PHYTOSTEROLS & OMEGA-3 POLYUNSATURATED FATTY ACIDS FOR CARDIOVASCULAR HEALTH IN HYPERLIPIDEMIA

By

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A thesis submitted for the degree of

Doctor of Philosophy, Human Physiology

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June, 2010

Statement of Originality

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Michelle Micallef

Acknowledgements

It is a pleasure to thank those who made this thesis possible.

First of all, I am indebted to my wonderful family, who let me leave home, but was just as much apart of this journey as if they were here with me. Thank you to mum, dad and Nicole for your support, encouragement and patience, through the good times and the bad. Whether it was on the phone or trips home, you were always there for me.

I am thankful to my supervisor, Manohar Garg, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of my research field and produce this thesis. Thank you for always having the door open for discussion.

Thank you to Doug Smith for providing his time, skills and use of the laboratory. Thanks to Maggie Soliman for her help, it was great working together.

I offer my sincere thanks to all of those who supported me in any respect during the completion of my studies, especially to James for your friendship and strength.

Lastly, I would like to show my gratitude to the many volunteers who donated their time and being part of this study.

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List of Abbreviations

AA	Arachidonic Acid
ABC	ATP-Binding Cassette
ACAT	AcylCoA:cholesteryl acyltransferase
AHA	American Heart Association
AI	Adequate Intake
ALA	Alpha Linolenic Acid
ANZCTR	Australian & New Zealand Clinical Trials Registration
APOA5	Apolipoprotein A5
apoB	Apolipoproetin B
APOC3	Apolipoprotein C3
apoE	Apolipoprotein E
ATP	Adenosinetriphosphate
ATPIII	Adult Treatment Panel Step III
BIA	Bioelectrical Impedance Apparatus
BMI	Body Mass Index
BP	Blood Pressure
CE	Cholesterol Ester
CETP	Cholesterol Ester Transport Protein
CHD	Coronary Heart Disease
CKK	Cholecystokinin
COX	Cyclooxygenase
CRP	C-Reactive Protein
CSANZ	Cardiac Society of Australia and New Zealand
CV	Coefficient of Variation
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
ELISA	Enzyme Linked Immunosorbet Assay
EPA	Eicosapentaenoic Acid

FA-CoA	Fatty Acyl-CoA Thioesters
FAO	Fatty Acid Oxidation
FFA	Free Fatty Acid
FFM	Fat Free Mass
FM	Fat Mass
GC	Gas Chromatography
HDL	High-Density Lipoprotein
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
HPL	Hyperlipoproteinemia
HR	Heart Rate
HSPG/LRP	Heparin Sulfate Proteoglycan/LDL Receptor Related Pathway
IL-6	Interleukin-6
LA	Linoleic Acid
LCAT	Lecithin-Cholesterol Acetyltransferase
LCFA	Long Chain Fatty Acid
LDL	Low-Density Lipoprotein
LDLr	Low-Density Lipoprotein Receptor
LOX	Lipooxygenase
LPL	Lipoprotein Lipase
LT	Leukotriene
MCFA	Medium Chain Fatty Acid
MUFA	Monounsaturated Fatty Acid
NCEP	National Cholesterol Education Program
NHFA	National Heart Foundation Australia
NHMRC	National Health and Medical Research Council
NPC1L1	Niemann-Pick C1-Like 1 Protein
NRV	Nutrient Reference Value
PG	Prostaglandin
PPARa	Peroxisome Proliferator-Activated Receptor Alpha
PS	Phytosterol
PUFA	Polyunsaturated Fatty Acid
RFLP	Restriction Fragment Length Polymorphism

SBP	Systolic Blood Pressure
SCFA	Short Chain Fatty Acids
SEM	Standard Error of Mean
SFA	Saturated Fatty Acid
TNF-a	Tumor Necrosis Factor-Alpha
TNFRSF	Tumor Necrosis Factor Superfamily
TXA	Thromboxane
USF1	Upstream Stimulatory Factor 1
VLDL	Very Low-Density Lipoprotein

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Publications from This Thesis

Journal Articles

1. **Micallef, M.A** & Garg, M.L (2009). "Beyond blood lipids: Phytosterol and omega-3 polyunsaturated fatty acid combination therapy for hyperlipidemia." Journal of Nutritional Biochemistry, 20: 927-39.

2. **Micallef, M.A** & Garg, M.L (2009). "Anti-inflammatory and cardio-protective effects of n-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals." Atherosclerosis, 204: 476-82.

3. **Micallef, M.A** & Garg, M.L (2008). "Synergistic and lipid-lowering effects of (n-3) polyunsaturated fatty acids and phytosterols in hyperlipidemic men and women." The Journal of Nutrition, 138: 1086-90.

4. **Micallef, M.A** & Garg, M.L (2009). "Phytosterols and eicosapentaenoic acid reduces cardiovascular risk in hyperlipidemia." (Submitted).

Other Related Articles

5. **Micallef M.A.,** Munro I, Phang M & Garg M.L (2009). "Plasma *n*-3 polyunsaturated fatty acids are negatively associated with obesity." British Journal of Nutrition, 102: 1370-1374.

6. **Micallef, M.A.,** Munro, I & Garg, M.L (2009). "An inverse relationship between plasma n-3 fatty acids and C-reactive protein in healthy individuals." European Journal of Clinical Nutrition, 63: 1154-1156.

7. Lazarus, S.A., **Micallef, M.A** & Garg, M.L (2008). "Synergistic effects of tomato serum and long chain omega-3 fatty acids on platelet aggregation." (Hamazaki, T., Nagasawa, T & Okuyama, H., eds.) pp. 57-73.

Conference Abstracts

1. **Micallef, M.A** & Garg, M.L (2009). "Phytosterols combined with oils rich in EPA or DHA as a treatment for hyperlipidemia." Nutrition Society of Australia & New Zealand Nutrition Society, Newcastle, December 8-11.

2. **Micallef, M.A** & Garg, M.L (2009). "Phytosterols combined with oils rich in eicosapentaenoic or docosahexaenoic acid: a potential treatment for hyperlipidemia." World Congress on Oils and Fats & 28th ISF Congress, Sydney, September 27-30.

3. **Micallef, M.A** & Garg, M.L (2009). "Nutraceuticals for reducing the risk of CVD in hyperlipidemic individuals." 5th International Congress on Cardiovascular Disease, Kosice, June 4-7.

4. **Micallef, M.A** & Garg, M.L (2009). "Cardioprotective effects of n-3PUFA and phytosterols in hyperlipidemia." International Atherosclerosis Society, Boston, June 14-18.

5. **Micallef, M.A** & Garg, M.L (2009). "Lipid-lowering and anti-inflammatory effects of n-3PUFA and phytosterols in hyperlipidemia." Heart Foundation Conference, Brisbane, May 14-16.

6. **Micallef, M.A** & Garg, M.L (2008). "Combined phytosterols and omega-3 fatty acid supplementation in the management of hyperlipidemia: effects on plasma lipids, inflammation and cardiovascular risk." XIII Congress on Clinical Nutrition, Xalapa, Mexico, Jan 29-Feb 2.

7. **Micallef, M.A** & Garg, M.L (2008). "Anti-inflammatory and cardio-protective effects of omega-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals." Asia Pacific Journal of Clinical Nutrition, 17(*suppl 3*) S80.

8. **Micallef, M.A.,** Munro, I.A & Garg, M.L (2008). "Relationship of obesity and abdominal adiposity with plasma C-reactive protein, interleukin-6 and omega-3 polyunsaturated fatty acids." Asia Pacific Journal of Clinical Nutrition, 16 (*suppl 3*) S130.

9. Phang, M., Munro, I.A., **Micallef, M.A** & Garg, M.L (2008). "Gender differences in the relationship between plasma leptin and long chain omega-3 polyunsaturated fatty acids." Asia Pacific Journal of Clinical Nutrition, 17(*suppl 3*) S82.

10. Phang, M., **Micallef, M.A.,** Munro, I.A & Garg, M.L (2008). "Plasma n-3 polyunsaturated fatty acids and weight status in free living adults." Asia Pacific Journal of Clinical Nutrition, 16 (*suppl 3*) S113.

11. **Micallef, M.A** & Garg, M.L (2008). "Anti-inflammatory and cardio-protective effects of omega-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals." Functional Foods & Edible Oils, Auckland, New Zealand, Nov 11-13.

12. Phang, M., **Micallef, M.A.,** Munro, I.A & Garg, M.L (2008). "Plasma long chain omega-3 polyunsaturated fatty acids and obesity." Functional Foods & Edible Oils, Auckland, New Zealand, Nov 11-13.

13. **Micallef, M.A** & Garg, M.L (2007). "Long chain omega-3 polyunsaturated fatty acids in the management of hyperlipidemia: A pilot study." XIII Congress on Clinical Nutrition, Alberta, Canada, Jun 17-21.

14. **Micallef, M.A** & Garg, M.L (2007). "Synergistic lipid-lowering effects of phytosterols and long chain omega-3 fatty acids in hyperlipidemic individuals." Australian Atherosclerosis Society, Fremantle, Perth, Oct 23-26.

15. **Micallef, M.A** & Garg, M.L (2007). "Lipid-lowering potential of combined phytosterols and long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA) in hyperlipidemia." Asia Pacific Journal of Clinical Nutrition, 16(*suppl*): S65.

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Synopsis

Atherosclerosis is a major factor influencing morbidity and mortality worldwide. The pathogenesis of atherosclerosis has been extensively investigated however treatment modalities have not changed much over the past decade. Prevention of atherosclerosis and its complications, both primary and secondary, are based mainly on controlling the various cardiovascular risk factors. Treating combined hyperlipidemia, and in particular reducing LDL-cholesterol and triglyceride levels, is established as a highly efficacious means of reducing both morbidity and mortality from cardiovascular disease. With the increased emphasis on various lipoprotein sub fractions, many patients need to consider combining treatments to achieve recommendations. Although statins can be an effective treatment for hyperlipidemia, they may not be sufficient to achieve the recommended LDL-cholesterol and triglyceride goals as set out by national governing bodies.

This thesis examines the lipid, inflammatory and cardiovascular response to concomitant supplementation with phytosterols and omega-3 fatty acids in combined hyperlipidemia. Phytosterols and omega-3 fatty acids are functional ingredients with potential cardiovascular benefits. Phytosterols inhibit cholesterol absorption, thereby reducing total-cholesterol and LDL-cholesterol. The consumption of 1.5-2.0g/day of phytosterols can result in a 10-15% reduction in LDL-cholesterol within a three week period, in hyperlipidemic populations. The added benefit of phytosterol supplementation has been demonstrated in individuals already taking statin medications. Omega-3 fatty acid supplementation has strong hypotriglyceridemic properties, and provides benefits in other risk factors associated with cardiovascular disease, such as anti-thrombotic and anti-inflammatory function. Given the propensity of hyperlipidemia to manifest in high risk individuals and populations alike, there is a plausible role for combining phytosterols and omega-3 fatty acids supplementation.

A series of clinical trials were undertaken to explore the plasma lipid, inflammatory and overall cardiovascular response to combined supplementation with phytosterols and omega-3 fatty acids rich in either EPA or DHA.

This particular dietary combination of functional ingredients was designed to optimise improvements in plasma lipid profile in individuals with combined hyperlipidemia. Findings from this thesis show that the combined supplementation of phytosterols and various omega-3 fatty acids reduces total-cholesterol, LDL-cholesterol, triglycerides and increases HDL-cholesterol, greater than the supplementation of either function ingredient alone.

Furthermore, a number of circulating inflammatory mediators were analysed showing significant reductions in response to the combined dietary treatment. Overall cardiovascular risk was reduced by an average of 20%. Interestingly, the combination of phytosterols and DHA was most effective in reducing triglyceride levels and inflammatory mediators, compared to the EPA combination. The phytosterol and DHA combination showed synergistic total-cholesterol and LDL-cholesterol lowering effects.

The apolipoprotein E genotype represents one of the most widely investigated genotypes with respect to lipid concentration. In this thesis, we examine the genotype within our cohort of combined hyperlipidemic individuals, who represent a population with an atherogenic lipoprotein phenotype. Whilst this genotype represents an obvious potential genetic modulator of lipid response to dietary therapies, it is yet to be explored in a case of concomitant supplementation with phytosterols and omega-3 fatty acids.

In conclusion, while this study has highlighted the potential of phytosterols and omega-3 fatty acids as a preventative strategy in combined hyperlipidemia, the data prompts further examination of the relative importance of individual fatty acids and fatty acid combinations with phytosterols.

Chapter 1

INTRODUCTION

Excerpts From This Chapter Have Been Published:

Micallef, M.A & Garg, M.L., (2009), "Beyond blood lipids: Phytosterol and omega-3 polyunsaturated fatty acid combination therapy for hyperlipidemia." Journal of Nutritional Biochemistry, 20: 927-39.

1.1 Dietary Lipids

Lipids represent all water insoluble compounds, such as triglycerides, phospholipids, cholesterol, cholesterol esters, free (un-esterified) fatty acids, fat soluble vitamins and carotenoids. Fats present in the diet, are primarily (>95%) made up of triglycerides. The amount and type of fats and oils consumed in the diet continue to be a focus of human nutritionists because of the relationship between fat intake and chronic diseases. Over the past several decades, national dietary recommendations have focused on reducing fat intake. The notion that dietary fat is synonymous with obesity and heart disease, has led manufacturers to market products as "low-fat" or "fat-free". Ironically, while dietary fat intake as a percentage of energy has reduced over time, total caloric intake has not and the prevalence of obesity, heart disease and diabetes continues to escalate [1].

Research questions the specific type of dietary fat being consumed and the interaction of these fat components involved in the current epidemic [2]. The study of the major structural elements of lipids is one of the most enigmatic research fields in human nutrition to date. Fats contain the most concentrated form of energy (37kJ/g), found in animal and plant-based foods containing varying proportions of saturated and unsaturated forms. An imperative component in the diet, fats provide essential fatty acids, assist in the absorption of fat-soluble vitamins, aid in metabolic pathways and membrane synthesis and provide structural elements in cells and tissues [3]. The biosynthetic pathway of one lipid class is dissimilar to another, suggesting their considerable variation in the complexity of structure and diversity of function.

1.1.1 Lipid Metabolism

Most dietary lipid digestion begins within the stomach and ends at the lumen of the small intestine. Lipids in the stomach are hydrolysed by lingual lipase, an enzyme secreted by serous glands beneath the tongue [4]. This enzyme accounts for the limited digestion of fats in the stomach, due to the acidic environment of gastric juices [5].

These gastric juices act upon triglycerides containing medium-chain fatty acids (MCFA) and short-chain fatty acids (SCFA), releasing fatty acid products. Further emulsification via gastric distension and contraction of the stomach muscles, push the chyme into the duodenum of the small intestine [6].

In order for dietary lipids to be absorbed by the intestine they must to be emulsified. The hydrolysis of long-chain fatty acids (LCFA) requires a less acidic environment, alternate lipases and effective emulsifying agents. These conditions are provided within the duodenum and hence digestion of lipids continue with the secretion of bile salts from the gallbladder, via the stimulation of the hormone cholecystokinin (CKK) [7]. Bile salts have highly effective emulsifying properties (containing both hydrophilic and hydrophobic ends) and act by converting lipids into smaller lobules, ready for further hydrolysis by pancreatic lipase [8].

Pancreatic lipase and phospholipase A_2 activation, requires the participation of bile salts, calcium ions and protein co-lipase [9]. The hydrolysis of triglycerides at positions 1 and 3 result in a mixture of diacylglycerols, monoacylglycerols and free fatty acids (FFA) (Figure 1.1).

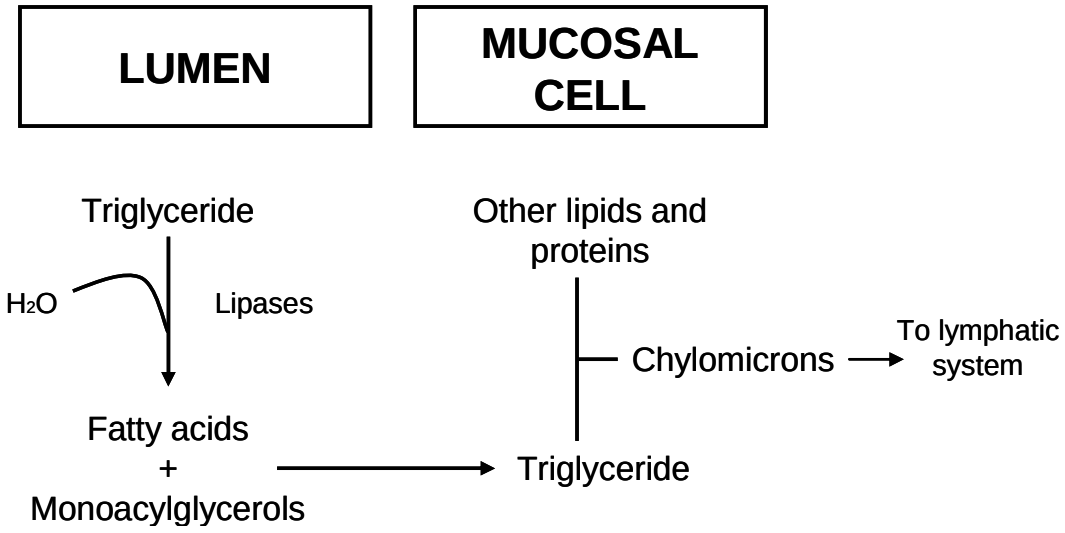

Figure 1.1 *Micelle lipid particles are taken up by intestinal mucosal cells and reassembled as lipid components into chylomicrons [10].*

Only a small percentage of triglycerides are hydrolysed into free glycerol [11]. Esterified cholesterol is further hydrolysed into free cholesterol and a fatty acid, catalysed by cholesterol esterase. The by-product of partially digested lipids is conjugated with bile salts to form polymolecular aggregates, more commonly known as micelles.

1.1.2 Lipid Absorption

Micelles are very small in diameter (~5nm) allowing them access the intra-microvillus spaces of the small intestine [12]. They interact with intestinal mucosal cells (enterocyte), where their lipid contents (FFA, monoacylglycerols and cholesterol) diffuse into the enterocyte. It is here that the LCFA are coupled with acyl–CoA synthetase and converted to fatty acyl-CoA thioesters used for further lipid synthesis [13]. SCFA pass directly into the portal blood stream. The re-synthesised lipids collect within the endoplasmic reticulum, attach to a protein and are exocytosed into the lymphatic circulation in the form of a chylomicrons [14]. These lipoproteins contain mainly triglycerides that have been produced by the intestinal mucosa from newly absorbed fat. Chylomicrons contain lipid droplets surrounded by polar lipids and finally a layer of proteins. Chylomicrons are the primary form of lipoprotein produced from exogenous lipids, and exist in other several forms to transport endogenous lipids. These include:very low-density lipoprotein; VLDL, low-density lipoprotein; LDL, and high-density lipoprotein; HDL [15]. These various lipoproteins differ in chemical composition, physical properties (e.g. density) and metabolic function.

The major lipoprotein secreted by the liver is the triglyceride-rich VLDL, which ranges in size from 300-500 Å, and is predominantly composed of triglycerides with little to no cholesterol ester [16]. The surface coats of these particles contain unesterified cholesterol, phospholipids, and apolipoproteins including the C and E apolipoprotein, as well as apolipoprotein B-100 (apo B-100)[17, 18]. Although the primary function of VLDL-cholesterol is to transport triglycerides, this lipoprotein plays a definite role in the metabolism of cholesterol. A major portion of cholesterol in VLDL particles are transformed to LDL-cholesterol in the normal conversion of VLDL to LDL [14].

LDL particles are heterogenous in size, density and composition, ranging in size from 175-200 Å [19]. Studies indicate that the size of the LDL confers an independent risk, with small and dense particles being more atherogenic than are larger, less dense particles [20-22]. Not only do high intakes of cholesterol increase the number of circulating LDL-cholesterol, but they can also change the size and composition, becoming enriched with cholesteryl esters [23]. LDL-cholesterol contain mostly cholesterol ester in their lipid core and more than 95% of their protein content is apo B-100 [17, 18]. Apo B is the major structural apoprotein of VLDL-cholesterol and LDL-cholesterol, regulated by hepatic cholesterol concentration and dietary cholesterol consumption [24].

HDL-cholesterol represents a group of smaller lipoproteins with diameters ranging from 70-100 Å [25]. These particles mainly contain cholesterol ester in their neutral lipid core and are coated with unesterified cholesterol, phospholipids and apolipoproteins. The major apoproteins found on HDL-cholesterol are apo A-I and apo A-II, both of which are water soluble apoproteins, and smaller amounts of apo C and E are also present [25].

1.1.3 Lipid Transport

Chylomicrons are the primary form of lipoprotein formed from exogenous lipids.. To some extent HDL-cholesterol is synthesised within enterocytes and released directly into the mesenteric lymph, whilst VLDL particles and LDL-cholesterol are formed endogenously by the liver and directly transported to adipose and muscle tissue [26].

Chylomicrons and VLDL-cholesterol, which are formed endogenously in the liver, pass through the circulation while undergoing intravascular hydrolysis. During their circulation in plasma, they undergo several changes. Firstly, VLDL particles acquire cholesterol ester in their neutral lipid core, derived mainly from HDL-cholesterol by way of exchange with triglycerides [27]. Secondly, they are lipolysed by lipoprotein lipase (LPL) found on the surface of endothelial cells, causing degradation of triglyceride molecules, and release of fatty acids and glycerol [14]. These now chylomicrons remnants, which are rich in cholesterol, are released back into the circulation and are cleared rapidly by the liver [28].

Lipoproteins other than chylomicrons do not arise directly from intestinal absorption, but are processed through other tissues such as the liver [29]. The fatty acids are oxidised for energy (β-oxidation) and the glycerol returns to the liver and kidneys, where it is converted to glycolytic intermediates. Both VLDL-cholesterol and LDL-cholesterol provide fatty acids for tissues and organs throughout the body [14].

Chylomicron remnants and SCFA are presented to the liver via apoE receptors. The lipid portion is hydrolysed and re-synthesised by hepatocytes, after which it is converted into bile salts and secreted within bile or may be incorporated into HDL-cholesterol or VLDL-cholesterol and released back into circulation. An important function of HDL-cholesterol is to remove unesterified cholesterol from cells and other lipoproteins, where it may have accumulated, and return it to the liver for excretion in the bile. This process is mediated by through its apo A-I component, which stimulates the activity of the enzyme lecithin: cholesterol acyltransferase (LCAT) [12]. This enzyme forms cholesteryl esters from free cholesterol by catalyzing the transfer of fatty acids, enabling it to be readily exchanged among plasma lipoproteins, mediated by the transfer protein cholesteryl ester transfer protein (CETP) [12].

1.2 Dietary Cholesterol

There are two main sources of cholesterol in the human body are dietary are endogenously produced cholesterol [30]. Biliary cholesterol output ranges from 800-1200mg/day and is totally unesterified, whereas dietary cholesterol ranges from 400-500mg/day and is partly esterified by LCFA [29]. Only about 40-65% of dietary cholesterol is absorbed, as the body makes most of what it needs. Table 1.1 gives some examples of the cholesterol content of selected foods [31].

Cholesterol forms structural elements within every cell of the human body and is integral for the synthesis of many hormones (estrogen, testosterone and adrenaline), the production of bile acids and the metabolism of many essential nutrients. Prior to absorption esterified dietary cholesterol is hydrolysed to unesterified cholesterol by cholesterol esterase in the small intestine [32].

Table 1.1 *Cholesterol content of selected foods [31].*

Food Item	Measure	Cholesterol (mg)
Skim milk	1 cup	4
Mayonnaise	1 tbsp	10
Butter	5 g	11
Low-fat milk (2%)	1 cup	22
Ice cream	0.5 cup	30
Whole milk	1 cup	34
Oysters, salmon	3 oz	40
Tuna	3 oz	55
Chicken, turkey	3 oz	70
Beef, pork	3 oz	75
Lamb, crab	3 oz	85
Shrimp, lobster	3 oz	110
Egg yolk	1	210
Liver	3 oz	410
Kidney	3 oz	540
Brains	3 oz	2640

Within the lumen of the small intestine this biliary cholesterol mixes with dietary cholesterol and a significant proportion is absorbed across the intestinal mucosa. In the mucosal cells, some of the absorbed cholesterol is esterified to cholesteryl esters by the enzyme acylCoA:cholesteryl acyltransferase (ACAT) prior to incorporation into the chylomicrons for transport via the lymphatic system. The enterohepatic movement of biliary and dietary cholesterol is of fundamental importance, in governing cholesterol balance throughout the body.

Cholesterol is transported throughout the body in the form of LDL-cholesterol and HDL-cholesterol. These lipoproteins differ according to their ratio of lipid-to-protein within the particle, as well as having different proportions of lipid types. Plasma levels should be >1.0mmol/L, whereas plasma LDL-cholesterol is recommended to be < 2.5mmol/L. Plasma cholesterol levels should be kept < 4.0mmol/L in order to minimise the risk for cardiovascular disease (CVD). The regulation of cholesterol homeostasis has been elucidated in early biochemical studies, showing enzymatic pathways involved in cholesterol synthesis, transport and feedback systems [32].

1.2.1 The Fate of Cholesterol Esters

Plasma cholesterol homeostasis is the net result of all cholesterol input (endogenous synthesis and dietary consumption) and cholesterol output (bile secretion) [33]. Cholesterol is maintained at a steady level by three regulatory mechanisms: (1) 3-hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity, (2) intracellular free cholesterol through the activity of ACAT and, (3) LDL-cholesterol uptake and HDL-cholesterol transport [34].

The primary means for governing plasma cholesterol level is via the regulation of HMG-CoA reductase activity, a rate-limiting enzyme involved in cholesterol synthesis, which is controlled by feed-back inhibition, gene expression and enzyme-degradation. The gene expression for HMGCoA reductase is reduced, hence reducing cholesterol synthesis [35]. Plasma cholesterol is also governed by LDL-cholesterol receptor number and activity. Elevated plasma levels of cholesterol will initiate the removal of LDL-cholesterol from plasma, by means of producing LDL-cholesterol receptors (LDLr) on the surface of the liver [36]. The level of LDLr is dictated by the influx of cholesterol from the intestine and the abundance in the liver [32, 37].

The process by which cholesterol is returned to the liver for excretion or degradation is commonly refered to as reverse cholesterol transport. The secretion of nascent HDL particles from the liver, containin apo A or E and phospholipids, and have a high affinity for unesterified cholesterol, which are acquired from other lipoproteins [38]. Unesterfied cholesterol in the outer coat of the lipoprotein is esterified through the reaction with LCAT, resulting in a HDL_3 particles, which has a molecular weight of 175,000 [38]. As these small spherical particles circulate, they are able to take on other consituents such as unesterified cholesterol, phospholipids, and other apoproteins (C and E) released from the degredation of VLDL or chylomicrons [28]. These changes to its structure can expand the HDL_3 particle, producing larger particles, HDL_2, which has a molecular weight of about 340,000. HDL can transfer some of its cholesterol ester to VLDL in exchange for triglyceride, whereby it re-enters the liver via catabolism of VLDL or by hepatic uptake of of LDL, whereby apolipoprotein E plays an important role in the removal of cholesterol from extrahepatic cells [39].

1.3 Apolipoprotein E (apoE) Polymorphism

Apolipoprotein E (apoE) is a 299 amino acid long glycoprotein with a molecular mass of ~ 34kDa [40]. There are three isoforms of apoE which reside on chromosome 19 in humans and give rise to three homozygous (apoE2/2, apoE3/3 and apoE4/4) and three heterozygous (apoE2/3, apoE2/4 and apoE3/4) genotypes [41]. Carriers of the E3 allele represent ~ 55-60% of the population and ~ 22% in Caucasian populations are E4 carriers [41, 42]. ApoE is secreted by many tissues, primarily the liver, brain, skin and macrophages [43-45]. The amino-terminal domain of apoE exists as a four-helix bundle and the structure of the three isoforms vary in subtle, yet important ways (Figure 1.2).

In vitro assays show that E3 and E4 bind with similar affinity to LDLr of cultured cells, whereas E2 has < 2% of this binding capacity, which is thought to be associated with the genetic disorder type III hyperlipoproteinemia (HPL) associated with the development of atherosclerosis [46, 47]. The major reason for this being that E2 has a reduced positive ion potential and hence a reduced ionic interaction with the negatively charged ligand binding regions of the LDLr [43].

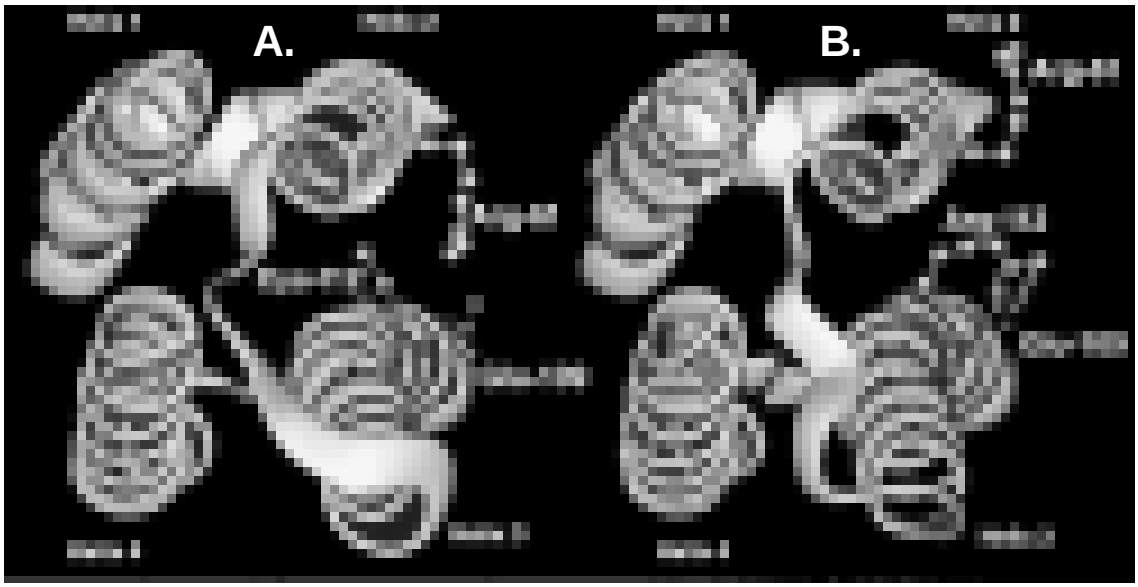

Figure 1.2 *The three-dimensional structure of apoE highlighting differences in isoforms. The four-helix bundle of apoE3 (A) and apoE4 (B) [43].*

1.3.1 Role of Apolipoprotein E in Lipoprotein Metabolism

The primary metabolic role of apoE is to transport and deliver lipids from one tissue or cell type to another [48]. As a component of VLDL particles, apoE are secreted by the liver and taken up by chylomicrons. As they circulate through capillaries, they become enriched in apoE and are eventually lipolysed on the surface of endothelial cells. Through this process apoE directs the metabolism of both endogenous triglycerides and cholesterol and dietary triglycerides and cholesterol by delivering them to either extrahepatic cells or the liver. ApoE complements the lipid transport system through two receptor pathways: (1) the LDLr on hepatic and extrahepatic cells and, (2) the heparin sulfate proteoglycan/LDL receptor-related protein (HSPG/LRP) pathway, which functions mainly in the liver for chylomicron remnant metabolism (Figure 1.3) [49, 50].

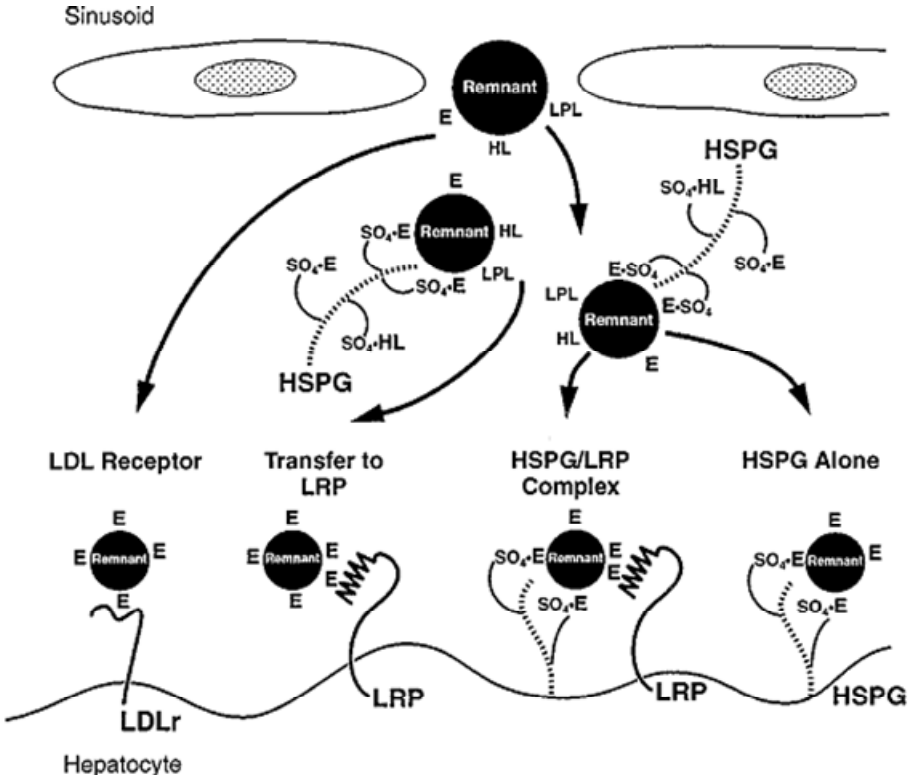

Figure 1.3 *Receptor pathways involved in the clearance of lipoprotein remnants by the liver [43]. HL, hepatic lipase; LPL, lipoprotein lipase; HSPG, heparin sulfate proteoglycan; LDL, low-density lipoprotein; LDLr, LDL receptor; HSPG/LRP,heparin sulfate proteoglycan/ LDL receptor-related protein pathway.*

The protective effect of apoE in atherosclerosis is apparent from its role in normal lipoprotein metabolism, especially in targeting chylomicron remnants for removal from circulation, however the benefit depends on the apoE isoform, the total plasma apoE concentration and the cell type responsible for the synthesis and secretion [43]. For example, animal studies show that the E2 and E4 alleles are associated with an increase in cholesterol levels and hence an increased CVD risk [51-53]. Conversely, apoE-null mice develop extremely high lipid concentrations and severe atherosclerosis, even whilst consuming a low-fat diet.

1.4 Dyslipidemia

Dyslipidemia is a heterogeneous disorder involving multiple aetiologies. It is commonly characterised by, (1) an increased flux of FFA, (2) raised triglycerides, LDL-cholesterol and apolipoprotein B (apoB) concentration and, (3) reduced plasma HDL-cholesterol concentration [54]. Changes in blood lipids may be a consequence of metabolic effects (primary cause), or dietary and lifestyle choices (secondary cause) (Table 1.2). Evidence from several prominent epidemiological studies (Framingham Heart Study, US Physicians Health Study and the Atherosclerosis Risk in Communities Study) show that HDL-cholesterol and LDL-cholesterol concentration contribute greatly to the risk of cardiovascular complications, diabetes and the metabolic syndrome (as reviewed by: [55]).

Table 1.2 *Some examples of primary and secondary aetiologies in dyslipidemia [56].*

Primary Causes	**Secondary Causes**	
LDL-cholesterol	Obesity	Nephrotic syndrome
receptor deficiency	Atherogenic diet	Anabolic steroids
Familial homozygous	Hyperthyroidism	Progestin
Hyperlipidemia	Diabetes mellitus	Pregnancy
	Alcohol	

In a recent study involving 29,972 subjects from 52 countries, it was shown that the strongest predictive risk factor for CVD and myocardial infarction was hyperlipidemia, followed by smoking, hypertension, diabetes, abdominal obesity, low fruit and vegetable consumption, excessive alcohol consumption and lack of regular exercise [57].

The most prominent genetic form of hyperlipidemia is familial hypercholesterolemia. This autosomal dominant condition, resulting from abnormalities in the gene coding for the cell surface receptor which removes LDL-cholesterol from circulation [58]. Clinical features include elevations in plasma LDL-cholesterol, tendon xanthomas (especially in the achilles tendon and extensor tendons of the hand) and a severe risk of premature coronary heart disease (CHD) [59].

Genes which are reportedly involved in clinical features of familial hyperlipidemia include LPL, lecithin-cholesterol acetyltransferase (LCAT), peroxisome proliferator-activated receptor alpha (PPARα), tumor necrosis factor superfamily , member 1B (TNFRSF1B), apolipoprotein C3 (APOC3), apolipoprotein A5 (APOA5) and upstream stimulatory factor 1 (USF1) [60].

1.4.1 The Pathophysiology of Hyperlipidemia

The primary defect involved in combined hyperlipidemia is the increased concentration of FFA, caused by inadequate esterification. As a consequence, a reduction in the retention of fatty acids by adipose tissue, leads to an increased flux of fatty acids returning to the liver [54]. In turn, this stimulates hepatic triglyceride synthesis, promoting the assembly and secretion of VLDL-particles and the production of apoB [61].

In the presence of increased plasma triglyceride concentration, cholesterol ester transfer proteins (CETP) mediate triglyceride cholesterol ester exchange between LDL-cholesterol and HDL-cholesterol, forming triglyceride rich HDL-cholesterol particles. These particles are more likely to be catabolised and hence HDL-cholesterol is reduced in the presence of elevated plasma fatty acids. Alternatively, VLDL-cholesterol particles are also lipolysed and hence fail to bind efficiently to LDLr. Importantly, CETP mediates the exchange of triglycerides from VLDL to HDL in exchange for CE (Figure 1.4).

Studies have shown that these LDL-cholesterol particles have been implicated in the development of CVD, characterised by reduced receptor-mediated clearance, increased arterial wall retention and an increased susceptibility of LDL-cholesterol to peroxidation [62].

The two general strategies for the primary prevention of hyperlipidemia are widely recognised. The “high risk” approach, in which individuals who are at an increased risk of developing hyperlipidemia, are identified and targeted for prevention. The “population” approach, where relatively small population-wide changes in risk factors of hyperlipidemia are implemented, leads to large reductions in the prevalence of the disease [63, 64]

Guidelines for prevention require continual reassessment of the response to research in the pattern of disease associated with hyperlipidemia. The primary focus is to stabilise blood lipid profiles at a healthy level, which is mostly governed by dietary and lifestyle choices. Obesity, particularly abdominal adiposity, is associated with an increased prevalence of diabetes, hypertension, insulin resistance and above all, hyperlipidemia [65]. Hence, a hallmark adaptation of exercise and appropriate dietary balance is a reduction in the risk of hyperlipidemia. Exercise is often a recommended approach to the modulation of blood lipid profiles, as a consequence of body weight loss [66]. A 10% reduction in body weight will result in changes in fat mass and abdominal visceral fat, such as reductions in VLDL-cholesterol secretion and up-regulation of LDL-cholesterol catabolism [54].

It is difficult to predict the compensatory effects of macronutrients, namely dietary fat, carbohydrate and protein on body weight adjustments and lipoprotein patterns. Weight reduction and increased physical activity are the most important and effective preventative techniques for improving blood lipid profiles.

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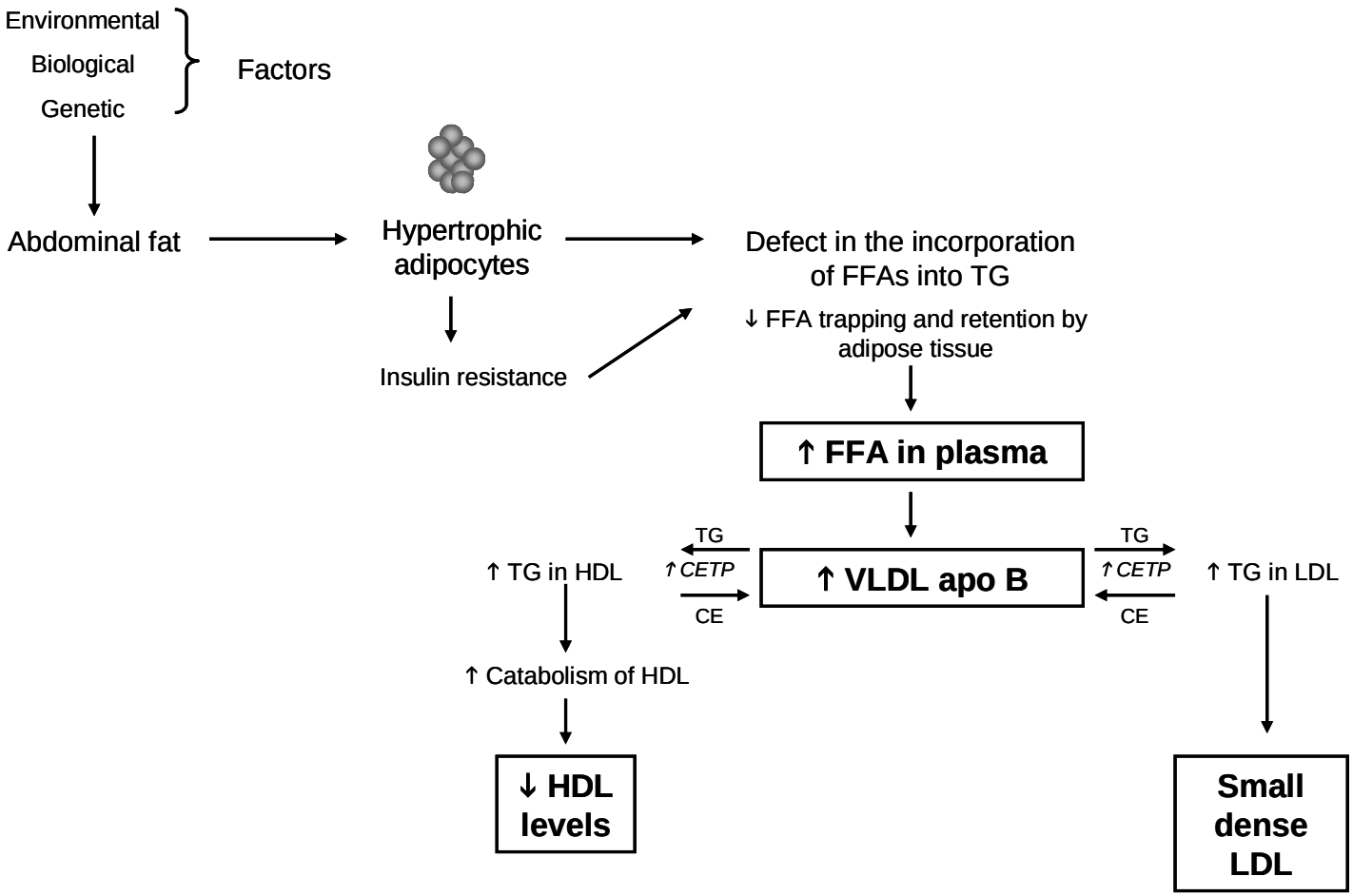

Figure 1.4 *Schematic representation of hyperlipidemia [54]. FFA, free fatty acid; TG, triglyceride; apo, apolipoprotein; HDL, high density lipoprotein; CETP, cholesterol ester transfer protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein.*

1.5 Implications of Lipid Modifying Therapy

In 2005 the National Heart Foundation of Australia (NHFA), along with the Cardiac Society of Australia and New Zealand (CSANZ), released a position statement on lipid management with the aim to establish a cost-effective risk factor management strategy to identify those individuals at higher absolute risk of a cardiovascular event [64]. The implementation of an absolute risk approach, is expressed as the chance of experiencing a predefined outcome as a percentage over a particular period of time (i.e. 5 or 10 years). The interventions outlined by the NHFA and CSANZ are aimed to achieve a 25% relative risk reduction in high risk groups, with an emphasis on LDL-cholesterol, HDL-cholesterol and total-cholesterol [67]. The American National Cholesterol Education Program suggests that an aggressive LDL-cholesterol reduction could in large ameliorate the risks attributable to diabetes, coronary artery disease and insulin sensitivity combined with hyperlipidemia [68].

In light of evidence surrounding the benefits of maintaining healthy plasma lipoprotein levels, body weight and blood pressure, the adoption of healthy practices such as physical activity and a balanced dietary intake contribute substantially to reducing the burden of disease.

1.5.1 Lifestyle Approach

Appropriate lifestyle interventions are an integral part of risk management. Protocols are based upon recommendations to follow a low-saturated fat diet, which incorporates moderate amounts of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), oils, marine and plant fatty acids, a wide variety of fruit, vegetables, whole grains and cereal products [69]. Other lifestyle interventions include moderate-intensity physical activity on most days of the week and weight loss/maintenance. A consistent body of evidence from both observational and experimental studies indicate that weight is positively associated with blood pressure and hypertension [70]. Lifestyle interventions in addition to pharmacological approaches may reduce absolute disease risk over and above lowering plasma lipids.

1.5.2 Dietary Manipulation of Lipids

Reasons that some dietary patterns, such as those rich in fruits, vegetables and fish are associated with reduced disease risk is largely understood. Foods contain variable mixtures of macro and micronutrients, which may impact risk singly or in combination [71]. Dietary manipulation of plasma lipid profiles are based on the effects of known food components, with emphasis placed on an individuals overall eating pattern.

Low fat diets are commonly prescribed and if followed, do elicit significant reductions in LDL-cholesterol [72-74]. The American Heart Association (AHA) has devised a very-low saturated fat, low cholesterol diet in an effort to achieve healthy dietary practices whilst maintaining cardiovascular and overall health. The general principle of the AHA diet is the endorsement of fruits, vegetables, fat-free and low-fat dairy products, cereal and grain products, legumes nuts and fish, poultry and lean meats (Table 1.3) [71].

Table 1.3 *Composition of the American Heart Association (AHA) diet [75].*

Nutrient	% of Total Energy Intake
Total fat	< 30
Saturated fats	8-10
Polyunsaturated fats	< 10
Monounsaturated fats	< 15
Carbohydrates	55
Protein	15
Cholesterol	< 300 (mg/d)
Alcohol	0

Hunninghake *et al* [76] found a 5% reduction in LDL-cholesterol in hyperlipidemic patients following the AHA diet, conversely a 6% reduction in HDL-cholesterol therefore the LDL:HDL ratio remained unchanged.

Modest weight-loss in obese subjects is associated with an overall improvement in CVD risk, including hyperlipidemia. Weight reduction in viscerally obese men of approximately 10kg in 12 weeks, showed a decrease in hepatic apoB secretion and an up regulation of LDL-cholesterol catabolism by 50 and 125%, respectively [69]. The reduction of visceral adipose tissue is the largest indicator of changes in VLDL-cholesterol secretion and LDLr clearance.

A comparative study of four popular diets: Atkins (carbohydrate restriction), Zone (macronutrient balance), Weight Watchers (calorie restriction) and the Ornish diet (fat restriction), assessed for one year, showed reductions in weight and cardiac risk factors [77]. All diets reduced plasma cholesterol (although not statistically significant), increased HDL-cholesterol and reduced C-reactive protein (CRP) at one year.

The low carbohydrate diets (Atkins and Zone) were most likely to reduce triglycerides, diastolic blood pressure and insulin in the short term (2 months). Therefore, for each diet, weight loss predicted the amount of improvement of several cardiovascular risk factors, namely total/HDL-cholesterol (r=0.36), CRP (r=-0.37) and insulin (r=-0.39).

1.5.3 Pharmacological Therapy

Lifestyle intervention remains the corner stone in treating hyperlipidemia, however when cholesterol goals are not achieved, pharmacological therapy is usually prescribed. The main approach is to reduce LDL-cholesterol levels and total-cholesterol to ensure the management of hyperlipidemia.

1.5.3.1 Bile Acid Sequestrant Resins

The biosynthesis of cholesterol *de novo* accounts for slightly less than half of the body's cholesterol content. Inputs of biliary cholesterol are about twice of that of dietary cholesterol, with intestinal perfusion studies showing endogenous cholesterol absorption between 60-80% [14].

The exogenous movement of dietary and biliary cholesterol out of the intestine and the formation of fecal bile acids for excretion, are pathways affected by bile acid sequestrant resins (i.e. cholestyramine, colestipol, colesevalem) [78]. At high doses (approximately 22g) these drugs are shown to reduce total-cholesterol and LDL-cholesterol by 15-17% and 18-25%, respectively [79].

These drugs bind to bile acids preventing their solubilisation into micelles. This reduction in hepatic bile acids consequently activates the rate-limiting enzyme 7-α-hydroxylase, an enzyme which converts cholesterol to bile acid in the liver. For every molecule of bile produced, one molecule of cholesterol is used, thus reducing circulating cholesterol. This need for cholesterol activates 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. This enzyme also induces the number of LDLr, therefore leading to a reduction in circulatory LDL-cholesterol [35].

1.5.3.2 Cholesterol Absorption Inhibitors

Cholesterol absorption inhibitors such as ezetimibe are available for the management of hyperlipidemia. Ezetimibe is a selective inhibitor of intestinal cholesterol absorption which specifically binds to cell surface receptors deemed for dietary cholesterol, without affecting triglyceride, fatty acid or fat-soluble vitamin absorption [80]. Following ingestion, ezetimibe is gluccoronidated. This compound is active in blocking cholesterol absorption by around 50% and as it is not stored by the body, it is excreted in the urine and the feces [81].

1.5.3.3 HMG-CoA Reductase Inhibitors

The primary means for governing plasma cholesterol level is via the regulation of HMG-CoA reductase activity. As discussed in detail further, HMG-CoA reductase is a rate-limiting enzyme involved in cholesterol synthesis, which is controlled by feed-back inhibition, gene expression and enzyme-degradation mechanisms [35].

The newest class of cholesterol-lowering drug, HMG-CoA reductase inhibitors, or more commonly, statins, show more potential as lipid-lowering agents then previous drugs. HMG-CoA reductase is the rate limiting enzyme for the synthesis of mevalonate, a precursor of cholesterol synthesis [82]. Therefore statins competitively inhibit cholesterol synthesis and actively reduce total-cholesterol and LDL-cholesterol [83]. Common side-effects of lipid-lowering medications may include headaches, upset stomach, fatigue, flu-like symptoms and myalgia (muscle aches) [84].

A meta-analysis of five major statin trials with a mean follow-up time of 5.4 years and a mean age of 59 years, show a mean reduction (weighted by sample size) in total-cholesterol, LDL-cholesterol and triglyceride levels of 20%, 28% and 13% respectively and HDL-cholesterol was increased by an average of 5% [85]. There was a consistent reduction in the odds of coronary events compared with controls and the proportion of risk reduction was similar for women (29%; 95% CI, 13-42%) and men (31%; 95% CI, 26-35%). This meta-analysis is also supported by a review of randomised trials by Herbert *et al* [86].

1.5.4 Interactions Between Extrinsic Factors

Variations in individual responses to lipid-lowering therapy are primarily due to extraneous factors (i.e. erratic consumption, discontinuation or intolerability). A large Australian study by Simons *et al* (2000) showed that 30% of patients discontinue taking their medication within 6-7 months of commencement. This was despite the excellent tolerability and safety profile of the drug [87]. The time of administration and concomitant drug therapy may also be accountable for the inter-individual variability in response. An individual's background diet is also extremely important in optimising the reduction in LDL-cholesterol. The effect of a low-fat diet coupled with reduced cholesterol intake is additive rather then synergistic to pharmacological treatment of hyperlipidemia [88-91].

In determining the inter-individual responses to dietary cholesterol absorption, genetic studies suggest there are differences in both the magnitude and response to dietary cholesterol [92]. They suggest that people are either hypo-responders (have higher basal rates of cholesterol synthesis) or hyper-responders (have lower basal rates of cholesterol synthesis) due to an apoE polymorphism. To elaborate, sub-group analysis of the Scandinavian Simvastatin Survival Study showed that people who were hyper-responders, showed a lesser decrease in cholesterol synthesis when treated with the drug. The study concludes by suggesting that hyper-absorbers of dietary cholesterol showed no reduction in coronary events when treated with simvastatin and had the same relative risk rate of CHD as those in the placebo group [93].

The reduction in coronary events is largely affected by the extent to which these drugs lower LDL-cholesterol in hyperlipidemic individuals. A meta-analysis of the five major drug trials indicates a reduction in coronary events (31%), fatal coronary disease (29%) and total mortality (21%) and an overall risk reduction of 29% and 31 % in women and men respectively [85]. Despite the present data about statin therapy and other pharmacological interventions for treating elevated plasma lipids, many patients are still reluctant to use these prescribed drugs or they may not be able to reach an adequately low LDL-cholesterol level using statins alone. This warrants a review of the efficacy of alternative non-pharmacological treatments for managing elevated plasma lipids.

1.5.5 Complementary Alternatives to Drug Therapy

Pharmacological therapies including bile acid sequestrant resins, statins, fibrates, niacin and cholesterol absorption inhibitors are common treatment options for hyperlipidemia, however the use of alternative therapies is also becoming increasingly popular. Controlled trials using a range of complementary alternatives to drug therapy (i.e. policosanols, flaxseed, red yeast rice, guggulipid, garlic, viscous fiber, almonds, macadamia nuts and soy proteins) have been examined as potential functional foods in the management of hyperlipidemia [94].

The two components of soy, including protein and isoflavones are responsible for the lipid-lowering effect, possibly by their ability to modulate LDLr in the liver [95, 96]. A meta-analysis of 38 randomised controlled trials with an average daily soy intake of 47g, showed a reduction in total-cholesterol and triglyceride levels by 9.3% and 10.5%, respectively [94]. Dietary fibers, particularly soluble (viscous) fiber has also been associated with strong cholesterol-lowering properties [97]. It appears that soluble fiber affects hepatic cholesterol and lipoprotein metabolism. As a result, there is an increase in the loss of bile acids and an up-regulation of LDLr in the liver, thus a reduction in total circulating cholesterol becomes evident. A meta-analysis of eight controlled trials demonstrated a mean dose-dependent relationship between soluble fiber intake (7.2g/day) and reductions in total-cholesterol (4%) and LDL-cholesterol (7%) after 8-26 weeks of supplementation in moderately hyperlipidemic subjects, also prescribed to a low-fat diet [97]. In a study by Jenkins *et al* [98] a dietary portfolio approach to cholesterol reduction was applied to the diets of thirteen hypercholesterolemic subjects for one month. The test diet was a combination of phytosterols (1g/1000kcal), soy protein (23g/1000kcal) and viscous fiber (9g/1000kcal) obtained from normally purchased foods. Significant reductions in total-cholesterol, LDL-cholesterol and LDL: HDL-cholesterol was found to be 22.3, 29.0 and 26.5% respectively. A 30% reduction in calculated CHD risk was also reported. Other complementary functional ingredients include policosanols, which are very-long-chain alcohols derivatives of sugarcane. These are shown to reduce total-cholesterol and LDL-cholesterol in the range of 10-23% in a recent animal study by Marinangeli *et al* [99], but had no effect in an earlier study in hypercholesterolemic subjects [100]. Also of interest is the Chinese red yeast rice (*Monascus purpureus*) a fermented product, documented from the Tang Dynasty 800AD. This extract is used as a food preservative but also for its medicinal properties, as in Chinese and Japanese populations where red yeast rice is a dietary staple, studies have shown its cholesterol (11-32%) and triglyceride (12-19%) lowering properties [101]. Nowadays, red yeast rice is a key ingredient in various cholesterol-lowering medications (i.e. Cholestin, Lovastatin and Mevinolin). Guggul extract (guggulipid) from the resin of the mukul myrrh tree (*Commiphora mukul*) is a well established Ayurvedic medicinal compound [102]. The extract is an antagonist of the bile acid receptor and has been shown to reduce cholesterol (11%), LDL-cholesterol (12%) and triglycerides (15%), however this is contradicted by another study showing the extract increased LDL-cholesterol [103, 104].

1.6 Phytosterols

Whilst cholesterol is the predominant sterol of mammalian cells, another group of sterols which are structurally and functionally analogous exists [105]. These are a group of non-nutritive compounds produced by plants collectively known as plant sterols or phytosterols. Recent convention divides phytosterols into two categories. Phytostanols are characterised by a reduction at the double bond and are consequently saturated versions of phytosterols and are therefore less abundant in the food supply [106].

1.6.1 Dietary Sources and Consumption

The primary dietary sources of phytosterols are vegetables, vegetable products including vegetable oils, fruit, nuts, legumes and cereals (Table 1.4) [107]. Notably, vegetable oils (e.g. corn oil) are a highly concentrated source, for example consuming 30g/day of corn oil would provide 286mg of phytosterols, an amount which has been shown to reduce cholesterol absorption efficiency by 12% [108]. The typical Western dietary consumption of phytosterols is approximately 100-300mg/day (160-350mg/day of sitosterol and campesterol and 20-50mg/day of sitostanol) and 20-50mg/day of phytostanols, in which the body retains < 5% [109].

Table 1.4 *Phytosterol content in a variety of foods [110].*

Food Source	Phytosterol Content	Food Source	Phytosterol Content
Corn oil	952	Corn	70
Sunflower oil	725	Wheat	69
Safflower oil	444	Palm oil	49
Soybean oil	221	Lettuce	38
Olive oil	176	Banana	16
Almonds	143	Apple	12
Beans	76	Tomato	7

Includes β-sitosterol, campesterol and stigmasterol (mg/100g).

Of this amount, most is rapidly excreted by the liver with < 1% remaining [30, 111, 112]. Evidently, a vegetarian diet will contain higher amounts of phytosterols, compared to that of a conventional Western diet. Phytosterol content of a traditional Asian diet is 350-400mg/day, whereas a vegetarian diet may contain as much as 600-800mg/day [113].

The most common phytosterols are β-sitosterol (24-ethylcholesterol), campesterol (24-methylcholesterol) and stigmasterol (Δ^{22}-24-ethylcholesterol) and on average they comprise 65, 30 and 3% of the total dietary phytosterol intake, respectively [105, 114]. Nuts and seeds are rich sources of phytosterols and are consistently shown to be associated with reducing plasma cholesterol levels [115-117]. The quantification of phytosterols in commonly consumed nuts and seeds show that sesame seeds and wheat germ have the highest phytosterol content (400-413mg/100g), whilst Brazil nuts have the lowest (95mg/100g). β-sitosterol, campesterol and stigmasterol are the predominant sterols in foods and therefore often the only sterols routinely measured. Figure 1.5 illustrates that numerous sterols comprise a significant proportion of total phytosterols in most nuts and seeds.

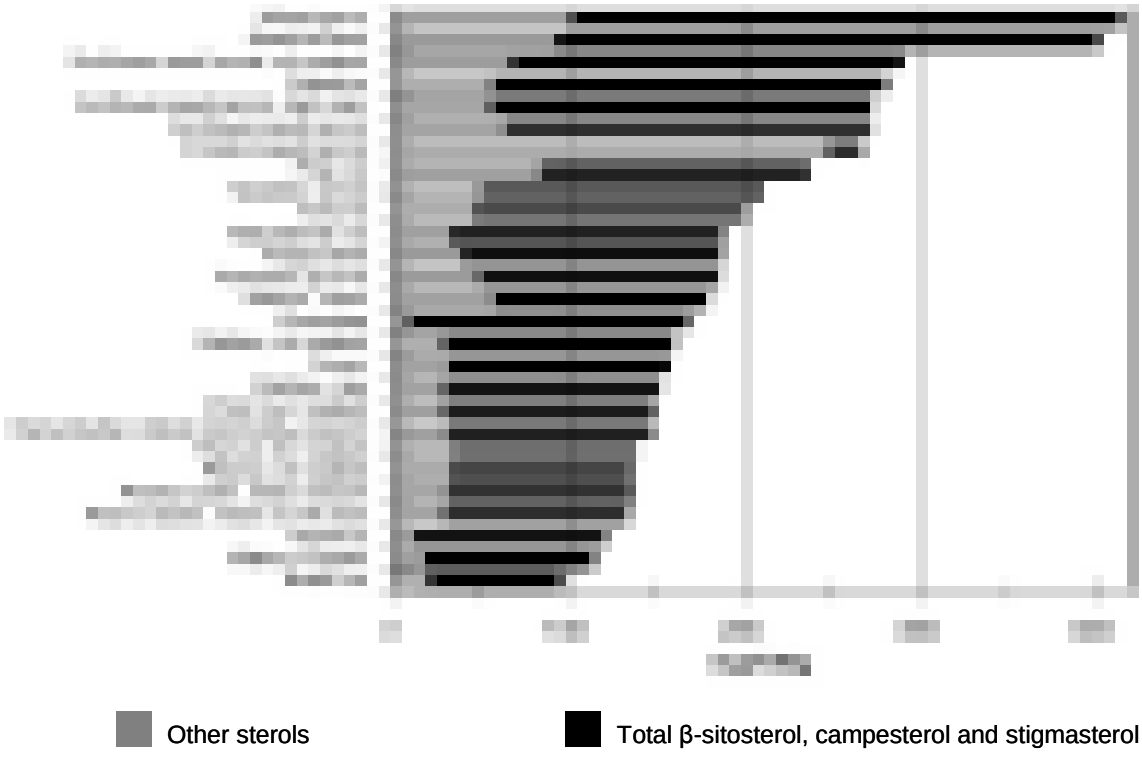

Figure 1.5 *Phytosterol content of various nuts, seeds and food products [118].*

Phytosterols are structurally related to cholesterol, however they include the presence of a modified side chain configuration at carbon C-24. The absorption of phytosterols depends upon the nature of the C-24 side chain. Increases in the complexity of this side chain (as seen in phytosterols) increases its hydrophobicity, thereby reducing or impairing absorption. Sitosterol and campesterol have an ethyl and methyl substitute at C-24, respectively, whereas stigmasterol contains a double bond at C-22 (Figure 1.6) [119].

Sterols which are not absorbed undergo intestinal bacterial transformation to produce metabolites, such as corposterol and coprostanone [120]. The body is unable to endogenously synthesise phytosterols, therefore they are solemnly derived from the diet. Ultimately phytosterols compete with dietary and biliary cholesterol and hence have long been known to reduce serum LDL-cholesterol via the interference with cholesterol absorption within the intestine [121]. Phytosterols encompass a wide variety of biological interactions. Above all they are known for their efficacious cholesterol-lowering properties [108, 122-124].

1.6.2 Phytosterol Balance

Phytosterols were traditionally thought to be non-absorbable, as it was shown that humans consuming a solid food diet excreted more than 90% of sitosterol in their stool [125]. However, a small percentage is absorbed by the intestine, usually less then 5% of dietary levels, considerably lower than that of cholesterol ($\geq$ 40%). The most viable method for measuring phytosterol absorption is analysis of serum, which measures absorption into the systemic circulation rather then just intestinal mucosa [110]. Under normal conditions serum phytosterol levels are around 0.3-1.7mg/dl with a daily dietary intake of 160-360mg/day [126]. These levels have been shown to increase up to two-fold in addition to dietary supplementation (> 1.5g/day) [126-128]. Serum phytosterol concentration is also dependent upon serum lipoprotein metabolism, meaning those with hypercholesterolemia not only have significantly higher cholesterol levels, but also have significantly reduced plasma phytosterol concentration [129].

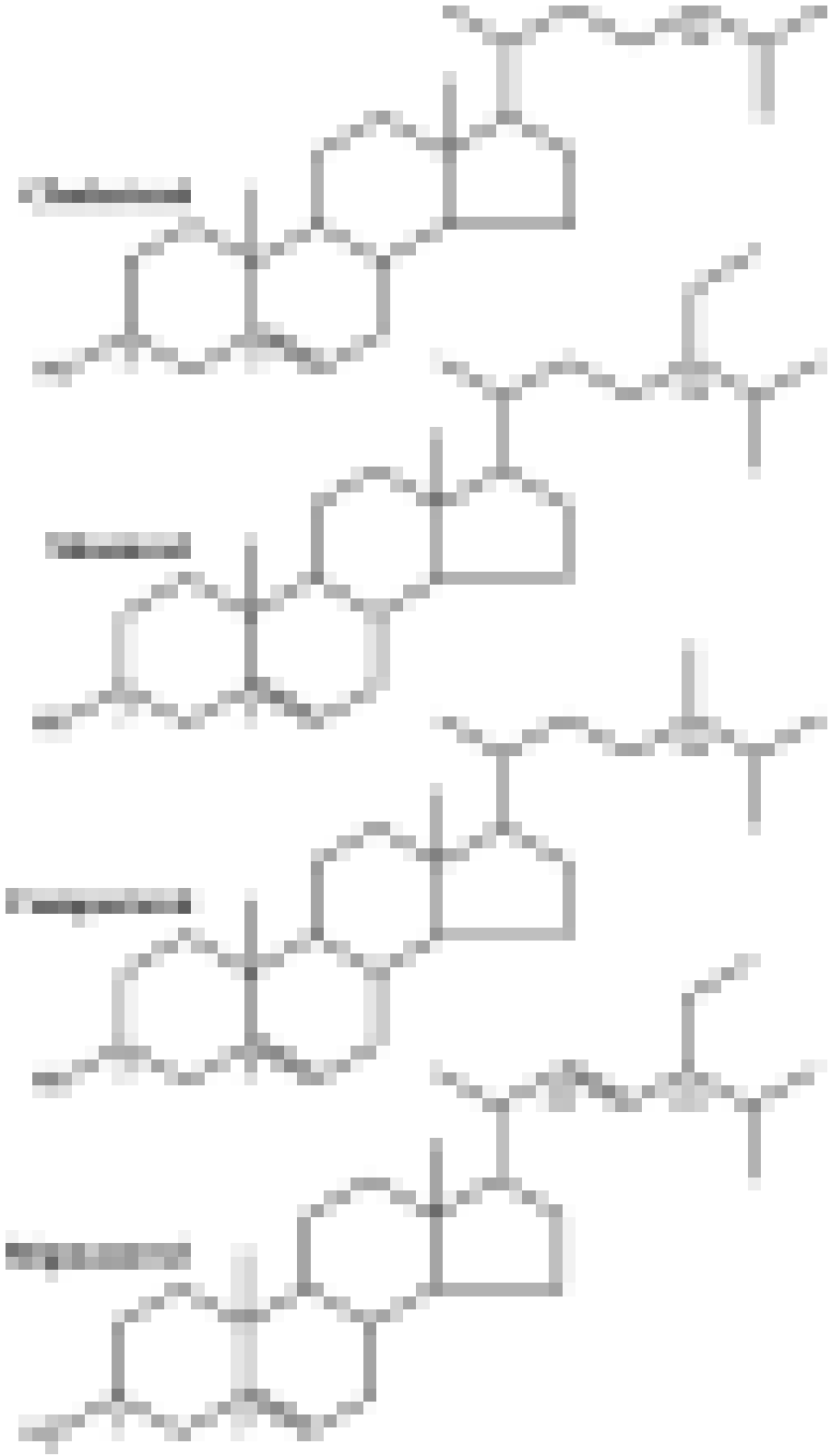

Figure 1.6 *Biochemical structure of cholesterol and common phytosterols [110].*

Dietary and biliary absorption of phytosterols requires the solubilisation in micellular form for efficient intestinal absorption. The sterol-laden micellular particle interacts with the intestinal brush border, thereby facilitating the uptake of phytosterols by enterocytes [105]. Unlike cholesterol absorption (40-60%), intestinal phytosterol absorption is selective. Campesterol absorption is three times lower than that of cholesterol (9-18%) and sitosterol absorption is three times lower than that of campesterol (4-8%) [129, 130]. The discrimination of phytosterol absorption involves the variation in the side chain, which affects the uptake by villus cells and brush border membranes. Uptake is decreased with an increase in the number of carbon atoms at C-24 [131]. The exact mechanism for absorption has not been well defined, however the uptake of cholesterol and phytosterols by enterocytes both involve similar pathway mechanisms. Phytosterol elimination takes place via the biliary route, which occurs more rapidly than cholesterol excretion due to poor intestinal absorption [120]. The biliary excretion rate of sitosterol is faster then that of campesterol and campesterol excretion is faster then that of cholesterol.

Phytosterol balance studies have shown that decreased blood cholesterol levels are attributable, at least in part, to an inhibition of cholesterol absorption. This inhibition has been ascribed to a number of mechanisms, including partitioning in the micellular phase of the intestinal lumen, presence of mucosal barriers which might limit trans-membrane transport and alterations in the rates of cholesterol esterification in the intestinal wall [114].

1.6.3 Absorption of Phytosterols and Cholesterol

Prior to intestinal absorption, cholesterol and phytosterol esters must be solubilised into micellular form. Micelles promote absorption through the facilitation of transport across the unstirred water layer adjacent to the surface of the intestinal luminal cells [132]. Here the micelles interact with the microvillus brush border of the enterocyte, allowing cholesterol and phytosterols to passively diffuse across the membrane via the Niemann-Pick C1-Like 1 Protein (NPC1L1) [133, 134]. Essentially, NPC1L1 is a trans-membrane regulator of cholesterol homeostasis, whose function is to traffic intracellular cholesterol.

A study by Altman and colleagues [134] showed that NPC1L1 is critical for the uptake of cholesterol across the membrane of the intestinal enterocyte, where NPC1L1-deficient mice exhibited a substantial reduction in cholesterol absorption, which was unaffected by dietary supplementation of bile acids.

Once inside the enterocyte there are ultimately two pathways for cholesterol and phytosterols. Firstly, they may be esterified by ACAT and packaged into chylomicrons at the basolateral membrane (Figure 1.7). The chylomicrons are secreted into the lymphatic system for portage to the left subclavian vein and ultimately the peripheral circulation, where they are hydrolysed and transported to the liver [14]. Secondly, unesterified cholesterol and phytosterols may be transported back into the intestinal lumen by trans-membrane ATP-Binding Cassette (ABC) proteins 5 (ABCG5) and 8 (ABCG8) [135].

These transporters selectively pump phytosterols and cholesterol back from the enterocyte into the intestinal lumen, thereby regulating the rate of absorption [130]. These phytosterol and cholesterol esters are transported in lipoproteins to the liver. The sterol esters, along with lipoprotein remnants, are in an un-esterified state. Once phytosterols reach the liver they are rapidly excreted through the bile. Once cholesterol is within the liver there are five potential fates: (1) re-esterified and stored as cholesterol esters, (2) secreted back into plasma along with lipoproteins (mostly VLDL-cholesterol), (3) converted into bile acids, (4) secreted into bile as cholesterol or (5) they may inhibit the synthesis of cholesterol by the liver [14].

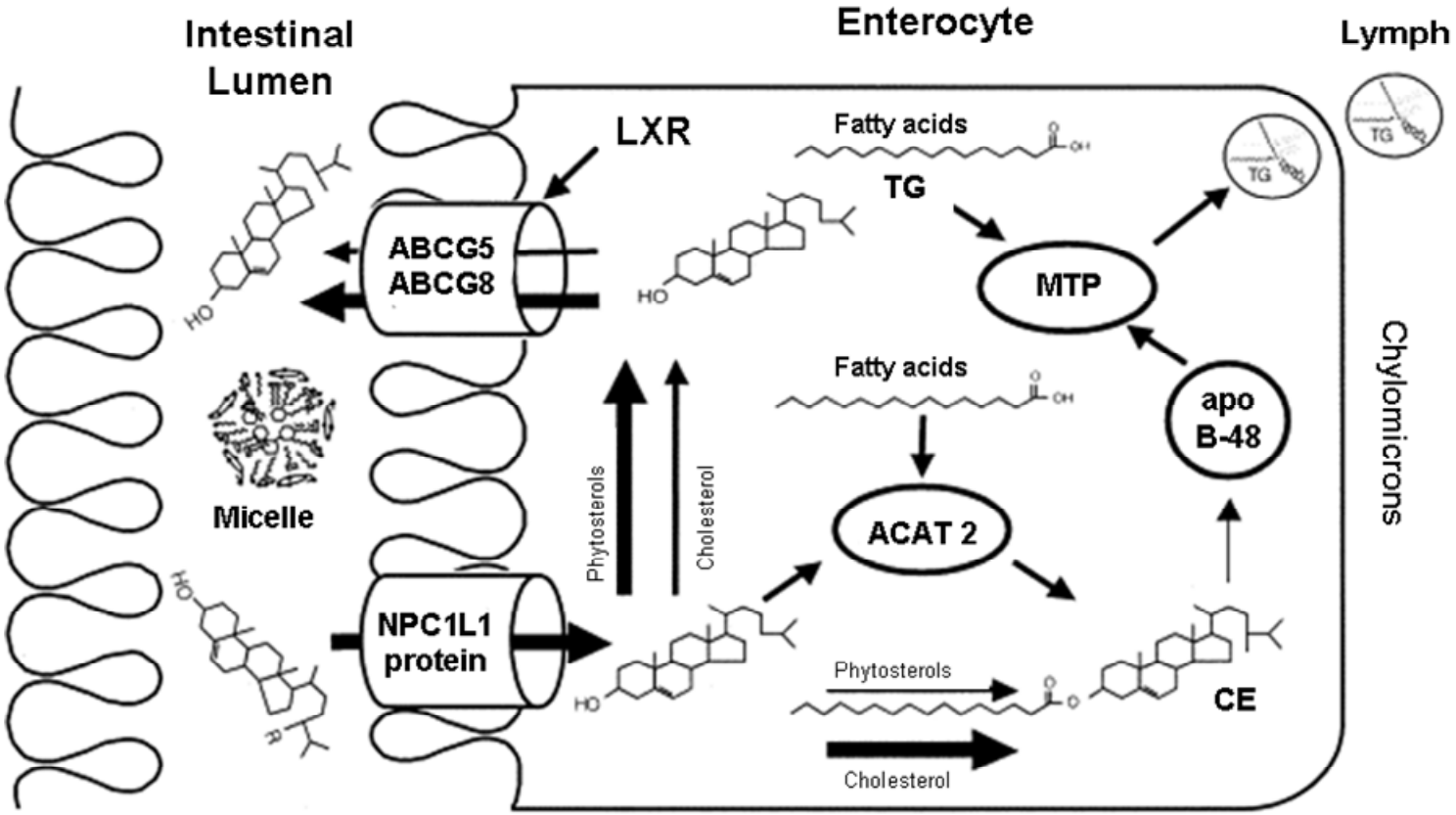

Figure 1.7 *Major pathways in the absorption and intracellular traffic of cholesterol and phytosterols in the intestinal mucosal cell [129]. ABCG5/G8, adenosine triphosphate-binding cassette G5/G8; ACAT, acyl coenzyme A cholesterol acyltransferase; apo, apolipoprotein; CE, cholesterol ester; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick C1-Like 1 protein; TG, triglyceride.*

1.6.4 Phytosterolemia

Phytosterolemia is an extremely rare inherited metabolic disorder, characterised by an increased absorption of phytosterol esters [136]. Normally, ATP-binding cassette transporters prevent the absorption of phytosterols, as they selectively mediate their efflux back into the intestinal lumen [92]. In the case of phytosterolemia, a loss of selectivity due to mutations of ABCG5/G8 leads to atherosclerosis and xanthomatosis at an early age [137]. Total-cholesterol is only moderately elevated in phytosterolemic patients, however sterol esters can be found in high concentrations within atherosclerotic lesions and depositions within other tissues throughout the body [138].

1.6.5 Phytosterols in Hyperlipidemia

Phytosterol consumption has been shown to reduce circulating lipid levels in different treatment populations [139-141], making it an attractive functional ingredient in the management of hyperlipidemia. The mechanism is primarily through the competitive inhibition of micellular solubilisation and hence intestinal absorption of both dietary and biliary cholesterol [142, 143]. Phytosterol balance studies show marked decreases in intestinal cholesterol absorption after the administration of phytosterols, despite increases (10-20%) in lathosterol, a biomarker of cholesterol synthesis [94, 108, 144].

A meta-analysis of 14 randomised trials of phytosterol and phytostanol efficacy by O'Neil *et al* [145] showed a strong correlation between serum levels of 7α-hydroxy-4-cholesten-3-one (7α-OHC), a well-validated marker of bile acid synthesis and phytosterol consumption. A 27% decrease ($P=0.01$) in 7α-OHC levels were evident in subjects supplemented with phytosterols (1.6g/day) for two-months [146], which is supported by supplementation trials by Westrate and colleagues [147] suggesting phytosterols suppress bile acid synthesis.

Phytosterols have been found to reduce total-cholesterol and LDL-cholesterol without any significant effects on HDL-cholesterol, triglycerides or lipid-soluble vitamins (A, D, E, K) [148]. The optimum dose-response concentration of phytosterols is 2g/day (1.5 – 3.0g/day) to elicit a reduction in total-cholesterol and LDL-cholesterol of 10-12% (Figure 1.8) [147, 149, 150].

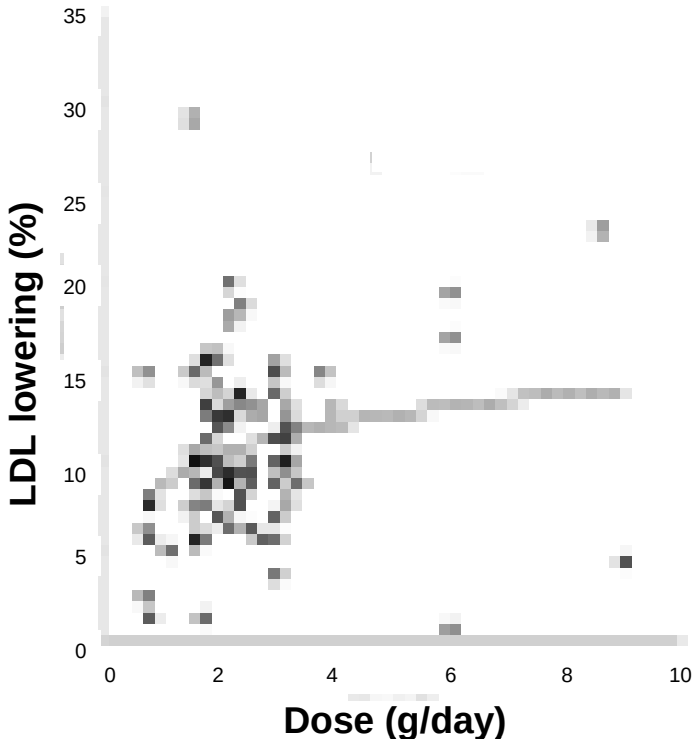

Figure 1.8 *Results of LDL-cholesterol lowering (% from baseline) following phytosterol consumption in 55 clinical trials (n=2930 subjects)* [151].

At higher concentrations, changes in lipid profiles seem to plateau with an average reduction in serum LDL-cholesterol of 0.54mmol/L (14%, 95% confidence interval 0.46-0.63mmol/L) [151]. Data from these observational studies and randomised trials suggest a reduction in LDL-cholesterol of 0.5mmol/L would sufficiently reduce the risk of CVD by 25% in two years [151].

1.6.6 Phytosterol-Enriched Functional Foods

Numerous randomised controlled studies have shown that the consumption of phytosterol esters (around 2g/day) can achieve reductions in LDL-cholesterol in the order of 10-15% in about 90% of individuals [152, 153]. This is equivalent to doubling the dose of statins. The comparable LDL-cholesterol lowering capabilities of phytosterols and phytostanols, reflects their strong individual ability to reduce cholesterol absorption and compensatory increase in cholesterol synthesis.

Numerous studies have introduced phytosterols and phytostanols into regularly consumed foods such as cream cheese, salad dressing, yoghurt, milk, cereal bars and margarine shown to be an effective means of reducing total- and LDL-cholesterol in adults and children with hyperlipidemia [151, 154, 155].

In an effort to increase dietary consumption of phytosterols, they have been introduced into fat spreads. As fats are needed to solubilise sterols, margarines are an ideal vehicle as they increase the lipid solubility and facilitate the incorporation into the gut. The estimated effects apply to an average serve of 20-25g/day providing 1.6-2g of sterol esters per serve. In a study by Nestel *et al* [156] the consumption of 20g/day of sterol ester fortified margarine (2.4g/day) for four-weeks, provided a median reduction in total-cholesterol (12.2%) and LDL-cholesterol (13.6%) in line with that reported by Westrate and Meijer [147] and Gylling and Miettinen [140]. A recent systemic review of the efficacy of phytosterols in lowering total-cholesterol and LDL-cholesterol concentration in familial hyperlipidemic subjects shows differences between treatment and control groups for total-cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides [121].

Fat spreads enriched with phytosterols (2.3 ± 0.5g/d) significantly reduced total-cholesterol (7-11%) with a mean reduction of 0.65mmol/L [95% CI -0.88, -0.42mmol/L], ($p<0.00001$) and LDL-cholesterol (10-15%) with a mean reduction of 0.64mmol/L [95% CI -0.86, -0.43mmol/L], ($p<0.00001$) after 6.5 ± 1.9 weeks compared to the control group, without any adverse effects. Triglycerides and HDL-cholesterol concentration were not affected.

These studies suggest that the cholesterol-raising effects of margarine and butter products may be counteracted by the inclusion of phytosterols, making them an effective food for the management of hyperlipidemia.

In a long-term (12 months) study by Hendriks *et al* [157], mildly hyperlipidemic participants consuming 20g/day of a phytosterol-enriched spread (providing 1.6g phytosterols), showed a consistent reduction in total and LDL-cholesterol, 4 and 6% on average over one year. Phytosterol supplementation did not affect red blood cell deformability, hormone level, clinical and haematological parameters or fat-soluble vitamin concentration. However, lipid adjusted α- and β- carotene concentration was reduced by 15-25%, relative to control. A simple method of preventing [158] changes in carotenoid concentration is to increase fruit and vegetable consumption during phytosterol supplementation [158-160]. It has also been suggested that carotenoid responses to phytosterols may vary according to apoE genotype, more specifically carriers of apoE may have a tapered response to phytosterol consumption [161]. Although there are a limited number of studies investigating the long-term effects of phytosterol-enriched foods, there is no evidence to suggest their consumption to be unsafe, making them an effective functional food for the management of hyperlipidemia.

In a community intervention program based in Maastricht, Netherlands, observations on the voluntary use and effectiveness of phytosterol/stanol-enriched fat spreads were made during the time of their introduction to the Dutch market [162]. Blood samples taken in 1999 and again in 2003, show significant changes in total-cholesterol concentration in non-users (+2%), enriched-spread users (-4%), cholesterol-lowering drug users (-17%) and combination users (spread + statin) (-29%). Although the recommended dose was not consumed (1.4 ± 9g/day), a modest reduction in cholesterol is apparent in this free-living community setting.

The enrichment of margarine products with phytosterols has shown to be an affective vehicle for the management of total-cholesterol and LDL-cholesterol. When comparing phytosterol enriched margarine products with other enriched foodstuffs, it is clear that margarine is an appropriate dietary therapy for hyperlipidemia. In a study by Noakes *et al* (2005) either a phytosterol-enriched milk (300ml/d; 2.0g plant sterols/d) or yoghurt

(300g/d; 1.8g plant sterols/d) product was supplemented into the diets of modestly hypercholesterolemic (total-cholesterol 5-7.5mmol/L) participants for three weeks. The milk and yoghurt products yielded reductions in LDL-cholesterol of 6-8% and 6% respectively. In another study [156] a breakfast cereal and bread product enriched with phytosterols (2.4g/d) showed a reduction in total-cholesterol (8.5%) and LDL-cholesterol (13.6%) after four weeks supplementation. This was comparable with results of a study by Clifton [163] which supplemented milk, yoghurt, bread and cereal products (1.6g/d) for three weeks in mildly hypercholesterolemic subjects.

1.6.6.1 The Role of ApoE in Response to Phytosterols

Several studies have investigated the plasma lipid response to phytosterol supplementation in the context of apoE genotype, with most studies showing LDL-cholesterol lowering irrespective of the apoE allele [164-168]. In a recent study by Sanchez-Muniz *et al* [161] where subjects receiving a phytosterol-enriched spread (1.1g/day or 2.2 g/day) for five weeks in conjunction with the NCEP-step I diet, showed a variation in lipid and lipoprotein response between different apoE alleles. ApoE2 carriers experienced the greatest reduction in LDL-cholesterol (12.7%), when compared to the control, whereas E3 and E4 carriers showed LDL-cholesterol reductions of 5.5% and 5.6%, respectively. Conflicting findings from a study by Plat and Mensink [165] showed no significant differences in lipoprotein response to 4g/day phytostanol esters, between apoE alleles. Geelan *et al* [169] also observed that in healthy normo-cholesterolemic individuals of apoE3/E4 and apoE3/E3 genotype responded similarly to 3.2g/day of a phytosterol-enriched spread [162].

The specific role of apoE as a determinant of plasma lipid response to phytosterol supplementation is largely unknown. Due to considerable differences in study design, populations and participant numbers, further investigation is warranted.

1.6.7 Phytosterols as an Adjunct to Statin Therapy

Given that statin therapies are the most widely used method for lipid-lowering in hypercholesterolemic patients, these drugs may only be used at suboptimal doses. Under these conditions, the added benefit of phytosterols to inhibit cholesterol absorption seems reasonable [170]. This additive nature is convincingly demonstrated by Simons [171] in a study with four-parallel treatment arms. Hypercholesterolemic participants were asked to consume 25g/day of a sterol-ester enriched margarine (providing 2g/day) in addition to a lipid-lowering drug *Cerivastatin* (40mg/day) for four weeks. This combination of phytosterols and statins produced an 8% reduction in LDL-cholesterol. The additive effect of this combination is equivalent to doubling the dose of a statin [172]. Similarly, Neil *et al* [173] showed an 11% reduction in LDL-cholesterol with a statin plus sterol ester margarine (2.5g/day) versus placebo, for eight weeks. These studies show that the combination of phytosterols as an adjunct to statin therapy is additive rather then synergistic, resulting in incremental decreases in LDL-cholesterol ranging from 10-20%, as reviewed by Thompson *et al* [87].

A series of randomised, controlled trials [174-176] combining phytosterols with statin medication shows a 4.5 ± 2.4% additive effect, which translates to an additional 9-14% reduction in cardiovascular events, using risk modeling equations [177, 178]. Accordingly, statin-induced inhibition of cholesterol synthesis may insufficiently reduce LDL-cholesterol, produce adverse effects and may be uneconomical, such that suboptimal doses may be required [170]. The addition of phytosterol-enriched foods as an adjuvant to statin therapy may provide sufficient improvement to total- and LDL-cholesterol to counteract the reduction in lipid-lowering medication.

1.6.8 Sterols and Stanols: Comparisons and Contrasts

It is surely questionable whether saturated sterols (phytostanols) or esterified, unsaturated phytosterols are most effective as lipid-lowering agents. The absorption rates for phytosterols differ markedly to phytostanols. For example, the saturated derivative of sitosterol, sitostanol, is virtually un-absorbable (< 2%) (Table 1.5) [109]. Small amounts of phytostanols can be recovered from serum or bile as they are effectively excreted after consumption [179].

Table 1.5 *Physiological aspects of cholesterol, phytosterols & phytostanols [109].*

	Cholesterol	**Phytosterols**	**Phytostanols**
Dietary intake	300-500mg/d	200-400mg/d	< 10mg/d
Rate of absorption	40-60%	< 5%	0.1-2%
Plasma concentration	140-320mg/dl	0.3-1.7mg/dl	0.3-0.6mg/dl
Rate of excretion	40-60%	> 95%	> 98%

The mechanisms by which phytosterols and phytostanols reduce circulating cholesterol are effectively the same. They inhibit cholesterol absorption, solubility and hydrolysis at the intestine [127]. The degree to which they alter total-cholesterol and LDL-cholesterol is comparable. In a head-to-head randomised trial of the efficacy of phytosterols and phytostanols, LDL-cholesterol was reduced by 8-13% respectively, and cholesterol absorption was reduced by 36-25% respectively, by a phytosterol or phytostanol-enriched product (1.8-2.5g/day) [109]. There were no changes in HDL-cholesterol and triglyceride concentration.

The consumption of phytostanols also appears to reduce the absorption of phytosterols as-well-as cholesterol [179, 180]. Whereas the consumption of phytosterols, produced a 71.6 and 32.5% increase is campesterol and β-sitosterol [144]. The concern is, in the circumstance of phytosterolemia, in which patients tend to hyper-absorb cholesterol and phytosterols alike, there is the tendency to develop atherogenic traits and ultimately CVD.

Therefore, there is concern as to whether increasing consumption may be atherogenic rather then cardio-protective. The safety aspects of phytosterols have been discussed by Plat and Mensink [181] and Sehayek and Breslow [182] who conclude that there are no reports of serious side-effects resulting from phytosterol consumption, also supported by numerous efficacy studies [183-185].

1.7 Omega-3 Polyunsaturated Fatty Acids

The common components in major classes of dietary fats (triglycerides, phospholipids, cholesterol esters) are fatty acids [186]. Fatty acids vary in both the chain length and the number of double bonds as well as positions of double bonds. There are three major types of fatty acid families, saturated fatty acids (SFA), MUFA and PUFA [187]. Fatty acids are designated as X:Y (n- or ω-z) where X represents the number of carbon atoms, Y represents the number of double bonds and, z represents the position of the first double bond from the methyl (n or ω) end of the carbon chain [187].

SFA acids do not contain any double bonds between their carbon atoms and can be classed into three sub-categories. SCFA (2-4 carbon atoms) include acetic, propionic and butyric acid. MCFA (4-12 carbon atoms) include caproic, caprylic, carpric and lauric acid. LCFA (> 14 carbon atoms) include myristic, palmitic and stearic acid [188]. Most animal fats (i.e. bacon grease, lard, tallow and butter) are saturated and are solid at room temperature. Many plant fats such as coconut oil, palm kernel oil and palm oil are also saturated. An increased consumption of total and SFA have been widely recognised to contribute to the development of obesity and cardiovascular complications [186, 189, 190].

Unsaturated fatty acids are a group of fats with one or more double bonds, either of a *cis* or *trans* isomer. The greater the degree of unsaturation in a fatty acid the more vulnerable it is to peroxidation. Most unsaturated fats are plant-based (corn, soybean, cottonseed, sunflower seed, peanut, tree nuts and olive oil) and tend to be a liquid at room temperature [191, 192]. The double bonds present in these fats prevent them packing together and solidifying [193].

MUFA with one double bond, include palmitoleic, oleic and erucic acids, are generally found in olive, sunola, canola and several tree-nut oils. Naturally occurring fatty acids have double bonds in their *cis* configuration [194]. *Trans* fatty acids are popular amongst food manufacturers as these are produced during the partial hydrogenation of fats/oils for the manufacturing of margarines, shortenings and peanut butter to improve plasticity.

During partial hydrogenation, some of the double bonds are reconfigured to form a *trans*-alignment, much like a SFA [195]. Another source of *trans* fatty acids in the human diet are ruminant meats and dairy products. Partial hydrogenation of fats/oil by micro-organisms in ruminant stomachs generates *trans* fatty acids that are incorporated into milk and muscle meats. The major *trans* fatty acid present in foods is elaidic acids [2].

PUFA are molecules containing more then one double bond and are classified into two sub-categories: omega-6 (or n-6) and omega-3 (or n-3) [196]. Omega-6 fatty acids are found in plant-derived oils including soybean, corn, sunflower and cottonseed oils, while omega-3 fatty acids originate from either plant-derived oils such as canola, flaxseed and soybean or from seafood (herring, tuna, salmon and mackerel etc) [178, 197, 198]. LA and ALA are essential in human diets as humans lack enzymes to synthesise these fatty acids.

Generally unsaturated fatty acids are labeled as the “healthy fats”. Plant-based diets are considered healthy compared to a typical Western diet, as they are generally low in SFA and high in PUFA. In addition to the favorable fatty acid composition of plant-based diets, the phytochemical and antioxidant nutrients of diets high in grains, seeds, fruit and vegetables has been shown to promote good cardiovascular health [192].

1.7.1 Dietary Sources and Consumption

The two most important metabolically active PUFA are the parent fatty acids, linoleic acid (LA) and α-linolenic acid (ALA) of the omega-6 and the omega-3 families respectively [199]. LA and ALA are elongated and desaturated in animal cells, forming the metabolically active omega-6 and omega-3 families [200].

ALA is a key constituent of dark green leafy vegetables (i.e. broccoli, cabbage and spinach), many seed oils, meats and cereal products (Table 1.6). The longer chain omega-3 fatty acids are predominantly found in oil-rich fish products such as, tuna, salmon, trout, herring and sardines [201]. There is constant growth in the availability of foods fortified with omega-3 fatty acids in the consumer market.

Enriched sources include eggs produced by chicken's fed omega-3 rich diets, fish oils or algae-derived omega-3 fatty acid supplements, margarine spreads (Seachange), milk (Dairy Farmers, Heart Plus) and breads (HighTop) [202, 203].

Table 1.6 *Dietary sources of omega-3 and omega-6 fatty acids [196, 202, 204, 205].*

Dietary Source	Omega-3	Omega-6
Oil	Flaxseed, canola, linseed, rapeseed, soybean, walnut, cod liver and fish	Sunflower, soybean, corn, cottonseed, peanut, safflower and sesame
Meat, poultry & seafood	Tuna, salmon, trout, crab, mackerel, prawns and liver	Lean meat, offal and poultry
Vegetables	Dark green leafy vegetables: broccoli, cabbage and spinach	
Other	Fortified food products: bread, eggs, milk, margarine and fish oil supplements	Margarine spreads

The nematode *Caenorhabditis elegans* (*C. elegans*) are able to synthesise both LA and ALA, due to the presence of an endogenous omega-3 fatty acid desaturase which recognises a range of 18 and 20-carbon omega-6 fatty acid substrates and a Δ^{12} fatty acid desaturase, which converts 16 and 18-carbon omega-6 fatty acid to omega-3 fatty acid [206]. The generation of PUFA is achieved through the action of desaturase which directs the conversion of MUFA to PUFA [207].

To demonstrate the functionality of the expression of the *C. elegan* desaturases, the cDNA coding sequence could be introduced into livestock [206]. The pattern of dietary change over the past 40 years has shown that the consumption of vegetable oil-based products has increased and the consumption of omega-3 fatty acids has decreased.

Hence, the balance of fatty acid consumption within of the Western diet has shifted away from omega-3 to omega-6 domination. Our modern diet is full of complex, processed and synthetic foods, which differ considerably compared with the more traditional diets, rich in fresh fruit, vegetables and fish. Today, farming practices mean animal products are much less rich in omega-3 fatty acids, the price of fresh oil-rich fish is rather a luxury then a staple and developments in food technology mean there are a lot more omega-6 oil products on the market [202].

In 1992 the National Health and Medical Research Council (NHMRC) promoted the increase in the intake of omega-3 fatty acids from plant foods and fish products within the Australian diet [208]. The Food and Agriculture Organisation (FAO) of the United Nations (WHO Consultation on Fats and Oils) suggests an omega-6 to omega-3 ratio in the diet to be 5:1 to 10:1 [209].

The Australian dietary intake of LA is approximately 10-20g/day, constituting more then 85% of total PUFA intake. The Australian recommended adequate intake (AI) for LA is 13g/day and 8g/day for males and females respectively. The average dietary intake of arachidonic acid (AA) for males and females is 130mg/day and 96mg/day, respectively [210, 211]. This equates to approximately 4-5% of dietary energy intake. The chief consumption of omega-6 fatty acids are margarine products, which Australians consume 1.9kg/year/capita [202, 204].

Meat and poultry products account for at least one-fifth of the Australian average intake of ALA and its long chain derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [212]. The Australian AI for ALA is 1.3g/day for men and 0.8g/day for women, which equates to 0.4-0.5% of total dietary energy [213]. The Australian mean consumption of omega-3 fatty acids (EPA+DPA+DHA) in the adult population is 190mg/day, which is comparable to the average American intake (140mg/day), although lower then habitual fish-eating countries like Japan (1600mg/day) [214, 215].

At present, dose-response relationships between EPA, DPA and DHA are yet to be established therefore it is difficult to differentiate between their individual requirements.

Hence the Australian Nutrient Reference Values (NRV) for EPA, DOA and DHA are based on the highest median population intake (males, 160mg/day and females 90mg/day) [213]. These changes fuel growing concern as to whether our diet may become insufficient to meet the omega-3 fatty acid dietary requirements, particularly since omega-3 fatty acids are involved in highly significant developmental and regulatory processes (Table 1.7).

Table 1.7 *Internationally recommended daily intakes of omega-3 fatty acids [216, 217].*

Organisation	**EPA+DHA (mg/day)**	**Population**
NHMRC (Australian NRV)	160 90	Males Femaile
British Nutrition Foundation Task Force	500-1000	People at risk of CVD
UK Department of Health	200	General population
European Academy of Nutritional Science	200	General population
ISSFAL	500	General population
AHA	1000 Oily fish (2/week)	At risk of CVD General population
NIH	300	Pregnant/lactating
WHO	1-2% energy	General population

NHMRC, National Health and Medical Research Council; NRV, Nutrient reference value; ISSFAL, International Society for the Study of Fatty Acids and Lipids; AHA; NIH, National Institute of Health; WHO, World Health Organisation; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

1.7.2 Metabolism of Omega-3 Fatty Acids

The two essential fatty acids (LA and ALA) cannot be synthesised *de novo* within humans. Hence it is important that they are consumed as part of a habitual diet. LA is the parent fatty acid of the omega-6 fatty acid, described by its shorthand notation C18:2n-6 and ALA is the parent fatty acid of the omega-3 fatty acids, with the shorthand notation C18:3n-3. The location of the carbon double bonds within the PUFA is important, as it affects metabolism of the molecule [200]. If the first double bond is located six-carbons from the methyl (omega) end of the fatty acid, it is an omega-6 fatty acid. Hence, if the first double bond is located three-carbons from the omega end, it is an omega-3 fatty acid.

Importantly, both LA and ALA are further converted into their respective long-chain fatty acids, which are biologically important. Upon entering the cell via fatty acid transporters, they are rapidly converted by acyl-CoA synthetases to fatty acyl-CoA thioesters (FA-CoA), which are substrates for lipid synthesis, elongation, desaturation, β-oxidation and protein acylation reactions [10]. Upon entering the cell, they are desaturated by Δ6-desaturase, and then elongated by microsomal enzymes, producing highly unsaturated, long-chained fatty acids.

The desaturation process of each of the fatty acids causes the carbon chain to develop a curvature, where the hydrophobic ends bend inwards within the cell membrane [218]. It is the curvature of the fatty acid once incorporated into the plasma membrane phospholipid, which is said to increase the fluidity and functionality of the cell membrane [207, 219]. The most important long-chain fatty acid of the omega-6 series is AA (C20:4n-6), whereas EPA (C20:5n-3) and DHA (C22:6n-3) are the major omega-3 fatty acids (Figure 1.9) [10, 220]. The metabolism of the omega-6 and omega-3 fatty acid is intensely competitive, as both pathways employ the same set of enzymes for the desaturation and elongation processes [221].

Figure 1.9 *The chemical structure of EPA and DHA [31].*

Several studies have investigated the metabolism of omega-3 fatty acids in humans using labeled isotopes [222, 223]. These studies consistently show that ≈ 15-35% of dietary ALA is catabolised to carbon dioxide for energy, ≈ 8% is converted to EPA and < 4% to DHA [224, 225]. This fractional conversion tends to be more efficient in women compared to men, with up to 21% and 9% converted to EPA and DHA, respectively [226]. Nonetheless, doses of ALA up to 14g/day, provide linear increases in EPA, however plasma phospholipid DHA concentration does not increase

1.7.3 Omega-3 Fatty Acids and Eicosanoid Production

The essentiality of LA and ALA is that they are precursors for some of the more important highly unsaturated fatty acids (AA, DHA and EPA), which are necessary for the synthesis of the family of bioactive mediators known as the eicosanoids. The term "eicosanoids" is used as a collective name for molecules derived from twenty-carbon fatty acids. They are recognised as intercellular messengers travelling short distances to their target cells [227, 228]. The half life of eicosanoids varies. Thromboxanes and prostaglandins are relatively unstable with a half-life of 30 seconds and 3 minutes, respectively [228].

The metabolism of AA via the cyclooxygenase (COX) pathway generates the synthesis of the pro-inflammatory 2-series prostanoids, including prostaglandins (PGE_2 and PGI_2) and thromboxanes (TXA_2). The metabolism of AA via the lipoxygenase (LOX) pathway, generates the 4-series leukotrienes (LTB_4, LTC_4 and LTE_4) [229]. Alternatively, the metabolism of EPA and DHA generates an anti-inflammatory response by competitively inhibiting the production of 2-series eicosanoids and producing the less biologically active 3-series prostaglandins (PGE_3 and PGI_3) and thromboxanes (TXA_3) via the COX pathway and the 5-series leukotrienes (LTB_5, LTC_5 and LTE_5) via the LOX pathway (Figure 1.10).

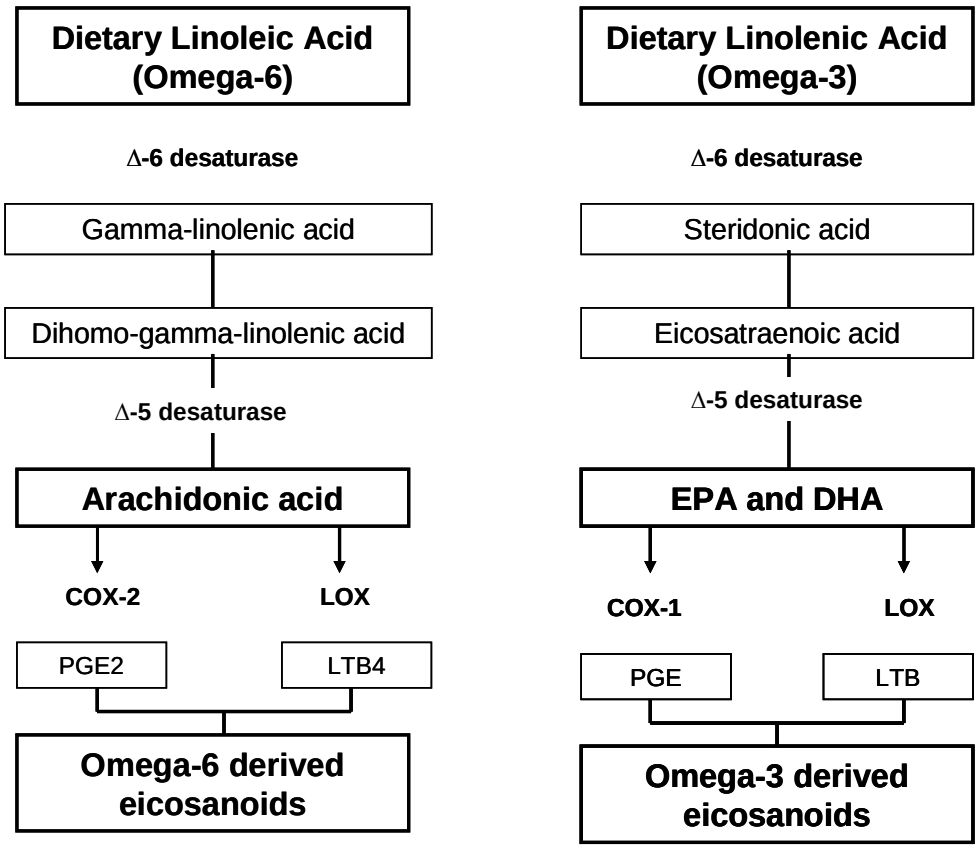

Figure 1.10 *The metabolic pathway of omega-6 and omega-3 fatty acids. The enzymes involved in the elongation and desaturation of the fatty acids are highly competitive [187, 200]. COX, cycloxygenase; LOX, lipoxygenase; PGE, prostaglandin; LTB, leukotriene.*

Inflammatory and immune cells cultivated from humans consuming a typical Western diet contain high amounts of LA and low proportions of EPA and DHA [230, 231]. Intakes of LA are approximately 10g/day, whilst intakes of AA are 50-100mg/day, predominantly coming from the elongation and desaturation of LA and AA [200].

1.7.4 Hypolipidemic Effect of Omega-3 Fatty Acids

Omega-3 fatty acids are pleiotropic molecules with a broad variety of biological actions including hypotriglyceridemic, anti-aggretory, anti-inflammatory and anti-arrhythmic [203]. Epidemiological and experimental evidence suggests that the consumption of omega-3 fatty acids are associated with a reduced risk of CVD, certain types of cancer, inflammatory disease, diabetes mellitus, multiple sclerosis and clinical depression [202]. These effects are mediated by alterations in circulating plasma lipids, eicosanoids, cytokines and physico-chemical properties in the membranes [232].

Supplementation with omega-3 fatty acid favorably modifies many adverse serum and tissue lipid alterations, the most consistent finding is a drastic reduction in fasting and postprandial serum triglycerides and FFA [233]. This has been observed with EPA and DHA alone [234] and with their combination in fish oil. Reduced VLDL-cholesterol production by the liver [235] largely results from: (1) decreased availability of FFA released from adipose stores, (2) suppression of lipogenic activity, (3) an increase in the activity of triglyceride-synthesising enzymes (DGAT and PAP), (4) the induction of genes involved in fatty acid oxidation and (5) an increase in phospholipid synthesis [236]. This regulation of gene expression proceeds through the inhibition of sterol regulatory element binding protein 1 (SREBP-1) and the activation of PPARα, which omega-3 fatty acids can interact [199]. Net production of apoB is also reduced. An increased lipolytic activity of lipoprotein lipase (LPL) in extra-hepatic tissues completes the hypotriglyceridemic effect.

As a consequence of the reduction of triglycerides and VLDL-cholesterol, HDL-cholesterol synthesis is indirectly affected. The cholesteryl ester transfer protein (CETP) plays a pivotal role in HDL-cholesterol metabolism, as evidenced by the CETP amino acid polymorphism [237]. This transfer protein enables cholesteryl esters to be transferred from HDL-cholesterol to VLDL- and LDL-cholesterol and the synthesis of triglyceride-rich HDL-cholesterol [238]. In the absence or reduction of circulating triglycerides, CETP is reduced thereby reducing the amount of triglycerides being transferred from VLDL- to HDL-cholesterol, which results in a modest increase in triglyceride-poor HDL-cholesterol and possibly apoAI concentration (Figure 1.11).

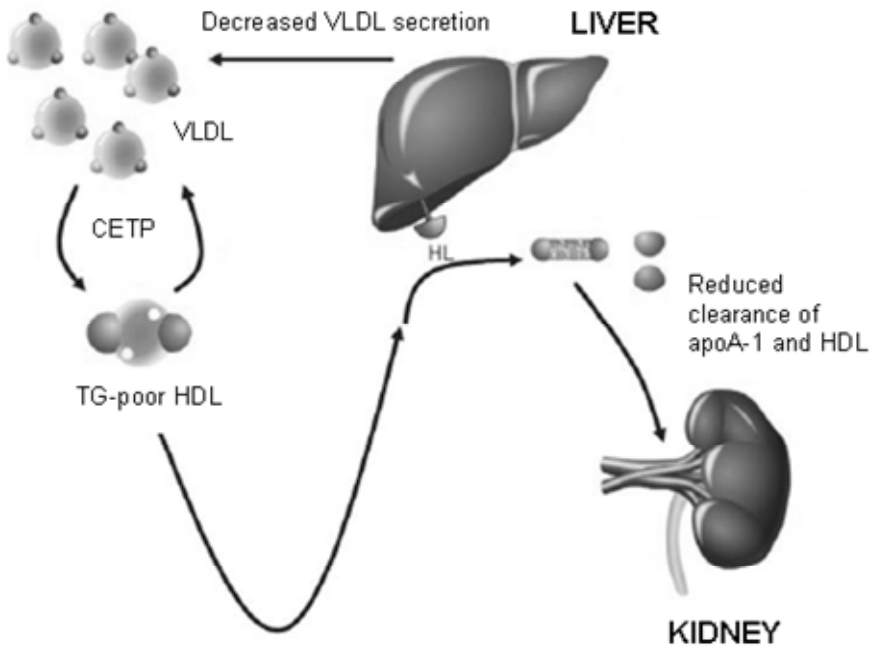

Figure 1.11 *Mechanisms of reducing HDL-cholesterol in a hypertriglyceridemic state with the use of omega-3 fatty acids (adapted from [25]). Cholesteryl ester transfer protein (CETP) mediates the exchange of cholesteryl esters (CE) and triglycerides (TG), producing TG-poor HDL-cholesterol. This reduces hepatic lipase (HL) activity and clearance of HDL-cholesterol. This is thought to be an important role in the elevation of HDL-cholesterol via the administration of dietary omega-3 fatty acids.*

Omega-3 fatty acids have contrasting effects on LDL-cholesterol, with a general tendency toward slightly increased LDL-cholesterol concentrations, however the size of the LDL molecule is also increased, which is thought to be less atherogenic [239]. The mechanism by which this occurs is largely unknown and many speculations have been made. It is thought that LDL synthesis rates are increased, rather than the receptors themselves, however, the potential associated cardiovascular risk is largely compensated for by the incorporation of omega-3 fatty acids into the surface phospholipids of small, dense lipoprotein particles, which change their structure to exhibit antioxidant properties [240].

Since omega-3 fatty acids are shown to have beneficial effects on other CVD risk factors such as, HDL-, VLDL-cholesterol, triglycerides, CETP, triglyceride-synthesising enzymes (DGAT and PAP), fatty acid oxidation, lipogenesis, blood pressure and inflammatory biomarkers, this may offset any potentially negative effects in changes in LDL-cholesterol. Moreover, the LDL particle size is increased following dietary supplementation with omega-3 fatty acids to reduce the atherogenetic properties of these particles.

It is largely agreed that omega-3 fatty acids reduce hepatic secretion of triglyceride-rich lipoproteins (VLDL & LDL) in the order of about 32% [241, 242]. These studies in part, firmly establish that this mechanism involves the reduction of triglyceride concentrations via the inhibition of VLDL secretion rates. Grimsgaard *et al* [243] reported on the effects of supplementation with highly purified EPA (3.8g/d) or DHA (3.6g/d) for seven weeks in healthy, non-smoking male volunteers. They found a reduction in plasma triglycerides that was at least as marked in the DHA group (26%) as in the EPA group (21%), in addition, HDL-cholesterol increased only in the DHA group [243]. These results provide convincing evidence that EPA and DHA are equally effective at reducing serum triglycerides, but that DHA may raise HDL-cholesterol as well as LDL particle size (i.e. both anti-atherogenic outcomes).

1.7.4.1 Omega-3 Fatty Acids in Hyperlipidemia

An important action of omega-3 fatty acids is that they could play a key role in the prevention and management of several diseases such as CHD, diabetes mellitus, insulin resistance, hypertension and above all, hyperlipidemia [244]. These effects are mediated by alterations in circulating plasma lipids, eicosanoids, cytokines and physico-chemical properties in the phospholipid membrane. When added to the diet, EPA and DHA present within fish oil can alter the membrane phospholipid composition of the cells, impact eicosanoid synthesis and action and regulate transcription factor activity and abundance [203]. Secondary prevention trials have shown omega-3 fatty acid supplementation, reduce premature mortality from coronary artery disease, reduce risk of impaired glucose tolerance and diabetes and have beneficial effects on thrombosis and arterial compliance. It has been shown that high-doses of omega-3 fatty acids reduce triglycerides and improve HDL-cholesterol [245, 246].

1.7.5 Omega-3 Fatty Acid Supplementation

Secondary prevention trials [247-249] have shown that omega-3 fatty acid supplementation reduces premature mortality from coronary artery disease, reduces risk of impaired glucose tolerance and diabetes and has beneficial effects on thrombosis and arterial compliance. It is well accepted that omega-3 fatty acids are hypotriglyceridemic and improve HDL-cholesterol concentrations (Table 1.8) [250, 251]. Epidemiological and experimental evidence suggests that the consumption of omega-3 fatty acids is associated with a reduced risk of CVD, certain types of cancer, inflammatory disease, multiple sclerosis and clinical depression [202, 252]. These effects are mediated by alterations in circulating plasma lipids, eicosanoids, cytokines and physico-chemical properties in the membrane [232].

Table 1.8 *Clinical trials of triglyceride (TG) reducing effects of omega-3 fatty acids in hyperlipidemic subjects [253].*

Study Design	N	Intervention	Weeks	EPA/DHA[1]	ΔTG	Ref.
Cross over	18	Fish oil	6	1.5/1.0	-30[2]	[254]
Cross over	14	Fish oil	8	1.8/1.2	-27[2]	[255]
Parallel	31	Fish oil	6	2.0/3.0	-30[2]	[256]
Parallel	26	DHA-rich	6	-/1.2	-24[2]	[257]
				-/2.5	-21[2]	
Parallel	55	EPA-rich	6	3.8/-	-18[2]	[258]
		DHA-rich		-/3.7	-20[2]	
Parallel	40	Fish oil	6	1.8/1.4	-33[2]	[259]
Parallel	5664	Fish oil	100	0.6/0.3	-4.8[2]	[260]
Cross over	10	Fish oil	3-5	5% total en	-66[2]	[261]

[1]g/day. [2]Significantly different from control. Ref, reference; en, energy.

1.7.5.1 The Role of ApoE in Response to Omega-3 Fatty Acids

Responsiveness of plasma lipids to omega-3 fatty acid supplementation can be highly variable, with diet-gene interactions thought to explain, in part, interindividual responses. Population studies have shown that plasma cholesterol, LDL-cholesterol and apoB concentrations are highest in individuals carrying the E4 allele, intermediate in individuals with the E3 alleles and lowest in those with the E2 allele [41, 262, 263]. In a study supplementing hyperlipidemic subjects with 6g/day fish oil (3g EPA+DHA), reductions in triglycerides were greatest in men and women with an apoE2 allele (32.5 ± 4.6%), who also tended to have a higher fasting HDL-cholesterol concentration [264].

The hypotriglyceridemic properties of omega-3 fatty acid supplementation may be counteracted by a potential shift in cholesterol profile in individuals with apoE4. In a study by Caslake *et al* [265] where participants consumed either 0.7g/day or 1.8g/day fish oil capsules for eight weeks, showed the greatest hypotriglyceridemic response in male carriers of apoE4 (15% and 23% for 0.7g and 1.8g, respectively). The selective affinity of the E4 protein isoforms for VLDL, in contrast with the E2 and E3 isoforms, may help explain the apparently greater hypotriglyceridemic potential of omega-3 fatty acids in persons with an E4 allele. In comparison to Minnihanes study [264], this study was only able to show significant differences in male participants with the apoE4 isoform, hence the differences in findings may be attributed to differences in the genders of the the two populations and warrants further investigation.

1.7.5.2 Omega-3 Fatty Acids and Statin Combinations

The combination of statins and fibrates are usually prescribed in patients with combined hyperlipidemia to reduce circulating pools of triglyceride and cholesterol concentration. While these combinations have been shown to reduce triglycerides by up to 50% [266, 267], there are reports of adverse events, such as liver and muscle toxicity [268-270]. When omega-3 fatty acids are administered as an adjunct to a statin therapy in hypercholesterolemic patients with persistent hypertriglyceridemia, benefits in lipid parameters (total-cholesterol and triglycerides) are enhanced [271, 272].

In the Combination of Prescription Omega-3 with Simvastatin study the efficacy of a highly purified omega-3 fatty acid (465mg EPA + 375mg DHA per 1g capsule) was trialed in 254 patients on a stable statin therapy with persistent hypertriglyceridemia [273]. Non HDL-cholesterol was significantly reduced after treatment with omega-3 fatty acids and statins, compared to the placebo group (9.9% vs. 2.2%). Also, reductions in triglycerides and VLDL-cholesterol had a median decrease of 30% and 28%, respectively. These findings have been found in a number of other studies [274, 275]. In another omega-3 (8g/day DHA-rich supplement) and statin combination study, a 27% reduction in triglycerides after three months was found [276].

Also a significant correlation between dose of fish oil and the extent of cholesterol reduction (r = -0.344, $P < 0.05$) was found, however no significant changes in VLDL-, IDL- and LDL-cholesterol were present. There is a significant amount of research to show that statin therapy in combination with omega-3 fatty acids significantly reduces lipids compared to a placebo, particularly triglycerides and lipoprotein sub-fractions [272, 277, 278].

This supports data showing that omega-3 fatty acid supplementation decreases coronary mortality in established CHD patients and reduces long-term risk of CVD [279].

1.8 Potential Health Benefits of Combination Therapies

In 2005 the NHFA along with the CSANZ [280], released a position statement on lipid management in an aim to establish a cost-effective risk factor management strategy for those individuals at a high risk of a cardiovascular event. The interventions outlined by the NHFA and CSANZ to achieve a 25% relative risk reduction in high risk groups, places emphasis on LDL-cholesterol, HDL-cholesterol and total-cholesterol [67]. The NCEP suggests that an aggressive LDL-cholesterol reduction could in large ameliorate the risk of diabetes, coronary artery disease and insulin sensitivity associated with combined hyperlipidemia [68].

In light of evidence surrounding the benefits of maintaining healthy plasma lipoprotein levels, body weight and blood pressure, the adoption of healthy practices such as physical activity and a balanced dietary intake contribute substantially to reducing the burden of disease.

The benefits of cholesterol-lowering treatments on the risk of CHD and mortality have been clearly established in large clinical trials involving the use of inhibitors of cholesterol synthesis (statins) [175, 281-283]. However, a monotherapy of statins is frequently insufficient for reducing plasma cholesterol concentration to target levels in practice, especially in hypercholesterolemic patients with increased intestinal absorption [179].

1.9 Phytosterols and Omega-3 Fatty Acids: A Treatment for Hyperlipidemia

In combination, phytosterols and omega-3 fatty acids may offer a more comprehensive strategy for not just optimising circulating lipid levels, but also to provide additional health benefits via anti-inflammatory,-hypertensive and -arrhythmic effects. There is considerable evidence to suggest that omega-3 fatty acid supplementation is involved in improved vascular function and lipoprotein profile, lower arterial pressure, diminished thrombogenicity and modification of atherogenic processes, all of which are important cardiovascular preventative actions. Additionally, inflammatory cytokines, adhesion molecules and vasoconstrictive eicosanoids have all shown to be beneficially reduced with the consumption of omega-3 fatty acids [284]. In the GISSI Prevenzione Trial, 11,324 patients were randomised to receive 1g/day of EPA plus a DHA supplement or placebo [285]. After 3.5 years there was a remarkable reduction in most cardiovascular endpoints and ultimately a 20% reduction in cardiovascular related deaths, nonfatal infarctions and nonfatal strokes [285]. This clinical trial provides evidence-based medicine which underpins the validity of epidemiological findings and physiological plausibility of omega-3 fatty acids being protective against CVD. Comparably, very little work has been done to investigate the cardioprotective effects of phytosterols in human nutrition, apart from its hypocholesterolemic effect. The findings from a study by Madsen *et al* [286] suggest that the consumption of 2g/day of phytosterols had no effect on apoA-1, Lp(a) and CRP, but significantly decreased apoB (4.6%), a strong predictor of coronary events, these findings are also supported by Ridker [287] and Yusuf [57].

The dose-response effect of phytosterols and omega-3 fatty acids is also an important issue to address when considering the efficacy of such nutritional therapies. A large majority of studies provide evidence of both phytosterols and omega-3 fatty acids to be effective lipid-lowering agents over the short term, however long-term studies are needed to establish sustained, continued efficacy of such functional ingredients. In a 6 week dose–response study by Clifton *et al* [288], responses from soybean oil, tall oil, and a mix of tall oil and rapeseed oil were tested in free-living subjects with hypercholesterolemia.

LDL-cholesterol was modestly reduced in a dose-response manner and after phytosterol withdrawal, plasma sterols decreased by 50% within 2 weeks, dependent upon baseline concentrations. In a long-term, open-label, cross-over study by Amundsen [149], children and their parents with familial hypercholesterolemia were asked to consume 20g/day of a phytosterol-enriched spread (1.76g phytosterols). Significant reductions in total lipid profile were achieved with a mean consumption of 1.2 and 1.5g/day of phytosterols in children and their parents, respectively. Sustained efficacy of cholesterol reduction and long-term compliance of a phytosterol-enriched spread were demonstrated in this study, with a 26-week follow-up. To date, this is the only open-label trial to investigate the long-term compliance of phytosterol-enriched spreads. Whether lipid lowering or health benefits of phytosterols and omega-3 fatty acids can be achieved in free-living populations, needs to be established.

Since the 1970's, phytosterols have been esterified to improve their functionality, solubility and incorporation into food products [289, 290]. The esterification of phytosterols to long-chain omega-3 fatty acids, does not impair their hypolipidemic property, yet enhances their solubility in oil by 10-fold [291]. The extent to which the fatty acid moiety of phytosterol esters influences cholesterol absorption has been examined in a limited number of animal and human trials. In a study by Rasmussen *et al* [292] phytosterols were esterified with fatty acids from soybean oil, beef tallow or purified stearic acid and tested in 35 male F_1B Syrian hamsters for 4-weeks (50g/kg phytosterol esters esterified with fatty acids). This study showed that beef tallow and stearic acid are more effective then soybean oil in reducing cholesterol absorption, liver cholesterol and plasma non HDL-cholesterol concentration in hamsters. In a similar study by Ewart *et al* [293], male hamsters were fed phytosterol esters esterified to fish oil, which showed a significant reduction in non HDL-cholesterol concentration compared to the control. Furthermore, in insulin-resistant rats fed the same phytosterol-fish oil esters, serum triglyceride and total-cholesterol levels were significantly reduced compared to the control rats [294]. Unfortunately, in the studies by Ewart [293] Russell [294] and Demonty [295], their study design did not allow for a phytosterol only or fish oil only group for comparison. Therefore, it is difficult to determine whether the combined phytosterol-fish oil esters were the most effective compared with phytosterol esters or fatty acid esters alone.

To date, phytosterols provided as food matrices, mostly as spreads or soft-gel capsules have been shown to affect LDL-cholesterol concentrations in both normo- and hyperlipidemic individuals [296-299]. In a cross-over design study by Jones *et al* [295, 300] 21 moderately overweight hypercholesterolemic subjects consumed an isoenergetic diet for 28 days, each supplemented with foods containing one of three phytosterol ester preparations (fish oil, sunola oil and olive oil). Each treatment contained 1.7 g/day phytosterols and the phytosterol-fish oil treatment contained 5.4g/day fish oil (EPA + DHA). The changes in total-cholesterol, LDL-cholesterol and HDL-cholesterol did not significantly differ between the three diets, however the phytosterol-fish oil ester significantly reduced fasting and postprandial triglyceride concentration. Furthermore, plasma TNF-α, IL-6, CRP, prostate specific antigen and fibrinogen concentrations were unaffected by the three phytosterol preparations.

These studies taken together, suggest that the phytosterol carrier may indeed play a role in the exerted effects of various phytosterol preparations and the interaction between phytosterols and various fatty acid esters needs to be further explored. To a large extent, reducing the inflammatory milieu to provide benefit among cardiovascular risk factors is yet to be fully understood in the context of hyperlipidemia and given the lack of evidence, it is difficult to speculate the exact mechanism by which phytosterols are anti-inflammatory.

1.10 Conclusion

It is well established that dietary supplementation with phytosterols reduce blood levels of total- and LDL-cholesterol, with no effects on HDL-cholesterol and triglyceride concentration. Phytosterols do not have any significant effects on plasma triglycerides, nor do they have anti-inflammatory or anti-aggregatory properties. Conversely, there is ample evidence in the literature to demonstrate the hypotriglyceridemic, as well as anti-inflammatory potential of omega-3 fatty acids.

Therefore, it is conceivable that a combined dietary therapy of phytosterols and omega-3 fatty acids may offer not only complementary lipid-lowering effects but may also provide improvements in inflammatory milieu, resulting in greater cardiovascular risk reduction in individuals with combined hyperlipidemia.

The aims of this thesis are:

1) To investigate the effect of combined supplementation with a phytosterol-enriched spread combined with EPA or DHA in people with combined hyperlipidemia.

2) To examine the effect of this dietary combination on major cardiovascular risk factors.
3) To determine whether changes in these risk factors affect overall cardiovascular risk.

4) To compare the response of EPA-rich fish oil or DHA-rich fish oil in combination with phytosterols, on plasma lipid and inflammatory profiles.

Chapter 2

GENERAL METHODS

2.1 Clinical Assessment

All data were collected at two clinical visits (baseline and post intervention). All measurements were made with participants dressed in light clothing and without shoes.

2.1.1 Medical Questionnaire

A general medical questionnaire was collected from all participants upon entry into the study [Appendix 1]. The questionnaire includes medical history, current medical conditions and current prescribed medications.

2.1.2 Anthropometry

Height (m) and weight (kg) were measured using a calibrated balance beam scale (PCS Measurement, NSW, Australia), calculated to the nearest 0.1. Height and weight were also expressed as body mass index (BMI) calculated as weight/height^2 (kg/m^2). BMI was categorized according to NHFA [64].

- Underweight = $\leq$ 18.5kg/m^2
- Healthy weight = 18.5 – 24.9kg/m^2
- Overweight = 25 – 29.9kg/m^2
- Obese = $\geq$ 30.0 kg/m^2

Waist circumference was measured at the mid-point between the lowest rib and the illiac crest. Hip measurement was taken at the fullest point of the hip, as viewed from the side. Waist to hip ratio was calculated as waist girth (cm) divided by hip girth (cm) [301, 302]. A healthy waist circumference was considered to be $\leq$ 94cm for males and $\leq$ 80cm for women.

2.1.3 Bioelectrical Impedance Analysis

Single frequency bioelectrical impedance apparatus (BIA) was performed using a tetrapolar Maltron Bioscan 916 (Maltron International, Essex, UK) with the emission of a low electrical current (50 kHz). BIA measurements were taken in the morning after a ≥10 hour fast and participants were asked to refrain from physical exertion and alcohol consumption 24-hours prior to testing. Subjects were studied in the supine position, without shoes and socks. The upper and lower limbs were arranged at an angle of approximately 30^{o} from the midline. Electrodes were placed on the left hand (centrally directly below the third knuckle of the middle finger and on the crease of the wrist) and left foot (centrally directly where the second and third toe meet the foot and at the crease of the ankle in line with the shin bone) after the areas were cleaned with alcohol wipes. Body composition was then recorded. Body fat mass (FM) and fat free mass (FFM) were recorded as percentage of total body mass.

BIA is a non-invasive method of measuring body composition and fluid changes. Several prediction equations have been derived using linear regression, in order to estimate body composition from BIA measurements of tissue hydration and integrity, including resistance (R) and reactance (Xc) [303]. R is the restriction to the flow of the electrical current through the body, which primarily relates to the amount of water present in tissues and Xc is the resistive effect produced by the tissue interfaces and cell membranes [303]. Part of the electrical current is stored by cell membranes, creating a phase shift, which is quantified as a phase angle (PA). In conclusion, PA differs between gender, decreases with age and has a positive association with physical activity [304].

2.1.4 Blood Pressure

Blood pressure (BP) and pulse were measured using an automated BP monitor (Microlife BP 3AD1-A, Heerbrugg, Switzerland), validated by the British Hypertension Society and compliant with the Association of the Advancement of Medical Instrumentation International Protocol (pressure ± 3mmHg; pulse ± 5%).

This method of BP measurement is also compliant with the "gold" standard for instantaneous BP measurement by the intra-arterial technique [305].

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were taken in the supported left arm of the rested (5 minutes), seated participant. Cuff size adjustment was based on arm circumference. SBP was recorded at the first appearance of Korotkoff sounds and DBP recorded at the disappearance (phase V) of Korotkoff sounds. SBP and DPB were based on the average of two separate measurements (5 minutes apart) taken by the investigating researcher. A healthy BP reading was considered to be (SBP (mmHg)/DBP (mmHg)) < 130/85mmHg for adults < 65 years and < 140/90mmHg for adults ≥ 65 years in accordance with the NHFA [64].

2.1.5 Cardiovascular Disease Risk Analysis

Ten year risk of coronary artery disease was measured using the NCEP-ATPIII [Appendix 2] [306, 307]. This risk factor sum model is an adaptation of the Framingham Study risk equations based on ten year risk of hard cardiac points, including coronary artery disease and myocardial infarction in patients without diabetes mellitus or clinically evident CVD. The prediction equation has taken the form of gender-specific equations, using continuous variables such as age, total-cholesterol level (mmol/L), HDL-cholesterol level (mmol/L) and SBP (mm Hg) and smoking as a dichotomous variable (yes/no). The model is age-adjusted for cholesterol and smoking status and corrects for treatment of blood pressure. A risk factor weighting approach is assigned to each variable and when totaled, correspond to estimates of absolute ten year risk % [308]. A high cardiovascular risk is considered > 15%, an increased risk is considered 10-15% and a low risk is considered < 10%.

2.2 Dietary Analysis

Participants did not receive additional dietary counseling and were asked to maintain their normal dietary and exercise patterns throughout the study period.

2.2.1 Dietary Recall (24-hour)

At baseline and post intervention, the previous day's food intake was collected using a 24-hour food recall [Appendix 3]. This procedure was kept informal, using a semi-structured, three-phase protocol to enhance standardization and completeness (Figure 2.1) [309]. The initial food consumption list included brief accounts of foods from the previous day's intake. Open question techniques were then used to gain information on food brands and portion sizes and additions made to items eaten. The third phase of the interview involved a review of all items on the recall form, providing an opportunity to correct details. The questionnaire contained seven meal times and participants were asked "if they considered this to be a typical day's food consumption?"

The 24-hour food recalls were entered into a food consumption database system (FoodWorks, Xyris®, QLD, Australia) by the same researcher, which was cross checked by a colleague. All data was entered into an Australian Standards database and a Fatty Acids database for analysis. Possible changes in dietary intake were registered by comparing the difference of macro and micronutrient food intakes entered at baseline and post intervention on the Australian Standards database, which references the Food Statndard Australia New Zealand (FSANZ) NUTTAB 2006 food composition tables [310]. The Fatty Acids database was used to estimate dietary fatty acid intake only [311].

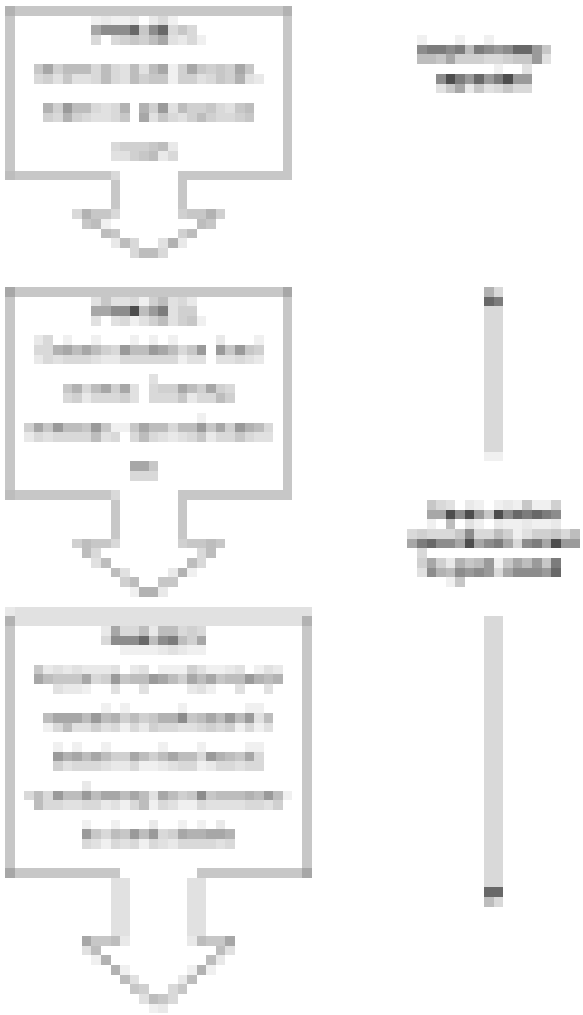

Figure 2.1 *Three-phase 24-hour food recall technique (adapted from [309]).*

2.3 Biochemical Analysis

Blood collection: Fasting (> 10 hours) venous blood samples were collected from participants at baseline and post intervention. Blood was collected into lithium heparin tubes (Becton Dickinson, Chicago, USA) for glucose analysis and sodium heparin tubes for plasma total-cholesterol, HDL-cholesterol and triglyceride analysis. Blood collected into ethylenediaminetetraacetic acid (EDTA) coated tubes were used for further analysis.

Plasma preparation: Blood collected into EDTA coated tubes were prepared by centrifuging (Heraeus Biofuge Stratos) for ten minutes x 3000 g at 4^{o}C. Aliquots of plasma, buffy coat and red blood cells were collected and stored at -80^{o}C until further analysis.

2.3.1 Plasma Lipid Analysis

Plasma total-cholesterol, HDL-cholesterol, triglycerides and glucose were measured by automated methods on a VP auto-analyzer, using standardized reagents (Hunter New England Area Health Services, Newcastle, NSW, Australia). LDL-cholesterol concentration was calculated using the Friedwald equation (*total-cholesterol – HDL-cholesterol – (triglycerides/2.2)*) [312].

2.3.2 Fatty Acid Determination

Plasma fatty acid concentration was determined using the method established by Lepage and Roy [313]. 2mL of a methonal/toluene mixture (4:1*v/v*), containing C19:0 (0.02mg/mL) was added to 200µL of plasma. Fatty acids were methylated by adding 200µL acetyl chloride drop wise while vortexing and heating to 100^{o}C for one hour in a bench top heating block. After cooling, the reaction was stopped by adding 5mL 6% K_2CO_3. The sample was centrifuged at 3000rpm at 4^{o}C for ten minutes to facilitate separation of the layers. The upper toluene layer was used for gas chromatorgraphy (GC) analysis of the fatty acid methyl esters, using a 30m x 0.25mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J & W Scientific, Folsom, CA). Both injector and detector port temperatures were set at 250^{o}C. The oven temperature was 170^{o}C for two minutes, increased 10^{o}C/minute to 190^{0}C, held for one minute, then increased to 3^{o}C/minute up to 220^{o}C and maintained to give a total run time of 30 minutes. A split ratio of 10:1 and an injection volume of 5µL were used. The chromatograph was equipped with a flame ionization detector, autosampler and auto detector.

Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture of fatty acid methyl esters and quantified using a Hewlett Packard 6890 Series Gas Chromatograph with Chemstations Version A.04.02 for GC analysis.

2.3.3 Interleukin-6 (IL-6) Analysis

Plasma samples were analysed using a 5.5 hour solid-phase high-sensitivity IL-6 quantitative enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) [314]. Absorbance values were measured using a plate reader at a wavelength of 490nm, and the raw data corrected for recovery. The mean minimal detection limit of this assay is 0.039pg/mL, with an intra and inter assay coefficient of variance (CV) of < 9%.

Kit Validation: The following procedure was undertaken to validate the ability of the kit to measure pure IL-6. To each well 100µL of assay diluent was added. In addition, another 100µL of sample/standard IL-6 was added to each cell, at various known concentrations. The ELISA kit was used to quantify the concentration of IL-6 in each cell. Each concentration was analysed in duplicate. The concentrations obtained using the ELISA kit were plotted against the known concentration of standard IL-6 that had been added. There was a high correlation between the known concentrations and the concentrations determined using the kit ($R^2 = 0.996$) (Figure 2.2).

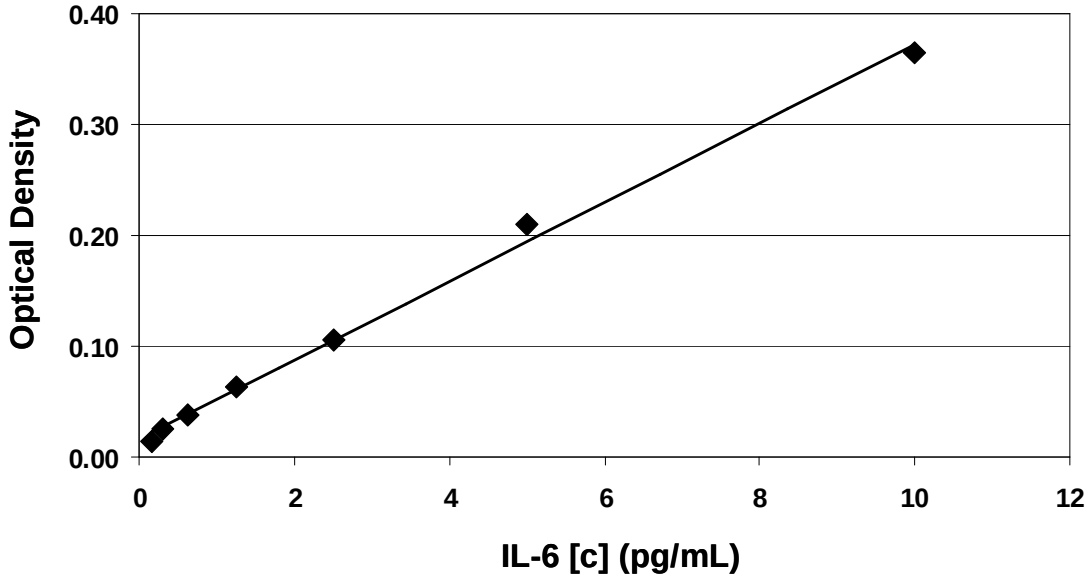

Figure 2.2 *Validation of IL-6 enzyme linked immunosorbent assay kit.*

2.3.4 Tumor Necrosis Factor Alpha (TNF-α) Analysis

Plasma samples were analysed using a 6.5 hour solid-phase high-sensitivity TNF-α quantitative ELISA kit (R&D Systems, Minneapolis, MN, USA)[314]. Absorbance values were measured using a plate reader at a wave length of 490nm, and the raw data corrected for recovery. The mean detection limit of this assay is 0.106pg/mL with an intra and inter assay CV of < 9%.

Kit Validation: In order to validate the ability of the kit to measure pure TNF-α, the following validation protocol was employed. To each well, 50µL of assay diluent (buffered protein base) was added, in addition to 200µL of sample/standard TNF-α at various known concentrations. The ELISA kit was used to quantify the concentration of TNF-α well. Each concentration was analysed in duplicate. The concentrations obtained using the kits were plotted against the known concentration of standard TNF-α. There was a high correlation between the known concentrations and the concentrations determined using the kit ($R^2 = 0.998$) (Figure 2.3).

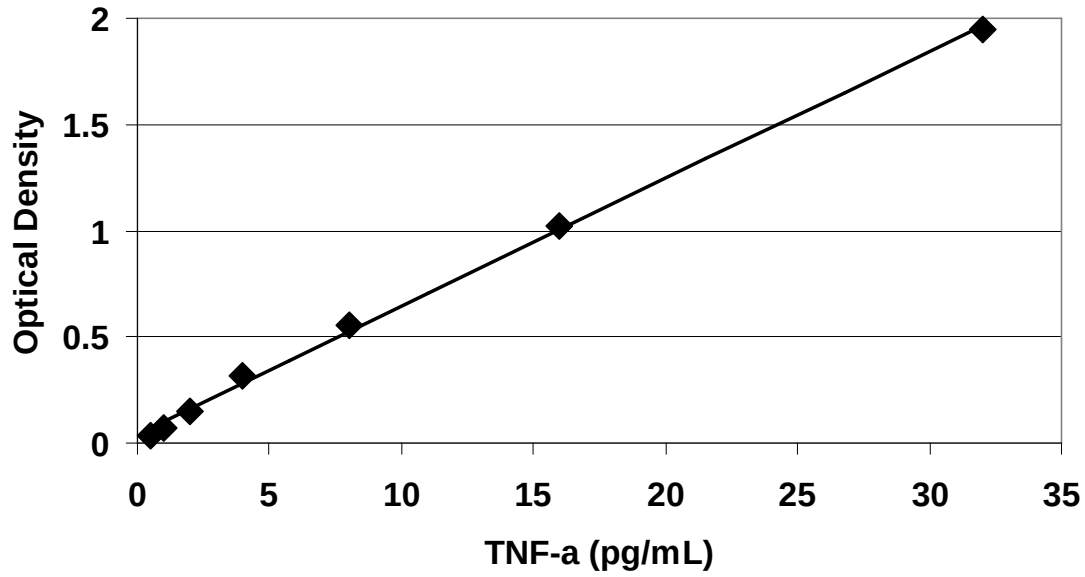

Figure 2.3 *Validation of TNF-α enzyme linked immunosorbent assay kit.*

2.3.5 Leukotriene B_4 (LTB_4) Analysis

Plasma samples were analysed with a LTB_4 ELIZA (Cayman Chemical, Ann Arbor, MI, USA) [314]. Absorbance values were measured using a plate reader at a wavelength of 412nm, and the raw data was corrected for recovery. The antiserum used in this assay has 100% cross-reactivity with LTB_4 and < 0.01% for each of LTC_4, LTD_4 and LTE_4. The mean detection limit of this assay is 13pg/mL, and has an intra and inter assay CV of <6%.

Kit Validation: The following procedure was undertaken to validate the ability of the kit to measure pure LTB_4. To each cell, 50µL of sample/standard LTB_4 was added to each well, at various known concentrations. The ELIZA kit was used to measure the concentration of LTB_4 in each well. Each sample was analysed in duplicate. The concentrations obtained using the ELIZA kit were plotted against the known concentration of standard LTB_4 that had been added. There was a high correlation between the known concentration and the concentration determined using the kit (R^2 = 988) (Figure 2.4) [314].

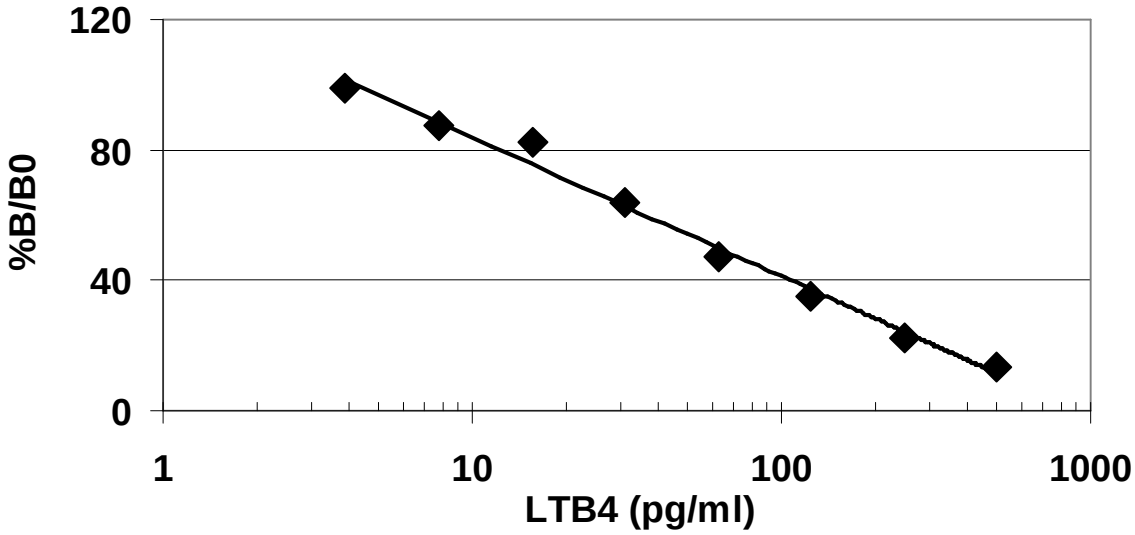

Figure 2.4 *Validation of LTB_4 enzyme linked immunoassay kit.*

2.3.6 C-Reactive Protein (CRP) Analysis

High sensitivity C-reactive protein (hs-CRP) was measured using an immuno-turbidimetric method (Hunter Area Pathology Service, Newcastle, NSW, Australia) from plasma samples collected into lithium heparin tubes (Becton Dickinson). The minimal detection concentration for this method is 0.5µg/mL.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago). Based on previous estimates of variance in plasma total-cholesterol and triglyceride concentration, sixty participants provide 80% power at $P < 0.05$ for detection of a 0.60 mmol/L (10%) and a 0.15 mmol/L (10%) change in total-cholesterol and triglyceride, respectivley. All data are presented as means ± SEM. Significance was set at P value < 0.05. Data was tested for normality. Baseline characteristics of each group were compared using one-way analysis of variance (ANOVA).

Changes from baseline were determined using paired samples t-test or non-parametric analysis (Wilcoxon signed-rank test). The effect of each treatment on the percentage change on the dependent variable between groups was explored using two-way ANOVA. The 95 percent confidence intervals (95% CI) for the differences in the changes between the groups are also given. Post hoc comparisons (Tukey's HSD) were used when significance was found. This analysis was also used to determine whether there was a significant main effect of each independent variable (omega-3 fatty acid or phytosterol) by testing for between-subject effects. Also, an interaction effect was tested between the two independent variables (omega-3 fatty acid x plant sterol) in their effect on the dependent variable.

2.5 Ethics

For all the studies described in this thesis, informed written consent was obtained from the subjects [Appendix 4]. Approval for all studies was obtained from the University of Newcastle Human Research Ethics Committee (HREC H-291-0906). The studies described in chapters three, four and five were undertaken at the University of Newcastle. These studies were also registered with the Australian and New Zealand Clinical Trials Registry (ANZCTR) (trial # 00081597).

Chapter 3

PHYTOSTEROLS AND DOCOSAHEXAENOIC ACID IN HYPERLIPIDEMIA

Excerpts From This Chapter Have Been Published:

- **Micallef, M.A** & Garg, M.L (2008). "Synergistic and lipid-lowering effects of (n-3) polyunsaturated fatty acids and phytosterols in hyperlipidemic men and women." The Journal of Nutrition, 138: 1086-90.

- **Micallef, M.A** & Garg, M.L (2009). "Anti-inflammatory and cardioprotective effects of n-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals." Atherosclerosis, 204: 476-482.

- **Micallef, M.A** & Garg, M.L (2009). "Cardioprotective effects of n-3PUFA and phytosterols in hyperlipidemia." Atherosclerosis Supplements, Vol 10, Is 2.

3.1 Introduction

Hyperlipidemia is associated with an increased risk in the development of CVD [57]. Dietary and/or pharmacological management of hyperlipidemia remains an effective strategy to reduce overall cardiovascular risk [63]. It is rather evident that lipid aberrations rarely occur in isolation and are highly interactive; therefore in order to effectively treat combined hyperlipidemia, the simultaneous management of all plasma lipids including lipoproteins are required.

Phytosterols are structurally analogous to cholesterol and have been shown to substantially reduce intestinal cholesterol absorption by 30-40 % [180, 315]. Phytosterol-enriched foods such as margarine spreads, have shown a 4-9 % and 10-15 % reduction in total and LDL-cholesterol respectively, with an average dosage of 2 g/day of phytosterols [147, 156]. Phytosterols however, do not affect plasma concentrations of other lipids; triglyceride or HDL-cholesterol. A recently renewed interest in phytosterols as a 'nutraceutical', stems from its efficacious lipid-lowering property and heightened by its commercial availability, now readily added to fat spreads, yoghurt and milk products [124, 316]. It has been shown that plant sterols lower plasma total and LDL-cholesterol, by competing with dietary and biliary cholesterol for intestinal absorption, but research on their effect in the inflammatory process, is scarce [110]. Given that patients with elevated inflammatory levels are at an increased risk of developing diabetes, hypertension and CVD, the effect of lipid-lowering foods on circulating inflammatory markers warrants investigation. The possibility that phytosterols may improve cardiovascular risk factors is speculative, but deserves further consideration.

The principle omega-3 fatty acids in marine oils: EPA and DHA, have been shown to possess a wide range of physiological effects, from alterations in circulating plasma lipids to eicosanoid and cytokine production [317]. Current literature supports reductions in triglycerides and improvements in circulating HDL-cholesterol, in response to high dosage (1-5g/day) omega-3 fatty acid supplementation [245, 246]. An increased consumption of omega-3 fatty acids results in increased proportions of those fatty acids in immune cell phospholipids, partly at the expense of AA [318].

The functional significance of this is that mediators formed from omega-3 fatty acids are deemed to inhibit early atherogenic events, by reducing cytokine-induced expression of pro-atherogenic/inflammatory proteins in the endothelium [319]. Epidemiological evidence shows the consumption of omega-3 fatty acids protects against CVD within Western populations [320] and has the capacity to amend several cardiovascular risk factors, including elevated blood pressure, plasma lipid profile, platelet aggregation and endothelial dysfunction [321].

Current evidence supports a central role for inflammation in all phases of the development of atherosclerosis [230]. Circulating markers of inflammation, such CRP, TNF-α, and some interleukins (IL-6, IL-1) correlate with propensity to develop cardiovascular events. The relationship between inflammation and plasma lipid aberrations, as seen in hyperlipidemia, is not as yet known. The tendency of cardiovascular risk factors to cluster in hyperlipidemic individuals, would suggest that many of these patients are likely to benefit from interventions targeted at multiple risk factors.

The present study was conducted to evaluate the lipid-lowering and cardio protective effects as measured by improvements in plasma lipid profile, blood pressure, body composition, markers of systemic inflammation and overall CVD risk, subsequent to combined supplementation with omega-3 fatty acids and phytosterols in hyperlipidemic individuals.

3.2 Methods

3.2.1 Study Population

Participants with established combined hyperlipidemia (total-cholesterol >6.0mmol/L and triglyceride >1.5mmol/L) were recruited from the general community of Newcastle, Australia from advertisements in local papers, the university, and interviews on local radio stations and through the Hunter Medical Research Institute (HMRI) volunteer database.

The primary selection criteria included: (1) plasma total-cholesterol concentration ≥ 6.0mmol/L (231mg/dL), (2) triglyceride concentration ≥ 1.5mmol/l (132mg/dL), (3) aged between 35 and 70 years, (4) no previous cardiovascular events, diabetes mellitus, chronic inflammatory disease, untreated hypertension (≥ 140/95mm Hg) or liver/renal disease, (5) not taking anti-inflammatory or lipid-lowering medication, consuming a phytosterol-enriched spread and/or fish oil supplements and, (6) no strong aversion or any known allergies/intolerances to the foods involved in the study. On the basis of these criteria, sixty (male n=27 and female n=33) individuals were enrolled and completed the trial. All participants had a usual weekly consumption of no more than two fish meals per week and maintained their habitual diet throughout the study.

3.2.2 Study Design

This study was a randomised, single-blind, placebo-controlled, 2 x 2 factorial intervention trial with four parallel groups. The study period was three consecutive weeks. Participants were randomised using permuted stratified block randomisation, controlled for gender. Supplement containers were labeled before commencing the trial with a blind code, so that neither principle researchers nor volunteers knew what capsules were being consumed.

Participants were randomised to receive either sunola oil alone (placebo group) or in combination with 25g/day of a spread containing 2g phytosterols per day (phytosterol group), or 4g/day omega-3 fatty acid supplements (EPA and DHA) alone (fish oil group) or in combination with 25g/day of a phytosterol-enriched spread (combination group) (Figure 3.1).

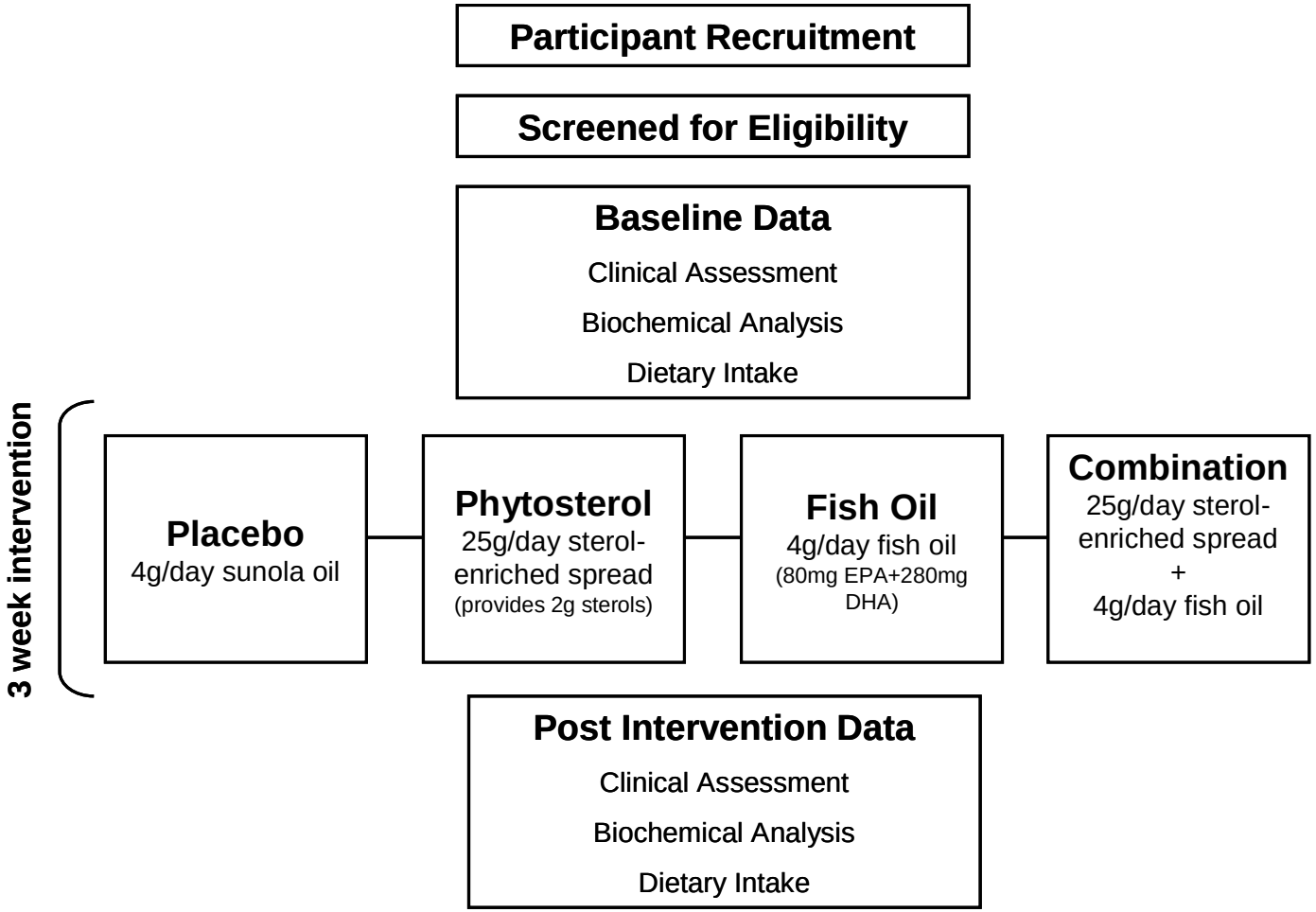

Figure 3.1 *Study design for intervention trial.*

Fifteen participants were recruited in each group. The omega-3 fatty acid supplements were provided as four 1g capsules (NuMega Ingredients Pty Ltd, QLD, Australia) providing 1.4g/day of omega-3 fatty acids (80mg EPA + 280mg DHA/capsule). The control oils were 1g capsules of sunola oil. Oils were flavoured with peppermint to disguise the taste of the oil and were encapsulated in opaque gelatin capsules. Participants were instructed to take four capsules with their main meals each day. The phytosterol spread was provided as individually measured tubs (25g each) to replace all habitual margarine/butter (Table 3.1). Participants were instructed to use the spread on bread/crackers, melted over vegetables, mixed into mashed potatoes/pumpkin, rice etc. and not to use it for high temperature cooking or frying. Unintentional intake of phytosterols from other food sources was not possible to monitor or restrict.

Table 3.1 *Energy and nutrient composition of the phytosterol-enriched spread*

	Amount/25g	**Amount/100g**
Energy, kJ	625	2500
Total Fat, g	16.7	67.0
SFA, g	4.2	16.5
MUFA, g	8.5	33.8
PUFA, g	4.0	16.2
Omega-3 fatty acids, g	1.2	5.2
ALA, g	1.2	5.2
Phytosterols, g	2.0	8.0

Compliance was monitored by regular telephone contact with participants, weighing of tubs and capsule count-back before and after the trial period, interviewing volunteers about their use of the spread at the end of the trial, evaluating their dietary records, and analysis of plasma fatty acid composition. Volunteers also recorded whether they had taken their supplements and/or consumed the spread on a diary card each day.

3.2.3 Clinical Assessment

A medical questionnaire [Appendix 1], anthropometric measurements, bioelectrical impedance and blood pressure were collected at baseline and post intervention. Cardiovascular risk [Appendix 2] was also calculated. The methods used are outlined in Chapter 2.1 Clinical Assessment.

3.2.4 Biochemical Assessment

Plasma concentrations of total-cholesterol, LDL-cholesterol, triglycerides, HDL-cholesterol and glucose were determined. Plasma fatty acid composition and plasma concentration of IL-6, TNF-α, LTB_4 and CRP were measured. The methods used are outlined in Chapter 2.2 Biochemical Analysis.

3.2.4.1 Adiponectin Analysis

Plasma samples were analysed with an adiponectin ELISA kit (Sapphire Bioscience, Redfern, NSW, Australia) [314]. Absorbance vales were measured using a plate reader at a wave length of 450nm. The minimal detection limit of the assay is 0.7µg/mL. This kit has been used to measure adiponectin concentration in human plasma samples.

Kit Validation: To validate this kit, 50µL of various known concentrations of adiponectin were added to each well. In addition, 50µL of quality control samples were added to wells (0.1 – 10µg/mL). The ELISA kit was used to quantify the concentration of adiponectin in each sample. Each sample was measured in duplicate at baseline and again at post intervention. The concentrations obtained from the kit were plotted against the known concentration of the standards. There was a high correlation between the known concentrations and the concentrations determined using the kit (R^2 =0.992) (Figure 3.2).

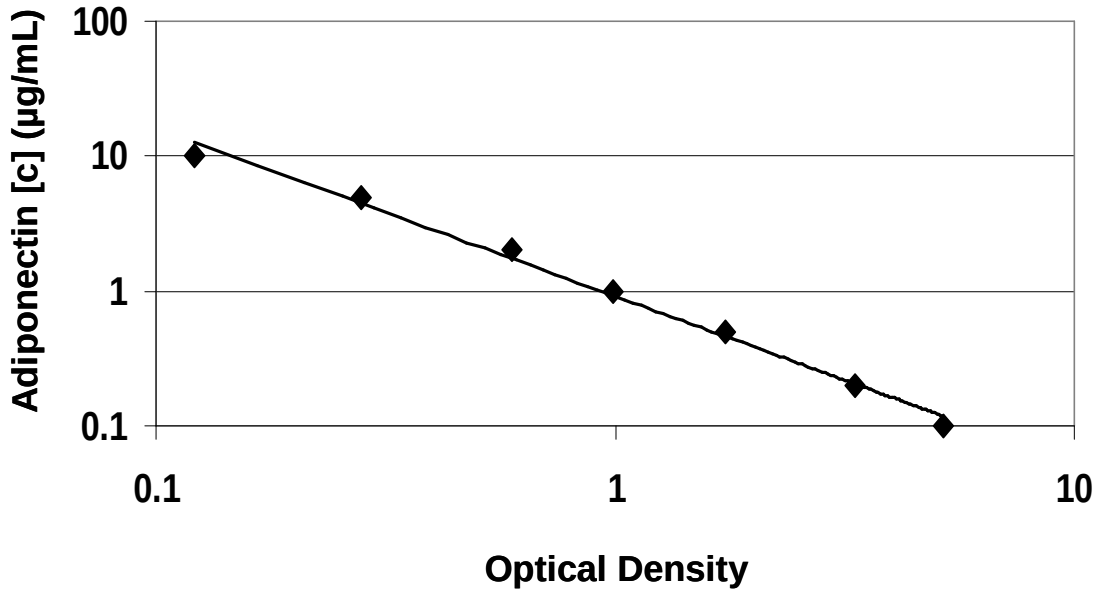

Figure 3.2 *Validation of adiponectin enzyme linked immunosorbent assay.*

3.2.4.2 Leptin Analysis

Plasma samples were analysed with a leptin ELIZA kit (Cayman Chemical, Ann Arbor, MI, USA) [314]. Absorbance values were measured using a plate reader at a wave length of 450nm and corrected for recovery. The minimal detection limit of the assay is 0.1ng/mL and has an inter and intra assay CV of < 9%. This kit has been used to measure leptin concentration in human plasma samples.

Kit Validation: To validate this kit, 100µL of various known concentrations of leptin (1 – 50ng/mL) were added to each well. In addition, 50µL of quality control samples were added to wells. The EIA kit was used to quantify the concentration of leptin in each sample. Each sample was measured in duplicate at baseline and again at post intervention. The concentrations obtained from the kit were plotted against the known concentration of the standards. There was a high correlation between the known concentrations and the concentrations determined using the kit (R^2 =0.991) (Figure 3.3).

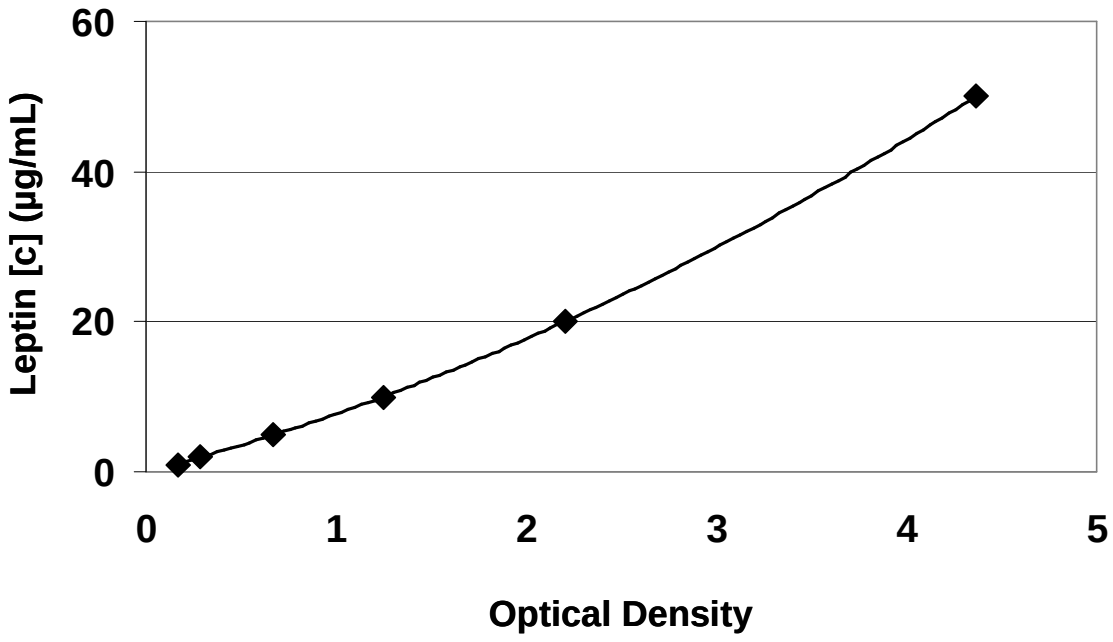

Figure 3.3 *Validation of leptin enzyme linked immunosorbent assay.*

3.2.5 Dietary Analysis

Dietary intake was assessed using the 24-hour food recall method, outlined in Chapter 2.2 Dietary Analysis [Appendix 3].

3.2.6 Statistical Analysis

Results were analysed using SPSS version 15.0 for windows. All data are presented as means ± SEM. The 95 percent confidence intervals (95% CI) for the differences in the changes between the groups are also given. Statistical significance was set at P value <0.05. Changes from baseline were determined using non-parametric analyses (Wilcoxon signed-rank test). The effect of treatment on the percentage change in variables between groups was determined using two-way between-group analysis of variance (ANOVA) with post-hoc comparisons (Tukey HSD).

A two-way ANOVA was used to determine whether there was a significant main effect for each independent variable by testing for between-subject effects, furthermore an interaction effect (phytosterol x omega-3 fatty acid) was tested between the two independent variables in their effect on the dependent variable. This method was also used to test for synergistic/complementary effects of the two independent variables.

3.3 Results

This study included sixty participants (male n = 27 and female n = 33), having a mean age (± SEM) of 55.4 ± 1.0 years and BMI of 26.9 ± 0.5kg/m^2. Participant demographics are reported in Table 3.2. Each of the four treatment groups were well matched and did not differ in general anthropometric measures at baseline (one-way ANOVA). Body composition did not differ among the groups at baseline, with an average body FM of 26.0 ± 1.2kg (33.3 ± 1.0%) and FFM of 50.8 ± 1.2kg (66.6 ± 1.0%).

Participants had an average systolic blood pressure of 134 ± 1.9mm Hg, a diastolic blood pressure of 82.1 ± 1.7mm Hg, and a heart rate of 69.4 ± 1.2bpm at baseline. Consistent with current literature, those treated with omega-3 fatty acids tended to exhibit reductions in systolic (3.1 ± 1.7 and 1.5 ± 1.3 %) and diastolic blood pressure (4.13 ± 1.2 and 3.56 ± 1.7 %) over the three weeks; however this failed to reach significance. Participant demographics, body composition and blood pressure did not significantly change throughout the intervention.

The capsules were well tolerated, with an overall compliance (as determined by capsule count-back) of 98 ± 0.5%. Mean spread consumption (analyzed by 24-hour food recall) was 23.5 ± 0.8g/d, mostly accompanied by bread/crackers. Dietary consumption of macronutrients and fatty acid intake was analysed using 24-hour food recalls at baseline and again at post intervention (Table 3.3).

Table 3.2 *Participant baseline demographics, body composition and blood pressure*[1]

	Placebo	Fish Oil (DHA-rich)	Phytosterol	Combination
N	15	15	15	15
Age (years)	54.9 ± 2.6	56.6 ± 2.0	57.6 ± 1.5	52.6 ± 2.2
Gender (M/F)	7/8	6/9	7/8	7/8
Body weight (kg)	78.3 ± 4.5	73.5 ± 4.0	79.1 ± 3.7	78.9 ± 3.4
Height (m)	1.7 ± 2.5	1.6 ± 0.02	1.7 ± 0.02	1.7 ± 0.02
BMI (kg/m^2)	26.6 ± 1.0	26.4 ± 1.3	27.4 ± 1.4	27.2 ± 0.9
Waist circumference (cm)	92.8 ± 3.3	88.2 ± 3.1	92.0 ± 3.3	95.6 ± 2.7
Hip circumference (cm)	98.7 ± 2.5	98.5 ± 2.8	101.4 ± 3.8	99.8 ± 2.5
Waist-to-hip ratio	0.9 ± 0.02	0.8 ± 0.02	0.9 ± 0.01	0.9 ± 0.01
Fat mass (%)	33.6 ± 1.5	32.3 ± 2.4	33.1 ± 2.5	34.2 ± 1.7
Fat free mass (%)	66.3 ± 1.5	67.6 ± 2.4	66.6 ± 2.5	65.8 ± 1.7
Systolic blood pressure (mm Hg)	137.3 ± 2.9	133.0 ± 4.0	134.2 ± 3.7	131.4 ± 3.8
Diastolic blood pressure (mm Hg)	79.6 ± 5.1	83.3 ± 2.6	83.4 ± 2.4	82.2 ± 2.5
Heart rate	71.0 ± 2.5	71.4 ± 2.3	68.6 ± 2.8	66.5 ± 2.3

[1]Values are reported as mean ± SEM. There were no significant differences between groups (one-way ANOVA).

Table 3.3 *Reported macronutrient and fatty acid intake, assessed by 24-hour food recall at baseline and post intervention*[1]

	Placebo		**Fish oil (DHA-rich)**		**Phytosterol**		**Combination**	
	BL	PI	BL	PI	BL	PI	BL	PI
Energy, kJ/day	8567 ± 652	8667 ± 892	6659 ± 566	7466 ± 1045	9256 ± 990	8932 ± 863	8929 ± 576	9256 ± 895
Protein, g/day (% en)	102 (21)	103 (20)	78.0 (20)	84.0 (20)	106 (21)	103 (20)	88.2 (17)	93.0 (18)
Carbohydrate, g/day (% en)	226 (44)	235 (42)	178 (47)	199 (46)	213 (39)	216 (40)	248 (48)	264 (46)
Sugar, g/day	72.1 ± 10.4	90.2 ± 14.9	78.5 ± 10.3	70.3 ± 10.9	77.7 ± 6.9	91.1 ± 17.2	87.8 ± 12.7	126 ± 26.3
Starch, g/day	108 ± 16.2	143 ± 23.1	81.0 ± 8.9	121 ± 28.9	133 ± 24.1	124 ± 15.1	119 ± 13.5	127 ± 14.9
Total Fat, g/day (% en)	72.8 (31)	73.1 (32)	56.4 (31)	60.4 (30)	96.9 (38)*	84.6 (36)	78.6 (32)	76.5 (33)
SFA, g/day (% fat)	28.0 (38)	27.5 (37)	19.9 (35)	22.4 (37)	38.0 (39)*	26.0 (30)	30.0 (38)	22.5 (29)
MUFA, g/day (% fat)	27.1 (37)	26.6 (36)	21.3 (37)	22.6 (37)	37.8 (39)*	26.3 (31)	29.9 (38)	22.7 (29)
PUFA, g/day (% fat)	12.5 (17)	18.4 (25)	8.6 (15)	8.5 (14)	12.8 (13)	10.7 (12)	11.4 (14)	9.9 (13)
Cholesterol, mg/day	276 ± 28	297 ± 40	188 ± 41	219 ± 30	370 ± 63*	277 ± 42	257 ± 41	279 ± 49
Fibre, g/day	22.9 ± 2.4	23.8 ± 3.7	19.3 ± 1.8	23.4 ± 3.6	25.5 ± 2.6	25.2 ± 3.0	27.9 ± 3.0	29.5 ± 7.0

[1] Values represented as mean ± SEM, unless other wise indicated. No significant within group differences between baseline and post intervention were found, using non-parametric analyses (Wilcoxon signed-rank test). A one-way ANOVA was used to explore difference between the four groups at baseline and post intervention. *$P<.05$ value is significantly different to fish oil group at baseline. BL, baseline; PI, post intervention (3-weeks); % en, percentage of total energy intake; % fat, percentage of total fat intake; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

The mean energy intake at baseline was 8352kJ/day, comprising of 19, 45 and 33% protein, carbohydrates and fat, respectively. Macro/micro nutrients and fatty acid intake between each of the groups was compared at baseline and post intervention. Dietary consumption of total fat, SFA, MUFA and cholesterol were significantly different between the phytosterol and fish oil group at baseline.

Plasma lipid concentrations did not differ among the groups at baseline (Table 3.4). Plasma lipid variables did not change in the placebo group. Plasma HDL-cholesterol increased (P=0.01) and triglycerides decreased from baseline in the fish oil group (P=0.004). Total-cholesterol (P=0.12), LDL-cholesterol (P=0.12) and triglycerides (P=0.07) tended to decrease from baseline in the phytosterol group. In the combination group, plasma total-cholesterol (P=0.001), LDL-cholesterol (P=0.002) and triglyceride (P=0.005) was lowered and HDL-cholesterol (P=0.04) was increased from baseline.

Table 3.4 *Participant baseline plasma lipid and glucose profile*[1]

		Fish Oil		
	Placebo	**(DHA-rich)**	**Phytosterol**	**Combination**
Cholesterol (mmol/L)	6.4 ± 0.2	6.6 ± 0.1	6.6 ± 0.1	6.9 ± 0.2
LDL-cholesterol (mmol/L)[2]	4.2 ± 0.2	4.4 ± 0.1	4.3 ± 0.3	4.6 ± 0.2
HDL-cholesterol (mmol/L)	1.3 ± 0.07	1.3 ± 0.1	1.3 ± 0.07	1.4 ± 0.08
Triglyceride (mmol/L)	1.5 ± 0.1	1.6 ± 0.2	1.4 ± 0.1	1.5 ± 0.1
Glucose	5.0 ± 0.1	5.2 ± 0.1	5.4 ± 0.8	4.9 ± 0.05

[1]Values are reported as mean ± SEM. [2] LDL-cholesterol calculated using the Friedewald equation [312]. There were no significant differences between groups (one-way ANOVA).

The treatment effects on the percentage change from baseline in each of the groups was explored. The percentage change in plasma total-cholesterol from baseline in the combination group differed from that in the placebo group (P=0.002, 95% CI: -20 to -3.5) and fish oil group (P<0.001, 95% CI: -24 to -8.5) (Figure 3.4).

Similarly, the percentage change in LDL-cholesterol in the combination group differed from that of the fish oil group (P<0.001, 95% CI: -29 to -7) (Figure 3.5). Also, the reduction in LDL-cholesterol in the phytosterol group was significantly different to the fish oil group (P=0.04, 95% CI:-22 to -0.3).

A decrease in plasma triglyceride concentration in the fish oil group (P=0.005) was greater than that in the placebo group. A reduction in triglyceride concentration in the combination group, proved to be significantly different to the placebo group (P=0.003) (Figure 3.6). Changes in HDL-cholesterol in the combination group was significantly different to the placebo group (P=0.05, 95% CI: -0.2 to 21) (Figure 3.7).

An exploration of possible interaction effects of phytosterols and omega-3 fatty aids was conducted using two-way ANOVA with post-hoc analysis. A main effect for phytosterols (P<0.001) and an interaction effect between omega-3 fatty aids and phytosterols (P=0.009) was found for plasma total-cholesterol. Similarly, there was a main effect for phytosterols (P=0.001) and an interaction effect for LDL-cholesterol (P=0.01).

Main effects for HDL-cholesterol and triglyceride was found for omega-3 fatty aids (P=0.011, P<0.001 and P=0.023, respectively).

Plasma fatty acid concentration did not differ among groups at baseline (Table 3.5). In both the fish oil and combination groups, both EPA (44.5%, P=0.002; 44.7%, P=0.002) and DHA (41.1%, P=0.02; 54.5%, P=0.001) increased from baseline, demonstrating compliance to the omega-3 fatty acid supplementation. The percentage change in plasma EPA concentration in the fish oil group differed from that of the placebo and phytosterol groups (P<0.001, 95% CI: 26 to 127; P=0.007, 95% CI: 13 to 115) (Figure 3.8). This was also the case with the combination group (P<0.001, 95% CI: 26 to 127; P=0.007, 95% CI: 13 to 115). Similarly, the percentage change in plasma DHA concentration in the fish oil and combination groups differed from that of the placebo (P<0.001 95% CI: 26 to 122; P<0.001, 95% CI: 64 to 160) and phytosterol groups (P=0.008, 95% CI: 12 to 107; P<0.001, 95% CI: 49 to 145). An interaction effect was assessed for EPA and DHA, with a significant main effect on plasma concentration of EPA (P<0.001) and DHA (P<0.001).

There were no significant differences in inflammatory markers between each of the four groups at baseline (Table 3.6). Percentage change from baseline was examined for each group (Figure 3.9 -14). Plasma inflammatory markers did not significantly change in the placebo group. In the fish oil group, hs-CRP and TNF-α were significantly decreased from baseline (2.1 ± 0.4 to 1.7 ± 0.3μg/mL, P = 0.02 and 3.0 ± 0.2 to 2.8 ± 0.2pg/mL, P=0.002, respectively). IL-6, LTB_4 and leptin decreased (1.7 ± 0.1 to 1.5 ± 0.1pg/mL, P=0.14; 27.2 ± 3.5 to 24.2pg/mL ± 3.3 P=0.30; and 19.1 ± 5.2 to 16.1 ± 4.6ng/mL, P=0.33, respectively), whilst adiponectin increased (1.5 ± 0.2 to 1.7 ± 0.2μg/ml; P = 0.39) in the fish oil group, however failed to reach significance. There were no significant changes in any of the inflammatory markers in the phytosterol group. Conversely, for the combination group there was a significant reduction in hs-CRP (2.9 ± 0.5 to 2.6 ± 0.5μg/mL, P=0.009), TNF-α (3.8 ± 0.5 to 3.4 ± 0.5pg/mL, P=0.02), IL-6 (1.7 ± 0.2 to 1.5 ± 0.2pg/mL, P=0.009), and LTB_4 (19.6 ± 3.4 to 16.2 ± 2.6pg/mL, P=0.01) and an increase in adiponectin (1.7 ± 0.2 to 1.9 ± 0.1μg/mL, P = 0.05). There was no change in leptin.

The change in hs-CRP in the fish oil and combination groups were significantly different to the placebo group (P=0.007, 95 % CI, 0.76 to 100.1 and P=0.004, 95% CI, -1.6 to 82.7, respectively). No other between-group differences were found. Post hoc analysis found a significant main effect of fish oil for hs-CRP and TNF-α (P<0.0001 and P=0.05, respectively).

Cardiovascular risk, as determined by the NCEP ATP-III model, showed no significant changes from baseline among the placebo (P=0.08), fish oil (P=0.10) and phytosterol (P=0.06) groups (Figure 3.15). However, a significant risk reduction (22.6 ± 5.1%, P=0.006) was found in the combination group. ANOVA failed to find any significant between-group differences in percentage change from baseline and post hoc analysis did not find any fish oil x phytosterol interactions for overall risk.

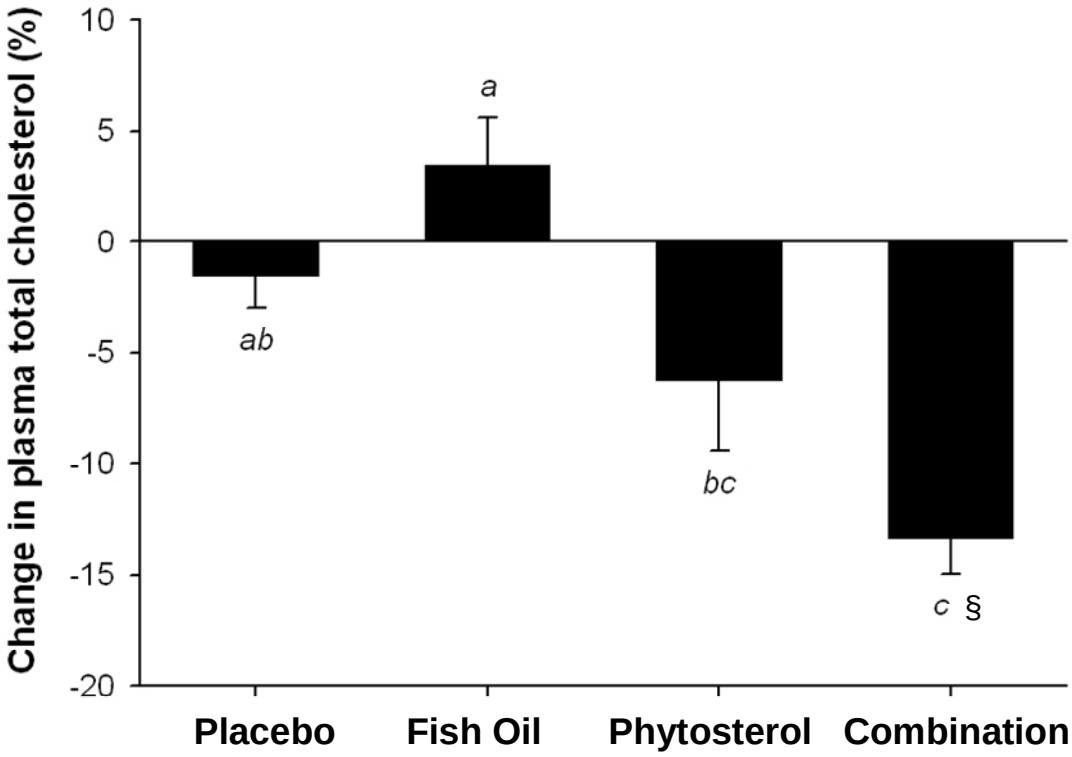

Figure 3.4 *Change (%) from baseline in plasma total-cholesterol concentration, in hyperlipidemic subjects consuming sunola oil (Placebo), 4g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ, P < 0.05.*

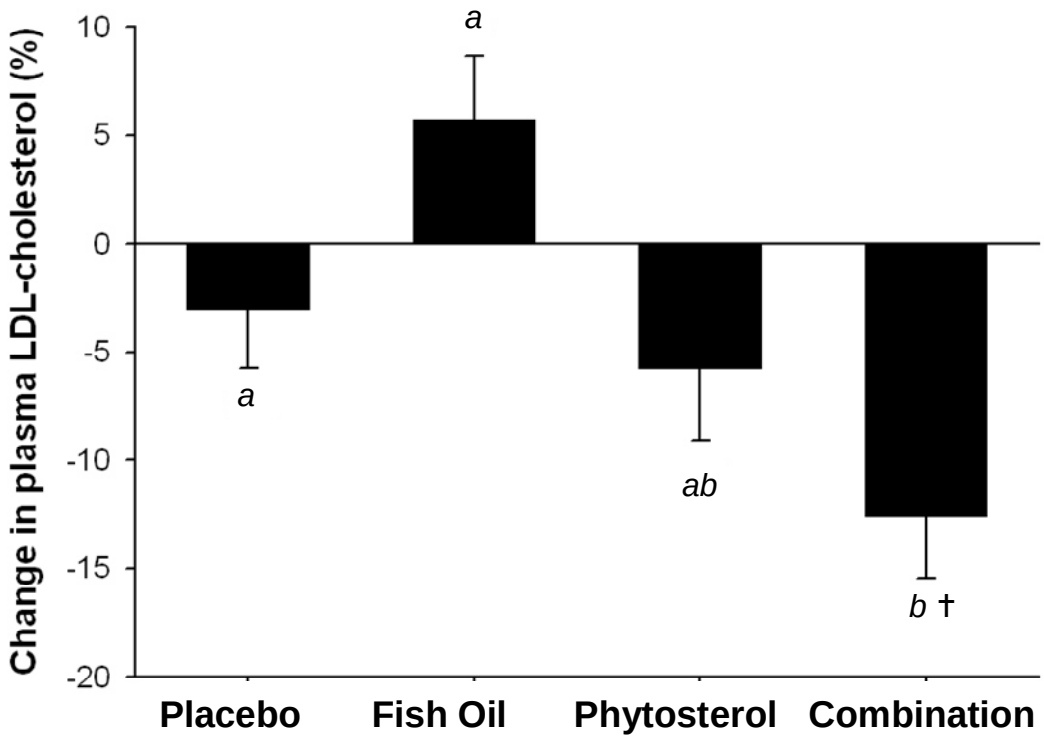

Figure 3.5 *Change (%) from baseline in plasma LDL-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), 4g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ,* $P < 0.05$.

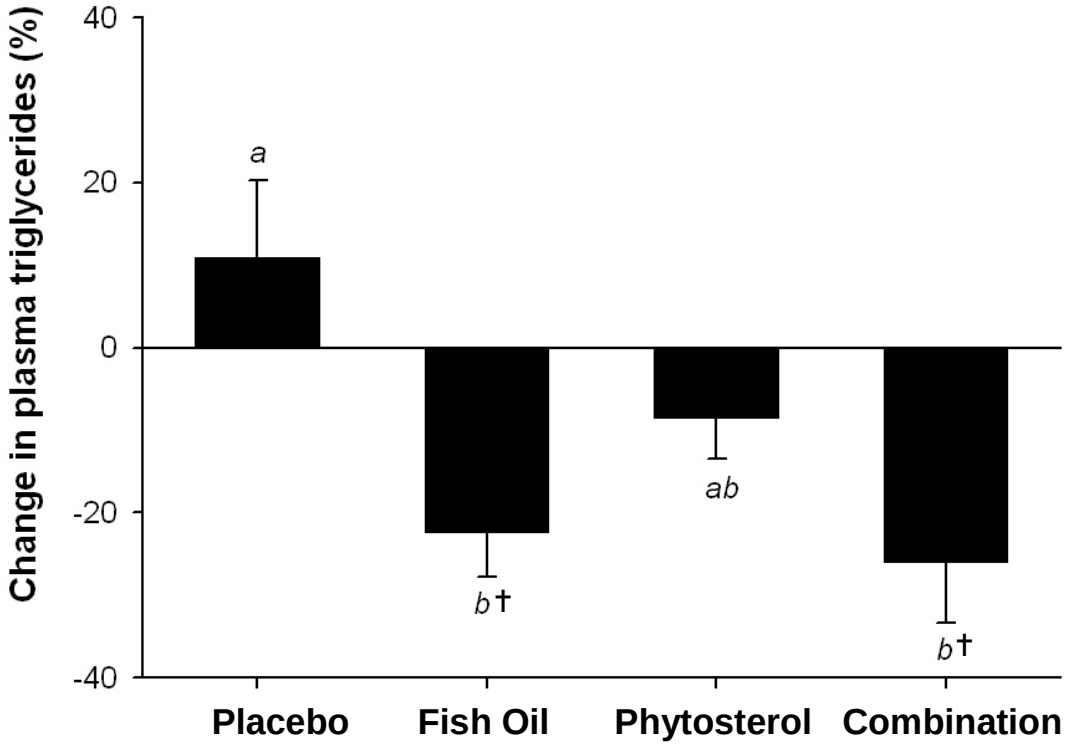

Figure 3.6 *Change (%) from baseline in plasma triglyceride, in hyperlipidemic subjects consuming sunola oil (Placebo), 4g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ,* $P < 0.05$.

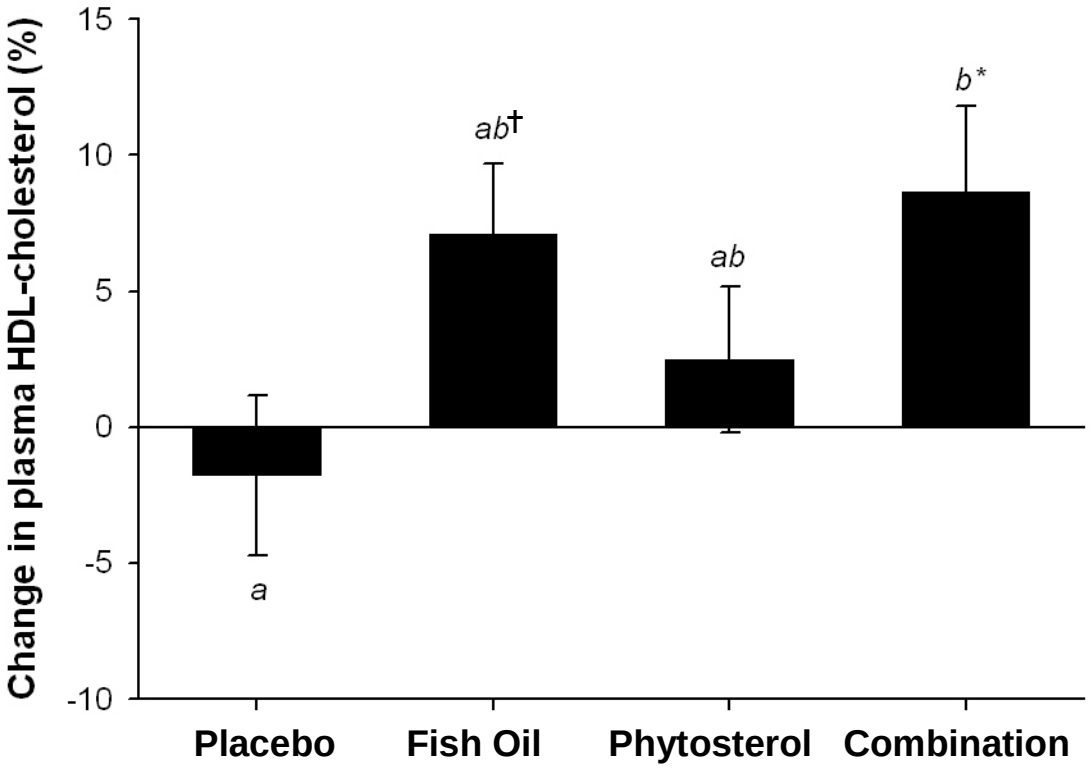

Figure 3.7 *Change (%) from baseline in plasma HDL-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), 4g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ, $P < 0.05$.*

Table 3.5 *Participant plasma fatty acid composition at baseline*[1]

	Placebo	Fish Oil (DHA-rich)	Phytosterol	Combination
C16:0	20.9 ± 0.5	19.6 ± 0.5	21.2 ± 0.6	21.5 ± 0.4
C18:0	6.6 ± 0.1	6.4 ± 0.1	6.9 ± 0.1	7.1 ± 0.1
C18:1n-9	21.0 ± 0.7	20.6 ± 0.7	21.8 ± 0.8	23.5 ± 0.7
C18:1n-7	2.4 ± 0.2	2.7 ± 0.2	3.6 ± 1.8	1.9 ± 0.1
C18:2n-6	25.7 ± 1.0	23.5 ± 1.1	25.7 ± 1.3	23.7 ± 1.0
C18:3n-3	0.43 ± 0.04	0.53 ± 0.03	0.54 ± 0.06	0.63 ± 0.03
C20:0	0.4 ± 0.09	0.3 ± 0.1	0.3 ± 0.02	0.3 ± 0.1
C20:3n-6	1.5 ± 0.06	1.5 ± 0.08	1.5 ± 0.07	1.8 ± 0.07
C20:4n-6	6.1 ± 0.2	6.1 ± 0.2	6.8 ± 0.3	6.4 ± 0.3
C20:5n-3 (EPA)	1.61 ± 0.22	1.21 ± 0.12	1.44 ± 0.15	1.23 ± 0.11
C22:0	0.7 ± 0.04	0.7 ± 0.05	0.8 ± 0.05	0.7 ± 0.03
C22:5n-3	0.7 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	0.6 ± 0.03
C22:6n-3 (DHA)	3.0 ± 0.2	2.2 ± 0.2	2.4 ± 0.2	2.2 ± 0.1
C24:0	0.7 ± 0.05	0.7 ± 0.05	0.8 ± 0.06	0.7 ± 0.04
C24:1n-9	1.5 ± 0.09	1.2 ± 0.08	1.2 ± 0.09	1.2 ± 0.05
SFA	30.3 ± 0.4	28.7 ± 0.4	31.2 ± 0.6	31.5 ± 0.3
MUFA	27,2 ± 0.8	26.8 ± 0.8	28.4 ± 0.8	30.5 ± 0.9
n-6 PUFA	34.1 ± 1.0	31.9 ± 1.1	34.8 ± 1.3	32.9 ± 1.1
n-3 PUFA	6.0 ± 0.4	6.9 ± 0.5	5.2 ± 0.3	4.8 ± 0.2

[1]Values are reported as mean (% of total fatty acids) ± SEM. There were no significant differences between groups (one-way ANOVA).

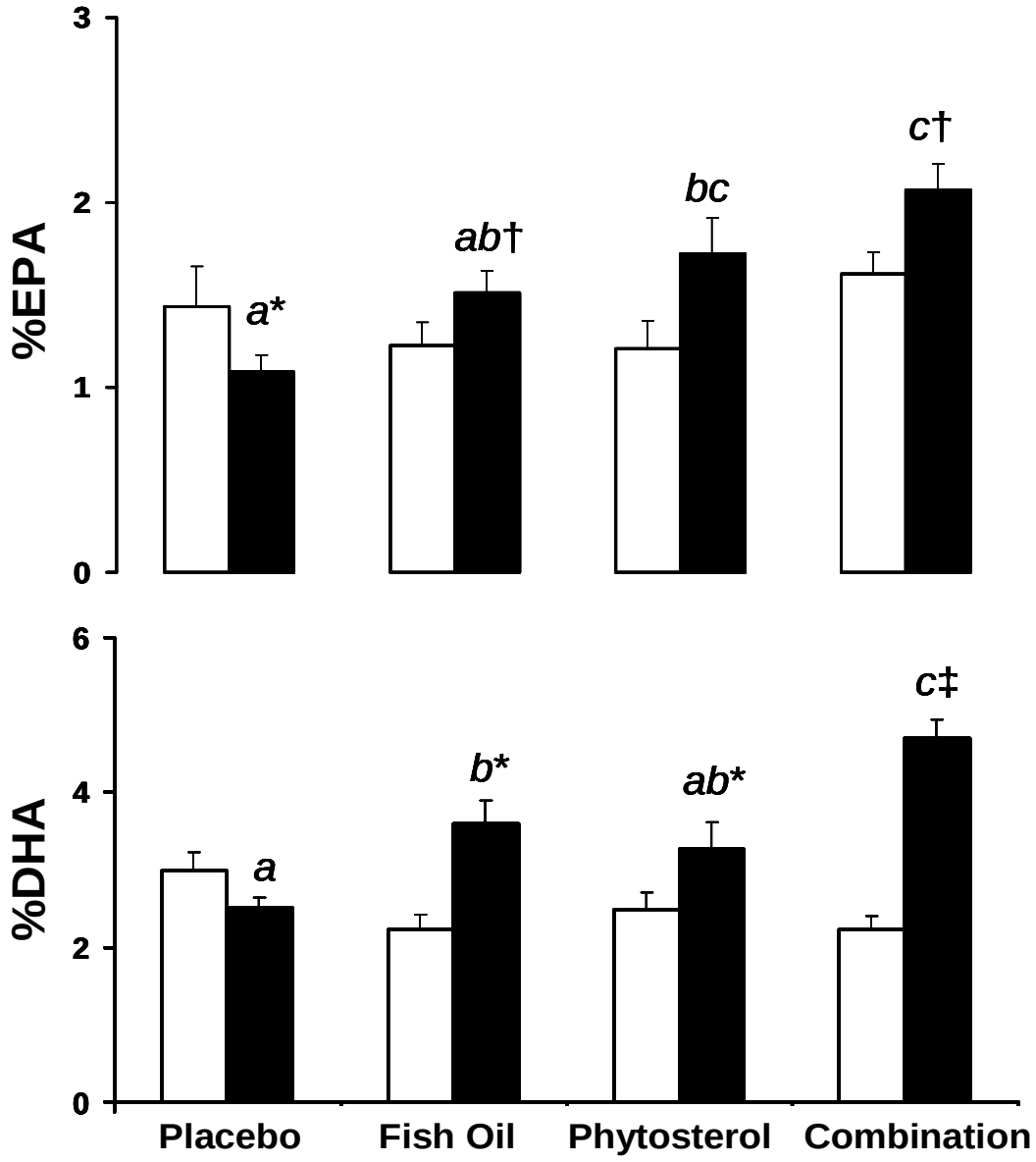

Figure 3.8 *Plasma concentration (% of total fatty acids) at baseline (■) and post intervention (□) of EPA and DHA in hyperlipidemic subjects consuming sunola oil (Placebo), 4g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Student-t test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$. Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ, $P < 0.05$.*

Table 3.6 *Participant concentration of plasma inflammatory markers at baseline*[1].

	Placebo	Fish Oil (DHA-rich)	Phytosterol	Combination
CRP (µg/mL)	3.2 ± 1.0	2.1 ± 0.4	2.9 ± 0.9	3.0 ± 0.5
IL-6 (pg/mL)	1.8 ± 0.4	1.7 ± 0.1	1.4 ± 0.2	1.7 ± 0.2
TNF-α (pg/mL)	3.8 ± 0.4	3.0 ± 0.2	2.6 ± 0.1	3.8 ± 0.5
LTB_4 (pg/mL)	20.4 ± 5.2	27.2 ± 3.5	24.9 ± 3.6	19.6 ± 3.4
Adiponectin (µg/mL)	13.4 ± 1.3	10.9 ± 1.9	11.2 ± 1.9	10.4 ± 1.2
Leptin (ng/mL)	15.1 ± 2.7	19.1 ± 5.2	14.4 ± 3.2	19.7 ± 4.8

[1]Values are reported as mean ± SEM. There were no significant differences between groups (one-way ANOVA).

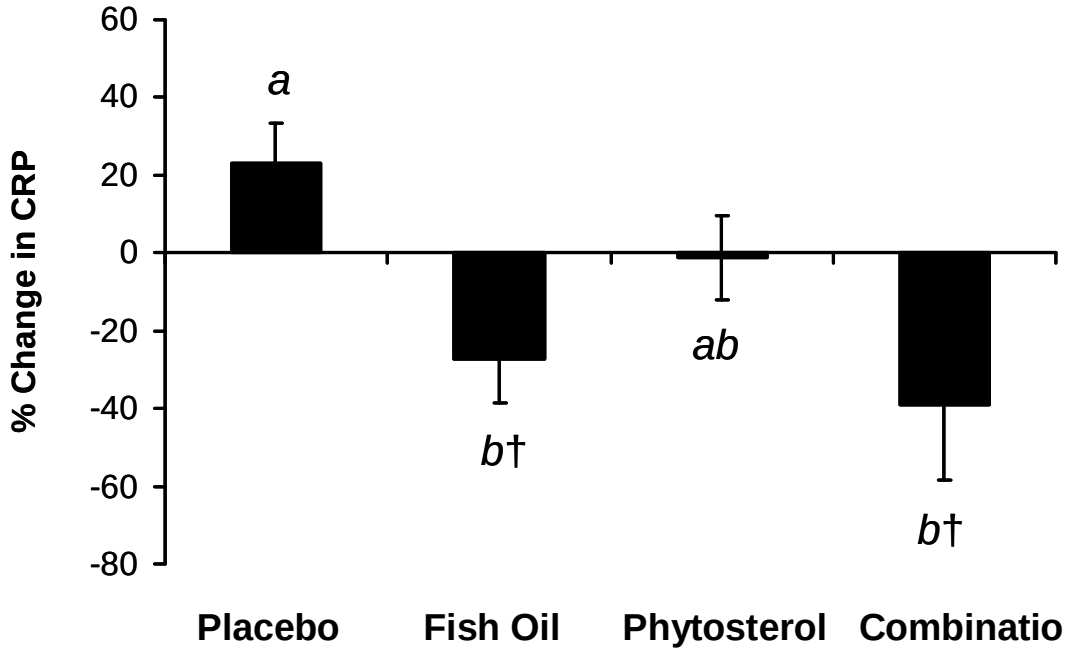

Figure 3.9. *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on high-sensitivity C-reactive protein (CRP). Bars represent change (%) from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, ‡ P <0.001 vs. baseline. Between-group differences were analysed using two-way ANOVA. Where significance was found, Tukey's HSD post hoc analysis was used for multiple comparisons. Bars without a common letter differ, P < 0.05.*

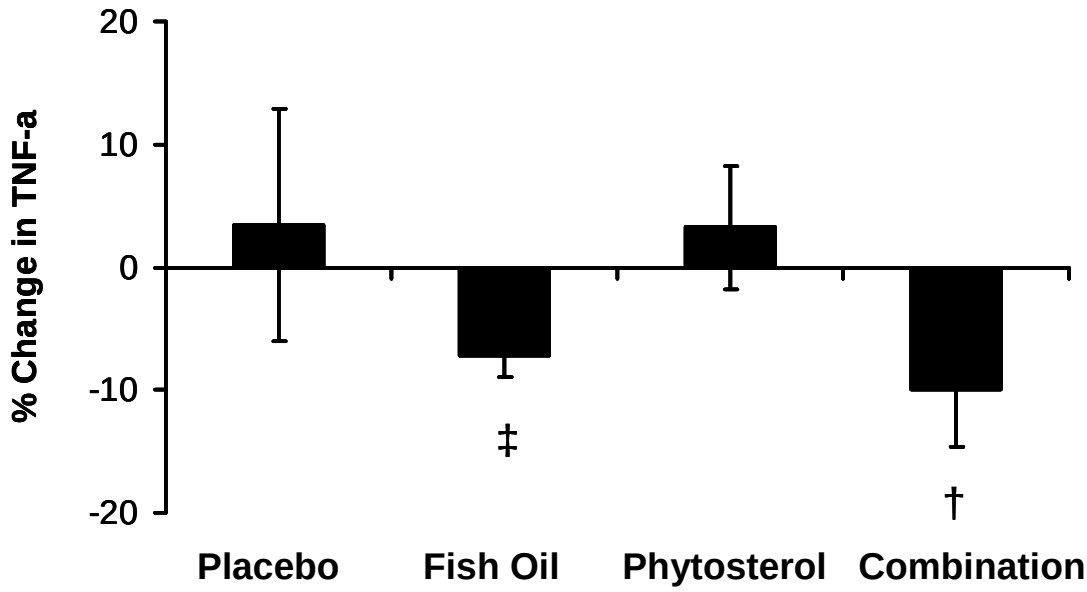

Figure 3.10. *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on tumor necrosis factor-alpha (TNF-a). Bars represent change (%) from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, ‡ P <0.001 vs. baseline. Between-group differences were analysed using two-way ANOVA. Where significance was found, Tukey's HSD post hoc analysis was used for multiple comparisons. Bars without a common letter differ, P < 0.05.*

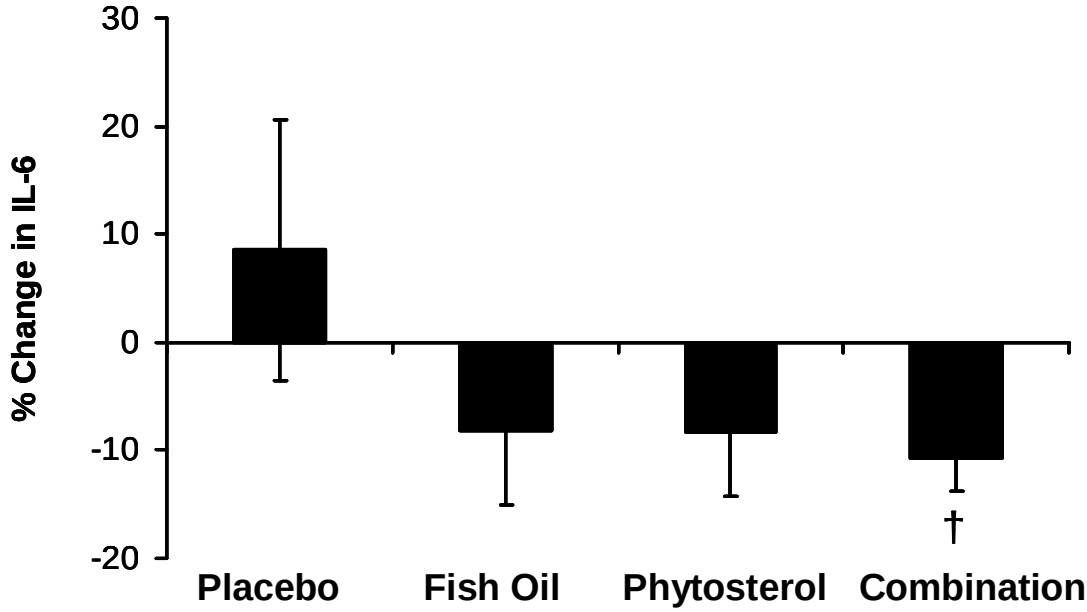

Figure 3.11 *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on interleukin-6 (IL-6). Bars represent change (%) from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, ‡ P <0.001 vs. baseline.*

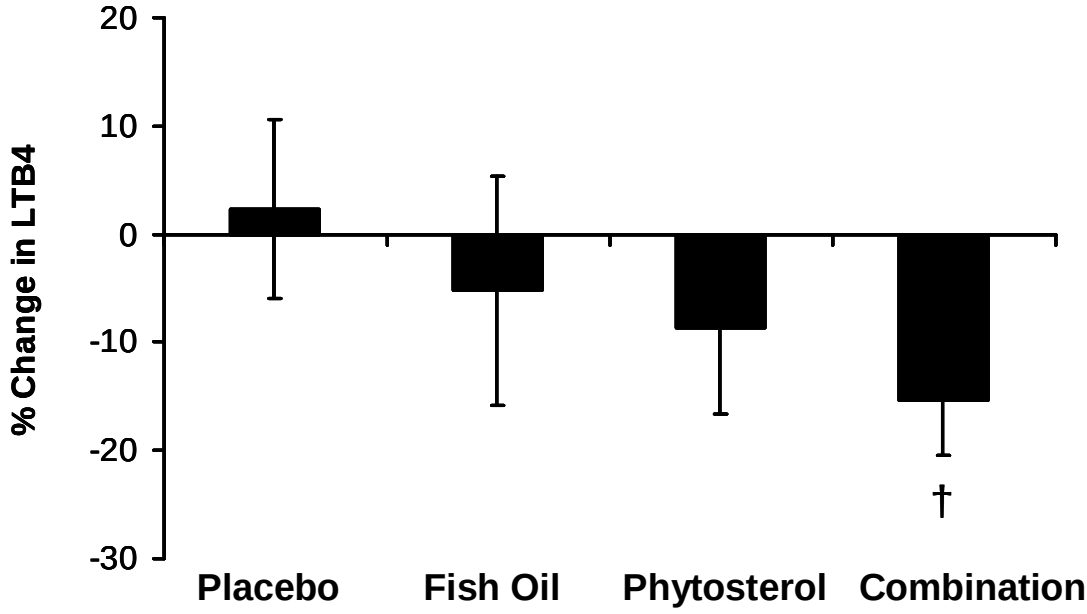

Figure 3.12 *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on leukotriene B_4 (LTB_4). Bars represent change (%) from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test, * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. baseline.*

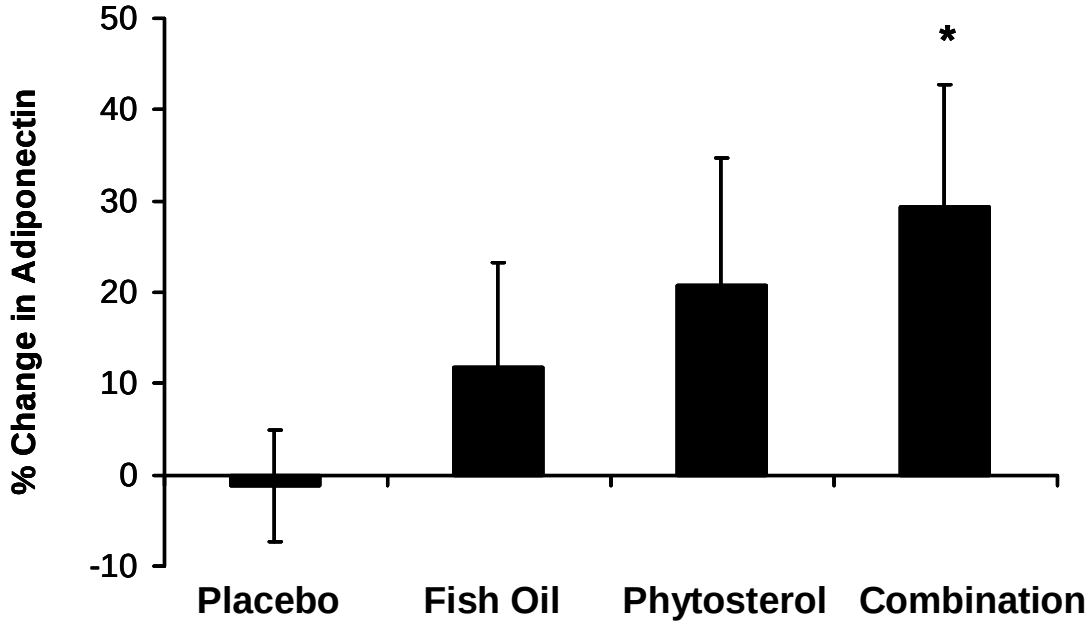

Figure 3.13 *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on adiponectin. Bars represent change (%) from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, ‡ P <0.001 vs. baseline.*

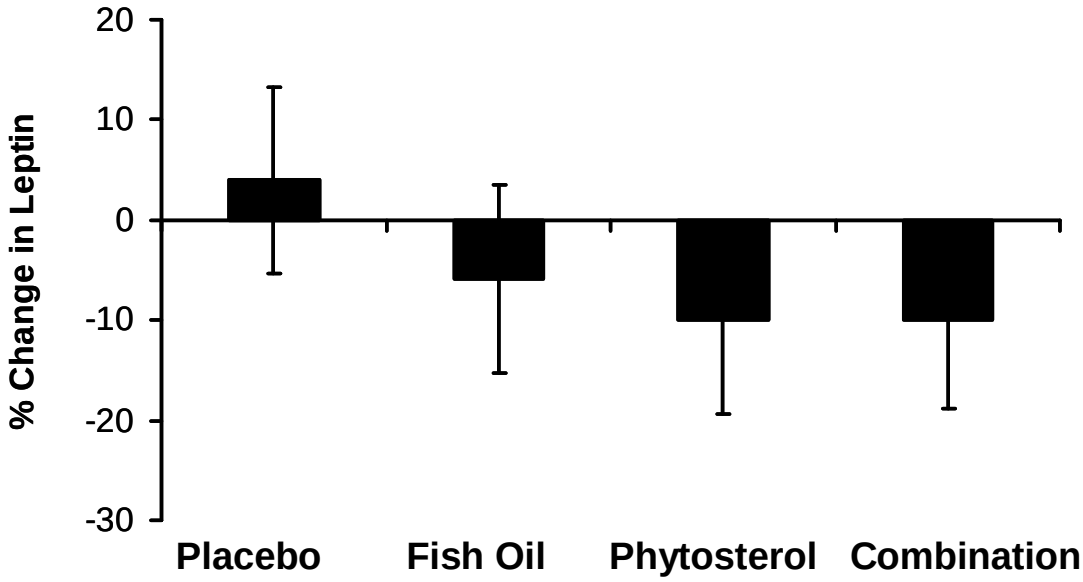

Figure 3.14 *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on leptin. Bars represent change (%) from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, ‡ P <0.001 vs. baseline.*

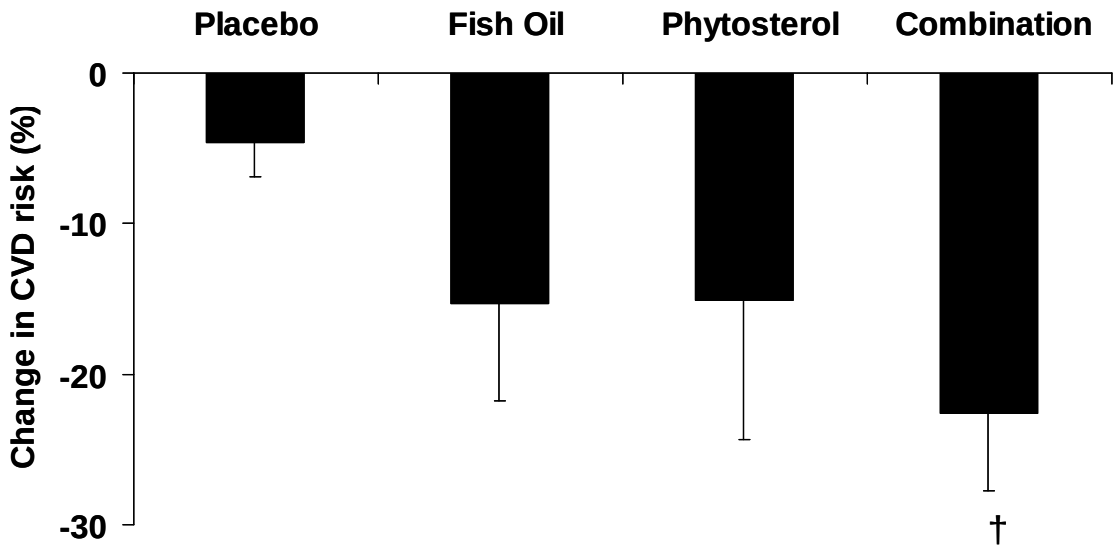

Figure 3.15 *Effect of dietary intervention with sunola oil (Placebo), 4g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 4g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on 10-year CVD risk. Bars represent percentage change from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, ‡ P <0.001 vs. baseline.*

3.4 Discussion

This is the first study to demonstrate the efficacy of concomitant supplementation with a phytosterol-enriched spread and omega-3 fatty acid supplementation, in individuals with hyperlipidemia. Our findings provide evidence of a synergistic reduction in total and LDL-cholesterol and complementary effects on triglycerides and HDL-cholesterol concentration, using concomitant dietary supplementation with phytosterols and omega-3 fatty acid. It would appear therefore, that the combined therapy is ideal for optimisation of plasma lipid fractions for maximum cardio-protection.

The reductions in total-cholesterol (6.2%) and LDL-cholesterol (5.7%) seen in the placebo group, are consistent with other studies of similar duration and intervention [90, 148, 167, 322, 323]. This study also showed that combining a phytosterol-enriched spread with omega-3 fatty acid supplements, significantly reduces total-cholesterol and LDL-cholesterol concentration by 13.3 and 12.5%, respectively. Moreover, the combined treatment provided a synergistic reduction in plasma total-cholesterol ($P=0.009$), which was greater than omega-3 fatty acid ($P<0.001$, 95 % CI: -24 to -8) or sunola oil alone ($P=0.002$, 95% CI: -20 to -3) as well as a synergistic reduction in LDL-cholesterol concentration ($P=0.011$), which was greater than omega-3 fatty acid alone ($P<0.001$, 95% CI: -29 to -7).

A number of studies have investigated the effect of phytosterols on lipids and lipoproteins in conjunction to a low fat diet [90, 139, 156, 160, 286, 324]. In the study by Cleghorn *et al* [90] moderately hypercholesterolemic participants following a reduced fat diet (< 30% energy from fat) and replaced their butter consumption with 25 g/d phytosterol-enriched spread (2g/d sterol esters) for four weeks. They showed an 8.9 and 12.3% reduction in total-cholesterol and LDL-cholesterol. Three studies [160, 286, 324] which used the NCEP Step 1 diet in combination with a phytosterol spread (range 1.6-2.3g/d), had a mean total and LDL-cholesterol reduction of 5.4 and 7.6%, respectively. It is conceivable that the impact of a reduced fat diet is in itself enough to attain appreciable reductions in plasma lipid profile.

Conversely, our combination group had a considerably greater reduction in total and LDL-cholesterol, whilst maintaining a habitual diet (33.3% energy from fat). Our combination treatment also differs from such studies, with important respect to the practicality of its application and minimal dietary constraint.

The mechanism by which phytosterols reduce total-cholesterol and LDL-cholesterol is not entirely understood, however their competition with and displacement of cholesterol from bile salts and micelles is well recognized [129, 137, 183, 315, 325, 326]. Given the discrepancies within omega-3 fatty acid studies, which generally show a small increase or no effect on plasma total and LDL-cholesterol [232, 240, 327], the mechanism by which the combination of phytosterols and omega-3 fatty acid elicit reductions in plasma total-cholesterol remains unclear. Omega-3 fatty acids can alter hepatic regulation of LDL-cholesterol, via alterations in the rate of LDL formation and receptor-dependent LDL uptake [37, 328, 329]. Nonetheless, the authors speculate that these findings may be associated with the combined effects of both phytosterols and omega-3 fatty acid in their higher affinity for micellular absorption and hence increases in cholesterol displacement and clearance. This mechanism is subject to further investigation. It is likely that the co-existence of omega-3 fatty acid and phytosterols in the gastrointestinal tract may cause greater micellar displacement of cholesterol, resulting in greater inhibition of cholesterol absorption. A small amount of dietary phytosterols do get absorbed into the circulation, therefore it is also likely that lipoprotein displacement (analogous to micellar competition) occurs, resulting in a greater reduction of total and LDL-cholesterol. Omega-3 fatty acid supplementation is associated with an increase in LDL particle size, while phytosterols are known to reduce LDL concentration. The net effect of the phytosterol treatment may be a reduction in both circulating LDL concentration and an increase in LDL particle size, contributing to a reduction in atherogenic risk.

Moreover, the hypotriglyceridemic and HDL raising effects of omega-3 fatty acid are sustained even in presence of phytosterol. In the current study, it was noted that optimisation of the cholesterol-lowering effect of phytosterols, by combining omega-3 fatty acid for triglyceride lowering and HDL-cholesterol improvements. A sizeable reduction in triglycerides (22.3 and 25.9%) and an increase in HDL-cholesterol (7.1 and 8.6%), was shown for the fish oil and combination groups, respectively.

The relatively short duration of this intervention trial is acknowledged, however, changes in lipids and lipoproteins in response to phytosterols and omega-3 fatty acid have been highly responsive. It is also acknowledged that the habitual consumption of dietary fats was significantly different between the phytosterol and combination groups. It is suggested that future studies may benefit by controlling the participants diets, hence controlling for this confounding factor. Considering the already established anti-inflammatory, anti-aggregatory and anti-hypertensive effects of omega-3 fatty acid, the synergistic and complementary cholesterol and triglyceride lowering effects along with the increases in HDL-cholesterol demonstrated in this study, makes the combined phytosterol and omega-3 fatty acid therapy an ideal alternative or adjunct treatment for maximum reduction in cardiovascular risk. It is likely that the effects on inflammatory mediators were additive, when omega-3 fatty acids were combined with phytosterols, however this is difficult to deduce, given the lack of studies that investigate this combination dietary modality. Perhaps functional foods containing the two active components; i.e. phytosterols and omega-3 fatty acid, in appropriate dose levels can be developed for the ease of consumption and improved compliance. Indeed, long-term safety and efficacy as well as compliance need to be demonstrated in a larger multi-centered trial to confirm our findings from this study.

We also demonstrated a 39% reduction in circulating hs-CRP, 10% reduction in TNF-α, 10.7% reduction in IL-6, 15.3% reduction in LTB_4 and a 29.5% increase in adiponectin levels, in response to combined omega-3 fatty acid and plant sterol supplementation for three weeks. Participant inflammatory markers were within the normal reference ranges [1, 330]. Evidence suggests that the omega-3 fatty acid DHA and to a lesser extent EPA, inhibit early atherogenic events by reducing cytokine expression of pro-inflammatory proteins in the endothelium, however it is not yet clear whether this effect is associated with individual or combined effects of the two fatty acids [331]. In a study by Thies *et al*. [332] comparing the effects of supplementation with fish oil (1g/d EPA + DHA), highly-purified DHA (720mg/d) and a placebo oil on lymphocyte proliferation in healthy subjects, it was shown that the fish oil combination significantly reduced lymphocyte proliferation, while DHA alone had no effect. Another study by Halvorsen *et al*. [333] compared the effects of 3.8g/d EPA and 3.6g/d DHA on phagocytic activity of monocytes, reporting no effect.

These findings may be taken to suggest that neither EPA nor DHA is responsible for an immunomodulatory effect alone, but required together for effective treatment. In a more recent study [334] on purified EPA and DHA (5g/d for 4 weeks), it was found that neither fatty acid affected monocyte nor neutrophil phagocytosis, however DHA did appear to decrease the production of some inflammatory cytokines. In our study, we supplemented hyperlipidemic participants with DHA-rich tuna oil, which was effective in reducing several inflammatory markers [335]. Interestingly, when plant sterols were combined with omega-3 fatty acid, a greater anti-inflammatory effect was seen, whereas plant sterols alone had no effect.

Although it is hard to speculate on the exact mechanism by which plant sterols are anti-inflammatory, it should be noted that plant sterol supplementation did have a significant main effect on DHA concentration (P=0.04). The plant sterol-enriched spread used in this study provided an additional 1.5g/d of omega-3 fatty acid ALA. The authors speculate that the conversion of ALA to DHA may be a possible mechanism by which the combination of the two supplements elicits anti-inflammatory effects. We acknowledge the relatively short duration of this intervention trial; however, changes in inflammatory markers in response to omega-3 fatty acid and plant sterol supplementation have been highly responsive.

Our current knowledge of the interaction between plant sterols and inflammatory markers is poor. In a recent study by Clifton *et al*.[288], hypercholesterolemic individuals were supplemented with 1.6g/d plant sterols for three weeks, followed by 3g/d plant sterols for three weeks, compared to a control. In this study, no significant changes in hs-CRP were found, although there was a modest reduction trend (P=0.07). In a similar study with hypercholesterolemic men, a four week supplementation period with a plant sterol-enriched spread (2.0g/d) did not significantly change CRP levels [336]. More recently, De Jong and colleagues [337] provided statin treatment patients with 2.5g/d of plant sterols as margarine for 16 weeks. No effects were found for soluble adhesion molecules, CRP or monocyte chemotactic protein-1 concentrations. These studies support our findings of a non-significant reduction in hs-CRP with 2g/d plant sterols for three weeks.

Conversely, a study by Devaraj *et al.* [338] a median reduction in hs-CRP of 12% (P=0.02) was found with 2g/d plant sterol supplementation provided as a reduced-calorie orange juice beverage, perhaps due to large inter-individual variations. The role of plant sterols in the inflammatory process is largely unknown; however given their interaction with the regulation of cholesterol influx and efflux at the phospholipid membrane, there is some degree of interaction with receptor activity. Potentially these could influence gene expression of COX-2 and IL-6, thereby having a direct impact on plasma markers of inflammation [339].

The relationship between plasma lipids and inflammatory cytokines, suggests that hyperlipidemia and enhanced inflammation are separate but interactive processes [340]. We have previously reported significant reductions in plasma total- and LDL-cholesterol and triglycerides and increases in HDL-cholesterol after three weeks of omega-3 fatty acid and plant sterol supplementation [341]. Further exploration of the data found no correlation between reductions in inflammatory markers and reductions in plasma lipid profile (data not shown), suggesting that the anti-inflammatory effects of combined omega-3 fatty acid and phytosterol. To a large extent, reducing the inflammatory milieu to provide benefit amongst cardiovascular risk factors is yet to be fully understood in the context of hyperlipidemia.

It is evident that markers of systemic inflammation such as hs-CRP, TNF-α, IL-6 and several adipokines are elevated in hyperlipidemic individuals [342]. The relevance of this remains in the setting of primary prevention, as many large-scale studies have shown baseline levels of such markers can independently predict future CVD events, many with adequate power after adjustment for all Framingham covariates [343].

An additional objective of this study was to attenuate a possible reduction in overall cardiovascular risk. A growing body of evidence indicates an association between the consumption of fish and relative risk of sudden death. In the US Physicians Health Study, an inverse relationship between plasma levels of omega-3 fatty acid and risk of sudden death in men without a history of CVD was found [344].

In the Diet and Reinfarction Trial (DART) a 29% reduction in all-cause mortality over 2 years in male myocardial infarction survivors was found, following an increase of oily fish intake (200 to 400g/week) [345]. In one of the largest randomised controlled trials, the GISSI-Prevention Study with 11,324 patients with pre-existing CHD randomised to 300mg vitamin E, 850mg omega-3 fatty acid, both or neither [285]. After 3.5 years, the omega-3 fatty acid alone group experienced a 15% reduction in the primary end point of death, nonfatal myocardial infarction and nonfatal stroke ($P < 0.02$), compared with the control.

In our study we showed a 22.6% (P=0.006) reduction in overall cardiovascular risk using the combination of omega-3 fatty acid and plant sterols. To date, this is the first study to investigate the combined cardioprotective effects of these two functional ingredients in hyperlipidemic individuals with no history of cardiovascular events. We speculate that the improvement in cardiovascular risk is primarily representative of subsequent improvements in plasma lipid profile, given we did not see significant changes in blood pressure [341], a risk factor usually affected by omega-3 fatty acid supplementation [346]. Dietary supplementation longer than three weeks in duration may be needed to significantly influence other cardiovascular risk factors such as blood pressure, which merits further investigation.

We have demonstrated that dietary intervention with combined omega-3 fatty acid and phytosterols significantly reduces the inflammatory markers hs-CRP, TNF-α, IL-6 and LTB_4 and significantly increases adiponectin in hyperlipidemic individuals. More importantly, the data demonstrates a 22.6% reduction in overall cardiovascular risk. Together, these findings suggest that reducing systemic inflammation in hyperlipidemia represents an important mechanism by which omega-3 fatty acid and phytosterols confer their putative cardiovascular benefits. Collectively, our data is supportive of the cardioprotective benefits of combined omega-3 fatty acid and plant sterol supplementation for hyperlipidemic individuals. It is the first study to demonstrate overall lipid-lowering benefits, reduce markers of systemic inflammation and reduce overall cardiovascular risk, using a non-pharmacological dietary approach. This makes the combined omega-3 fatty acid and plant sterol therapy an ideal alternative or adjunct to pharmacological treatment, for maximum cardio protection in high risk individuals.

Chapter 4

PHYTOSTEROLS AND EICOSAPENTAENOIC ACID IN HYPERLIPIDEMIA

- **Micallef, M.A** & Garg, M (2009). “Phytosterols and eicosapentaenoic acid reduces plasma lipids and inflammatory markers in hyperlipidemia.” (submitted).

4.1 Introduction

In light of the increasing prevalence and health consequences associated with CVD, there is an emerging need to identify treatments which alleviate risk factors associated with its development and progression. Modifiable cardiovascular risk factors such as hyperlipidemia, contribute to the underlying mechanisms of atherosclerotic disease, promoting endothelial dysfunction, oxidative stress and pro-inflammatory pathways [57, 63, 67, 347, 348].

Phytosterols are becoming increasingly popular for their hypocholesterolemic properties [110, 349]. The consumption of 2g per day reduces LDL-cholesterol by 10% on average, and more over has been shown to reduce plasma concentrations of inflammatory markers, such as IL-6, TNF-α and CRP [296, 350-352].

Fish oil rich in the omega-3 fatty acids (EPA and DHA) has been shown to have hypotriglyceridemic and immunomodulatory properties [232, 246, 353, 354]. EPA is one of the omega-3 fatty acids found in large quantities in fish oil and its preventative cardiovascular properties have been examined in many epidemiological and clinical studies. Most studies have found that an increased intake of fish and fish oil is related to a reduced risk of CVD, coronary artery disease, stroke, total mortality and sudden death [265, 295, 345, 355].

In combination, phytosterols and omega-3 fatty acids may offer a more comprehensive strategy for not just optimising circulating lipid levels, but also to provide additional health benefits via anti-inflammatory,-hypertensive and -arrhythmic effects in hyperlipidemic patients. We have recently shown that the combination of a phytosterol-enriched spread in addition to fish oil supplementation (DHA-rich) provides a synergistic and complementary lipid lowering and anti-inflammatory effect in hyperlipidemic adults. Given the continued interest of omega-3 fatty acids and phytosterols in the diet as food additives or functional ingredients, it is important to determine the extent of any differential effects between EPA and DHA when combined with phytosterols.

The aim of this study was to directly investigate the effect of fish oil rich in EPA in combination with phytosterol supplementation, on plasma lipid profile, inflammatory status, and cardiovascular risk as a possible therapeutic treatment for hyperlipidemia.

4.2 Methods

4.2.1 Study Population

Individuals with combined hyperlipidemic (total-cholesterl >6.0mmol/L and triglyceride > 1.5mmol/L) but otherwise healthy men and women (25-70 years) were recruited through a volunteer database (Hunter Medical Research Institute) and advertisements/interviews in the local media in the Hunter region, Newcastle area, Australia. Potential candidates were screened via telephone contact. The primary inclusion criteria included a fasting plasma cholesterol concentration ≥ 6.0mmol/L (231mg/dL), triglyceride concentration ≥ 1.5mmol/l (132mg/dL), no previous cardiovascular events, diabetes mellitus, chronic inflammatory disease, hypertension or liver/renal disease. Subjects were excluded if they were taking anti-inflammatory medication, anti-hypertensive medication or hypolipidemic agents, also if they regularly consumed phytosterol-enriched foods and/or fish oil and flaxseed oil supplements. Subjects were also excluded if they had a strong aversion or any known allergies/intolerances to the foods involved in the study, and a usual weekly consumption of no more than two fatty fish meals per week.

4.2.2 Study Design

Fifty-eight subjects were recruited for the study and were randomly assigned to 1 of 4 intervention groups (n = 14-15 per treatment group). Randomisation was stratified for gender. Each subject was asked to consume three oil capsules per day (one 1g capsule with each meal).

Each capsule contained 1g sunola oil (control) or 1g EPA-rich fish oil (providing 550mg EPA and 120mg DHA in a triacylglycerol form, EPAX 5510 TG/N) (see Appendix 5).

Subjects were randomly assigned to consume the capsules alone or in conjunction with 25g/day of a phytosterol-enriched fat spread (providing 2g/day phytosterols) (Table 3.1). The four groups were identified as (1) placebo (sunola-oil), (2) EPA, (3) phytosterol and (4) combination (phytosterol plus EPA-rich oil capsules) (Figure 4.1). The phytosterol spread was provided as individually measured tubs (25g each) to replace all habitual margarine/butter. Subjects were instructed to use the spread on bread/crackers, melted over vegetables, mixed into mashed potatoes/pumpkin, rice etc. and not to use it for high temperature cooking or frying. Unintentional intake of phytosterols from other food sources was not possible to monitor or restrict. Compliance was monitored by regular telephone contact with participants, weighing of tubs and capsule count-back before and after the trial period, interviewing volunteers about their use of the spread at the end of the trial and evaluating their dietary records. Volunteers also recorded whether they had taken their supplements and/or consumed the spread on a diary card each day.

4.2.3 Clinical Assessment

A medical questionnaire [Appendix 1], anthropometric measurements, bioelectrical impedance and blood pressure were collected at baseline and post intervention. Cardiovascular risk [Appendix 2] was also calculated. The methods used are outlined in Chapter 2.1 Clinical Assessment.

4.2.4 Biochemical Assessment

Plasma concentrations of total-cholesterol, LDL-cholesterol, triglycerides, HDL-cholesterol and glucose were determined. Plasma fatty acid composition and plasma concentration of IL-6, TNF-α, LTB_4 and CRP were measured. The methods used are outlined in Chapter 2.2 Biochemical Analysis.

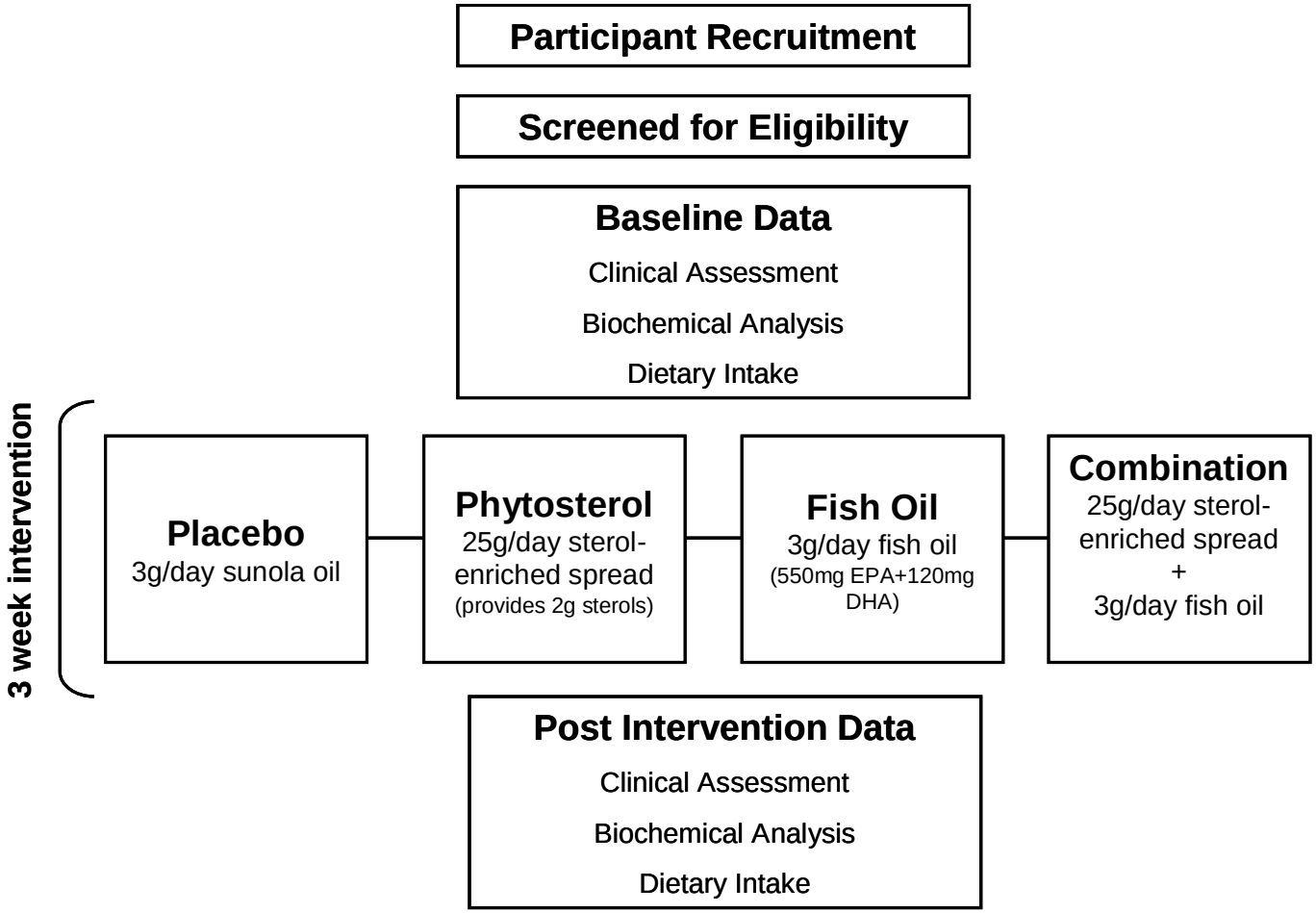

Figure 4.1 *Study design for intervention trial.*

4.2.5 Dietary Analysis

Dietary intake was assessed using the 24-hour food recall method, outlined in Chapter 2.2 Dietary Analysis [Appendix 3].

4.2.6 Statistical Analysis

Results were analysed using SPSS version 15.0 for Windows. All data are presented as means ± SEM. The 95 percent confidence intervals (95% CI) for the differences in the changes between the groups are also given. Statistical significance was set at P value < 0.05. Changes from baseline were determined using non-parametric analyses (Wilcoxon signed-rank test). The effect of treatment on the percentage change in variables between groups was determined using two-way between-group analysis of variance (ANOVA) with post-hoc comparisons (Tukey HSD). A two-way ANOVA was used to determine whether there was a significant main effect for each independent variable by testing for between-subject effects, furthermore an interaction effect (phytosterol x omega-3 fatty acid) was tested between the two independent variables in their effect on the dependent variable. This method was also used to test for synergistic/complementary effects of the two independent variables.

4.3 Results

Participants (male n=21, female n=37) had a mean (± SEM) age of 56.7 ± 2.2 years and a mean BMI of 25.8 ± 1.0kg/m^2. Participant demographics at baseline are reported in Table 4.1. Participant characteristics of the four groups at baseline were well matched and were not significantly different (one-way ANOVA). Analysis of body composition showed no significant differences between each of the groups, with a mean (± SEM) body fat mass of 25.6 ± 2.3 kg (33.4 ± 2.1%) and fat free mass of 49.8 ± 2.4 (66.5 ± 2.1%). Participant demographics and body composition did not significantly change throughout the intervention. Blood pressure and heart rate were also not significantly different between groups and did not significantly change over the course of the intervention period. Participants had an average SBP of 133.4 ± 4.1mm Hg a DBP of 82.2 ± 2.6mm Hg and a HR of 67 ± 2.8bpm at baseline.

Table 4.1 *Participant baseline demographics, body composition and blood pressure*[1]

	Placebo	Fish Oil (EPA-rich)	Phytosterol	Combination
N	14	15	14	15
Age (years)	53.9 ± 2.5	57.4 ± 1.9	55.7 ± 3.0	59.9 ± 1.2
Gender (M/F)	3/11	5/10	7/7	6/9
Body weight (kg)	71.7 ± 2.1	79.2 ± 2.8	76.5 ± 2.6	77.9 ± 5.8
Height (m)	1.3 ± 1.1	1.2 ± 1.1	1.2 ± 1.1	1.2 ± 0.03
BMI (kg/m^2)	24.9 ± 0.9	26.7 ± 0.9	25.6 ± 0.9	25.9 ± 1.4
Waist circumference (cm)	86.2 ± 2.5	89.7 ± 2.0	88.8 ± 2.2	92.3 ± 4.7
Hip circumference (cm)	96.8 ± 2.3	100.6 ± 2.5	95.7 ± 2.6	100.6 ± 2.4
Waist-to-hip ratio	0.8 ± 0.02	0.8 ± 0.02	0.9 ± 0.02	0.9 ± 0.03
Fat mass (%)	34.2 ± 1.7	35.3 ± 2.2	30.4 ± 2.4	33.9 ± 1.8
Fat free mass (%)	65.7 ± 1.7	64.6 ± 2.2	69.5 ± 2.4	66.1 ± 1.8
Systolic blood pressure (mm Hg)	127.0 ± 3.4	135.2 ± 3.8	133.8 ± 3.4	137.9 ± 5.7
Diastolic blood pressure (mm Hg)	79.2 ± 2.8	83.2 ± 2.4	81.7 ± 2.3	84.9 ± 2.9
Heart rate	68.1 ± 3.1	67.1 ± 3.6	65.4 ± 2.4	67.3 ± 2.3

[1]Values are reported as mean ± SEM. There were no significant differences between groups (one-way ANOVA).

Evidence of adherence to the study was analysed by dietary records, capsule counts, weighing of spread containers and analysis of plasma fatty acid concentration. The capsules were well tolerated and compliance as determined by capsule count-back was 97 ± 0.03%. The mean amount of spread consumed (analysed by 24-hour food recall) was 24.2 ± 0.2g/day, mostly consumed at breakfast and lunch on bread and crackers.

Dietary consumption of macronutrients and fatty acids was analysed using the 24-hour food recall at baseline and post intervention (Table 4.2). The mean energy intake at baseline was 7822kJ/day, comprising of 19.5, 44 and 33.5% protein, carbohydrate and fat, respectively. Dietary macronutrient and fatty acid intake were not significantly different between groups at baseline and did not change significantly during the intervention. Consumption of alcohol and physical activity was also unchanged.

Participants had a mean ± SEM cholesterol and triglyceride concentration of 6.4 ± 0.1mmol/L and 1.6 ± 0.1mmol/L, respectively. Plasma lipid profile did not differ among the four groups at baseline (Table 4.3). There were no significant changes in plasma lipid profile in the placebo group. Fish oil (1.6g/day EPA) supplementation alone, caused a significant reduction in plasma triglyceride concentration ($P=0.02$) and an increase in HDL-cholesterol concentration ($P<0.01$). In the phytosterol group (2g/day) plasma total-cholesterol and LDL-cholesterol were significantly reduced from baseline in three-weeks ($P<0.01$ and $P<0.01$, respectively). In the combination group (1.6g/day EPA + 2g/day phytosterols), plasma total-cholesterol ($P<0.01$), LDL-cholesterol ($P<0.01$) and, triglycerides ($P<0.01$) were significantly reduced from baseline and HDL-cholesterol was significantly increased ($P<0.01$).

A comparison between the four treatment groups by one-way ANOVA was performed on percentage change in plasma lipids. The percentage change from baseline in plasma total-cholesterol in the combination group differed from that in the placebo group ($P=0.04$, 95% CI: -14.1 to -0.1) and fish oil group ($P<0.001$, 95% CI: -18.5 to -4.7) (Figure 4.2). Also, the change in plasma total-cholesterol in the fish oil group differed to that of the phytosterol group ($P<0.01$, 95% CI: 3.2 to 17.3). There were no differences between each of the four groups for change in LDL-cholesterol concentration (Figure 4.3). The reduction in plasma triglyceride concentration in the combination group was significantly different to the placebo group ($P<0.01$, 95% CI: 6.1 to 25.1) (Figure 4.4).

Similarly, the percentage change in HDL-cholesterol in the combination group differed from that of the placebo group (P=0.04, 95% CI: 0.1 to 18.7) (Figure 4.5).

Possible treatment effects of phytosterol and fish oil supplementation alone, as well as their interaction (phytosterol x fish oil), was examined using two-way ANOVA with post-hoc analysis. A significant main effect for phytosterol supplementation was found for total-cholesterol (P<0.001), and triglycerides (P=0.04). A significant main effect for fish oil (EPA-rich) supplementation was found for triglycerides (P=0.01) and HDL-cholesterol (P=0.02). No significant interactions between the two dietary variables (phytosterol x EPA) were found.

Plasma fatty acid concentration did not differ amongst groups at baseline (Table 4.4). Plasma concentration of both EPA and DHA were significantly increased from baseline in the fish oil (44.6%, P<0.01; 36.6%, P=0.05) and combination (45.8%, P<0.01; 31.3%, P=0.03) groups, demonstrating compliance to the omega-3 fatty acid supplementation. The percentage change in plasma EPA concentration in the fish oil group, was significantly different to the placebo and phytosterol groups (P=0.01, 95% CI: 28 to 162; P=0.01, 95% CI: 77 to 181) (Figure 4.6). Also the change in EPA in the combination group differed to the placebo and phytosterol groups (P<0.05, 95% CI: 63 to 109; P<0.05, 95% CI: 72 to 108). Similarly, the percentage change in plasma DHA concentration in the fish oil and combination groups differed significantly to the change in the placebo and phytosterol groups. An interaction effect was assessed for omega-3 fatty acid supplementation, with a significant main effect on plasma EPA (P<0.001) and DHA (P<0.001) concentration.

There were no significant differences in inflammatory mediators between groups at baseline (Table 4.5). Percentage change from baseline was examined for each group. Plasma inflammatory markers did not significantly change in the placebo group. In the fish oil group and hs-CRP and TNF-α significantly changed (4.8 ± 3.7µg/mL, P=0.02 and 2.2 ± 0.3pg/ml to 1.6 ± 0.1pg/ml, P=0.02, respectively) from baseline. In the phytosterol group TNF-α was significantly increased from baseline (1.7 ± 0.1pg/ml to 2.1 ± 0.3pg/ml, P=0.01). In the combination group, plasma hs-CRP concentration was significantly reduced (3.4 ±0.5 to 3.0 ± 0.5µg/mL, P=0.02) from baseline. The change in TNF-α in the fish oil group was significantly different to the phytosterol group (P<0.01, 95 % CI: -0.5 to -0.1). No other between-group differences were found.

Cardiovascular risk, as determined by the NCEP ATP-III model, calculated a 10-year risk of CVD of 3.0, 6.6, 6.7 and 7.0% in placebo, fish oil, phytosterol and combination groups at baseline (Figure 4.7). After the three-week intervention cardiovascular risk was increased by 5.7 ± 1.1% (P=0.7) in the placebo group, 3.5 ± 1.1% (P=0.1) in the fish oil group, 12.8 ± 1.1% (P=0.1) in the phytosterol group and 19.5 ± 5.3% (P=0.01) in the combination group, from baseline. ANOVA failed to find any significant between-group differences in percentage change from baseline and post hoc analysis did not find any phytosterol x fish oil interacts for overall risk.

Table 4.2 *Reported macronutrient and fatty acid intake, assessed by 24-hour food recall at baseline and post intervention*[1]

	Placebo		**Fish oil (EPA-rich)**		**Phytosterol**		**Combination**	
	BL	PI	BL	PI	BL	PI	BL	PI
Energy, kJ/day	7493 ± 702	7181 ± 577	7684 ± 800	8194 ± 968	7517 ± 574	7038 ± 505	8594 ± 794	9526 ± 945
Protein, g/day (% en)	94.4 (21)	76.8 (19)	87.8 (19)	98.9 (22)	93 (21)	88.3 (21)	90.1 (17)	118 (21)
Carbohydrate, g/day (% en)	188 (43)	207 (48)	196 (45)	197 (42)	183 (42)	165 (40)	233 (47)	249 (45)
Sugar, g/day	90.1 ± 9.4	97.2 ± 17.5	86.7 ± 9.3	92.4 ± 11.3	75.7 ± 14	60.7 ± 8.1	107 ± 9.8	122 ± 25.6
Starch, g/day	96.2 ± 13.9	107.9 ± 14	107.1 ± 17	104 ± 18.4	103 ± 11.1	103 ± 8.4	123 ± 8.2	124 ± 12
Total Fat, g/day (% en)	68.7 (34)	60.3 (32)	70.2 (33)	76.2 (34)	68.7 (34)	66.5 (35)	78.3 (33)	82.2 (32)
SFA, g/day (% fat)	28.1 (44)	23.6 (41)	23.3 (38)	28.4 (42)	28.7 (45)	25.3 (42)	29.6 (42)	35.1 (46)
MUFA, g/day (% fat)	24.2 (38)	22.5 (40)	25.9 (40)	26.2 (39)	23.1 (39)	23.4 (38)	30.4 (41)	29.5 (38)
PUFA, g/day (% fat)	10.4 (17)	9.0 (17)	14.4 (20)	14.7 (19)	9.2 (15)	11.6 (18)	11.3 (16)	10.7 (15)
Cholesterol, mg/day	279 ± 32.2	220 ± 31.4	277 ± 54.4	354 ± 53.6	274 ± 41.5	334 ± 36.6	231 ± 37.8	269 ± 43
Fibre, g/day	22 ± 2.5	22 ± 2.0	23 ± 4.2	19.7 ± 3.4	18.9 ± 2.0	18.4 ± 0.9	24 ± 2.7	28.1 ± 3.8

[1] Values represented as mean ± SEM, unless other wise indicated. No significant within group differences between baseline and post intervention were found, using non-parametric analyses (Wilcoxon signed-rank test). A one-way ANOVA was used to explore difference between the four groups at baseline and post intervention. BL, baseline; PI, post intervention (3-weeks); % en, percentage of total energy intake; % fat, percentage of total fat intake; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 4.3 *Participant baseline plasma lipid and glucose profile*[1]

	Placebo	Fish Oil (EPA-rich)	Phytosterol	Combination
Cholesterol (mmol/L)	6.5 ± 0.1	6.2 ± 0.2	6.5 ± 0.2	6.3 ± 0.1
LDL-cholesterol (mmol/L)[2]	4.2 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	4.0 ± 0.1
HDL-cholesterol (mmol/L)	1.5 ± 0.1	1.3 ± 0.09	1.2 ± 0.08	1.4 ± 0.1
Triglyceride (mmol/L)	1.5 ± 0.3	1.4 ± 0.1	1.5 ± 0.2	1.8 ± 0.3
Glucose	5.0 ± 0.09	5.4 ± 0.1	5.4 ± 0.1	5.4 ± 0.1

[1]Values are reported as mean ± SEM. [2] LDL-cholesterol calculated using Friedewald equation [312]. There were no significant differences between groups (one-way ANOVA).

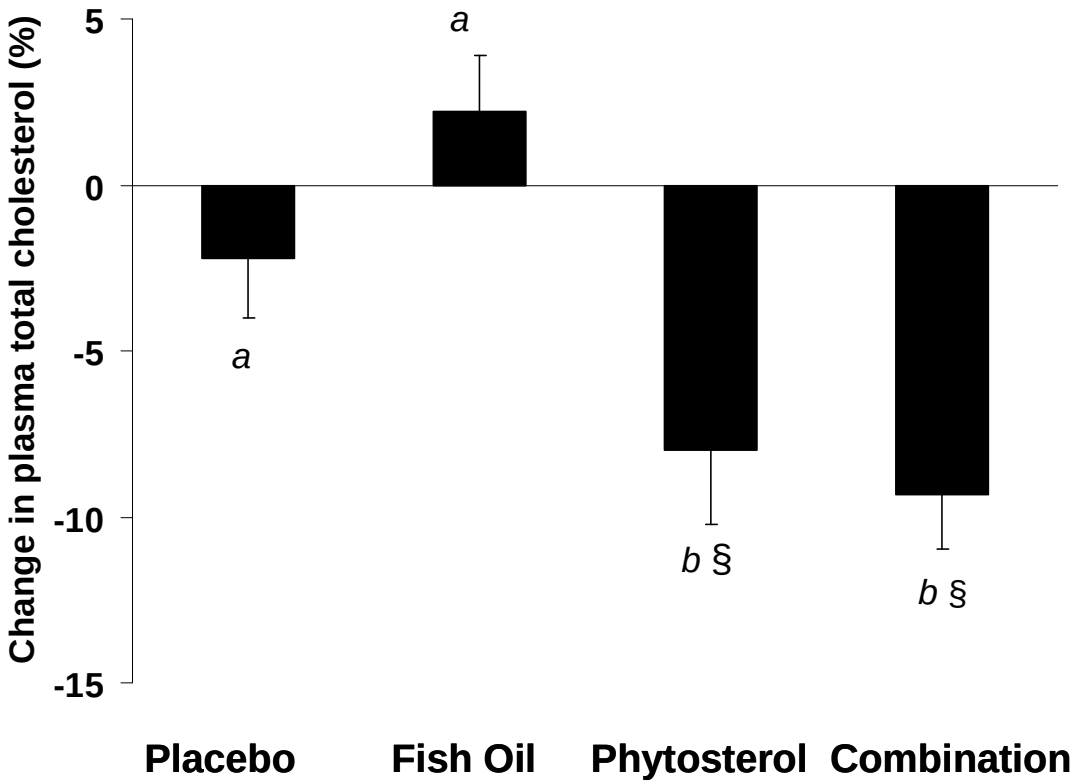

Figure 4.2 *Change (%) from baseline in plasma total-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), 3g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 3g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ,* $P < 0.05$.

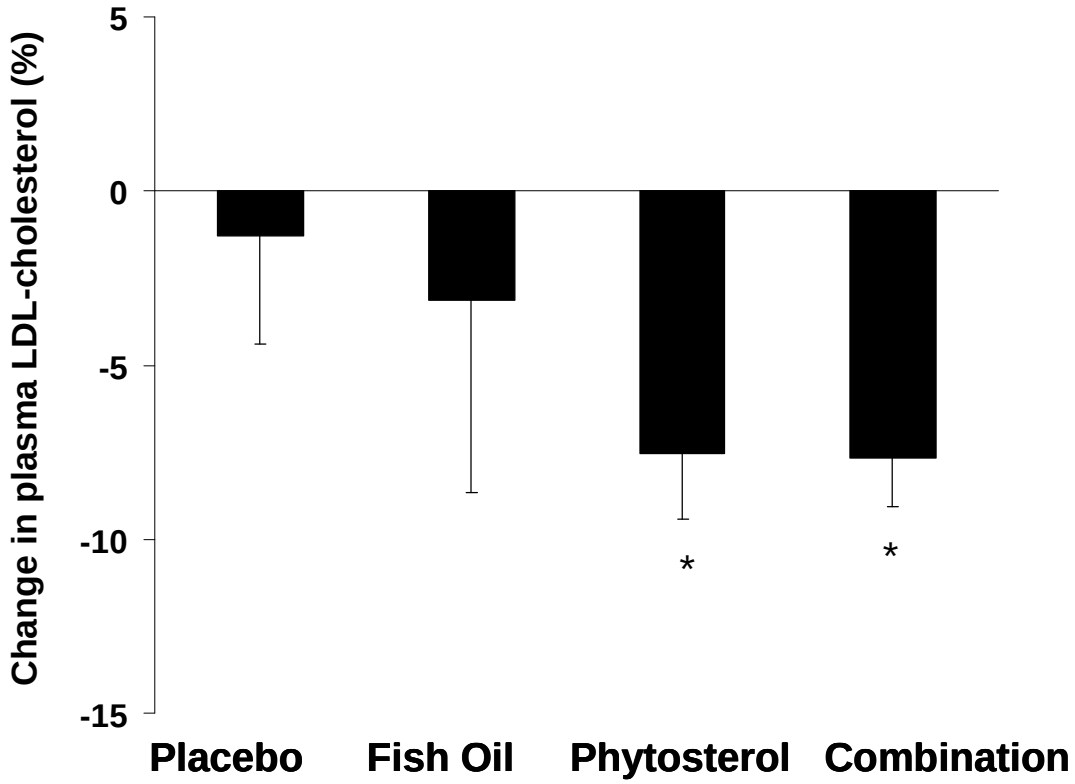

Figure 4.3 *Change (%) from baseline in plasma LDL-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), 3g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 3g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ, P < 0.05.*

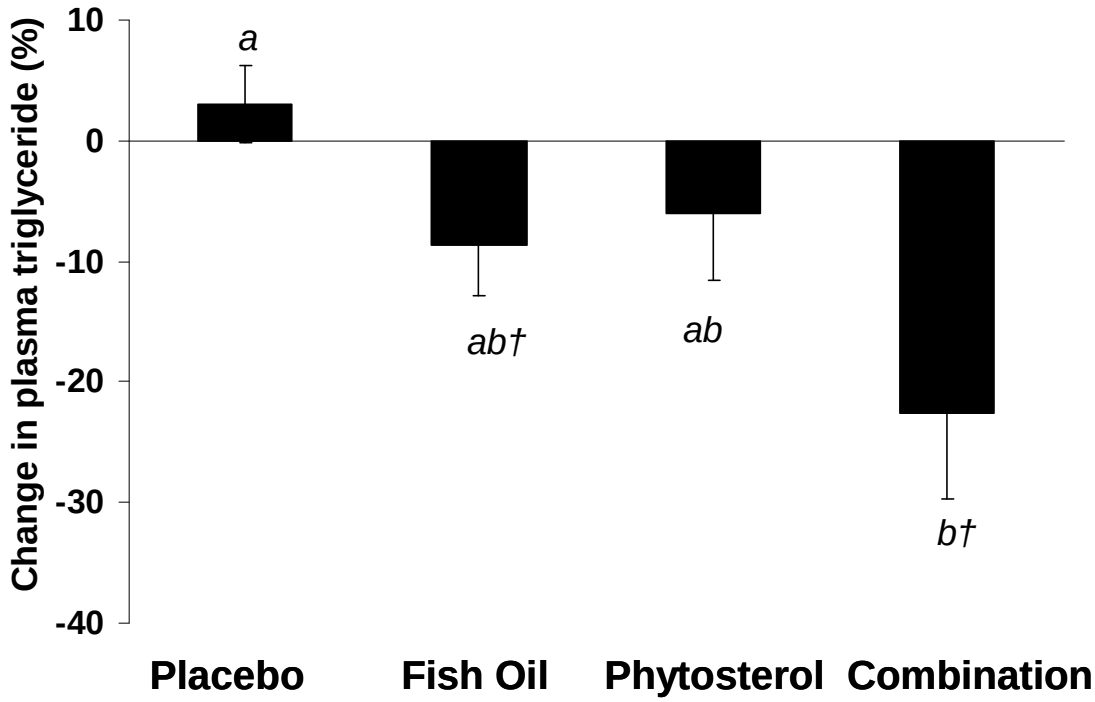

Figure 4.4 *Change (%) from baseline in plasma triglyceride, in hyperlipidemic subjects consuming sunola oil (Placebo), 3g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 3g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ,* $P < 0.05$.

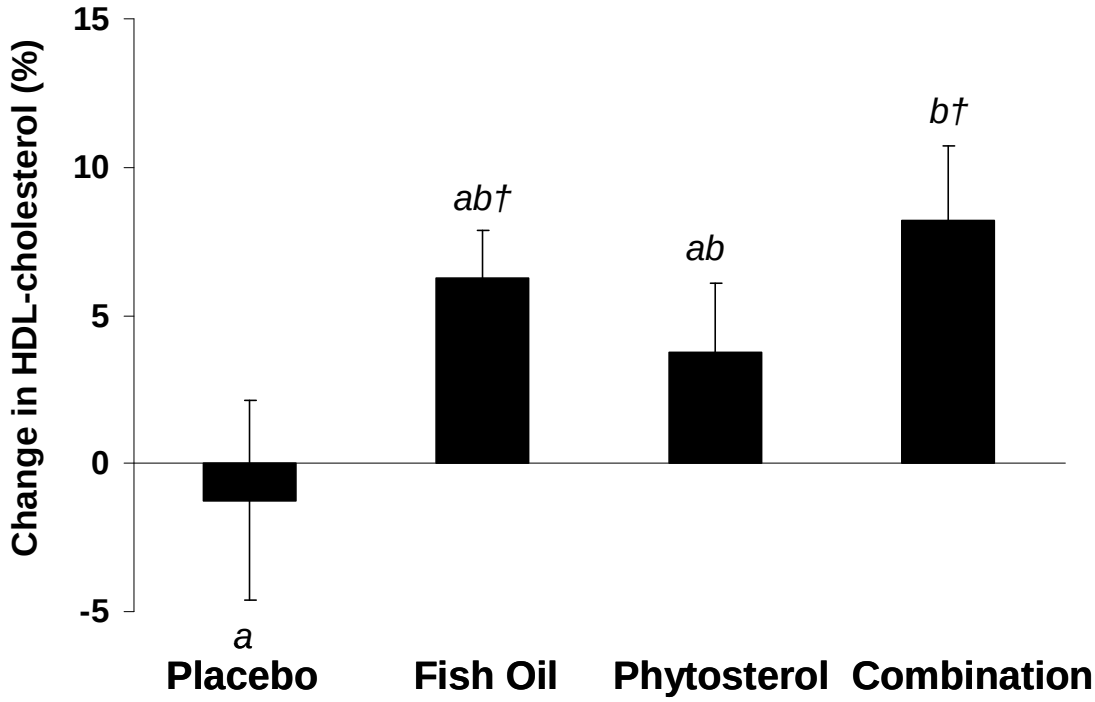

Figure 4.5 *Change (%) from baseline in plasma HDL-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), 3g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 3g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=15 per group. Wilcoxon signed-rank test was used to test within group changes from baseline,* $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. *Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ,* $P < 0.05$.

Table 4.4 *Participant plasma fatty acid composition at baseline*[1]

	Placebo	Fish Oil (EPA-rich)	Phytosterol	Combination
C16:0	15.0 ± 1.3	15.3 ± 0.3	15.3 ± 0.5	16.7 ± 0.6
C18:0	4.6 ± 0.4	5.1 ± 0.5	6.5 ± 0.3	5.6 ± 0.3
C18:1n-9	13.1 ± 1.3	15.5 ± 1.4	17.0 ± 0.6	17.6 ± 1.2
C18:1n-7	9.5 ± 2.4	12.5 ± 2.3	9.6 ± 2.6	10.6 ± 2.3
C18:2n-6	12.0 ± 2.6	7.8 ± 2.6	12.1 ± 2.8	10.5 ± 2.4
C20:0	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.01	0.2 ± 0.01
C20:3n-6	0.6 ± 0.4	0.2 ± 0.06	1.2 ± 0.6	0.2 ± 0.09
C20:4n-6	6.2 ± 0.4	7.0 ± 0.3	6.1 ± 0.8	6.0 ± 0.4
C20:5n-3 (EPA)	1.4 ± 0.19	1.1 ± 0.18	1.5 ± 0.2	1.4 ± 0.2
C22:0	0.6 ± 0.06	0.7 ± 0.03	0.7 ± 0.08	0.7 ± 0.03
C22:5n-3	1.6 ± 0.2	0.7 ± 0.05	1.1 ± 0.02	0.8 ± 0.08
C22:6n-3 (DHA)	2.6 ± 0.2	2.6 ± 0.2	2.9 ± 0.3	2.3 ± 0.1
C24:0	3.5 ± 0.3	3.8 ± 0.1	3.3 ± 0.3	3.4 ± 0.3
C24:1n-9	0.7 ± 0.08	0.6 ± 0.03	0.8 ± 0.07	0.6 ± 0.03
SFA	41.8 ± 2.2	42.6 ± 0.7	43.3 ± 0.5	42.8 ± 0.9
MUFA	30.8 ± 2.2	35.2 ± 2.4	33.5 ± 2.5	35.9 ± 2.3
n-6 PUFA	17.3 ± 2.3	12.9 ± 2.8	14.1 ± 3.1	12.9 ± 2.5
n-3 PUFA	9.1 ± 1.0	6.6 ± 0.4	8.1 ± 0.6	7.0 ± 0.5

[1]Values are reported as mean (% of total fatty acids) ± SEM. There were no significant differences between groups (one-way ANOVA).

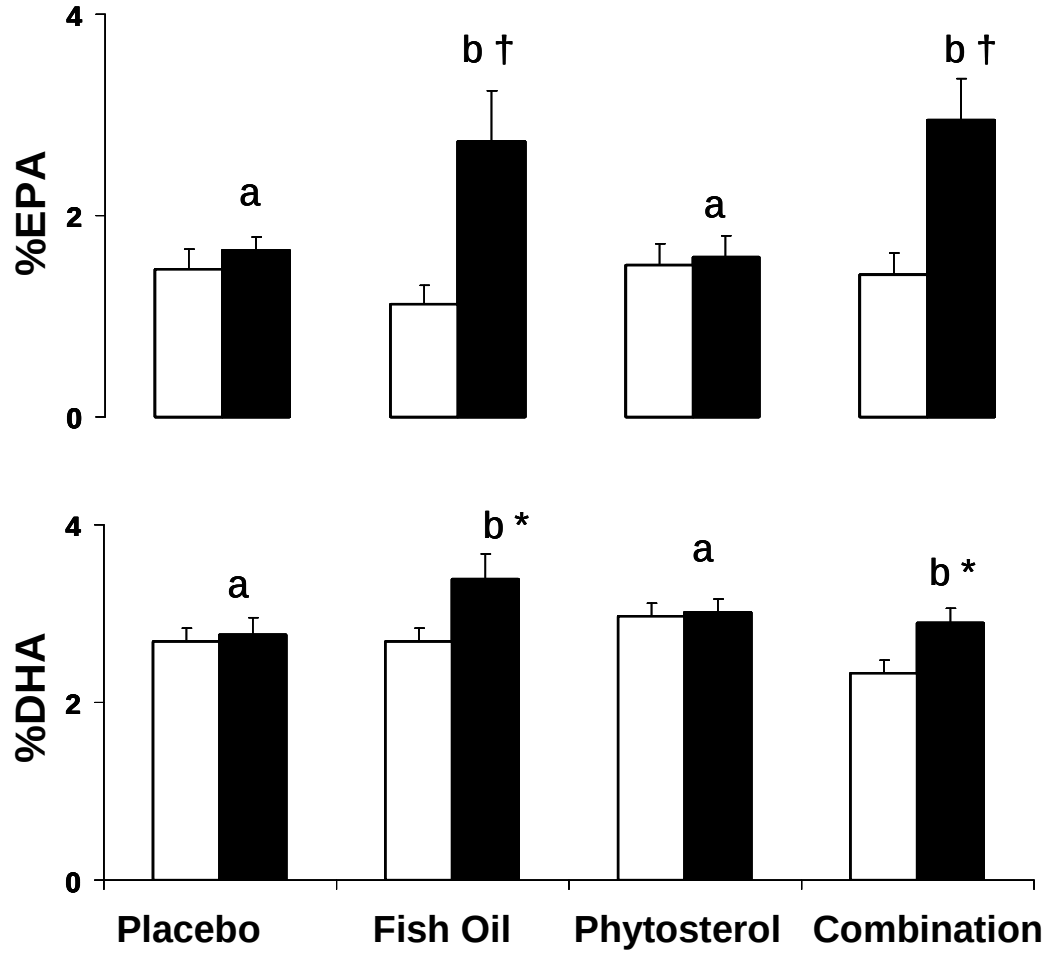

Figure 4.6 *Plasma concentration (% of total fatty acid) at baseline (■) and post intervention (□) of EPA and DHA in hyperlipidemic subjects consuming sunola oil (Placebo), 3g/day omega-3 fatty acids (Fish Oil), 2g/day phytosterols (Phytosterol) or 3g/day omega-3 fatty acid + 2g/day phytosterols (Combination). Values are mean ± SEM, n=14-15 per group. Students-t test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. Two-way ANOVA was used to test for between-group differences. Bars without a common letter differ, $P < 0.05$.*

Table 4.5 *Participant concentration of plasma inflammatory markers at baseline and post intervention*[1]

	Placebo		Fish oil (EPA-rich)		Phytosterol		Combination	
	BL	PI	BL	PI	BL	PI	BL	PI
CRP (µg/mL)	2.9 ± 0.3	3.0 ± 0.3	4.8 ± 1.0	3.7 ± 0.7	2.6 ± 0.4	2.8 ± 0.6	3.4 ± 0.5	3.0 ± 0.5*
IL-6 (pg/mL)	1.5 ± 0.3	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.2
TNF-α (pg/mL)	2.2 ± 0.2	2.0 ± 0.2	2.2 ± 0.3	1.6 ± 0.1*	2.1 ± 0.1	1.7 ± 0.3*	1.9 ± 0.4	1.5 ± 0.2
LTB_4 (pg/mL)	39.6 ± 8.9	36.6 ± 6.8	54.9 ± 7.1	52.7 ± 7.8	39.9 ± 6.0	33.4 ± 7.8	46.4 ± 7.2	43.9 ± 6.5

[1]Values are reported as mean ± SEM. There were no significant differences between groups (one-way ANOVA). Differences between baseline and post intervention within-groups was analysed using paired samples t-test, * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. baseline. BL, baseline; PI, post intervention (3-weeks)

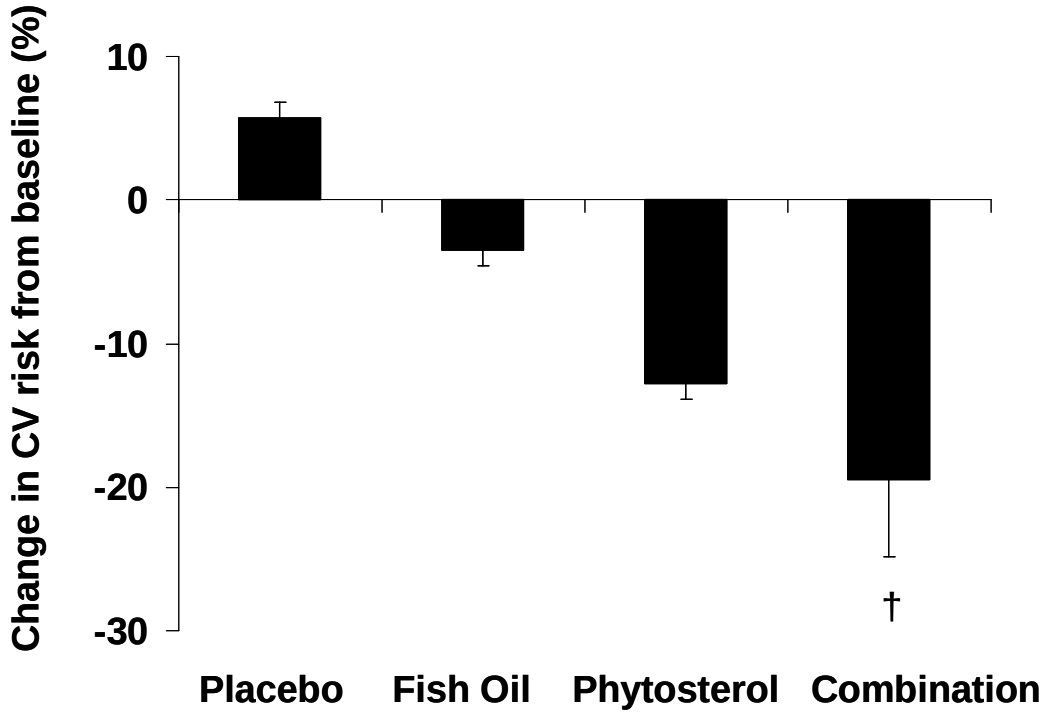

Figure 4.7 *Effect of dietary intervention with sunola oil (Placebo), 3g/day omega-3 fatty acid (Fish Oil), 2g/day phytosterols (Phytosterol), or 3g/day omega-3 fatty acid + 2g/day phytosterols (Combination) on cardiovascular risk. Bars represent percentage change from baseline (mean ± SEM), following 3 weeks of dietary supplementation. Statistical analyses were performed using paired samples t-test,* * *P < 0.05,* † *P < 0.01, § P <0.001 vs. baseline.*

4.4 Discussion

Although several studies have examined the individual effects of dietary phytosterols and EPA supplementation on plasma lipids, few have assessed the combined effects of these functional ingredients together. The present study was designed to assess whether phytosterols and EPA are more effective when consumed as single functional ingredients or in combination to modulate plasma lipid profile and inflammatory status in mildly hyperlipidemic Australian adults. The consumption of phytosterols in conjunction with EPA-rich oil provided the greatest overall improvement in plasma lipid profile compared to either of these functional ingredients taken alone. Moreover, nearly a 20% reduction in cardiovascular risk was found in the combination group.

Phytosterols inhibit cholesterol absorption, thereby reducing total- and LDL-cholesterol concentration. A number of clinical trials have established that the consumption of 1.5-2.0g per day of phytosterols can result in a 10-15% reduction in LDL-cholesterol in as short as a three week period. The benefits of phytosterol consumption have been demonstrated in normolipidemic, hyperlipidemic and individuals already taking lipid-lowering medications (i.e. statin drugs) [147, 322, 356]. In this study, a significant 8% reduction in total-cholesterol, 7.5% reduction in LDL-cholesterol and a 7% reduction in triglyceride concentration after 3 weeks of supplementation with 2g/day phytosterols. These findings are comparable to other studies with similar phytosterol dose and duration of supplementation. In a study by Clifton *et al* [163], hyperlipidemic patients were supplemented with 1.6g/day phytosterol-enriched foods (bread, cereal, milk and yoghurt) for 3 weeks, showing an average reduction in serum LDL-cholesterol of 9%, with milk providing the greatest reduction (16%). The hypotriglyceridemic effect of phytosterols has been thought to be minimal in previous studies. In a recently published meta-analysis by Naumann *et al* [357], individuals having greater baseline triglyceride concentrations were found to have a greater hypotriglyceridemic response to phytosterol supplementation. In response to 2g phytosterols daily, reductions in plasma triglycerides of 1.0, 3.8 and 4.7% were observed respective to baseline concentrations of 1.0, 2.0 and 3.0mmol/L [357].

The hypotriglyceridemic properties of omega-3 fatty acids have long been elucidated [240], In a study by Mori *et al* [232], DHA provided the greatest reduction in triglyceride concentration (20%) in hyperlipidemic adults. In another study a significant within-group reduction in triglyceride concentration was seen (22%) from baseline with EPA supplementation [358]. In our study, the EPA-rich oil group had reductions in plasma triglyceride levels (8.6%) and HDL-cholesterol was significantly increased by 6.0%. The contradictory findings between our study and previous findings may be explained in part by the concentration of consumed fish oil and duration of supplementation. In another placebo-controlled study carried out in our laboratory, it was shown that in hyperlipidemic patients, triglyceride concentration could be reduced by 22.3% (P=0.004) in three weeks, with 3g/day DHA-rich oil [335]. Grimsgaard *et al* [359] reported that EPA and DHA have similar hypotriglyceridemic-lowering effects compared with a placebo. However, there are a few studies to suggest that EPA alone can significantly reduce triglyceride concentration [360] and VLDL-cholesterol and increase LDL-cholesterol and HDL-cholesterol [361], with no changes in total-cholesterol.

In combination, phytosterols and EPA-rich oil may offer a more comprehensive strategy for the optimisation of plasma lipid profile. In this study, total-cholesterol, LDL-cholesterol and triglycerides were significantly reduced by 9.3 ± 1.6%, 7.6 ± 1.4% and 22.5 ± 3.1%, respectively and HDL-cholesterol was increased significantly by 8.1 ± 2.5%. Further analysis of the data showed there were no significant interaction effects of phytosterol x EPA. There is very limited published data, which evaluates the independent and interactive effects of phytosterols and omega-3 fatty acids. In a recently published study by Khandelwal *et al* [362]**,** showed significant reductions in triglyceride concentration in response to phytosterol and omega-3 fatty acids alone and in combination. Compared to our study, they showed no changes in total-cholesterol, LDL-cholesterol and HDL-cholesterol. This could have been due to the difference in subject population, with our study having a higher percentage of women (76% vs. 11%), also our mean baseline total-cholesterol was higher at 6.4mmol/L and the fish oil supplementation had a higher concentration of EPA.

The genetic makeup of the study participants may also be responsible for the differences observed in the two studies [60, 161, 265, 358].

The esterification of phytosterols to long-chain omega-3 fatty acids, does not impair their hypolipidemic properties, yet enhances their solubility in oil by 10-fold [291]. The extent to which the fatty acid moiety of phytosterol esters influences cholesterol absorption has been examined in a limited number of animal and human trials. In a study by Rasmussen *et al* [292] phytosterols were esterified with fatty acids from soybean oil, beef tallow or purified stearic acid and tested in 35 male F_1B Syrian hamsters for 4-weeks (50 g/kg phytosterol esters esterified with fatty acids). This study showed that beef tallow and stearic acid are more effective then soybean oil in reducing cholesterol absorption, liver cholesterol and plasma non HDL-cholesterol concentration in hamsters. In a similar study by Ewart *et al* [293], male hamsters were fed phytosterol esters esterified to fish oil, which showed a significant reduction in non HDL-cholesterol concentration compared to the control. Furthermore, in insulin-resistant rats fed the same phytosterol-fish oil esters, serum triglyceride and total-cholesterol levels were significantly reduced compared to the control rats [294]. Unfortunately, in the studies by Ewart [293] Russell [294] and Demonty [295], their study design did not allow for a phytosterol only or fish oil only group for comparison. Therefore, it is difficult to determine whether the combined phytosterol-fish oil esters were the most effective compared with phytosterol esters or fatty acid esters alone.

In combination, phytosterols and omega-3 fatty acids may offer a more comprehensive strategy for not just optimising circulating lipid levels, but also to provide additional health benefits via anti-inflammatory, -hypertensive and –arrhythmic effects. There is evidence to suggest that omega-3 fatty acid supplementation is involved in improved vascular function and lipoprotein profile, lower arterial pressure, diminished thrombogenicity and modification of atherogenic processes, all of which are important cardiovascular preventative actions [363]. The evidence seems to be contradictory in animal studies comparing the anti-inflammatory effects of EPA and DHA [364, 365], however in human clinical trials no significant difference is seen between the two fatty acids [334, 366, 367].

In this study, phytosterols significantly increased the cytokine TNF-α (2.4% P=0.01), whilst EPA-rich oil significantly reduced TNF-α (-2.3% P=0.02), however had no effect on IL-6, CRP or LTB_4. The combination of the two functional ingredients only showed a

significant reduction in hs-CRP (-2.1% *P*=0.02). To date, our laboratory is the only group to have investigated the anti-inflammatory effects of combined phytosterol and omega-3 fatty acid supplementation in hyperlipidemia [314]. In a previous study combining phytosterols with DHA-rich oil supplementation for three-weeks, hs-CRP, TNF-α, IL-6 and LTB_4 were all significantly reduced (39, 10, 10.7 and 29.5%, respectively) [314]. A possible explanation for the difference in anti-inflammatory effects between phytosterols combined with EPA or combined with DHA could be the limited metabolism of omega-3 fatty acids beyond DPA. Given a large portion of clinical trials are more supportive of the anti-inflammatory properties of DHA [334, 368, 369] and the continued interest in the immuno-modulatory effects of omega-3 fatty acids, further studies are needed to clarify the anti-inflammatory properties of these fatty acids in different models of disease.

When omega-3 fatty acids are administered as an adjunct to a statin therapy in hypercholesterolemic patients with persistent hypertriglyceridemia, benefits in lipid parameters (total-cholesterol and triglycerides) can be enhanced by up to 50% [271, 272]. In the Combination of Prescription Omega-3 with Simvastatin study highly purified omega-3 fatty acid (465mg EPA + 375mg DHA per 1g capsule) and simvastatin lowered triglyceride concentration by 30% [273]. In another omega-3 (8g/day DHA-rich supplement) and statin combination study, a 27% reduction in triglycerides after 3 months was found [276]. There is a significant amount of research to show that statin therapy in combination with omega-3 fatty acids significantly reduces lipids compared to a placebo, particularly triglycerides and lipoprotein sub-fractions [274, 275]. Compared to our study, the combination of omega-3 fatty acids and phytosterols is just as effective in reducing triglyceride concentration in a hyperlipidemic population as is omega-3 fatty acids combined with statins. Our findings also support data showing that omega-3 fatty acid supplementation decreases coronary mortality in established CHD patients and reduces long-term CVD risk [279]. Conditions associated with plasma lipid lipoprotein metabolism, such as hyperlipidemia, play a major role in the development and progression of atherosclerotic disease. Dietary supplementation studies with omega-3 fatty acids, demonstrate their hypotriglyceridemic and anti-inflammatory properties, whilst supplementation with phytosterol esters show hypocholesterolemic effects. The present study adds to the current body of knowledge, demonstrating a natural, safe and efficacious combined dietary treatment for hyperlipidemia.

Chapter 5

APOLIPOPROTEIN E POLYMORPHISM IN HYPERLIPIDEMIA

5.1 Introduction

First recogonised as a major determinant in lipoprotein metabolism and CVD, apoE has emerged as an important protein in several biological processes. ApoE has been found to play a major role in lipid transport and several diseases including hyperlipidemia, Alzheimer's disease, cognitive function and immuno-regulation [43]. ApoE is a polymorphic protein arising from three alleles at a single gene locus. The three major isoforms, E2, E3 and E4, differ from one another only by single amino acid substitutions, yet these changes have profound functional consequences at both the cellular and molecular level [43]. ApoE3 has cysteine at residue 112 and arginine at residue 158, while apoE4 has arginine and apoE2 has cysteine at both sites. In a general Caucasian population, the approximate frequency for the various apoE genotypes are 1% for apoE2/E2, 11% for apoE2/E3, 2% for apoE2/E4, 60% for apoE3/E3, 23% for apoE3/E4, and 3% for apoE4/E4 [263, 370].

Studies have shown that plasma cholesterol, LDL-cholesterol and apoB levels are highest in subjects with an apoE4 allele, intermediate for individuals with apoE3, and lowest in those with an apoE2 allele [41, 262, 263]. The effect of apoE genotype on lipoprotein metabolism and responsiveness to dietary fat restriction has been extensively studied. Carriers of the apoE4 allele, which represent ~ 22% of Caucasians, are believed to be most responsive to a restriction in dietary total and saturated fat and cholesterol intake [42]. The role of apoE in lipoprotein metabolism highlights an obvious potential genetic modulator, of the lipoprotein response to omega- 3 fatty acids and phytosterol supplementation. Studies have shown that an individual's apoE genotype influences their responsiveness to dietary treatment with fish oil, with the greatest benefits for triglyceride reduction in carriers of apoE2 [264]. Conversely, studies show lipid responses to phytosterol supplementation bear no relationship to specific apoE alleles; however this relationship needs further investigation [164-167].

Although the relationship between apoE genotype and lipoprotein metabolism has been investigated in relation to fish oil supplementation and phytosterol supplementation alone, no study has examined the influence of apoE genotype on the combined supplementation of omega-3 fatty acids and phytosterols in hyperlipidemia.

The present study aims to examine the impact of the apoE genotype on plasma lipid profile at baseline and responsiveness to the dietary intervention of phytosterols and omega-3 fatty acids.

5.2 Methods

Buffy coat samples collected from participants in chapters three and four were analysed for apoE genotyping [371].

5.2.1 DNA Extraction

Leukocyte DNA was extracted from 200µL of buffy coat, isolated from a 10mL blood draw into tubes coated with EDTA, using the Qiagen DNA blood mini kit (Qiagen Ltd, Crawley, United Kingdom). 20µl of a protease solution and 200µl of a buffer solution was added to 200µl of the buffy coat sample. The contents of the tube was mixed by pulse-vortexing for 15 seconds, then incubated at 56^{o}C for 10 minutes in a bench top heating block. DNA reaches a maximum yield after lysis for 10 minutes. The sample was centrifuged for one minute at 6,000 g to remove precipitation drops which may have formed inside the lid. 200µl of ethanol (96%) was added to the tube, vortex mixed for 15 seconds, then centrifuged at 6,000 g for a further minute. The entire contents of the tube was then carefully transferred into a clean 2mL QIAamp spin column and centrifuged at 6,000 g for one minute.

The QIAamp spin column containing the DNA precipitate was carefully placed into a clean 2mL collection tube and the filtrate discarded. 500µl of a buffer solution was added to the precipitate, then centrifuged at 6,000 g for one minute. The QIAamp spin column was transferred into a clean collection tube and the filtrate discarded. 500µL of a buffer solution was added to the precipitate, which was then centrifuged at 20,000 g for three minutes. The QIAamp spin column was transferred into a clean 1.5mL tube and the previous step was repeated, to further purify the DNA sample.

The QIAamp column was transferred into a clean 1.5mL microcentrifuge tube. 200µL of a buffer solution was added to the DNA precipitate. The tube was incubated at room temperature (15-25^{o}C) for one minute and then centrifuged at 6,000 g for one minute. The eluted DNA sample was stored at -20^{o}C until further analysis.

5.2.2 Determination of Concentration, Yield and Purity

DNA yield was determined from the concentration of DNA in the extracted elution, as measured using a nanodrop 1000 spectrophotometer (version 3.7.1) at 260nm (Thermo Fischer Scientific, Wilmington, DE, USA). DNA samples were brought to room temperature. Distilled water was used as a blank or control. One drop of distilled water was placed on the sensor pad of the nanodrop and the concentration was analysed. Approximately 3µL of extracted DNA sample was placed on the sensor pad of the nanodrop. DNA concentration was analysed. The nanodrop was blanked between each DNA sample and the sensor pad was cleaned thoroughly.

The purity of the DNA samples was calculated at an absorbance of 260nm and 280nm. Pure DNA has an A_{260}/A_{280} of 1.7-1.9. Each DNA sample was diluted to a concentration of 20ng/µL using nuclease free water.

5.2.3 Polymerase Chain Reaction (PCR)

ApoE genotyping was determined using a derivation of the restriction fragment length polymorphism (RFLP) technique of Hixon and Vernier [372]. A 218-bp-long DNA fragment containing two polymorphic sites at positions 112 (Arg→Cys) and 158 (Arg→Cys) was amplified. The following apoE sense and antisense primers were used (GeneWorks, SA, Australia).

– 5’-ACA GAA TTC GCC CCG GCG TGG TAC ACT GCC A 3’

– 5’ TAA GCT TGG CAC GGC TGT CCA A 3’

A PCR mix containing: 2µL 10 x buffer (Invitrogen, Australia), 0.4µL (2 U) accuprime taq DNA polymerase high fidelity (Invitrogen, Australia), 4µL Q-solution (Qiagen, NSW, Australia), 2µL of oligonucleotide pairs (at 20µmol/L each) and 6.6µL nuclease-free water, was made for each sample. 15µL of the PCR mix was pipetted into each well of a 96 well plate (Scientific Specialities Inc., CA, USA) and 5µL of DNA sample was added. The plate was capped (Scientific Specialities Inc., CA, USA), vortex mixed and centrifuged at 3,700 g for 1 minute in preparation for thermal cycling (Eppendorf, Hamburg, Germany). The following cycling conditions were applied: 94^{o}C for 10 min (denaturation), 94^{o}C for 30 sec, 56^{o}C for 30 sec (annealing), 72^{o}C for 1 min (extension) (32 cycles) and, 72^{o}C for 4 min. Plates were kept at 4^{o}C until further use.

5.2.4 Restriction Digestion

The amplified DNA was then digested with 4U of *HahI* (20 000 kU/L, New England Biolabs, Hitchin, UK). For each well, a preparation containing: 5µL 10 x NE buffer 4 (500 mmol/L potassium acetate, 200 mmol/L Tris-acetate, 100 mmol/L magnesium acetate, 10mmol/L dithiothreitol, pH 7.9 at 25^{o}C) (New England Biolabs, Hitchin, UK), 0.5µL 100 x bovine serum albumin (BSA) (10g/L, New England Biolabs, Hitchin, UK), 0.5µL *HahI* (20 MU/L) and, 24µL nuclease-free water was prepared. 30µL of the digest mix was pipetted into each well, to make a total product volume of 50µL. The plate was capped, vortexed and centrifuged at 3700 g for 1 minute and the contents of each well were digested in an incubator (Eppendorf, Hamburg, Germany) at 37^{o}C for 16 hours. Plates were kept at 4^{o}C until further use.

5.2.5 Electrophoresis, Imaging and Analysis

A 4% agarose solution was prepared by combining 8g low-melting agarose (Bioline, NSW, Australia) with 192ml of 1 x tris-borate-EDTA (TBE) buffer (Sigma Aldrich, Mo, USA). Mixed thoroughly and heated in a microwave oven until the agarose has completely desolved.

Added to the agarose mixture is 10µL of 10mg/ml ethidium bromide (Sigma, MO, USA), which is mixed evenly with the liquid gel, and cast immediately in a gel tray of an appropriate size. Combs for 40 wells were inserted.

Once the gel had polymerized, each well was loaded with 50µL of a mixture of the cleaved PCR product and 10µL of 5 x tri-colour gel-loading buffer (Bioline, NSW, Australia). The gel was run at 100V for ~ 40 minutes at room temperature, along side a 10bp DNA ladder (1µg/µl) (Invitrogen, Australia) in an electrophoresis tank (BioRad, VIC, Australia) containing 1 x TBE. The gel was immediately analysed under an ultraviolent transilluminator and acquired a digital image of the gel**.** The detection of apoE genotype was determined (Table 5.1).

Table 5.1 *Apolipoprotein E genotypes as determined by the weight of their base pair (bp) fragments.*

Genotype	48bp	72bp	83bp	91bp
ApoE2			✓	✓
ApoE3	✓			✓
ApoE4		✓		✓
ApoE2/E3	✓		✓	✓
ApoE2/E4		✓	✓	✓
ApoE3/E4		✓		✓

5.2.6 Statistical Analysis

Results were analysed using SPSS version 15.0 for Windows. All data are presented as means ± SEM. Statistical significance was set at P value < 0.05. Data was separated by genotype and gender and the change from baseline was determined using non-parametric analyses (Wilcoxon signed-rank test). The difference between the genotypes was analysed using analysis of variance (ANOVA).

5.3 Results

ApoE genotype was successfully measured in 77 samples. The mean ± SEM of A_{260}/A_{280} was 1.81 ± 0.01 and the mean DNA concentration of extracted samples was 72.46 ± 4.5µg/mL. Of these samples, 73 (95%) were homozygous for apoE3, and four were heterozygous (apoE2/E3) (Figure 5.1). There were no participants who were homozygous or heterozygous for apoE4.

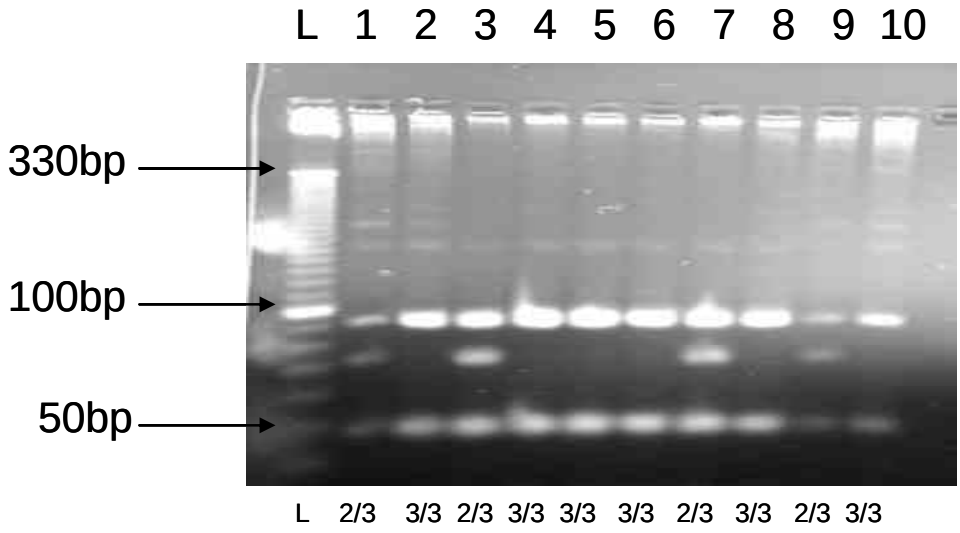

Figure 5.1 *View of 12-well 4% agarose gel. 10-bp ladder (L) and a set of 10 HahI digests. The direction of electrophoresis is from top to bottom. The identifying arrows are placed next to the migrating bands at 330bp, 100bp and 50bp. Tracer of nondigesting higher molecular mass PCR products are common from the PCR and are evident just below the loading wells. Lower numbers indicate the detected genotypes of each sample.*

Baseline characteristics of participants according to their genotype can be seen in Table 5.2. Participants had a mean ± SEM total-cholesterol and triglyceride concentration of 6.45 ± 0.02mmol/L and 1.45 ± 0.15mmol/L, respectively (Table 5.2). There were no statistically significant differences among the two genotype groups. The LDL-cholesterol concentration was similar among the two apoE genotype groups. Furthermore, analysis also showed that CRP concentration differed among the two apoE genotypes (2.3 ± 0.6 vs. 3.4 ± 0.6), albeit non-significant (P=0.20).

Table 5.2 *Baseline anthropometric and fasting lipid levels of subjects by genotype*[1]

	ApoE2	**ApoE3**
N (%)	4 (5)	73 (95)
Age (years)	58.2 ± 2.7	56.06 ± 3.4
Gender (M/F)	0/4	28/45
Body weight (kg)	71.1 ± 6.7	77.0 ± 2.7
Height (m)	1.7 ± 0.03	1.7 ± 0.02
BMI (kg/m^2)	23 ± 1.3	26.4 ± 0.8
Fat mass (%)	33.3 ± 2.0	36.2 ± 2.3
Fat free mass (%)	66.6 ± 2.0	63.8 ± 2.5
SBP (mm Hg)	123.5 ± 4.7	134.1 ± 4.1
DBP (mm Hg)	78.2 ± 5.2	82.5 ± 2.6
Heart rate	70.5 ± 9.5	66.7 ± 2.7
Total-cholesterol (mmol/L)	6.5 ± 0.2	6.4 ± 0.2
LDL-cholesterol (mmol/L)	4.4 ± 0.2	4.3 ± 0.2
Triglycerides (mmol/L)	1.2 ± 0.1	1.6 ± 0.2
HDL-cholesterol (mmol/L)	1.5 ± 0.06	1.4 ± 0.1
CRP (µg/mL)	2.3 ± 0.6	3.4 ± 0.6

[1]Values are reported as mean ± SEM. ApoE2 indicates apoE2/E3 and apoE3, apoE3/E3. There were no significant differences between groups.

It so happened that the apoE2/E3 individuals were all female participants. Further analysis of the data, split by gender, did not show any significant differences, when comparing lipid characteristics between male and female apoE3/E3 individuals and female apoE3/E3 and apoE2/E3 participants.

5.4 Discussion

The aim of the present study was to determine the apoE genotype of participants from the previous two studies outlined in chapter 3 and chapter 4. The impact of the common apoE polymorphism on fasting lipid profile was also investigated. All subjects in this study were either heterozygous or homozygous for the apoE3 allele. Based on the study by Brown and Roberts [373], who showed a comparable distribution of apoE alleles (n = 25, 14(56%) apoE3/E3, 6(24%) apoE2/E3, 4(16%) apoE3/E4 and 1(4%) apoE4/E4) to that of the general Caucasian population, it was thought that the distribution of various apoE genotypes would also be obtained from our cohort. Unfortunately the proportion of subjects who were homozygous for apoE3, greatly out numbered those who were heterozygous for apoE2. Despite differences in subject numbers, there were no significant differences in baseline clinical characteristics (anthropometrics and fasting lipids). The homozygous apoE3 genotype occurs most frequently in the population and is considered 'normal'. Since the homozygous apoE3 allele was evenly distributed amongst the treatment groups, it was possible to evaluate the influence of the genotype.

The apoE polymorphism is one possible genetic variant which may impact on the response to dietary treatment for hyperlipidemia. Its influence as a source of genetic variability has been widely investigated. Studies have shown that the response in total-cholesterol and LDL-cholesterol to changes in dietary cholesterol [374], dietary fat alone [375] or in combination with dietary cholesterol consumption [376, 377], are influenced by the apoE genotype. Carriers of the apoE4 genotype respond better than carriers of other isoforms to the manipulation of dietary fat consumption.

ApoE plays an essential role in the metabolism of cholesterol and triglycerides and is a major constituent of triglyceride-rich lipoprotein particles (chylomicron remnants and VLDL-cholesterol), as well as HDL-cholesterol. The role of apoE in lipoprotein metabolism and clearance has been extensively studied in recent years [41, 261-263, 378, 379].

In our study, carriers of the apoE2/E3 allele tended to have a slightly higher plasma LDL-cholesterol concentration. Carriers of the E2 allele are less efficient in the removal of

VLDL and chylomicron remnants, due to their inherent lower binding affinity [380]. Brenninkmeijer *et al* [381] showed that normolipidemic apoE2 homozygote's have reduced catabolism of chylomicron remnants when compared with apoE2/E3. On the contrary, findings from Weintraub *et al* [382] report that apoE2/E3 carriers did not have defective chylomicrons remnant removal. Slower rates of hepatic clearance of remnants may lead to lower hepatic cholesterol levels, more expression of LDLr, an increase in LDL-cholesterol metabolism and thus lower plasma LDL-cholesterol concentration. This finding is supported by Tzaikas *et al* [383], showing slightly higher LDL-cholesterol concentrations in acute coronary syndrome patients carrying the apoE2/E3 allele compared to the apoE3/E3 allele.

The nutrigenetic interaction between apoE polymorphisms and omega-3 fatty acids has not been extensively investigated, despite apoE having a strong role in lipid metabolism. One of the most well know studies investigating the effect of the apoE isoform on the response to modest fish oil consumption (< 2g/day), is the FINGEN Study [265]. Comprising of 312 healthy participants consuming various (0.7g/day or 1.8g/day) concentrations of omega-3 fatty acids, this study showed a 3.10% and 3.86% increase, respectively in total plasma fatty acid concentration, which equates to a 43% and 69% increase, respectively in long chain omega-3 fatty acid (EPA + DPA +DHA) concentration after eight weeks of administration. Despite an 8% reduction in triglyceride concentration, there were no significant effects of genotype; however carriers of the apoE4 polymorphism tended to be more responsive than other genotypes [265]. The hypotriglyceridemic effect of omega-3 fatty acids is know to be mediated in part, by apoE, as observed in apoE knockout rodents, however this was not found in the FINGEN Study [384, 385].

A gender x genotype x treatment interaction was found for male apoE4 carriers, who experienced greater (≈ 3 times) triglyceride lowering effects compared with female apoE4 carriers [265]. The relationship between apoE polymorphism and gender, in relation to the hypotriglyceridemic effects of fish oils may be due to less contribution to omega-3 storage pools and greater metabolism and utilistaion of fatty acids by males, hence leading to greater hypotriglyceridemic outcome. Conversely, the apoE4 allele has been shown to contribute to the LDL-cholesterol raising effects.

This is supported in a study by Minihane *et al* [264], where the strongest increase in LDL-cholesterol was seen in apoE4 carriers (16% increase), following six weeks of fish oil supplementation. This pro-atherogenic increase in LDL-cholesterol concentration may be offset by a reduction in small dense LDL particles and an increase in the percentage of large buoyant LDL_3 particles [240, 386-390].

Studies investigating the influence of apoE polymorphism on plasma lipid changes in phytosterol supplementation are somewhat limited. Most studies show a LDL-cholesterol lowering effect with phytosterols, irrespective of an individual's apoE genotype [164-168, 391, 392]. Baseline fasting serum phytosterol concentrations do not appear to be related to a specific apoE genotype, perhaps due to the fact that phytosterol consumption in Westernised diets are quite low. Conversely, studies show that apoE4 carriers have higher serum campesterol and sitosterol levels than apoE3 carriers [393], whilst Miettinen *et al* [166] found lower circulating phytosterol concentrations in apoE4 subjects. In a study by Sanchez-Muniz *et al* [161] serum phytosterol concentration increased in subjects with apoE3 and apoE4 genotypes. Overall, the findings suggest that phytosterol supplementation in hypercholesterolemic individuals with an apoE4 genotype, may not be beneficial, due to reductions in circulating carotenoids and a reduced effect on reductions in total-cholesterol, LDL-cholesterol and apoB concentration.

The impact of apoE genotype in the risk of developing CHD has been widely investigated and has been the subject of recent meta-analysis [394]. The apoE4 allele confers an approximately 40% increase in the risk of CHD as compared with the common apoE3 genotype and apoE2 carriers.

It is generally considered to protect against the development of atherosclerosis, but the benefit depends upon the apoE isoform, circulating plasma concentration and the cell type responsible for the secretion and synthesis of apoE [43]. Furthermore, apoE regulates the inflammatory response, by modifying the expression of T-lymphocytes and macrophage cytokine production [395]. In our study, carriers of the apoE2 allele tended to have a lower CRP concentration than carriers of the apoE3 allele.

It has been proposed, that apoE has an allele-specific antioxidant activity, with apoE2 found to be the most effective in protecting against oxidative damage, whereas the apoE4 allele was the least effective [396].

In the present study, it was observed that no relationship between apoE polymorphism and baseline fasting lipids, in subjects with a combined hyperlipidemic profile. Further analysis (not shown) also failed to show any interaction between gender x genotype x treatment. There were also no significant differences in the lipid response to each of the dietary treatments (placebo, fish oil, phytosterol or combination), when individuals were separated by genotype. Given the somewhat divergent results reported in the literature regarding the impact of the apoE genotype and lipid response, it is acknowledge that our study, having a small sample size, limits the ability to further explore this relationship. Most nutrigenomic studies involve a screening process prior to commencement; however our study was not designed with the primary intention to investigate polymorphisms. Our findings do however concur with the established literature, to support that the most common apoE polymorphism is the homozygous apoE3. Furthermore, this negates any reservations regarding the influence of apoE genotype in our study participants, particularly its influence as a confounding factor of individual response to dietary supplementation of phytosterols and omega-3 fatty acids alone or in combination.

In conclusion, both omega-3 fatty acids and phytosterols are effective in improving specific lipids and lipoproteins in a hyperlipidemia state. Despite our inability to draw any conclusions regarding the relationship or interaction between apoE polymorphism and response to supplementation, the genotype is an important determinant of responsiveness to dietary intervention and warrants further investigation.

Chapter 6

GENERAL DISCUSSION & FUTURE DIRECTION

Hyperlipidemia rarely exists in isolation and is usually accompanied by increases in inflammation. The hypolipidemic properties of both phytosterols and omega-3 fatty acids have been well documented. Dietary supplementation with phytosterols reduces the circulating concentration of total-cholesterol and LDL-cholesterol, albeit with no significant effect on HDL-cholesterol or triglycerides. Conversely, there is ample evidence to demonstrate the triglyceride lowering, as well as anti-aggregatory, anti-inflammatory and anti-thrombotic potential of omega-3 fatty acids. In addition several studies have also shown the HDL-cholesterol is elevated following dietary supplementation with omega-3 fatty acids.

The hypothesis underlying this thesis is that the combined supplementation of phytosterols and omega-3 fatty acids may provide clinically significant improvements in plasma lipid profile and inflammatory status, in individuals with combined hyperlipidemia. The proposed dietary combination is both novel and efficacious and has the potential to improve the cardiovascular risk profile of hyperlipidemic individuals, resulting in improved quality of life and reduced burden of cardiovascular related disease (Figure 6.1).

Diet has a substantial effect on the progression of atherosclerosis and CVD. For example, dietary sources of phytosterols and omega-3 fatty acids, such as nuts, seeds, fruits and vegetables, fish, and vegetable oils, are important components of a healthy diet, as they contribute towards lipid metabolism and excretion pathways, eicosanoid production, cell membrane phospholipid composition and gene expression [397]. In this thesis it is demonstrated that a significant improvement in plasma lipid profile and inflammatory mediators, providing an overall reduction in cardiovascular risk. These findings confirm our hypothesis, that the combination of phytosterols and omega-3 fatty acids can indeed improve major cardiovascular risk factors in hyperlipidemia.

In chapters three and four, the cardiovascular benefits from concurrent supplementation with phytosterols and varying types of omega-3 fatty acids, show significant improvements in total- and LDL-cholesterol, triglycerides and HDL-cholesterol. In some cases (i.e. triglycerides and HDL-cholesterol), reductions were somewhat comparable to that found with pharmacotherapeutic drugs.

In addition, it is demonstrated that a synergistic reduction in total- and LDL-cholesterol, following the combination of DHA-rich fish oil and phytosterols and a complementary reduction in LDL-cholesterol, following EPA-rich fish oil and phytosterol combination. However, this combination did provide a reduction in LDL-cholesterol, albeit non-significant. These findings are interesting and challenge our current thinking on two levels.

Firstly, it suggests that the mechanisms, by which omega-3 fatty acids and phytosterols provide their hypolipidemic effect, may be interactive or potentially improve one-another's efficiency. It is likely that omega-3 fatty acids replace cholesterol from micelles, more efficiently compared to SFA, MUFA or omega-6 fatty acids, resulting in enhanced reduction in cholesterol absorption. Phytosterols are absorbed, albeit inefficiently from the gastrointestinal tract, therefore, long-term supplementation may result in considerable increases in plasma phytosterol concentration and may influence metabolic pathways, other than simply reducing cholesterol absorption. However, to date, no such evidence has been presented in the literature. Any interactive influence of phytosterols and omega-3 fatty acids following absorption from the gut on haemostatic factors, inflammatory mediators and eicosanoid metabolism, remains to be examined.

Secondly, our findings highlight the differential impact of EPA-rich and DHA-rich fish oil combined with phytosterols, on lipid and lipoprotein fractions. Growing evidence supports the notion that EPA and DHA may have specific, yet partly different effects on lipid metabolism. These differences may be important in the subsequent utilization and metabolism of both EPA and DHA *in vivo* [398, 399].

Although the majority of literature focuses on the impact of co-feeding EPA and DHA, a number of studies have also compared their differential effects on plasma lipid response. Based on the information obtained from the clinical interventions described in this thesis, a direct comparative analysis between the EPA-rich and DHA-rich fish oils combined with phytosterols, was undertaken. As can be seen in table 6.1, each of the six treatment groups had a similar demographic (mean ± SEM age, 56.2 ± 2.0 years; BMI, 26.4 ± 1.0 kg/m^2; W:H, 0.85 ± 0.01), body composition (FM, 33.5 ± 2.0 %; FFM, 66.3 ± 2.0), and blood pressure (SBP, 133.9 ± 3.9mm Hg; DBP, 82.5 ± 2.7mm Hg) at baseline.

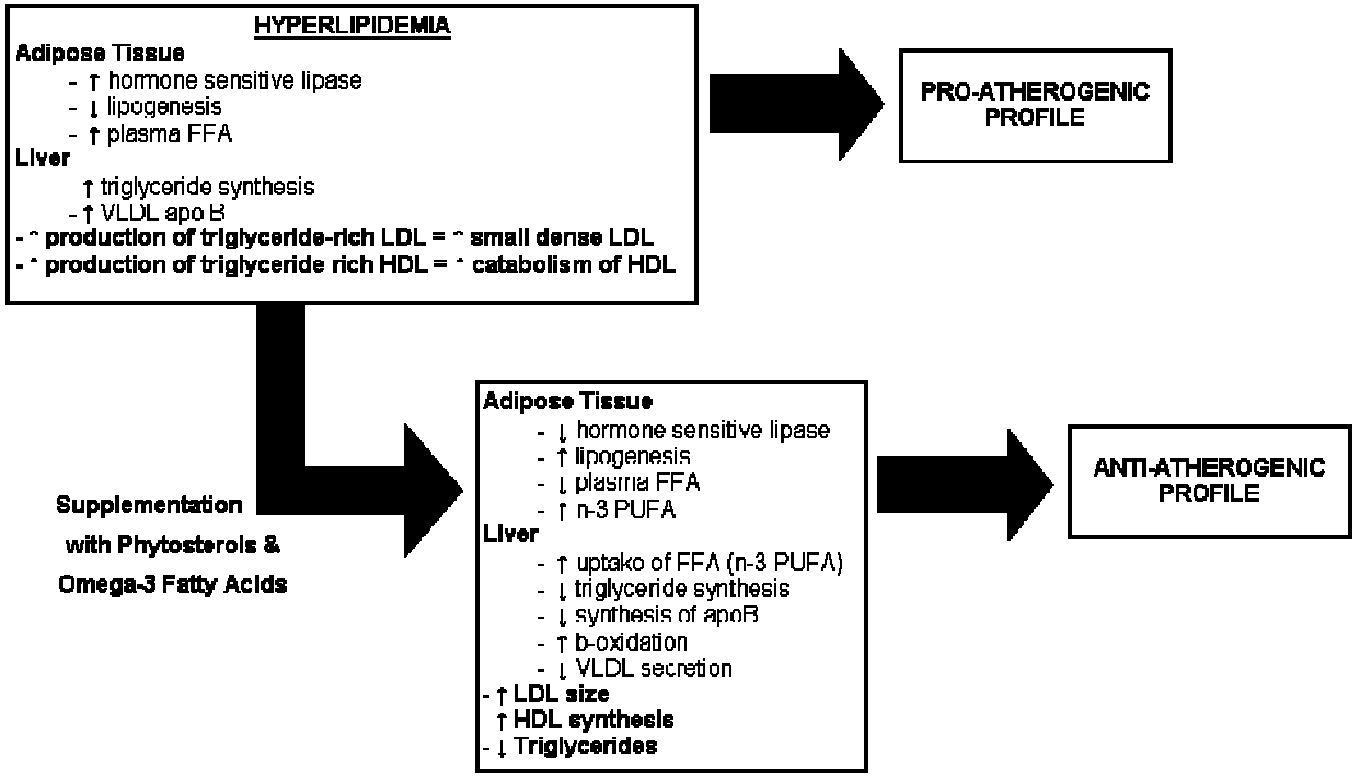

Figure 6.1 *The mechanism by which hyperlipidemia provides a pro-atherogenic environment. Concurrent dietary supplementation with phytosterols and omega-3 fatty acids optimise the lipid profile, thereby creating an anti-atherogenic environment [400].*

Upon direct comparison, it was found that the combination of DHA-rich fish oil with phytosterols, improved plasma lipid status, in particular, total-cholesterol, LDL-cholesterol and triglyceride concentration, more so than the combination of EPA-rich fish oil and phytosterols (Figure 6.2-6.5). Although the difference between the two fish oils was not significant, DHA did provide the greatest overall optimization in lipid profile. Sunola oil supplementation did not alter plasma lipid concentration and other fatty acid concentrations; therefore its use as a placebo was justified.

Neither EPA nor DHA alone significantly affected total-cholesterol or LDL-cholesterol concentration. The consumption of EPA-rich fish oil provided an LDL-cholesterol lowering effect, however DHA-rich fish oil had a LDL-cholesterol elevating effect. This is a striking finding, considering the relatively modest DHA intake. Results from the GISSI study showed a 3-5% elevation in LDL-cholesterol concentration with 0.85g/day ethyl esters of DHA and EPA, however these findings were confounded by the use of statins throughout the study [401]. Higgins *et al* [402] failed to show a significant change in plasma LDL-cholesterol concentration, following 0.3, 0.6 and 0.9g/day omega-3 fatty acid supplementation for 16 weeks. Our observation of a 5% increase in LDL-cholesterol, albeit non-significant, is not likely to be due to chance, because the study had sufficient power to detect the observed change. This observation is supported by others [327]. Minihane *et al* [264] suggests that the increase in LDL-cholesterol is greatest in men carrying the apoE4 allele, but not significant in men who are homozygous for the apoE3 allele. In the present thesis, LDL-cholesterol concentration increased following DHA supplementation in both men and women (data not shown), who were homozygous for the apoE3 allele.

DHA generally increases LDL-cholesterol levels and this increase is generally proportional to an increase in the size of the LDL-cholesterol, which may represent a shift to a less atherogenic LDL particle. Unfortunately in this study, LDL molecule size was not measured, and therefore are not able to conclude whether a reduction in overall LDL-cholesterol, as opposed to a reduction in LDL particle size, is more beneficial to overall cardiovascular risk profile.

Table 6.1 *Participant baseline demographics, body composition and blood pressure*[1]

	Placebo	Fish oil (EPA-rich)	Fish oil (DHA-rich)	Phytosterol	EPA + Phytosterol	DHA + Phytosterol
N	29	15	15	29	15	15
Age (years)	54.1 ± 2.6	57.4 ± 1.9	56.6 ± 2.0	56.6 ± 2.2	59.9 ± 1.2	52.6 ± 2.2
Gender (M/F)	10/19	5/10	6/9	14/15	6/9	7/8
Body weight (kg)	75.0 ± 3.3	79.2 ± 2.8	73.5 ± 4.0	77.8 ± 3.1	77.9 ± 5.8	78.9 ± 3.4
Height (m)	1.5 ± 1.8	1.2 ± 1.1	1.6 ± 0.02	1.4 ± 0.5	1.2 ± 0.03	1.7 ± 0.02
BMI (kg/m^2)	25.7 ± 0.9	26.7 ± 0.9	26.4 ± 1.3	26.5 ± 1.1	25.9 ± 1.4	27.2 ± 0.9
Waist circumference (cm)	89.5 ± 2.9	89.7 ± 2.0	88.2 ± 3.1	90.4 ± 2.7	92.3 ± 4.7	95.6 ± 2.7
Hip circumference (cm)	97.7 ± 2.4	100.6 ± 2.5	98.5 ± 2.8	98.5 ± 3.2	100.6 ± 2.4	99.8 ± 2.5
Waist-to-hip ratio	0.8 ± 0.02	0.8 ± 0.02	0.8 ± 0.02	0.9 ± 0.01	0.9 ± 0.03	0.9 ± 0.01
Fat mass (%)	33.9 ± 1.6	35.3 ± 2.2	32.3 ± 2.4	31.7 ± 2.4	33.9 ± 1.8	34.2 ± 1.7
Fat free mass (%)	66.1 ± 1.6	64.6 ± 2.2	67.6 ± 2.4	68.0 ± 2.4	66.1 ± 1.8	65.8 ± 1.7
SBP (mm Hg)	132.1 ± 3.1	135.2 ± 3.8	133.0 ± 4.0	134.0 ± 3.5	137.9 ± 5.7	131.4 ± 3.8
DBP (mm Hg)	79.4 ± 3.9	83.2 ± 2.4	83.3 ± 2.6	82.5 ± 2.3	84.9 ± 2.9	82.2 ± 2.5
Heart rate	69.5 ± 2.8	67.1 ± 3.6	71.4 ± 2.3	67.0 ± 2.6	67.3 ± 2.3	66.5 ± 2.3

[1]Values are reported as mean ± SEM. There were no significant differences between groups (one-way ANOVA).

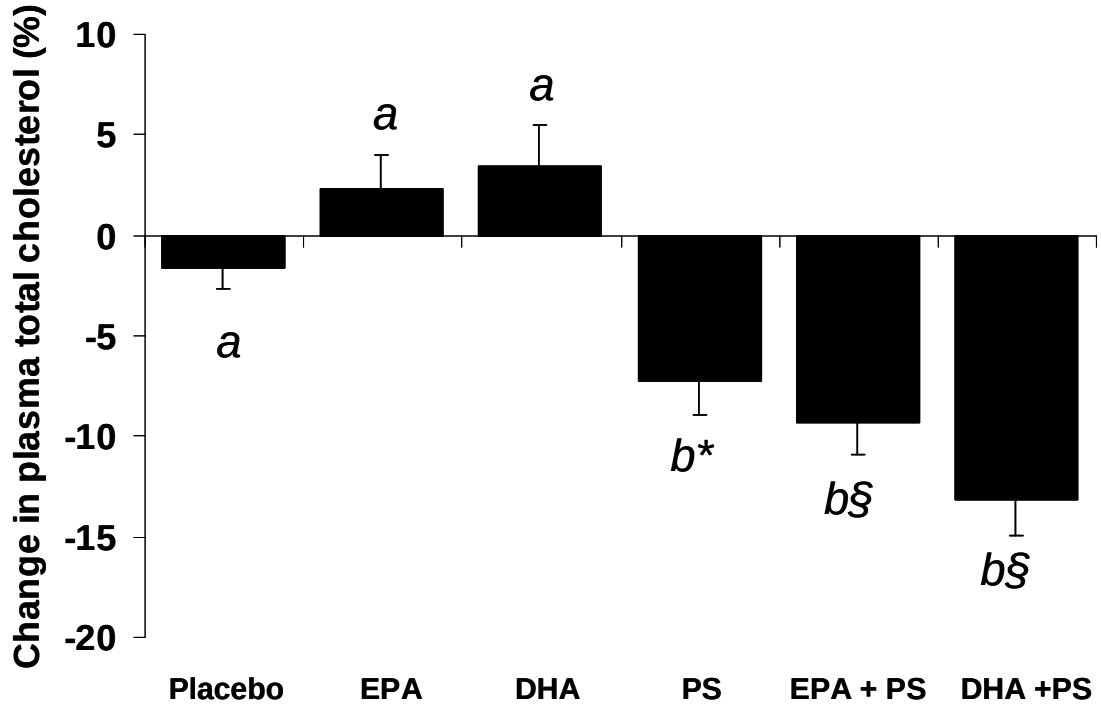

Figure 6.2 *Change (%) from baseline in plasma total-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), EPA-rich fish oil (EPA), DHA-rich fish oil (DHA), 2g/day phytosterols (PS), EPA + PS, or DHA + PS. Values are mean ± SEM. Wilcoxon signed-rank test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. ANOVA was used to test for between-group differences. Bars without a common letter differ, P < 0.05. PS, phytosterol*

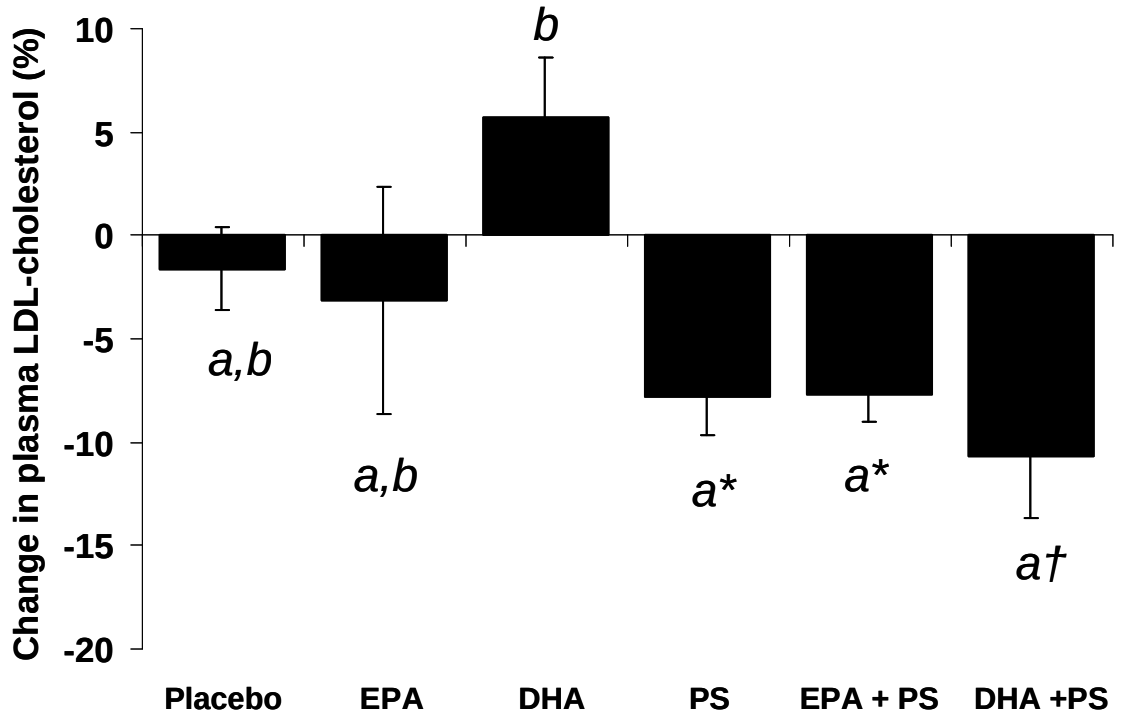

Figure 6.3 *Change (%) from baseline in plasma LDL-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), EPA-rich fish oil (EPA), DHA-rich fish oil (DHA), 2g/day phytosterols (PS), EPA + PS, or DHA + PS. Values are mean ± SEM. Wilcoxon signed-rank test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. ANOVA was used to test for between-group differences. Bars without a common letter differ, $P < 0.05$. PS, phytosterol*

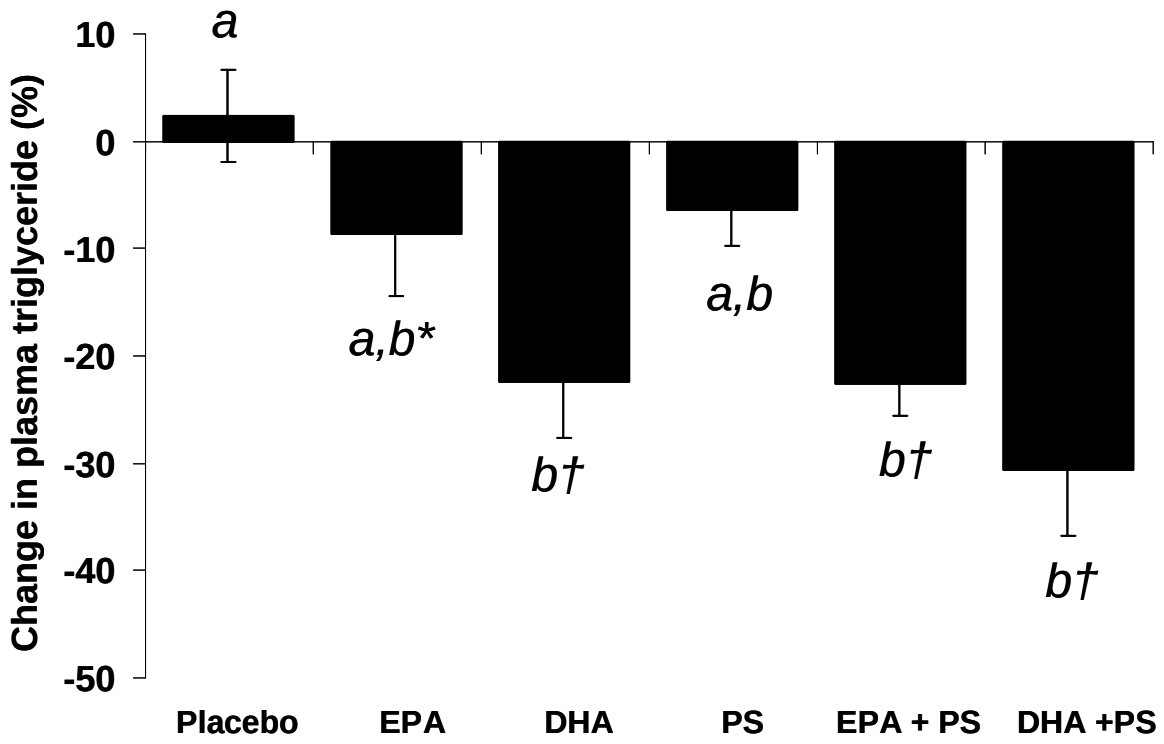

Figure 6.4 *Change (%) from baseline in plasma triglyceride, in hyperlipidemic subjects consuming sunola oil (Placebo), EPA-rich fish oil (EPA), DHA-rich fish oil (DHA), 2g/day phytosterols (PS), EPA + PS, or DHA + PS. Values are mean ± SEM. Wilcoxon signed-rank test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. ANOVA was used to test for between-group differences. Bars without a common letter differ, P < 0.05. PS, phytosterol*

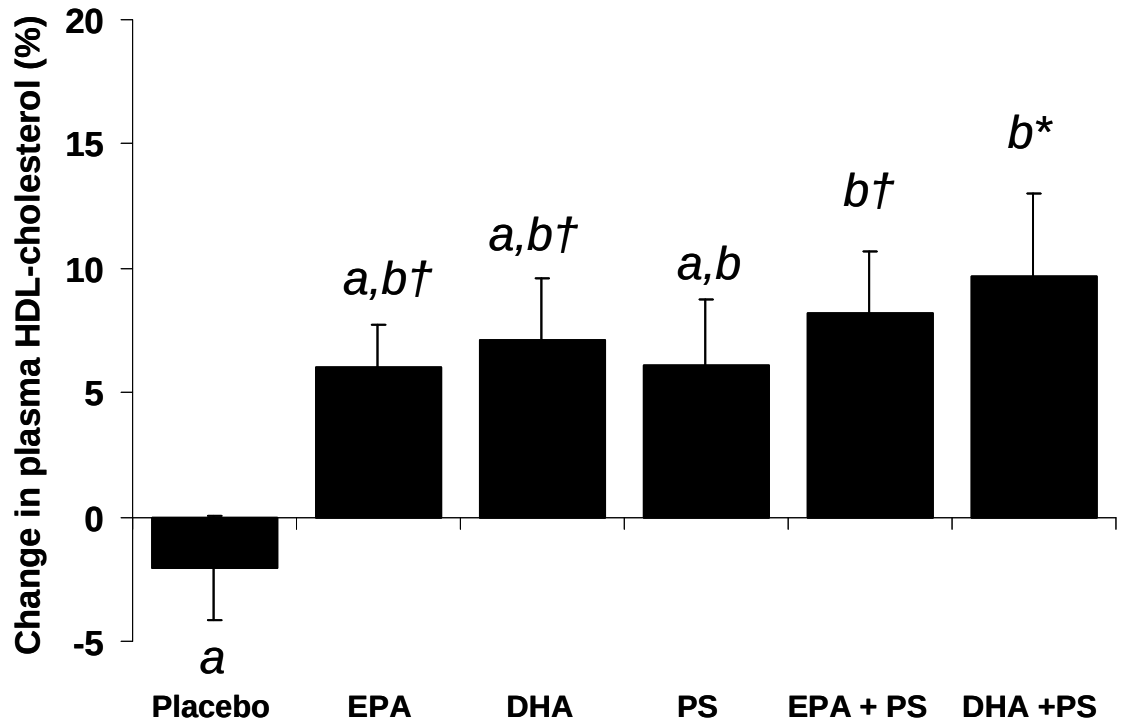

Figure 6.5 *Change (%) from baseline in plasma HDL-cholesterol, in hyperlipidemic subjects consuming sunola oil (Placebo), EPA-rich fish oil (EPA), DHA-rich fish oil (DHA), 2g/day phytosterols (PS), EPA + PS, or DHA + PS. Values are mean ± SEM. Wilcoxon signed-rank test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. ANOVA was used to test for between-group differences. Bars without a common letter differ, $P < 0.05$. PS, phytosterol*

Both EPA and DHA significantly reduced triglyceride concentration, both alone and in combination with phytosterols. The greatest reduction in triglycerides was provided by the consumption of DHA-rich fish oil (~ 22%). In support of our findings, a four week supplementation trial (4.8g/day EPA and 4.9g/day DHA, in triglyceride form) in normolipidemic adults, showed a 15% (P=0.2) and 22% (P=0.03) reduction in fasting triglyceride concentration [358]. Participants with combined hyperlipidemia consuming 1.25 or 2.5g/day of a DHA supplement administered as a triglyceride form, compared to a control oil (vegetable oil), also showed significant reductions in triglyceride concentration and significant increases in LDL-cholesterol concentration [403].

In another study using purified ethyl ester concentrates of EPA and DHA, of 3.8 and 3.6 g/day, respectively, for seven weeks, a reduction in triglyceride concentration of 26% and 21%, respectively was found [359]. Also supporting these findings is a six week supplementation trial of 4g/day EPA or DHA (ethyl esters), showing reductions in triglyceride concentration of 18% and 20%, respectively [232]. In a study by Hansen *et al* [369], five weeks consumption of an EPA and DHA supplement (4g/day ethyl ester form), significantly reduced triglyceride concentration (19% and 49% respectively). There generally appears to be a lack of convincing evidence pinpointing whether EPA or DHA is responsible for the hypotriglyceridemic properties of fish oil, and furthermore whether the form it is administered in (triglyceride or ethyl ester) is more efficacious.

Whilst the supplementation of fish oil has undoubtedly shown to be responsible for various cardiovascular health benefits, it is only the recent availability of pure forms of EPA and DHA, which provide avenues to better clarify the physiological role of these fatty acids. Investigations demonstrate that supplementation with fish oils, consistently show increases in both EPA and DHA plasma concentration, in a somewhat dose-dependent manner [404-407]. When comparing the plasma concentration of fatty acids from studies described in chapter three and chapter four, it was found that significant increases in both EPA and DHA concentration, following their respective supplementation (Figure 6.6). EPA supplementation increased plasma EPA, and to a lesser extent DHA. DHA supplementation increased plasma DHA, as well as plasma EPA concentration, suggesting a possible retro conversion of DHA to EPA.

These findings agree with those of previous reports which suggest that ≈ 9% of dietary DHA is retro converted to EPA [198, 359, 408]. Previous studies have described an apparent saturable increase in DHA concentration, at a dose of 1.2g/day, following combined fatty acid supplementation, however a saturation level for EPA is yet to be defined [224]. Supplementation with DHA generally results in a dose dependent, saturable increase in plasma DHA concentration and a modest increase in EPA concentration. This response pattern was evident in our study (Figure 6.6). Plasma DHA concentrations equilibrate in approximately three to four weeks and then remain at a steady level throughout supplementation. Hence the reason this study was a three week trial. DHA doses of ~ 2g/day results in a near maximal plasma DHA response (normal range is 1.5 – 7.5% of total fatty acids) [224].

The lipid and lipoprotein response to increased EPA and DHA consumption is highly heterogeneous, with genetic variability thought to be largely responsible. Here, the importance of inter-individual response to phytosterol and omega-3 fatty acid supplementation has been explored and discussed with reference to apoE genotype in this thesis. ApoE has been shown to be a determining factor for the plasma lipid-lowering and carotenoid depletion following phytosterol supplementation [265]. Chapter five explores the influence of apoE genotype in our study cohort. A relationship between apoE polymorphism and baseline fasting lipids was not observed in subjects with a combined hyperlipidemic profile. The studies in this thesis were not powered to detect differences in response between genotypes, and further research using a much larger sample size is required to establish whether there is a difference in response according to apoE genotype.

Undertaking dietary supplementation trials in hyperlipidemic populations does have its difficulties, due to several practical constraints specific to this population. Firstly, in order to observe the therapeutic benefits of the dietary supplementation studied in this thesis, it is important that participants are not taking lipid-lowering pharmacotherapeutics, which may interfere with results; this however is difficult in a hyperlipidemic population. Furthermore, studies manipulating fatty acid intake are also difficult, as many hyperlipidemic individuals already take fish oil supplements routinely.

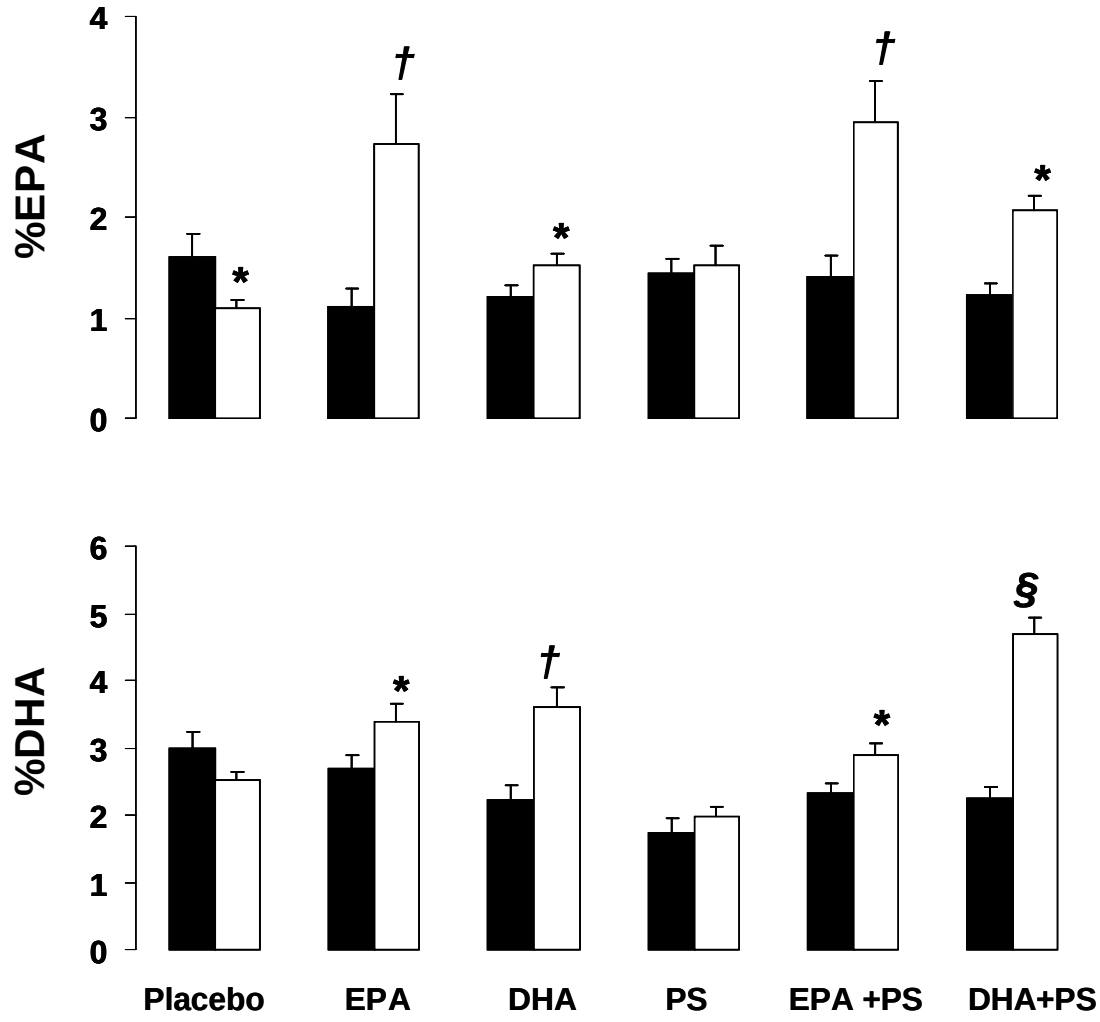

Figure 6.6 *Plasma concentration (% of total fatty acids) at baseline (■) and post intervention (□) of EPA and DHA in hyperlipidemic subjects consuming sunola oil (Placebo), 3g/day EPA-rich fish oil (EPA), 4g/day DHA-rich fish oil (DHA), 2g/day phytosterols (PS), EPA+PS, or DHA PS. Values are mean ± SEM. Students-t test was used to test within group changes from baseline, $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\S}P<0.001$. ANOVA was used to test for between-group differences. Bars without a common letter differ, $P<0.05$.*

Luckily however, in Australia the dietary omega-3 fatty acid consumption and phytosterol consumption tend to be low [212]. Lastly, there are ethical issues to consider when attempting to trial alternative dietary therapies. A randomised controlled trial using a true placebo group was not possible, particularly for our phytosterol group. It is acknowledged that it would have been unethical to warrant the consumption of a control fat spread in a hyperlipidemic population.

Dietary data from the studies described in chapter three and chapter four were collected using the 24-hour recall method [409]. While this method is reasonably well established for determining average intake levels of groups, the limitations of a method relying on subject recall and a short time frame are acknowledged. It is also acknowledged that analysis of dietary records does not provide definitive information on macro- and micronutrient intake, as well as fatty acid intake.

The majority of biochemical analyses presented in this thesis was applied to plasma samples, as opposed to red blood cells. While much useful information has been derived from examining RBC, plasma analysis is more indicative of the immediate/current changes in circulating fatty acid concentration, which was evaluated in this thesis. Others have reported a strong correlation between the combined concentration of EPA and DHA in RBC and plasma, whilst others report significant correlations between concentrations measured in cheek cells and plasma [410, 411]. Because plasma is highly responsive to dietary intake of omega-3 fatty acids, circulating tissue fatty acid concentration was measured. The accumulation and retention of both EPA and DHA are also related to the lipid moieties in which these fatty acids are stored. EPA is equally distributed among neutral lipids (sterol esters and triglycerides) and phospholipids, whilst DHA is predominantly carried within phospholipids [412-414]. Hence only small amounts are present as a nonesterified free fatty acid form [415].

In our study, each participant was randomly assigned to one of the dietary supplementation groups, and each subject served as their own control. The benefit of this design is the limitation of between individual variations so that the main comparison was made between the mean parameter values from baseline to post intervention (three weeks).

The dose period of three-weeks was considered to be sufficient to observe the effects of the dietary treatment. In earlier studies, it has been shown that phytosterols reduce cholesterol concentration within 2-3 weeks of commencing supplementation [416, 417]. Similarly, DHA concentration reaches its saturable point after 3-4 weeks of supplementation. In our study, the mean baseline DHA concentration was 2.55% of total fatty acid concentration, which was somewhat lower than data from Arterburn *et al* [224], suggesting that the mean fasting DHA concentration in healthy adults not taking fish oil supplements is 3.5% of total fatty acids (range is 1.5 – 7.5%). Given that in our study, DHA concentration was significantly lower, this may have influenced the responsiveness of our cohort to omega-3 fatty acid supplementation. Clinical data has shown concentrations reaching 12% for DHA, following chronic consumption (1.50 - 1.62g/day) [198, 418].

Should further research be successful in establishing a definite role for the combined supplementation of phytosterols and omega-3 fatty acids in hyperlipidemia, it would be desirable to develop a single functional food incorporating both functional ingredients for ease of consumption and improved compliance. However, there are technological difficulties in achieving this due to limited solubility of phytosterols and oxidising potential of omega-3 fatty acids, particularly at dose levels required to optimise blood lipids and influence inflammatory and aggregatory pathways. Further research is needed to successfully incorporate phytosterols and omega-3 fatty acids into food matrices and to develop industrial processes to optimise the retention of these ingredients in the final products.

It would also be advantageous to explore the functionality of other phytosterol-enriched foods, in conjunction with omega-3 supplementation, such as the phytosterol-enriched yoghurt or milk products currently available on the Australian consumer market. However, the hypocholesterolemic effects provided by phytosterols may be reduced or lessened, if provided in a low-fat product, given that the mechanism by which phytosterols exert their cholesterol-lowering properties, involves the stimulation of micelles from fat ingestion. Whether the administration of phytosterols in low-fat products changes the efficacy of phytosterols, is not entirely understood, however it is plausible that the time of consumption or intake with a meal, may affect efficacy.

Conversely, efficacy of phytosterol-enriched margarine spreads was shown to be similar irrespective of the frequency of consumption, highlighting that dose is the main contributing factor towards phytosterol efficacy [419]. Whilst the long-term effect of phytosterols on atherosclerosis or coronary artery disease are yet to be investigated, the metabolic steady state in plasma cholesterol concentration is usually attained after 3-4 weeks of continued phytosterol supplementation and cholesterol levels return within 2-3 weeks of cessation [420, 421]. The studies discussed in this thesis were 3 weeks in duration, as this is the optimal time to observe changes in cholesterol and LDL-cholesterol concentrations.

As well as reducing plasma lipid profile, supplementation with phytosterols and omega-3 fatty acids have also demonstrated an anti-inflammatory effect. This occurs, due to the nature of the eicosanoids and inflammatory proteins produced from these fats, having a subdued inflammatory response in comparison to those produced from omega-6 fatty acids or SFA. Hence, the cardiovascular benefits provided by the combined supplementation of phytosterols and omega-3 fatty acids, may be enhanced further by a diet containing higher proportions of MUFA, also shown to reduce inflammatory susceptibility of lipids and lipoprotein sub fractions [422, 423].

In conclusion, the combination of phytosterols and omega-3 fatty acids offer lipid-lowering effects but also provide anti-aggregatory and anti-inflammatory improvements, resulting in greater cardiovascular risk reduction in individuals with combined hyperlipidemia (Figure 6.7). Our main hypothesis assumed a simple relationship between the consumption of phytosterols and omega-3 fatty acids, and their potential therapeutic role in hyperlipidemia.

The data described in this thesis reveals a more complex state, highlighting the important differences between the individual fatty acids, EPA and DHA, and also emphasizing the complexity of hyperlipidemia with its concurrent presence of multiple abnormalities. Given that treatment modalities for hyperlipidemia have not changed extensively over time, an exploration and investigation of dietary combination therapies is warranted. The efficacy of combining phytosterols and omega-3 fatty acids to offer greater cardiovascular benefits than either of the supplements alone has been explored.

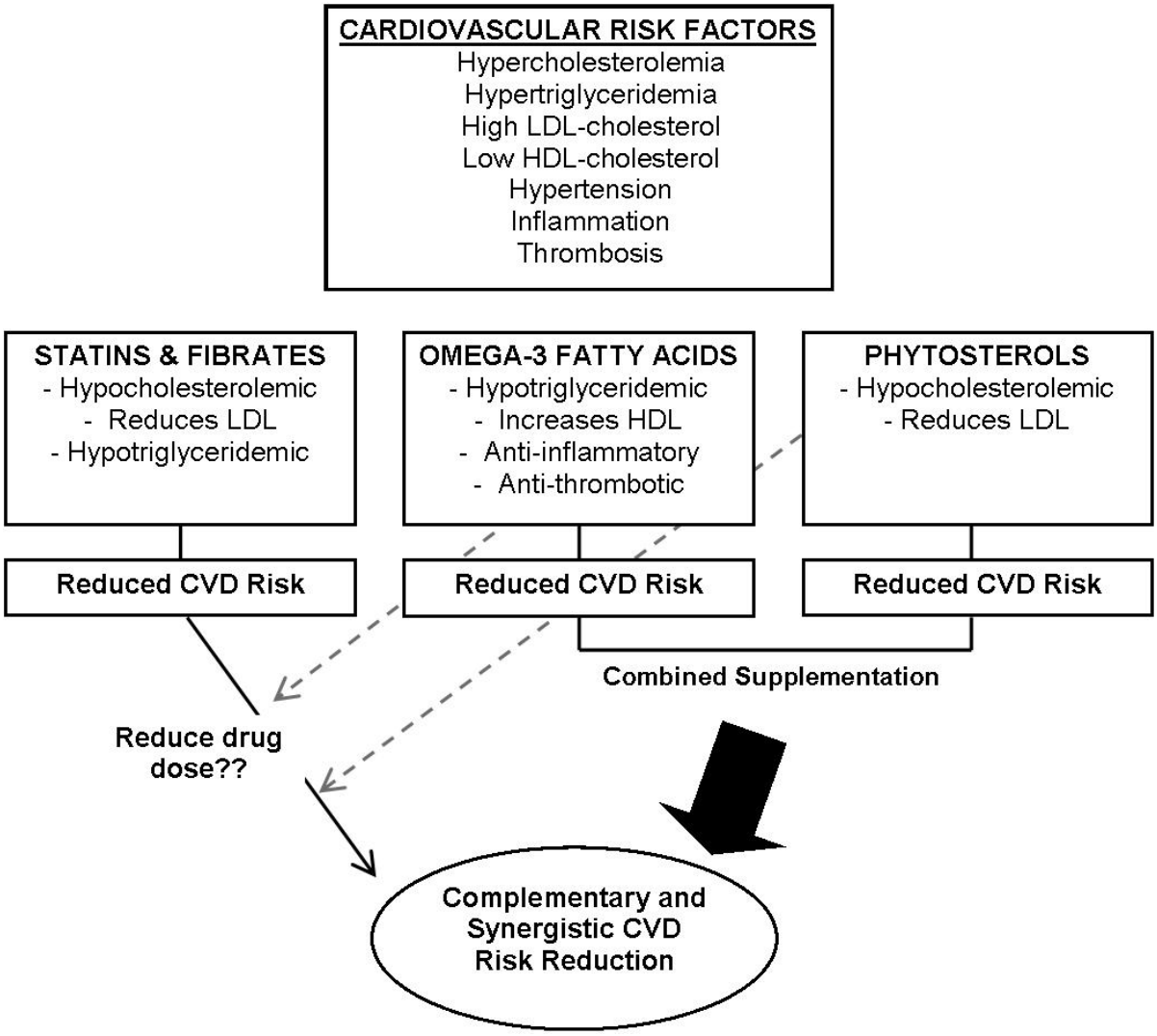

Figure 6.7 *The consumption of omega-3 fatty acids and phytosterols (± statins) may reduce CV risk, via generation of an anti-atherogenic profile [400].*

The relevance of these findings needs to be confirmed in other high risk populations (e.g. diabetics, metabolic syndrome). This thesis demonstrates an efficacious effect on overall cardiovascular risk profile in a hyperlipidemic population, following phytosterol and omega-3 fatty acid supplementation. Our observation indicates that DHA-rich fish oil is as effective as EPA in reducing plasma lipid profile and inflammatory markers, however further studies are needed to establish the possible separate effects on lipid metabolism between the two major fatty acids.

Furthermore, findings from this thesis contribute to our current understanding of the significance and potential use of dietary therapeutics in human disease, however the effect of combined phytosterols and omega-3 fatty acid supplementation on the development and progression of cardiovascular risk factors needs to be verified by further investigation and long-term intervention trials.

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Appendices

Appendix 1: Medical Questionnaire

The UNIVERSITY *of* NEWCASTLE

Lipid lowering potential of combined phytosterols and long chain omega-3 fatty acids
Investigators: Professor Manohar Garg, Miss Michelle Micallef

Pre-trial Medical Questionnaire - Page 1 of 2

Participant ID Number: _____ Date: __ / __ / ____

Date of Birth: __ / __ / ____ Height (cm): _____

Sex: Male / Female Weight (kg): _____

1. ***Please list all current medical conditions (conditions for which you are presently receiving treatment or advice).***

Condition:	_____	Condition:	_____
Year Diagnosed:	_____	Year Diagnosed:	_____
Condition:	_____	Condition:	_____
Year Diagnosed:	_____	Year Diagnosed:	_____

2. ***Have you ever had*** *(please circle)*

A stroke?	Yes / No
A heart attack?	Yes / No
Angina?	Yes / No
Other heart-related problems?	Yes / No
Angioplasty?	Yes / No
By-pass surgery?	Yes / No
Other heart/vascular surgery?	Yes / No

3. ***Please list all medications that you take as prescribed by a doctor.***

Brand Name:	_____	Brand Name:	_____
Dose:		Dose:	
Frequency:	_____	Frequency:	_____

4. Please list all over-the-counter medications you take regularly.

Brand Name:	_____	Brand Name:	_____
Dose:		Dose:	
Frequency:	_____	Frequency:	_____

5. Please list all vitamin, mineral, and/or herbal supplements you take regularly.

Brand Name:	_____	Brand Name:	_____
Dose:		Dose:	
Frequency:	_____	Frequency:	_____

6. Please indicate the number of alcoholic beverages you normally consume per week *(please circle)* 0 1-3 4-7 8-10 >10

Do you smoke? Yes / No

7. Please describe how much exercise you normally conduct each week *(please circle)**?***

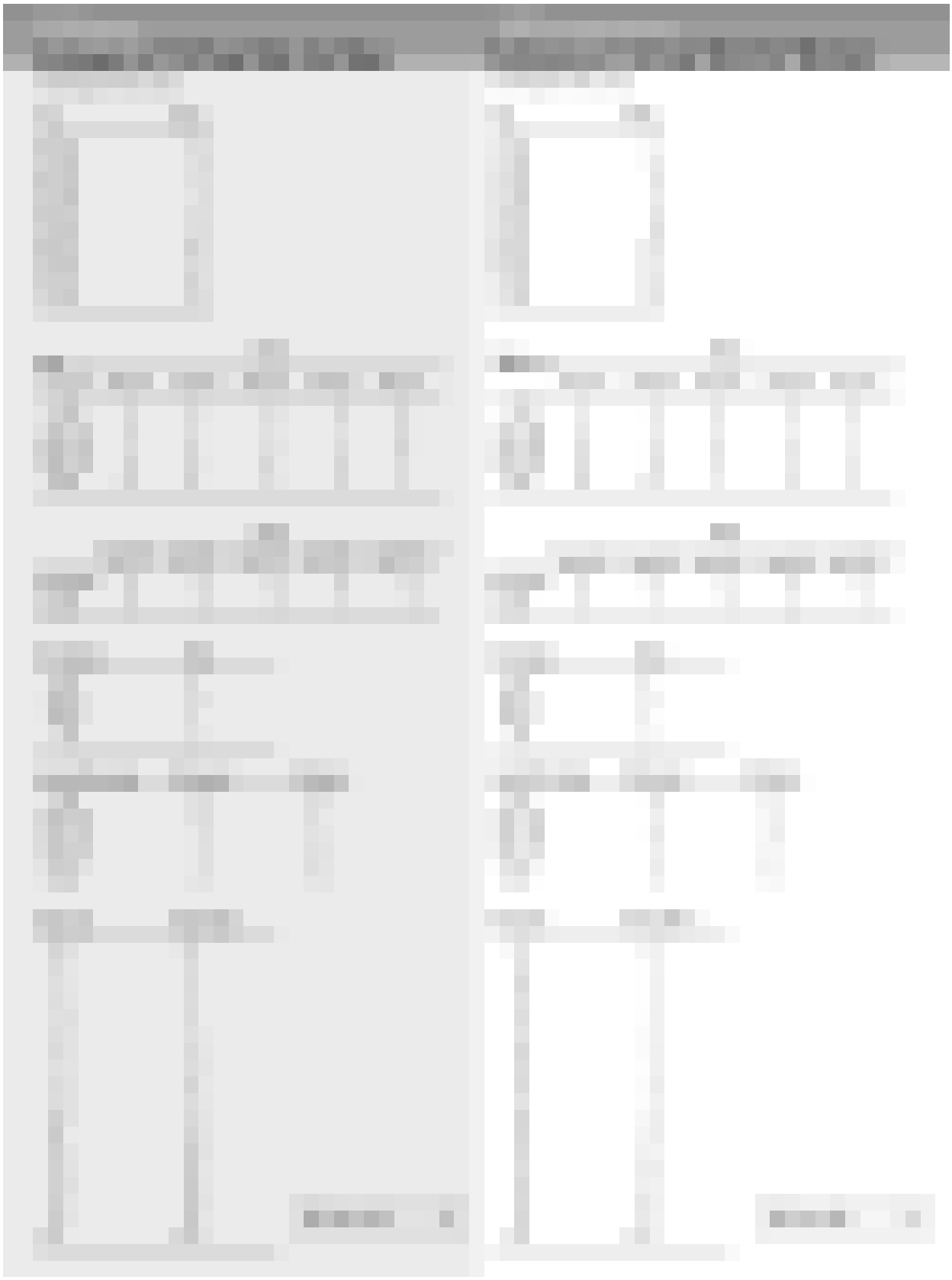
Light	Moderate	Heavy	Very Heavy

8. Please tell us how many minutes you exercised in the last 24-hours *(please circle)**?***

0 mins	1-15 mins	16-30 mins	> 30 mins

Thank you for taking the time to fill in this questionnaire.

Appendix 2: Cardiovascular Risk Assessment



Appendix 3: Food Recall (24-hour)

FUNCTIONAL FOODS AND BLOOD CHOLESTEROL STUDY

Date: _____ **Time:** _____

Before Breakfast

_____
_____

Breakfast

_____
_____
_____
_____

Snack

_____
_____
_____

Lunch

_____
_____
_____

Afternoon Snack

_____
_____
_____

Dinner

_____
_____
_____
_____
_____

Evening Snack

_____
_____

Would you consider this to be a typical day's food consumption? **Yes** **No**

Appendix 4: Participant Consent Form

Approval Number: H-291-0906

Consent Form "Lipid lowering potential of combined phytosterols and long chain omega-3 fatty acids"

Investigators: A/Professor Manohar Garg and Miss Michelle Micallef
Version 4; Dated: 13th October 2006

I give my consent to participate in the above research project designed to test the effectiveness of a phytosterol enriched spread and fish oil on plasma lipid profile. I understand that the following information has been provided to me in the Information Statement, a copy of which I have retained;

- I understand that my participation is **voluntary** and entirely my choice.
- I understand I can **withdraw** my consent for participation at any time and **do not** have to give any **reason** for withdrawing.
- I understand that I will consume one of following supplements for a three week period;
    - 25 grams of plant sterol rich margarine (Logical brand) per day plus 8 x 0.5 gram of monounsaturated fat (sunola oil) capsules per day; or
    - 8 x 0.5 g fish oil capsules per day; or
    - 25 grams of plant sterol rich margarine (Logical) plus 8 x 0.5g fish oil capsules per day; or
    - 8 x 0.5g monounsaturated fat (sunola oil) capsules per day
- I understand that I will donate blood on 2 occasions: 25 ml (after an overnight fast) at start of the study and another 25 ml at 21 days after consumption of the supplements.
- I understand that I do not need to change or stop any medications without prior consent of my doctor.
- I understand that my personal information will remain **confidential** to the researchers.
- Complete a medical questionnaire, provide a recall of foods/drinks consumed over 24-hours and allow body weight, height and body fat measurements.
- The outcome of this research **will be freely available** to me and will be posted to me at the completion of the study.

Signed by Participant	**Signed by Person Asking for Consent**
Print Name: _____	Print Name: _____
Signed: _____	Signed: _____
Date: / /	Date: / /

Appendix 5: Certification of Placebo and EPA-rich Fish Oil

Specification

Product: EPAX 5510 TG/N	Specification no.: 05510-20	Previous specification no.: N/A	
This edition approved: 260607	Previous edition approved: 070507	First edition approved: 060906	Shelf life/retest period: 00730 days
Prepared by: Kari Ann Paulsen Vik	Verified by: Per Arve Kleiven	Authorized by: Trine Hagen Lie	

Test	Min. value	Max. value	Unit	Method
Appearance Characteristics; bland, mild fish like			Clear oil	611059
Acid Value		1,0	mg KOH/g	611002
Colour		5	Gardner	611065
Peroxide value		3,0	meq/kg	611009
Anisidine value		20	-	611014
Total oxidation		20	.	611136
Absorbance 233 nm		0,60	AU	611047
Eicosapentaenoic acid C20:5 (TG)	500		mg/g	611129
Docosahexaenoic acid C22:6 (TG)	100		mg/g	611129
Total n-3 (7 TG) Total n-3: EPA, DHA, 18:3, 18:4, 20:4, 21:5, 22:5			mg/g	611129
Eicosapentaenoic acid C20:5 (FA)	480		mg/g	611129
Docosahexaenoic acid C22:6 (FA)	90		mg/g	611129
Total n-3 (7 FA) Total n-3: EPA, DHA, 18:3, 18:4, 20:4, 21:5, 22:5			mg/g	611129
Eicosapentaenoic acid C20:5	55		A%	611129
Docosahexaenoic acid C22:6	12		A%	611129
Total n-3 (7) Total n-3: EPA, DHA, 18:3, 18:4, 20:4, 21:5, 22:5			A%	611129
Saturated fatty acid			A%	611129
Monounsaturated fatty acids			A%	611129
Polyunsaturated fatty acids			A%	611129
Triglycerides	90		A%	611064
Oligomers		1,0	A%	611064
Mixed tocopherol	3,0	4,5	mg/g	611081

Specification

Product:	Specification no.:	Previous specification no.:	
EPAX 5510 TG/N	05510-20	N/A	
This edition approved:	**Previous edition approved:**	**First edition approved:**	**Shelf life/retest period:**
260607	070507	060906	00730 days
Prepared by:	**Verified by:**	**Authorized by:**	
Kari Ann Paulsen Vik	Per Arve Kleiven	Trine Hagen Lie	

Test	Min. value	Max. value	Unit	Method
Frequency analyses from date:			ddmmyy	.
The following frequency analyses are analysed 3 times pr. year with the latest results as reported below:				
As, Arsene		0,1	mg/kg	611500
Cd, Cadmium		0,01	mg/kg	611500
Hg, Mercury		0,005	mg/kg	611500
Pb, Lead		0,05	mg/kg	611500
DDT (sum: o,p' + p,p')		0,005	mg/kg	611500
DDD (sum: o,p' + p,p')		0,005	mg/kg	611500
DDE p,p'		0,005	mg/kg	611500
HCB		0,005	mg/kg	611500
PCBs		0,01	mg/kg	611500
IUPAC no. 28, 52, 101, 118, 138, 153, 180				
Benzo(a)pyrene		1,0	ng/g	611500
Dioxinlike PCBs (TE WHO)		3,0	pg/g	611516
Dioxins + furans (TE WHO)		1	pg/g	611516
CO for Dioxins: Result complies with Council regulation 2375/2001				
Dioxins + furans + dioxinlike PCBs (TE WHO)		4,0	pg/g	611516

Shelf life is guaranteed if product is stored in unopened original closed containers, protected from heat and light.

EPAX do not contain any components of GMO origin, IP certified tocopherols are added

Publications from This Thesis

Three articles removed from this section due to copyright and poor quality of scan.

1. Micallef,M.A & Garg, M.L (2009). “Beyond blood lipids: Phytosterol and omega-3 polyunsaturated fatty acid combination therapy for hyperlipidemia.” Journal of Nutritional Biochemistry, 20: 927-39.
http://dx.doi.org/10.1016/j.jnutbio.2009.06.009

2. Micallef,M.A & Garg, M.L (2009). “Anti-inflammatory and cardioprotective effects of n-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals.” Atherosclerosis, 204: 476-82.
http://dx.doi.org/10.1016/j.atherosclerosis.2008.09.020

3. Micallef,M.A & Garg, M.L (2008). Lipid-lowering effects of Phytosterols and (n-3) polyunsaturated fatty acids are synergistic and complementary in hyperlipidemic men and women.” The Journal of Nutrition, 138: 1086-90.
http://dx.doi.org/10.1093/qjmed/hcr007

British Journal of Nutrition (2009), **102**, 1370–1374
doi:10.1017/S0007114509382173

British Journal of Nutrition

Plasma *n*-3 polyunsaturated fatty acids are negatively associated with obesity

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(Received 3 October 2008 – Revised 19 March 2009 – Accepted 22 April 2009 – First published online 19 May 2009)

The objective of the present study was to investigate the relationship between plasma *n*-3 PUFA composition and weight status. A total of 124 adults, stratified by weight status: healthy weight (*n* 21), overweight (*n* 40) and obese (*n* 63) were recruited. Fasting blood samples, anthropometric measures and body composition were collected. Plasma fatty acid composition was determined by GC. BMI, waist circumference and hip circumference were inversely correlated with *n*-3 PUFA, EPA and DHA ($P<0·05$ for all) in the obese group. Obese individuals had significantly lower plasma concentrations of total *n*-3 PUFA, compared with healthy-weight individuals (4·53 (SD 1·11) *v.* 5·25 (SD 1·43) %). When subjects were pooled and stratified into quartiles of total *n*-3 PUFA, a significant inverse trend was found for BMI ($P=0·002$), waist circumference and hip circumference ($P=0·01$ and $P<0·001$ respectively). Higher plasma levels of total *n*-3 PUFA are associated with a healthier BMI, waist circumference and hip circumference. Our findings suggest that *n*-3 PUFA may play an important role in weight status and abdominal adiposity.

***n*-3 Fatty acids: Obesity: Lipids**

Obesity is a consequence of the excessive accumulation of fat in adipose tissue which can result in significant morbidity and mortality. Health problems associated with obesity include cardiovascular disorders such as hypertension, stroke and CHD, conditions associated with insulin resistance such as type 2 diabetes, and certain types of cancers[1,2]. A weight loss of between 5 and 10 % can substantially reduce these risks[3,4]; however, successfully maintaining weight loss, in the long term, is difficult[5]. Hence, effective strategies to improve adherence to weight loss and weight maintenance are needed[6].

The consumption of *n*-3 PUFA, namely EPA and DHA, have been linked to reduced CVD risk[7–9], and to reduced fasting glucose levels, providing a protective effect against the development of type 2 diabetes[10]. There is also continuing debate as to whether or not *n*-3 PUFA contribute to weight loss.

Dietary fatty acids are an important source of adipose tissue fatty acids and play a significant role in adipose tissue metabolism[11]. Intake of *n*-3 PUFA has been shown to influence the fatty acid composition of membrane phospholipids, thus modulating several metabolic processes that take place in the adipocyte[12–15]. Lipid management at the cellular level influences the degree of the development of disease and co-morbidities in obesity[16]. Indeed, abnormal *n*-3 PUFA metabolism in studies of obese children has been suggested[17–20]; therefore, not only the amount of dietary fat, but also the composition of dietary fat, plays an important role in adipose tissue metabolism and thus on body fat accumulation.

In the present study, we investigate the relationship between plasma *n*-3 PUFA concentration and various anthropometric measures in healthy-weight, overweight and obese adults. We hypothesise that plasma *n*-3 PUFA is associated with weight status, more specifically obesity. Perhaps *n*-3 PUFA could assist weight loss by complementing existing weight-loss approaches through their influence on biomarkers of obesity[13,21,22]. We also examine whether the contribution of *n*-3 PUFA concentration to covariates of body composition is independent of weight status.

Experimental methods

Participants

A total of 124 male and female free-living participants, aged 18–70 years were recruited from the university campus and the general community of Newcastle, Australia. Exclusion criteria for participation were: diagnosed diabetes mellitus; liver or other endocrine dysfunction; evidence of CVD, including angina or hypolipidaemic medication; chronic inflammatory disease; consumption of fish oil supplements; consumption of more than two fatty fish meals per week; on a restricted diet; BMI < 20 or > 40 kg/m^2; tobacco smoking. Further biochemical exclusion criteria included fasting glucose $> 6·8$ mmol/l (1225 mg/l).

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki; all procedures involving human subjects were approved by the Human Research Ethics Committee of the University of Newcastle, Australia. Written informed consent was obtained from all subjects.

Abbreviation: FM, fat mass.

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Anthropometry assessment

All anthropometric measurements were made with participants wearing light clothing and no shoes. BMI was calculated as body mass in kilograms (kg) divided by the square of height in meters (m) to the nearest 0·1 (kg/m^2) using a calibrated balance beam scale (PCS Measurement, NSW, Australia). Waist circumference was measured at the mid-point between the lowest rib and the top of the hipbone; the hip measurement was taken at the fullest point of the hip, as viewed from the side. The waist:hip ratio was calculated as waist girth in centimetres (cm) divided by hip girth (cm). Single-frequency bioelectrical impedance was used to determine fat mass (FM) and fat-free mass (Maltron International, Rayleigh, Essex, UK). Measurements were taken in the supine position following a > 10 h fast with no physical activity or alcohol consumption 24 h before testing. Calculations determined percentage FM ((FM/body weight) × 100)[23].

Plasma fatty acid analyses

Fasting (> 10 h) blood samples were collected into tubes precoated with EDTA by venepuncture. Samples were prepared by centrifuging for 10 min at 3000 *g* at 4°C. Plasma samples were collected and stored at −80°C until further analysis.

The fatty acid composition of plasma lipids was determined according to a modification in the method of Lepage & Roy[24], using an acetyl chloride methylation procedure. Fatty acid methyl esters were quantified using GC (Hewlett Packard 6890; Hewlett Packard, Palo Alto, CA, USA). The identity of each fatty acid peak was ascertained by comparison of the peak's retention time with the retention times of synthetic standards of known fatty acid composition (Nu Check Prep, Elysian, MN, USA). The relative amount of each fatty acid was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids. Concentrations of *n*-3 PUFA were calculated by summing the respective 18–22 carbon atom fatty acids (linolenic acid, EPA, docosapentaenoic acid and DHA). Fatty acid results are reported as percentage of total fatty acids.

Statistical analysis

Data are presented as mean values and standard deviations. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance, with no serious violations noted for anthropometric and body composition measurements. Variables that were not normally distributed were log-transformed before analysis. Comparisons between the different groups were made with one-way ANOVA and *post hoc* testing. $P<0·05$ was considered significant. Data were further explored with all weight-status groups pooled and stratified into quartiles of *n*-3 PUFA. All statistical analyses were carried out with SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Among the 124 adults, the average age was 49·5 (SD 10·7) years, with 37 % being male. Participants were stratified into weight status according to BMI (healthy weight 20–24·9 kg/m^2 (*n* 21), overweight 25–29·9 kg/m^2 (*n* 40) and obese 30–40 kg/m^2 (*n* 63)). Anthropometric characteristics of the three groups are presented in Table 1. The healthy-weight group had a significantly lower body weight, BMI, waist circumference, hip circumference and FM ($P<0·001$ for all) compared with the obese group. The overweight group had a significantly lower body weight, BMI, waist circumference, hip circumference ($P<0·001$ for all) and FM ($P=0·03$) compared with the obese group.

The correlation between plasma *n*-3 PUFA concentration and features of anthropometry were explored separately for each weight status. No significant correlation was observed for the healthy-weight and overweight groups (data not shown). Correlations between plasma *n*-3 PUFA concentration and BMI, waist circumference, hip circumference, waist:hip ratio and FM in the obese group were analysed (Table 2). Total *n*-3 PUFA, EPA and DHA were inversely correlated with BMI ($P=0·004$, $P=0·009$, $P=0·004$, respectively), waist circumference ($P=0·03$, $P=0·05$, $P=0·02$, respectively) and hip circumference ($P<0·001$, $P=0·009$, $P=0·002$, respectively).

When participants were stratified into quartiles according to total *n*-3 PUFA composition (thirty-one subjects per quartile (quartile 1: 3·4 (SD 0·06); quartile 2: 4·1 (SD 0·02); quartile 3: 4·9 (SD 0·06); quartile 4: 6·7 (SD 0·1)% total fatty acids)), a highly significant inverse trend was found for BMI ($P=0·002$), waist circumference ($P=0·01$) and hip circumference ($P<0·001$) (Fig. 1). No trends were found for waist:hip

Table 1. Subject characteristics
(Mean values and standard deviations)

	Healthy weight (*n* 21)		Overweight (*n* 40)		Obese (*n* 63)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	55·28[a]	8·56	49·87[a]	11·46	43·79[b]	12·22
Body weight (kg)	66·34[a]	9·23	80·54[b]	8·08	95·53[c]	14·50
BMI (kg/m^2)	23·12[a]	1·61	27·74[b]	1·56	33·59[c]	2·72
Waist (cm)	83·07[a]	9·37	94·05[b]	7·84	104·49[c]	8·86
Hip (cm)	91·36[a]	6·45	104·31[b]	8·18	118·63[c]	8·94
Waist:hip ratio	0·91	0·09	0·90	0·08	0·98	0·08
Fat mass (%)	33·81[a]	7·37	37·36[a]	7·38	40·90[b]	6·70
Fat-free mass (%)	66·57[a]	8·07	62·89[a]	7·90	59·10[b]	6·70

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

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Table 2. Associations between plasma *n*-3 PUFA (% of total fatty acids) and measures of anthropometry in obese subjects (Pearson's correlations)

	Total *n*-3 PUFA	Linolenic acid	EPA	DHA
BMI (kg/m^2)	−0·40*	−0·11	−0·32*	−0·36*
Waist (cm)	−0·27*	−0·01	−0·24*	−0·28*
Hip (cm)	−0·41*	−0·07	−0·32*	−0·38*
Waist:hip ratio	0·17	0·12	0·10	0·11
Fat mass (%)	−0·03	−0·12	0·14	−0·16

* $P<0·05$.

ratio ($P=0·50$) and FM ($P=0·16$). *Post hoc* analysis shows a significant difference between the lowest and highest quartiles for BMI ($P=0·004$), waist circumference ($P=0·03$) and hip circumference ($P=0·001$).

Discussion

In the present cross-sectional study, we observed significantly lower plasma concentrations of *n*-3 PUFA in obese men and women compared with healthy-weight individuals. The finding of the present study is that measures of weight status were correlated with plasma *n*-3 PUFA composition when participants were stratified into quartiles of total *n*-3 PUFA concentration.

Similar findings were reported in a study comparing serum phospholipid fatty acids with adipose tissue in twenty-five normal-weight and obese adolescents, which found that concentrations of *n*-3 PUFA were significantly lower in obese *v.* lean, age-matched, females[17]. Another study involving 120 normal-weight and overweight adolescents found that overweight adolescents had lower total *n*-3 PUFA and lower DHA concentrations compared with normal-weight adolescents, independent of body fat and fat distribution[25]. When dietary intake was also considered in a study of 134 age- and sex-matched normal-weight and overweight children, the BMI *z*-score of the obese children was negatively associated with plasma *n*-3 PUFA and DHA, despite obese children having higher intakes of the main fatty acid families, including PUFA[26].

Previous observational studies which also considered food intake indicate a negative association of fish consumption with central obesity measures[27]. In rodents, feeding fish oil-enriched diets have been shown to prevent abdominal fat accumulation compared with other types of dietary oils[28–30]. In human subjects, replacement of 6 g visible fat/d with 6 g fish oil/d for 3 weeks resulted in reduced fat mass and increased basal lipid oxidation[31]. A recent study has shown that the inclusion of lean fish, fatty fish or fish oil to a nutritionally balanced diet resulted in a greater weight loss within 4 weeks compared with diets devoid of seafood or marine supplements[32]. Another study which investigated the dietary intake of 132 children aged 4 years reported that a low *n*-3 PUFA intake was associated with higher body weight[33]. These studies, along with our observations, suggest that *n*-3 PUFA supplementation may play an important role in preventing weight gain and improving weight loss when *n*-3 PUFA are supplemented concomitantly with a structured weight-loss programme. Furthermore, inclusion of *n*-3 PUFA in a weight-loss programme may provide additional health benefits[8].

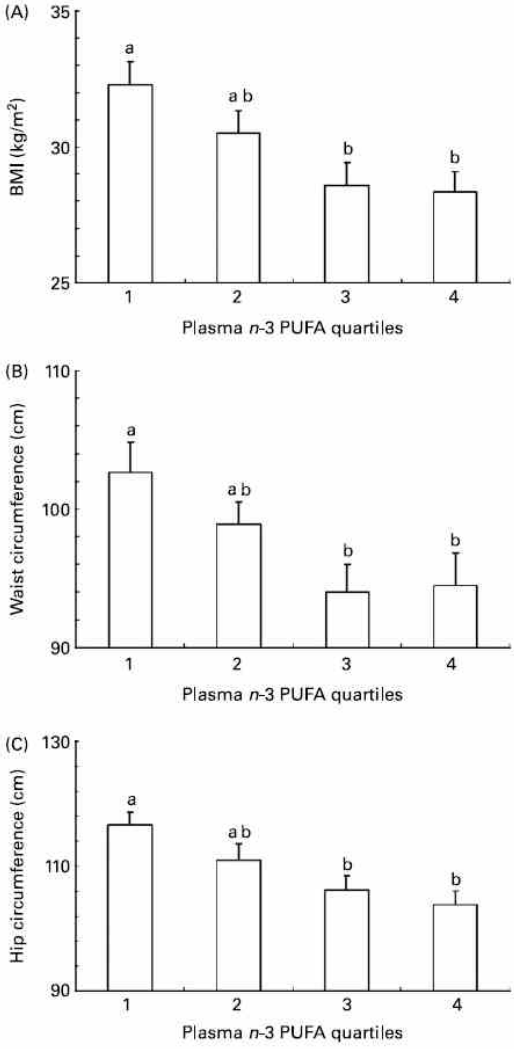

Fig. 1. Quartiles of plasma *n*-3 PUFA concentration for (A) BMI (kg/m^2), (B) waist circumference (cm) and (C) hip circumference (cm). Values are means (*n* 31 per quartile), with standard deviations represented by vertical bars. ^{a,b}Mean values with unlike letters were significantly different ($P<0·05$). For BMI, *P* for trend=0·002; for waist circumference, *P* for trend=0·01; for hip circumference, *P* for trend<0·001.

The results presented are biologically plausible because several mechanisms underlying the association between *n*-3 PUFA and obesity have been shown. One possibility is that *n*-3 PUFA could increase basal fat oxidation which may in turn reduce fat mass[9,31]. Animal studies have shown that *n*-3 PUFA supplementation may be associated with increased expression of mitochondrial uncoupling protein[34], a system of thermogenesis that can provide a defence against obesity. Furthermore, a recent study has shown that *n*-3 PUFA intake increases postprandial satiety in overweight and obese individuals during weight loss[35]. Fatty acids may interact with

neuroendocrine factors including insulin[36,37], ghrelin[38,39] and leptin[40–42] to modulate brain–intestinal loop signals for energy metabolism and appetite control. A recent study found that ghrelin is negatively correlated with body weight and total *n*-3 PUFA in normal-weight subjects[43], suggesting that *n*-3 PUFA can modulate appetite. Thus, the idea that fish oil can regulate weight status via improved appetite control along with a subsequent reduction in energy intake is plausible and worthy of further investigation.

A limitation of our study is that it does not explain why plasma *n*-3 PUFA concentration was lower in obese individuals. A possible reason could be that the diets of obese adults are such that their intake of *n*-3 PUFA (marine foods) is lower than in normal-weight individuals. Alternatively, lower plasma *n*-3 PUFA levels in obese individuals may be a reflection of increased utilisation or oxidative damage to these highly unsaturated fatty acids. Indeed, obesity has been associated with increased oxidative stress[44]. Future studies should also examine long-term biomarkers of *n*-3 PUFA status, such as the *n*-3 index, to further explore the relationship with obesity. We also acknowledge the small sample size of the present study, and recognise that a large-scale multicentre trial would be appropriate; certainly future studies should account for ethnicity and family history of disease and obesity.

Regardless of the mechanisms by which *n*-3 PUFA may assist in the maintenance of weight status[16,35], a significant inverse trend for BMI, waist circumference and hip circumference was observed when participants were stratified into quartiles of plasma *n*-3 PUFA concentration. Whether improvements in plasma concentration of *n*-3 PUFA by dietary supplementation with marine oils may reduce abdominal adiposity, or obesity in general, merits investigation. Interestingly, anthropometric measures correlated with the major *n*-3 PUFA (EPA and DHA) but not the parent *n*-3 PUFA (linolenic acid).

In summary, we have reported an inverse relationship between plasma concentrations of *n*-3 PUFA and anthropometric measures of obesity including BMI, waist circumference and hip circumference. Previous studies involving children and adolescents have shown a negative correlation between adiposity and plasma *n*-3 PUFA and DHA concentrations, but there appears to be a paucity of research in adults. These studies make the basis for conducting more intervention trials in adults examining the influence of dietary supplementation with *n*-3 PUFA-rich fats/oils in assisting weight loss and weight maintenance.

Acknowledgements

M. M. participated in the conception and design of the study, data collection and performed the statistical analysis and drafting of the manuscript. I. M. participated in the conception and design of the study, data collection and in drafting the manuscript. M. P. analysed the plasma fatty acid composition and was involved in drafting the manuscript. M. G. was involved in the coordination of the study, provided significant advice and consultation and participated in drafting the manuscript.

The authors have no conflict of interest to disclose.

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