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Functional Food Synergies: improving  
the effect of the omega-3 fatty acid  
docosahexaenoic acid on cardiovascular  
disease risk factors through concurrent  
dietary consumption of canola or soy  
isoflavones

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Ridges, Leisa, Functional Food Synergies: improving the effect of the omega-3 fatty acid docosahexaenoic acid on cardiovascular disease risk factors through concurrent dietary consumption of canola or soy isoflavones, PhD thesis, School of Health Sciences, University of Wollongong, 2007. <http://ro.uow.edu.au/theses/218>

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Functional Food Synergies: Improving the effect of the omega-3 fatty  
acid docosahexaenoic acid on cardiovascular disease risk factors  
through concurrent dietary consumption of canola or soy isoflavones.

A thesis submitted in (partial) fulfilment of the  
requirements for the award of the degree

DOCTOR OF PHILOSOPHY (PhD)

From

UNIVERSITY OF WOLLONGONG

By

Leisa Ridges (BSc Hons)

School of Health Sciences  
2007

## **Certification**

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I, Leisa Anne Ridges, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Health Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Leisa Anne Ridges

14 September 2007

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## List of Abbreviations

AA	Arachidonic acid
ALA	Alpha linolenic acid
CAD	Coronary artery disease
CHD	Coronary heart disease
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DHAc-s	Daily DHA-rich oil supplementation for twelve weeks with concurrent consumption of control cereal for the first six weeks followed by consumption of soy cereal between six and twelve weeks of the intervention period.
DHAs-c	Daily DHA-rich oil supplementation for twelve weeks with concurrent consumption of soy cereal for the first six weeks followed by consumption of control cereal between six and twelve weeks of the intervention period.
DPA	Docosapentaenoic acid
FXR	Farnesol X receptor
HDL	High density lipoprotein
HNF-4 $\alpha$	Hepatocyte nuclear factor 4 $\alpha$
IDL	Intermediate density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LXR	Liver X receptor
OOc-s	Daily olive oil supplementation for twelve weeks with concurrent consumption of

	control cereal for the first six weeks followed by consumption of soy cereal between six and twelve weeks of the intervention period.
OOs-c	Daily olive oil supplementation for twelve weeks with concurrent consumption of soy cereal for the first six weeks followed by consumption of control cereal between six and twelve weeks of the intervention period.
PPAR	Peroxisome proliferator - activated receptor
SBP	Systolic blood pressure
SR-B1	Scavenger receptor B class-1
SREBP	Sterol regulatory element binding protein

## **Abstract**

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Ischaemic heart disease and cerebrovascular disease are among the leading causes of death in Australian men and women with heart diseases being the third highest cause of death in Australian women and fourth highest cause of death in Australian men (AIHW, 2006). In Australia more than 50% of all adults have two or three (out of a possible nine) risk factors for cardiovascular disease and 15% having four or more risk factors.

It has long been recognised that diet modification can reduce these risks. Recently dietary advice has moved from an “exclusionary” to an “inclusionary” paradigm. That is, rather than identify dietary items to avoid, current guidelines recommend incorporating advice to increase the consumption of a range of functional foods including marine sourced omega-3 fatty acids EPA and DHA and vegetable oils.

EPA and DHA are effective functional foods in reducing CVD mortality, cardiac death, sudden death and myocardial infarction. EPA and DHA provide this cardiovascular benefit by improving several risk factors including: fasting plasma triglycerides, blood pressure and arterial compliance. However, a safety concern of dietary EPA and DHA supplementation is their capacity to cause a significant increase in LDL cholesterol concentrations.

Dietary intake levels of EPA and DHA in the Australian diet are well below those associated with reductions in CVD risk. In 50% of the population a potential 20 – fold increase in EPA and DHA would be required to increase intake levels to those commensurate with reduced CVD risk.

While dietary supplementation with EPA and DHA is one means of increasing dietary intake levels, strategies to increase the efficacy of EPA and DHA would also be advantageous and could reduce supplement doses. Dietary strategies that could simultaneously counteract the rise in LDL cholesterol caused by DHA would also be beneficial.

The research described in this thesis aimed to modify the bioavailability and cardiovascular effects of DHA by modifying other dietary factors and combining DHA with other active ingredients. To address these aims two human clinical trials were conducted. The first examined the effect of altering the types of oil and margarine consumed in the diet with view to reducing the dietary intake of omega-6 fatty acids while supplementing the diet with DHA-rich fish oil (MOFO study). This study showed that replacing usual dietary oil and margarine with canola products while supplementing the diet with 1.1g/d of DHA favourably improved total omega-3 fatty acid incorporation and reduced the omega-6: omega-3 fatty acid ratio in plasma and erythrocyte membrane phospholipids as effectively as double the supplement dose of DHA. Additionally, there was a similar rise in erythrocyte membrane and plasma DHA when either safflower or sunola oil, which contain very different amounts of linoleic acid, were consumed concurrently with a daily dietary supplementation of 1.1g/d of DHA. A distinguishing feature of canola is its relatively high omega-3 ALA content. Thus, these findings add to the body of scientific evidence supporting the view that the total amount of dietary omega-3 consumed has greater impact on the bioavailability of supplemented DHA than the ratio of dietary omega-6: omega-3 fatty acids.

The MOFO study also showed that the combination of canola plus 1.1g/d of DHA is equally as effective as daily supplementation with 2.2g/d of DHA at reducing fasting plasma triglyceride concentrations with the added benefit of preventing the significant rise in both LDL and total cholesterol caused by both doses of DHA alone. While further research is warranted based on the findings from animal studies, it can reasonably be proposed that the findings from the MOFO study may be an example of a synergistic effect of canola phytosterols and DHA, rather than ALA and DHA, working together to significantly reduce fasting plasma triglyceride concentrations while preventing detrimental effects on LDL cholesterol in people with mild hypertriglyceridemia.

The second human clinical trial conducted as part of this thesis examined the effect of combining omega-3 fatty acids with soy isoflavones on fasting blood lipids, blood pressure and arterial compliance (Omega-Soy study). The Omega-Soy study showed that the



combined consumption of DHA with soy isoflavones resulted in an 8-10% improvement in HDL cholesterol, an 18-20% reduction in plasma triglyceride concentrations and the absence of a 10.8% rise in LDL cholesterol observed with DHA supplementation alone. Furthermore, the increases in LDL and total cholesterol observed with DHA supplementation in the first six weeks were reversed and significantly reduced when soy cereal was concurrently consumed. The results showed that the dietary combination of soy isoflavones and DHA improve the lipid profile of moderately hyperlipidemic individuals more favourably than either constituent alone.

While further research is warranted based on evidence from *in vitro* cell culture and *in vivo* animal models demonstrating functional effects of soy isoflavones and DHA on lipid metabolism pathways, it can reasonably be proposed that the findings from the Omega-Soy study demonstrate a synergistic effect of soy isoflavones and DHA, working together to significantly reduce fasting plasma triglyceride concentrations without detrimental effects on LDL cholesterol in people with mild hyperlipidemia.

The findings from this thesis support two functional food synergies for effective improvement of blood lipid concentrations when consumed as part of the usual diet of men and postmenopausal women with moderately elevated blood lipids. These functional food combinations are DHA with canola and DHA with soy isoflavones. The findings of this thesis sheds some light on how isoflavones may be actively involved in reducing plasma cholesterol concentrations when consumed with soy protein or in soy containing foods, furthermore the findings of this thesis provide strategies for ultimately reducing the negative side effects of dietary DHA supplementation and for achieving a better outcome in overall lipid profile improvements than could be achieved with DHA supplementation alone. Future research into these synergistic combinations of functional food ingredients with DHA may lead to new directions in functional food development by food manufacturers to enable more consumers to manage their blood lipid concentrations with minimal or without drug therapy requirements.

## **Acknowledgements**

---

I commenced my PhD in 1999 so this thesis has been 8 years in the making, give or take a couple of years of leave. There are many people who have supported me tirelessly throughout this long stretch to whom I am forever indebted.

The person I need to thank first and foremost for her tolerance, love and support is my lovely wife Kellie Ridges. I could not have finished this thesis if it were not for her constant support and clever tactics to get me into the study and writing. Kellie's determination to see my thesis finished provided me with the motivation necessary to inhibit my procrastination trends, forcing me to write. I appreciate the great amount of patience and the many sacrifices of fun times and holidays that Kellie made for me during this long candidature.

I would like to thank Professor Lee Astheimer and Associate Professor Peter McLennan for putting up with me during my candidature and helping me to get over the finish line. I am especially appreciative of Peter's kind offer to take over my supervision at a late stage in my thesis after having taken leave. Peter has been a wonderfully inspiring and supportive supervisor and I am very grateful for his guidance. I am also extraordinarily fortunate that Lee continued to support me throughout the full 8 years of my candidature trying every tactic she could muster to get this thesis finished. I owe Lee many good publications from this thesis to try and repay her commitment to my doctoral endeavors. I would also like to thank Professor Peter Howe and Associate Professor Barbara Meyer for all of their support and guidance at the commencement and first few years of my candidature. I learnt a great deal from both Peter and Barbara and am appreciative of the many things they taught me. I also need to thank the University of Wollongong, the Heads of Department, Heads of Postgraduate Studies and the staff at the Postgraduate Student Research Centre who continued to support my candidature even after I had exceeded my five year timeframe.

I would like to thank Associate Professor Ken Russell for all of his expert advice and guidance on how to statistically analyse my data in Chapter 3. I am extremely grateful for his advice and patience.

I would like to give a very big thank you to Judy Martin and to John, Elise, Andrew and Allyson for allowing me to take over Judy's study or Allyson's bedroom whenever I visited over the past two years and for the many cups of excellent coffee, biscuits, snacks and meals provided to me as I typed away long hours at the computer. A very special thank you also to the many long chats that I shared with Judy about the thesis journey. The Martin family support was incredibly helpful and I am extremely appreciative of the support they have provided me.

I would like to thank all of my friends who never stopped believing in my ability to finish this thesis and who never got cross at me for missing numerous catch-ups and get-togethers because I had to stay home and write. A big thank you must go to John Byron and Angela Pratt for their constant support throughout my candidature. They always boosted my self confidence and belief in my abilities whenever I saw them and that assisted me greatly in getting my thesis finished. I must also give a big thanks to my brother Malcolm Ridges who was also wonderfully supportive in sharing his PhD experiences with me and being constantly understanding of where I was at during each phase of the thesis process. I truly appreciate his support.

A special thank you must also go to my work supervisors Peter Williams and Troy Coyle. Both provided me with fantastic support and encouragement to get my thesis finished. I am especially thankful for having a day a week throughout 2007 to write. I cannot thank Troy enough for providing me with the opportunity to have solid time periods for thesis writing and thinking.

Finally, I would like to thank my parents for encouraging me throughout my thesis and constantly believing in my ability to complete my PhD, no matter how long it took me. I am very appreciative of their support.

## **1 Literature review**

---

## **1.1 Cardiovascular disease prevalence**

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Cardiovascular disease (CVD) is defined as all diseases of the heart and blood vessels (Australian Institute of Health and Welfare, 2005). Atherosclerosis which is the narrowing and hardening of the arteries over time is the underlying cause of most cardiovascular disease.

In 1999 when work on this thesis commenced, CVD was the leading cause of death amongst Australians accounting for 40% of all deaths, and coronary heart disease and stroke were the first and second highest causes of death, respectively (Australian Institute of Health and Welfare, 2001). Cardiovascular disease was also the leading cause of long-term disability in adults and the most costly disease for the health system in Australia. Earlier figures show that CVD was responsible for 12% (\$3.9 billion) of total recurrent health expenditure in 1993-94 with coronary heart disease (\$894 million), high blood pressure (\$831 million) and stroke (\$630 million) consuming most health system resources.

The most recent Australian data shows that in 2004, ischaemic heart disease (coronary heart disease, heart attack and related disorders), cerebrovascular disease (primarily stroke) and other heart diseases (including heart failure) were responsible for 31.1% of all deaths in men and 35.8% of all deaths in women. Ischaemic heart disease and cerebrovascular disease were the leading causes of death in men and women with other heart diseases being the third highest cause of death in women and fourth highest cause of death in men (Australian Institute of Health and Welfare, 2006). The total number of deaths from cardiovascular disease has significantly declined in recent years, but remains the leading cause of death amongst Australians.

## **1.2 Cardiovascular disease risk factors**

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Based on an analysis of the 2001 National Health Survey data, it was reported by the Australian Institute of Health and Welfare (2005) that in 2001 more than fifty percent of all adults had two or three (out of a potential nine) risk factors for cardiovascular disease and that one in six adults had four or more risk factors. On average, and regardless of age, adults were most likely to have two risk factors. In 1999-2000, over six million Australian

adults aged 25 years and over (approximately 23%) had blood cholesterol concentrations higher than 5.5 mmol/L; the upper limit of the recommended healthy range (NCEP Expert Panel, 2002).

The primary preventable risk factors for cardiovascular diseases are tobacco smoking, high blood pressure, raised low-density lipoprotein (LDL) cholesterol, raised triglycerides, low concentrations of high density lipoprotein (HDL) cholesterol, insufficient physical activity, poor nutrition, risky alcohol consumption, increasing age, male gender, family history, excess body weight and type 2 diabetes (Australian Institute of Health and Welfare, 2005; Grundy et al., 1999). Of these, blood lipids and blood pressure have been popular targets for dietary intervention and drug therapy to reduce and manage the risk of CVD.

### **1.2.1 Total and LDL cholesterol**

The relationship between cholesterol concentrations and the risk of CVD has been extensively evaluated and as such the evidence supporting this relationship is very strong. A report by Mensink et al. (2003) that systematically and critically evaluated the science to validate biomarkers linking diet and cardiovascular disease risk, concluded that an extensive amount of research has demonstrated unequivocally that serum cholesterol is a biomarker for CVD risk (Mensink et al., 2003). This conclusion was based on consistent reports from human observation studies conducted in a wide range of populations (including individuals with late stage CVD and varying blood lipid profiles), across a range of age groups showing serum cholesterol concentrations accurately predict risk of CVD (Mensink et al., 2003). Several key prospective human population studies commencing as far back as 1948 have also consistently demonstrated that a positive curvilinear relationship exists between plasma cholesterol concentrations and rates of death from coronary heart disease (CHD) (Stamler et al., 1986; Assman et al., 1998a; Castelli, 1984). Furthermore, this relationship is present across both elevated and normal cholesterol concentrations (Stamler et al., 1986).

In plasma, the primary lipoprotein carriers of cholesterol are low density lipoproteins (LDL). The evidence substantiating the positive curvilinear correlation between plasma

total cholesterol concentration and risk of CVD also exists for LDL cholesterol (Stamler et al., 1986; Castelli, 1984; Assman et al., 1998a). Double blind, four year, placebo controlled intervention studies of more than 2000 patients, with adequate statistical power to detect a change in LDL cholesterol confirm the findings from prospective studies and suggest that a 1.0 mmol/L reduction in plasma cholesterol corresponds with an approximate 20% reduction in the risk of a future cardiovascular event (Lipid Research Clinics Program, 1984a and 1984b; Sheperd et al., 1995; Downs et al., 1998). Supporting the role of LDL cholesterol as a biomarker of CVD risk are mechanistic studies demonstrating the important role of LDL cholesterol in atherosclerotic plaque formation (Smith and Slater, 1972; Steiberg and Witztum, 1990; Ballantyne et al., 1997).

There have been several reviews published on the mechanisms of atherosclerotic plaque development (Berliner et al., 1995; Ross, 1999), all of which emphasise the role of LDL cholesterol in the plaque development process. Ross (1999) claims that LDL, which may be modified, is a major cause of injury to the endothelium and underlying smooth muscle while Berliner and co-workers (1995) demonstrate that there is strong evidence showing elevations in plasma concentrations of LDL are sufficient to induce all of the components of the atherosclerotic reaction.

Studies conducted in the 1980's and early 1990's demonstrated that LDL particles enter the artery wall in a concentration-dependent manner without the requirement for receptor-mediated endocytosis (Berliner et al., 1995). Once in the wall, LDL particles are susceptible to becoming oxidised by oxidative products from different pathways. Oxidation of the lipid within the LDL particle can also modify the receptor binding proteins on the particle making it susceptible to uptake by macrophages in the endothelium. The consequence of this process is the deposition of lipid filled foam cells and development of the fatty streak which is the early phase of plaque formation.

### **1.2.2 HDL cholesterol**

Plasma HDL are thought to be cardio-protective, with HDL cholesterol being inversely associated with the incidence of coronary artery disease independent of changes in other

blood lipids such as LDL cholesterol or triglycerides (Franceschini, 2001; Bass et al., 1993). Across a range of prospective human population studies, serum HDL cholesterol has been shown to be a strong lipid predictor of future cardiovascular events and of CVD risk (Castelli, 1984; Stamler et al., 1986; Assman et al., 1996; Bainton et al., 1992). These studies also report that low concentrations of HDL cholesterol are inversely related to the risk of developing premature CVD. HDL cholesterol data from several prospective trials (Castelli, 1984; Lipid Research Clinics Program, 1984b) provides evidence that an increase in HDL cholesterol of 0.025mmol/L is associated with a 2-5% reduction in CHD risk (Gordon et al., 1989). Conversely, an HDL cholesterol concentration of less than 1.0 mmol/L is associated with an increased risk of CVD (Mensink et al., 2003, Barter et al., 2001).

There is strong evidence to support the mechanisms by which HDL exerts its cardio-protective effects. Primarily, HDL is responsible for reverse cholesterol transport whereby cholesterol is removed from cells, tissues and even atherosclerotic plaques and transported to the liver (Dietschy, 1997; Franceschini et al., 1991). This process reduces the quantity of cholesterol that is available for deposition and oxidation in arterial walls, reducing plaque formation. Additionally, proteins within and associated with HDL have been shown to have antioxidant capabilities enabling HDL to inhibit the oxidation of LDL particles and subsequently foam cell formation. Finally, HDL may also reduce the expression of endothelial cell adhesion molecules required for monocyte uptake into the artery wall, thus, inhibiting the early stages of plaque formation (Barter, 2004).

### **1.2.3 Triglycerides**

Elevated plasma triglycerides have also been shown to be a CVD risk factor (Bass et al., 1993; Assman et al., 1998b). An early review of 18 case control studies published between 1959 and 1981 and 8 prospective studies published prior to 1989 examining the relationship between blood triglyceride concentrations and CVD risk concluded that in the case control studies a univariate association exists between hypertriglyceridemia and coronary heart disease. This relationship however was shown to be less consistent in long term prospective studies and the increased risk was not always independent of HDL cholesterol measures



(Austin, 1989). In 1993, the National Institutes of Health Consensus Panel on Triglycerides, HDL and Coronary Heart Disease concluded that case control observational studies consistently showed a strong association between elevated fasting triglycerides and CHD, a finding that is confirmed in prospective cohort studies. It was also concluded that a dose response effect exists between plasma triglyceride concentrations and the development of CVD (NIH Consensus Development Panel, 1993). A later meta-analysis of 17 population based prospective studies in 1996 reported that fasting triglycerides are a risk factor for CVD in both men and women and that this association is independent of HDL cholesterol concentrations (Hokanson & Austin, 1996).

Mechanistically, elevated triglyceride concentrations may contribute to atherosclerosis and coronary artery disease (CAD) through the enhanced uptake by macrophages of triglyceride rich lipoproteins and deposition of these in the artery walls (NIH Consensus Development Panel, 1993), an increased production of pro-thrombotic factors and associated small dense LDL particles with enhanced atherogenic potential (Godsland et al., 2007).

#### **1.2.4 Blood pressure**

Sustained high blood pressure or hypertension is defined as systolic blood pressure (SBP) > 140 mmHg and diastolic blood pressure (DBP) > 90mmHg (The Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, 1997). In their review, Mensink et al. (2003) concluded that there is consistent evidence to demonstrate that reducing blood pressure significantly decreases the risk of cardiovascular events. Ten prospective population studies have shown that by lowering DBP and SBP by 5 and 9mmHg, respectively, the incidence of stroke and CHD is reduced by over 25% (MacMahon et al., 1990; Castelli, 1984). Clinical trials have confirmed these findings. Two reviews including 25 large clinical trials assessing the effect of anti-hypertensive medications reported that reductions of 5 mmHg in DBP and 10 mmHg in SBP decreased the incidence of stroke by more than 30% and CHD by almost 20% (Collins et al., 1990; Staessen et al., 2000). The reduced risk of CVD associated with significant reductions in blood pressure or lower blood pressure is consistent across different populations and subgroups (Mensink et al., 2003).

There are clear mechanisms to explain the relationship between blood pressure and CVD risk. Sustained high blood pressure increases the after-load on the heart and can result in left ventricular hypertrophy and subsequent heart failure (Bakris & Mensah, 2003). The risk of stroke is also enhanced by high blood pressure due to the sheer forces produced in the blood vessels which can lead to damaged epithelium as well as destabilisation and dislodgement of atherosclerotic plaque causing total or partial arterial occlusion (Luscher, 1992).

### **1.3 Guidelines for reducing CVD risk**

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In the *Prevention of Cardiovascular Disease: an evidence based clinical aid (2004)*, a guideline for Australian medical practitioners, three interventions, are advocated for the prevention of CVD. These are: lifestyle changes (increasing exercise, improving diet and decreasing stress), cessation of smoking and treatment of hypertension and dyslipidemia (Practical Implementation Taskforce for the Prevention of Cardiovascular Disease, 2004).

Lifestyle modifications are the first line of treatment for all adults that present with one or more CVD risk factors, a primary component of which is dietary modification to encourage healthy eating. Dietary treatment for reducing the risk of CVD has predominantly been aimed at improving blood lipids, but also maintaining or reducing body weight and improving blood pressure (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001; Krauss et al., 2000; Dwyer, 1995). For blood lipid improvement (with a focus primarily on reducing plasma total and LDL cholesterol concentrations), dietary advice both in Australia and the US has been to reduce total fat, saturated fat and dietary cholesterol intake (The Expert Panel, 1988 and 2001; Barter et al., 2001; Practical Implementation Taskforce for the Prevention of CVD, 2004). With the exception of increasing fruit and vegetable consumption, the dietary advice to reduce CVD risk up until recently has focused predominantly on limiting or excluding certain foods. However, dietary advice to reduce the risk of CVD, according to Kris-Etherton et al. (2002) has since 2000, moved from an ‘exclusionary’ to an ‘inclusionary’ paradigm. The dietary recommendations from the American Heart Association and National Cholesterol

Education Program Adult Treatment Panel III (NCEP ATP III) focus on foods that can be included in a heart healthy risk reduction diet, rather than foods that should be excluded. These newer guidelines are designed to assist individuals and guide physicians about foods people can eat rather than on foods people shouldn't eat.

The 'inclusive' guidelines for CVD risk reduction are based on a growing amount of epidemiological and clinical trial data providing high quality evidence to support reduction in various CVD risk factors with a growing number of functional food components. Included in the current guidelines in addition to the historical advice to reduce total fat, saturated fat and dietary cholesterol intake is advice to consume: monounsaturated fatty acids, omega-3 fatty acids from fish and vegetable oils, folate, potassium, calcium, antioxidant-rich foods, soy protein, soluble fibre and plant stanols and sterols (Kris-Etherton et al., 2002).

While dietary and lifestyle changes are the first line of management for lowering cholesterol and reducing CVD risk in all individuals, for people who are at high risk of CVD and in whom diet treatment is ineffective at lowering blood cholesterol levels toward target concentrations, drug therapy is prescribed (Barter et al., 2001).

Cholesterol and triglyceride reducing drugs were the leading class of top selling prescription medications worldwide in 2005, up from being second ranked in 2003 (IMS Health, 2006). In 2006, Lipitor (Pfizer, New York, USA), a cholesterol and triglyceride lowering medication was the top selling drug world wide for the fifth year in a row (Herper and Keng, 2006). In Australia more than 43.4 million prescriptions for cardiovascular drugs were dispensed in 1998 representing almost a quarter of all prescriptions (Australian Institute of Health and Welfare, 2001). Simvastatin-RL™ (GlaxoSmithKline Australia Pty Ltd, Boronia, VIC), a cholesterol-lowering drug, was the top drug cost to the Australian Government, amounting to \$192 million in that same year.

Antihyperlipidemic medications are not free from causing side effects. These can include, but are not limited to: hepatitis, gallstones (fibrates), gastrointestinal upset, erectile dysfunction in men (fibrates), muscle aches, elevated blood triglycerides and intense

flushing of the skin often requiring the co-consumption of aspirin (Knopp, 1999). According to Anderson (2003) statin intolerance in particular, is a reason some patients are referred to specialty lipid clinics and that “*statin-intolerant patients, especially those with serum LDL cholesterol levels greater than 5.18 mmol/L need intensive nutritional management*” (p 532, Anderson, 2003).

Patient compliance to anti-hyperlipidemic drugs is low, both in clinical trials and in clinical practice (Kiortsis et al., 2000; Andrade et al., 1995). Two Australian studies have reported that the majority of Australian patients are non-compliant with taking lipid lowering medication (Simons et al., 1996; Simons et al., 2000). In a study of 610 patients newly prescribed lipid-modifying drug therapy, 60% of patients had stopped taking their medication after twelve months. In a second study conducted on 32,384 patients, 30% of patients had stopped taking their lipid lowering medication after 6-7 months. Reasons for non-compliance included patients being unconvinced about the need for treatment (32%), poor efficacy (32%) and adverse effects (7%). Kiortsis et al. (2000) have reported that a perceived high frequency of side effects and prescription of multiple medications are reasons that have been found to contribute to such non-compliance.

With such a low level of compliance to CVD risk-lowering medication it is important that dietary and lifestyle strategies are found that are both easy to adhere to and maximise CVD risk reduction.

#### **1.4 Functional foods for CVD risk reduction**

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The term ‘functional foods’ was coined in the 1990’s as studies identified the potential and capability of foods and food components to reduce disease risk factors. Numerous organisations across various countries have developed working definitions of functional foods (Clydesdale, 2004; American Dietetic Association, 2004; Ohama et al., 2006; Tapsell et al., 2005). Based on these definitions functional foods can be described as *‘foods that are whole, fortified, enriched or enhanced foods or food ingredients that may provide a health benefit beyond basic nutrition when consumed regularly as part of a varied diet at effective levels’*.

There has been extensive research undertaken into the health benefits and disease risk reduction achievable with specific functional foods and functional food ingredients. For some functional foods this research has culminated in substantiated health claims based on convincing evidence that meets the criteria of Significant Scientific Agreement (U.S Food and Drug Administration, 1999a; Food Standards Australia New Zealand, 2005).

Individual functional food ingredients can impact on biochemical pathways and disease biomarkers via similar or diverse mechanisms. Combinations of functional ingredients that improve CVD risk factors have the potential therefore, to act in synergy, enhance bioavailability, produce cumulative benefits or simply provide a combined effect that is greater than can be achieved from the individual contributions of the functional ingredients. In recent years the volume of research has grown in the area of functional food combinations for reducing disease risk factors, especially as new scientific techniques and technologies allow critical analysis of these combinations. However, to date there is insufficient scientific evidence to demonstrate that any particular functional food combination (with the exception of the broader category of ‘fruit and vegetables’) has the capacity to reduce cardiovascular disease risk factors by a greater extent than could be achieved with the individual functional foods.

In the 1930’s and 1940’s the key functional ingredients in foods were considered to be vitamins and minerals and the macronutrients, fat, carbohydrate and protein. At that time, a

balanced diet was about ensuring an adequate intake of these dietary factors to avoid deficiency diseases. In the 1950's and 1960's it became clear, due to the findings of several large epidemiological studies (Caggiula and Mustad, 1997) that the levels of consumption of some dietary factors such as saturated fat, cholesterol and total energy and the under-consumption of other dietary factors such as dietary fibre were related to increased blood cholesterol concentrations and the subsequent risk of serious diseases such as CVD. This link between dietary factors and risk of CVD became known as the diet-heart hypothesis (Gordon, 1988). One of the key dietary factors that met this hypothesis was polyunsaturated fatty acids (PUFAs) based on the cardiovascular benefit achieved with their replacement of saturated fat in the diet.

#### **1.4.1 The shift to polyunsaturated fats**

Early intervention studies were instrumental in showing effects of different types of fatty acids on plasma cholesterol concentrations (Ahren et al., 1954). This work showed that corn oil, rich in PUFAs, consumed in place of animal fat, rich in saturated fatty acids, significantly reduced total plasma cholesterol. Subsequent work by Keys et al. (1959, 1965) and Hegsted et al. (1965) established that PUFAs significantly decreased and saturated fatty acids, excepting stearic acid, significantly increased blood cholesterol concentrations while monounsaturated fatty acids when consumed in place of saturated fatty acids had a neutral effect on plasma cholesterol concentrations.

Polyunsaturated fatty acids have more than one double bond and can be categorised as either omega-3 or omega-6 fatty acids. Omega-3 fatty acids have their last double bond located at the third carbon from the methyl end of the carbon chain while omega-6 fatty acids have their last double bond located at the sixth carbon from the methyl end of the carbon chain. The omega-3 fatty acids include alpha-linolenic acid (ALA), found in walnuts, canola, linseeds and green leafy vegetables, and the very long chain eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids found predominantly in oily fish and various strains of algae. The omega-6 fatty acids include the plant-sourced linoleic acid (LA) which is found predominantly in plant oils such as sunflower, soybean and safflower oils and the very long chain arachidonic acid (AA) which is provided in the diet

via red and white meat sources as a result of animals feeding on grains rich in LA (Simopolous, 1991).

In the early 1950's, Hugh Sinclair proposed that a diet rich in seal meat, fish and seafood as consumed by the Greenland Eskimos was protective against the incidence of heart disease. Additionally, studies in the 1950's examined the effect of fish oil and corn oil as replacements for butter and lard in the diet and showed that both sources of PUFAs could significantly reduce total plasma cholesterol in individuals with atherosclerosis (Ahrens et al., 1954; Keys et al., 1957; Ahrens et al., 1959; Keys et al., 1959). Despite this, the hypocholesterolemic effect of corn oil and other vegetable oils rich in LA received a lot more attention in the scientific literature prior to 1970. Subsequent to the publication of research showing that PUFAs both replacing saturated fat and partially replacing dietary carbohydrate could reduce plasma cholesterol concentrations, medical practitioners and nutritionists began recommending diets high in polyunsaturated fat and reduced saturated fat to reduce CVD risk (Ginsberg et al., 1994). This growing public awareness coupled with proficient processing techniques for generating oils from grains (Simopoulos, 1991; Krishenbauer, 1960) created a strong market for vegetable oil and spreads and an enhanced role for these products in the everyday diet (Simopolous, 1991). This trend was observed in many countries. In the United States (USA) corn oil consumption quickly replaced animal fats in the food supply, to the extent that corn and other vegetable oils now represent over 70% of dietary PUFAs in the USA diet (Taubes, 2001; Ailhaud et al., 2006). In 1940, dietary PUFA intake provided 2.5% of energy in the USA diet. By 1985 this had increased to 7.5% (Stephen and Wald, 1990). A similar increase in the use of LA-rich vegetable oils and margarines occurred in the United Kingdom (UK). In UK males, daily LA intake between 1980 and 1992 increased 50%, from 10g/day up to 15g/day (Ailhaud et al., 2006). Additionally, in France, between 1960 and 2000, triglyceride consumption from plant oils and margarines, increased from 19g/day to 37g/day due to increased consumption of peanut oil and sunflower oil (Ailhaud et al., 2006). Ailhaud et al. (2006) highlight in their review that in France LA is the most consumed fatty acid (42%) compared to LNA (1%), oleic acid (33%) and saturated fatty acids (18%).

There have been some inconsistencies in the scientific evidence regarding the cardio-protection offered by a diet rich in LA. These inconsistencies may be because early studies conducted in the 1960's examining the cholesterol lowering effects of replacing saturated fat with predominantly LA from vegetable oils were based on high dietary concentrations of PUFAs (20-25% of energy) in high fat diets (30-40% of energy) (Hegsted et al., 1993); Further, many studies had limited subject numbers, providing potentially low statistical power (Keys et al., 1957; Hegsted et al., 1965).

Early clinical trials reported a reduced prevalence of atherosclerosis with the increased dietary consumption of LA-rich polyunsaturated fats (Leren, 1966; Miettinen et al., 1972). Large prospective cohort studies also reported fewer cardiovascular events (Djousse et al., 2001; Oomen et al., 2001) including a reduced incidence of stroke (Iso et al., 2002) with higher dietary LA intake, however not all prospective cohort studies have made the same observations (Hu et al., 2001). Laakson et al. (2005) showed that men with the highest intake of dietary LA compared with those with the lowest intake had a significantly reduced risk of dying from CVD. They also showed that increased serum concentrations of LA were inversely associated with CVD mortality. Subsequent clinical trials have shown LA-rich vegetable oil consumption, especially when consumed as a replacement for dietary saturated fatty acids, is beneficial in lowering total and LDL cholesterol (Hodson et al., 2001). A quantitative meta-analysis of metabolic ward studies in which LA rich vegetable oils were consumed in place of dietary sources of saturated fat, concluded that PUFA consumption decreases while saturated fat increases LDL cholesterol concentrations (Clarke et al., 1997). Some studies have indicated that LA-rich oils may be more effective than oleic acid-rich oils at lowering LDL cholesterol when replacing saturated fat in the diet (Howard et al., 1995; Mensink & Katan, 1992). This suggests that LA may have an LDL cholesterol lowering effect that is independent of that achieved by lowering dietary saturated fat intake. Other studies however, suggest LA may not have an independent cholesterol lowering effect, reporting that PUFA- or MUFA-rich vegetable oils are equally as effective at lowering plasma LDL cholesterol when replacing sources of dietary saturated fat (Mattson & Grundy, 1985; Grundy et al., 1986; Mensink & Katan, 1989).



A systematic review of 27 randomized controlled trials reducing or modifying dietary fat consumption in healthy adults (Hooper et al., 2001) provided some support to the suggestion that it is the reduction in saturated fat intake rather than the increase in dietary LA that is most important for lower LDL cholesterol and CVD risk. The review found that increasing dietary consumption of polyunsaturated fatty acids (predominantly LA) was not associated with a statistically significant reduction in either the incidence of cardiovascular events or mortality. Other studies (Yam et al., 1996; Dubnov and Berry, 2003; Pella et al., 2003) that have examined populations with a high LA-rich diet, such as in Israel, have also shown no associated reduction in the incidence of CAD or diabetes with an LA-rich diet. A large review of ecological, cross-sectional, cohort and case-control studies also failed to find a protective effect from increasing the intake of dietary PUFAs which are LA-rich with or without a reduction in the dietary consumption of saturated fatty acids (Ravnskov, 1998).

In summary, dietary LA intake has increased significantly in the past 50 years as a result of increasing consumption of LA-rich vegetable oils and margarines, a change originally advocated on the basis of reducing plasma cholesterol concentrations. This reduction in cholesterol has been shown to be created by a reduction in dietary saturated fat intake as a consequence of replacing food sources of saturated fat with vegetable oils and margarines. While this outcome is beneficial, the evidence from human clinical trials suggests that LA may not itself have any intrinsic cholesterol lowering capacity other than as a replacement for saturated fat in the diet. As discussed later in this chapter, a high intake of dietary LA may not be as favourable as first thought due to the competition between LA and omega-3 fatty acids EPA and DHA and the increased evidence demonstrating more favourable cardiovascular functions of omega-3 over omega-6 fatty acids.

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### **1.5 The Cardiovascular Benefits of Omega-3 Fatty Acids**

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In the early 1950's Hugh Sinclair's work with Inuit Eskimos highlighted their unusual dependence on fish, seal and whale blubber. He correlated this very high dietary omega-3 fatty acid intake with a very low incidence of heart disease suggesting for the first time that dietary omega-3 fatty acids had CVD protective qualities (Sinclair, 1956). It wasn't until the studies of Bang and Dyerberg however in the 1970's that the scientific community revisited the potential for omega-3 fatty acids to have cardiovascular benefits on a much

larger scale. Bang and Dyerberg compared the diets, blood lipid concentrations and fatty acid profiles of Greenland Inuit with Danish men and women living in Denmark. They showed that Greenland Inuit had significantly lower concentrations of plasma cholesterol and triglycerides and had significantly higher (30-50% higher) levels of plasma EPA and lower plasma LA levels compared to the Danish reference population (Bang et al., 1971; Bang and Dyerberg, 1972; Dyerberg et al., 1975). Subsequently additional studies demonstrated that Inuit populations from Greenland, Alaska and Canada have a significantly higher dietary consumption of seafood and very long chain omega-3 fatty acids and significantly lower mortality from ischemic heart disease than non-native populations in Denmark, Alaska and Canada (Bjerregaard et al., 1997; Dewailly et al., 2001; Middaugh, 1990; Davidson et al., 1983, Bjerregaard & Dyerberg, 1988). Similarly, in Japan where dietary consumption of fish and very long chain omega-3 fatty acids is considerably high in comparison to most other western countries, the incidence of thrombotic disease is very low (Hirai et al., 1989).

Following on from this early work in Greenland Inuit was a plethora of ecological, epidemiological and observational studies on fish and omega-3 fatty acid consumption and the incidence of heart disease and heart disease mortality. Zhang et al. (1999) conducted an ecological study examining the relationship between fish consumption and mortality data from 36 countries across three time periods: 1961-1963, 1979-1981, and 1981-1991. They observed that fish consumption across all time periods was independently and inversely associated with all-cause-mortality, ischemic heart disease mortality and stroke mortality in men and women.

An inverse relationship between fish consumption and mortality from CVD has been demonstrated in several large cohort studies. Based on a 20-year follow up on dietary data obtained in 1960, Kromhout et al. (1985) discovered an inverse dose-response relation between fish consumption and death from coronary heart disease in 852 men in the Netherlands; this relationship which was maintained following multiple logistic regression analyses. Men consuming at least 30g of fish, daily, had a 50% reduction in mortality from coronary heart disease compared with men that consumed no fish. In the Chicago Western Electric study Davignus et al. (1997) also reported an inverse relationship between fish

consumption and coronary heart disease in 1822 men, free of cardiovascular disease at baseline, that were followed over 30 years. Reaffirming these findings were the Seven Countries and Nurses' Health studies (Sandler et al., 1993; Hu et al., 2002). In the former study consumption of fatty fish was associated with lower CHD mortality compared with consumption of non-fatty fish in 1088 Finnish, 1097 Italian and 553 Dutch men aged 50-69 years, free of CHD at baseline and followed up over 20 years. This finding implicated the omega-3 fatty acids as playing an active role in reducing the incidence of CHD which is associated with increased fish consumption due to the higher content of omega-3 fatty acids in fatty fish (Sandler et al., 1993). The Nurses' Health Study followed 84,688 healthy women aged 34-59 years over 16 years and observed that the relative risk of CHD progressively declined with increasing frequency of fish consumption. Further, in women with a higher intake of omega-3 fatty acids, the risk of CHD progressively decreased over quintiles of intake (Hu et al., 2002). The Multiple Risk Factor Intervention Trial (MRFIT) was a randomized clinical trial in coronary heart disease primary prevention conducted in 12,866 men at high risk of developing CVD. Analysis of data from that study showed that in men allocated to usual dietary care for CVD risk factors, progressively higher EPA and DHA intake over 10.5 years was associated with a progressive reduction in CHD and total mortality (Dolecek et al., 1992).

Two observational studies have shown an inverse relationship between an established biomarker for dietary omega-3 fatty acid intake and cardiac arrest and risk of sudden cardiac death (Morris et al., 1995; Siscovick et al., 2000). The US Physician's Health Study conducted in 20,551 male physicians aged 40-85 years followed for 11 years showed that consumption of one fish meal a week was associated with a significantly lower risk of sudden cardiac death and a 30% reduction in total mortality compared with consumption of less than one fish meal/month. Subsequent prospective analysis of healthy physicians followed up after 17 years showed whole blood long chain omega-3 fatty acid content was significantly inversely correlated with the risk of sudden cardiac death. This corresponded with an 81% reduced risk of sudden death amongst men with the highest quartile of whole blood omega-3 fatty acid content compared with men in the lowest quartile (Morris et al., 1995).

There have been many systematic reviews examining the effect of omega-3 fatty acids (consumed as fish, fish oil or ALA) on mortality and cardiovascular events (Wang et al., 2006; Hooper et al., 2006; Balk et al., 2006; He et al., 2004a; He et al., 2004b). These reviews, summarized in **Table 1.1**, consistently report that in the majority of secondary prevention studies increased intake of fish or fish oil supplementation in subjects with CVD was associated with a clinical reduction in all cause mortality and CVD events. Most of the reviews also reported that data from primary prevention studies is more varied and less strong than for secondary intervention studies; while the majority of primary prevention cohort studies show that increasing fish consumption can significantly reduce the relative risk of CVD mortality some studies do not report this relationship and others suggest a potential negative effect of fish or fish oil on CVD events (**Table 1.1**).

Only one review, a meta-analysis of 48 randomized clinical trials and 41 cohort studies published prior to February 2002, concluded that omega-3 fatty acid intake for six months or more is associated with a 14% reduction in the risk of total mortality or cardiovascular events, however, the risk reduction is not statistically significant (Hooper et al., 2006). Considerable concerns were raised about the integrity of this review by a large number of experienced scientists with extensive experience in omega-3 fatty acid research. Quality concerns focused primarily on the inclusion of several confounding factors in the analyses. Such factors included combining randomized clinical trial data from studies using ALA supplementation with studies using EPA and DHA and combining data from secondary and primary prevention studies (Decklebaum and Akabas, 2006). Additionally, this review included a large clinical trial that has been strongly criticized for being of poor scientific quality and is subsequently implicated as the primary cause for the absence of a statistically significant finding (Scholl, 2006; Astrup, 2006; Lund, 2006). Based on these design limitations, the implications of the meta-analyses' findings should be interpreted cautiously and in the context of the contrasting results of several other systematic reviews.

Table 1.1 Review of outcomes from systematic reviews of the scientific literature on fish, fish oil and omega-3 fatty acids and the risk of CVD mortality and events.

Review	Period of review	Type of review	Inclusion criteria	Number of studies	Outcomes
Wang et al. (2006)	1966 - 2006	Systematic review	Study duration $\geq$ 1 year Quantified intake of fish, fish oil, EPA and/or DHA or ALA. Supplementation with $< 6\text{g/d}$ of fish oil.	46	<p><b>Secondary prevention</b></p> <ul style="list-style-type: none"> <li>- 14 RCTs and 1 cohort study (mostly fish oil some high dietary omega-3 fatty acid intake)</li> <li>- Most trials reported fish oil reduces myocardial infarction, all-cause mortality, cardiac and sudden death or stroke.</li> </ul> <p><b>Primary prevention</b></p> <ul style="list-style-type: none"> <li>- 1 RCT, 25 prospective cohorts, 7 case-control studies</li> <li>- In most cohort studies increased frequency of fish consumption was associated with reduced rates of all cause mortality and adverse cardiac outcomes.</li> <li>- The effect of fish consumption on stroke was inconsistent</li> </ul> <p>The evidence for a cardiovascular benefit of fish oil is stronger in secondary prevention than primary prevention.</p>
Hooper et al. (2006)	1966 - 2004	Meta-analysis	Study duration $\geq$ 6 months, assessed intake of omega-3 fatty acids	48	<ul style="list-style-type: none"> <li>- Pooled estimate of 41 cohort studies (secondary and primary prevention and ALA and fish oil studies combined) showed no statistically significant reduction in the risk of total mortality or combined cardiovascular events.</li> <li>- In no subgroup or refined analysis of the data set was a clear CVD risk reduction observed with increased consumption of omega-3 fatty acids.</li> </ul>
He et al.(2004a)	1966 - 2003	Meta-analysis	Cohort studies reporting relative risk and corresponding 95% confidence intervals (CI) of stroke relating to frequency of fish consumption	8	<ul style="list-style-type: none"> <li>- Compared with no fish or fish less than 1/ month the relative risk (RR) of developing stroke was:</li> <li>- 0.91 fish 1-3 / month</li> <li>- 0.87 fish 1/ week</li> <li>- 0.82 fish 2-4 / week</li> <li>- 0.69 fish <math>\geq 5/</math> week</li> <li>- Non-significant trend toward greater reduction in RR of developing ischemic stroke with increasing frequency of fish intake.</li> </ul>

Table 1.1 Review of outcomes from systematic reviews of the scientific literature on fish, fish oil and omega-3 fatty acids and the risk of CVD mortality and events.

Review	Period of review	Type of review	Inclusion criteria	Number of studies	Outcomes
He et al. (2004b)	1966 - 2003	Meta-analysis	Studies reporting relative risk and corresponding 95% CI for CHD mortality in relation to fish consumption and the frequency of fish intake	11	<ul style="list-style-type: none"> <li>- Compared with no fish or fish less than 1/month, higher fish intake related to lower CHD mortality</li> <li>- Each 20g/d increase in fish intake was related to a 7% lower risk of CHD mortality (<math>p = 0.03</math> for trend).</li> </ul>
Balk et al. (2006)	1966 - 2003	Systematic review	Study duration $\geq 1$ year Quantified intake of fish, fish oil, EPA and/or DHA or ALA	39	<p><b>Secondary Intervention trials (11 RCT, 1 cohort)</b></p> <ul style="list-style-type: none"> <li>- Largest RCT and most other trials reported reduced all cause mortality and CVD events with EPA and DHA</li> <li>- One RCT comparing ALA and fish oil showed both were effective at reducing CVD outcome</li> </ul> <p><b>Primary intervention trials (22 cohort, 1 RCT)</b></p> <ul style="list-style-type: none"> <li>- RCT showed no significant effect of EPA and DHA or ALA on CVD outcomes</li> <li>- Most large cohort studies reported fish consumption reduces all-cause mortality and CVD events</li> <li>- Several studies reported no significant or negative results.</li> </ul>
Zhang et al. (1999)	1961 - 1963 1979 - 1981 1989 - 1991 (fish consumption data) 1992 - 1993 (mortality data)	Ecological study	All cause and ischemic heart disease mortality for from World Health Organisation in 36 countries for age groups: 45 – 54, 55 – 64 and 65 – 74 years of age.	N/A	<ul style="list-style-type: none"> <li>- Fish consumption across all time units assessed was inversely associated with all cause and ischemic heart disease mortality in men and women.</li> <li>- Accounting for all confounding factors, fish consumption was independently, significantly related inversely to all cause, ischemic heart disease and stroke mortality in all 3 time periods in men and women.</li> </ul>

The most recent, systematic review (Wang et al., 2006) included 48 studies of one year duration or longer, published between 1966 and 2005. That review found that the evidence on the primary prevention of CVD events by very long chain omega-3 fatty acids consists almost entirely of cohort studies with no randomized clinical trials. Based on the outcomes of the cohort studies as summarized in **Table 1.2**, there is good evidence for very long chain omega-3 fatty acids (EPA and DHA) to reduce the incidence of cardiac death, sudden death and myocardial infarction. However, this evidence requires substantiation in well designed, appropriately powered, randomized clinical trials.

Table 1.2 Number of studies based on data contained in the systematic review by Wang et al. (2006) that reported significant reductions, trend toward a reduction, no effect or potential negative effect of dietary omega-3 fatty acid intake, fish oil or fish consumption on CVD outcomes in the general population<sup>1</sup>

Please see print copy for table 1.2

1. Adapted from: Wang C, Harris WS, Chung M, Lichtenstein AH, Balk EM, Kupelnick B, Jordan HS, J. Lau. 2006 N-3 fatty acids from fish or fish-oil supplements, but not  $\alpha$ -linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *American Journal of Clinical Nutrition* 84: 5-17.

Overall, the scientific evidence is strong for fish and fish oil to have a protective effect in the secondary prevention of CVD and there is a moderate level of evidence supporting this role of omega-3 fatty acids in primary CVD prevention. Inconsistencies in the evidence, where present, reside mostly in the heterogeneity of the different study designs used to examine the effect of EPA and DHA on cardiovascular outcomes.

## **1.6 Mechanisms of omega-3 fatty acid CVD protection**

There is no single mechanism by which omega-3 fatty acids reduce the risk of cardiovascular disease. However, there are a range of risk factors that are improved and a number of physiologically relevant transcription and biochemical pathways that are impacted by EPA and DHA to bring about these changes, which when summed, offer significant cardio-protective potential. This potential stems from the anti-arrhythmic, anti-thrombotic and anti-inflammatory effects of EPA and DHA, as well as the hypotriglyceridemic effects, reduced blood pressure, improved endothelial function, and retarded growth of atherosclerotic plaque which have been observed with EPA and DHA supplementation (Balk et al., 2006). This thesis will focus on clinically relevant and measurable cardiovascular disease risk factors that have been shown to be significantly influenced by EPA and DHA. Specifically, lipid and lipoprotein concentrations, blood pressure, heart rate and arterial compliance.

### **1.6.1 Blood lipid effects**

The most consistently reported effect of omega-3 fatty acid supplementation on cardiovascular risk factors is a significant reduction in fasting plasma triglyceride concentrations, while the effect of EPA and DHA on other lipid parameters is more varied.

Harris (1989) conducted a review of thirty-one studies reporting the effects of fish oil supplementation on lipids and lipoproteins. The studies were conducted in normo-lipidemic subjects (n = 596) in which the average dose of fish oil was 24.3g/d (range: 1.6 – 100g/d) providing 5.3g/d of omega-3 fatty acids (range: 1.1 – 30g/d). Overall, these studies showed no change in total or LDL cholesterol, a 25% reduction in triglyceride concentrations and a slight rise in HDL cholesterol (3%). Eleven studies were summarized which examined the effect of fish oil in hyperlipidemic subjects (n=194) in which the average dose of fish oil was 21.8 g/d (range: 5 – 112 g/d) providing 6.4g/d of omega-3 fatty acids (range: 1.7 – 25g/d). Those studies showed that total cholesterol was not affected by fish oil, however both LDL and HDL cholesterol increased 5-7% and triglyceride concentrations decreased 38%. Ten studies were summarized which examined the effect of fish oil in subjects with hypertriglyceridemia (n = 101) in which the average dose of fish oil was 29.8g/d (range: 11 – 75g/d)



providing 8.0g/d of omega-3 fatty acids (range: 2.9 – 25g/d). In these hypertriglyceridemic subjects fish oil decreased total cholesterol by 8%, triglycerides by 52% and increased LDL and HDL cholesterol by 30% and 10% respectively. Harris' early review was targeted at addressing the variability in trials studying the effect of fish oil on plasma lipid concentrations. Harris identified several factors in human clinical trials that were likely impacting on the lipid effects of fish oil supplementation. These factors included: the presence or absence and types of lipoprotein disorders; the relationship between VLDL cholesterol and total cholesterol; the type of fat consumed in the control diets, dose of fish oil supplementation and the varying effects of EPA and DHA. It was concluded from the 1989 review that the dose of fish oil had little impact on explaining the variance in lipid responses amongst studies, however saturated fat consumption in both the fish oil and control groups was an influential factor on lipid responses. When the studies conducted in normo-lipidemic subjects were separated based on whether saturated fat intake remained constant or changed, it was clearly demonstrated in these studies where saturated fat remained constant, LDL cholesterol levels increased with fish oil supplementation and when saturated fat intake was lower during the fish oil versus control diet, reductions in LDL and HDL cholesterol were observed. Among the studies in hyperlipidemic subjects, saturated fat intake was held constant across all of the studies reviewed and in every case, LDL cholesterol increased. Harris concluded that "since fish oil may cause LDL cholesterol levels to rise in hypertriglyceridemic patients it is possible that fish oil consumption might raise cardiovascular risk in this group" (Harris, 1989).

At the time the Harris review was published there was little data to explain differences between EPA and DHA on blood lipid parameters and the effect of fish oil on lipoprotein metabolism. Harris undertook a later review (Harris, 1996) which included 72 placebo-controlled human clinical trials on the effect of fish oil on lipids and lipoproteins. Studies were included in the review if they were placebo-controlled, used no more than 7g/d of EPA and DHA and were of at least 2 weeks duration. The studies used slightly lower doses of fish oil supplementation than in the previous review with the average intake of EPA and DHA ranging between 3.7 and 4.0g/d. The review found that in normo-lipidemic subjects, fish oil supplementation decreased triglyceride concentrations by 25% and increased total cholesterol by 2%, likely due to a 4% increase in LDL cholesterol and 3% increase in HDL cholesterol. In hyper-

triglyceridemic subjects fish oil supplementation decreased triglyceride concentrations by 28% and did not change total or HDL cholesterol. The second Harris review demonstrated that a statistically significant inverse relationship exists between omega-3 fatty acid supplementation and percentage reduction in fasting plasma triglyceride concentrations both in normo-lipidemic ( $r = 0.35$ ,  $p < 0.001$ ) and hyper-lipidemic subjects ( $r = 0.50$ ,  $p < 0.001$ ) (Harris, 1996), thus concluding that EPA and DHA lower plasma triglyceride concentrations in a dose-dependent manner. Furthermore, the review showed that the reduction in triglyceride concentrations persists as long as fish oil supplementation is continued. The rise in LDL cholesterol concentration occurred in 18% of studies in normo-lipidemic subjects and in 32% of studies in hyper-triglyceridemic patients. While many of the studies were not statistically powered to observe changes in plasma lipids, no study reported a reduction in LDL cholesterol with fish oil supplementation compared to a placebo group. Similarly HDL cholesterol increased significantly in only 3% of studies reviewed and decreased in none of the studies (Harris, 1996).

In a third review (Harris, 1997) of 36 cross-over, randomized, human clinical trials, fish oil (predominantly EPA-rich) supplementation at 10-12g/d providing an average intake of 4g/d of EPA and DHA reduced fasting triglycerides by 25% and 34%, respectively, in healthy and hyper-triglyceridemic subjects. LDL cholesterol however, increased 4.5% in healthy subjects and 10.8% in hyper-triglyceridemic subjects. HDL cholesterol did not show significant changes in either subject group. The results from parallel-designed studies were similar to those observed with cross-over studies.

A recent meta-analysis (Balk et al., 2006) was undertaken on 21 human clinical trials examining the effect of EPA, DHA or ALA on blood lipids. The results showed that fish oil consumption reduced plasma triglyceride concentrations by 0.31 mmol/L, increased HDL cholesterol by 0.04 mmol/L and increased LDL cholesterol by 0.16 mmol/L. Fish oil had no net effect on total cholesterol concentrations. The reductions in serum triglycerides observed across the 21 studies were significantly associated with the dose of fish oil supplementation and baseline concentrations of serum triglyceride. Thus a dose-response effect of fish oil supplementation on fasting plasma triglyceride concentrations was evident and more pronounced in individuals with higher baseline triglyceride concentrations. Furthermore, study duration (between 4 weeks and 2 years)

was not associated with the effect of fish oil supplementation on fasting plasma triglyceride concentrations suggesting that once a maximal reduction is achieved, it is maintained over the course of the intervention period.

A review of studies that have examined the effect of purified EPA and DHA on plasma lipids showed that both are equally effective at lowering plasma triglyceride concentrations across a range of subject populations (Mori and Woodman, 2006). Controlled studies using EPA reported 12-24% reductions, while uncontrolled studies reported an average reduction of 24% (range 14 – 35%). In controlled trials using DHA, the reduction in triglyceride concentrations ranged from 17-33% and in uncontrolled studies the average reduction was 20% (Mori and Woodman, 2006). In the same review no real differences were observed between studies using EPA compared with those using DHA in total or LDL cholesterol changes. In three randomized, placebo-controlled, parallel intervention trials that have compared EPA with DHA supplementation, both EPA and DHA reduced triglyceride concentrations by a similar amount (Buckley et al., 2004; Nestel et al., 2002; Mori et al., 2000a). In two studies, EPA and DHA had no effect on total, LDL or HDL cholesterol (Buckley et al., 2004; Nestel et al., 2002). In overweight, mildly hyperlipidemic men, 4g/d of DHA significantly increased LDL cholesterol (8%) and LDL particle size while EPA had no effect. Additionally, HDL<sub>2</sub> cholesterol increased with DHA supplementation and HDL<sub>3</sub> decreased with EPA supplementation, while neither EPA nor DHA resulted in a significant change in plasma HDL cholesterol concentrations (Mori et al., 2000a).

From the small number of studies that have compared EPA with DHA supplementation on plasma lipid parameters, it appears that both omega-3 fatty acids have similar effects on triglyceride concentrations but DHA may have a greater propensity to increase LDL cholesterol. It is unclear what mechanisms might be responsible for this difference between EPA and DHA on LDL metabolism.

### 1.6.2 Blood pressure and vascular function

Evidence from randomized human clinical trials demonstrates that fish oil supplementation can decrease blood pressure. A meta-analysis of such trials published prior to 1993, showed a dose-response effect of EPA and DHA on blood pressure is evident and that this dose effect appears to be more pronounced with DHA than with EPA (Morris et al., 1993). Mori et al. (1999) showed that 6 weeks of DHA (4g/d) but not EPA supplementation significantly decreased ambulatory 24-hr blood pressure (-5.8/-3.3 mmHg) and daytime (awake) blood pressure (-3.5/-2.0 mmHg) in overweight, mildly hyper-cholesterolemic subjects. In a hypertensive rat model, McLennan et al. (1996) showed that DHA is more efficacious than EPA at suppressing the development of hypertension in spontaneously hypertensive rats (McLennan et al., 1996). Mori and Woodman (2006) summarized five controlled trials examining the effect of EPA supplementation on blood pressure, showing that in all of those studies, doses of EPA ranging from 1.8 – 4g/d taken over 6-16 weeks had no effect on blood pressure.

Studies of omega-3 fatty acid intake levels ranging from 2 – 15g/d have reported significant reductions in blood pressure in both healthy (Bach et al., 1989) and mildly hypertensive individuals (Knapp and Fitzgerald, 1989; Radack et al., 1991; Bonaa et al., 1990). Several studies have also shown that daily fish consumption (3.65g of EPA and DHA) and fish oil supplementation (3.4g of EPA and DHA) in hypertensive individuals already taking pharmacological agents to manage their blood pressure, can provide a significant reduction in blood pressure additional to that achieved with drug therapy alone (Bao et al., 1998; Lungershausen et al., 1994).

The mechanisms by which fish oil or DHA in particular, reduces blood pressure may be related to effects on arterial structure and function. Chin and colleagues (1993) showed that forearm vasoconstriction in response to various vaso-constricting agents was significantly reduced by EPA and DHA ingestion. Similarly, Mori et al. (2000b) reported that supplementation with 4g/d of DHA, but not EPA, by overweight, mildly hyperlipidemic men significantly enhanced forearm vasodilatory mechanisms and restricted the degree of vascular constriction that occurred in response to norepinephrine stimulation. In other studies omega-3 fatty acid supplementation has reduced the relative risk of occlusion in coronary venous bypass grafts (Eritsland et al., 1996) and in

heart transplant patients, has improved endothelium-dependent vasodilation (Fleischhauer et al., 1993). McVeigh and colleagues (1994) have also shown that fish oil supplementation in subjects with diabetes enhanced endothelial nitric oxide production in association with an improvement in pulsatile arterial function (McVeigh et al., 1994). Thus, DHA may improve blood pressure via its direct or indirect effects on the arterial wall. Indirect effects may relate to cardiac or autonomic functions while direct effects may relate to alterations in cell membrane structure due to membrane incorporation of DHA altering fluidity and membrane protein functions or to modification of endothelial and platelet factors such as nitric oxide, thromboxane, prostacyclin or endothelium-derived hyperpolarizing factor (Mori, 2006; Chin et al., 1993a; Chin et al., 1993b; Chin et al., 1994; Mori et al., 2000; Harris et al., 1997).

DHA and EPA may also have beneficial effects on haemodynamics that could be independent of their effects on blood pressure. McVeigh et al. (1994) demonstrated that six weeks of daily supplementation with 4g of fish oil by subjects with type II diabetes significantly improved non-invasive estimates of vascular compliance in both large and small arteries and that these changes occurred in the absence of improved blood pressure, cardiac output or stroke volume. Nestel et al. (2002) also demonstrated that both EPA and DHA, taken as 3g daily for 7 weeks, were equally as effective in increasing systemic arterial compliance in dyslipidemic subjects and, similar to McVeigh et al. (1994), that blood pressure remained unchanged. These findings support the role of EPA and DHA in favourably influencing vascular reactivity and arterial wall characteristics via mechanisms that may be independent to their effect on blood pressure.

It is unclear whether reduced arterial compliance contributes toward the development of CVD (risk factor) or if it develops as a consequence of CVD (Glasser et al., 1997). Several techniques are now available for estimating arterial compliance measures non-invasively with reproducibility similar to that achieved with invasive techniques (Finkelstein et al., 1988; Glasser et al., 1997; McVeigh et al., 1999), making arterial compliance a valuable outcome measure for assessing the potentially varied cardiovascular effects of dietary interventions such as EPA and DHA. McVeigh et al. (1999) showed via multiple regression analysis that an increase in SBP significantly reduced estimates of large artery compliance, while increases in systolic, diastolic and

pulse pressure had no effect on small artery compliance (McVeigh et al., 1999). Compliance changes in large arteries are usually caused by structural modifications such as alteration in elastin and collagen fibres and smooth muscle cell structure thus resulting in alterations to the media thickness: lumen ratio (Hutchins et al., 1996). Ageing is associated with structural changes to large arteries corresponding with a significant reduction in larger artery compliance (Seals, 2003; McVeigh et al., 1999; Glasser et al., 1997). Compliance of small arteries may be more related to functional modifications caused by endothelial and smooth muscle responses to such factors as nitric oxide, norepinephrine and thromboxane. Thus, improvements in arterial compliance in the absence of a change in blood pressure with EPA and DHA may relate to small artery compliance changes caused by mechanisms independent of those utilised to change blood pressure.

### **1.7 Summary of cardiovascular risk factor effects of EPA and DHA**

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EPA and DHA are effective in reducing several CVD risk factors including fasting plasma triglycerides, blood pressure and arterial compliance. Reductions in these risk factors can be observed with doses of EPA and DHA as low as 0.94 – 4.0 g/d in healthy individuals and people with elevated cardiovascular risk factors. Primary prevention and epidemiological studies provide evidence to demonstrate that the reduction in risk factors achieved with dietary EPA and DHA supplementation can contribute towards a significant reduction in CVD mortality, cardiac death, sudden death and myocardial infarction with as little as 30-35g/day of fish, or with fish oil supplementation (Wang et al., 2006).

Both EPA and DHA appear to be equally as effective at reducing fasting plasma triglyceride concentrations but may both result in a significant increase in plasma LDL cholesterol concentrations. While there is no clear difference in the effect of EPA and DHA on LDL and total cholesterol, there is some evidence to suggest DHA may lead to a greater rise in LDL cholesterol than EPA (Mori et al., 2000a). Clear evidence exists to demonstrate that DHA is considerably more effective than EPA at reducing blood pressure (Morris et al., 1993; Mori and Woodman, 2006) and this may be due to enhanced vascular and endothelial effects of DHA (Mori et al., 2000b). This thesis will focus predominantly on the effects of dietary DHA supplementation on CVD risk factors.

### 1.8 Omega-6 versus omega-3 fatty acids

Harris and Von Shacky (2004) recently proposed that the sum of EPA and DHA in red blood cell membranes, which they refer to as the “Omega-3 Index”, is a biomarker for CVD risk. They examined this relationship in several large prospective cohort studies, a randomized clinical trial and secondary intervention studies and concluded that an Omega-3 Index of  $\geq 8\%$  was associated with the highest level of cardiovascular protection while an index of  $\leq 4\%$  was associated with the lowest level of cardiovascular protection. Depending on the baseline Omega-3 Index, a daily intake of approximately 800 mg of EPA and DHA would likely increase the Omega-3 Index to 8% or more (Harris and Von Schacky, 2004). Currently in Australia it is estimated that the mean dietary intake of EPA and DHA is 189 mg/d with the median intake showing that 50% of the population is consuming no more than 30 mg of EPA and DHA daily (Meyer et al., 2003). Thus, for many Australians increasing the daily intake of EPA and DHA to a level sufficient to raise the Omega-3 Index to a level commensurate with reduced risk of CVD would require a significant increase; potentially a 20-fold increase in dietary EPA and DHA intake for most of the population.

An examination of domestic food supplies in 38 countries to determine dietary allowances of omega-3 fatty acids associated with reduced incidence of CVD reported that omega-3 fatty acid intake levels of 0.08% of dietary energy were only associated with a potential protection against mortality from stroke. However, intake levels of 0.34% of dietary energy were associated with a reduction in the risk of mortality and morbidity from all illness models for over 98% of the population. These latter intake levels were associated with cell and tissue very long chain fatty acid content being made up of 60% long-chain omega-3 fatty acids and only 40% long chain omega-6 fatty acids (Hibbeln et al., 2006). Such dietary intake and tissue omega-3 levels correspond with those observed in Japan (Kobayashi et al., 2001; Iso et al., 1989; Okita et al., 1995; Sasaki, et al., 2000) where the dietary intake of omega-3 fatty acids is high and CVD mortality low compared with most other countries (Zhang et al., 1999). Interestingly, Hibbeln et al. (2006) reported that the amount of dietary EPA and DHA that needed to be consumed in order to get tissue EPA and DHA levels as high as 60% of very long chain fatty acids varied by as much as 13-fold among countries depending on the

background dietary omega-6 fatty acid content. Hibblen et al. (2006) estimated that to achieve these tissue levels the quantity of dietary EPA and DHA that would need to be consumed in the average American diet could be reduced by as much as 10% if dietary LA intake could also be significantly reduced.

Omega-3 and omega-6 fatty acids compete for binding sites on membrane phospholipids (Budowski and Crawford, 1985; Harris, 2006; Arterburn et al., 2006; Nestel et al., 1996). Due to this competition, there is a limit to the amount of PUFAs that can be incorporated into membrane phospholipids at a given time. Subsequently, the binding affinity of the acyl-transferases for the different PUFA and the quantity of each PUFA available for incorporation will determine the dietary PUFA composition of phospholipid membranes (Budowski and Crawford, 1985). The amount and ratio of omega-6 and omega-3 fatty acids in the diet will thus directly influence the cellular membrane content of these two families of fatty acids.

It has been shown in studies with rats and monkeys that a significantly greater incorporation of erythrocyte membrane EPA can occur when dietary intake levels of LA are reduced (McMurchie et al., 1990; Garb et al., 1990). In humans Cleland et al. (1992) showed that high dietary consumption of linoleic acid inhibited the amount of dietary EPA that was incorporated into the cell membrane. Hatala et al. (1994) also showed that membrane phospholipid EPA and LA each increased in direct response to exogenous exposure to these fatty acids. However, these increases were associated with a concurrent dose-dependent reduction in the opposite fatty acid, such that increased EPA caused a dose-dependent reduction in phospholipid LA and vice versa. A reduction in the amount of EPA and DHA that can be incorporated into cell membranes could have an impact on the cardiovascular benefits related to a given dietary dose of these fatty acids. These findings therefore provide a basis for reducing dietary LA intake concurrent to increasing dietary EPA as a strategy for ensuring that the cellular uptake and efficacy of EPA is not compromised.

In Australia the mean dietary intake of LA is estimated as being 10.8g/day with individual intakes ranging from 2.8 – 37g/day; the main contributor to dietary LA intake being fats and oils (Meyer et al., 2003; Ollis et al., 1999). In a recent paper discussing the best ways for delivering recommended levels of omega-3 fatty acids in the diet,



Garg and colleagues (2006) concluded that “it is imperative that the delivery and the bioavailability of omega-3 PUFA are optimized” and that to optimize the benefits from omega-3 supplements, such as fish oil, dietary omega-6 fatty acid intake should be as low as possible and omega-3 supplements should be consumed with a background diet rich in monounsaturated fatty acids (Garg et al., 2006). A strategy therefore to increasing the bioavailability of the low quantities of dietary EPA and DHA consumed in the Australian diet and reducing the degree to which dietary omega-3 fatty acid supplementation needs to be increased in the Australian population, is for Australians to replace LA-rich oils and margarines with monounsaturated-rich varieties, to limit dietary LA intake, while increasing dietary omega-3 fatty acid intake.

The competition between omega-3 and omega-6 fatty acids extends beyond their incorporation into cellular membranes. Arachidonic acid, EPA and DHA also compete for a variety of enzymes including phospholipase A<sub>2</sub> for release from phospholipids and eicosanoid-converting enzymes such as cyclooxygenase and lipoxygenase (Harris, 2006).

It is generally agreed that a high intake of omega-6 fatty acids in the diet and subsequently high tissue AA leads to the formation of eicosanoids in response to physiological stressors that are proinflammatory, proaggregative, and vasoconstrictive (Harris et al., 2006; Blok et al., 1996). In contrast when fish consumption is increased and tissue EPA levels are high, the eicosanoids formed are less inflammatory, aggregatory and vasodilatory and the level of the AA derived eicosanoids under the same conditions of physiological stress are reduced (Harris et al., 2006; Lands, 1992).

Arachidonic acid is the predominant substrate for eicosanoid production. Arachidonic acid is released from membrane phospholipids by the phospholipase A<sub>2</sub> (Muller, 1991). Once released from the phospholipid, arachidonic acid can be acted upon by either cyclooxygenase or lipoxygenase to form prostaglandins, prostacyclin and thromboxane A<sub>2</sub> from the former and leukotrienes from the latter (Muller, 1991; Leaf and Weber, 1988). Thromboxane A<sub>2</sub> is formed predominantly in platelets and is responsible for platelet aggregation and arterial vasoconstriction (Lands, 2001). Prostacyclin is formed predominantly in endothelial and vascular smooth muscle cells and inhibits platelet activation and promotes vasodilation (Lands, 1979). The eicosanoids formed by lipoxygenase activation of arachidonic acid include leukotrienes that are produced in

white blood cells. The leukotrienes promote vasoconstriction, leukocyte activation and vascular permeability and are implicated in atherosclerosis development (Muller, 1991; Simopolous, 2002; Lefer, 1986).

It is thought that part of the cardiovascular benefit of omega-3 fatty acids in addition to their reduction in fasting plasma triglycerides and blood pressure and improvement in arterial compliance, comes from their ability to compete with AA for eicosanoid synthesis and as such reduce levels of vasoconstricting, pro-inflammatory and pro-thrombotic eicosanoids. Thus a high omega-6 fatty acid diet may be unfavourable for CVD risk management due to both the adverse effects of AA-derived eicosanoids and the potential inhibition of the cardiovascular efficacy and impact of low levels of EPA and DHA contained in the typical Australian diet.

In 1999, a workshop was held in the USA to establish recommended dietary intakes for omega-6 and omega-3 fatty acids. Nutrition scientists with extensive knowledge on the health benefits of these fatty acids agreed that in conjunction with increasing dietary omega-3 fatty acid consumption it was important that omega-6 fatty acid intake is reduced to decrease the adverse effects of eicosanoids formed from excess arachidonic acid (Simopolous et al., 1999). The workshop recommended two interdependent strategies for reducing excess cellular membrane arachidonic acid content. The first was decreasing the consumption of dietary oils and margarines high in linoleic acid. The second was to increase omega-3 fatty acid intake in the diet. Concurrent undertaking of these strategies, however, was the preferred option.

The rationale behind this recommendation comes under question when studies have shown that reducing dietary LA intake does not necessarily result in a reduction in phospholipid AA (Raatz et al., 2001; Sarkkinen et al., 1994). Thus, reducing dietary LA may not directly reduce the adverse effects of AA derived eicsoanoids. A more practical rationale for the same recommendation may be that reducing dietary LA intake while increasing the dietary consumption of omega-3 fatty acids can enhance the phospholipid incorporation of EPA and DHA for a given dietary dose of these fatty acids due to competition for the Sn-2 position in membrane phospholipids. Greater EPA and DHA may then provide competition with AA for phospholipase A<sub>2</sub> mediated phospholipid

release and the eicosanoid forming enzymes of cyclooxygenase and lipoxygenase, altering the type of eicosanoids formed.

This thesis will examine the effectiveness of replacing omega-6 - rich oil and margarine in the background diet with two different types of oleic acid - rich vegetable cooking oils, one of which is also rich in the plant sourced omega-3 fatty acid ALA as a mechanism for enhancing the uptake and effectiveness of DHA supplementation on CVD risk factors.

### **1.9 Soy Isoflavones as a means for reducing LDL cholesterol**

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Dietary DHA and EPA supplementation can effectively reduce fasting plasma triglyceride concentrations but may result in a significant increase in plasma LDL cholesterol concentrations. In 1999, the US Food and Drug Administration concluded that the rise in LDL cholesterol often observed with fish oil supplementation was a safety concern and this contributed to the FDA not approving a high level health claim based on Significant Scientific Agreement for reduced heart disease and dietary supplementation with EPA and DHA (U.S Food and Drug Administration, 2000).

There is clear evidence demonstrating that increased LDL cholesterol is a strong and well established biomarker of increased CVD risk. Thus any dietary factors which may be able to diminish or reverse the rise in LDL cholesterol observed with EPA and DHA supplementation would be beneficial. One such dietary factor may be soy isoflavones.

### 1.9.1 Introduction to soy protein and isoflavones

In 1995 a landmark meta-analysis reviewing 29 human clinical trials conducted since 1960 revealed that dietary consumption of soy protein was associated with a significant reduction in total cholesterol, LDL cholesterol and fasting plasma triglyceride concentrations. This review concluded that replacement of animal protein with soy protein successfully lowered plasma lipid concentrations and with this reduction was strongly influenced by baseline plasma lipid concentrations (Anderson et al., 1995).

Many subsequent human clinical trials confirmed the cholesterol lowering effects of soy protein using doses ranging from 30-50g/d in moderately hypercholesterolemic men and women and normocholesterolemic men (Wong et al., 1998; Teixeira et al., 2000; Potter et al., 1993; Tonstad et al., 2002; Baum et al., 1998). In 1999, the US Food and Drug Administration approved a health claim about soy protein and reduced risk of heart disease. The claim stated: “Diets low in saturated fat and cholesterol that include 25 grams of soy protein a day may reduce the risk of heart disease” (U.S Food and Drug Administration, 1999b).

While there is considerable scientific evidence to support a lipid lowering effect of soy protein on plasma lipid concentrations in subjects with elevated blood lipids, the degree of cholesterol reduction has been demonstrated to be considerably smaller based on studies published after the Anderson et al. (1995) meta-analysis and it is still unclear which components of soy might be responsible for the cholesterol reduction (Sacks et al., 2006).

Soybeans and soy foods are rich sources of isoflavone phytoestrogens (King and Bignell, 2000; Reinli & Block, 1996). The isoflavones associated with soy proteins are daidzein, genistein and glycitein. These isoflavones can be found in four different chemical forms which include: the aglycone or unconjugated form; the glucoside or conjugated form (daidzin, genistin, and glycitin); acetylglucoside and malonylglucoside forms. Typically, isoflavones contained in soy protein and most soy foods are conjugated to sugars and thus are present in the glucoside form as daidzin and genistin, respectively (Ren et al., 2001; Piskula et al., 1999; Setchell, 2000).

Within the digestive tract, the glycoside form of isoflavones are not readily absorbed and require hydrolysis from the sugar moiety before they can be absorbed and

metabolized (Setchell et al., 2002a). The conjugated form of isoflavones is efficiently hydrolyzed along the entire length of the intestinal tract, by bacterial  $\beta$ -glucosidases releasing the bioactive aglycones, daidzein, genistein and glycitein (Day et al., 1998; Setchell et al., 2002a). These aglycones may be absorbed or further metabolized in the distal intestine and potentially in the colon by the gut microflora to form isoflavone metabolites which include equol and desmethylangolensin produced from daidzein and p-ethylphenol formed from genistein (Rowland et al., 2003). Glycitin has been shown to be metabolically stable. While glycitin is a 6-methoxy analog of daidzin, the aglycone glycitein does not appear to be readily converted to daidzein and as such is not considered a precursor of equol (Setchell et al., 2001; Zhang et al., 1999). The aglycone isoflavones and their metabolites once absorbed from the intestinal tract are transported via the portal venous system to the liver, where they are predominantly conjugated with glucuronic acid (95%) or to a lesser extent may form sulfate conjugates (Day et al., 1998; Lundh, 1995; Rowland et al., 2003). These isoflavone conjugates are then excreted in the urine or in the bile (Ren et al., 2001; Zhang et al., 1999).

The chemical structures of isoflavones, especially the daidzein metabolite equol, are very similar to that of mammalian estrogens (Setchell, 2002b). Clinical and nutritional interest in soy isoflavones increased when it was realized that levels of isoflavones in urine and blood following soy food consumption were well in excess of those of endogenous estrogens (Setchell and Cassidy, 1999; Setchell et al., 1984). It was subsequently suggested that the isoflavone component of soy protein may have hormonal effects and via these effects could potentially be responsible for the cholesterol lowering properties of soy protein (Setchell, 1984).

### **1.9.2 The cardiovascular protection offered by isoflavones**

Anthony and colleagues (1996, 1997) conducted a series of pivotal studies in monkeys which provided early evidence supporting soy isoflavones as the likely component of soy protein responsible for its cardiovascular health benefits. Anthony and colleagues (1996) showed that in monkeys fed diets containing 200g/kg of soy protein, LDL cholesterol was lower and HDL cholesterol was higher compared with monkeys fed the same amount of soy protein without the soy isoflavones (Anthony et al., 1996). In a subsequent study this group also showed that monkeys fed a high level of soy

isoflavones in an atherogenic diet had fewer and smaller atherosclerotic lesions compared to those consuming less or no isoflavones (Anthony et al., 1997).

Numerous human clinical trials following Anthony's studies have demonstrated that soy isoflavones contribute to cholesterol reduction. Sanders et al. (2002) found that in young healthy normolipidemic men and women that daily consumption of soy protein containing 52mg of isoflavones produced a significantly greater increase in HDL cholesterol and Apo A-1 concentrations by 4% and 6%, respectively, than consumption of soy protein containing only 2% isoflavones. In a study conducted in pre-menopausal women, Merz-Demlow et al. (2000) showed that 53g/d of soy protein containing 128.7mg of isoflavones but not lower amounts of isoflavones significantly lowered LDL cholesterol, total:HDL cholesterol ratio and LDL:HDL cholesterol ratio during specific phases of the menstrual cycle. Gardner et al. (2001) reported a significantly greater reduction in LDL cholesterol with 42g/d of soy protein containing 80mg of isoflavones compared with almost no isoflavones. However, neither soy diet reduced LDL cholesterol more than the reduction observed with milk protein. Wangen et al. (2001) showed that 63g/d of soy protein with 132mg/d of isoflavones significantly reduced LDL cholesterol (6.5%) and the ratio of LDL:HDL cholesterol (8.5%), while soy protein with 65mg/d of isoflavones significantly reduced the LDL:HDL cholesterol ratio by 7.7%. Soy protein without isoflavones had no effect on plasma lipid concentrations. This study concluded that "although the effects were small, it is possible that isoflavones may contribute to a lower risk of coronary heart disease if consumed over many years in conjunction with other lipid-lowering strategies" (Wangen et al., 2001).

A study conducted by Crouse et al. (1999) was one of the strongest demonstrations of the importance of soy isoflavones in the cholesterol reduction achieved with soy protein consumption. In that study healthy men and women consuming the National Cholesterol Education Program Step I diet consumed 25g/d of casein containing varying quantities of soy isoflavones. In patients with the highest baseline LDL cholesterol concentrations there was a dose-response effect of increasing the amounts of isoflavones on total and LDL cholesterol with reductions in total and LDL cholesterol ranging from 8-10%. Soy protein with minimal isoflavones had no effect on blood lipids.

In a meta-analysis of 23 randomized controlled trials, Zhan and Ho (2005) reported that daily consumption of soy protein with less than 80mg of isoflavones showed a significant and greater reduction in LDL cholesterol than soy protein with less isoflavones. Zhuo et al. (2004) demonstrated in their review of eight randomized clinical trials that constant soy protein (50g/d) intake with a high isoflavone content (96mg/d) reduced LDL cholesterol by a significantly greater amount than if the soy protein contained minimal isoflavones (6mg/d). The findings from these meta-analyses provide evidence for an LDL cholesterol lowering effect of soy isoflavones achieved either independently or in combination with soy protein.

To determine whether soy isoflavones can reduce LDL cholesterol independent of soy protein studies were conducted that examined the cholesterol lowering effects of supplements with soy isoflavone or red clover extracts. To date, seven human clinical trials have been published which have examined the effect of an isolated isoflavone extract on blood lipids (Simons et al., 2000; Hsu et al., 2001; Hale et al., 2002; Squadrito et al., 2002; Dewell et al., 2002; Hall et al., 2006; Garrido et al., 2006). In three of these (Hall et al., 2006; Simons et al., 2000; Dewell et al., 2002), subjects had moderately elevated (greater than 5.5 mmol/L) total cholesterol concentrations ranging from 5.9 – 6.8 mmol/L while in 4 others (Hall et al., 2006; Simons et al., 2000; Hale et al., 2002; Squadrito et al., 2002) subjects had mildly elevated (greater than 3.5 mmol/L) LDL cholesterol concentrations ranging from 3.6 -3.9 mmol/L. The dose of isoflavones examined ranged from 50-150mg/day supplemented over 2 weeks – 6 months. To date no study examining the effect of isolated soy isoflavone supplements on blood lipid concentrations has been conducted in men with either normal or elevated blood lipids.

In all of the soy isoflavone supplementation studies no statistically significant reduction in total or LDL cholesterol or an increase in HDL cholesterol was observed. While these results may suggest that soy isoflavones do not alter blood lipid concentrations independently, most of the studies had some limitations in their design which may have impacted on their findings. In four of the studies a quantitative compositional analysis of the background diet was not undertaken and thus changes in background dietary macro- and micro-nutrient intake and the impact that any such changes may have had on blood lipids was not determined (Simons et al., 2000; Hale et al., 2002; Squadrito et al., 2002; Garrido et al., 2006). In one study the duration of isoflavone supplementation was only two weeks which may not have been long enough to detect a significant

change in plasma lipid concentrations. In that same study there was no measurement of either urine or plasma isoflavone concentrations and thus no quantitative assessment of subject compliance to the isoflavone supplements (Hale et al., 2000). Similarly, in the study by Dewell et al. (2002) in which subjects consumed an isoflavone supplement providing 150mg/d of isoflavones for 6 months, subject compliance to the isoflavone supplement was not assessed.

The absence of changes in blood lipids with dietary soy isoflavone supplementation could be interpreted to mean that soy isoflavones do not influence blood lipids independent of soy protein. However, there are some additional explanations that should be examined. Firstly, it is difficult to determine what the appropriate size of a study population should be to detect a statistically significant change in plasma lipids with soy isoflavone supplementation, when it is unclear what contribution isoflavones make to the cholesterol reduction observed with soy protein. Thus, it may be that studies using isolated soy isoflavone supplements have not been appropriately powered to detect a statistically significant change in plasma lipids when soy protein is not concurrently consumed. Second and somewhat related, is the issue of between-subject variability in gut microflora and the subsequent variation in isoflavone metabolite concentrations especially those of equol which has been implicated as playing an important role in the cholesterol lowering effects of isoflavones (Setchell et al., 2002b; Meyer et al., 2004). Equol has been shown to have a significantly greater affinity for estrogen receptor binding than the aglycone isoflavones and as such is likely to have a stronger estrogen or antiestrogen effect depending on the background hormonal milieu, than the isoflavones. While early clinical trials have indicated that equol production is associated with greater improvements in blood lipids in response to the dietary consumption of soy protein containing isoflavones (Lydeking-Olsen et al., 2004; Meyer et al., 2004), other studies have not supported this finding (Greany et al., 2004; Hall et al., 2006). It is estimated that approximately 20 - 35% of the population are able to produce equol from the dietary consumption of daidzein (Setchell et al., 2006). In the studies which examined the lipid effects of isolated soy supplements the percentage of equol producers was not assessed and it is therefore unclear if this may have been an influencing factor in these studies.



It has been suggested that a high dietary carbohydrate intake which can increase intestinal fermentation can enhance gut microflora metabolism of isoflavone aglycones to increase equol formation and potentially enhance the cholesterol lowering effect of dietary isoflavone consumption (Setchell and Cassidy, 1999). This suggestion is supported by two dietary intervention trials (Slavin et al., 1998; Lampe et al., 1998) conducted in women, one of which reported a significant association between equol excretion and a higher intake of dietary fiber and carbohydrate (Lampe et al., 1998) and the other which reported that women who were classified as equol excreters consumed a significantly higher amount of dietary carbohydrate and dietary fibre (both soluble and insoluble) compared with women who did not excrete equol (Slavin et al., 1998). The findings from these studies suggest, that isolated isoflavones may be more efficacious at improving blood lipids when consumed in a food matrix rich in carbohydrate and fibre potentially via promoting the production of equol.

A recent study (Hall et al., 2006) has examined the effect of consuming two soy-isoflavone enriched cereal bars (providing 50mg of isoflavones/day) on markers of lipid and glucose metabolism in postmenopausal women with mildly elevated total and LDL cholesterol concentrations. After 8 weeks, total, LDL and HDL cholesterol as well as fasting plasma triglyceride concentrations were unchanged. In that study only 33 of 117 women (28.2%) were classified as equol producers and the effect of dietary isoflavone consumption was not different between equol producers and non-producers. It was concluded by the authors of the study that amongst other reasons, that although consumed in a food matrix, the dose of 50mg/day of isoflavones may not have been sufficient to cause a statistically significant improvement in blood lipids in the presence of a low dietary content of soy protein.

*In vivo* and *in vitro* studies have definitely provided evidence to support an independent effect of soy isoflavones on cholesterol metabolism and other cardiovascular end points. Human, animal and *in vitro* studies have demonstrated that soy isoflavones can increase LDL receptor mRNA, increase LDL receptor binding and hepatic cellular uptake and degradation (Baum et al., 1998; Borradaile et al., 2002, Kirk et al., 1998). Genistein and daidzein may also reduce apolipoprotein B secretion (Borradaile et al., 2002). *In vitro* studies provide evidence to suggest that soy isoflavones may favourably alter LDL metabolism via their impact on sterol regulatory element binding proteins (SREBPs)

and peroxisome-proliferator activated receptors (PPAR) (Mezei et al., 2003; Ricketts et al., 2005).

In addition to blood lipid changes soy isoflavones have also been shown to have a positive effect on endothelial function. Human clinical trials have reported an improvement in arterial vascular compliance with soy isoflavone consumption (Nestel et al., 1997; Teede et al., 2003). This outcome may reflect the beneficial effect of genistein on endothelial dependent vasodilation which has been demonstrated in human and animal *in vitro* cell studies (Squadrito et al., 2002; Molsiri et al., 2004; Li et al., 2004; Khemapech et al., 2003). In monkeys, dietary consumption of isoflavones within an atherogenic diet dose-dependently reduced the degree of atherosclerosis developed (Anthony et al., 1997), while *in vitro* studies have shown that both genistein and daidzein can cause concentration-dependent relaxation of rat aorta (Mishra et al., 2000) suggesting both isoflavones are capable of influencing endothelial function.

There is clear mechanistic evidence to support an independent effect of soy isoflavones on cardiovascular disease risk factors; however it is unclear how to optimize these effects through the dietary consumption of these functional ingredients. While there is strong evidence to support that soy protein combined with soy isoflavones is effective in improving blood lipid parameters in individuals with elevated cholesterol concentrations, it is still unclear whether soy isoflavones consumed independently of soy protein or in combination with other functional ingredients can favourably influence cardiovascular parameters when provided within a food matrix.

### **1.10 Complementary effects of soy isoflavones and DHA**

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To date there have been few studies conducted that have examined the combined health benefits of soy isoflavones and marine sourced omega-3 fatty acids. Two studies have been conducted in animal models which examined the combination of soy protein and fish oil. A pilot study in ovariectomized mice showed that the combination of soy protein (containing some isoflavones) with fish oil significantly decreased cholesterol levels and reduced bone mineral density loss compared with a diet enriched with corn oil and casein protein (Fernandes et al., 2003). This study concluded that fish oil may be acting in similar ways to oestrogen to improve bone mineral density as fish oil prevented the activation of RANKL (the receptor activator of nuclear factor - $\kappa$ B (NF-

κB) ligand) induced by dietary protein in ovariectomized mice and lowered pro-inflammatory cytokines shown to increase osteoclast activation and reduce bone mineral density. Estrogen has also been shown to suppress pro-inflammatory cytokines (Pacifi et al., 1991; Ralston et al., 1990; Shanker et al., 1994) and to reduce blood cholesterol concentrations, thus Fernandes and co-workers (2003) hypothesised that both soy and fish oil were potentially demonstrating oestrogenic effects. This is supported by studies conducted in human breast cancer cells which demonstrated that genistein and EPA acted in synergy in a time- and dose-dependent manner to inhibit cancer cell growth and to initiate cell cycle arrest and apoptosis (Nakagawa et al., 2000). These effects were observed in both oestrogen receptor -positive and -negative cells, suggesting that a synergy between genistein and EPA may involve estrogenic and non-estrogenic pathways. While an interaction between DHA and daidzein has not yet been tested, synergy between genistein and EPA suggests it may be possible.

A second study (Mitsugi et al., 2004) conducted in rats and tumor-bearing mice, supported the *in vitro* results of Nakagawa and colleagues (2000) and demonstrated that a diet enriched with soy protein and fish oil successfully prevented gastrointestinal toxicity associated with methotrexate anti-tumor treatment. In contrast to these findings the control group in this study consuming a casein enriched diet all died following methotrexate treatment while 90% of animals on the soy protein and fish oil diet survived.

Findings from studies examining the independent effects of soy isoflavones and EPA or DHA suggest some potential synergies between these functional ingredients could be expected. In a recent study conducted in postmenopausal women, daily supplementation with 100mg of soy isoflavones significantly reduced platelet thromboxane A<sub>2</sub> receptor density and that this change was negatively correlated with serum isoflavone concentrations (Garrido et al., 2006). Thromboxane A<sub>2</sub> is a cyclooxygenase metabolite of AA and is a potent stimulator of platelet adhesion and thrombosis formation and endothelial smooth muscle vasoconstriction (Narumiya et al 1999). Dietary supplementation with the omega-3 fatty acids EPA and DHA has been shown to reduce levels of thromboxane A<sub>2</sub> formation from AA and in human platelet *in vitro* studies, phospholipid enrichment with EPA and DHA has resulted in a significant inhibition of thromboxane A<sub>2</sub> and prostaglandin H<sub>2</sub> receptor binding and function (von

Schacky et al., 1985; Swann et al., 1990). Combining these effects of soy isoflavones and EPA/DHA suggests an enhanced reduction in thromboxane A<sub>2</sub> driven processes beyond what might be achieved with these ingredients independently.

Complementary benefits achieved with DHA and isoflavones may also be achieved via their effects on peroxisome proliferator – activated receptors (PPARs). The PPAR receptors are associated with a variety of metabolic processes including adipogenesis, glucose control, expression of lipoprotein lipase (LPL), cholesterol efflux, fatty acid metabolism and cholesterol metabolism (Barbier et al., 2002; Fajas et al., 2001; Lee et al., 2003; Kersten et al., 2000). In a human cell culture study Mezei et al. (2003) reported that an isoflavone-containing soy extract doubled PPAR-directed gene expression, while genistein or daidzein alone doubled PPAR $\alpha$  and PPAR $\gamma$  directed gene expression, with the latter as high as 400%.

Somewhat similar to soy isoflavones, long chain omega-3 fatty acids have been shown to activate all three isoforms of PPAR - PPAR $\gamma$ , PPAR $\alpha$  and PPAR $\delta$  (Li et al., 2005; Dreyer et al., 1993; Kliewer et al., 1997; Forman et al., 1997; Diep et al., 2000; Gottlicher et al., 1993; Willumsen et al., 1992). Several studies have demonstrated that omega-3 fatty acids activate PPAR $\alpha$  levels in hepatic cells (Ren et al., 1997; Kersten et al., 1999; Kliewer et al., 1997; Krey et al., 1997). It is thought that this may be the mechanism by which omega-3 fatty acids increase the transcription of genes encoding enzymes involved in fatty acid oxidation (Ren et al., 1997; Clarke, 2000; Willumsen et al., 1992), potentially leading to the hypotriglyceridemic effect typical of omega-3 fatty acid supplementation (Davidson, 2006). The PPAR $\gamma$  receptor is an integral component of inflammatory control pathways (Ricote et al., 1999; Tononoz and Nagy, 1999). Both isoflavones and omega-3 fatty acids have been shown to influence this receptor and instigate anti-inflammatory responses. Genistein has been shown *in vitro* and *in vivo* to facilitate anti-inflammatory responses via binding and activating PPAR $\gamma$  (Mezei et al., 2003). More specifically, genistein has been shown to inhibit monocyte rolling and adhesion to cytokine-activated endothelial cells via activation of PPAR $\gamma$ , a protective anti-adhesion effect important for reducing atherosclerotic plaque development in response to endothelial flow or shear stress (Chacko et al., 2005). Similarly, incubation of human monocytic cells with DHA has been shown to have a strong anti-

inflammatory response by decreasing interleukin – 6, interleukin - 1 $\beta$  and TNF $\alpha$  gene expression as a consequence of increasing the activation of PPAR $\gamma$  (Zhao et al., 2005). The effect of soy isoflavones and very long chain omega-3 fatty acids on PPAR receptors appears to be very similar with complementary changes in CVD risk being observed.

To date no *in vitro*, *in vivo* or human clinical studies have examined the potential health benefits of combining the dietary consumption of soy isoflavones with EPA or DHA. *In vivo* and *in vitro* studies examining the independent effects of these functional ingredients or clinical trials examining the combination of soy isoflavones provided with soy protein in combination with fish oil suggest that there is great potential for these functional ingredients to have a synergistic beneficial effect on cardiovascular risk factors if consumed concurrently in the diet.

### **1.11 Thesis Aims**

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This thesis aimed to assess if the effectiveness of DHA on CVD risk factors can be enhanced by:

1. Changing dietary fatty acid composition to potentially improve the bioavailability of supplemented DHA,
2. Combining DHA with a functional ingredient that has potentially complementary effects on similar or different CVD risk factors

Specifically this thesis will:

1. Examine the influence of other fatty acids on the ability of omega-3 fatty acids to modify blood lipids, arterial compliance and blood pressure,
2. Examine the effect of combining omega-3 fatty acids with soy isoflavones on fasting blood lipids, lipoprotein composition, blood pressure and arterial compliance.

## **2 Monounsaturated oils and fish oil (MOFO) study**

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## 2.1 Introduction

The long chain omega-3 fatty acid content of the current Australian diet is estimated to be on average 0.189g/d with approximately 50% of the population consuming no more than 0.03g/d of EPA and DHA. In contrast the dietary intake of omega-6 fatty acids by the average Australian adult is 10.9g/day of which 10.8g is LA, predominantly provided by dietary fats and oils (Meyer et al., 2003). Currently, doses of fish oil or long chain omega-3 fatty acids used to reduce CVD risk factors are relatively high, with doses of 3-5.6g/d of EPA and DHA used to treat hypertriglyceridemia and to significantly reduce blood pressure in people with hypertension (Breslow, 2006). Due to competition between omega-6 and omega-3 fatty acids for uptake into cellular phospholipids and enzymes involved in metabolic pathways and cellular responses to physiological stress (Harris et al., 2006), a high omega-6 fatty acid diet may significantly impact the dose of omega-3 fatty acids required to reach levels necessary to achieve cardiovascular benefits (Ackabas & Decklebaum, 2006). Furthermore, increased doses of omega-3 fatty acids are associated with an increased propensity for causing side effects. Breslow (2006) claims that doses of omega-3 fatty acids greater than 3g/d can lead to gastrointestinal upset, clinical bleeding, fishy aftertaste, worsening hyperglycemia, and increased LDL cholesterol all of which are more pronounced than at lower doses (Kris-Etherton et al., 2003; Harris et al., 1997c).

One approach for increasing the effectiveness of a given dose of omega-3 fatty acids in providing CVD benefits is to reduce omega-6 fatty acid intake concurrent with dietary omega-3 fatty acid supplementation. This may be achieved by altering the type of oil and margarine consumed in the typical Australian diet while simultaneously supplementing that diet with EPA and/or DHA rich oil.

Cleland et al. (1992) conducted a study in which subjects consumed an EPA-rich fish oil supplement providing 1.6g of EPA and 0.32g of DHA against a background diet containing an oil and margarine either rich in the omega-6 LA or low in LA (olive oil). In the diet with low LA intake neutrophil membrane EPA uptake was significantly higher compared with the high LA diet and a 20% reduction in the AA: EPA ratio was achieved providing great potential for reducing the production of pro-inflammatory eicosanoids. Their study demonstrated that relatively unobtrusive modifications to the

diet can have a favourable influence on the dietary fatty acid profile and can successfully improve the omega-6: omega-3 fatty acid ratio of cell membranes when combined with a relatively low dose of EPA-rich fish oil supplementation.

Hwang et al. (1997) conducted two experiments that assessed the blood lipid effect of different omega-6: omega-3 fatty acid ratios achieved by either altering the intake levels of dietary omega-6 fatty acids while keeping a constant dose of omega-3 fatty acids or by changing the dose of fish oil supplementation while keeping dietary omega-6 fatty acid intake constant. They found that the reduction in plasma triglycerides with fish oil was not affected by altering dietary omega-6 fatty acid intake amounts. However, a weakness of that study was the very high doses of fish oil used, ranging from 4.8 – 12g/d of DHA and EPA. Such high doses of DHA and EPA produced dietary omega-6: omega-3 fatty acid ratios of 2.9:1, 2.1:1 and 1.25:1 which are 5-6-fold lower than the omega-6: omega-3 fatty acid ratio of current western diets (Simopolous, 2006). Subsequently their conclusions - that the total amount of omega-3 fatty acids in the diet, rather than the dietary ratio of omega-6: omega-3 fatty acids is important for modifying certain cardiovascular disease risk factors - requires further examination using lower doses of omega-3 supplementation and more realistic dietary omega-6: omega-3 fatty acid ratios.

Under conditions of high dietary LA, conversion of dietary ALA to EPA and DHA can be reduced by up to 40%, resulting in net reductions in EPA and DHA accumulation of 70% (Arteburn et al., 2006, Emken et al., 1994). Furthermore, the uptake of ALA into phospholipids is also reduced (Goyens and Mensink, 2006). Conversely, when dietary LA is reduced, ALA and endogenously generated EPA uptake into phospholipids can be promoted (Goyens and Mensink, 2006; Lasserre et al., 1985), leading to an increased level of total omega-3 fatty acids in cellular pools and reducing the omega-6 fatty acid content of phospholipids. Thus, in addition to reducing dietary omega-6 fatty acids through increased intake of monounsaturated-fatty acid rich oils and margarines, dietary omega-3 intake may also be increased through consumption of a low-LA oil and margarine that is also rich in ALA, such as canola.

It is unclear whether DHA incorporation from supplements or the diet is readily influenced by the background dietary omega-6 fatty acid content. DHA is incorporated



more predominantly in cellular phospholipids and stable lipid fractions in plasma while EPA is distributed more evenly between neutral lipids (sterol esters and triglycerides) and phospholipids (Vidgren et al., 1997; Zuijdgeest-van Leeuman et al., 1999; Subbaiah et al., 1993). Thus DHA uptake into cellular stores appears to be relatively more stable than EPA and thus less likely to be altered by background dietary fatty acid compositions, especially a high omega-6 fatty acid diet. It is known that plasma phospholipid DHA concentrations increase in a dose-dependent and saturable manner (Arteburn et al., 2006). However, it is still unclear what influence background dietary omega-6 fatty acid intake has on the cellular uptake and effectiveness of dietary DHA supplementation to improve CVD risk factors. To date, there have been no studies that have examined the effect of altering dietary omega-6 fatty acid intake on the uptake of DHA from DHA-rich oil supplements into phospholipid stores. Furthermore, it is unclear if reducing dietary LA while supplementing the diet with DHA will influence the effect of DHA on cardiovascular risk factors.

This study aimed to determine whether the consumption of vegetable oils and margarines, rich in oleic acid (sunola and canola sourced) in place of an omega-6- rich oil and margarine in the background diet, concurrent with daily DHA-rich oil supplementation can enhance the cellular uptake and effectiveness of DHA to improve CVD risk factors compared with the independent effect of supplementing a typical Australian diet with double the dose of DHA.

## 2.2 Study hypothesis

The primary hypothesis of this study is that in individuals with mildly elevated plasma triglyceride concentrations, consumption of monounsaturated vegetable oils (sunola and canola) as a means of reducing dietary omega-6 fatty acid intake, compared with omega-6-rich vegetable oil (safflower), will enable greater incorporation of omega-3 fatty acids from fish oil supplements into plasma and erythrocyte membrane phospholipids.

Specifically, it is hypothesised that:

- Supplementing the diet with DHA-rich oil will increase levels of EPA and DHA in plasma and in erythrocyte membranes in a dose-dependent manner,
- Dietary consumption of either canola or sunola oil and margarine will result in greater DHA-rich oil-induced increases of phospholipid EPA and DHA than dietary consumption of safflower oil,
- Dietary consumption of canola oil and margarine will result in greater DHA-rich oil induced increased in phospholipid EPA and DHA as a consequence of the ALA content of canola.

It is also hypothesised that omega-3 fatty acids from DHA rich oil will result in:

- A significant and dose-dependent reduction in fasting plasma triglyceride concentrations,
- A significant improvement in HDL cholesterol, heart rate, blood pressure and estimated arterial compliance,
- A significant increase in LDL cholesterol.

And that concurrent consumption of dietary monounsaturated-rich canola and sunola oil with 4g/d of DHA-rich oil supplementation will:

- Improve fasting plasma triglyceride concentrations, heart rate, blood pressure and estimated arterial compliance more favourably than consumption of dietary safflower oil with 4g/d of DHA-rich oil supplementation,
- Improve fasting plasma triglyceride concentrations, heart rate, blood pressure and estimated arterial compliance by an amount similar to that achieved with 8g/d of DHA-rich oil supplementation.

## **2.3 Methods**

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### **2.3.1 Subjects**

Male and female subjects who were forty years of age or older were recruited by newspaper, television or radio announcement. Eligibility for study participation was assessed using fasting plasma triglyceride concentrations determined from a finger-prick blood sample using an Accutrend GCT (Boehringer Mannheim, Germany), responses to a health and lifestyle questionnaire and 3-day records of daily oil and margarine consumption. The exclusion criteria were fasting plasma triglyceride concentrations below 1.5 mmol/L, use of lipid lowering medications, daily omega-3 supplementation, regular use of nonsteroidal antiinflammatory medication, a diet low in oil or margarine (<30g /day) and/or daily consumption of more than 5 standard alcoholic drinks.

The study was approved by the Human Ethics Committee of the University of Wollongong (HE98/252). Informed consent forms were signed by all subjects before the start of the study.

### **2.3.2 Study design and protocol**

This study followed a double-blind, parallel design. Subjects were assigned to one of four six-week interventions based on fasting plasma triglyceride levels, body mass index (BMI), age and gender (**Table 2.1**). The interventions consisted of: 4g/d of DHA-rich fish oil (27.3 % DHA and 6.9 % EPA) with 30g/d of one of the following oils and margarines: a) canola b) sunola or c) safflower or d) 8g/d of FO with 30g/d of safflower oil and margarine - (intervention groups: Can(4g), Sun(4g) saff(4g) and saff(8g) respectively). The study design was such that the only dietary changes associated with the intervention were a replacement of subject's current oil and margarine with either safflower, canola or sunola and the addition of fish oil supplements to the diet. All other aspects of subjects' diets were to remain unchanged. Subjects were encouraged to maintain their usual diets, levels of physical activity and exercise habits.

Table 2.1 Blocking characteristics of the four intervention groups<sup>1</sup>

Please see print copy for table 2.1
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1. Mean (range)
2. Fasting plasma triglyceride concentrations determined from a finger-prick blood sample using an Accutrend GCT (Boehringer Mannheim, Germany)

Study participants visited the clinic following an overnight fast, on two consecutive mornings, at both the commencement of the study and after six weeks. Height was measured at the first clinic appointment. Weight, supine blood pressure and arterial compliance were measured at all clinic visits. Fasted blood (20mL) was also collected at every clinic appointment by venipuncture into evacuated tubes containing EDTA. Subjects were provided with fish oil capsules, oil and margarine and instructions about their consumption at the second of the two initial clinic appointments. Additionally, subjects were instructed to maintain their normal diets and current levels of physical activity throughout the study. Arterial compliance and blood pressure were re-assessed after twelve weeks (six weeks after the completion of the intervention) in forty seven subjects to determine whether any seasonal changes had occurred in these parameters.

The number of fish oil capsules supplied to subjects was in excess of the required amount. Capsules remaining at the end of six weeks were returned and counted, serving as a measure of subject compliance with the fish oil intervention. Additional measures of dietary compliance included unannounced telephone calls made fortnightly during the six weeks and the daily recording by subjects of their oil, margarine and fish oil capsule intake. Assessment of plasma and erythrocyte membrane fatty acid compositions was also used as a measure of subject compliance to the fish oil capsules.

### 2.3.3 Food and supplements

The Hi DHA Tuna Oil supplement (Clover Corporation, Victoria Australia) was taken as either two or four 1g capsules twice daily providing a total of 1.1g of DHA and 0.28g of EPA or 2.2g of DHA and 0.55g of EPA as triglycerides, respectively (**Table 2.2**). The subjects were provided with margarine for use as a spread and oil for use in

cooking. Subjects were required to substitute their current dietary oils and margarines with one of three oils and one of three margarines - canola, sunola or safflower (**Table 2.3**). All vegetable oils and margarines were supplied by Meadow Lea Foods Ltd. (Sydney, Australia). Both the subjects and study coordinator were blinded to the type of oil and margarine provided. To blind the study coordinator and subjects, all interventions were colour coded by the study coordinator's supervisors such that pink represented Can(4g), green represented Sun(4g), yellow represented saff(4g) and blue represented saff(8g). All vegetable oil bottles and margarine and fish oil containers were labelled by these colours – pink, green, yellow or blue. The labelling on all fish oil containers provided daily dose details which were not shown to the study coordinator. After all laboratory analyses had been completed and the data checked for consistency, the study coordinator was unblinded and informed of the intervention associated with each colour. Subjects were then unblinded when they received letters informing them of their individual study results.

Table 2.2 Fatty acid profile of Hi-DHA Tuna Oil<sup>1-2</sup>

Please see print copy for table 2.2						
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1. Each capsule contained 1000mg of tuna oil and fatty acid analyses is based on 95% triglycerides.
2. Data is reproduced from a Certificate of Analysis for Hi-DHA Tuna Oil 1000mg Caps with 10 mg Vit. E. Batch number 61108. (Clover Corporation Limited, Altona North, Victoria, Australia, 2000).

Table 2.3 Fatty acid content of dietary oils and margarines<sup>1</sup>

	safflower		canola		sunola	
	Oil	Margarine	Oil	Margarine	Oil	Margarine
	<i>High Linoleic Acid</i>		<i>High Oleic acid</i>			
Linoleic Acid	77	44	20	17	8	12
Alpha-linoleic acid	0	3	10	8	0	3
Oleic acid	14	31	63	58	85	65
Saturated fat	9	22	7	17	7	20

1. Values represent percent of total fat.

### 2.3.4 Arterial compliance instrumentation

Arterial compliance was assessed non-invasively using a HDI/ *PulseWave*™ Model CR-2000 CardioVascular Profiling Instrument (Hypertension Diagnostics Inc. Eagan, Minnesota, USA). With this instrument, radial artery blood pressure waveforms are obtained using a piezoelectric-based, acoustic sensor placed in direct contact with the skin, over the right radial artery. Systolic and diastolic blood pressure data are derived from changes in the inflation of a blood pressure cuff on the left upper arm as detected by an oscillometric blood pressure module incorporated into the instrument. The waveforms are calibrated to the oscillometrically-determined systolic and diastolic cuff pressure values. A pulse contour analysis is performed on a collection of radial artery blood pressure waveforms collected over 30 seconds. Calculations describing diastolic pressure contours during the diastolic decay portion of the cardiac cycle are based on a modified Windkessel model that incorporates two assessments of compliance – capacitive which resides in the large arteries (large artery compliance) and reflective compliance of the smaller vessels (small artery compliance).

In a study population of 115 subjects McVeigh et al. (1999) compared non-invasive estimates of large and small artery compliance using the HDI/ *PulseWave*™ Model CR-2000 CardioVascular Profiling Instrument with invasive measurements of arterial compliance. They reported that the relationship between estimated arterial compliance and increasing age was consistent and predictable with both invasive and non-invasive measures made from the brachial and radial arteries, respectively (McVeigh et al., 1999). Comparison of the slope of the regression lines for age and estimates of systemic vascular resistance (large artery compliance) were not significantly different between invasive and non invasive measures. A significant difference was found for linear regression slopes of small artery compliance, such that while the direction of the slope and trend were the same, a steeper decline in compliance with age was observed with the non invasive assessment. Despite this one difference all trends and relationships observed in small and large artery compliance and systemic vascular resistance over the age group of the study population were clearly apparent with either the invasive or non-invasive techniques.

There are many indirect methods used to estimate arterial compliance. These can include pulse wave velocity, pulse pressure, pulse contour analysis and Fourier

transformation and can provide estimates of global compliance, compartmental compliance or large or small artery compliance (Glasser et al., 1997). While there is currently no “gold standard” methodology for the non-invasive assessment of arterial compliance (Glasser et al., 1997), an advantage of the HDI instrument and method of calculation is that it is able to provide an assessment of capacitive compliance which resides in the large arteries and reflective compliance of the smaller vessels. A range of additional measures including pulse pressure, heart rate, estimated cardiac ejection time, estimate stroke volume, estimated stroke volume index, estimated cardiac output, cardiac index and systemic vascular resistance and total vascular impedance can also be made using the HDI/ *PulseWave*<sup>TM</sup> Model CR-2000 Research CardioVascular Profiling Instrument (Hypetension Diagnostics Inc., Eagan, Minnesota, USA).

### **2.3.5 Methodology for the assessment of arterial compliance**

Subjects were provided with an information sheet explaining the necessary preparation required for measurement of both blood pressure and arterial compliance. Subjects were asked to take the following preparatory steps prior to day one of each clinic visit:

1. allow enough time to get to the clinic without rushing
2. take time to visit the toilet if their bladder felt full
3. avoid smoking and drinking coffee, tea, alcohol, or other drinks containing stimulants (eg., sports drinks) at least 1-hour before the clinic visit
4. avoid any strenuous activity prior to the clinic visit
5. take their regular medications, but avoid taking stimulants such as pseudoephedrine and other medications for colds, flu, or asthma within 1-hour of the clinic visit.

Upon arrival at the clinic subjects were required to lie supine with their right arm relaxed out on the bed beside them at a 45 degree angle from their body with open palm facing the ceiling. A blood pressure cuff was applied to the left arm and a baseline blood pressure reading taken. Three subsequent blood pressure readings were taken at three minute intervals. Subjects were required not to speak and to remain still during all measurements of blood pressure and arterial compliance. In between blood pressure measurements, the right radial pulse was found and a small pen mark made for later reference. The right wrist was then placed in a stabiliser and the arterial compliance sensor attached to the wrist with the sensor tip directly above the marked position of the radial artery pulse. After the third blood pressure reading the sensor was adjusted down



over the artery and tightened very slowly until a reproducible trace appeared on the monitor. At no time was the sensor tightened to a point of discomfort to the subject. Once a reproducible trace was obtained the subject was informed that a blood pressure reading would be taken first followed by a thirty second period during which all waveforms will be recorded. Subjects were reminded to remain still, not talk and to maintain their normal breathing patterns. An arterial compliance reading was then taken. An estimate of systolic blood pressure, diastolic blood pressure, pulse pressure, systemic vascular resistance and total vascular impedance were obtained from each reading. This process was repeated the next morning on the second clinic visit day. The values obtained on the two days of each clinic visit (baseline, 6 and 12 weeks) for each parameter were averaged and compared with readings obtained at other study time points.

Results of preliminary tests indicated that many factors could contribute to variation within an individual; thus extreme attention was given to developing a meticulous procedure that enabled subjects to be studied under consistent conditions. All efforts were made to minimize sources of intervisit variation.

### **2.3.6 Laboratory analysis**

Following blood collection, blood was spun in a centrifuge at 4°C (1000 x g, 10 min) and plasma was harvested. Plasma and unwashed erythrocytes were stored at -80°C for future analysis. HDL cholesterol was separated from fresh plasma by dextran sulphate magnesium chloride precipitation (Warnick et al., 1982) and plasma samples stored at -80°C for subsequent analysis. Plasma total cholesterol, HDL-cholesterol and triglycerides were quantified using a COBAS MIRA Plus automated analyser (Roche Diagnostics Ltd, Rotkreuz, Switzerland) with commercially available kits (Cholesterol CHOD-PAP, Cat # 1489232 Boehringer Mannheim, Germany; Unimate 5 Trig, art 0736791, Roche Diagnostics, Australia). LDL cholesterol levels were estimated from total and HDL cholesterol and triglyceride concentrations according to the Friedewald equation (Friedewald et al., 1972). Non-HDL cholesterol concentrations were calculated by subtracting HDL-cholesterol from total plasma cholesterol.

### **2.3.7 Plasma and erythrocyte membrane analysis**

The fatty acid compositions of plasma and erythrocyte membranes were measured after extraction and direct trans-esterification (Lepage & Roy, 1986), followed by capillary gas-liquid chromatography (Ridges et al., 2001). The Lepage & Roy method of direct trans-esterification is a simple, quick, cost-effective and accurate method of fatty acid extraction. Lepage and Roy (1986) compared their method for the direct methylation of non-esterified fatty acids (NEFA) on five plasma samples, with the Folch extraction method (Folch et al. 1957) and Tserng's specific methylation of NEFA (Tserng et al., 1981). The recovery of both saturated and unsaturated fatty acids with the latter two methods was significantly less than those obtained by the shorter method of Lepage and Roy, the most significant of the differences being in the long-chain unsaturated fatty acid recoveries. The Lepage and Roy method resulted in almost 100% recoveries of all NEFA standards and was completed within one hour.

A recent well controlled validation analysis of plasma and erythrocyte membrane fatty acid content demonstrated that both plasma and erythrocyte membrane DHA, EPA and LA content were strongly correlated with dietary intake data generated via a validated food frequency questionnaire (Sun et al., 2007). Furthermore, significant dose-response relationships were observed between DHA and LA plasma and erythrocyte membrane content and dietary intake. It has been demonstrated in a human clinical dietary intervention trial that serum omega-3 fatty acid concentrations increased much sooner after dietary omega-3 fatty acid supplementation compared with erythrocyte membrane omega-3 fatty acid content (Katan et al., 1997). As the half-life of erythrocytes is 120 days it was proposed that erythrocyte membrane fatty acid composition is a good reflection of dietary fatty acid intake over past months, while plasma fatty acid content reflects dietary fatty acid intake over recent weeks (Katan et al., 1997; Sun et al., 2007). Several human clinical trials have validated significant correlations between dietary polyunsaturated fatty acid intake assessed by food frequency questionnaires and plasma or erythrocyte membrane fatty acid content (Ma et al., 1995; Olsen et al., 1995; Parra et al., 2002; Stanford et al., 1991). These findings substantiate the use of plasma and erythrocyte membrane omega-6 and omega-3 fatty acid content as biomarkers of the dietary intake of these fatty acids.

Subsequently, in this study, plasma and erythrocyte fatty acid composition were measured to assess dietary compliance with the DHA-rich oil supplements and to assess the level of cellular incorporation achieved from the supplementation.

#### **2.3.7.1 Plasma fatty acid extraction procedure**

This procedure began with the addition of 2mL of methanol:toluene (4:1) to 200uL of plasma. The samples were mixed using a vortex and 200uL of acetyl chloride concurrently added. Samples were then placed on a heat block (Thermoline BTC-9090 Dry Block Heater, Thermoline Scientific Equipment, Smithfield, NSW) at 100° C for one hour. Upon cooling, 3mL of 10% K<sub>2</sub>CO<sub>3</sub> was added slowly and the samples vortex-mixed. An additional 200uL of toluene was added and tubes mixed thoroughly. These were spun at 3578 x g at 5° C for eight minutes in a bench-top centrifuge (Hettich Zentrifugen, Rotanta 460 Centrifuge, GMI Inc., Minnesota, USA) and the supernatant removed and placed in a 2.5 mL glass vial for gas chromatography analysis. Samples were frozen at -20° C until later analysis.

#### **2.3.7.2 Red blood cell membrane fatty acid extraction procedure**

A 500uL aliquot of red blood cells (RBC) (harvested from fasting blood samples) were resuspended in a TRIS solution (10mM BIS TRIS, 2mM EDTA Na<sub>2</sub>, pH 7.4) transferred to ultracentrifuge tubes (size 16X76mm, lot # 089619, Nalgen® Brand products, USA). The tubes were filled with additional TRIS solution and left at room temperature for forty minutes, after which the tubes were spun in an ultracentrifuge (Beckman model L-80 OPTIMA, USA) at 315,000 x g for thirty minutes at 4° C and the supernatant removed. The remaining membrane pellet was resuspended in 200uL of distilled water and 150uL transferred to another tube. The one step reaction procedure used for plasma fatty acid extraction was then followed.

#### **2.3.7.3 Flame-ionization capillary gas chromatography**

Plasma and erythrocyte membrane fatty acid methyl esters were analysed by flame-ionization capillary gas chromatography (model GC-17A, Shimadzu) using a 30 m x 0.25 mm internal diameter capillary column (Ridges et al., 2001). Specifically, individual fatty acids were identified by comparison with known fatty acid standards (F.A.M.E Mix C4-C24, SUPELCO, Bellefonte, PA, USA). The temperature program consisted of an initial temperature of 185° C, ramp function of 5° C/minute for 15 min,

maintaining 260° C for 5 min, resulting in a total run time of 20min. Injector and detector temperature were 260° C. The carrier gas was ultra high purity hydrogen (BOC Gases, Sydney, Australia) and the column flow rate was 1.54mL/min. Peak quantification was calculated by area for corrected normalisation.

## **2.4 Statistical analysis**

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Kolmogorov-Smirnov tests were used to assess normality of data. A 2-factor, repeated measures analysis of variance (ANOVA) with type of intervention as the between-subject effect and time as the within-subject effect was conducted to determine significant changes in outcome measures within the four groups over time and whether such changes were varied between the four interventions over time (intervention x time). Where a significant time effect was observed, paired-samples t-tests were undertaken to determine within-group changes. A one-way, between-groups ANOVA with Tukey's post hoc test were used to compare within-group changes in the relevant parameters where a significant interaction x time effect was observed. Correlations between changes in different variables and baseline values or changes in plasma or red blood cell fatty acid composition and changes in blood lipids were determined using linear regression.

A p-value of less than 0.05 was considered statistically significant, while p-values between 0.05 and 0.085 were considered to represent a trend. All data are presented as mean  $\pm$  standard error of the mean (SEM). All data were analysed using SPSS version 11.5 for Windows.

The sample size for this study was based on the number of subjects required to detect a 15% reduction in fasting plasma triglyceride concentrations. The number of subjects required to have a 95% power to detect a 0.15 mmol/L reduction in fasting triglyceride concentrations was calculated to be 14. This calculation assumed a standard deviation of 3.0mmol/L for the estimated reduction in fasting triglyceride concentrations. The data used for this calculation was based on a study using 1.65g/d of DHA supplementation.

## 2.5 Results

Seventy three people were enrolled in the study. Six people chose to withdraw, 3 for personal reasons, 2 due to side effects of the fish oil supplements and 1 due to commencement of lipid lowering medication. Four subjects were excluded from data analysis due to low compliance with the fish oil supplements (as determined by returned capsule count and erythrocyte membrane fatty acid analysis), while another subject was excluded due to a very large increase ( $> 2$  standard deviations from the mean) in the proportion of EPA in both plasma and erythrocyte membranes after 6 weeks. Consequently, data from 63 people were included in the final analyses.

Subject compliance to the fish oil capsules as assessed by measurement of excess capsules remaining after six weeks was very good with compliance being no less than 92% of the prescribed dose in any of the four intervention groups.

Daily records of oil and margarine consumption showed that subjects had complied well in consuming 30g/d of their allocated oil and margarine. Qualitative information (such as: type of meals, food varieties, frequency of meals and eating out, frequency of exercise, types of physical activity) obtained from unannounced telephone calls during the six-week intervention, indicated subjects did not change their diets or exercise habits during the course of the study.

Body weight increased significantly ( $t = -2.17$ ,  $df = 15$ ,  $p = 0.047$ ) and by approximately 1.0 kg with the Can(4g) intervention but this change was not statistically different when compared with the other three interventions in which body weight remained unchanged (**Table 2.4**).

Table 2.4 Body weight at baseline and after six week with the four interventions<sup>1-3</sup>

Groups	0 wks	6 wks	0-6 wks	t-stat	df	P
	<i>kg</i>					
saff(8g)	87.69 $\pm$ 2.45	88.09 $\pm$ 2.45	0.41 $\pm$ 0.38	-1.06	15	0.31
saff(4g)	80.11 $\pm$ 3.94	80.44 $\pm$ 3.88	0.33 $\pm$ 0.35	-0.93	15	0.37
Can(4g)	89.53 $\pm$ 3.34	90.59 $\pm$ 3.54	1.06 $\pm$ 0.49	-2.17	15	0.047
Sun(4g)	90.22 $\pm$ 4.03	90.70 $\pm$ 4.02	0.48 $\pm$ 0.33	-1.46	14	0.17
All groups	86.83 $\pm$ 1.78	87.41 $\pm$ 1.79	0.57 $\pm$ 0.20	-2.91	62	0.005

1. Data are mean  $\pm$  SEM

2. One way between-groups ANOVA showed there was no difference in the change in body weight between the four interventions ( $F = 0.72$ ,  $df = 3$ ,  $p = 0.54$ ).

3. Shading denotes a statistically significant difference (paired t-tests,  $p < 0.05$ )

### 2.5.1 Plasma fatty acids

A significant time effect was observed in plasma DHA ( $F = 568.8$ ,  $df = 59$ ,  $p < 0.001$ ), DPA ( $F = 11.49$ ,  $df = 59$ ,  $p = 0.001$ ) and EPA ( $F = 110.80$ ,  $df = 59$ ,  $p < 0.001$ ) across the four interventions. Plasma DHA ( $p < 0.01$ ), EPA ( $p \leq 0.003$ ) and total omega-3 fatty acids ( $p < 0.001$ ) increased significantly with all four interventions. There was a significant time x intervention effect in plasma DHA with the elevation in DHA after six weeks being greater ( $F = 6.91$ ,  $df = 1$ ,  $p < 0.001$ ) with the 8g/d dose of fish oil compared with the 4g/d dose regardless of the type of dietary oil consumed (**Figure 2.1**). The increase in EPA observed with the four interventions was not affected by the type of oil and margarine consumed or the dose of fish oil supplemented. Plasma DPA decreased significantly ( $-0.11 \pm 0.03$  percent of total fatty acids;  $t = 3.64$ ,  $df = 15$ ,  $p = 0.002$ ) with the saff(4g) intervention but remained unchanged in the other groups (**Figure 2.1**). The reduction in DPA with the saff(4g) intervention was significantly different ( $F = 2.71$ ,  $df = 3$ ,  $p = 0.03$ ) to the absence of change observed in the Can(4g) group. There was a trend toward an increase in ALA with the Can(4g) intervention ( $0.13 \pm 0.07$  percent of total fatty acids;  $t = -1.92$ ,  $df = 15$ ,  $p = 0.07$ ) reflecting the higher ALA content of the canola oil and margarine. However, plasma ALA remained unchanged with the other interventions. Plasma total omega-3 fatty acids increased significantly ( $p < 0.001$ ) after six weeks of each intervention. The increase in total omega-3 fatty acids observed with 8g/d of fish oil was similar to that observed with the Can(4g) intervention but was significantly larger ( $F = 6.00$ ,  $df = 3$ ,  $p = 0.001$ ) than the increases observed with both the Sun(4g) ( $p = 0.012$ ) and saff(4g) ( $p = 0.001$ ) interventions (**Table 2.5**).

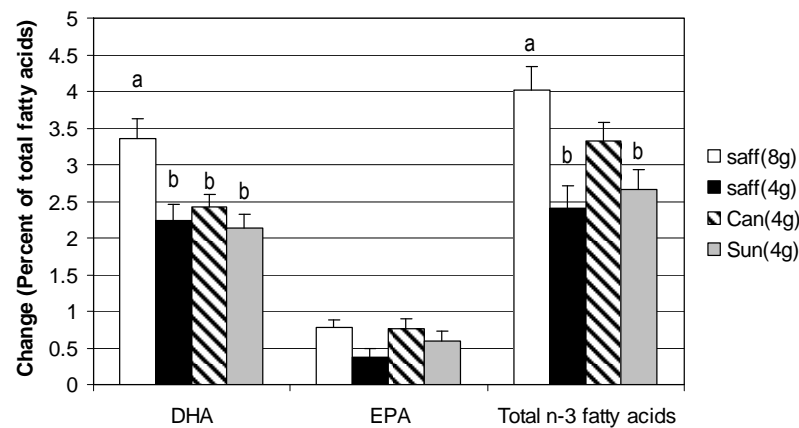


Figure 2.1 Change in plasma EPA and DHA (% of total fatty acids) after 6 weeks of dietary intervention<sup>1,2</sup>

1. Total n-3 = the sum of the change (in the percent of total fatty acids) in EPA (Eicosapentaenoic acid), DPA (Docosapentaenoic acid) and DHA (Docosahexaenoic acid) in plasma.
2. Changes within each fatty acid which do not share the same superscript letter are significantly different (ANOVA, Tukeys post-hoc test,  $p < 0.05$ ).

Table 2.5 Plasma omega-3 fatty acids at baseline and after six weeks of the four interventions<sup>1-3</sup>

	T=0	T=6w	6w-0	t	df	p
<b>DHA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	1.39 ± 0.11	4.75 ± 0.29	3.35 ± 0.27 <sup>a</sup>	-12.57	15	< 0.001
saff(4g)	1.95 ± 0.17	4.18 ± 0.28	2.24 ± 0.22 <sup>b</sup>	-10.38	15	< 0.001
Can(4g)	1.68 ± 0.14	4.10 ± 0.22	2.43 ± 0.16 <sup>b</sup>	-14.96	15	< 0.001
Sun(4g)	1.69 ± 0.12	3.82 ± 0.16	2.13 ± 0.19 <sup>b</sup>	-11.29	14	< 0.001
<b>EPA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	0.61 ± 0.05	1.40 ± 0.12	0.78 ± 0.10	-7.70	15	< 0.001
saff(4g)	0.88 ± 0.08	1.27 ± 0.15	0.38 ± 0.11	-3.52	15	0.003
Can(4g)	0.84 ± 0.12	1.60 ± 0.21	0.76 ± 0.13	-6.02	15	< 0.001
Sun(4g)	0.82 ± 0.07	1.42 ± 0.17	0.59 ± 0.14	-4.22	14	0.001
<b>DPA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	0.41 ± 0.03	0.37 ± 0.02	-0.04 ± 0.03 <sup>ab</sup>	1.40	15	0.18
saff(4g)	0.52 ± 0.03	0.41 ± 0.03	-0.11 ± 0.03 <sup>a</sup>	3.64 <sup>a</sup>	15	0.002
Can(4g)	0.45 ± 0.02	0.45 ± 0.04	0.001 ± 0.03 <sup>b</sup>	-0.05 <sup>b</sup>	15	0.96
Sun(4g)	0.46 ± 0.02	0.41 ± 0.03	-0.04 ± 0.02 <sup>ab</sup>	1.87	14	0.083
<b>ALA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	0.59 ± 0.06	0.50 ± 0.06	-0.09 ± 0.08	1.12	15	0.28
saff(4g)	0.55 ± 0.06	0.45 ± 0.06	-0.11 ± 0.06	1.73	15	0.10
Can(4g)	0.55 ± 0.05	0.68 ± 0.07	0.13 ± 0.07	-1.92	15	0.07
Sun(4g)	0.56 ± 0.04	0.53 ± 0.05	-0.02 ± 0.05	0.44	14	0.67
<b>Total omega-3 fatty acids</b>	<i>Percent of total fatty acids</i>					
saff(8g)	3.00 ± 0.16	7.01 ± 0.39	4.01 ± 0.33 <sup>a</sup>	-12.21	15	< 0.001
saff(4g)	3.91 ± 0.23	6.31 ± 0.44	2.40 ± 0.32 <sup>b</sup>	-7.59	15	< 0.001
Can(4g)	3.51 ± 0.25	6.83 ± 0.42	3.32 ± 0.25 <sup>ab</sup>	-13.29	15	< 0.001
Sun(4g)	3.52 ± 0.18	6.18 ± 0.28	2.66 ± 0.27 <sup>b</sup>	-9.70	14	< 0.001

1. Data are mean ± SEM

2. Shading represents statistically significant changes after six weeks compared to baseline (paired-samples t-tests,  $p < 0.05$ )

3. Different superscripts indicate statistically significant between-group difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

There was a significant time effect in plasma LA ( $F = 31.29$ ,  $df = 59$ ,  $p < 0.001$ ), AA ( $F = 4.18$ ,  $df = 59$ ,  $p = 0.045$ ) and total omega-6 fatty acids ( $F = 19.65$ ,  $df = 59$ ,  $p < 0.001$ ) across the four interventions. As was expected, LA increased significantly in the two groups consuming safflower (saff(8g):  $3.95 \pm 0.55$ ,  $t = -7.19$ ,  $df = 15$ ,  $p < 0.01$ ; saff(4g):  $4.92 \pm 1.22$ ,  $t = -4.02$ ,  $df = 15$ ,  $p = 0.01$ ), however, this did not translate into an increase in AA for either group. Despite the absence of change in AA, plasma total n-6 fatty acids increased significantly (saff(8g):  $3.65 \pm 0.71$ ,  $t = -5.12$ ,  $df = 15$ ,  $p < 0.001$ ; saff(4g):  $4.43 \pm 1.36$ ,  $t = -3.24$ ,  $df = 15$ ,  $p = 0.005$ ) with the two safflower interventions. Both LA and AA and subsequently, plasma total omega-6 fatty acids remained unchanged with the Sun(4g) and Can(4g) interventions (**Table 2.6**). The increase in LA with the two safflower interventions was significantly different ( $F = 11.80$ ,  $df = 3$ ,  $p < 0.001$ ) to the absence of change observed with the Sun(4g) and Can(4g) interventions. Similarly, the increase in plasma total omega-6 fatty acids was significantly higher ( $F = 8.55$ ,  $df = 3$ ,  $p < 0.002$ ) with the saff(8g) intervention compared with Can(4g) and was significantly higher with the saff(4g) intervention compared with both the Sun(4g) ( $p = 0.02$ ) and Can(4g) ( $p < 0.001$ ) interventions.

There was a significant time effect ( $F = 299.05$ ,  $df = 59$ ,  $p < 0.001$ ) in the ratio of plasma total omega-6: omega-3 fatty acids with a significant reduction ( $p < 0.001$ ) occurring in the ratio with all four interventions (**Figure 2.2**). The reduction in the ratio that occurred with the saff(8g) intervention was double ( $F = 11.50$ ,  $df = 3$ ,  $p = 0.003$ ) that observed with saff(4g). The reduction in the omega-6: omega-3 ratio observed with the Can(4g) intervention was also significantly greater ( $p = 0.002$ ) than that seen with the saff(4g) intervention.



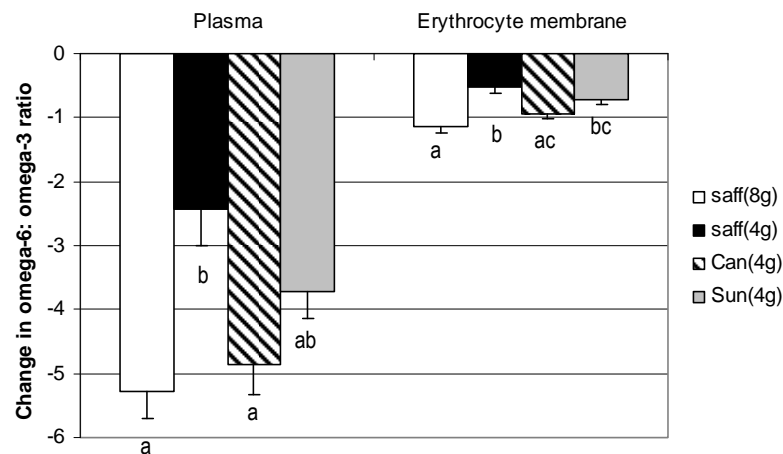


Figure 2.2 Change in the plasma and erythrocyte membrane omega-6: omega-3 fatty acid ratio after six weeks with the four interventions<sup>1</sup>

<sup>1</sup> Changes which do not share the same superscript letter are significantly different (ANOVA, Tukeys post-hoc test,  $p < 0.05$ ).

Table 2.6 Plasma omega-6 fatty acids at baseline and after six weeks of each intervention<sup>1-3</sup>

	T=0	T=6w	6w-0	t	df	p
<b>LA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	24.76 ± 1.08	28.70 ± 1.05	3.95 ± 0.55 <sup>a</sup>	-7.19	15	< 0.001
saff(4g)	25.51 ± 1.02	30.43 ± 1.22	4.92 ± 1.22 <sup>a</sup>	-4.02	15	0.001
Can(4g)	25.96 ± 0.84	25.14 ± 0.71	-0.82 ± 0.69 <sup>b</sup>	1.20	15	0.25
Sun(4g)	24.19 ± 0.77	24.94 ± 0.71	0.75 ± 0.38 <sup>b</sup>	-2.01	14	0.06
<b>AA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	5.46 ± 0.34	5.17 ± 0.24	-0.30 ± 0.08	1.04	15	0.31
saff(4g)	6.15 ± 0.36	5.66 ± 0.38	-0.49 ± 0.29	1.69	15	0.11
Can(4g)	5.59 ± 0.27	5.39 ± 0.25	-0.20 ± 0.16	1.23	15	0.24
Sun(4g)	5.66 ± 0.25	5.61 ± 0.32	-0.05 ± 0.26	0.21	14	0.84
<b>Total omega-6 fatty acids</b>	<i>Percent of total fatty acids</i>					
saff(8g)	30.22 ± 1.15	33.87 ± 0.95	3.65 ± 0.71 <sup>ac</sup>	-5.12	15	< 0.001
saff(4g)	31.66 ± 1.21	36.09 ± 1.32	4.43 ± 1.36 <sup>a</sup>	-3.24	15	0.005
Can(4g)	31.55 ± 0.97	30.53 ± 0.86	-1.02 ± 0.68 <sup>b</sup>	1.49	15	0.157
Sun(4g)	29.84 ± 0.78	30.55 ± 0.68	0.70 ± 0.37 <sup>bc</sup>	-1.87	14	0.082
<b>Omega-6:omega-3 fatty acid ratio</b>	<i>Percent of total fatty acids</i>					
saff(8g)	10.37 ± 0.55	5.09 ± 0.34	-5.28 ± 0.42 <sup>a</sup>	12.43	15	< 0.001
saff(4g)	8.52 ± 0.58	6.07 ± 0.41	-2.44 ± 0.57 <sup>b</sup>	4.30	15	0.001
Can(4g)	9.56 ± 0.67	4.69 ± 0.31	-4.87 ± 0.45 <sup>a</sup>	10.82	15	< 0.001
Sun(4g)	8.84 ± 0.55	5.13 ± 0.33	-3.71 ± 0.42 <sup>ab</sup>	8.87	14	< 0.001

1. Data are mean ± SEM
2. Shading represents statistically significant changes after six weeks compared to baseline (paired-samples t-tests,  $p < 0.05$ )
3. Different superscripts indicate statistically significant between-group difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

A significant time effect was observed in plasma oleic ( $F = 119.55$ ,  $df = 59$ ,  $p < 0.001$ ), palmitic ( $F = 8.057$ ,  $df = 59$ ,  $p = 0.006$ ) and stearic acid ( $F = 15.03$ ,  $df = 59$ ,  $p < 0.001$ ) across the four interventions. Plasma oleic acid decreased significantly ( $p \leq 0.01$ ) with all four interventions (**Table 2.7**). The reductions in oleic acid in the two groups consuming the monounsaturated-rich oils and margarines: Can(4g) [ $-1.53 \pm 0.45$  percent of total fatty acids,  $p = 0.004$ ] and Sun(4g) [ $-1.69 \pm 0.60$  percent of total fatty acids,  $p = 0.01$ ] were significantly smaller than the reductions observed with the two safflower interventions (saff(8g):  $-5.90 \pm 0.77$  percent of total fatty acids;  $p < 0.001$ ; saff(4g):  $-5.05 \pm 0.71$  percent of total fatty acids;  $p \leq 0.003$ ).

Plasma palmitic acid decreased significantly with the saff(4g) ( $T = 7.12$ ,  $df = 15$ ,  $p < 0.001$ ) and Sun(4g) ( $T = 2.49$ ,  $df = 14$ ,  $p = 0.03$ ) interventions and plasma stearic acid decreased significantly with the saff(8g) ( $T = 3.33$ ,  $df = 15$ ,  $p = 0.005$ ) and Sun(4g) ( $T = 4.05$ ,  $df = 14$ ,  $p = 0.001$ ) interventions (**Table 2.7**). These changes however were not significantly different to those observed with the other interventions.

Table 2.7 Plasma monounsaturated and saturated fatty acids at baseline and after six weeks of intervention<sup>1-3</sup>

	T=0	T=6w	6w-0	t	df	p
<b>Oleic Acid</b>	<i>Percent of total fatty acids</i>					
saff(8g)	25.43 ± 0.89	19.53 ± 0.57	-5.90 ± 0.77 <sup>a</sup>	7.62	15	< 0.001
saff(4g)	23.38 ± 0.81	18.33 ± 0.89	-5.05 ± 0.71 <sup>a</sup>	7.12	15	< 0.001
Can(4g)	23.52 ± 0.67	21.99 ± 0.58	-1.53 ± 0.45 <sup>b</sup>	3.40	15	0.004
Sun(4g)	24.00 ± 0.62	22.31 ± 0.58	-1.69 ± 0.60 <sup>b</sup>	2.80	14	0.01
<b>Palmitic Acid</b>	<i>Percent of total fatty acids</i>					
saff(8g)	1.31 ± 0.08	1.13 ± 0.06	-0.18 ± 0.09	2.05	15	0.06
saff(4g)	1.21 ± 0.12	1.14 ± 0.12	-5.05 ± 0.71	7.12	15	< 0.001
Can(4g)	1.23 ± 0.07	1.16 ± 0.09	-0.07 ± 0.05	1.45	15	0.17
Sun(4g)	1.48 ± 0.06	1.28 ± 0.09	-0.20 ± 0.08	2.49	14	0.03
<b>Stearic Acid</b>	<i>Percent of total fatty acids</i>					
saff(8g)	24.87 ± 0.47	23.68 ± 0.40	-1.19 ± 0.36	3.33	15	0.005
saff(4g)	23.77 ± 0.51	23.09 ± 0.49	-0.67 ± 0.59	1.14	15	0.27
Can(4g)	24.41 ± 0.50	24.16 ± 0.51	-0.25 ± 0.33	0.76	15	0.46
Sun(4g)	25.22 ± 0.33	24.16 ± 0.34	-1.05 ± 0.26	4.05	14	0.001

1. Data are mean ± SEM
2. Shading represents statistically significant changes after six weeks compared to baseline (paired-samples t-tests,  $p < 0.05$ );
3. Different superscripts indicate statistically significant between-group difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

### 2.5.2 Erythrocyte membrane fatty acids

A significant time effect was observed in erythrocyte membrane EPA ( $F = 248.55$ ,  $df = 59$ ,  $p < 0.001$ ), DPA ( $F = 162.43$ ,  $df = 59$ ,  $p < 0.001$ ), DHA ( $F = 1023.13$ ,  $df = 59$ ,  $p < 0.001$ ) and total omega-3 fatty acids ( $F = 855.79$ ,  $df = 59$ ,  $p < 0.001$ ). Erythrocyte membrane DHA ( $p < 0.001$ ) and EPA ( $p \leq 0.006$ ) both increased significantly in all four interventions. The increase in DHA was significantly greater ( $F = 15.07$ ,  $df = 3$ ,  $p < 0.001$ ) with the 8g/d dose of fish oil compared with the 4g/d dose regardless of the type of oil and margarine consumed (**Table 2.8**). Erythrocyte membrane EPA increased similarly with the saff(8g) and Can(4g) interventions with the increase observed in both groups being significantly larger ( $F = 12.72$ ,  $df = 3$ ,  $p < 0.001$ ) than that observed with either the saff(4g) ( $p < 0.001$ ) or Sun(4g) ( $p \leq 0.008$ ) interventions. A significant reduction ( $p \leq 0.03$ ) in erythrocyte membrane DPA occurred with all interventions. The reduction observed with the Can(4g) intervention was significantly smaller ( $F = 4.61$ ,  $df = 3$ ,  $p = 0.006$ ) than reductions observed with either the saff(8g) ( $p = 0.004$ ) or saff(4g) ( $p = 0.04$ ) interventions (**Table 2.8**).

Erythrocyte membrane total omega-3 fatty acids (EPA + DPA + DHA) increased significantly with all four interventions. The increase in total omega-3 fatty acids was significantly greater ( $F = 15.69$ ,  $df = 3$ ,  $p < 0.001$ ) with the saff(8g) intervention compared with all three interventions using 4g/d of fish oil. Within these three groups, the increase in erythrocyte membrane total omega-3 fatty acids was significantly larger ( $p = 0.03$ ) with the Can(4g) intervention compared with the saff(4g) intervention (**Figure 2.3**).

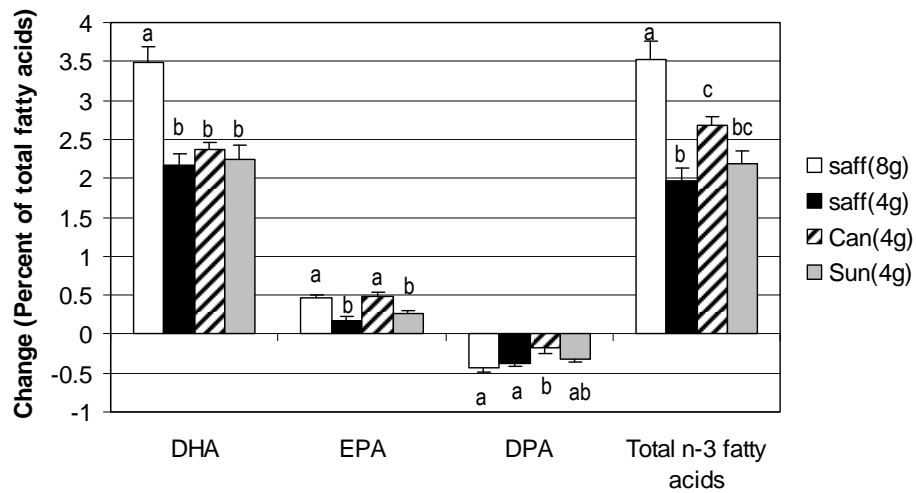


Figure 2.3 Changes in erythrocyte membrane omega-3 fatty acids with the four interventions<sup>1,2</sup>

1. Changes which do not share the same superscript letter are significantly different (ANOVA, Tukeys post-hoc test,  $p < 0.05$ ).
2. Total n-3 = the sum of the change (in the percent of total fatty acids) in EPA (Eicosapentaenoic acid), DPA (Docosapentaenoic acid) and DHA (Docosahexaenoic acid) content of erythrocyte membranes.

Table 2.8 Erythrocyte membrane omega-3 fatty acid content at baseline and after six weeks with the four interventions <sup>1-4</sup>

	T=0	T=6w	6w-0	t	df	p
<b>DHA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	4.44 ± 0.22	7.92 ± 0.25	3.49 ± 0.20 <sup>a</sup>	-17.16	15	< 0.001
saff(4g)	5.25 ± 0.27	7.41 ± 0.23	2.16 ± 0.15 <sup>b</sup>	-14.23	15	< 0.001
Can(4g)	4.89 ± 0.34	7.26 ± 0.34	2.37 ± 0.10 <sup>b</sup>	-23.21	15	< 0.001
Sun(4g)	5.01 ± 0.26	7.27 ± 0.21	2.25 ± 0.17 <sup>b</sup>	-13.35	14	< 0.001
<b>EPA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	0.65 ± 0.04	1.12 ± 0.06	0.47 ± 0.04 <sup>a</sup>	-12.55	15	< 0.001
saff(4g)	0.79 ± 0.05	0.96 ± 0.06	0.17 ± 0.05 <sup>b</sup>	-3.18	15	0.006
Can(4g)	0.73 ± 0.09	1.22 ± 0.10	0.49 ± 0.04 <sup>a</sup>	-11.47	15	< 0.001
Sun(4g)	0.79 ± 0.06	1.05 ± 0.06	0.26 ± 0.04 <sup>b</sup>	-6.46	14	< 0.001
<b>DPA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	2.68 ± 0.09	2.25 ± 0.06	-0.43 ± 0.05 <sup>a</sup>	9.31	15	< 0.001
saff(4g)	2.77 ± 0.07	2.39 ± 0.06	-0.37 ± 0.04 <sup>a</sup>	9.54	15	< 0.001
Can(4g)	2.62 ± 0.11	2.44 ± 0.08	-0.17 ± 0.07 <sup>b</sup>	2.34	15	0.03
Sun(4g)	2.79 ± 0.07	2.46 ± 0.06	-0.33 ± 0.03 <sup>ab</sup>	9.93	14	< 0.001
<b>Total omega-3 fatty acids</b>	<i>Percent of total fatty acids</i>					
saff(8g)	7.77 ± 0.27	11.29 ± 0.32	3.53 ± 0.23 <sup>a</sup>	-15.46	15	< 0.001
saff(4g)	8.80 ± 0.31	10.76 ± 0.30	1.96 ± 0.17 <sup>b</sup>	-11.49	15	< 0.001
Can(4g)	8.24 ± 0.43	10.92 ± 0.45	2.68 ± 0.11 <sup>c</sup>	-24.05	15	< 0.001
Sun(4g)	8.59 ± 0.28	10.77 ± 0.26	2.18 ± 0.18 <sup>bc</sup>	-12.31	14	< 0.001

1. Data are mean ± SEM
2. Shading denotes a statistically significant change from baseline ( $p < 0.05$ , paired t-test)
3. Different superscripts indicate statistically significant difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)
4. Note: ALA omega-3 fatty acid values are not shown because the levels for most subjects were below the detection limit of the method utilized for fatty acid analyses.

Erythrocyte membrane LA increased significantly with the saff(4g) ( $0.86 \pm 0.21$  percent of total fatty acids;  $t = -4.14$ ,  $df = 15$ ,  $p = 0.001$ ) intervention but remained unchanged with saff(8g) ( $-0.05 \pm 0.13$  percent of total fatty acids;  $t = 0.42$ ,  $df = 15$ ,  $p = 0.68$ ). Similar to the reductions seen in plasma, the Sun(4g) ( $t = 5.79$ ,  $df = 14$ ,  $p < 0.001$ ) and Can(4g) ( $t = 0.42$ ,  $df = 15$ ,  $p < 0.001$ ) interventions both resulted in a significant reduction in erythrocyte membrane LA. These reductions in LA were significantly different ( $F = 23.01$ ,  $df = 3$ ,  $p < 0.001$ ) to the increase in LA observed with saff(4g) and the absence of change in LA that occurred with the saff(8g) intervention. Despite the varying changes in LA, erythrocyte membrane AA decreased significantly ( $p < 0.001$ ) with all four interventions. The reduction in AA with saff(8g) was significantly greater ( $F = 3.15$ ,  $df = 3$ ,  $p = 0.03$ ) than the reduction observed with the Sun(4g) intervention (Table 2.9).

Erythrocyte membrane total omega-6 fatty acids increased significantly ( $p < 0.001$ ) with all interventions except Saff(4g) ( $t = 0.88$ ,  $df = 15$ ,  $p = 0.40$ ). The erythrocyte membrane omega-6: omega-3 fatty acid ratio decreased significantly ( $p < 0.001$ ) with all four interventions (**Figure 2.2**). The reduction in the ratio that occurred with the 8g/d dose of fish oil was significantly greater ( $F = 11.50$ ,  $df = 3$ ,  $p < 0.001$ ) than the reduction observed with either the saff(4g) ( $p < 0.001$ ) or Sun(4g) ( $p = 0.003$ ) interventions. The reduction in the omega-6: omega-3 fatty acid ratio observed with the Can(4g) intervention was also significantly greater ( $p = 0.002$ ) than that seen with saff(4g). The Omega-3 Index at baseline averaged 5.63% across the four intervention groups. The Omega-3 Index increased significantly ( $p < 0.001$ ) with all four interventions and after six weeks the Index had increased to 8.55% across the four groups. The increase in the Omega-3 Index was significantly greater ( $F = 17.91$ ,  $df = 3$ ,  $p < 0.001$ ) with the 8g/d dose of fish oil compared with the 4g/d dose regardless of the type of oil and margarine consumed (**Table 2.9**).

Table 2.9 Erythrocyte membrane omega-6 fatty acids, omega-6: omega-3 ratio and Omega-3 Index at baseline and after six weeks of the interventions<sup>1-3</sup>

	T=0	T=6w	6w-0	t	df	p
<b>LA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	9.38 ± 0.31	9.32 ± 0.29	-0.05 ± 0.13 <sup>a</sup>	0.42	15	0.68
saff(4g)	8.90 ± 0.28	9.76 ± 0.34	0.86 ± 0.21 <sup>c</sup>	-4.14	15	0.001
Can(4g)	9.18 ± 0.16	8.35 ± 0.16	-0.83 ± 0.17 <sup>b</sup>	4.91	15	< 0.001
Sun(4g)	8.74 ± 0.16	8.05 ± 0.14	-0.69 ± 0.12 <sup>b</sup>	5.79	14	< 0.001
<b>AA</b>	<i>Percent of total fatty acids</i>					
saff(8g)	14.82 ± 0.21	13.33 ± 0.20	-1.49 ± 0.10 <sup>a</sup>	14.70	15	< 0.001
saff(4g)	14.50 ± 0.27	13.46 ± 0.26	-1.05 ± 0.13 <sup>ab</sup>	8.09	15	< 0.001
Can(4g)	14.63 ± 0.40	13.55 ± 0.35	-1.07 ± 0.18 <sup>ab</sup>	6.01	15	< 0.001
Sun(4g)	14.55 ± 0.30	13.61 ± 0.27	-0.94 ± 0.12 <sup>b</sup>	7.86	14	< 0.001
<b>Total omega-6 fatty acids</b>	<i>Percent of total fatty acids</i>					
saff(8g)	24.20 ± 0.26	22.66 ± 0.27	-1.54 ± 0.12 <sup>a</sup>	12.74	15	< 0.001
saff(4g)	23.40 ± 0.33	23.22 ± 0.33	-0.18 ± 0.21 <sup>b</sup>	0.88	15	0.40
Can(4g)	23.81 ± 0.42	21.90 ± 0.38	-1.90 ± 0.17 <sup>a</sup>	11.02	15	< 0.001
Sun(4g)	22.29 ± 0.32	21.66 ± 0.32	-1.63 ± 0.12 <sup>a</sup>	14.05	14	< 0.001
<b>Omega-6: omega-3 fatty acid ratio</b>	<i>Percent of total fatty acids</i>					
saff(8g)	3.18 ± 0.13	2.04 ± 0.07	-1.14 ± 0.09 <sup>a</sup>	12.42	15	< 0.001
saff(4g)	2.72 ± 0.12	2.19 ± 0.09	-0.53 ± 0.08 <sup>b</sup>	6.93	15	< 0.001
Can(4g)	3.01 ± 0.16	2.06 ± 0.10	-0.95 ± 0.07 <sup>ac</sup>	12.68	15	< 0.001
Sun(4g)	2.76 ± 0.12	2.03 ± 0.07	-0.73 ± 0.07 <sup>bc</sup>	10.40	14	< 0.001
<b>Omega-3 Index</b>	<i>Percent of total fatty acids</i>					
saff(8g)	5.08 ± 0.24	9.04 ± 0.29	3.96 ± 0.23 <sup>a</sup>	-17.55	15	< 0.001
saff(4g)	6.03 ± 0.29	8.37 ± 0.27	2.33 ± 0.16 <sup>b</sup>	-14.22	15	< 0.001
Can(4g)	5.62 ± 0.40	8.48 ± 0.41	2.86 ± 0.09 <sup>b</sup>	-32.25	15	< 0.001
Sun(4g)	5.80 ± 0.29	8.31 ± 0.25	2.52 ± 0.19 <sup>b</sup>	-13.49	14	< 0.001

1. Data are mean ± SEM
2. Shading denotes a statistically significant change from baseline ( $p < 0.05$ , paired t-test)
3. Different superscripts indicate statistically significant difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

Erythrocyte membrane oleic acid decreased significantly ( $t = 9.92$ ,  $df = 15$ ,  $p < 0.001$ ) with the saff(8g), saff(4g) ( $t = 9.06$ ,  $df = 15$ ,  $p < 0.01$ ) and Can(4g) ( $t = 2.81$ ,  $df = 15$ ,  $p = 0.01$ ) interventions. Similar to changes in plasma fatty acids, the reduction in erythrocyte membrane oleic acid in the two safflower groups was significantly greater ( $F = 17.66$ ,  $df = 3$ ,  $p \leq 0.003$ ) than that seen with either the Can(4g) or Sun(4g) interventions (**Table 2.10**).

There was a small but significant reduction ( $t = 2.29$ ,  $df = 15$ ,  $p = 0.04$ ) in erythrocyte membrane palmitic acid with the saff(8g) intervention however, this was the only change observed in erythrocyte membrane saturated fatty acids (palmitic and stearic acids) with any of the interventions (**Table 2.10**).

The Unsaturation Index (UI – the sum of (percent of total fatty acids x number of double bonds) for each fatty acid identified) is an indicator of potential changes in membrane fluidity. The UI increased significantly ( $p < 0.001$ ) with all of the interventions. The increase in the UI was significantly greater ( $F = 8.68$ ,  $df = 3$ ,  $p < 0.001$ ) with the 8g/d dose of fish oil compared with the 4g/d dose regardless of the type of oil and margarine consumed.

Table 2.10 Erythrocyte membrane monounsaturated and saturated fatty acid content at baseline and after six weeks of the interventions<sup>1-3</sup>

	T=0	T=6w	6w-0	t	df	p
<b>Oleic Acid</b>	<i>Percent of total fatty acids</i>					
saff(8g)	13.05 ± 0.23	11.74 ± 0.23	-1.31 ± 0.13 <sup>a</sup>	9.92	15	< 0.001
saff(4g)	12.96 ± 0.20	11.50 ± 0.15	-1.46 ± 0.16 <sup>a</sup>	9.06	15	< 0.001
Can(4g)	13.11 ± 0.17	12.61 ± 0.23	-0.50 ± 0.18 <sup>b</sup>	2.81	15	0.01
Sun(4g)	12.79 ± 0.19	12.76 ± 0.16	-0.04 ± 0.16 <sup>b</sup>	0.24	14	0.82
<b>Palmitic Acid</b>	<i>Percent of total fatty acids</i>					
saff(8g)	0.37 ± 0.02	0.34 ± 0.01	-0.02 ± 0.01	2.29	15	0.04
saff(4g)	0.39 ± 0.02	0.38 ± 0.02	-0.01 ± 0.02	0.69	15	0.50
Can(4g)	0.36 ± 0.01	0.35 ± 0.02	-0.00 ± 0.01	1.25	15	0.23
Sun(4g)	0.39 ± 0.01	0.39 ± 0.02	-0.00 ± 0.02	6.16	14	0.88
<b>Stearic Acid</b>	<i>Percent of total fatty acids</i>					
saff(8g)	22.16 ± 0.20	22.20 ± 0.22	0.04 ± 0.13	-0.28	15	0.78
saff(4g)	22.29 ± 0.17	22.18 ± 0.17	-0.11 ± 0.09	1.24	15	0.23
Can(4g)	22.00 ± 0.21	22.09 ± 0.21	0.09 ± 0.11	-0.83	15	0.42
Sun(4g)	22.21 ± 0.15	22.23 ± 0.19	0.02 ± 0.11	-0.16	14	0.88
<b>Unsaturation Index</b>	<i>Percent of total fatty acids</i>					
saff(8g)	134.35 ± 1.12	148.12 ± 1.47	13.77 ± 1.10 <sup>a</sup>	-12.49	15	< 0.001
saff(4g)	138.04 ± 1.21	146.06 ± 1.08	7.97 ± 0.89 <sup>b</sup>	-8.97	14	< 0.001
Can(4g)	136.07 ± 1.24	145.39 ± 1.55	9.33 ± 0.91 <sup>b</sup>	-10.23	15	< 0.001
Sun(4g)	136.45 ± 0.99	144.42 ± 0.82	8.02 ± 0.79 <sup>b</sup>	-10.10	15	< 0.001

1. Data are mean ± SEM
2. Shading denotes a statistically significant change from baseline ( $p < 0.05$ , paired t-test)
3. Different superscripts indicate statistically significant difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

### 2.5.3 Plasma lipids

A significant time effect ( $F = 22.995$ ,  $p < 0.001$ ) was observed in fasting plasma triglycerides across the four groups. Fasting plasma triglycerides decreased significantly (ranging from 14% - 22%) with all interventions except saff(4g) in which there was a trend toward an 18% reduction ( $t = 1.95$ ,  $df = 14$ ,  $p = 0.07$ ) (**Table 2.11**). A two – factor, repeated measures ANOVA did not show a significant time x intervention ( $F =$



0.878,  $df = 3$ ,  $p = 0.11$ ) effect or between-group effect ( $F = 0.594$ ,  $df = 3$ ,  $p = 0.11$ ) indicating that there were no differences in fasting plasma triglycerides between the four groups at baseline or after 6 weeks of DHA supplementation. Furthermore, the reductions in fasting plasma triglycerides achieved with saff(8g), Can(4g) and Sun(4g) were not significantly different from one another and were not significantly different from the trend toward an 18% reduction observed with saff(4g) ( $F=0.226$ ,  $df = 3$ ,  $p = 0.878$ ).

When the data for all groups was combined ( $n = 63$ ) fasting plasma triglyceride levels dropped 14.6% from 1.99 mmol/L to 1.69 mmol/L ( $t = 4.88$ ,  $df = 62$ ,  $p < 0.001$ ) following six weeks of fish oil supplementation. This overall reduction in triglycerides ( $n=63$ ) correlated negatively with baseline concentrations ( $r = -0.67$ ,  $p < 0.05$ ). When subjects were categorised into low, mid and high tertiles based on baseline triglycerides (**Figure 2.4**), significant reductions of 17% ( $t = 4.36$ ,  $df = 20$ ,  $p < 0.001$ ) and 22% ( $t = 6.30$ ,  $df = 20$ ,  $p < 0.001$ ) respectively, were observed in the two higher tertiles (tertile 2 [1.5 - 2.1 mmol/L], tertile 3 [2.2 – 4.6 mmol/L]) with baseline concentrations greater than 1.5mmol/L irrespective of the dose of fish oil consumed. No change ( $t = -0.96$ ,  $df = 20$ ,  $p = 0.35$ ) in triglycerides was observed in subjects with baseline triglyceride concentrations below 1.5 mmol/L (tertile 1 [0.74 – 1.5 mmol/L]).

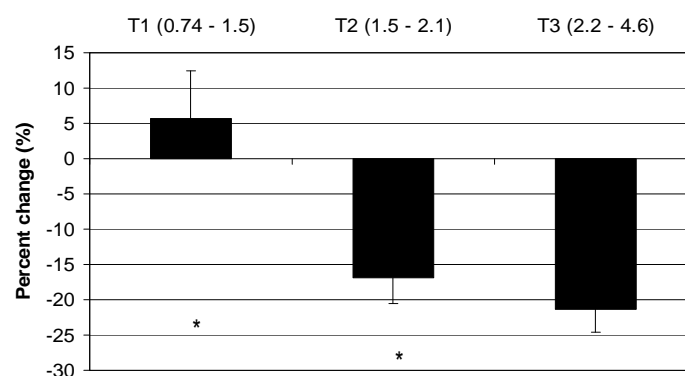


Figure 2.4 Percent change in fasting plasma triglycerides based on tertiles of baseline triglyceride concentrations<sup>1-5</sup>

1. T1 represents subjects with baseline triglyceride concentrations ranging between 0.74 and 1.5 mmol/L
2. T2 represents subjects with baseline triglyceride concentrations ranging between 1.5 and 2.1 mmol/L
3. T3 represents subjects with baseline triglyceride concentrations ranging between 2.2 and 4.6 mmol/L
4.  $n = 21$  in each tertile
5. Asterisk represents a statistically significant change ( $p < 0.05$ )

A significant time and time x intervention effect was observed in total (time:  $F = 12.30$ ,  $p = 0.001$ ; time x intervention:  $F = 12.30$ ,  $df = 59$ ,  $p = 0.001$ ) and LDL cholesterol (time:  $F = 17.30$ ,  $p < 0.001$ ; time x intervention:  $F = 3.26$ ,  $df = 59$ ,  $p = 0.03$ ) concentrations across the four groups. Fasting total plasma cholesterol increased significantly with the saff(8g) (10%,  $t = -4.44$ ,  $df = 15$ ,  $p < 0.001$ ) and Sun(4g) (9%,  $t = -2.44$ ,  $df = 14$ ,  $p = 0.029$ ) interventions. Similarly, LDL cholesterol increased by 16% ( $t = -3.35$ ,  $df = 15$ ,  $p < 0.001$ ) with saff(8g) and by 9% ( $t = -2.95$ ,  $df = 14$ ,  $p = 0.01$ ) with the sun(4g) intervention. A trend toward an increase (8.8%,  $t = -2.02$ ,  $df = 15$ ,  $p = 0.06$ ) in LDL cholesterol was also observed with the saff(4g) intervention. In contrast, LDL cholesterol remained unchanged with the Can(4g) intervention. The increase in total and LDL cholesterol observed with the saff(8g) (total cholesterol,  $p = 0.03$ ; LDL cholesterol,  $p = 0.04$ ) and Sun(4g) (total cholesterol,  $p = 0.05$ , LDL cholesterol  $p = 0.046$ ) interventions were significantly different (total cholesterol:  $F = 3.45$ ,  $df = 3$ ,  $p = 0.02$ ; LDL cholesterol:  $F = 3.26$ ,  $df = 3$ ,  $p = 0.03$ ) to the absence of change seen in these two parameters with the Can(4g) intervention (**Figure 2.5 and 2.6**).

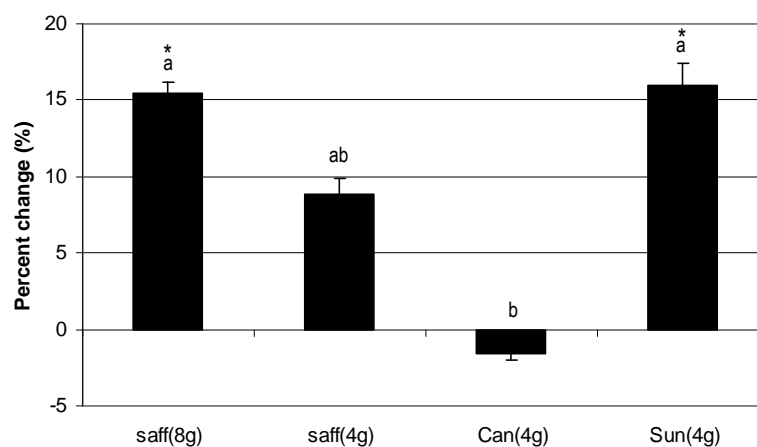


Figure 2.5 Change in LDL cholesterol after six weeks with the four interventions <sup>1-2</sup>

1. Asterisk represents a statistically significant change ( $p < 0.05$ )
2. Different subscripts indicate statistically significant between-group differences ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

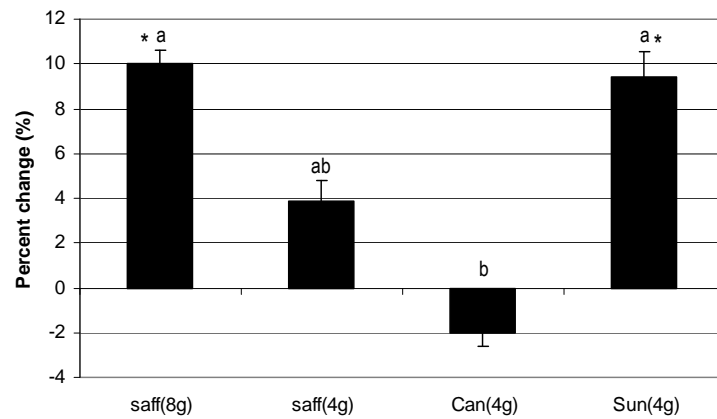


Figure 2.6 Change in total cholesterol after six weeks with the four interventions <sup>1-2</sup>

1. Asterisk represents a statistically significant change ( $p < 0.05$ )
2. Different subscripts indicate statistically significant between-group differences ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

A significant time effect ( $F = 9.04$ ,  $p = 0.004$ ) was observed in HDL cholesterol across the four groups. HDL cholesterol increased significantly with the saff(8g) (10.8%,  $t = -2.13$ ,  $p = 0.05$ ) and Sun(4g) (8.2%,  $t = -2.78$ ,  $df = 14$ ,  $p = 0.02$ ) interventions and remained unchanged with saff(4g) and Can(4g). Despite the changes observed, there was no between-group difference in the increase in HDL cholesterol observed after six weeks of dietary intervention. A significant inverse linear correlation ( $r = -0.404$ ,  $p = 0.001$ ,  $n = 63$ ) was found between baseline HDL cholesterol and triglyceride concentrations when data from all subjects in this study was combined. At the end of the six week intervention this correlation was still present ( $r = -0.505$ ,  $p < 0.001$ ,  $n = 63$ ).

A significant time effect ( $F = 7.63$ ,  $df = 59$ ,  $p = 0.008$ ) was observed in non-HDL cholesterol across the four interventions. Non-HDL cholesterol increased significantly ( $t = -3.32$ ,  $df = 15$ ,  $p = 0.005$ ) with the saff(8g) intervention and there was a trend toward an increase ( $t = -2.08$ ,  $df = 14$ ,  $p = 0.06$ ) with the Sun(4g) intervention. A significant time x intervention effect ( $F = 3.00$ ,  $df = 59$ ,  $p = 0.04$ ) was observed in non-HDL cholesterol with the increase observed with the saff(8g) intervention being significantly different ( $F = 3.00$ ,  $df = 3$ ,  $p = 0.05$ ) to the absence of change in non-HDL cholesterol observed with the Can(4g) intervention (**Table 2.11**).

There was no linear correlation between the reductions observed in plasma palmitic or stearic acid and the increases observed in plasma total and LDL cholesterol with the saff(8g) or Sun(4g) interventions. When the data from the four groups was combined there was a weak but significant linear correlation between the increase in erythrocyte membrane DHA and change in LDL cholesterol ( $r = 0.28$ ,  $n = 63$ ,  $p = 0.02$ ) after six weeks of intervention.

Table 2.11 Fasting plasma lipid concentrations at baseline and after six weeks of dietary supplementation<sup>1-3</sup>

	0 weeks	6 weeks	0-6 wks	t	df	P
<b>Triglycerides</b>	<i>mmol/L</i>					
Saff(8g)	1.97 ± 0.20	1.70 ± 0.13	- 0.28 ± 0.11	2.51	15	0.02
Saff(4g)	1.84 ± 0.26	1.49 ± 0.17	-0.34 ± 0.18	1.95	15	0.07
Can(4g)	2.13 ± 0.20	1.91 ± 0.18	-0.22 ± 0.09	2.48	15	0.03
Sun(4g)	2.03 ± 0.24	1.69 ± 0.19	-0.34 ± 0.09	3.68	14	0.002
<b>Total cholesterol</b>	<i>mmol/L</i>					
saff(8g)	5.09 ± 0.21	5.60 ± 0.22	0.51 ± 0.12 <sup>a</sup>	-4.44	15	<0.001
saff(4g)	5.38 ± 0.17	5.59 ± 0.12	0.21 ± 0.15 <sup>ab</sup>	-1.44	15	0.17
Can(4g)	5.61 ± 0.26	5.50 ± 0.30	-0.11 ± 0.16 <sup>b</sup>	0.71	15	0.49
Sun(4g)	4.98 ± 0.17	5.45 ± 0.24	0.47 ± 0.19 <sup>a</sup>	-2.44	14	0.03
<b>LDL cholesterol</b>	<i>mmol/L</i>					
saff(8g)	3.36 ± 0.25	3.88 ± 0.24	0.52 ± 0.16 <sup>a</sup>	-3.35	15	0.04
saff(4g)	3.40 ± 0.14	3.70 ± 0.13	0.30 ± 0.15 <sup>ab</sup>	-2.02	15	0.06
Can(4g)	3.85 ± 0.28	3.79 ± 0.27	-0.06 ± 0.13 <sup>b</sup>	-0.48	15	0.64
Sun(4g)	3.25 ± 0.13	3.77 ± 0.21	0.52 ± 0.18 <sup>a</sup>	-2.95	14	0.01
<b>HDL cholesterol</b>	<i>mmol/L</i>					
saff(8g)	1.02 ± 0.04	1.13 ± 0.07	0.11 ± 0.03	-2.13	15	0.05
saff(4g)	1.30 ± 0.09	1.34 ± 0.09	0.04 ± 0.04	-1.20	15	0.25
Can(4g)	0.97 ± 0.05	1.01 ± 0.07	0.03 ± 0.05	-0.64	15	0.53
Sun(4g)	0.98 ± 0.06	1.06 ± 0.08	0.08 ± 0.03	-2.78	14	0.02
<b>Non-HDL cholesterol</b>	<i>mmol/L</i>					
saff(8g)	4.07 ± 0.21	4.47 ± 0.21	0.40 ± 0.12 <sup>a</sup>	-3.32	15	0.005
saff(4g)	4.08 ± 0.16	4.25 ± 0.14	0.17 ± 0.14 <sup>ab</sup>	-1.19	15	0.25
Can(4g)	4.64 ± 0.27	4.49 ± 0.28	-0.15 ± 0.14 <sup>b</sup>	1.06	15	0.31
Sun(4g)	4.00 ± 0.19	4.39 ± 0.25	0.40 ± 0.19 <sup>ab</sup>	-2.08	14	0.06

1. Data are mean ± SEM
2. Shading denotes a statistically significant change from baseline ( $p < 0.05$ , paired t-test)
3. Different superscripts indicate statistically significant difference ( $p < 0.05$ , one-way ANOVA with Tukeys post hoc test)

#### **2.5.4 Arterial compliance and blood pressure**

There was a trend toward a time effect ( $F = 3.55$ ,  $df = 59$ ,  $p = 0.06$ ) in large artery compliance across the four interventions, however, large artery compliance remained unchanged with each intervention. Small artery compliance decreased by a small but significant amount ( $t = 2.60$ ,  $df = 14$ ,  $p = 0.02$ ) with the Sun(4g) intervention but remained unchanged with the remaining three interventions. The reduction in small artery compliance with Sun(4g) was not significantly different ( $F = 0.52$ ,  $df = 3$ ,  $p = 0.67$ ) to any of the other interventions. Both small and large artery compliance remained unchanged during the six weeks following the intervention period (data not shown). Systolic and diastolic blood pressure and pulse pressure remained unchanged with all interventions and during the six weeks following the intervention period when subjects returned to their normal diets (**Table 2.12**).

Total vascular impedance, cardiac ejection time, stroke volume, stroke volume index and systemic vascular resistance were unchanged with the four interventions (data not shown).

Table 2.12 Arterial compliance, blood pressure and heart rate at baseline and after six weeks with each intervention<sup>1-2</sup>

	Baseline	6 wks	0 – 6wks	T	df	p
<b>Large artery compliance (ml/mmHg)</b>						
saff(8g)	1.14 ± 0.09	1.23 ± 0.08	0.09 ± 0.09	-0.95	15	0.36
saff(4g)	1.15 ± 0.13	1.26 ± 0.08	0.11 ± 0.07	1.59	15	0.13
Can(4g)	1.17 ± 0.09	1.18 ± 0.07	0.01 ± 0.09	-0.12	15	0.90
Sun(4g)	1.21 ± 0.07	1.29 ± 0.06	0.08 ± 0.18	-1.75	14	0.10
<b>Small artery compliance (ml/mmHg)</b>						
saff(8g)	0.055 ± 0.005	0.054 ± 0.006	-0.0008 ± 0.003	0.24	15	0.82
saff(4g)	0.049 ± 0.007	0.050 ± 0.005	-0.00009 ± 0.0004	-0.02	15	0.98
Can(4g)	0.048 ± 0.004	0.048 ± 0.006	-0.0003 ± 0.003	-0.09	15	0.93
Sun(4g)	0.056 ± 0.004	0.050 ± 0.004	-0.005 ± 0.002	2.60	14	0.02
<b>Systolic blood pressure (mmHg)</b>						
saff(8g)	136.7 ± 3.3	136.3 ± 3.3	-0.34 ± 2.31	0.15	15	0.88
saff(4g)	133.1 ± 4.9	132.3 ± 4.4	-0.88 ± 2.31	0.38	15	0.71
Can(4g)	134.5 ± 4.0	136.9 ± 3.6	2.41 ± 2.93	-0.82	15	0.42
Sun(4g)	138.5 ± 2.7	135.8 ± 3.2	-2.70 ± 2.50	1.08	14	0.23
<b>Diastolic blood pressure (mmHg)</b>						
saff(8g)	77.6 ± 1.6	78.3 ± 5.7	0.75 ± 1.16	-0.65	15	0.53
saff(4g)	77.6 ± 3.1	77.9 ± 3.1	0.31 ± 1.72	-0.18	15	0.86
Can(4g)	79.7 ± 2.3	80.2 ± 2.5	0.50 ± 1.86	-0.27	15	0.79
Sun(4g)	79.8 ± 1.5	78.0 ± 1.6	-1.80 ± 1.26	1.43	14	0.18
<b>Pulse pressure (mmHg)</b>						
saff(8g)	59.1 ± 2.6	58.0 ± 2.6	-1.09 ± 1.86	0.59	15	0.57
saff(4g)	55.5 ± 2.5	54.3 ± 2.1	-1.19 ± 1.26	0.94	15	0.36
Can(4g)	54.8 ± 2.4	56.7 ± 2.1	1.91 ± 2.09	-0.91	15	0.38
Sun(4g)	58.7 ± 2.2	57.8 ± 2.3	-0.90 ± 1.93	0.47	14	0.65
<b>Heart rate (beats/min)</b>						
saff(8g)	63.22 ± 1.69	64.41 ± 2.47	1.19 ± 1.76	-0.67	15	0.51
saff(4g)	68.25 ± 2.62	67.00 ± 2.20	-1.25 ± 1.50	-0.83	15	0.42
Can(4g)	70.56 ± 2.08	70.03 ± 2.14	-0.53 ± 1.71	0.31	15	0.76
Sun(4g)	63.37 ± 1.97	64.23 ± 1.97	-2.13 ± 1.77	1.21	14	0.25

1. Data are mean ± SEM
2. Shading represents statistically significant changes after six weeks compared to baseline (paired-samples t-tests,  $p < 0.05$ )

## 2.6 Discussion

This study aimed to test whether modifying the type of oil and margarine in the diet as a means for reducing dietary LA intake in combination with dietary supplementation with DHA could increase the bioavailability of DHA and improve the effect of DHA on CVD risk factors.

### 2.6.1 Fatty acids

Two doses of DHA-rich oil supplementation were utilised in this study – 8g/d and 4g/d providing 2.2 and 1.1g/d of DHA respectively. Plasma and erythrocyte membrane fatty acid compositions are established markers of dietary fatty acid intake (Glatz et al 1989; Arab, 2003; Parra et al., 2002; Kuriki et al., 2003; Frost Anderson et al., 1996). Both plasma and erythrocyte membrane DHA increased dose dependently in response to daily fish oil supplementation. In plasma phospholipids DHA increased 2.4-fold with 2.2g/d of DHA and 1.35-fold with 1.1g/d of DHA. In erythrocyte membranes the proportion of DHA increased 0.4-fold with 4g/d and 0.8-fold with 8g/d of DHA-rich fish oil. These increases are very similar to those observed by Davidson et al. (1997). They reported a 1.7-fold increase in plasma DHA following six weeks of daily supplementation with 1.25g of DHA and a 2.2-fold increase in DHA following daily supplementation with 2.5g of DHA. Arterburn et al. (2006) conducted a cross-study, meta-regression analysis on plasma phospholipid DHA content using data from 12 studies in which doses of DHA supplementation ranged from 0.2 – 6 g/d of DHA consumed over 1 – 6 months. Their analysis showed that plasma DHA concentrations increase in a dose-dependent and saturable manner (Arterburn et al., 2006). The results from the current study support these meta-regression findings. Several studies using similar doses (range: 0.7 – 3.0 g/d) of DHA-rich oil supplementation to those used in this study have also reported similar increases in plasma (range: 0.7 – 2.5 – fold increase) (Theobald et al., 2004; Sanders and Hinds, 1992; Conquer and Holub, 1996; Conquer and Holub, 1998; Rambjor et al., 1996) and erythrocyte membrane DHA (range: 0.2 – 0.6 – fold increase) (Theobald et al., 2004; Sanders and Hinds, 1992).

The observed increase in DHA in plasma and erythrocyte membrane phospholipids was not influenced by the type of dietary oil and margarine consumed. To date no studies have been conducted which have examined the effect of DHA-rich fish oil supplementation on DHA uptake into plasma and cellular phospholipids under conditions of altered dietary LA intake or reduced dietary omega-6 intake. In a study by Brady et al. (2004) in which subjects consumed an EPA-rich fish oil (1.47g EPA, 1.00g DHA) with either a moderate (15g/d of total omega-6 fatty acids) or high (26g/d of total omega-6 fatty acids) LA background diet, platelet membrane DHA increased significantly irrespective of dietary LA intake. However, DHA increased by only 42% on the moderate omega-6 fatty acid diet and by 75% on the high LA diet. A significant time x treatment interaction was observed suggesting greater DHA uptake was achieved with the high LA background diet. Thus based on the findings by Brady et al. (2004) and of this study, it would appear that the incorporation of dietary DHA into membrane phospholipids may not be enhanced by reducing dietary LA intake.

Plasma EPA increased significantly in all four groups and similarly, irrespective of daily fish oil dose or type of oil and margarine consumed. In erythrocyte membranes EPA increased by a similar amount with the saff(8g) and Can(4g) interventions and this increase was significantly greater than the increase in EPA observed with the saff(4g) and Sun(4g) interventions. While the EPA content of the fish oil used in this study was low relative to the DHA content (providing only 0.28g and 0.55g of EPA with the 4g/d and 8g/d doses, respectively), it was predicted that in the two groups consuming the monounsaturated oils and margarines that phospholipid incorporation of EPA from the fish oil supplements would be enhanced as a consequence of their lower LA content. Human and animal studies have reported a suppressed uptake of EPA from fish oil supplementation when dietary LA intake is high or an enhanced EPA uptake when dietary LA is reduced (Garg et al., 1988a; McMurchie et al., 1990; Cleland et al., 1992; James et al., 1991). While the increase in erythrocyte membrane EPA content was enhanced with the Can(4g) intervention compared with saff(4g), this was not the case with Sun(4g) despite similarly low LA content. This finding suggests that the low LA content of canola was not responsible for enhancing dietary EPA uptake into cellular phospholipid pools.



Plasma LA increased by 4 and 5 percent of total fatty acids with the saff(8g) and saff(4g) interventions, respectively, while plasma LA content did not change with either the Sun(4g) or Can(4g) interventions. Erythrocyte membrane LA content in contrast to plasma decreased significantly in the Can(4g) and Sun(4g) groups and increased significantly with the saff(4g) intervention. These results demonstrate that replacing LA rich oil and margarine with canola or sunola is an effective strategy for reducing dietary LA intake and for reducing the long term uptake of LA into cellular membrane phospholipid pools.

Erythrocyte membrane LA increased significantly with the saff(4g) intervention however it remained unchanged following the same daily intake level of safflower oil and margarine in the saff(8g) intervention. This finding suggests that if dietary intake levels of EPA are high enough, EPA will be preferentially taken up into cellular membranes despite dietary LA intake also being high. In the Australian population which has a high dietary LA relative to EPA intake (Meyer et al., 1999), this strategy may be one way to increase cellular EPA content, however such levels of fish oil supplementation as those used in this study (8g/d of fish oil consumed as eight one gram capsules) are unlikely to be achieved or sustained by the majority of the Australian population. A more successful strategy may be to combine a dietary source of EPA and DHA such as fish oil supplementation consumed at a lower dose with regular dietary consumption of canola oil and margarine. This combination as demonstrated in the Can(4g) intervention was equally as successful as double the daily dose of fish oil at increasing erythrocyte membrane EPA content and increasing the total omega-3 fatty acid content of membrane phospholipids. A similar pattern was observed with plasma EPA although statistical significance was not achieved.

The increase in EPA with the Can(4g) intervention is likely to be a result of the ALA content of the canola oil and margarine and elongation of ALA to EPA. Arterburn et al. (2006) reviewed studies (Burge et al., 2003; Burge et al., 2002a; Emken et al., 1994; Hussein et al., 2005; Burge et al., 2002b) that have examined the fractional conversion of dietary ALA to EPA and concluded that for men between 0.3 and 8% of LNA is converted to EPA while for women up to 21% of LNA is converted to EPA. Numerous dietary clinical trials (Mantzioris et al., 1994; Finnegan et al., 2003; Freese and Mutanen, 1997; Harper et al., 2006; Sinclair et al., 1999; Adam et al., 1986; Allman et

al., 1995; Goyens et al., 2006; Weaver et al., 1990; Wilkinson et al., 2005) have shown that over an extended period (ranging from 2 weeks to 6 months) of dietary ALA supplementation the small amount of ALA converted to EPA can add up to a significant increase in plasma or membrane phospholipid EPA content when ALA is provided in the diet as food or supplements based on linseeds, linseed oil or canola oil and margarine. The inclusion of 25g/d of Canola oil and margarine in the diet during the MOFO study would have provided approximately 2.25g/d of ALA. Assuming a conversion of approximately 10% of ALA to EPA, Canola oil and margarine may have provided additional EPA for incorporation into phospholipid pools equivalent to almost 0.23g/d of supplemented EPA.

Kinetic and compartmental modelling studies have demonstrated that less than 1% of ALA is likely to be elongated to DHA, at least under conditions of high dietary LA intake (Emkin et al., 1994; Palowsky et al., 2003; Goyens and Mensink, 2005). Dietary studies demonstrating an increase in EPA with dietary ALA supplementation reported no change in plasma or membrane phospholipid DHA (Freese and Mutanen, 1997; Finnegan et al., 2003; Harper et al., 2006; Mantzioris et al., 1994; Sinclair et al., 1999; Allman et al., 1995). These findings explain why phospholipid DHA, did not increase significantly more with the Can(4g) intervention despite the greater increase in EPA, compared with the Sun(4g) and saff(4g) interventions.

In other dietary intervention trials where EPA increased with ALA dietary supplementation, the intermediary very long chain omega-3 fatty acid - DPA increased significantly, suggesting some elongation of dietary ALA to DPA (Freese and Mutanen, 1997; Finnegan et al., 2003; Harper et al., 2006; Allman et al., 1995). In this study, plasma DPA decreased significantly with the saff(4g) intervention, while erythrocyte membrane DPA decreased significantly with all of the interventions. The reduction in erythrocyte membrane DPA with the Can(4g) intervention was significantly less than the reduction observed with either the saff(4g) or saff(8g) interventions. This may be explained by a potential increase in DPA caused by the elongation of ALA supplied by the canola oil and margarine partially counteracting the reduction in DPA resulting from the DHA-rich fish oil supplements.

Many other studies using doses of DHA supplementation similar to those used in this study have reported similar reductions in plasma or serum DPA (Agren et al., 1996; Maki et al., 2005; Geppert et al., 2006; Grimsgaard et al., 1997; Conquer and Holub, 1996; Conquer and Holub, 1998) or erythrocyte membrane DPA (Sanders et al., 2006). Not all studies have reported this reduction, with some reporting no change in plasma or serum DPA following DHA supplementation (Buckley et al., 2004; Nestel et al., 2002; Mori et al., 2000c). It is unclear why DHA supplementation may result in a reduction in plasma and membrane phospholipid DPA. It is established that accumulation of DHA in plasma and phospholipids can lead to the retroconversion of DHA to DPA and EPA (Brossard et al., 1996). An increase in DHA can also lead to a significant reduction in the elongation of DPA to DHA (Pawlosky et al., 2003). It is possible therefore that a reduction in DPA in response to increased DHA in the circulation and in membrane phospholipids may be caused by reduced elongation of DPA coupled with increased conversion of DPA to EPA and/or suppressed elongation of EPA to DPA via a controlled inhibitory feed-back path from DHA (Brossard et al., 1996; Conquer and Holub, 1996; Agren et al., 1997). These proposals while potentially feasible require further investigation and validation as does the clinical significance of a reduction in plasma and erythrocyte DPA in response to DHA supplementation.

All interventions used in this study irrespective of the type of oil and margarine consumed or dose of fish oil were successful at increasing the Omega-3 Index above 8% which is associated with the greatest degree of cardioprotection. Geppert et al. (2005) reported similar increases from 4.8 to 8.4% in the Omega-3 Index following 8 weeks of daily supplementation with 0.94g of DHA in healthy vegetarian adults (Geppert et al., 2005). The greater increase in the Index with 8g/d of fish oil compared with 4g/d suggests that it may be the total amount of dietary omega-3 fatty acids that has the greatest positive effect on the Omega-3 Index rather than alterations in dietary omega-6: omega-3 fatty acid ratio.

Erythrocyte membrane AA decreased significantly with all four interventions in this study while plasma AA remained unchanged. In the majority of studies using similar doses of DHA supplementation plasma or serum AA decreased significantly (Buckley et al., 2004; Davidson et al., 1997; Geppert et al., 2006; Grimsgaard et al., 1997; Nelson et al., 1997; Hamazaki et al., 1996; Conquer and Holub, 1996; Sanders and Hinds,

1992; Mori et al., 2000c and Conquer and Holub, 1998), however, the results are not consistent with some studies reporting no change in plasma AA (Maki et al., 2005; Rambjor et al., 1996).

Earlier studies suggested that limited amounts of EPA are utilised in the formation of eicosanoids suggesting eicosanoids derived from EPA may be in low concentrations with little clinical importance (Kivits & Hugteren, 1988; Harris, 2006). Eicosanoids derived from AA constitute the majority of eicosanoids present in the body at any given time, suggesting that the availability of AA as the predominant substrate is the most important factor influencing eicosanoid biosynthesis (Garg et al., 1990). As it is well established that eicosanoids derived from AA have potent vasoconstrictive, aggregatory and inflammatory capabilities (Simopoulos et al., 2006), a reduction in phospholipid AA such as the reduction observed in erythrocyte membrane composition in this study, can be interpreted as a potentially beneficial marker for reduced cardiovascular disease risk.

Arachidonic acid is made available for phospholipid incorporation from predominantly two pathways – by direct dietary consumption (dietary sources can include white and red meat and dairy products) (Simopolous, 1991; Sinclair and Mann, 1996; Meyer et al., 1999) and via the elongation and desaturation of LA (Arterburn et al., 2006). A reduction in AA with DHA supplementation is likely a result of direct competition for incorporation into the Sn-2 position in membrane phospholipids as DHA, EPA and ALA, as well as AA all compete for this position (Arterburn et al., 2006). However, it may also be due to reduced  $\Delta 6$  or  $\Delta 5$  desaturase activity caused by a possible inhibitory feed-back mechanism by DHA (Grimsgaard et al., 1997; Garg et al., 1988b; Garg et al., 1988c). Garg et al. (1990) demonstrated that the efficacy of DHA in reducing  $\Delta 6$  desaturase activity and thus reducing AA levels is related to the dietary LA to saturated fatty acid ratio. This factor, could explain why not all studies using DHA supplementation report a reduction in phospholipid AA.

Plasma LA content increased significantly with safflower oil and margarine consumption in this study, however plasma AA concentrations did not change. This corresponds with the results of other dietary intervention studies in which plasma AA did not change despite significant reductions in dietary LA intake (Raatz et al., 2001;

Sarkkinen et al., 1994). In erythrocyte membrane phospholipids, AA decreased significantly with all of the interventions, while the change in LA was more varied. These findings suggest that this dietary biomarker is more influenced by changes in EPA and DHA intake provided by the fish oil supplementation than to changes in dietary LA content. It could also be proposed from this finding that strategies to reduce excess cellular AA content may be more successful if aimed at increasing dietary EPA and DHA intake rather than only reducing dietary LA content.

Both the plasma and erythrocyte membrane omega-6: omega-3 fatty acid ratio decreased significantly with the four interventions in this study. The saff(8g) intervention produced a significantly greater reduction in the ratio than the saff(4g) (erythrocyte membrane and plasma) or Sun(4g) interventions (plasma only). In contrast the Can(4g) intervention produced a similar reduction (59% decrease) in the ratio to the higher 8g/d dose of fish oil. Conquer and Holub (1998) reported a 60% reduction in the serum omega-6: omega-3 fatty acid ratio with daily supplementation of 0.75g of DHA and a 67% reduction following 1.5g of DHA per day. These reductions in the ratio are similar to those observed in this study with the Can(4g) and saff(8g) interventions. The similar reduction in the omega-6: omega-3 fatty acid ratio with these two interventions but not Sun(4g) suggests that in the context of trying to reduce dietary omega-6 fatty acid intake the total amount of dietary omega-3 fatty acids remains the most important factor in reducing the omega-6: omega-3 fatty acid ratio.

A reduction in the omega-6: omega-3 fatty acid ratio has been implicated as being beneficial for reducing inflammation (Harris, 2006; Simopolous, 2006). As inflammation is developing as a potential risk factor for cardiovascular disease due to its influence in the initiation of atherosclerosis (Ross, 1999), a reduction in the omega-6: omega-3 fatty acid ratio in plasma and erythrocyte membranes is a positive finding. However, the specific clinical relevance of a reduction in the omega-6: omega-3 fatty acid ratio as achieved in the current study is not known.

In a review by Harris et al. (2006) of 13 studies (11 case-control and 2 cohort studies) in which plasma or erythrocyte fatty acid composition was measured in people who had or were destined to have a cardiac event, cellular membrane composition of individual fatty acids did not differ between cardiac cases and control. However, the Omega-3

Index and total omega-3 fatty acids were both significantly lower in cases than in controls while the omega-6: omega-3 fatty acid ratio was significantly higher (by 5.6%) in cases compared with controls. This finding suggests that a reduction in the omega-6: omega-3 fatty acid ratio may be beneficial for potentially reducing CVD risk, however Harris et al. (2006) made the strong case that the difference in total omega-3 fatty acids between cases and control were twice the difference in the omega-6: omega-3 fatty acid ratio suggesting that total omega-3 fatty acids is a much more sensitive marker of CVD risk.

Irrespective of which is the better marker for determining CVD risk, in the current study the combined consumption of canola oil and margarine with daily dietary supplementation with 1.1g/d of DHA was equally as effective as double the dose of fish oil supplementation with safflower oil at reducing the omega-6: omega-3 fatty acid ratio, increasing total plasma and erythrocyte membrane total omega-3 fatty acids and increasing the Omega-3 Index. The absence of a similar finding with the Sun(4g) intervention suggests it is more important to increase overall dietary omega-3 fatty acid content than it is to reduce dietary LA intake for enhancing the bioavailability of a given dose of EPA and DHA in the diet. The combination of canola and DHA-rich fish oil supplementation at the dose used in this study may be a feasible strategy for increasing EPA and DHA intake in the Australian diet to a level that corresponds with a reduction in CVD risk, without the requirement for unsustainably high fish oil supplements.

### 2.6.2 Lipids

In this study mean fasting plasma triglycerides decreased significantly with 8g/d of DHA-rich fish oil (-14%) and with 4g/d of DHA-rich fish oil when dietary canola, sunola and safflower oil were consumed (10%, 17%, 18%, respectively). The majority of studies that have examined the effects of DHA on blood lipids have (Sanders et al., 2006; Maki et al., 2005; Agren et al., 1996; Nestel et al., 2002; Geppert et al., 2006; Davidson et al., 1997; Conquer and Holub, 1996; Buckley et al., 2004) reported similar reductions (-14% to -32%) in plasma triglycerides with 0.94 - 3.0g/d of DHA over 4-15 weeks in healthy and hyperlipidemic subjects (**Table 2.13**).

A dose-response effect in fasting triglyceride concentrations in response to DHA supplementation was not observed in this study. In a meta-analysis of 72 data sets from 60 publications, Harris (1996) reported a significant inverse relationship between the dose of omega-3 fatty acid intake and reduction in fasting triglycerides in both healthy and hyperlipidemic subjects. He concluded that supplementation with EPA and DHA at doses of 7g/d or less reduces fasting triglyceride concentrations in a dose-dependent manner. However, many of the studies in that review used fish oil supplements containing more EPA than DHA. Few studies have examined the dose-response effect of DHA supplementation on triglyceride reduction. Davidson et al. (1997) examined the effect of daily supplementation with 1.25 or 2.5g of DHA on blood lipids in hyperlipidemic subjects and reported similar reductions (-18% and -20%, respectively) in plasma triglycerides with both DHA doses. Conquer and Holub (1998) also examined the effect of two doses of 0.75g and 1.5g/d of DHA on blood lipids in healthy men and women and reported no change in plasma triglycerides after six weeks with either dose of DHA. In the current study the reduction in plasma triglycerides caused by DHA supplementation was inversely correlated with baseline triglyceride concentrations. Significant reductions in triglycerides were observed in subjects with baseline concentrations above but not below 1.5mmol/L. Geppert et al. (2006) also reported a similar significant inverse correlation ( $r = -0.627$ ,  $p < 0.01$ ) between the reduction in plasma triglycerides following daily supplementation with 0.94g of DHA and baseline triglyceride concentrations in their study in vegetarian men and women. In the dose response study by Conquer and Holub (1998), baseline triglyceride concentrations averaged 1.0 and 1.40 mmol/L in the two intervention groups, which coupled with low subject numbers on each intervention may have accounted for the absence of change in plasma triglycerides observed with both doses of DHA in that study.

Comparison of the triglyceride reduction reported in thirteen human clinical trials (including the current study) with doses of DHA supplementation ranging from 0.94 – 6.0g/d shows a negative linear correlation ( $r = -0.525$ ,  $p = 0.045$ ,  $n = 15$ ) between the dose of DHA supplementation and reduction in fasting plasma triglyceride concentrations (**Figure 2.7**).

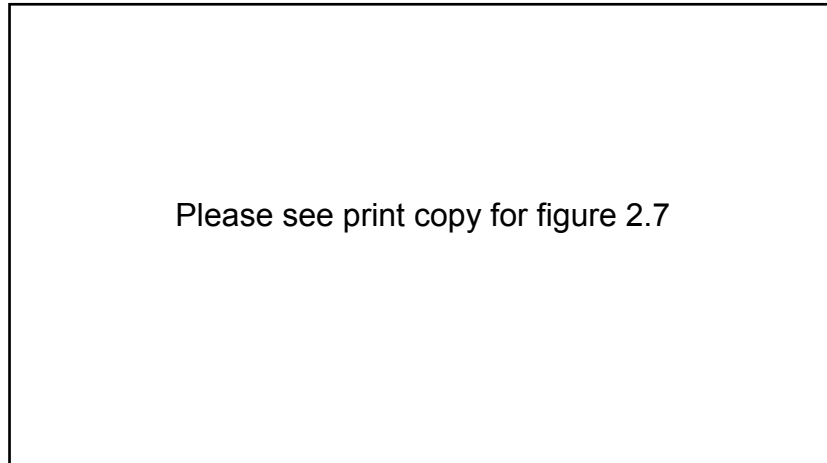


Figure 2.7 Comparison of percent change in fasting plasma triglycerides and dose of DHA supplementation from eleven human clinical trials<sup>1</sup>

1. Data in the graph was obtained from the following studies – Mori et al. (2000c); Grimsgaard et al. (1997); Rambjor et al. (1996); Sanders et al. (2006); Maki et al. (2005); Agren et al. (1996); Nestel et al. (2004); Geppert et al. (2006); Davidson et al. (1997); Conquer and Holub (1996); Buckley et al. (2004).

The correlation, while significant, is not strong. The absence of a dose response effect in this study and that of Davidson et al. (1997) suggests other factors may influence the triglyceride lowering effect of DHA. Baseline triglyceride concentrations of subjects appears to be one such factor, however other elements in the background diet may also impact on the effectiveness of DHA in reducing plasma triglyceride concentrations and other lipid changes.



Table 2.13 Changes in blood lipids in human clinical trials using DHA supplementation

Study	Design <sup>1</sup>	Duration	DHA dose	Subjects	LDL	Total cholesterol	HDL	Trigs
		<i>Wks</i>	<i>(g/day)</i>		Change <i>mmol/L (% change)</i>			
Mori et al. (2000)	RCT, DB, parallel	6	4.0	59 overweight mildly hyperlipidemic men <sup>3</sup>	0.34 (8%)	NC	0.03 (3%)	-0.45 (-20%)
Grimsgaard et al. (1997)	RCT, DB, parallel	7	3.6	234 healthy men	NC	NC	0.06 (4%)	-0.22 (-18%)
Rambjor et al. (1996)	RCT, DB, parallel	3	3.0	49 healthy men & women	NC	NC	NC	NC
Sanders et al. (2006)	RCT, DB, parallel	4	1.5	79 healthy men & women	0.17 (7%)	0.19 (4%)	0.10 (7%)	-0.15 (-14%)
Maki et al. (2005)	RCT, DB, parallel	6	1.52	57 men & women with low HDL	0.43 (12%)	0.32 (6%)	NC	-0.48 (-24%)
Agren et al. (1996)	RCT, SB, parallel	15	1.68	55 healthy men	NC	NC	0.09 (10%)	-0.20 (-17%)
Nestel et al. (2002)	Controlled, DB, parallel	7	2.8	38 dyslipidemic men & women	NC	NC	NC	-0.63 (-32%)
Geppert et al. (2006)	Controlled DB, parallel	8	0.94	114 vegetarian men & women	0.26 (11%)	0.27 (6%)	0.12 (7%)	-0.25 (-23%)
Davidson et al. (1997)	RCT, DB, Parallel	6	1.25	27 hyperlipidemic	NC	no data	0.07 (6%)	-0.59 (-21%)
			2.5		0.56 (14%)	no data	0.06 (6%)	-0.59 (-18%)
Conquer and Holub (1996)	Controlled, parallel	6	1.6 <sup>2</sup>	24 vegetarian men & women	NC	NC	0.20 (17%)	-0.16 (-17%)
Conquer and Holub (1998)	Controlled, parallel	6	0.75	22 healthy men & women	NC	NC	NC	NC
			1.5		NC	NC	NC	NC
Buckley et al. (2004)	Controlled, DB, parallel	4	4.9	42 healthy men & women	NC	NC	NC	-0.72 (-62%)
Hamazaki et al. (1996)	Controlled DB, parallel	13	1.5 -1.8	24 healthy men & women	NC	NC	NC	NC
Theobald et al. (2004)	RCT, DB, crossover	12	0.7	38 healthy men & women	0.23 (7%)	0.22 (4.2%)	NC	NC
This study	Controlled, DB, parallel	6	1.1 <sup>1</sup>	47 men & women with mildly elevated triglycerides	0.25 (7%)	0.19 (3.6%)	0.06 (6%)	-0.30 (-15%)
			2.2 <sup>2</sup>	16 men & women with mildly elevated triglycerides	0.52 (16%)	0.51 (10%)	0.11 (11%)	-0.28 (-14%)

1. n = 47, data from the three interventions using 4g/d of fish oil – saff(4g), Sun(4g) and Can(4g).

2. n =16, data from the saff(8g) intervention

In this study the reduction in triglycerides was not influenced by the type of oil and margarine consumed. Brady et al. (2004) demonstrated in their study in 2004 that increasing dietary consumption of LA through incorporating an LA-rich oil and margarine into a background diet supplemented with 4.0g/d of fish oil (2.5g EPA & DHA) did not attenuate the beneficial effect of fish oil supplementation on fasting plasma triglycerides. Hwang et al. (1997) also showed that the triglyceride lowering effect of fish oil was not influenced by varying the amount of dietary omega-6 fatty acids consumed in the diet. A reduction in dietary LA concurrent with DHA supplementation may be anticipated to result in a greater triglyceride reduction than if LA intake remains high due to the potentially greater availability of DHA in hepatic and phospholipid pools. In this study inclusion of canola or sunola oil in the diet as a means of reducing dietary LA intake do not influence plasma and erythrocyte membrane DHA content. This may explain why the Can(4g) and Sun(4g) interventions did not result in a greater reduction in plasma triglycerides compared with the two groups consuming safflower oil and margarine. While erythrocyte membrane EPA and plasma and erythrocyte membrane total omega-3 fatty acids were higher with the Can(4g) intervention, these increases did not translate into an increased triglyceride reduction compared with the other interventions. This may suggest that a potential threshold for maximum triglyceride reduction was reached with the 4g/d dose of fish oil. Akabas and Deckelbaum (2006) highlighted in their recent overview on current status and future recommendations for omega-3 fatty acid research that ‘dose response data for EPA and DHA are limited and should be studied’ (p 1536S, Akabas & Deckelbaum, 2006). Such future research could be helpful in providing answers as to why a dose-dependent reduction was not observed with plasma triglyceride reduction in this study.

Plasma total and LDL cholesterol increased significantly by 9-10% and 15-16% respectively, with the saff(8g) and Sun(4g) interventions while in the saff(4g) intervention there was a trend toward an increase in LDL cholesterol. Studies examining the effect of DHA supplementation on blood lipids have reported either no change (Grimsgaard et al., 1997; Rambjor et al., 1996; Agren et al., 1996; Nestel et al., 2002; Conquer and Holub, 1996; Buckley et al., 2004; Hamazaki et al., 1996) or a significant increase (Mori et al., 2000c; Sanders et al., 2006; Maki et al., 2005; Geppert et al., 2006; Theobald et al., 2004) in LDL cholesterol. The increases in LDL cholesterol

reported previously in healthy and hyperlipidemic subjects have ranged from 7-14% with doses of DHA ranging from 0.7 – 4.0g/d (**Table 2.13**).

In this study halving the dose of DHA supplementation resulted in a smaller increase in LDL cholesterol with only a trend toward an 8% increase in LDL cholesterol being observed with the saff(4g) intervention compared with the 15.5% increase observed with the saff(8g) intervention. A dose-response effect of DHA supplementation on raising LDL cholesterol has not previously been established.

Replacing safflower oil and margarine with either canola or sunola as a means of reducing dietary LA intake had a mixed effect on the LDL raising response to DHA supplementation. The increase in LDL cholesterol with the Sun(4g) intervention replicates the findings of Rivellese et al. (2003). In that study, a reduction in LDL cholesterol achieved with consumption of a high-oleic sunflower oil and margarine spread in the diet, with negligible amounts of omega-3 fatty acids (similar to the sunola oil used in this study) was completely cancelled out by dietary omega-3 supplementation (3.6g/d fish oil), the latter causing a significant increase in LDL cholesterol. Thus, it would appear that replacing dietary LA with monounsaturated fatty acids is not an effective approach to minimising the LDL cholesterol raising potential of dietary DHA supplementation.

Replacing safflower oil and margarine with canola however, appears to be a more effective strategy as the Can(4g) intervention cancelled out the LDL cholesterol-raising effect of DHA supplementation, with LDL cholesterol remaining unchanged after six weeks. Canola oil and margarine produced the same increase in erythrocyte membrane EPA and plasma and erythrocyte membrane total omega-3 fatty acids and reduction in the omega-6: omega-3 fatty acid ratio as the saff(8g) intervention, but importantly prevented an increase in LDL cholesterol. The potential mechanisms that may be responsible for this finding are discussed in detail in Chapter 4 of this thesis.

Due to the fact that background dietary composition was not analysed in this study it cannot be known with complete certainty that other dietary factors were not contributing to the changes or absence of change in LDL cholesterol observed with the four interventions. Thus, it could be beneficial to reassess the effect of combined

consumption of canola oil and margarine and DHA-rich oil supplementation on LDL cholesterol concentrations in a study where background dietary intake is quantitatively assessed.

While LDL cholesterol increased significantly with the saff(8g) and Sun(4g) interventions, this change occurred concurrent to an increase in HDL cholesterol. Increases in HDL cholesterol ranging from 3-17% have been reported in both healthy and hyperlipidemic subjects following supplementation with 0.94 – 4.0g/d of DHA (Mori et al., 2000c; Grimsgaard et al., 1997; Agren et al., 1996; Geppert et al., 2006; Davidson et al., 1997; Conquer and Holub, 1996; Sanders et al., 2006). However, an absence of change in HDL cholesterol has just as frequently been reported in studies using doses of DHA supplementation in the range of 0.7 – 4.9g/d again in both healthy and dyslipidemic subjects (Rambjor et al., 1996; Maki et al., 2005; Nestel et al., 2002; Conquer and Holub, 1998; Buckley et al., 2004; Hamazaki et al., 1996; Theobald et al., 2004). A significant inverse linear correlation ( $r = -0.404$ ,  $p = 0.001$ ,  $n = 63$ ) was found between baseline HDL cholesterol and triglyceride concentrations when data from all subjects in this study was combined. At the end of the six week intervention this correlation was still present ( $r = -0.505$ ,  $p < 0.001$ ,  $n = 63$ ). This relationship has also been reported in previous studies using DHA supplementation (Grimsgaard et al., 1997; Geppert et al., 2006). This finding suggests that the increase in HDL cholesterol may be due to a reduction in lipid transfer protein activity promoting increased HDL cholesterol concentrations and reduced VLDL triglyceride concentrations, which has been previously demonstrated with omega-3 fatty acid supplementation (Abbey et al., 1990). However, in this study and in a previous study by Grimsgaard et al. (1997) in which HDL cholesterol increased with daily supplementation of 3.8g/d of DHA, there was no correlation found between the change in triglyceride and HDL cholesterol concentrations ( $r = 0.079$ ,  $p = 0.54$ ,  $n = 63$ ). This outcome suggests that DHA may be influencing triglyceride and HDL cholesterol metabolism by separate mechanisms (Grimsgaard et al., 1997), although these are currently unknown.

It is unclear why HDL cholesterol increased significantly with the Sun(4g) intervention but not with the saff(4g) or Can(4g) interventions. The varied responses to a range of DHA supplementation doses from previous clinical trials suggests that changes in HDL cholesterol with DHA supplementation are not dose-dependent. Both the Sun(4g) and

Can(4g) interventions had similar effects on plasma and erythrocyte membrane LA and oleic acid content after six weeks of dietary intervention. Thus it is unlikely that changes in LA or oleic acid contributed to increasing HDL cholesterol in the Sun(4g) intervention.

The Can(4g) intervention resulted in a similar increase in plasma and erythrocyte membrane total omega-3 fatty acids to the saff(8g) intervention and yet HDL cholesterol remained unchanged. Several human clinical trials using diets enriched with ALA (Kestin et al., 1990; Bemelmans et al., 2002; Zhao et al., 2004) or ALA-rich flaxseed oil (Wilkinson et al., 2005) have reported a significant reduction in HDL cholesterol compared to either an LA-rich diet or average American diet (Zhao et al., 2004). In one of these trials conducted in hypercholesterolemic subjects, an ALA-enriched diet significantly reduced apoA-1 levels in association with a reduction in HDL cholesterol (Zhao et al., 2004). However, not all studies using ALA supplementation have reported a reduction in HDL cholesterol (Mantzioris et al., 1997; Li et al., 1999; Harper et al., 2006; Goyens and Mensink, 2005; Goyens and Mensink, 2006; Finnegan et al., 2003). In a study conducted in hamsters, Dorfman et al. (2005) reported that a diet enriched with canola oil or soybean oil (both contain equally low levels of LA and canola has high ALA) resulted in a significant reduction in HDL cholesterol which was associated with a higher exogenous lecithin-cholesterol acyltransferase (LCAT) activity, and increased hepatic apo A-1 and scavenger receptor B class-1 (SR-B1) mRNA compared with a saturated or trans fat enriched diet. The authors from that study concluded that canola and soybean oils may have positive effects on components in the reverse cholesterol transport pathway, changes that have been associated with reduced CVD risk (Spady et al., 1999; Landschultz et al., 1996). These findings suggest the increased ALA intake associated with canola oil and margarine in this study was unlikely to be responsible for the suppression of a rise in HDL cholesterol observed with the Can(4g) intervention. Further research is required to explain this finding.

### **2.6.3 Blood pressure, arterial compliance and heart rate**

Blood pressure, arterial compliance and heart rate remained unchanged with all four interventions in this study. Studies over a broad range of omega-3 fatty acid intake levels ranging from 2 – 15g/d have reported significant reductions in blood pressure in both healthy (Bach et al., 1989) and mildly hypertensive individuals (Knapp and Fitzgerald, 1989; Radack et al., 1991; Bonaa et al., 1990). However, to date the minimum dose of EPA and DHA or appropriate ratio of DHA: EPA required to produce a significant reduction in blood pressure in both hypertensive and normo-tensive individuals has not been determined.

Previous studies which have examined the effect of DHA supplementation on blood pressure have reported mixed results. Geppert et al. (2006) reported no significant changes in blood pressure or heart rate following 8 weeks of daily supplementation with 0.94g of DHA in normolipidemic, vegetarian men and women. Similarly, Conquer and Holub (1998) reported no change in heart rate, SBP or DBP after three or six weeks of daily supplementation with 0.75 or 1.5g of DHA. Sanders et al. (2006) reported a slightly more positive result with significant treatment effect in pulse pressure (4 mmHg reduction) and a trend toward a reduction (-5 mmHg) in SBP (which failed to reach statistical significance when compared with placebo) being observed after 4 weeks of daily supplementation with 1.5g of DHA. Sanders and Hinds (1992) in their uncontrolled trial in 9 hyperlipidemic men reported a significant 3 mmHg reduction in both SBP and DBP after 6 weeks of 2.1g/d of DHA, with blood pressure returning to baseline values post-treatment. The most significant finding, however, was in the study by Mori et al. (1999) in which daily supplementation with 4.0g of DHA for six weeks in mildly hyperlipidemic, overweight men resulted in a significant reduction in 24 hour (-5.8/3.3 mmHg) and daytime (-3.5/2.0 mmHg) blood pressure and a significant reduction in 24 hour, daytime, night-time (asleep) and ambulatory heart rate. Taken together, results from these studies suggest that DHA supplementation below 1.5g/d may not produce a significant reduction in blood pressure but doses over 1.5g/d may be consistently more effective.

In the study by Mori et al. (1999) where ambulatory blood pressure decreased significantly, plasma DHA phospholipids increased from 4.0 to 10.93% of total fatty

acids with 4.0g/d of DHA supplementation. Additionally, plasma LA decreased to 18% and AA decreased to 8.82% of total fatty acids. In the current study plasma DHA content was considerably lower at baseline averaging 1.68% of total fatty acids and only increased to 4.75% with 2.2g/d of DHA and a maximum of 4.18% with 1.1g/d of DHA. Furthermore, plasma AA did not significantly decrease. While plasma AA was 5.5% of total fatty acids after six weeks, the LA content of plasma averaged 26.1% of total fatty acids. It is possible therefore that the dose of DHA utilised in this study was insufficient to reduce AA and LA by an amount adequate enough to produce a significant reduction in blood pressure or heart rate. Additionally, while subject numbers in this study matched those in the Mori et al. (1999) study, higher subject numbers may have been required to detect a significant change with the lower doses of DHA supplementation tested.

Only one other human clinical trial has examined the effect of DHA supplementation on arterial compliance. Nestel et al. (2002) in their study of 38 dyslipidemic men and women reported a significant 27% increase in systemic arterial compliance following 7 weeks of daily supplementation with 3g of DHA. The improvement in arterial compliance was accompanied by trends toward reductions in systolic and pulse pressures and a reduction in total vascular resistance, though all failed to reach statistical significance. In contrast, the subject population in the current study was not hypercholesterolemic, a condition which can potentially lead to reduced arterial compliance. These improvements in arterial compliance may have been more easily detected in a dyslipidemic population. Further investigation into the minimum dose of DHA required to improve arterial compliance in different study populations is needed.

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## **2.7 Conclusion**

This study showed that replacing usual dietary oil and margarine with canola while supplementing the diet with 1.1g/d of DHA favourably improved total omega-3 fatty acid incorporation and reduced the omega-6: omega-3 fatty acid ratio in plasma and erythrocyte membrane phospholipids by proportions not dissimilar to those achieved with double the dose of fish oil consumed with safflower oil that has a lower ALA and higher LA content. Similarly, the combination of canola and 1.1g/d of DHA significantly decreased fasting plasma triglyceride concentrations by an amount not less

than that achieved with double the daily dose (2.2g) of DHA. An added benefit of combining fish oil with dietary canola consumption, potentially attributed to the ALA content of the canola was the cancellation of the significant rise in both LDL and total cholesterol caused by the DHA supplementation. However, despite these benefits, 1.1g/d of DHA with canola oil and margarine failed to produce the significant increase in HDL cholesterol achieved with 2.2g/d of DHA supplementation.

A daily dose of 8g of DHA-rich oil (2.2g/d of DHA) is quite a high dose to sustain over a long time period. This study's findings raise the potential for lower doses of fish oil to be equally as beneficial under circumstances of reduced dietary omega-6 fatty acid intake and increased plant sourced ALA intake. Further research is required however and the results of this study warrant further investigation in a double blind controlled human intervention trial measuring all aspects of background diet to confirm that dietary canola oil and margarine consumption improves the effect of DHA-rich fish oil supplementation on CVD risk factors.



### **3      Omega-Soy study**

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#### ***Study Context***

*This dietary intervention study was conducted by three PhD students, Leisa Ridges, Theresa Larkin, and Gina Martin, enrolled in the Biomedical Science Department at the University of Wollongong. I managed subject screening, blocking, clinic visits, lipid and cardiovascular analysis, while Theresa Larkin managed the urinary and plasma isoflavone analysis and Gina Martin conducted all dietary analysis. With Theresa Larkin, I designed the intervention study under the supervision of PRC Howe, LB Astheimer and BJ Meyer, and managed all aspects of subject recruitment. Although I did not conduct the original plasma and urine analysis of soy isoflavones and their metabolites, I used the raw data for subsequent analysis and included the analysis in this thesis. Dietary data has also been included in this thesis; however, all such data was collected and analysed by Gina Martin and has been referenced accordingly.*

### 3.1 Introduction

Combined intake of functional ingredients capable of reducing cardiovascular risk factors may work synergistically accomplishing greater benefits than if such functional foods are consumed independently. Two ingredients which have a demonstrated impact on cardiovascular disease risk factors and may compliment one another to produce an overall favourable reduction in cardiovascular risk profile are the marine sourced omega-3 fatty acids DHA and/or EPA and soy isoflavones.

Dietary supplementation with the marine sourced long chain omega-3 fatty acids DHA and/or EPA has been clearly demonstrated to significantly reduce plasma triglyceride concentrations (Harris, 1996; Harris, 1997; Nelson et al., 1997; Mori et al., 2000c; Grimsgaard et al., 1997; Davidson et al., 1997; Sanders and Hinds, 1992; Conquer and Holub, 1996). However, DHA and EPA appear to have little benefit for reducing plasma cholesterol concentrations, in fact earlier reviews (Harris, 1996; Harris, 1997) and later human clinical trials (Hsu et al., 2000; Rivellesse et al., 2003; Mori et al., 2000c; Theobald et al., 2004; Putadechakum et al., 2005) have reported significant increases in plasma LDL cholesterol concurrent with reductions in plasma triglyceride concentrations. While some reviews have considered this increase to be transient (Harris, 1996; Weber and Raederstorff, 2000) results have not been consistent. From a systematic evaluation of the scientific literature as a means of evaluating a health claim on omega-3 fatty acids and reduced risk of heart disease, the US Food and Drug Administration ruled that increased levels of LDL cholesterol especially amongst individuals with hyperlipidemia was an unresolved safety concern associated with long-chain omega-3 fatty acid dietary supplementation (Hasler, 2002; U.S Food and Drug Administration, 2000). An increase in LDL cholesterol has been clearly established as a risk factor for CVD (Bertolotti et al., 2005; Mensink, 2003), thus, while the cardiovascular benefits of omega-3 fatty acids could be considered to far out-weigh the negative impact of increased LDL cholesterol, dietary strategies to counteract this effect would be beneficial (Krauss et al., 2000; NCEP Expert Panel, 2001).

Soy isoflavones in contrast to omega-3 fatty acids have been shown to contribute to cholesterol reduction. Crouse et al. (1999) demonstrated in 156 healthy men and women that plasma LDL and total cholesterol concentrations decreased dose-

dependently with increasing quantities (ranging from 3-62mg) of isoflavones provided in a 25g daily intake serve of soy protein. In a meta-analysis of 23 randomized controlled trials, Zhan and Ho (2005) reported that daily consumption of soy protein with greater than 80mg of isoflavones showed a significant and greater reduction in LDL cholesterol than soy protein with less isoflavones. Zhuo et al. (2004) demonstrated in their review of eight randomized clinical trials that regular consumption of soy protein (50g/d) with high isoflavone content (96mg/d) reduces LDL cholesterol by a significantly greater amount than if the soy protein has a low isoflavone content (6mg/d). The results from these two meta-analyses demonstrate that soy isoflavones, either independently or in concert with soy protein, significantly contributes to reducing LDL cholesterol concentrations.

It has been demonstrated *in vivo* in mice and *in vitro* in HepG2 cells that soy isoflavones independent of soy protein can increase hepatic LDL uptake by increasing LDL receptor expression and activity (Kirk et al., 1998; Borradaile et al., 2002). However, human intervention studies using extracted soy isoflavones devoid of soy protein have not reported significant reductions in LDL cholesterol or improvements in other blood lipids (Dewell et al., 2002; Simons et al., 2000; Nestel et al., 1997). The evidence *in vitro* of a valid LDL lowering mechanism supports the cholesterol lowering potential of soy isoflavones *in vivo* but perhaps only in partnership with other dietary factors such as components of soy protein (Zhan and Ho, 2005). Should soy isoflavones require soy protein *in vivo* to successfully reduce LDL cholesterol, the mechanisms and minimum dose of protein required, have not been determined.

In addition to influencing LDL cholesterol metabolism, it has recently been demonstrated in US women with coronary risk factors undergoing coronary angiography for suspected ischemia, that higher plasma daidzein concentrations were significantly associated with lower triglycerides, higher HDL cholesterol and a beneficial total to HDL cholesterol ratio. Furthermore, the strength of this relationship was incrementally related to increasing plasma daidzein concentrations independent of other lipoprotein modulators (Merz et al., 2006).

Dietary soy isoflavone consumption has also been reported to improve arterial compliance (Nestel et al., 1997), favourably influence endothelial function (Mahn et al.,

2005; Mishra et al., 2000) and reduce the incidence and development of atherosclerosis in animal models (Anthony et al., 1997; Honore et al., 1997) potentially via their activation of nitric oxide production (Mahn et al., 2005; Park et al., 2005). This latter mechanism may also be responsible for an improvement in blood pressure (Park et al., 2005; Mahn et al., 2005). Omega-3 fatty acids have also been shown to significantly improve systemic arterial compliance and blood pressure (Mori et al., 1999; Goodfellow et al., 2000; McVeigh et al., 1994) potentially by similar mechanisms (Harris et al., 1997b). There is also recent evidence emerging to strongly support a reduction in heart rate with dietary long chain omega-3 fatty acid consumption (Mozaffarian et al., 2005). Thus the combined dietary intake of soy isoflavones and long-chain omega-3 fatty acids could potentially improve several CVD risk factors.

The dietary combination of soy isoflavones and omega-3 fatty acids was recently examined as a dietary treatment to enhance bone conservation in a rat model of postmenopausal bone loss (Watkins et al., 2005). The actions of soy isoflavones and omega-3 fatty acids were complementary in attenuating bone mineral reduction. These findings replicated those of an earlier study in the same rat model, in which cholesterol levels were concurrently reduced (Fernandes et al., 2003).

In humans, the combination of soy protein, rich in isoflavones, and linseed, rich in the plant sourced omega-3 fatty acid ALA, favourably improved fasting plasma total and LDL cholesterol in hypercholesterolemic postmenopausal women (Ridges et al., 2001). To date, however, there have been no human studies that have examined the cardiovascular benefits of a diet combining soy isoflavones with long chain marine sourced omega-3 fatty acids on cardiovascular end points.

This study aimed to determine whether the dietary supplementation with a marine sourced, omega-3, DHA-rich oil in combination with soy isoflavones by men and women with moderately elevated blood lipids, could improve CVD risk factors more favourably than either soy isoflavones or omega-3 fatty acids alone.

### **3.2 Study hypothesis**

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The primary hypothesis of this study is that in mildly hyperlipidemic individuals, consumption of a soy cereal high in isoflavones and low in soy protein in conjunction with DHA-rich oil supplementation over six weeks will result in an improvement in the following cardiovascular risk factors: fasting plasma triglycerides, LDL cholesterol, HDL cholesterol, blood pressure and arterial compliance.

Specifically, it is hypothesised that:

Consumption of soy isoflavones alone for six weeks by mildly hyperlipidemic individuals will:

- Significantly decrease plasma LDL cholesterol,
- Prevent or minimise the rise in LDL cholesterol often seen with DHA-rich oil consumption
- Potentially improve arterial compliance and/or blood pressure

Consumption of DHA-rich oil alone for six weeks by mildly hyperlipidemic individuals will:

- Significantly decrease plasma triglycerides
- Significantly increase LDL cholesterol
- Improve arterial compliance, heart rate and blood pressure

The combined dietary consumption of DHA-rich oil and soy isoflavones will result in:

- A smaller rise in LDL cholesterol than is seen with DHA –rich oil alone or no change or a reduction in LDL cholesterol concentrations
- A significant improvement in HDL cholesterol and fasting plasma triglyceride concentrations
- A significant improvement in blood pressure, heart rate and arterial compliance measurements

### **3.3 Methods**

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#### **3.3.1 Subjects**

Male and female subjects older than 40 years were recruited from the Illawarra region. Potential study participants were assessed for eligibility via a telephone questionnaire. Suitable subjects visited the University Clinic for weight and blood pressure measurement, a fasting blood sample and to complete a comprehensive health and lifestyle questionnaire which included questions about dietary intake of soy-based or omega-3 fatty acid rich foods.

Inclusion criteria were total plasma cholesterol  $> 5.5$  mmol/L, LDL cholesterol  $> 3.5$  mmol/L (assessed via the Friedewald calculation, (Friedewald et al., 1972)) triglycerides  $> 2.0$  mmol/L, and blood pressure  $> 140/90$ , although the latter two criteria were not essential. Exclusion criteria consisted of medically diagnosed cardiovascular disease or diabetes, the use of medication that influenced blood lipids, hormones or blood pressure and the use of fish oil supplements in the previous six months; consumption of more than two standard alcoholic drinks on average per day; an aversion to the daily consumption of breakfast cereal or not eating breakfast on a regular basis; a high habitual intake of soy-based or omega-3 fatty acid-rich foods; and for women, if they had menstruated in the previous twelve months or were taking hormone replacement therapy.

One hundred and seventy one people enquired about the study. Eighty-four people met the entry criteria and were invited for further screening of blood lipids. Of these, forty three subjects were eligible for inclusion.

The study met the National Health and Medical Research Council ethics requirements and was approved by the Human Ethics Committee of the University of Wollongong (HE00/222); all subjects signed informed consent forms prior to study commencement.

### 3.3.2 Study design

This study followed a placebo-controlled, double-blind, cross-over design. Eligible subjects were allocated to one of four cross-over dietary intervention groups (**Figure 3.1**) using stratified randomisation based on their screening plasma cholesterol, LDL cholesterol, HDL cholesterol and triglyceride values and clinic systolic (SBP) and diastolic blood pressure (DBP) (**Table 3.1**).

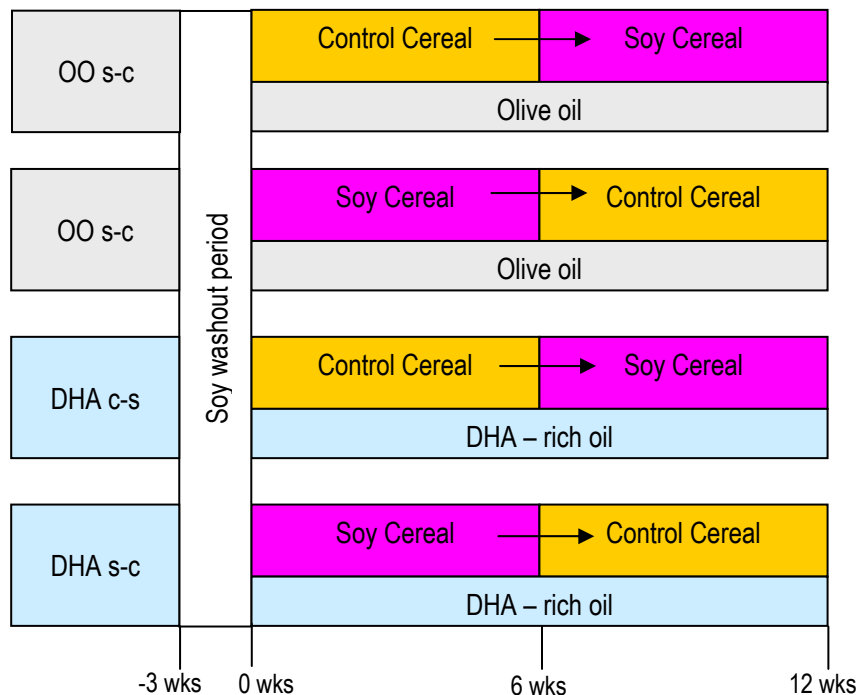


Figure 3.1 Diagrammatical representation of the four dietary interventions

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption).

Table 3.1 Blocking characteristics for the four intervention groups<sup>1</sup>

Groups <sup>2</sup>	Females / males	Total cholesterol	HDL <sup>3</sup>	Trigs <sup>4</sup>	LDL <sup>5</sup>	SBP <sup>6</sup>	DBP <sup>7</sup>
		<i>mmol/L</i>				<i>mmHg</i>	
OOc-s	5 / 6	6.6 ± 0.8	1.1 ± 0.1	2.4 ± 0.6	3.9 ± 0.2	141.3 ± 6.9	78.8 ± 1.5
OOs-c	3 / 8	6.6 ± 0.3	1.1 ± 0.1	2.6 ± 0.4	4.1 ± 0.2	143.5 ± 7.3	84.8 ± 4.6
DHAc-s	3 / 6	6.7 ± 0.4	1.3 ± 0.1	2.8 ± 1.0	4.1 ± 0.2	138.6 ± 6.6	81.0 ± 3.7
DHAs-c	4 / 8	6.7 ± 0.4	1.1 ± 0.1	2.6 ± 0.3	4.2 ± 0.4	137.5 ± 4.7	82.1 ± 3.9
Total/mean	15 / 28	6.7 ± 0.2	1.2 ± 0.1	2.6 ± 0.3	4.1 ± 0.1	140.4 ± 3.2	81.9 ± 1.8

1. Data are mean ± SEM

2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption).

3. HDL cholesterol was separated from fresh plasma by dextran sulphate magnesium chloride precipitation (Warnick et al., 1982)

4. Triglyceride

5. LDL cholesterol was calculated using the Friedewald calculation (Friedewald et al., 1972).

6. Systolic blood pressure

7. Diastolic blood pressure

Subjects consumed an oil supplement for 12 weeks combined with either control or soy cereal for 6 weeks, followed by cross-over to the alternate cereal for 6 weeks. The four intervention groups (**Figure 3.1**) consisted of:

**OOc-s** – Daily olive oil supplementation for twelve weeks with concurrent consumption of control cereal for the first six weeks followed by consumption of soy cereal between six and twelve weeks of the intervention period.

**OOs-c** – Daily olive oil supplementation for twelve weeks with concurrent consumption of soy cereal for the first six weeks followed by consumption of control cereal between six and twelve weeks of the intervention period.

**DHAc-s** – Daily DHA-rich oil supplementation for twelve weeks with concurrent consumption of control cereal for the first six weeks followed by consumption of soy cereal between six and twelve weeks of the intervention period.

**DHAs-c** – Daily DHA-rich oil supplementation for twelve weeks with concurrent consumption of soy cereal for the first six weeks followed by consumption of control cereal between six and twelve weeks of the intervention period.

Prior to study commencement, all subjects were interviewed by a dietitian and provided information about their usual diet in the form of a diet history. Subjects found to be consuming any known soy-containing foods in their diets were asked to cease such consumption for the duration of the study period. Alternative foods were recommended to replace the soy items. All subjects had a three week wash-in period to ensure complete removal of soy from the diet and for such modified diets to stabilise prior to commencement of the intervention period.



### 3.3.3 Food and supplements

All subjects consumed eight 1.0g capsules daily of either olive oil (Omega Tech Inc., Boulder, Colorado, USA) or DHA Gold (Omega Tech Inc., Boulder, Colorado, USA). Daily supplementation with DHA Gold provided a total of 2.9g/day of DHA and 0.06g/day of EPA (**Table 3.2**).

For each six week intervention period subjects consumed either 45g of a soy or control breakfast cereal daily (Specialty Cereals Pty Ltd, Mt Kuring-Gai, NSW Australia). The cereal was provided to subjects in pre-packaged 45g serves. The soy containing cereal supplied 90.3mg/d of isoflavones (supplied from 3g of Soy Isolife providing 46.4mg daidzein & daidzin, 31.5mg of glycitein & glycitin and 12.4mg of genistein & genistin) and 1.2g/d soy protein (**Table 3.3**). This cereal formulation was chosen as it was similar to a commercially available breakfast cereal and provided a high level of isoflavones with a relatively low level of soy protein. The control cereal was prepared specifically for this study and had the same composition as the soy cereal with the omission of the Soy Isolife™. The cereal packages and containers of oil capsules were unmarked except for a numerical code.

Both the study investigators and subjects were blinded to the types of oil and cereal provided, however, the oils were not modified to mask identification from its aftertaste. Despite this few subjects accurately guessed which oil they were taking during the study. Subjects were encouraged to consume the cereal as part or all of their breakfast meal. Not all subjects were able to do this for the entire length of the study. Where this was the case subjects were provided suggestions by the dietitian on when and how to consume the cereal across the course of their daily diet. Consumption of the olive and DHA oil capsules occurred at meal times: 2 capsules at breakfast, 3 each with lunch and dinner.

Table 3.2 Fatty acid profile of DHA Gold

	DHA Gold <sup>1</sup>
Fatty Acids	(mg/g)
C12	3.5
C14	89
C14:1	0.9
C15	3.0
C16	228.5
C16:1	5.6
C18	5.7
C18:1	12.6
C18:2	2.9
C18:3n6	1.4
C18:4	2.0
C20	1.0
C20:4n6	3.5
C20:4n3	7.8
C20:5n3	7.6
C22	0.5
C22:5n6	135.1
C22:6n3	365.1
C24	1.4
C24:1	2.0
Others	33.1
Total	912.7

1. Data reproduced from a Certificate of Analysis Addendum for DHA Gold Oil, lot number DHAL0K0014A. (Omega Tech Inc., Boulder, Colorado, USA, December, 2000).

Table 3.3 Ingredient profile of the soy and control breakfast cereals

	Soy Cereal <sup>1</sup>	Control Cereal <sup>1</sup>
Ingredients	<i>g/45g serve</i>	
Pasta bran	12.62	13.54
Bakers Flour	9.47	10.15
Malted wheat	9.47	10.15
Hi-Maize flour	3.16	3.38
Caster sugar	6.31	6.77
SoyLife™	3.03	0.00
Tricalcium phosphate	0.44	0.47
Salt	0.34	0.37
Emulsifier	0.13	0.14
Vitamin E	0.02	0.02
Annato	0.01	0.01

1. Data reproduced from formulation data provided by Specialty Cereals Pty Ltd (Sydney, Australia)

Subjects were provided with surplus bags of cereal and oil capsules and were requested to return any remaining capsules or cereal packs at the end of each six week intervention period. The degree of subject compliance to the oil supplements and cereal was estimated from the number of packs and capsules returned. Due to fire damage in the clinical area however, data on capsule and cereal return was destroyed and cannot be reported. Subject compliance to the cereal and oil supplements was further tested by analysis of urinary isoflavone concentrations and erythrocyte membrane fatty acid levels after six and twelve weeks of the intervention period.

Subjects were encouraged to maintain their usual level of physical activity throughout the study.

#### **3.3.4 Clinical assessment protocol**

Prior to commencement of the study, all subjects attended an individual diet interview with a dietitian to establish their habitual dietary intake and completed a 3-day food record. At study commencement (week 0) and at the end of 6 and 12 weeks, subjects visited the clinic following a 10-12 hours fast, on two consecutive mornings. **Figure 3.2** shows the measurements taken at each clinic visit. Height was measured on day one of the first clinic appointment only. On the first day of each clinic visit weight, percent body fat composition, supine blood pressure and arterial compliance were measured and a venous blood sample was collected for determination of plasma levels of isoflavones, lipids, lipoprotein composition and erythrocyte membrane fatty acids. Subjects were also provided with urine collection bottles and asked to collect urine for the 24 hour period between clinic visits. Each subject was fitted at this time with an ambulatory blood pressure monitoring unit to their left upper arm, which they were required to wear for the same time period. On the second morning of each clinic visit weight and percent body fat composition were measured and a fasted blood sample was taken for repeat lipid assay. Subjects returned their 24 hour urine sample and ambulatory blood pressure unit and were provided sufficient cereal and oil capsules for the subsequent six weeks.

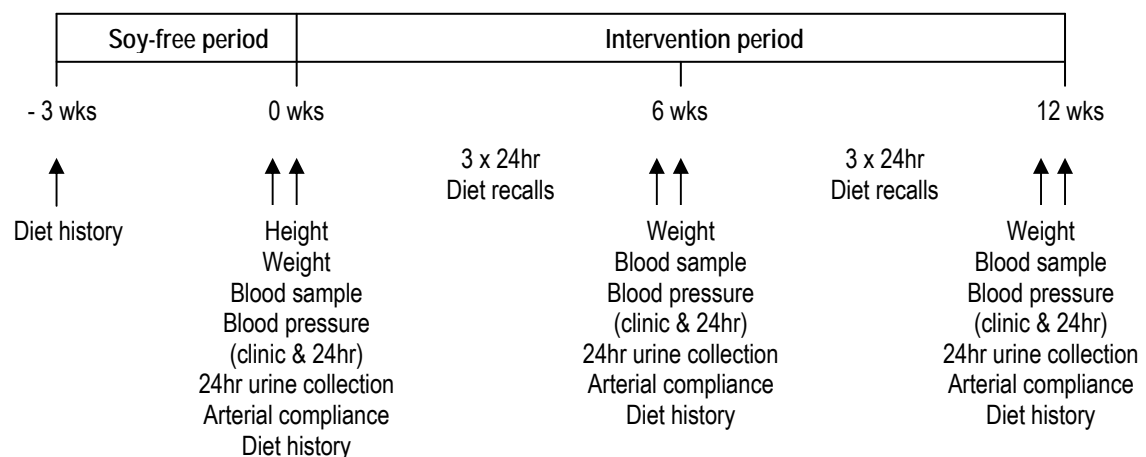


Figure 3.2 A diagrammatical representation of study timeline showing clinic visit measurements and dietary monitoring tools. Each arrow represents one clinic visit.

### 3.3.5 Dietary compliance assessment

Prior to study commencement and on one morning of each clinic visit, subjects met with the dietitian for a diet history interview and were provided advice on necessary dietary modifications to ensure the macronutrient profile of their diet remained stable throughout the study and to maintain their usual use of oils and spreads and intake of fish, seafood and nuts. In addition to a diet history interview, details about subjects' level of exercise during the preceding six weeks were rated on a scale of light, light-moderate, moderate, moderate-heavy or heavy at baseline, six and twelve weeks. Examples were provided to explain each category.

As additional measures of dietary compliance subjects received three unannounced telephone calls requesting 24-hour dietary recalls during each six-week intervention and were required to record on a daily basis, the number of capsules consumed and their intake of cereal.

All dietary data obtained was entered into FoodWorks Nutrient Analysis Software and analysed in two databases, the AUSNET and NUTTAB 95 Australian food composition databases to obtain information on macronutrient intake and generally recognized nutrients, and the Australian fatty acid database (RMIT, Melbourne, 2002), for more accurate information on the fat composition of the diets.

### 3.3.6 Clinical measurements

Height without shoes was measured in the clinic using a stadiometer. Weight in light indoor clothing and percent body fat composition were measured via bioelectric impedance using a calibrated Tanita body fat monitor (Model BF-664, Tanita Corporation, Tokyo, Japan).

Clinic blood pressure at the screening clinic visits was assessed with a Dinamap (Dinamap 1846 SX/P; Critikon Inc, Tampa FL) blood pressure recording unit. Subjects had blood pressure recorded in a reclining chair. A cuff was placed on subjects' left arm with a suitable cuff size used to ensure a snug fit. After the subject had rested in the reclined chair for five minutes a blood pressure reading was taken followed by three subsequent readings taken at two minute intervals. The last three readings were averaged to provide measures of systolic and diastolic blood pressure.

Supine clinic blood pressure at the three intervention clinic visits was assessed using the HDI/ *PulseWave*<sup>TM</sup> Model CR-2000 Research CardioVascular Profiling Instrument (HDI Hypertension Diagnostics Inc., Eagan, Minnesota, USA).

### 3.3.7 Twenty-four hour blood pressure assessment

Subjects were fitted with SpaceLabs 90207 ambulatory blood pressure monitor (SpaceLabs Inc., Hillsboro, Oregon, USA). The inflatable cuff was placed on the left upper arm and an appropriate cuff size was chosen to ensure a snug fit. Subjects wore the monitor for 24hrs and kept a diary of activities and sleep times. Readings were made every twenty minutes from 7am -11pm (day) and every 30 minutes from 11pm-7am (night). Subjects maintained their usual daily activities and were asked to maintain consistency in the nature and type of activities undertaken on all three 24hr blood pressure measuring days. Subjects were instructed to keep their arm as stationary as possible during cuff inflation and deflation and were notified of the relevant alarms which indicate a successful or unsuccessful reading. In the case of an unsuccessful reading, each subject was encouraged to keep their arm as stationary as possible for the two subsequent repeat readings which would take place at one minute intervals until a correct reading was obtained. If both subsequent readings were unsuccessful, then that reading would be missed and the next reading taken twenty minutes later as scheduled.

Data was downloaded from the SpaceLab 90207 monitor using ABP Communications software (1994 Spacelabs Medical, Inc USA) and was only used for data analysis if  $\geq 80\%$  were successful. If the success level was less than eighty percent than subjects were re-fitted with the SpaceLab 90207 monitor for a repeat 24hr blood pressure measurement.

### **3.3.8 Non-invasive arterial compliance assessment**

Arterial compliance was assessed non-invasively using a HDI/ *PulseWave*<sup>TM</sup> Model CR-2000 Research CardioVascular Profiling Instrument (HDI Hypertension Diagnostics Inc., USA). The methodology utilised for this assessment is described in Chapter 2 of this thesis. In the MOFO study one measure of arterial compliance was taken on both days of each clinic visit. The data collected on the two days was then averaged and compared with the mean data from the other time points. In the Omega-Soy study, three measures of arterial compliance were taken 5 minutes apart on the first day only of each two day clinic visit period using the same methodology as described for the MOFO study in an effort to try to minimise variability in the arterial compliance data. These three readings were then averaged and compared with the average of the three readings obtained at the six week time point.

### **3.3.9 Sample collection and analyses**

Blood samples were kept on ice until the completion of each morning's clinic visits after which they were centrifuged at 1000g at 4°C (Hettich, Universal 16R) for 10 min. Plasma and red blood cells were removed and aliquots stored at -80°C prior to analysis.

At each clinic visit, blood samples were collected by a qualified venipuncturist into tubes (Sarstedt Monovette, Germany) containing ethylenediamine tetraacetic acid (EDTA). Blood samples were kept on ice until the completion of each morning's clinic visits after which they were centrifuged at 1000 g at 4 °C (Hettich, Universal 16R) for 10 minutes. Plasma and erythrocytes were removed and aliquots stored at -80 °C for future analysis of fasting blood lipids (triglycerides, total cholesterol and HDL cholesterol), erythrocyte membrane fatty acid composition and plasma isoflavone concentrations. Fresh plasma (10mL) from the first day of each clinic visit was used for isolation of lipoproteins.

Urine samples were collected in 2-litre plastic bottles containing 1.2 mg sodium azide and 1 g ascorbic acid as preservatives. The total volume of urine collected over each of three 24-hour collection periods was measured and aliquots stored in sealed glass vials at -80 ° C until subsequent analysis.

The methods used for the measurement of plasma lipids and erythrocyte membrane fatty acid compositions are described in Chapter 2 of this thesis.

### **3.3.10 Lipoprotein composition**

Plasma was separated from fasted blood samples obtained at the first day of each clinic visit at baseline, six and twelve weeks. For each subject, 10 mL of fresh plasma (density of plasma = 1.006g/mL) was placed in a 13.5mL Type 70.1Ti ultracentrifuge tube (Beckman Coulter Australia Pty Ltd, Gladesville, NSW) and overlayed with solution of density 1.006g/mL (22.8g NaCl, 0.2g EDTANa<sub>2</sub>, 1L H<sub>2</sub>O, 2mL of 1M NaOH and 0.4g NaN<sub>3</sub> in 2L of distilled water) until tube was filled. Tubes were then heat sealed and spun in an ultracentrifuge (Beckman model L-80 OPTIMA, USA) at 250,770 x g at 15 ° C for a minimum of 16 hours.

After spinning, tubes were sliced to remove VLDL contained in the top portion of the tube. The volume of the remaining plasma (now at a density > 1.006g/mL) was then measured and the required volume of solution with density 1.478g/mL (100mL x density 1.006 solution) + (5mL x density 1.478 solution) = 1.030g/mL) was added to bring the sample to a density of 1.030g/mL. The plasma was then overlayed with solution of density 1.478g/mL. The tube was heat sealed and spun in the ultracentrifuge at 15 ° C at 250,770 x g for a minimum of 18 hours.

Following spinning tubes was sliced to remove the IDL contained in the upper portion of the tube. The volume of the remaining plasma was measured and the relevant volume of solution with density 1.478g/mL as determined by the formula: [(1mL x density solution 1.478) + (10.25mL x density solution 1.030) = 1.063g/mL] was added to the tube to adjust the density to 1.063g/mL. The sample was then overlayed with density solution 1.063g/mL, heat sealed and spun in the ultracentrifuge at 15 ° C at 250,770 x g for 18 hours.

After spinning the tube was sliced to recover the LDL located in the top portion of the tube. The remaining plasma was adjusted to density 1.25g/ml (density of HDL) with the addition of potassium bromide (KBr). The plasma was then overlaid with a solution of density 1.25g/ml and spun under the conditions above for 24 hours. After spinning the tube was sliced to remove the HDL.

Total cholesterol (Cholesterol CHOD-PAP, Cat # 1489232, Boehringer Mannheim, Germany), triglyceride (Unimate 5 Trig, art 0736791 Roche Diagnostics, Australia), free cholesterol (Cat No 274-47109, Wako Pure Chemical Industries Ltd, Alpha Laboratories Ltd, Hampshire, UK), protein (Unimate 7TP, art 0736783, Roche Diagnostics, Australia) and apolipoprotein B (Unimate 3ApoB, art. 0736899, Roche Diagnostics, Australia) in lipoprotein sub-fractions were quantified using a COBAS MIRA Plus automated analyser (Roche Diagnostics Ltd; Rotkreuz, Switzerland) with commercially available kits. Cholesterol ester was calculated by subtracting free cholesterol from total cholesterol.

### **3.3.11 Isoflavone analysis**

The methodology for isoflavone analysis of plasma and urine samples was adapted from that of Gamache and Acworth (1998) and is described in Larkin (2005).

#### **3.3.11.1 Extraction and hydrolysis**

Ethanol (1.2mL) was added to 0.2ml of thawed plasma or urine samples and mixed by vortex for 10 minutes. Samples then underwent centrifugation at 4° C at 1000g for a further 10 minutes. A 1.0mL sample of the derived supernatant was subsequently removed and evaporated under nitrogen gas. Once evaporated the samples were reconstituted in 0.1mL of methanol by 5 minutes of sonication and diluted with 0.3mL of water. An aliquot (50µL) of the sample then underwent enzymatic hydrolysis with 50mL of beta-glucuronidase and 0.2mL buffer (0.1M sodium acetate, pH 5.0, containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA) and was incubated at 37 ° C for 3 hours. Following a repeat round of centrifugation for 10 minutes at 4° C and 1000g, 1.0ml of supernatant was evaporated to dryness under nitrogen gas. The extracts were then redissolved once more in 0.1mL of methanol by 5 minutes of sonication and diluted with 0.3mL of water.



### 3.3.11.2 Analysis of isoflavones

Samples were separated by HPLC (Shimadzu, Sydney, Australia) using a C<sub>18</sub> column (5µM, 4.6mm by 250 mm, SGE, Melbourne, Australia) with mobile phase flowing at a rate of 1mL/min. Samples were analysed by electrochemical detection (ESA, Coulochem) at a potential of 520 mV, with the conditioning set at -50mV and the analytical cell at 470 mV. An injection volume of 20mL of extracted urine was applied to the column and measured at a sensitivity of 10 µA. Peak areas of daidzein, genistein and equol (Sigma, Australia) were used to determine concentrations from standard curves formulae (Larkin, 2005).

### 3.3.12 Statistical analysis

Kolmogorov-Smirnov(a) tests were used to assess normality of data. For data that were normally distributed, paired-samples t-tests were used to determine within-group changes. For data that were not normally distributed or when the sample number was very small, the Wilcoxon Signed Rank Test was used to determine within-group changes. Between-group differences over time were determined using between-group repeated ANOVA analysis with a Tukey HSD post hoc test.

A 3-factor ANOVA with oil and sequence as between subject effects and time as a within subject effect was conducted to explore the impact of sequence of cereal consumption and type of oil on fasting plasma lipids, lipoprotein composition, arterial compliance measures and clinic and ambulatory blood pressure. Data from the soy cereal period and control cereal period were analysed separately in the 3-factor ANOVA. Data from zero and six weeks in the intervention period were considered the data for the start and end of the soy cereal period for Groups OOs-c and DHAs-c, respectively, while data from six and twelve weeks were considered the data for the start and end of the soy cereal period in Groups OOC-s and DHAc-s, respectively. Multivariate tests carried out in the mixed within-between ANOVA included the effect of time, time\*oil interaction and time\*sequence interaction. Between-group differences tested included oil, sequence, and oil \*sequence interaction.

A statistical analysis was undertaken to determine the effect of the DHA and olive oils on all outcome measures (fasting blood lipids, lipoprotein composition, blood pressure, arterial compliance, and other cardiovascular parameters) when the soy and control cereals were consumed, correcting for baseline values, age, BMI and gender.

The analysis used a Repeated Measures, 2 Factor ANCOVA with oil and gender as the between-group factors, cereal as the within-subject factor, and age and BMI as covariates.

For each outcome measure, the response variable was calculated as the change given by subtracting the end-of-treatment value (i.e after 6 weeks of that cereal) from baseline (time = 0) irrespective of whether the cereal was consumed between 0 and 6 weeks or 6 and 12 weeks of the intervention period. This ensured that baseline values were corrected for in the analysis. This was done to remove any influence of the preceding treatment on the change in each outcome measure between 6 and 12 weeks.

The BMI values calculated at baseline (time = 0) were used in this analysis.

The results of this analysis included the effect of cereal, and cereal\*oil, cereal\*BMI, cereal\*age and cereal\*gender interactions on each of the outcome measures. The effects of oil, age, BMI and gender on all outcome measures were also determined.

SPSS Version 11.5 was used for all statistical analysis (SPSS Inc, Chicago, USA) and a p-value less than 0.05 was considered statistically significant while a p-value between 0.05 and 0.09 was considered a trend. All data are presented as mean  $\pm$  standard error of the mean (SEM).

At the time that the Omega-Soy study was conducted only one other human clinical trial had been published (Simons et al., 2000) using soy isoflavone supplementation independent of soy protein. That study was conducted in 20 healthy post-menopausal women using 80mg/d of soy isoflavones over 8 weeks and reported no significant changes in total or LDL cholesterol. As such, it was difficult to obtain relevant data to perform a power calculation and determine the required number of subjects to observe a statistically significant change in plasma total and LDL cholesterol. The sample size for

this study was therefore based on the results from the MOFO study. That study was based on a power calculation where the number of subjects to have a 95% power to detect a 0.15 mmol/L reduction in fasting plasma triglyceride concentrations (estimated 15% reduction) was calculated to be 14. In this study the total number of subjects consuming DHA or olive oil supplements was 17 and 18, respectively. The Omega-soy study used similar entry criteria to the MOFO study with the additional criteria of mildly elevated fasting plasma cholesterol in addition to fasting plasma triglyceride concentrations. Furthermore, in the MOFO study a statistically significant reduction was observed in fasting plasma triglyceride concentrations and a significant increase in LDL cholesterol was observed with both 1.1 and 2.2g/d of DHA supplementation. It was therefore decided that these subject numbers would be sufficient to detect changes in fasting plasma triglyceride and LDL cholesterol concentrations in the Omega-Soy study with DHA supplementation.

### 3.4 Results

Four subjects were excluded from parts of the data set as a result of being more than 2 standard deviations above or below the mean. Four subjects were only able to complete the first six weeks of the study and two subjects dropped out of the study for personal reasons prior to completing the first six weeks. Subsequently data from thirty-eight subjects were available at baseline and six weeks, and data from thirty-three subjects were available at twelve weeks for use in statistical analysis.

#### 3.4.1 Anthropometric results

Body weight was similar between the four groups at the commencement of the intervention period (**Table 3.4**) and remained unchanged in all groups after six weeks. In both DHA groups (DHAc-s  $1.2 \pm 0.2$  kg,  $t = -4.88$ ,  $p = 0.002$ ; DHAs-c  $1.0 \pm 0.4$  kg,  $t = -2.39$ ,  $p = 0.04$ ) body weight increased significantly between 6 and 12 weeks while remaining stable in the olive oil groups. Despite the increase in the DHA groups, body weight was not significantly different between the four groups after six or twelve weeks.

Percent body fat was similar between the four groups at the start of the intervention period. After six weeks, body fat increased significantly in OOc-s (increased  $1.5 \pm 0.6$  % total body fat,  $t = -2.71$ ,  $p = 0.03$ ) and in DHAc-s (increased  $1.3 \pm 0.4$  % total body fat,  $t = -3.31$ ,  $p = 0.01$ ). Percent body fat remained unchanged in all groups between six and twelve weeks. Despite the changes seen in OOc-s and DHAc-s in the first six weeks, percent body fat remained similar between the four groups after six and twelve weeks of the intervention.

Seventy-one percent of subjects reported a consistent level of physical activity at zero, six and twelve weeks of the intervention period. The remaining 29% of subjects reported changes from 'light' to 'light-moderate' or vice versa or 'light-moderate' to 'moderate'. The average activity level in OOc-s was 'light' at zero, six and twelve weeks and in the remaining three groups was 'light-moderate' at all time points. Based on these findings, body weight and percent body fat results, it can be concluded that subjects were consistent in their physical activity levels during the twelve weeks of the intervention period.

Table 3.4 Body weight at zero, six and twelve weeks of the intervention period and changes in body weight after six and twelve weeks, in the four intervention groups<sup>1-3</sup>

Groups	n	0 wks	6 wks	12 wks	0-6w	t-stat	P	6-12w	t-stat	P
		(kg)						(kg)		
OOc-s	9	85.7 ± 3.9	85.1 ± 3.8	85.7 ± 3.7	-0.6 ± 0.3	1.90	0.09	0.6 ± 0.4	-1.49	0.18
OOs-c	9	90.4 ± 5.4	91.1 ± 5.2	89.3 ± 5.4	0.7 ± 0.6	-1.13	0.29	0.4 ± 0.3	-1.18	0.28
DHAc-s	8	86.0 ± 3.1	86.1 ± 3.1	87.2 ± 3.2	0.03 ± 0.3	-0.08	0.94	1.2 ± 0.2	-4.89	0.002
DHAs-c	9	86.7 ± 4.0	87.4 ± 4.0	88.4 ± 4.3	0.6 ± 0.3	-1.85	0.10	1.0 ± 0.4	-2.39	0.04

1. Data are mean ± SEM
2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)
3. Shading denotes a statistically significant change (paired t-tests,  $p < 0.05$ )

### 3.4.2 Dietary intake

Dietary intake data at baseline after six and twelve weeks based on diet history interviews are provided in **Table 3.5** and **3.6**. At baseline, total energy intake was significantly higher ( $F = 3.021$ ,  $df = 3$ ,  $p = 0.03$ ) in DHAs-c compared to OOc-s, a difference that remained after twelve weeks; while total fat ( $F = 2.98$ ,  $df = 3$ ,  $p = 0.047$ ) and monounsaturated fat ( $F = 3.26$ ,  $df = 3$ ,  $p = 0.035$ ) intake were significantly higher in OOs-c compared to OOc-s. These latter differences were not maintained over the course of the study.

In OOc-s, monounsaturated fat intake increased by 19% ( $t = -3.66$ ,  $df = 8$ ,  $p = 0.006$ ) and protein intake decreased by 8% ( $t = 2.35$ ,  $df = 8$ ,  $p = 0.046$ ) after six weeks of olive oil supplementation. In OOs-c during the first six weeks, dietary carbohydrate ( $t = -2.70$ ,  $df = 8$ ,  $p = 0.03$ ) intake increased by 10% while total energy intake decreased by 6% ( $t = 3.67$ ,  $df = 7$ ,  $p = 0.008$ ). The change in carbohydrate intake was reversed between six and twelve weeks (**Table 3.5**).

In DHAc-s dietary polyunsaturated fat intake increased by 18% ( $t = -2.67$ ,  $df = 7$ ,  $p = 0.03$ ) and in DHAs-c total energy intake increased by 5% ( $t = 2.48$ ,  $df = 8$ ,  $p = 0.04$ ) during the first six weeks (**Table 3.6**) of the study. These changes remained after twelve weeks. Dietary polyunsaturated fat intake was significantly higher in the two DHA groups compared to OOc-s after six ( $F = 4.05$ ,  $df = 3$ , DHAc-s:  $p = 0.031$ , DHAs-c:  $p = 0.046$ ) and twelve weeks ( $F = 5.32$ ,  $df = 3$ , DHAc-s:  $p = 0.019$ , DHAs-c:  $p = 0.011$ ) and at twelve weeks, total energy intake was significantly higher ( $F = 2.83$ ,  $df = 3$ ,  $p = 0.04$ ) in DHAs-c compared to OOc-s.

Table 3.5 Dietary intake data obtained from diet histories for the two olive oil groups at zero, six and twelve weeks of the intervention period<sup>1-3</sup>

Group	Dietary component	Time 0	6 weeks	12 weeks	0 – 6wks Change	t	df	p	6 - 12 wks Change	t	df	p
OOc-s (n = 9)	Energy (KJ)	9435.7±774.3	9451.5 ± 776.3	8836.7 ± 741.9	15.9 ± 387.6	-0.41	8	1.00	- 614.8 ± 273.6	2.25	8	0.055
	Protein (g)	106.3 ± 8.9	103.6 ± 8.4	95.4 ± 6.3	- 2.8 ± 4.3	0.65	8	0.54	- 8.1 ± 3.5	2.35	8	0.046
	Total fat (g)	72.2 ± 7.4	77.9 ± 8.2	71.5 ± 7.8	5.7 ± 4.3	-1.33	8	0.22	- 6.3 ± 3.4	1.88	8	0.097
	Saturated fat (g)	25.3 ± 3.0	26.2 ± 3.2	23.3 ± 2.6	0.9 ± 1.9	-0.48	8	0.65	- 2.9 ± 1.5	2.02	8	0.078
	Monounsaturated fat (g)	26.2 ± 2.5	31.0 ± 3.0	29.1 ± 3.2	4.9 ± 1.3	-3.66	8	0.006	- 2.0 ± 1.4	1.41	8	0.195
	Polyunsaturated fat (g)	13.4 ± 2.2	13.2 ± 2.1	12.5 ± 2.1	- 0.2 ± 1.0	0.23	8	0.82	- 0.7 ± 0.5	1.49	8	0.175
	Cholesterol (mg)	242.3 ± 20.3	231.6 ± 20.5	212.3 ± 15.5	- 10.7 ± 17.7	0.60	8	0.56	- 19.3 ± 15.0	1.29	8	0.234
	Carbohydrate (g)	281.7 ± 27.8	279.1 ± 24.5	265.4 ± 26.5	- 2.6 ± 16.8	0.16	8	0.88	-13.7 ± 10.4	1.31	8	0.23
	Fibre (g)	28.6 ± 2.6	26.2 ± 1.9	23.8 ± 1.9	- 2.4 ± 2.3	1.04	8	0.11	- 2.4 ± 1.1	2.06	8	0.42
	Alcohol (g)	6.8 ± 1.9	4.9 ± 1.4	4.2 ± 1.6	- 1.9 ± 1.0	1.82	8	0.33	- 0.7 ± 0.8	0.85	8	0.07
OOs-c (n = 9)	Energy (KJ)	11336.0 ± 656.6	11561.2 ± 568.7	10832.5 ± 656.3	225.2 ± 460.0	-0.49	8	0.64	-693.1 ± 174.1	3.67	7	0.008
	Protein (g)	119.2 ± 8.5	115.3 ± 8.8	112.6 ± 7.4	- 3.9 ± 4.7	0.82	8	0.44	- 0.4 ± 3.2	0.12	7	0.91
	Total fat (g)	105.9 ± 11.1	101.2 ± 6.6	93.4 ± 6.8	- 4.7 ± 7.3	0.64	8	0.54	- 5.0 ± 3.1	1.60	7	0.16
	Saturated fat (g)	36.7 ± 4.6	34.5 ± 2.5	30.8 ± 1.9	- 2.2 ± 3.5	0.63	8	0.55	- 1.8 ± 1.4	1.26	7	0.25
	Monounsaturated fat (g)	42.4 ± 5.9	41.7 ± 3.6	38.4 ± 3.9	- 0.7 ± 3.0	0.23	8	0.82	- 2.8 ± 1.3	2.20	7	0.06
	Polyunsaturated fat (g)	17.3 ± 1.6	16.2 ± 1.6	15.7 ± 1.5	- 1.1 ± 0.9	1.28	8	0.24	- 0.4 ± 1.1	0.34	7	0.75
	Cholesterol (mg)	310.6 ± 34.1	288.6 ± 21.9	278.9 ± 23.8	- 22.1 ± 25.6	0.86	8	0.41	- 0.1 ± 10.7	0.01	7	0.99
	Carbohydrate (g)	297.6 ± 20.4	328.6 ± 23.6	307.1 ± 24.8	31.0 ± 11.4	-2.70	8	0.03	- 27.7 ± 7.2	3.86	7	0.006
	Fibre (g)	31.7 ± 2.4	29.6 ± 3.1	30.4 ± 2.6	- 2.1 ± 1.6	1.33	8	0.22	0.01 ± 2.0	-0.01	7	1.00
	Alcohol (g)	13.8 ± 3.1	12.2 ± 3.5	10.8 ± 2.3	- 1.7 ± 2.6	0.65	8	0.54	0.2 ± 4.1	-0.04	7	1.00

1. Data are mean ± SEM

2. OO (olive oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by control cereal consumption).

3. Shading represents statistically significant changes (paired-samples t-tests,  $p < 0.05$ )

Table 3.6 Dietary intake data obtained from diet histories in the two DHA groups at zero, six and twelve weeks of the intervention period <sup>1-3</sup>

Group	Dietary component	0 weeks	6 weeks	12 weeks	0 – 6wks Change	t	df	p	6 – 12wks Change	t	df	p
DHAc-s (n = 8)	Energy (KJ)	10651.1 ± 736.3	10437.6 ± 93.2	10249.8 ± 807.1	-213.5 ± 612.1	0.35	7	0.74	-324.1 ± 157.2	2.06	6	0.09
	Protein (g)	105.8 ± 7.0	103.3 ± 7.2	100.5 ± 6.3	-2.5 ± 5.3	0.47	7	0.65	-3.9 ± 2.5	1.53	6	0.18
	Total fat (g)	88.8 ± 8.3	91.3 ± 7.1	90.5 ± 6.3	2.5 ± 6.1	-0.42	7	0.69	0.9 ± 5.0	0.18	6	0.86
	Saturated fat (g)	30.1 ± 3.1	30.3 ± 2.2	30.5 ± 2.0	0.2 ± 2.4	-0.07	7	0.95	0.3 ± 1.1	-0.26	6	0.80
	Monounsaturated fat (g)	31.2 ± 2.7	31.0 ± 2.7	30.7 ± 2.8	-0.2 ± 3.1	0.05	7	0.96	0.04 ± 2.9	-0.02	6	0.99
	Polyunsaturated fat (g)	19.3 ± 4.5	22.7 ± 3.8	22.5 ± 3.5	3.4 ± 1.2	-2.76	7	0.03	-0.9 ± 1.4	0.60	6	0.57
	Cholesterol (mg)	244.9 ± 20.2	235.6 ± 12.8	226.5 ± 11.8	-9.8 ± 16.4	0.60	7	0.57	-12.0 ± 8.9	1.35	6	0.23
	Carbohydrate (g)	298.4 ± 21.2	286.2 ± 23.3	273.0 ± 29.7	-12.3 ± 23.9	0.51	7	0.62	-18.6 ± 10.2	1.83	6	0.12
	Fibre (g)	31.4 ± 3.2	28.8 ± 3.2	27.6 ± 3.8	-2.6 ± 1.9	1.36	7	0.22	-1.8 ± 0.8	2.28	6	0.06
	Alcohol (g)	20.5 ± 8.6	17.0 ± 5.4	21.1 ± 9.0	-3.4 ± 3.8	0.91	7	0.39	3.3 ± 4.3	-0.77	6	0.47
DHAs-c (n = 9)	Energy (KJ)	12374.5 ± 702.5	11736.4 ± 808.7	11723.1 ± 775.5	- 638.1 ± 256.9	2.48	8	0.04	-13.2 ± 349.4	0.04	8	0.97
	Protein (g)	130.7 ± 10.5	127.9 ± 9.9	1203. ± 9.5	- 2.8 ± 6.5	0.43	8	0.68	- 7.6 ± 4.4	1.75	8	0.12
	Total fat (g)	101.0 ± 7.9	96.0 ± 9.5	98.5 ± 10.1	- 5.0 ± 6.9	0.74	8	0.48	2.5 ± 3.3	-0.76	8	0.47
	Saturated fat (g)	31.7 ± 2.8	34.1 ± 4.7	33.9 ± 4.1	2.4 ± 2.2	-1.09	8	0.31	- 0.18 ± 1.3	0.13	8	0.90
	Monounsaturated fat (g)	38.4 ± 4.0	31.7 ± 3.9	34.1 ± 4.2	- 6.7 ± 3.4	2.00	8	0.08	2.4 ± 1.6	-1.53	8	0.17
	Polyunsaturated fat (g)	21.7 ± 2.1	21.9 ± 1.2	22.5 ± 1.7	0.15 ± 2.1	-0.71	8	0.95	0.62 ± 1.1	-0.57	8	0.59
	Cholesterol (mg)	285.4 ± 33.3	272.4 ± 39.2	265.2 ± 32.0	- 13.0 ± 28.2	0.46	8	0.66	- 7.2 ± 14.9	0.49	8	0.64
	Carbohydrate (g)	365.3 ± 24.0	338.6 ± 21.5	343.9 ± 21.8	- 26.7 ± 12.8	2.08	8	0.07	5.3 ± 12.2	-0.43	8	0.68
	Fibre (g)	34.8 ± 3.1	31.9 ± 2.5	31.4 ± 2.7	- 2.9 ± 2.0	1.60	8	0.15	- 0.5 ± 1.9	0.52	8	0.62
	Alcohol (g)	10.7 ± 6.4	11.4 ± 6.0	9.5 ± 4.4	- 0.5 ± 1.8	-0.37	8	0.72	0.76 ± 0.9	0.98	8	0.735

1. Data are mean ± SEM
2. DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by control cereal consumption).
3. Shading represents statistically significant changes (paired-samples t-tests,  $p < 0.05$ )

### 3.4.3 Initial plasma and urinary isoflavones

The levels of urinary and plasma isoflavone concentrations for each group at the commencement of the study intervention period are shown in **Table 3.7** and are indicative of compliance to soy cereal consumption.

Subjects commenced the intervention phase of the study following a 3-week wash-in period during which they were asked not to consume any soy-containing foods. At the commencement of the study intervention phase the mean concentration of plasma genistein across the four groups was  $8.6 \pm 2.8$  ng/mL with no difference being detected between groups. Plasma daidzein was not detected in any group, however, detectable levels of plasma equol were found in some subjects in all four groups, with the mean concentration of all subjects at the start of the intervention period being  $31.7 \pm 7.9$  ng/mL. Plasma equol concentrations in each group were similar.

Mean 24hr urine mean isoflavone concentrations provides a more generalized picture of isoflavone consumption over the previous 48 hours than do plasma concentrations which fluctuate more widely with meals (Kelly et al., 1995; Setchell et al., 2003a; Setchell et al., 2003b). In mean 24hr urine samples daidzein was detected in all groups, with the mean for the four groups being  $2.8 \pm 0.4$  µg/mL. Genistein was also detected at very low concentrations in all groups with the mean concentration  $0.11 \pm 0.05$  µg/mL. Equol was not detected in the urine of any subjects during the wash-in period.

**Table 3.7** Concentration of daidzein, genistein and equol in plasma and urine for each group and all groups combined at the commencement of the intervention period <sup>1</sup>.

Groups <sup>2</sup>	n <sup>3</sup>	Plasma (ng/mL)			Urine (µg/mL)		
		Daidzein <sup>4</sup>	Genistein	Equol	Daidzein	Genistein	Equol <sup>4</sup>
OOc-s	9	nd	$17.1 \pm 7.2$	$30.6 \pm 15.6$	$1.8 \pm 0.4$	$0.02 \pm 0.0$	nd
OOs-c	9	nd	$8.4 \pm 9.9$	$42.9 \pm 18.3$	$3.0 \pm 1.0$	$0.31 \pm 0.2$	nd
DHAc-s	8	nd	$0.00 \pm 0.00$	$9.1 \pm 9.1$	$1.8 \pm 0.4$	$0.04 \pm 0.0$	nd
DHAs-c	10	nd	$8.0 \pm 4.1$	$40.6 \pm 17.2$	$4.3 \pm 0.9$	$0.07 \pm 0.0$	nd
All Gps	36	nd	$8.6 \pm 2.8$	$31.7 \pm 7.9$	$2.8 \pm 0.4$	$0.11 \pm 0.0$	nd

1. Data are mean  $\pm$  SEM

2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption).

3. Number of subjects

4. nd = not detected



#### 3.4.4 Isoflavones following soy cereal consumption

The plasma and 24hr urine concentrations of daidzein, genistein and equol at the start and end of the soy cereal intervention period were similar between the four groups (**Table 3.8** and **3.9**).

Plasma daidzein concentrations increased significantly with soy cereal consumption in OOs-c ( $t = -3.01$ ,  $df = 8$ ,  $p = 0.02$ ) and almost significantly ( $t = -2.26$ ,  $df = 7$ ,  $p = 0.058$ ) in DHAc-s. Plasma daidzein increased by a similar magnitude in DHAs-c, but due to high within-group variability, this increase was not statistically significant ( $t = -1.84$ ,  $df = 9$ ,  $p = 1.0$ ). In OOc-s only very low daidzein concentrations were detected in plasma following soy cereal consumption (**Figure 3.3**); while the concentration of plasma daidzein appeared lower ( $10.1 \pm 6.7$  ng/mL) in OOc-s than in any of the other three groups (mean of the other three groups:  $65.6 \pm 28.7$  ng/mL) there were no differences in plasma daidzein concentrations between the four groups ( $F = 1.065$ ,  $df = 3$ ,  $p = 0.38$ ) after soy cereal.

Plasma genistein increased significantly in the two DHA groups (DHAc-s:  $t = -2.70$ ,  $df = 7$ ,  $p = 0.03$ ; DHAs-c:  $t = -2.49$ ,  $df = 9$ ,  $p = 0.04$ ) and Group OOs-c ( $t = -3.23$ ,  $df = 8$ ,  $p = 0.01$ ) and was near significance ( $t = -2.17$ ,  $df = 8$ ,  $p = 0.06$ ) in OOc-s with the intake of soy cereal (**Figure 3.4**).

Plasma equol increased significantly in OOs-c ( $75.6 \pm 27.8$  ng/mL,  $t = -2.72$ ,  $df = 8$ ,  $p = 0.03$ ) and there was a trend toward a similar increase in DHAs-c ( $75.7 \pm 69.8$ ,  $t = -1.08$ ,  $df = 9$ ,  $p = 0.31$ ) although the variability was too high for the increase to be statistically significant. Plasma equol remained unchanged in the two groups (OOc-s and DHAc-s) that consumed soy during the second six weeks of the intervention period (**Figure 3.5**).

Table 3.8 Plasma isoflavone concentrations in the four groups before and after six weeks of soy cereal consumption<sup>1-4</sup>

Groups	Start of soy period	End of soy period	Change	t	p	df
<b>Daidzein plasma (ng/mL)</b>						
OOc-s	nd	10.1 ± 6.7	10.1 ± 6.7	-1.51	0.17	8
OOs-c	nd	65.6 ± 21.4	65.6 ± 21.4	-3.01	0.02	8
DHAc-s	nd	66.5 ± 29.4	66.5 ± 29.4	-2.26	0.06	7
DHAs-c	nd	64.7 ± 35.2	64.7 ± 35.2	-1.84	1.00	9
<b>Genistein plasma (ng/mL)</b>						
OOc-s	6.3 ± 6.3	34.2 ± 11.7	28.0 ± 12.9	-2.17	0.06	8
OOs-c	8.4 ± 6.9	61.9 ± 20.1	53.5 ± 16.6	-3.23	0.01	8
DHAc-s	0.0 ± 0.0	73.9 ± 27.3	73.9 ± 27.3	-2.70	0.03	7
DHAs-c	8.0 ± 4.1	43.6 ± 17.5	35.6 ± 14.3	-2.49	0.04	9
<b>Equol plasma (ng/mL)</b>						
OOc-s	30.1 ± 10.7	30.1 ± 17.9	-0.03 ± 23.2	0.00	1.00	8
OOs-c	42.9 ± 18.3	118.6 ± 33.5	75.6 ± 27.8	-2.72	0.03	8
DHAc-s	18.5 ± 9.7	5.1 ± 5.1	-13.4 ± 10.3	1.31	0.23	7
DHAs-c	40.6 ± 17.2	116.2 ± 84.8	75.7 ± 69.8	-1.08	0.31	9

1. Data are mean ± SEM
2. OO (olive oil, DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption).
3. nd = not detected
4. Shading represents statistically significant changes (paired-samples t-tests,  $p < 0.05$ )

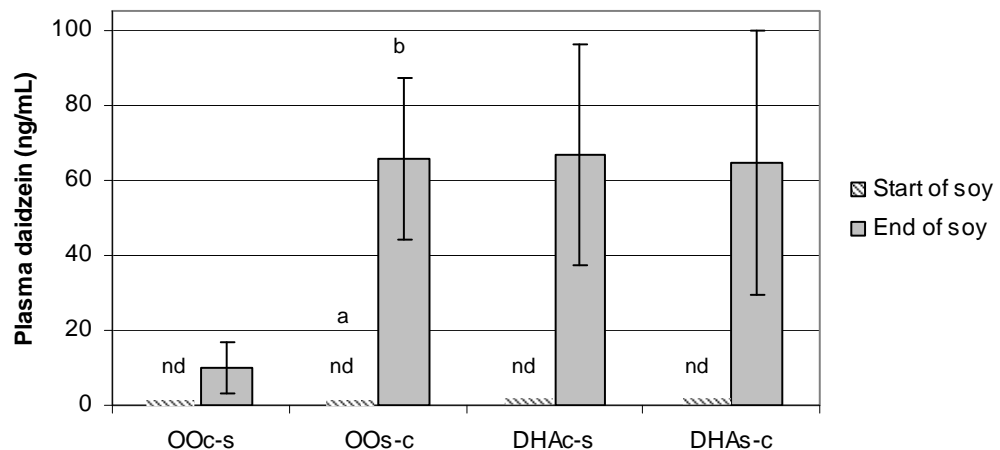


Figure 3.3 Plasma daidzein concentrations in the four groups before and after soy cereal consumption

1. nd = not detected
2. Different subscripts depict statistically significant differences
3. OO (olive oil, DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
4. OOc-s (n=9), OOs-c (n=9), DHAc-s (n=7), DHAs-c (n=10)

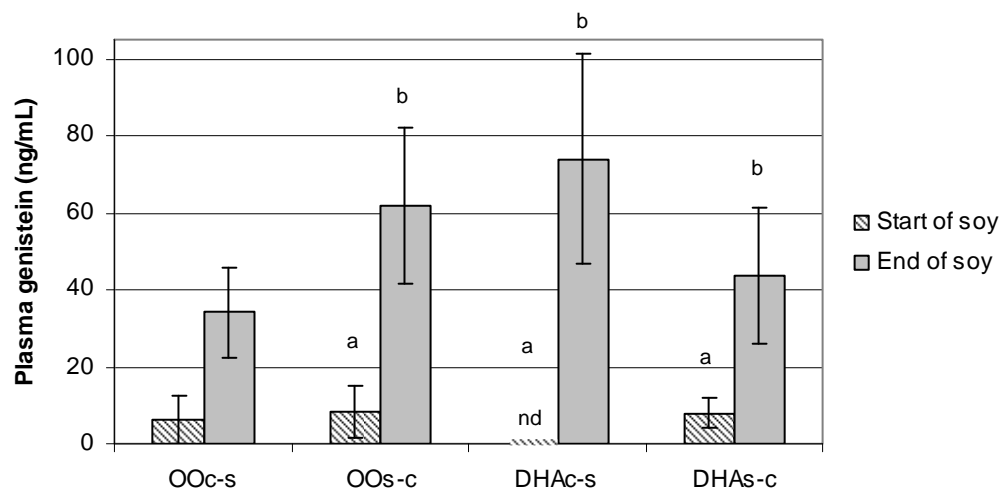


Figure 3.4 Plasma genistein concentrations in the four groups before and after soy cereal consumption

1. nd = not detected
2. Different subscripts depict statistically significant differences
3. OO (olive oil, DHA (DHA-rich oil, c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
4. OOc-s (n=9), OOs-c (n=9), DHAc-s (n=7), DHAs-c (n=10)

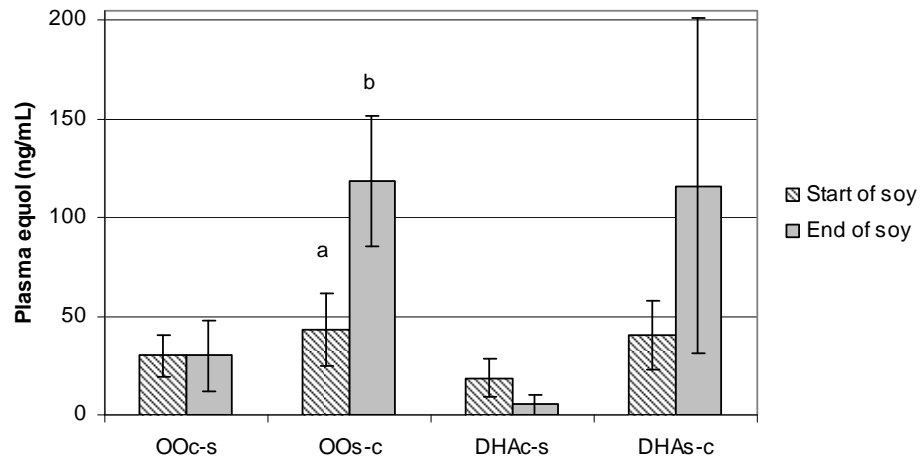


Figure 3.5 Plasma equol concentrations in the four groups before and after soy cereal consumption

1. Equol concentrations were calculated using data from all subjects in each group. At the commencement of the soy cereal intervention: 5, 4, 3 and 6 subjects, respectively, from OOc-s, OOs-c, DHAc-s and DHAs-c had detectable concentrations of equol in plasma. At the completion of the soy cereal intervention, equol concentrations were detected in 3, 8, 1 and 5 subjects in OOc-s, OOs-c, DHAc-s and DHAs-c, respectively.
2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
3. Different subscripts depict a statistically significant difference at  $p < 0.05$

In 24hr urine samples daidzein concentrations increased significantly and similarly in all four groups after six weeks of soy cereal consumption (**Table 3.9**). There was a trend toward an increase in urinary genistein in all groups however only the increase observed in DHAc-s ( $9.8 \pm 3.8 \mu\text{g/mL}$ ) reached statistical significance ( $t = -3.27$ ,  $df = 7$ ,  $p = 0.04$ ). Unlike the plasma findings, equol was detected in the urine samples of only five subjects after soy cereal consumption: one in OOC-s and four in OOs-c, representing 13.8 percent of the study population. Subsequently there was no significant increase in urine equol concentrations in any group; there was a trend toward an increase in OOs-c ( $0.8 \pm 0.4$ ,  $t = -1.94$ ,  $df = 8$ ,  $p = 0.09$ ) but no equol was detected in urine samples from any subjects in the two DHA groups following soy cereal intake.

The mean percent recovery from 24hr urine samples of the estimated dietary consumption of daidzein, genistein and total isoflavones was similar between the four groups (**Table 3.10**).

Table 3.9 Concentrations of isoflavones in 24hr urine samples in the four groups before and after six weeks of soy cereal consumption<sup>1-3</sup>

Groups	Start of soy period	End of soy period	Change	t	p	df
<b>Daidzein urine (<math>\mu\text{g/mL}</math>)</b>						
OOC-s	$3.3 \pm 1.4$	$8.1 \pm 2.2$	$4.8 \pm 2.1$	-2.30	0.05	8
OOs-c	$3.0 \pm 0.9$	$9.3 \pm 1.2$	$6.4 \pm 1.2$	-5.27	0.00	8
DHAc-s	$3.0 \pm 0.4$	$12.9 \pm 4.0$	$9.8 \pm 3.8$	-2.56	0.04	7
DHAs-c	$4.3 \pm 0.9$	$11.1 \pm 3.0$	$6.9 \pm 3.0$	-2.27	0.049	9
<b>Genistein urine (<math>\mu\text{g/mL}</math>)</b>						
OOC-s	$0.1 \pm 0.1$	$0.2 \pm 0.1$	$0.1 \pm 0.1$	-0.81	0.44	8
OOs-c	$0.3 \pm 0.2$	$0.9 \pm 0.4$	$0.6 \pm 0.3$	-2.07	0.07	8
DHAc-s	$0.04 \pm 0.04$	$1.0 \pm 0.3$	$1.0 \pm 0.3$	-3.27	0.01	7
DHAs-c	$0.07 \pm 0.04$	$0.6 \pm 0.3$	$0.5 \pm 0.3$	-1.73	0.12	9
<b>Equol urine (<math>\mu\text{g/mL}</math>)</b>						
OOC-s	$0.01 \pm 0.01$	$1.1 \pm 1.1$	$1.1 \pm 1.1$	-0.99	0.35	8
OOs-c	$0.0 \pm 0.0$	$0.83 \pm 0.43$	$0.8 \pm 0.4$	-1.94	0.09	8
DHAc-s	$0.03 \pm 0.03$	$0.0 \pm 0.0$	$-0.03 \pm 0.03$	1.00	0.35	7
DHAs-c	$0.0 \pm 0.0$	$0.0 \pm 0.0$	$0.0 \pm 0.0$	1.68	0.13	9

1. Data are mean  $\pm$  SEM
2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption).
3. Shading represents statistically significant changes (paired-samples t-tests,  $p < 0.05$ )

Table 3.10 Estimated percent of total isoflavones consumed that were recovered in urine<sup>1-2</sup>

		Daidzein	Genistein	Total Isoflavones
Groups	n	Percent recovery in 24hr urine (%)		
OOc-s	9	43.5	6.2	35.4
OOs-c	9	41.6	15.7	36.0
DHAc-s	8	56.7	14.9	47.7
DHAs-c	9	51.8	7.0	42.1
All Groups	35	48.2	10.8	40.1

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption).
2. n = number of subjects

A 3-factor ANOVA detected a significant time effect for urine and plasma concentrations of daidzein and genistein over the six week soy cereal intervention period, however, there was no time x oil, time x order (of soy cereal consumption) interaction, or between group oil or order effects detected (**Table 3.11**). These findings suggest that the type of oil supplementation and order of soy cereal consumption did not significantly influence the change in urine or plasma daidzein or genistein concentrations after soy cereal consumption. A one-way ANOVA using oil or sequence as the between group factor, also found no significant differences in urine or plasma concentrations of daidzein, genistein or equol with soy cereal consumption, confirming that oil supplementation and order of soy cereal consumption had no measurable effect on these parameters.

Table 3.11 Results from a 3 Factor ANOVA for plasma and urine concentrations of daidzein, genistein and equol using type of oil supplementation and order of soy cereal consumption as between group factors and time as the within group factor<sup>1-4</sup>

	Plasma			Urine		
	Daidzein	Genistein	Equol	Daidzein	Genistein	Equol
time	0.001	< 0.001	0.141	< 0.001	< 0.001	0.138
time x oil	0.327	0.419	0.866	0.371	0.179	0.124
time x order <sup>1</sup>	0.317	0.684	0.073	0.879	0.986	0.848
Between Group effects						
oil	0.327	0.713	0.769	0.282	0.914	0.131
order <sup>1</sup>	0.317	0.913	0.081	0.853	0.592	0.788
oil x order	0.311	0.242	0.826	0.917	0.143	0.830

1. The term oil refers to the type of oil supplementation – olive oil or DHA-rich oil.
2. The term order refers to the order of soy cereal consumption. For OOc-s and DHAc-s the soy cereal was consumed in the second six weeks of the intervention period, while in OOs-c and DHAs-c the soy cereal was consumed in the first six weeks.
3. OOc-s: n = 9; OOs-c: n = 9; DHAc-s: n = 8; DHAs-c: n = 10, n = 36 in total.
4. Shading represents statistically significant changes (paired-samples t-tests,  $p < 0.05$ )

### 3.4.5 Erythrocyte membrane fatty acids

There were no differences in erythrocyte membrane fatty acid composition between the four groups (**Table 3.12**) at baseline. In the olive oil groups, there was no change in erythrocyte membrane DHA, DPA, EPA, total omega-3 fatty acids, Omega-3 Index or omega-6: omega-3 ratio after six or twelve weeks.

Erythrocyte membrane DHA was similar between the two DHA groups and significantly higher than in the olive oil groups after six ( $F = 41.84$ ,  $df = 3$ ,  $p < 0.001$ ) and twelve ( $F = 172.11$ ,  $df = 3$ ,  $p < 0.001$ ) weeks of DHA oil supplementation (**Figure 3.6**). Erythrocyte membrane DHA increased by 70 percent, from  $4.7 \pm 0.2$  to  $8.1 \pm 0.3$  percent of total fatty acids after six weeks and by a further 16 percent to  $9.6 \pm 0.2$  percent of total fatty acids, between six and twelve weeks with DHA-rich oil supplementation (**Table 3.12**).

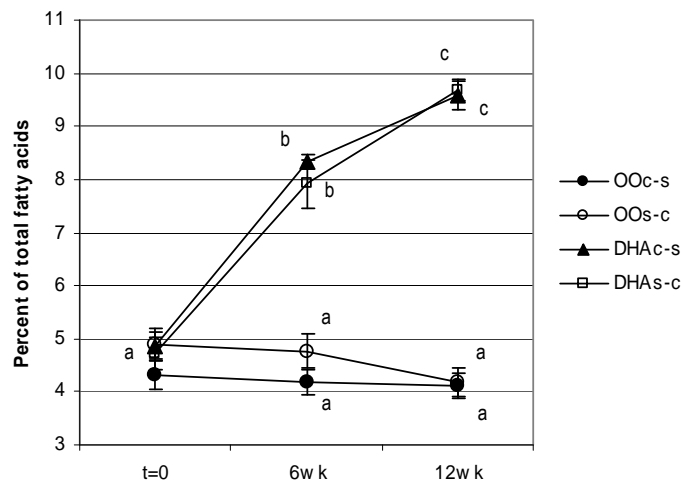


Figure 3.6 Erythrocyte membrane DHA in the four groups before, and after six and twelve weeks of the intervention period<sup>1-2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA.

Table 3.12 Erythrocyte membrane omega-3 fatty acid content in the four groups before and after six and twelve weeks of either olive oil or DHA-rich oil supplementation<sup>1-3</sup>

	T=0	T=6w	T=12w	6w-0	t	df	p	12w-6w	t	df	p
<b>DHA</b>	Percent of total fatty acids										
OOc-s	4.33 ± 0.30	4.18 ± 0.25	4.11 ± 0.23	-0.15 ± 0.14	1.06	8	0.32	-0.07 ± 0.10	0.63	8	0.55
OOs-c	4.89 ± 0.30	4.77 ± 0.31	4.17 ± 0.27	-0.13 ± 0.08	1.59	8	0.15	-0.68 ± 0.35	1.96	7	0.09
DHAc-s	4.86 ± 0.27	8.33 ± 0.13	9.57 ± 0.27	3.47 ± 0.31	-11.09	7	<0.001	1.22 ± 0.19	-6.31	6	0.001
DHAs-c	4.71 ± 0.30	7.92 ± 0.46	9.67 ± 0.20	3.21 ± 0.36	-8.96	9	<0.001	1.34 ± 0.16	-8.61	8	<0.001
<b>EPA</b>											
OOc-s	0.77 ± 0.08	0.73 ± 0.06	0.72 ± 0.05	-0.05 ± 0.03	1.61	8	0.15	-0.01 ± 0.03	0.30	8	0.77
OOs-c	0.94 ± 0.15	1.14 ± 0.41	1.11 ± 0.43	-0.40 ± 0.44	0.73	8	0.49	0.41 ± 0.46	-0.31	7	0.11
DHAc-s	0.85 ± 0.08	1.03 ± 0.08	0.93 ± 0.08	0.18 ± 0.02	-7.72	7	<0.001	-0.22 ± 0.13	2.93	6	0.03
DHAs-c	0.77 ± 0.08	0.97 ± 0.04	1.04 ± 0.05	0.20 ± 0.06	-3.04	9	0.01	0.06 ± 0.04	-1.23	8	0.25
<b>DPA</b>											
OOc-s	2.53 ± 0.15	2.51 ± 0.15	2.46 ± 0.15	-0.02 ± 0.06	0.22	8	0.83	-0.06 ± 0.03	2.03	8	0.08
OOs-c	2.65 ± 0.37	2.60 ± 0.37	2.64 ± 0.38	0.40 ± 0.44	1.88	8	0.10	0.49 ± 0.46	-1.51	7	0.17
DHAc-s	2.61 ± 0.10	1.76 ± 0.08	1.19 ± 0.07	-0.86 ± 0.07	11.64	7	<0.001	-0.72 ± 0.13	13.98	6	<0.001
DHAs-c	2.43 ± 0.08	1.82 ± 0.13	1.49 ± 0.14	-0.61 ± 0.13	8.41	9	<0.001	-0.47 ± 0.20	3.13	8	0.01
<b>Total omega-3 fatty acids</b>											
OOc-s	7.65 ± 0.44	7.43 ± 0.29	7.29 ± 0.30	-0.21 ± 0.19	1.12	8	0.30	-0.15 ± 0.11	1.30	8	0.23
OOs-c	8.33 ± 0.47	8.05 ± 0.42	7.52 ± 0.25	-0.27 ± 0.15	1.83	8	0.10	-0.66 ± 0.32	2.08	7	0.08
DHAc-s	8.32 ± 0.28	11.12 ± 0.17	11.69 ± 0.33	2.80 ± 0.24	-11.61	7	<0.001	0.54 ± 0.22	-2.45	6	0.050
DHAs-c	7.96 ± 0.31	10.60 ± 0.41	12.08 ± 0.25	2.66 ± 0.37	-7.10	9	<0.001	1.12 ± 0.21	-5.44	8	0.001
<b>Omega-6: omega-3 ratio</b>											
OOc-s	3.40 ± 0.22	3.48 ± 0.15	3.60 ± 0.17	0.08 ± 0.10	-0.74	8	0.48	0.12 ± 0.07	-1.76	8	0.12
OOs-c	3.01 ± 0.19	3.20 ± 0.21	3.35 ± 0.13	0.16 ± 0.08	-1.96	7	0.09	0.13 ± 0.15	-0.88	6	0.42
DHAc-s	2.95 ± 0.09	2.04 ± 0.04	1.88 ± 0.07	-0.91 ± 0.09	10.36	7	<0.001	-0.15 ± 0.04	3.91	6	0.008
DHAs-c	3.18 ± 0.14	2.21 ± 0.12	1.84 ± 0.05	-0.97 ± 0.14	6.99	9	<0.001	-0.26 ± 0.04	6.96	8	<0.001
<b>Omega-3 Index</b>											
OOc-s	5.12 ± 0.32	4.92 ± 0.23	4.84 ± 0.23	-0.20 ± 0.23	1.21	8	0.26	-0.08 ± 0.09	0.85	8	0.42
OOs-c	5.83 ± 0.44	5.66 ± 0.41	4.97 ± 0.28	-0.17 ± 0.12	1.42	8	0.19	-0.81 ± 0.40	2.05	7	0.08
DHAc-s	5.71 ± 0.32	9.36 ± 0.15	10.50 ± 0.29	3.66 ± 0.31	-11.86	7	<0.001	1.12 ± 0.21	-5.36	6	0.002
DHAs-c	5.52 ± 0.34	8.89 ± 0.46	10.70 ± 0.23	3.37 ± 0.39	-8.56	9	<0.001	1.39 ± 0.18	-7.74	8	<0.001

1. Data are mean ± SEM
2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)
3. Shading represents statistically significant changes (paired samples t-tests,  $p < 0.05$ )

Eicosapentaenoic acid accounted for only 0.83 percent of total erythrocyte membrane fatty acids at baseline when averaged across the four groups. Erythrocyte membrane EPA increased similarly and by approximately 23% from  $0.80 \pm 0.05$  to  $0.99 \pm 0.04$  percent of total fatty acids in the two DHA groups after six weeks however, this increased erythrocyte membrane EPA was similar to that seen in the two olive oil groups after six weeks (**Figure 3.7**). Between six and twelve weeks, erythrocyte membrane EPA decreased by a very small amount ( $-0.22 \pm 0.13$  percent of total fatty acids,  $F = 2.93$ ,  $df = 6$ ,  $p = 0.026$ ) in DHAc-s, however, after twelve weeks EPA was significantly higher ( $F = 4.57$ ,  $df = 3$ ,  $p = 0.009$ ) as a percent of total fatty acids in DHAs-c than OOc-s.

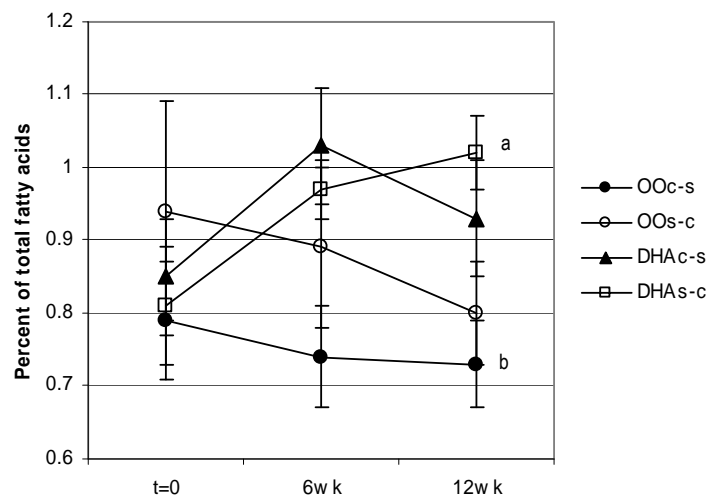


Figure 3.7 Erythrocyte membrane EPA in the four groups before, and after six and twelve weeks of oil supplementation<sup>1,2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA.



In contrast to DHA and EPA changes, erythrocyte membrane DPA decreased by approximately one third of baseline values in both DHA groups after six weeks ( $t = 13.30$ ,  $df = 17$ ,  $p < 0.001$ ) and continued to decrease by a similar proportion between six and twelve weeks ( $t = 6.28$ ,  $df = 15$ ,  $p < 0.01$ ) (**Table 3.12**). Erythrocyte membrane DPA was similar between the two DHA groups and was significantly lower ( $p < 0.001$ ) than erythrocyte membrane DPA in the two olive oil groups, after six ( $F = 16.05$ ,  $df = 3$ ,  $p < 0.001$ ) and twelve weeks ( $F = 31.38$ ,  $df = 3$ ,  $p < 0.001$ ) (**Figure 3.8**).

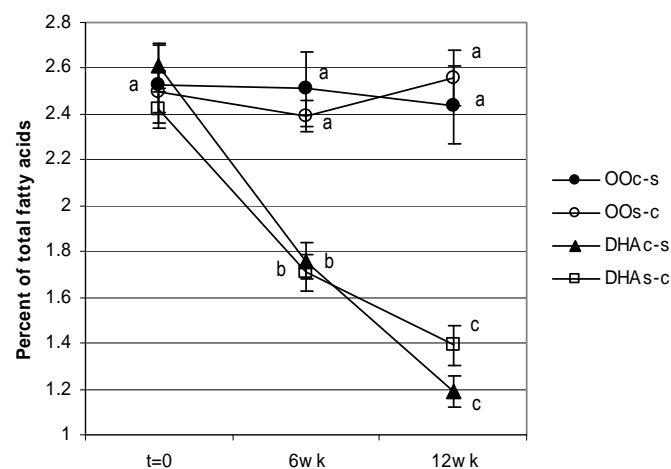


Figure 3.8 Erythrocyte membrane DPA in the four groups before, and after six and twelve weeks of oil supplementation <sup>1-2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA.

Levels of erythrocyte membrane alpha-linolenic acid (ALA) were only detectable in two out of eighteen subjects in OOC-s and OOS-c, and in twelve out of eighteen subjects in DHAc-s and DHAS-c. Consequently, the mean ALA levels in DHAc-s and DHAS-c were less than 0.05 percent of total fatty acids at any given time point. Due to the small quantity of ALA present in the erythrocyte membranes, statistical analysis of ALA was not undertaken and ALA levels were not included in total omega-3 fatty acid calculations.

### 3.4.6 Total omega-3 fatty acids

After six weeks erythrocyte membrane total omega-3 fatty acids (DHA, EPA and DPA) in the two DHA groups increased by one third of baseline values ( $t = -11.91$ ,  $df = 17$ ,  $p < 0.001$ ) from  $8.11 \pm 0.21$  to  $10.83 \pm 0.24$  percent of total fatty acids. Between six and twelve weeks, the rise in total omega-3 fatty acids was much smaller ( $t = -5.29$ ,  $df = 15$ ,  $p < .0001$ ), rising to  $11.91 \pm 0.20$  percent of total fatty acids (**Table 3.12**) in the two DHA groups however, total omega-3 fatty acids were still significantly higher ( $p < 0.001$ ) in the DHA groups compared with the olive oil groups after both six ( $F = 75.65$ ,  $df = 1$ ,  $p < 0.001$ ) and twelve weeks ( $F = 258.76$ ,  $df = 1$ ,  $p < 0.001$ ) (**Figure 3.9**).

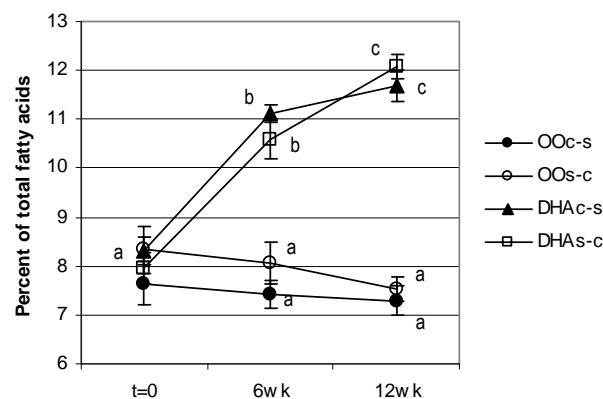


Figure 3.9 Total omega-3 fatty acids in the four groups before, and after six and twelve weeks of oil supplementation <sup>1-2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)

2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA.

#### 3.4.6.1 Omega-3 Index

At the commencement of the intervention period the Omega-3 Index averaged across the four groups was 5.5 percent of total erythrocyte membrane fatty acids. After six ( $t = -13.81$ ,  $df = 17$ ,  $p < 0.001$ ) and twelve weeks ( $-9.36$ ,  $df = 15$ ,  $p < 0.001$ ) of DHA-oil supplementation the Index increased to 9.1 and 10.6 percent of total erythrocyte membrane fatty acids (data from the two DHA groups combined), respectively (**Table 3.12**).

### 3.4.6.2 Omega-6 fatty acids

The erythrocyte membrane content of all omega-6 fatty acids was similar between the four groups at baseline (**Table 3.13**). After six weeks linoleic acid (LA) as a proportion of total erythrocyte membrane fatty acids had significantly decreased ( $t = 5.35$ ,  $df = 17$ ,  $p < 0.0001$ ) by a similar proportion (mean decrease of  $0.72 \pm 0.20$  percent of total fatty acids) in both DHA groups. In contrast, LA significantly increased ( $t = -0.367$ ,  $df = 8$ ,  $p = 0.006$ ) in OOs-c after six weeks (**Figure 3.10**) and subsequently, LA as a percentage of total membrane fatty acids was significantly lower ( $F = 5.40$ ,  $df = 3$ ,  $p < 0.02$ ) in the two DHA groups compared to OOs-c. Between six and twelve weeks, LA remained unchanged in all four groups. Erythrocyte membrane LA was significantly lower ( $F = 4.93$ ,  $df = 3$ ,  $p = 0.03$ ) in DHAs-c after twelve weeks than in both olive oil groups.

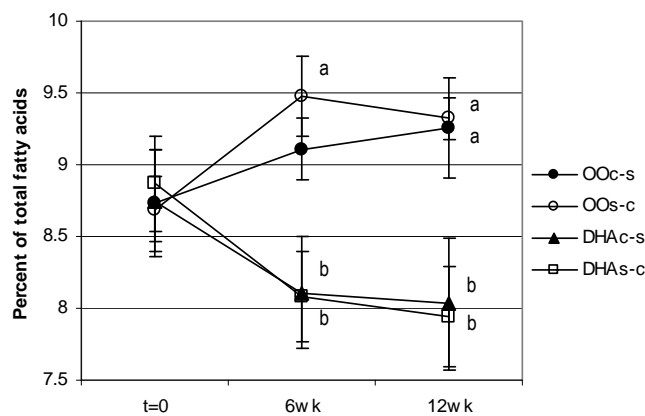


Figure 3.10 Erythrocyte membrane LA in the four groups before and after six and twelve weeks of oil supplementation <sup>1-2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)

2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA

Table 3.13 Erythrocyte membrane omega-6 fatty acid content in the four groups before and after six and twelve weeks of either olive oil or DHA-rich oil supplementation<sup>1-3</sup>

	T=0	T=6w	T=12w	6w-0	t	df	p	12w-6w	t	df	p
LA	Percent of total erythrocyte membrane fatty acids										
OOc-s	8.73 ± 0.37	9.11 ± 0.22	9.26 ± 0.35	0.38 ± 0.28	-1.35	8	0.21	0.16 ± 0.18	-0.89	8	0.40
OOs-c	8.69 ± 0.23	9.48 ± 0.28	9.32 ± 0.15	0.79 ± 0.21	-3.67	8	0.006	-0.16 ± 0.26	0.60	7	0.57
DHAc-s	8.75 ± 0.36	8.11 ± 0.39	8.03 ± 0.46	-0.64 ± 0.21	2.99	7	0.02	-0.09 ± 0.23	0.37	6	0.72
DHAs-c	8.87 ± 0.33	8.08 ± 0.31	7.94 ± 0.35	-0.80 ± 0.18	4.38	9	0.002	-0.04 ± 0.13	0.28	8	0.79
AA											
OOc-s	14.40 ± 0.23	14.34 ± 0.22	14.41 ± 0.25	-0.05 ± 0.19	0.26	8	0.80	0.07 ± 0.16	-0.43	8	0.68
OOs-c	13.74 ± 0.46	13.64 ± 0.54	13.81 ± 0.48	-0.31 ± 0.17	1.88	8	0.10	0.29 ± 0.24	-1.21	7	0.27
DHAc-s	13.63 ± 0.25	12.12 ± 0.21	11.05 ± 0.20	-1.52 ± 0.12	12.18	7	<0.001	-0.98 ± 0.13	7.52	6	<0.001
DHAs-c	13.75 ± 0.45	12.51 ± 0.42	11.46 ± 0.42	-1.24 ± 0.13	9.57	9	<0.001	-1.07 ± 0.12	9.04	8	<0.001
Total omega-6 fatty acids											
OOc-s	25.25 ± 0.41	25.25 ± 0.28	25.83 ± 0.27	0.27 ± 0.29	-0.94	8	0.38	0.30 ± 0.13	-2.33	8	0.048
OOs-c	24.39 ± 0.51	25.17 ± 0.60	25.03 ± 0.58	0.51 ± 0.30	-1.69	8	0.14	0.00 ± 0.59	0.00	7	0.36
DHAc-s	24.39 ± 0.19	22.69 ± 0.23	21.82 ± 0.32	-1.71 ± 0.14	11.86	7	<0.001	-0.78 ± 0.27	2.83	6	0.03
DHAs-c	24.90 ± 0.21	23.00 ± 0.24	22.10 ± 0.30	-1.90 ± 0.26	7.31	9	<0.001	-0.84 ± 0.15	5.49	8	0.001

1. Data are mean ± SEM
2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)
3. Shading represents statistically significant changes (paired samples t-tests,  $p < 0.05$ )

Arachidonic acid (AA) as a proportion of total fatty acids decreased significantly and similarly in both DHA groups after six weeks ( $t = 14.46$ ,  $df = 17$ ,  $p < 0.001$ ) and again between six and twelve weeks ( $t = 12.06$ ,  $df = 15$ ,  $p < 0.001$ ). Olive oil supplementation did not result in any change in AA (**Figure 3.11**).

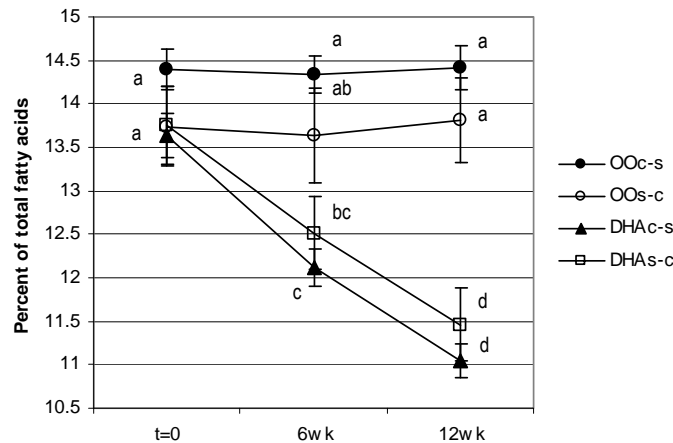


Figure 3.11 Erythrocyte membrane AA in the four groups before and after six and twelve weeks of oil supplementation<sup>1,2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA.

Overall, total erythrocyte membrane omega-6 fatty acids (LA, AA, DPA and dihomo- $\gamma$ -linolenic acid) decreased significantly and by a similar amount in both DHA groups after six ( $t = 11.67$ ,  $df = 17$ ,  $p < 0.001$ ) and twelve weeks ( $5.71$ ,  $df = 15$ ,  $p < 0.001$ ) (**Table 3.13**). The reduction seen in the first six weeks ( $-1.81 \pm 0.20$  percent of total fatty acids) however, was almost double that seen between six and twelve weeks ( $-0.81 \pm 0.21$  percent of total fatty acids). In contrast to the reductions seen with DHA oil supplementation, total erythrocyte membrane omega-6 fatty acids increased in OOc-s ( $t = -2.33$ ,  $df = 8$ ,  $p = 0.048$ ) between six and twelve weeks of the intervention period (**Figure 3.12**).

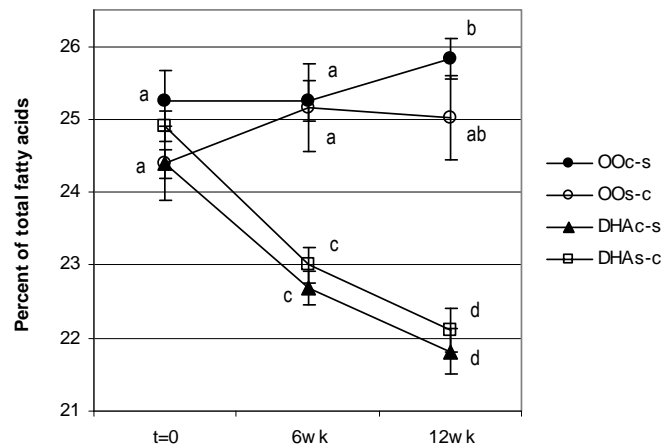


Figure 3.12 Total erythrocyte membrane omega-6 fatty acids in the four groups before and after six and twelve weeks of oil supplementation<sup>1-2</sup>

1. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in the first six weeks followed by six weeks of soy cereal consumption), s-c (soy cereal consumed in the first six weeks followed by six weeks of control cereal consumption)
2. Different subscripts depict a statistically significant between-group difference at  $p < 0.05$  as determined by ANOVA.

#### 3.4.6.3 Omega-6:omega-3 fatty acid ratio

At baseline, the erythrocyte membrane omega-6: omega-3 fatty acid ratio was similar between the four groups. After six weeks, DHA supplementation resulted in a significant reduction in the ratio from  $3.08 \pm 0.09$  to  $2.14 \pm 0.07$  percent of total fatty acids ( $t = 11.15$ ,  $df = 17$ ,  $p < 0.001$ ) and a smaller but further reduction after twelve weeks ( $-0.20 \pm 0.04$ ,  $p < 0.009$ ) (**Table 3.12**).

#### 3.4.6.4 Oleic acid (OA)

In OOC-s erythrocyte membrane OA remained unchanged after six and twelve weeks. In OOS-c, erythrocyte membrane OA significantly increased ( $t = -2.94$ ,  $df = 8$ ,  $p = 0.02$ ) after six weeks but remained unchanged between six and twelve weeks (**Table 3.14**). In contrast, erythrocyte membrane OA levels decreased ( $t = 5.79$ ,  $df = 17$ ,  $p < 0.001$ ) by a similar but small amount from  $12.94 \pm 0.19$  to  $12.33 \pm 0.18$  percent of total fatty acids in the two DHA groups in the first six weeks and remained unchanged between six and twelve weeks. Erythrocyte membrane OA was significantly higher ( $F = 5.54$ ,  $df = 3$ ,  $p = 0.003$ ) in OOS-c than DHAS-c after twelve weeks (**Table 3.14**).

#### **3.4.6.5 Saturated Fatty acids**

In DHAc-s palmitic acid as a proportion of total erythrocyte membrane fatty acids increased by a small but statistically significant ( $t = 5.60$ ,  $df = 7$ ,  $p = 0.001$ ) amount after six weeks but remained unchanged between six and twelve weeks. In OOs-c there was a trend ( $p = 0.06$ ) toward a decrease ( $t = 2.22$ ,  $df = 8$ ,  $p = 0.06$ ) in stearic acid after six weeks of olive oil supplementation and soy cereal (**Table 3.14**).

Table 3.14 Erythrocyte membrane saturated and monounsaturated fatty acid content in the four groups before and after six and twelve weeks of either olive oil or DHA-rich oil supplementation<sup>1-3</sup>

	T=0	T=6w	T=12w	6w-0	t	df	p	12w-6w	t	df	p
<b>Oleic acid</b>	<i>Percent of total fatty acids</i>										
OOc-s	13.05 ± 0.43	13.14 ± 0.30	13.23 ± 0.15	0.09 ± 0.34	-0.27	8	0.79	0.09 ± 0.20	-0.46	8	0.66
OOs-c	13.12 ± 0.48	13.49 ± 0.52	13.91 ± 0.59	0.36 ± 0.12	-2.94	8	0.02	0.31 ± 0.27	-1.15	7	0.29
DHAc-s	13.02 ± 0.35	12.39 ± 0.25	12.63 ± 0.12	-0.62 ± 0.18	3.40	7	0.01	0.10 ± 0.18	-0.56	6	0.59
DHAs-c	12.88 ± 0.22	12.27 ± 0.27	12.12 ± 0.24	-0.60 ± 0.13	4.62	9	0.001	-0.04 ± 0.13	0.29	8	0.78
<b>Palmitic acid</b>											
OOc-s	21.6 ± 0.23	21.58 ± 0.28	21.54 ± 0.19	-0.04 ± 0.18	0.21	8	0.84	-0.04 ± 0.16	0.28	8	0.79
OOs-c	21.41 ± 0.30	21.58 ± 0.35	21.15 ± 0.25	0.17 ± 0.19	-0.90	8	0.39	-0.51 ± 0.31	1.67	7	0.14
DHAc-s	21.21 ± 0.13	21.93 ± 0.20	22.24 ± 0.42	0.72 ± 0.13	-5.60	7	0.001	0.38 ± 0.41	-0.94	6	0.39
DHAs-c	21.26 ± 0.25	21.69 ± 0.39	21.72 ± 0.41	0.43 ± 0.27	-1.60	9	0.15	0.30 ± 0.17	-1.75	8	0.12
<b>Stearic acid</b>											
OOc-s	15.88 ± 0.28	15.74 ± 0.18	15.77 ± 0.19	-0.14 ± 0.21	0.67	8	0.52	0.03 ± 0.06	-0.54	8	0.60
OOs-c	16.07 ± 0.43	15.59 ± 0.33	15.44 ± 0.30	-0.48 ± 0.22	2.22	8	0.06	-0.03 ± 0.09	0.32	7	0.76
DHAc-s	15.88 ± 0.28	15.81 ± 0.16	15.54 ± 0.13	-0.07 ± 0.20	0.33	7	0.75	-0.16 ± 0.12	1.33	6	0.23
DHAs-c	15.91 ± 0.19	15.79 ± 0.23	15.84 ± 0.17	-0.13 ± 0.10	1.28	9	0.23	-0.10 ± 0.08	1.31	8	0.23

1. Data are mean ± SEM
2. OO (olive oil), DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)
3. Shading represents statistically significant changes (paired samples t-test,  $p < 0.05$ )



### 3.4.7 Plasma lipids

Fasting plasma concentrations of total cholesterol, HDL cholesterol, triglycerides and calculated LDL cholesterol were similar between the four groups at the commencement of the intervention period (**Table 3.15**).

In the olive oil groups, soy cereal consumption had no effect on plasma total cholesterol, HDL cholesterol, LDL cholesterol or triglycerides irrespective of whether or not the soy was consumed before or after the control cereal. In OOC-s, HDL cholesterol increased significantly ( $t = -2.56$ ,  $df = 9$ ,  $p = 0.03$ ) during the first six weeks when the control cereal was consumed and remained elevated during the subsequent six weeks (**Table 3.15**).

There was a significant time x order interaction during the soy cereal intervention in the two DHA groups for total cholesterol ( $F = 8.82$ ,  $df = 32$ ,  $p = 0.006$ ), HDL cholesterol ( $F = 8.82$ ,  $df = 32$ ,  $p = 0.006$ ) and LDL cholesterol ( $F = 7.45$ ,  $df = 32$ ,  $p = 0.01$ ) suggesting that the order of soy cereal consumption had an impact on these parameters. In DHAc-s, there was a trend towards an increase in both total ( $t = -2.08$ ,  $df = 7$ ,  $p = 0.077$ ) and LDL cholesterol ( $t = -2.22$ ,  $df = 6$ ,  $p = 0.06$ ) during the first six weeks when DHA-rich oil and control cereal were consumed concurrently; however, after soy cereal was consumed in the subsequent six weeks, total ( $t = 2.60$ ,  $df = 7$ ,  $p = 0.04$ ) and LDL cholesterol ( $t = 2.87$ ,  $df = 6$ ,  $p = 0.03$ ) were significantly reduced. In contrast, total and LDL cholesterol remained unchanged in DHAs-c, during the first six weeks when soy cereal was consumed concurrently with the DHA-rich oil and remained unchanged between six and twelve weeks when control cereal was consumed (**Figure 3.13** and **3.14**). Despite the trend toward an increase in the first six weeks and the significant decrease in total and LDL cholesterol between six and twelve weeks in DHAc-s, there were no differences in these parameters between the four groups after six (Total cholesterol:  $F = 0.64$ ,  $df = 3$ ,  $p = 0.77$ ; LDL cholesterol:  $F = 2.34$ ,  $df = 3$ ,  $p = 0.09$ ) or twelve weeks (total cholesterol:  $F = 0.15$ ,  $df = 3$ ,  $p = 0.59$ ; LDL cholesterol:  $F = 2.34$ ,  $df = 3$ ,  $p = 0.23$ ).

Table 3.15 Fasting plasma lipids in the two olive oil groups at baseline and after six and twelve weeks<sup>1-3</sup>

	0 weeks	6 weeks	12 weeks	0-6 wks	t	df	P	6-12 wks	t	df	P
<b>Total cholesterol (mmol/L)</b>											
OOc-s	6.61 ± 0.74	6.33 ± 0.49	6.25 ± 0.53	-0.28 ± 0.37	0.76	9	0.47	-0.19 ± 0.14	1.37	8	0.21
OOs-c	6.06 ± 0.32	6.27 ± 0.37	6.37 ± 0.41	0.21 ± 0.20	-1.03	8	0.33	0.16 ± 0.25	-0.65	7	0.54
<b>HDL cholesterol (mmol/L)</b>											
OOc-s	0.98 ± 0.13	1.12 ± 0.10	1.12 ± 0.08	0.14 ± 0.06	-2.56	9	0.03	-0.04 ± 0.04	1.12	8	0.30
OOs-c	1.06 ± 0.11	1.07 ± 0.09	1.30 ± 0.25	0.02 ± 0.04	-0.42	8	0.69	0.27 ± 0.28	-0.98	7	0.36
<b>Triglycerides (mmol/L)</b>											
OOc-s	3.02 ± 0.94	2.17 ± 0.44	2.28 ± 0.56	-0.85 ± 0.53	1.60	9	0.14	0.05 ± 0.13	-0.37	8	0.72
OOs-c	2.17 ± 0.41	2.60 ± 0.65	3.23 ± 0.86	0.43 ± 0.46	-0.92	8	0.38	0.34 ± 0.28	-1.22	7	0.26
<b>LDL cholesterol (mmol/L)</b>											
OOc-s	3.82 ± 0.10	3.98 ± 0.25	3.80 ± 0.24	0.16 ± 0.22	0.11	9	0.48	-0.20 ± 0.13	1.60	8	0.19
OOs-c	3.80 ± 0.20	3.90 ± 0.28	3.41 ± 0.53	0.10 ± 0.23	0.001	8	0.69	-0.25 ± 0.42	0.86	7	0.57

1. Data are mean ± SEM
2. OO (olive oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)
3. Shading represents statistically significant changes (paired samples t-tests,  $p < 0.05$ )

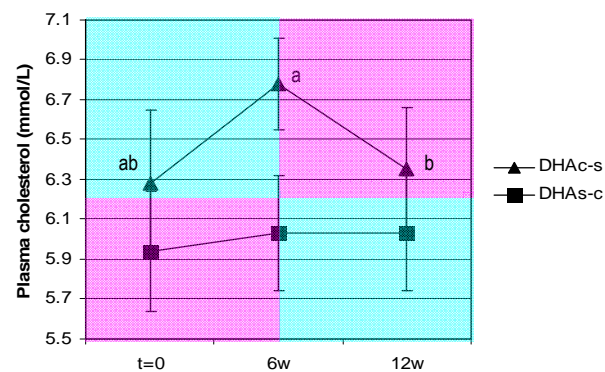


Figure 3.13 Total cholesterol concentrations in the two DHA groups at baseline and at six and twelve weeks of the intervention period<sup>1-4</sup>

1. DHAc-s, n = 8 (0-6wks) and n=7 (6-12wks); DHAs-c: n= 10 (0 – 6wks) and n = 9 (6-12wks)
2. Different subscripts indicate a statistically significant within-group difference ( $p < 0.05$ ). Paired t-test showed a trend toward an increase ( $p = 0.08$ ) in total cholesterol between t=0 and 6w and a significant reduction ( $p = 0.04$ ) in total cholesterol between 6w and 12w in DHAc-s.
3. Independent samples t-test showed a trend toward a difference ( $p = 0.087$ ) in total cholesterol between DHAc-s and DHs-c after 6wks. Total cholesterol concentrations were similar between the two groups at t=0 and 12w.
4. Periods of soy cereal consumption are highlighted in pink shade.

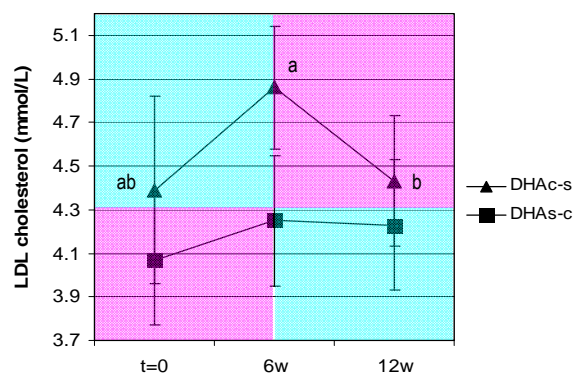


Figure 3.14 LDL cholesterol concentrations in the two DHA groups at baseline and at six and twelve weeks of the intervention period<sup>1-4</sup>

1. DHAc-s, n = 8 (0-6wks) and n=7 (6-12wks); DHAs-c: n= 10 (0 – 6wks) and n = 9 (6-12wks)
2. Different subscripts indicate a statistically significant within-group difference ( $p < 0.05$ ). Paired t-test showed a trend toward an increase in LDL cholesterol between t=0 and 6w ( $p = 0.07$ ) and a significant reduction ( $p = 0.03$ ) between 6w and 12w in DHAc-s.
3. Independent samples t-test found no differences in LDL cholesterol concentrations between the two groups at t=0, 6w or 12w.
4. Periods of soy cereal consumption are highlighted in pink shade.

Table 3.16 Fasting plasma lipids in the DHA groups at zero, six and twelve weeks of the intervention period<sup>1-3</sup>

	0 weeks	6 weeks	12 weeks	6wk – 0wks	t	df	P	12 - 6wks	t	df	P
<b>Total cholesterol (mmol/L)</b>											
DHAc-s	6.28 ± 0.37	6.78 ± 0.23	6.35 ± 0.31	0.50 ± 0.24	-2.08	7	0.08	-0.44 ± 0.17	2.60	6	0.04
DHAs-c	5.94 ± 0.30	6.03 ± 0.29	6.03 ± 0.29	0.09 ± 0.12	-1.15	9	0.47	0.00 ± 0.20	-0.02	8	0.99
<b>HDL cholesterol (mmol/L)</b>											
DHAc-s	1.20 ± 0.09	1.32 ± 0.12	1.24 ± 0.13	0.12 ± 0.06	-2.01	7	0.08	-0.04 ± 0.03	1.55	6	0.17
DHAs-c	1.07 ± 0.07	1.17 ± 0.09	1.21 ± 0.08	0.09 ± 0.03	-4.14	9	0.007	0.04 ± 0.04	-1.21	8	0.26
<b>Triglycerides (mmol/L)</b>											
DHAc-s	1.87 ± 0.38	1.61 ± 0.32	1.85 ± 0.42	-0.26 ± 0.15	1.76	7	0.12	0.16 ± 0.18	-0.87	6	0.42
DHAs-c	2.15 ± 0.30	1.64 ± 0.24	1.60 ± 0.24	-0.51 ± 0.15	3.22	9	0.009	-0.04 ± 0.10	0.44	8	0.67
<b>LDL cholesterol (mmol/L)</b>											
DHAc-s	4.39 ± 0.43	4.86 ± 0.28	4.43 ± 0.30	0.47 ± 0.22	-2.22	7	0.07	-0.46 ± 0.16	2.87	6	0.03
DHAs-c	4.07 ± 0.30	4.25 ± 0.30	4.23 ± 0.30	0.19 ± 0.14	-1.90	9	0.21	-0.02 ± 0.21	0.09	8	0.91

1. Data are mean ± SEM
2. DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)
3. Shading represents statistically significant changes (paired samples t-tests,  $p < 0.05$ )

DHA oil and soy cereal, in the first six weeks of the study resulted in a significant reduction (-23.7%,  $p = 0.009$ ) in plasma triglyceride concentrations, while DHA oil alone had no significant effect (-16.5%,  $p = 0.12$ ) on plasma triglycerides; despite this, triglyceride concentrations were similar between the two groups after six weeks. Between six and twelve weeks plasma triglyceride concentrations remained unchanged in both groups (**Table 3.16**). There was an almost significant between-group oil effect ( $F = 3.97$ ,  $df = 1$ ,  $p = 0.055$ ) in plasma triglyceride concentrations during the control cereal intervention period. An unpaired t-test showed triglyceride concentrations were significantly lower ( $t = 2.098$ ,  $df = 33$ ,  $p = 0.048$ ) after six weeks of DHA-rich oil consumption compared with olive oil supplementation and control cereal.

HDL cholesterol increased significantly in DHAs-c (8.4%,  $t = -4.14$ ,  $df = 9$ ,  $p = 0.007$ ) and there was a trend toward an increase in DHAc-s (9.1%,  $t = -2.01$ ,  $df = 7$ ,  $p = 0.08$ ) after six weeks, however concentrations remained unchanged between six and twelve weeks in both groups (**Table 3.16**). Despite this increase in plasma HDL cholesterol and that observed in OOC-s, there was no change in either HDL<sub>2</sub> or HDL<sub>3</sub> sub-fractions in any group (data not shown).

Repeated measures analysis of change from baseline in plasma total cholesterol, HDL cholesterol and triglyceride, with oil and gender as between-group factors and age and BMI as covariates, found no significant within- or between-group effects. The same analysis performed on LDL cholesterol found a significant oil ( $F = 4.305$ ;  $df = 1,28$ ;  $p = 0.047$ ) and BMI effect ( $F = 5.099$ ;  $df = 1,28$ ;  $p = 0.021$ ). A mean reduction in LDL cholesterol was found for olive oil ( $-0.305 \pm 0.165$  mmol/L), while a small increase in LDL cholesterol was found with DHA oil ( $0.168 \pm 0.171$  mmol/L).

### 3.4.8 Lipid correlations with isoflavones and fatty acids

Pearson's correlation tests revealed no significant associations between plasma or urine concentrations of daidzein, genistein or equol and blood total, LDL, HDL or triglyceride concentrations after six weeks of consuming soy cereal. This finding was similar for men and women (data not shown).

In DHAc-s there was a significant negative correlation ( $r = -0.725$ ,  $p = 0.04$ ) between the percentage of erythrocyte membrane EPA and fasting plasma triglyceride concentrations at six weeks. In contrast, percentages of erythrocyte membrane DHA at twelve weeks were positively correlated with fasting plasma triglyceride concentrations ( $r = 0.764$ ,  $p = 0.046$ ) and the change in this parameter ( $r = 0.76$ ,  $p = 0.046$ ) between six and twelve weeks. Similarly the percentage of DPA in erythrocyte membranes at six ( $r = 0.78$ ,  $p = 0.02$ ) and twelve weeks ( $r = 0.86$ ,  $p = 0.01$ ) was positively correlated with fasting plasma triglyceride concentrations at both time points. In DHAc-s, there was a positive correlation between the percentage of erythrocyte membrane DHA ( $r = 0.73$ ,  $p = 0.04$ ) and DPA ( $r = 0.87$ ,  $p = 0.005$ ) at six weeks and the trend toward an increase in LDL cholesterol concentrations between baseline and six weeks.

In DHAs-c there were no significant correlations between DHA, EPA or DPA and LDL Cholesterol or fasting plasma triglyceride concentrations.

### 3.4.9 Plasma lipoprotein composition

#### 3.4.9.1 Very low density lipoprotein (VLDL)

Concentrations of VLDL total cholesterol, free cholesterol, cholesterol ester, triglyceride, phospholipid and protein were similar between the four groups at zero, six and twelve weeks. VLDL ApoB was similar between the four groups at zero and six weeks of the intervention period and while there was a trend toward a difference between the groups at twelve weeks ( $F = 2.85$ ,  $df = 3$ ,  $p = 0.058$ ), a Tukey HSD post-hoc test found no significant between-group differences.

During six weeks of oil supplementation with control cereal consumption, VLDL phospholipid significantly decreased ( $t = 2.40$ ,  $df = 9$ ,  $p = 0.04$ ) in OOC-s and VLDL protein significantly increased ( $t = -2.70$ ,  $df = 7$ ,  $p = 0.03$ ) in OOs-c; In DHAc-s there was an almost significant reduction ( $t = 2.33$ ,  $df = 6$ ,  $p = 0.058$ ) in VLDL Apolipoprotein B (ApoB) and in DHAs-c there was an almost significant reduction in VLDL triglycerides ( $t = 2.23$ ,  $df = 8$ ,  $p = 0.057$ ).

When the data from the two DHA groups were combined to assess the effect of DHA oil supplementation on VLDL composition, there was a trend toward a reduction in VLDL triglyceride ( $-0.33 \pm 0.16$  mmol/L,  $t = 2.03$ ,  $df = 16$ ,  $p = 0.06$ ), total cholesterol ( $-0.14 \pm 0.007$  mmol/L,  $t = 1.95$ ,  $df = 16$ ;  $p = 0.07$ ) and cholesterol ester ( $-0.09 \pm 0.04$ ,  $t = 2.07$ ,  $df = 16$ ,  $p = 0.055$ ) concentrations after six weeks with control cereal. No significant changes were observed with olive oil supplementation. When the DHA and olive oil groups were compared, concentrations of VLDL triglyceride, total cholesterol, cholesterol ester, free cholesterol and phospholipid were significantly lower after six weeks of DHA oil supplementation and control cereal (**Table 3.17**). These differences were not present following soy cereal consumption.

Table 3.17 Components in VLDL in which a significant difference was found between the groups taking olive oil and the groups taking DHA-rich oil at the end of the control cereal period<sup>1</sup>

VLDL Component	Control Cereal (t = 0)				Control Cereal (t = wk 6)				Control Cereal (t = 0)			Control Cereal (t = wk 6)		
	DHA Groups (n=17)				Olive oil Groups (n = 18)				Between-group difference (t = 0) <sup>3</sup>	F <sup>4</sup>	P <sup>4</sup>	Between-group difference (t = wk 6) <sup>3</sup>	F <sup>4</sup>	P <sup>4</sup>
	T = 0	T = wk 6	6wk - 0	P <sup>2</sup>	T = 0	T = wk 6	6wk - 0	P <sup>2</sup>						
Triglyceride (mmol/L)	1.3 ± 0.2	1.0 ± 0.2	-0.3 ± 0.2	0.06	2.3 ± 0.7	1.9 ± 0.4	-0.4 ± 0.5	0.48	-39%	1.36	0.25	-49%	4.131	0.05
Total cholesterol (mmol/L)	0.7 ± 0.1	0.5 ± 0.1	-0.1 ± 0.07	0.07	1.4 ± 0.4	1.2 ± 0.3	-0.2 ± 0.2	0.32	-50%	2.35	0.13	-56%	4.565	0.04
Cholesterol ester (mmol/L)	0.4 ± 0.05	0.3 ± 0.04	-0.1 ± 0.04	0.055	0.8 ± 0.3	0.7 ± 0.2	-0.08 ± 0.1	0.58	-43%	1.50	0.23	-60%	4.172	0.049
Free Cholesterol (mmol/L)	0.3 ± 0.05	0.2 ± 0.04	-0.1 ± 0.03	0.10	0.6 ± 0.2	0.5 ± 0.1	-0.1 ± 0.1	0.21	-47%	3.15	0.08	-51%	4.655	0.04
Phospholipid (mg/L)	13.2 ± 2.4	10.9 ± 1.5	-2.3 ± 1.7	0.19	21.1 ± 3.8	18.7 ± 3.4	-2.5 ± 1.6	0.15	-34%	2.58	0.12	-42%	4.234	0.048

1. Data are mean ± SEM
2. Values derived from paired t-tests.
3. Percent differences reflect comparison of DHA vs Olive oil, thus negative changes indicate value in DHA group is less than value in olive oil group and vice versa for positive changes.
4. Values derived from one-way ANOVA. P < 0.05 signifies statistical difference
5. Shading represents statistically significant differences (p < 0.05), or trends (p > 0.05 and p < 0.09)



Table 3.18 Change from baseline (t=0) in VLDL total cholesterol, cholesterol ester, triglyceride and apolipoprotein B as determined by repeated measures, 2 factor ANCOVA with age and BMI as covariates <sup>1</sup>

	Control Cereal	Soy Cereal
<b>VLDL Total Cholesterol <sup>2</sup></b>	<i>mmol/L</i>	
Olive oil (n = 17)	-0.138 ± 0.190	-0.328 ± 0.221
DHA oil (n = 17)	-0.280 ± 0.197	-0.109 ± 0.229
<b>VLDL Cholesterol Ester <sup>3</sup></b>	<i>mmol/L</i>	
Olive oil (n = 17)	-0.094 ± 0.116	-0.218 ± 0.117
DHA oil (n = 17)	-0.181 ± 0.119	-0.074 ± 0.119
<b>VLDL Triglycerides <sup>4</sup></b>	<i>mmol/L</i>	
Olive oil (n = 17)	-0.242 ± 0.491	-0.566 ± 0.579
DHA oil (n = 17)	-0.623 ± 0.509	-0.273 ± 0.600
<b>VLDL ApoB <sup>5</sup></b>	<i>mg/L</i>	
Olive oil (n = 17)	-3.371 ± 6.660	-12.928 ± 7.376
DHA oil (n = 17)	-11.085 ± 6.900	-3.684 ± 7.642

1. All data are mean ± SEM

2. Covariates appearing in the repeated measures, 2 factor model are evaluated at the following values: age = 52.86 and BMI = 30.22 as determined by SPSS.

3. Covariates appearing in the repeated measures, 2 factor model are evaluated at the following values: age = 52.85 and BMI = 30.22 as determined by SPSS.

4. Covariates appearing in the repeated measures, 2 factor model are evaluated at the following values: age = 52.61 and BMI = 30.35 as determined by SPSS.

5. Covariates appearing in the repeated measures, 2 factor model are evaluated at the following values: age = 52.61 and BMI = 30.22 as determined by SPSS.

Repeated measures analysis of change from baseline in VLDL composition with oil and gender as between-group factors and age and BMI as covariates found a significant oil x cereal interaction in VLDL total cholesterol ( $F = 6.13$ ,  $df = 1$ ,  $p = 0.02$ ), cholesterol ester ( $F = 7.34$ ;  $df = 1,29$ ;  $p = 0.01$ ), triglyceride ( $F = 6.97$ ;  $df = 1,29$ ;  $p = 0.013$ ) and apolipoprotein B ( $F = 9.10$ ;  $df = 1,29$ ;  $p = 0.005$ ) (**Table 3.18**). In all four variables, a greater reduction was observed with DHA than with olive oil when control cereal was consumed. This appeared to be reversed when the soy cereal was consumed (**Figure 3.15 – 3.18**).

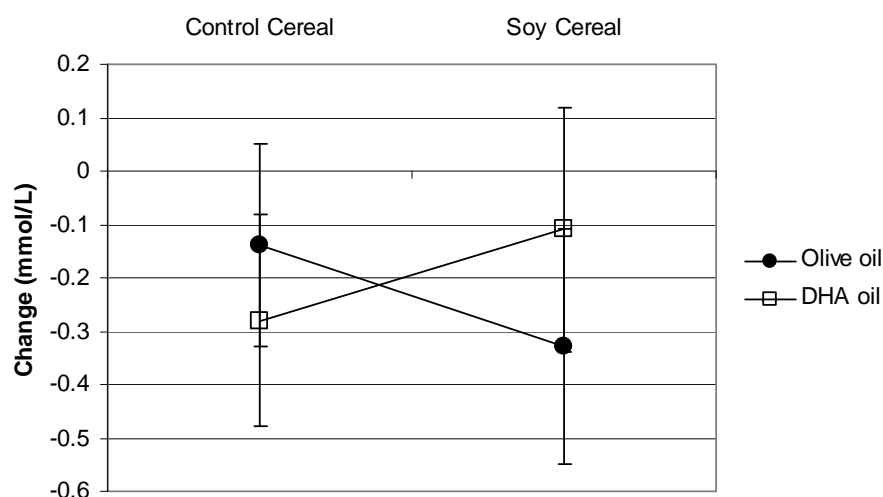


Figure 3.15 Change from baseline (t=0) in VLDL total cholesterol in the olive oil and DHA oil groups during the 6 weeks of control cereal and soy cereal

1. Data derived from repeated measures analysis of change from baseline in VLDL total cholesterol with oil and gender as between-group factors and age and BMI as covariates
2. Data from the OOC-s and OOC-c groups (n = 17) were combined for olive oil analysis
3. Data from the DHAc-s and DHAc-c groups (n = 17) were combined for the DHA analysis

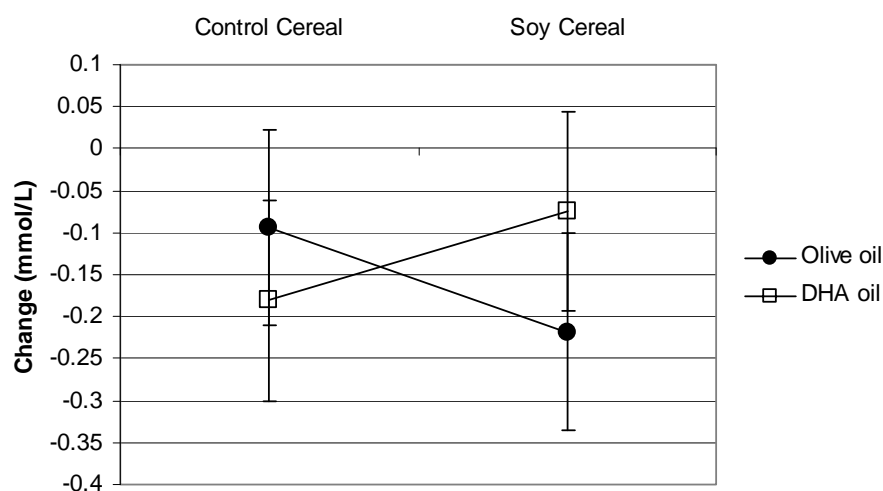


Figure 3.16 Change from baseline (t=0) in VLDL cholesterol ester in the olive oil and DHA oil groups during the 6 weeks of control cereal and soy cereal

1. Data derived from repeated measures analysis of change from baseline in VLDL cholesterol ester with oil and gender as between-group factors and age and BMI as covariates
2. Data from the OOC-s and OOC-c groups (n = 17) were combined for olive oil analysis
3. Data from the DHAc-s and DHAc-c groups (n = 17) were combined for the DHA analysis

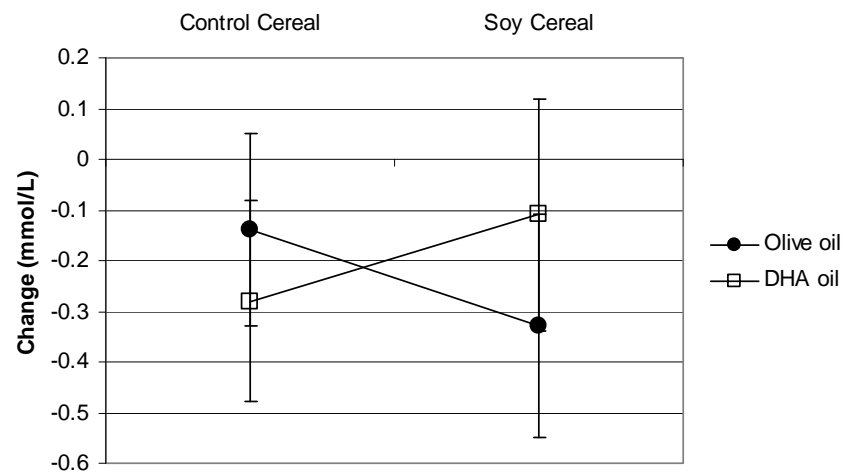


Figure 3.17 Change from baseline (t=0) in VLDL triglyceride in the olive oil and DHA oil groups during the 6 weeks of control cereal and soy cereal

1. Data derived from repeated measures analysis of change from baseline in VLDL triglyceride with oil and gender as between-group factors and age and BMI as covariates
2. Data from the OOC-s and OOs-c groups (n = 17) were combined for olive oil analysis
3. Data from the DHAc-s and DHAs-c groups (n = 17) were combined for the DHA analysis

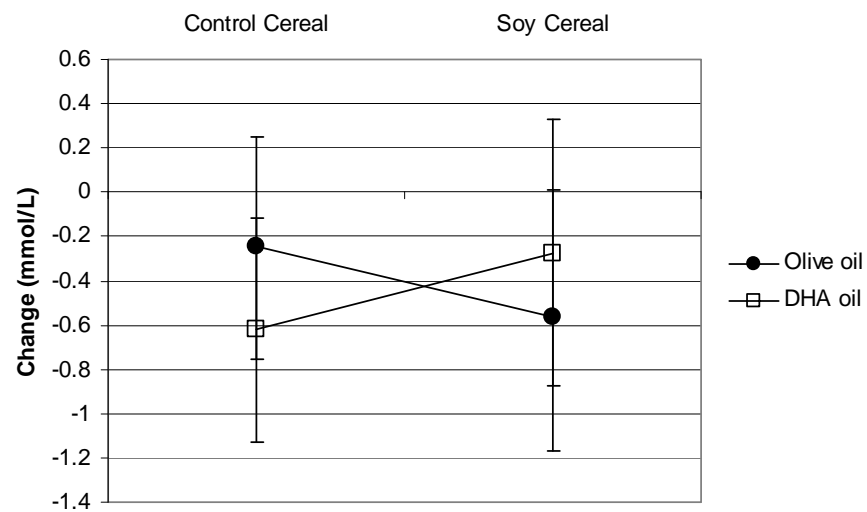
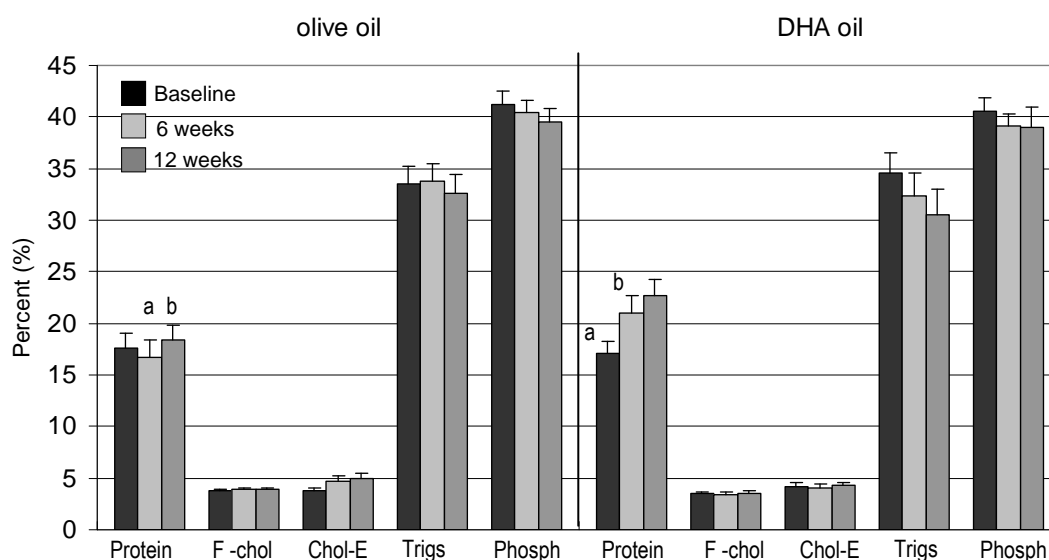


Figure 3.18 Change from baseline (t=0) in VLDL apolipoprotein B in the olive oil and DHA oil groups during the 6 weeks of control cereal and soy cereal

1. Data derived from repeated measures analysis of change from baseline in VLDL apolipoprotein B with oil and gender as between-group factors and age and BMI as covariates
2. Data from the OOC-s and OOs-c groups (n = 17) were combined for olive oil analysis
3. Data from the DHAc-s and DHAs-c groups (n = 17) were combined for the DHA analysis

When lipoprotein components were assessed as percentages of the sum of all components, the proportion of VLDL free cholesterol and phospholipid were significantly decreased after six weeks of olive oil supplementation in OOC-s (free cholesterol:  $t = 2.69$ ,  $df = 8$ ,  $p = 0.03$ ; phospholipid:  $t = 2.43$ ,  $df = 8$ ,  $p = 0.04$ ) while free cholesterol increased significantly in OOs-c ( $t = -3.00$ ,  $df = 8$ ,  $p = 0.02$ ) over the same time period.

In DHAc-s the percentage of VLDL protein increased significantly ( $t = -2.26$ ,  $df = 8$ ,  $p = 0.05$ ) during the first six weeks of oil supplementation while there was a trend toward a decrease in the percentage of cholesterol ester ( $t = 2.22$ ,  $df = 8$ ,  $p = 0.058$ ). When the data from the DHA and olive oil groups were respectively combined, the percentage of VLDL protein was shown to increase significantly during the first six weeks with DHA oil (3.92%,  $t = -2.24$ ,  $df = 19$ ,  $p = 0.04$ ) and increase significantly between six and twelve weeks with olive oil supplementation (2.89%,  $t = -2.06$ ,  $df = 17$ ,  $p = 0.055$ ). After six ( $F = 3.39$ ,  $df = 40$ ,  $p = 0.07$ ) and twelve weeks ( $f = 3.92$ ,  $df = 36$ ,  $P = 0.056$ ) however there was a trend toward a higher percentage of VLDL protein in the DHA groups compared with the olive oil groups (**Figure 3.19**).



**Figure 3.19** Percentage composition of VLDL in the two olive oil groups and two DHA groups at baseline and after six and twelve weeks of oil supplementation<sup>1-3</sup>

1. F-chol = free cholesterol; Chol-E = cholesterol ester, Phosph = phospholipid.
2. Different subscripts represent statistically significant differences, ( $p < 0.05$ )
3. Olive oil groups:  $n = 18$ ; DHA oil groups:  $n = 20$ .

In the two DHA groups, a significant negative correlation was found between the percentage of erythrocyte membrane EPA at six weeks and VLDL total and free cholesterol ( $r = -0.58$ ,  $p = 0.01$ ), phospholipid ( $-0.58$ ,  $0.01$ ), protein ( $r = -0.48$ ,  $p = 0.04$ ) and triglyceride concentrations ( $r = -0.66$ ,  $p = 0.003$ ). At twelve weeks, a similar negative correlation was found between erythrocyte membrane EPA and VLDL triglyceride concentrations ( $r = -0.55$ ,  $p = 0.03$ ).

When the core (cholesterol ester and triglyceride) to surface (free cholesterol, phospholipid and protein) ratio was assessed as a measure of particle size (Inagaki & Harris, 1990), there were no significant within-group changes in the VLDL core to surface ratio or between-group differences at baseline or after six or twelve weeks. When the groups were compared based on the type of oil supplementation the VLDL core to surface ratio was significantly lower ( $F = 4.412$ ,  $df = 36$ ,  $p = 0.04$ ) after six weeks of DHA oil ( $0.54 \pm 0.03$ ) compared with olive oil ( $0.66 \pm 0.05$ ) and control cereal. This difference was not present at the beginning of the control cereal period.

In OOs-c, there was a trend toward an increase ( $0.07 \pm 0.04$ ,  $t = -1.92$ ,  $df = 9$ ,  $p = 0.087$ ) in the ratio of VLDL total cholesterol: VLDL ApoB between baseline and six weeks and a trend toward a decrease ( $-0.05 \pm 0.02$ ,  $t = 2.11$ ,  $df = 7$ ,  $p = 0.073$ ) in the ratio of VLDL free cholesterol: VLDL ApoB between six and twelve weeks. In DHAc-s there was a trend toward a decrease ( $-0.07 \pm 0.03$ ,  $t = 2.19$ ,  $df = 7$ ,  $p = 0.065$ ) in the ratio of VLDL cholesterol ester: ApoB between baseline and six weeks and a significant increase ( $0.07 \pm 0.02$ ,  $t = -2.78$ ,  $df = 6$ ,  $p = 0.032$ ) in that same ratio between six and twelve weeks.

#### **3.4.9.2 Intermediate density lipoprotein (IDL)**

When the data from the two olive oil groups were combined there was a significant increase ( $t = -2.14$ ,  $df = 17$ ,  $p = 0.05$ ) in IDL triglyceride concentrations after six weeks and as no change was observed with DHA supplementation, IDL triglyceride concentrations were significantly lower ( $F = 4.12$ ,  $df = 37$ ,  $p = 0.05$ ) following six weeks of DHA compared with olive oil supplementation. In DHAc-s, concentrations of IDL triglyceride increased by a small but significant amount ( $0.10 \pm 0.04$ ,  $t = -2.75$ ,  $df = 6$ ,  $p = 0.03$ ) and a trend toward an increase in IDL triglyceride concentrations with DHA oil

supplementation between six and twelve weeks (0.05 mmol/L,  $t = -1.95$ ,  $df = 15$ ,  $p = 0.07$ ). As a percentage of total IDL composition, in DHAc-s, IDL protein decreased (-2.1%,  $t = 3.21$ ,  $df = 8$ ,  $p = 0.01$ ) significantly between six and twelve weeks when soy cereal was consumed, while the percentage of IDL triglyceride significantly increased (2.5%,  $t = -2.74$ ,  $df = 8$ ,  $p = 0.03$ ).

At six weeks there was a significant negative correlation ( $r = -0.741$ ,  $p < 0.01$ ) between the percentage of erythrocyte membrane EPA and IDL triglyceride concentrations. At twelve weeks there was a negative correlation between erythrocyte membrane EPA and IDL total cholesterol ( $r = -0.522$ ,  $p = 0.04$ ), free cholesterol ( $r = -0.51$ ,  $p = 0.04$ ), phospholipid ( $r = -0.605$ ,  $p = 0.01$ ), protein, ( $r = -0.57$ ,  $p = 0.02$ ), and triglyceride ( $r = -0.61$ ,  $p = 0.01$ ) concentrations.

#### **3.4.9.3 Low density lipoproteins (LDL)**

In DHAc-s, LDL free cholesterol (19%,  $t = -2.94$ ,  $df = 7$ ,  $p = 0.02$ ) and triglyceride concentrations (12.5%,  $t = -4.76$ ,  $df = 7$ ,  $p = 0.002$ ) both increased significantly after six weeks of DHA oil. In the same group between six and twelve weeks, the total cholesterol content of LDL significantly decreased (-16%,  $t = 2.96$ ,  $df = 6$ ,  $p = 0.025$ ) when soy cereal was consumed with the DHA oil. In DHAs-c, LDL composition remained unchanged with either DHA oil alone or DHA plus soy cereal.

Apart from a small increase (0.43%,  $t = -2.86$ ,  $df = 9$ ,  $p = 0.019$ ) in the percentage of LDL triglycerides in OOC-s between six and twelve weeks, LDL composition remained unchanged in the two olive oil groups after soy and control cereal consumption.

LDL ApoB, total cholesterol, cholesterol ester, free cholesterol, phospholipid and protein concentrations were all significantly higher in DHAc-s after six weeks of control cereal compared with either OOC-s or OOC-c at the completion of their respective control cereal periods (week six for OOC-s and week twelve for OOC-c, respectively). Such differences were not present at the beginning of the control cereal period (**Table 3.19**). These differences between DHAc-s and the two olive oil groups were not present when data was expressed as a percentage of LDL composition (data not shown).

Table 3.19 LDL composition in DHAc-s compared with the two olive oil groups after six weeks of oil supplementation and control cereal consumption<sup>1,23,3a,3b</sup>.

LDL component	DHAc-s (n = 8)	OOc-s (n = 10)	p <sup>3a</sup>	OOs-c (n = 8)	p <sup>3b</sup>	One-way ANOVA			
						Control (t = 0)		Control (Wk 6)	
						F	p	F	p
Apolipoprotein B ( <i>mg/L</i> )	33.9 ± 3.7 <sup>a</sup>	23.9 ± 1.8 <sup>b</sup>	0.02	26.4 ± 1.18	NS	0.85	0.48	3.40	0.03
Total Cholesterol ( <i>mmol/L</i> )	3.5 ± 0.3 <sup>a</sup>	2.5 ± 0.2 <sup>b</sup>	0.02	2.6 ± 0.2 <sup>b</sup>	0.04	0.77	0.52	3.91	0.02
Cholesterol Ester ( <i>mmol/L</i> )	2.6 ± 0.2 <sup>a</sup>	1.8 ± 0.2 <sup>b</sup>	0.02	2.0 ± 0.3 <sup>b</sup>	0.05	0.80	0.51	3.85	0.02
Free Cholesterol ( <i>mmol/L</i> )	1.0 ± 0.1 <sup>a</sup>	0.7 ± 0.1	0.057	0.6 ± 0.1 <sup>b</sup>	0.03	0.70	0.56	3.61	0.02
Phospholipid ( <i>mg/L</i> )	30.9 ± 2.0 <sup>a</sup>	21.9 ± 1.8 <sup>b</sup>	0.003	22.1 ± 1.3 <sup>b</sup>	0.007	0.78	0.51	5.97	0.002
Protein ( <i>μg/L</i> )	3.7 ± 0.4 <sup>a</sup>	2.3 ± 0.3 <sup>b</sup>	0.006	2.7 ± 0.2	NS	0.89	0.46	3.66	0.02

1. Data are mean ± SEM

2. Values in the same row with different superscripts are significantly different, p &lt; 0.05.

3. p-values obtained from one-way ANOVA with Tukeys HSD post-hoc analysis.

3a. DHAc-s vs OOc-s

3b. DHAc-s vs OOs-c

Repeated measures analysis of change from baseline in LDL composition with oil and gender as between-group factors and age and BMI as covariates found a significant between oil effect in LDL total cholesterol ( $F = 4.45$ ;  $df = 1,28$ ;  $p = 0.04$ ), LDL free cholesterol ( $F = 7.04$ ;  $df = 1,28$ ;  $p = 0.013$ ) and LDL phospholipid ( $F = 4.38$ ;  $df = 1,28$ ;  $p = 0.046$ ). A reduction in these parameters was observed with olive oil while a small increase occurred with DHA supplementation (**Table 3.20**).

Table 3.20 Mean change in LDL total cholesterol, free cholesterol and phospholipid following six weeks of olive oil and DHA oil supplementation as determined by repeated measures ANCOVA with age and BMI as covariates <sup>1,2,3</sup>

	Olive Oil (n = 17)	DHA Oil (n = 17)	F	df	p
LDL Total Cholesterol (mmol/L)	-0.27 ± 0.13	0.11 ± 0.14	4.45	1,28	0.04
LDL Free Cholesterol (mmol/L)	-0.08 ± 0.04	0.06 ± 0.04	7.04	1,28	0.013
LDL Phospholipid (mg/L)	-27.87 ± 11.28	4.66 ± 11.69	4.38	1,28	0.046

1. Data are mean ± SEM

2. Data derived from a repeated measures analysis of change from baseline in LDL composition with oil and gender as between-group factors and age and BMI as covariates

3. Covariates appearing in the repeated measures, 2 factor model are evaluated at the following values: age = 52.61 and BMI = 30.35

The ratios of LDL total cholesterol: ApoB, free cholesterol: ApoB or cholesterol ester: ApoB were similar between the four groups and did not change in any groups throughout the study. There were no within-group, between-group or oil differences in the LDL core: surface ratio after six or twelve weeks.

At six weeks there was a significant positive correlation ( $r = 0.69$ ,  $p = 0.002$ ) between the percentage of erythrocyte membrane EPA and LDL total cholesterol, while at twelve weeks there was a positive correlation ( $r = 0.70$ ,  $p = 0.003$ ) between EPA and LDL free cholesterol concentrations. There were no significant correlations between erythrocyte membrane DHA and LDL composition.

#### 3.4.10 Arterial compliance

A 3 –factor ANOVA showed a significant between-group oil effect ( $F = 4.75$ ,  $df = 1$ ,  $p = 0.037$ ) in large artery compliance and a between-group order effect ( $F = 5.46$ ,  $df = 1$ ,  $p = 0.026$ ) in small artery compliance during the six weeks of control cereal consumption. Large artery compliance was significantly higher ( $F = 5.902$ ,  $p = 0.02$ ,  $df$



= 1) in the two DHA groups ( $1.51 \pm 0.08$  ml/mmHg) compared with the two olive oil groups ( $1.24 \pm 0.08$  ml/mmHg) after six weeks of oil supplementation and control cereal consumption, however, this difference was not due to a change in large artery compliance with DHA-rich oil (**Table 3.21**).

Small and large artery compliance remained unchanged in all groups over the course of the intervention period and did not change with soy cereal consumption.

Table 3.21 Large artery compliance in the two olive oil groups compared with the two DHA-rich oil groups before and after six weeks of oil supplementation and control cereal consumption<sup>1</sup>.

	Start of control cereal period	End of control cereal period <sup>2</sup>	Change	t	df	p
	<i>ml/mmHg</i>					
Olive oil	$1.32 \pm 0.07$	$1.24 \pm 0.08^a$	$-0.08 \pm 0.06$	-1.78	17	0.17
DHA-rich oil	$1.55 \pm 0.11$	$1.51 \pm 0.08^b$	$-0.04 \pm 0.09$	0.56	16	0.59
Difference b/w oils	$0.23 \pm 0.13$	$0.27 \pm 0.11$				

1. Data are mean  $\pm$  SEM

2. Values with different superscripts are significantly different as determined by unpaired t-test ( $t = -2.43$ ,  $df = 33$ ,  $p = 0.02$ )

### 3.4.11 Additional cardiovascular measures

There were no significant differences between the four groups in heart rate, cardiac ejection time, cardiac index, stroke volume, stroke volume index, cardiac output, or systemic vascular resistance at the commencement of the intervention period.

With the exception of changes in heart rate, there were no within-group changes in any of the other cardiovascular measures after six or twelve weeks.

A 3-factor ANOVA showed a significant between-group oil effect ( $F = 12.07$ ,  $df = 1$ ,  $p = 0.02$ ) and a significant between-group oil  $\times$  order interaction ( $F = 5.97$ ,  $df = 1$ ,  $p = 0.02$ ) when control cereal was consumed. Heart rate decreased significantly in DHAc-s ( $-4.6 \pm 1.2$  beats/min,  $t = 3.80$ ,  $df = 6$ ,  $p = 0.009$ ) in the first six weeks and in OOC-s ( $-2.4 \pm 1.0$  beats/min,  $p = 0.046$ ) heart rate decreased significantly between six and twelve weeks. Despite these changes there were no differences in heart rate between the two DHA groups after six weeks or between the two olive oil groups after twelve weeks.

When the data were combined, heart rate was significantly lower with DHA compared with olive oil supplementation after six ( $F = 9.88$ ,  $df = 1$ ,  $p = 0.003$ ) and twelve weeks ( $F = 6.15$ ,  $df = 1$ ,  $p = 0.019$ ) (**Table 3.22**).

Table 3.22 Heart rate in the olive oil and DHA oil groups at baseline and after six and twelve weeks<sup>1,2</sup>

	Baseline	6 wks	12 wks	F	df	p
	<i>Beats/min</i>					
Olive oil	65.0 ± 1.8	68.1 ± 1.7 <sup>a</sup>	66.3 ± 2.5 <sup>a</sup>	2.34	9	0.15
DHA oil	60.9 ± 2.2	59.1 ± 2.4 <sup>b</sup>	57.9 ± 2.2 <sup>b</sup>	1.08	10	0.38
	NS	F = 9.88, df = 1, p = 0.003	F = 6.15, df = 1, p = 0.019			

1. Data are mean ± SEM

2. Values with different subscripts are significantly different (one-way ANOVA, olive vs DHA oil).

There were no between-group differences in stroke volume, cardiac output, cardiac index, systemic vascular resistance or total vascular impedance after six or twelve weeks.

When the effect of oil supplementation was examined, there was a trend toward a higher cardiac ejection time and significantly higher stroke volume index after six and twelve weeks with DHA compared with olive oil. Cardiac index was also significantly lower with DHA compared with olive oil after six weeks and stroke volume was significantly higher with DHA compared with olive oil after twelve weeks (**Table 3.23**).

Table 3.23 Cardiovascular parameters which were significantly different after six or twelve weeks of DHA supplementation compared with olive oil supplementation<sup>1,2</sup>

	Baseline	6 wks	12wks	Time	F	df	p
Cardiac Ejection Time ( <i>msec</i> )							
Olive oil	352.9 ± 5.6	350.7 ± 4.1	352.0 ± 5.7	6wk	3.57	1	0.067
DHA oil	358.4 ± 4.1	361.7 ± 4.0	366.4 ± 5.3	12wk	3.45	1	0.07
Stroke Volume ( <i>ml</i> )							
Olive oil	86.0 ± 3.1	83.1 ± 2.4	82.9 ± 3.0 <sup>a</sup>	12wk	5.71	1	0.02
DHA oil	90.2 ± 2.9	89.1 ± 2.5	92.8 ± 2.8 <sup>b</sup>				
Stroke Volume Index ( <i>ml/beat/m<sup>2</sup></i> )							
Olive oil	43.2 ± 1.2	42.0 ± 1.0 <sup>a</sup>	42.5 ± 1.5 <sup>a</sup>	6wk	3.67	1	0.064
DHA oil	45.2 ± 1.1	44.9 ± 1.1 <sup>b</sup>	46.9 ± 1.2 <sup>b</sup>	12wk	5.23	1	0.03
Cardiac Index ( <i>L/min/m<sup>2</sup></i> )							
Olive oil	2.8 ± 0.03	2.9 ± 0.03 <sup>a</sup>	2.8 ± 0.03	6wk	6.95	1	0.01
DHA oil	2.7 ± 0.06	2.7 ± 0.05 <sup>b</sup>	2.7 ± 0.05				

1. Data are mean ± SEM

2. Values with different subscripts are significantly different (one-way ANOVA, olive vs DHA oil)

### 3.4.12 Blood pressure – clinic and ambulatory

Clinic systolic, diastolic and mean arterial blood pressure were similar between the four groups at the commencement of the intervention period. In OOs-c there was a trend toward an increase ( $t = -2.31$ ,  $df = 6$ ,  $p = 0.06$ ) in mean arterial blood pressure after six weeks and a significant increase ( $t = -3.90$ ,  $df = 5$ ,  $p = 0.01$ ) after twelve weeks. There were no changes in systolic or diastolic blood pressure in OOs-c after six or twelve weeks. Systolic, diastolic and mean arterial blood pressure remained unchanged in the other three groups after six and twelve weeks (**Table 3.24**).

Table 3.24 Clinic blood pressure in the four intervention groups at baseline and after six and twelve weeks<sup>1</sup>

	Baseline	6 wks	12wks
<b>SBP (mmHg)</b>			
OOc-s	136.2 ± 4.4	141.23 ± 6.1	135.2 ± 6.1
OOs-c	135.6 ± 5.0	134.2 ± 4.6	141.1 ± 5.3
DHAc-s	137.4 ± 6.2	137.4 ± 4.5	139.4 ± 8.1
DHAs-c	127.0 ± 4.1	127.1 ± 4.3	126.8 ± 3.0
<b>DBP (mmHg)</b>			
OOc-s	78.2 ± 2.2	79.3 ± 2.6	76.2 ± 3.0
OOs-c	83.9 ± 4.5	83.9 ± 4.1	88.7 ± 4.6
DHAc-s	81.0 ± 3.4	80.3 ± 3.0	82.8 ± 4.8
DHAs-c	77.5 ± 2.6	74.9 ± 1.9	77.3 ± 2.8
<b>MAP (mmHg)</b>			
OOc-s	103.0 ± 3.6	105.9 ± 5.1	100.9 ± 3.8
OOs-c	98.5 ± 4.9	101.2 ± 4.5	107.5 ± 4.2
DHAc-s	106.5 ± 4.9	105.5 ± 4.9	110.1 ± 7.9
DHAs-c	93.6 ± 3.0	94.1 ± 2.4	96.4 ± 2.2

1. Data are mean ± SEM

2. DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)

Ambulatory systolic blood pressure was similar between the four groups at the commencement of the intervention period, however, ambulatory diastolic blood pressure averaged over the 24hr recording period ( $F = 2.97$ ,  $df = 3$ ,  $p = 0.046$ ) and daytime diastolic blood pressure ( $F = 3.39$ ,  $df = 3$ ,  $p = 0.03$ ) were significantly higher in OOs-c compared with OOc-s at the start of the intervention period. This difference in ambulatory daytime diastolic blood pressure between the two olive oil groups remained ( $F = 2.70$ ,  $df = 3$ ,  $p = 0.046$ ) after six weeks of oil supplementation (**Table 3.25**). There were no other between- or within-group differences in ambulatory blood pressure after six or twelve weeks.

Table 3.25 Ambulatory systolic and diastolic blood pressure in the four intervention groups at baseline and after six and twelve weeks<sup>1,2</sup>

	Baseline	6 wks	12wks	Baseline	6 wks	12wks
	<i>Systolic blood pressure</i>			<i>Diastolic blood pressure</i>		
<i>Mean (mmHg)</i>						
OOc-s	122.8 ± 6.0	122.3 ± 4.6	122.2 ± 4.1	74.2 ± 2.7	74.0 ± 2.2	74.7 ± 2.3
OOs-c	128.0 ± 3.6	127.1 ± 5.0	123.5 ± 4.0	82.9 ± 2.1	82.0 ± 2.9	80.9 ± 2.2
DHAc-s	123.6 ± 4.2	122.6 ± 4.0	127.0 ± 6.3	75.4 ± 2.6	75.0 ± 2.0	77.1 ± 3.5
DHAs-c	118.8 ± 2.7	118.9 ± 2.4	120.3 ± 2.8	76.3 ± 1.5	74.9 ± 1.9	76.2 ± 1.5
<i>Awake (mmHg)</i>						
OOc-s	127.8 ± 6.6	126.7 ± 4.7	127.3 ± 4.3	77.5 ± 2.9	77.1 ± 2.3	78.3 ± 2.4
OOs-c	134.7 ± 3.9	132.4 ± 5.0	129.6 ± 4.9	87.4 ± 2.5	86.0 ± 2.8	84.6 ± 2.2
DHAc-s	129.4 ± 4.6	129.4 ± 5.0	133.3 ± 7.7	78.9 ± 2.6	80.0 ± 2.3	81.4 ± 4.1
DHAs-c	123.4 ± 2.7	124.8 ± 2.7	125.6 ± 2.5	80.0 ± 1.4	79.3 ± 1.9	80.2 ± 1.3
<i>Asleep (mmHg)</i>						
OOc-s	110.0 ± 5.2	109.1 ± 5.4	108.2 ± 4.5	64.9 ± 2.5	64.4 ± 3.1	64.9 ± 2.9
OOs-c	107.8 ± 2.7	112.6 ± 5.4	110.4 ± 4.7	67.4 ± 2.0	70.4 ± 3.4	70.9 ± 3.1
DHAc-s	105.6 ± 3.6	103.1 ± 2.0	108.3 ± 3.9	64.3 ± 3.0	61.6 ± 1.8	64.7 ± 2.5
DHAs-c	106.1 ± 3.1	104.0 ± 2.8	105.4 ± 4.8	66.0 ± 1.6	64.5 ± 2.4	64.9 ± 2.4

1. Data are mean ± SEM
2. DHA (DHA-rich oil), c-s (control cereal consumed in first six weeks followed by six weeks of soy cereal consumption); s-c (soy cereal consumed in first six weeks followed by six weeks of control cereal consumption)

### **3.5 Discussion**

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The findings from this study provide the first preliminary evidence from a human clinical trial of a potential benefit to blood lipids and lipoproteins in mildly hyperlipidemic individuals of combining in the diet soy isoflavones with the very long chain omega-3 fatty acid DHA.

Dietary supplementation with DHA oil in the first six weeks of the study resulted in no significant change in fasting plasma triglyceride concentrations, however VLDL ApoB decreased almost significantly suggesting fewer VLDL particles in the circulation. DHA also resulted in a trend toward an increase in HDL cholesterol. Concurrent with these changes however, DHA resulted in a trend toward an increase in LDL and total cholesterol and these changes corresponded with significantly higher LDL ApoB, cholesterol ester, free cholesterol, phospholipid and protein compared with the olive oil controls, suggesting more LDL particles and cholesterol in circulation. Such changes in LDL increase the trend toward, rather than away from, cardiovascular risk.

When soy was combined with the DHA during the first six weeks of the study, plasma triglyceride concentrations significantly decreased (-24%), and HDL cholesterol significantly increased (8.4%). The increases in plasma LDL cholesterol concentrations and LDL composition components observed with DHA alone were not seen and plasma total cholesterol concentrations remained unchanged.

When DHA was consumed with control cereal between six and twelve weeks of the study plasma lipid concentrations remained unchanged however there was an almost significant reduction in VLDL triglyceride concentrations. When soy was combined with DHA between six and twelve weeks, plasma triglyceride and HDL cholesterol concentrations remained unchanged, however, plasma LDL and total cholesterol were significantly reduced. This corresponded with a significant reduction in LDL cholesterol content.

These findings show that some beneficial lipid changes can be achieved with DHA supplementation alone however they provide preliminary evidence for greater improvement to the overall lipid profile when soy isoflavones and DHA are combined.

Table 3.26 Summary of the effects of DHA supplementation alone and the combination of DHA and soy isoflavones on plasma lipids between baseline and six weeks (0-6wks) and between six and twelve weeks of the study (6 – 12 wks)

	0 – 6wks	
	DHA + Control	DHA + Soy
Triglycerides	No change	↓ (24%) p = 0.009
HDL cholesterol	↑ (9.1%) p = 0.08	↑ (8.4%) p = 0.007
LDL cholesterol	↑ (10.7%) p = 0.07	No change
Total cholesterol	↑ (8.0%) p = 0.08	No change
	6 – 12wks	
	DHA + Control	DHA + Soy
Triglycerides	No change	No change
HDL cholesterol	No change	No change
LDL cholesterol	No change	↓ (9.5%) p = 0.03
Total cholesterol	No change	↓ (6.5%) p = 0.04

### 3.5.1 DHA supplementation

Fasting plasma triglyceride concentrations decreased 24% when DHA oil supplementation was combined with soy cereal consumption. As this change was not observed with DHA oil supplementation and control cereal this finding may suggest an influence of soy isoflavones on plasma triglyceride concentrations. A meta-analysis of 17 studies with 853 subjects examining the effect of isoflavone interventions in the form of tablets on plasma lipid concentrations found no effect of soy isoflavones on plasma triglyceride concentrations. Recently however, Merz et al. (2006) reported that higher plasma daidzein concentrations were significantly associated with lower triglyceride concentrations and that the strength of this relationship was incrementally related to the magnitude of daidzein levels independent of other lipoprotein modulators (Merz et al., 2006). While a significant correlation between plasma daidzein and triglyceride concentrations was not observed in this study, it is possible that the soy isoflavones may have influenced plasma triglyceride concentrations when consumed in conjunction with DHA supplementation. The majority of studies that have examined the effects of DHA on blood lipids have reported similar reductions to those observed in this study, ranging from 14% to 32% in plasma triglycerides with 0.94 - 3.0g/d of DHA over 4-15 weeks in healthy and hyperlipidemic subjects (Sanders et al., 2006; Maki et al., 2005; Agren et al., 1996; Nestel et al., 2002; Geppert et al., 2006; Davidson et al., 1997; Conquer and Holub, 1996; Buckley et al., 2004). Not all studies using DHA oil supplementation have reported a reduction in triglyceride concentrations (Rambjor et al., 1996; Conquer &

Holub, 1998; Hamazaki et al., 1996; Theobald et al., 2004). However, low subject numbers, low baseline triglyceride concentrations ( $< 1.5$  mmol/L) (Rambjor et al., 1996; Conquer and Holub, 1998) and daily supplementation with less than 1.0g/d of DHA (Theobald et al., 2004) have likely contributed to these findings.

The current study was powered to observe a change in fasting plasma triglyceride concentrations with DHA oil supplementation. However, that was based on combining the data from the two DHA groups. The absence of a reduction in plasma triglycerides in the first six weeks with DHA alone may be due to insufficient statistical power. In this study plasma triglyceride concentrations did not change between six and twelve weeks of DHA supplementation. This may be because the maximum reduction in triglycerides was achieved after six weeks. This is supported by the fact that the 24% reduction in triglycerides observed with DHA and soy in the first six weeks was similar to reductions reported from other studies using similar doses of DHA supplementation (Sanders et al., 2006; Maki et al., 2005; Agren et al., 1996; Nestel et al., 2002; Geppert et al., 2006; Davidson et al., 1997; Conquer and Holub, 1996; Buckley et al., 2004). Erythrocyte membrane DHA increased by 70% after six weeks and by 100% of baseline values after twelve weeks in both DHA groups. These increases are similar to those reported in other studies where erythrocyte membrane DHA increased by 58 - 91% from baseline values after 6 - 14 weeks of daily supplementation with 0.7 - 2.2g/d of DHA (Theobald et al., 2004; Vidgren et al., 1997). The continued increase in erythrocyte membrane DHA over the twelve weeks suggests subjects were compliant with ingesting the prescribed quantity of DHA oil over the course of this study. Thus an absence of change in plasma triglyceride concentrations between six and twelve weeks is not due to decreased subject compliance to the DHA oil supplements.

DHA supplementation resulted in significantly lower VLDL triglyceride, total cholesterol, cholesterol ester, free cholesterol, and phospholipid and a significantly lower VLDL core: surface ratio, suggesting smaller VLDL particles, when compared with the olive oil control. Few studies have examined the effect of a DHA rich oil supplement on VLDL triglyceride concentration or composition however two studies which did, reported a significant reduction in VLDL triglyceride concentrations. Agren et al. (1996) reported a significant reduction in fasting plasma triglyceride concentrations, VLDL triglyceride content and VLDL ApoB after 15 weeks of dietary

supplementation with 1.68g/d of DHA in healthy men, while Nestel et al. (2002) reported a significant reduction in plasma and VLDL triglyceride concentrations after 7 weeks of daily supplementation with 3g/d of DHA in dyslipidemic men. Additionally, two studies have reported no change (Rambjor et al., 1996; Agren et al 1996) while one study reported a significant reduction in VLDL cholesterol (Sanders and Hinds, 1996) following DHA (1.68 – 3.0g/d) supplementation. Smaller, less triglyceride-rich VLDL particles as observed in this study is a frequently reported outcome following fish oil supplementation (Price et al., 2000; Chan et al., 2003; Bordin et al., 1998; Lu et al., 1999; Hsu et al., 2000; Contacos et al., 1993; Tato et al., 1993; Abbey et al., 1990; Tsai and Lu, 1997; Hau et al., 1996, Inagaki et al., 1988). In association with a reduced VLDL triglyceride content a reduction in VLDL apoB is often observed with fish oil supplementation (Abbey et al., 1990; Tato et al., 1993; Hsu et al., 2000). In this study there was a trend toward a reduction in VLDL apoB after six weeks in DHAc-s supporting these findings.

Hau et al. (1996) reported in healthy male subjects, that daily consumption of 1.4g EPA and 1.2g DHA for six weeks reduced VLDL cholesterol and triglycerides by almost 50%, however examination of the percent composition of the VLDL particles showed that VLDL became enriched with cholesterol ester and protein but depleted of triglyceride. In the current study, the percentage of VLDL protein increased significantly in DHAc-s after six weeks while the percentage of cholesterol ester increased almost significantly in DHAs-c. These findings and those of Hau et al. (1996) could suggest increased VLDL triglyceride catabolism by lipoprotein lipase with omega-3 supplementation which has been reported previously (Rivellese et al., 2003; Park et al., 2004; Khan et al., 2002). LDL is formed by the process of VLDL triglyceride hydrolysis which is mediated by the enzymes lipoprotein lipase and hepatic triglyceride lipase (Nilsson-Ehle et al., 1980; Nozaki et al., 1986). In DHAc-s there was a trend toward a 17% reduction in VLDL apoB and LDL apoB was 30% higher than in the control group OOC-s after six weeks suggesting an increased conversion of VLDL to LDL over that period. Several studies have shown that smaller VLDL particles that contain less triglyceride are more readily catabolized to LDL than larger, triglyceride-rich VLDL particles (Lu et al., 1999; Packard et al., 1984; Oschry et al., 1985; Inagaki and Harris, 1990) and several studies have demonstrated an increased propensity for



VLDL particles to be converted to LDL with fish oil supplementation (Lu et al., 1999; Huff and Telford, 1989; Chan et al., 2003; Harris et al., 1990).

In this study IDL triglyceride increased significantly between six and twelve weeks in DHAc-s and there was a trend toward an increase in IDL total cholesterol, free cholesterol and phospholipid over the same time period. No changes were seen in IDL composition in DHAs-c. Other studies that have isolated IDL and examined composition changes with fish oil supplementation have reported no change (Contacos et al., 1993), a significant or trend toward a decrease in IDL triglyceride and cholesterol concentrations (Kasim-karakas et al., 1995; Abbey et al., 1990); a reduction in IDL particle size (Fisher et al., 1998) or increased conversion of IDL to LDL (Chan et al., 2003). IDL is the intermediate product of triglyceride hydrolysis from VLDL in the formation of LDL. Thus a trend toward an increase in IDL triglyceride, cholesterol and phospholipid content may be the result of increased conversion of VLDL to LDL although this cannot be determined from the current study.

There was a trend toward a 10.7% increase in LDL cholesterol in the first six weeks of DHA supplementation in this study. This increase although not quite reaching statistical significance is similar to reports from other studies in which increases in LDL cholesterol of 7-14% using doses of DHA ranging from 0.7 – 4.0g/d taken over 6-12 weeks have been reported in healthy and hyperlipidemic subjects (Mori et al., 2000c; Sanders et al., 2006; Maki et al., 2005; Geppert et al., 2006; Theobald et al., 2004) (see Table 2.11 in Chapter 2). In conjunction with the trend toward an increase in plasma LDL cholesterol concentrations as calculated by the Friedewald formula, LDL Apo B, cholesterol ester, free cholesterol, phospholipid and protein were all significantly higher after six weeks of DHA supplementation with control cereal compared with six weeks of supplementation with the olive oil control. Few studies have examined the effect of DHA, EPA-rich, fish or algal oil supplementation on LDL composition. Two studies which examined the effect of DHA supplementation on LDL cholesterol content reported no change following 1.68g/d (Agren et al., 1996) and 3g/d (Rambjor et al., 1996) of DHA. Lindsey et al. (1992) reported a significant increase in LDL cholesterol ester, a reduction in LDL protein content and a significant increase in the LDL core: surface ratio following two weeks of daily supplementation with 3.6g of EPA and 2.9g of DHA. Thomas et al. (2004) however reported no change in LDL cholesterol content

following four weeks of supplementation with 2.4g EPA and 1.6g of DHA, while Hsu et al. (2000) reported a significant increase in LDL cholesterol content and LDL ApoB following daily intake of 1.45g EPA and 1.55g DHA over four weeks. Similar to the findings of this study, several studies have reported increased LDL ApoB (Radack et al., 1990; Fisher et al., 1998; Hsu et al., 2000) following fish oil supplementation.

While there was an increase in the number of LDL particles in this study demonstrated by a rise in LDL apoB, the size of the LDL particles as estimated by the core: surface or LDL cholesterol: ApoB ratio did not change. Numerous studies have reported similar findings with fish oil (predominantly EPA-rich) supplementation (Hsu et al., 2000; Fisher et al., 1998; Rivellesse et al., 2003; Lu et al., 1999; Hau et al., 1996; Calabresi et al., 2000; Radack et al., 1990), however, this finding is not consistent especially amongst studies using DHA-rich oil (Engler et al., 2005; Theobald et al., 2004; Mori et al., 2000; Suzukawa et al., 1995; Lindsey et al., 1992). In these latter studies EPA or DHA-rich oil supplementation resulted in an increase in LDL size or reduced LDL density.

The number of LDL particles can increase in the blood due to either the increased production or decreased clearance of LDL. As already discussed an increase in LDL cholesterol may occur as a result of increased catabolism of triglyceride in VLDL possibly via increased lipoprotein lipase activity induced by the DHA supplementation. While evidence from the current study supports this mechanism, a previous kinetic study found no change in fractional catabolic rates of ApoB in VLDL, IDL or LDL following fish oil supplementation (Chan et al., 2003). Kasim-Karakas et al. (1995) reported that fish oil supplementation caused a significant increase in LDL cholesterol which correlated with a reduction in VLDL cholesterol however this was not associated with an increase in VLDL lipolysis to LDL. While no studies have examined the effect of DHA supplementation on LDL receptor activity and hepatic uptake there is evidence from human fish oil supplementation, *in vitro* and animal studies that EPA and DHA may inhibit LDL receptor activity (Wong and Nestel, 1987; Roach et al., 1987; Hannah et al., 1993), reduce LDL receptor mRNA abundance (Lindsey et al., 1992) and potentially reduce LDL binding to LDL receptors (Hsu et al., 2000); resulting in an increase in plasma LDL cholesterol and LDL apoB. Further research is required examining the effect of DHA on LDL receptor activity.

Six weeks of DHA supplementation in this study resulted in a trend toward a 9% increase in HDL cholesterol when control cereal was consumed and a significant 8.4% increase in HDL cholesterol when soy cereal was consumed. An increase in HDL cholesterol is a typical outcome of omega-3 fatty acid supplementation (Harris, 1996). In several studies that have examined lipid responses to DHA oil supplementation (1.6-4.0g/day) a significant increase (3-17%) in HDL cholesterol has been reported (Mori et al., 2000; Grimsgaard et al., 1997; Davidson et al., 1997; Sanders and Hinds, 1992; Conquer and Holub, 1996). In several studies (Mori et al., 2000; Grimsgaard et al., 1997; Rambjor et al., 1996) HDL subfractions were measured and DHA supplementation significantly increased concentrations of HDL<sub>2</sub> cholesterol (Mori et al., 2000; Agren et al., 1996; Sanders and Hinds, 1992) sometimes concurrently with a reduction in HDL<sub>3</sub> cholesterol (Rambjor et al., 1996). However total plasma HDL cholesterol was not always reduced in these studies despite the change in HDL subfractions. In the current study there were no changes in HDL subfractions concurrent with the rise in plasma HDL in Group DHAs-c or with the trend toward an increase in Group DHAc-s after six weeks of DHA supplementation. This finding is identical to the results of Maki et al. (2005) in which 1.52g/d of algal-sourced DHA resulted in a trend toward a 9% increase in HDL cholesterol without a change in the concentrations of cholesterol carried by the HDL<sub>2</sub> or HDL<sub>3</sub> subfractions. Maki et al. (2005) did not provide an explanation for their findings.

### **3.5.2 Soy isoflavone consumption**

Urinary isoflavone concentrations are considered primary biomarkers of dietary consumption of soy isoflavones (Ritchie et al., 2004; Xu et al., 2000; Lampe et al., 1999). The soy cereal used in this study provided 47mg of daidzein and 13mg of genistein per 45g serve. Soy cereal consumption resulted in a 1.5 - 3 fold increase in urinary daidzein concentrations demonstrating good compliance by the subjects to regular consumption of the soy cereal. Forty eight percent of ingested daidzein and 10.8% of ingested genistein was recovered in 24 hour urine samples in this study which are similar to recoveries of 38 - 66% for daidzein and 9 - 24% for genistein reported in other studies (Xu et al., 2000; Lu and Anderson, 1998). A difference in isoflavone metabolism between men and women has been identified (Lu and Anderson, 1998). In the current study there was no difference in urinary isoflavone recovery between men

and women (data not shown), however, there were significantly fewer women than men in the study.

In this study a daily serve of 90mg/d of soy isoflavones combined with olive oil supplementation did not result in any improvements in plasma lipids. The absence of an effect on blood lipids in the two olive oil groups however is a finding not dissimilar to studies that have used isoflavone supplements devoid of soy protein (**Table 3.24**).

The soy cereal used in the current study was high in glycitein (making up one third (35%) of the total isoflavone content) and had a low ratio of genistein: daidzein (1:3.7) compared with the other intervention studies using extracted soy isoflavone supplements (Dewell et al., 2002; Hale et al., 2002). There have been few studies which have examined the cholesterol lowering effects of glycitein. Sirtori et al. (2002) reported no change in plasma lipids in twenty subjects with type II hypercholesterolemia following daily supplementation with a soy milk containing 25g of protein and equal amounts of glycitein (24mg), daidzein (28mg) and genistein (25mg). The authors of that study attributed the absence of a cholesterol reduction to the high level of glycitein contained in the milk as a previous study following the same protocol apart from the presence of glycitein, conducted within the same research unit, showed a significant reduction in plasma cholesterol (Sirtori et al., 1999). It could be possible that in the current study the high glycitein content of the soy cereal and subsequent lower levels of daidzein and genistein may have limited the amount of cholesterol reduction achievable in the two olive oil groups when the soy cereal was consumed. While the soy cereal provided 90mg of isoflavones only 58mg was comprised of daidzein and genistein which may be the more active isoflavones for influencing cholesterol reduction. This dose of daidzein and genistein is representative of a typical daily intake level in Asian countries that are high soy consumers (Wakai et al., 1999; Somekawa et al., 2001). While epidemiological studies suggest this may be sufficient to reduce the incidence and risk of developing CVD it may not have been sufficient to demonstrate a significant reduction in plasma cholesterol concentrations after only six weeks.

Table 3.27 Comparison of lipid outcomes from clinical trials testing the effect of soy isoflavone supplementation (in the absence of soy protein) on plasma lipids, with the findings from the current study<sup>1-4</sup>

Study	Design	Subjects	Isoflavone dose (in tablet)	Study duration	Baseline Total & LDL chol	Total chol	LDL chol
						Change (mmol/L)	
Garrido et al. (2006)	controlled parallel	29 healthy postmenopausal women	100 mg/d	12 wks	5.5 ± 1.0 3.4 ± 0.4	0.03 5% (NS)	0.30 9% (NS)
Hall et al. (2006)	controlled cross-over	117 healthy postmenopausal women	50 mg/d	8 wks	6.0 ± 1.3 3.9 ± 1.1	0.10 2% (NS)	0.04 1% (NS)
Simons et al. (2000)	controlled cross-over	20 healthy post-menopausal women	80 mg/d	8 wks	5.9 ± 0.3 3.9 ± 0.3	-0.41 -7% (NS)	-0.33 -8% (NS)
Hale et al. (2002)	controlled parallel	29 menopausal women	80 mg/d	2 wks	5.0 ± 1.0 3.9 ± 1.3	0.38 8% (NS)	-0.03 -0.5% (NS)
Hsu et al. (2001)	uncontrolled single group	37 post-menopausal women	150 mg/d	6mths	5.1 ± 1.0 3.1 ± 0.9	-0.11 -2% (NS)	-0.1 (3%) (NS)
Squadrito et al. (2002)	controlled parallel	60 post-menopausal women	54 mg/d	6 mths	5.3 ± 1.1 3.6 ± 0.4	+0.2 4% (NS)	0.1 3% (NS)
Dewell et al. (2002)	controlled parallel	36 elderly post-menopausal women	150 mg/d	6 mths	6.8 ± 0.2	-0.40 -6% (NS)	no data
Tablet supplementation with isoflavones from red clover							
Howes et al. (2000)	controlled crossover	66 post menopausal women	43.5 mg	5 wks	6.3 ± 1.0 4.3 ± 1.1	-0.21 -3% (NS)	-0.06 -1% (NS)
			87mg			-0.18 -3% (NS)	-0.02 -0.5% (NS)
Hodgson et al. (1998)	controlled parallel	46 men and 13 post menopausal women	55 mg	8 wks	5.4 ± 0.2 3.5 ± 0.2	-0.06 -1% (NS)	-0.01 -0.3% (NS)
Samman et al. (1999)	randomised crossover	14 pre-menopausal women	86 mg	8 weeks	4.1 ± 0.2 2.5 ± 0.2	-0.03 -0.7% (NS)	-0.06 -2%(NS)
Nestel et al. (1999)	controlled crossover	17 menopausal women	40mg	5wks	6.1 ± 0.8 4.0 ± 0.8	-0.17 -3% (NS)	-0.23 -6% (NS)
			80mg			-0.2 -3%(NS)	-0.24 -6% (NS)
Omega-Soy study	controlled crossover	38 men and women	90 mg	6 wks	6.3 ± 0.5 3.8 ± 0.2	0.01 0.2% (NS)	-0.05 1.3% (NS)

1. Baseline total and LDL cholesterol data are mean (mmol/L) ± SEM

2. Total and Total chol = Total cholesterol

3. LDL and LDL chol = LDL cholesterol

4. NS = not significant

It has been suggested that equol, a metabolite of daidzein due to its high estrogenicity may be partly responsible for the cholesterol lowering effects of soy consumption (Setchell et al., 2002b). Approximately 25 - 35% of the population are capable of producing equol in response to dietary daidzein intake (Setchell et al., 2002b) and it has been proposed (Setchell et al., 2002b; Meyer et al., 2004) that this may explain the variable blood lipid responses seen in clinical trials involving dietary soy consumption. Two clinical trials (Lydeking-Olsen et al., 2002; Meyer et al., 2004) have reported that the greatest hypocholesterolemic responses to a soy diet occurred in the proportion of the study population that were equol producers. A later study by Greany et al. (2004) however, reported that soy consumption reduced plasma LDL and total cholesterol in mildly hypercholesterolemic postmenopausal women independent of equol production status.

In the current study, there was no correlation found between plasma or urine equol concentrations and plasma total or LDL cholesterol. Plasma equol concentrations increased significantly ( $75.6 \pm 27.8$  ng/mL) and in 89% of subjects in OOs-c and there was a trend toward a similar increase ( $75.7 \pm 69.8$  ng/mL) in DHAs-c following soy cereal consumption, with mean plasma equol concentrations being similar in both groups ( $118.6 \pm 33.5$  ng/mL and  $116.2 \pm 84.8$  ng/mL, respectively). LDL cholesterol remained unchanged in OOs-c following soy cereal consumption demonstrating that the increase in equol concentrations did not have any significant effect on LDL cholesterol. Furthermore, equol was detected at very low concentrations in only one subject in DHAc-s following soy cereal consumption, but despite this LDL cholesterol decreased significantly by 9.5%. These findings support those of Greany et al. (2004) suggesting that equol is not an influencing factor on blood lipids.

Several meta-analyses of human clinical trials have reported that between 25 – 60g/d of soy protein can significantly reduce total and LDL cholesterol and that this reduction improves significantly as the isoflavone content of the soy protein increases (Zhan and Ho, 2005; Zhuo et al., 2004; Anderson et al., 1995; Reynolds et al., 2006). In this study 1.2g of soy protein was consumed each day with 90mg of isoflavones. If soy protein is required for soy isoflavones to have an impact on plasma cholesterol concentrations which has been suggested previously (Erdman et al., 2000) and is supported by animal models (Clarkson, 2002; Clarkson et al., 2001), the minimum amount of soy protein

required for this to occur has not been determined. Based on the absence of change in blood lipids observed with the soy isoflavone consumption it may be suggested that a higher quantity of protein is required to observe a significant reduction in cholesterol concentrations with this dose of isoflavones.

### **3.5.3 Combined effect of isoflavones and DHA on lipid metabolism**

The addition of isoflavones to the diet in combination with DHA supplementation was associated with a significant reduction in total and LDL cholesterol between six and twelve weeks in DHAc-s and a suppression of an expected rise in LDL cholesterol in the first six weeks in DHAs-c. These results suggest a potential interaction effect or facilitation between DHA and soy isoflavones which enabled the isoflavones to have a beneficial effect on plasma cholesterol concentrations. The changes in LDL cholesterol are unlikely to be due to other factors. A significant weight loss of 1kg was observed in each of the two DHA groups between six and twelve weeks of the intervention; however, in DHAs-c plasma total and LDL cholesterol remained unchanged despite the weight loss. This finding suggests that the 1kg weight loss seen in DHAc-s is unlikely to have contributed to the significant cholesterol reduction observed. Dietary intake and composition did not change in either of the DHA groups between six and twelve weeks of the intervention ruling out any background dietary factors contributing to the reduction in total or LDL cholesterol in DHAc-s.

It has been suggested that the rise in LDL cholesterol associated with fish oil or DHA supplementation may be transient and that levels plateau or even return to baseline with extended supplementation periods (Harris, 1996; Weber and Raederstorff, 2000). Such transience in the effect of DHA on LDL cholesterol could potentially explain the significant reduction in LDL cholesterol observed in DHAc-s between six and twelve weeks. The evidence for this suggestion however is relatively weak. Harris (1996) reported that studies in hyperlipidemic subjects of less than ten weeks duration (n=31) resulted in a fairly consistent rise of approximately 30% in LDL cholesterol while in studies six and twelve months duration (n=3), the rise in LDL cholesterol was approximately 5% and was often statistically insignificant. These findings from Harris (1996) relating to long term studies, should be considered in the context of study data relating to potential changes in dietary composition, subject compliance to the oil supplements and other factors such as changes in body weight and exercise regime.

Changes in any of these parameters are more likely to occur over longer study durations and could influence blood lipid results. Furthermore, there have been several studies not included in the Harris (1996) review in which a significant increase in LDL cholesterol was maintained after 24 weeks of fish oil (Connor et al., 1993; Radack et al., 1990) or DHA supplementation (Theobald et al., 2004).

With other factors excluded, it is appropriate to suggest that the changes in LDL cholesterol observed when DHA was consumed with soy isoflavones in this study may have been due to some type of synergistic effect between these two functional ingredients. A detailed discussion of the potential mechanisms by which this could occur is provided in Chapter 4 of this thesis.

#### **3.5.4 Arterial compliance, blood pressure, heart rate and other cardiovascular measures**

DHA supplementation resulted in a trend toward a higher cardiac ejection time compared with olive oil supplementation after both six and twelve weeks of the intervention period. Cardiac ejection time is a calculated value representing left ventricular ejection time. A trend toward an increase in this parameter with DHA supplementation is likely to be associated with the concurrent reduction in heart rate achieved. It may also reflect a potential increase in left ventricular ejection fraction produced by enhanced ventricular filling which has been observed in marmoset monkeys fed tuna oil (McLennan et al., 1992) and in healthy men after seven weeks of taking 4g/day of DHA (Grimsgaard et al., 1998). McLennan et al. (1992) also reported that in association with increased left ventricular ejection fraction, in animals fed tuna oil, was a 40-70% increase in stroke volume. Additionally, tuna oil supplementation resulted in a significantly lower resting heart rate. In the current study stroke volume and stroke volume index were 12% and 10.4% higher, respectively, after twelve weeks of DHA-rich oil compared with olive oil supplementation.

Reflecting on the findings of McLennan et al. (1992) in the marmoset monkeys, Grimsgaard et al. (1998) reported a 2.2 beats/min reduction in resting heart rate in association with improved ventricular filling in men taking DHA supplements. In this study, resting heart rate when DHA-oil was consumed in the absence of soy cereal decreased 4.6 beats/min ( $p = 0.01$ ) in DHAc-s and 2.8 beats/min ( $p = 0.6$ ) in DHAs-c.



When soy cereal was consumed concurrent with DHA oil supplementation there was an insignificant increase of approximately 1 beat/min in resting heart rate. No other studies could be found which have measured the effect of soy isoflavone supplementation on heart rate. It is therefore unclear as to whether isoflavone intake might affect this parameter.

A meta-analysis (Mozaffarian et al., 2005) of 30 randomised trials that tested the effect of fish oil supplementation on heart rate, showed that fish oil reduced heart rate by 1.6 beats/min compared with placebo and that the reduction in heart rate achieved with fish oil supplementation was influenced by a higher baseline heart rate ( $\geq 69$  beats/min) and duration of supplementation ( $\geq 12$  weeks). Furthermore, changes in heart rate were not dependent on fish oil dose, population age or health, or type of control oil (Mozaffarian et al., 2005). In the current study heart rate was 4.3 beats/min lower after twelve weeks of DHA-oil compared with olive oil supplementation. This reduction is similar to that reported by others using 4g/day of DHA supplementation (Mori et al., 1999; Woodman et al., 2002). In those studies included in the meta-analysis which used a dose of omega-3 fatty acids (2.7 – 3.0g/d) similar to that used in the current study (2.7g/d), heart rate irrespective of the proportion of DHA:EPA contained in the oils predominantly decreased by a varied amount (range = 0.0 to -6.0 beats/min), with an average reduction calculated at -1.8 beats/min (Conquer and Holub, 1998; Deslypere, 1992; McVeigh et al., 1994; Mills et al., 1989; Miyajima et al., 2001; Nestel, 2002; Stark and Holub, 2004).

Mozaffarian et al. (2005) determined that, based on work by Jouven et al. (2001), a reduction of 1.6 beats/min with fish oil supplementation could be viewed as a 5% reduction in the risk of sudden death. Based on the same assumptions, the 4.6 beats/min reduction in heart rate observed in this study with DHA supplementation could potentially account for a 14% reduction in sudden death risk.

Arterial compliance of the small and large arteries did not change in this study with either DHA-rich oil supplementation or soy cereal consumption. Other studies using a similar dose (3g/day) of either DHA or fish oil (1.8g EPA and 1.2g DHA) supplementation have shown significant improvements in non-invasively assessed arterial compliance (McVeigh et al., 1994; Nestel et al., 2002). McVeigh et al. (1994)

reported a significant increase in small and large artery compliance in men and women with type 2 diabetes after six weeks of fish oil (1.8g EPA and 1.2g of DHA) supplementation. In that study, blood pressure, cardiac output, stroke volume and systemic vascular resistance remained unchanged. The authors suggested that the improvement in arterial compliance may have been due to direct endothelial influence of the omega-3 fatty acids altering the production, release or breakdown of vasoactive substances. In an earlier study by Simon et al. (1982) intravenous administration of nitroglycerin (an exogenous nitric oxide donor), significantly decreased systolic blood pressure but did not change diastolic or mean arterial pressure, cardiac index, stroke index or total peripheral resistance. This finding, which is similar to that reported by McVeigh et al. (1994), suggested that omega-3 fatty acids may have improved arterial compliance via increasing the endothelial release of nitric oxide, a mechanism that has been shown in other studies to occur with omega-3 fatty acid supplementation (Harris et al., 1997b; McVeigh et al., 1993).

It is unclear why in the current study DHA-rich oil supplementation did not result in a reduction in arterial compliance. The dose and duration of DHA or omega-3 fatty acid supplementation, baseline values of arterial compliance and number of subjects, were all similar to those reported by Nestel et al. (2002) and McVeigh et al. (1994) in which improvements in arterial compliance were observed. One potential explanation for the absence of a beneficial effect of DHA on arterial compliance in this study could be the high level of palmitate contained within the DHA-rich oil supplements. Palmitate has been shown *in vitro* to be associated with the promotion of microvascular endothelial cell oxidation and apoptosis (Yamagishi et al., 2002) and both *in vitro* and *in vivo* to induce high levels of interleukin-6 in coronary artery endothelial cells and to subsequently promote vascular inflammation (Staiger et al., 2004). While these effects may not necessarily impact directly on the measurement of arterial compliance they do suggest increased levels of palmitate may influence endothelial function in a dissimilar way to omega-3 fatty acids. Thus the high content of palmitate in the DHA oil may have potentially counteracted the beneficial effects DHA may have had on arterial compliance. This explanation however, is potentially weakened by the fact that the significant improvement in arterial compliance reported by McVeigh et al. (1994) occurred via dietary supplementation with MaxEpa oil, which has also been shown to contain relatively high levels of saturated fat and cholesterol (Harris et al., 1989).

In this study arterial compliance remained unchanged with soy cereal consumption. Several studies have reported a significant increase in arterial compliance with isoflavone supplementation (Nestel et al., 1997; Teede et al., 2003; Nestel et al., 1999). In menopausal women, daily consumption of 80mg/d of soy isoflavones significantly improved systemic arterial compliance by 26% compared with placebo (Nestel et al., 1997). In men and postmenopausal women, six weeks of supplementation with 80mg/d of formononetin or biochanin (daidzein and genistein precursors, respectively) significantly improved systemic arterial compliance and reduced total peripheral resistance and central pulse wave velocity compared to placebo (Teede et al., 2003). In that study the arterial compliance improvements were attributed to the formononetin-enriched isoflavone supplement. 80mg/d of isoflavones from red clover, providing predominantly formononetin and minimal genistein and daidzein was also reported to reduce systemic arterial compliance in menopausal women compared with placebo however, the placebo group in that study by Nestel et al. (1999) consisted of only three subjects compared with the thirteen – fourteen women in the active treatment groups, weakening the strength of this finding. The results suggest that formononetin or genistein may be beneficial in improving arterial compliance. Human and animal *in vitro* studies confirm the beneficial effect of genistein on endothelial dependent vasodilation (Squadrito et al., 2002; Molsiri et al., 2004; Li et al., 2004; Khemapech et al., 2003). While both genistein and daidzein have been shown *in vitro* to cause a similar concentration-dependent relaxation of rat aorta (Mishra et al., 2000), human studies examining the effect of soy isoflavone supplements containing equal quantities of genistein and daidzein on vascular reactivity, have provided mixed results as to a beneficial effect (Lissin et al., 2004; Hale et al., 2002).

In the current study, daily intake of genistein was quite low compared to daidzein and glycitein intake, when soy cereal was consumed. The absence of an effect of the soy isoflavones on arterial compliance may be due to the low genistein content of the cereal. It is unlikely that the low soy protein content of the cereal was responsible for the lack of effect of the soy isoflavones on arterial compliance. Improvements in arterial compliance have been seen with 80mg doses of isoflavone supplementation in the absence of soy protein (Nestel et al., 1997; Teede et al., 2003; Nestel et al., 1999) and in at least one study using the same dose of isoflavones (80mg/d) in which 30g/d of soy

protein was also consumed for five weeks, arterial compliance did not change (Meyer et al., 2004).

In this study blood pressure did not change with either DHA-rich oil supplementation or soy cereal consumption. A significant reduction in blood pressure has been reported in one study, following four weeks of supplementation with 50mg/d of isoflavones from red clover in postmenopausal women with type 2 diabetes (Howes et al., 2003). However, there have been a considerable number of studies using isoflavone supplementation from soy and clover sources, over a range of doses (43.5 – 114mg/d) that have reported no improvement in either clinical or ambulatory blood pressure (Simons et al., 2000; Hodgson et al., 1999; Atkinson et al., 2004; Teede et al., 2003; Han et al., 2002; Nikander et al., 2003). One study using a pharmacological dose of soy isoflavones (5mg/kg of body weight) in postmenopausal women even reported an upward trend in systolic blood pressure with isoflavone supplementation (Hutchins et al., 2005).

While these findings suggest that isoflavones may have little effect on blood pressure, it should be considered that most of these studies were conducted in normotensive individuals where blood pressure was not the primary outcome of the study. Thus, while the current study also found an absence of a blood pressure lowering effect of soy isoflavones, further research in normo- and hyper-tensive individuals designed to measure blood pressure as a primary outcome, is warranted.

In a 2002 American Heart Association Scientific Statement on dietary fatty acids and cardiovascular disease it was reported that based on cellular, animal, observational and clinical investigations, supplementation with doses of approximately 3g/d of marine sourced omega-3 fatty acids are required to produce a minimal effect on blood pressure in non-hypertensive individuals and modest effects in hypertensive individuals (Kris-Etherton et al., 2002). Furthermore, DHA may be more effective than EPA in lowering blood pressure (Mori et al., 1999). An earlier meta-analysis (Morris et al., 1993) of thirty-one placebo-controlled human trials including 1356 subjects, showed that a mean reduction of -3.0mmHg in systolic pressure and -1.5mmHg in diastolic pressure can be achieved with fish oil supplementation. A dose-response effect was also reported such that doses of fish oil supplementation less than 3g/d were shown to reduce systolic

blood pressure by 1.3 mmHg and diastolic blood pressure by 0.7 mmHg. The duration of fish oil treatment was not found to be an influential factor in altering the degree of blood pressure reduction achieved with fish oil supplementation. In a sub-analysis of studies that recruited or screened for hypercholesterolemic subjects, similar to the current study, fish oil supplementation caused a significant reduction of 4.4 mmHg in SBP and did not change DBP (Cobaic et al., 1991; Demke et al., 1988; Bach et al., 1989; Kestin et al., 1990; Wilt et al., 1989; Dart et al., 1989).

In this study, 2.9g/day of DHA consumed over twelve weeks did not reduce clinic supine or ambulatory blood pressure in people with normal or slightly elevated systolic blood pressure and mildly elevated cholesterol. Based on the findings of the meta-analysis which showed a dose-response effect of fish oil supplementation on blood pressure, a higher dose of DHA-rich oil may have been required to see a detectable change in blood pressure.

### **3.6 Conclusion**

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The daily consumption of soy isoflavones (90mg) by mildly hypercholesterolemic men and women had no effect on fasting plasma lipids, lipoprotein composition or blood pressure and arterial compliance in this study. However, the daily consumption of soy isoflavones in conjunction with DHA supplementation (2.9g) during the first six weeks of the study resulted in an 8-10% increase in HDL cholesterol, an 18-20% reduction in plasma triglycerides and what appeared to be a suppression of an increase in LDL cholesterol which was observed with DHA supplementation alone. When consumed in the second six week period, the combination of soy and DHA resulted in a 9.5% reduction in LDL cholesterol and a 7% reduction in total plasma cholesterol concentrations.

These findings suggest that the combination of dietary soy isoflavones and DHA-rich oil supplementation may have a more favourable effect on CVD risk factors than if just isoflavones or DHA-rich oil are consumed independently. This functional food combination requires further investigation to determine the mechanisms by which soy isoflavones and DHA may result in an improvement in blood cholesterol concentrations.

## **4 Discussion**

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#### **4.1 Food synergies and functional food combinations**

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This thesis aimed to assess whether the effectiveness of DHA on CVD risk factors could be enhanced by firstly, changing dietary fatty acid composition to increase the bioavailability of supplemented DHA and secondly, by combining DHA with another functional ingredient capable of independently complementing the effects of DHA on CVD risk factors. In both studies the effect of DHA on CVD risk factors was improved, although not as a result of the original expectations. This chapter will explore the mechanistic hypotheses that might explain the unexpected synergies observed when two different functional ingredients were combined separately with DHA and the effects achieved on LDL cholesterol.

A starting point for this theoretical exploration is the understanding that functional ingredients are dietary substances that provide health benefits beyond basic nutrition, based on significant scientific evidence. Individual functional food ingredients can achieve these health benefits via a myriad of diverse mechanisms which may involve similar and interconnected biochemical pathways. As such, combinations of functional ingredients with beneficial effects on cardiovascular risk factors have the potential to act in synergy, enhance bioavailability and produce cumulative benefits greater than could be achieved from the individual contributions.

In the two human clinical trials conducted for this thesis, the combination of canola with DHA (MOFO study) and soy isoflavones with DHA (Omega-Soy study) improved the blood lipid profile more favourably than DHA alone, leading to the hypothesis that a synergistic interaction took place between the whole foods or parts thereof to achieve this outcome.

In the MOFO Study, the combination of 1.1g/d of DHA with canola, sunola or safflower oil and margarine was equally as effective as 2.2g/d of DHA at reducing fasting plasma triglyceride concentrations. However, 1.1g/d of DHA supplementation resulted in a smaller increase in LDL cholesterol with only a trend toward an 8% increase in LDL cholesterol being observed with saff(4g) compared with a 15.5% increase in LDL with saff(8g). Replacing safflower oil and margarine with either canola or sunola as a means of reducing dietary LA intake had a mixed effect on the LDL

raising response to DHA supplementation. The rise in LDL and total cholesterol observed with 2.2g/d of DHA supplementation equalled that observed with Sun(4g), suggesting that replacing dietary LA with monounsaturated fatty acids is not an effective approach to minimising the LDL cholesterol raising potential of dietary DHA supplementation. However, the dietary combination of canola with 1.1g/d of DHA effectively cancelled the LDL rise in response to DHA, with LDL cholesterol concentrations remaining unchanged compared with the 15-16% increase in LDL cholesterol observed with saff(8g) or Sun(4g).

In the Omega-Soy study the daily consumption of soy isoflavones (90mg) had no effect on fasting plasma lipids or lipoprotein composition while DHA supplementation alone (2.9g/d) resulted in a 10.8% increase in LDL cholesterol. The daily consumption of soy isoflavones in combination with DHA supplementation resulted in an 18-20% reduction in plasma triglycerides with no change in LDL cholesterol when consumed in the first six weeks and a 9.5% reduction in LDL cholesterol and a 7% reduction in total plasma cholesterol concentrations when consumed in the second six weeks.

The results of the MOFO study and Omega-Soy study suggest that the combinations of canola and DHA and soy isoflavones and DHA are more effective at improving plasma lipid concentrations than DHA alone. A number of mechanisms can be proposed to support a favourable synergistic effect of these functional food combinations on lipid concentrations.

## **4.2 How could EPA and DHA decrease fasting triglyceride concentrations?**

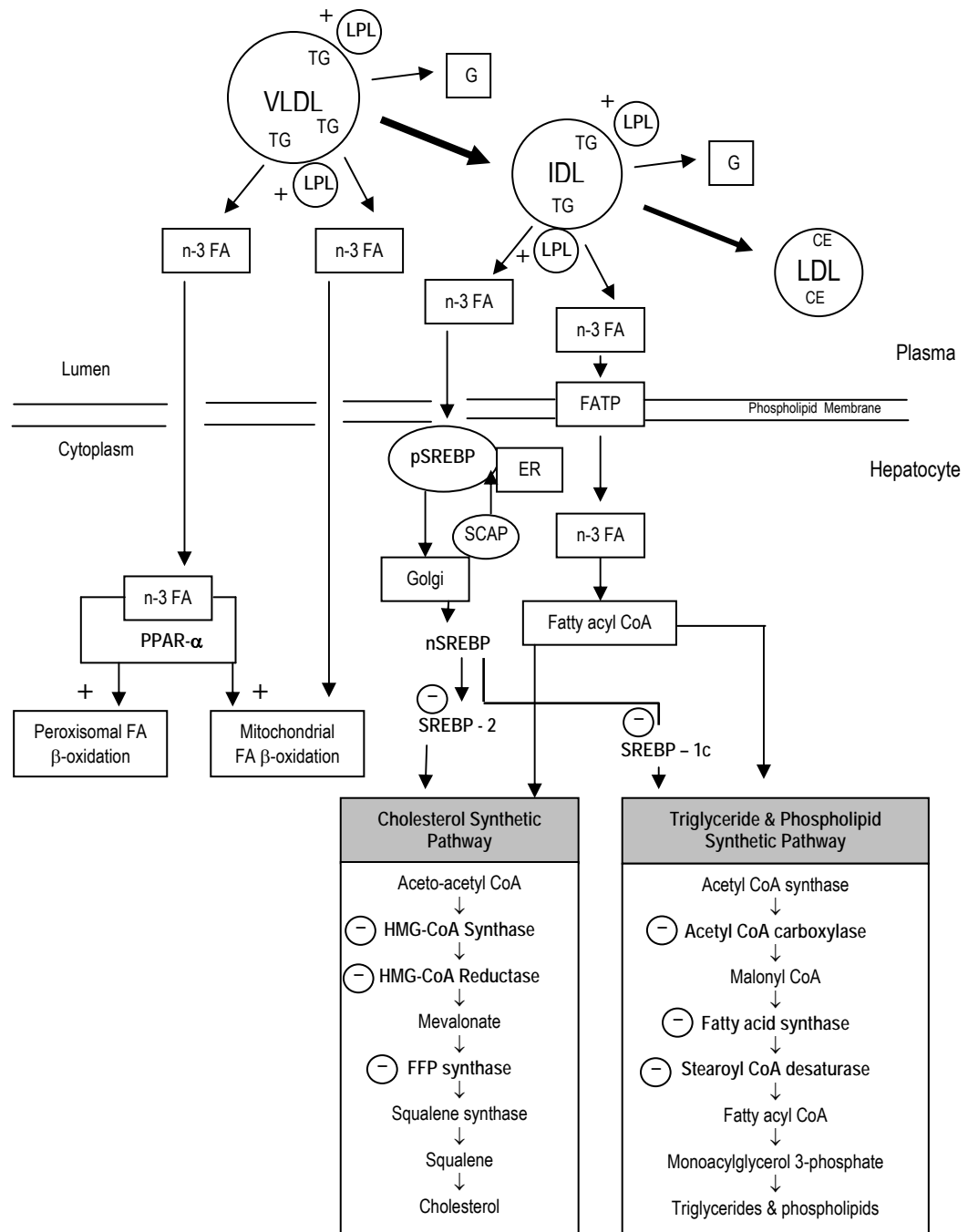
Reductions in fasting plasma triglyceride concentrations can be achieved as a result of either enhanced clearance (increased degradation) or reduced synthesis. Increased triglyceride clearance from the circulation can occur as a result of increased lipoprotein lipase activity which breaks down triglyceride in circulating lipoproteins or increased cellular uptake of triglyceride-rich lipoproteins. As these particles are usually converted to cholesterol-rich lipoproteins via the actions of lipoprotein lipase, increased hepatic uptake of triglyceride may be achieved by enhanced cholesterol ester transfer protein (CETP) activity and increased cellular HDL uptake. Increased CETP activity would result in an increase in LCAT-generated cholesterol ester from HDL being transferred to



VLDL and LDL in exchange for triglyceride. This would enable triglycerides to be removed from the circulation via the hepatic removal of HDL. While studies using fish oil supplementation have reported an increase in VLDL triglyceride catabolism by lipoprotein lipase (Rivellese et al., 2003; Park et al., 2004; Khan et al., 2002), no studies to date have reported an increase in CETP activity or an associated increase in HDL hepatic clearance (Thomas et al., 2004; Calabresi et al., 2004).

Reduced synthesis of triglyceride can occur as a result of suppressed intracellular lipogenesis and subsequent reduction in VLDL formation and release from the liver. This would result in less endogenously produced triglyceride entering the circulation. Several human clinical trials have reported a reduction in VLDL apoB and reduced VLDL triglyceride content following dietary fish oil supplementation (Abbey et al., 1990; Tato et al., 1993; Hsu et al., 2000) suggesting this may be a feasible mechanism by which EPA and DHA lower fasting plasma triglyceride concentrations.

Human *in vitro* and animal *in vivo* studies have demonstrated that EPA and DHA can suppress the hepatic production of triglyceride and VLDL (Nestel et al., 1984; Kendrick and Higgins 1999; Rustan et al., 1988), by increasing peroxisomal and mitochondrial fatty acid oxidation and diminishing fatty acid synthesis (Clarke, 2001; Ide et al., 2004; Halvorsen et al., 2001; Rustan et al., 1992). The increased fatty acid oxidation may be due to an increase in peroxisome proliferator-activated receptors (PPAR) while a reduction in fatty acid and triglyceride synthesis may be caused by a reduction in sterol receptor element binding protein – 1c (SREBP-1c) (**Figure 4.1**).



**Figure 4.1** Omega-3 fatty acids are released from triglyceride-rich lipoproteins by the actions of lipoprotein lipase resulting in the formation of LDL from VLDL. Free omega-3 fatty acids can enter cells via transporters or by diffusion through the phospholipid membrane. Once in the cell omega-3 fatty acids are ligands for PPARs and can bind directly with PPAR- $\alpha$  which promotes peroxisome and mitochondrial  $\beta$ -oxidation. Additionally omega-3 fatty acids suppress the formation of nuclear SREBP-1c and SREBP-2. In association with this, omega-3 fatty acids have been shown to decrease some of the gene expression of enzymes associated with cholesterol synthesis including suppressing LDL receptor gene expression and gene expression of enzymes involved in fatty acid synthesis and triglyceride formation. LPL – lipoprotein lipase; TG – triglyceride; G – glycerol; n-3 FA – omega-3 fatty acid; CE – cholesterol ester; FATP – fatty acid transport protein; ER – endoplasmic reticulum; pSREBP – precursor sterol regulatory binding protein; n-SREBP – nuclear sterol regulatory binding protein; SCAP – SREBP-cleavage activating protein; PPAR – peroxisome proliferators-activated receptor.

+ = symbolizes promotion or activation by omega-3 fatty acids. ⊖ = symbolizes a reduction or suppression of activity by omega-3 fatty acids

Peroxisome proliferator-activated receptors, in particular PPAR $\alpha$ , are highly expressed in the liver and enhance mitochondrial, microsomal and peroxisomal fatty acid oxidation (Pegorier et al., 2004; Clarke, 2001; Issemann et al., 1990; Schoonjans et al., 1996). Activation of PPARs also results in increased lipoprotein lipase and fatty acid transport protein (FATP) as well as enhanced adipocyte differentiation all of which can result in a reduction in plasma triglyceride concentrations (Davidson, 2006; Clarke, 2001; Pegorier et al., 2004). Several studies have demonstrated that omega-3 fatty acids activate PPAR $\alpha$  levels in hepatic cells (Ren et al., 1997; Kliewer et al., 1997; Krey et al., 1997) and this may be a means by which omega-3 fatty acids increase the transcription of genes encoding enzymes involved in fatty acid oxidation (Ren et al., 1997; Clarke, 2000; Willumsen et al., 1992) potentially leading to the hypotriglyceridemic effect seen with omega-3 fatty acid supplementation (Davidson, 2006).

Sterol regulatory element binding proteins (SREBP's) are synthesised as precursors (pSREBP) bound to the endoplasmic reticulum in the hepatocyte cytoplasm. The precursor SREBP is then bound to the SREBP-cleavage activating protein (SCAP) which carries the pSREBP to the Golgi complex for proteolytic processing. Following this post-translational process, nuclear SREBP (nSREBP) is transported to the nucleus with the assistance of inportin- $\beta$  (Nagoshi et al., 1999) where nSREBP then binds to sterol regulatory elements (SRE) in promoters of a variety of genes involved in lipogenesis (Jump et al., 2005; Bennet et al., 2004) (**Figure 4.1**). There are several isoforms of SREBP expressed in the liver, SREBP-1a and SREBP-1c which are primarily involved in regulating genes for lipogenesis and SREBP-2 which regulates genes involved in cholesterol biosynthesis and metabolism (Davidson, 2006; Le-Jossic-Corcus et al., 2005).

It has been consistently demonstrated in human *in vitro* studies and animal models that EPA and DHA reduce levels of membrane (precursor) and nuclear (mature) SREBP-1c in the liver and levels of hepatic mRNA SREBP-1a (Yoshikawa et al., 2002; Le Jossic-Corcus et al., 2005; Xu et al., 1999; Kim et al., 1999; Yahagi et al., 1999). *In vivo* studies in rats and mice and *in vitro* studies in human liver and kidney cells have also demonstrated that EPA and DHA can reduce SREBP-2 mRNA and protein (Kim et al.,

1999; Le Jossic-Corcos et al., 2005). In the study conducted in rats, FFP synthase mRNA and protein an enzyme involved in cholesterol synthesis, was also significantly reduced with a fish oil diet. The authors concluded that this reduction in FFP synthase could be explained by a decrease in the nuclear form of SREBP-2 which they suggested was likely as a result of the reduction in SREBP mRNA that they observed (Le Jossic-Corcos et al., 2005). The same authors also reported significantly reduced nuclear mature forms of both SREBP-1a and SREBP-2 in human HEP-G2 cells following exposure to EPA and DHA (Le Jossic-Corcos et al., 2005). Several other studies have shown that a reduction in SREBP-1 and/or SREBP-2 with EPA and DHA is associated with reductions in SRE-dependent gene expression and mRNA levels of lipogenic enzymes including, in association with SREBP-1c, fatty acid synthase, Acetyl-CoA carboxylase and stearoyl-CoA desaturase-1. In association with SREBP-2 reductions in hepatic LDL receptor, 3-hydroxy-3methylglutaryl-CoA (HMG-CoA) reductase, HMG-CoA synthase, and FFP synthase mRNA and protein have been observed with EPA and DHA (Kim et al., 1999; Le Jossic-Corcos et al., 2005; Yahagi et al., 1999; Ren et al., 1997) (**Figure 4.1**). The findings of these studies demonstrate that omega-3 fatty acids can suppress the actions of both nuclear SREBP-1 and SREBP-2 subsequently reducing hepatic triglyceride output (Kim et al., 2001).

A recent review by Davidson (Davidson, 2006) outlines a molecular model based around four nuclear receptors which can describe how EPA and DHA reduce fasting plasma triglyceride concentrations at pre- and post-transcription levels and potentially alter other blood lipids (**Figure 4.2**). The four receptors included in Davidson's model are liver X receptor (LXR), hepatocyte nuclear factor-4 $\alpha$  (HNF-4 $\alpha$ ), farnesol X receptor (FXR), and peroxisome proliferators-activated receptors (PPARs); these may directly or indirectly impact on or be influenced by omega-3 fatty acids effects on SREBP-1c (Davidson, 2006). Transcription of the SREBP-1c gene appears to require sterol activation of LXR suggesting that SREBP-1c is under the control of LXR. One of the primary roles of LXR is to prevent the build up and potential toxicity of excess cellular cholesterol. LXR is therefore activated by the binding of oxysterol. LXR can also bind non-esterified fatty acids (NEFA) and these may compete with oxysterol for LXR binding. Two studies have shown that EPA and DHA both bind to LXR and by doing so suppress LXR activity resulting in an inhibition of SREBP-1c (Yoshikawa et al., 2002; Ou et al., 2001). Thus one mechanism by which omega-3 fatty acids may reduce plasma

triglyceride levels is by binding to LXR and subsequently suppressing triglyceride synthesis induced by SREBP-1c. Other actions of LXR include promotion of cholesterol efflux into HDL and increased cholesterol efflux from hepatic and intestinal cells to reduce cellular cholesterol levels (Davidson, 2006). It is unclear if the binding of EPA or DHA to LXR impacts on these LXR-related functions. Similar to the function of fibrates which significantly reduce fasting plasma triglyceride concentrations, EPA and DHA also bind to HNF-4 $\alpha$  inhibiting its gene transcription (Clarke, 2001). Hepatocyte nuclear factor-4 $\alpha$  enhances genes encoding key enzymes involved in carbohydrate metabolism and thus suppression of HNF-4 $\alpha$  by EPA and DHA may reduce the level of citrate that can enter the triglyceride synthesis pathway reducing fasting plasma triglyceride concentrations. It has been demonstrated that EPA and DHA inhibit HNF-4 $\alpha$  binding activity resulting in a reduction in glucose-6-phosphatase (Horton et al., 1998). It has been demonstrated that EPA and DHA are FXR ligands and that binding to FXR may promote the transcription of FXR-encoded genes (Zhao et al., 2004) and may reduce fasting plasma triglyceride concentrations by influencing lipoprotein lipase activity, inducing PPAR $\alpha$  and inhibiting SREBP-1c (**Figure 4.2**). The effect of EPA and DHA binding to PPAR $\alpha$  has already been discussed.

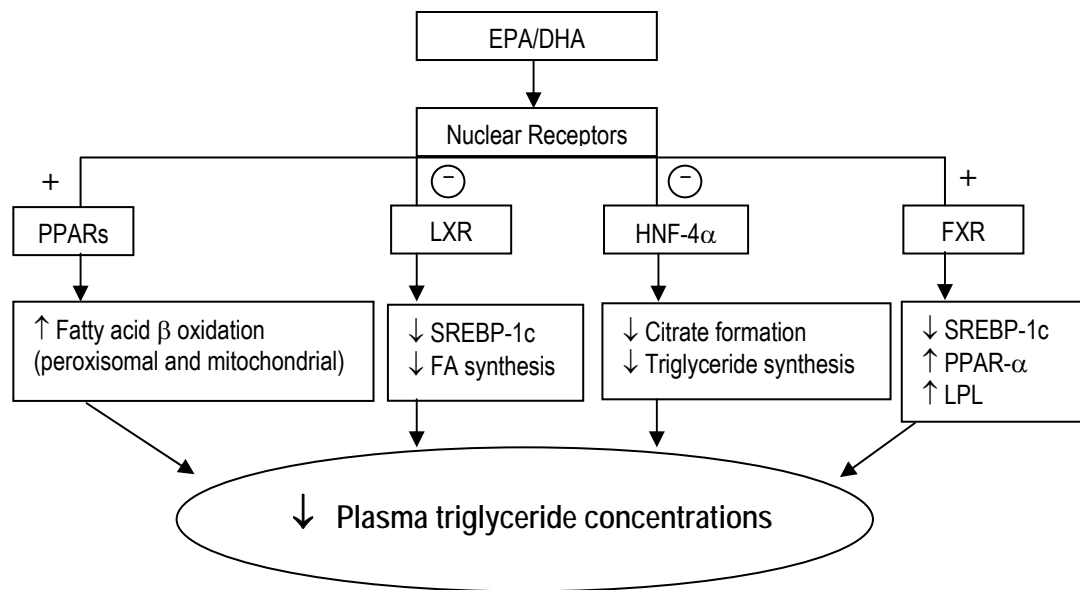


Figure 4.2 Model as proposed by Davidson (2006) for reduced plasma triglyceride concentrations with omega-3 fatty acids based on their coordinated effect on four nuclear receptors.

EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; PPAR = peroxisomal proliferator-activated receptor; LXR = liver X receptor; HNF- $\alpha$  = hepatocyte nuclear factor -  $\alpha$ ; FXR = farnesol X receptor; SREBP = sterol regulatory binding protein; FA = fatty acid; LPL = lipoprotein lipase.

+ = symbolizes promotion or activation by omega-3 fatty acids.  $\ominus$  = symbolizes a reduction or suppression of activity by omega-3 fatty acids

Davidson (2006) proposes that it is the coordinated effect of omega-3 fatty acids on these four receptors that suppresses hepatic lipogenesis and increase fatty acid oxidation to explain the reduction in fasting plasma triglyceride concentrations observed so consistently and dose-dependently with dietary supplementation with EPA and DHA.

### 4.3 How could EPA and DHA cause an increase in LDL cholesterol?

The mechanisms by which EPA and DHA might result in an increase in LDL cholesterol concentrations have not yet been defined. LDL cholesterol can increase due to either reduced clearance of LDL from the circulation due to reduced LDL receptor abundance, reduced LDL receptor mRNA expression, alterations to the LDL particle which reduces its capacity to bind to the hepatic LDL receptor and be taken up into the liver or increased hepatic cholesterol concentrations resulting in reduced expression of hepatic LDL receptors (increased intracellular lipogenesis). Alternatively plasma LDL cholesterol concentrations can increase due to increased synthesis of LDL. This can

occur when catabolism of VLDL to LDL, by the actions of lipoprotein lipase, increases. All of these mechanisms may be influenced by EPA and DHA (**Figure 4.3**).

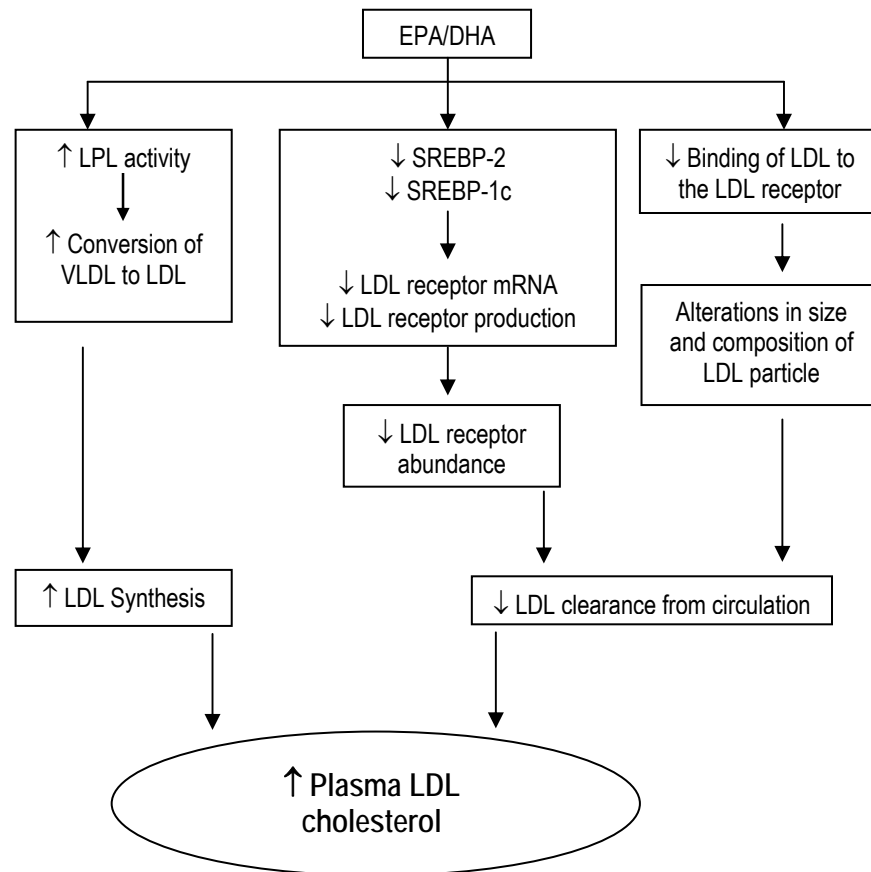


Figure 4.3 Diagram of mechanisms by which EPA and DHA have been shown to increase plasma LDL cholesterol concentrations

EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; LPL = lipoprotein lipase; SREBP = sterol regulatory binding protein.

*In vitro* and *in vivo* evidence suggests EPA and DHA may inhibit LDL receptor number (Wilkinson et al., 1998) and activity (Wong et al., 1987; Roach et al., 1987; Hannah et al., 1993), reduce LDL receptor mRNA abundance (Lindsey et al., 1992) and potentially reduce LDL binding to LDL receptors (Hsu et al., 2000; Schectman et al., 1996). However, the effect of EPA and DHA has been shown to be different in different animal species (Spady et al., 1995). In LDL receptor knockout mice, EPA and DHA increased

hepatic LDL uptake and clearance by a mechanism that is independent to the LDL receptor (Vasandani et al., 2002), while in rats fish oil supplementation doubled hepatic LDL uptake without altering levels of LDL receptor protein or mRNA (Spady et al., 1995). In contrast, in hamsters, that metabolise dietary fat and have plasma LDL concentrations similar to humans, omega-3 fatty acids increase LDL cholesterol concentrations (Surette et al., 1992; Lin et al., 1995; Kubow et al., 1996; Lu et al., 1996) and reduced LDL receptor number and LDL receptor mRNA (de Silva et al., 2004; Spady et al., 1995).

The nuclear mature form of SREBP-2 activates transcription of the gene encoding the LDL receptor (Shimano et al., 1997; Yokoyama et al., 1993; Hannah et al., 2001). It has been shown in cultured hamster cells that SREBPs are essential for cholesterol uptake from LDL (Brown and Goldstein, 1997) and that any disruptions to the functioning of SREBP-1 and -2 can inhibit a cells capacity to synthesize cholesterol or take it up from plasma LDL (Brown and Goldstein, 1997; Sakai et al., 1996). Vallet et al. (1996) showed *in vitro* using human HepG2 cells that both SREBP-2 and SREBP-1c can strongly activate expression of the LDL receptor. Furthermore, activation by the SREBPs of cholesterol regulated genes including LDL receptor, HMG-CoA synthase and HMG-CoA reductase are expressed at a high level independent of the cholesterol content of the cell (Vallet et al., 1996). Studies in animal models and human Hep-G2 cells have demonstrated that EPA and DHA can cause a significant reduction in hepatic levels of both mRNA and nuclear, mature forms of SREBP-1a and SREBP-2 (Le-Jossic-Corcus et al., 2005; Kim et al., 2005). Kim et al. (1999) observed in mice consuming a fish oil enriched diet that mRNA of SREBP-1c and SREBP-2 decreased significantly but that the SREBP-2 protein levels remained unchanged while nuclear levels of SREBP-1c significantly decreased. These changes were associated with a significant reduction in LDL receptor gene expression.

It is possible therefore that the rise in LDL cholesterol sometimes observed with DHA and/or EPA dietary supplementation may be caused by the suppression of SREBP-1 and/or SREBP-2 produced by these omega-3 fatty acids.

*In vitro* evidence also suggests that EPA and DHA may directly or indirectly increase levels of lipoprotein lipase which may lead to increased catabolism of VLDL



triglyceride and enhanced formation of LDL (Rivellese et al., 2003; Park et al., 2004; Khan et al., 2002) (**Figure 4.1**). Two nuclear receptors included in Davidson's model for triglyceride reduction with omega-3 fatty acids can influence lipoprotein lipase when bound to EPA or DHA. These receptors include PPAR $\alpha$  and FXR. While not proposed by Davidson, it is possible that while EPA and DHA may reduce fasting plasma triglycerides via their interaction with these receptors they may also induce an increase in LDL cholesterol especially when their impact on PPAR $\alpha$  and FXR is combined with their suppression of SREBP-1 and SREBP-2 (**Figure 4.3**).

#### **4.4 Theoretical framework for combined effect of canola and DHA on LDL cholesterol metabolism**

In the MOFO study, dietary supplementation with 2.2g/d of DHA resulted in a 15% increase in LDL cholesterol while dietary supplementation with 1.1g/d of DHA consumed with dietary sunola oil and margarine resulted in a 16% increase in LDL cholesterol. When canola oil and margarine were consumed in a diet supplemented with 1.1g/d of DHA LDL cholesterol concentrations did not change. These findings suggest that some synergy exists between canola and DHA to cancel out the LDL – raising effect of DHA and that this synergistic effect relates to either a unique biochemical capability of canola or to unique components of canola not shared by the other vegetable oils which failed to suppress a rise in LDL cholesterol.

A few biochemical scenarios could explain a synergistic effect of canola and DHA on LDL cholesterol concentrations. These scenarios include:

- a) Canola consumption as a whole within a normal diet or consumption of one or more components of canola, producing a reduction in LDL cholesterol that is significant enough to counteract the rise in LDL cholesterol produced by the DHA supplementation (an approximate 15% reduction in LDL cholesterol).
- b) One or more components of canola altering biochemical pathways that directly counteract the LDL raising mechanisms of DHA
- c) One or more components of canola preventing DHA from causing a rise in LDL cholesterol

#### **4.4.1 Evidence to support a cholesterol lowering effect of canola or its components**

Studies that have observed the effect of consuming canola oil and margarine in the diet on total and LDL cholesterol concentrations have provided mixed findings. Evidence from human (Vega-Lopez et al., 2006; Truswell et al., 1998; Sundram et al., 1995; Bemelmans et al., 2002; Nydahl et al., 1994; Indu, 1992) and animal studies (Dorfman and Lichtenstein, 2006; Dorfman et al., 2005; Aguila et al., 1998) has been consistent in demonstrating a reduction in LDL cholesterol concentrations when canola oil replaces saturated fat in the diet. Parallel designed, controlled animal studies have revealed potential mechanisms by which this might occur. In hamsters, a good model due to their similarity to humans in cholesterol metabolism (Spady et al., 1995; Ohtani et al., 1990), dietary consumption of canola oil has produced significant increases in LDL receptor abundance and SREBP-2 mRNA levels when compared to a diet enriched in saturated fat sources (Fernandez et al., 1996; Dorfman and Lichtenstein, 2006). What is unclear from these study designs however is whether or not canola produces an increase in LDL receptor abundance and SREBP mRNA or whether it just appears that way when in fact these changes are a consequence of reduced dietary consumption of saturated fatty acids.

If canola can independently reduce LDL cholesterol by increasing LDL receptor abundance and SREBP-2 mRNA, it is likely that the components of canola responsible for these changes are oleic acid and ALA as these fatty acids replace saturated fats. Animal studies show that plasma cholesterol concentrations are lowered with both canola and soybean oil by similar mechanisms (Dorfman and Lichtenstein, 2006; Dorfman et al., 2005). Canola oil contains double the amount of oleic acid as soybean oil while both oils contain similar quantities of ALA (Dorfman et al., 2005). This suggests that it is not the oleic acid content of canola that lowers cholesterol. Confirming this point are findings from studies conducted in guinea pigs, also a good model for cholesterol metabolism in humans (Fernandez and Volek, 2006) showing higher LDL cholesterol and reduced hepatic LDL receptor abundance following diets enriched with olive or safflower oil compared with canola (Fernandez et al., 1996). Combining these results with those from the MOFO study it could be suggested that the suppressed rise in LDL cholesterol observed with the Can(4g) intervention was due to the ALA content of canola. It is only when canola and not safflower or sunola oil and

margarine were combined with DHA supplementation that LDL cholesterol concentrations did not increase. In comparison to safflower and sunola with no ALA, the ALA content of canola oil is 10-fold higher and almost 3-fold higher in margarine.

Human and animal studies using ALA enriched diets have demonstrated inconsistency in the LDL cholesterol lowering effects of ALA. The majority of studies have failed to demonstrate a cholesterol reduction especially when an ALA enriched diet is compared with an oleic acid rich diet (Kestin et al., 1990; Harper et al., 2006; Freese and Mutanen, 1997; Li et al., 1999; Mantzioris et al., 1994; Finnegan et al., 2003). The majority of these studies used linseed and linseed oil as the source of ALA as opposed to canola, suggesting there may be something unique about the provision of ALA via canola compared with linseed. Two studies by Goyens and Mensink (2005 and 2006) suggest this may be the case as both reported reductions in LDL cholesterol of between 4.5 and 5.0% following ALA enriched diets of mildly hypercholesterolemic and healthy adults in which canola oil was used as the dietary source of ALA. In one of those studies (Goyens and Mensink, 2005), the canola sourced ALA diet lowered the proportion of small VLDL particles which are the preferred substrate for conversion of VLDL to LDL; Goyens and Mensink (2005) hypothesised that this may be a mechanism by which ALA reduces LDL cholesterol. While this hypothesis requires further empirical examination, knowledge of ALA metabolism and its effects on biochemical pathways actually make this a weak candidate to explain suppression of the rise in LDL cholesterol typical with DHA supplementation.

In humans, ALA once consumed can be incorporated into cell membranes, undergo  $\beta$ -oxidation or undergo elongation and desaturation to form the longer chain omega-3 fatty acids EPA and DPA (Burge, 2004). The proportion of ALA found in healthy human adults is relatively low compared with other fatty acids and typically below 0.5% of total fatty acids (Burge, 2004). A large proportion (up to 24 – 33%) of ALA is typically preferentially utilised for  $\beta$ -oxidation (Bretillon et al., 2001; DeLany et al., 2000; Burge et al., 2003) while only 8-21% of ALA is likely to be elongated to EPA (Burge et al., 2003; Burge and Wootton, 2002a). Subsequently, the bioavailability of dietary ALA appears low and limits the probability of ALA impacting on lipid pathways with any significance following dietary consumption.

Furthermore, if ALA was an important component of canola involved in suppressing the LDL cholesterol raising effect of DHA, then ALA should reduce LDL cholesterol independently or in combination with other Canola components, or prevent DHA from causing a rise in LDL cholesterol.

On a molecular level, evidence suggests that ALA either has no effect or the same effect as EPA and DHA on nuclear factors such as SREBP, PPAR $\alpha$  or HNF-4 $\alpha$  at altering lipogenic enzymes, fatty acid oxidation, or lipogenesis (Ou et al., 2001; Jump et al., 2005; Hertz et al., 2001). Alpha linolenic acid has also been shown to have no effect on SREBP-2 protein levels the transcription factor for SRE induced gene expression of the LDL receptor (Hannah et al., 2001). Thus, increased ALA is an unlikely candidate to cause increased LDL receptor expression as a mechanism for increasing hepatic LDL uptake and reducing plasma LDL cholesterol concentrations, and hence suppression of the rise in LDL cholesterol that occurred with the Can(4g) intervention.

Thus it also seems unlikely that ALA has pre or post translational or biochemical affects that are significantly different to EPA and DHA, such that those effects might work in synergy to explain the suppressed rise in LDL cholesterol observed with the combined consumption of canola and DHA-rich oil supplementation. While ALA may reduce the proportion of small VLDL particles and thus the conversion of VLDL to LDL subsequently lowering LDL cholesterol concentrations, the degree of cholesterol reduction achievable via this ALA mechanism is likely to be small. It is therefore more probable that the suppressed rise in LDL cholesterol was due to some other component in the canola independently or in combination with the effects of ALA. One such component may be phytosterols.

#### **4.4.2 Canola phytosterols and their potential for cholesterol reduction**

In a study conducted in healthy young men, Pedersen et al. (2000) showed that daily consumption of 75g of canola oil and sunflower oil resulted in significantly lower VLDL, IDL and LDL apoB concentrations compared with olive oil consumption. Canola oil also significantly lowered LDL cholesterol, triglyceride and phospholipid concentrations compared with olive oil consumption. The plasma cholesterol differences between the olive oil and canola oil diets could not be entirely explained by differences in fatty acid composition (Pedersen et al., 2000). However, plasma

concentrations of the phytosterols sitosterol and campesterol were significantly higher following consumption of both canola and sunflower oil compared with olive oil, with campesterol concentrations after canola consumption being double those observed with the safflower oil. Pedersen et al. (2000) proposed that the differences in phytosterol content and subsequent plasma phytosterol concentrations achieved after olive, canola and sunflower oil consumption may have contributed to the differences in blood lipid concentrations and lipoprotein composition.

A variety of studies have been conducted comparing the phytosterol content of a broad range of vegetable oils including corn, cottonseed, canola, olive, soybean, sunflower, palm oil, oleic sunflower oil, linoleic sunflower oil and groundnut oil (Gordon and Miller, 1997; Gul and Seker, 2006; Gul and Amar, 2006; Vlahakis and Hazebroek, 2000). These studies have consistently demonstrated that canola oil has by far one of the highest phytosterol contents of all vegetable oils tested. The majority of vegetable oils contain between 1 – 5g of phytosterols/kg of oil, while canola contains double this level with up to 10g of phytosterols/kg (Piiroven et al., 2000). Specifically, Vlahakis and Hazebroek (2000) compared the phytosterol content of canola, sunflower and soybean oil. While these oils can be distinguished by their fatty acid profiles they also differ in phytosterol content. Canola, despite containing the same quantity of ALA as soybean but half the LA and significantly less LA than sunflower oil, contained double the phytosterols compared with both other oils. Similarly, Gul and Seker (2006) showed that canola oil contained between 4.25 and 11.37g of phytosterols/kg of oil in comparison to olive oil which contained just 1.3 – 2.4g/kg. Gul and Seeker (2006) showed that sitosterol followed by campesterol were the phytosterols within canola that were present in highest amounts. If the phytosterol content of the canola oil and margarine in the MOFO study was contributing to a reduction in LDL cholesterol, the considerably higher levels of phytosterols in canola compared with sunola or safflower oil may explain why a suppressed reduction in LDL cholesterol was not observed with these interventions when combined with dietary DHA supplementation.

According to a review by Ostlund (2004) phytosterol esters reduce LDL cholesterol by 10% at a maximum effective dose of 2g/day. In another review of human clinical trials, Normen et al. (2004) reported that 1.5 - 3.0g/d of phytosterols can lower LDL cholesterol concentrations by 10-15%. Hallikainen et al. (2000) reported from their

human study that 1.6g/d of phytosterols was sufficient to significantly reduce plasma cholesterol by a maximum proportion, such that additional phytosterols caused no further clinically relevant cholesterol reductions (Hallikainen et al., 2000). Baseline diets can provide anywhere between 150 – 450mg of phytosterols per day before the addition of phytosterol rich foods (Ostlund, 2002). Furthermore, it has been shown that relatively small amounts of phytosterols in foods such as those provided by 25-30g of corn oil or daily consumption of a wheat germ muffin, are bioactive and can contribute to an approximately 40% reduction in dietary cholesterol absorption (Ostlund et al., 2002; Ostlund et al., 2003). Such reductions in cholesterol absorption can result in a reduction in LDL cholesterol concentrations (Perez-Jimenez et al., 2001; Howell et al., 1998).

Based on the phytosterol concentrations reported in varieties of canola, it could be estimated that consumption of 25g/d of canola would provide between 0.195g – 0.285g per day of phytosterols. This added to a background diet providing up to 450mg/day of phytosterols could have raised daily consumption levels of phytosterols to 735mg/day in the Can(4g) intervention. It is unclear whether this relatively low dose of phytosterols could reduce LDL cholesterol by up to 15%, counteracting the rise in LDL cholesterol caused by the DHA supplementation in the MOFO study. However, future studies should examine this possibility. If this daily dose of phytosterols is not sufficient to substantially reduce plasma LDL cholesterol levels independently it may reduce LDL cholesterol in combination with daily DHA supplementation.

It has been demonstrated in animal studies, though not yet tested in humans, that dietary consumption of the phytosterols  $\beta$ -sitosterol and campesterol esterified to fish oil rich in EPA and DHA significantly reduced both total plasma cholesterol and fasting plasma triglyceride concentrations (Ewart et al., 2002; Demonty et al., 2005). Demonty and co-workers (2005) showed in hamsters that a five week diet containing phytosterols esterified to EPA and DHA was more effective at reducing total plasma cholesterol concentrations than a diet providing non-esterified plant sterols or no plant sterols. They also reported a significantly higher content of sitosterol in erythrocyte membranes following dietary consumption of omega-3 esterified phytosterols compared to the dietary consumption of free phytosterols. This led the authors to conclude that omega-3

esterified phytosterols may be more bioavailable than non-esterified phytosterols and more effective at lowering plasma cholesterol concentrations (Demonty et al., 2005).

Findings from human studies demonstrate that plant sterol esters, stanol esters and free sterol/stanol mixtures consumed as part of vegetable-oil based margarines have similar efficacy for lowering total and LDL cholesterol (Moreau et al., 2002; Jones, 1999; Jones and Raeini-Sarjaz, 2001). It has been known for some decades that phytosterols can reduce plasma LDL cholesterol concentrations. Phytosterols can achieve this effect via several mechanisms all of which impact on cholesterol absorption (Trautwein et al., 2003). These include: co-crystallisation with cholesterol to form insoluble mixed crystals (Trautwein et al., 2003; Jandacek et al., 1977; Christiansen et al., 2001; Mel'nikov et al., 2004a); competition with cholesterol for solubilisation in mixed micelles (Trautwein et al., 2003; Ikeda and Sugano, 1983; Mel'nikov et al., 2004b); and enhanced intestinal cell transport protein expression for sterol efflux in the intestinal tract (Plat and Mensink, 2002). Normen and co-workers (2006) demonstrated in ileostomy subjects that phytosterol- and phytostanol- esters are both hydrolysed to free phytosterols in the intestinal tract where they enter micelles and subsequently compete with cholesterol for solubilisation in mixed micelles (Normen et al., 2006). When DHA and canola are consumed concurrently in the diet the phytosterols contained within canola are either esterified to fatty acids present in canola oil such as oleic acid or ALA or they are free phytosterols. The findings from Normen et al. (2006) suggest that for phytosterol esters in canola to be absorbed they enter the stomach and duodenum where they are emulsified and hydrolysed allowing solubilisation into free phytosterols and subsequent competition with cholesterol for uptake into mixed micelles. Once in micelles or taken up into the intestinal wall free phytosterols are likely re-esterified as this assists with their transport. When this occurs, if there is an increase in the availability of EPA and/or DHA due to dietary supplementation with these fatty acids then it is possible that the phytosterols may be re-esterified with DHA forming omega-3 esterified phytosterols. These may be more bioavailable and result in a greater reduction in LDL cholesterol despite the mechanisms for why this might occur being unclear.

A reduction in cholesterol absorption with phytosterol consumption would result in a reduction in circulating, hepatic and intracellular cholesterol concentrations. This would likely lead to an increase in LDL receptor expression to increase the hepatic uptake of

cholesterol. Plat and Mensink (2002) showed in healthy adults that 8 weeks of plant sterol ester consumption significantly reduced plasma LDL cholesterol concentrations which correlated negatively with the degree of increase in cell surface LDL receptor protein and mRNA in monocytes and T-lymphocytes, both of which increased significantly with plant sterol consumption. These findings suggest that increased LDL receptor expression contributes to the reduction in LDL cholesterol observed with dietary plant sterol consumption.

Thus, there are three potential mechanisms by which the combined consumption of canola phytosterols and DHA-rich oil may suppress the rise in LDL cholesterol observed with DHA alone. These mechanisms are:

1. That the phytosterols provided by 20-25g/d of canola oil potentially increasing dietary phytosterol intake to an estimated 735mg/d could independently decrease LDL cholesterol by an amount equivalent to the rise in LDL cholesterol caused by the DHA-rich oil.
2. That increased concurrent consumption of canola phytosterols and DHA could result in DHA esterification of the phytosterols which increases the bioavailability of the phytosterols and the LDL cholesterol lowering capacity of the phytosterols resulting in a significant reduction in LDL cholesterol – beyond what could have been achieved with canola alone.
3. That the esterification of DHA to canola phytosterols could impact on DHA's effects on LDL cholesterol preventing DHA from increasing LDL cholesterol concentrations. This effect may come about because the DHA that is esterified to phytosterols is no longer available as a free fatty acid or fatty acid esterified to a membrane phospholipid reducing the amount of free DHA that is available to influence the SREBP pathways which may impact on LDL cholesterol. Given omega-3 esterified phytosterols can reduce plasma triglyceride and LDL cholesterol concentrations it suggests that the phytosterols esterified to DHA do not impinge on the mechanisms by which omega-3 fatty acids reduce triglycerides.

While further research is required and warranted based on the findings from animal studies conducted to date, it can reasonably be proposed that the findings from the MOFO study may be an example of a synergistic effect of canola phytosterols and



DHA, rather than ALA and DHA, working together to significantly reduce fasting plasma triglyceride concentrations but not detrimentally affecting LDL cholesterol in people with mild hypertriglyceridemia.

#### **4.5 Theoretical framework for combined effect of DHA and isoflavones on cholesterol metabolism**

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In the Omega-Soy study, DHA rich oil supplementation alone during the first six weeks caused a 10.7% increase in LDL cholesterol while the combined consumption of soy isoflavones and DHA caused no change. Between six and twelve weeks when DHA was consumed with soy isoflavones, LDL cholesterol decreased 9.5% while DHA consumption alone had no effect on LDL cholesterol. Soy isoflavones consumed independent of DHA had no effect on blood lipid parameters. These findings suggest a synergistic effect of DHA and soy isoflavones on LDL cholesterol metabolism. Both soy isoflavones and DHA are known to influence the expression and activity of nuclear receptors and subsequently may impact on the same metabolic pathways with complementary or contrasting effects. Some effects include, but may not be limited to: - direct or indirect effects on LDL receptor activity and number, activation of nuclear peroxisome-proliferator activated receptors (PPARs), hepatic gene transcription factors - sterol regulatory element binding proteins (SREBP's) and potential estrogenic effects.

Animal and *in vitro* studies have already reported potential synergistic estrogenic effects of omega-3 fatty acids and soy isoflavones demonstrated in different tissues such as bone and breast cancer cells (Fernandes et al., 2002; Nakagawa et al., 2000). Human and *in vitro* studies have also reported that omega-3 fatty acids and soy isoflavones both influence a range of cardiovascular and anti-inflammatory outcomes such as thromboxane A2 (Garrido et al., 2006; Swann et al., 1990; Von Schacky, 1985), PPARs (Mezei et al., 2003; Li et al., 2005; Dreyer et al., 1993; Kliewer et al., 1997; Forman et al., 1997; Diep et al., 2000; Gottlicher et al., 1993; Willumsen et al., 1992), monocyte activity (Chacko et al., 2005) and cytokine and interleukin expression (Fernandez et al., 2002; Zhao et al., 2005).

Of particular interest to a synergistic effect on LDL cholesterol is the observation that both soy isoflavones and DHA influence LDL receptor expression, potentially via the

same pathways, although their effects are in opposite directions, with DHA reducing LDL receptor expression and soy isoflavones increasing it.

#### 4.5.1 Effect of isoflavones and DHA on LDL receptor activity

In humans, dietary consumption of soy isoflavones with soy protein has been shown to significantly increase mononuclear cell LDL receptor mRNA (Baum et al., 1998). It is thought that this effect may be produced through stimulation of estrogenic pathways since estrogen administration can stimulate hepatic LDL-receptor expression and HMG-CoA reductase activity in men (Angelin et al., 1992) and increase hepatic LDL-receptor expression in rats (Kovanen et al., 1979) and cultured human hepatic cells (Croston et al., 1997). Borradaile et al. (2002) showed that incubation of human HepG2 cells with high doses of daidzein and genistein (100 $\mu$ M) increased LDL receptor mRNA by 3- to 6-fold and significantly increased LDL-receptor binding, cellular uptake and degradation. Similarly, Kirk et al. (1998) showed that in LDL receptor deficient mice a high isoflavone diet significantly increased plasma cholesterol concentrations, the susceptibility of LDL to oxidative modification and the development of atherosclerosis compared with reductions in all of these parameters in control mice (Kirk et al., 1998).

While no human intervention studies have examined the effect of DHA supplementation on LDL receptor activity and hepatic uptake there is evidence from *in vitro* and animal studies suggesting that EPA and DHA may inhibit LDL receptor activity (Wong et al., 1987; Roach et al., 1987; Hannah et al., 1993), reduce LDL receptor mRNA abundance (Lindsey et al., 1992) and potentially reduce LDL binding to LDL receptors (Hsu et al., 2000). All of these results can cause an increase in plasma LDL cholesterol and LDL apoB.

The LDL receptor gene is one of several genes regulated by sterol regulatory element binding proteins (SREBP's). Mullen et al. (2004) demonstrated *in vitro*, that treatment of HepG2 cells with soy isoflavone extract and individual isoflavones increased levels of mature SREBP-2, HMG CoA reductase protein and HMG CoA synthase mRNA but did not affect levels of SREBP-1. Subsequent isoflavone treatment of HepG2 cells incubated with LDL molecules significantly increased intracellular LDL compared with non-isoflavone treated cells. This finding suggests that the increased SREBP-2 levels produced by the isoflavones enhanced surface LDL receptor expression and increased

LDL receptor-mediated uptake (Mullen et al., 2004). Genistein and daidzein both increased SREBP-2 maturation and SREBP-2 regulated gene expression, while glycitein had very little effect. An absence of an effect of soy isoflavones on SREBP-1 has also been reported by Tovar et al. (2005) in human hepG2 cells. These findings suggest that soy isoflavones may impact on LDL cholesterol receptor expression via its effects on SREBP-2 but not by influencing SREBP-1.

In contrast, EPA and DHA can cause a significant reduction in hepatic levels of both mRNA and nuclear, mature forms of SREBP-1a and SREBP-2 (Le-Jossic-Corcus et al., 2005; Kim et al., 2005). These changes have been associated with a significant reduction in LDL receptor gene expression, as explained earlier in this chapter. Thus, the opposing effects of DHA and isoflavones on LDL receptor expression and activity may reflect the different and opposing effects that these two functional food ingredients have on SREBP mRNA and protein concentrations.

#### **4.5.2 Potential explanations for a combined effect of soy isoflavones and DHA on LDL cholesterol**

The potential for a synergistic effect of DHA and soy isoflavones on LDL cholesterol metabolism is evident. However, the results from the Omega-Soy study and numerous other human clinical trials have failed to demonstrate that independent dietary consumption of isolated soy isoflavones can significantly reduce LDL cholesterol concentrations. It is unclear why soy isoflavones do not reduce LDL cholesterol independently, since they clearly play an integral role in the LDL cholesterol reduction achieved with soy protein. It has been proposed that there is some degree of interaction and synergy between soy protein and isoflavones (Taku et al., 2007; Torres et al., 2006; Clair and Anthony, 2005; Peluso et al., 2000). A recent meta-analysis determined that soy isoflavones and isoflavone depleted soy protein accounted for up to 72% and 56%, respectively, of the LDL cholesterol reduction observed with soy protein enriched with isoflavones (Taku et al., 2007). These findings suggest that concurrent consumption of isoflavones with soy protein results in a synergistic or additive cholesterol lowering effect beyond what is observed when either is consumed independently.

Thus, it could be hypothesised that DHA, like soy protein might interact directly or indirectly with soy isoflavones in a way that enables them to favourably impact on LDL cholesterol metabolism. Based on this hypothesis, I have developed three potential mechanisms that attempt to explain the potential for a synergistic reduction in LDL cholesterol observed with the concurrent dietary consumption of DHA and soy isoflavones in the Omega-Soy study. These mechanisms are presented schematically in **Figure 4.4** and are explained below.

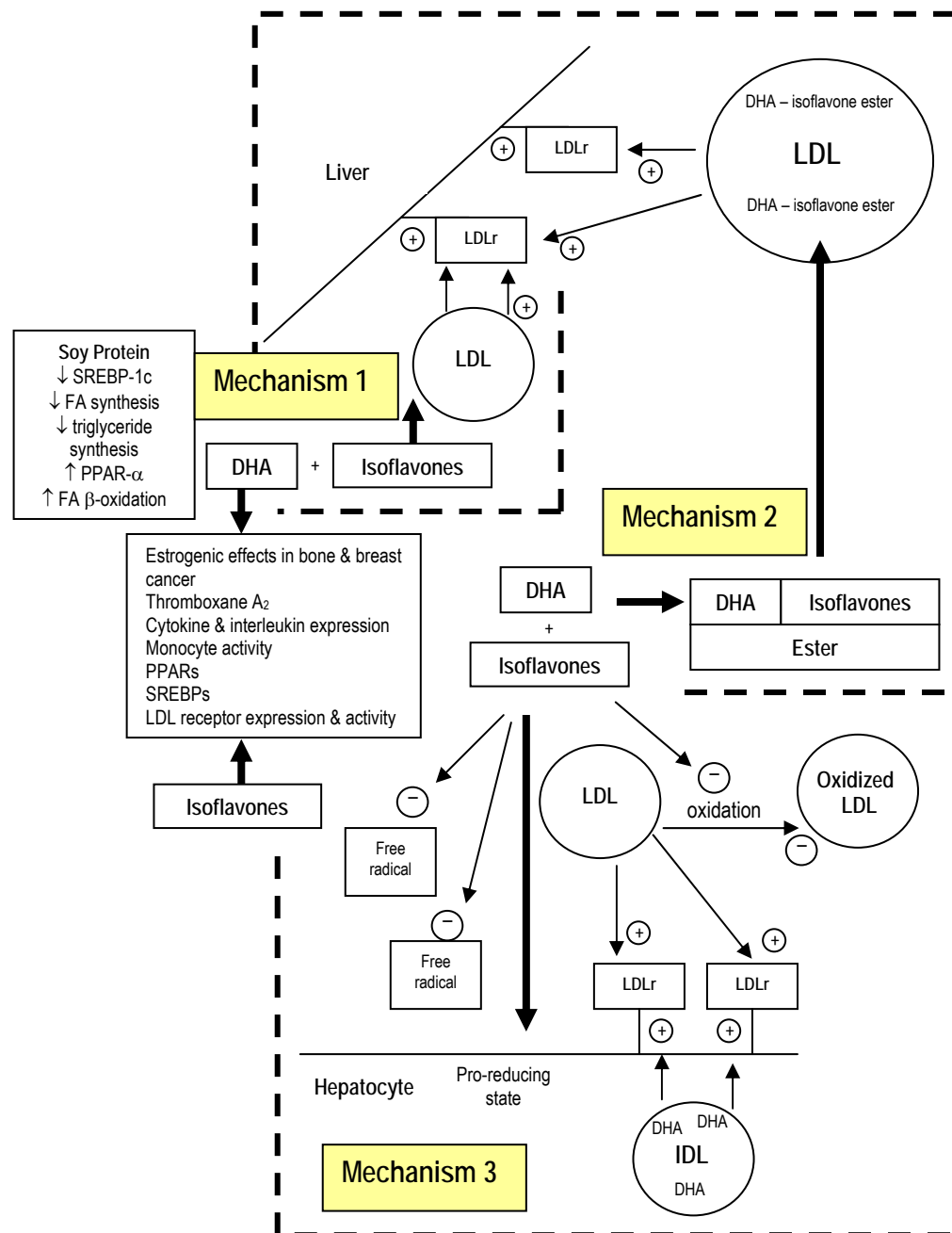


Figure 4.4 Schematic representation of the 3 proposed mechanisms by which DHA and soy isoflavones may synergistically reduce plasma LDL cholesterol concentrations. DHA = docosahexaenoic acid; LDL = low density lipoprotein; IDL= intermediate density lipoprotein; LDLr = Low density lipoprotein receptor; PPAR = peroxisome proliferators-activated receptor; SREBP = sterol regulatory element binding protein; FA = fatty acid.

### 4.5.3 Proposed mechanism #1 – An isoflavone enabling effect of DHA similar to soy protein.

Dietary consumption of soy isoflavones combined with a casein enriched diet, animal protein or a phytosterol ester enriched diet do not affect plasma cholesterol concentrations (Lin et al., 2004; Lichtenstein et al., 2002). However isoflavones in combination with soy protein significantly lower LDL cholesterol. Soy protein appears to have a number of effects on biochemical pathways that are different to animal sourced proteins. Dietary soy protein consumption has been shown in rat models to suppress the expression of SREBP-1 mRNA and to reduce the expression of hepatic fatty acid synthase, malic enzyme, adipose tissue fatty acid synthase mRNA and mature SREBP-1 (Ascencio et al., 2004; Nagasawa et al., 2003; Moriyama et al., 2004; Tovar et al., 2002; Tovar et al., 2005). Additionally soy protein has been shown to upregulate PPAR $\alpha$  gene expression in the liver, thereby increasing the gene transcription for some enzymes associated with beta-oxidation, such as acyl-CoA oxidase (Moriyama et al., 2004; Tovar et al., 2002), and preventing hepatic triglyceride accumulation (Torres et al., 2006). Soy protein has also been shown to reduce hepatic ApoB secretion (Lovati et al., 1998; Lovati et al., 2000). Such effects are not observed with casein (Tovar et al., 2005; Torres et al., 2006). Soy protein has also been shown to increase the nuclear mature form of SREBP-2 subsequently increasing the gene expression of HMG-CoA reductase and LDL receptor and to increase LDL receptor activation (Lovati et al., 1998; Lovati et al., 2000; Tovar et al., 2002). Casein, in contrast does not have an impact on SREBP-2 or the cholesterol synthesis pathway (Ascencio, 2004).

While the mechanisms are not clear, it is possible that these biochemical differences between soy protein and animal protein are the distinguishing factors that enable soy isoflavones when combined with soy protein to favourably influence LDL cholesterol concentrations. Interestingly, these distinguishing effects of soy protein, especially the effect of soy protein on SREBP-1 (potentially via an LXR- $\alpha$  receptor related mechanism; Torres et al., 2006; Davidson, 2006), the expression of fatty acid synthesis enzymes and PPAR- $\alpha$  and fatty acid oxidation pathways are similar to those observed with EPA and DHA (**Table 4.1**). Similarly, both soy protein and omega-3 fatty acids influence the nuclear form of SREBP-2 and LDL receptor activity although their effects on these parameters are opposite.

Table 4.1 Similarities between soy protein and EPA and DHA effects on lipid metabolism enzymes

EPA/DHA	Biochemical Lipid Parameter <sup>1</sup>	Soy Protein
↑	SREBP-1 expression (potentially via LXR- $\alpha$ mechanism)	↑
↑	Nuclear mature form of SREBP-1c	↑
↑	Fatty acid synthesis gene expression (fatty acid synthase, malic enzyme)	↑
↑	Activation of PPAR $\alpha$	↑
↑	$\beta$ -oxidation of fatty acids	↑
↓	Nuclear mature form of SREBP-2	↑
↓	HMG-CoA reductase	↑
↓	LDL receptor expression	↑

<sup>1</sup> Abbreviations: SREBP = sterol regulatory element binding protein; LXR = liver X receptor; PPAR= peroxisome proliferator-activated receptor; HMG-CoA = hydroxy-3-methylglutaryl-CoA; LDL = low density lipoprotein. The dark shading represents those biochemical lipid parameters that are influenced by EPA/DHA in an opposite direction compared to soy protein.

It could therefore be hypothesised that via these or other mechanisms both omega-3 fatty acids (DHA in particular) and soy protein may work in synergy with or enable soy isoflavones to significantly reduce plasma cholesterol metabolism. The underlying assumption of this proposal is that the dietary combination of soy isoflavones and DHA may be similar to that of soy protein and isoflavones in that the influence of DHA and soy protein in both combinations could be activating or working in synergy with isoflavones to reduce LDL cholesterol.

With respect to the findings of the Omega-Soy study, this proposed mechanism is supported by the fact that olive oil, specifically oleic acid, does not have the same effects or is less effective than omega-3 fatty acids and soy protein, in influencing SREBP-1 levels and lipogenic enzymes (Xu et al., 1999; Ren et al., 1997; Le Jossic-Corcus et al., 2005). Accordingly, if this activity is required to assist isoflavones in lowering plasma cholesterol levels it could explain why there was no change in plasma cholesterol in the two olive oil groups when soy cereal was consumed in the Omega-Soy study.

#### 4.5.4 Proposed mechanism #2: Formation of isoflavone-omega-3 fatty acid esters

Soy isoflavones have been shown *in vitro* and *in vivo* to reduce LDL oxidation (Tikkanen et al., 1998; Kerry and Abbey, 1998; Jenkins et al., 2000; Wiseman et al., 2000). However, in cellular studies it was shown that only very small quantities of unmodified isoflavones were identified in the LDL particles which were less susceptible to oxidation (Tikkanen et al., 1998). It was subsequently hypothesised by Meng et al. (1999a) that this antioxidant capacity of isoflavones might occur via the incorporation of isoflavone fatty acid esters into LDL particles. This hypothesis was based on the fact that estrogen fatty acid esters are found in LDL particles which are less susceptible to oxidation when human estrogens are incubated with lipoproteins *in vitro* (Meng et al., 1999a). Meng et al. (1999b) synthesised a number of fat-soluble isoflavone esters (esterifying free isoflavones with oleic acid or linoleic acid) and demonstrated that isoflavones made fat soluble by esterification were successfully incorporated into LDL particles.

Esterification of isoflavones with omega-3 fatty acids may be a mechanism by which isoflavones are activated and thus might influence LDL cholesterol metabolism. Larner et al. (1993) demonstrated that estradiol fatty acid esters served as a source of estrogen which could potentially be delivered throughout the body via lipoprotein transport. Furthermore, it has been shown *in vitro* that esterification of 17- $\beta$ -estradiol can occur in HDL particles mediated by LCAT and that these esters can be transferred to LDL particles via a cholesterol ester transfer protein (CETP)-mediated mechanism (Herlisten et al., 2001)

Similar to estrogen, isoflavones once converted to polar sulfate or glucuronide conjugates upon digestion, become inactivated (Loukovaara et al., 1995). This was confirmed in cellular studies undertaken by Rimbach et al. (2004) which demonstrated that sulfate conjugation in place of the hydroxyl group at either position 4' or 7' decreased the antioxidant capabilities, anti-aggregatory effects and the ability of isoflavones to impact on monocyte or endothelial function. Loukovaara et al., (1995) showed *in vitro* that human HepG2 cells metabolised predominantly (63%) sulfate (61%) or glucuronidase (2%) conjugates of daidzein, equol and genistein, with approximately 37% remaining unconjugated and in the active form. Therefore it is



possible that a considerable quantity of isoflavones upon reaching the liver after dietary consumption may require cleavage from sulfate or glucuronidase conjugates before they can be activated and subsequently influence cholesterol metabolism. This may explain (at least partially) why *in vitro* effects observed when cells are incubated directly with aglycone isoflavones, demonstrating several mechanisms by which isoflavones influence cholesterol metabolism, are not replicated *in vivo* in human trials involving extracted soy isoflavone supplements.

The results from these studies provide the basis for the hypothesis that the combination of DHA oil supplementation and isoflavones could promote fatty acid esterification of isoflavones to form DHA-isoflavone esters. These esters may be more potent than unesterified isoflavones and may facilitate improvements in plasma LDL cholesterol concentrations.

The dietary combination of olive oil and soy isoflavones is less likely to produce a reduction in LDL cholesterol via this proposed mechanism compared with DHA and isoflavones. This is because unlike DHA, which is poorly oxidized in mitochondria and peroxisomes, oleic acid is preferentially oxidized (Madsen et al., 1998). The bioavailability of oleic acid could therefore be considerably lower than that of DHA for isoflavones esterification. DHA may actually be a preferred substrate for isoflavone-fatty acid ester formation as increased cellular and plasma levels of DHA have been shown to increase the gene expression of several beta-oxidation enzymes (Ren et al., 1997; Clarke et al., 2000; Willumsen et al., 1992). As DHA is a poor substrate for beta-oxidation, it may be preferentially available for isoflavone-fatty acid ester formation when present in levels similar to those achieved in this study after a significant period of supplementation.

#### **4.5.5 Mechanism #3: Modification of hepatic redox state via antioxidant effect of isoflavones promoting hepatic LDL uptake by DHA**

It has been demonstrated in several studies (Kim et al., 1999; Lindsey et al., 1992; Roach et al., 1987) that omega-3 fatty acid supplementation results in a down-regulation of LDL receptor activity. Zheng et al. (2002) however, demonstrated that incubation of human hepatocytes which were in a pro-reducing state (as opposed to a pro-oxidising state), with chylomicron remnants enriched with omega-3 fatty acids, increased the

abundance of mRNA for the LDL receptor, LDL receptor protein and PPAR $\alpha$  (Zheng et al., 2002). These findings suggest that the hepatic effects of omega-3 fatty acid enriched lipoproteins and their effect on hepatic gene expression may be modulated by the redox state of the cell.

Dietary consumption of soy isoflavones has been shown to decrease free radicals in plasma and in liver (Yousef et al., 2004, Banz et al., 2004) in animal models, and to favourably influence cellular redox status (Caccamo et al., 2005; Davis et al., 2001). Thus, it could be hypothesised that soy isoflavones may modify the oxidative state of hepatic cells enabling DHA to increase mRNA for LDL receptor, LDL protein and PPAR $\alpha$ . This combined with increased LDL receptor activity brought about by the action of the isoflavones (potentially via mechanisms 1 and 2 above) may result in increased cellular LDL uptake and a subsequent reduction in plasma total and LDL cholesterol concentrations.

#### 4.5.6 Summary of proposed mechanisms

All of the proposed mechanisms have the potential to explain a synergistic effect of DHA and soy isoflavones on LDL cholesterol metabolism. However, all require further examination to determine their validity and clinical significance. Several recommendations can be made for future research –

1. *In vitro* studies in human HepG2 cells examining the effect of isoflavones alone and in combination with soy protein on nuclear receptors SREBPs and PPAR $\alpha$  and subsequent effects on lipogenic enzyme pathways (cholesterol and triglyceride synthesis and  $\beta$ -oxidation) and LDL receptor expression and activity, may provide some useful information on the interaction between soy protein and isoflavones. To provide insight into proposed mechanism #1 these studies could be repeated with soy isoflavones alone and in combination with DHA and the results compared with the isoflavone and soy protein findings.
2. As a first step to assessing the feasibility of proposed mechanism #2, measurement of the fatty acid-isoflavone ester content of isolated lipoprotein subfractions following dietary supplementation with DHA and soy isoflavone consumption could provide insight as to whether or not DHA-isoflavone esters are formed and incorporated into lipoproteins. One such study has been completed, assessing the uptake of aglycone isoflavones, some isoflavone

metabolites and lignans into lipoprotein subfractions (Owen, Astheimer, Larkin and Ridges, (unpublished)). Further studies should examine the presence, proportion and types of isoflavone fatty acid esters in lipoproteins. In particular, the presence of DHA-isoflavone esters should be evaluated.

3. To test the feasibility of proposed mechanism #3, *in vitro* studies using human HepG2 cells measuring changes to the redox state of cells when isoflavones are added to the cell culture medium would be illuminating. Depending on the findings from those studies, further assessment of the uptake of DHA enriched lipoproteins and their effect on LDL receptor expression and activity may be warranted.

Further research is clearly required. However, based on evidence from *in vitro* and *in vivo* studies, it can reasonably be proposed that the findings from the Omega-Soy study may be an example of a synergistic effect of soy isoflavones and DHA, working together to significantly reduce fasting plasma triglyceride concentrations but not detrimentally affecting LDL cholesterol in people with mild hyperlipidemia.

## 4.6 Summary

The research undertaken in this thesis aimed to assess whether: a) DHA could improve CVD risk factors more effectively when the background fatty acid composition is modified (MOFO study); and b) combining DHA with soy isoflavones could complement the actions of DHA to result in a greater improvement in CVD risk factors (Omega-Soy study).

The results from the two human clinical trials conducted for this thesis demonstrate that combining functional foods that have the capacity to reduce cardiovascular risk factors can enhance the effectiveness of one or both foods on CVD risk factors and complement the effects of the other functional food such that the sum of their actions is greater than their individual contributions.

In the MOFO Study, the combination of canola oil and margarine with 1.1g/d of DHA was equally as effective as double the dose of DHA supplementation at enhancing the amount of EPA and total omega-3 fatty acids and at reducing the omega-6: omega-3 fatty acid ratio in membrane phospholipid pools. This outcome was not achieved with the combined consumption of DHA and sunola oil and margarine. The inclusion of 25g/d of canola oil and margarine in the diet provided approximately 2.25g/d of ALA. Assuming a conversion of approximately 10% of ALA to EPA, canola oil and margarine may have provided additional EPA for incorporation into phospholipid pools equivalent to almost 0.23g/d of supplemented EPA. Thus the changes in membrane fatty acids observed with canola and DHA can be attributed to the increased consumption of omega-3 fatty acid with this dietary combination. Additionally, daily supplementation with 1.1g/d of DHA resulted in a similar rise in erythrocyte membrane and plasma DHA irrespective of the type of oil and margarine consumed in the diet or its LA content. These findings add to the body of evidence advocating that it is the total amount of dietary omega-3 fatty acids consumed rather than the ratio of dietary omega-6: omega-3 fatty acids that has the greatest impact on the bioavailability of supplemented very long chain omega-3 fatty acids.

The MOFO study demonstrated that replacing LA rich oil and margarine in the diet with canola or sunola is an effective strategy for reducing dietary LA intake and for

reducing the long term uptake of LA into cellular membrane phospholipid pools. The MOFO study also demonstrated that if the dose of EPA provided by fish oil supplementation is high enough, then EPA will be preferentially taken up into the membrane phospholipids displacing any additional LA provided by LA-rich oils and margarines in the diet. It could therefore be advocated that a successful strategy for increasing cellular uptake of dietary EPA may be via significantly increasing the dietary intake levels of EPA without any other dietary modifications. However, such levels as those used in the MOFO study (8g/d of fish oil consumed as eight one gram capsules) are not easily achievable or sustainable by the majority of the Australian population with the current low dietary intake of marine sourced omega-3 fatty acids (Meyer et al., 1999). Thus a more successful strategy as demonstrated by the MOFO study could be to combine a lower dose fish oil supplement (1.1g/d rather than 2.2g/d) with regular dietary consumption of canola oil and margarine.

An added benefit of the functional food combination of canola and DHA is the ability of the combination to reduce fasting plasma triglyceride concentrations as effectively as double the dose of DHA, while simultaneously preventing a 15-16% increase in LDL cholesterol caused by both 1.1 and 2.2g/d of DHA. The high phytosterol content of canola is likely to be responsible for this effect although this claim was not investigated further in this thesis. The phytosterol content of the vegetable oils used in the MOFO study were not evaluated directly; plasma phytosterol concentrations were not determined and in backing up the proposal that the bioavailability of canola phytosterols is enhanced with DHA consumption, cellular membrane fatty acid phytosterol complexes were also not assessed. Thus future research should specifically measure these factors in addition to plasma lipid changes to test the validity of this hypothesis.

It remains unclear why soy isoflavones consumed independently of soy protein have no significant effect on plasma LDL cholesterol as is demonstrated by the findings from the Omega-Soy study and numerous other human clinical trials, while they contribute significantly to the cholesterol reduction achieved with soy protein intake. Insights may come from the synergistic effect of soy isoflavones and DHA on LDL cholesterol metabolism observed in the Omega-Soy study. The results showed that the dietary combination of soy isoflavones and DHA improve the lipid profile of moderately hyperlipidemic individuals more favourably than either constituent alone. Several

mechanistic hypotheses were developed in this Chapter to explain how DHA and soy isoflavones may act synergistically to reduce LDL cholesterol and future research is warranted to substantiate these mechanisms.

An important design feature of the two clinical trials conducted in this thesis was that subjects consumed oil supplements or an isoflavone-rich breakfast cereal as part of their usual diet. Study subjects did not modify their diet to adopt the low fat, low sugar and low energy diets that are commonly advocated for people with elevated blood lipids. This design was chosen to examine whether as a relatively simple dietary solution these functional ingredients could elicit cardiovascular benefits when consumed as part of the usual diet. Thus, the findings from the two studies in this thesis have broad application to the proportion of the Australian population that have elevated blood lipids.

#### **4.7 Implications of this research**

The two clinical trials conducted in this thesis tested the effect of daily DHA-rich oil supplementation on cardiovascular disease risk factors while either the type of oil and margarine consumed in the diet was varied or a soy isoflavone-rich breakfast cereal was consumed.

In the MOFO study the saff(8g) intervention group consumed 8g/d of a high DHA tuna oil providing 2.2g/d of DHA for six weeks. In the Omega-Soy study the DHAc-s and DHAs-c intervention groups consumed 8g/d of a DHA-rich algal-derived oil providing 2.9g/d of DHA for six weeks. The fatty acid composition of the DHA-rich oil used in each study was similar with the exception of the omega-6 fatty acid DPA which represented only 1.8% of total fatty acids in the tuna oil (MOFO study) but 14.8% of total fatty acids in the algal-oil (Omega-soy study). It is hypothesised (Conquer and Holub, 1997; Arterburn et al., 2006; Arterburn et al., 2007) that the omega-6 fatty acid DPA is retroconverted to arachidonic acid similar to the retroconversion of the omega-3 DHA to EPA and while it has also been suggested in the scientific literature that the omega-6 DPA may interfere with DHA accretion the evidence is not conclusive (Sanders et al., 2006; Lim et al., 2005; Arterburn et al., 2007).

While it is difficult to make direct comparison between the two studies due to different inclusion criteria and potential differences in dietary intake (the most predominant being

a concurrent consumption of soy isoflavones in the Omega-Soy study), observation of the erythrocyte membrane fatty acid composition of the subjects in each study suggests that the difference in omega-6 DPA content of the two oils may have had very little impact on DHA accretion.

After six weeks of DHA supplementation, DHA as a percentage of total fatty acids in erythrocyte membranes increased by 3.34% in the Omega-Soy study and by 3.49% in the MOFO study. Total omega-6 fatty acid content of erythrocyte membranes was 22.66% of total fatty acids in the MOFO study and 22.85% in the Omega-Soy study representing reductions in total omega-6 fatty acid content of 1.54% and 1.81%, respectively. Similarly, the Omega-3 Index after six weeks of DHA supplementation was 9.04 in the MOFO study and 9.13 in the Omega-Soy study. This data suggests that the DHA-rich algal oil used in the Omega-Soy study may be as effective as the tuna-oil for being a bioequivalent food source of DHA. However, without data to show direct comparison of these two oil sources on the effect of DHA uptake in plasma and erythrocyte membrane in the same study population, bioequivalence of the two oils can only be suggested not proven.

A study by Arterburn and colleagues (2007) showed that two algal sources of DHA differing only in their content of the omega-6 fatty acid DPA (one oil contained 16% DPA, the other less than 1% DPA), were bioequivalent sources of DHA as demonstrated by either proportions or absolute concentrations of fatty acids in plasma or erythrocyte membranes. These findings suggest that concurrent consumption of dietary omega-6 DPA with DHA may have little impact on the bioavailability of DHA and combined with the erythrocyte membrane fatty acid data from the two clinical trials conducted in this thesis, suggests that a DHA-rich algal oil with a high content of DPA may be as effective as a DHA-rich tuna oil as a food source of DHA.

Some concerns have been raised about potential heavy metal contamination of some fish and fish oils and Food Standards Australia New Zealand released warnings in 2004 for consumers to reduce the dietary intake of predatory fish species due to their high mercury levels (Food Standards Australia New Zealand, 2004). Demonstrating bioequivalence of a variety of fish and non-fish sources of DHA would therefore be helpful in providing a range of options for consumers to include DHA in their diet and

for food manufacturers interested in developing functional foods fortified with DHA. Future research comparing the DHA uptake in plasma and erythrocyte membrane fatty acids to confirm the bioequivalence of the algal and tuna oil examined in this thesis would be advantageous.

The benefits of enhanced improvements in CVD risk factors with combinations of functional foods as demonstrated in this thesis are that they enable lower doses of one or both functional foods to be consumed for the same or greater benefit. In the case of the Australian population that consumes very low levels of omega-3 fatty acids in the diet such simple functional food combinations as those identified in this thesis would be especially beneficial. These combinations can also provide a potential competitive advantage to functional food manufacturers developing foods targeting cardiovascular health. This is especially relevant for omega-3 fatty acids given that their popularity is significantly increasing as a functional food fortificant. Being able to distinguish a product from others on the basis of DHA and/or soy content, for example, could be advantageous. The findings of this thesis also provide exciting opportunities for advancing the scientific understanding of the potential mechanisms by which synergistic reductions in LDL cholesterol can occur. These mechanisms, once understood, could provide the impetus for exploring other functional food combinations for reducing CVD risk factors or other health outcomes and further the scientific understanding of the interrelationship between nuclear receptor pathways in lipid metabolism.

The findings from the Omega-Soy study have potential policy implications in use of the term 'soy' in food labeling. Under the Australia New Zealand Food Standards Code food manufacturers are able to label foods as containing 'soy' and use 'soy' in the name of the food product irrespective of the amount of soy present in the food, or the composition of the soy ingredients. This Omega-Soy study examined the effect of a breakfast cereal containing 90mg of soy isoflavones with almost no soy protein on cardiovascular risk factors. While the level of soy isoflavones was increased for the purposes of the study, a similar product is commercially available on supermarket shelves and labelled as a soy cereal. However when the soy cereal was consumed, no change in plasma total or LDL cholesterol or any other CVD risk factor was observed.



This may be due to elements of the study design including inadequate statistical power to detect a change in these parameters. However, when compared within the context of the broader scientific literature it would appear that soy isoflavones may have little independent cardiovascular benefit unless consumed with another functional ingredient that may act in synergy with the isoflavones, such as soy protein or, as indicated by the Omega-Soy study, DHA supplementation. Thus, it could be considered somewhat misleading to a consumer if a breakfast cereal is labelled as being a “soy cereal” that is “rich in soy isoflavones” but does not contain soy protein or other active ingredients known to facilitate the cardiovascular or other health benefits of isoflavones. On this basis it may be worthwhile for Food Standards Australia New Zealand to consider defining the term ‘soy’ and developing criteria which must be met before manufactured foods can include soy in the food product name or marketing. The results of the Omega-Soy study in conjunction with other data in the scientific literature also suggests that in future food manufacturers may want to ensure that their soy containing products contain soy protein or some other ingredient which may facilitate the actions of soy isoflavones to promote health benefits related to their consumption.

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#### **4.8 Conclusion**

Despite advances in drug therapies for hyperlipidemia and cardiovascular disease risk management, the cost of long term drug therapy and management of side effects may limit use by people needing these medications. Furthermore, the poor rates of compliance to these medications suggest they may not be an effective treatment strategy for all people with moderately elevated blood lipids. Therefore, functional food combinations that may be easy to implement, and readily adhered to at little additional cost relative to drug therapy either alone or along with limited drug therapy requirements would be beneficial.

The findings from this thesis support two functional food combinations for effective improvement of blood lipid concentrations when consumed as part of the usual diet of men and postmenopausal women with moderately elevated blood lipids. These functional food combinations are DHA and canola and DHA and soy isoflavones. The findings of this thesis shed some light on how isoflavones may be actively involved in reducing LDL cholesterol levels when consumed with soy protein or in soy containing

foods. Furthermore, the results provide strategies for ultimately reducing a side effect of dietary DHA supplementation and for achieving a better outcome in overall lipid profile improvements than could be achieved with DHA supplementation alone. Future research into these synergistic combinations of functional food ingredients with DHA may lead to new directions in functional food development by food manufacturers to enable more consumers to potentially manage their blood lipid concentrations with minimal or without drug therapy requirements.

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