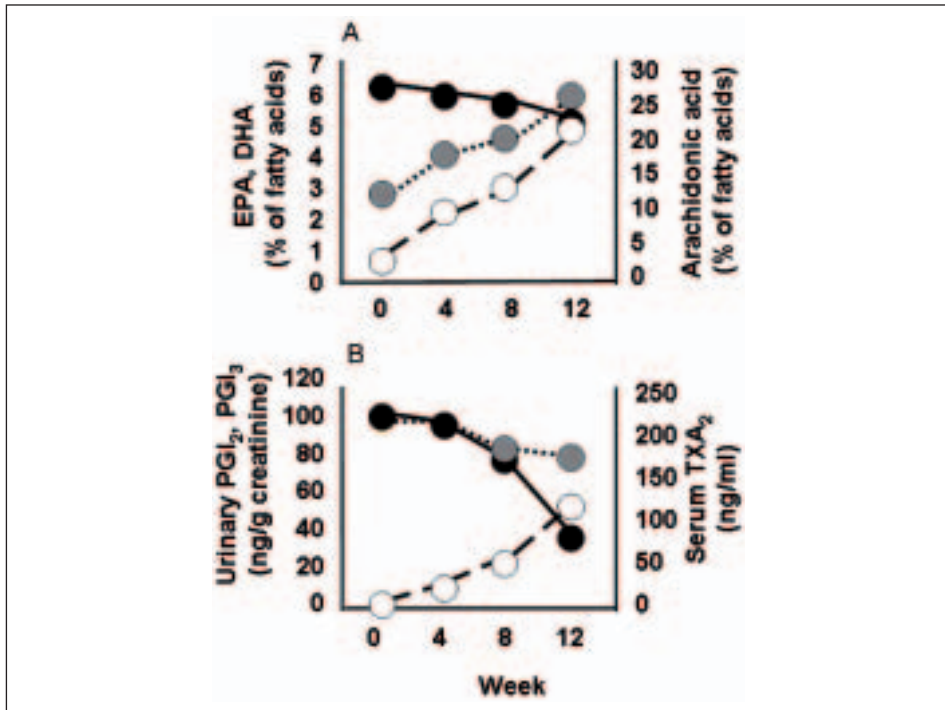


Figure 3.5 — Effect of long chain n-3 polyunsaturated fatty acids on platelet fatty acid composition and production of eicosanoids involved in regulation of platelet aggregation.

Healthy volunteers consumed 10 ml cod liver oil/day for 4 weeks, then 20 ml/day for 4 weeks, and then 40 ml/day for 4 weeks. Platelet EPA (○), DHA (●) and arachidonic acid (●) contents were determined by gas chromatography (A). Production of TXA₂ by clotting blood (●) and the urinary appearance of PGI₂ (●) and PGI₃ (○) were determined (B). Data are from VON SCHACKY *et al.*, 1985.

induced by endotoxin [CHU *et al.*, 1997]. HANSEN *et al.* [1989] showed that tissue factor expression on unstimulated and endotoxin-stimulated mononuclear cells was decreased by consumption of cod liver oil (25 ml/day for 8 weeks) by healthy adults. Similarly ABBATE *et al.* [1996] and TREMOLI *et al.* [1994] reported that 3.0 or 3.4 g EPA+DHA/day for 18 weeks decreased monocyte procoagulant activity (both unstimulated and endotoxin stimulated) by over 60%. One cross-sectional study revealed significant negative associations between fish or n-3 PUFA intake and concentrations of fibrinogen, factor VIII, and von Willibrand factor, but no association with factor VII [SHAHAR *et al.*, 1993].

Another study revealed no association between fish, EPA or DHA intake and concentrations of fibrinogen, factor VII, factor VIII or von Willebrand factor [ARCHER *et al.*, 1998]. Several studies report no effect of fish oil, even at fairly high doses, on factor VII concentration or coagulant activity [see British Nutrition Foundation, 1992; NETTLETON, 1995 for references]. YAHIA and SANDERS [1997] reviewed nine studies of fish oil and two of oily fish that provided between 1.3 and 9 g EPA+DHA/day for between 10 days and 9 months and identified only two studies that reported effects (both increases) on Factor VII coagulant activity. A number of studies report no effect of fish oil (providing 1.6 to 5.1 g EPA+DHA/day) on fibrinogen concentration, although some studies using high doses (> 4.5 g EPA+DHA/day) or long durations report decreases [see British Nutrition Foundation, 1992; NETTLETON, 1995 for references].

- **Long chain *n*-3 PUFAs and fibrinolytic factors**

Some studies have reported an increase in PAI-1 activity after fish oil supplementation, whereas others report a decrease or no effect [see NETTLETON, 1995]. In most studies fish oil did not affect tPA concentrations, although some studies have reported an increase or a reduction [see British Nutrition Foundation, 1992].

3-2-5 Inflammation

Atherosclerosis is now considered to be an inflammatory disease resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T cells and the normal cellular elements of the vessel wall [ROSS, 1993; 1999; BLAKE, RIDKER, 2001; GLASS, WITZTUM, 2001] (**Figures 3.1 and 3.2**). The earliest lesions, fatty streaks, are pure inflammatory lesions consisting only of monocyte-derived macrophages and T lymphocytes. Endothelial injury increases the adhesiveness of the endothelium to leukocytes (and platelets) by upregulating expression of adhesion molecules as well as its permeability. Mice deficient in intercellular adhesion molecule 1 (ICAM-1), E-selectin or P-selectin show less atherosclerosis when fed an atherosclerotic diet [see GLASS, WITZTUM, 2001; BLAKE, RIDKER, 2001], indicating the importance of endothelial-leukocyte adhesive interactions in the atherosclerotic process. The injured endothelium also produces vasoactive molecules, cytokines and growth factors. Some of these molecules such as monocyte chemoattractant protein 1 (MCP-1), act as chemoattractants attracting monocytes and T cells to the vessel wall (**Figures 3.1 and 3.2**). The resulting inflammatory response stimulates migration and proliferation of smooth muscle cells that become mixed into the area of inflammation (**Figure 3.2**). The

inflammatory response within the vascular wall is mediated by monocyte-derived macrophages and T cells [see ROSS, 1993; 1999; GLASS, WITZTUM, 2001]. Continued inflammation results in increased numbers of inflammatory cells that migrate from the bloodstream and proliferate within the lesion. Activation of these cells leads to release of enzymes, cytokines, chemokines, eicosanoids and growth factors continuing the inflammatory process and contributing to the build up of the plaque. C reactive protein (CRP) is an acute phase protein produced by the liver; interleukin (IL)-6 is the major inducer of CRP synthesis. CRP has been considered to be simply a marker of inflammation. However more recent studies have indicated that CRP may play an active role in inflammation by stimulating monocytes to release inflammatory cytokines and by increasing upregulation of adhesion molecule expression and MCP-1 synthesis by endothelial cells [see BLAKE, RIDKER, 2001 for references].

In accord with the proposed central role of inflammation in development of the atherosclerotic plaque, several inflammatory markers present in the serum or plasma are associated with cardiovascular disease risk. Soluble P-selectin was identified as an independent predictor of future CVD risk in the Women's Health Study [RIDKER *et al.*, 2001]. sICAM-1 concentration was shown to be an independent predictor of CHD risk [HWANE *et al.*, 1997; RIDKER *et al.*, 1998]. Serum IL-6 concentrations may also be predictive [RIDKER *et al.*, 2000]. However, of the inflammatory markers, CRP has been most investigated, and has been found to be predictive of future myocardial infarction, stroke, peripheral vascular disease and CVD mortality [see BLAKE, RIDKER, 2001 for references]. CRP has been compared with other inflammatory and lipid risk factors and found to be the most powerful predictor of risk [see BLAKE, RIDKER, 2001].

Long chain *n*-3 fatty acids decrease production of some chemoattractants including LTB₄ and MCP-1 [LEE *et al.*, 1985; SPERLING *et al.*, 1993; WALLACE *et al.*, 1995; BAUMANN *et al.*, 1999] and growth factors including platelet derived growth factor [WALLACE *et al.*, 1995; BAUMANN *et al.*, 1999] and the expression of some adhesion molecules on endothelial cells and leukocytes [HUGHES *et al.*, 1996; see also Section 5-4-2]. Thus long chain *n*-3 PUFAs could down-regulate processes leading to leukocyte and smooth muscle migration into the vessel wall intima. Long chain *n*-3 fatty acids are also anti-inflammatory exerting effects on the production of inflammatory eicosanoids and cytokines (see Sections 5-3 and 5-4-1) and so they could decrease inflammatory processes within the vessel wall, which are now recognised to be a major contributory factor in the atherosclerosis. Long chain *n*-3 PUFAs have been reported to decrease plasma concentrations of some adhesion molecules [see MILES *et al.*, 2001].

3-3 Long chain *n*-3 PUFAs and cardiovascular risk: evidence from ecological, epidemiological and case-control studies

As indicated in Section 3-2, long chain *n*-3 fatty acids favorably affect a number of risk factors involved in the development of atherosclerosis (Table 3.3), indicating that they will most likely slow the progression of the disease. Indeed, including long chain *n*-3 PUFAs in the diet has been demonstrated to decrease atherosclerosis in a variety of animal models [LANDYMORE *et al.*, 1985; WEINER *et al.*, 1986; DAVIS *et al.*, 1987; CAHILL *et al.*, 1988; RENIER *et al.*, 1993; MORTENSEN *et al.*, 1998].

Table 3.3 — Effects of long chain *n*-3 PUFAs on selected cardiovascular risk factors.

Risk factor	Effect of long chain <i>n</i> -3 PUFAs
Hypercholesterolaemia	-/↑
Fasting hypertriglyceridaemia	↓
Post-prandial hypertriglyceridaemia	↓
Hypertension	↓
Vascular dysfunction	↓
Propensity towards thrombosis	↓
Inflammation	↓

If long chain *n*-3 PUFAs do slow atherosclerosis, they might be expected to decrease risk of CVD mortality. It has been known for many years that Inuit populations in Greenland, Northern Canada and Alaska consuming their traditional diet had much lower cardiovascular mortality than predicted [DYERBERG *et al.*, 1978; KROMANN, GREEN, 1980; BJERREGAARD, DYERBERG, 1988; NEWMAN *et al.*, 1993]. Typically the rate of cardiovascular mortality was < 10% of that predicted [e.g. KROMANN, GREEN, 1980]. This was surprising since these populations consumed a high fat diet. The protective component was

suggested to be the long chain *n*-3 fatty acids consumed in very high amounts as a result of the regular intake of seal and whale meat and whale blubber [BANG *et al.*, 1976, 1980]. Intake of these fatty acids was estimated to average as much as 5 to 15 g/day amongst such populations [BANG *et al.*, 1976, 1980]. The Japanese also exhibit a low cardiovascular mortality [YANO *et al.*, 1988] and the traditional Japanese diet is rich in seafood including oily fish, which contain significant amounts of EPA and DHA. CVD mortality was different between Japanese fishing and farming villages [TOSHIMA *et al.*, 1995], supporting a protective role of fish. Substantial evidence from epidemiological and case-control studies has now accumulated indicating that consumption of fish or of long-chain *n*-3 fatty acids reduces the risk of cardiovascular mortality in Western populations [1]; [see CALDER, 2004 for details] and elsewhere [YUAN *et al.*, 2001]. KROMHOUT *et al.* [1985] demonstrated a significant effect of consuming fish with a 40% lower 20-year coronary heart disease mortality among men in The Netherlands who ate 1 to 14 g fish/day in 1960 compared with those men who did not eat fish at all in 1960. Men who consumed more than 30 g fish/day had a 60% lower risk of 20-year mortality from coronary heart disease compared with non-fish eaters. DAVIGLUS *et al.* [1997] reported a dose-dependent effect of fish consumption upon 30-year mortality among men in the USA. Men who ate 1 to 17 g fish/day in 1957 had a 10% lower risk of death from coronary heart disease or myocardial infarction compared with those who did not eat fish. Men who ate more than 34 g fish/day had a 40% lower risk of mortality from coronary heart disease or myocardial infarction compared with those who did not eat fish [DAVIGLUS *et al.*, 1997]. Although not all studies agree [e.g. ASCHERIO *et al.*, 1995], the protective effects of the fish and/or long chain *n*-3 fatty acids have been confirmed by a number of recent studies [ISO *et al.*, 2001; TAVANI *et al.*, 2001; ALBERT *et al.*, 2002; HE *et al.*, 2002; GUALLAR *et al.*, 2002; ERKILA *et al.*, 2003; LEMAITRE *et al.*, 2003; MOZAFFARIAN *et al.*, 2003]. Data from the Nurses' Health Study revealed that fish and long chain *n*-3 PUFA intake decreased risk of coronary heart disease, fatal coronary heart disease and non-fatal myocardial infarction [HU *et al.*, 2002]. In an Italian study, fish and long chain *n*-3 PUFA intake were lower in patients who suffered a non-fatal myocardial infarction compared with age- and gender-matched controls [TAVANI *et al.*, 2001]. Data from the Physicians' Health Study showed that the long chain *n*-3 PUFA status of whole blood at study entry was strongly inversely related to the risk of sudden death over the follow-up period of 0.7

[1] KROMHOUT *et al.*, 1985, 1995; SHEKELLE *et al.*, 1980; NORELL *et al.*, 1986; DOLECEK, 1992; FESKENS *et al.*, 1993; KELI *et al.*, 1994; SISCOVIC *et al.*, 1995; GILLUM *et al.*, 1996; DAVIGLUS *et al.*, 1997; ZHANG *et al.*, 1999; OOMEN *et al.*, 2000; PEDERSEN *et al.*, 2000.

to 16.9 (mean 8.7) years [ALBERT *et al.*, 2002]. The relative risk of sudden death was lower by about 50% in those men in the second quartile of whole blood total long chain *n*-3 PUFA content compared with those in the lowest quartile. The relative risk of sudden death in the highest quartile of blood long chain *n*-3 PUFA content was 70 to 90% lower (depending upon adjustment for other factors) than in the lowest quartile [ALBERT *et al.*, 2002]. A European multi-centre study identified that adipose tissue DHA content, a marker of DHA intake, was inversely associated with risk of first myocardial infarction, even when toenail mercury level (associated with increased risk) was adjusted for [GUALLAR *et al.*, 2002]. The relative risk of first myocardial infarction was 40% lower in men in the highest quintile of adipose tissue DHA content compared with those in the lowest quintile [GUALLAR *et al.*, 2002]. In another study, the combined EPA and DHA content of plasma phospholipids was lower in cases of fatal coronary heart disease than in matched controls [LEMAITRE *et al.*, 2003]. The authors identified that the odds ratio for fatal coronary heart disease was decreased by 70% with a one standard deviation increase in plasma phospholipid EPA plus DHA content.

3-4 Secondary prevention studies in post-MI patients

A secondary prevention study providing long chain *n*-3 fatty acids in the form of oily fish or fish oil capsules to patients who had already suffered a myocardial infarction demonstrated a significant reduction (29%) in mortality compared with the control group [BURR *et al.*, 1989]. More recently the GISSI Prevenzione study investigated the effect of *n*-3 fatty acids (0.885 g EPA plus DHA/day in the form of Omacor, a pharmaceutical-grade preparation of long chain *n*-3 PUFAs) on 3.5-year mortality outcomes in post-myocardial infarction patients in a placebo-controlled study also investigating vitamin E and involving approximately 11,000 patients in Italy. The major findings are shown in **table 3.4**, the most remarkable of which is the approximately 45% reduction in risk of sudden death. The effects observed occurred in the absence of lipid lowering [GISSI Prevenzione, 1999] and occurred in a relatively short period of time after starting supplementation. The reduction in risk of sudden death at 3.5 years in those patients consuming long chain *n*-3 fatty acids was already apparent at 4 months and the reductions in risk of cardiovascular mortality and coronary heart disease mortality were apparent within 6 to 8 months of initiating *n*-3 fatty acid treatment [MARCHIOLI *et al.*, 2002].

Table 3.4 — Summary of the findings of the GISSI Prevenzione study [1999].

	Control group (%)	<i>n</i>-3 PUFA group (%)	Risk reduction (%)	<i>P</i>
Primary outcomes				
All cause mortality, non-fatal MI and non-fatal stroke	14.8	12.6	16	0.02
Cardiovascular mortality, non-fatal MI and non-fatal stroke	11.7	9.4	20	0.006
Secondary outcomes				
All cause mortality	10.6	8.4	21	0.0004
Cardiovascular death	7.2	5.1	30	< 0.001
Cardiac death	6.1	4.0	35	< 0.001
Coronary death	5.2	3.6	32	< 0.01
Sudden death	3.3	1.8	44	0.0006
Non-fatal cardiovascular events	4.9	4.9	2	NS

Despite the potential for protection against atherosclerosis (Sections 3-2 and 3-3), much interest has been focused on the potent protective effect of *n*-3 fatty acids towards fatal myocardial infarction [BUUR *et al.*, 1989; SISCOVICK *et al.*, 1995; DAVIGLUS *et al.*, 1997; GISSI Prevenzione, 1999], and particularly towards sudden death [ALBERT *et al.*, 1998, 1999; GISSI Prevenzione, 1999; HU *et al.*, 2002], suggesting that they influence acute events. Several studies also report protection against non-fatal myocardial infarction [SISCOVICK *et al.*, 1995; PEDERSEN *et al.*, 2000; HU *et al.*, 2002; LEMAITRE *et al.*, 2003], which is consistent with a lowered risk of acute events be they non-fatal or fatal.

Two mechanisms have been proposed to contribute to the strong protective effect of long chain *n*-3 fatty acids towards acute cardiovascular events, especially those that are fatal. The first is the anti-thrombotic effect of long chain *n*-3 fatty acids already described in Section 3-2. However, some acute damage to the endothelium must occur to precipitate the thrombosis and so some other event must precede thrombosis (Figure 3.3).

Electrical impulses in the heart can become rapid (ventricular tachycardia) or chaotic (ventricular fibrillation) or both. The resulting irregular heart rhythm (arrhythmia) can cause the heart to suddenly stop beating, causing an infarction frequently in the form of sudden death (**Figure 3.6**). Studies in rats, pigs, dogs and marmosets suggest that long chain *n*-3 fatty acids from fish oil have anti-arrhythmic effects [e.g. McLENNAN *et al.*, 1988; 1990; McLENNAN, 1993] and so would act to counter this cause of sudden death. NAIR *et al.* [1997] reviewed 20 dietary studies with fish oil in animal models, almost all of which demonstrate benefits on outcomes such as ventricular fibrillation and ventricular tachycardia. These effects of dietary fish oil can be mimicked in cultured cardiomyocytes exposed to EPA or DHA [KANG, LEAF, 1994; LEAF, XIAO, 2001]. The presence of *n*-3 fatty acids in cardiomyocyte membrane phospholipids decreases electrical excitability and modulates the activity of ion (e.g. sodium, potassium, calcium) channels [XIAO *et al.*, 1997; 1998], effects that are claimed to promote electrical stability in the cell and prevent arrhythmias. SELLMAYER *et al.* [1995] reported that 2.4 g EPA+DHA/day decreased by 48% the frequency of premature ventricular complexes after 16 weeks in patients who experienced frequent but not life threatening ventricular arrhythmias. A recent study has indicated that long chain *n*-3 fatty acids may be able to alter arrhythmias acutely. In the study, patients with ventricular tachycardia

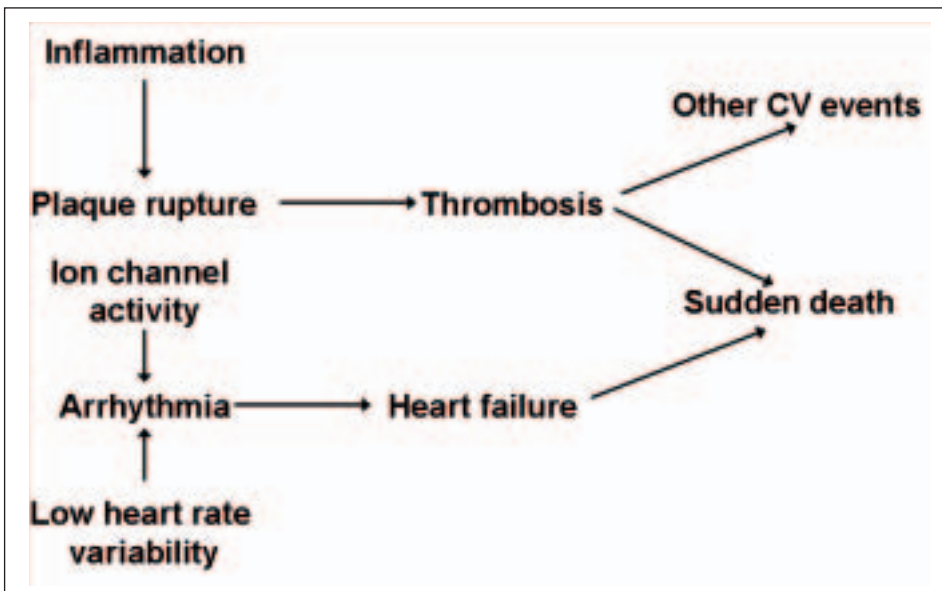


Figure 3.6 — Model linking inflammation, plaque rupture and thrombosis and arrhythmia to sudden death and other cardiovascular (CV) events.

and with implanted defibrillators were infused with an emulsion containing 3.8 g long chain *n*-3 fatty acids [SCHREPF *et al.*, 2004]. The infusion rendered five out of seven patients in this uncontrolled and unblinded study non-responsive to the induction of sustained monomorphic ventricular tachycardia.

In addition to possible anti-arrhythmic actions due to their effects on ion channels, long chain *n*-3 fatty acids might influence heart rate variability (low heart rate variability is believed to be associated with increased mortality post-myocardial infarction [TSUJI *et al.*, 1996; HUIKURI *et al.*, 1998]), and this might have an anti-arrhythmic effect *via* the autonomic nervous system (**Figure 3.6**). CHRISTENSEN *et al.* [2001] reported a positive correlation between the *n*-3 fatty acid content of platelets and heart rate variability in patients with type-1 diabetes mellitus. BROUWER *et al.* [2002] reported a significant positive association between the DHA, but not EPA, content of serum cholesteryl esters and heart rate variability in healthy subjects, although there was no association with the so-called QT interval (corrected for heart rate). CHRISTENSEN *et al.* [1996] reported increased heart rate variability in myocardial infarction survivors given 5.2 g EPA + DHA/day for 12 weeks. However, GEELLEN *et al.* [2002] found no effect of 1.5 g EPA+DHA/day for 12 weeks on QT interval (corrected for heart rate) or on other electrocardiographic characteristics in healthy elderly subjects. This may be because of the lower dose of EPA+DHA used. These studies are suggestive of two potential ways by which long chain *n*-3 fatty acids could affect cardiac arrhythmias: *via* modulation of ion channels and by increased heart rate variability (**Figure 3.6**). However, it is still unclear whether moderate intakes of long chain *n*-3 PUFAs do affect arrhythmias and so the results of new and ongoing studies are eagerly awaited.

An additional third mechanism may play an important role in the protective effects of long chain *n*-3 fatty acids towards acute cardiovascular events: the well-documented anti-inflammatory effects of long chain *n*-3 fatty acids may be important (**Figure 3.6**). The rupture of an atherosclerotic plaque, which is the acute event that exposes the plaque contents to the highly pro-thrombotic environment of the vessel lumen, is, essentially, an inflammatory event [PLUTZKY, 1999; GLASS, WITZTUM, 2001] (**Figure 3.3**). The characteristics of an atherosclerotic plaque that make it vulnerable to rupture include a thin fibrous cap and increased numbers of inflammatory cells such as macrophages [STARY *et al.*, 1995; FELTON *et al.*, 1997; PLUTZKY, 1999; GLASS, WITZTUM, 2001]. Long chain *n*-3 fatty acids might act to stabilize atherosclerotic plaques by decreasing infiltration of inflammatory and immune cells (e.g. monocyte/macrophages and lymphocytes) into the plaques and/or by decreasing the activity of those cells once in the plaque. Unstable regions of plaques have a lower *n*-3 PUFA content, and a higher *n*-6 PUFA content, than stable

areas [FELTON *et al.*, 1991]. RAPP *et al.* [1991] showed that high dose fish oil supplementation resulted in higher EPA and DHA contents of plaques in various sites of the vasculature than seen in the absence of supplementation. A recent study confirmed that long chain *n*-3 fatty acids are readily incorporated from dietary fish oil supplements (providing 1.4 g EPA+DHA/day) into advanced atherosclerotic plaques and showed that this incorporation is associated with structural changes consistent with increased plaque stability [THIES *et al.*, 2003] (**Table 3.5**). The

Table 3.5 — Summary of the findings of the study of THIES *et al.* [2003].

	EPA in carotid plaque phospholipids (% of total FAs)*	Plaque morphology		Macrophage staining	
		Type IV (% of plaques)	Type V (% of plaques)	Moderate (% of plaques)	Heavy (% of plaques)
Placebo group	0.6 ± 0.4	59.6	29.8	13.2	84.2
Fish oil group	1.1 ± 0.6	71.7	15.1	38.1	61.9
Significance of difference between groups (<i>P</i>)	< 0.0001	0.041	0.027	0.011	0.026

*mean ± SD; FAs=Fatty acids

morphology of carotid plaque sections was characterized according to the American Heart Association (AHA) classification [STARY *et al.*, 1995]. Plaques from patients treated with fish oil were more likely to be Type IV (fibrous cap atheromas: well-formed necrotic core with an overlaying thick fibrous cap) than those from the placebo group (odds ratio 1.19; **Table 3.5**). Conversely, plaques from patients treated with fish oil were less likely to be Type V (thin fibrous cap atheromas – thin fibrous cap infiltrated by macrophages and lymphocytes) than those from the placebo group (odds ratio 0.52). Thus, there were more plaques with a well formed fibrous cap, rather than a thin inflamed cap, in the fish oil group. Infiltration by macrophages was investigated using immunohistochemistry. It was found that plaques from patients given fish oil were more likely to be less heavily infiltrated with macrophages than those in the placebo group (**Table 3.5**). Since it is the vulnerability of the plaque to rupture rather than the degree of atherosclerosis that is the primary determinant

of thrombosis-mediated acute cardiovascular events, it is likely that the findings of THIES *et al.* [2003] are clinically relevant. If carotid plaques really are stabilized by fish oil, then the risk of neurological events in patients with advanced carotid atherosclerosis may be reduced. If a similar stabilizing effect of *n*-3 PUFAs occurs in coronary plaques then these too might be stabilized. This might explain the significant protective effects of *n*-3 PUFAs towards both fatal and non-fatal cardiovascular events [see earlier], which are so far not fully explained.

3-5 Conclusions

Cardiovascular disease is accompanied by significant morbidity and remains the biggest cause of mortality in Western populations. Some of the risk factors involved were known many years ago, but in recent times new risk factors have been identified. Many of these risk factors are related to, or can be influenced by, diet. There is considerable evidence from ecological, epidemiological and case control studies that consumption of fish, oily fish or long chain *n*-3 PUFAs decreases risk of cardiovascular events and of cardiovascular mortality. Secondary prevention trials in survivors of myocardial infarction have shown that long chain *n*-3 PUFAs decrease mortality with a particularly potent effect on sudden death. Studies in experimental animals and in humans have demonstrated beneficial effects of long chain *n*-3 PUFAs on cardiovascular risk factors including hypertriacylglycerolemia, post-prandial hypertriacylglycerolemia, hypertension, vascular dysfunction, thrombosis and inflammation. Since these are involved in initiating and progressing atherosclerosis (i.e. the buildup of the fibrous plaque in the vessel wall), the reduction in risk factor burden may explain the benefits of long chain *n*-3 PUFA consumption. However, effects on sudden death and sudden death mortality suggest effects on cardiovascular events and on event severity even in subjects with advanced atherosclerosis. Anti-thrombotic effects of long chain *n*-3 PUFAs may decrease event severity, and so decrease the likelihood of dying. Anti-arrhythmic actions are believed by many to decrease the likelihood and severity of myocardial infarction particularly of the type that leads to sudden death. *In vitro* and animal studies and a limited number of human studies support this mechanism of action. Finally, the anti-inflammatory actions of long chain *n*-3 PUFAs may be important in maintaining the stability of advanced plaques, so preventing their rupture. In reality all of these mechanisms may operate in concert.

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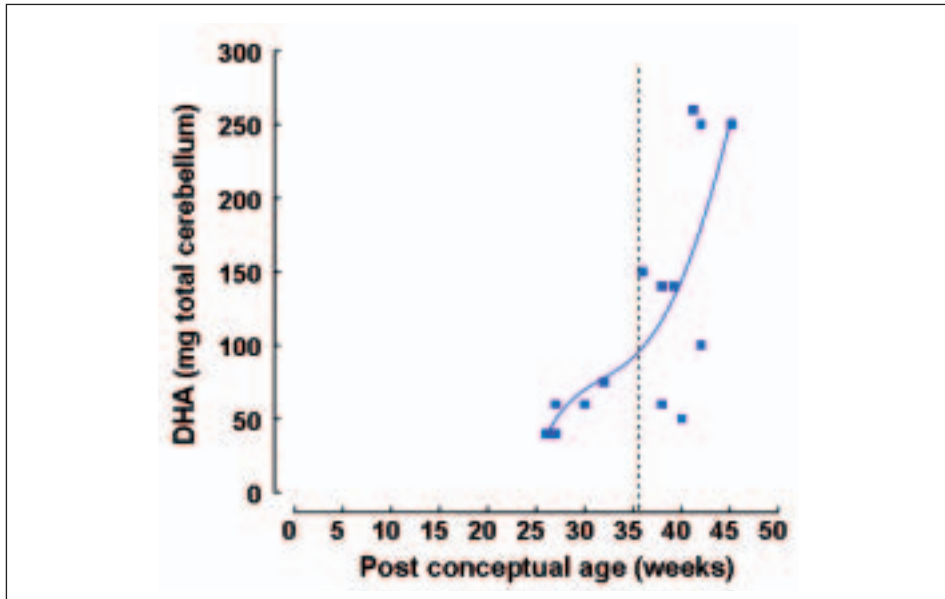
4 – Long chain *n*-3 fatty acids and the brain

4-1 The relationship between maternal and fetal docosahexaenoic acid status

4-1-1 Fetal and infant brain growth creates a demand for DHA

More than 50% of the dry weight of the brain is lipid, particularly structural lipid (i.e. phospholipids). The most abundant fatty acids in the brain are docosahexaenoic acid (DHA), arachidonic acid and adrenic acid [O'BRIEN, SAMPSON, 1965; CRAWFORD *et al.*, 1976]. The human brain and retina contain an especially high proportion of DHA relative to other tissues and little eicosapentaenoic acid (EPA) [O'BRIEN, SAMPSON, 1965; ANDERSON, 1970; CRAWFORD *et al.*, 1976]. Grey matter phosphatidylethanolamine contains 24% of fatty acids as DHA while grey matter phosphatidylserine contains 37% fatty acids as DHA [O'BRIEN, SAMPSON, 1965; CRAWFORD *et al.*, 1976]. DHA contributes 50 to 70% of the fatty acids present in the rod outer segments of the retina [ANDERSON, 1970]. These rod outer segments contain the eyes' photoreceptors. The human brain growth spurt occurs from approximately the beginning of the third trimester of pregnancy to 18 months after birth [MARTINEZ, 1992]. The amount of DHA in the brain increases dramatically during the brain growth spurt (**Figure 4.1**). In humans, brain weight increases from about 100 g at 30 weeks of gestation to about 1100 g at 18 months of age [DOBBING, SANDS, 1973]. Over this period the DHA content of the brain increases from 900 µg/g (90 mg in total) to 3,000 µg/g (3,300 mg total) [CUNNANE *et al.*, 2000; see also MARTINEZ, MOUGAN, 1998; LAURITZEN *et al.*, 2001]. This represents a 35-fold increase in total brain DHA. The estimated rate of accretion of DHA into the human brain in the last trimester of pregnancy is 15 to 22 mg/week [CLANDININ *et al.*, 1980]. This

is also the most active period of brain cell division. Thus it is believed that an adequate supply of DHA during this period is essential for normal growth, neurological development and function, and learning behaviour.



76

Figure 4.1 — Accumulation of docosahexaenoic acid (DHA) by the human cerebellum during the period before and soon after birth.

Drawn by Dr G.C. BURDGE using data from MARTINEZ, MOUGAN [1998].

4-1-2 Maternal supply of DHA to the fetus during pregnancy

There is a significant linear relationship between the DHA contents of maternal and umbilical cord plasma phospholipids (Figure 4.2). This suggests that maternal plasma phospholipids are an important source of DHA for the fetus and that maternal plasma phospholipid DHA concentration determines DHA supply to the fetus. A model for supply of fatty acids from mother to fetus is shown in figure 4.3. An increase in maternal plasma DHA concentration occurs during pregnancy and this increase precedes the increase in DHA

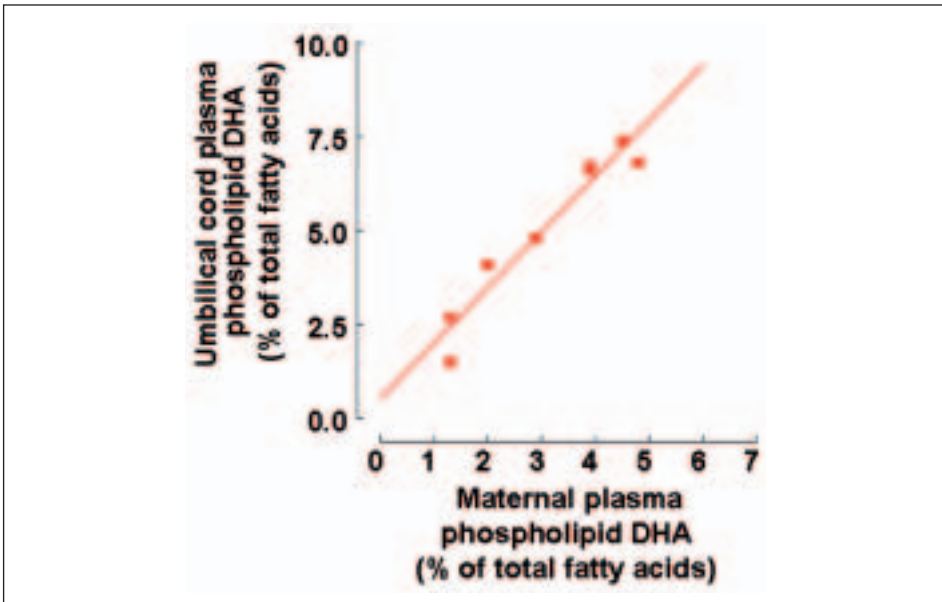


Figure 4.2 — The relationship between the docosahexaenoic acid contents of maternal and umbilical cord blood phospholipids.

Drawn by Dr. G.C. BURDGE using data from SHERMAN *et al.* [2001].

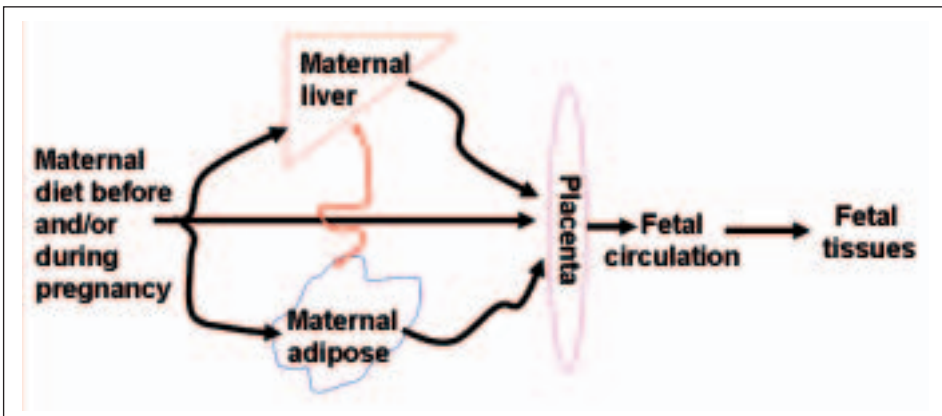


Figure 4.3 — General model for the transfer of nutrients from mother to fetus.

Drawn by Dr G.C. BURDGE.

accretion by the brain. The extent of the increase differs among pregnant women in different countries [OTTO *et al.*, 1997], perhaps indicating dietary differences during the pre-pregnancy period. AL *et al.* [1997] reported that maternal plasma phospholipid DHA content was lower in women who had had multiple pregnancies than in those in their first pregnancy. This may indicate that maternal body stores are important in maintaining plasma DHA status but that these may be eroded by multiple pregnancies.

4-1-3 Maternal supply of DHA to the infant after birth

Human breast milk contains DHA, although the amount differs among populations and sub-groups of the population, one important influence being diet (**Table 4.1**) [see LAURITZEN *et al.*, 2001 for a discussion]. However, a typical breast milk DHA concentration in Western populations is 0.2 to 0.3% of fatty acids or about 0.02% of milk. Note that Inuit women have a much higher breast milk DHA content than women consuming a typical Western diet (**Table 4.1**). Breast milk DHA content decreases with duration of lactation [see LAURITZEN *et al.*, 2001].

4-2 DHA plays special roles in brain and eye development (and function)

Studies in rats, mice, guinea-pigs, cats, pigs and monkeys demonstrate that maternal deprivation of *n*-3 fatty acids results in decreased accumulation of DHA into the brain and eye, and in reduced visual responses and discrimination [WHEELER *et al.*, 1975; FRANÇOIS *et al.*, 1980; NEURINGER *et al.*, 1984; 1986; 1988; REISBICK *et al.*, 1990; WEISINGER *et al.*, 1996] and in impaired learning [LAMTEY, WALKER, 1976; YAMAMOTO *et al.*, 1988]. Some studies demonstrated that supplementation with DHA or fish oil after birth could restore the abnormalities [CONNOR, NEURINGER, 1988]. A study in monkeys showed that maternal deprivation of *n*-3 PUFAs also resulted in behavioural deficits in the offspring such as increased drinking frequency, increased anxiety, depression and impaired socialisation [REISBICK *et al.*, 1992]. Thus, an adequate supply of *n*-3 PUFAs, and perhaps DHA in particular, appears essential for visual, neural and behavioural development to occur in an appropriate way. In humans, maternal dietary intake of DHA (and also of arachidonic acid) is related to head circumference at birth [CRAWFORD *et al.*, 1986; DOYLE *et al.*, 1990; KOLETZKO, BRUNN, 1991], indicating the importance of DHA supply to the fetus to *in utero* brain growth.

Table 4.1 — Human breast milk fatty acid composition (% of total fatty acids).

Country	Linoleic acid	α -Linolenic acid	Arachidonic acid	DHA	Reference
USA	14.5	0.7	0.68	0.29	SPECKER <i>et al.</i> [1987]
USA	14.7	1.5	0.28	0.06	FINLEY <i>et al.</i> [1985]
USA	15.3	0.8	0.4	0.1	HARRIS <i>et al.</i> [1984]
USA	15.6	1.0	0.6	0.23	BITMAN <i>et al.</i> [1983]
UK	10.9	0.49	0.38	0.37	SANDERS, REDDY [1992]
UK*	19.5	1.25	0.38	0.30	SANDERS, REDDY [1992]
Australia	10.8	0.6	0.4	0.32	GIBSON, KNEEBONE [1981]
Canada	12.7	0.6	0.7	0.4	INNIS, KUHNLEIN [1988]
Canada**	11.5	0.5	0.6	1.4	INNIS, KUHNLEIN [1988]
Finland	11.4	-	-	-	VUORI <i>et al.</i> [1982]
Sweden	12.9	0.7	0.4	0.3	JANSSON <i>et al.</i> [1981]
Germany	10.0	0.8	0.39	0.16	HARZER <i>et al.</i> [1983]
Germany	10.8	0.8	0.36	0.22	KOLETZKO <i>et al.</i> [1988]

* Vegetarians

** Arctic

n-3 PUFAs, most likely DHA, appear to be required for several aspects of normal brain development and function. During the structural development of the brain neurones develop by outgrowth of extensions called neurites and by establishing connections between neurites. Nerve growth factor (NGF) promotes the differentiation of neurones. Deficiency of *n*-3 PUFAs in pregnant rats has been demonstrated to decrease the NGF concentration in the brain of the offspring [IKEMOTO *et al.*, 2000] and to decrease neurone length in the hippocampus and cortex, although not in the hypothalamus, of the offspring at 6 weeks of age [IKEMOTO *et al.*, 1997; AHMAD *et al.*, 2002], suggesting an impairment of neurite outgrowth. DHA deficiency also results in impaired dopaminergic and serotonergic neurotransmission [ZIMMER *et al.*, 2000] and decreased neuronal Na⁺/K⁺ ATPase activity [BOWEN, CLANDININ, 2002].

The role of DHA in the eye appears to be related to providing an environment that allows the protein rhodopsin to undergo conformational changes necessary for signal transduction [VAIDYANATHAN *et al.*, 1994] (**Figure 4.4**).

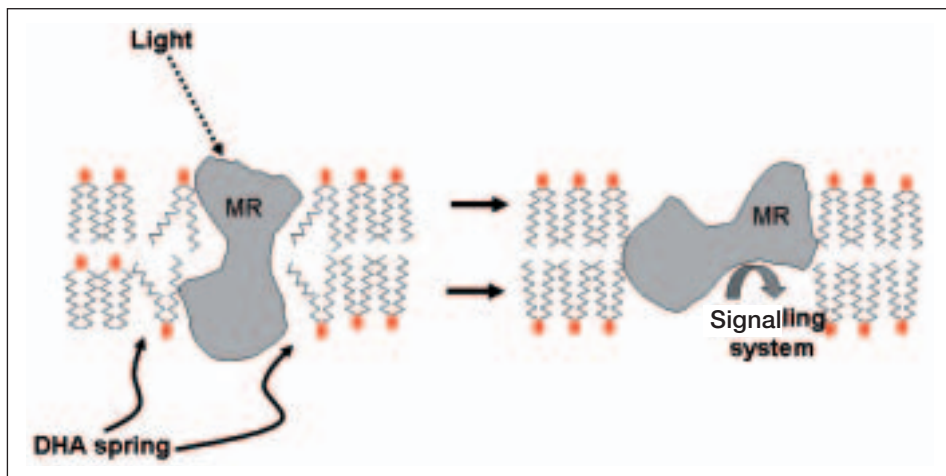


Figure 4.4 — Model of the role of docosahexaenoic acid (DHA) in the membrane of retinal rod outer segments in allowing metarhodopsin (MR) to respond to visual signals.

The structure of DHA allows phospholipids containing two DHA moieties to act like a spring enabling metarhodopsin to change conformation upon receipt of a visual stimulus. The conformational change initiates the signaling events.

Drawn by Dr G.C. BURDGE.

Studies with rhodopsin imbedded in artificial membrane bilayers demonstrate that phospholipids containing two DHA molecules are superior to phospholipids containing one or no DHA molecules [MITCHELL, LITMAN, 1998].

4-3 DHA and visual function

4-3-1 Pre-term human infants

Pre-term birth results in interruption of DHA supply from the mother and so the infant is at great risk of decreased DHA status. Supply of human breast milk, which contains long chain PUFAs including DHA, results in improved visual development in preterm infants compared with feeding formula that does not contain long chain PUFAs [UAUY *et al.*, 1990; BIRCH DG *et al.*, 1992; BIRCH EE *et al.*, 1992]. A number of studies reported that addition of DHA to pre-term formulas resulted in improved visual acuity [UAUY *et al.*, 1990; BIRCH DG *et al.*, 1992; BIRCH EE *et al.*, 1992; CARLSON *et al.*, 1993; 1996; CARLSON, WERKMAN, 1996; WERKMAN, CARLSON, 1996; FALDELLA *et al.*, 1996; O'CONNOR *et al.*, 2001] compared with infants receiving standard formula, although one study was negative [VAN WEZEL-MEIJLER *et al.*, 2002] and the positive effects on visual acuity eventually diminished [CARLSON *et al.*, 1993; CARLSON, WERKMAN, 1996; WERKMAN, CARLSON, 1996]. A meta-analysis including studies published up to 1996 (4 studies) showed differences between DHA-supplemented formula and standard formula in visual acuity at 2 and 4 months of age; improvement with DHA containing formula was roughly 23 and 14% at 2 and 4 months, respectively. It was concluded that DHA is efficacious with respect to early visual system development, but whether this advantage is maintained remains to be determined [SANGIOVANNI *et al.*, 2000].

4-3-2 Term infants

WILLIAMS *et al.* [2001] observed that children whose mothers ate oily fish during pregnancy tended to have better visual acuity at 3.5 years than children whose mothers did not. JORGENSEN *et al.* [2001] observed a positive association between DHA intake from breast milk and visual acuity at 4 months of age. Some studies show better visual acuity in term infants fed breast milk than in those fed standard formula [MAKRIDES *et al.*, 1995; 2000; JORGENSEN *et al.*, 1996; BIRCH *et al.*, 1998; HOFFMAN *et al.*, 2000]. Other studies show better visual acuity

in infants fed milk formula supplemented with DHA than without DHA [MAKRIDES *et al.*, 1995; 2000; CARLSON *et al.*, 1996; BIRCH *et al.*, 1998; HOFFMAN *et al.*, 2000], although some studies do not show such differences [JORGENSEN *et al.*, 1996; AUESTAD *et al.*, 1997; 2001; 2003; MAKRIDES *et al.*, 2000]. These data have been subjected to meta-analysis. One meta-analysis concluded that DHA-containing formula results in better visual acuity at 2 months, and perhaps 4 months, of age than seen with non-DHA containing formulas [SANGIOVANNI *et al.*, 2000]. However a second meta-analysis concluded that there is little evidence in favour of benefit for visual development of term infants [SKIMMER, 2001].

4-4 DHA and cognitive development

4-4-1 Pre-term infants

LUCAS *et al.* [1992] reported that pre-term infants who had received breast milk for one month post-partum had a significantly higher IQ at 8 years of age than infants who received standard formulas. They speculated that the difference may be due to the presence of long chain PUFAs, including DHA, in breast milk but not formula. However, a later study by the same researchers found that breast milk was not superior to standard formula with respect to Bayley Mental Development Index at 1 or 1.7 years of age [LUCAS *et al.*, 2001], although FEWTRELL *et al.* [2002] did show such superiority of breast milk at the same ages.

4-4-2 Term infants

DANIELS *et al.* [2004] reported that children of mothers who ate fish four times per week during pregnancy had higher developmental scores at 18 months than children of mothers who did not eat fish. HELLAND *et al.* [2003] supplemented the diet of pregnant women with 2.4 g/day long chain *n*-3 PUFAs (including 1.2 g/day DHA) from week 18 of pregnancy until 3 months post-partum. They assessed the intelligence and achievement of the children born to these mothers at 4 years of age using the Kaufman Assessment Battery for Children. This has four components: sequential processing, simultaneous processing, achievement, non-verbal abilities. The authors combined results from the first two components to give a "mental processing score" which is indicative of intelligence. Children of mothers in the control group scored 102.3 ± 11.3 while those of mothers in the fish oil group scored 106.4 ± 7.4 , the difference between the two groups being

significant ($P = 0.049$). Thus, in this study early exposure to long chain *n*-3 PUFAs augmented children's intelligence at 4 years of age.

Consistent with the idea that early DHA supply promotes cognitive and intellectual development, GIBSON *et al.* [1997] reported significant correlations between breast milk or infant cord blood erythrocyte DHA contents and mental development at one year of age as assessed by the Bayley Mental Development Index. Breast feeding or long chain PUFA supplemented formula was shown to promote cognitive development at 4 months of age [AGOSTONI *et al.*, 1995] but not at 2 years [AGOSTONI *et al.*, 1997]. WILLATTS *et al.* [1998] reported that a DHA containing formula given from birth to 4 months of age improved problem solving at 10 months. However, two studies found no relationship between umbilical cord plasma DHA levels and cognitive function in 4 [GHYS *et al.*, 2002] or 7 [BAKKER *et al.*, 2003] year olds, and several studies fail to show effects of breast feeding or long chain PUFA containing formula on measures of cognitive development assessed at between 2 and 4 years of age [SCOTT *et al.*, 1998; MAKRIDES *et al.*, 2000; AUESTAD *et al.*, 2001; 2003; GHYS *et al.*, 2002; BAKKER *et al.*, 2003].

4-5 Dietary strategies to increase maternal DHA status

With the recognition that maternal supply of DHA to the fetus is important (Section 4-1) and that fetal and infant DHA status is important for optimal visual and brain development and function (Sections 4-2, 4-3, 4-4), attempts to increase maternal DHA status have been made. Clearly the simplest way to do this would be to increase maternal DHA intake from fish or from fish oils or from other long chain *n*-3 PUFA rich oils (e.g. algal oil rich in DHA). Regular consumption of oily fish results in higher maternal erythrocyte DHA content [OLSEN *et al.*, 1991; SANJURJO *et al.*, 1995]. CONNOR *et al.* [1996] reported that consumption of sardines and fish oil by women from week 30 of pregnancy resulted in higher DHA contents of maternal plasma and erythrocytes 4 weeks later than observed in women taking placebo. In that study a total of 3 g long chain *n*-3 PUFAs including 1.1 g DHA were supplied per day. A similar intake of long chain *n*-3 PUFAs (2.7 g/day) and of DHA (1.1 g/day) provided as fish oil from week 30 of pregnancy resulted in higher DHA contents of maternal plasma phospholipids and of umbilical cord vein and artery at birth [VAN HOUWELINGEN *et al.*, 1995]. Fish oil providing 3.3 g/day long chain *n*-3 PUFAs (2.2 g/day as DHA) from week 20 of

pregnancy resulted in higher DHA contents of maternal erythrocytes at weeks 30 and 37 of pregnancy and at 6 weeks post-partum [DUNSTAN *et al.*, 2004]. Furthermore the DHA content of cord blood erythrocytes was 40% higher than in the placebo group [DUNSTAN *et al.*, 2004]. A much lower intake of DHA (200 mg/day) from week 15 of pregnancy resulted in higher DHA content of maternal plasma erythrocytes at week 28 of pregnancy and at birth, although there was no effect on DHA content of cord blood plasma or erythrocytes [MONTGOMERY *et al.*, 2003].

Breast milk DHA content can be increased by maternal supplementation with fish oil [HARRIS *et al.*, 1984], DHA-rich oil [JENSEN *et al.*, 2000; HAWKES *et al.*, 2002] or long chain *n*-3 PUFA rich eggs [JENSEN *et al.*, 2000]. HARRIS *et al.* [1984] gave lactating women 5 g/day fish oil for 28 days or 10 g/day fish oil for 14 days or 47 g/day fish oil for 8 days. Levels of DHA in breast milk were 0.1% of total fatty acids at baseline and 0.5, 0.8 and 4.8% of fatty acids after 5, 10 and 47 g/day fish oil, respectively. HAWKES *et al.* [2002] gave lactating women placebo, “low” dose DHA (300 mg/day) or “high” dose DHA (600 mg/day) from day 3 post-partum for 4 weeks. They found that the DHA content of maternal plasma, maternal mononuclear cells, breast milk and breast milk cells increased in relation to DHA intake. In another low dose approach, JENSEN *et al.* [2000] compared fish oil, algal oil and eggs as a source of DHA for incorporation into breast milk: lactating women (two weeks after giving birth) received about 200 mg/day DHA from the oils or eggs for 4 weeks. This resulted in an increase in DHA in maternal plasma phospholipids (from 2.5 to about 4% of total fatty acids) and in breast milk (from about 0.2 to about 0.4% of total fatty acids). Furthermore infant plasma phospholipid DHA increased from about 3.6 to about 5% of total fatty acids. This demonstrates that increased consumption of DHA by lactating women results in increased DHA in breast milk, subsequently elevating infant DHA status.

4-6 Long chain *n*-3 PUFAs and childhood developmental disorders

Children with attention deficit hyperactivity disorder (ADHD) or autistic spectrum disorders appear to show lower levels of long chain *n*-3 PUFAs in their plasma or erythrocytes than control children [MITCHELL *et al.*, 1987; STEVENS *et al.*, 1996; BELL *et al.*, 2000; 2004; BURGESS *et al.*, 2000; VANCASSEL *et al.*, 2001], leading to the

suggestion that these and other developmental disorders such as dyslexia and dyspraxia are related to some sort of fatty acid deficiency state [RICHARDSON, 2004]. In one study behavioural and learning problems were greater in boys (a mix of controls and those with ADHD) with low levels of long chain *n*-3 PUFAs in their plasma phospholipids [BELL *et al.*, 2000]. Therefore, normalisation of fatty acid levels or proportions might lead to clinical benefit. This has been examined in a limited number of trials.

A study of 0.345 g DHA/day for four months showed no effect on measures of inattention and impulsivity or on parent-rated symptoms among children with ADHD aged 6 to 12 years [VOIGT *et al.*, 2001]. Another study using foods providing children aged 6 to 12 years with about 0.5 g DHA/day for two months showed no improvements in the ADHD outcome measures made [HIRAYMA *et al.*, 2004]. In another study 0.56 g/day long chain *n*-3 PUFAs (mostly in the form of DHA and in combination with some γ -linolenic acid and arachidonic acid) resulted in improvements in only two out of sixteen outcomes [STEVENS *et al.*, 2003].

One small study of long chain *n*-3 PUFAs (666 mg/day, mostly as DHA) in combination with γ -linolenic acid reported significant beneficial effects after 12 weeks on 6 out of 14 outcomes in dyslexic children [RICHARDSON, PURI, 2002]. RICHARDSON [2004] also discusses data from unpublished studies in dyslexia and dyspraxia, which suggest some clinical benefits.

4-7 Long chain *n*-3 PUFAs and psychiatric and psychological disorders in adults

RUDIN [1981] was the first to suggest that mental disorders might result from a deficiency in *n*-3 PUFAs and might respond to provision of these fatty acids. Schizophrenic patients have lower levels of long chain *n*-3 PUFAs in their erythrocytes than do controls [GLEN *et al.*, 1994; YAO *et al.*, 1994; PEET *et al.*, 1995; EDWARDS *et al.*, 1998]. In a study of nine countries, HIBBELN [1998] demonstrated a significant correlation between high annual fish consumption and lower prevalence of major depression, an observation that is compatible with a proposed protective effect of long chain *n*-3 PUFAs. There was also a significant correlation between low fish consumption and depressive

symptoms among Finnish adults [TANSKANEN *et al.*, 2001]. Other studies show that fish consumption is associated with lower risk of bipolar disorder [NOAGHIUL, HIBBELN, 2003] and homicide [HIBBELN, 2001]. A recent study showed that consumption of fish, oily fish or DHA is associated with lower risk of hostility in young adults [IRIBARREN *et al.*, 2004].

A small study using a very high dose of long chain *n*-3 PUFAs (9.6 g/day) reported a reduction in depressive symptoms [SU *et al.*, 2003], while a study using a lower dose of DHA alone (2 g/day) found no such effect [MARANGELL *et al.*, 2003]. Intervention with 6.2 g/day EPA+DHA in patients with bipolar maniac depression resulted in significant improvements in nearly all outcomes, especially with respect to depressive symptoms, after 4 months [STOLL *et al.*, 1999]. Likewise, 2 g/day EPA as the ethyl ester improved symptoms in patients with unipolar depressive disorder after 4 weeks [NEMETS *et al.*, 2002]. The first trial of long chain *n*-3 PUFAs in schizophrenia identified clinical improvement with EPA (2 g/day), but not with DHA [PEET *et al.*, 2001]. Subsequently, trials have examined the effect of EPA as an ethyl ester using doses of 1, 2 [PEET, HORROBIN, 2002], 3 [FENTON *et al.*, 2001; EMSLEY *et al.*, 2002] or 4 [PEET, HORROBIN, 2002] g/day. Two of these studies [PEET, HORROBIN, 2002; EMSLEY *et al.*, 2002] demonstrated clinical improvement with EPA. Although these findings are encouraging, a Cochrane review concluded that long chain *n*-3 PUFAs should be regarded only as an experimental treatment for schizophrenia at this stage [JOY *et al.*, 2003]. One study reported significant benefit from 1 g/day EPA in borderline personality disorder [ZANARINI, FRANKENBURG, 2003], while two studies report anti-aggressive effects of DHA [HAMAZAKI *et al.*, 1996; 2002].

4-8 Long chain *n*-3 PUFAs and neurodegenerative diseases of ageing

Fish intake (> 20 g/day) was identified as a protective factor against cognitive decline, dementia and Alzheimer's disease among older Dutch subjects [KALMIJN *et al.*, 1997]. A prospective study of elderly subjects found that those who ate oily fish at least once per week had a 60% lower risk of Alzheimer's disease over the following 4 years [MORRIS *et al.*, 2003]. DHA rather than EPA appeared to be the protective component. Post-mortem studies

showed that the brains of Alzheimer's disease sufferers contain less DHA than those without the disease [PRASAD *et al.*, 1998; TULLY *et al.*, 2003]. Some studies have linked low plasma long chain *n*-3 PUFA status to dementia [SODERBERG *et al.*, 1991] and cognitive impairment [CONQUER *et al.*, 2000].

4-9 Conclusions

DHA is a key structural component of the brain and retina, where it plays particular, unique functional roles. A supply of DHA is very important early in life, especially during the fetal and early infant periods when the eye and central nervous system are developing. Since the supply must come from maternal sources (*via* the placenta and breast milk), maternal DHA status is likely to be important in determining eye and brain development early in life. Thus maintenance of maternal DHA status is the key to optimising DHA supply to the developing fetus and newborn infant. Animal experiments indicate that a lack of *n*-3 PUFAs in the diet results in poor visual development and in learning and behavioural abnormalities. There is evidence that preterm infants, in whom this early DHA supply is interrupted, have poorer visual and cognitive development than term infants, although the provision of breast milk or DHA-supplemented formulas improves these outcomes. Increased consumption of DHA during pregnancy or lactation increases the DHA content of maternal and cord plasma and of breast milk, indicating a greater ability of the mother to supply DHA. There is some evidence for benefits of increased DHA supply either through the maternal diet during pregnancy or breast feeding or in formulas in term infants, although this is less strong than the evidence in pre-term infants. Newly emerging areas of interest relate to the influence of long chain *n*-3 PUFAs on childhood developmental disorders, adult psychiatric and psychological disorders, and neurodegenerative diseases of ageing. These conditions appear to be associated with a lowered long chain *n*-3 PUFA status. Additionally, there is epidemiological evidence for a lowered risk of psychiatric, psychological disorders, and neurodegenerative disorders with increased consumption of fish. Intervention studies indicate clinical benefit from long chain *n*-3 PUFAs in childhood developmental and adult psychiatric and psychological disorders, although the evidence base is not yet sufficiently robust for clear recommendations to be made. Interestingly many of these studies are indicative that EPA is more important than DHA, which contrasts with the relative roles of the fatty acids in very early brain development.

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OMEGA-3 FATTY ACIDS: THE GOOD OIL?

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5 – Long chain *n*-3 fatty acids and inflammation

5-1 Inflammation in health and disease

Inflammation is the body's immediate response to infection or injury; as such it is a component of the immune response. It is typified by redness, swelling, heat and pain. These occur as a result of increased blood flow, increased permeability across blood capillaries, which permits large molecules (e.g. complement, antibodies, cytokines) to leave the bloodstream and cross the endothelial wall, and increased movement of leukocytes from the bloodstream into the surrounding tissue. Inflammation functions to begin the immunological process of elimination of invading pathogens and toxins and to repair damaged tissue. These responses must be ordered and controlled. The movement of cells into the inflammatory/infected site is induced by the up-regulation of adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) on the surface of endothelial cells allowing leukocyte binding and subsequent diapedesis. The earliest cells appearing at inflamed sites are granulocytes, with monocyte/macrophages and lymphocytes appearing later. Granulocytes and monocyte/macrophages are involved in pathogen killing, in clearing up cellular and tissue debris, and in tissue repair. The activity of these cells is induced by certain triggers. One important exogenous trigger is bacterial endotoxin (also known as lipopolysaccharide or LPS), a component of the cell wall of Gram-negative bacteria. LPS can trigger complement activation (resulting in vasodilation and increased vascular permeability), coagulation, fibrinolysis and the kinin cascade. LPS can directly activate monocyte/macrophages inducing them to form cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6 and IL-8; eicosanoids, such as prostaglandin (PG) E₂; nitric oxide; matrix metalloproteinases (MMPs); and other mediators. LPS also induces adhesion

molecule expression on the surface of endothelial cells and leukocytes. The cytokines produced by monocyte/macrophages also serve to regulate the whole body response to infection and injury (Figure 5.1). Thus, inflammation and the inflammatory response are part of the normal, innate immune response; inflammatory mediators also provide a link between the innate and acquired immune responses (Figure 5.1). However, when inflammation occurs in an uncontrolled manner disease ensues. High levels of TNF- α ,

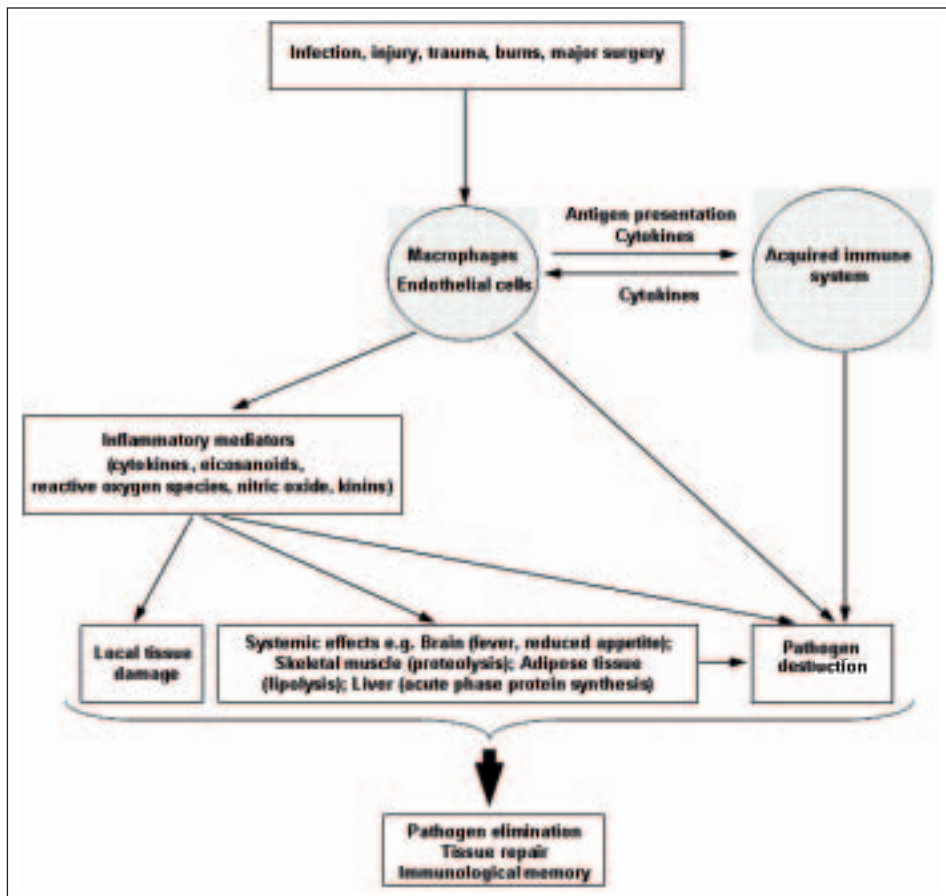


Figure 5.1 — The role of inflammation in host defense and in causing tissue damage. Modified from P.C. CALDER. Polyunsaturated fatty acids and immunity. *Lipids*, 2001; 36, 1007-1024, with permission from the American Oil Chemists' Society.

IL-1 β and IL-6 are particularly destructive. Chronic overproduction of TNF- α and IL-1 may cause muscle wasting and loss of bone mass. TNF- α , IL-1 and IL-6 are implicated in causing some of the pathological responses that occur in endotoxic shock, in adult respiratory distress syndrome and in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. It is this highly destructive nature of these and other inflammatory mediators that prompted THOMAS [1972] to say «Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are more in danger from them than from the invaders. We live in the midst of explosive devices; we are mined».

5-2 Arachidonic acid-derived eicosanoids and inflammation

Inflammatory cells typically contain a high proportion of the *n*-6 polyunsaturated fatty acid (PUFA) arachidonic acid and low proportions of *n*-3 PUFAs especially eicosapentaenoic acid (EPA). The exact proportion of arachidonic acid in human inflammatory cells varies according to cell type and the lipid fraction examined [DINARELLO *et al.*, 1983; LEWIS *et al.*, 1990]. The principal functional role for arachidonic acid is as a substrate for synthesis of the eicosanoid family of bioactive mediators according to the metabolic processes described in **figures 2.4** and **2.5**. Eicosanoids are involved in modulating the intensity and duration of inflammatory responses [see LEWIS *et al.*, 1990; TILLEY *et al.*, 2001 for reviews]. However, these mediators have cell- and stimulus-specific sources and frequently have opposing effects. For example, PGE₂ is produced mainly by monocytes, macrophages and, to a lesser extent, neutrophils and inhibits the production of TNF- α and IL-1 β [DINARELLO *et al.*, 1983; KNUDSEN *et al.*, 1986; KUNKEL *et al.*, 1988; MEJA *et al.*, 1997; DOOPER *et al.*, 2002; MILES *et al.*, 2002], while leukotriene (LT) B₄ is produced mainly by neutrophils, other granulocytes and, to a lesser extent, monocytes and macrophages, and increases the production of TNF- α and IL-1 β [ROLA-PLESZCZYNSKI, LEMAIRE, 1985; GAGNON *et al.*, 1989; SCHADE *et al.*, 1989]. Thus, the overall physiological (or pathophysiological) outcome will depend upon the cells present, the nature of the stimulus, the timing of eicosanoid generation, the concentrations of different eicosanoids generated and the sensitivity of target cells and tissues to the eicosanoids generated. Recent studies have demonstrated that PGE₂ induces cyclooxygenase (COX)-2 in fibroblasts cells and so upregulates its own production [BAGGA *et al.*, 2003], induces production of IL-6 by macrophages

[BAGGA *et al.*, 2003], inhibits 5-lipoxygenase (LOX) and so decreases production of 4-series LTs [LEVY *et al.*, 2001], and induces 15-LOX so promoting the formation of lipoxins [VACHIER *et al.*, 2002] that have been found to have anti-inflammatory effects [GEWIRTZ *et al.*, 2002; SERHAN *et al.*, 2003]. Thus PGE₂ possesses both pro- and anti-inflammatory actions.

5-3 Long chain *n*-3 PUFAs and inflammatory eicosanoid production

Increased consumption of long chain *n*-3 PUFAs such as EPA and docosahexaenoic acid (DHA), results in increased proportions of those fatty acids in inflammatory cell phospholipids, partly at the expense of arachidonic acid (**Figures 2.10** and **5.2**). Since significantly increased consumption of fish oil results in a decrease in the amount of arachidonic acid in the membranes of inflammatory cells, there will be less substrate available for synthesis of eicosanoids from arachidonic acid (**Figure 5.3**). Thus, fish oil supplementation of the human diet has been shown to result in decreased production of PGE₂ [ENDRES *et al.*, 1989; MEYDANI *et al.*, 1991; CAUGHEY *et al.*, 1996; TREBBLE *et al.*, 2003], TXB₂ [CAUGHEY *et al.*, 1996], LTB₄ and 5-HETE [LEE *et al.*, 1985; SPERLING *et al.*, 1993] (**Figure 5.4**) and LTE₄ [VON SCHACKY *et al.*, 1993] by inflammatory cells. Since EPA is able to act as a substrate for both COX and 5-LOX (**Figure 2.6**), fish oil supplementation of the human diet has been shown to result in increased production of LTB₅, LTE₅ and 5-HEPE by inflammatory cells [LEE *et al.*, 1985, SPERLING *et al.*, 1993; VON SCHACKY *et al.*, 1993] (**Figure 5.4**), although generation of PGE₃ has been more difficult to demonstrate [HAWKES *et al.*, 1991]. The functional significance of this is that the mediators formed from EPA are believed to be less potent than those formed from arachidonic acid. For example, LTB₅ is 10- to 100-fold less potent as a neutrophil chemotactic agent than LTB₄ [GOLDMAN *et al.*, 1983; LEE *et al.*, 1984]. Recent studies have compared the effects of PGE₂ and PGE₃ upon production of cytokines by cells lines and by human cells. BAGGA *et al.* [2003] reported that PGE₃ was a less potent inducer of COX-2 gene expression in fibroblasts and of IL-6 production by macrophages. However, PGE₂ and PGE₃ had equivalent inhibitory effects upon production of TNF- α [DOOPER *et al.*, 2002; MILES *et al.*, 2002] and IL-1 β [MILES *et al.*, 2002] by human mononuclear cells stimulated

with endotoxin. The reduction in generation of arachidonic acid-derived mediators which accompanies fish oil consumption has led to the idea that fish oil is anti-inflammatory (Figure 5.5).

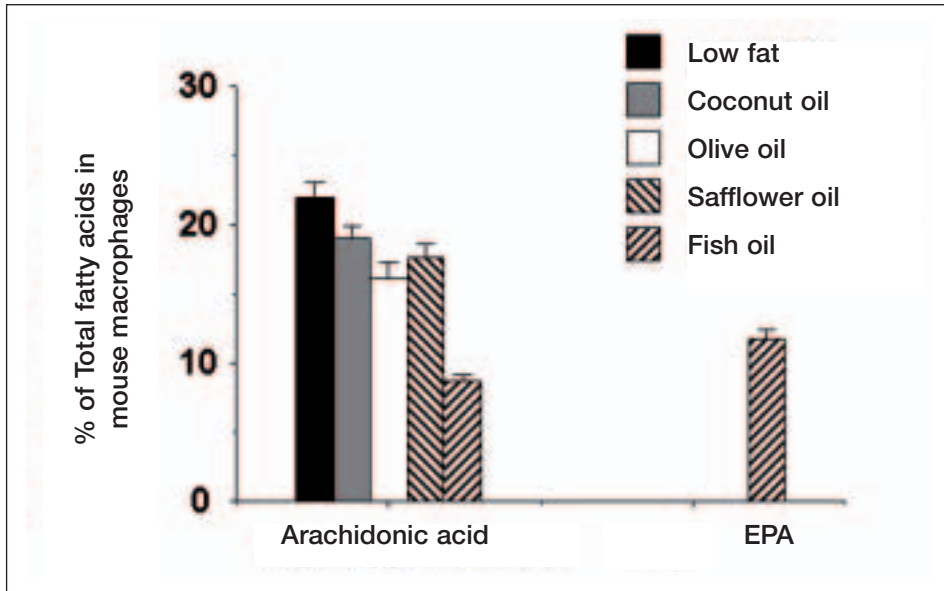


Figure 5.2 — Effect of dietary fats on the fatty acid composition of mouse macrophages. Male C57Bl6 mice were fed on a low fat (LF; 2.5% by weight mixed oils) diet or on one of four high fat (21% by weight fat) diets in which the bulk of the fat was as coconut oil, olive oil, safflower oil or fish oil. After 6 weeks the mice received an intraperitoneal injection of Brewer's thioglycollate broth and 4 days later peritoneal macrophages were isolated. The fatty acid composition of the macrophages was determined by gas chromatography. Data are mean \pm SEM% of arachidonic acid or eicosapentaenoic acid (EPA) from 6 mice per diet and are from WALLACE *et al.* [2000].

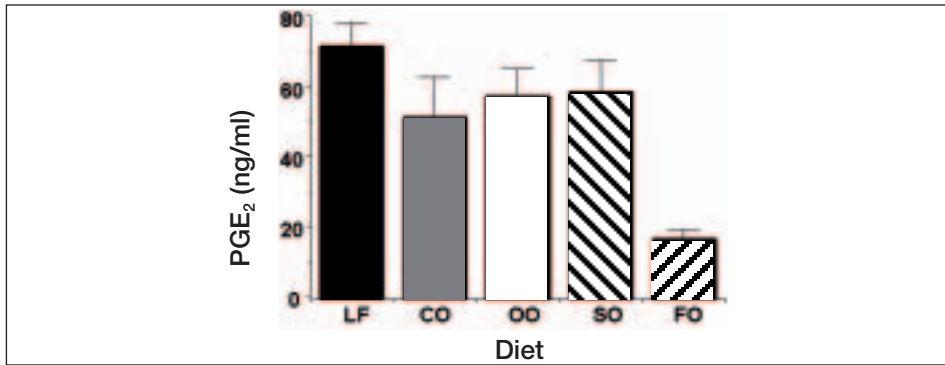


Figure 5.3 — Effect of dietary fats on prostaglandin E₂ production by mouse macrophages. Male C57Bl6 mice were fed on a low fat (LF; 2.5% by weight mixed oils) diet or on one of four high fat (21% by weight fat) diets in which the bulk of the fat was as coconut oil (CO), olive oil (OO), safflower oil (SO) or fish oil (FO). After 8 weeks the mice received an intraperitoneal injection of Brewer's thioglycollate broth and four days later peritoneal macrophages were isolated. Macrophages were incubated with bacterial endotoxin and prostaglandin (PG) E₂ concentrations in the culture medium measured 8 hours later. Data are mean ± SEM from 3 mice per diet and are from YAQOUB, CALDER [1995].

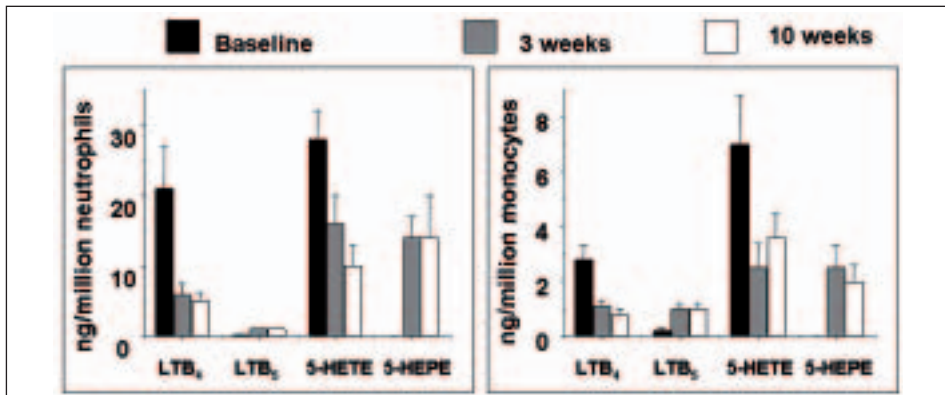


Figure 5.4 — Effect of fish oil supplementation on production of lipoxigenase metabolites by human neutrophils and monocytes. Healthy subjects supplemented their diet of fish oil capsules providing 9.4 g EPA plus 5.0 g DHA per day for a period of 10 weeks. Blood neutrophils and monocytes were isolated at baseline and after 3 and 10 weeks and stimulated with calcium ionophore for 15 minutes. The concentrations of lipoxigenase metabolites of arachidonic acid and eicosapentaenoic acid were measured by reverse phase HPLC. Data are mean ± SEM from 8 subjects and are from SPERLING *et al.* [1993]. HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene.

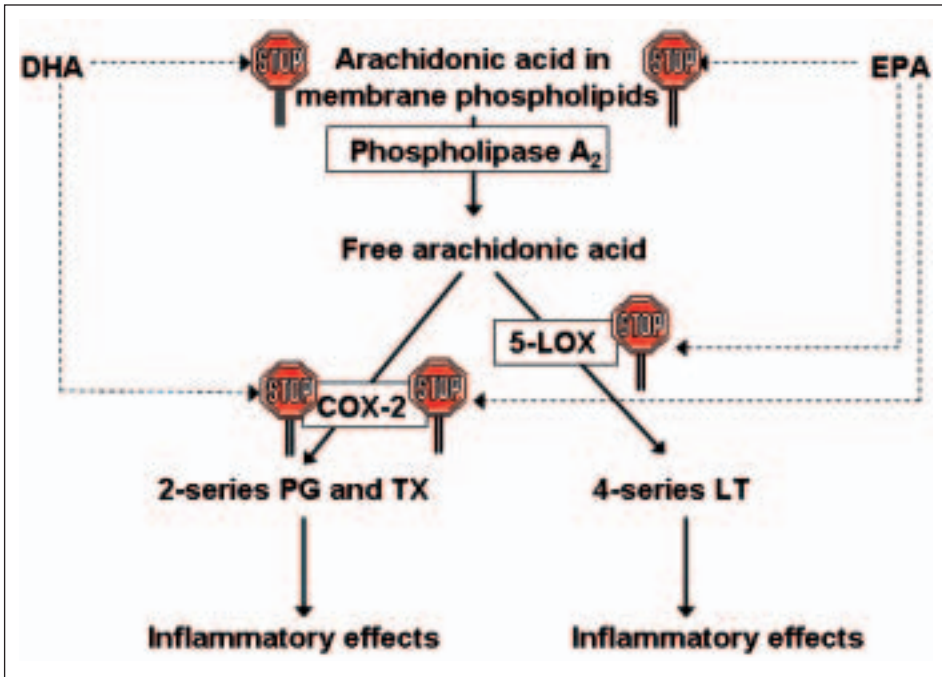


Figure 5.5 — Classic view of the anti-inflammatory actions of long chain *n*-3 PUFAs. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) antagonise the incorporation of arachidonic acid into inflammatory cell membrane phospholipids and inhibit the metabolism of arachidonic acid by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. LT, leukotriene; PG, prostaglandin; TX, thromboxane.

In addition to long chain *n*-3 PUFAs modulating the generation of eicosanoids from arachidonic acid and to EPA acting as substrate for the generation of alternative eicosanoids, recent studies have identified a novel group of mediators, termed E-series resolvins, formed from EPA by COX-2 that appear to exert anti-inflammatory actions [SEHRAN *et al.*, 2000a; 2000b; 2002]. In addition, DHA-derived mediators termed D-series resolvins, docosatrienes and neuroprotectins also produced by COX-2 have been identified and these too appear to be anti-inflammatory [HONG *et al.*, 2003; MARCHESELLI *et al.*, 2003; MUKHERJEE *et al.*, 2004]. This is an exciting new area of *n*-3 fatty acids and inflammatory mediators and the implications to a variety of conditions may be of great importance.

5-4 Anti-inflammatory effects of long chain *n*-3 PUFAs other than altered eicosanoid production

Although their action in antagonizing arachidonic acid metabolism is a key anti-inflammatory effect of *n*-3 PUFAs, these fatty acids have a number of other anti-inflammatory effects that might occur downstream of altered eicosanoid production or might be independent of this. For example, animal and human studies have shown that dietary fish oil results in suppressed production of pro-inflammatory cytokines and can modulate adhesion molecule expression (**Table 5.1**).

5-4-1 *n*-3 PUFAs and inflammatory cytokine production

104

Cell culture studies demonstrate that EPA and DHA can inhibit the production of IL-1 β and TNF- α by monocytes [CHU *et al.*, 1999], and the production of IL-6 and IL-8 by venous endothelial cells [DE CATERINA *et al.*, 1994; KHALFOUN *et al.*, 1997]. Fish oil feeding decreased *ex vivo* production of TNF- α , IL-1 β and IL-6 by rodent macrophages [BILLIAR *et al.*, 1988; RENIER *et al.*, 1993; YAQOUB, CALDER, 1995]. Supplementation of the diet of healthy human volunteers with fish oil providing more than 2.4 g EPA plus DHA per day decreased production of TNF, IL-1 and IL-6 by mononuclear cells [ENDRES *et al.*, 1989; MAYDANI *et al.*, 1991; GALLAI *et al.*, 1993; ABBATE *et al.*, 1996; CAUGHEY *et al.*, 1996]. CAUGHEY *et al.* [1996] reported a significant inverse correlation between the EPA content of mononuclear cells and the ability of those cells to produce TNF- α and IL-1 β in response to endotoxin (**Figure 5.6**). Recent studies have confirmed the ability of dietary fish oil to decrease production of TNF α [TREBBLE *et al.*, 2003] and IL-6 [TREBBLE *et al.*, 2003; WALLACE *et al.*, 2003] by human mononuclear cells. Furthermore these studies provide for the first time information on the dose-response relationship between dietary intake of long chain *n*-3 fatty acids and production of these cytokines. It should be noted that there are also several studies that fail to show effects of dietary long chain *n*-3 fatty acids on production of inflammatory cytokines in humans [see CALDER, 2001 for references]. It is not clear what the reason for this is but dose of *n*-3 fatty acids used and other technical factors are likely to be contributing factors.

Table 5.1 — Summary of the anti-inflammatory effects of long chain *n*-3 PUFAs.

Anti-inflammatory effect	Mechanism(s) likely to be involved
Decreased generation of arachidonic acid-derived eicosanoids (many with inflammatory actions)	Decreased arachidonic acid in cell membrane phospholipids; Inhibition of arachidonic acid metabolism; Decreased induction of COX-2, 5-LOX and 5-LOX activating protein
Increased generation of EPA-derived eicosanoids (many with less inflammatory and some with anti-inflammatory actions)	Increased cell membrane phospholipid content of EPA
Increased generation of EPA and DHA-derived resolvins (some with anti-inflammatory actions)	Increased cell membrane phospholipid content of EPA and DHA
Decreased generation of inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-8)	Decreased activation of NF κ B (via decreased phosphorylation of I κ B); Activation of PPAR γ ; Altered activity of other transcription factors; Differential effects of arachidonic acid- vs. EPA-derived eicosanoids
Decreased expression of adhesion molecules	Decreased activation of NF κ B (via decreased phosphorylation of I κ B); Altered activity of other transcription factors
Decreased leukocyte chemotaxis	Not clear – perhaps decreased expression of receptors for some chemoattractants

Abbreviations used: COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; I κ B, inhibitory subunit of NF κ B; IL, interleukin; LOX, lipoxygenase; NF κ B, nuclear factor κ B; PPAR, peroxisome proliferators activated receptor; TNF, tumour necrosis factor.

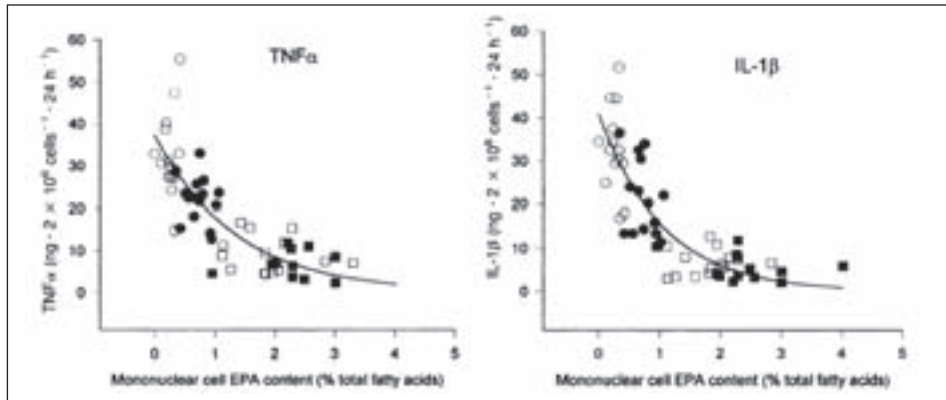


Figure 5.6 — The inverse relationship between the eicosapentaenoic acid (EPA) content of human mononuclear cells and their production of inflammatory cytokines.

Healthy subjects modified their diet to include increased amounts of α -linolenic acid and/or long chain n -3 PUFAs for 4 weeks. Blood mononuclear cells were isolated and their fatty acid composition determined by gas chromatography. They were also incubated with bacterial endotoxin for 24 hours and the concentrations of tumour necrosis factor (TNF)- α and interleukin (IL)-1 β measured.

Reproduced from CAUGHEY *et al.*, 1996 with permission by American Journal of Clinical Nutrition © Am J Clin Nutr. American Society for Clinical Nutrition.

5-4-2 n -3 PUFAs and adhesion molecule expression

Although it was shown some years ago that culture of murine macrophages with n -3 PUFAs decreased their ability to bind to various surfaces [CALDER *et al.*, 1990], the first demonstration that these fatty acids could affect the expression of adhesion molecules on the cell surface was by DE CATERINA *et al.* [1994]. These authors showed that culture of human venous endothelial cells with DHA significantly decreased cytokine-induced surface expression of E-selectin, ICAM-1 and VCAM-1, and impaired the ability of ligand-bearing monocytes to adhere [DE CATERINA *et al.* 1995]. EPA also inhibited LPS-induced expression of these three adhesion molecules on human venous endothelial cells, and again this had the functional effect of decreasing binding of monocytes [WEBER *et al.*, 1995]. In another cell culture study, EPA decreased surface expression of ICAM-1 on monocytes stimulated with interferon (IFN)- γ [HUGHES *et al.*, 1996a]. Studies of dietary fatty acids and adhesion molecule expression are few. Dietary fish oil decreased expression of ICAM-1 on the surface of murine macrophages [MILES *et al.*, 2000]. Supplementing the diet of healthy humans with fish oil providing about 1.5 g EPA plus DHA per day resulted in a lower level of expression of ICAM-1 on the surface of blood monocytes stimulated *ex vivo* with IFN- γ [HUGHES *et al.*, 1996b]. Recently, dietary fish oil was found to decrease circulating levels of

soluble VCAM-1 in elderly subjects [MILES *et al.*, 2001], but it is not clear if this represents decreased surface expression of VCAM-1.

5-5 *n*-3 PUFAs and inflammatory gene expression

Many of the anti-inflammatory effects of *n*-3 PUFAs appear to be exerted at the level of altered gene expression. However, these effects have been demonstrated only a limited number of times and often in artificial *in vitro* settings, and thus the extent of these effects *in vivo* is not yet clear. Nevertheless, cell culture and animal feeding studies indicate potentially very potent effects of *n*-3 PUFAs on expression of a range of inflammatory genes (**Table 5.2**).

Culturing bovine chondrocytes with EPA or DHA markedly decreased cytokine-mediated induction of expression of the COX-2 (but not COX-1), TNF- α and

Table 5.2 — Inflammatory genes downregulated by long chain *n*-3 PUFAs.

Gene expression	Genes downregulated
Enzymes of eicosanoid metabolism	COX-2 5-LOX FLAP
Inflammatory cytokines	TNF- α IL-1 β IL-6 MCP-1
Enzymes of arginine metabolism	iNOS
Adhesion molecules	VCAM-1 ICAM-1
Matrix proteinases	Aggrecanase-1 Aggrecanase-2 MMP-2 MMP-3 MMP-13

Abbreviations used: COX, cyclooxygenase; FLAP, 5-lipoxygenase activating protein; ICAM, intercellular adhesion molecule; IL, interleukin; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

IL-1 α genes [CURTIS *et al.*, 2000]. The same study investigated the influence of *n*-3 PUFAs upon the expression of aggrecanase-1 and -2 genes. Aggrecanase-1 and -2 degrade cartilage proteoglycan and their expression in cartilage is upregulated in response to the pro-inflammatory cytokines TNF- α and IL-1. *n*-3 PUFAs, but not other fatty acids, inhibited cytokine-mediated upregulation of expression of the aggrecanase-1 and aggrecanase-2 genes and of aggrecanase activity [CURTIS *et al.*, 2000]. This study has been extended by one using cultured explants of human osteoarthritic cartilage [CURTIS *et al.*, 2002]. Including EPA or DHA in the culture medium markedly decreased the cytokine-induced upregulation of expression of the COX-2, IL-1 α , IL-1 β , TNF- α , 5-LOX, 5-LOX activating protein (FLAP), MMP-3, MMP-13, and aggrecanase-1 genes in these cells. The *n*-3 PUFAs did not affect expression of the COX-1, 12-LOX or 15-LOX genes, which were not induced by cytokines [CURTIS *et al.*, 2002]. Also, there was little effect of *n*-3 PUFAs on the expression of genes for the tissue inhibitor of metalloproteinase (TIMP)-1, -2 or -3, which again were not cytokine-inducible [CURTIS *et al.*, 2002]. These studies indicate a marked capacity of *n*-3 PUFAs to suppress the expression of inflammatory genes, with little effect on the expression of housekeeping (e.g. COX-1) or anti-inflammatory (TIMP) genes. They also indicate that one potential contributor to the reduction in generation of arachidonic acid-derived eicosanoids after fish oil feeding may be decreased expression of the enzymes and proteins responsible for producing these mediators (e.g. COX-2, 5-LOX, FLAP). In an earlier study, DE CATERINA *et al.* [1994] had demonstrated that the down-regulation of VCAM-1 expression on endothelial cells caused by DHA was exerted at the level of VCAM-1 gene expression, and that this effect was independent of effects on eicosanoid production and on antioxidant status. Furthermore, culture of murine peritoneal macrophages with DHA decreased the level of mRNA for inducible nitric oxide synthase (iNOS) after stimulation with LPS and IFN- γ [KHAIR-EL-DIN *et al.*, 1996]. This effect correlated with decreased production of nitric oxide and was due to decreased transcription of the iNOS gene [KHAIR-EL-DIN *et al.*, 1996].

A limited number of feeding studies have demonstrated an effect of dietary fish oil on inflammatory gene expression. Inclusion of fish oil in the diet completely abolished mRNA for TNF- α , IL-1 β and IL-6 in the kidneys of autoimmune disease-prone mice [CHANDRASEKAR, FERNANDES, 1994]. Feeding mice a fish oil-rich diet significantly decreased the level of IL-1 β mRNA in LPS- or phorbol ester-stimulated spleen lymphocytes [ROBINSON *et al.*, 1996]; the lower IL-1 β mRNA level was not due to accelerated degradation but to impaired synthesis. Fish oil feeding to mice lowered basal and LPS-stimulated TNF- α mRNA levels in peritoneal macrophages [RENIER *et al.*, 1993]. ICAM-1 mRNA levels were lower in fresh peritoneal macrophages from mice fed fish oil [MILES *et al.*, 2000].

Since eicosanoids derived from arachidonic regulate inflammatory gene expression, the effects of *n*-3 PUFAs might come about through antagonism of the

effects of arachidonic acid-derived mediators. However, at least some (if not all) of these effects seem to occur in an eicosanoid-independent manner [e.g. DE CATERINA *et al.*, 1994]. It is now emerging that *n*-3 PUFAs might exert their effects through direct actions on the intracellular signalling pathways which lead to activation of one or more transcription factors such as nuclear factor kappa B (NFκB).

NFκB plays a role in inducing a range of inflammatory genes including COX-2, ICAM-1, VCAM-1, E-selectin, TNF-α, IL-1β, IL-6, iNOS, acute phase proteins and MMPs in response to inflammatory stimuli [CHRISTMAN *et al.*, 1998; CHEN *et al.*, 1999] (Figure 5.7). The signalling pathway leading to NFκB activation

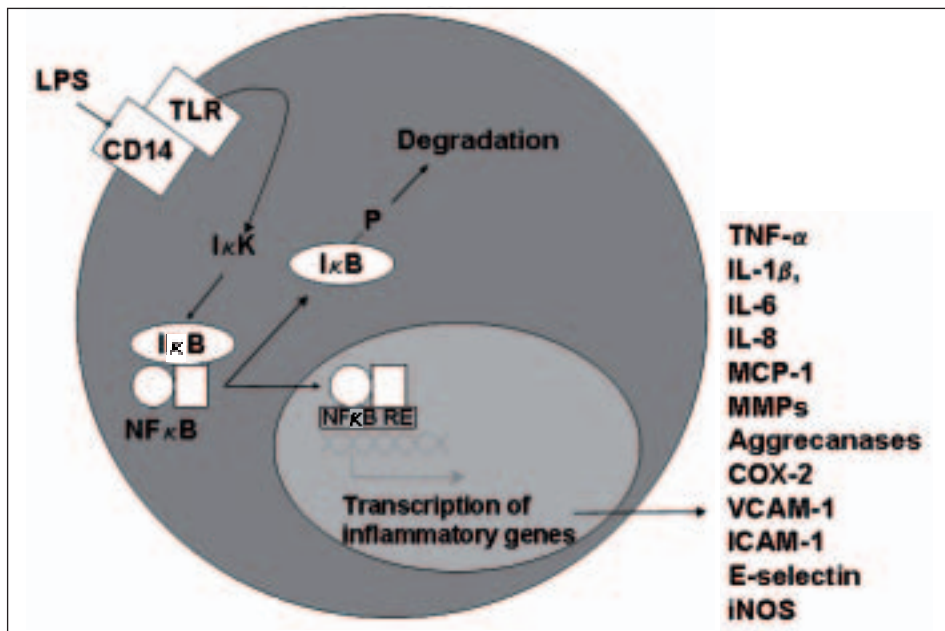


Figure 5.7 — Outline of the pathway of upregulation of inflammatory gene expression via nuclear factor kappa B.

CD14, cluster of differentiation 14 (the LPS receptor); COX, cyclooxygenase; ICAM, intercellular adhesion molecule; IκB, inhibitory subunit of NFκB; IκK, IκB kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MMPs, matrix metalloproteinases; NFκB, nuclear factor kappa B; RE, response element; TLR, toll-like receptor; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

Modified from P.C. CALDER, *n*-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids*, 2003; 38: 342-352, with permission from the American Oil Chemists' Society.

is becoming better understood [KARIN, BEN-NERIAH, 2000; KARIN, DELHASE, 2000]. NF κ B exists as an inactive heterotrimer in the cytosol of resting inflammatory cells; one of the subunits is called inhibitory subunit of NF κ B (I κ B). Upon stimulation, a signalling cascade activates a protein complex known as I κ B kinase (I κ K). Activated I κ K phosphorylates I κ B on two N-terminal serine residues, thus promoting its dissociation from the rest of the inactive NF κ B trimer. The remaining NF κ B heterodimer is rapidly translocated to the nucleus where it binds to response elements in target genes, so regulating their transcription. The phosphorylated I κ B undergoes polyubiquitination, targeting it for degradation by the 26S proteasome. Recent studies have shown that *n*-3 PUFAs can down-regulate the activity of NF κ B. Feeding mice fish oil resulted in a lower level of NF κ B in the nucleus (i.e. activated NF κ B) of LPS-stimulated spleen lymphocytes compared with feeding corn oil [XI *et al.*, 2001]. Infecting the mice with the murine AIDS virus resulted in increased NF κ B in the nucleus, but the level was lower in fish oil fed mice [XI *et al.*, 2001]. The mechanism by which *n*-3 PUFAs decrease the activation of NF κ B is not clear. However, incubating human monocytes with EPA or fish oil decreased LPS-induced activation of NF κ B [CAMANDOLA *et al.*, 1996; LO *et al.*, 1999; NOVAK *et al.*, 2003; ZHAO *et al.*, 2004] and this was associated with decreased phosphorylation of I κ B [ZHAO *et al.*, 2004]. This suggests an effect of *n*-3 PUFAs on the signalling process leading to activation of I κ K. Incubation of a pancreatic cell line with TNF- α markedly upregulated degradation of I κ B, and this could be totally abolished by prior incubation of the cells with EPA [ROSS *et al.*, 1999]. This effect could be due to inhibition of phosphorylation of I κ B, so preventing it from being targeted for degradation, or to inhibition of the degradation process itself.

A second group of transcription factors currently undergoing scrutiny for their role in inflammation are the peroxisome proliferator activated receptors (PPARs). The main members of this family are PPAR α and PPAR γ . Although PPAR α and γ play important roles in liver and adipose tissue, respectively [SCHOONJANS *et al.*, 1996], they are also found in inflammatory cells [CHINETTI *et al.*, 1998; RICOTE *et al.*, 1998]. PPARs dimerise with the retinoid-X-receptor to regulate gene expression, and they can bind, and appear to be regulated by, PUFAs and eicosanoids [KLEIWER *et al.*, 1995; DEVCHAND *et al.*, 1996] (see Section 2-4). PPAR α deficient mice have a prolonged response to inflammatory stimuli [DEVCHAND *et al.*, 1996], suggesting that PPAR α activation might be «anti-inflammatory». More recently, activators of both PPAR α and γ have been shown to inhibit the activation of inflammatory genes including TNF- α , IL-1 β , IL-6, IL-8, COX-2, VCAM-1, iNOS, MMPs and acute phase proteins [RICOTE *et al.*, 1998; JIANG *et al.*, 1998; POYNTER, DAYNES, 1998; JACKSON *et al.*, 1999; MARX *et al.*, 1999; TAKANO *et al.*, 2000; WANG *et al.*, 2001; XU *et al.*, 2001]. Two mechanisms for the anti-

inflammatory actions of PPARs have been proposed [see CHINETTI *et al.*, 2000; DELEVIRE *et al.*, 2001 for reviews]. The first is that PPARs might stimulate the breakdown of inflammatory eicosanoids through induction of peroxisomal β -oxidation. The second is that PPARs might interfere with/antagonize the activation of other transcription factors, including NF κ B. *n*-3 PUFAs might act by increasing the level of these anti-inflammatory transcription factors.

There are a number of other transcription factors that are activated by inflammatory signals and which play a role in expression of inflammatory genes [see HWANG, RHEE, 1999 for a review]. It is possible that *n*-3 PUFAs might affect the activation of these factors, but this has not been studied to detail. However, effects of *n*-3 PUFAs on signalling processes that lead to activation of various transcription factors have been reported. For example, incubation of murine macrophages with EPA was found to decrease LPS-induced phosphorylation and activation of mitogen-activated protein kinase [LO *et al.*, 2000]. Thus, various intracellular signalling steps appear to be partly inhibited by the presence of increased amounts of *n*-3 PUFAs in cells.

5-6 Clinical applications of the anti-inflammatory effects of *n*-3 PUFAs

5-6-1 Introductory comments

Although the inflammation may afflict different body compartments, one common characteristic of inflammatory conditions and diseases is excessive or inappropriate production of inflammatory mediators including eicosanoids and cytokines. The roles of *n*-6 and *n*-3 PUFAs in shaping and regulating inflammatory processes and responses suggest that the balance of these fatty acids might be important in determining the development and severity of inflammatory diseases. The recognition that the long chain *n*-3 PUFAs have anti-inflammatory actions has led to the idea that supplementation of the diet of patients with inflammatory diseases may be of clinical benefit. Possible therapeutic targets for long chain *n*-3 PUFAs are listed in **table 5.3**. Supplementation trials have been conducted in many of these diseases. Those dealing with rheumatoid arthritis, inflammatory bowel diseases (Crohn's disease and ulcerative colitis) and asthma will be

discussed in some detail here. This is because a larger number of trials have been conducted in these diseases than in the others.

Table 5.3 — List of diseases and conditions with an inflammatory component in which long chain *n*-3 PUFAs might be of benefit.

Disease
Rheumatoid arthritis
Crohn's disease
Ulcerative colitis
Systemic lupus erythematosus
Type-1 diabetes
Cystic fibrosis
Childhood asthma
Adult asthma
Allergic disease
Psoriasis
Multiple sclerosis
Atherosclerosis
Acute cardiovascular events
Obesity
Systemic inflammatory response to surgery, trauma and critical illness

5-6-2 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disease characterized by joint inflammation that manifests as swelling, pain, functional impairment, morning stiffness, osteoporosis and muscle wasting. Joint lesions are characterized by infiltration of activated macrophages, T lymphocytes and plasma cells (antibody producing B lymphocytes) into the synovium (the tissue lining the joints) and by proliferation of synovial cells called synoviocytes. Synovial biopsies from patients with rheumatoid arthritis contain high levels of TNF- α , IL-1 β , IL-6, IL-8 and granulocyte/macrophage-colony stimulating factor (GM-CSF), and synovial cells cultured *ex vivo* produce TNF- α , IL-1 β , IL-6, IL-8 and GM-CSF for extended periods of time without additional stimulus [FELDMANN, MAINI, 1999]. COX-2 expression is increased in the synovium of rheumatoid arthritis patients, and in the joint tissues in rat models of arthritis [SANO *et al.*, 1992]. PGE₂, LTB₄, 5-hydroxyeicosatetraenoic acid and also platelet activating factor are found in the synovial fluid of patients with active rheumatoid arthritis [SPERLING, 1995]. The efficacy of non-steroidal anti-

inflammatory drugs in rheumatoid arthritis indicates the importance of pro-inflammatory COX pathway products in the pathophysiology of the disease. Increased expression of E-selectin, VCAM-1 and ICAM-1 is found in patients with arthritis, and blocking ICAM-1 or VCAM-1 with antibodies reduces leukocyte infiltration into the synovium and synovial inflammation in animal models of the disease [see FAULL, 1995 for references].

Dietary fish oil has been shown to have beneficial effects in animal models of arthritis. For example, compared with vegetable oil, feeding mice fish oil delayed the onset (mean 34 days vs. 25 days) and reduced the incidence (69% vs. 93%) and severity (mean peak severity score 6.7 vs. 9.8) of type II collagen-induced arthritis [LESLIE *et al.*, 1985]. Both EPA and DHA suppressed Streptococcal cell wall-induced arthritis in rats, but EPA was more effective [VOLKER *et al.*, 2000].

Several studies report anti-inflammatory effects of fish oil in patients with rheumatoid arthritis, such as decreased LTB₄ production by neutrophils [KREMER *et al.*, 1985; 1987; CLELAND *et al.*, 1988; VAN DER TEMPEL *et al.*, 1990] and monocytes [CLELAND *et al.*, 1988; TULLEKAN *et al.*, 1990], decreased IL-1 production by monocytes [KREMER *et al.*, 1990], decreased plasma IL-1 β concentrations [ESPERSON *et al.*, 1992], decreased serum C-reactive protein concentrations [KREMER *et al.*, 1985] and normalization of the neutrophil chemotactic response [SPERLING *et al.*, 1987]. A number of randomized, placebo-controlled, double-blind studies of fish oil in rheumatoid arthritis have been reported. The characteristics and findings of these trials are summarized in **table 5.4**. The dose of long chain *n*-3 PUFAs used in these trials was between 2.1 and 7.1 g/day and averaged about 3.9 g/day. Each of these trials showed some benefit of fish oil. Such benefits include reduced duration of morning stiffness, reduced number of tender or swollen joints, reduced joint pain, reduced time to fatigue, increased grip strength and decreased use of non-steroidal anti-inflammatory drugs. A number of reviews of these trials have been published [VOLKER, GARG, 1996; JAMES, CLELAND, 1997; GEUSENS, 1998; KREMER, 2000; CALDER, 2001; CALDER, ZURIER, 2001] and each has concluded that there is benefit from fish oil, as has a meta-analysis that included data from nine trials published between 1985 and 1992 inclusive, and from one unpublished trial [FORTIN *et al.*, 1995]. In an editorial commentary discussing the use of fish oil in rheumatoid arthritis it was concluded that «the findings of benefit from fish oil in rheumatoid arthritis are robust», «dietary fish oil supplements in rheumatoid arthritis have treatment efficacy», and «dietary fish oil supplements should now be regarded as part of the standard therapy for rheumatoid arthritis» [CLELAND, JAMES, 2000]. Thus, there is fairly strong evidence that long chain *n*-3 PUFAs have some clinical benefits in rheumatoid arthritis.

Table 5.4 — Summary of placebo-controlled trials of long chain n-3 PUFAs in rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and asthma.

Disease	Number of trials	Dose of EPA + DHA used (g/day)	Duration (weeks)	Outcomes improved
Rheumatoid Arthritis	15	2.1 to 7.1 (av. 3.9)	12 to 52	Number of tender joints (10 studies) Number of swollen joints (6 studies) Severity of tender joints (3 studies) Joint pain index (1 study) Duration of morning stiffness (7 studies) Grip strength (2 studies) Time to fatigue (1 study) Physician's pain assessment (2 studies) Physician's global assessment (6 studies) Use of non-steroidal anti-inflammatory drugs (3 studies) Patient's pain assessment (2 studies) Patient's global assessment (4 studies) Health assessment (1 study)
Inflammatory bowel diseases	11 (8 in UC; 2 in CD; 1 in UC & CD)	2.7 to 5.6 (av. 4.6)	12 to 104	Sigmoidoscope score (3/5 studies) Gut mucosal histology (2/5 studies) Use of steroids (3/3 studies) Disease activity (4/5 studies in UC; 0/1 study in CD) Relapse (1/6 studies) Remission (2/2 studies)
Asthma	6 in adults 2 in children	1 to 6 (av. 4.4) < 1.2	8 to 52 24 or 40	Improved PEF (1 study) Improved FEV ₁ (1 study) Decreased asthma symptom scores and bronchial hyper-responsiveness to acetylcholine challenge (1 study)

Abbreviations: CD, Crohn's Disease; FEV₁, forced expiratory volume at one second; PEF, peak expiratory flow; UC, ulcerative colitis.

5-6-3 Inflammatory bowel diseases

Ulcerative colitis and Crohn's disease are chronic inflammatory diseases of the alimentary tract. In ulcerative colitis the mucosa of the colon is mainly affected, while in Crohn's Disease any part of the alimentary tract from the mouth to the anus can be affected, although it is usually the ileum and colon. In both diseases the intestinal mucosa contains elevated levels of inflammatory cytokines and eicosanoids such as LTB₄ [SHARON, STENSON, 1984]. Dietary fish oil has been shown to have beneficial effects in animal models of colitis [WALLACE *et al.*, 1989; VILASECA *et al.*, 1990]. Long chain *n*-3 PUFAs are incorporated into gut mucosal tissue of patients with inflammatory bowel disease who supplement their diet with fish oil [LORENZ *et al.*, 1989; HILLIER *et al.*, 1991; HAWTHORNE *et al.*, 1992], and there are reports that this results in anti-inflammatory effects, such as decreased LTB₄ production by neutrophils [MCCALL *et al.*, 1989; HAWTHORNE *et al.*, 1992; SHIMIZU *et al.*, 2003] and colonic mucosa [STENSON *et al.*, 1992; SHIMIZU *et al.*, 2003], decreased PGE₂ and TXB₂ production by colonic mucosa [HILLIER *et al.*, 1991] and decreased production of PGE₂ by blood mononuclear cells [TREBBLE *et al.*, 2004]. Small open-label or pilot studies reported clinical benefit of fish oil supplementation in ulcerative colitis [MCCALL *et al.*, 1989; SALOMON *et al.*, 1990]. A number of randomized, placebo-controlled, double-blind studies of fish oil in inflammatory bowel disease have been reported. The characteristics and findings of these trials are summarized in **table 5.4**. The dose of long chain *n*-3 PUFAs used in these trials was between 2.7 and 5.6 g/day and averaged about 4.6 g/day. Some of these trials indicate benefits of fish oil which include improved clinical score, improved gut mucosal histology, improved sigmoidoscopic score, lower rate of relapse, and decreased use of corticosteroids. One study of special note is that of BELLUZZI *et al.* [1996] in which patients with Crohn's disease in remission were randomized to receive placebo or 2.7 g long chain *n*-3 PUFAs/day from an enterically coated fish oil preparation for one year. The primary outcome was relapse. There was a significant difference in the proportion of patients who relapsed over 12 months: 11/39 (28%) in the fish oil group vs. 27/39 (69%) in the placebo group ($P < 0.001$). Likewise there was a significant difference in the proportion of patients who remained in remission at 12 months: 59% in the fish oil group vs. 26% in the placebo group ($P = 0.003$). Several reviews of trials of fish oil in inflammatory bowel diseases have been published [RODGERS, 1998; BELLUZZI, 2000; 2002] and, although these conclude that there is some benefit from fish oil, the overall view at the moment must be that there is only weak evidence that long chain *n*-3 PUFAs have clinical benefits in inflammatory bowel diseases. However, the apparent ability of long chain *n*-3 PUFAs to retain Crohn's Disease patients in remission [BELLUZZI *et al.*, 1996] is a striking finding.

5-6-4 Asthma

Arachidonic acid-derived eicosanoids such as PGD_2 , LTC_4 , D_4 and E_4 are produced by the cells that mediate pulmonary inflammation in asthma (e.g. mast cells) and are believed to be major mediators of asthmatic bronchoconstriction. 4-series LT have been detected in the blood, bronchoalveolar lavage fluid and urine of asthmatics [HENDERSON, 1994]. In addition to the role of arachidonic acid-derived eicosanoids as mediators of asthma, PGE_2 is also involved in regulating the development of the T helper type 2 phenotype of T lymphocyte that predisposes to allergic inflammation [MILES *et al.*, 2003] and promotes the formation of immunoglobulin E by B lymphocytes [ROPER, PHIPPS, 1994] (Figure 5.8). Thus a hypothesis has evolved that an increased intake of *n*-6 PUFAs has played a causal role in increased asthma incidence [HODGE *et al.*, 1994; BLACK, SHARP, 1997]. There are epidemiologic data

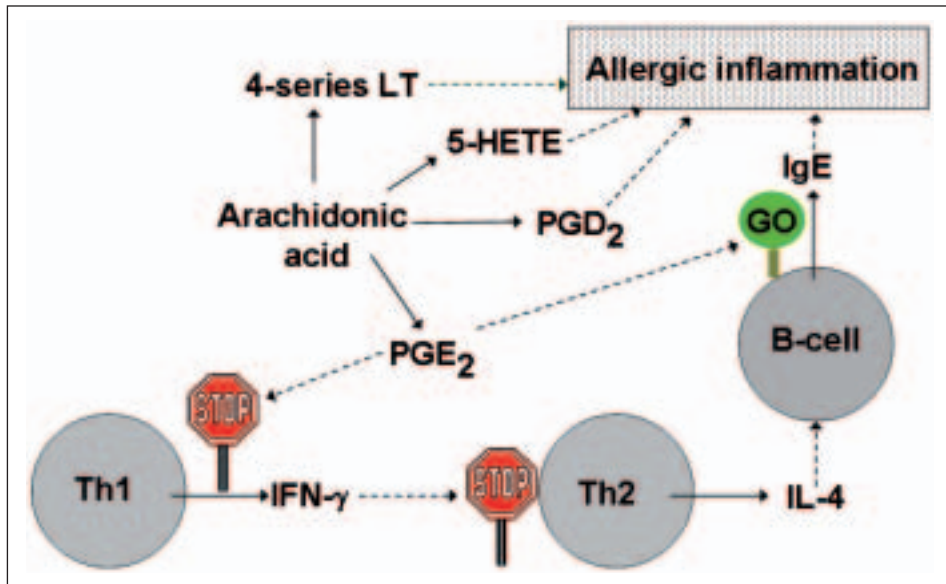


Figure 5.8 — The role of arachidonic acid-derived eicosanoids in allergic inflammation. Several arachidonic acid-derived eicosanoids are mediators of allergic inflammation. In addition prostaglandin₂ promotes immunoglobulin E production by B lymphocytes and inhibits the production of the Th-1 type cytokine interferon- γ , which normally acts to down regulate the pro-allergic Th2 type response. HETE, hydroxyeicosatetraenoic acid; IFN, interferon; IgE, immunoglobulin E; IL, interleukin; LT, leukotriene; PG, prostaglandin. Modified from P.C. CALDER, Polyunsaturated fatty acids and immunity. *Lipids*, 2001, 36, 1007-1024, with permission from the American Oil Chemists' Society.

linking high *n*-6 PUFA or low *n*-3 PUFA consumption with childhood asthma [HODGE *et al.*, 1994; DUNDER *et al.*, 2001]. Early exposure to long chain *n*-3 PUFAs does appear to alter cytokine production by neonatal T cells [DUNSTAN *et al.*, 2003a, 2003b] although the longer-term clinical impact of this is not yet clear. Nevertheless, the role of arachidonic acid-derived eicosanoids in asthma has prompted a series of studies attempting to modify the disease with fish oil treatment. Several studies report anti-inflammatory effects of fish oil in patients with asthma, such as decreased 4-series LT production [PAYAN *et al.*, 1986; ARM *et al.*, 1988; KIRSH *et al.*, 1988], and leukocyte chemotaxis [ARM *et al.*, 1988; KIRSH *et al.*, 1988]. Several uncontrolled or open-label trials of fish oil reveal clinical benefit of fish oil. However, randomized, placebo-controlled, double-blind studies of fish oil in asthma (characteristics and findings of these trials summarized in **table 5.4**) reveal little overall benefit. THIEN *et al.* [2002] included eight studies published between 1988 and 2000 in a systematic review, and identified “no consistent effect on forced expiratory volume at one second, peak flow rate, asthma symptoms, asthma medication use or bronchia hyper-reactivity”. They conceded that one study in children showed improved peak flow and reduced asthma medication use. However, studies by BROUGHTON *et al.* [1997] and NAGAKURA *et al.* [2000] indicate that there may be subgroups of asthmatic subjects who may benefit greatly from long chain *n*-3 PUFAs.

In the study by BROUGHTON *et al.* [1997], lung function worsened in adult asthmatics on low *n*-3 PUFA intakes, consistent with a protective effect of *n*-3 PUFAs (**Table 5.5**). However, lung function improved in only some (ca. 40%) of

Table 5.5 — Summary of findings from the study of BROUGHTON *et al.* [1997].

Outcome	Cumulative dose of methacholine (units) to cause a 20% reduction			
	Baseline (n = 19)	Low dose fish oil (n = 19)	High dose fish oil	
			Responders (n = 9)	Non-responders (n = 10)
FVC	24.1	11.8	> 68	3.7
PEF	17.1	5.9	> 68	3.5
FEV ₁	16.9	1.9	> 68	4.9
FEF ₂₅₋₇₅	9.0	0.7	> 68	9.9

Adult asthmatics consumed fish oil capsules for 4 weeks such that the *n*-6 to *n*-3 PUFA ratio of their diet was 10 (low dose fish oil) or 2 (high dose fish oil). At baseline, after consuming the low dose fish oil and after consuming the high dose fish oil, subjects underwent challenge with increasing amounts of methacholine to give cumulative doses up to 68 units. Data are the mean cumulative dose of methacholine required to cause a 20% reduction in each indicator of lung function.

Abbreviations used: FEV₁, forced expiratory volume at one second; FEF₂₅₋₇₅, maximum forced expiratory flow; FVC, forced vital capacity; PEF, peak expiratory flow.

the subjects when on high *n*-3 PUFA intakes. In fact in the other 60% of subjects, lung function was again worsened from baseline by a high *n*-3 PUFA intake. The difference between responders and non-responders to *n*-3 PUFAs appeared to relate to an alteration in production of 5-series LTs from EPA; those subjects who had a urinary ratio of 4- to 5-series LTs less than 1 after high *n*-3 PUFA ingestion were those whose lung function improved. In the study by NAGAKURA *et al.* [2000] asthmatic Japanese children benefited significantly from fish oil supplementation in terms of improved asthma score and better lung function. Clearly more and better studies need to be done in this area, the effects of *n*-3 PUFAs in children and adults need to be discriminated, and the possibility of responder populations needs to be confirmed.

5-7 Conclusions

118

Inflammation is a part of normal host defense but is also a component of a range of acute and chronic human diseases. Inflammation is characterized by the production of inflammatory cytokines, arachidonic acid-derived eicosanoids, other inflammatory mediators (e.g. platelet activating factor) and adhesion molecules. *n*-3 PUFAs decrease the production of many inflammatory mediators and the expression of some adhesion molecules. They act both directly (e.g. by replacing arachidonic acid as an eicosanoid substrate and inhibiting arachidonic acid metabolism) and indirectly (e.g. by altering the expression of inflammatory genes through effects on transcription factor activation). Long chain *n*-3 PUFAs also give rise to anti-inflammatory mediators (resolvins). Thus, *n*-3 PUFAs are potentially potent anti-inflammatory agents. As such, they may be of therapeutic use in a variety of acute and chronic inflammatory settings. Evidence of their clinical efficacy is strong in some settings (e.g. in rheumatoid arthritis) but weak in others (e.g. in asthma). More, better designed and larger trials are required in inflammatory diseases to assess the therapeutic potential of long chain *n*-3 PUFAs in these conditions.

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6 – Strategies to increase long chain *n*-3 PUFA status in humans

6-1 Recommendations for long chain *n*-3 PUFA intake and possible strategies to achieve these

Long chain *n*-3 PUFAs exert a range of physiological functions that result in real or potential benefits to human health (**Table 6.1**). These benefits have long been recognized and have resulted in a series of recommendations to increase the intake of long chain *n*-3 PUFAs by various government and professional bodies (**Table 6.2**). Current intakes of long chain *n*-3 PUFAs in the United Kingdom are < 0.25 g/day [Scientific Advisory Committee, 2004] and these are likely representative of many Western countries. Thus, to meet even the most conservative of recommendations will require a doubling of intake, while a six-fold increase in intake will be required to meet the less conservative recommendations [British Nutrition Foundation, 1999]. A limited number of strategies is currently available to increase the intake of long chain *n*-3 PUFAs. It is evident that frequent consumption of oily fish could substantially increase long chain *n*-3 PUFA intake (and so status) in all subgroups of the population. However there has been an unwillingness for many consumers to eat fish. The latest figures for the United Kingdom indicate that about 20% of adults and < 10% of adolescents and children consume fish (excluding battered fish etc.) [Scientific Advisory Committee, 2004], although it is encouraging that average fish consumption has increased in the United Kingdom over the last 10 years. Reasons for this may be publicity about the health benefits of eating fish, a greater appreciation of the importance of healthy eating amongst some sections of the population, the proliferation of popular cooking programmes on television, a temporary move away from eating meat in some places, and increased availability and lower cost of some types of fish. In the

future, fish farming will make fish more readily available and at a lower cost. However, changes in feeding regimens of farmed fish may impact on the long chain *n*-3 PUFA content of the flesh (Section 1-3). An alternative way of meeting recommendations that would not require dietary change is the consumption of fish oil capsules (or liquid); consumption of a single typical one gram fish oil capsule per day would allow the majority of consumers to meet the most conservative of recommendations for long chain *n*-3 PUFA intake.

Table 6.1 — Summary of the physiological roles and potential clinical benefits of long chain *n*-3 PUFAs.

Physiological role of long chain <i>n</i> -3 PUFAs	Potential clinical benefit	Target
Regulation of blood pressure	Decreased blood pressure	Hypertension; CVD
Regulation of platelet function	Decreased likelihood of thrombosis	CVD
Regulation of blood coagulation	Decreased likelihood of thrombosis	CVD
Regulation of plasma triacylglycerol concentrations	Decreased plasma triacylglycerol concentrations	Hypertriacylglycerolemia; CVD
Regulation of vascular function	Improved vascular reactivity	CVD
Regulation of cardiac rhythm	Decreased arrhythmias	CVD
Regulation of inflammation	Decreased inflammation	Inflammatory diseases (see Table 5.3); CVD
Regulation of immune function	Improved immune function	Compromised immunity?
Regulation of bone turnover	Maintained bone mass	Osteoporosis
Regulation of insulin sensitivity	Improved insulin sensitivity	Type-2 diabetes
Regulation of tumour cell growth	Decreased tumour cell growth & survival	Some cancers
Regulation of visual signalling (rhodopsin)	Optimised visual signalling	Poor infant visual development (especially pre-term)
Structural component of brain and central nervous system	Optimised brain development – cognitive and learning processes	Poor infant and childhood cognitive processes and learning

Abbreviation used: CVD, cardiovascular disease.

Table 6.2 — Some recommendations for the intake of long chain *n*-3 PUFAs in adult humans.

Recommended intake of long chain <i>n</i> -3 PUFAs (g/day)	Reference
0.2	DE DECKERE <i>et al.</i> [1998]
0.45	SCI. ADVISORY COMMITTEE ON NUTR. [2004]
0.65	SIMOPOLOUS <i>et al.</i> [1999]
1.0*	KRIS-ETHERTON <i>et al.</i> [2002]
1.0*	VAN DE WERF <i>et al.</i> [2003]
1.2 to 1.6	BRITISH NUTRITION FOUNDATION [1999]
2 to 4**	KRIS-ETHERTON <i>et al.</i> [2002]

* for secondary prevention of myocardial infarction; **for hypertriglycerolemia.

A third strategy to increase long chain *n*-3 PUFA intake would be to increase the content of those fatty acids in habitually consumed foods that normally lack these fatty acids (e.g. spreads, milk, meat, bread, eggs). Such enrichment could occur through fortification, through altered poultry or animal feeding practices, or, ultimately, through the application of biotechnology in crops [SAYANOVA, NAPIER, 2004]. FINNEGAN *et al.* [2003] were able to use a spread modified to include long chain *n*-3 PUFAs such that consumption of 25 g/day of the spread as part of a typical diet (in the United Kingdom) would increase intake of those fatty acids to 0.7 g/day. This resulted in significant enrichment of plasma phospholipids in EPA and DHA [FINNEGAN *et al.*, 2003]. Thus it is possible to fortify some food products such that they can deliver sufficiently increased amounts of long chain *n*-3 PUFAs to increase the long chain *n*-3 PUFA content of at least some body lipid pools. However, altered feeding practices in farms may be the key to population wide increases in long chain *n*-3 PUFA intake. Feeding flaxseed to laying hens increases the long chain *n*-3 PUFA content of the eggs produced, such that one egg can provide 115-140 mg EPA+DPA+DHA. Various strategies have been used to increase the long chain *n*-3 PUFA content of chicken meat and of goat's, sheep and cow's milks, the most successful being the inclusion of fish meal, fish oil or *n*-3 PUFA-rich algal oil in the diet [CANT *et al.*, 1997; FRANKLIN *et al.*, 1999; OFFER *et al.*, 1999; DONOVAN *et al.*, 2000; KEADY *et al.*, 2000; BAER *et al.*, 2001; GULATI *et al.*, 2003]. In a recent study, dairy cows were fed on a diet containing rumen-protected tuna oil [KITESSA *et al.*, 2004]. EPA and DHA appeared in the milk from 36 hours after the start of feeding and plateaued at day 6. At this stage EPA and DHA contributed 0.6 and 1.1% of milk fatty acids, respectively. This is equivalent to 25 and 45 mg per 100 ml milk, respectively. Thus one 250 ml glass of this milk could provide between 35 and 40% of the current UK recommended intake of long chain *n*-3 PUFAs.

Feeding fish oil to pigs results in increased contents of EPA, DPA and DHA in edible muscle and fat, in edible offal such as liver and kidney, and in meat products such as sausages [IRIE, SAKIMOTO, 1992; LESKANICH *et al.*, 1997]. In another study pigs were fed two levels of flaxseed providing 6 and 10-times more dietary α -linolenic acid (α LNA) than the control animals received [MATTHEWS *et al.*, 2000]. This dietary strategy resulted in greatly enhanced amounts of α LNA, EPA and DPA in the meat, liver and kidney.

An alternative to the strategy of increasing intake of preformed long chain *n*-3 PUFAs is to increase consumption of precursor fatty acids such as α -linolenic acid (α LNA) in order to promote their endogenous synthesis using the pathway shown in **figure 1.5 (Table 6.3)**. The potential for this strategy to be successful is discussed in Section 6-2.

Table 6.3 — Strategies to increase the status of long chain *n*-3 PUFAs in human plasma, cells and tissues.

Metabolic strategy	Dietary strategy
Provide pre-formed long chain <i>n</i> -3 PUFAs	Eat oily fish Supplement diet with fish oil capsules Eat enriched or fortified foods
Provide precursor fatty acids (mainly α -linolenic acid)	Use α -linolenic acid containing vegetable oils Supplement diet with flaxseeds Eat enriched or fortified foods

6-2 Is α -linolenic acid a suitable substitute for long chain *n*-3 PUFAs?

6-2-1 Introductory comments

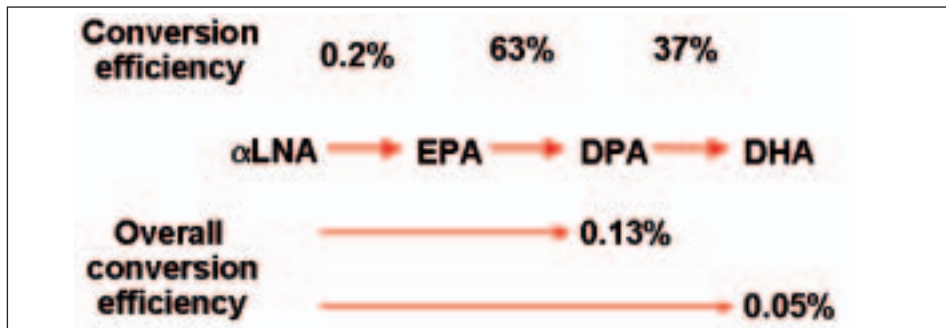
The major dietary sources of α LNA are green leaves, and oils used in cooking such as rapeseed oil and soybean oil where it accounts for up to 10% of total fatty acids. Flaxseed (also known as linseed) oil is particularly rich in α LNA (> 50% total fatty acids). Typical consumption of α LNA in Europe, Australia and North America ranges between 0.6 to 2.2 g/day in men and 0.5 to 2.1 g/day in women (see **Table 1.4**).

α LNA can be converted to the long chain *n*-3 PUFAs using the pathway outlined in **figure 1.5**. Thus, the question of whether α LNA is a suitable substitute for long chain *n*-3 PUFAs actually has two components. The first is whether α LNA in its own right can elicit the physiological effects of long chain *n*-3 PUFAs, as outlined in Sections 3, 4 and 5. If this is the case then α LNA could be used as a genuine substitute for its longer chain derivatives. The second component to the question is whether α LNA can be converted in humans to long chain *n*-3 PUFAs in sufficient amounts for the latter to elicit their effects. In other words, this is a passive role for α LNA of being the substrate for the synthesis of physiologically active products. In practice, it has proved difficult to separate these two components of the question: in cell culture, animal feeding and human experiments a proportion of α LNA provided is converted to long chain *n*-3 PUFAs, and so it is difficult to ascribe with great certainty any biological effects seen to α LNA *per se* or to its derivatives. However attempts to answer this question in humans have used one of two approaches: acute stable-isotope studies or chronic intervention studies.

6-2-2 Stable isotope studies

A number of studies using α LNA labeled with either [^{13}C] or deuterium have provided estimates of the extent of conversion to long chain PUFAs in humans [EMKEN *et al.*, 1994; 1999; SALEM *et al.*, 1999; VERMONT *et al.*, 2000; PAWLOSKY *et al.*, 2001; 2003a,b; BURDGE *et al.*, 2002; 2003]. The general consensus of these studies is that the proportion of α LNA entering the desaturation/elongation pathway and converted to EPA and DPA is low. The extent of conversion of α LNA to DHA is even lower. The highest estimated fractional conversion is 4% [EMKEN *et al.*, 1994], while most other studies have reported lower estimates of conversion (0.05% or less) [VERMONT *et al.*, 2000; PAWLOSKY *et al.*, 2001; 2003a,b; BURDGE *et al.*, 2003] and one study failed to detect significant incorporation of stable isotope into DHA in men [BURDGE *et al.*, 2002]. PAWLOSKY *et al.* [2001] have suggested estimates for the efficiency of conversion of individual steps in the desaturation/elongation pathway from kinetic analysis based on the concentrations of individual deuterated fatty acids in plasma from a mixed group of men and women. The findings of this study were that the efficiency of conversion of α LNA to EPA was 0.2%, of EPA to DPA 65% and of DPA to DHA 37%. Thus producing an overall efficiency of conversion from α LNA is 0.2% to EPA, 0.13% to DPA and 0.05% to DHA (**Figure 6.1**). However, BURDGE AND WOOTTON [2002] showed that conversion of α LNA to EPA and DHA in women aged about 28 years was substantially greater (2.5-fold and >200-fold, respectively) than in a comparable study of men of similar age [BURDGE *et al.*, 2002]. This finding is strongly supported by kinetic analysis which showed that the rate constant coefficient for the conversion of DPA to DHA was approximately 4-fold greater in women compared

to men [PAWLOSKY *et al.*, 2003b]. This may reflect greater availability of α LNA for conversion in women than in men perhaps, in part, due to lower partitioning towards β -oxidation. One possible explanation for the greater synthesis of EPA and DHA from α LNA in women compared to men is the action of estrogen, since DHA synthesis was almost 3-fold greater in women using an oral contraceptive pill containing 17 α -ethinyloestradiol than in those who did not [BURDGE, WOOTTON, 2002]. One possible biological role for greater capacity for α LNA conversion in women may be in meeting the demands of the fetus and neonate for DHA. Since circulating estrogen concentration rises during pregnancy due to synthesis and secretion by the placenta, one possibility is that α LNA conversion may increase during gestation.



134

Figure 6.1 — Estimated efficiency of the different steps of the pathway of conversion of α -linolenic acid to its longer chain more unsaturated derivatives in humans. Data are from PAWLOSKY *et al.* [2001].

6-2-3 Effects of chronically increased α -linolenic acid consumption

A number of studies have reported the effects of consuming increased amounts of α LNA, usually via consumption of oils with a high α LNA content or of products made with those oils (for example spreads), on the fatty acid composition of plasma [1] or cell lipids [2]. These studies were conducted either in men or in

[1] SANDERS, YOUNGER, 1981; KELLEY *et al.*, 1993; MANTZIORIS *et al.*, 1994; CUNNANE *et al.*, 1995; LI *et al.*, 1999; BEMELMANS *et al.*, 2002; FINNEGAN *et al.*, 2003; JAMES *et al.*, 2003; WALLACE *et al.*, 2003.
 [2] SANDERS, YOUNGER, 1981; SANDERS, ROSHANAI, 1983; WEAVER *et al.*, 1990; KWON *et al.*, 1991; MUTANEN *et al.*, 1992; KELLEY *et al.*, 1993; MANTZIORIS *et al.*, 1994; CAUGHEY *et al.*, 1996; ALLMAN-FARINELLI *et al.*, 1999; LI *et al.*, 1999; HEALY *et al.*, 2000; BEMELMANS *et al.*, 2002; JAMES *et al.*, 2003; KEW *et al.*, 2003; WALLACE *et al.*, 2003

mixed groups of men and women, used intakes of α LNA ranging from less than one to 20 g/day, and were of a few weeks to many months duration. Despite differences in the study design, physical form in which the α LNA was presented and the duration of the studies, overall they consistently demonstrate increased proportions of EPA in both plasma and cell lipids when α LNA intake is increased (e.g. **Figures 6.2 and 6.3**). Using such data it is clear that the relationship between increased α LNA

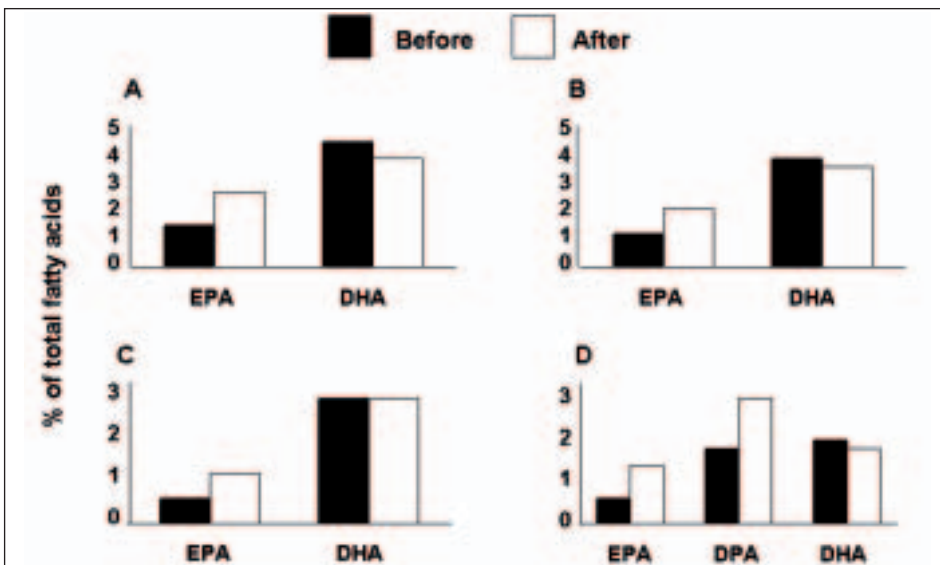


Figure 6.2 — Effect of increased α -linolenic acid consumption on the long chain *n*-3 PUFA contents of human plasma phospholipids and platelets.

Healthy volunteers consumed 6.5 (A), 9 (B), 9.4 (C) or 18 (D) g/day α -linolenic acid for 2 (A, C), 3 (D) or 4 (B) weeks. The fatty acid compositions of plasma phosphatidylcholine (A), plasma phospholipids (B), or platelets (C, D) were determined. Data are from SANDERS, YOUNGER [1981] (A), CUNNANE *et al.* [1995] (B), SANDERS, ROSHANAI [1983] (C), and ALLMAN *et al.* [1995] (D).

intake and increased EPA concentration in plasma phospholipids is a significant linear one ($r = 0.846$, $P = 0.004$). The relationship for cell phospholipids is also likely to be linear, but there are insufficient data for a single cell type to allow this to be clearly identified at this stage. Several studies also demonstrate increased proportions of DPA in plasma and cell lipids when α LNA consumption is increased (e.g. **Figure 6.2D**). The studies also consistently demonstrate that increased

consumption of α LNA does not result in increased proportions of DHA in plasma or cell lipids (e.g. **Figures 6.2 and 6.3**). Indeed many studies report a tendency for DHA to decline when α LNA consumption is markedly increased, although few studies have identified this as a statistically significant effect.

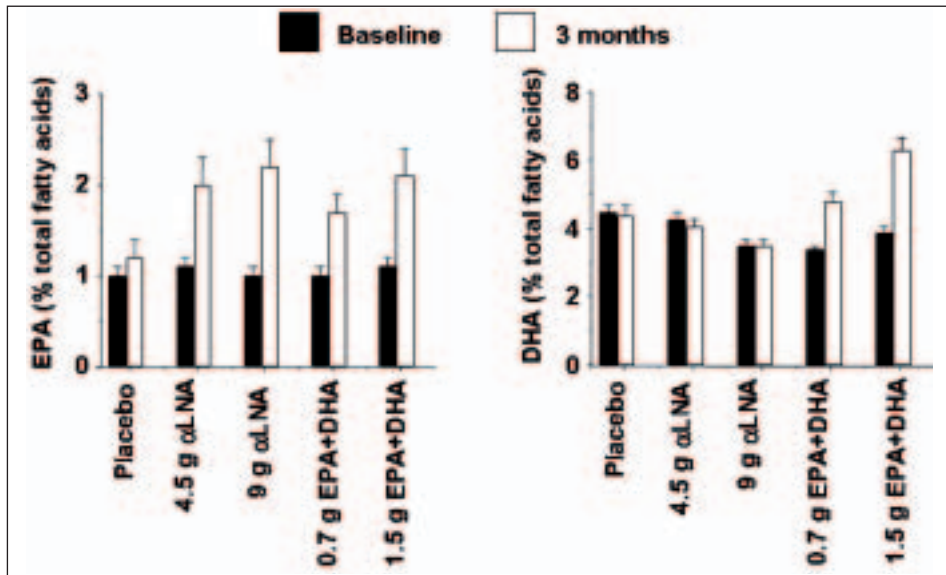


Figure 6.3 — Effect of increased α -linolenic acid or long chain n -3 PUFA consumption on the long chain n -3 PUFA content of human plasma phospholipids. Healthy volunteers consumed diets providing 4.5 or 9 g/day α -linolenic acid or 0.7 or 1.5 g/day EPA+DHA for 3 months. The fatty acid composition of plasma phospholipids was determined. Data are from FINNEGAN *et al.* [2003].

Consumption of 10.7 g α LNA/day by lactating women for 4 weeks increased maternal plasma, erythrocyte and breast-milk α LNA concentration [FRANÇOIS *et al.*, 2003]. Breast milk EPA increased by about 35% while DPA concentration did not change. Increased consumption of α LNA did not alter breast-milk DHA concentration, which tended to decrease at 4 weeks.

Because of competition for metabolism between linoleic acid and α LNA, the linoleic acid content of the diet may influence conversion of α LNA to longer chain derivatives. If this is so then the EPA content of blood and cell lipids should be greater at a given intake of α LNA if linoleic acid intake is decreased. A study by

CHAN *et al.* [1993] demonstrated that this is indeed the case. Subjects consumed diets providing 7 g α LNA/day for 18 days against a background of either 21 or 50 g linoleic/day. The proportion of EPA was greater in plasma and platelets after the low compared with the high linoleic acid background (Figure 6.4).

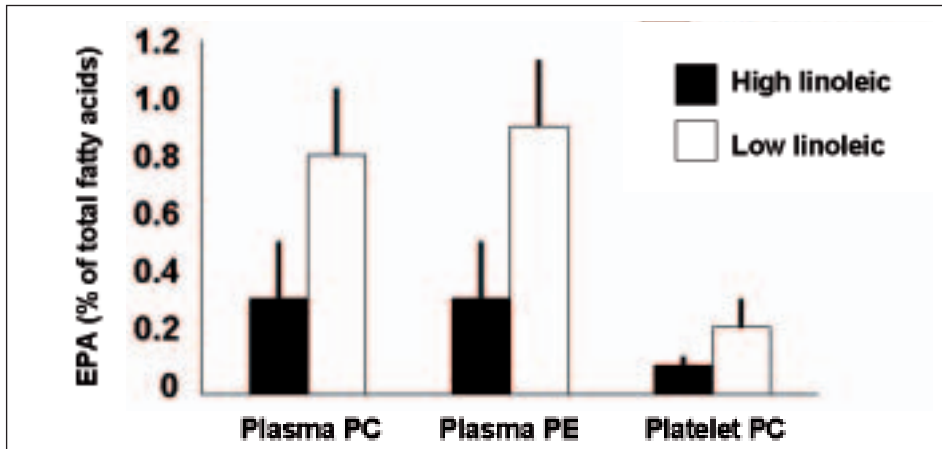


Figure 6.4 — Effect of linoleic acid consumption on human plasma phospholipid and platelet contents of eicosapentaenoic acid after consumption of a diet rich in α -linolenic acid.

Healthy volunteers consumed a diet containing 7 g/d α -linolenic acid and either 21 or 50 g/day linoleic acid for 18 days. The fatty acid compositions of plasma phosphatidylcholine (PC), plasma phosphatidylethanolamine (PE), or platelets (PC) were determined. Data are from CHAN *et al.* [1993].

Overall, these studies demonstrate that chronically increased consumption of α LNA results in conversion to EPA resulting in increases in EPA concentration in plasma and cell pools, while the extent of conversion to DHA is insufficient to increase the concentration of this fatty acid. One limit on the conversion of α LNA to EPA may be Δ 6-desaturase activity. If this is so then the product of Δ 6-desaturase (stearidonic acid; 18:4 n -3) should be converted to EPA more efficiently than α LNA is. JAMES *et al.* [2003] compared the appearance of EPA in plasma and cell phospholipids after supplementation of the diet with 0.75 g/d for 4 weeks and then 1.5 g/day for 4 weeks of α LNA, stearidonic acid and EPA. They found that stearidonic acid resulted in greater appearance of EPA than α LNA did (Figure 6.5), thus supporting the notion that Δ 6-desaturase is a limit to α LNA conversion.

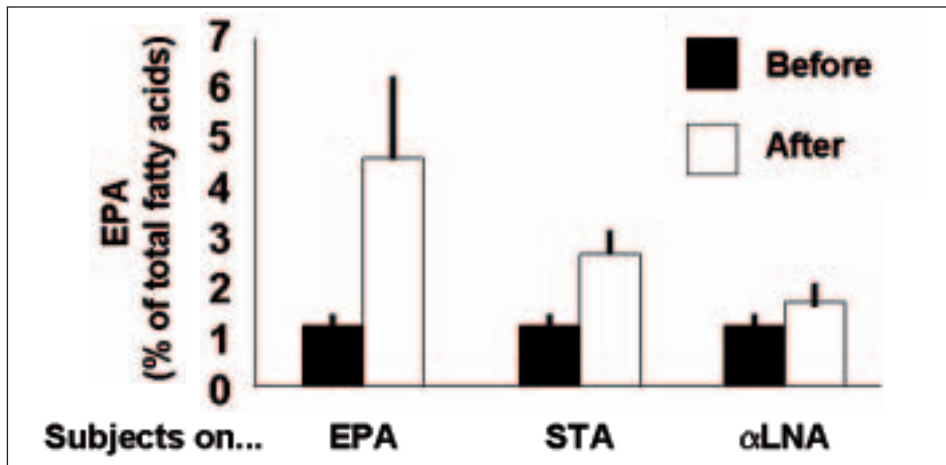


Figure 6.5 — Plasma phospholipid eicosapentaenoic acid content after consumption of increased amounts of α -linolenic acid (α LNA), stearidonic acid (STA) or eicosapentaenoic acid (EPA).

Healthy volunteers consumed 0.75 g/day α -linolenic acid, or stearidonic acid or eicosapentaenoic acid for three weeks and then 1.5 g/day of the same fatty acid for 3 weeks. The fatty acid composition of plasma phospholipids was determined. Data are from JAMES *et al.* [2003].

6-2-4 α -Linolenic acid and human health-related outcomes

- **α -Linolenic acid and cardiovascular disease**

A population based case-control study in Costa Rica reported that high adipose tissue α LNA content, a marker for intake, was associated with lower risk of myocardial infarction [BAYLIN *et al.*, 2003]. Dietary α LNA intake was significantly inversely associated with mortality from coronary heart disease in the Multiple Risk Factor Intervention Trial [DOLECEK, 1992]. The highest quintile of α LNA intake (1.36 g/day) was associated with 45% fewer coronary deaths in women compared with the lowest quintile (0.71 g/day) [HU *et al.*, 1992]. Dietary α LNA intake was inversely associated with risk of myocardial infarction among men [ASCHERIO *et al.*, 1996]; there was a relative risk of 0.41 for each 1% increase in energy intake as α LNA. In a study of over 4400 men and women the highest quintile of α LNA intake (1.1 g/day) was associated with 40% lower mortality from coronary artery disease compared with the lowest quintile of intake (0.5 g/day) [DJOUSSE *et al.*, 2001]. In addition, dietary α LNA

intake was inversely related to carotid atherosclerosis [DJOUSSE *et al.*, 2003]. In contrast to these findings, other epidemiological studies found no association between α LNA intake and coronary heart disease risk [SIMON *et al.*, 1995; OOMEN *et al.*, 2001] or mortality [PIETINEN *et al.*, 1997; OOMEN *et al.*, 2001]. A recent meta-analysis of five prospective cohort studies [DOLECEK *et al.*, 1992; ASCHERIO *et al.*, 1996; PIETINEN *et al.*, 1997; HU *et al.*, 1999; OOMEN *et al.*, 2001] concluded that high α LNA intake is associated with decreased risk of fatal coronary heart disease [BROUWER *et al.*, 2004].

The well-known Lyon Heart Study reported a substantial reduction in coronary events and deaths among myocardial infarction-survivors following a Mediterranean style diet which included an α LNA rich margarine providing 1.5 g α LNA/day [DE LORGERIL *et al.*, 1994]. However, this intervention also involved several other dietary changes and so the contribution, if any, of increased α LNA intake to the positive outcomes cannot be identified.

- **α -Linolenic acid and cardiovascular risk factors**

Blood lipids

A number of studies have investigated the effect of increased α LNA consumption of blood cholesterol, LDL and HDL concentrations. While some of these studies report little effect of α LNA intervention, several indicate that α LNA is similar to linoleic acid with respect to blood cholesterol concentration [KESTIN *et al.*, 1990; CHAN *et al.*, 1991; MANTZIORIS *et al.*, 1994; ARJMANDI *et al.*, 1998; PANG *et al.*, 1998; SODERGREN *et al.*, 2001; BEMELMANS *et al.*, 2002; FINNEGAN *et al.*, 2003; ZHAO *et al.*, 2004]. Likewise some of these studies indicate similar effects of α LNA and linoleic acid on LDL and HDL cholesterol concentrations [KESTIN *et al.*, 1990; CHAN *et al.*, 1991; ARJMANDI *et al.*, 1998; SODERGREN *et al.*, 2001; BEMELMANS *et al.*, 2002; FINNEGAN *et al.*, 2003; ZHAO *et al.*, 2004]. However two studies [MANTZIORIS *et al.*, 1994; PANG *et al.*, 1998] suggest that α LNA is not as effective as linoleic acid in lowering LDL cholesterol concentrations. Furthermore, studies reporting that α LNA decreased HDL concentration [NESTEL *et al.*, 1997; RALLIDIS *et al.*, 2003] did not see this effect with linoleic acid, while BEMELMANS *et al.* [2002] found that HDL concentration was significantly lower (by 4% or 0.05 mM) after α LNA than after linoleic acid. Taken together these data would suggest that, while α LNA is cholesterol and LDL lowering, it is less effective than linoleic acid, and that α LNA, unlike linoleic acid, may decrease HDL concentration.

Studies investigating the effect of α LNA on fasting plasma TAG concentrations are contradictory, with most reporting no change from baseline, several reporting an increase or a tendency to an increase, and two reporting a decrease. These differences in outcome may relate to the fat content of the

background diet, to habitual dietary fat composition, to whether subjects were placed on a standardized diet prior to the α LNA intervention, to the amount of α LNA and of other fatty acids supplied, and to the degree of triacylglycerolemia at baseline. It is interesting that the two studies reporting decreased plasma TAG concentrations used very high α LNA intakes [SINGER *et al.*, 1986; ZHAO *et al.*, 2004]. Some studies permit the effect of α LNA on fasting TAG concentrations to be directly compared with that of linoleic acid. Several found that the effects of a high α LNA diet were not different from those of linoleic acid-rich diet [KESTIN *et al.*, 1990; NESTEL *et al.*, 1997; PANG *et al.*, 1998; RALLIDIS *et al.*, 2003; ZHAO *et al.*, 2004]. However, ARJMANDI *et al.* [1998] and FINNEGAN *et al.* [2003] who found that α LNA tended to increase TAG concentration, did not see this effect with a high linoleic acid diet, while BEMELMANS *et al.* [2002] reported that plasma TAG concentration was significantly higher (12% or 0.24 mM) after α LNA than after linoleic acid. These studies are suggestive that α LNA has a TAG raising effect compared with linoleic acid. FINNEGAN *et al.* [2003] compared diets with high α LNA content to those with modest long chain *n*-3 PUFA content; long chain *n*-3 PUFAs tended to lower plasma TAG concentration in contrast to α LNA, which tended to be TAG raising.

Post-prandial lipemia

FINNEGAN *et al.* [2003] found no effect of diets containing 4.5 or 9 g α LNA/day on the post-prandial TAG response to a test meal, compared with study entry or with a linoleic acid rich diet.

LDL oxidation

NESTEL *et al.* [1997] found that the lag time of copper-induced oxidation of LDL was significantly lower (by 14 minutes; about 22%) after consumption of an α LNA rich (20 g/day) low fat (26% energy) diet compared with a monounsaturated fat rich low fat diet. In addition, the content of thiobarbituric acid reactive substances in the oxidised LDL was higher if the LDL came from subjects on the α LNA rich diet [NESTEL *et al.*, 1997]. However, FINNEGAN *et al.* [2003] reported no effect of diets containing 4.5 or 9 g α LNA/day on lag time of LDL oxidation, compared with study entry or with a linoleic acid rich diet. EZAKI *et al.* [1999] found no effect of 4.2 g α LNA/day on plasma lipid peroxide or oxidized LDL concentrations in elderly Japanese subjects.

Blood pressure

BERRY and HIRSCH [1986] noted that a 1% increase in adipose tissue α LNA content was associated with a 5 mm Hg decrease in systolic and diastolic blood pressure. SALONEN *et al.* [1988] reported an inverse association between α LNA consumption and blood pressure in a large prospective study. However, 38 g

α LNA/day for 2 weeks did not affect systolic or diastolic blood pressure in normotensive or hypertensive subjects [SINGER *et al.*, 1986]. Likewise, 9.2 g α LNA/day did not affect systolic or diastolic blood pressure in a 6 week intervention study in normotensive hypercholesterolemic subjects [KESTIN *et al.*, 1990]. Furthermore, 6.3 g α LNA/day for 2 years did not affect systolic or diastolic blood pressure in subjects with multiple cardiovascular risk factors [BEMELMANS *et al.*, 2002], although some of these subjects were under antihypertensive therapy.

Vascular reactivity

NESTEL *et al.* [1997] found increased arterial compliance (aortic flow velocity and aortic root driving pressure) after four weeks of 20 g α LNA/day (9% energy and 2.7% energy as LA) in exchange for oleic acid in obese subjects.

Haemostasis and platelet aggregation

Increased consumption of α LNA results in an increased content of EPA and DPA in platelets (see **Figure 6.3**) in parallel with a decreased content of arachidonic acid. This might be expected to affect platelet aggregation, since this is regulated in part by arachidonic acid- and EPA-derived eicosanoids. Indeed healthy men consuming a high α LNA diet (8.5% energy from α LNA; approx. 18 g/day) had decreased collagen-induced platelet aggregation compared with those on a linoleic acid-rich diet (12% energy) [ALLMAN *et al.*, 1995]. FREESE *et al.* [1994] reported a decrease in ADP-induced platelet aggregation in hyperlipemic subjects consuming 2.1% energy as α LNA (and 6.5% energy as linoleic acid) compared with a low α LNA diet (0.3% energy from α LNA and 8% energy from linoleic acid). However there are reports that substantially increased consumption of α LNA (3.7, 5.9 or 15.4 g/day) does not affect platelet aggregation induced by collagen [FREESE *et al.*, 1997; LI *et al.*, 1999]. Furthermore, there was no effect of 6.8 or 10 g/day α LNA on ADP-induced platelet aggregation [JUNKER *et al.*, 1991; WENSING *et al.*, 1999].

A number of intervention studies report little effect of α LNA on coagulation and fibrinolytic factors, including factor VII, factor XII, fibrinogen, plasminogen activator inhibitor (PAI)-1 or tissue plasminogen activator (t-PA) concentration or factor VII, PAI-1 or t-PA activity. Intakes of α LNA studied have been in the range of 3.7 to 15.4 g/day.

Inflammation

Increased consumption of α LNA results in an increased content of EPA and DPA in the membranes of inflammatory cells like neutrophils and monocytes [KELLEY *et al.*, 1993; MANTZIORIS *et al.*, 1994; CAUGHEY *et al.*, 1996; HEALY *et al.*, 2000; KEW *et al.*, 2003] in parallel with a decreased content of arachidonic acid. This might be

expected to affect inflammation, since this is regulated in part by arachidonic acid- and EPA-derived eicosanoids. Studies investigating the effect of α LNA on inflammatory outcomes in humans have examined the circulating concentrations of inflammatory markers such as CRP, soluble adhesion molecules or cytokines [JUNKER *et al.*, 1991; THIES *et al.*, 2001; RALLIDIS *et al.*, 2003; 2004; BEMELMANS *et al.*, 2004; ZHAO *et al.*, 2004], while other studies have examined inflammatory cell responses *ex vivo* [CAUGHEY *et al.*, 1996; HEALY *et al.*, 2000; THIES *et al.*, 2001; KEW *et al.*, 2003; WALLACE *et al.*, 2003]. CAUGHEY *et al.* [1996] reported that 13.7 g α LNA/day for 4 weeks resulted in a decrease in *ex vivo* production of prostaglandin E₂, TNF- α and IL-1 β by endotoxin-stimulated mononuclear cells by 33, 27 and 30%, respectively. By comparison fish oil providing 2.7 g EPA+DHA/day decreased production of these inflammatory mediators by 55, 70 and 78% respectively. Thus on a g/day basis, long chain *n*-3 PUFAs are about 8 to 14-times more potent than α LNA with respect to this outcome in healthy subjects. In contrast to the observations of Caughey *et al.* [44], several studies using lower intakes of α LNA (2 to 9.5 g/day) did not find effects on neutrophil chemotaxis, neutrophil respiratory burst, monocyte respiratory burst, TNF- α , IL-1 β and IL-6 production by endotoxin stimulated mononuclear cells, all studied *ex vivo*, or on soluble adhesion molecule concentrations [JUNKER *et al.*, 1991; HEALY *et al.*, 2000; THIES *et al.*, 2001; KEW *et al.*, 2003; WALLACE *et al.*, 2003]. Furthermore, a study by RALLIDIS *et al.* [2004] reported no effect of 8 g α LNA/day on sICAM-1 or sE-selectin concentrations and a similar decrease in sVCAM-1 concentration in both α LNA and control groups. Likewise BEMELMANS *et al.* [2004] found no effect of 6.3 g α LNA/day on sICAM-1 concentration at one and two years of intervention. Taken together, these data suggest that increasing α LNA intake to >9 g/d is required in order for marked anti-inflammatory effects to be seen. Even then the effects will be much more modest than those exerted by long chain *n*-3 PUFAs [CAUGHEY *et al.*, 1996]. However, both RALLIDIS *et al.* [2003] and BEMELMANS *et al.* [2004] did find a significant decrease in CRP concentration, suggesting that this may be a marker that is more sensitive to intakes of α LNA that do not affect soluble adhesion molecule or cytokine concentrations or *ex vivo* inflammatory cell responses. One study using a very high intake of α LNA (approx. 17.5 g/day) reported significant decreases in both CRP and soluble adhesion molecule concentrations [ZHAO *et al.*, 2004]. Interestingly, the authors found that the changes in these inflammatory markers were significantly related to changes in serum concentrations of EPA or EPA plus DPA, but not of α LNA. This suggests that the observed effects are due to the long chain *n*-3 PUFAs rather than to α LNA *per se*. Thus the likely explanation for the lack of anti-inflammatory effect of α LNA at modest [HEALY *et al.*, 2000; THIES *et al.*, 2001; KEW *et al.*, 2003; WALLACE *et al.*, 2003] and even at rather high [KEW *et al.*, 2003] intakes, is that there has been insufficient conversion of α LNA to the more active EPA.

6-2-5 To summarise

Epidemiological studies show that α LNA intake is inversely associated with risk of cardiovascular disease. This inverse relationship may explain the benefit on mortality seen in an intervention study that involve increased α LNA intake [DE LORGERIL *et al.*, 1994]. Intervention studies are indicative that substantially increased α LNA intake can beneficially affect a range of cardiovascular risk factors including blood cholesterol, LDL cholesterol and TAG concentrations, vascular reactivity, platelet aggregation and inflammation. However, the effect on cholesterol concentration is similar to that of linoleic acid, while α LNA is less potent than linoleic acid at decreasing LDL concentration. Furthermore α LNA can decrease HDL concentration, which linoleic acid does not do and, in contrast to the effects of long chain *n*-3 PUFAs, may increase TAG concentration. More modest increases in α LNA intake do not affect blood lipid concentrations, post-prandial lineman, blood pressure, platelet aggregation, haemostatic factors, or inflammation. This was also the view reached by a workshop on this subject held in the United Kingdom which concluded “the studies suggested little, if any, benefit of α LNA, relative to linoleic acid, on risk factors for cardiovascular disease” [SANDERSON *et al.*, 2002].

The effects of α LNA contrast with those of long chain *n*-3 PUFAs (see Sections 3 and 5) and it is apparent from studies where α LNA and long chain *n*-3 PUFAs have been compared that α LNA is substantially less potent [e.g. CAUGHEY *et al.*, 1996]. The reason why very high intakes of α LNA may induce qualitatively similar effects to those of long chain *n*-3 PUFAs (e.g. decreasing TAG concentration, platelet aggregation and inflammation) while lower intakes do not, may be that the high intakes allow sufficient synthesis of EPA to occur to induce biologically effective changes in EPA concentration. This is borne out by the observations of ZHAO *et al.* [2004] that the α LNA-induced changes in inflammatory markers were significantly related to changes in serum concentrations of EPA or EPA plus DPA, but not of α LNA. Similar to this, CAUGHEY *et al.* [1996] related the effects of α LNA on inflammatory cytokine production to the increased content of EPA in mononuclear cells. Thus, the likely explanation for the lack of biological effect of more modest intakes of α LNA is that there has been insufficient conversion of α LNA to the more active EPA. From this point of view increased intake of α LNA offers little real advantage compared with increased intake of long chain *n*-3 PUFAs, and may even be disadvantageous if α LNA does increase blood TAG concentration. One clear advantage of increased α LNA intake is that it seems unlikely to make LDL more sensitive to oxidation, a process which can be promoted by long chain *n*-3 PUFAs. Once again, the fact that one study using a

very high intake of α LNA reported increased susceptibility of LDL to oxidation [NESTEL *et al.*, 1997] may reflect the fact that the LDL may have been significantly richer in EPA and DPA as a result of α LNA conversion.

6-3 Conclusions

α LNA is converted to EPA and DPA in humans. This process is promoted if linoleic acid intake is decreased. Stearidonic acid appears better converted than α LNA, indicating that poor $\Delta 6$ -desaturase activity plays a role in limiting α LNA conversion. Conversion of α LNA to DHA, is limited in humans although conversion is much greater in women than men. The limited extent to which α LNA is converted to its longer-chain metabolites may explain, at least in part, the relative lack of effectiveness of increased consumption of α LNA on risk factors for cardiovascular disease and on inflammatory markers. However α LNA at sufficiently high intakes can mimic the effects of long chain *n*-3 PUFAs, at least qualitatively, probably as a result of conversion to EPA. It is important to note that most studies have been conducted in men or in mixed groups of men and middle aged or older women, in whom conversion of α LNA to long chain *n*-3 PUFAs is not so good as it is in younger women. Therefore the effect of increased α LNA consumption, in combination with decreased linoleic acid consumption, on health-related outcomes in young women needs to be investigated.

The best strategy by which to increase the intake of long chain *n*-3 PUFAs and to increase the content of long chain *n*-3 PUFAs in blood, cells and tissues is to eat oily fish regularly (**Table 6.4**). An alternative is to consume fish oil capsules, which is a highly effective way of increasing long chain *n*-3 PUFA status in human blood and cells (**Figures 2.10 to 2.12**). It is apparent that foods either naturally enriched or fortified with long chain *n*-3 PUFAs will become increasingly available over the next 5 years and these will represent a very good strategy by which to increase intakes of these fatty acids, and will provide greater choice for those consumers who do not eat fish and who do not wish to take capsules. It is also clear that greatly increased intake of α LNA is a viable strategy to increase the EPA and DPA content of blood, cells and tissues, but this appears to be very poor strategy to increase DHA content, and may even decrease it. In this regard α LNA is greatly inferior to either fish or fish oil capsules. As might be expected conversion of α LNA to EPA is improved if linoleic acid intake is decreased in parallel with increased α LNA intake.

Table 6.4 — Comments on strategies to increase the status of long chain n-3 PUFAs in human plasma, cells and tissues

Metabolic strategy	Dietary strategy	Comments on the strategy
Provide pre-formed long chain n-3 PUFAs	<p>Eat oily fish</p> <p>Supplement diet with fish oil capsules</p> <p>Eat enriched or fortified foods</p>	<p>Excellent strategy but fish eating not popular and may not be sustainable</p> <p>Excellent strategy but may not be desirable</p> <p>Very good strategy but limited foods currently available; good future potential</p>
Provide precursor fatty acids (mainly α -linolenic acid)	<p>Use α-linolenic acid containing vegetable oils</p> <p>Supplement diet with flaxseeds</p> <p>Eat enriched or fortified foods</p>	<p>α-Linolenic acid containing oils are widespread</p> <p>May be a viable way to increase EPA (and DPA) status but requires quite high intake</p> <p>Does not increase DHA status (may even decrease it)</p> <p>Works better if linoleic acid intake is decreased at the same time</p> <p>Stearidonic acid is more effective than α-linolenic acid</p>

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OMEGA-3 FATTY ACIDS: THE GOOD OIL?

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OMEGA-3 FATTY ACIDS: THE GOOD OIL?

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OMEGA-3 FATTY ACIDS: THE GOOD OIL?

7 – Long chain *n*-3 fatty acids in artificial nutrition with an emphasis on immuno-inflammatory outcomes

7-1 Septic syndromes

153

The systemic inflammatory response syndrome is the name given to the uncontrolled inflammatory response to insult or injury involving excessive production of inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and IL-8 [BONE *et al.*, 1997]. Sepsis has been defined as “the systemic inflammatory response syndrome that occurs during infection” [BONE *et al.*, 1997]. Sepsis is the leading cause of death in critically ill patients in Western countries. Using records from 1995 for state hospitals in the United States it was estimated that there were over 750,000 cases of sepsis with a 28.6% mortality rate (215,000 deaths) and a total cost of almost US\$17 billion [ANGUS *et al.*, 2001]. Death from septic shock is the result of multiple organ failures and represents the extreme end of a continuum of events of increasing severity and decreasing likelihood of survival [FRIEDMAN *et al.*, 1998; BRUN-BUISSON, 2001] (**Figure 7.1**). The systemic inflammatory response syndrome (SIRS), sepsis and septic shock may together be termed as “septic syndromes”.

The involvement of inflammatory cytokines in septic syndromes has been long recognized and VERVLOET *et al.* [1998] wrote “these mediators (i.e. inflammatory cytokines) are largely, if not completely, responsible for the clinical signs and symptoms of the septic response to bacterial infection”. In support of this idea, patients with sepsis were found to have markedly elevated

STAGE	SYMPTOMS	MORTALITY RISK
Insult		
SIRS	2 or more of: Temperature $\geq 38^{\circ}\text{C}$ or $\leq 36^{\circ}\text{C}$ Heart rate ≥ 90 bpm Respiratory rate $\geq 20/\text{min}$ Leukocytes $\geq 12000/\mu\text{l}$ or $\leq 4000/\mu\text{l}$	10%
Sepsis	SIRS with assumed or certain infection	20%
Severe sepsis	Sepsis with ≥ 1 organ failure	20 – 40%
Septic shock	Metabolic acidosis Refractory hypotension	40 – 80%

Figure 7.1 — The progression to septic shock and the risk of mortality at the different stages.

bpm, beats per minute; SIRS, systemic inflammatory response syndrome. Reprinted from P.C. CALDER. *n-3 fatty acids, inflammation and immunity – relevance to postsurgical and critically ill patients. Lipids* 2004; 39: 1147-1161, with permission from the American Oil Chemists' Society.

circulating concentrations of TNF- α , TNF-receptor 1, IL-1 β , IL-1 receptor antagonist (IL-1ra) and IL-6, and those patients with the highest concentrations were more likely to die [GIRARDIN *et al.*, 1988; VERVLOET *et al.*, 1998; ARNALICH *et al.*, 2000; HATHERILL *et al.*, 2000]. In addition, circulating white cells from septic patients exhibit high levels of activated nuclear factor kappa B (NF κ B), a transcription factor that promotes the expression of numerous genes associated with inflammation (see **Figure 5.7**), and again levels of activated NF κ B were higher in those patients who went on to die [ARNALICH *et al.*, 2000]. Animal studies also support a role for inflammatory cytokines in the septic response. These studies have often used bacterial endotoxin (also called lipopolysaccharide; LPS) as a surrogate for infection, although endotoxin is a fragment of the Gram-negative bacterial cell wall and not a viable organism. Mice injected with endotoxin exhibit high circulating concentrations of TNF- α , IL-1 β , IL-6 [see SADEGHI *et al.*, 1999] and IL-8 and survival of these animals can be dramatically improved by administering anti-cytokine antibodies [BEUTLER *et al.*, 1985; TRACEY *et al.*, 1987], cytokine receptor antagonists [ALEXANDER *et al.*, 1991], or anti-

inflammatory cytokines such as IL-10 [MARCHANT *et al.*, 1994], or by knocking out the TNF- α receptor [PFEFFER *et al.*, 1993]. Despite this evidence, it is important to note that some studies report that many septic patients do not show detectable or elevated circulating concentrations of TNF- α or IL-1 β [see CALDER, 2004 for references]. Furthermore, it appears that inflammatory cytokines do play a beneficial role in sepsis. For example, in some animal models blocking TNF- α increases mortality [ESKANDARI *et al.*, 1992; OPAL *et al.*, 1996; ECHTENACHER *et al.*, 2001], while a TNF- α antagonist increased mortality in a clinical trial [FISHER *et al.*, 1996]. Thus, the situation regarding the pathological role of inflammatory cytokines in sepsis is unclear; it may be that a little is beneficial but that excess is harmful and that complete blocking negates the beneficial effects. Another consideration is that there may be large between-individual differences in the generation of inflammatory cytokines, in the sensitivity to the harmful effects of these cytokines and in the effects of blocking these cytokines. Thus, there may be significant variation in the susceptibility of individuals to exhibit the systemic inflammatory response syndrome and to progress towards septic shock. This may partly relate to the extent and site of the initial injury, partly to the nature and site of the infection, if any, and partly to aspects of the patient's wellbeing prior to receiving the injury (e.g. nutritional state). It is now recognized that genetics may also play a role. For example genotypes affecting TNF- α production appear to be of relevance with respect to sepsis mortality [STUBER *et al.*, 1996; MIRA *et al.*, 1999; KAHLKE *et al.*, 2004].

Although there has been much focus on the potential detrimental role of inflammatory cytokines in sepsis, other mediators including arachidonic acid-derived eicosanoids, reactive oxygen species, nitric oxide and adhesion molecules are involved in the pathological processes that accompany critical illness. Prostaglandin (PG) E₂ is implicated in sepsis, burns and critical illness [GRBIC *et al.*, 1991; Ertel *et al.*, 1992], while leukotriene (LT) B₄ and oxidants released by neutrophils are involved in acute respiratory distress syndrome [see KOLLEF, SCHUSTER, 1995].

In addition to hyperinflammation, patients with sepsis also display immunosuppression [MEAKINS *et al.*, 1977; LEDERER *et al.*, 1999; KAHLKE *et al.*, 2004]. There are reports that septic patients have high circulating concentrations of the anti-inflammatory cytokine IL-10 and that these are strongly correlated with mortality [O'SULLIVAN *et al.*, 1995; OPAL, DEPALO, 2000]. Note that this is contrary to the predicted effect of IL-10 since this cytokine down-regulates TNF- α production and its early administration is protective in murine endotoxemia [GERARD *et al.*, 1993; HOWARD *et al.*, 1993; SMITH *et al.*, 1994]. However, the apparently harmful effect of

IL-10 may relate to the timing of its production. Lymphocytes from patients with burns or trauma produce low levels of the T helper (Th) 1-type cytokines (e.g. interferon (IFN)- γ) associated with host defence against bacteria and viruses but high levels of the Th2- and Treg-type cytokines (IL-4, IL-10) associated with inhibition of host defence against bacteria and viruses [O'SULLIVAN *et al.*, 1995; LEDERER *et al.*, 1999]. There also appears to be decreased monocyte expression of human leukocyte antigens (HLA) [HERSHMAN *et al.*, 1990; WAKEFIELD *et al.*, 1993; ASTIZ *et al.*, 1996; MANJUCK *et al.*, 2000], the proteins involved in antigen presentation to T cells, and this is associated with impaired ability of monocytes to stimulate T cells [MANJUCK *et al.*, 2000]. Interestingly, IL-10 downregulates both Th1-type cytokine production and HLA expression [MUNOZ *et al.*, 1991; BRANDTZAEG *et al.*, 1996] and this might be the origin of the harmful effect of this cytokine in septic patients. Recent studies have revealed impaired proliferative or secretory functions of T cells from patients with sepsis, trauma or burns [HEIDECHE *et al.*, 1999; PELLEGRINI *et al.*, 2000].

156

The traditional view is that the immunosuppressed phase of septic syndromes lags behind the hyperinflammatory phase (**Figure 7.2**) i.e. initially sepsis is characterised by increased generation of inflammatory mediators (the systemic inflammatory response syndrome) but as it persists there is a shift towards an anti-inflammatory, immunosuppressed state sometimes called the compensatory anti-inflammatory response syndrome. However some recent

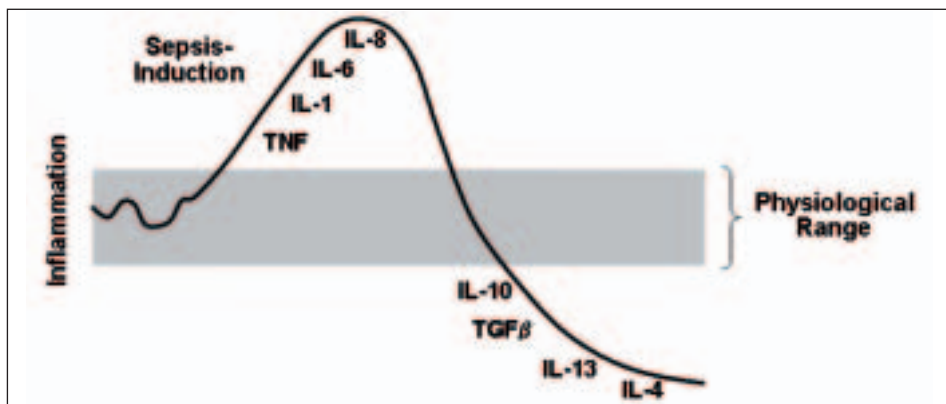


Figure 7.2 — Hypothetical biphasic immunoinflammatory response to a traumatic insult. IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor. Reprinted from P.C. CALDER. *n*-3 fatty acids, inflammation and immunity – relevance to postsurgical and critically ill patients. *Lipids* 2004; 39: 1147-1161, with permission from the American Oil Chemists' Society.

studies challenge this and suggest that the hyperinflammatory and immunosuppressed states co-exist. Some authors report that immunosuppression is present at the onset of sepsis [HEIDECKE *et al.*, 1999; WEIGHARDT *et al.*, 2000; TSCHAIKOWSKY *et al.*, 2002], rather than being a later compensatory response. For example, TSCHAIKOWSKY *et al.* [2002] identified that significantly decreased monocyte expression of HLA-DR was evident at the onset of severe sepsis in post-surgical patients; in survivors there was some recovery of expression but in non-survivors there was a further decrease or even a permanent suppression of HLA-DR expression. These authors identified that the timing of the peak of the systemic inflammatory reaction (identified as the time of maximum C-reactive protein concentration) coincided with the timing of the lowest monocyte expression of HLA-DR. From this they concluded that decreases in monocyte HLA-DR expression occur simultaneously with “signs of hyperinflammation” and as early as the onset of severe sepsis [TSCHAIKOWSKY *et al.*, 2002].

Thus, it appears that immune cells and cytokines have both detrimental and protective roles in patients as they move through the stages of sepsis. However, the traditional view that hyperinflammation precedes immunosuppression, as shown in **Figure 7.2**, may be a simplification of the real situation and this increases the challenge to finding interventions that might benefit high risk patients.

7-2 Potential relevance of long chain *n*-3 fatty acids to immuno-inflammatory responses in post-surgical and critically ill patients

The anti-inflammatory actions of long chain *n*-3 fatty acids (Section 5; **Table 5.1**) may be of benefit in sepsis, particularly during the “early” hyperinflammatory phase. For example, studies using the isolated, perfused rabbit lung have identified contrasting effects of arachidonic acid- and EPA-derived eicosanoids on inflammatory responses. Infusion of *Escherichia coli* hemolysin caused hypertension mediated by thromboxane (TX) B₂ and increased vascular leakage mediated by 4-series LTs [GRIMMINGER *et al.*, 1997a]. Inclusion of arachidonic acid in the perfusate increased TXB₂ and 4-series LT generation,

Table 7.1 — Summary of the effects of dietary fish oil on responses to endotoxin in experimental animals.

Response	Effect of fish oil
Fever	Decrease
Anorexia	Decrease
Weight loss	Decrease
Acidosis	Decrease
Hypotension	Decrease
Impaired heart function	Improve
Impaired lung function	Improve
Lung oedema	Decrease
Circulating inflammatory mediators	Decrease
Impaired bacterial killing	Improve
Death	Decrease

arterial pressure and vascular leakage [GRIMMINGER *et al.*, 1997a,b]. In contrast, inclusion of EPA in the perfusate decreased TXB₂ and 4-series LT generation, decreased arterial pressure and vascular leakage and increased generation of TXB₃ and 5-series LTs [GRIMMINGER *et al.*, 1997a]. Perfusion with fish oil attenuated the hypertension induced by calcium ionophore [BREIL *et al.*, 1996]. Compared with soybean oil infusion, fish oil decreased the concentration of LTC₄ by 50% and increased the concentration of LTC₅ from barely detectable to very similar to that of LTC₄ [BREIL *et al.*, 1996]. A number of benefits of fish oil in animal models of experimental endotoxemia have also been demonstrated, including diminished metabolic perturbations [POMPOSELLI *et al.*, 1990, 1991; TEO *et al.*, 1991], improved heart and lung function and decreased lung oedema [MURRAY *et al.*, 1993, 1995, 2000; MANCUSO *et al.*, 1997a,b; SANE *et al.*, 2000] (**Table 7.1**). These metabolic improvements and maintenance of organ function may relate to the reported decreased circulating concentrations of inflammatory eicosanoids (PGE₂, TXB₂, 6-keto-PGF_{1α}) [UTSUNOMIYA *et al.*, 1994; SANE *et al.*, 2000] and cytokines [SADEGHI *et al.*, 1999]. The real evidence of benefit comes from mortality studies that show that dietary fish oil or fish oil infused intravenously significantly enhances survival of guinea pigs to intraperitoneal endotoxin compared with safflower oil [MASCIOLO *et al.*, 1988, 1989] (**Figure 7.3**).

In addition to effects on production of inflammatory eicosanoids and inflammatory cytokines, long chain *n*-3 PUFAs also exert effects on cell-mediated immunity [see CALDER, 2001; CALDER *et al.*, 2002 for reviews]. Large amounts of fish oil in the diet of laboratory animals have been reported to exert immunosuppressive effects [e.g. YAQOUB *et al.*, 1994a,b; SANDERSON *et al.*, 1995, 1997]. Clearly such effects

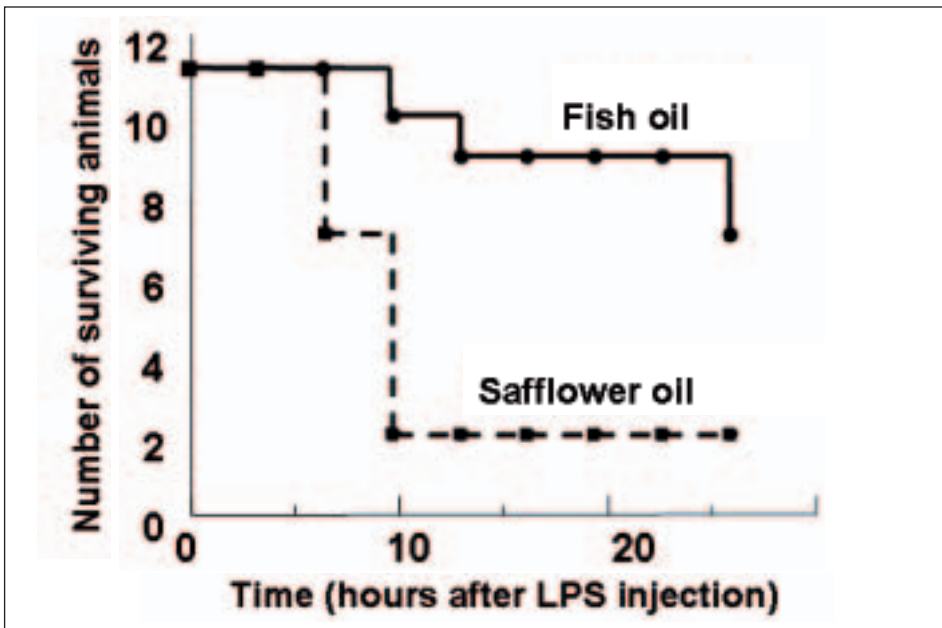


Figure 7.3 — Effect of intravenous fish oil on survival to endotoxin in guinea pigs.

Male Hartley guinea pigs received infusions of either safflower oil or fish oil (10% vol) for two days. Then endotoxin (0.35 mg/100 g body weight) was given intraperitoneally. Survival was monitored over 4 days.

Data are redrawn from E.A. MASCIOLI, L. LEADER, E. FLORES, S. TRIMBO, B. BISTRAN, G. BLACKBURN. Enhanced survival to endotoxin in guinea pigs fed fish oil emulsion, *Lipids* 1988; 23: 623-625, with permission from the American Oil Chemists' Society.

are to be avoided in patients with, or at risk of, sepsis. However it is the effects of lower amounts of long chain *n*-3 fatty acids that are of relevance to that setting and these effects appear to be limited. In fact a recent study reported that enhanced T cell responses (proliferation and IFN- γ production) occur at modest doses so long as antioxidants are also given [TREBBLE *et al.*, 2003]. In terms of sepsis, the true test of immunocompetence occurs when live pathogens are administered. This is a different situation from using endotoxin which is not living and which therefore does not require a robust cell-mediated immune response to eliminate it. As indicated above, it is clear that long chain *n*-3 fatty acids protect against the deleterious effects of endotoxin. The same appears to be true for many live pathogens. Infusion of fish oil into rats also receiving low-dose endotoxin decreased the number of viable bacteria in mesenteric lymph nodes and liver [PSCHIEDL *et al.*,

2000]. Fish oil did not decrease bacterial translocation across the gut and so the authors concluded that fish oil must have improved bacterial killing. Compared with linoleic acid-rich vegetable oils, fish oil fed to rats prior to exposure to live bacteria [BARTON *et al.*, 1991; RAYON *et al.*, 1997] resulted in increased survival, which was associated with decreased production of PGE₂. More recently infusion of fish oil after induction of sepsis by cecal ligation and puncture decreased mortality (and PGE₂ production) compared with vegetable oil [LANZA-JACOBY *et al.*, 2001]. Intragastric administration of fish oil into chow-fed rats prior to cecal ligation and puncture improved survival compared with saline or vegetable oil infusion [JOHNSON *et al.*, 1993]. Thus, the picture that emerges from a range of animal studies is that administration of long chain *n*-3 fatty acids in the form of fish oil increases survival upon exposure to live pathogens. From this it can be inferred that host immune defences are likely to have been improved by long chain *n*-3 fatty acids. Interestingly several studies have focussed upon the fish oil-induced decrease in PGE₂ production as being a key mechanistic effect, suggesting that the immunosuppressive effect of PGE₂ generated in response to infection might be deleterious to host survival.

7-3 Studies of long chain *n*-3 fatty acids in surgical patients

7-3-1 Introductory comments

Surgery is typically accompanied by an inflammatory response that may be exaggerated in some patients, especially if the surgery is major. If the patient is exposed to pathogenic organisms and is unable to cope with these, then sepsis may develop. Artificial nutrition is frequently used post-surgery and this may involve parenteral (i.e. intravenous) infusions, especially where the gastrointestinal tract is not fully functional (e.g. post abdominal surgery). Lipids are included in parenteral nutrition to provide an alternative source of calories to glucose and the lipid source used most frequently has been soybean oil, which is rich in the *n*-6 fatty acid linoleic acid, although it also contains a proportion of α -linolenic acid. A meta-analysis of total parenteral nutrition suggested that inclusion of lipids might be detrimental ($P = 0.09$ for lipids vs. no lipids) [HEYLAND *et al.*, 1998], at least in very ill patients. It is not clear why this is, although a number of *in vitro* experiments have shown that soybean oil-based lipid emulsions can exert immunosuppressive effects [see CALDER *et al.*, 1994 for references], which would clearly be detrimental in patients at risk of infection and sepsis. Clinical trials provide conflicting evidence some

showing some immunosuppressive effects [BATTISTELLA *et al.*, 1997; FURUKAWA *et al.*, 2002] and others not [GOGOS *et al.*, 1990; SEDMAN *et al.*, 1991; LENSSEN *et al.*, 1998], at least in some patient groups. The concern about potential harm, the view of sepsis as a hyperinflammatory state followed by an immunosuppressed state (Figure 7.2), and the idea that *n*-6 fatty acids might be “pro-inflammatory and immunosuppressive” has led to the development of alternative lipid emulsions for parenteral applications. Emulsions using a mix of medium-chain triglycerides and soybean oil or based upon olive oil instead of soybean oil have been developed, but these will not be discussed here. However, of relevance to the present discussion is the development of emulsions that include fish oil as a partial replacement for soybean oil. Several such emulsions have been tested in surgical patients.

7-3-2 Parenteral *n*-3 fatty acids

Intravenous infusion of a lipid emulsion containing fish oil for 5 days into patients who had undergone major abdominal surgery resulted in much higher LTC₅ production by blood leukocytes stimulated *ex vivo* at 6 days postoperation [MORLION *et al.*, 1996]. In another study, patients who had undergone abdominal surgery received soybean oil or a mix of medium-chain triglycerides, soybean oil and fish oil (50:40:10 vol:vol:vol) for 5 days post-surgery [KOLLER *et al.*, 2003]. Leukocytes from these patients produced more LTB₅ and LTB₅-isomers at postoperative days 6 and 8. Patients who had undergone major gastrointestinal surgery received a medium-chain triglyceride-soybean oil mix (50:50 vol:vol) or a mix of medium-chain triglycerides, soybean oil and fish oil (50:30:20 vol:vol:vol) for 5 days post-surgery [WACHTLER *et al.*, 1997]. Patients receiving fish oil got 3 g (days 1 and 2) and 6 g (days 3, 4 and 5) long chain *n*-3 fatty acids per day. Neutrophils from these patients produced less LTB₄ and more LTB₅ at postoperative days 6 and 10. Plasma TNF- α concentrations were lower in the fish oil group at day 6, while plasma IL-6 concentrations were lower at day 10. The study did not report clinical outcomes. A more recent study infused a fish oil-rich formula on the day before abdominal surgery and on days 1 to 5 following abdominal surgery [WEISS *et al.*, 2002]. On days 4 and 5 the patients also received standard total parenteral nutrition which included 50 g fat/day. TNF- α production by endotoxin-stimulated whole blood tended to be lower at postoperative day 5 in the fish oil group, but this was not significant. Serum IL-6 concentrations were significantly lower at days 0, 1 and 3 in the fish oil group. Monocyte expression of HLA-DR was preserved in the fish oil group but declined at postsurgery days 3 and 5 in the control group. No differences in infection rates or mortality were observed. However postoperative stay in intensive care tended to be shorter in the fish oil group (4.1 vs. 9.1 days) as did total hospital

stay (17.8 vs. 23.5 days), although neither of these was a significant effect. Postoperative stay on medical wards was significantly shorter in the fish oil group. Another recent study compared the effects of lipid-free total parenteral nutrition or parenteral nutrition including 10% soybean oil or 8.3% soybean oil plus 1.7% fish oil for 5 days after large bowel surgery [SCHAUDER *et al.*, 2002]. There were no differences between the groups with respect to the numbers of circulating lymphocytes, B cells, CD4⁺ cells, CD8⁺ cells or natural killer cells before surgery or at days 3 and 6 postsurgery, although these were affected by surgery itself. There were no differences between groups with respect to T lymphocyte proliferation, but IL-2 production was increased in the fish oil group and the postsurgery decline in IFN- γ production was prevented by fish oil. These studies indicate that inclusion of fish oil in parenteral nutrition regimens for gastrointestinal surgical patients modulates generation of inflammatory eicosanoids [MORLION *et al.*, 1996; WACHTLER *et al.*, 1997; KOLLER *et al.*, 2003] and cytokines [WACHTLER *et al.*, 1997; WEISS *et al.*, 2002] and may help to counter the surgery-induced declines in antigen presenting cell activity [WEISS *et al.*, 2002] and T cell cytokine production [SCHAUDER *et al.*, 2002]. Importantly these studies do not reveal deleterious immunologic effects of fish oil infusion in these patients. Furthermore, the only one of these fairly small studies to have examined hard endpoints like length of hospital stay suggests some clinical benefit from fish oil infusion in these patients [WEISS *et al.*, 2002]. However, larger studies are required to evaluate the effects of this approach on complication rates, hospital stay and mortality. A very recent report from a larger cohort of patients receiving parenteral nutrition post-surgery does indicate benefit of inclusion of fish oil in the regimen [TSEKOS *et al.*, 2004]. Patients received fish oil post-operatively and controls received a 50:50 medium-chain triglyceride-soybean oil mix. There were no differences between the two groups with respect to the proportions of patients who died or developed wound infections or with respect to length of hospital stay. However the proportion of patients who were readmitted to intensive care was significantly lower in the fish oil than in the control group. A group of patients also received the fish oil containing emulsion for two days preoperatively. Here there were a number of significant benefits, including decreased need for mechanical ventilation, a shorter length of hospital stay, less need for readmission to intensive care and lower mortality. This study demonstrates a benefit from the inclusion of long chain *n*-3 fatty acids in parenteral nutrition regimens used in abdominal surgery patients. However it also demonstrates a much greater benefit if the fatty acids are additionally provided pre-surgery, which, of course, is only possible in elective surgery. The greater benefit of preoperative infusion of long chain *n*-3 fatty acids may relate to better incorporation of the fatty acids into leukocytes and other tissues.

7-3-3 Enteral *n*-3 fatty acids

Enteral nutrition is an alternative form of artificial nutrition. It describes provision of nutrients directly into the gastrointestinal tract *via* a tube and is sometimes referred to as “tube feeding”. Enteral nutrition is used in patients with a functional gastrointestinal tract and is considered preferential to parenteral nutrition. The influence of enteral feeds including long chain *n*-3 fatty acids in their composition has been examined in surgical patients, generally in those who have undergone surgery to remove cancerous regions of the intestine. These studies have frequently used an enteral formula named Impact[®] which contains arginine, long chain *n*-3 fatty acids, and nucleotides, each of which is lacking from control formulas. Thus, any effects observed cannot be ascribed to a particular component of Impact[®]. The effects of Impact[®] on immuno-inflammatory outcomes in surgical patients have been widely examined [DALY *et al.*, 1992; 1995; KEMEN *et al.*, 1995; SENKAL *et al.*, 1995; SCHILLING *et al.*, 1996; BRAGA *et al.*, 1996; 1999; GIANOTTI *et al.*, 1999]. Overall it appears that Impact[®] exerts anti-inflammatory and immune enhancing effects in surgical (or at least gastrointestinal surgical) patients. Several of these studies report significantly improved clinical outcomes related to lower infection rate [DALY *et al.*, 1992; 1995; BRAGA *et al.*, 1996; 1999] and decreased length of hospital stay [DALY *et al.*, 1995; BRAGA *et al.*, 1996; 1999]. Studies of Impact[®] and similar enteral formulas investigating clinical outcomes in post-surgical patients have been subject to meta-analyses [HEYS *et al.*, 1999; BEALE *et al.*, 1999; HEYLAND *et al.*, 2001], which conclude that this approach to enteral nutrition significantly decreases infectious complications and length of hospital stay in elective surgery patients. It is possible that the modulation of inflammation and the improvements in immune function reported in these patients receiving Impact[®] contribute to the improved clinical outcomes. However, it is not possible to ascribe these benefits to long chain *n*-3 fatty acids.

7-4 Studies of long chain *n*-3 fatty acids in critically ill patients

Critically ill patients frequently require artificial support depending upon the extent of organ damage or failure and this will include nutritional support. The influence of enteral feeds including long chain *n*-3 fatty acids has been examined in critically ill patients; again many of these studies have involved Impact[®]. A study in intensive care unit patients (a mix of trauma,

sepsis and major surgery patients) reported that Impact® resulted in higher T cell proliferation at days 3 and 7 [CERRA *et al.*, 1990], while a study of severe trauma patients reported greater HLA-DR expression at day 7 [WEIMANN *et al.*, 1998]. These studies did not report improvements in clinical outcomes. However, other such studies have reported on clinical outcomes and have been subject to meta-analysis [HEYS *et al.*, 1999; BEALE *et al.*, 1999; HEYLAND *et al.*, 2001]. The most recent of these concluded that this approach to enteral nutrition decreases length of hospital stay but has no effect on infectious complications or mortality in critically ill patients [HEYLAND *et al.*, 2001].

A trial performed in patients with moderate and severe acute respiratory distress syndrome used an enteral preparation that differed mainly in lipid source from the control [GADEK *et al.*, 1999]. The control group of patients received a formula where the lipid source was 97% corn oil plus 3% soy lecithin. The experimental group received a lipid source that was 32% canola oil, 25% medium-chain triglycerides, 20% borage oil, 20% fish oil and 3% soy lecithin. The experimental formula also contained more vitamin C and vitamin E than the control and it contained β -carotene, taurine and carnitine, which the control formula did not. Patients receiving the experimental formula got about 7 g EPA, 3 g DHA, 6 g γ -linolenic acid, 1.1 g vitamin C, 400 IU vitamin E and 6.6 mg β -carotene per day for 6 days. By 4 days the numbers of total leukocytes and of neutrophils in the alveolar fluid declined significantly in the experimental group and were lower than in the control group. Arterial oxygenation and gas exchange were improved in the experimental group. Clinical outcomes from the study are shown in **Table 7.2**. It is clear that there was substantial clinical improvement with a trend towards decreased mortality with the *n*-3 PUFA containing formula. More recently, new data from this study have become available [PACHT *et al.*, 2003]. Patients receiving the experimental formula had significantly lower concentrations of IL-8 in their alveolar fluid and tended to have lower concentrations of LTB₄ and TNF- α . It is possible that the lower concentrations of LTB₄ and IL-8, both of which are potent leukocyte chemoattractants, may have been responsible for the lower neutrophil infiltration reported in the experimental group, and indeed neutrophil counts were significantly associated with these concentrations. This study establishes that the experimental treatment decreases production of inflammatory mediators and infiltration of inflammatory leukocytes and that this can result in significant clinical improvement in extremely ill patients. Because of the many differences in composition between the experimental and control formulas used it is not possible to ascribe the effects and benefits to any particular nutrient. However the effects upon LTB₄, IL-8 and TNF- α concentrations are consistent with effects of long chain *n*-3 fatty acids reported elsewhere (see Section 5).

Table 7.2 — Effect of an enteral formula containing long chain *n*-3 PUFAs on clinical outcomes in adults with acute respiratory distress syndrome.

Outcome	Control formula	<i>n</i> -3 PUFA containing formula
Number of patients evaluated	47	51
Time on supplemental oxygen (days)	20.2	15.8
Time on ventilator (days)	16.3	11.0
Length of ICU stay (days)	17.5	12.8
Length of hospital stay (days)	34.6	29.4
New organ failures (%)	28	8
Mortality (%)	19	12

Adults with acute respiratory distress syndrome received a standard enteral formula or an experimental formula containing long chain *n*-3 PUFAs and a number of other ingredients (see text) for 4 to 7 days.

Data are from GADEK *et al.* [1997]. Abbreviations used: ICU, intensive care unit; PUFA, polyunsaturated fatty acid.

Recently data from studies using parenteral nutrition with fish oil in sepsis patients have become available. Patients received a standard soybean oil-based emulsion or an emulsion containing fish oil for 5 [MAYER *et al.*, 2003a] or 10 [MAYER *et al.*, 2003b] days. Blood leukocyte counts and serum C-reactive protein concentration tended to be lower and production of LTB₅ by stimulated neutrophils was significantly higher in patients receiving long chain *n*-3 fatty acids [MAYER *et al.*, 2003]. Production of TNF- α , IL-1 β , IL-6, IL-8 and IL-10 by endotoxin-stimulated mononuclear cells did not increase during infusion of the fish oil containing emulsion whereas production of the four pro-inflammatory cytokines was markedly elevated during the first two days of soybean oil infusion [MAYER *et al.*, 2003a]. These studies establish that infusion of long chain *n*-3 fatty acids into patients with sepsis can modulate inflammatory mediator production and related inflammatory processes. However, the impact of this on hard clinical outcomes in these patients is not yet clear.

7-5 Conclusions

Because long chain *n*-3 PUFAs are potentially useful anti-inflammatory agents they may be of benefit in patients at risk of developing sepsis. An emerging application of *n*-3 PUFAs is in surgical or critically ill patients where they may be added to parenteral or enteral formulas. There appear to be no

adverse effects of inclusion of long chain *n*-3 PUFAs in such formulas. Indeed, parenteral or enteral nutrition including *n*-3 PUFAs preserves immune function better than standard formulas and partly prevents some aspects of the inflammatory response. Studies to date are suggestive of clinical benefits from these approaches, especially in post-surgical patients. However, more and bigger studies are required before the clinical benefit of *n*-3 PUFAs in these settings can be accepted with certainty. The contribution that *n*-3 PUFAs make to the benefits of enteral nutrition will remain unclear because experimental formulas have several important differences from control formulas.

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