The Role Of n-3 Fatty Acids

In Cardiometabolic Risk

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A thesis submitted in partial fulfilment of

the requirements of Liverpool John Moores University

for the Degree of Doctor of Philosophy

September 2014

To Peter Jones (‘Doctor Pete’),
who encouraged me to undertake this project,
and promised to check my spellings,
but sadly did not live long enough to do so.

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ABSTRACT

The work described in this thesis addresses two questions relating to benefits of dietary omega-3 fatty acids (n-3 LCPUFA). Accretion of research knowledge indicates that adequate n-3 LCPUFA intakes may be instrumental in maintaining good health throughout life, including in the optimisation of cardiovascular health. Sources of n-3 LCPUFA traditionally include fish oils but concern regarding long-term sustainability of fish stocks has led to investigation of alternative sources. Krill oil, obtained from the crustacean *Euphausia superba*, contains n-3 fatty acids as phospholipids and triglycerides, astaxanthin and related carotenoid pigments, and has been proposed as a more effective alternative than triglyceride oils.

Some foods rich in n-3-LCPUFA, including prawns, also contain cholesterol; concern regarding possible adverse health effects of such dietary cholesterol has led to public health advice to limit intake of these potentially beneficial foodstuffs.

These questions are addressed in two food intake studies in male volunteers by monitoring markers of cardiovascular health, food intake and biochemical markers of compliance. Firstly, prawns and a white fish control were compared for effects on lipoprotein profiles and other markers. Dietary supplementation with 225 g prawns daily for 4 weeks was associated with a decrease in ApoB of 7.8mg/dL compared an increase (+2.4 mg/dL) for the white fish control. Participants with BMI > 25 kg/m^2 also showed a reduction in plasma TAG (0.17 mmol/L) compared with baseline following prawn intake; those receiving the control showed an increase in plasma TAG (0.30 mmol/L), a decrease in HDL-C, and increases in VLDL-C and sdLDL-C. No overall adverse effect was found for prawns compared with processed white fish.

Secondly, effects were compared of fish oil and krill oil on cardiometabolic profiles of a cohort of men with metabolic syndrome, using anthropometric measurements, biochemical markers of cardiovascular health and assessments of food intake. Krill oil and fish oil at the doses used showed no overall difference in effect on MetS markers after 6 weeks; correlations between magnitude of reduction and baseline measurement were observed in both groups for TAG, fasting glucose, NEFA and sdLDL after 3 weeks on both supplements, and for glucose and cholesterol after 6 weeks of fish oil.

Comparison of analytical methods was also undertaken during both studies; use of a point-of-care system using capillary blood correlated well with plasma biochemistry using venous blood, while the Hirano method for sdLDL measurement correlated favourably with more labour-intensive centrifugation techniques. Limitations of the methods used are discussed, and proposals put forward for future work, including improving compliance through the use of newly emerging technologies such as metabolomics.

ACKNOWLEDGEMENTS

My principal and heartfelt thanks go to my supervisor, Ian Davies, who kindly gave me the opportunity to take on this project, have stood by me and has been no less than a tower of strength through some very challenging personal situations.

I would like to thank others who have assisted with the supervision of the work described in this thesis; in particular Leo Stevenson, Allan Hackett and Julie Abayomi. I am grateful too to Gordon Lowe for advice and facilitating the use of space and equipment in his laboratory at Byrom Street, and to Deborah Scott who helped with the insulin assay and with coordinating participants at LJMU.

Part of the work described here was carried out in collaboration with Professor Bruce Griffin and colleagues at the University of Surrey, and my thanks go to them for their assistance. Fish oil capsules used in the study described in Chapter 4 were provided by Efamol, and I am grateful to my long-time associate Peter Clough for facilitating this. Krill oil and partial funding towards this study was provided by Azantis (USA), and I would like to thank Dr Michael Schmidt for his assistance in this and for useful and constructive debate about the study design and results. Professor Seppo Salminen (University of Turku and European Food Safety Agency) has also furnished lively and constructive debate on several of the issues discussed here.

Dr John Lodge at the University of Northumbria, Professor Rick Dunn of Warwick University, and Professor Roy Goodacre and his team at Manchester University have provided helpful advice regarding the potential use of metabolomics as a tool for the future.

Professor Stan van den Berg (University of Liverpool) has helped with advice and useful discussion on a range of topics.

Finally I would like to thank members of my family and circle of friends who, like Ian, have provided support and encouragement during this project.

ABBREVIATIONS

ALA	α-linolenic acid
ArA	Arachidonic acid
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
BMI	Body Mass Index
CHD	Coronary heart disease
COX	Cyclooxygenase
CVD	Cardiovascular disease
DGUC	Density gradient ultracentrifugation
DHA	Docosahexaenoic acid
DHAP	Dihydroxyacetone phosphate
DPA	Docosopentaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FO	Fish Oil
HDL	High density lipoprotein
HDL-C	HDL-cholesterol
IDL	Intermediate density lipoprotein
IL	Interleukin
IR	Insulin resistance
KO	Krill oil
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acid
LDL	Low density lipoprotein
LDL-C	LDL-cholesterol
LOX	lipoxygenase
LT	Leukotriene
MetS	Metabolic syndrome
MI	Myocardial infarction
n-3	Omega-3
n-6	Omega-6
NEFA	Non-esterified fatty acid
PC	Phosphatidylcholine
PG	Prostaglandin
PL	Phospholipid
RCF	Relative centrifugal force
sdLDL	small dense low density lipoprotein
SFA	Saturated fatty acid
T2D	Type 2 diabetes
TAG	Triacylglycerol
TC	Total cholesterol
TFA	Trans fatty acid
TNF	Tumour necrosis factor
TOOS	N-ethyl-N-sulphohydroxypropyl-m-toluidine
VLDL	Very low density lipoprotein

CHAPTER 1

Introduction and Background

1. 1. LONG-CHAIN OMEGA-3 FATTY ACIDS

1.1.1. Nomenclature

The omega-3 (n-3) series of fatty acids (FA) are synthesised by single-celled organisms and marine organisms such as shrimp, which provide the major source of these FAs to fish and mammals further up the food chain (Calder, 2004). They are long-chain carboxylic acids characterised by repeated *cis*-carbon-carbon double bonds (-CH=CH-) separated by methylene ($-CH_2-$) groups. The structural formulae for eicosapentaenoic and docosohexaenoic acids (EPA and DHA) are shown in Figure 1.1.

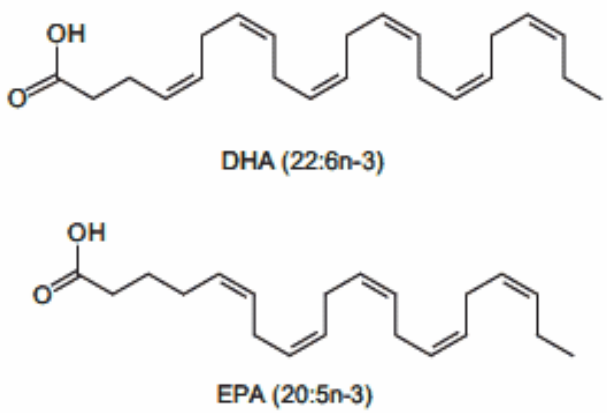

Figure 1.1. Structure of DHA and EPA (from Calder, 2004)

Together with arachidonic acid (ArA) (Figure 1.2), the major elongation product of the n-6 series of FAs, EPA and DHA have key roles in the structure and function of cell membranes and modulation of inflammatory processes. Some features are highly specific to either the n-3 or the n-6 family, and between individual FAs (Calder & Grimble, 2002; Calder, 2006a; 2006b; 2013; Maskrey *et al.*, 2013). In the nomenclature used here, the n-number refers to the first unsaturated carbon atom,

counting from the end furthest away from the carboxyl group: for EPA, 20:5 gives the length of the carbon backbone followed by the number of double bonds.

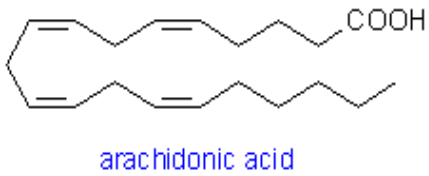

Figure 1.2. Structure of arachidonic acid (from Calder, 2004)

1.1.2. Biosynthesis

LCPUFAs of the n-3 and n-6 series are synthesised *in vivo* by elongation and desaturation of the essential FAs linoleic acid (LA) and α-linolenic acids (ALA) respectively, as shown in Figures 1.3 and 1.4 (Simopoulos, 1991; Calder, 2012a & 2012b; Calder & Yaqoob, 2012). Whereas marine organisms are capable of synthesising LA and ALA *in situ*, many mammals are not, and an adequate dietary supply of these FAs is therefore essential.

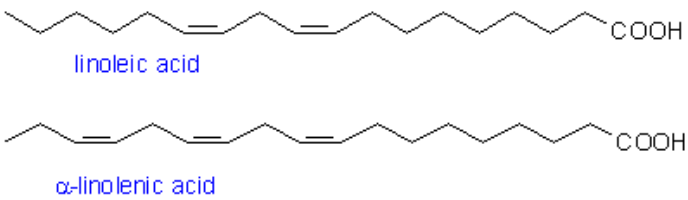

Figure 1.3. Structure of linoleic and α-linolenic acid *(from lipidlibrary.aocs.org)*

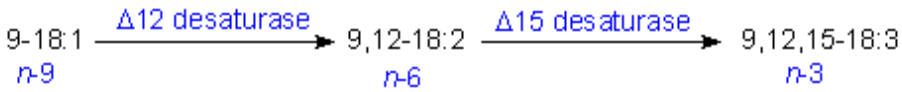

Figure 1.4. Biosynthesis of LA and ALA (from lipidlibrary.aocs.org)

The elongation and desaturation pathways are similar, but not identical, for the n-6 and n-3 series, as shown in Figure 1.5. This similarity may result in competitive inhibition if the components of one pathway are present in much larger concentration. In humans, the rate of conversion of ALA to EPA and DHA is about 10%, less efficient in males

than females (Calder, 2004; Burdge & Calder, 2005). ALA is utilised by the skin, and may be more rapidly oxidised than EPA and DHA, thus being less available for elongation in the liver (Burdge & Calder, 2005; Burdge, 2006; Brenna *et al*., 2009). This supports the view that the majority of these long-chain FAs should normally be furnished from dietary sources containing EPA and DHA such as meat, fish and eggs (Givens *et al*., 2006; Elwood *et al*., 2008).

Components of the n-3 series up to and including DHA, with information about occurrence and relative importance, are shown in Table 1.1.

1.1.3. Destination and catabolism of n-3 FAs

Dietary FAs are obtained in the form of phospholipids (PL) or triglycerides (triacylglycerols, TAG), which are hydrolysed in the gut to produce free or non-esterified FAs (NEFA). These enter intestinal cells via FA transport systems and are re-esterified to fatty acyl-Coenzyme A thioesters (catalysed by acyl-CoA synthetases); FA-CoAs form the substrate for tissue synthesis of neutral lipids (TAG and cholesterol esters) and polar lipids (PLs, sphingolipids and plasmalogens) (Kinsella *et al*., 1990). Re-esterified FAs are packaged into very low density lipoprotein particles (VLDL) in the liver or chylomicrons (in the gut) for transport (Jump, 2004, 2011: Jacome-Sosa & Parks, 2014).

LCPUFA are integral components of cell and tissue structures, and also essential precursors for signalling molecules controlling developmental, neurological, and inflammatory processes. These will be briefly summarised here with reference to human health and disease, with particular emphasis on cardiovascular disease (CVD) risk.

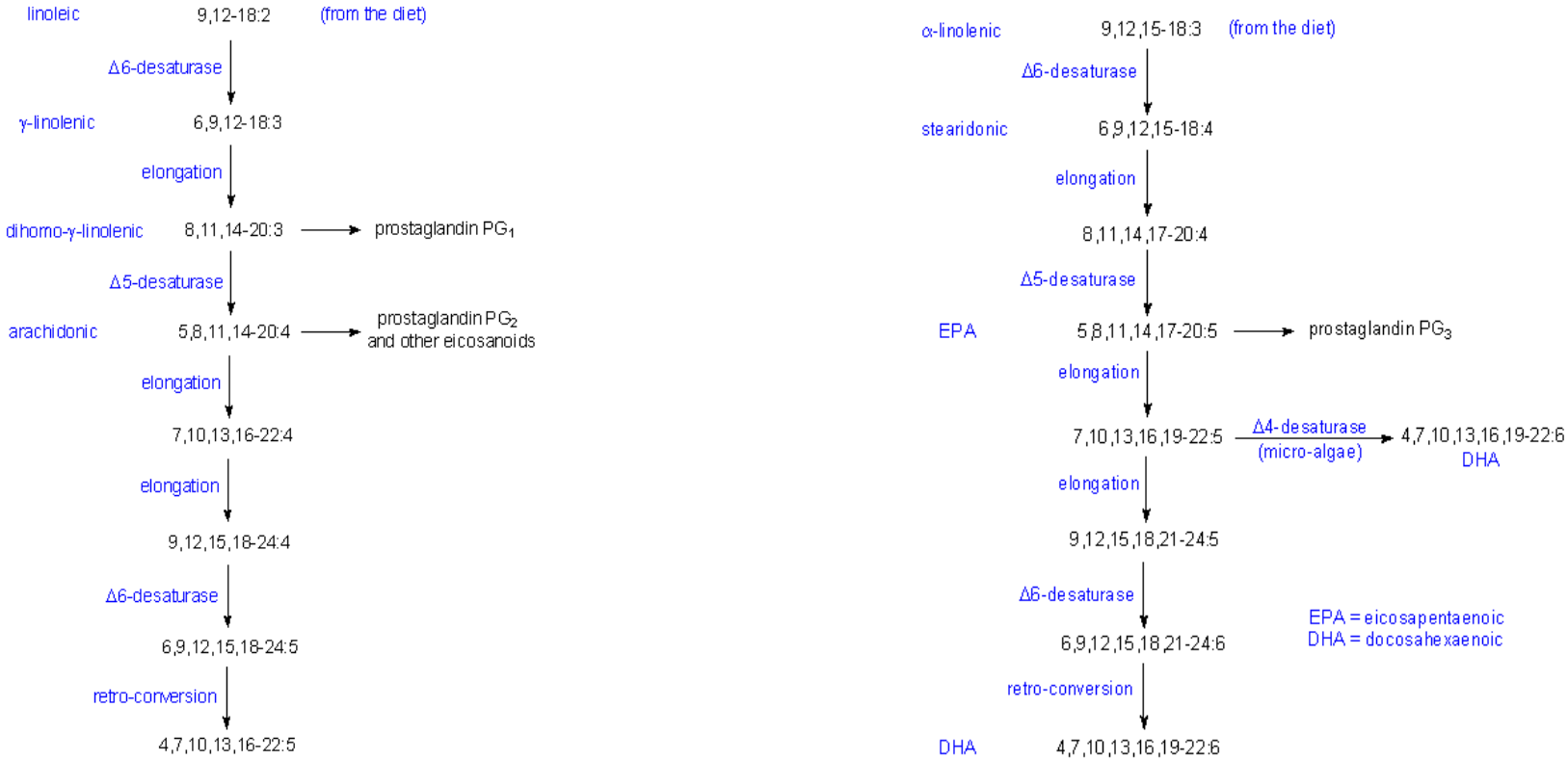

Figure 1.5: Elongation and desaturation pathways for n-6 and n-3 fatty acids *(from lipidlibrary.aocs.org)*

	Name	Occurrence	Major dietary source	Importance
18:3(n-3):	α-linolenic acid (ALA)	Algae & higher plants. Animal tissue lipids (<1%)	Linseed oil (65%), Soybean (7%), Rapeseed (7%)	Precursor of other n-3 fatty acids, also oxidised to shorter chain molecules.
20:3(n-3):	Eicosatrienoic acid	Animal tissue PLs (up to 1%), higher in fish oils.	No significant source	
18:4(n-3):	Stearidonic acid	Some plants, also algae and fish oils	Echium oil Blackcurrant seed oil	May be a more efficient source of n-3 LCPUFAs than α-linolenic acid
18:5(n-3):	Octadecapentaenoic acid	Dinoflagellates: enters marine food chain from this source	No significant source in human diet	
20:4(n-3):	Eicosatetraenoic acid	Most fish oils, animal PLs, algae & mosses. Rarely in higher plants.	No significant source	
20:5(n-3):	Eicosapentaenoic acid (EPA)	Occurs widely in fish oils and algae, Rarely if at all in higher plants.	Oily fish & marine organisms. Oils extracted from algae, fish & crustacea (e.g. krill)	Important component of PLs in animal tissues, especially brain. Precursor of PG3 prostaglandins & resolvins
22:5(n-3):	Docosapentaenoic acid	Important component of fish oils; animal PLs (2-5%)	Fish oil and PL	Possible source of EPA by interconversion; little evidence of interconversion with DHA
22:6(n-3):	Docosahexaenoic acid (DHA)	Fish oils & animal PLs (brain synapses and retina). Algae, especially marine	Oily fish & marine organisms. Oils extracted from algae, fish & crustacean (krill)	Correlated with improved cognitive & behavioural function in development, also with reduction in cardiovascular disease risk.

Table 1.1: Fatty acids of the n-3 series *(From: AOCS Lipid Library, 2013)*

Both ArA and EPA are precursors of leukotrienes and prostaglandins. The PG_2 series of prostaglandins and LT_4 leukotrienes derived from ArA are mainly pro-inflammatory in nature; they contribute to formation of blood clots and atheromas (plaques), resulting in increased blood viscosity, vasoconstriction, and reduced bleeding times (Serhan *et al*., 2008; Kris-Etherton *et al*., 2002; Rabkin *et al.,* 2013; Harris & Shearer, 2014). Those derived from EPA are PG_3 prostanoids and LT_5 leukotrienes (LTA_5, LTB_5 and LTC_5), which are anti-inflammatory or contribute to the resolution of inflammatory episodes. The relationship between arachidonate and eicosanoate–derived signalling molecules is shown in Figure 1.6.

The longer n-3 LCPUFA, DHA, is incorporated into PLs in cell membranes, mainly at the sn2 position; it is particularly important in neurological tissues, and imparts an increased level of fluidity to cell membranes compared with saturated or n-6 FAs.

1.1.4. The role of n-3 fatty acids in inflammatory processes

Inflammation protects an organism against pathogens, but is also a common feature of chronic diseases, including cardiovascular disease (CVD) (Sprecher & Chen, 1999; Tricon *et al*., 2004; Markovic *et al*., 2004). The n-6 LCPUFA ArA and n-3 LCPUFAs released from the sn-2 position of membrane PLs by the action of phospholipase, provide substrates for cycloxygenases (COX), lipoxygenases (LOX) and cytochrome P450 monoxygenases, resulting in production of prostanoids and thromboxanes. COX products modulate thrombotic, inflammatory and chemotactic responses, whereas LOX products play a role in vascular permeability, vasoconstriction and bronchoconstriction (Jump, 2002; Spite & Serhan, 2010; Stables & Gilroy, 2011). Inhibition of COX-1 and COX-2 has been a focus of anti-inflammatory drug development in recent years. The effect of aspirin in CVD prevention is attributed to inhibition of prostaglandin and prothrombotic thromboxane (TXA_2) generation by binding to COX-1. Statins, used in reduction of low-density lipoproteins in hyperlipidaemic patients, have also been reported to reduce inflammation *in vivo* (Spite & Serhan, 2010).

Anti-thrombotic and anti-inflammatory effects of n-3 LCPUFAs had been attributed to inhibition of pro-inflammatory cytokine synthesis. EPA appears to compete with ArA to bind to COX-1, but not to COX-2, reducing the production of ArA-derived prostanoids (Agarwal *et al*., 2009; Calder *et al*., 2009). More recently EPA and DHA have been shown to be precursors for the E and D series of resolvins, respectively (Stables &

Gilroy, 2011). The role of these molecules is to resolve the initial acute inflammatory response and effectively bring an episode of inflammation to a structured conclusion. They act on specific cell types to increase anti-inflammatory cytokine production and decrease pro-inflammatory cytokine production in macrophages.

Extensive reviews of n-3 PUFA in chronic disease (Sijben & Calder, 2007) and CVD (Lovegrove & Griffin, 2013) suggest that the effects of n-3 PUFA on inflammatory function are not dose-dependent, that local effects may be more significant than systemic effects, and that clinical endpoints may be more valuable measures of success than cytokine measurements. Key cytokines related to inflammation and atherosclerosis are listed in Tables 1.2 and 1.3.

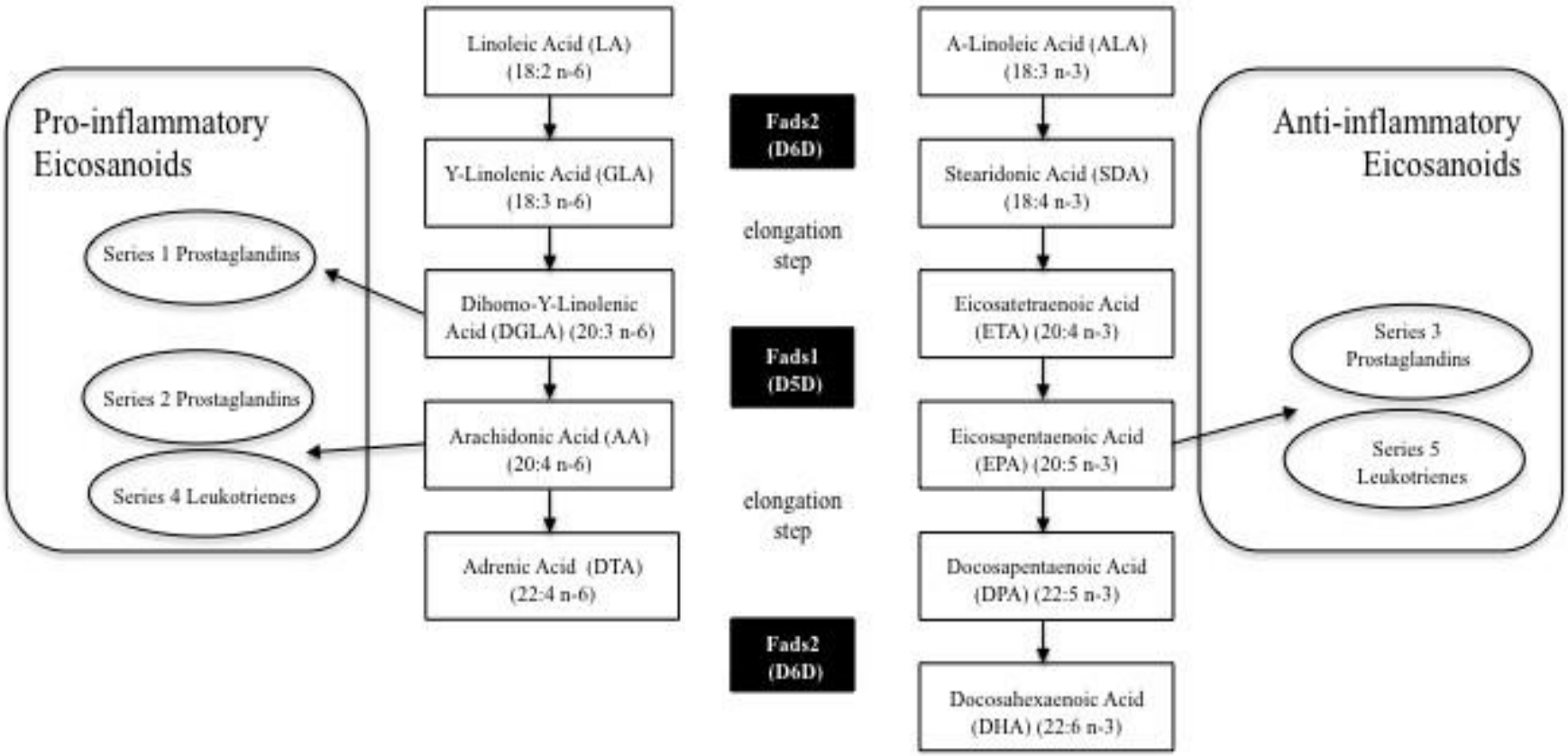

Figure 1.6: Derivation of signalling molecules from n-3 and n-6 LCPUFAs *(from Merino et al., 2010; lipidworld.com).*

Cytokine		**Site(s) of production**	**Role**	**Reference**
IL-1β (Interleukin - 1β)	17kDa	Monocytes and macrophages	Increases expression of adhesion molecules	Garlanda *et a*l., 2013
IL-6 (Interleukin-6)	2kDa	Lymphocytes, monocytes, fibroblasts, vascular smooth muscle cells (VSMC) & endothelial cells. Production of IL-6 is stimulated by angiotensin II. Expression in VSMCs may be upregulated by ANg II type 1 receptor (AT1R) and NF-κB.	Maintenance of inflammatory state: important local & circulating marker for inflammation in cardiovascular tissues. Stimulates: • expression of tissue factor, matrix degrading enzymes & LDL receptors in macrophages • aggregation of platelets • proliferation of VSMCs • Enhances expression of adhesion molecules & other cytokines (IL-1β & TNF-α in endothelial cells).	Scheller & Rose-John, 2006; Tanaka *et al*., 2012
IL-8 (Interleukin-8)	8.3kDa	Monocytes & macrophages, many tumour cells	Recruits neutrophils to the site of inflammation. Triggers adhesion of rolling monocytes to vascular endothelium, in the earliest step of atherosclerosis. Mitogen and chemoattractant factor for VSMCs. May reduce cholesterol efflux from macrophages.	Xie, 2001; Ito & Ikeda, 2003; Chen *et al*., 2014
TNF**α (**Tumour necrosis factor–**α)**	17kDa	Macrophages (foam cells), monocytes	Effects include increased body temperature, reduced appetite and stimulation of other cytokines.	Grimble, 1996; Baker *et al*., 2011; Zheng *et al*., 2013

Cytokine		Site(s) of production	Role	Reference
PDGF (Platelet-derived growth factor)	32-25kDa	Platelets, vascular cells, lymphocytes	Stimulates smooth muscle cell proliferation & has a role in cell migration into new muscle tissue following injury. Also thought to play a major role in the proliferation of atherosclerotic lesions.	Raines, 2004; Murray & Wynn, 2011
MCP-1 (Monocyte chemoattractant protein)	9-15kDa	Lymphocytes, monocytes	Thought to play an early role in the recruitment of monocytes to atherosclerotic lesions, & in development of intimal hyperplasia after injury. Induces the expression of tissue factor, superoxide anions and pro-inflammatory genes.	Ito & Ikeda, 2003; Charo & Taubman, 2004; Yadav *et al*., 2010
CRP (C-reactive protein)		Hepatic by-product of IL-6	Commonly used as a marker of systemic inflammation; with IL-6, raised levels are thought to indicate an increased risk of CVD.	Tsimikas *et al*., 2006; Kaptoge *et al*.,2012

Table 1.2. Key pro-inflammatory cytokines

Cytokine	Site(s) of production	Role	Reference
IL-10 (Interleukin-10)	Monocytes, macrophages and T cells	Produced by monocytes, macrophages & T cells during inflammatory processes to limit the release of pro-inflammatory mediators.	Ouchi *et al*., 2011; Jung & Choi, 2014
Adhesion molecules			
Endothelial adhesion molecules VCAM-1 ICAM-1 E-selectin	Endothelial cells	Facilitate recruitment and attachment of circulating leukocytes to the walls of blood vessels. Endothelial cell activation may lead to increased vasoconstriction, proliferation of smooth muscle, platelet aggregation, leukocyte adhesion, LDL oxidation, and MMP activation.	Zhang *et al*., 2011; Liao, 2013

Table 1.3. Key anti-inflammatory cytokines

1.2. THE IMPORTANCE OF n-3 FATTY ACIDS IN HUMAN HEALTH

Fish and marine produce have historically been major components of the diet of coastal populations. PUFA constitute approximately 20% of the dry weight of the human brain, and a third of FAs in the central nervous system (Bourre, 2004). The diet and exercise habits of much of the human population now, particularly in respect of fat intake and metabolism, are quite different from those that applied to our ancestors (Simopoulos, 2008); specifically, the proportion of n-3 FAs in the diet has fallen dramatically over the past century, especially in the western world. This is partly due to increased use of n-6 vegetable oils in processed foods, and changes in animal-rearing practices allowing greater access to animal protein and fat sources (Simopoulos, 2002a; 2003; 2011). A recent survey of FA intakes in 266 countries reported that despite some success of public health initiatives to reduce saturated fat intake, seafood n-3 consumption remains below 250 mg per day in nearly 80% of the world's population, with increases mainly in countries where consumption is already higher than average (Micha *et al.,* 2014). The n-6:n-3 ratio in the North American diet is estimated to be about 20:1, compared with a probable optimum of about 2:1 (Simopoulos, 2003; Wijendran & Hayes, 2004). The OPTILIP study in the UK (Griffin *et al*., 2006) reported a mean ratio of n-6:n-3 of approximately 8:1 in 258 men and women between 45 and 70 years of age. An increase in overall intake of n-3 LCPUFA and reduction in the dietary n-6:n-3 FA ratio are seen as desirable objectives in reducing the risk of many chronic diseases.

Children in Britain in the period following the 2nd world war were routinely fed cod liver oil supplements in primary school, primarily aimed at prevention of rickets (cod liver oil is rich in Vitamin D as well as n-3 LCPUFAs) (Rajakumar, 2003). The oil used was a by-product of fish protein (fillet) production, and as such was regarded of lower commercial importance; it would be susceptible to oxidation because of the high proportion of unsaturated FAs, and was in poor condition and unpleasant to consume (Kaitaranta, 1992). More recently, attention has focussed on the benefits of eating oily fish, and of the 'Mediterranean diet' for cardiovascular health, reduction of obesity and certain types of cancers (Estruch *et al*., 2013a; 2013b). This is typically described as rich in fruit, vegetables, legumes, nuts and olive oil, with moderate intakes of fish, dairy products and wine, and low consumption of meat, poultry and sweets; benefits are attributed to high levels of fibre and antioxidants, but also relatively low levels of saturated FAs and high levels of oleic acid, n-6 and n-3 FAs (Sofi *et al*., 2010, Trichopoulou *et al*., 2014).

There is a strong perception of the importance of LCPUFAs in maintaining fluidity of cell membranes and moderating inflammatory processes; dietary supplementation with n-3 LCPUFAs may directly affect gene expression (Calder, 2012b). Recent reports infer a direct association between intake of FO or DHA alone and regulation of several transcription factors related to TAG or lipoprotein metabolism, with increased expression of antioxidative enzymes and decreased expression of pro-oxidative enzymes (Dawson *et al*., 2012; Schmidt *et al*., 2012a; 2012b; 2012c). It has also been suggested, however, that high levels of LCPUFA in cell membranes may render them more susceptible to lipid peroxidation, leading to increased damage to intracellular components. Valencak and Ruf (2007) correlated PL membrane composition in 42 mammalian species with maximum lifespan and basal metabolic rate; they reported a decrease in lifespan with an increased n-6:n-3 ratio, but no specific correlation with membrane unsaturation or concentration of DHA rather than EPA. It was suggested that this refutes a direct link between high amounts of membrane PUFAs, elevated basal metabolic rate and hence impaired longevity. The role of n-3 PUFAs on membrane function may be partly via their effect on 'lipid rafts', cholesterol- and sphingolipid-rich regions within the exoplasmic leaflet of the plasma membrane, which selectively incorporate proteins (including cell-signalling proteins) and control protein-protein and protein-lipid interactions (Ikonen, 2001; Adkins & Kelley, 2010; Calder, 2013). Some of the evidence linking diet and lifestyle factors, cytokine production and inflammatory processes is summarised in Tables 1.4 and 1.5.

The balance between FOs and vegetable oils in the diet may be important in inflammation. Wada *et al*. (2007) compared the effects of vegetable oil, FO and combinations of both on prostanoid formation by cultured cells; prostanoids help control blood pressure, reduce incidence of allergies and modulate inflammation, but those derived from vegetable oils can also increase levels of pain, swelling and redness in some tissues. It was suggested that increasing PL EPA:ArA ratios (by increasing the relative proportion of FO to vegetable oil in the diet) may reduce series 2 prostanoids (PGD, PGE and PGF).

Cytokine	Factors increasing levels	Factors decreasing levels	Reference
IL-1β	Increased in response to strenuous exercise: not mitigated by previous ingestion of 3.6 g EPA plus DHA. This may demonstrate that IL-1β responds to the stronger stimulus of the exercise stress.	IL-1β levels reduced by between 61 and 90% in patients with rheumatoid arthritis taking oral doses of 2.7-5.8 g n-3 LCPUFA (fish oil) per day.	James *et al*., 2000; von Schacky & Harris, 2007
IL-6	Increased as a result of hyperglycaemia	Reduced (with TNF-alpha) following n-3 supplementation (ex vivo analysis). It is not clear whether this is dose-related.	Esposito *et al*., 2002; Trebble *et al*., 2003
PDGF	Increased in diabetes in granulation tissue in wounds associated with impaired vascularisation	DHA and EPA supplementation (7g per day) in volunteers resulted in reduction of mRNA coding for PDGF-A and –B.	Kaminski *et al*., 1993; Baumann *et al*., 1999; Galiano *et al*., 2004
MCP-1	Increases correlated with obesity and increased BMI	DHA and EPA supplementation (7g/day) reduced mRNA coding for MCP-1 in unstimulated mononuclear cells ex vivo by 40%. This effect of EPA/DHA may be linked to a decrease in activation of NF-κB.	Baumann *et al*., 1999; Kim *et al*., 2006
CRP	Increased levels associated with development of diabetes mellitus	An inverse relationship between EPA and DHA intake and levels of CRP was shown in an epidemiological study. Inverse relationship between plasma n-3 LCPUFA levels and CRP. Production reduced by statins.	Pradhan *et al*., 2001; Pischon *et al*., 2003; Mori & Beilin, 2004; Micallef *et al*., 2009; Spite & Serhan, 2010

Table 1.4 Factors affecting expression of pro-inflammatory cytokines and markers

Cytokine	Factors increasing levels	Factors decreasing	Reference
IL-10	Low levels of IL-10, (a potent anti-inflammatory cytokine) are associated with low levels of DHA	Daily administration of 3.4 g EPA & DHA to heart transplant patients resulted in a decrease in IL-10, although a supplementation study in healthy volunteers failed to show any change in IL-10 levels in stimulated or unstimulated mononuclear cells *ex vivo*.	Ferrucci *et al*., 2006; Hamer, 2007
Endothelial activation molecules			
VCAM-1 **ICAM-1** **E-selectin**	Production enhanced by pro-inflammatory cytokines TNF-α, IFN and IL-6	*In vitro* & animal studies have demonstrated reductions in plasma levels of sVCAM, sICAM and sE-selectin in the presence of n-3 FAs. *In vivo* results in humans have so far produced mixed results: this may be due to disparities in the precise conditions and doses used.	von Schacky, 2007; Zhang *et al.,* 2011
Lipoxins	Generated from ArA; production may be triggered by aspirin, production may be promoted by statins	May be inhibited by selective COX-2 inhibitors	Spite & Serhan, 2010
Resolvins	Generated from EPA or DHA by enzymatic modification: production may be triggered by aspirin	Counter-regulate excessive acute inflammation: stimulate molecular and cellular events that define resolution of inflammatory processes	
Protectins	Generated from DHA		
Maresins	Generated from DHA in macrophages		

Table 1.5 Factors influencing expression of cytokines with a role in reducing or resolving inflammatory processes

There is at present no internationally accepted recommendation for fat or cholesterol intake. A recent review (Aranceta & Perez-Rodrigo, 2012) included reports of recommendations for intake of n-3 and n-6 FAs, summarised in Table 1.6. To date there has been little formal recommendation regarding intake of individual PUFAs. Informal recommendations for n-3 intake in the UK range up to 1.4 g per day (British Nutrition Foundation, 2006) for adults, with a consensus that 0.2 g per day is a minimum health requirement (Calder, 2004). Specific recommendations for mitigation of cardiovascular risk are discussed later in this chapter.

	PUFA	**n-6**	**n-3**	**ALA**	**EPA**	**DHA**	**EPA +DHA**
FAO/WHO 2008	6-11% energy	2.5-9% energy	0.5-2% energy		None	None	0.25-2g/day
UK COMA 1991	6% energy	1% energy	none	0.2% energy	None	none	450mg/2 servings fish weekly
USA AHA 2006, 2009	5-10% energy	5-10% energy	none	None	None	None	0.5-1g/day or 2 portions of fatty fish a week
Australia/NZ	6-8% energy	13 g/day for males, 8 g/day for females	increase	Males 1.3g/day, Females 0.8 g/day	None	none	Males: 160mg/d, Females: 90mg/day
ISSFAL	2% energy	none	none	0.7% energy	None	200-300 mg/day	None

Table 1.6 Recommendations for n-3 PUFA and n-6 PUFA intake

The role of LCPUFA at all stages of life has been extensively reviewed. In relation to pregnancy and the potential effects on neonatal health, a recent meta-analysis of 15 randomised controlled trials (Imhoff-Kunsch *et al*., 2012) reported that n-3 LCPUFA supplementation in pregnancy is associated with a reduced rate of premature labour, but there is no clear association with birthweight or the immediate health of the neonate. The predominant emphasis of research into the nutritional effects of n-3 LCPUFA in infants and young children has been associated with issues of visual and

cognitive development or reduction of allergic disease (Ciccone *et al*., 2013). It has been suggested that n-3 LCPUFA could be an important factor in reducing cardiovascular risk factors in children and thus in the prevention of CVD later in life (Miles & Calder, 2013). Improvements in indices of CVD risk have been demonstrated in studies combining exercise and diet including n-3 LCPUFA; it has been suggested that the effects of n-3 LCPUFA and exercise could be synergistic (Carpentier *et al*., 2006; Hill *et al*., 2007).

Maintenance of good health into old age has been attributed to genetic as well as to lifestyle and dietary factors (Haveman-Nies *et al*., 2003). Degenerative diseases associated with ageing have come to prominence in Western society with the dramatic extension of life expectancy during the 20th century. An inflammatory component is recognised in the majority of these including CVD (Gustafson, 2010; Libby, 2012); n-3 FAs are believed to have a role in ameliorating susceptibility and progression across the spectrum (Simopoulos, 2008; Loef & Walach, 2013). The dramatic increase in the incidence of cardiometabolic disease and identification of risk factors has become an important public health issue in many countries.

1.3. CARDIOMETABOLIC DISEASE

Cardiometabolic disease describes the spectrum of conditions including obesity, metabolic syndrome (MetS), pre-diabetes, Type 2 diabetes (T2D) and cardiovascular disease (CVD) (Despres *et al*., 2008). The global epidemic of overweight and obesity has been identified as a major public health challenge, with estimates of 1.5 billion overweight and obese adults worldwide, and 68% of US adults and 31% of US children and adolescents overweight or obese in 2011 (Golub *et al*., 2011). Obesity has serious health consequences, including increased risk of T2D and CVD (Reaven, 2011). The mechanism of progression from obesity to dyslipidaemia, MetS and thence CVD has been extensively reviewed; risk factors and patterns of phenotype associated with increased CVD risk are now well understood.

1.3.1. Metabolic Syndrome

MetS is characterised by a number of strongly inter-related risk factors for CVD, including obesity, dyslipidaemia, insulin resistance (IR) and hypertension. IR occurs when adipose, liver and muscle cells do not respond appropriately to insulin, resulting

in a failure to process circulating glucose (Huang, 2009). Risk factors for development of MetS and CVD are evident from gestation onwards. Impaired foetal growth is associated with an increased risk of later metabolic disease, while accelerated growth in the postnatal period and ongoing increased energy intake result in reduced insulin sensitivity (Cottrell & Ozanne, 2008). Obesity in childhood is a risk factor for development of MetS, as shown in a systematic review of 378 studies published between 2003 and 2013 (Friend *et al*., 2013). Overall prevalence of MetS was reported at 3.3%, increasing in overweight and obese populations to 11.9% and 29.2% respectively. The criteria for diagnosis of MetS in children have been inconsistent, so that trends in prevalence over time were not clear; notwithstanding this, central obesity at the age of 9-10 years is a risk factor for MetS in early adulthood (Morrison *et al*., 2008). Incidence of MetS was approximately 3-fold higher in women with previous gestational diabetes than in a control group (Lauenborg *et al*., 2005), with obesity an additional risk factor.

Genetic factors and ethnicity contribute to a predisposition to MetS: a genome-wide association study of genetic determinants of lipid metabolism in over 17000 individuals (Waterworth *et al*., 2010) identified 4 novel loci associated with circulating lipids, including low and high density lipoprotein-cholesterol (LDL-C and HDL-C) and circulating TAG. MetS affects a higher proportion of the South Asian population (approximately 30% compared with approximately 20% for European or African-Caribbean groups) (Tillin *et al*., 2005). Low dietary intakes of n-3 LCPUFA may be a contributing factor: some intervention studies have resulted in strong recommendations for increased n-3 PUFA in the diet of South Asians in Britain (Lovegrove, 2007). Other lifestyle factors including smoking and physical inactivity are reported to increase the odds ratio of MetS (Park *et al*., 2003). The impact of diet on obesity and hence MetS is multifactorial and is addressed in Section 1.4.

Prevention and mitigation of MetS and sequelae is approached by medication and/or lifestyle changes. A series of recommendations for screening, diagnosis and management of the spectrum of cardiometabolic diseases has recently been published (Anderson *et al*., 2013; Ryden *et al*., 2014). An alternative and possibly controversial view (Kraushaar & Kramer, 2009) is that widespread screening is inefficient and costly in acute care consequences, and that encouraging engagement in population-wide lifestyle and diet modification programmes would be a preferable and more affordable public health strategy. The effectiveness of lifestyle interventions has been positively reported (reviewed in the ECS/EASD taskforce and others).

1.3.1.1. Definition of metabolic syndrome (MetS)

The definition of MetS arose from the recognition of a cluster of risk factors for T2D and CVD. Laws and Reaven (1993) initially studied a small group of non-diabetic, overweight, sedentary men and observed that although there was little difference in BMI, waist/hip ratio or physical endurance capacity, those with higher steady-state plasma glucose levels also showed higher fasting insulin and TAG, and lower fasting HDL-C. They suggested that IR affects the modulation of plasma insulin, TAG and HDL-C, independent of overall obesity or general physical fitness.

Characteristic findings in MetS include increased anthropometric measures (waist, weight, BMI), blood pressure and changes in plasma TAG and lipoproteins. Comparison of definitions of IR or MetS highlighted variation in the criteria used and also the relative complexity of some of the measures (Grundy *et al*., 2004a; 2004b). The International Diabetes Federation, recognising the need for a simple diagnostic tool convenient to use in clinical practice, and which would enable comparisons between data from different countries, proposed a definition of MetS requiring increased central obesity according to waist measurement, with two additional criteria (Alberti *et al*., 2006). This was subsequently adopted as the standard definition for MetS (Alberti *et al*., 2009), recognising that central obesity as defined by waist measurement varies between ethnic groups. Criteria compared by the American Heart Association (Grundy et al., 2004a; 2004b), with the IDF definition of MetS, are summarised in Table 1.7.

	ATPIII	WHO CRITERIA	AACE	IDF (2009)
Insulin resistance	-	**Yes, plus two of the following**:	-	-
Abdominal obesity (waist circumference)	Men: >102cm Women: >88cm	-	-	Men: ≥94cm Women: ≥80cm (European) ***plus 2 of the following:***
BMI	-	>30 kg/m^2	≥ 25 kg/m^2	-
Waist/hip ratio	-	Men: >0.9 Women >0.85	-	-
TAG	≥ 1.7 mmol/L	≥ 1.7 mmol/L	≥ 1.7 mmol/L	≥ 1.7 mmol/L
HDL-C	Men: <1.04 mmol/L Women: < 1.30 mmol/L	< 0.9 mmol/L <1.0 mmol/L	Men: <1.04 mmol/L Women: < 1.30 mmol/L	<1.0 mmol/L < 1.3 mmol/L
Blood pressure **Systolic** **Diastolic**	≥ 130 mm Hg ≥85 mm Hg	≥ 140 mm Hg ≥ 90 mm Hg	≥ 130 mm Hg ≥ 85 mm Hg	≥ 130mm Hg ≥ 85 mm Hg
Fasting glucose	≥ 6.1 mmol/L	≥ 6.1 mmol/L	6.1- 6.9 mmol/L	≥ 5.6 mmol/L (100mg/dL)
2-hour post glucose challenge	-	-	>7.8 mmol/L	-
Urinary albumin excretion	-	≥ 20 ug/min,	-	-
Albumin:creatinine ratio	-	≥ 3.4 mg/mmol	-	-
Other risk factors	**-**	**-**	May include family history, ethnicity	-

Table 1.7 Alternative criteria for diagnosis of metabolic syndrome (MetS)

1.3.2. Type 2 Diabetes

The development of T2D often follows from MetS; estimated worldwide diabetes prevalence in 2011 was 366 million, projected to rise to 552 million by 2030 (Whiting *et al*., 2011). Recent estimates for the United Kingdom indicated the frequency of diabetes to be greater than 5%, of whom the majority are Type 2: the number of diagnosed diabetics rose from 1.4 million to 2.9 million between 1996 and 2012, and is expected to reach 5 million by 2025. It is believed that there may be as many as 850,000 undiagnosed diabetics in the UK at the present time (*information from www.diabetes.org.uk, September 2013*). The health economic implications of providing care for the increasing numbers of diabetic individuals are significant, as well as indirect costs to the national economy from supporting people unable to work productively (Hex *et al*. (2012): these are summarised in Table 1.8.

	2010/2011	**2035/2036**
Direct costs	£9.8bn	£16.9bn
Type 1	£1bn	£1.8bn
Type 2	£8.8bn	£15.1bn
Indirect costs	£13.9bn	£22.9bn
Type 1	£0.9bn	£2.4bn
Type 2	£13bn	£20.5bn
Percentage of health expenditure	10%	17%

Table 1.8 Costs of diabetes to the UK healthcare economy

The major complications of diabetes and associated healthcare costs arise from disturbances in lipid and glucose metabolism; these result in overproduction of reactive oxygen species, disruption of endothelial cell function and inflammation, manifesting in diabetic vascular disease. Microvascular complications include retinopathy leading to blindness, nephropathy (with associated kidney dysfunction and failure) and the potential of amputation resulting from decreased blood supply to major limbs. Macrovascular complications cover the range of cardiovascular problems including myocardial infarction (MI) and stroke (Paneni *et al*., 2013).

1.3.3. Cardiovascular disease (CVD)

CVD and cancer are major causes of death worldwide; CVD, including MI and stroke, was in 2005 reported to be responsible for 3 out of 10 deaths (WHO report, 2013). As described above for T2D, the health economic costs of CVD are considerable. Recent figures of CVD incidence and related costs for the UK (Coronary Heart disease Statistics, www.bhf.org.uk) are shown in Table 1.9.

Coronary heart disease	Reported numbers
Deaths in 2010	180,000
Prescriptions in 2011	292 million
People living with CHD	2.3 million
Heart attacks in 2012	103,000
Incidence of stroke in 2012	152,000
Cost to UK health economy	£9 bn
Hospital costs (64%)	£5.76bn
Medication (23%)	£2.07 bn

Table 1.9: Reported statistics for CHD in the UK *(www.bhf.org.uk, 2012)*

1.3.4. Pathogenesis of MetS, T2D and CVD

The mechanism by which obesity and MetS lead to T2D is via dysregulation of plasma lipoproteins: increased levels of free FAs promote fat deposition and lipotoxicity in muscle, liver and pancreatic β-cells (Cusi, 2010; Donath & Shoelson, 2011). The accumulation of cholesterol in β-cells causes disruption of glucose metabolism and reduces insulin secretion, thus potentially leading to a diabetic phenotype (Fryirs *et al.,* 2009). Cholesterol is important in regulating β -cell membrane organisation: the balance between lipoprotein classes and concomitant effect on cholesterol transport may be important in determining β -cell function and survival (Rothblat & Phillips, 2010). Lipid dysregulation in MetS is augmented by the presence of large numbers of small, dense, low-density lipoprotein particles (sdLDL) (Subramanian & Chait, 2012); in combination with raised serum TAG and low concentrations of HDL-C, this has been called the 'atherogenic lipoprotein phenotype' (Austin, 1991) or 'atherogenic lipid triad' (Toth, 2005). These small particles may be more atherogenic than larger, more buoyant particles: it appears that they are less easily cleared by the LDL receptor and are more susceptible to oxidation and scavenging by macrophages (Toth, 2013).

1.3.4.1. Role of lipoproteins in CVD

Lipoproteins are complex aggregates of lipids and proteins, synthesised mainly in the liver and intestines, that enable lipids to be transported through the body. Once in the circulation, they are continually changing in structure and composition as they deliver lipid components (TAG, cholesterol, cholesterol esters and PLs) to tissues or return to the liver for removal those components that are not required. The principal PL component of all lipoproteins is phosphatidylcholine (PC) (Cole *et al*., 2012). The key protein components, the apolipoproteins, determine overall structure and metabolism of lipoprotein aggregates, although the presence of other 'associated proteins' has also been identified. It is common to use a classification based on the methods of separating lipoprotein classes or subclasses by size and density. Generally speaking, lower density particles contain a higher proportion of TAG and a lower proportion of PL. The main groups are:

- Chylomicrons (CM)
- Very low density lipoproteins (VLDL)
- Low density lipoproteins (LDL)
- High density lipoproteins (HDL)

The identification of subclasses within the low density lipoproteins is well established (Davies *et al*., 2003), while the use of computational biology techniques have allowed the identification of many distinct apolipoprotein and associated protein species within these groups: for example, there are now known to be at least 53 proteins in HDL particles (Davidsson *et al*., 2010). Although lipoproteins were previously believed to be microemulsions with a central core of non-polar lipids (TAG and cholesterol esters) surrounded by polar lipids and apolipoproteins, recent advances in modelling have suggested that a significant proportion of TAG may also be located near the surface in small HDL subclasses (Kumpula *et al*., 2008).

Simplistically, the role of chylomicrons and VLDL is to transport TAG from the liver and intestines to peripheral tissues for energy production, synthesis into cell membranes or as precursors of signalling molecules, and cholesterol for cell membrane synthesis. The functions of HDL are in the removal of excess cholesterol, transportation to the liver for processing and excretion as bile acids or recirculation, in the transfer of TAG between lipoprotein classes and the removal of VLDL and chylomicron particles that are no longer needed (Hodson *et al*., 2008). Proteins in HDL aggregates have

important anti-inflammatory functions, limiting the effects of the pro-inflammatory cytokines in CVD (Brown, 2007). Some of the key components of lipoprotein aggregates and known associations with CVD risk (Krauss, 2010) are summarised in Table 1.10.

The major protein component of lipoproteins, the apolipoproteins, are simple polypeptides with little tertiary structure, whose functions are to solubilise non-polar lipids for transport in the circulation, and to recognise specific cell receptors. ApoA1 and ApoA2 are the main HDL apolipoproteins; with ApoC, these are found in all lipoprotein classes (Davidsson *et al*., 2010). ApoE is mainly present in lipoproteins delivered from the liver; in addition to lipid homeostasis it is believed to have some functions on immune response and inflammation. All the apolipoproteins in HDL particles are exchangeable: the particles are subject to continuous remodelling and lipid and apolipoprotein exchange with other circulating lipoproteins and tissues. ApoB100 is present in VLDL, IDL and LDL, while ApoB48 is present in chylomicrons and TAG-rich particles: these are considered to be 'non-exchangeable', meaning that the lipid component is not exchanged until the lipoproteins are eventually removed from circulation. In addition to apolipoproteins, lipoproteins contain important enzymes, transport and anti-oxidative/anti-inflammatory proteins (Chait *et al*., 2005; Podrez, 2010).

A relationship between the levels of lipoprotein subclasses and risk of CVD has been the subject of extensive research, although lack of standardisation between analytical methods has complicated this (Krauss, 2010). Despite this, there is a strong association between LDL levels and the risk of coronary heart disease, although less so for stroke (Helfand *et al*., 2009).

	Key role	Key components	Association with CVD	Reference
Chylomicron (CM)	Transport of TAG and cholesterol from liver and intestines to peripheral tissues and cell membranes	ApoB48		Davidsson *et al*., 2010
Very low density lipoprotein (VLDL	Transport of TAG and cholesterol from liver and intestines to peripheral tissues and cell membranes	ApoB100, ApoB48	Increased hepatic production in T2D	Krauss, 2010
VLDL1		Large buoyant particles high in TG		
VLDL2				
Low density lipoprotein (LDL)		ApoB100, lipoprotein-associated phospholipase A_2	ApoA1 reduced in T2D, ApoB100 and ApoCIII increased in T2D	
Small dense LDL (sdLDL)			Increased production in T2D associated with hypertriglyceridaemia	Ip *et al*., 2009; Subramanian &Chait, 2012
IDL		ApoB100		

	Key role	Key components	Association with CVD	Reference
High density lipoprotein (HDL)	Removal of excess cholesterol to the liver, transfer of TAG between classes, removal of redundant VLDL and chylomicron particles	Proteins with negative inflammatory effects ApoA1, ApoA2, ApoE Serum Amyloid A, serum phospholipase A_2 Clusterin (ApoJ)	Limiting inflammatory effects in CVD. ApoE in HDL3 reduced by statin treatment ApoA1-containing HDL reduced in T2D and during inflammation	Chait *et al*., 2005; Brown, 2007; Davidsson *et al*, 2010; Krauss, 2010

Table 1.10 Key lipoproteins and associations with CVD risk

1.3.4.2. Inflammatory processes in pathogenesis of CVD

There is extensive evidence that atherosclerosis is primarily an inflammatory disease, with early infiltration by inflammatory cells and subsequent expression of inflammatory cytokines. Proteins associated with lipoprotein and other non-associated inflammatory proteins (complement, fibrinogen, ferritin and caeruloplasmin) are believed to have atherogenic potential (Chait *et al*., 2005). The key initiating step in atherosclerosis is the sub-endothelial accumulation of apoB-containing lipoprotein; this triggers an inflammatory response (which may be enhanced if the lipoproteins are oxidised), attracting monocytes to the endothelium. Activated endothelial cells secrete chemokines (including MCP and IL-8) that interact with monocyte receptors and promote migration. Fatty streaks, which are early atherosclerotic lesions, become the focus of a non-resolving inflammatory response involving the accumulation of cells, lipid and matrix in the subendothelial layer. These may expand to form a necrotic core, which is the trigger for an acute vascular incident (MI, stroke or sudden cardiac death) (Moore & Tabas, 2011).

The role of cytokines as pro-and anti-inflammatory regulators has been summarised in Tables 1.2 and 1.3 (Donath & Shoelson, 2011, McLaren *et al*., 2011). There is clear evidence for the role of pro-inflammatory cytokines in the development of T2D and the development and persistence of atherosclerotic lesions and pathology. Increased plasma concentrations of IL-6 and its hepatic degradation product CRP, are observed in acute MI and in unstable angina, often associated with a poor prognosis in coronary artery disease (Tousoulis *et al*., 2007); correlation with ischaemic stroke is less reliable.

1.4. DIET & n-3 LCPUFA IN PREVENTION OF CARDIOVASCULAR DISEASE

Given the association between obesity and development of MetS and CVD, it is reasonable to infer the relevance of lifestyle factors, including diet and physical exercise. Many adults and children in the western world engage in less physical activity than their predecessors, while easy accessibility of convenient food and changes in eating patterns encourage higher than necessary consumption (Simopoulos, 2008). An additional question is whether the type of food consumed may exacerbate or mitigate disease risk (Cho *et al*., 2013, van Dam *et al*., 2013, Mirrahimi *et al*., 2014).

High levels of fat in the diet may significantly increase the severity of a heart attack, but the type of fat in the diet may also be important. Avoidance of both saturated fat (SFA) and trans-fatty acids (TFA) has been widely recommended, although in a recent meta-analysis Hoenselaar (2012) has questioned the basis of the recommendations in respect of SFA. Dietary SFA are associated with an increase in both LDL-C and HDL-C, and the research on which much of the advice was based is open to interpretation. Sacks and Katan (2002) reviewed the merits of replacing saturated fat with carbohydrate, monounsaturated or polyunsaturated fat, and deduced that LDL-C is decreased by all three substitutions, with the relative effect carbohydrate<MUFA<PUFA; unsaturated fat decreased LDL-C more than HDL-C, resulting in an overall reduction in the LDL-C/HDL-C ratio. A multi-ethnic study of SFA intake (de Oliveira Otto *et al*., 2012) concluded that SFA *per se* may not be as important in predicting cardiovascular risk as the source of the fat, with dairy products associated with lower risk than red meat. The evidence in respect of TFA suggests that those derived from industrial hydrogenation processes pose a higher CVD risk than those occurring naturally in ruminant-derived foods, especially grass-fed animals (Bendsen *et al*., 2011; McAfee *et al*., 2010).

The investigations described in Chapters 3 and 4 of this thesis are focussed on two dietary components, n-3 LCPUFA and cholesterol. Cholesterol build-up in arteries has been shown to be a risk factor for CVD (as described in Section 1.3) but it is not clear whether dietary cholesterol may directly contribute to this. An understanding of the importance of n-3 FA in development, membrane structure and in cell signalling has given rise to speculation and accumulation of evidence regarding benefits in reducing cardiometabolic risk, including whether one food source of n-3 FA may be more beneficial than another.

1.4.1. Dietary cholesterol

Reduction of CVD risk has been attributed in part to reduction of plasma cholesterol. Cholesterol is an essential component of cell membranes and a necessary precursor for steroid synthesis; it is not an essential nutrient, since adults and children over 2 years of age are capable of sufficient production in the liver to satisfy normal requirements. The potential of dietary cholesterol in increasing CVD risk is not clear. It has been reported that a Western-type diet is associated with a higher CVD risk than a Mediterranean-type diet (Estruch *et al*., 2013b). Although such a diet has been variously defined, one consistent aspect is that it is lower in dietary cholesterol than a

Western-type diet (Djousse & Gaziano, 2009). Cholesterol in the diet is often associated with other potentially atherogenic components which may be found in red meat, poultry, and dairy products, (although also with fish and shellfish) and the distinction between the relative risk of the various components is not clear: additionally, genetic factors may influence cholesterol absorption and synthesis; studies of statins have identified variability in response due to differences in absorption and in vivo synthesis (Lecerf & de Lorgeril, 2011). The role of ApoE genes in modulating lipoprotein metabolism is also of interest; carriers of the ApoE4 gene are most responsive to impact of fat and cholesterol intake (Minihane, 2013). Dietary guidelines for adults in the USA (The National Cholesterol Education Program Adult Treatment Panel III) (2005) recommended that daily cholesterol intake should not exceed 300 mg/day for healthy adults, and 200 mg/day for those with elevated LDL cholesterol. There are no comparable guidelines in the UK or Europe.

Evidence for a specific link between dietary cholesterol and CVD risk has been limited and inconsistent. Studies where a link has been reported (Puddu *et al*., 2011, Sakurai *et al*., 2011) have not eliminated possible contributions from confounding factors (Djousse & Gaziano, 2009), while in other large studies of CVD risk factors, no association was found after adjustment of possible confounding factors including other dietary components (Kanter *et al*., 2012). A review of dietary intake and CVD risk (MI, coronary death, or stroke) in adults between 70 and 79 years of age (Houston *et al*., 2011) found an increased risk of CVD correlated with dietary cholesterol or egg intake, but further analysis suggested that this applied only to those with T2D. A study of 34,670 women in Sweden over a 10-year period (Larsson *et al*., 2012) reported a positive association between dietary cholesterol from reported intake and risk of stroke (RR = 1.2). Overall it appears that while there may be some individuals (up to a quarter) in whom dietary cholesterol may increase LDL-C, HDL-C is also increased, so that the LDL/HDL ratio is maintained at a fairly constant level with little attendant increase in CVD risk (Fernandez, 2012).

1.4.2. n-3 LCPUFA

A major emphasis in nutrition research has been on the relative role of PUFAs, in particular the potential mitigation of disease risk by n-3 LCPUFA. Some of the evidence in this respect is presented below.

1.4.2.1. Reviews

Harris (1996) performed a meta-analysis on seventeen intervention studies; FO was shown to reduce plasma TAG and increase LDL-C but have little effect on HDL-C (Harris, 1996). Comparison of later studies (Kelley *et al*., 2007) suggested that the effects of DHA alone on plasma LDL-C and HDL-C and LDL particle diameter are less clear-cut, with some studies showing an increase in both, while others have failed to demonstrate any change. Rizos *et al*. (2012) reviewed 20 publications (selected from 3635 citations) covering 6860 participants and reported no statistically significant correlation between n-3 LCPUFA supplementation and risk of mortality from sudden cardiac death, MI or stroke. However, another meta-analysis in the same year (Chowdhury *et al*., 2012) showed a reduced risk of cardiovascular disease (RR 0.94) in individuals eating 2-4 servings of fish weekly. Benefits were less significant in individuals taking FO supplements, which raises the question as to whether nutrients in fish besides the n-3 LCPUFA may be providing additional benefits.

A review of 21 randomised controlled trials reported that FO intake (Balk *et al*., 2006; Wang *et al*., 2006) may reduce TAG (0.31 mmol/L), increase HDL-C (0.04 mmol/L) and LDL-C (0.16 mmol/L): ranges of n-3 LCPUFA in the studies reviewed were between 0.045 and 5 g per day, but a beneficial TAG reduction was noted at an intake of 0.9 g per day. Based on another review of 4 major trials covering 40,000 participants a recommendation was made for target EPA plus DHA consumption of 500 mg/day for individuals without underlying overt CV disease and 800 mg/day for individuals with known coronary heart disease and heart failure (Lavie *et al*., 2009).

1.4.2.2. Individual studies of intake and intervention

There is extensive data from intervention and food intake studies with n-3 LCPUFA over at least the past 30 years, including comparisons of the relative importance of DHA and EPA and total intakes. Oils enriched with EPA or DHA are reported to be comparable in respect of TAG reduction (Mori & Beilin, 2001). The DART (Harris, 2007) and GISSI intervention trials (Marchioli *et al.,* 2007) reported that dietary intake of between 0.3 and 1 g per day of n-3 LCPUFA for 3 months or more showed a correlation with reduced mortality from CVD, particularly in individuals who had already experienced one MI.

The Japan EPA Lipid Intervention Study (JELIS) (Yokoyama *et al*., 2007) followed 5,859 men between 40 and 75 and 12,786 postmenopausal women up to 75 years of age for an average of 4.6 years, randomised to treatment with statins alone or statins

with EPA (600mg as ethyl ester daily). This group reported an improvement in the incidence of cardiac events, although a less significant reduction in sudden cardiac death than the GISSI study, an observation attributed to the cardioprotective effect of the higher baseline fish consumption in the Japanese study. In a smaller study, EPA supplementation (1.8g/day as ethyl ester) was shown to reduce the progression of atherosclerosis in T2D (Mita *et al*., 2007). Thies *et al.* (2003) reported that an intake of 2g per day EPA and DHA resulted in a 25-30% reduction in fasting TAG, while 1.4 g EPA plus DHA per day resulted in increased incorporation and improved stability of atherosclerotic plaques. A detailed study of the effects of DHA supplementation (3g DHA per day) (Kelley *et al*., 2007) showed:

- ***Reduction in:*** fasting TAG, concentration of large VLDL (including apoCIII) and IDL particles, mean diameter of VLDL particles and TAG:HDL ratio.
- ***Increase in:*** fasting LDL-C, LDL-C/HDL-C, HDL-C/ApoA1, LDL-C/ApoB, concentration of sVLDL, large LDL and HDL particles and in the mean diameter of LDL particles.
- ***Postprandial decrease*** in TAG, IDL, sLDL, large VDL and sHDL
- ***Postprandial increase*** of large LDL, HDL and sVLDL
- ***No change*** in fasting or postprandial total and HDL-C
- ***LDL-C*** increased in fasting but not post-prandial plasma.

Maximum effects were observed within 45 days from the start of the study and maintained for the next 45 days except for heart rate, which decreased by 8.3% at 45 days and by 5% at 91 days. Overall, DHA intake was correlated with a reduction in atherogenic lipids and lipoproteins and an increase in cardioprotective proteins, particularly ApoCIII.

The OPTILIP study (Griffin *et al*., 2006, Sanders *et al*., 2006; Griffin, 2008) aimed to optimise the n-6:n-3 intake ratio by dietary changes in a cohort of men and post-menopausal women between 45 and 70, an age range that typically has an increased risk of CVD; 28% of the participants fell within the definition of MetS. This group looked for associated changes in IR, plasma lipoproteins and indices of clotting function (fibrinogen, factors VII and XII); they found that reduction of the n6:n3 ratio from a baseline ratio of 6.7-8.3 to 2.2-2.3 by increasing n-3 PUFA intake (but not merely by changing the ratio of precursor LA and ALA) was associated with beneficial decreases in basal and post-prandial plasma TAG, and improvement in the profile of lipoprotein particle size. However, there was no significant effect on insulin sensitivity or clotting function. This was proposed by the authors to be the maximum effect on n-6:n-3 ratios

that can be achieved by dietary management. Later work has suggested that it may be the total n-3 intake rather than the n-6:n-3 ratio that is important in modulation of CVD risk (Lovegrove & Griffin, 2011, Griffin, 2012).

A survey comparing consumption of n-3 LCPUFAs with other dietary variables for 1441 individuals on the Diabetic Control and Complications (DCTT) database (Cundiff *et al*., 2007) was interpreted as showing that the benefits of n-3 LCPUFA may be due to a strong correlation between higher fish/fish oil intakes and an overall healthier dietary pattern.

1.4.3. Recommendations of n-3 LCPUFA intake for cardioprotection

Consumption of EPA and DHA is now widely recommended by international authorities and national cardiac societies (von Schacky & Harris, 2007) to prevent cardiovascular incidents, for treatment after MI and prevention of sudden death and secondary disease. The American Heart Association recommends a daily intake of 2-4 g EPA plus DHA for patients with high TAG, which is predicted to reduce plasma TAG by 20-40%. The National Institute for Clinical Excellence (UK) (2012) recommended lifestyle changes for patients following MI, including dietary intake of 7 g of n-3 LCPUFA per week from oily fish, or at least 1 g daily of n-3 PUFAs as ethyl esters if the former is not achievable. These recommendations were based on a wide portfolio of literature describing mechanisms of actions, animal models, and also large intervention trials, although an earlier Cochrane analysis of available data (Hooper *et al*., 2006) showed a null result.

1.4.4. Mechanism of action of n-3 LCPUFA

It is reported that n-3 LCPUFAs act as mediators of the nuclear events governing specific gene expression in lipid and glucose metabolism and adipogenesis (Lombardo and Chicco, 2006). Regulation of several transcription factors related to TAG or lipoprotein metabolism is observed following FO supplementation. Increased expression of antioxidative enzymes was also reported in both normo- and dyslipidaemic men, and decreased expression of pro-oxidative enzymes (P450 enzymes and matrix metalloproteinases). In an extension of this study it was suggested that there was evidence for differences between normolipidaemic and dyslipidaemic

men in gene expression following FO supplementation (Schmidt *et al.,* 2012a; 2012b; 2012c). Possible routes by which EPA and DHA may reduce CVD risk include:

1.4.4.1. Antiarrhythmic effect

Sudden cardiac deaths associated with fatal arrhythmia are a frequent cause of death in industrialised societies, with up to 80% of sudden deaths due to ventricular fibrillation, and only a small effect on mortality due to use of anti-arrhythmic drugs. Studies of n-3 supplementation have suggested a beneficial reduction in tachycardia and ventricular fibrillation, attributed to changes in cell membrane composition (Leaf *et al*., 2005, Raitt *et al*., 2005). A recent review of clinical endpoint data reports a small, non-significant reduction in sudden cardiac death despite demonstration of biochemical changes which would be considered beneficial, most specifically reduction In plasma TAG (Khoueiry *et al*., 2013).

1.4.4.2 Anti-thrombotic effect

Increased availability of n-3 LCPUFAs results in a reduction in ArA in membrane PLs in platelets, endothelial cells and in heart muscle (Garg *et al*., 2006 Metcalf *et al*., 2007), which directly decreases the amount of this FA available for eicosanoid synthesis, so that the levels of the highly pro-aggregatory TXA_2 are greatly reduced (Calder, 2004). EPA and DHA are reported to reduce levels of the pro-inflammatory cytokines IL-1ß, IL-6 and TNFα, and reduce the mRNA levels in mononuclear cells of the pro-atherosclerotic growth factors PDGF-A, PDGF-B and MCP-1. There is also some *in vitro* evidence for the effects of EPA and DHA in reducing levels of sICAM, sVCAM and sE-selectin, cytokines with a role in endothelial activation although a recent meta-analysis of n-3 supplementation effects on soluble adhesion molecules reported reduction in plasma levels of sICAM-1 but not VCAM, p-selectin or E-selectin (Yang *et al*., 2012).

1.5. SOURCES OF n-3 FATTY ACIDS

The potential health benefits of n-3 LCPUFA have given rise to extensive commercial development with concomitant marketing publicity. Traditionally n-3 LCPUFAs were obtained from fish liver oil supplements such as cod liver oil as by-products of fish-meat harvesting. It is now impossible to supply global consumer needs from the current global fish harvest, leading to a requirement for new sources, which could include

microalgae, krill oil, calamari oil and genetically engineered land plant crops (Ryckebosch *et al*., 2014).

1.5.1. Relative efficacy of different n-3 LCPUFA sources

Evidence from feeding studies in pigs indicates that the form in which n-3 or n-6 LCPUFA are provided may influence their ultimate distribution in plasma lipoproteins (Amate *et al*., 2001), with PL-derived FAs partitioning to HDL rather than LDL lipoprotein. It has been suggested that LCPUFA in PL form may be associated with benefits in infant feeding, with a reduction in necrotising enterocolitis and enhanced intestinal repair. Sala-Vila *et al*. (2004), however, reported that the FA composition of plasma lipids did not vary according to the source of the FAs in formula-fed infants, and that bioavailability was comparable from PLs and TAG sources, although they did not fractionate out the lipoprotein subclasses to determine ultimate distribution. Plant lecithins, which contain high concentrations of PC in addition to choline and betaine, are commonly used to improve emulsification of processed foods, but a nutritional role in mitigation of alcoholic liver disease (Lieber, 2004) and CVD risk (Sahebkar, 2013) has also been suggested.

1.5.1.1. Triglycerides

Fish oils (FO) are the primary source of n-3 LCPUFA in TAG form, and the most extensively studied in respect of health benefits. Fatty acid composition varies between species, but is also sex- and season- dependent. A portion of fish may contain between 0.06 and 2.2 g n-3 PUFA (British Nutrition Foundation, 1999), and the relative proportions of EPA:DHA may range from 3:1 to 1:4 between species. 'White' fish species such as cod, haddock and halibut concentrate oil in the liver, and the oil derived from these species also contains high natural levels of fat-soluble vitamins, whereas 'oily' fish, such as salmon and mackerel, have higher levels of oil in the flesh; oil from some species such as tuna is extracted from the eye sockets. These oils are widely available in health food shops or pharmacies as either free bottled oils or in capsules (usually gelatine). Industrial fermentation processes are used to manufacture oils rich in DHA, EPA and ArA from microalgae, which are also obtainable as food supplements, and are used in supplementation of infant formulae to achieve PUFA levels comparable with human breast milk.

1.5.1.2. Krill oil (KO)

Krill (*Euphausia superba*), a small shrimp-like marine crustacean, forms the world's largest animal biomass, and provides the major nutrient supply for the blue whale. Harvested krill contains about 15% high quality protein, with approximately 3% fat and minerals, which on a dry-weight basis is equivalent to 60-78% protein, and between 7-26% fat; KO contains significant proportions of DHA and EPA, partly as TAG but with 30-58% in the form of PL (PC, phosophatidylethanolamine and phosphatidylinositol) (Suzuki, 1981). The other notable component of KO is the antioxidant astaxanthin (Figure 1.7), present with other antioxidants including vitamin E, Vitamin A and β-carotene.

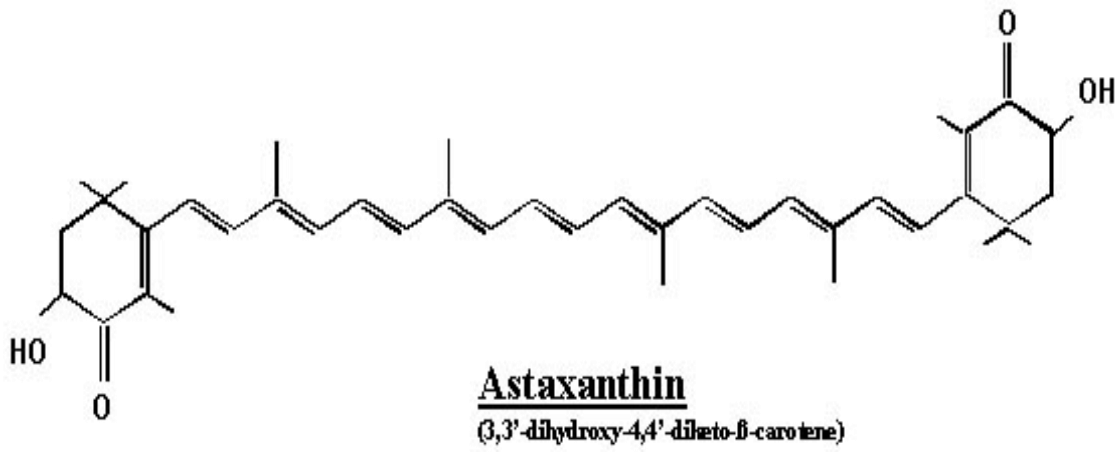

Figure 1.7: Structure of astaxanthin

The composition of KO is dependent on factors including seasonal variation, location, sex, nutrient availability and condition (Savage & Foulds, 1987; Grantham, 1977; van Niewerburgh *et al*., 2005). Once harvested, krill deteriorate rapidly due to the release of visceral enzymes, and must be processed or deep frozen immediately to prevent compromise to quality (Suzuki, 1981; *personal communication, O Hoestmaelingen & E Lied);* the PUFAs are reported to be stable for only about 15 days, and astaxanthin is less stable. Indeed, 48 hours is considered to be the maximum time that krill can be kept before the sensory characteristics deteriorate to an extent that makes it unfit for consumption (Kolokowska, 1988).

Analysis of commercially available KO reveals wide variation in levels of PL, specific FAs and astaxanthin (Kusumoto *et al*., 2004; Fricke *et al*., 1984) (Table 1.11).

	Aquasource	Neptune	Enzymotec (2 products)	Healthspan
N-3 fatty acids, total	180 mg	300mg	190mg/300mg	240mg
EPA	100 mg	150mg	90mg/150mg	140mg
DHA	60 mg	90 mg	50mg/90mg	64mg
Astaxanthin	0.2 mg	1.3mg	0.2mg/1.5mg	0.1mg
PLs	100 mg	400mg	400mg/420	430mg

Table 1.11: Composition of krill oils *(from Manufacturers' data)*

Krill forms a critical link between plankton and higher forms of marine life in the Antarctic and elsewhere, and there are concerns regarding sustainability of the species, but with careful management it is thought to be capable of providing krill for human and animal nutrition while maintaining stocks adequate to support marine life (Bender, 2006). The fishery for Antarctic krill is the largest by tonnage in the Southern Ocean (Nicol *et al.*, 2012). The annual catch remained relatively stable at around 120 000 tonnes for 17 years until 2009, but has recently increased to more than 200 000 tonnes. This is well within the precautionary catch limits set by the Commission for the Conservation of Antarctic Marine Living Resources (8.6 million tonnes). However, recent developments in harvesting technology and in product development (some 200 patents in 2013 alone) indicate renewed interest in exploiting this resource. Environmental changes in the Southern Ocean are likely to affect both krill and the fishery.

1.5.2. Comparison of FO and KO in relation to lipid metabolism

1.5.2.1. Animal studies

Possible effects and mechanisms of action of KO on lipid metabolism have been investigated in animal models. In rats fed with a diet enriched with 2.5% KO, KO inhibited hepatic lipid synthesis to a greater extent than FO, by reducing the expression of the mitochondrial tricarboxylate carrier (Ferramosca *et al*., 2012a; 2012b). This was associated with a greater reduction in levels of hepatic TAG and cholesterol in KO-fed than in FO-fed animals, as well as reduction of plasma TAG and glucose, and a smaller increase in plasma insulin. A decrease in the mitochondrial citrate carrier and the

cytosolic acetyl-CoA carboxylase and FA synthetase enzymes were observed. Increased carnitine palmitoyl-transferase I activity and levels of carnitine were interpreted to indicate stimulation of hepatic FA oxidation. Animals supplemented with KO also maintained efficient mitochondrial oxidative phosphorylation, with less accretion of body weight, and less oxidative damage of lipids and proteins, than are often found in high-fat fed animals. A similar study in mice transgenic for human TNFα (a model of chronic inflammation and poor lipid metabolism) investigated the effects of krill powder (6.4% lipids, 4.3% protein, w/w) on markers of lipid metabolism (Vigerust *et al*., 2013, Bjorndal *et al*., 2012), over a 6-week feeding period. Those mice receiving KO powder displayed lower hepatic and plasma TAG, with increased β-oxidation and reduced hepatic expression of genes involved in lipogenesis and glycerolipid synthesis. Plasma cholesterol, enzymes involved in cholesterol synthesis, and hepatic gene expression of SREBP2 were also reduced, as were genes involved in glycolysis and gluconeogenesis. An anti-inflammatory effect was indicated by the reduction of endogenous hepatic TNFα. The inference from this data was that KO may have a greater potential than FO to promote lipid catabolism. Comparison of TAG with PL in n-3 LCPUFA supplementation of corn-oil based high fat diets in C57BL/6J mice (9 weeks) (Rossmeisl *et al*., 2012), reported that the PL was more effective than TAG in prevention of glucose intolerance and reduction of obesity. Lipaemia and hepatosteatosis were also more effectively suppressed by dietary PLs. This was thought to correlate with better bioavailability of DHA and EPA, resulting in higher DHA accumulation in the liver, white adipose tissue, and muscle PLs; inflammation in white adipose tissue was also reduced.

Finally, it has been suggested that there may be an association between elevated peripheral levels of endogenous ligands of cannabinoid receptors (endocannabinoids) and MetS. Banni *et al.* (2011) reported more efficient downregulation of the endocannabinoid system by KO than by FO in tissues of obese zucker rats. The same group (Piscitelli *et al*., 2011) investigated the dose-dependent effects of KO on metabolic parameters in high fat diet-fed mice, and measured levels of the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) in inguinal and epididymal adipose tissue liver, gastrocnemius muscle, kidneys and heart. An eight-week high-fat diet increased endocannabinoid levels in all tissues except the liver and epididymal adipose tissue: KO reduced anandamide and/or 2-AG levels in all tissues studied except the liver, usually in a dose-dependent manner. Levels of endocannabinoid precursors were also generally down-regulated.

1.5.2.2. Human studies

An early comparison between KO and FO in humans was a small study to observe effects on symptoms of premenstrual syndrome and dysmenorrhoea (Sampalis *et al*., 2003). The same group (Bunea *et al*., 2004) subsequently compared intake of KO at 2-3 or 1-1.5 g per day with FO containing EPA and DHA for effects on hyperlipidaemia. They reported more effective reduction of total cholesterol (TC), TAG, LDL-C, HDL-C and glucose by KO than FO, and proposed that KO is an effective alternative n-3 LCPUFA source for the management of hyperlipidaemia. A subsequent comparison of KO and FO (menhaden) was carried out by Maki *et al*. (2009), with FO, KO and olive oil (control) supplementation at 2g per day for 4 weeks and measurements of plasma n-3 LCPUFA and indicators of safety, tolerability and CVD risk. Plasma EPA and DHA concentrations increased significantly more ($p < 0.001$) in the KO (178.4 ± 38.7 and 90.2 ± 40.3 µmol/L, respectively) and FO groups (131.8 ± 28.0 and 149.9 ± 30.4 µmol/L, respectively) than in the control group (2.9 ± 13.8 and -1.1 ± 32.4 µmol/L, respectively). A reduction in systolic blood pressure was more significant ($p < 0.05$) in the FO (-2.2 ± 2.0 mm Hg) group than in the control group (3.3 ± 1.5 mm Hg); the effect of KO was between the other two groups and was not significantly different from either (-0.8 ± 1.4 mm Hg). Apart from blood urea, which reduced slightly in the KO group compared with FO, there were no other significant differences for any other safety parameters. This study demonstrated comparable uptake of n-3 LCPUFA from KO and FO over 4 weeks, although with little difference in CVD risk markers.

Ulven *et al.* (2011) compared KO and FO supplementation to evaluate effects on markers of oxidative stress and circulating n-3 LCPUFA levels. 113 adults with normal or slightly elevated TC and/or TAG were randomised into three groups; 1) 3g KO (543mg EPA/DHA) daily, 2) 1.8g FO daily (EPA plus DHA 864mg), and 3) no supplement. After 7 weeks, similar increases in plasma EPA, DHA and DPA were seen in both supplemented groups, with no significant differences in markers of oxidative stress or oxidation between any groups. This was taken to demonstrate that KO was as effective at two-thirds the concentration of DHA/EPA of FO in increasing n-3 LCPUFA uptake, possibly associated with a higher bioavailability of the KO n-3 LCPUFA (being partly present as PL) compared with the TAG form of FAs in FO. Three EPA+DHA formulations derived from FO (re-esterified TAG [rTAG], ethyl-esters) and KO were compared for effects on their effects on fatty acid composition of plasma PLs (Schuchardt *et al*., 2011) over a period of 72 hours. Single doses of 1680 mg EPA given as TAG, rTAG, ethyl ester or KO were taken by each of 12 young men (4 in each group). Higher incorporation of n-3 LCPUFA was seen in those receiving KO, followed

by FO TAG; lowest incorporation was seen from the ethyl ester preparation. The KO in this study contained relatively high levels (about 22%) of the n-3 LCPUFA as free FAs: the possible effect of this is not known.

More recently, Trepanowski *et al*. (2012) compared the effects of KO or coconut oil supplementation (2g/day) on 12 men and 27 women carrying out a 21-day Daniel fast. This is a highly restricted vegan diet in which participants avoid animal products, refined foods, white flour, preservatives, additives, sweeteners, flavourings, caffeine, and alcohol. Although adherence to such a diet for 21 days has been demonstrated to improve blood pressure, LDL-C, and certain markers of oxidative stress, it has also been shown to lower HDL-C. There was no measurable difference between KO and coconut oil in the effects on a range of markers of metabolic and CVD risk, although overall the effect of the Daniel Fast on a number of these markers was significant. The cohort of men and women showed wide initial variation in BMI, blood lipids, blood glucose and blood pressure, and it is not unreasonable to assume that in such a relatively small sample with such a high level of heterogeneity, subtle alterations in metabolism from KO supplementation might be obscured by the baseline 'noise' and changes in metabolic activity due to the change in diet. Also, the study was only pursued for 3 weeks, which is not long enough to achieve maximum effect on lipid markers.

Possible interactions of KO with the endocannabinoid system (ECS) have been investigated in human studies (Banni *et al*., 2011; Silvestri & Di Marzo, 2012; 2013). These investigators compared the changes in plasma endocannabinoids resulting from supplementation of 2 g/d of KO (309mg EPA/DHA in 2:1 ratio) or FO (menhaden) (390 mg EPA/DHA in 1:1 ratio) over a period of 4 weeks in obese and overweight subjects. The results showed increased levels of endocannabinoids in overweight and obese compared with normal weight subjects. KO (but not FO or olive oil control) decreased 2-arachidonoylglycerol (2-AG) in obese but not overweight individuals. The decrease of 2-AG was correlated to the plasma n-6:n-3 PL LCPUFA ratio. It is suggested that decreased 2-AG biosynthesis may be caused by the replacement of the ArA precursor by n-3 LCPUFAs.

1.6. WORK DESCRIBED IN THIS THESIS

The experimental work described in this thesis addresses two aspects of the application of n-3 LCPUFA in mitigation of CVD.

The first piece of work aimed to establish whether prawns, as a source of n-3 LCPUFA, but also rich in cholesterol, may be beneficial or harmful; the hypothesis for this was that daily ingestion of a standard weight of prawns would not confer any increased CVD risk compared with a similar weight of a control food (processed white fish meat). In addition to measurement of blood indices of CVD risk, food diaries were obtained from participants as a measure of compliance. Recruitment and management of participants, and some of the biochemical measurements were carried out by colleagues at the University of Surrey; determination of lipid profiles, statistical analysis and interpretation of the food diaries was carried out by this investigator, as was the comparison of methods for measurement of sdLDL.

In the second piece of work, the potential effects of KO and FO are compared for their effect on some indices of cardiovascular health. The aim of this investigation was to determine whether krill oil (KO) may be more beneficial than fish oil (FO) in effect on indices of CVD risk; it was hypothesised that KO may be as beneficial as FO at a lower (2/3 w/w) daily dose. Measurements of blood indices of CVD risk were undertaken, using both venous blood and a point-of-care capillary blood measurement system; food diaries were also completed to provide a measure of compliance. Recruitment and management of participants in this study (including food diaries and collection and preparation of blood samples) were carried out by this investigator, with assistance from colleagues at LJMU; the automated biochemical assays were carried out by Randox, Northern Ireland, and insulin assays by colleague Debbie Scott. Interpretation and analysis of food diaries and all statistical analysis were carried out by the main investigator.

CHAPTER 2

Materials And Methods

2.1. NUTRITION INTAKE STUDIES; CONDUCT OF INVESTIGATIONS

Two nutrition intervention studies were carried out, a) to compare the effects of prawns (Pr) and a processed white fish foodstuff (OC) on lipoprotein and CVD markers, and b) to compare the effects of FO and KO on CVD risk markers. A range of techniques was employed in these studies, and a brief explanation of application in similar studies is given where relevant.

2.1.1. Recruitment of participants

Participants for both studies were recruited by invitation from among volunteers responding to advertisement; the demographics of each group are described in subsequent chapters. Recruitment, project information and all protocols were in conformity with the requirements of the Research Ethics Committee appropriate for the study in question (detailed in Chapter 3 and 4 respectively). Volunteers were provided with information about the nature and purpose of the study in question and were screened for suitability before commencement of the relevant study, as described in Chapters 3 (Section 3.3.1) and 4 (section 4.2.1). All results were anonymised and treated with the highest possible level of security and confidence.

2.1.2. Preparation of plasma samples

At each visit, blood samples were taken by standard venepuncture procedure using a vacutainer and anticoagulant tubes suitable for the specific subsequent investigations (EDTA, citrate or heparin) as indicated in the table below:

Anticoagulant	**Assay**
Potassium-EDTA	Cholesterol, TAG, LDL-C, HDL-D, NEFA, Lipoprotein subclasses, plasma fatty acid profiles,
Sodium citrate	Glucose
Lithium Heparin	Insulin

Table 2.1. Anticoagulants and assays

For the KO/FO study, capillary samples of blood were also obtained and used for immediate measurement of lipid biochemistry markers using a cassette-based screening system (Cholestech LDX, Alere), as described in Section 2.2.2.1. Plasma for other analyses was separated by centrifugation in a refrigerated benchtop centrifuge (2000 g for 15 minutes at 4^{o}C), removed by Pasteur pipette into a cryovial, and stored at -80^{o}C until all samples were available for batch analysis.

2.2. MARKERS OF OBESITY, MetS AND CVD RISK

2.2.1. Physiological measurements

2.2.1.1. Blood pressure

Measurement of blood pressure reflects the force that blood exerts against the wall of a large artery. The pressure of the blood is highest (systolic) at the point when it leaves the left ventricle during contraction of the heart, and lowest during relaxation of the heart muscle (diastolic). For the studies described in this thesis, blood pressure was measured using an automated sphygmamometer (Omron Healthcare Europe B.V., Hoofddorp, Netherlands).

2.2.1.2. Anthropometric measures

The body mass of participants was measured using standard platform scales accurate to within 0.5kg, and height using a stadiometer to the nearest whole cm. Body mass index (BMI) was derived by the standard equation:

$$BMI = \text{Weight (kg)}/(\text{height (m)})^2$$

Waist circumference was measured using a standard tape measure, midway between the uppermost border of the iliac crest and the lower border of the costal margin (rib cage). BMI Categories (as defined by the National Heart Lung and Blood Institute, USA) in standard use by advisory bodies in the UK were used as guidelines:

- Underweight: < 18.5 kg/m^2
- Normal weight: 18.5–24.9 kg/m^2
- Overweight: 25–29.9 kg/m^2
- Obesity: > 30 kg/m^2

2.2.2. Biochemical methods for measurement of CVD risk; general principle

Biochemical markers of CVD risk were measured in the course of the studies described in the subsequent chapters. Although these were conducted using different analytical instruments, the principles employed were common to all. The basis of each was:

1. To separate out the molecular species of interest if needed (to remove species that might interfere with the assay)
2. To release the species from any other ligands bound to it (by enzymatic methods)
3. To react the species of interest in an enzymatic reaction to produce an active molecule (in this series of reactions, hydrogen peroxide)
4. To react the peroxide with a chromogenic compound to produce a coloured quinoneimine dye; the dye produced can be measured by absorbance of reflectance photometry at a set wavelength. The methods have been designed so that the absorbance or reflectance is linear over the concentration range under investigation.

2.2.2.1. Screening markers using LDX screening method

Screening of whole blood lipid markers was carried out at all screening visits on capillary blood samples using the Cholestech® LDX system (Alere, UK). This is a 'point-of-care' analytical system designed to permit clinical measurement of selected lipid-related parameters using small samples (40 *μ*L) of capillary blood applied to a solid-phase cassette, and giving results within a few minutes of sampling. Cassettes are available to provide measurements of total cholesterol (TC), HDL-C, TAG, TC/HDL-C ratio, LDL-C and glucose, using a combination of established enzymatic methodology (Siedel *et al*., 1983) and solid-phase technology. This system has been certified for use by the CDC Cholesterol Reference Method Laboratory Network in the USA, and approved for use by the UK MHRA. Pluddemann *et al*. (2012) validated the Cholestech LDX device for TC, LDL-C, HDL-C and TAG; correlation with laboratory analyses were reported to be 0.91, 0.88. 0.77 and 0.93 respectively.

Procedure: 40*μ*L capillary blood from a finger-prick was applied to an Alere Cholestech LDX® cassette, which was placed in the analyser. The plasma was separated from the blood cells in the sample by membrane filtration; one portion of plasma was directed to a cholesterol/TAG analysis pad. A second portion of plasma was directed

to the other side of the cassette, where low and very low-density lipoproteins were precipitated with dextran sulphate (MW 50,000) and magnesium acetate (Warnick *et al*., 1982, Warnick *et al*., 1983).

The analytical methods underpinning the Alere LDX cassette system are based on similar enzyme-catalysed reaction series to generate an active molecule (hydrogen peroxide). For all of these measurements the hydrogen peroxide is reacted in the same colorimetric reaction to produce a coloured adduct which is estimated by reflectance photometry at a chosen wavelength. Each cassette has a brown magnetic strip containing the calibration information required to convert the reflectance reading to the total cholesterol, HDL cholesterol, triglycerides and glucose concentrations. The techniques are summarised below:

Measurement of total cholesterol and HDL cholesterol (HDL-C)

This is based on the enzymatic method developed by Allain and Roeschlau (Allain *et al*., 1974; Roeschlau *et al*., 1974), as shown in Figure 2.1.

1. Cholesterol esters in plasma are hydrolysed to free cholesterol,
2. Free cholesterol is oxidised by cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide.
3. The hydrogen peroxide generated reacts with 4-aap and N-ethyl-N-sulphohydroxypropyl-m-toluidine (TOOS) to form a purple-coloured quinoneimine dye (in a reaction catalysed by horseradish peroxidase). The colour intensity is proportional to the total cholesterol and HDL cholesterol concentration of the sample.

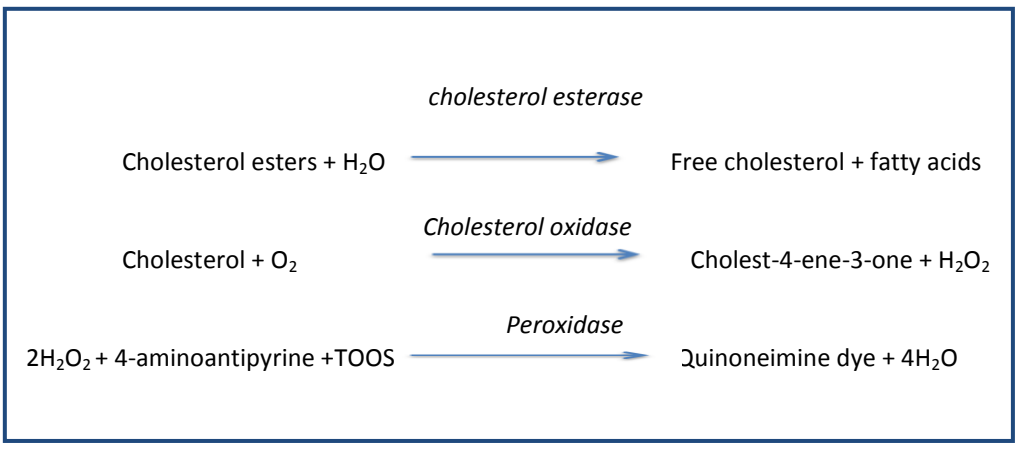

Figure 2.1 Measurement of total cholesterol (TC) and HDL-C

Measurement of TAG is by an enzymatic method (Figure 2.2) based on

1. The hydrolysis of TAG by a mixture of lipases to glycerol and free fatty acid
2. Glycerol is converted to glycerol-3-phosphate (catalysed by glycerol kinase),
3. Glycerol-3-phosphate is oxidised to dihydroxyacetone phosphate (DHAP) and hydrogen peroxide (Fossati and Prencipe, 1982).
4. Hydrogen peroxide generation is measured by the same colour reaction as for total cholesterol.

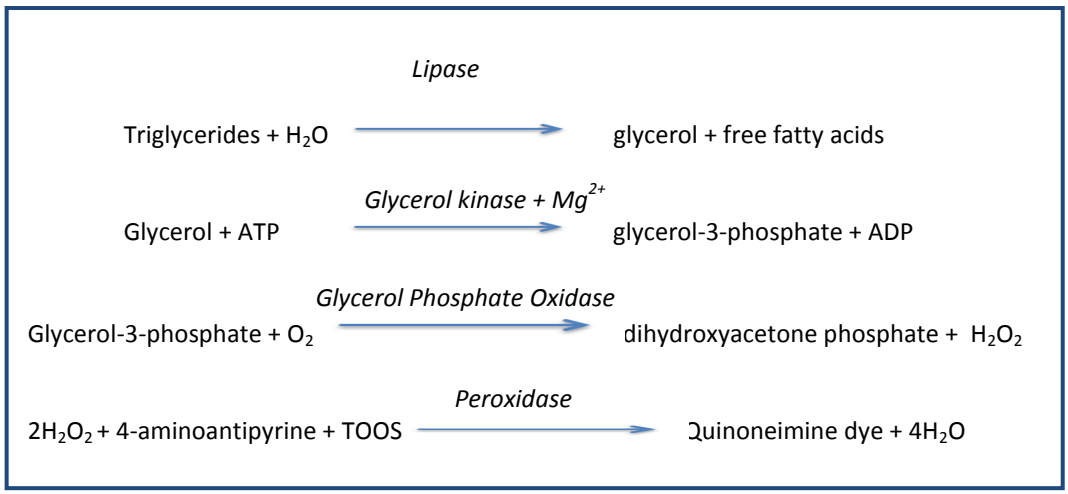

Figure 2.2 Measurement of TAG

Glucose is measured by a similar enzymatic method (Figure 2.3);

1. The oxidation of glucose by glucose oxidase to generate gluconolactone and hydrogen peroxide.
2. Colour reaction utilising horseradish peroxidase as described above.

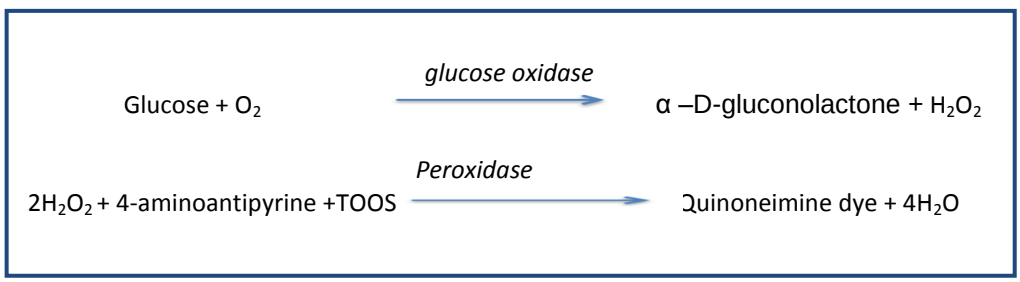

Figure 2.3 Measurement of plasma glucose

2.2.2.2. Analyses using a RANDOX auto-analyser

Plasma samples were analysed using a RANDOX auto-analyser (Randox Laboratories, Co. Antrim, N. Ireland): for each assay specific quality controls (QCs) were measured at 2-hour time intervals. Each assay was based on an enzymatic reaction, linked to endpoint production of a coloured pigment, the concentration of which was proportional to absorption at a specific wavelength (Trinder, 1969a; 1969b; Siedel *et al*., 1983; Kattermann *et al*., 1984; Munz *et al*., 1974). The biochemical principles of these assays are the same as those described for the Alere LDX analyser, but employing different initial separation techniques.

Total cholesterol (TC): The measurement of TC includes estimation of all lipoprotein subclasses using an enzymatic process as described in 2.2.2.1, with the absorbance of the quinoneimine pigment measured bichromatically at 505 and 692 nm. The test reagents as provided are stated by Randox to provide a wide measuring range (0.865-16.6 mmol/L), within-run precision of 3.7% at 1.7 mmol/L and 3.8% at 7.7 mmol/L, with between-run precision of 1.3% at 1.7 mmol/L and 1.4% at 7.5 mmol/L.

HDL-C: Measurement of HDL-C depends on isolation and quantitation of the cholesterol component of the lipoprotein aggregate. The Randox Direct Clearance method completely removes all non-HDL components (chylomicrons, VLDL-C and LDL-C) from plasma using cholesterol esterase, cholesterol oxidase, catalase and a selective reagent, followed by release of HDL-C by detergents containing sodium azide to inhibit any remaining catalase (Lawlor *et al*, 1998; Warnick *et al*., 2001; Mather, 2013). The intensity of the quinoneimine dye product is directly proportional to the cholesterol present at 600 nm within a concentration range of 0.10-2.4 mmol/L. Intra- and inter-assay precision gave CVs for low, medium and high and quality controls of < 5% respectively. Other methods of measuring HDL-C depend either on ultracentrifugation to separate lipoprotein subclasses, or masking of the non-HDL subclasses; the method used here has a high (99%) concordance with the ultracentrifugation reference method, but the masking methods have been reported to underestimate the concentration of HDL-C. The Randox technique has a measuring range of 0.189 - 3.73 mmol/L (7.30 - 144 mg/dL), with intra-assay precision 1.8% at 0.8 mmol/L and 3.1% at 2.0 mmol/L, and inter-assay precision 2.8%.

LDL-C: As for HDL-C, measurement of LDL-C is a way of quantitating low-density lipoprotein aggregates by measurement of the cholesterol present in the aggregates.

The Randox LDL-C kit utilises a similar direct clearance method to remove interfering lipoprotein subclasses prior to analysis, analogous to that described for HDL-C.

Step 1: Elimination of chylomicrons, VLDL-Cholesterol and HDL-Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase.

Step 2. Release of LDL-C by detergents, followed by estimation of LDL-C as for HDL-C (azide is used to inhibit catalase in this step). TOOS (N-Ethyl-N-(2 hydroxy-3 sulphopropyl)-3–methylaniline) is the pigment used in the colorimetric reaction. The measurement range (linearity of reaction) is 0.189 - 22.2 mmol/L (7.3 - 860 mg/dL). Intra and inter-assay precision gave CVs for low, medium and high and quality controls of < 5% respectively.

An alternative practice is to calculate LDL-C using the Friedewald equation, which enables the estimation of LDL when triglyceride and HDL levels are known. This was used for the study described in Chapter 3. However, this calculation is only accurate if plasma TAG levels are <400mg/dL (22 mmol/L), chylomicrons are not present and the sample does not contain beta-VLDL.

Glucose: Glucose is determined in the Randox system by enzymatic oxidation in the presence of glucose oxidase (GOD), as described in section 2.2.2.1. The absorbance of the red/violet quinoneimine dye is measured bichromatically at 505/692 nm. Intra- and inter-assay precision gave CVs for high and low quality controls of < 5% respectively.

Triglycerides (TAG): The Randox TAG assay is based on the principle described in section 2.2.2.1, with measurement of the resulting quinoneimine pigment measured bichromatically at 505 and 692 nm. The measuring range is reported to be 0.134 - 12.7 mmol/L, within run precision 3.3% at 0.3 mmol/L and 1.8% at 5.6 mmol/L, and between run precision 3.5% at 0.6 mmol/L and 1.3% at 3.0 mmol/L.

ApoA1 and ApoB: The Randox assays for ApoA1 and ApoB are latex-enhanced immunoturbidimetric assays; the apolipoprotein present in the plasma samples combines with a highly specific polyclonal rabbit antibody present in the reagent to form an insoluble complex. The turbidity, which is increased by the presence of latex in the reagent, is measured at 340nm, and is proportional to the apolipoprotein concentration in the sample (Contois *et al*., 1996a; 1996b).

Non-esterified fatty acids (NEFA): NEFA were measured using the enzymatic reactions of Acyl CoA synthetase and Acyl CoA oxidase in sequence to produce peroxide, and using the same principle for estimation of peroxide as outlined above (Figure 2.4). The intensity of the colour is proportional to the NEFA concentration. The assay is linear up to 2.0 mmol/L with a sensitivity of 0.10 mmol/L. Low and high quality controls were employed with intra-assay CVs of 3.4% and 2.3% respectively (low QC, n = 11 and high QC, n = 9) and inter-assay CVs of 8.9% and 7.4% respectively (low QC, n = 22 and high QC n = 18).

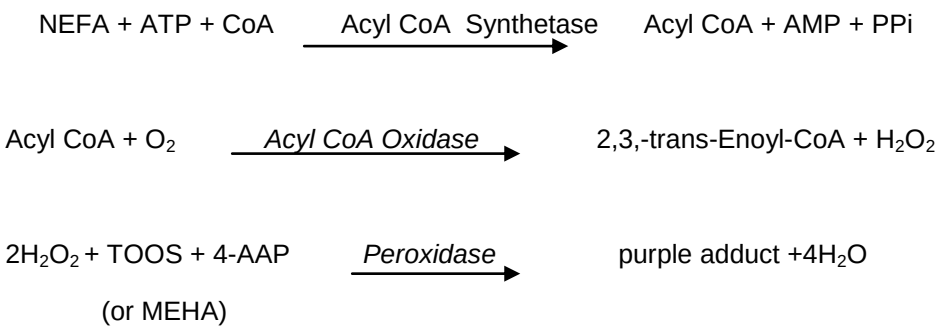

Figure 2.4 Measurement of non-esterified fatty acids

(4-AAP = 4-aminoantipyrine, TOOS = N-ethyl-N-(2-hydroxy-3-sulphopropyl) m-toluidine or 3-Methyl-N-Ethyl-N-(beta-Hydroxyethyl)-Aniline (MEHA)

2.2.2.3. Insulin

Plasma insulin was measured using the Mercodia Insulin ELISA, a solid phase two-site enzyme immunoassay. In this assay, based on the sandwich technique, two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. One anti-insulin antibody in the microtitration plate is bound to the insulin in the sample and a second peroxidase-conjugated anti-insulin antibody is in solution. A sample of serum (25 μL) was added to the antibody-containing well, 100 μL enzyme conjugate solution added and the plate incubated on a shaker for 1 hour at room temperature. The plate was washed six times, and 200 μL substrate (tetramethylbenzidine, TMB) added. After 15 minutes on a shaker at room temperature, the reaction was stopped by addition of 50uL Stop solution (pH 0.5), and colour development at 450nm was estimated. The sensitivity of detection was1 pU/L.

2.2.2.4. Ultracentrifugation methods: Iodixanol gradient ultracentrifugation for the separation and estimation of lipoproteins (DGUC)

A useful method for separation and identification of lipoprotein subclasses is iodixanol gradient ultracentrifugation (DGUC), followed by individual analysis and summation of subfractions.

Principle of method: Ultracentrifugation has been used for many years to separate lipoprotein classes and subclasses on the basis of density (Havel *et al*., 1955, Wilcox & Heimberg, 1970). Early methods were very labour-intensive and time-consuming. The development of density gradient ultracentrifugation (DGUC) depends on the formation of a continuous density gradient during the centrifugation process, with lipoprotein classes and subclasses stratified according to density (Krauss & Burke, 1982; Swinkels *et al*., 1987, Griffin *et al*., 1990; Krauss, 1994). Advances in the design of rotors from swing-out design to short path-length vertical or near-vertical rotors allowed a reduction in centrifugation times from over 12 hours to under 4 (Chung *et al*., 1986). The high salt concentrations in earlier methods (sodium or potassium bromides) risked disruption of apolipoproteins (Murdoch & Breckenridge, 1994): the technique described here used iodixanol, which rapidly forms a continuous density gradient, is non-toxic and iso-osmotic, and reduces the risk of apoliprotein disruption (Ford *et al*., 1994; Graham & Billington, 1996). The method used was that developed by Davies *et al*. (2003), based on that of Graham *et al*. (1996) with modifications to save time, reduce inter-run variation and improve LDL subclass separation, using smaller samples (3 mL rather than 5 mL plasma) which would be more appropriate for large-scale intervention studies. A near-vertical rotor rather than a vertical rotor was used to reduce the chylomicron adherence to tube walls seen with vertical rotors, while still maintaining the necessary relative centrifugal force (RCF).

Materials and equipment:

- Beckman-Coulter ultracentrifuge
- Beckman-Coulter ultracentrifuge NT65 rotor, with places for 8 Optiseal tubes
- Optiprep™ (Axis-Shield): This is a commercially available solution (60% w/v) of iodixanol, used as a density gradient medium: a 9% solution was prepared from this using phosphate-buffered saline as diluent.
- Optiseal™ tubes (11.2 mL), with caps, plugs and spacers
- Stainless-steel cannula (1)
- NVT 65 Beckman-Coulter rotor
- Beckman-Coulter Ultracentrifuge XL series.

Procedure

Blood was obtained from participants by venepuncture at study visits, into EDTA anticoagulant tubes. Plasma was obtained by centrifugation at 4^{o}C in a bench centrifuge (1500 rpm for 20 minutes), transferred to clean sample tubes and stored at -80^{o}C until use. On the day of lipoprotein analysis, samples were thawed on ice prior to gradient formation and centrifugation. Plasma (2.8 mL) was added to 0.7 mL of Optiprep^{TM} (iodixanol) in a bijou tube (7 mL), and mixed by gentle inversion. The density of the resulting plasma/Optiprep mixture was checked using a refractometer (Density = 1.050 g/ml, refractive index = 1.3498). A 7.8 mL portion of the 9% iodixanol/PBS solution was placed in an 11.2 ml Beckman Optiseal centrifuge tube.

A steel cannula and syringe were used to under-layer 3 mL of the denser working sample beneath the 9% Optiprep solution (making sure there were no air bubbles). The tubes were left for 30 minutes to settle, before fitting plugs into the tubes, securing in the NVT65 rotor and fitting spacers and caps to each tube. Caps were tightened to 180 inch pounds using a torque wrench, before centrifugation at 65 000 RPM, 16°C, for 3h 10 min. After centrifugation, caps were removed with torque wrench and adapter; spacers and plugs were also removed from the centrifuge tubes as they were taken out of the rotor and placed in a rack for fractionation. The products of each gradient were divided sequentially into 0.5ml fractions in microcentrifuge tubes using an Eppendorf fraction collector, and the lipoprotein concentration of each fraction was estimated by an automated technique for cholesterol estimation as described earlier in this chapter. Density ranges for lipoprotein subclasses (Davies *et al.*, 2003) are shown in Table 2.2.

Lipoprotein subclass	Density (kg/L)
HDL	>1.063
LDL	1.021-1.041
LDL-I	1.022- 1.025
LDL-II	1.025-1.028
LDL-III	1.028-1.036
LDL-IV	1.036
VLDL	1.009

Table 2.2: Density ranges for lipoprotein subclasses by DGUC

2.2.2.5. An alternative method for measurement of sdLDL-C

As described in section 1.3.4.1, it is now recognised that elevated levels of LDL present a major CVD risk factor, and that a prevalence of small dense particles (sdLDL) presents a greater atherogenic risk than large buoyant particles (lbLDL). Methods for sdLDL-C measurement have until recently depended on laborious ultracentrifugation and electrophoresis-based methods. A simple and rapid method for sdLDL-C quantification based on Mg–heparin precipitation (Hirano *et al*., 2005) was compared with the DGUC method (Davies *et al*., 2003) for some of the samples obtained in the study described in Chapter 3. Blood sampled into tripotassium citrate anticoagulant was obtained from nine adults, and plasma removed by centrifugation and separated into two portions that were used for sdLDL analysis by one of the two methods. The two methods were carried out by different operators and results compared subsequently. For the Hirano method, heparin–$MgCl_2$ was added to plasma to separate VLDL-C, IDL-C and large buoyant LDL-C, while sdLDL-C and HDL-C remained in the infranatant fraction. LDL-C in this fraction was determined by the direct LDL-C method on an ILAB 650 autoanalyser (Randox Laboratories Ltd, UK).

Comparison of methods: Ultracentrifugation of one portion in an iodixanol gradient was followed by fractionation and measurement of the cholesterol in twenty fractions as described in Section 2.2.2.4. A plot of results from the heparin method against those from the iodixanol method was linear, with a gradient of 1.026 (R^2 = 0.8766) (Jones *et al*, 2009), as shown in Figures 2.5 and 2.6.

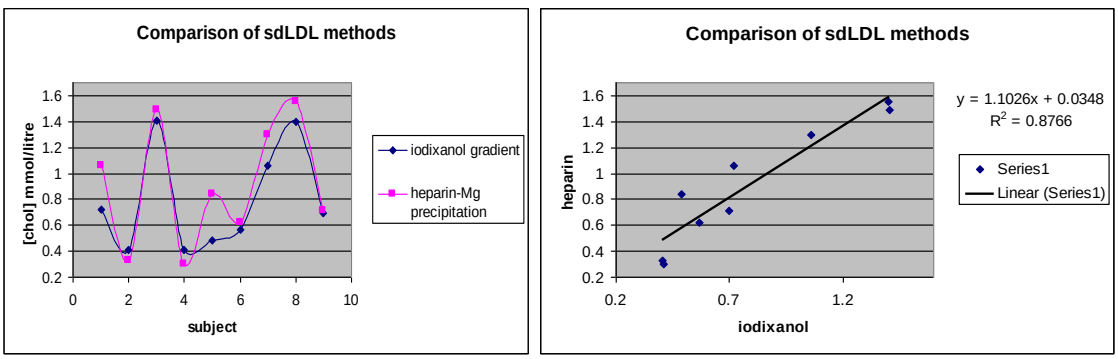

Figure 2.5 Comparison of DGUC (iodixanol) and Hirano methods for sdLDL estimation

2.2.2.6. Measurement of plasma fatty acid profiles

Plasma samples were stored at -80°C until analysis, then thawed at room temperature, vortexed for 5 seconds and centrifuged for 4 minutes at 2500rpm. Aliquots of 400 µL were placed in 10mL glass tubes and 40 µg 1-2-dipentadecanoyl-sn-glycero-3-

phosphocholine added. Total lipids were extracted following a modified method of Folch *et al*. (1957). 400µL of 0.9% saline solution was added to the plasma and vortexed for 30 seconds. 5ml chloroform:methanol (2:1 v/v)/ BHT (0.01% w/v), followed by 1mL 1M NaCl, was added to the plasma and vortexed for 30 sec. Samples were centrifuged at 2500rpm for 10 minutes; the lower organic phase was transferred into new glass tubes and evaporated to dryness under a stream of nitrogen.

Phospholipids (PL) were isolated using amino propyl columns (NH_2). Samples were resuspended in 1mL of hexane and loaded onto the columns (500mg, Waters) conditioned with 20mL of hexane. Neutral lipids were washed out with 3ml hexane. Free fatty acids were washed out with 6ml diethyl ether/acetic acid (10%). PLs were eluted with methanol (3mL). After evaporation to dryness under nitrogen, PLs were transmethylated to fatty acid methyl esters (FAMEs). The Phospholipids were resuspended in 2mL toluene:methanol (4:1 v/v)/BHT (0.01% w/v) and transmethylated with 200µl of acetyl chloride for 1 hour at 100°C.; 5mL of 0.25M K_2CO_3 was added, and centrifuged for 2mins at 2500rpm. The upper toluene layer was removed for analysis by GCMS. FAMEs were stored at -80°C until GCMS analysis.

FAMEs were analysed by GCMS (Agilent Technologies 7890a GC system and Agilent Technologies 5975c MS system) with a fused silica capillary column (60m x 0.25mm x 0.15µm). The injector temperature was 250°C and helium was used as the carrier gas. The FAMEs (1µL) were injected into the GC in split mode with a split ratio of 20:1 and a flow rate of 23.9 ml/min. The oven temperature was initially set at 50°C for 1 min, ramping to 175°C (25°C/min) and then to 230°C (2.2°C/min) and held for 9 min; run time was 40 mins. The GCMS was run in SIM/Scan mode; selected ion monitoring (SIM) was used to monitor 9 ions (m/z 41, 43, 5, 67, 69, 74, 79, 87, 91). SIM data was used for quantification of FAMEs using Chemstation software. FAMEs were identified by comparison of retention time, Mass Spectra authenticated standards and the NIST database.

FAMEs of the fatty acids 16:0, 18:0, 18:1*n*-9c, 18:2*n*-6c,18:3*n*-3, 20:4*n*-6, 20:5*n*-3 and 22:6*n*-3 were well separated from each other and clearly identifiable. PL-derived FA concentrations were calculated by comparison of the peak area of the individual FAs with the peak area of the internal standard using external calibration curves. Standards were run before and after each sample batch and an analytical quality control was run with each batch. The quality control was a pooled plasma sample taken from a selection of healthy adults.

2.2.3. Derivation of indices of CVD Risk

Compound indices postulated to provide diagnostic criteria for cardiometabolic risk, were calculated as shown in the relevant sections of Chapter 3 and 4. These were: ApoB/ApoA1 ratio (Walldius *et al*., 2004), HOMA-IR and HOMA- β status, QUICKI and R-QUICKI.

2.3. COMPARISON OF CAPILLARY AND PLASMA MEASUREMENTS

In the study described in Chapter 4, measurements obtained using the Cholestech point-of-care system were compared with those obtained from Autoanalyser techniques to provide information about relative accuracy and comparability.

Mean values (with SD) for the Cholestech analysis are presented in Table 2.3. Inspection of the data revealed no significant outliers in any category. No deviation from normality was observed using either K-S or Shapiro-Wilk tests, except for endpoint TAG/HDL-C ratio (FO intervention), for which one individual presented with a high value (although less than 2SD from the mean). Correlation between capillary and venous blood results was carried out using the Pearson correlation, as presented in Table 2.4.

	FO n = 18		**KO n = 18**	
	Baseline	**End**	**Baseline**	**End**
TC (mmol/L)	5.71 (1.24)	5.65 (1.03)	5.44 (1.01)	5.48 (1.07)
HDL-C (mmol/L)	1.13 (0.29)	1.13 (0.28)	1.09 (0.27)	1.14 (0.26)
TAG (mmol/L)	1.82 (0.75)	1.78 (0.80)	1.58 (0.51)	1.56 (0.61)
LDL-C (mmol/L)	3.74 (1.09)	3.71 (1.17)	3.63 (0.98)	3.63 (0.96)
Non HDL-C (mmol/L)	4.58 (1.23)	4.52 (1.09)	4.35 (1.04)	4.34 (1.09)
TC/HDL	5.36 (1.69)	5.29 (1.53)	5.27 (1.52)	5.07 (1.39)
TAG/HDL	1.77 (0.90)	2.07 (1.74)	1.58 (0.76)	1.67 (0.96)
Glucose (mmol/L)	5.41 (0.47)	5.45 (0.58)	5.49 (0.57)	5.32 (0.35)

Table 2.3: Mean capillary blood measurements (SD in brackets)

	Pearson correlation		*p*	
	Baseline	**Endpoint**	**Baseline**	**Endpoint**
TC	0.768	0.655	.000	0.000
HDL-C	0.705	0.805	.000	0.000
TAG	0.79	0.975	0.000	0.000
LDL-C	0.837	0.810	0.000	0.000
TC/HDL-C	.800	0.891	0.000	0.000
TAG/HDL-C	0.892	0.987	0.000	0.000
Glucose	0.561	0.649	0.001	0.000
Non-HDL-C	0.830	0.698	0.000	0.000

Table 2.4: Correlations between capillary and blood testing methods

(Pearson correlation, with significance for all parameters)

These results lead to the following observations:

1. Correlations are generally good in linear terms although not in absolute values.
2. Correlation varies for certain measurements between batches of measurements (e.g. TC, correlation for baseline measurements 0.768, for endpoint 0.655).
3. The highest and more reproducible between-batch correlations are observed for the TAG/HDL and TC/HDL ratios.

There were no discrepancies observed in relative magnitude between capillary and venous blood measurements. Systematic over-estimation of TAG (0.3g/L, 0.34 mmol/L) and underestimation of LDL-C (0.043g/L, 0.011 mmol/L) has been reported for capillary measurements in hyperlipidaemic individuals (Stein *et al*., 2002; Mayer *et al*., 2007; Parikh *et al*., 2009; Pluddemann *et al*., 2012).

2.4. MEASURES OF COMPLIANCE

In dietary intervention studies, it is important to understand whether participants comply with the intervention process, and consistency or changes in diet or physical activity during the course of the study, as such alterations may confound any findings. Participants in both studies described here were asked not to alter their normal dietary and physical exercise patterns. In order to evaluate compliance, participants were asked to return unused capsules at the end of the krill/fish oil study, and were asked to

complete food diaries during each study to enable an approximate estimation of major changes in dietary habits. Unfortunately, the majority of the participants did not return unused capsules, so that it was not possible to compare compliance and relate this to biochemical parameters; full compliance was therefore assumed in the data analysis.

In addition, retrospective inspection of some of the biochemical data (glucose, insulin and plasma FAs) provided indications as to the extent of compliance with supplementation and maintenance of normal dietary habits.

2.4.1. Food diaries

Participants in both studies recorded dietary intakes over periods of 3 or 7 days before and during the supplementary intervention times, as described in the relevant chapters. For both studies participants were provided with a printed food diary originally designed and used at the University of Surrey, set out with a matrix to be filled in for each day of the food record period (Appendix A). This food diary included guideline pictures taken from The Food Atlas (Nelson *et al.,* 1997) of common foods in different portion sizes to help them in estimating quantities and portion sizes consumed. The data from the work described in Chapter 3 was analysed using Windiet, and that in Chapter 4 using Microdiet V. These are both commercially available computer programs which allow estimation of major nutrient groups: Microdiet uses data derived from standard data sources of food composition, while Windiet uses data from online food composition sources. Nutrient intakes were subjected to statistical analysis using SPSS v20.

2.5. STATISTICAL ANALYSIS

Data from all studies were subjected to analysis using the SPSS20 statistical program (SPSS Inc., Chicago, USA). Initially, descriptive statistics were undertaken, to characterise the study populations at each time point and assess normality of the data distribution (Kolgorov-Smirnoff or Shapiro-Wilks test). Within an intervention group, paired *t* tests were applied for comparisons by time, and independent *t* tests were used to investigate any significant differences in outcome measures between intervention groups at each time point. Statistical comparison of changes from beginning to end, and in the second study, from beginning to midpoint, of dietary interventions, was also carried out.

Split-plot analysis of variance (SPANOVA) allowed composite investigation of time and treatment effects for all repeated outcome measures. When controlling for additional confounding variables (e.g. baseline measures, gender, body weight) and adjusting for baseline measures analysis of covariance (ANCOVA) was employed. Where data could not be normalised, it was not possible to employ ANCOVA or ANOVA, and in these cases non-parametric statistics were used. To explore the relationship between variables Pearson correlation and product moment (r^2) coefficients were determined. To test for the relationship between two categorical variables a chi-squared test was employed.

The detail of the statistical tests employed is provided in Chapter 3 for the prawn/OC comparison and in Chapter 4 for the KO/FO comparison.

CHAPTER 3

The effect of dietary Prawn intake on Cardiovascular Disease Risk

3.1. INTRODUCTION

Prawns and other shellfish are a source of n-3 LCPUFA (Ackman, 2000) but also contain significant amounts of cholesterol (140-190 mg/100g) (Oehlenschlager, 2006). Potential benefits of n-3 LCPUFA consumption have been addressed in Chapter 1. Dietary guidelines for minimising CVD risk have historically advised against the consumption of cholesterol-rich foods on the grounds that they may increase serum LDL-C (Weggemans *et al*., 2001; Tanasescu *et al*., 2004), although it has long been apparent that any CVD risk associated with cholesterol intake may be less clear-cut than the association between raised LDL-C and intake of SFA or TFA (Beveridge, 1964; Kratz, 2005). The American Heart Association report in 2000 (Krauss *et al*., 2000a; 2000b) recommended dietary cholesterol < 300mg per day for healthy individuals, with a limit of 200 mg/day cholesterol for those with raised LDL-C, pre-existing CVD or T2D. Studies of egg consumption have overall failed to provide convincing support for a link with CVD (Djousse & Gaziano, 2008; Harman *et al*., 2008; Gray & Griffin, 2009; Djousse *et al*., 2010), as summarised in Table 3.1.

A study of 21327 male doctors (Djousse & Gaziano, 2008) suggested an adjusted hazard ratio of 1.41 for all cause mortality in those eating more than one egg per day, in contrast to earlier studies (Dawber *et al*., 1982; Nakamura *et al*., 2004) reporting no association. Djousse & Gaziano reported no association between egg consumption and CVD risk overall, but noted an effect for a cohort with diabetes. An association between egg consumption and CVD risk in diabetics has been noted in several other reports. A cohort of 5672 T2D women was followed prospectively for 8 years, with diet questionnaires every 2 years (Tanasescu *et al*., 2004). During this period, 619 cases of CVD were recorded (including 268 MI, 183 fatal CHD and 168 strokes). The relative risk of a cardiovascular incident was estimated at 1.37 for an increase of 200mg cholesterol per 1000 kcal (95% CI: 1.12 -1.68; p = 0.003). Higher cholesterol intake was associated with increased BMI, which appeared to be specific for egg intake rather than overall cholesterol. The threshold for increased risk was at the level of 3 eggs per week (Table 3.2), lower than that reported by Djousse & Gaziano (1 egg per day).

A study of 45 individuals comparing intake of 2 eggs per day with an egg-free diet, combined with energy restriction (Harman *et al*., 2008), revealed no significant difference in LDL-C, plasma lipoproteins, or LDL particle size between groups after 12 weeks. This was interpreted to indicate that egg intake in conjunction with energy restriction does not increase total plasma or LDL-C, and that advice to avoid eggs in a weight loss programme may not be necessary. More recently however, inferences from food frequency questionnaires (Larsson *et al*., 2010; Houston *et al.,* 2011) have supported a possible association between CVD risk and egg consumption.

In summary, evidence from retrospective and prospective cohort and intervention trials up to the commencement of this study suggested that dietary cholesterol has a clinically insignificant effect on blood LDL-C and CVD risk in healthy individuals (Hu *et al*., 1999; Lee & Griffin, 2006; Harman *et al*., 2008) but there was no specific evidence to support or refute a possible link between prawn consumption, LDL and CVD risk.

3.1.1. Dietary cholesterol metabolism: implications for CVD risk

Dietary cholesterol is absorbed from the small intestine, taken up into lipoprotein aggregates with apolipoproteins and FAs (both as TAG and PLs) and delivered to the peripheral tissues where it is an important constituent of cell membranes. It is also an important precursor for a range of steroid hormones in the adrenal glands and gonads (Hu *et al*., 2010). Although the regulation of cholesterol metabolism appears to be tightly controlled the reported association between increased plasma cholesterol, especially LDL-C, and increased CVD risk, has led to a cautionary approach in dietary advice (Krauss, 2000a; 2000b). Evidence accruing suggests that the response to dietary cholesterol and the homeostatic mechanisms are affected by genetic characteristics which ultimately determine the balance of lipoprotein subclasses and relative CVD risk (Hopkins, 1992). Plasma cholesterol levels are regulated by different genetic factors from those which control cholesterol absorption and synthesis (Lecerf & de Lorgeril, 2011). Indeed, polymorphisms in ApoE are reported to be responsible for 7% of the variation in cholesterol levels, with contributing effects from ApoA4, ApoB and Apo C3 (Kanter *et al*., 2012).

Type of study	Number of participants	Duration	Outcome	Reference
Dietary survey (Framingham)	912	24-year follow-up	No association between egg consumption and serum cholesterol or incidence of heart disease	Dawber *et al*., 1982
Prospective cohort study	37,851 men 80,052 women	8 years (men), 14 years (women)	No overall association at 1 egg per day (RR = 1.08 for men, 0.82 for women). For diabetic men, RR = 2.02, women RR = 1.49	Hu *et al*., 1999
Epidemiological survey	5,186 men, 4077 women	14 years	No significant association between egg consumption and CHD risk for men or women	Nakamura *et al.,* 2004
Prospective study of food intake	5,672 women with T2D	8 years, review every 2 years	RR of CVD for increase of 200mg per day dietary cholesterol per 1000kcal = 1.37 (95% CI 1.12-1.68; p = 0.003). *Relative risks from egg consumption: < 1 egg per week: 1.0 1- <3 eggs per week: 1.10 3. eggs per week 1.08 3-6 eggs per week 1.39 >6 eggs per week 1.44	Tanasescu *et al*., 2004 Kratz, 2005
Retrospective review of food intake	21,327 male doctors	Average 20 years	Hazard ratio 1.41 for all CVD-related mortality from >1 egg per day. Hazard ratio for diabetic subjects 2.27-2.48 for consumption of 5-6 eggs per week (p <0.01)	Djousse & Gaziano, 2008

Type of study	Number of participants	Duration	Outcome	Reference
Dietary intervention (2 eggs per day) plus energy restriction	45	12 weeks	No difference in CVD markers following consumption of 2 eggs per day compared with null egg consumption.	Harman *et al.,* 2008
Food frequency questionnaire	34,670 women 49-83 years	10.4 years	Increased risk of stroke (RR = 1.20 for 300mg cholesterol per day compared with 170mg/day, 95% CI = 1.00-1.44, *p* for trend 0.01)	Larsson *et al*., 2012
Food questionnaire	1,941 adults between 70-79 years	3 years	Overall positive association between egg consumption (> 3 per week compared with <1 per week) and CVD risk (HR = 1.68, *p* = 0.01. Subgroup analysis indicated increased risk only in T2D (HR 3.66 for dietary cholesterol, 5.02 for >3 eggs per week)	Houston *et al*., 2011

Table 3.1: Studies of the relationship of dietary cholesterol or egg consumption and CVD

(*95% CI: 1,05, 1.98: *p* for trend 0.001)

3.1.2. Prawns as a source of n-3 LCPUFA

Many people do not enjoy eating oily fish, which are rich in n-3 LCPUFA, but shellfish (including prawns), which contain both n-3 LCPUFA and cholesterol, are popular foods. The cholesterol and n-3 LCPUFA content of some common foods are summarised in Table 3.2. The cholesterol content of 200g prawns is equivalent to that of a large egg yolk, with associated n-3 LCPUFA content of the order of 600mg, a level reported to favourably influence CVD risk (Calder, 2004, Kris-Etherton *et al*., 2002; 2004).

Typical recommendations for dietary n-3 LCPUFA supplementation to mitigate CVD risk are of the order of 1g per day of EPA plus DHA (Balk *et al*., 2006; Marchioli *et al*., 2007; Yokoyama *et al*., 2007; Lavie *et al*., 2009; Rauch *et al*., 2010). Intakes of 2 g per day EPA plus DHA (Thies *et al*., 2003) have been used in studies aimed at reducing CVD risk. At these levels of supplementation, compliance with the large volumes of FO may become an issue; an alternative is to use highly concentrated FA esters but these are expensive and, in some countries, require a medical product licence for administration. Prawns and other shellfish represent a more palatable source of n-3 LCPUFA, but have not been widely recommended because of the cautionary approach to the perceived risk from dietary cholesterol.

	n-3 LCPUFA mg/100g	**Cholesterol (mg/100g)**
Eggs		350
Prawns (processed, as eaten)	267-332	143-162
Oily fish	1600-2600	Approximately 70
Seafood sticks	Trace	16
Light spread		0.7-5
White fish (cod, haddock)	170-260	49-66
Crabmeat (brown)	2450	271
Crabmeat (white)	174	66
Processed chicken	60	36-48
Butter		153

Table 3.2: Cholesterol and n-3 LCPUFA content of some common foods

(Isherwood *et al*., 2010; *https://www.gov.uk/government/policies/reducing-obesity-and-improving-diet, 2013)*

3.2. AIM OF INVESTIGATION

This study was conceived to address the question as to whether dietary cholesterol in the form of prawns has an adverse effect on indices of cardiovascular health. The specific aim was to assess the effects of cold water prawns on lipoprotein fractions and LDL subclasses, (with an emphasis on sdLDL), in a randomly controlled dietary intervention trial in healthy male volunteers: the null hypothesis was that no difference would be observed between prawns and a processed white fish in effects on cardiometabolic risk measurements.

3.3. STUDY DESIGN

3.3.1. Recruitment and testing procedures

This study was carried out in collaboration with the University of Surrey (UoS). Recruitment of the participants was carried out at UoS, as were study visits and the biochemical assays. Lipoprotein analysis, comparison of sdLDL methods, and the analysis of biochemical data and food diaries, were carried out at LJMU.

3.3.1.1. Recruitment and study visits

A cohort of 21 healthy, male volunteers aged between 19 and 67 years was recruited from existing subject databases at UoS. The power and sample size for the study was based on a previous US study in normolipidaemic men (De Oliveira e Silva *et al*., 1996) reporting that daily intake of 225g shrimp for 21 days resulted in a 5% reduction (0.16 mmol/L) in plasma LDL-C (the description of shrimp in the USA would be equivalent to that of prawns in the UK). From this it was estimated that a sample size of 19 participants would be required to detect a significant difference at the 5% level. Allowing for a dropout rate of 30%, the initial aim was to recruit 30 participants, but from an initial 25, only 21 of those screened were suitable and willing to undertake the study. The criteria for inclusion in the study are shown in Figure 3.1. At the time of recruitment, all participants were normolipidaemic (TC <6.5 mmol/L, TAG <2.3 mmol/L), had normal blood pressure (<125/73 mm Hg), were not suffering from any medical conditions known to affect lipid metabolism, and had no significant history of recent weight loss (> 3kg in the preceding three months). Criteria for inclusion in the study also excluded current intake of dietary supplements such as FO, or specific

medications which would be likely to influence lipid metabolism, and previous fish allergy.

PRAWN STUDY: Inclusion characteristics for participants

Healthy males

Age 19-67

Total cholesterol < 6.5mM/L,

Plasma triglycerides < 2.3 mM/L

Normal blood pressure (SBP/DBP <125/73 mm Hg)

No recent weight loss history

Not taking any lipid-modifying dietary supplements or medications

No history of diabetes or cardiovascular disease

No previous fish allergy

Figure 3.1: Criteria for inclusion in study

Participants attended an initial pre-screening visit (following a 12-hour overnight fast), at which the following investigations were completed (as described in Section 2.2.1):

- Anthropometric and blood pressure measurements
- Blood sample
- 3-day food diary

Those who met the inclusion criteria attended for 4 further such visits, at the beginning and end of each intervention period.

The study was a crossover design, as shown in Figure 3.2; participants received both prawns (Pr) and processed white fish for separate periods with a washout period between. Participants were randomised, using a random number generator, to receive a daily portion of either prawns (225g/day) or an equivalent weight of white fish provided in the form of crabsticks, also known as ocean sticks (OC) for 4 weeks. The investigator analysing results was not aware of the order in which dietary interventions were carried out. Following a 4-week washout period (no prawns or OC), participants changed to the alternative protein portion for a further 4 weeks. They were requested

to follow their habitual diet throughout the study, not to consume additional shellfish or fish, and not to change other lifestyle variables such as physical exercise.

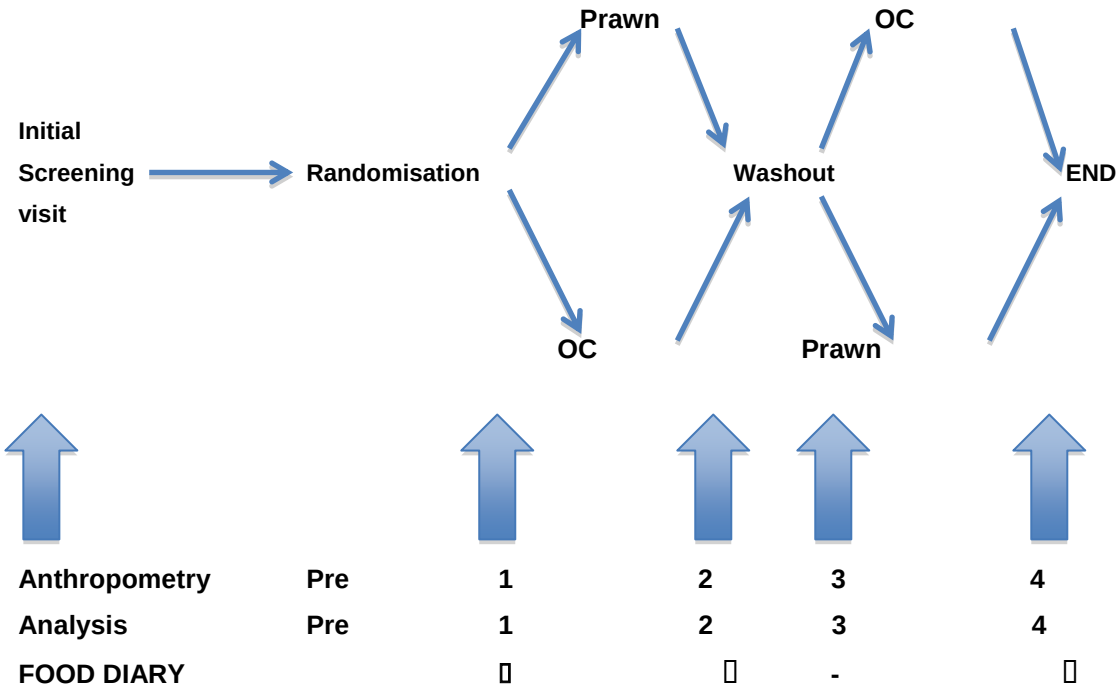

Figure 3.2. Diagram of intervention protocol

Blood samples were obtained from subjects at the start and end of each intervention stage (4 in total) and collected into EDTA; plasma was separated by centrifugation and stored in 1 mL aliquots at -80^{o}C until analysis (as described in Section 2.1.2.).

3.3.1.2.Biochemical markers of CVD risk

The following were measured as described in Section 2.2.2:

- TC, TAG, HDL-C, sdLDL-C (enzymatic assay)
- ApoA1, ApoB (immunoturbidimetric assay)
- Plasma Insulin

LDL-C was calculated from the Friedewald formula (Friedewald *et al*., 1972).

3.3.1.3. Lipoprotein analysis by DGUC

Lipoprotein fractions (LDL, HDL, VLDL) and LDL subclasses were separated from plasma by iodixanol density gradient centrifugation (Davies *et al*., 2003) followed by fractionation and estimation of cholesterol in fractions using the cholesterol oxidase

method of Roschlau (Roschlau *et al*., 1974), as described in Section 2.2.2.4. Inter and intra- assay coefficients of variation for all autoanalyser assays were less than 2%, and for iodixanol separations were <3% and <1% (Davies *et al*., 2003).

3.3.1.4. Food intake assessment

Participants were asked to complete 3-day food intake diaries as described in Chapter 2, section 3: these were analysed using WinDiet software (Robert Gordon University, 2005). The parameters estimated from food diaries completed prior to and during each of the intervention stages are listed in Table 3.3.

Macronutrients	Energy, Fat, Protein and Carbohydrate
Fat components	SFA, PUFA, MUFA cholesterol
Carbohydrate components	dietary fibre, non-starch polysaccharide (NSP), sugars, non-milk extrinsic sugars (NMES)
Alcohol	-

Table 3.3 Nutrients estimated from food intake diaries

3.4. STATISTICAL ANALYSIS

All data were subjected to statistical analysis using SPSS v20. Outliers (more than 2SD from the mean) were removed from further analysis. The data were examined for normality using the Kolgorov-Smirnov test before applying appropriate significance test. Data that could be normalised were tested parametrically using the paired student *t*-test; data that could not be normalised were analysed non-parametrically using the Wilcoxon ranked sign test. Split-plot ANOVA (SPANOVA) and ANCOVA were used to compare outcomes between and within groups. The changes (Δ) between start and end of interventions were also compared between Pr and OC groups. Correlations were carried out between baseline and baseline-endpoint change (Δ) for biochemical and lipoprotein measurements. Pearson correlation coefficients were determined to compare relationships between selected variables. For all statistical analyses a probability of $p < 0.05$ was regarded as significant.

3.5 RESULTS

3.5.1. Composition of Pr and OCs

The macronutrient composition of Pr and OCs, as provided by the manufacturers, is summarised in Table 3.4, together with an estimate of n-3 LCPUFA content and cholesterol for each.

OCs are manufactured from white fish meat, flavoured to taste similar to crabmeat. They are low in cholesterol and in n-3 LCPUFA; the manufacturer's label indicates only a trace of these in 100 g. Overall protein, energy and carbohydrate contents of the two foods was significantly different; prawns contained approximately double the amount of protein, and half the fat of OC, but very little carbohydrate. By contrast, OC contained approximately 1/8 weight as carbohydrate, and nearly double the level of sodium compared with Pr. Assuming full compliance, these differences in composition may have been significant factors; the possibility that this may have influenced or confounded the outcome of the study is discussed at the end of this chapter.

Nutrient	**Prawns**		**OCs**	
	Per 100g	Per 225g (daily intake)	Per 100g	Per 225g (daily intake)
Energy (kcal)	62	140	115	259
Protein (g)	17.9	40.3	9.0	20.3
Carbohydrate (g)	0.1	0.23	15.5	34.9
Total sugars (g)	0.1	0.23	3.9	8.9
Fat (g)	0.74	1.67	1.6	3.6
Saturates (g)	0.4	0.9	0.4	0.9
Monounsaturates (g)	0.3	0.7	0.3	0.7
Polyunsaturates (g)	0.1	0.23	0.5	1.1
n-3 LCPUFA (DHA plus EPA) (mg)	267-311	600-700	Trace	Trace
Cholesterol (mg)	130	295	Trace	trace
Fibre (g)	0.5	1.1	0.6	1.4
Salt (g)	1.1	2.45	1.9	4.3
Sodium (mg)	440	990	760	1710

Table 3.4 Composition of Prawns and OCs

3.5.2. Anthropometry

3.5.2.1: Baseline anthropometry

The anthropometric details for all participants at the start of the study are summarised in Table 3.5. Participants were recruited on the basis of normal healthy profile; however, of the 21 individuals, only 9 fell within the recommended range for a normal BMI (between 19 and 25). For a further 9, BMI was between 25 and 30, and for 3 in excess of 30 kg/m^2. Although the mean systolic blood pressure at the start of the study was <125mm Hg, one individual had an initial systolic/diastolic BP of 171/93. This was less than 2SD from the mean: this individual was not excluded from the study.

	Range	**Mean**	**SD**
Age (yrs)	19-67	41	
Weight (kg)	59.6 – 109.0	82.1	12.2
Height (m)	1.65-1.94	1.79	0.08
BMI	18.9-35.1	25.7	4.3
BP (systolic)	98-171	125	14
BP (diastolic)	45-101	73	11
Pulse	51-101	67	10

Table 3.5. Anthropometric details for participants (n = 21)

3.5.2.2. Changes in anthropometric characteristics

Changes in anthropometric measurements are shown in Table 3.6. Distribution for all measures was normal ($p > 0.05$) according to Kolgorov-Smirnoff test except for post-intervention systolic blood pressure (OC), and pre- and post-intervention pulse (Pr). There was no significant change in body weight, BMI, systolic or diastolic blood pressure between baseline measurements and those at the end of either dietary intervention ($p > 0.05$). The pre-post intervention difference was compared for each measure using the Wilcoxon Signed Rank test; there was no significant difference in changes between interventions except for pulse rate. Pulse measurements showed a mean increase during OC intervention compared with a decrease during Pr intake, which was significant (independent *t*-test, $p = 0.016$). A decrease in heart rate was previously noted by Kelley *et al*. (2007) following supplementation with DHA at 3g per day: heart rate decreased by 8.3% after 45 days and by 5% at 91 days. This compares with a mean decrease in pulse rate in the group described here of 8.0%, with a combined EPA plus DHA intake estimated at 600-700 mg daily.

	OC n = 21					Prawn n = 21					Significance ΔOC vs ΔPr[2]
	Pre intervention	Post intervention	[1] *p*	Change (ΔOC)	% change	Pre intervention	Post intervention	[1] *p*	Change (ΔPr)	% change	*p*
Weight (kg)	82.0 (12.6)	81.6 (12.4)	0.21	-0.43 (1.5)	-0.5 (1.8)	82.1 (12.2)	81.8 (12.2)	0.205	-0.27 (0.96)	-0.3 (1.2)	0.642
BMI (kg/m^2)	25.7 (4.3)	25.6 (4.3)	0.196	-0.12 (0.45)	-0.5 (1.8)	25.7 (4.3)	25.6 (4.3)	0.226	-0.09 (0.31)	-0.3 (1.2)	0.696
BP Systolic	124 (14)	126 (16)	0.788	1.76 (9.1)	1.6 (7.7)	125 (14)	124 (13)	0.383	-0.57 (9.6)	-0.2 (7.3)	0.715
BP Diastolic	72 (12)	73.1 (11)	0.989	1.07 (6.3)	2.1 (9.4)	73 (11)	73 (9)	0.443	0.02 (8.2)	1.1 (13.1)	0.497
Pulse	69 (12)	73.1 (18)	0.106	4.3 (14.2)	6.6 (21.9)	66 (8)	61 (16)	0.180	-5.3 (14.3)	-7.8 (23.0)	0.016

Table 3.6 Changes in anthropometric characteristics following dietary intervention

All measurements are given as mean with SD in brackets; absolute measures were compared using paired t-test[1]: Changes (ΔOC vs ΔPr) could not be normalised and therefore were compared using Wilcoxon test[2]

3.5.3. Biochemical markers of MetS and CVD risk

3.5.3.1. Measurement of atherogenic markers

Mean values pre- and post- intervention of all lipid biochemistry measurements, with mean changes, standard deviations and significance for difference between groups, are shown in Table 3.7. Comparison of the baseline values for the prawn and OC groups revealed no significant difference in baseline measurements between interventions, and SPANOVA analysis revealed no overall significant difference between endpoint measurements between groups ($p > 0.12$ for all measurements, assuming equality of variances using Levene's test). No significant change between baseline and post-intervention measurements was observed for either prawns or OCs for cholesterol, TAG, HDL, sdLDL or ApoA1. For ApoB, a mean negative change (**Δ)** was observed between start and end of the prawn intervention (-7.8 mg/dL, $p = 0.047$), with a small increase during the OC intervention (2.4 mg/dL, $p = 0.274$); the difference in overall mean change (ΔOC vs ΔPr) in ApoB was significant ($p = 0.023$) using Wilcoxon rank test.

Marker	Prawn (n = 18)						OC (n = 18)						
	Pre-intervention		Post-intervention		[1]p	Mean Change (ΔPr)	Pre-intervention		Post-intervention		[1]p	Mean Change (ΔOC)	Significance ΔOC vs ΔPr [2]p
	Mean	SD	Mean	SD			Mean	SD	Mean	SD			
TAG (mmol/L)	1.06	0.32	1.23	0.50	.459	.03	1.29	0.50	1.25	0.42	.636	-.04	.715
TC (mmol/L)	5.15	1.00	4.98	0.91	.106	-.17	4.94	0.91	5.29	0.67	.979	0.05	.124
HDL-C (mmol/L)	1.50	0.34	1.44	0.31	.092	-.03	1.52	0.36	1.50	0.36	.582	-.02	.626
ApoA1 (mg/dL)	141.4	23.4	130.9	19.5	.114	-5.1	135.9	17.6	137.0	19.6	.876	0.48	.336
ApoB (mg/dL)	97.6	22.4	89.7	16.2	***.047***	-7.8	91.4	17.6	94.7	16.3	.274	2.4	***.023****
sdLDL-C (mmol/L)	16.1	12.1	15.8	8.4	.914	-0.3	15.39	6.61	17.5	6.6	.297	7.2	.145

Table 3.7. Lipid metabolism profiles from Autoanalyser techniques

*All measurements are given as mean and SD; absolute measures were compared using paired t-test[1]. Changes (ΔOC vs ΔPr) could not be normalised and therefore were compared using Wilcoxon test[2]**:** * non-equality of variance assumed (Levene)*

3.5.3.2. Lipoprotein profiling by DGUC

Lipoproteins were separated by ultracentrifugation for 18 of the 21 participants. The results of estimation of lipoproteins and subclasses are shown in Table 3.8. There was no significant difference in mean baseline measurements of any lipoprotein group ($p > 0.31$), nor in mean endpoint measurements of any lipoprotein type between interventions ($p > 0.11$). Differences between pre- and post-intervention measurements were not significant for any lipoprotein during either intervention. The change of each lipoprotein class from start to finish was also calculated. No significant difference was noted for any subclass for either intervention, as shown in the final column of Table 3.8.

	Prawn (n=18)				OCs (n = 18)				
	Pre-intervention	**Post-intervention**	**Change ΔPr**		**Pre-intervention**	**Post-intervention**	**Change ΔOC**		**ΔPr vs ΔOC**
	Mean (SD)	Mean (SD)	Mean Change	***P***	Mean (SD)	Mean (SD)	Mean Change	***p***	***p***
TC (DGUC) (mmol/L)	6.39 (1.41)	6.29 (1.48)	-0.09	.723	5.87 (1.04)	5.84 (1.01)	-0.07	.735	.938
VLDL-C (mmol/L)	0.63 (0.21)	0.67 (0.24)	0.04	.280	0.55 (0.25)	0.57 (0.21)	0.01	.459[1]	[2].656
LDL-C (mmol/L)	3.42 (0.94)	3.19 (0.78)	-0.23	.228	3.14 (0.79)	3.22 (0.80)	0.04	.731	.227
sdLDL-C (mmol/L)	0.98 (0.37)	0.98 (0.35)	<0.01	.974	1.01 (0.44)	1.11 (0.49)	0.12	***.050***	.187
lbLDL-C (mmol/L)	2.44 (0.74)	2.21 (0.60)	-0.23	.134	2.13 (0.61)	2.10 (0.63)	-0.08	.492	.425
HDL-C (mmol/L)	1.98 (0.59)	1.91 (0.43)	0.09	.701	1.84 (0.39)	1.73 (0.28)	-0.10	.527[1]	.429[2]
HDL/SDL ratio	0.87 (0.31)	0.89 (0.20)	0.03	.287[1]	0.93 (0.30)	0.88 (0.24)	-0.02	.616	.146[2]
VLDL % of total	10.1 (3.2)	10.9 (3.4)	0.84	.100	9.4 (3.4)	9.8 (3.6)	0.02	.906[1]	.443
LDL % of total	52.9 (6.6)	50.9 (6.7)	-1.98	.236	52.9 (6.5)	54.4 (5.7)	1.29	.073	.074
lbLDL (%of LDL)	71.4 (8.0)	69.3 (8.0)	-2.0	.237	68.1 (10.4)	65.7 11.1)	-3.1	.068[1]	.638

	Prawn (n=18)				OCs (n = 18)				
	Pre-intervention	**Post-intervention**	**Change ΔPr**		**Pre-intervention**	**Post-intervention**	**Change ΔOC**		**ΔPr vs ΔOC**
	Mean (SD)	Mean (SD)	Mean Change	***P***	Mean (SD)	Mean (SD)	Mean Change	***p***	***p***
sdLDL (% of LDL)	28.6 (8.0)	30.7 (8.0)	2.0	.237	31.9 (10.4)	34.3 (11.1)	3.1	.055	.638
HDL % of total	31.3 (7.0)	32.5 (7.0)	1.2	.403	31.9 (6.3)	30.0 (4.9)	-1.4	.144	.128
Total cholesterol	6.60 (1.18)	6.38 (0.72)	<0.01	.798	6.50 (0.94)	6.43 (1.04)	-0.1	.445	.605

TABLE 3.8: Lipoprotein classes and subclasses (DGUC)

All measurements are given as mean with SD in brackets; absolute measures were compared using paired t-test for normal distributions, except for [1]Wilcoxon rank (non-parametric for non-normal distribution),: Changes (ΔOC vs ΔPr) could not be normalised and therefore were compared using Wilcoxon test[2]

3.5.3.3. Correlations between plasma biochemistry and lipoprotein measurements

The data presented in sections 3.5.3.1.and 3.5.3.2 were further investigated for correlation between a) different biochemical measurements at baseline and b) between measurements of lipoproteins obtained by autoanalyser and DGUC techniques. These results are presented in Table 3.9.

A strong correlation (r > 0.5) was observed for TC and HDL-C estimated from ultracentrifugation and autoanalyser techniques (0.798, $p < 0.001$) and (0.527, $p = 0.001$) respectively. Strong positive correlations (rho > ρ0.5) were also noted for TAG with VLDL-C and sdLDL-C measurements (both by DGUC and autoanalyser technique). TC measurements also showed strong correlation with those for ApoB, LDL-C, sdLDL-C and lbLDL-C, as did measurement of HDL-C (by autoanalyser technique or DGUC) with ApoA1, and ApoB with sdLDL-C, VLDL-C, and lbLDL-C.

Moderately positive correlations (rho = 0.3 to 0.49) were noted for TC with HDL, ApoA1, sdLDL-C and VLDL-C, for HDL-C with lbLDL-C, for ApoA1 with ApoB, HDL/SDL and HDL-C as a percentage of total lipoprotein, and for ApoB with VLDL-C.

Strong negative correlations (r > -0.50) were noted for plasma TAG with HDL-C, ApoA1, HDL-C, HDL-C (as percentage of TC), lbLDL-C (as a percentage of LDL-C), for TC with HDL/SDL ratio and HDL (as percentage of total), and for ApoB with HDL/SDL ratio, HDL as percentage of total. A moderate negative correlation (r between -0.30 and -0.49) was observed for HDL-C with LDL-C and also with sdLDL-C when these were expressed as a percentage of total. Although absolute values for lipoprotein measurements differed between methods used, the correlation between values was very strong ($p < 0.001$).

The data were also inspected for correlation between baseline measurements and changes (delta), using the Spearman correlation coefficient (non-parametric): results are shown in Table 3.11. There was a significant negative correlation between baseline measurements and Δ during intervention for TC, ApoB, sdLDL-C, LDL-C, lbLDL-C and the HDL/SDL ratio ($p < 0.001$in all cases) and a less significant correlation for plasma TAG ($p = 0.011$). Correlations for other measurements (HDL-C, ApoA1, VLDL-C and sdLDL-C by DGUC) were not significant.

Use of ANOVA or ANCOVA techniques to compare between-subject effects was limited by the non-normality of some of the data, in particularly plasma TAG and HDL-C

measurements. It was not possible to normalise data for baseline-endpoint differences (Δ measurements).

	Positive correlation			Negative Correlation		
		rho	P		rho	P
Plasma TAG	VLDL	.530	.001	HDL	-.576	<.001
	sdLDL(DGUC)	.549	.001	ApoA1	-.451	.004
	VLDL (%)	.521	.002	HDL (DGUC)	-.361	.036
	sdLDL (%) (DGUC)	.632	<.0001	lbLDL (%)	-.632	<.001
				HDL (%)	-.486	.004
TC (autoanalyser)	HDL	.421	.006	HDL/sdLDL ratio	-.553	<.001
	ApoA	.409	.007	HDL (%)	-.544	.001
	ApoB	.803	<0.001			
	sdLDL	.309	.047			
	TC (DGUC)	.798	<0.001			
	VLDL	.478	.003			
	LDL	.872	<0.001			
	sdLDL (DGUC)	.558	<0.001			
	lbLDL	.711	<0.001			
	LDL (%)	.572	<0.001			
HDL (autoanalyser)	ApoA1	.843	<0.001	LDL (%)	-.334	.050
	HDL (DGUC)	.527	.001			
	lbLDL (%)	.383	.023	sdLDL (%)	-.383	.023
	HDL (%)	.341	.045			
ApoA	ApoB	.357	.020			
	HDL (DGUC)	.585	< 0.001			
	HDL/SDL ratio	.341	.042			
	HDL%	.351	.036			
ApoB	sdLDL	.508	.001	HDL/SDL	-.517	.001
	TC (DGUC)	.680	<0.001	HDL %	-.580	<.001
	VLDL	.449	.006			
	LDL	.795	<0.001			
	sdLDL (DGUC)	.645	<0.001			
	lbLDL	.598	<0.001			
	LDL (%)	.604	<0.001			

Table 3.9: Correlations between biochemical and lipoprotein measurements

(Spearman correlation used for all comparisons)

	Correlation coefficient	Spearman's rho
TAG	-.398	.011
TC	-.526	.000 *
HDL-C	-.291	.065
ApoA1	-.252	.108
ApoB	-.564	.000 *
sdLDL-C	-.573	.000 *
VLDL-C	-.304	.076
sdLDL-C (DGUC)	-.271	.110
LDL-C	-.574	.000*
lbLDL-C	-.599	.000 *
HDL-C	-.291	.085
HDL/SDL ratio	-.518	.001*

Table 3.10. Correlation between baseline value and change for biochemical and lipoprotein measurements

(Spearman rank significant at 0.01 level (2-tailed).*

3.5.4. Impact of baseline measurements on outcome

3.5.4.1. Baseline BMI and changes in markers of CVD risk

To determine whether there might be any impact of baseline BMI on the outcome of either dietary intervention, participants were subdivided by BMI, and comparisons of post-intervention measurements and changes in biochemical/lipoprotein measurements were repeated. Significant changes are tabulated in Table 3.11.

Pr intervention: the only significant difference in ΔPr was for plasma TAG, which showed a mean increase of 0.30 mmol/L (SD = 0.51) for participants with BMI ≤ 24.9, and a mean decrease of 0.17 mmol/L for participants with BMI ≥ 25.00 *(p* = 0.028). This represented a mean increase of 32.3% (SD 45.2) from baseline value for the lower BMI group, compared with a mean percentage decrease of -7.7% (SD 22.3%) for the higher BMI group (*p* = 0.033). There were no other significant differences between ΔPr values between subgroups during the prawn intervention.

OC intervention: By contrast, differences between ΔOC of BMI subgroups were observed, as follows:

ΔApoB: A mean increase of 6.09 mmol/L (SD = 9.38) for BMI ≤ 24.9, and a mean decrease of 1.70 mmol/L (SD = 8.70) for BMI ≥ 25.0, (*p* = 0.064). When considered in terms of percentage changes, the difference between BMI groups was significant, (+8.16% (SD = 10.86) and -1.55% (SD = 8.51) respectively, (*p* = 0.036)).

ΔVLDL: a mean increase of 0.08 mmol/L (SD 0.97) for BMI < 24.9, compared with a decrease of 0.07 mmol/L for BMI > 25.0, (p = 0.091). As for ApoB changes, the

difference between BMI groups achieved significance when calculated as a percentage of the baseline value, (+20.79 % (SD 23.54) for the lower BMI group, compared with -12.31% (SD 40.26) for the higher BMI group, (p = 0.049). ***ΔVLDL*** as a percentage of total lipoprotein cholesterol showed a mean increase of 2.22% (SD 3.43) for the lower BMI subgroup compared with a mean decrease of 2.18% (SD 3.39) for the higher BMI subgroup (p = 0.015).

ΔHDL: a mean decrease of 0.32 mmol/L (SD 0.44) or 13.47% (SD 18.67) for the lower BMI group, compared with an increase of 0.12 mmol/L (7.27%) for the higher BMI group (p = 0.007 and 0.005 respectively). ΔHDL calculated as a percentage of total lipoprotein cholesterol also showed a significant difference between the two BMI subgroups, with a mean decrease of 3.78% (SD 2.63) for the lower BMI subgroup, and a mean increase of 0.94% (SD 3.69 for the higher BMI subgroup (p = 0.007). The differences in HDL and sdLDL for the two BMI subgroups were also reflected in a significant difference between groups in the ΔHDL/sdLDL ratio (p = 0.043).

The higher BMI group showed a beneficial change in plasma TAG compared with the lower BMI group during the Pr intervention. For the OC group, the significant differences in lipoprotein subclasses and ApoB also indicate a small improvement in profile for the higher BMI group relative to the lower BMI group, or may be indicative of a small increase in atherogenic risk in this latter group.

Measurement	Prawn			OC		
	BMI≤24.9	**BMI≥25.0**	***p***	**BMI<24.9**	**BMI>25.0**	***p***
Δ TAG (mmol/L)	0.30 (0.51)	-0.17 (0.38)	***0.028***	0.08 (0.30)	-0.17 (0.48)	0.168
Δ TAG (% of baseline)	32.3 (45.2)	-7.7 (22.3)	***0.033***	11.55 (30.40)	-6.44 (33.51)	0.213
ΔHDL (mmol/L)	-0.11 (0.17)	0.02 (0.14)	0.067	-0.02 (0.18)	-0.01 (0.10)	0.916
ΔApoB (mg/dL)	-1.39 (13.45)	-12.67 (18.25)	0.135	6.09 (9.38)	-1.70 (8.70)	0.064
ΔApoB (%of baseline)	1.48 (19.00)	-10.23 (17.06)	0.136*	8.16 (10.86)	-1.55 (8.51)	***0.036***
ΔsdLDL (AA) (mmol/L)	4.54 (10.70)	-3.95 (13.84)	0.227*	3.04 (5.14)	11.79 (27.25)	0.342**
ΔsdLDL (% of baseline)	87.05 (126.52)	2.68 (58.94)	0.093 **	24.06 (45.49)	1.29 (36.29)	0.267
ΔVLDL (mmol/L)	0.09 (0.16)	0.01 (0.15)	0.291	0.08 (0.97)	-0.07 (0.23)	*0.091*
ΔVLDL (% of baseline)	16.65 (30.48)	3.01 (25.92)	0.324	20.79 (23.54)	-12.31 (40.26)	***0.049***
ΔsdLDL (DGUC) (mmol/L)	0.14 (0.25)	-0.09 (0.29)	0.104	0.06 (0.21)	0.19 (0.27)	0.285
ΔsdLDL (% of baseline)	18.8 (31.1)	-6.87 (23.15)	0.071	12.52 (24.65)	18.61 (24.44)	0.606
ΔHDL (DGUC) (mmol/L)	0.10 (0.56)	0.08 (0.42)	0.946	-0.32 (0.44)	0.12 (0.07)	***0.007****
ΔHDL (DGUC) (% of baseline)	9.92 (33.00)	2.38 (18.55)	0.541	-13.47 (18.67)	7.27 (3.89)	***0.005****
ΔHDL/SDL ratio	0.03 (0.22)	0.02 (0.29)	0.938	-0.11 (0.13)	0.07 (0.20)	***0.043***
ΔVLDL % of total lipoprotein	0.91 (2.48)	0.80 (1.87)	0.917	2.22 (3.43)	-2.18 (3.39)	***0.015***
ΔHDL % of total lipoprotein	-0.03 (4.02)	2.01 (7.04)	0.498	-3.78 (2.63)	0.94 (3.69)	***0.007***

Table 3.11: Changes in biochemical measurements and lipoprotein subclasses stratified by BMI

(Non parametric test for significance, ** Levene test <0.05, equality of variances not assumed)*

3.5.4.2. ApoB/ApoA1 ratio

The ApoB/ApoA1 ratio was estimated for both prawn and OC interventions. No significant differences were observed between mean values for the two groups, and the mean values within groups also showed no significant differences. Estimation of the change in ApoB/ApoA1 ratio was also calculated for each participant, and a comparison of these results is shown in Figure 3.3 and in Table 3.12. One individual (case 11/32) showed an increase in ApoB/ApoA1 ratio during OC (but not Pr) which was > 2SD from the mean, although measurements for ApoA1 and ApoB individually were within 2SD of the mean for both baseline and post-intervention measurements. TC following OC (but not prawn) intervention was also more than 2SD above the mean for this individual. This may indicate some abnormality in cholesterol or lipidaemic metabolism compared with other members of the cohort.

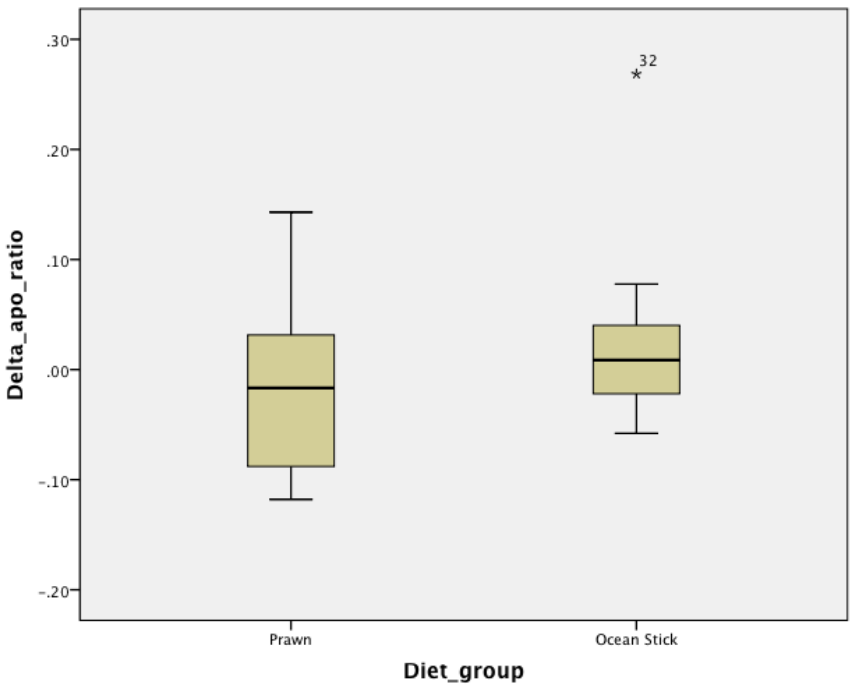

Figure 3.3 Comparison of changes in ApoB/ApoA1 ratio between interventions

Diet Group	Baseline ApoB/ApoA1	Endpoint ApoB/ApoA1	Δ ApoB/ApoA1
Prawn	0.698 (0.16)	0.683 (0.131)	-.0213 (.073)
OC	0.675 (0.135)	0.693 (0.140)	0.018 (0.070)
p *(between groups)*	[1]0.613	[1]0.815	[2]0.081

Table 3.12: Changes in ApoB/ApoA1 ratio

(*mean with SD in brackets; [1]paired t-test, [2]Wilcoxon test for non-parametric distribution*)

3.5.5. FOOD INTAKES

Eighteen participants completed the initial food record, with 11 completing during the prawn intervention and 14 during the OC intervention. Significant outliers for specific nutrients (>2SD away from the mean) were excluded from overall analysis.

3.5.5.1. General observations

Mean nutrient intakes as calculated from the information provided by the participants are shown in Table 3.13. Nutrient intakes were normal in distribution, with the exception of energy intake (both in kcal and as a percentage of RNI) post intervention for the OC period.

The contribution of macronutrients to overall energy intake showed differences between baseline and endpoint of interventions, as shown in Table 3.14. Paired *t*-tests between baseline and endpoint showed a significant decrease in carbohydrate between baseline and end of the OC intervention (p = 0.047), although change in carbohydrate as a percentage of total energy was not significant (p = 0.44). Intakes of fibre (p = 0.01), non-starch polysaccharide (p = 0.001), sugars (p = 0.015) and non-milk extrinsic sugars (p = 0.032) were all significantly lower at the end of the OC intervention; cholesterol intake also decreased between start and end of this intervention, from a daily mean of 331 mg (SD 223) to 275 mg (SD 92), (p = 0.01).

By contrast, significant changes noted during the prawn intervention were an increase in protein both as a percentage of RNI and of total energy (p = 0.024 and 0.021 respectively), and in mean cholesterol intake from 331 to 794 mg (SD 186, p <0.001). Alcohol intake was not significantly altered between baseline and the end of either intervention, although the number of participants reporting zero alcohol intake rose from 8 at baseline to 12 (OC) and 15 (Pr).

Differences in mean nutrient intake between the diaries completed at the end of the OC and prawn intervention accord fairly closely with the differences in nutrient composition of the daily OC and prawn portions for energy, protein, fat and carbohydrate. The mean intake of sugars was higher by about 8g per day for the prawn than for the OC records, whereas sugar intake due to the OC would have been approximately 8g higher than the sugar intake due to prawns.

	Baseline (n = 18)		Post intervention OC (n = 14)			Post intervention Pr (n= 11)			Pr/OC endpoints
	Mean	**SD**	**Mean**	**SD**	**[1]p =**	**Mean**	**SD**	**[1]p =**	**[2]p =**
Energy (kcal)	2205	458	2099	630	.207	1940	393	.457	.661
Energy as % EAR	88.1	18.2	83.9	24.4	.196	78.6	15.3	.453	.701
Protein (g)	86.4	23.0	92.2	27.6	.098	109.1	21.7	***.020***	.052
Protein as % RNI	***155.1***	***42.4***	***167.4***	***49.2***	***.099***	***199.7***	***37.1***	***.020***	***.024***
Protein as % energy	15.8	3.3	19.2	4.9	.086	23.7	4.3	***.007***	.021
Carbohydrate (g)	277.0	77.3	233.2	68.8	***.047***	208.1	52.9	.083	.324
Carbohydrate as % energy	50.2	8.7	46.3	6.7	.441	42.9	6.7	.039	.259
Fat (g)	76.4	21.7	66.1	26.1	.168	63.7	18.2	.810	.870
Fat as % DRV	76.7	21.5	61.5	18.6	.132	64.7	17.8	.798	.601
Fat as % energy	31.2	5.6	29.8	3.1	.100	29.4	4.6	.841	.460
Saturated fatty acids (g)	24.6	8.8	20.7	10.0	.110	18.9	4.7	.187	>0.99
SFA as % DRV	78.4	27.5	66.1	31.6	.105	61.3	15.2	.188	>0.99
PUFA (g)	***15.1***	***6.1***	***9.4***	***3.5***	***.161***	***13.5***	***7.9***	***.570***	***.147***
PUFA as % DRV	***81.6***	***32.4***	***51***	***19.6***	***.158***	***74.0***	***43.1***	***.579***	***.173***
MUFA (g)	25.3	8.0	22.3	10.8	.276	21.2	5.9	.912	.511
Cholesterol (mg)	***331***	***223***	***275***	***91.7***	***.010***	***794.1***	***186.0***	***.000***	***.000***
Fibre (g)	17.3	5.7	13.1	2.9	***.008***	15.7	5.0	.410	.213
NSP (g)	13.8	4.1	11.2	3.2	***.001***	11.2	3.4	.121	.784
NSP as % DRV	76.3	22.8	61.5	17.7	***.001***	61.5	19.0	.116	.784

	Baseline (n = 18)		Post intervention OC (n = 14)			Post intervention Pr (n= 11)			Pr/OC endpoints
	Mean	**SD**	**Mean**	**SD**	**[1]*p* =**	**Mean**	**SD**	**[1]*p* =**	**[2]*p* =**
Sugars (g)	117.6	38.8	87.7	30.3	***.015***	95.3	31.0	.105	.469
NMES (g)	81.9	38.0	56.4	18.0	***.032***	62.5	31.9	.237	.706
NMES as % DRV	112.7	51.6	78.3	25.1	***.031***	87.2	43.6	.231	.643
Alcohol*	22.6	12.5	29.3	18.6	.302	33.7	18.8	.710	.723
Alcohol as % energy	7.1	4.5	9.9	4.5	.138	12.0	5.9	.445	.409

Table 3.13 Dietary intake data

*(*Only those with positive alcohol consumption were recorded (n = 13, 9 and 6 respectively)*

[1]Significance – paired t-test between initial and final records. [2]Wilcoxon non-parametric test for non-normal distribution.

3.5.5.2. Intake of n-3 LCPUFA and cholesterol

The key nutrients of interest were n-3 LCPUFA and cholesterol. Intake of cholesterol was derived from the information provided in the food diaries and assuming full compliance with the supplementation of Pr or OC. Overall intake of PUFA (n-6 plus n-3), excluding Pr or OC intake, was not significantly different in total weight or as a percentage of DRV between baseline and end of either intervention. It was not possible to make an accurate overall assessment of n-3 LCPUFA (total or LCPUFA) intake based on food intake records, using the Windiet program version available at the time. The decision was therefore taken to make an approximation based on expected intakes of PUFA. It has been suggested based on population surveys that the baseline n-3 PUFA intake of these individuals would be of the order of 100-200mg per day (Calder, 2004), although there is little data to confirm this. Calder suggested in this review that typical intakes of LCPUFA for adult males in the UK were approximately: 13.5g LA, 1.7g ALA, 0.3g ArA and 0.25g DHA+EPA (totalling 15.75g). This corresponds reasonably well with the mean baseline PUFA intake from the food diaries of the participants (15.1g), which would have provided DHA+EPA at approximately 1.6% of total PUFA. Based upon this, assuming daily n-3 LCPUFA intake from OC to be <20 mg, and from prawns to be 650mg, estimates of intake and the changes for the two groups were derived, as shown in Table 3.14.

		OC (n = 14)	**Prawn (n = 11)**	**OC (n = 14)**	**Prawn (n = 11)**
	Pre	**Post**	**Post**	**ΔOC**	**ΔPr**
Mean daily intake (mg)	242	169	856	-80	664
SD	97	55	135	90	148
Significance Pre/Post intervention		0.016	<0.001		
Significance between groups				<0.001	

Table 3.14: Estimates of n-3 LCPUFA intake and changes

The mean daily intake was based on records from food intake diaries, assuming n-3 LCPUFA to be 1.6% of recorded PUFA intake, and with calculated additional intake for prawn intervention assuming full compliance.

3.5.5.3. Changes in food intake

Difference in nutrient intake between start and finish of each intervention was calculated for each individual who completed both baseline and final food records, and the mean of this difference then calculated. Mean changes in intake for macronutrients

were calculated as total intake, percentage of daily recommended intakes (RNI, EAR or DRV as appropriate) and as percentage of total energy intake for protein, carbohydrate, total fat and alcohol. These are shown in Table 3.15.

Both groups showed a mean reduction in energy intake between start and finish of the intervention (171 vs 199 kcal, 5.5% and 5.4% of baseline value for Pr and OC respectively), but the difference in changes was not significant between interventions (p = 0.928). Protein intake increased both as percentage of RNI and as percentage of total energy intake for those consuming prawns but less so for the OC intervention; this difference was significant as measured by *t*-test, (p = 0.038 for total protein and 0.046 for protein as percentage of RNI). Differences between interventions for other nutrients were not significant, except for total PUFA, where an increase during Pr intervention is contrasted with a decreased during OC intervention (p = 0.048), and the highly significant changes in cholesterol and n-3 LCPUFA intake (as described above).

	Prawn (n = 8)				OC (n= 12)					
	Mean change	**SD**	**% change**	**SD**	**Mean change**	**SD**	**% change**	**SD**	***p* mean change**	***p* % change**
Energy kcal	-171.5	623.1	-5.5	26.6	-199.2	684.4	-5.4	32.0	0.928	0.993
Energy as % of EAR	-7.0	25.2			-8.3	27.4			0.919	
Protein (g)	31.3	28.8	45.1	47.8	2.6	27.7	6.7	35.2	***0.038***	0.052
Protein (%RNI)	57.1	52.1	45.7	48.3	4.6	52.7	7.3	36.8	***0.046***	0.065
Protein as % of energy	7.2	6.2			2.2	4.1			***0.038***	
Carbohydrate (g)	-70.3	98.1	-18.8	32.1	-30.2	97.9	-6.0	36.9	.382	.436
Carbohydrate as % energy	-7.9	9.6			-1.3	5.7			.065	
Fat (g)	-2.5	28.1	1.4	45.9	-12.5	25.9	-10.9	30.7	0.42	0.482
Fat % of DRV	-2.6	28.2	1.6	46.0	-12.8	25.9	-10.9	30.6	.419	.472
Fat (% energy)	0.5	7.5			-2.1	4.7			.343	
SFA	-4.46	8.7	-35.6	53.6	-2.6	9.3	-22.1	53.2	.654	.588
SFA (% DRV)	14.5	28.2	14.2	36.4	-8.5	29.8	-0.4	49.1	.658	.508
PUFA (g)	1.9	9.1	21.0	83.6	-5.3	6.2	-28.1	31.1	***.048***	.078
PUFA (% DRV)	10.0	49.2	21.1	84.2	-29.0	33.4	-28.3	31.3	***.048***	.078
MUFA (g)	0.36	9.0	7.2	40.4	-4.7	11.7	-9.7	46.1	.312	.410
Cholesterol (mg)	553.5	169.1	258.1	160.0	-69.6	192.5	3.75	51.7	***<.001***	<.001
Dietary fibre (g)	-1.9	6.2	-0.7	46.2	-5.6	4.9	-24.7	22.0	.156	.134
NSP (g)	-2.3	3.7	-12.1	32.4	-3.5	3.2	-19.2	18.2	.446	.538

	Prawn (n = 8)				OC (n= 12)					
	Mean change	**SD**	**% change**	**SD**	**Mean change**	**SD**	**% change**	**SD**	***p* mean change**	***p* % change**
NSP (% DRV)	-13.0	20.5	-12.6	33.1	-19.4	18.0	-19.4	18.4	.470	.559
Sugars (g)	-28.0	42.5	-19.1	30.4	-21.6	40.0	-12.7	32.7	.737	.667
NMES (g)	-21.1	46.4	-18.1	42.8	-19.3	34.4	-14.3	36.4	.921	.857
NMEs (% of DRV)	-29.3	63.5	-18.2	42.8	-26.3	47.1	-14.7	36.5	.905	.846
Alcohol (g)	2.6	12.7	10.4	39.9	5.7	15.6	26.7	64.8	.741	.662
Alcohol (% of energy)	0.7	1.7			2.5	3.8			.421	

Table 3.15: Changes in nutrient intake between dietary interventions

(*t*-test assuming equality of variances (Levene test, p> 0.05 in all cases)

3.6. DISCUSSION

The aim of this pilot study was to determine whether prawns as a source of n-3 LCPUFA might present a risk to cardiovascular health because of the associated increase in dietary cholesterol. The participants were ostensibly healthy males between 19 and 67 years of age; however, of the 21 who completed the study, only 9 fell within the recommended range for a normal BMI (between 19 and 24.9). For 9, the BMI was between 25 and 30, and for 3 in excess of 30 kg/m^2. Benefits in dietary management might be more apparent for those with the most extreme baseline measurements; conversely, recent work (Tananescu *et al.,* 2004; Houston *et al.,* 2011) has pointed to a small increased relative risk of CVD associated with significant egg intake in individuals at risk of T2D.

The choice of OC as the control group for the dietary comparison provided a weight-for-weight foodstuff containing no cholesterol or n-3 LCPUFA, but the two foods were different in other ways. OC, derived from Asian cuisine, are manufactured from white fish combined with starch-based binding ingredients, flavoured to resemble crabmeat. A daily 'portion' of 225 g prawns provided approximately 300 mg dietary cholesterol and an estimated 6-700 mg DHA plus EPA, compared with only trace amounts of either from a 225 g portion of OC. However, a 225g portion of OC provided approximately twice as many calories (259 kcal compared with 140), approximately 35g carbohydrate compared with less than 1 g (0.23 g) for a prawn portion, but only half as much protein (approximately 20 g compared with 40 g). Daily fat intake from prawns and OCs was low (1.6 g and 3.6 g respectively), and apart from the difference in n-3 LCPUFA, similar in the context of total daily intake. The daily dietary contribution to salt intake was 1.1 g for a prawn portion and 1.9 g for OCs, both of which represent a significant intake compared with a recommended upper limit in the UK of 6 g per day (18% and 32% respectively). Total salt intake was not estimated in this study.

An alternative choice to OCs could have been filleted white fish, which would be high in protein, low in carbohydrate and also in salt (typically 20-22% w/w protein, < 1% carbohydrate and 0.2% salt), but this would have presented practical issues, making it more complicated to ensure a measured portion on a daily basis for a 4-week intervention study of this nature, in free-living participants.

3.6.1. Markers of CVD risk

3.6.1.1. Overall changes in CVD markers

No significant difference between intervention groups was noted for any anthropometric measures. There was a significant difference in the mean pre-post pulse rate, with an increase in the OC group compared with a small decrease in the prawn group (p = 0.016). This decrease of 8% in the mean pulse rate over an intervention period of 28 days compares favourably with that of 8.3% reported after 45 days intervention with 3g per day DHA (Kelley *et al*., 2007).

Mean TC at the start of prawn and OC interventions were 5.15 mmol/L (SD = 1.00) (range 3.1-7.0) and 4.94 mmol/L (SD = 0.91, range 3.4-7.1) respectively. Plasma TAG at the start of each intervention were 1.06 mmol/L (SD = 0.32, range 0.64-2.96) and 1.29 mmol/L (SD = 0.50, range 0.57-2.32) respectively. These figures compare with upper levels of 6.5 mmol/L for TC and 2.3 mmol/L for TAG for normolipidaemic individuals, as defined by Cleeman *et al*. (2001) and which were the criteria for inclusion in the study. Even at the start of the interventions there were one or two individuals in each case who had fulfilled the screening criteria but for whom TC or TAG were already higher than the inclusion criteria. This may represent failure to comply with the 8-hour fast prior to study visits.

A small increase in plasma TAG was noted during the Pr intervention; stratification by BMI revealed a significant difference between the groups. There was a small decrease in participants with BMI >25.0 (-0.17 mmol/L, SD 0.38, -7.7%, SD 22.3%), compared with a small increase for those participants with normal BMI (0.30 mmol/L, SD = 0.51 and 32.3%, SD = 45.2). A similar decrease was observed for the higher BMI group during the OC intervention, but the difference between subgroups did not reach significance.

3.6.1.2. Relation to n-3 LCPUFA intake

The n-3 LCPUFA content of the 225 g prawns is approximately 600mg, a level which has been reported to favourably influence CVD risk (Calder, 2004, Kris-Etherton *et al*., 2002; 2004). The estimated mean increase in daily intake of 664 mg n-3 LCPUFA for 4 weeks in this study was associated with a small decrease in plasma TAG (mean - 0.17 mmol/L, -7.7% of baseline) in those individuals with a BMI in excess of 25 kg/m^2 (p = 0.033). No other markers of CVD risk were significantly altered in either the normal or high BMI subgroup. This compares with the meta-analysis of 21

randomised controlled trials by Balk *et al*. (2006), which reported a positive effect of n-3 LCPUFA at an intake of 900 mg per day with a possible reduction up to 0.31 mmol/L. This review also reported an increase in HDL-C (0.04 mmol/L) and LDL-C (0.16 mmol/L) at n-3 LCPUFA intakes between 45mg and 5000 mg per day (Wang *et al*., 2006). Mean HDL-C in the present study was increased by 0.09 mmol/L, although this did not reach significance, but mean LDL-C decreased by 0.23 mmol/L. Kelley *et al*. (2007) in their study of DHA supplementation (3000 mg/day), reported a reduction in fasting TAG, TAG:HDL ratio, an increase in fasting LDL-C, LDL-C/HDL-C, but no change in fasting HDL-C. Maximum effects were observed within 45 days from the start of this high-dose intervention (3g DHA per day) and maintained for the next 45 days for these and other lipoprotein markers. Smith *et al*. (2011) however, compared intakes of DHA (1500 mg) or EPA esters (1860 mg) with corn oil over an 8 week period in a group of 16 elderly subjects as part of an investigation into muscle protein synthesis: after 8 weeks supplementation they found no significant change in fasting TAG levels (mean 0.92 mmol/L, 0.95 mmol/L respectively). The potential reduction in TAG estimated by Balk *et al.* was larger than that seen for the Pr intervention in this study, and the level of DHA supplementation reported by Kelley was approximately 4 times that of the combined DHA/EPA intake in this study. Up to 65% of n-3 LCPUFA in prawns may be present as PL rather than TAG (Tou *et al*., 2007). As discussed in Chapter 4, it might be expected that more efficient absorption of n-3 LCPUFA in this form would be reflected in a more beneficial effect on CVD markers, but this was not clearly observed in the study described in this chapter.

Changes in CVD risk markers and lipoprotein types and subclasses also showed a difference according to starting BMI during the OC intervention, with the higher BMI subgroup showing more beneficial changes in lipoprotein profile. An increase in VLDL-C (21% of baseline) for the lower BMI group contrasted with a decrease of 12.31% of baseline for the group with BMI > 25 kg/m^2 (p = 0.049), while an increase of 7.27% of baseline for the high BMI group contrasted with a decrease of 13.47% of baseline for the group with normal BMI (p = 0.005). The changes in VLDL-C and HDL-C as a percentage of total lipoprotein also reached significance between the two BMI groups for the OC intervention (p = 0.049 and 0.005 respectively). This may be inferred to indicate an overall improvement in cardiometabolic risk profile during the OC intervention for participants with a BMI in excess of 25 kg/m^2. There is no published data on the possible beneficial effects of OCs on CVD markers, although there have been reports that fish protein may play a role. The amount of protein in the OC supplementation was, however, only half that of the prawns, so it is suggested that this

would not have contributed to the effect described here. There were significant changes in intake of macronutrients during the OC intervention, which may have confounded any neutral effect of this supplementary food.

There was no overall difference between Pr and OC interventions on other markers of dyslipidaemia apart from ApoB, which showed a small mean decrease (-7.8 mmol/L, p = 0.047) during the Pr intervention compared with a small mean increase (2.4 mmol/L) following OC intake. The difference between mean changes was also significant (p = 0.023). Stratification into groups according to initial BMI revealed some further differences. In the OC intervention, an increase in mean ApoB for the higher BMI group (Δ = 6.09 mmol/L) and a small decrease for the lower BMI group (Δ = - 1.7 mmol/L) was noted; this was significant when expressed as a percentage relative to baseline measurement, (p = 0.036).

The potential mechanisms of n-3 PUFA in improving CVD risk profile are discussed in Chapter 5. Mori & Beilin (2001) suggested that EPA and DHA are comparable in respect of TAG reduction. The apparent relative reduction in CVD risk of the lipoprotein profile during OC intervention for the high BMI group compared with the normal BMI group is curious, and there is no easily obvious explanation, although this is discussed later in this chapter in the light of data on nutrient intakes. It is inferred from the results presented here that there is no disbenefit from prawn intake on overall lipidaemic profile.

The ApoB/ApoA1 ratio has been implicated as a significant index of CVD risk (Walldius *et al*., 2004; Walldius & Jungner, 2005). The changes in ratios during the two interventions (Table 3.15) indicate a small decrease in the ratio during the prawn intervention, compared with a small increase during the OC intervention, reflecting the changes in the individual apolipoproteins; the difference between mean changes (p = 0.081) did not reach significance, despite a significant difference in ApoB.

3.6.1.3. Relationship with cholesterol intake

The cholesterol content of 225 g prawns (295mg) is approximately equivalent to that of a large egg yolk (Table 3.2). Both eggs and prawns are good sources of important nutrients (high quality protein including essential amino acids). The evidence for a direct and specific link between dietary cholesterol and CVD risk has been limited and inconsistent, and has mainly focussed on egg consumption (Eckel, 2008). Studies where a link has been reported (Weggemans *et al*., 2001; Puddu *et al*., 2011; Sakurai

et al., 2011) have not eliminated possible contributions from confounding factors (Djousse & Gaziano, 2009). In an extensive review of the relationship between dietary cholesterol intake and CVD, Kanter *et al*. (2012) observe that restriction of dietary cholesterol may be associated with increased intake of alternative detrimental nutrients such as simple sugars, refined carbohydrates and saturated or trans fats; interestingly, in this context, dietary intake records indicated a higher intake of some carbohydrates and sugars following Pr than for OC intervention. It could be speculated that energy-dense salad dressings or sauces may have contributed to this.

Djousse & Gaziano (2008) derived an adjusted hazard ratio of 1.41 for all cause mortality in adult males eating more than one egg per day, although previous studies (Dawber *et al*., 1982; Nakamura *et al*., 2004) found no association. More specifically, and of direct relevance to the study described here, associations between egg consumption, CVD risk and diabetes have been noted in other reports. Nakamura *et al*., (2004) reported an increase in CVD risk in a cohort of diabetic individuals. An increased risk (RR= 1.37) of a cardiovascular incident in T2D women (n = 5672) was calculated for an increase of 200 mg cholesterol per 1000 kcal dietary intake (Tanasescu *et al*., 2004). (Table 3.1). Higher cholesterol intake was also associated with increased BMI, which appeared to be specific for egg intake rather than overall cholesterol. Later studies by the same group also suggested that significant egg intake (3 eggs per day) was associated with an increased relative risk of developing T2D (Tanasescu *et al*., 2006; Houston *et al*., 2011), although other factors such as increased energy intake and body weight may also play a part in this pattern. Houston *et al.* (2011) reviewed dietary intake over a period of 9 years and reported a significant increase in CVD risk associated with egg consumption (p = 0.02), in older people with T2D but none for those without T2D. The association with total dietary cholesterol and T2D was less significant (p = 0.08). A further meta-analysis of eight retrospective studies (Rong *et al*., 2013) covered 263,938 participants for CHD and 210,404 participants for stroke, over periods of between 8 and 22 years. A total 5,847 cases of CHD and 7,579 of stroke were recorded; no overall link between egg consumption and either was found, but subgroup analysis indicated a possible link between egg intake and risk for those with diabetes (RR = 1.54, p = 0.01). The relative risk of coronary heart disease from one egg per day was 0.99 (95% confidence interval 0.85-1.15,p = 0.88), while that for stroke was 0.91 (p = 0.1). A reduced risk of hemorrhagic stroke (25%, p = 0.04) was reported for those with higher egg consumption. A positive association between dietary cholesterol and risk of stroke (RR = 1.2 for 300 mg per day, 1.17 for 255 mg/day) was also reported from a study of 34,670 women in Sweden over

a 10-year period (Larsson *et al*., 2012). A review of 17 studies (556 subjects) investigating the effect of dietary cholesterol on the ratio of TC to HDL-C ((Weggemans *et al*., 2001) reported that an increase of 100mg dietary cholesterol per day is associated with a similar increase in HDL-C and TC (0.008 mmol/L). Overall it appears that while there may be some individuals (up to a quarter) in whom dietary cholesterol may increase LDL-C, HDL-C is also increased, so that the LDL/HDL ratio is maintained at a fairly constant level with little attendant increase in CVD risk (Fernandez, 2012). In comparing these reports, it should be borne in mind that the meta-analyses will have included data from the individual intervention or food frequency studies described.

In the study reported here, an increase in cholesterol consumption (from prawn intake) at the level of 1 egg per day was associated with a small increase in HDL-C (0.09 mmol/L) and a decrease in LDL-C (0.23 mmol/L), but the differences between pre- and post- supplementation were not significant, and the difference between Pr and OC interventions did not reach significance for either parameter. This is in concurrence with the findings of Harman *et al*. (2008): a study of 45 individuals comparing egg intake (2 per day) with no egg intake over a 12 week period, combined with an energy restricted diet, did not reveal any significant difference in LDL-C, plasma lipoproteins, or LDL particle size at the end of the intervention. The change in TC reduction of 0.09 mmol/L in the investigation reported in this thesis was not significant, and not greatly different from the very small increase of 0.008 mmol/L reported by Weggemans *et al.* (2001).

Beveridge, in 1964, suggested that CVD risk associated with cholesterol intake may be less clear-cut than the association with raised LDL-C from intake of SFA or TFA (Beveridge, 1964). Beveridge showed that intakes of dietary cholesterol up to 600 mg per day (2-3 times that used in the present study) resulted in an increase in TC, but that supplementation above this did not result in any further increase; no evaluation of lipoprotein subclasses was possible at that time. Increases in TC even from cholesterol supplementation were clearly also related to associated dietary fat. A subsequent analysis of 27 studies investigating the effect of dietary cholesterol with strict control of dietary intake (Hopkins, 1992) confirmed this and also reported that individuals with high baseline dietary cholesterol showed smaller changes on supplementation. Based on this work, a supplementary intake of 300 mg cholesterol might be expected to result in an increase in TC of approximately 0.1 mmol/L. This was not observed in the investigation reported in this chapter, in which no significant change was observed for TC during prawn intervention. Notwithstanding the

conflicting interpretation from meta-analyses and individual studies, it would appear that there is little demonstrable CVD risk to normolipidaemic individuals from dietary cholesterol consumption. Overall there may be an increased risk in those with MetS or T2D, but this could also be associated with a higher overall energy intake. By extrapolation, it may have been expected that in the higher BMI group of this study, prawn consumption would be less beneficial than in the lower BMI group. This was not the case; the majority of CVD markers showed no significant difference from start to finish, with the exception of TAG, which showed a decrease in the higher BMI compared with an increase in the normal BMI group (p = 0.033). There was an apparent improvement in cardiometabolic risk profile for the higher BMI group than the lower BMI group during the OC intervention, which was associated with no change in cholesterol intake. Given the changes in overall dietary intake in this group, described herein, it may be presumptuous to ascribe this to a reduced risk from lower cholesterol intake (or indeed the converse with respect to prawn/cholesterol intake).

The efficiency of cholesterol absorption in the human is genetically controlled (Hopkins 1992), ranging between 20 and 80% in normal subjects consuming a diet containing moderate levels of cholesterol (Lecerf & de Lorgeril, 2011). An increased absorption tendency is correlated with increased plasma LDL-C concentrations, a result of reduced catabolism of LDL-C rather than increased synthesis. Additionally, the ability to down-regulate HMG-CoA reductase activity may reduce the effect of increased LDL-C as a CVD risk factor. Hopkins (1992) suggested that for most people, hepatic cholesterol overload results in a reduced responsiveness to increased dietary cholesterol. There may remain some individuals for whom increased dietary cholesterol results in adverse lipoprotein profiles and associated CVD risk, due to effects on intestinal absorption rather than post-absorption synthesis. It has been reported that individuals carrying the ApoE4 allele are more sensitive than others to dietary cholesterol, with a possible increase of 10% in total cholesterol from an increase of 300 mg in daily intake (Lecerf & de Lorgeril, 2011). It is not known whether any of the participants in the present study were carrying this allele.

3.6.3. Food intakes

3.6.3.1. Patterns of food intake

Of the original cohort of 21, only 18 completed the pre-intervention food diaries, and the numbers completing food records was less post-intervention (14 for OC, 11 for Pr).

The variation in nutrient intake within this small sample was considerable (Table 3.13). Indeed, there were several individual nutrient intakes more than 2SD from the mean reported intakes, which were discarded from further statistical analysis. There is some evidence to suggest that participants may not have maintained a consistent lifestyle in terms of diet and other factors throughout the course of the study. Specifically, it was noted that the numbers of participants recording alcohol intake varied from one food diary stage to another (16 at baseline, 13 and 9 at end of prawn and OC intervention respectively). The food diaries used in this study were completed over only a 3-day period, however, and it is possible that this may be a contributory factor, as participants might well have different dietary tendencies at a weekend compared with midweek. Beside cholesterol and n-3 LCPUFA, significant differences were noted in other nutrient intakes between start and end of interventions. There was a mean increase in protein intake between baseline and end of Pr intervention, from 86.4 to 109.1 g (p = 0.02). This represented an increase from 155-200% of the RNI (p = 0.02), and an increase in the proportion of calories from protein from 15.8-23.7% of total energy intake (p = 0.007). The decrease in mean carbohydrate intake, from 277 to 208 g daily, reached significance when expressed as a percentage of total calorie intake (50.2% to 42.9%, p = 0.039), reflecting the change in proportions of carbohydrate and protein during the prawn intervention.

For the OC intervention, food intake reports showed a mean decrease in total daily intake in carbohydrate from 277 to 233 g (p = 0.047). This change, which was smaller than for the prawn intervention, represented a change in the energy contribution of dietary carbohydrate from 50.2% to 46.3%, which was not significant (p = *0.44).* This decrease was despite a contribution of approximately 35 g per day from the OCs themselves. Intake of all carbohydrate categories was also significantly reduced from baseline to intervention, including dietary fibre, non-starch polysaccharide (NSP), sugars and non-milk extrinsic sugars. The mean decrease in fibre was from 17.3 to 13.1 g (p < 0.01), NSP from 76.3 to 61.5 g (p < 0.01), sugars from 117.6 to 87.7 g (p = 0.015) and non-milk extrinsic sugars from 81.9 to 56.4 g (p = 0.032). These changes may represent additional significant change in dietary habits by some or all of the participants during the OC intervention stage. A decrease in carbohydrate intake has been linked to improvements in markers of cardiometabolic risk (Shai *et al.,* 2008).

The difference between post-intervention protein intakes for the two arms of the study was also significant *(*p = 0.024).

3.6.4. Conclusion

The results presented here do not show an overall beneficial effect of n-3 LCPUFA in lowering TAG, but also do not indicate any harmful effect resulting from the dietary cholesterol component. Although the numbers in the subgroups are very small, these findings appear to have some significance. Of the 21 individuals who completed the initial screening, only 9 fell within the recommended range for a normal BMI (between 19 and 25). For a further 9, the BMI was between 25 and 30, and for 3 in excess of 30 kg/m^2. This enabled a comparison between the small subgroups with BMI above and below 25 kg/m^2. The higher BMI group showed a beneficial change in plasma TAG compared with the lower BMI group during Pr intervention. The significant differences in lipoprotein subclasses and ApoB during OC intervention also indicate a small improvement in profile for the higher BMI group compared with the lower BMI group. Data from food diaries during the OC intervention indicated that some or all participants may have made significant changes to their diet during this intervention, which may have confounded these findings. In view of the small size of the groups, it was not felt that there would be validity in exploring individual carbohydrate intakes, although it may be interesting to compare between the two BMI groups. The two intervention foods were significantly different in composition in several aspects apart from cholesterol and n-3 LCPUFA, in protein, carbohydrate and salt, all of which may impact on CVD risk markers. It is also possible, that participants in the high BMI subgroup made significant changes to their lifestyle during the intervention, which may have had an additive beneficial effect on lipid profiles. Although the overall mean changes in BMI and biochemical parameters did not change to a great extent, changes for some individuals may confound the overall pattern.

In summary, this study has not demonstrated any clear disbenefit from prawns as a source of n-3 LCPUFA compared with a weight-for-weight food source containing only traces of n-3 LCPUFA and cholesterol (OCs).

CHAPTER 4

A Comparison Of Krill And Fish Oils In Metabolic Syndrome

4.1. INTRODUCTION

As introduced in Section 1.2, the consumption of oily fish in the general population is lower than estimated requirements for healthy living, including the reduction of CVD risk (Simopoulos, 2002a; 2002b). FO supplements have been used for many years as a source of n-3 LCPUFA (Wang *et al*., 2006; Anandan *et al*., 2009). There are concerns about the sustainability of FO sources, and about possible toxic effects from accumulation of heavy metals and dioxins in the marine food chain (Virtanen *et al*., 2005, Jenkins *et al*., 2009). KO has been suggested as an alternative source of n-3 LCPUFA. A high proportion of the n-3 LCPUFA in KO are in the form of PL rather than TAG, and it has been suggested that KO may have benefits over FO due to superior bioavailability of the n-3 LCPUFA (Bunea *et al*., 2004; Colussi *et al*., 2007). KO also contains astaxanthin, a naturally occurring carotenoid pigment (Tou *et al*., 2007); this may help to protect LCPUFA against oxidation and degradation, or may, contribute to enhanced antioxidant status in individuals taking KO supplements (Yang *et al*., 2013).

The metabolic syndrome (MetS) is strongly associated with increased CVD risk (Despres *et al*, 2008; Reaven, 2011). Characteristic findings include increased anthropometric measures (waist, weight, BMI), blood pressure and changes in plasma TAG and plasma lipoproteins (described in Section 1.3). The role of insulin is to provide a signalling and control mechanism to balance nutrient availability and demands, by storing excess dietary energy in times of plenty (as glycogen in liver and muscle, as fat in adipose tissue), or to mobilise these when needed by glycogenolysis and lipolysis. In insulin resistance (IR), excess lipid accumulation results in impairment of insulin action; dietary glucose is not cleared from the bloodstream even in the presence of insulin, and glycogen stores are not accumulated for later use. Impaired insulin action in adipose tissue also results in re-esterification of lipids in the liver and other tissues, which exacerbates the impairment of insulin action (Samuel & Shulman, 2012). IR is a feature of MetS contributing to CVD risk (Lebovitz, 2001, Grundy *et al*., 2005; Bradley *et al*., 2009; Reddy *et al*., 2010). Dyslipidaemia observed in IR and T2D is typically associated with increased TAG, reduced levels of HDL-C and increased levels of sdLDL particles (Adiels *et al*., 2008). Increased TAGs result in overproduction of VLDL particles, precursors to sdLDL particle formation. This perturbation of normal

lipid metabolism, sometimes referred to as the 'lipid triad', may precede diagnosis of T2D (Rizzo & Berneis, 2005).

4.2. AIM OF STUDY

The aim of this study was to determine whether KO as a dietary supplement may be more effective than FO in reducing CVD risk, specifically whether it may be effective in reducing CVD risk markers in MetS. For this investigation, the null hypothesis was that there would be no difference between KO and FO in effects on cardiometabolic risk measurements.

4.3. STUDY DESIGN

The pilot study described in this chapter was designed with the aim of comparing the effects of a low dose of KO versus FO in a group of middle-aged males with MetS, and specifically to investigate changes in the markers of IR, MetS, and CVD risk. At the point when this project was planned, there was no published work specifically addressing the effects of KO in obese or MetS individuals, but some evidence from animal studies (summarised in Section 1.5.2.1) and two small studies in individuals with menopausal symptoms and arthritis (Bunea *et al*., 2004; Deutsch, 2007). The decision to study males rather than females was to avoid the potential of menstrual cycle or menopause-related individual variances. The choice of participants with MetS was on the basis that effects may be more likely to be detected in a group of individuals whose glucose and lipid biochemistry is already perturbed from normal, rather than in a completely normal group. A recent rationale published for the design of a study of possible effects of vitamin D and n-3 fatty acids in prevention of CVD and cancer (Manson *et al*., 2012) proposed a 5-year study in 16000 participants without previous disease history or known risk, with associated blood sampling and analysis in 6000 of these. This may indicate the size of study required to observe a significant outcome in a sample with no known deviation from normal.

4.3.1. n-3 LCPUFA supplements

KO was obtained from Azantis, USA, and FO from EFAMOL, UK. The composition of the supplements, as provided by the suppliers, is shown in Table 4.1, and the PL composition in Table 4.2. Although there is some variation between the two methods

used, it is evident that the major component of the PLs is PC. The FA profiles of neutral and PL fractions as determined by an independent analytical laboratory (Avanti Polar Lipids Inc.) is shown in Table 4.3. The major components in both fractions are the unsaturated FA of the n-3 series, particularly EPA and DHA.

	FO per capsule (1000mg)	**KO per 1000mg (619mg per capsule)**	**Total dose from KO**
PL (mg)	None	460 (318mg per capsule)	1272
n-3 PUFA (mg)	577	140 (97mg per capsule)	388
EPA (mg)	290	90 (62mg per capsule)	250
DHA (mg)	214	50mg (38mg per capsule)	152mg
Astaxanthin	None	0.4 (0.37mg per capsule)	1.5mg
Vitamin E	8.0mg	Not stated	Not stated
Peroxide value (mEq/kg)	3.6	<0.5	
Totox	-	16.5	

Table 4.1: Composition of supplements used *(provided by manufacturers)*

Method	**HPLC/ESD**	**^{31}P-NMR**
Phosphatidylethanolamine	1.6	1.1
Lysophospatidylethanolamine	-	0.9
PC	28.4	42.1
Phosphatidic acid	ND	ND
Phosphatidylinositiol	2.0	ND
Phosphatidylserine	ND	ND
Lysophosphatidylcholine	1.9	1.6
Total	33.8	45.7

Table 4.2 PL composition of KO

(PLs in weight %, Avanti Polar Lipids Inc.)

Fatty acid (as methyl ester)	**Total**	**Neutral lipids**	**PL fraction**
C8:0	10.07	6.38	0.0
C10:0	4.72	3.31	0.0
C12:0	0.03	0.07	0.0
C13:1	0.00	0.11	0.0
C14:0	0.89	0.62	1.54
C15:0	0.16	0.09	0.18
C16:0	9.29	4.20	20.71
C16:1	1.01	0.48	1.23
C17:0	0.12	0.08	0.08
C17:1	005	0.00	0.00
C18:0	1.37	1.20	0.64
C18:1 cis (oleic acid)	**5.26**	**3.99**	**4.78**
C18:1 trans	3.14	1.87	3.70
C18:2 (Linoleic acid)	**1.44**	**1.07**	**1.13**
C18:3 (6.9.12) (GLA)	**0.16**	**0.28**	**0.27**
C18:3 (9,12,15) ALA	**0.74**	**0.48**	**0.57**
C19:0	0.17	0.12	0.0
C20:0	0.28	0.17	0.02
C20:1	1.43	1.18	0.38
C20:2	0.33	0.27	0.19
C20:3 (8,11,14) eicosatrienoic acid	**0.13**	**0.21**	**0.0**
C20:4 ArA	**1.10**	**0.94**	**0.0**
C20:3 (5,8,11) Mead acid	**0.19**	**0.11**	**0.0**
C22:0	0.12	0.11	0.06
C22:1	0.82	0.45	0.78
C20:5 EPA	**17.93**	**14.30**	**15.77**
C22:5 n3 DPA	**1.36**	**2.57**	**0.0**
C22:6 DHA	**13.53**	**21.63**	**12.26**
C24:0	0.06	0.0	0.0
C24:1	0.31	0.24	0.0
TOTAL	**76.43**	**66.53**	**64.30**

Table 4.3: Fatty acids of polar and neutral fractions of KO *(Avanti Polar Lipids, Inc.)*

4.3.2. Recruitment and testing procedures

This study was carried out at LJMU. Recruitment and management of study visits were managed by this investigator with assistance from Dr I.G. Davies and Deborah Scott. Analysis of lipid profiles was carried out by Randox Laboratories, Dublin, and insulin assays by Deborah Scott. Additional analyses, including plasma FA profiles, were carried out by colleagues of Professor Bruce Griffin at the University of Surrey. The analysis of data from food intake diaries and all data analysis was carried out by the investigator.

4.3.2.1. Recruitment and study visits

Male participants (n=18, 35-65 y) were recruited from LJMU staff and the UK Biobank (Liverpool) via poster advertisement. Criteria for inclusion were a waist measurement greater than 94 cm (approximately 37 inches) with one or more additional criteria of MetS as defined by the International Diabetes Federation (2006), but not taking any medication or food supplements to reduce CVD risk, or with any previously known abnormalities in liver function. Following initial screening, participants were randomly assigned to receive 390 mg of EPA/DHA from KO (4 capsules) or 580 mg of EPA/DHA from FO (1 capsule) daily for 6 weeks. The study was a single blind randomised controlled crossover trial, with a 6-week washout period between interventions, as shown in Figure 4.1.

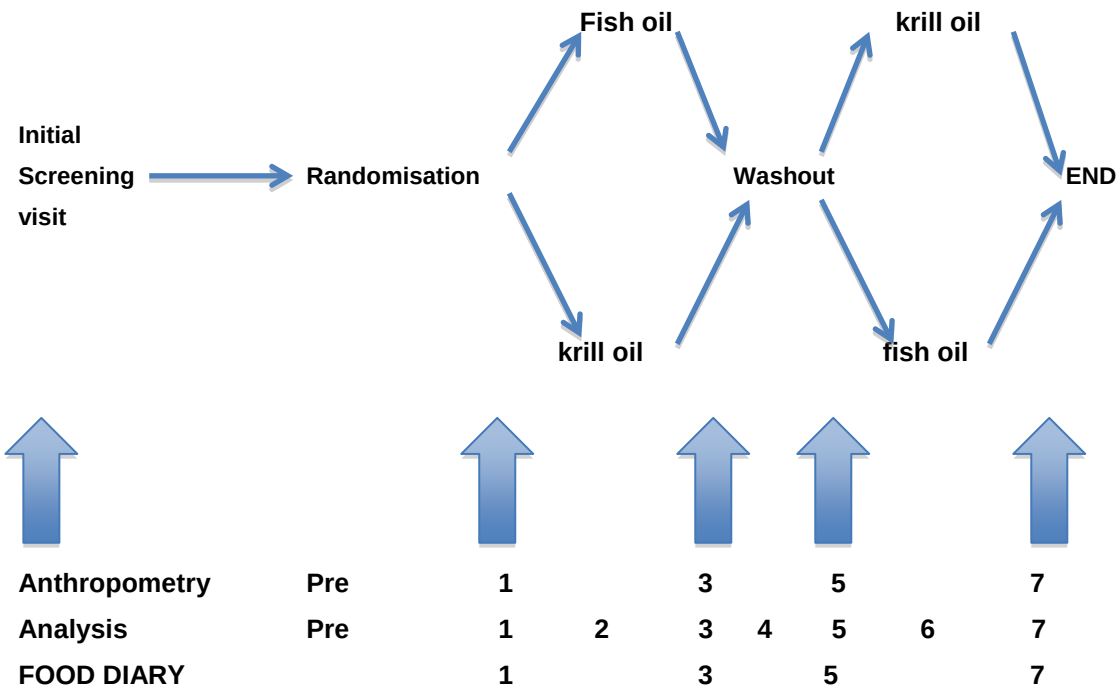

FIGURE 4.1: Diagram of intervention protocol

This was designed as a pilot study, with participants acting as their own controls to compare the FO and KO dietary interventions. No formal power calculation was undertaken; it was intended that this study may provide sufficient data to inform formal power calculations for the design of future larger randomised studies. The dose of supplement was also derived by discussion with a scientific consultant representing the supplier of the KO. Ethical committee approval (NRES) was obtained from the Sefton NHS Ethics Committee (approval number 09/H1001/48) prior to the commencement of the interventions, and the study was also registered with www.clinicaltrials.gov (NCT01705678). The schedule of investigations is summarised in Table 4.4.

	1st intervention			Washout	2nd intervention		
Height	✓						
Weight	✓	✓	✓	✓	✓	✓	✓
Waist	✓		✓		✓		✓
Blood pressure	✓		✓		✓		✓
BMI	✓	✓	✓	✓	✓	✓	✓
Capillary blood: **CVD risk profile**	✓		✓		✓		✓
MetS and CVD risk markers: Venous blood							
TAG	✓	✓	✓	✓	✓	✓	✓
Glucose	✓	✓	✓	✓	✓	✓	✓
TC	✓	✓	✓	✓	✓	✓	✓
HDL-C	✓	✓	✓	✓	✓	✓	✓
sdLDL	✓	✓	✓	✓	✓	✓	✓
Lipoprotein subclasses	✓	✓	✓	✓	✓	✓	✓
Plasma fatty acids (NEFA)	✓	✓	✓	✓	✓	✓	✓
Plasma FA profiles	✓		✓		✓		✓
7-day food diary completed	✓		✓		✓		✓

Table 4.4: Schedule of measurements and sampling

4.3.2.2. Anthropometric measurements

Anthropometric measurements (height, weight, waist measurement and blood pressure) were carried out at the start of the study as described in Section 2.2.1.2. With the exception of height, these were repeated at midpoint and end of each intervention and the midpoint of the washout period (7 in total). BMI was also derived from these measurements.

4.3.2.3. Biochemical markers of CVD risk

Capillary blood obtained at the beginning and end of each intervention was used to obtain a CVD risk profile using a Alere™ LDX point-of-care analyser as described in Section 2.2.2.1. Venous blood (from the antecubital vein) obtained at the start, midpoint and end of each intervention period and the midpoint of the washout period (7 in all at 3-week intervals), were used to measure plasma markers of MetS (Alberti *et al.*, 2006) and other features of IR indicative of CVD risk; TAG, glucose, TC, and lipoprotein subclasses. Plasma FA profiles were analysed as described in Section 2.2.2.6.

4.3.2.4. Food intake assessment

All participants were asked to fill in a 7-day food diary prior to starting each intervention and in the last week of each intervention (4 in total). Results of these were analysed for macronutrient and micronutrient intake using the Microdiet™ program (Downlee Systems Ltd, UK).

4.3.2.5. Indices of metabolic status

Some of the measurements described above were used to derive HOMA (homeostasis assessment model), QUICKI and R-QUICKI scores.

a) The homeostasis assessment model (HOMA) is a model devised to assess IR and β-cell function from measurement of fasting plasma glucose and insulin (Hanley *et al*., 2002; Grundy, 2006a; 2006b). A simple derivation of the original model gives the following equations:

$$\text{HOMA-IR} = [\text{glucose (mmol/L)} \times \text{insulin (mU/L)}]/22.5$$

A value greater than 2.5 was initially suggested to indicate IR.

$$\text{HOMA-}\beta\text{ (beta cell-) function} = [20 \times \text{insulin (mU/L)}]/\{\text{glucose (mmol/l)} - 3.5\]\%$$

This is expressed as a percentage assuming that normal β-cell function in non-diabetic subjects would be 100%.

b) QUICKI and R-QUICKI algorithms: An alternative index of MetS and CVD risk is the QUICKI algorithm (Katz *et al*., 2000) subsequently modified to R-QUICKI (Perseghin *et al*., 2001) by inclusion of FFA (NEFA) in the algorithm:

$$\text{QUICKI} = 1/[(\log_{10}\text{ glucose (mg/dL)}) + \log_{10}\text{ insulin (mU/ml)}]$$

$$\text{R-QUICKI} = 1/\{\log_{10}\text{ glucose (mg/dL)} + \log_{10}\text{ insulin (mU/ml)} + \log_{10}\text{ FFA (mg/dL)}\}$$

These indices were calculated at baseline, interim and endpoint of both interventions. Brady *et al*. (2004) reported that the R-QUICKI index correlates well with insulin sensitivity derived from intravenous glucose tolerance test. A later comparison of QUICKI and R-QUICKI (Singh *et al*., 2013) deduced that the inclusion of NEFA concentrations improved correlation with other insulin sensitivity measurements.

4.4. STATISTICAL ANALYSIS

All data were examined for normality and any extreme outliers, using the SPSSv20 statistics program. Means and standard deviations were derived, and those more than 2 SD from the mean were removed from further analysis. In particular, some extreme outliers were identified for n-3 LCPUFA intake, as discussed in the results below. After review of normal distributions, split-plot repeated measures ANOVA (SPANOVA) tests were used to investigate any differences within and between groups. Where distributions were not normal, comparison using non-parametric tests was used. As will be described, no differences between or within groups were observed: therefore, trends in changes from start to midpoint and start to end of interventions were also compared (Δ values). It was not possible to normalise these data, which were treated using non-parametric statistical tests.

4.5. RESULTS

4.5.1 Baseline measurements

The details of baseline weight, height and other anthropometric measurements, and of markers of MetS, are given in Table 4.5. Distribution was normal according to the Kolmogorov-Smirnov test of normality for all markers. Examination of biochemical data revealed one individual with significant deviations from normal distribution on a number of measurements, and this participant was removed from further analysis.

	Range (n=18)	Mean	SD	Metabolic syndrome range (IDF, 2006)
Age (yrs)	36-63	49	9.3	N/A
Height (m)	1.56-1.84	1.76	.07	N/A
Weight (kg)	80.0-124.5	96.7	13.1	N/A
BMI (kg/m^2)	27.3-38.4	31.3	3.3	>30[1]
Waist (cm)	94.1-128.4	106.1	8.7	>94
Systolic Blood pressure (mm Hg)	119-153	135.6	12.8	>130
Diastolic BP (mm Hg)	70-97	83.7	7.9	>85
Plasma TAG (mmol/L)	0.74-3.05	1.77	0.64	>1.7
Fasting plasma glucose (mmol/L)	2.7-9.4	5.45	0.47	>5.6
Serum cholesterol (mmol/L)	3.94 –7.46	5.55	0.95	N/A
HDL-C (mmol/L)	0.56-1.65	1.10	0.31	<1.03
LDL-C (mmol/L)	0.7-3.1	3.64	0.99	N/A
Non HDL-C (mmol/L)	1.9-5.6	4.45	1.00	N/A
TC/HDL ratio	2.5-3.8	5.50	1.86	N/A

Table 4.5 Initial anthropometric measurements, CVD and MetS markers (capillary blood measurements)

4.5.2. Intake of n-3 LCPUFA

Inspection of food and nutrient intakes revealed significant (>2SD) anomalies in food intakes of individual nutrients at different stages. It was not practicable with such a small starting cohort to eliminate participants completely, but individual data points more than 2SD away from the mean for particular measurements were removed. Special attention was paid to estimates of n-3 LCPUFA intake from the food diaries as this was key to the comparison of the supplements.

Intake of n-3 LCPUFA was estimated from food intake diaries prior to the start of supplementation and estimated from the food intake records and supplementation data, assuming full compliance with the supplement instructions. There was significant variation in baseline intakes at the start of both interventions, as illustrated in Figure 4.2 (mean daily intake 0.38g, SD = 0.446g for FO and mean 0.39g, SD = 0.397g for KO).

Furthermore, analysis of the daily intake in n-3 LCPUFA from food diaries (without allowance for supplement intake) revealed significant variation in endpoint intake, as shown in Figure 4.3 (mean daily intake for FO group 0.48g, SD = 0.53, mean for KO group 1.02g, SD =1.53). Inspection of the data revealed one participant who had a greatly increased intake of n-3 LCPUFA from dietary sources other than the supplements, particularly during KO intervention. This individual was therefore eliminated from further statistical analysis of food and biochemical data.

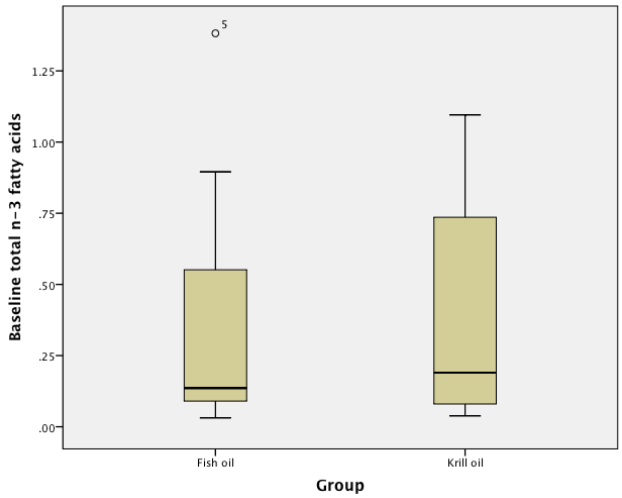

Figure 4.2: Baseline intakes of n-3 LCPUFA from food diaries
(intakes in mg per day, estimated from recorded data)

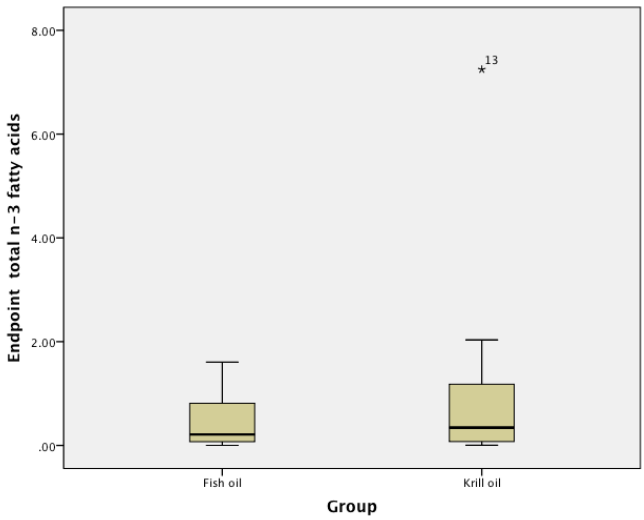

Figure 4.3: Endpoint intakes of n-3 LCPUFA from food diaries
(intakes in mg per day, including contribution from FO or KO supplements assuming full compliance)

A wide variation in n-3 LCPUFA intake from dietary sources was recorded, both at baseline and endpoint. Mean increases in n-3 LCPUFA intake were 620mg (SD = 353) and 1210 mg (SD = 1909) for the FO and KO groups, respectively. Thus, although the KO supplementation *per se* provided less n-3 LCPUFA than the FO, the mean increase for the KO group (ΔKO) was twice that for the FO group (ΔFO). When expressed as a percentage of baseline intake, mean ΔFO intake was 556% (a 5-fold increase in intake) compared with a mean ΔKO intake of 810%. Three individuals demonstrated changes in n-3 LCPUFA intake significantly higher than the mean. After removal of the most significant outlier, overall intakes of n-3 LCPUFA were recalculated (assuming full compliance with supplements), as shown in Figure 4.4. The recalculated mean daily n-3 LCPUFA intakes from dietary sources were 480mg (SD 528mg) and 580mg (SD 724) for FO and KO groups, respectively. Assuming full compliance with supplementation overall intakes were estimated to be 1060 mg (SD 529) and 970 mg (SD 725mg) respectively. Changes in n-3 LCPUFA intake for the FO group showed further individuals that were > 2SD from the recalculated mean (Figure 4.5), but as these were within the comparable range for the KO intervention, it was decided not to eliminate further data.

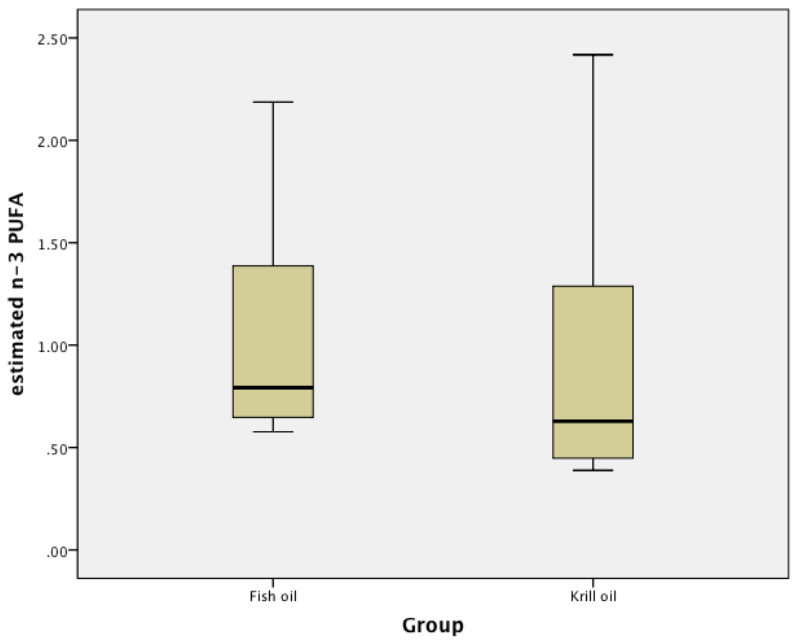

Figure 4.4 Recalculated n-3 LCPUFA intake for FO and KO groups

(in mg/day, after elimination of extreme outlier as identified from food diaries).

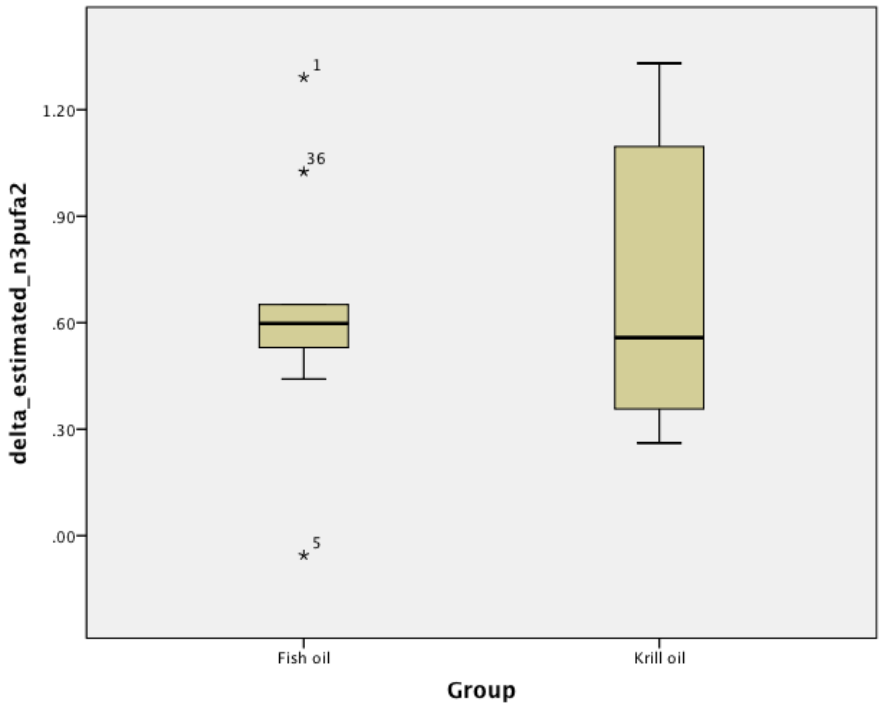

Figure 4.5 Changes in n-3 LCPUFA intake (Δ) for KO and FO interventions

(in mg per day, after elimination of extreme outlier identified from food diaries)

4.5.3. Anthropometric Data

Baseline, midpoint and endpoint measurements are summarised in Table 4.6, with mean changes and results of tests for significance (paired *t*-test for normal distributions and non-parametric Wilcoxon ranked test for pairs of variables with non-normal distribution). Distributions of anthropometric measurements were normal (Kolgorov-Smirnov test, $p > 0.05$), with the exception of baseline systolic blood pressure for KO intervention ($p = 0.024$). Results show very minor changes in mean anthropometric measurements; there was no significant difference between baseline and endpoint for any variables in either intervention, nor for the mean change (ΔBE) between interventions ($p > 0.23$ for all variables).

Split-plot analyses of variance (SPANOVA) were also conducted to compare weight and BMI at baseline, midpoint and end of each intervention, between the two interventions. There was no significant effect of intervention for either group or between groups for either variable (Wilks' Lambda >0.83 for all measurements).

	FO (n= 17)					KO (n = 17)					
	Baseline	**Interim**	**Endpoint**	***p***	**Overall change (ΔBE)**	**Baseline (n = 16)**	**Interim (n=12)**	**Endpoint (n = 15)**	***p***	**Overall change (ΔBE)**	**Significance between changes (*p*)**
Weight (kg)	96.6 (13.9)	96.4 (14.2)	97.1 (14.9)	0.35	0.4 (1.8)	97.3 (14.6)	99.9 (15.6)	97.2 (15.3)	0.40	-0.3 (1.4)	0.40
BMI	31.3 (3.4)	31.2 (3.5)	31.4 (3.7)	0.31	0.15 (0.57)	31.4 (3.7)	31.5 (3.8)	31.5 (3.8)	0.32	-0.1 (0.43)	0.32
BP s	134 (12)	-	134 (11)	0.75[1]	-0.3 (9.3)	133 (12)	-	134 (10)	0.97 [1]	0.8 (8.7)	0.66[1]
BP d	83 (8)	-	83 (6)	0.84	0.3 (6.6)	83 (6)	-	81 (6)	0.74	-2.2 (6.2)	0.75
Waist (cm)	106 (9)	-	107 (10)	0.46	0.5 (2.6)	107 (10)	-	107 (11)	0.23	-1.0 (3.1)	0.23

Table 4.6 Anthropometric measurements and changes

Values were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups. Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test.

	Fish oil (n = 16)				Krill oil (n = 15)			
	Baseline	**Interim**	**Endpoint**	**Significance (BE) *(p)***	**Baseline**	**Interim**	**Endpoint**	**Significance (BE) *(p)***
Plasma glucose (mmol/L)	5.12 (0.55)	5.32 (0.49)	5.63 (1.11)	0.28[1]	5.40 (0.79)	5.35 (0.59)	5.39 (0.53)	0.95[1]
Plasma insulin (pmol/L)	49.9 (22.4)	51.5 (33.9)	61.8 (38.4)	0.15	49.7 (27.1)	48.2 (18.2)	47.2 (23.2)	0.27
TAG (mmol/L)	1.68 (0.67)	1.45 (0.57)	1.79 (0.74)	0.39[1]	1.47 (0.50)	1.61 (0.42)	1.57 (0.62)	0.69[1]
Serum cholesterol (mmol/L)	5.27 (0.98)	5.31 (1.24)	5.29 (0.72)	0.38	4.90 (1.10)	5.04 (1.20)	4.99 (1.04)	0.56
HDL-C (mmol/L)	1.24 (0.25)	1.25 (0.30)	1.28 (0.24)	0.27	1.15 (0.29)	1.14 (0.31)	1.18 (0.26)	0.51
LDL-C (mmol/L)	3.71 (0.90)	3.82 (1.15)	3.99 (1.02)	0.07	3.49 (0.93)	3.60 (0.94)	3.53 (0.88)	0.75
NEFA (mmol/L)	0.66 (0.20)	0.55 (0.13)	0.68 (0.29)	0.74[1]	0.58 (0.24)	0.57 (0.14)	0.61 (0.20)	0.55[1]
sdLDL-C (mmol/L)	1.16 (0.59)	1.11 (0.57)	1.29 (0.55)	0.08	0.96 (0.44)	1.04 (0.39)	1.02 (0.44)	0.33
sdLDL-C (% of LDL)	30.0 (10.2)	28.3 (8.7)	32.4 (11.0)	0.09[1]	27.3 (9.8)	29.3 (8.1)	28.6 (9.7)	0.87
TC/HDL-C	4.35 (0.77)	4.32 (0.70)	4.35 (0.74)	0.78[1]	4.37 (0.75)	4.48 (0.51)	4.32 (0.69)	0.33[1]
TAG/HDL-C	1.41 (0.60)	1.27 (0.71)	1.51 (0.85)	0.41[1]	1.36 (0.59)	1.51 (0.49)	1.41 (0.65)	0.78[1]

Table 4.7: MetS markers (SD in brackets)

Values were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups (Wilks-Lambda > o.1 for all measurements). Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test.

	Baseline to endpoint (BE)			Baseline to interim (BI)		
	FO n = 16	**KO n = 15**	**Significance of change between interventions**	**FO n = 16**	**KO n = 15**	**Significance of change between interventions**
	ΔBE (SD)	**ΔBE (SD)**	***p***	**ΔBI (SD)**	**ΔBI (SD)**	***p***
Plasma glucose (mmol/L)	0.51 (1.37)	-0.01 (0.92)	0.22	0.21 (0.57)	0.02 (0.75)	0.36
Plasma insulin (pmol/L)	13.1 (33.2)	-4.5 (13.4)	0.08[2]	N/A	N/A	N/A
TAG (mmol/L)	0.12 (0.46)	0.09 (0.46)	0.90	-0.22 (0.58)	0.15 (0.44)	0.06
Total cholesterol (mmol/L)	0.14 (0.60)	0.10 (0.62)	0.85	0.04 (0.75)	0.07 (0.69)	0.89
HDL-C (mmol/L)	0.04 (0.15)	0.03 (0.16)	0.78	.014 (0.17)	0.02 (0.16)	0.93
LDL-C (mmol/L)	0.27 (0.55)	0.04 (0.52)	0.24	0.11 (0.68)	-0.01 (0.64)	0.64
Plasma NEFA (mmol/L)	0.02 (0.36)	0.03 (0.19)	0.93	-0.10 (0.20)	0.02 (0.20)	0.10
Plasma sdLDL-C (mmol/L)	0.14 (0.29)	0.06 (0.23)	0.34[1]	-0.04 (0.32)	0.05 (0.22)	0.40
Plasma sdLDL –C (percentage of LDL-C)	2.4 (9.2)	1.3 (5.8)	0.62	-1.6 (7.6)	1.8 (3.9)	0.14
TC/HDL-C	0.002 (0.25)	-0.045 (0.27)	0.63	-0.03 (0.29)	-0.01 (0.32)	0.88
TAG/HDL-C	0.10 (0.47)	0.06 (0.43)	0.95[1]	-0.14 (0.53)	0.12 (0.53)	0.12

Table 4.8 Changes in plasma measurements between start and end of interventions

Values were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups. Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test. [2]Assuming non-equality of variances (Levene test, p = 0.037)

4.5.4. Markers of metabolic syndrome

4.5.4.1 Biochemical measurements

Measurements obtained at baseline, midpoint and endpoint for KO and FO interventions are presented in Table 4.7. Inspection of these data revealed no significant outliers for variables except for endpoint plasma cholesterol, (two high outliers, FO intervention) and for endpoint glucose (one, KO intervention). However, these were within the overall range for the cohort as a whole and were not eliminated from analysis.

SPANOVA analyses were conducted to compare MetS markers within and between the two dietary interventions at each time point, for those variables with normal distribution. There was no significant effect of intervention for either group or between groups for any measurement (Wilks' Lambda $p > 0.1$ for all measurements). For those variables with non-normal distribution, the Friedman test indicated no significant difference in variables across the three time points for either intervention ($p > 0.19$ for all FO variables and > 0.22 for all KO variables).

No significant overall change was observed in TC, HDL-C, LDL-C, sdLDL-C (total or as percentage of LDL-C), NEFA, or in ratios of TC/HDL-C or TAG/HDL-C. No differences between baseline and endpoint reached statistical significance for any variable, nor was any significance detected between mean changes (ΔBE) for any variable between interventions.

Comparison between intervention groups of ΔBE as presented in Table 4.8, showed no significant difference between the groups on any variables measured. A similar comparison of changes from baseline to interim timepoint (ΔBI) also showed no significant differences between groups, although the difference for TAG approached significance ($p = 0.06$).

4.5.4.2. Derived indices of metabolic status

HOMA-IR and HOMA-β indices were calculated at baseline, interim and endpoint of both interventions. The mean values and changes from baseline-interim and baseline-endpoint for both interventions are presented in Table 4.9, with statistical comparison of measurements between time points. These data indicate an increase in the mean HOMA-IR as a measure of IR during FO intervention, and also between baseline and

endpoint in KO intervention, although with a small decrease at midpoint. Comparison of the mean values revealed no significant difference between measurements at different time points. The HOMA-β measure of β-cell function showed an overall increase in mean value for FO compared with a decrease in mean value for KO. For both interventions the mean interim value was lower than either baseline or endpoint. Notably, the HOMA-β values for two individuals at the end of the FO study were very high (in excess of 300) compared with the remainder of the group. SPANOVA was carried out, to compare HOMA-IR or HOMA-β measurements at baseline, midpoint and end of each intervention. There was no significant effect of intervention for either group or between groups for any measurement (Wilks' Lambda >0.16 for all measurements).

QUICKI and R-QUICKI indices were calculated at all time points. Inspection of the data showed that one individual presented with indices more than 2 SD outside the mean, and this individual was excluded from analysis. The mean and SD at each point are shown in Table 4.10. Comparison of means using both parametric and non-parametric analysis indicated no significant difference between indices calculated at different time points for either the KO or FO interventions (Table 4.12). The differences between these indices (delta-values) from baseline to interim and baseline to endpoint were also compared. No significant differences were observed between FO and KO for baseline-interim, baseline-endpoint or interim-endpoint changes.

	FO							KO						
	Baseline n = 16	**Interim n = 16**	**ΔBI**	***p* *B vs I***	**Endpoint n = 15**	**ΔBE**	***p* B vs E**	**Baseline n = 15**	**Interim n = 13**	**ΔBI**	***p* *B vs I***	**Endpoint n = 12**	**ΔBE**	***p* B vs E**
HOMA-IR	1.47 (0.70)	1.57 (1.02)	0.10 (0.94)	0.67	1.92 (1.19)	0.47 (1.11)	0.12	1.50 (0.81)	1.39 (0.50)	-0.03 (0.59)	0.87	1.81 (1.38)	0.08 (0.52)	0.62[1]
HOMA-β	104 (68)	88 (62)	-15.9 (56.9)	0.28	130 (139)	32 (132)	0.36	97 (75)	88 (64)	-8.9 (87.9)	0.65	92 (72)	-27 (52)	0.27[1]

Table 4.9. HOMA-IR and HOMA-β status and changes (SD in brackets)

Values are shown with SD in brackets. Data were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups. Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test.

	FO							KO						
	Baseline n = 16	**Interim n = 16**	**ΔBI**	***p*[1] *B vs I***	**Endpoint n = 15**	***p*[1] *B vs E***	**ΔBE**	**Baseline n = 15**	**Interim n = 13**	***p* *B vs I***	**ΔBI**	**Endpoint n = 12**	***p* *B vs E***	**ΔBE**
QUICKI	0.35 (0.03)	0.35 (0.03)	-0.005 (0.02)	0.88	0.36 (0.04)	0.28	-0.01 (0.03)	0.36 (0.04)	0.35 (0.02)	0.33	-.005 (0.028)	0.36 (0.03)	0.86	<0.01 (0.03)
R-QUICKI	0.38 (0.05)	0.39 (0.04)	-.01 (0.04)	0.72	0.37 (0.06)	0.17	-0.01 (-.02)	0.41 (0.07)	0.39 (0.03)	0.16	-.024 (0.05)	0.40 (0.05)	0.55	-.005 (0.03)

Table 4.10 QUICKI and R-QUICKI indices: values and changes

Values are shown with SD in brackets. Data were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups. Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test.

4.5.4.3. Plasma FA profiles

Plasma FA measurements are presented in Table 4.11. The principal components of the FA profile were palmitate (range of means 32.2 - 35.3%), LA (15.5 - 17.5%) and ArA (15.2 - 18.2%). Mixed between-within subjects analyses of variance (ANOVA) were conducted to compare FA percentages at baseline, midpoint and end of each intervention, for variables with normal distribution, as shown in Table 4.12. Inspection of the data revealed deviation from normality for several timepoints, for these the Friedman test was used (Table 4.13).

Changes in plasma n-3 and n-6 PUFA: Total n-3 PUFA (ALA plus EPA plus DHA) measurements revealed an increase between baseline and interim for FO (mean from 6.5 to 8.0 % of total), but no further increase from interim to endpoint (7.9%). The proportion of DHA and EPA followed a similar pattern; the contribution from ALA during FO intervention showed a small progressive increase from start to finish (0.22 to 0.36%), from which it may be inferred that the increase was due to FO and not increased plant-derived sources of ALA. However, comparable measurements for KO revealed a similar increase in total n-3 PUFA from baseline to interim (mean from 6.8 to 7.2 %) but a decrease from interim to endpoint, with the mean at endpoint lower than at baseline (mean 6.5 %); the concentrations of ALA, EPA and DHA changed in a similar pattern. With respect to n-6 PUFA, significant differences between baseline and interim measurements were noted for ArA (-2.41%) but not LA (-1.13%) for the FO intervention, and between baseline and endpoint for both LA and ArA. (an overall decrease in percentages of both, -2.07 and -1.55% respectively). In the KO intervention, percentages of both ArA and LA increased between baseline and interim, and decreased towards endpoint; the only significant difference noted during the KO intervention was for ArA between midpoint and endpoint. The n-6/n-3 ratios for both interventions reflected these changes in FA composition, falling from 5.7% to 4.1% from start to end of FO intervention, and from 5.1% to 4.9% at interim and back to 5.1% at endpoint for KO. Significant effects were observed using the Wilks' Lambda test, for total n-3 PUFA both with respect to time (p = 0.02) and to time x intervention (p = 0.03), and for the n-6/n-3 ratio (p < 0.001 and 0.004 respectively for time and time x intervention). No significant effect was noted for plasma DHA either by time or by intervention.

	FO						KO					
	Baseline n = 16	**Interim n = 16**	**Endpoint n = 15**	ΔBI	ΔBE	***[1]p*** ΔBI vs ΔBE	**Baseline n = 15**	**Interim n = 13**	**Endpoint n = 12**	ΔBI	ΔBE	***[1]p*** ΔBI vs ΔBE
Palmitate	33.1 (2.0)	34.2 (4.2)	33.8 (4.6)	0.99 (4.35)	0.66 (5.22)	0.59	34.6 (5.9)	32.2 (3.2)	35.3 (3.8)	-2.99 (6.98)	0.73 (6.72)	***0.05***
Stearate	16.6 (1.6)	17.9 (2.0)	18.2 (2.2)	1.28 (2.03)	1.56 (2.44)	0.94	18.2 (3.2)	16.1 (4.3)	18.6 (2.5)	-2.37 (4.79)	0.40 (4.54)	***0.05***
Oleate	8.1 (1.4)	7.9 (1.5)	8.3 (1.5)	-0.30 (1.64)	0.18 (1.68)	0.37	7.8 (1.4)	9.8 (5.5)	7.6 (0.9)	2.21 (5.12)	-0.20 (1.66)	0.20
Linoleate	17.5 (2.8)	16.4 (2.8)	15.5 (2.9)	-1.13 (3.28)	-2.07 (2.53)	0.52	15.8 (3.3)	17.2 (1.8)	16.3 (3.1)	1.67 (3.24)	0.56 (4.38)	0.55
α-linolenate	0.22 (0.10)	0.27 (0.14)	0.36 (0.42)	0.06 (0.16)	0.14 (0.41)	0.51[1]	0.20 (0.11)	0.28 (0.07)	0.25 (0.09)	0.06 (0.11)	0.04 (0.10)	0.40
Arachidonate	18.2 (3.0)	15.6 (2.9)	16.4 (3.5)	-2.41 (3.56)	-1.85 (3.26)	0.33	16.9 (4.4)	17.6 (2.8)	15.6 (3.4)	1.03 (5.32)	-1.25 (5.32)	***0.07***
EPA	1.84 (0.41)	3.02 (1.38)	2.76 (0.71)	-1.20 (1.16)	0.94 (0.93)	0.70[1]	2.36 (1.03)	2.40 (0.76)	2.23 (0.71)	<0.01 (1.27)	-0.13 (1.08)	0.74
DHA	4.44 (0.99)	4.76 (1.11)	4.74 (1.01)	0.33 (0.72)	0.43 (1.23)	0.56	4.20 (1.26)	4.56 (0.82)	4.04 (0.67)	0.36 (1.29)	-0.16 (1.13)	***0.05***
total n-6 FA (LA + ArA)	35.6 (2.4)	32.0 (4.6)	31.9 (4.9)	-3.54 (5.06)	-3.92 (4.97)	0.74	32.7 (6.7)	34.8 (2.9)	32.0 (5.1)	2.70 (7.77)	-0.70 (8.74)	***0.11***
Total n-3 FA (ALA + EPA + DHA)	6.5 (1.3)	8.0 (2.3)	7.9 (1.5)	1.80 (1.24)	1.52 (1.82)	0.97	6.8 (2.1)	7.2 (1.5)	6.5 (1.3)	0.35 (2.28)	-0.35 (1.65)	0.24
n-6/n-3 ratio	5.7 (1.3)	4.2 (0.9)	4.1 (0.7)	-1.58 (0.92)	-1.72 (1.04)	0.72	5.1 (1.3)	4.9 (0.8)	5.1 (1.2)	-0.18 (1.03)	-0.21 (1.04)	0.80

Table 4.11 Plasma FA profiles and changes during study

All data are shown as percentages of total Fas. Values are shown with SD in brackets. Data were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups. Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test.

	Time			Time * intervention		
	Wilks-Lambda	***p***	**Partial eta^2**	**Wilks-Lambda**	***p***	**Partial eta^2**
Palmitate	0.97	0.66	0.04	0.86	0.16	0.14
ArA	0.93	0.41	0.07	0.81	***0.08***	0.19
DHA	0.89	0.26	0.11	0.90	0.28	0.10
Total n-6 PUFA	0.94	0.50	0.06	0.79	***0.06***	0.21
Total n-3 PUFA	0.70	***0.02***	0.30	0.73	***0.03***	0.27
n6/n3 ratio	0.47	***<0.001***	0.53	0.60	***0.004***	0.40

Table 4.12 Dependence of FA concentrations on time and/or intervention

ANOVA for data with normal distributions.

	FO ***p* =**	**KO** ***p* =**
Stearate	0.15	0.13
Oleate	0.46	0.79
LA	0.26	0.37
ALA	0.84	0.35
EPA	***0.004***[1]	0.40

Table 4.13 Effect of intervention on plasma FAs

(Friedman test used for non-normality of distribution of data: the effect of time across three time points for EPA was significant using the Bonferroni correction, α = 0.017)

Changes in plasma FA between baseline and intervention were maintained to the endpoint for FO, but for several measures the FA profile reverted to baseline by the endpoint of KO intervention. Comparison of ΔBI with ΔBE for KO revealed a significant difference for DHA (p = 0.05). For EPA, where a non-normal distribution of values was observed, the Friedman test revealed a statistically significant difference across the three time points in the FO intervention (Table 4.13), (p = 0.004, significant using Bonferroni correction).

The significance of ΔFA at different time points are shown in Table 4.14. For n-6 PUFA, only ΔBI in the FO intervention reached significance for ArA (p = 0.04). Changes for total n-6 PUFA for the FO intervention reached significance for ΔBI (p = 0.023) and ΔBE (p = 0.011): those for the KO intervention were not significant. For n-3 PUFA, differences between ΔBI and ΔBE were significant for EPA in the FO intervention (p = 0.003 and 0.001 respectively), but there was no significance between measurements for KO, whereas for DHA only ΔIE was significant for KO (p = 0.04). Total n-3 PUFA showed a significant difference for FO intervention between baseline and interim (ΔBI) and between baseline and endpoint ΔBE (p = 0.002 and 0.008 respectively), but not for KO intervention. The ΔBI and ΔBE for the n-6/n-3 ratio were also significant for FO but not KO intervention.

Changes in other plasma FAs: In addition to changes in n-3 and n-6 FA, significant differences were noted between baseline and interim measurements (ΔBI), and between baseline and endpoint (ΔBE) for stearate for the FO intervention (p = 0.04 and 0.05 respectively). For the KO intervention, no significant differences between baseline and interim measurements or between baseline and endpoint were noted (p > 0.33 for all variables) but a significant difference between interim and endpoint (ΔIE) was noted for stearate (p = 0.05*)*. Comparison of the mean difference between baseline and interim or endpoint revealed a significant difference in the change for palmitate (p = 0.05) and stearate (p = 0.05). For variables with non-normal distribution, the Friedman test indicated no statistically significant difference in variables across the three time points for either intervention.

	FO				KO			
Fatty acid	**Baseline: interim n = 16** *p*	**Baseline: Endpoint *n = 15*** *p*	**Interim: Endpoint *n = 15*** ***p***	**ΔBE vs ΔBI *p***	**Baseline: Interim *n = 13*** ***p***	**Baseline: Endpoint *n = 12*** ***p***	**Interim: Endpoint *n = 12*** ***p***	**ΔBE vs ΔBI *p***
Palmitate	0.73	0.78	0.66	0.59	0.15	0.53	0.06	***0.05***
Stearate	***0.04***	***0.05***	0.59	0.94	0.13	0.82	***0.05***	***0.05***[1]
Oleate	0.64	0.69	0.16	0.37	0.09	0.76	0.20	0.20[1]
LA	0.19	***0.01***	0.68	0.52	0.78	0.91	0.55	0.55[1]
LNA	0.25	0.14	0.51	0.51[1]	0.10	0.26	0.13	0.40
ArA	***0.04***	***0.07***	0.33	0.33	0.60	0.23	***0.06***	***0.07***
EPA	***0.002***	***0.001***	0.68	0.70[1]	0.44	0.87	0.35	0.74
DHA	0.09	0.11	0.47	0.56	0.20	0.67	***0.04***	***0.05***
LA + LNA	***0.023***	***0.011***	0.47	0.74	0.42	0.33	0.15	***0.11***
n3 total	***0.002***	***0.008***	0.86	0.97	0.48	0.78	0.12	0.24
n6/n3 ratio[1]	**0.000**	**0.000**	0.72	0.72	0.55	0.46	0.94	0.80

Table 4.14: Comparison between plasma FAs at different time points

Values are shown with SD in brackets. Data from Table 4.11 were compared using paired t-test for normal distributions, and [1]Wilcoxon signed rank test for non-normal distributions. SPANOVA (not shown) revealed no significant difference between or within groups. Changes (Δ) between baseline and endpoint could not be normalized, and were also compared using Wilcoxon rank test

4.5.5. Relationship between changes in CVD risk measurements and baseline values

The effectiveness of pharmacological interventions to mitigate CVD risk may be related to the degree of aberrance from normal at the start of the intervention (Wang *et al*., 2003): thus, an individual with significantly elevated plasma TAG at baseline might be expected to show a larger effect from dietary (or indeed pharmacological) intervention than one with only moderately raised levels. In order to investigate whether this might be relevant to findings from this study, correlations were calculated between the change and baseline levels of plasma measurements, as set out in Tables 4.15 (FO) and 4.16 (KO). Significant correlations are highlighted in these tables.

	ΔBI		**ΔBE**	
	Pearson correlation	***p***	**Pearson correlation**	***P***
Cholesterol (mmol/L)	-.004	.989	**-.592**	**.020**
TAG (mmol/L)	**-.585**	**.017**	-.166	.540
HDL-C mmol/L	.001	.998	-.345	.191
LDL-C mmol/L	.039	.887	-.062	.819
NEFA mmol/L	**-.782**	**.000**	**-.601**	**.014**
sdLDL-C mmol/L	-.324	.221	-.379	.148
sdLDL (% LDL)	**-.558**	**.025**	-.177	.511
TC/HDL	-.411	.114	-.280	.293
TAG/HDL	-.222	.408	.254	.342
Glucose mmol/L	**-.616**	**.011**	**.626**	**.009**
Insulin pmol/L	-.106	.697	-.092	.744

Table 4.15: Correlation between baseline measurements and changes in plasma lipid markers at interim and endpoint of FO intervention

Data were compared using Pearson correlation; significant correlations are shown in bold print

	ΔBI		ΔBE	
	Pearson correlation	***p***	**Pearson correlation**	***P***
Cholesterol	-.177	.544	-.373	.171
TAG	**-.617**	**.019**	-.168	.550
HDL-C	-.100	.734	-.434	.106
LDL-C	-.224	.441	-.372	.172
NEFA	**-.772**	**.001**	**-.577**	**.024**
sdLDL-C	-.461	.097	-.269	.332
sdLDL (% LDL)	**-.660**	**.010**	-.319	.246
TC/HDL	-.469	.090	-.397	.143
TAG/HDL	**-.628**	**.016**	-.217	.437
Glucose	**-.721**	**.004**	**-.819**	**.000**
Insulin	**-.742**	**.002**	-.524	.081

Table 4.16: Correlation between baseline measurements and changes in plasma lipid markers at interim and endpoint of KO intervention

Data were compared using Pearson correlation; significant correlations are shown in bold print

For FO, the correlation between baseline measurement and both ΔBI and ΔBE was significant for NEFA ($p < 0.001$ at interim and $p = 0.014$ at endpoint) and for plasma glucose ($p = 0.011$ at interim and $p < 0.009$ at endpoint). The correlation between baseline and ΔBI but not baseline and ΔBE was significant for plasma TAG ($p = 0.017$) and for sdLDL (as percentage of LDL) ($p = 0.025$). Correlations between baseline measurements and subsequent changes in TC, HDL-C or LDL-C were not significant.

For KO, a significant correlation between baseline measurements and both ΔBI and ΔBE was found for NEFA ($p = 0.001$ and 0.024 respectively) and glucose ($p = 0.004$ and <0.001 respectively) (Table 4.16). The correlation between baseline and ΔBI but not ΔBE was revealed for TAG ($p = 0.019$), sdLDL-C (percentage of LDL-C) ($p = 0.010$), TAG/HDL-C ratio ($p = 0.016$) and insulin ($p = 0.002$).

Scatterplots showing individual changes were inspected to see whether any additional inferences could be drawn from these relationships. These are depicted in Figures 4.6 through to 4.15 for measurements achieving significance, with Pearson correlation and significance for each. Most participants with baseline TAG in excess of 1.7 mmol/L (the cutoff point for MetS) demonstrated a fall in plasma TAG from baseline to interim and also from baseline to endpoint (Figures 4.6 and 4.7); the trend was significant for

measurement at interim timepoint. For HDL-C, there was no significant correlation between baseline measurements and the extent of change either at interim or endpoint, and no evident effect for those individuals with baseline HDL-C below 1.03 mmol/L (MetS cutoff point). Scatterplots for the correlations between sdLDL-C are shown in Figures 4.8 and 4.9; visual inspection of these suggests that some individuals with baseline sdLDL at more than 30% of total LDL may show larger decreases with n-3 LCPUFA intervention.

Correlation was observed between the baseline TAG/HDL-C ratio and the magnitude of change at the interim time point for KO intervention; visual inspection of the scatterplots (Figures 4.10 and 4.11) suggests that most individuals with a baseline TAG/HDL-C ratio >1.6 (in accordance with MetS cutoff points for TAG and HDL-C) exhibit a fall in the TAG/HDL-C ratio after 3 weeks intervention.

Correlation between baseline and magnitude of change was also noted at interim and endpoint for both FO and KO interventions for plasma glucose (Figures 4.12 and 4.13) and plasma NEFA (Figures 4.14. and 4.15).

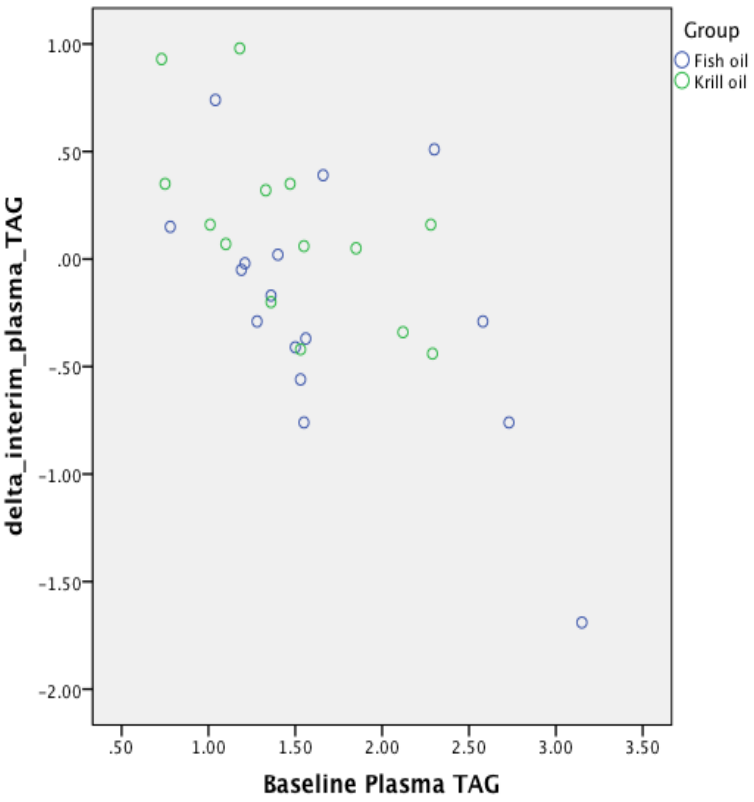

Figure 4.6 Correlation between baseline TAG and ΔBI
(all values in mmol/L; correlation = -0.61, p = 0.003)

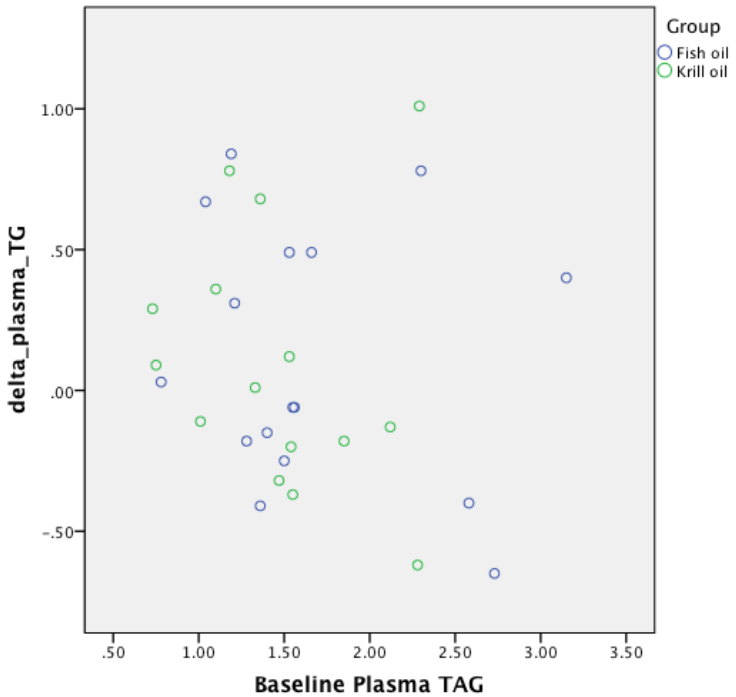

Figure 4.7. Correlation between baseline TAG and ΔBE
(all values in mmol/L, correlation = -0.158, p = 0.18)

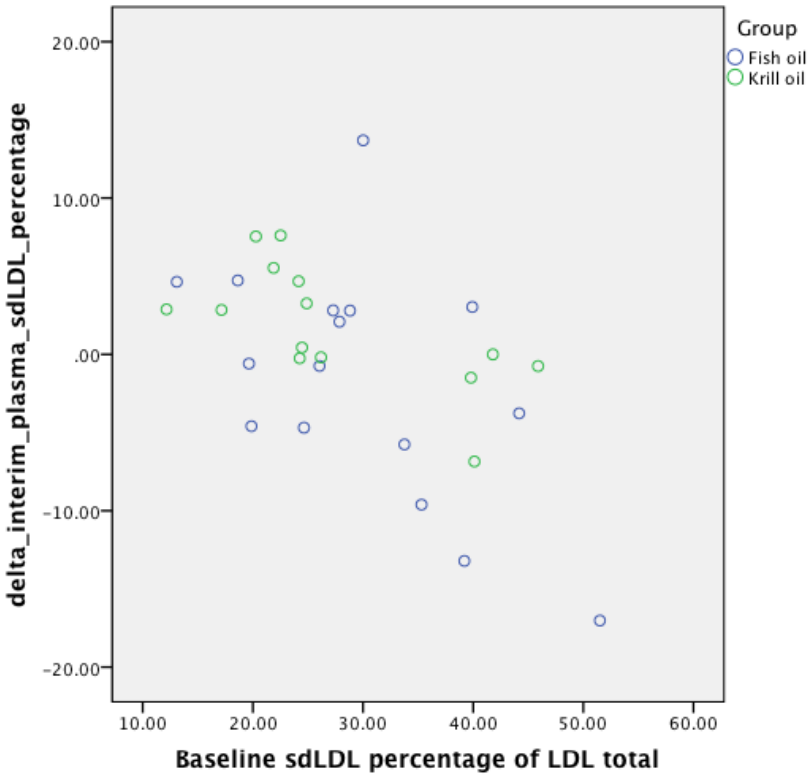

Figure 4.8: Relationship between baseline sdLDL (%) and ΔBI
(values expressed as percentages; correlation -0.660, p = 0.01)

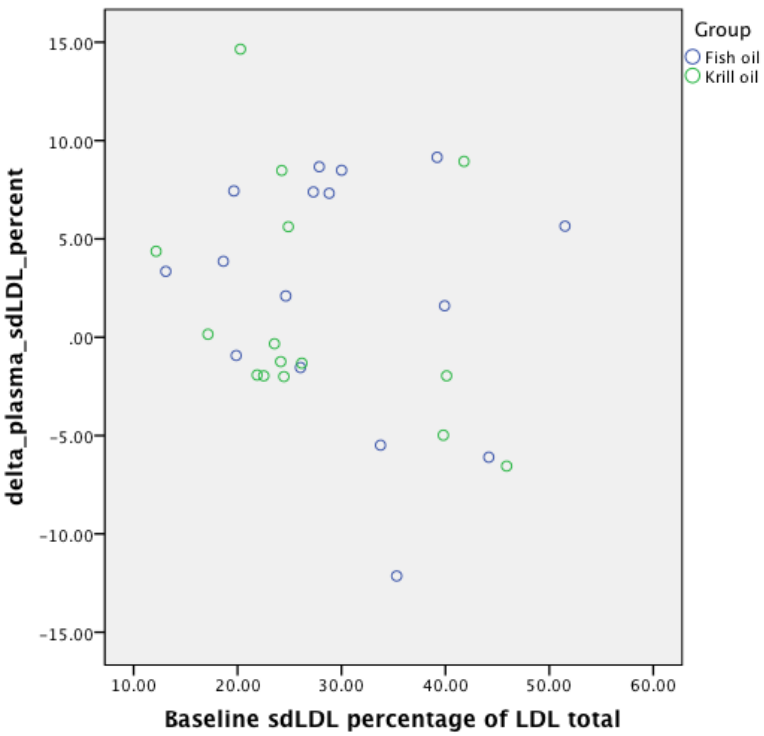

Figure 4.9: Relationship between baseline sdLDL (%) and ΔBE
(all values in percentages, correlation = - 0.32, p = 0.246)

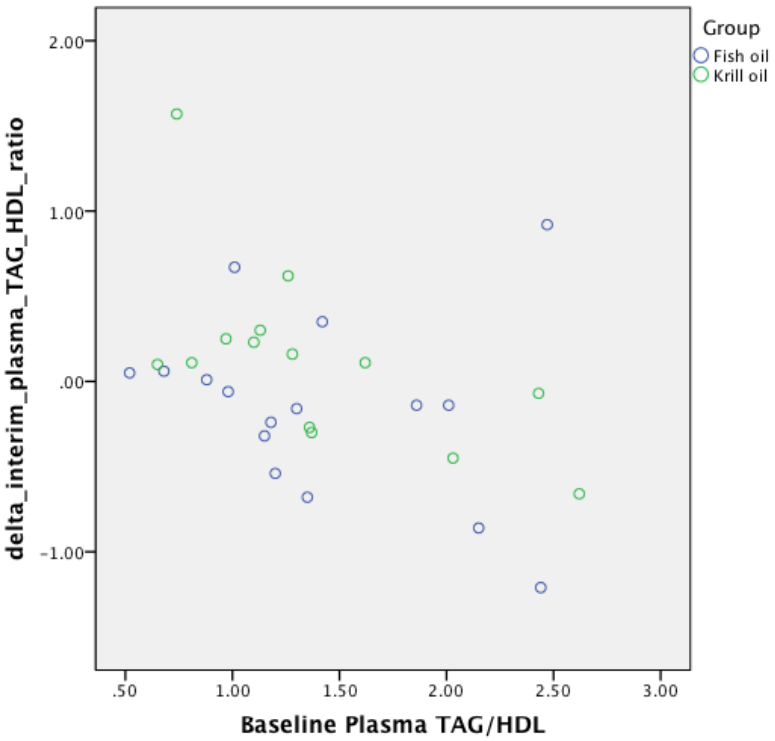

Figure 4.10 Relationship between baseline TAG/HDL ratio and ΔBI
(correlation = -0.628, p = 0.016)

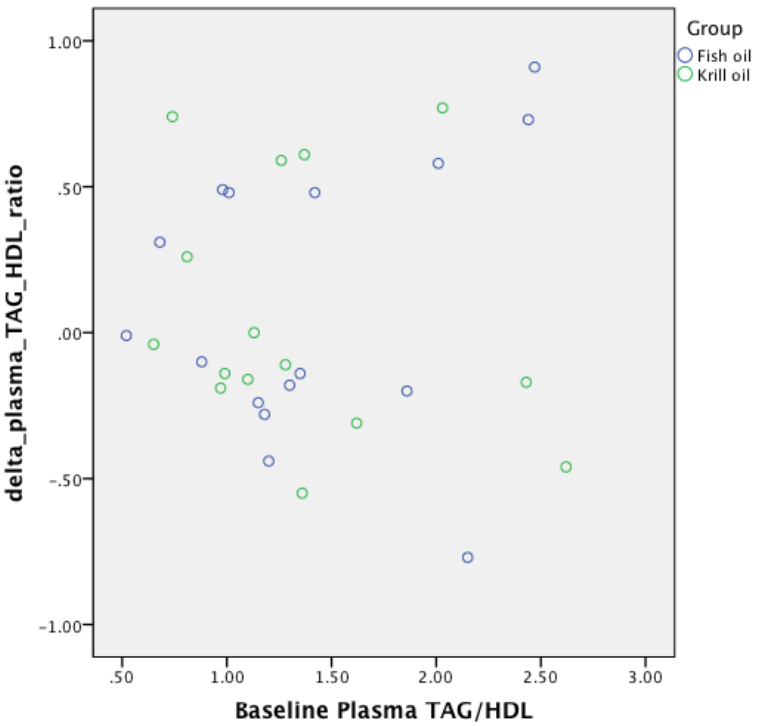

Figure 4.11 Relationship between baseline TAG/HDL and ΔBE
(correlation = - 0.217, p = 0.437)

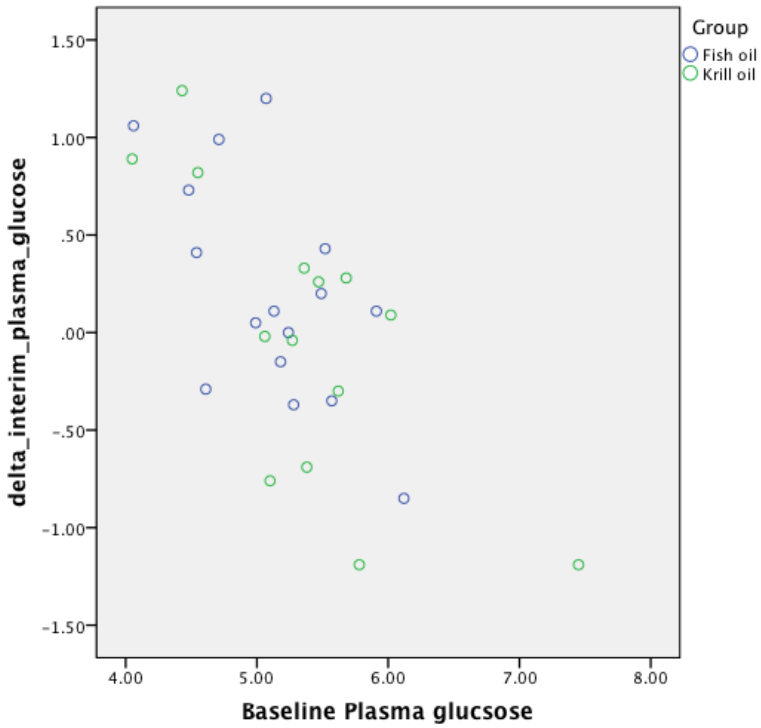

Figure 4.12 Relationship between baseline plasma glucose and ΔBI

(values in mmol/L, correlation = - 0.72, p = 0.004)

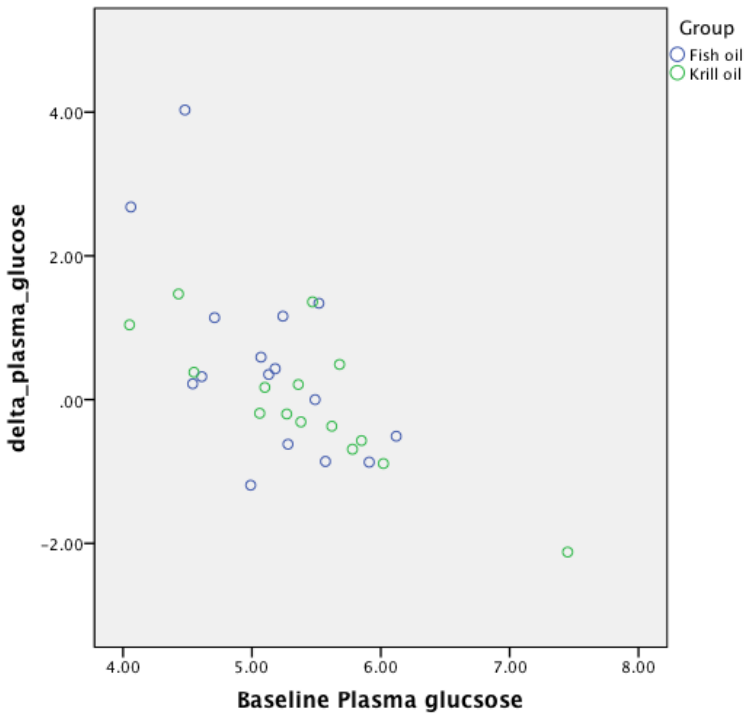

Figure 4.13 Relationship between baseline plasma glucose and ΔBE

(values in mmol/L, correlation = - 0.819, p <0.001)

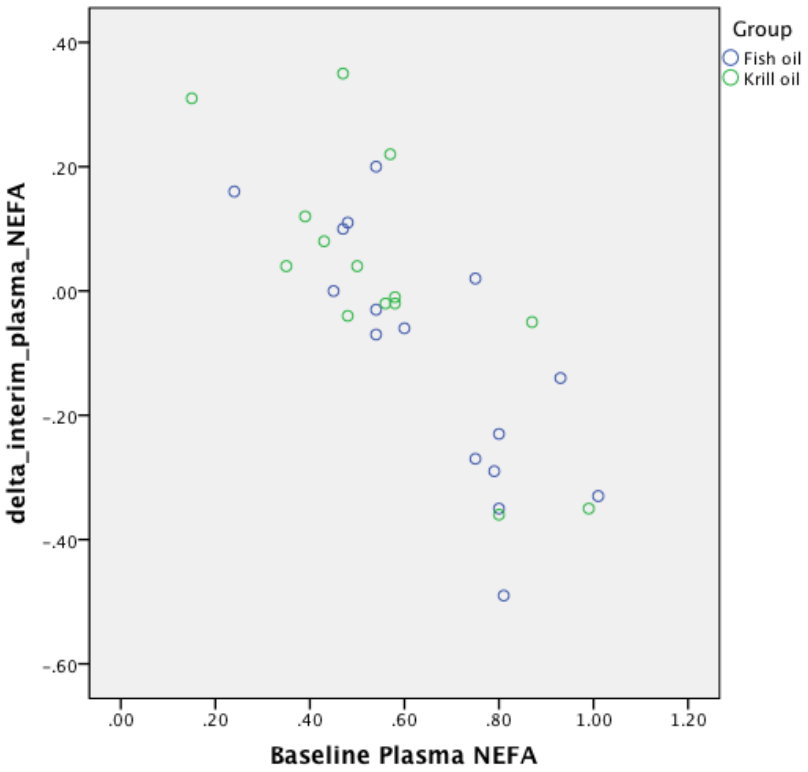

Figure 4.14 Relationship between baseline NEFA and ΔBI
(values in mmol/L, correlation = -0.77, p = 0.001)

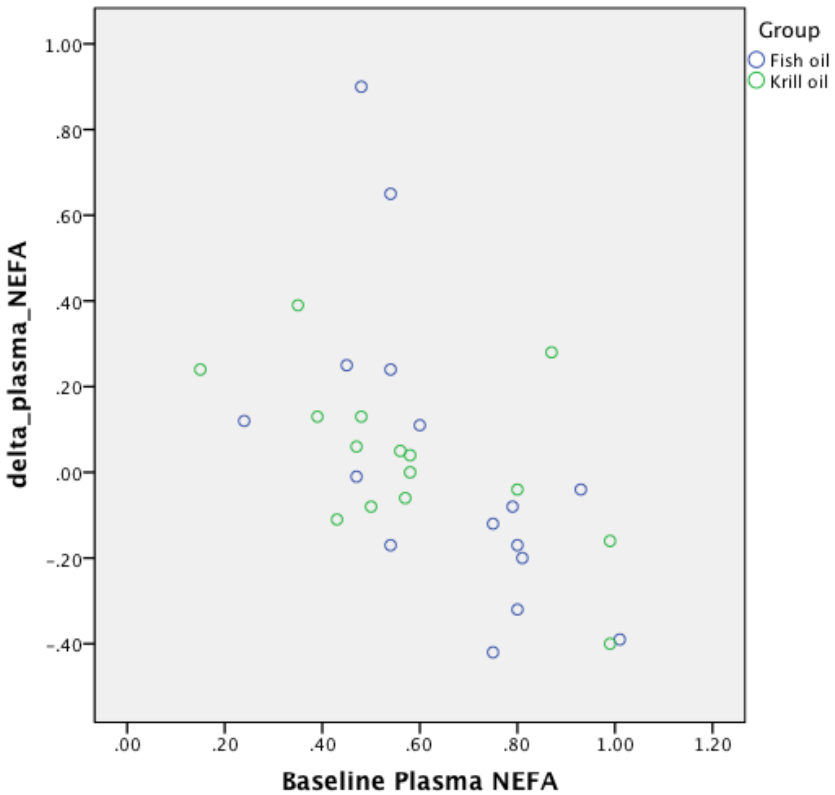

Figure 4.15 Relationship between baseline NEFA and ΔBE
(values in mmol/L, correlation - 0.58, p = 0.024))

4.5.6. Food intake records for nutrients other than-3 PUFA

Participants were requested to complete 7-day food diaries for the week before each intervention and the last week of each intervention (4 in total). The data from these was recorded and analysed using Microdiet[tm]. Of the 18 participants, only 10 completed all 4 weeks food records; the number completing the baseline and final diary for the first intervention was 14 and 15 respectively, and for the baseline and final diary for the 2[nd] intervention 11 and 14 respectively. Mean nutrient intakes with SD, and mean percentage changes from baseline to endpoint, are shown in Table 4.17.

A mean increase in overall energy intake was reported during the FO intervention (13%), whereas the change during the KO intervention was only 0.6%, although variation between participants was wide, and there were large percentage changes in several other nutrients, including individual sugars, and in alcohol intake. Alcohol intake was only analysed for those individuals who reported intake.

Sodium intake was not significantly changed during interventions, as deduced from food intake reporting, but potassium intake was increased during FO intervention, and fructose intake was also increased during FO but reduced during KO intervention.

	FO					KO				
	Baseline n = 14		Endpoint n = 15		Percentage Change	Baseline n = 11		Endpoint n = 14		Percentage change
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Energy (kcal)	2040	350	2304	643	+13.0	2197	389	2087	321	-0.6
Energy as % EAR	81.7	14.3	91.6	24.9	+12.1	87.6	15.3	83.2	12.0	-5.0
Nitrogen (g)	12.7	1.8	14.8	3.1		14.1	1.6	14.5	2.8	
Protein (g)	79.4	11.3	92.1	18.7	+16.0	88.4	11.3	91.4	17.8	+3.4
Protein as % RNI	146.6	20.2	169.4	32.6		163.3	22.1	168.3	31.7	
Protein as % energy	15.7	2.0	16.5	3.1		16.4	2.5	16.9	2.0	
Carbohydrate (g)	239.9	67.1	281.3	94.3	+17.3	267.0	56.1	256.8	59.7	-3.8
Carbohydrate as % energy	46.5	6.7	48.6	6.4		48.6	5.8	47.2	7.2	
Fat (g)	77.8	16.6	87.2	27.5	+12.1	82.1	21.2	81.1	21.8	-1.2
Fat as % energy	34.7	6.5	33.8	3.0		33.4	5.4	33.4	5.0	
Fat as % DRV	99.1	18.5	96.6	8.5		95.6	15.4	95.3	14.2	
Saturated fatty acids (g)	25.6	7.2	29.5	9.6	+15.2	30.2	9.9	27.8	8.8	-7.9
Saturated fat as % DRV	103.6	26.6	104.9	21.3		111.0	25.9	104.3	24.2	
PUFA (g)	12.0	2.5	14.7	5.3	+22.5	12.3	3.8	14.6	4.3	+18.7
PUFA as % DRV	83.3	24.5	87.4	17.4		78.0	19.7	92.6	21.6	
MUFA (g)	21.7	4.3	26.3	9.1	+21.2	23.0	7.6	24.5	5.3	+6.5
Cholesterol (mg)	203	67	167	86	-17.7	162	78	195	89	+20.4
Fibre (g)	11.0	5.0	12.4	4.2	+12.7	12.1	2.2	11.3	3.3	-6.6

	FO					KO				
	Baseline n = 14		Endpoint n = 15		Percentage Change	Baseline n = 11		Endpoint n = 14		Percentage change
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Soluble fibre (g)	3.8	2.3	4.1	1.5	-	4.1	2.1	4.7	2.4	-
NSP (g)	8.2	2.6	9.3	2.4	+13.4	9.1	3.4	9.5	3.7	+4.4
NSP as % DRV	45.5	14.4	51.5	13.4	-	50.6	19.1	52.8	20.5	-
Sugars (g)	90.2	45.0	100.5	47.9	+11.4	90.6	28.9	86.0	25.8	-5.1
Glucose (g)	10.2	7.2	10.4	6.3	+2.0	9.7	5.0	9.6	4.8	-1.0
Fructose (g)	10.6	7.3	12.5	8.8	17.9	11.1	6.6	10.4	5.9	-6.3
Sucrose (g)	19.4	15.0	18.1	13.1	-6.7	15.5	9.25.8	19.9	9.5	+28.4
Maltose (g)	5.8	2.9	5.2	3.4	-10.4	5.4	2.9	5.2	2.4	-3.7
Lactose (g)	9.0	4.0	10.6	5.2	+17.8	10.2	3.3	12.4	3.9	+21.6
Sodium (mg)	2889	552	2929	875	+1.4	3178	512	3234	786	+1.8
Potassium (mg)	2266	569	2534	461	+11.8	2455	653	2502	693	+1.9
Alcohol g*	18.2	20.2	17.3	22.9	-4.9	15.1	19.8	18.1	17.7	+19.9
Alcohol as % energy	8.9	6.7	7.3	6.4	-	7.1	5.6	8.0	5.2	-

Table 4.17 Changes in nutrient intakes

(Nutrient intakes derived from 7-day food intake records; all values as mean with standard deviations and percentage changes)

4.5.7. Influence of covariates

An ANCOVA analysis was conducted to investigate the possible relationship of dietary patterns on the overall effect of either n-3 supplement on plasma biochemical profiles. There was a small potential contribution of change in energy or protein intake to the overall changes observed, as indicated in Table 4.18.

	Change in plasma LDL		**Change in % plasma sdLDL**	
Component	*P*	**Partial eta^2**	*p*	**Partial eta^2**
Estimated n-3 LCPUFA	.522	.042	.302	.106
Change in energy (kcal)	.094	.255	**.049**	.335
Change in protein (g)	**.035**	.374	.069	.292
Change in total fat (g)	.316	.100	.152	.194
Change in carbohydrate (g)	.235	.318	.074	.284
Group effect	.873	.003	.292	.110

Table 4.18 Covariate effects of macronutrient intake on changes in plasma LDL and percentage sdLDL *(ANCOVA)*

4.6. DISCUSSION

The aims of this pilot study were to determine whether KO may be an effective alternative to FO as a source of n-3 LCPUFA in dietary mitigation of CVD risk. The study compared KO with a FO preparation, to observe effects of FO or KO on any perturbation of lipid and glucose metabolism by measurement of recognised markers and indices of MetS in conjunction with markers of dietary compliance (food diaries and plasma FA profiles). The main conclusion of this study is that KO vs FO at the doses used are comparable in respect of effects on CVD risk markers, and therefore the null hypothesis is accepted.

This small study was designed to obtain preliminary indications of effectiveness of KO compared with FO. The small cohort of 21 initial participants was further reduced by elimination of individuals for whom results of CVD markers and/or food intake data were more than 2 SD from mean measurements. At the time of embarking on this

study there were only a few studies of the effect of KO in animal models, and two reports of KO effects in dysmenorrhea and one in dyslipidaemia (Bunea *et al*., 2004; Deutsch, 2007).

4.6.1. Choice and dosage of supplements

Total n-3 LCPUFA concentration in the KO capsules (assuming full compliance) was 388 mg (250 mg EPA and 152 mg DHA, partly in PL). For FO, total n-3 LCPUFA in the form of TAG was 577mg (290 mg EPA and 214 mg DHA), thus providing approximately 150% n-3 LCPUFA compared with KO. A detailed comparison with the doses used by Bunea *et al* (2004) and Ulven *et al* (2011) is given in Section 4.6.2. KO also contained astaxanthin, at a level of 1.5 mg per daily intake. Astaxanthin is a powerful carotenoid antioxidant, with the potential to protect against lipid oxidation both in the supplement and *in vivo* (discussed in Section 4.6.2.3.). Preliminary experimental work suggested that this would still be available for absorption in the human intestine after exposure to the conditions of gastric acid and digestive enzymes; further work is required to extend and validate these findings, although recent publications are indicative of effective uptake of dietary astaxanthin.

4.6.2. Markers of CVD risk

Participants were recruited on the basis of fulfilling the criteria for MetS (IDF 2006), and all still fulfilled these criteria at the completion of the interventions. The mean changes were very small in comparison with the range of changes and the change in absolute measurements: there was no significant change in any anthropometric variable within or between interventions.

4.6.2.1. Overall changes in CVD markers

At the doses employed in this study, there was no significant overall effect on any markers of CVD risk for KO (388 mg DHA +EPA) or FO (588 mg EPA +DHA).

As the participants were recruited on the basis of a MetS profile, it was not possible to stratify the measurements in the same way as done for the prawn study described in Chapter 3. Correlations of baseline-interim and baseline-endpoint changes vs baseline

measurements were conducted, and visual inspection of these data was also carried out. Negative correlations with baseline were observed for FO intervention for baseline-interim change for glucose. Similar negative correlations between baseline and endpoint change were recorded for glucose (-0.626, p = 0.01) ad NEFA (-0.60, p = 0.01). For KO, significant negative correlations were observed between baseline and interim change for glucose, TAG, NEFA, sdLDL as % of LDL, and TAG/HDL-C, while for baseline-endpoint significant correlations were seen for glucose and NEFA only. Inspection of the scatterplots for these variables suggested that beneficial decreases in measurements may be achieved for participants with baseline TAG >1.7mmol/L, glucose > 6 mmol/L, NEFA > 0.75 mmol/L, sdLDL% >30% of total LDL and TAG/HDL-C > 1.6 mmol/L, arbitrary cutoff points representing values which correspond to the lower end of the range seen in MetS (IDF 2006).

While plasma FA measurements are not normally included in any description of MetS, it is suggested that individuals with baseline plasma NEFA > 0.6 mmol/L might be more likely to achieve benefit from n-3 LCPUFA intervention; NEFA might be a useful screening tool for future investigations of this type.

The use of compound indices of CVD risk has been proposed to provide more reliable markers for IR, T2D or CVD risk than stand-alone measurements while avoiding invasive and time-consuming diagnostic procedures such as the glucose clamp method (Vaccaro *et al.,* 2004). In this study the HOMA-IR index of IR, HOMA-β index of β-cell function and the QUICKI and R-QUICKI indices were calculated at baseline, interim and endpoint, and changes between baseline and both interim and endpoint also calculated. No significance was observed for comparisons of any time points for these indices, which appear to be less sensitive to changes in this study than stand-alone measurements.

Although participants were recruited on the basis of MetS, not all had a baseline TAG in excess of 1.7 mmol/L. From the data reported here, it is suggested that the effects of KO and FO at the doses used (FO n-3 LCPUFA 1.5 x that of KO n-3 LCPUFA) are similar, with more benefit for those with baseline TAG above 1.7 mmol/L, as shown in Figures 4.6 and 4.7.

The study by Bunea *et al*. (2004) reported effective reduction by KO of LDL-C and increase in HDL-C by KO. This study compared four groups each of 30 participants between 25 and 75 years of age over varying periods of at least 3 months: participants took KO (2-3g per day depending on body weight) for 90 days, KO (1-1.5g per day

depending on body weight) for 90 days followed by 500mg per day during a follow-up period, 3g FO for 90 days or a placebo (unspecified). Baseline TC was in the range 5.0 – 9.0 mmol/L and TAG in the range 2.3-9.2 mmol/L: reduction in TC was reported as between 13.4-13.7% for those receiving 1.0 or 1.5 g KO daily and 18.0-18.1% for those receiving 2-3g KO, compared with a 6% reduction for those receiving FO. LDL-C decreases of between 32 and 39% were reported, while HDL-C was reported to increase by 43-59%. Changes in TAG were reported to be non-significant for those receiving 1-1.5g KO or FO, compared with reductions of 27-28% for those receiving 2-2.5 g KO. Those receiving placebo exhibited a rise in TC (9.1%), an increase in LDL-C (13%), no change in HDL-C, but a 9.8% decrease in TAG. A fall in blood glucose levels was also observed for those taking KO: the reduction of 6.3% for the lower KO group compared with a 5.6% reduction for the higher KO group and 4.3% for FO, but it is interesting to note that the baseline glucose for the former KO group, at 5.8 mmol/L, was higher than that for the latter (5.1 mmol/L). This may be compared with the observation made in this thesis, of a potentially greater effect on plasma glucose by FO and KO for individuals with higher baseline plasma glucose levels (>6 mmol/L). The higher doses of KO used in the Bunea study were comparable with those used in the current study, but the study period at 12 weeks was considerably longer. Variable doses of KO were used according to starting BMI, which makes precise interpretation and comparison of the results difficult, but there would appear to be a level of agreement between the Bunea study and the study described in this thesis regarding beneficial effects on plasma TAG, LDL and glucose.

Maki *et al.* (2009) reported a comparison of daily supplementation of 2g KO, FO (menhaden) or olive oil in 3 separate groups of overweight and obese adults (76 in total) over a period of 4 weeks. Increases reported in plasma EPA and DHA were very much higher in percentage terms for FO and KO than reported in this chapter, although the daily intake of EPA and DHA (216 and 90 mg respectively) was lower than that in the present chapter (250 and 152 mg respectively). A decrease in TAG, and increases in glucose, insulin, HOMA- IR, TC, LDL-C and HDL-C were observed, but the differences between FO and KO were not significant. The finding of a reduction in plasma TAG associated with n-3 LCPUFA intake is in agreement with that observed in the investigation reported here for individuals with MetS.

Ulven *et al*. (2011) compared the effects of a daily intake of KO (3g, n-3 LCPUFA 543 mg) with FO (1.8g, n-3 LCPUFA 864 mg) over a 7 week period. They reported similar

increases in plasma concentrations of EPA, DHA and DPA between the two groups. Intakes of n-3 LCPUFA were similar in proportion (FO:KO) to those administered in the investigation described in this thesis, although approximately 1.5 times higher for both. No significant differences between the two supplements were noted for changes in HDL-C, TAG, HDL-C/TAG, TC, ApoA1 or ApoB. The participants in the Ulven study were of normal BMI and thus not directly comparable with the MetS participants described in this thesis. Small changes in levels of HDL-C, LDL-C and TAG were reported: the change in LDL-C was significant for FO compared with KO. A significant increase in the HDL-C/TAG ratio was observed for the KO intervention. Neither intervention was associated with a significant change in TAG, but it was noted that there was a greater reduction in TAG for those individuals with higher baseline values (those with TAG > 1.7 mmol/L). This finding is in agreement with the observations reported in this thesis.

It is of interest to note the very wide range of HOMA changes compared with the mean; this might indicate failure by some participants to comply with instructions to fast prior to blood tests, or a wide variation in individual response to the KO or FO interventions. Maki *et al* (2009) reported an increase in HOMA-IR status in obese individuals following 4-weekly intake of KO or FO (2 g daily), whereas in this investigation there was no significant difference. The use of HOMA modelling has been reviewed by Wallace *et al.* (2004), who suggested that use of a single sample for measurement of plasma glucose and insulin and hence HOMA-IR and HOMA-β indices may result in coefficients of variation up to 10.3% and 7.7% respectively, and that use of three samples taken at 5-minute intervals would be advisable.

4.6.2.2. Relation to n-3 LCPUFA intake

Given the difference in total n-3 LCPUFA, the higher dose of FO conferred no greater benefit overall than KO on cardiometabolic risk profile. At the interim time point, the effect from KO was greater than FO in certain respects. The reasons for the apparent fall-off in effectiveness may be related to lack of compliance for the full 6-weeks of the intervention, (perhaps combined with alterations in intake of other foods).

4.6.2.3. Relation to PL or astaxanthin component

It was not possible to determine in this study whether the apparently greater effect of KO over the first three weeks of intervention could be attributed to either n-3 LCPUFA in PL form or astaxanthin. In respect of a potential PL role, different formulations of n-3 LCPUFA were compared by Schuchardt *et al.* (2012) in 12 healthy men. Using doses

of 1680 mg EPA+DHA from FO, KO or as ethyl esters, they measured uptake of EPA and DHA in plasma lipids over a 72 hour period, and estimated relative mean uptakes from KO of approximately 80% compared with 60% from FO and 48% from ethyl esters. If this finding is representative for KO from all commercial sources, it is not unreasonable to expect that KO and FO administered at relative doses of 1:1.5 (as in the study described in this thesis) may have similar effects. Zhu *et al*. (2008) reported reductions of TAG, glucose, and LDL-C after 4 weeks administration of KO to a group of 10 hyperlipidaemic rats. KO supplementation of a high fat diet (1.25- to 5% w/w) in mice (Tandy *et al*., 2009) was associated with a reduction in mean TC (20-29%) and blood glucose (34-42%).

Earlier work carried out by Lieber (2004) suggested that PC from soybean has a protective effect in alcoholic liver damage. The effects of PC *in vitro* include reduction in TNF-α production by Kuppfer cells: dietary PC (not associated with n-3 LCPUFA) has also been reported to reduce liver cholesterol and liver TAG in hypercholesterolaemic rabbits and in hyperlipidaemic rats (Cohn *et al*, 2008). Supplementation with purified soybean PL (1800 mg/day for 6 months) has also been associated with reductions in TC, TAG and LDL-C in hyperlipidaemic individuals, with concomitant increase in HDL-C: an increase of 12-18% in HDL-C following administration of phosphatidylinositol (2800 mg or 5.6 g per day for 2 weeks) has also been reported (Cohn *et* al., 2008). The doses of PL used in these small uncontrolled studies were very much higher than in the investigation reported in this thesis (1272 mg).

Very few studies have so far been carried out on the effects of astaxanthin in human intervention studies. Hussein *et al*. (2005) reported that astaxanthin administration to spontaneously hypertensive rats was associated with a reduction in blood pressure, plasma nitrite/nitrate and lipid peroxidation, although the lipid peroxidation effect (at a dose of 5mg/kg/day) was no greater than for olive oil (1 mL/kg/day). Astaxanthin, being similar to other carotenoids such as β-carotene and canthaxanthin, might be expected to play a role in inhibition of LDL oxidation but this has not been fully substantiated *in vivo* in human studies (Higuera-Ciapara *et al*. 2006). Fassett & Coombes (2009) reviewed a total of 8 studies involving 180 human participants and reported that they demonstrated no adverse outcomes but did not comment on any positive findings.

Possible mechanisms by which KO may exert a beneficial effect compared with FO have been the subject of some speculation. A preliminary comparison of gene expression in mice and rats fed with FO and KO (Burri *et al*., 2011) has provided

evidence that KO may downregulate pathways involved in hepatic glucose production, and in synthesis of lipid and cholesterol. Greater reduction of lipogenic enzymes and the mitochondrial TCA carrier by KO than FO was also reported in a feeding study in rats (Ferramosca *et al*., 2012a). The potential of KO to downregulate the endocannabinoid system in obesity has also been investigated. Endocannabinoids are derived from ArA incorporated in the sn-1 or sn-2 position of PLs, and it has been proposed that increased activity in the endocannabinoid system may contribute to increased adiposity and other features of MetS. Increased levels of anandamide and 2-arachidonoylglycerol (2-AG) have been reported in overweight and obese individuals. There have been recent reports of downregulation of the endocannabinoid system in obese rats (Batetta *et al*., 2009), in mice and more recently in a cohort of 63 normolipidaemic men and women with raised BMI (Piscitelli *et al*., 2011, Banni *et al*., 2011). Increased levels of endocannabinoids were observed in this latter group; KO administration at 2g/day (306 mg n-LCPUFA) for 4 weeks was associated with a significant decrease of 2-AG in obese subjects compared with FO (390 mg n-3 LCPUFA). These authors suggested that a 4-week intervention is not long enough to achieve beneficial metabolic effects in MetS. In the investigation described in this thesis, small improvements in TAG, sdLDL and NEFA were evident after 3 weeks of FO and KO intervention. It would be interesting to ascertain the extent to which these changes might be enhanced after a longer period of full compliance.

4.6.2.4. Plasma FA profiles as measure of compliance

Inspection of data presented here indicate that for KO, changes in FA profile achieved at midpoint were reversed by the endpoint of the study. It may be interesting to speculate on the reasons for these observations. One explanation could be that changes in plasma FA during FO intervention indicate a continuing compliance with the supplement by the majority of participants, compared with a lower compliance by participants during the second half of KO intervention. Alternatively, this might reflect some other changes in dietary intake during the course of the KO but not the FO intervention. In the study reported by Maki *et al.* (2009) the percentage rise in plasma concentrations of EPA and DHA following 4 weeks KO intervention were very much higher (approximately 90% and 23% of baseline) than measured in the investigation reported in this chapter (both EPA and DHA changes less than 10% of total plasma FAs reported on a w/w basis. It is speculated that this could be indicative of greater compliance in the study reported by Maki.

4.6.3. Food intakes

It was evident from food diaries that there was a large variation in both baseline diet, and in changes during intervention periods. One participant showed evidence of very high n-3 LCPUFA intakes and was excluded from the study retrospectively, and another was excluded because of dietary intake and plasma FA measurements more than 2SD from the mean. Of the 18 initial participants, only 10 completed all 4 food records; the number completing the baseline and final diary for the first intervention was 14 and 15 respectively, and for the baseline and final diary for the 2nd intervention 11 and 14 respectively.

A mean increase in overall energy intake was reported during FO, and a slight decrease during KO intervention, although as with the prawn study, overall variation was very large, with large percentage changes in several other nutrients, including individual sugars, and alcohol intake. Sodium intake was not significantly changed during interventions, as deduced from food intake reporting, but potassium intake was increased during FO intervention; fructose intake was also increased during FO but reduced during KO intervention.

4.6.4. Comparison of capillary and plasma measurements

Comparison of measurements of CVD risk markers using the Cholestech LDX point-or care analytical system with those obtained by Autoanalyser techniques following separation of plasma from venous blood gave good linear correlations for all measures used, as described in Section 2.3. The advantages of the point-of-care method are of convenience for the participants; for the investigator also results can be obtained immediately at a study visit. This permits prompt identification of individuals who have been non-compliant (e.g. with pre-test fasting) allowing repeat testing or timely elimination of those who are repetitively non-compliant.

The accuracy of point-of-care techniques has been questioned. There is a need for caution in comparing absolute measurements from different studies where different analytical methods have been used, but it is suggested that for studies of the type described in this thesis, use of the point-of-care system provides a convenient and efficient monitoring method.

4.6.5. Conclusion

The overall conclusion from this investigation is that KO is comparable to FO in effects on markers of CVD risk at approximately 2/3 of the n-3LCPUFA concentration. There are concerns that participants in this study exhibited reduced compliance with the KO supplementation compared with FO, as inferred from the plasma FA profiles.

The cohort studied included only males with features of MetS according to the IDF (2006) (Alberti *et al., 2006)* definition. There was a wide variation in dietary habits within the cohort; this also varied significantly for individual participants between start and end or between interventions.

The timescale for intervention was not sufficient to obtain detailed analysis of erythrocyte membrane lipids, which would give a better overall view as to how dietary intervention of this nature might affect long-term physiology and cardiovascular risk.

It is of interest to note that an application was submitted to the European Food Safety Agency for consideration of health claims related to a KO product in relation to maintenance of normal blood TAG, and of normal blood LDL-C (EFSA Panel on NDA, 2012). The Panel adjudicated that maintenance of these parameters is a beneficial physiological effect, but that there are at present no published human studies which would justify scientific substantiation of these claims.

CHAPTER 5

SUMMARY AND DISCUSSION

The work described in this thesis has contributed to the body of knowledge in respect of dietary n-3 LCPUFA sources and human health. The aspect chosen for investigation was cardiovascular health; experimental work specifically compared a) the effects of prawns as a source of n-3 LCPUFA with a processed white fish source, and b) the effects of krill oil (KO) versus fish oil (FO). Two questions were addressed: a) whether there is a justification for advice to avoid prawns as a source of dietary n-3 LCPUFA because of associated dietary cholesterol, and b) whether KO may be a more effective n-3 LCPUFA supplement than FO in respect of CVD risk profile. The findings for both studies are summarised in Table 5.1.

From the results described here it is proposed that it is not necessary to avoid prawns (and by implication other shellfish) as a source of dietary n-3 LCPUFA because of possible adverse effects on cardiovascular health from cholesterol present in these foods; evidence also suggests that beneficial effects of prawns as a source of n-3 LCPUFA are more evident in individuals with clearly defined MetS. The comparison of KO with FO has confirmed previous reports by Bunea *et al.* (2004) and Ulven *et al*. (2011) that KO is equivalent in effect at approximately 2/3 the n-3 LCPUFA concentration of FO, with effects on markers of CVD risk more evident in those individuals with baseline measurements raised above normal, i.e. within the criteria for MetS.

5.1. RELEVANCE OF FINDINGS

The question should be asked as to what these studies have added to the already extensive body of knowledge/research data in respect of n-3 LCPUFA and human health. Key findings and conclusions are summarised in the following sections.

The potential importance of n-3 PUFA, in particular LCPUFA, in human health, has been extensively reviewed (Chapter 1, Section 1.2). There are clear arguments from an evolutionary perspective (Simopoulos 2003; 2011), from experimental evidence,

	Prawn/OC	KO/FO
Number	n = 21 healthy males (half fulfilled MetS criteria)	n = 18 males with MetS
Design	Crossover study, 4 weeks with washout	Crossover study, 6 weeks with washout
Measurements	Anthropometric, markers of CVD risk and food intake diaries	
Overall finding	No adverse effect from prawns compared with control	No difference in effects on MetS markers at doses used
Detailed observations	Pulse: 8% decrease following Pr vs 7% increase (OC), *p = 0.016*	No difference between groups
TAG	For high BMI group, ΔPr = -0.017 mmol/L vs ΔOC = +0.30 mmol/L, p = *0.033*	Correlation between baseline measurement and magnitude of effect: -0.611 after 6 weeks, *p < 0.01*
ApoB	**ΔPr = -7.8 mg/dL vs** ΔOC = + 2.4 mg/dL, *p = 0.023*	Not measured
	Differences also noted between high and low BMI groups for changes in VLDL and HDL	Correlation between baseline and magnitude of change also noted for NEFA, glucose and sdLDL

Table 5.1 Summary of findings from clinical studies

and reviews of food intakes and health, that adequate intakes of n-3 are needed for growth and development. At the other end of the human age range, there is increasing evidence that n-3 LCPUFA may play a role in risk reduction in a number of degenerative diseases of ageing, notably in the mitigation of inflammatory processes and the spectrum of cardiovascular diseases (including stroke and MI). Surveys of n-3 LCPUFA intake have demonstrated that many adults in developed countries have a suboptimal n-3 LCPUFA intake, resulting in a focus on ways to encourage adequate intakes. Reliance on intake of ALA-rich vegetable oils is not recommended, as the elongation-desaturation processes are not sufficiently efficient to fulfil the requirements for n-3 LCPUFA, particularly in the face of high intakes of n-6 PUFA commonly encountered in Western-style diets. Recommendations to increase consumption of oily fish find only limited compliance; possible reasons for this include resistance to flavour,

cost, or convenience of preparation. Prawns and shellfish are a popular and nutritious food option, but have not been widely recommended in the context of n-3 –rich foods because of concerns regarding risks from associated dietary cholesterol. The study described in Chapter 3 has addressed the question as to whether markers of CVD risk are adversely affected by prawn consumption and shown no clear disbenefit from prawn intake compared with a white fish control.

An alternative to n-3 rich foods is the use of n-3 supplements. Cod liver oil and other FOs are now widely available in pharmacies and health food stores, sold both in the form of capsules and in bottles, with a range of n-3 LCPUFA concentrations and EPA/DHA ratios. In some cases, n-3 LCPUFA content has either been concentrated from the original FO by distillation/chromatographic techniques, or derivatisation into ethyl esters. In the UK (as in the rest of the EU), health claims made for these products are required to satisfy the scrutiny of EFSA, the European Food Safety Agency, in respect of scientific substantiation.

An additional approach to achieving increased n-3 LCPUFA consumption is by design of foods containing n-3 LCPUFA or compositional modification of standard foods. In recent years, n-3 oils have been used to supplement poultry diets to increase n-3 PUFA content of the FAs of eggs: there is now a wide variation in n-3 FA content in eggs for sale in supermarkets in the UK (Gillian Butler communication at Nutrition Society meeting, Edinburgh 2013; Goldberg *et al.,* 2013). There is also evidence from the rapidly expanding aquaculture industry that the nutrient composition of farmed shellfish including prawns may vary according to the feedstock provided. There are practical issues associated with supplementation of standard foods while maintaining palatability, and commercial considerations of consumer acceptance, which have not been fully resolved (S. Salminen, personal communication).

An alternative development in n-3 LCPUFA ingredients has been that of the so-called ‘Single Cell Oils’, produced by fermentation from algae such as *Mortierella Alpina*. These have found application both in infant formulae and as supplements in capsules similar to the FOs. Incorporating higher levels of n-3 LCPUFA into plant phospholipids using GMO technologies could perhaps be an interesting and exciting way to enhance n-3 LCPUFA intake

The sustainability of FO as an n-3 LCPUFA source is open to question, due to concerns about sustainability of overfishing and fish stocks worldwide. KO has been identified as an alternative and possibly sustainable source of n-3 LCPUFA, as

described in Chapters 1 and 4 of this thesis. KO was chosen for this study on the basis that on a dose/dose basis of n-3 LCPUFA it may be a more effective mitigant for CVD risk than either FO or other n-3 LCPUFA preparations such as ethyl esters. The small study carried out here appears to corroborate this in respect of FO. Krill are found in oceans worldwide and although until recently commercial krill harvesting has been aimed at aquaculture and sport fishing applications, it is suggested (Tou *et al*., 2007) that a conscientious ecosystem approach to maintaining krill stocks should allow long-term sustainability of this as a source of n-3 LCPUFA for human consumption.

5.1.1. Health benefits or risks associated with prawn consumption

The work described in Chapter 3 indicates that prawns as a source of n-3 LCPUFA are of benefit in improving CVD health markers in normolipidaemic male volunteers. There is no direct evidence that the ingestion of dietary cholesterol from prawns adversely affected this cohort. This is consistent with findings from other cholesterol-containing foods, which do not adversely affect markers of cardiovascular disease in the majority of individuals (Kanter *et al.,* 2012; Tanasescu *et al.,* 2006; Houston *et al.,* 2011); while there may be some individuals in whom serum cholesterol may be increased (Fernandez, 2012), it is not clear at present whether the overall increase in LDL-C and HDL-C may obviate any increased CVD risk. This is an interesting topic for future work. As described in Section 3.1, evidence from prospective cohort and intervention trials had shown that dietary cholesterol has a clinically insignificant effect on blood LDL-C and associated CVD risk, but at the time of commencing this study there was no specific evidence to refute a link between prawn consumption, LDL-C and CVD risk.

Prawns are a popular foodstuff, often used in contemporary cuisine in the UK. Given recommendations to increase n-3 consumption, it may be that this is one way of providing variety in n-3 sources in the regular diet. The fact that approximately 2/3 of the n-3 LCPUFA in prawns are present as PL (Tou *et al.*, 2007) may confer benefits in terms of uptake of n-3LCPUFA compared with fish oil sources at similar concentrations of DHA and EPA. It would be interesting in a future study to provide a control supplement which contains n-3 LCPUFA in TAG form alongside a white fish protein source, to distinguish between the effects of n-3 LCPUFA and cholesterol content of the diets.

5.1.2. Relative efficacy of krill and fish oils as sources of n-3 LCPUFA

At the doses used, the KO and FO supplements used in this study show equivalent effects on CVD health markers in male volunteers with a profile of MetS as shown in overall changes after 6 weeks. There was evidence for both KO and FO that improvements in CVD markers (TAG, sdLDL as percentage and glucose, as well as NEFA) were more noticeable for those with a more dyslipidaemic baseline profile. These correlations were greater for KO in respect of the change at the interim time point than at the endpoint. Taken together with the plasma FA profiles, there is a strong supposition that compliance may have diminished in the second half of the KO intervention. It is interesting to speculate whether the KO would have demonstrated greater benefit compared with FO in those individuals with 100% compliance. The dose of KO used provided a lower daily intake of n-3 LCPUFA compared with FO (388 mg and 577 mg respectively): the mean calculated daily n-3 LCPUFA intakes including other dietary sources were 970 and 1060 mg respectively.

It was not possible to demonstrate in this study whether, as suggested elsewhere, the PL-derived n-3 LCPUFA may be absorbed more efficiently than the TAG in the FO. Mechanisms by which PL-derived n-3 LCPUFA may exert beneficial effects include reduction of TNF-α, and beneficial modifications of the lipoprotein profile, although at present most of the evidence in this respect comes from studies in animal models. The possibility that phospholipid-derived n-3 LCPUFA may influence the endocannabinoid system and thus contribute to control of obesity and dyslipidaemia through these pathways is an exciting avenue for future exploration.

The role of astaxanthin, which is present at significant concentrations in KO, might also be beneficial in protection of cardiovascular health: this is an area which requires further investigation. Some preliminary work was carried out which suggested, in an *in vitro* model of digestion, that astaxanthin in KO might reasonably be expected to be absorbed into the bloodstream and therefore exert some biological antioxidant function *in vivo*. A mechanism for the role of astaxanthin in protection against LDL oxidation merits further investigation. It is not known whether the benefits of n-3 LCPUFA and astaxanthin may be synergistic; a future study could include an additional group for intervention with an astaxanthin supplement alone. Another approach to this would be to compare FO alone with FO plus astaxanthin. It would be useful to be able to measure astaxanthin uptake in the blood following intake, and to compare varying intakes of astaxanthin to determine an optimum dose.

5.1.3. Mechanisms of n-3 LCPUFA in mitigation of CVD risk

A common effect observed in both investigations was a reduction of TAG, with a demonstrable correlation between baseline TAG level and magnitude of reduction. The role of n-3 LCPUFA in reducing CVD risk in dyslipidaemic individuals may be in modification of the lipoprotein profile to alter the balance of transport functions between lipoproteins, increasing the clearance of lipids from blood vessels to the liver compared with transport of lipids and cholesterol to the blood vessel and tissues (where they may contribute to development of atherosclerotic lesions). There is an increasing body of evidence for inflammatory processes in atherosclerotic disease, and for the role of n-3 LCPUFA in moderation of such processes in other degenerative inflammatory diseases. This may be by competition with ArA for eicosanoid synthesis, resulting in reduced synthesis of the highly pro-aggregatory TXA_2, by reduced synthesis of other pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, and reduced expression of PDGF-Q, PDGF-B and MCP-1. The main mechanism by which n-3 LCPUFA affect TAG levels is believed to be via upregulation of genes involved in TAG metabolism, particularly in dyslipidaemic individuals (Schmidt *et al.,* 2012c). The transcription factor peroxisome proliferator-activated receptor–α (PPAR-α) is up-regulated within days of n-3 LCPUFA supplementation; expression of ApoCIII and ApoB is reduced, resulting in both a reduction in VLDL synthesis and an increase in VLDL catabolism. Regulation of other transcription factors by n-3 LCPUFA has been reported, including hepatic nuclear factors involved in glucose and cholesterol metabolism, and other genes with an influence on cholesterol synthesis and efflux. The role of n-3 LCPUFA in increasing HDL-C levels in dyslipidaemia is not well understood; the existence of subclasses of HDL lipoproteins has only recently been identified, and elucidation of individual roles is yet to be established.

5.2. STUDY LIMITATIONS

There were some limitations to the studies performed which, together with the results obtained, may provide feedback for design of future studies of this kind.

In the first study, the effects of prawns vs a white fish product (OCs) were compared in 21 male volunteers, employing a cross-over study design. Both study components

were purchased from regular supermarket sources and provided for the participants to utilise as part of the normal diet. The KO study compared the effects of KO capsules vs FO capsules in a group of 18 men with indices of MetS, also using a cross-over study. The interventions were randomised so that half of each group received the intervention or 'placebo' first, with reversal for the second intervention phase. The advantage of this type of study is that it enables a direct comparison for each individual between the two interventions. Disadvantages are, from the point of view of the participants, that the study is more complicated and requires participation for, in this case, 18 weeks, allowing for a 6-week washout period. This reduces the potential for full compliance.

5.2.1. Selection of participants

Only male subjects were recruited for either study, the intention being to reduce individual variations due to hormonal fluctuations which might occur in a study involving female participants. In a small study it is desirable to limit confounding factors as far as possible. The KO study recruited men with two or more indices of MetS, anticipating that beneficial effects of n-3 LCPUFA interventions would be more evident than in individuals with no initial deviation from normal. However, this then begs the question as to whether a supplement which may be of benefit to an 'at risk' group of individuals may also be justifiably recommended for use in prevention of disease in a normal sample or population. For the Pr/OC study, the participants were not known to be at any increased risk of MetS, T2D or CVD compared with a random sample of the population, although approximately half of the participants in this study were overweight or fell within the MetS criteria as deduced from BMI measurements. One candidate who came forward for inclusion in the krill study had to be declined on the basis of screening tests which suggested that he may already have T2D. It has been suggested that the individual variation seen in response to diet and medication could be associated with specific genotypes; although it was not possible to pursue this line of enquiry in this study, a number of advances have been made in technologies which would enable this in future studies.

The initial screening data for participants in the prawn study enabled stratification of results between those with high and normal BMI, from which it became evident, even in the small numbers studied, that there were greater effects on CVD markers in those with higher baseline measurements. In the KO/FO study, volunteers were recruited on

the basis of fulfilling the criteria for MetS at initial screening. It was not possible, therefore, to carry out a stratification of results in the same way as for the prawn study. Nevertheless, inspection of the data and comparison of changes with baseline measurements also gave a clear indication that benefits of n-3 LCPUFA from either source were more evident in those with higher baseline measurements.

5.2.2. Size of studies

The number of participants in each clinical study was calculated as a minimum to obtain statistically significant data, based on previous published information. Of the number of potential participants, some were eliminated at an early stage due to unsuitability from screening tests, and others were excluded from the final analysis because either they demonstrated characteristics outside the expected range, or because it was evident from the results that they had failed to comply with the instructions provided (specifically failing to fast prior to blood tests). This reduced the numbers available for analysis. It was not possible to extend the time period for recruitment to include additional participants. Notwithstanding these limitations, it has been possible to derive conclusions in both intervention studies to provide a basis for future work.

5.2.3. Duration of interventions and washout periods

The intervention and washout period for the prawn study (chapter 3) was 4 weeks, and for the krill study (Chapter 4) 6 weeks. Although recent work has indicated that effects of n-3 LCPUFA on expression of transcription factors may be seen within one week of supplementation (Schmidt *et al.,* 2012a; 2012b; 2012c), a 4-week intervention period is a short length of time in which to reasonably determine dietary effects on lipidaemic profiles. A longer period would enable more detailed analysis of the uptake of FAs in, for example, red cell membranes or muscle PLs: the average lifespan of a human erythrocyte is 120 days (Shemin & Rittenberg, 1946), so that it may be unrealistic to estimate changes in turnover of erythrocyte membranes or lipid composition in less than a 3-month period. However, this may result in greater difficulties in recruitment, reduction in compliance and larger variations in individual habits, so that a much larger study would be required.

5.2.4. Clinical measurements

The measurements chosen were standard measures of dietary compliance, and of cardiovascular health such as are routinely used in clinical practice. These are therefore accessible for comparison between studies, although it is important to take varying methodologies into consideration when comparing absolute measurements. Reporting of percentage changes is a way of enabling such comparisons. Compound indices such as HOMA-IR and QUICKI have been developed and tested against the 'gold standard' techniques of measuring IR, such as the frequently-sampled intravenous glucose tolerance test or the glucose clamp (Brady *et al*., 2004). The advantage in that context is the avoidance of invasive techniques requiring specialist expertise (e.g. insertion of intravenous cannulas), and they have been shown to be useful comparators for assessment of IR. The use of compound indices (HOMA, QUICKI or R-QUICKI) did not, however, provide any additional insights to the simpler single-measurement indices in the studies reported here.

Central obesity as determined by waist measurement has previously been shown to be a valid risk indicator for lipid over-accumulation (Kahn & Valdez, 2003, Ross & Katzmarzyk, 2003). This simple measurement can be reliably compared with fasting TAG concentrations and with increased risk of mortality after middle age. Waist circumference has been reported to be a better predictor than BMI for hypertension and diabetes (Menke *et al*., 2007) although correlation with CRP is less reliable. A combination of high BMI (≥30kg/m^2) and waist circumference was linked to the development of MetS in 32% of subjects, compared with 10% of those with low BMI and waist circumference (Han *et al*., 2002). A more recent study in a cohort of 1758 middle-aged Swedish men has suggested that both overweight (as measured by high BMI) and MetS are risk factors for CVD (Arnlov *et al*., 2011), contradicting a perception that overweight in the absence of MetS is a benign condition.

Raised plasma TAGs have been consistently identified as a risk factor for development of CVD. Onat *et al*. (2006), in a cohort of 2682 Turkish adults initially free of CVD but with MetS, reported increased risk of CVD in the highest quintile of TAG measurements. Another prospective study of 926 adults in Argentina (Salazar *et al*., 2013), investigated whether the TAG/HDL-C ratio may be a more reliable indicator of CVD risk than a MetS diagnosis. They reported that MetS and elevated TAG/HDL-C ratio were equivalent in identification of individuals at high risk of CVD, and inferred that a single

TAG/HDL-C measurement is as effective a diagnostic tool as a MetS diagnosis. Given the observation reported in this thesis of a stronger correlation between capillary and venous blood measurements for TAG/HDL-C than for TAG, HDL-C or other single measures, the use of these ratio measurements may be more suitable for diagnostic/prognostic screening.

The association between impaired fasting glucose and the risk of incipient T2D and/or CVD has been widely studied. The Multi-Ethnic Study of Atherosclerosis (Yeboah *et al*., 2011) followed 6753 participants (45-84 years of age), initially free of clinical CVD, but 25% of whom had T2D or abnormal fasting glucose, for an average of 7.5 years. Those with T2D exhibited an increased CVD risk, while those with impaired fasting glucose had an increased risk of progressing to T2D, a correlation also noted by Park *et al*. (2013). In the Cooper Centre Longitudinal Study (DeFina *et al*., 2012), individuals with plasma glucose between 6.1-7.0 mmol/L exhibited a higher rate of conversion to T2D than those with normal (5.6 mmol/L) or slightly raised (5.6-6.0 mmol/L) glucose. Raised glucose in association with obesity or MetS doubled this risk. It is not possible to comment on the prognosis of the participants in the investigations reported in this thesis, except to note that those with higher plasma glucose showed greater reductions following n-3 LCPUFA intervention.

With respect to lipoprotein profiling, a relationship between CVD risk and LDL-C, as well as an inverse relationship with HDL-C has been reported (Parish *et al*., 2012): reduced HDL-C has been identified as a potential indicator of CVD risk. A low HDL-C phenotype has been associated with increased lipid peroxidation and platelet activation (Hoenselaar, 2012). Further subclassification of lipoprotein particles has led to reports of particularly high risk associated with sdLDL-C (Ip *et al*., 2009), although Parish *et al*., (2012) inferred little difference in predictive value between lipoprotein particles, apolipoproteins and cholesterol fractions of LDL, and therefore very little gain from LDL subclass analysis. Measurement of ApoB provides good correlation with LDL-C, since there is a good stoichiometric relationship (1:1), but ApoA1 concentrations are not directly proportional to HDL because the particles contain variable numbers of the apolipoprotein molecules.

The measurements used in the investigations reported in this thesis have a sound basis for use as markers in future studies. The measurement of sdLDL-C by the Hirano method gave good correlation with estimates of sdLDL obtained by the more time-consuming DGUC method (Jones *et al.*, 2009), suggesting that the Hirano method would be suitable for larger scale studies.

5.2.5. Quality of nutritional supplements

The prawns and OCs used in the study described in Chapter 3 were obtained from standard grocery sources. Instructions given to participants would have been as provided on the commercial packaging, but it was not possible to ensure that participants complied fully with these instructions.

The FO and KO supplements used in the study described in Chapter 4 were obtained from different suppliers. There seemed to be a lower degree of compliance with the KO supplement, possibly because of a larger number of capsule or a stronger fish odour from these compared with the FO. This odour suggests to the author that the quality of the KO may not have been as high as that of the FO. The degradation of n-3 FAs by oxidation results in the production of hydroperoxides and amines which are responsible for the 'fishy' odour. This necessarily indicates that the product contains less of the potentially beneficial n-3 LCPUFA than the original sample. It is not fully understood whether these degradation products are merely not beneficial, or indeed whether they may have adverse nutritional and health effects (Turner *et al.,* 2006).

5.2.6. Compliance

Participants in both studies were asked to adhere to their normal lifestyles and not to make any major modifications to diet or exercise regimes, and to include the nutritional ingredients (prawn/OCs) or n-3 LCPUFA supplements in addition to their normal diet. They were also asked to fast for 8 hours prior to attending for blood tests. It was not possible to assess with accuracy whether individuals had complied fully with the dietary interventions *per se*. Participants in the KO study were asked to return all unused capsules at the end of the krill/fish oil study, and were asked to complete food diaries to enable an approximate estimation of major changes in dietary habits at intervals during each study. Unfortunately, the majority of the participants did not return unused capsules: full compliance was therefore assumed in the data analysis, as it was for the prawn/OC study. The evidence regarding compliance and the issues highlighted by this are summarised here.

5.2.6.1. Biochemical measures of compliance

It was evident from fasting TAG analysis that some participants had failed to fast prior to blood testing. Measurements of plasma FA profiles in the KO study also provided an interesting insight into compliance over the 6-week interventions, suggesting that compliance with KO in particular decreased during the second half of the intervention period.

5.2.6.2. Food intake records

For both studies, food intake diaries revealed very large variations in nutrient intakes, and very large ranges compared with mean values for individual macronutrients. Bearing in mind the wide range and variety of foods on sale in the UK at the present time, this is to be expected in any study group of free-living participants. It may also be exacerbated by day-to-day or week-to-week changes for individuals during the course of a 12 or 18-week period, due to holidays or natural changes in work patterns. Additionally, individuals who enrol on dietary or other lifestyle research programmes may subconsciously make modifications to their lifestyle even when requested to maintain a constant pattern (Wrieden *et al.,* 2003*)*. In these studies, there was clear evidence of changes; in the prawn study, several of the participants changed their alcohol consumption dramatically during the study. Two participants in the KO/FO study were retrospectively withdrawn from analysis of results when it became apparent that n-3 LCPUFA intake for one, and overall nutrient intake and CVD markers for another, were more than 2SD away from the mean.

The design of these studies meant that the duration for each participant was 12 weeks for the prawn study and 18 for the FO/KO study. For a study of several months duration it is impractical to impose a strict baseline diet against which to measure changes. For participants accommodated in laboratory conditions such that all dietary intakes are quantitatively and qualitatively evaluated, it would be possible to make more accurate assessment of the effect of a particular dietary supplement, but this would be neither practical, acceptable, nor economic to implement, and would not reflect real life situations.

The use of food diaries as a tool in dietary interventions is the subject of some concern. For example, 24-hour records are known to be a poor indicator of dietary intake (Beaton *et al*., 1979; 1983; Dodd *et al*., 2006). It is recognised that under-reporting is common in dietary assessment methodology, especially by obese or potentially obese subjects. Yao *et al*. (2013) recently examined the challenges faced in the design and

reporting of dietary intervention trials, and point out that the relationship between food intake and the effects of bioactive components in the gut is an additional factor which should be addressed. They also highlight the difficulty of masking study foods and the difficulty of choosing appropriate placebo diets. In this case, there were notable differences in overall dietary habits between those receiving prawn and OC, which may have indicated some difference in palatability/satiety of the interventions.

Reporting of food intake may be inaccurate due to memory or diligence of the participants, and individuals may have different perceptions of portion size, although pictures of typical portion sizes were provided in the food diary booklets used. It is also evident that the exact composition of particular dishes may be highly variable, depending on whether the dish has been compiled at home from fresh ingredients, purchased in a restaurant or pub, or purchased as a 'ready meal' from a supermarket. Supermarkets typically offer ranges of ready meals including 'economy', 'luxury' and 'low fat', each distinct in portion size, fat and carbohydrate content. In both studies, transcription of data from the diaries into the program for analysis (Windiet or Microdiet) was performed by a single investigator to maximise consistency of interpretation. Nutrient data from supermarket labels (e.g. www.sainsburys.co.uk, www.tesco.com) was used wherever possible for composite foods, including, for example, the size of portions of snacks, bread, and biscuits. However, these did not always include complete nutritional data. For food and beverages obtained from retail outlets, information from the relevant website was used where available (e.g. http://www.costanutrition.co.uk/). If not, values obtained from suppliers of catering supplies to public houses (e.g. www.brakes.co.uk) were used to obtain the closest approximation. Some 500 foods at current nutrient values (2012) were thus added into the Microdiet^{TM} database. The database used by the MicroDiet program employed was not fully up to date: although composite foods are provided with compositional data, there have been changes in the use of ingredients in supermarket-purchased foods, including bakery products. For example, the use of hydrogenated margarines containing trans-fatty acids is now largely excluded from products sold in British food stores, and the use of vegetable oils in food preparation have replaced saturated animal fats to a large extent. Portion sizes indicated in the MicroDiet program in some cases did not correspond with values of portions normally available. For example, biscuits as sold in supermarkets are frequently 2g heavier (approximately 10%) than the weights suggested in the database. Wherever possible, new versions of such foods were entered into the database.

The process of transferring data from a 3- or 7-day food diary into the program for analysis is slow, and would not be efficient for a large-scale study. Although it might be desirable to monitor the behaviour of participants in studies of this nature, the extent to which additional compliance prompting (to take supplements, fast prior to attending for study visits, etc), may improve overall compliance, is open to question. The advent of smartphone technology with the ability to communicate by SMS and to easily obtain images of food consumed, admits the possibility of much closer day-to-day communication with volunteer participants but could be regarded as intrusive and might result in withdrawal from studies or refusal to participate. There are some initial reports of the use of wearable cameras to improve accuracy of dietary analysis or lifestyle monitoring (O'Loughlin *et* al. 2013; Gurrin *et al.,* 2013). Analysis of the information generated by such devices will require the ability to undertake image analysis and involve extensive calculation of nutrient intakes for each item.

5.3. SUGGESTIONS FOR FUTURE WORK

Consideration of the results presented in this thesis give rise to some suggestions to enhance the value of data obtained from future studies.

5.3.1. Study design

It would be preferable to carry out an intervention in a much larger sample, which would allow for elimination of individuals who had not complied in one or more respects and still allow sufficient numbers for robust statistical analysis of results. The use of longer intervention and washout periods would enable evaluation of the uptake of n-3 FA in muscle PLs and cell membranes, but in the author's view would also increase rates of non-compliance and would therefore also require larger starting sample sizes. A parallel study design where larger groups were used and compared directly against each other for a longer initial period, say 12 weeks, might overcome the difficulties of compliance and would reduce the need for a washout period.

5.3.1.1. Choice, dose and quality of supplements

The choice of supplements to achieve maximum ongoing compliance is a key issue for consideration in development of the work described here. The quality of supplements used in n-3 LCPUFA studies has not been widely studied and related to either

compliance or potential biochemical benefit. Even high quality supplements may deteriorate if not stored under ideal conditions, resulting in decreased palatability and potentially reduced compliance. In addition, it is not clearly understood what, if any, the health implications may be of consuming supplements which contain a significant percentage of degradation products. The free radical processes initiated by oxidation and ultraviolet radiation are self-perpetuating, resulting in generation of additional free radical species. It is recommended that storage of supplement capsules is carefully monitored and strategies put in place for recovering any unused capsules for analysis and determination of compliance. it may be interesting and informative to measure the quality of any n-3 LCPUFA supplements used at the start of an intervention and at the end, in particular the presence of volatile breakdown products of LCPUFA. It may then be possible to relate the quality of the supplements to any variation in compliance. Use of metabolomic analysis could also be valuable in detecting absorption of breakdown products.

The choice of 'active' supplement and placebo or control is an important factor in the design of nutritional intervention studies. Ideally supplement and placebo or control should be indistinguishable in sensory characteristics and of closely comparable nutritional content except in the parameter under study (in this case, n-3 LCPUFA and/or cholesterol). In the prawn/OC study, the use of OCs provided an equivalent weight of foodstuff with negligible cholesterol and/or n-3LCPUFA, but considerably higher carbohydrate content than the prawns. The two food materials were quite different in sensory characteristics. In the KO/FO study, the comparison between the two supplements may not have been at an ideal dosage, and the total daily quantity of n-3 LCPUFA was not exactly comparable. Also, the acceptability of the supplements may have been different, contributing to differences in compliance.

5.3.2. Clinical measurements

Based on the correlations between capillary and venous blood measurements reported here, it is suggested that a point-of-care system is an acceptable tool to use for future studies; this would also allow rapid screening of compliance by measurement of plasma glucose and TAG. The reporting of the TAG/HDL-C ratio appears to give good correlation between capillary and venous blood measurements. Other measurements could have included red cell membrane lipids. Measurement of sdLDL by the Hirano method is also proposed to be a useful and timesaving technique compared with DGUC. Methods of measuring HDL-C as described in Chapter 4 depend either on ultracentrifugation to separate lipoprotein subclasses, or masking of the non-HDL

subclasses; the method used in Chapter 4 has a high (99%) concordance with the DGUC reference method.

It has been suggested that measurement of clinical outcomes is more relevant than measurements of biochemical, lipidaemic or inflammatory status (Lovegrove & Griffin, 2013). While this is a reasonable viewpoint, it is not always practicable to achieve this in the relatively short timescale of studies such as described in this thesis, and therefore it is necessary to use appropriate biochemical and immunological measurements. Developing techniques to evaluate HDL and LDL subclasses in more detail may be useful in future studies. It is strongly proposed that measurement of inflammatory markers including IL-1, IL-6, MCP-1, CRP, TNF-α, IL-8, should be included. It would also be informative in any future studies of KO to measure astaxanthin uptake by participants.

5.3.3. Markers of compliance

The use of plasma FA profiling in KO/FO study described in Chapter 4 raised a question as to the compliance of some participants with the n-3 LCPUFA supplementation, particularly in the KO intervention. Further studies should have a more rigorous focus on establishing compliance, perhaps by questionnaires, reminders, return and counting of unused supplements, and also additional biochemical measurements (such as FA profiling or metabolomics analysis).

The use of food diaries in the studies described here revealed both a wide variation in baseline diet between participants, but also large changes in nutrients by some participants during the 4- or 6- week duration of interventions. There are several alternative methods of estimating food intake, but each have limitations of accuracy or difficulty of administration. The use of weighed food records, although more accurate, would not be applicable in a study of this length. An easier method from an administration point of view is that of estimated food records where participants or investigators estimate intakes based on the use of standard household measures and photographs or food models, although this is also subject to significant mis-reporting (Wrieden et al, 2003). A trained interviewer may be able to elicit an accurate record of food intake over a single 24-hour period, although this would be unlikely to be typical of intake averaged over days or weeks, and is still dependent on accurate recall by the interviewee. A further alternative is the use of a food frequency questionnaire, which

provides a list of foods for respondents to check intake against. This type of questionnaire can be administered by interview, telephone or by written response (e.g. by the use of an internet-based questionnaire, and could be a suitable option for future investigations. Wrieden reported two types of effect in mis-reporting of food intakes by participants: an observation effect, meaning that individuals may change their eating behaviour when asked to record intakes (as noted when intake activity is independently observed). Men tend to reduce alcohol intake, and women to reduce fat intake by approximately 12% when undertaking any type of food survey. Additionally, participants may mis-report changes in eating behaviour by up to 20%, especially when at home rather than in a 'laboratory' setting.

Possible ways to improve reporting of food intake in future studies might include the use of online food diaries, which might be linked to mobile phone technology. Use of a wearable camera to facilitate recording of meals of snacks (which could be combined with image analysis techniques) has been reported (O'Loughlin et al (2013), Gurrin et al (2013); comparison with use of smartphones indicates that this may be a viable mode of image capture for dietary studies. Another option proposed by Beckman *et al* (2013) is the metabolomic analysis of urine samples as a tool to supplement the use of food frequency questionnaires in epidemiological studies of food intake and disease risk. The use of urine signals for distinguishing between specific foods may allow more stringent evaluation of dietary influences on biomarkers and disease processes; markers reported for oily fish intake included methyl-histidine and anserine.

5.3.4. Use of metabolomic profiling in future studies

Metabolomic analysis may be a useful tool in future studies of this nature in monitoring compliance, and in beginning to understand differences in individual responses to interventions of this nature. The opportunity arose in the KO investigation for some preliminary metabolomic profiling of plasma and urine samples. Metabolomics is a branch of molecular biology which arose from the development of genome sequencing and identification technology (Venter *et al.,* 2001; Goodacre *et al.*, 2004). The sequencing of the human genome has opened up the potential for identification of genes which may be valid targets for pharmacological therapeutic intervention, by explaining the nature and role of protein expression and translation (proteomics) and the metabolic network (metabolomics). In metabolomics, a combination of molecular separation tools (mass spectrometry) with identification and analytical technologies (automated chromatographic methods), using advanced computational techniques to

interrogate the large volumes of data thus obtained, enables observations of subtle variations in individual molecular species, which may provide clues to important metabolic changes.

The proteome of a cell or organism is the full complement of proteins expressed in a cell at a particular state of development or activity; similarly the metabolome is idefined as the full complement of lower molecular weight molecules (Oliver *et al.*, 1998). These may participate in any of the many reactions required for the normal maintenance, growth and function of the cell (Beecher, 2003). The primary objective of a metabolomics experiment is to identify and quantify all metabolites present in a cell or tissue at a specific point in time; however, no automated analytical technology is capable of total capture and reproducibility of this information and metabolomic approaches may be targeted towards a specific aspect of cellular metabolism. The advantage of metabolomic techniques over traditional biochemical analyses is in quantification of large numbers of metabolites, permitting observation of perturbations in intermediates, accumulation or depletion of the endpoint products of biochemical pathways. It may thus be possible to observe more subtle effects of pharmacological, dietary or other health-related interventions than has previously been feasible.

A typical metabolomics analysis involves extraction of a metabolite fraction from cells, tissue sample, urine or plasma, followed by derivatisation, chemical treatment of the sample to render it suitable for mass spectrometry. The many molecular species are separated and identified by mass spectrometry coupled with an analytical technique such as gas chromatography. The large volume of data generated is interpreted by reference to one or more databases compiled by collaboration between systems biology researchers, and which are continually in the process of curation and classification (Mendes, 2002; Kanehisa *et al*., 2002; Famili *et al.,* 2003; Forster *et al*., 2003; Wishart et al., 2013). Validation of metabolite profiles is an ongoing challenge to ensure comparison between research groups using identical experimental protocols. Supervised machine learning algorithms may be used to transform data from metabolite profiles and identify patterns that may be interpreted on the basis of known variations or interventions (e.g. susceptibility to disease, pharmacologic or dietary treatment).

Early interpretations of metabolomics results has led away from the more traditional concepts of biochemical pathways to the concept of metabolite neighbourhoods and networks (i.e. that a metabolite is not necessarily involved in only one pathway at a time, but that the whole array of biochemical processes is far more dynamic than the models used in the mid to late 1900s). A number of novel pathways have already been

identified using metabolomic approaches (Kholodenko *et al*., 2002; Weckwerth & Fiehn, 2002; Steuer *et al*., 2003).

An advantage in the use of metabolomic techniques in nutritional studies is the ability to demonstrate subtle changes in small subject groups rather than the large cohorts customarily required. The first reported nutritional metabolomics study was reported in 2003 (Solanky *et al.* 2003), following ingestion of isoflavones in a group of healthy premenopausal women. Data from plasma analysis showed changes in lipoproteins, carbohydrates and proteins, while urinary analysis demonstrated changes considered to imply alterations in gut microflora. More recently, targeted approaches in nutritional metabolomics have allowed analysis of specific subsets of metabolic species, including lipids (German *et al*. 2007). Examples include the analysis of eicosanoid metabolism following FO interventions (Lankinen *et al*. 2009) and of PL metabolism (Wang *et al.* 2009).

Metabolomic analysis has recently been applied to the T2D-CVD spectrum. Shui & Lam (2013) profiled and compared over 300 plasma lipid species from subjects with and without T2D.They found increases in sphingolipids including ceramides, sphingomyelins, lactosylceramides (LacCer) and ganglioside GM3, with concomitant decreases in glucosylceramides. This was interpreted to indicate increased conversion of GluCer to LacCEr in milder T2D. Individual GM3 species were altered according to chain lengths – long chains (C>18) were increased, but 18:1/16:0 were decreased. Long-chain GM3 were negatively correlated with HOMA-β indices, and positively correlated with fasting blood glucose, allowing distinction between mildly diabetic patients with similar HOMA-β. Friedrich (2012) has reviewed metabolomics research in diabetes over the last few years, and pointed out findings indicating possible effects by alterations in gut flora, increased FA and changes in amino acid levels. It has been suggested that abnormal amino acid levels predictive of T2D development may be observed as much as 12 years before clinical presentation and diagnosis. Wang-Sattler *et al*. (2012) identified three metabolites with significantly altered levels in impaired glucose tolerance; glycine, lysophosphatidylcholine (LPC) 18:2 and acetylcarnitine. In addition to glycosuria, other plasma metabolites may be elevated in the urine of diabetic individuals ((Roussel *et al.,* 2007). This includes an increase in products of β-oxidation, as well as changes indicative of perturbation of nucleotide metabolism and gut microflora.

The possibility to observe changes in eicosanoid or PL metabolism in plasma or urine in MetS and T2D resulting from dietary intervention with FO, KO and/or astaxanthin is a

very exciting possibility for future research. In summary, the use of metabolomics analysis is seen as a key tool in future studies of the type described in this thesis.

5.4. FINAL SUMMARY

The investigations described in this thesis have provided answers to the research questions posed. It has been shown that there is no clear increase in cardiometabolic risk from prawns as a source of n-3 LCPUFA compared with a white fish food source, and that KO is comparable in effect on markers of cardiometabolic risk at an n-3-LCPUFA concentration approximately 2/3 that of a FO supplement. These results are in accordance with other published work, and may provide support to the pursuit of improving n-3 LCPUFA intake either for individuals or at a public health level. It is clear from both studies that greater benefits from n-3 LCPUFA supplementation are possible for those with more dyslipidaemic profiles. It is hoped in future studies to refine understanding of this by the use of nutrigenomic or metabolomic analysis.

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APPENDIX 1

PUBLICATIONS

JONES, W. S., DAVIES, I. G., & STEVENSON, L. (2008). Novel antioxidants for marine oils. In *Molecules & Ingredients Sante (MIS). Rennes, France*.

JONES, W. S., DAVIES, I. G., & STEVENSON, L. (2008). Plenty of fish in the sea; how sustainable is omega-3 supplementation? In *Molecules & Ingredients Sante (MIS). Rennes, France.*

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JONES, W. S., WONG, M., LOWE, G. M., DAVIES, I. G., ISHERWOOD, C., & GRIFFIN, B. A. 2010. The effect of prawn consumption on lipoprotein subclasses in healthy males. In *Proceedings of the Nutrition Society Vol. 69 (pp. E96).*

ISHERWOOD, C., WONG, M., **JONES, W. S.,** DAVIES, I. G. & GRIFFIN, B. A. 2010. Lack of effect of cold water prawns on plasma cholesterol and lipoproteins in normo-lipidaemic men. *Cell Mol Biol (Noisy-le-grand),* 56**,** 52-8.

JONES, W. S., SCOTT, D., LODGE, J. K., SCHMIDT, M., & DAVIES, I. G. (2013, July). The effect of fish oil versus krill oil on markers of metabolic syndrome and the plasma metabolome; a pilot study. In *Proceedings of the Nutrition Society Vol. OCE2 (pp. E67).*

APPENDIX 2

FOOD DIARY

Thank you for completing this diary

Please return it to the study team as requested

Study Contacts

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liverpool JMU

Food Diary

_____
Food Diary ID

_____
Volunteer ID

_____
Date Started

Details of how to fill in the diary

It is very important that you do not change what you normally eat and drink just because you are keeping this record. Please keep your usual food habits.

Each day is divided into sections, from the first thing in the morning to late evening and through the night.

When recording your food include the **brand name** (if known), **portion size** (using household measures, **weights** from labels or the picture examples to help), any additions to the food (fats, oils, sugars/sweeteners, sauces, salt, pepper etc), **cooking methods** (fried, grilled, baked, roasted etc) and any **leftovers**.

Please record everything at the time of eating, **not from memory** at the end of the day. The diary covers a 24h period, so please include any food or drinks that you may have had during the night. You may have had some foods and drinks between meals (snacks), or food that you have not recorded earlier, so please include these in the extras section.

It helps a great deal if you enclose labels from any unusual foods and also from any supplements you take when returning your completed food diary.

Overleaf you can see an example of how we would like you to record your food and drink intake.

.....

Day of Week Date

BETWEEN MEALS SNACKS & DRINKS if not already written in

Time	Food/Drink	Description & Preparation	Amount

Please name any medication, vitamins, minerals or food supplements and what they were taken with. Please give all details and enclose label(s) if possible

Brand	Name (in full)	Number: pills, capsules, teaspoons

EXAMPLE.....

Day of Week Date

BEFORE BREAKFAST

Time	Food/Drink	Description & Preparation	Amount
7.30	*Water*	*Tap water, no additions*	

BREAKFAST

Time	Food/Drink	Description & Preparation	Amount
8.00am	*Toast* *Margarine* *Marmalade*	*Hovis, wholemeal, pre sliced, thin cut* *Flora original* *Robetson's low sugar, thin cut orange and lime*	*2 slices (left crusts)* *2 medium spread* *2 thin spread*
8am	*Tea* *Milk* *Sugar*	*Medium strength Tetley* *Semi skimmed pasteurized* *White castor*	*1 mug (incl milk)* *Dash in tea* *1 teaspoon in tea (drank all)*
8am	*Breakfast Cereal* *Milk*	*Kellogg's cornflakes* *Semi skimmed pasteurized*	*1 pict 1 b (ate all)* *Approx 1/3 pint*

MID-MORNING—between breakfast time and lunch time

Time	Food/Drink	Description & Preparation	Amount
10.30 am *10.30 am*	*Coffee* *Milk* *Biscuit*	*Nescafe original caffein-ated* *Full fat pasteurized* *McVities chocolate digestive*	*1 cup, drank all* *1 average in coffee* *2 biscuits, ate all*

Day of Week Date

EVENING MEAL

Time	Food/Drink	Description & Preparation	Amount

LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount

EXAMPLE

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount
1.15 pm	*Ham Salad Sandwich*		
	Bread	*Tesco's own brand, medium sliced white*	*2 slices*
	Mayonnaise	*Hellman's ordinary mayonnaise*	*2 thin spreads on bread*
	Ham	*Bernard Matthews thin slices lean ham*	*2 thin slices*
	Lettuce	*Iceberg Lettuce*	*1 leaf*
	Cucumber	*Including Skin*	*3 thin slices*
	Seasoning	*Black Pepper, coarse ground*	*from mill*
1.15 pm	*Can of cola*	*Pepsi Max*	*1 can, 20% extra free (396ml) drank all*
1.30 pm	*Crisps*	*1 average pack, Walkers cheese and onion flavour*	*1 25g packet left half*

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount
4.00 Pm	*Coffee*	*Same as mid morning*	*2 cups*
4.10 Pm	*Apple*	*Golden Delicious*	*1 with skin peeled Didn't eat core*
4.15 pm	*Banana*	*Small*	*1*

EXAMPLE.....

Day of Week Date

EVENING MEAL

Time	Food/Drink	Description & Preparation	Amount
6.30 pm	*Lamb*	*Roast*	*3 thin slices*
	Potatoes	*Old, boiled in salt water, mashed with semis skimmed milk and anchor butter*	*Picture 4b*
	Cabbage	*Green, boiled in salt water*	*Picture 7a*
	Carrots	*Boiled in salt water*	*2 tablespoons*
	Potatoes	*Old, boiled in salt water, roasted in meat fat*	*3 medium potatoes, 1 left*
	Gravy	*Bisto Gravy granules made with veg water*	*4 tablespoons left approx $^{1}/_{4}$*
	Water	*Highland spring carbonated water*	*1 tumbler drank all*
			2 scoops
6.50 pm	*Ice cream*	*Tesco's vanilla non-dairy*	

LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount
7.30 pm	Tea	Same as breakfast	2 mugs
9.15 pm	Whiskey	Blackbush	2 shots in average size glass
	Lemonade	Diet own brand lemonade	To top of glass
9.30 pm	Chocolates	Cadburys roses	2 from box
12-6am	water	Tap water	1 average glass

.....

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

BEFORE BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

MID-MORNING—between breakfast time and lunch time

Time	Food/Drink	Description & Preparation	Amount

EXAMPLE

Day of Week Date

BETWEEN MEALS SNACKS & DRINKS if not already written in

Time	Food/Drink	Description & Preparation	Amount
8.30 pm	*Coffee*	*1 mug with cream, 1 tsp sugar*	*1 mug incl cream (drank all)*

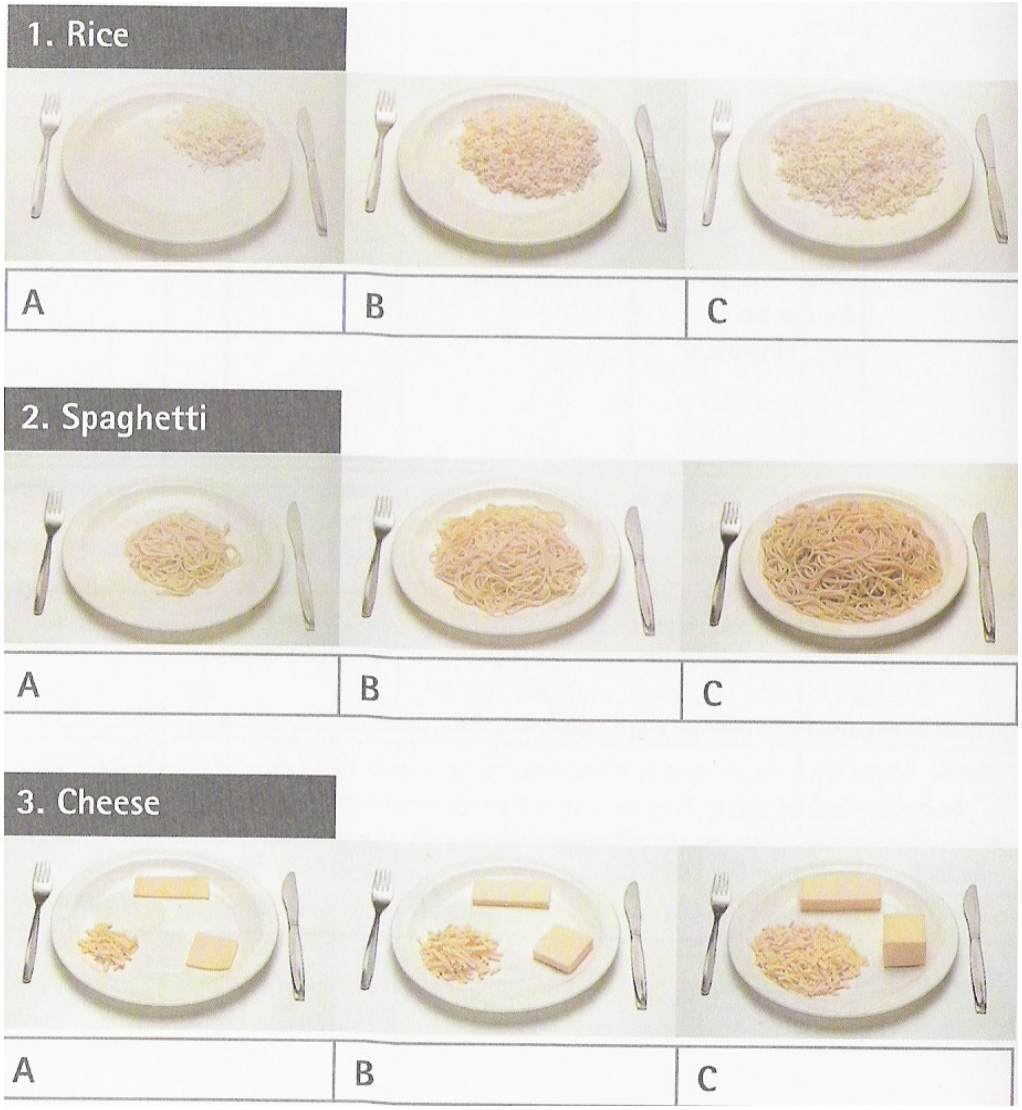
Please name any medication, vitamins, minerals or food supplements and what they were taken with. Please give all details and enclose label(s) if possible

Brand	Name (in full)	Number: pills, capsules, teaspoons
Sanatogen *Boots own brand*	*1 a day gold A-Z* *Cod liver oil capsule (normal strength)*	*1* *1 500mg capsule*

Use the pictures to help you to indicate the size of the portion you have eaten. Write on the food record the picture number and size A, B or C nearest to your own helping.

The pictures could also be used for foods not shown i.e. pasta shapes similar to spaghetti, ham pie similar to quiche and peas similar to baked beans.

Remember that the pictures are much smaller than life size. The actual size of the dinner plate is 10 inches (25cm), the side plate, 7 inches (18cm) and the bowl 6.3 inches (16cm).



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Day of Week Date

BETWEEN MEALS SNACKS & DRINKS if not already written in

Time	Food/Drink	Description & Preparation	Amount

Please name any medication, vitamins, minerals or food supplements and what they were taken with. Please give all details and enclose label(s) if possible

Brand	Name (in full)	Number: pills, capsules, teaspoons

Day of Week Date

EVENING MEAL

Time	Food/Drink	Description & Preparation	Amount

LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount

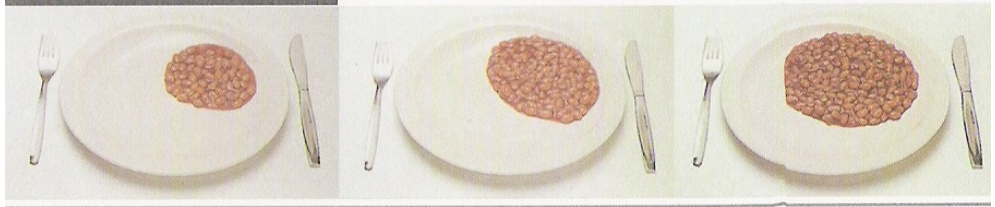
4. Boiled Potatoes

A B C

5. Chips

A B C

6. Baked Beans

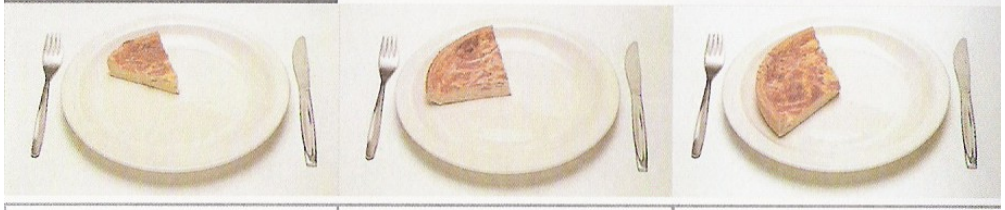


A B C

7. Broccoli

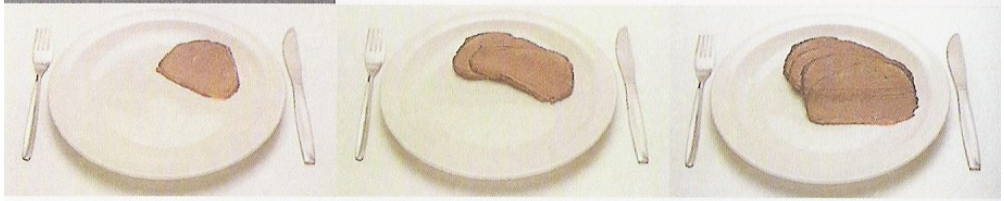
A B C

8. Quiche / Pie



A	B	C

9. Sliced Meat

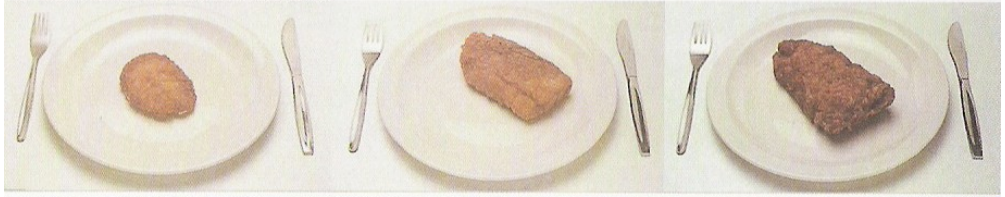


A	B	C

10. Stew

A	B	C

11. Battered Fish



A	B	C

.....

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

BEFORE BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

MID-MORNING—between breakfast time and lunch time

Time	Food/Drink	Description & Preparation	Amount

12. Cornflakes

A	B	C

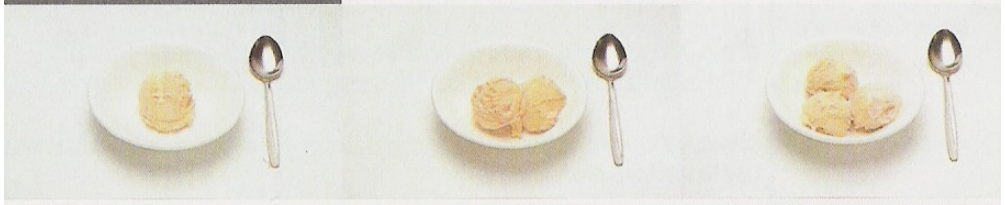
13. Fruit Cake

A	B	C

14. Sponge Cake

A	B	C

15. Ice Cream



A	B	C

Day of Week Date

BEFORE BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

MID-MORNING—between breakfast time and lunch time

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

BETWEEN MEALS SNACKS & DRINKS if not already written in

Time	Food/Drink	Description & Preparation	Amount

Please name any medication, vitamins, minerals or food supplements and what they were taken with. Please give all details and enclose label(s) if possible

Brand	Name (in full)	Number: pills, capsules, teaspoons

Day of Week Date

EVENING MEAL

Time	Food/Drink	Description & Preparation	Amount

LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

EVENING MEAL

Time	Food/Drink	Description & Preparation	Amount

LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

BEFORE BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

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Day of Week Date

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BREAKFAST

Time	Food/Drink	Description & Preparation	Amount

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EVENING MEAL

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LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

EVENING MEAL

Time	Food/Drink	Description & Preparation	Amount

LATER EVENING—and through the night

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

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Day of Week Date

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BREAKFAST

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MID-MORNING—between breakfast time and lunch time

Time	Food/Drink	Description & Preparation	Amount

Day of Week Date

LUNCH

Time	Food/Drink	Description & Preparation	Amount

TEA—between lunch and the evening meal

Time	Food/Drink	Description & Preparation	Amount