Comparison of rice bran oil margarine with Flora margarine and Flora pro-activ® margarine for lowering cholesterol

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Sarah Louise Eady

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Abstract

Phytosterols have been shown to be effective in reducing serum cholesterol levels in numerous human clinical studies and regular consumption is recommended as part of therapeutic lifestyle changes aimed at reducing low density lipoprotein (LDL-C) in the treatment of hyperlipidaemia, a risk factor for cardiovascular disease. Fat based spreads have been shown to be a very successful vehicle for delivery of plant sterols, readily accepted by consumers and efficacious in reducing cholesterol levels. Alfa One™ Rice Bran Oil (RBO) spread is a new product entering into the market place. It is derived from rice bran oil and contains high levels of unsaponifiable material rich in phytosterols, triterpene alcohols, ferulic acid esters (γ-oryzanol) and vitamin E isomers. As such it may have the potential to lower serum cholesterol levels when consumed on a daily basis.

In order to establish the effectiveness of Alfa One™ Rice Bran Oil (RBO) spread compared with Flora pro-activ® margarine, a well established brand of plant sterol margarine already proven to lower cholesterol, a randomised double blind cross-over human clinical trial over 12 weeks was conducted. The study was divided into two treatment arms. The first arm of the study was to determine whether Alfa One™ RBO spread (containing 1.5% plant sterols) could lower total and LDL cholesterol levels to a greater extent than standard Flora margarine (containing no plant sterols) or Flora Pro-activ® margarine (containing 8% plant sterols). The second study arm tested the proposition that daily consumption of Alfa One™ Rice Bran Oil (RBO) spread in conjunction with rice bran oil (containing 0.5% plant sterols) would lower total and LDL cholesterol to a greater extent than Alfa One™ RBO spread in isolation and more than Flora margarine in conjunction with sunflower oil.

Eighty mildly hypercholesterolaemic individuals (total cholesterol ≥ 5 mmol/L and ≤ 7.5 mmol/L) were recruited and randomised into two groups of forty. Participants were asked to continue with their normal dietary pattern but to

replace any margarine/butter/fat consumption with the trial products. One group of 40 were then assigned to the first treatment arm of the study (margarine-only group) and were randomised to consume 20 g (4 teaspoons) Alfa One™ RBO spread daily for 4 weeks, or 20 g Flora margarine daily for 4 weeks, or 20 Flora pro-activ® daily for 4 weeks. Phytosterol levels delivered in these amounts were: RBO margarine: 118mg phytosterol and 14 mg γ-oryzanol; Flora proactiv® 1600 mg phytosterol; Flora margarine 0mg phytosterol. The second group of 40 were allocated to the second arm of the trial (margarine and oil group) and consumed 20 g Alfa One™ RBO spread and 30 ml rice bran oil (RBO) daily for 4 weeks, or 20 g Flora margarine and 30 ml sunflower oil daily for 4 weeks, or 20 g Alfa One™ RBO spread daily for 4 weeks, changing treatment at the end of each 4-week period. Phytosterol amounts delivered in these amounts were: RBO margarine: 118 mg phytosterol and 14 mg γ oryzanol; RBO 222mg mg phytosterol, 150 mg γ oryzanol. Each participant consumed all three treatments in a random order over a 12 week period. At baseline and following each 4 week intervention period, measurements were made of weight and blood pressure. Venous blood samples were collected for analysis of total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total cholesterol: HDL-C, triglycerides and plasma phytosterols. Three-day diet records from each individual were also collected for analysis of normal dietary intake.

Results showed that compared to a standard Flora margarine, Alfa One™ RBO spread significantly reduced total cholesterol by 2.2% (P=0.045), total cholesterol:HDL by 4.1% (P=0.005) and LDL-C by 3.5% (P=0.016), but was not as effective overall as Flora Pro-activ® which reduced total cholesterol by 4.4% (P=0.001), total cholesterol:HDL by 3.4% (P=0.014) and LDL-C by 5.6% (P=0.001). Consumption of Flora margarine alone produced no significant decrease from baseline figures in any of the cholesterol parameters measured. Surprisingly, in group two, the addition of rice bran oil to the Alfa One™ RBO spread produced no differences in cholesterol levels. The reason for this unexpected result is being explored further.

These results confirm that Alfa One™ RBO spread is effective in lowering serum cholesterol levels when consumed as part of a normal diet. Studies have shown that a 1% reduction in LDL-C can equate to a 2% decrease in coronary heart disease (CHD) risk thus suggesting that the 3.5% reduction demonstrated by Alfa One™ RBO spread in this study could be effective in reducing CHD risk as much as 6% in a mildly hypercholesterolaemic population.

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Contents

List of Tables

List of Figures

Abbreviations

ABC	adenosine triphosphate binding cassette
Acetyl Co A	acetyl coenzyme A
AGE	advanced glycation end products
ALA	alpha linoleic acid
ALP	atherogenic lipoprotein phenotype
Apo	apolipoprotein
ATP	adenosine triphosphate
bHLH	basic helix-loop helix
BMI	body mass index
BP	blood pressure
CE	cholesterol ester
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
CHO	carbohydrate
CMR	chylomicron remnant
CTD	C-terminal domain
DHA	docosahexanoic acid
DNA	deoxyribonucleic acid
EPA	eicosapentaenoic acid
FDA	food and drug administration
FFA	free fatty acids
FH	familial hypercholesterolaemia
FPG	fasting plasma glucose
GI	glycaemic index
GL	glycaemic load
HDL-C	high density lipoprotein
HIV	human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HsCRP	C-reactive protein
ICAM-1	intracellular adhesion molecule -1
IDL	intermediate density lipoprotein
Insig-1	insulin induced gene 1
IL	interleukin
IR	insulin resistance
LCAT	lecithin: cholesterol acyltransferase
LDL-C	low density lipoprotein cholesterol
LDLr	low density lipoprotein receptor
Lp (a)	lipoprotein (a)
LPL	lipoprotein lipase
LRP	LDL receptor related protein
LXR	liver X receptor
MCP-1	monocyte chemo attractant protein-1
M-CSF	macrophage colony stimulating factor
MetS	metabolic syndrome
MOH	Ministry of Health
MUFA	monounsaturated fatty acids
NCEP	National cholesterol education programme
NFκβ	nuclear factor kappa beta

NHLBI	National Heart, Blood and Lung Institute
NIDDM	non insulin dependent diabetes mellitus
NO	nitric oxide
NOS	nitric oxide synthase
NZ	New Zealand
PAI-1	Plasminogen activator inhibitor 1
PPAR	peroxisome proliferator activated receptor
PPG	post prandial glucose
PUFA	polyunsaturated fatty acids
RAGE	receptor of advanced glycation end products
RAS	rennin angiotensin system
RBO	rice bran oil
SCAP	SREBP cleavage activating protein
SFA	saturated fatty acids
SREBP'S	sterol regulatory element binding proteins
S1P	site 1 protease
S2P	site 2 protease
TG	triglyceride
TLC	therapeutic lifestyle changes
TNF-α	tissue necrosis factor alpha
TRF	tocotrienol rich fraction
US	United States of America
VCAM-1	vascular cell adhesion molecule-1
VLDL-C	very low density lipoprotein cholesterol
WHO	World Health Organisation

Chapter 1

Introduction

In the industrialised world, coronary heart disease (CHD) remains a widespread and often untreated disorder despite many advances in prevention and treatment therapies thus it remains a major contributor to mortality and morbidity accounting for a large proportion of public health spending (Jones, 2001; Talbert, 2002). Globally, WHO currently attributes one third of all deaths to CHD and predicts that the future burden of this disease will shift to include developing nations as they increasingly adopt the behavioural risk factors such as inappropriate dietary practices, low physical activity levels and increased levels of smoking (WHO, 2003). In New Zealand, one out of five adults has been diagnosed with heart disease and CHD is the leading cause of death for both genders and all ethnic groups. It is clear there remain many challenges for improving health initiatives and policy relating to this disease (MOH, 2003a). There appears to be no single cause of CHD but several risk factors have been identified. These include non-modifiable factors such as age, gender and family history and modifiable risk factors including hypertension, cigarette smoking, type II diabetes, overweight/obesity, physical inactivity and, most notably consumption of an atherogenic diet high in saturated fat (Mann, 2002; Berry, 2005; MOH, 2003a). However, numerous epidemiological and clinical studies now show that the leading CHD risk is associated with lipid abnormalities encompassing hypercholesterolaemia (raised low density lipoprotein levels), reduced high density lipoprotein levels), elevated serum triglycerides and elevated triglyceride rich lipoproteins (remnant lipoproteins such as small very low density lipoproteins and intermediate lipoproteins) (Castro et al, 2005; Kannel 2006; National Heart, Lung, and Blood Institute ((NHLBI)) 2002). A major understanding of the connection between dyslipidaemia and CHD came from the results of the Framingham Heart Study, a program started in 1948 as a joint project between the National Heart, Lung, and Blood Institute of the National Institutes of Health and Boston University. The objective was to identify if there were any common factors relating to the establishment of CHD in a large population of healthy individuals studied over a

long time period. The original cohort of 5,209 men and women between the ages of 30 and 62 from the town of Framingham, Massachusetts underwent extensive physical examination and investigations into lifestyle patterns that were analysed for common factors which may be related to later development of CHD. These tests were repeated every two years and the study has been extended to an offspring cohort (1971) and a third generation cohort, which began in 2002. This expansive study has provided a large amount of information regarding the known risk factors for CHD that are widely recognised today and is still contributing to the wider understanding of CHD (Stamford and Moffat, 2006). Subsequently other studies such as the Multiple Risk Factor Intervention Trial (MRFIT) (Kannel et al, 1986) and the Lipid Research Clinics (LRC) trial (The Lipid Research Clinics Coronary Primary Prevention Trial, 1984) further substantiated the relationship between raised LDL cholesterol and increasing CHD risk (La Rosa, 2003; Stamford and Moffat, 2006). Consequently, LDL cholesterol (LDL-C) is now identified as the primary target for clinical management of CHD risk and forms the basis of the National Cholesterol Education Program (NCEP). These NHLBI guidelines are devised by a panel of experts in the United States using recent clinical evidence with the overall aim of reducing high blood cholesterol in the American population (NHLBI 2002). New Zealand guidelines for the assessment and management of cardiovascular disease risk follow the similar recommendations (New Zealand Guidelines Group, 2003). They take an intensive clinical approach for persons in whom LDL-C is significantly high, advising pharmacological therapy with lipid lowering agents such as statins, bile acid sequestrants, fibrates or niacin. Additionally they recommend a public health approach to prevent the incidence of CHD and maintain CHD risk factors such as LDL-C concentrations at a near optimal level. Intensified therapeutic lifestyle changes including a healthy diet, weight control and increased physical activity are viewed as the best strategies for reducing the extent of cardiovascular disease throughout the population as lifelong pharmacological therapy is not seen as a viable option (NHLBI, 2002; New Zealand Guidelines Group, 2003).

Currently, dietary cholesterol intake amongst the general NZ population is variable with average dietary intake being 381mg/d for males and 261mg/d for females. Recommended levels for dietary cholesterol intake is not presently included in the nutrient reference values for Australia and New Zealand (NHMRC, 2006) but

guidelines provided by the National Cholesterol Education Programme recommend that levels of no greater than 200mg/d should be consumed as part of therapeutic lifestyle changes to maximise the amount of LDL-C lowering that can be achieved through reduction in dietary cholesterol (NHLBI, 2002).

Plasma LDL-C levels are reported as 5.7 mmol/L for both genders in the New Zealand population (Russell, et al 1999) which is much higher than the recommendation of levels equal to or less than 4 mmol/L (Ministry of Health 2003b) and is also high in comparison to similar countries such as England (5.5mmol/L for males: 5.6 mmol/L for females) and Australia (5.5 mmol/L for males: 5.4 mmol/L for females (Ministry of Health 2003a). In the National Nutrition Survey of 1997, 23 percent of the New Zealand population had total cholesterol levels higher than 6.5 mmol/L (Ministry of Health 2003b). This may in part be due to the higher consumption of saturated fatty acids (SFA) in the diet derived mainly from animal fats. In 1997, saturated fatty acids contributed 15.1% of energy in males and 14.7% of energy in females, higher than the recommended levels of 8-12% of energy from SFA as recommended. Thus an approach to lowering SFA intake is desirable in the aim to lower plasma LDL-C levels. In New Zealand, the presence of high blood cholesterol levels was a contributing factor in 4721 deaths (17% of all deaths) during the year of 1997. A reduction in this mortality through the implication of policy initiatives such as lowering SFA, increasing soluble fibre, increasing physical activity and reducing obesity may translate into not only a reduction in mortality but a reduction in co-morbidities to cardiovascular disease such as ischaemia and stroke (Ministry of Health 2003b). The cost of cardiovascular disease, both human and financial, has a great impact on public health in New Zealand. CHD is the leading cause of death accounting for 41% of all deaths in 1999 and the burden is greatest amongst Maori and Pacific people. The financial cost for CHD (including stroke and drug treatments) is estimated to be between $306 and $467 million dollars. Thus this condition has a major impact on the delivery of health services in New Zealand (New Zealand Health Strategy, 2003).

Numerous studies have shown that dietary intake of specific food groups can play a major role in influencing the development of CHD impacting on a majority of the known risk factors (De Caterina et al, 2006; Mann 2002). Interventions such as reducing intake of saturated fat and cholesterol and increasing intake of dietary

soluble fibre form the basis of the dietary advice, in addition to the recent recommendation of including plant sterols/stanols into the daily food intake (NHLBI, 2002). Plant sterols are naturally occurring compounds found in food such as vegetable oils, breads, fruit and nuts and are essential components of plant cell membranes. Plant stanols are the completely saturated form of plant sterols and are less abundant in nature. Collectively the two groups are known as phytosterols (Fernandes and Cabral, 2006; Ostlund, 2007). The most abundant phytosterols are the 4-desmethyl sterols comprising sitosterol, stigmasterol and campesterol (Piironen et al, 2000). Currently a typical Western diet is thought to contain approximately 100-300mg sterols and 20-50mg stanols per day and these compounds have been attributed with producing a wide spectrum of biological activities in humans; most notably they reduce plasma LDL-C concentrations (Woodgate et al, 2006; Lichtenstein and Deckelbaum, 2001). This is credited to their structural similarity to cholesterol (differing only in the number of carbons in the aliphatic side chain) which affords them greater affinity for the mixed micelle formed in the gut by bile acids and phospholipids, allowing them to displace cholesterol and reduce intestinal cholesterol absorption. Subsequently LDL-C is lowered through increased LDL receptor expression and greater clearance of circulating LDL-C (Ling and Jones, 1995; Plat and Mensink, 2005). Plant sterols themselves have minimal absorption from the gut (Von Bergmann et al, 2005). Research currently suggests that phytosterols may produce up to a 15% reduction in plasma LDL-C at a dosage of 2g/day without any reported side effects (Law, 2000). Greater dosages do not appear to confer any further reductions in LDL-C and may contribute to a reduction in plasma fat soluble vitamins and carotenoid levels, a documented side effect of phytosterol consumption (Hendricks et al, 1999). As a 1% reduction in plasma LDL-C is calculated to result in a 2% decreased in CHD risk, this functional aspect of phytosterols has made them an attractive proposition for the primary prevention of CHD (Ostlund, 2007; NHLBI, 2002; Plat and Mensink, 2005). Food sources alone cannot provide the recommended 2g daily dose and thus some form of phytosterol supplementation is required (Devaraj and Jialal, 2006). In the “free” unesterified form, plant sterols are hydrophobic and have limited solubility thus plant sterols currently incorporated into foods are esterified to unsaturated fatty acids (creating sterol esters), increasing lipid solubility and the ability to incorporate theses compounds into fat based foods. Plant sterol enriched margarines are a popular

vehicle for delivery of these compounds and functional foods of this nature have been available to consumers since 1995 (Fernandes and Cabral, 2006; Salo and Wester, 2005). They have been widely studied for their effects on LDL-C lowering producing consistent reductions of between 9 to 15% (Salo and Wester, 2005; Katan et al, 2003; St Onge and Jones, 2003). Vegetable oils such as corn, soybean, safflower, sunflower and rapeseed (canola) are most commonly used as a base of these functional plant sterol based margarines however rice bran oil is now emerging as an effective alternative for vegetable oils both in this process and as an everyday cooking oil (Nicolosi et al, 2001; Wilson et al, 2007). Rice bran is a by product of the rice milling industry, separating from the white portion of the rice in the polishing process. Human consumption has been traditionally limited due to its instability and the rapid onset of rancidity produced by a high lipase activity in the bran causing deterioration of the lipids. However improved oil extraction methods have reduced this problem and in Asian countries such as Japan, China, Korea and Thailand, rice bran oil (RBO) is being increasingly consumed (Lee et al, 2005). Studies have verified that RBO demonstrates the ability to lower LDL-C levels to an equal if not better standard as other vegetable oils and this effect is largely attributed to the unusually high levels of unsaponifiable material found in RBO (Sugano and Tsuji, 1997; Most et al, 2005). This is composed of phytosterols, triterpene alcohols, ferulic acid esters (γ-oryzanol) and vitamin E isomers (tocopherols and tocotrienols). In addition, RBO contains up to 20% saturated fatty acids and equal amounts of monosaturated fatty acids and polyunsaturated fatty acids (Wilson et al, 2007). Collectively these components confer health benefits to RBO that include antioxidant potential, improved lipid metabolism, anti-cancer action, anti-atherogenic action and improved immune function (Cicero and Gaddi, 2001). The oil is also attractive to consumers having a mild flavour and high smoke point making it suitable for use in many cooking methods and importantly, no adverse effects have been reported. The potential availability of this product is huge as rice is a major cereal crop in many countries thus interest in this product is rising (De Deckere and Korver, 1996; Jariwalla, 2001; Wilson et al, 2007).

The aim of this study is to determine the effect of including a margarine based on rice bran oil that may have LDL cholesterol lowering ability into the daily diet. It aims to establish its potential when compared against standard polyunsaturated

margarines and other commercially available plant sterol containing spreads. Additionally, the study will examine if any cholesterol lowering activity can be enhanced by including a supplementary serving of the corresponding base oil of the margarines to the diet as it is envisaged that this may more closely mimic consumer behaviour when aiming to achieve maximum benefit from the plant sterol containing product.

In brief, eighty mildly hypercholesterolaemic individuals (total cholesterol ≥ 5 mmol/L and ≤ 7.5 mmol/L) were recruited and randomised into two groups of forty. Participants were asked to continue with their normal dietary pattern but to replace any margarine/butter/fat consumption with the trial products. One group of 40 were then assigned to the first treatment arm of the study (margarine-only group) and were randomised to consume 20 g (4 teaspoons) Alfa One™ RBO spread daily for 4 weeks, or 20 g Flora margarine daily for 4 weeks, or 20 g Flora pro-activ® daily for 4 weeks. The second group of 40 were allocated to the second arm of the trial (margarine and oil group) and consumed 20 g Alfa One™ RBO spread and 30 ml rice bran oil (RBO) daily for 4 weeks, or 20 g Flora margarine and 30 ml sunflower oil daily for 4 weeks, or 20 g Alfa One™ RBO spread daily for 4 weeks, changing treatment at the end of each 4-week period. The phytosterol content of these amounts of product were: Alfa One™ rice bran oil spread, 118mg phytosterol and 14mg γ-oryzanol; Alfa One™ rice bran oil, 150mg phytosterol. Following each 4 week intervention period, measurements were made of weight and blood pressure. Venous blood samples were collected for analysis of total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total cholesterol: HDL, triglycerides and plasma phytosterols. Three-day diet records from each individual were also collected for analysis of normal dietary intake.

1.1 Study Aims

- To determine whether Alfa One™ rice bran oil spread lowers cholesterol to a greater extent than Flora margarine.
- To determine whether Alfa One™ rice bran oil spread lowers cholesterol to a greater extent than Flora pro-activ® margarine.
- To determine whether Alfa One™ rice bran oil spread in conjunction with Alfa One™ rice bran oil lowers cholesterol to a greater extent than Alfa One™ rice bran oil spread alone.
- To determine whether Alfa One™ rice bran oil spread in conjunction with Alfa One™ rice bran oil lowers cholesterol to a greater extent than Flora margarine in conjunction with sunflower oil.

1.2 Study Hypothesis

The hypothesis is that Alfa One™ rice bran oil spread will be able to lower total and LDL cholesterol levels to a greater extent than Flora margarine or Flora pro-activ® margarine. Additionally it proposes that daily consumption of Alfa One™ rice bran oil spread in conjunction with Alfa One™ rice bran oil will lower total and LDL cholesterol to a greater extent than Alfa One™ rice bran oil spread in isolation and also to a greater extent than Flora margarine in conjunction with sunflower oil.

Chapter 2

Literature Review

For this review, a search of the National Library of Medicine Databases (PubMed) and the Web of Knowledge database was conducted. Epidemiologic and clinical investigations of disease pathologies and dietary factors (cholesterol, fat, fatty acids, coronary heart disease, rice bran oil, phytosterols) were collected. Original investigations, reviews, meta-analysis, epidemiologic studies and dietary intervention trials were examined for relevance and quality with greater weight given to randomised placebo controlled trials with clinical end points, large prospective cohort studies reporting disease outcomes and metabolic studies with established intermediate end points. Reviews and meta analyses that were selected were restricted to more recently published work to ensure the more recent theories relating to the topic were considered.

2.1 Introduction

Coronary Heart Disease (CHD) is a prevalent, major public health issue in most industrialised countries and despite advances in treatment and prevention, it remains a leading cause of premature morbidity and mortality, accountable for one third of all global deaths. By 2020 it is predicted that CHD will be the leading cause of death and disability worldwide as it continues to spread even within developing nations (De Caterino, 2006; Jones, 2001; WHO, 2003; Mitka, 2004). The disease is characterised by atherosclerosis, a multi-focal disease stemming from a chronic inflammatory response in the walls of the artery causing the hardening of arteries through the formation of atheromatous plaques of three distinct components. Firstly, the atheroma which is an accumulation of soft flaky yellow material at the centre of a large plaque that is composed of macrophages situated nearest the lumen of the artery. Underlying this is a layer of cholesterol crystals followed by calcified advanced lesions at the outer surface. The presence of these plaques makes coronary heart disease distinct from other vascular diseases such as arteriosclerosis (the hardening and loss of elasticity of medium or large arteries) and arteriolosclerosis

(the hardening and loss of elasticity of arterioles) (Libby and Theroux, 2005; Falk, 2006).

Epidemiological studies have identified several risk factors including genetic, nutritional and lifestyle factors (table 2.1) that contribute to the pathogenesis of atherosclerosis (Glass and Witztum, 2001; Mann, 2002; Carter and Jones, 2006).

Table 2.1: **Risk factors for the development of atherosclerosis**
(Taken from Glass and Witztum, 2001)

Factors with a significant genetic component **Non-modifiable factors** Male Gender Family History Increasing age	 **Modifiable factors** Elevated levels of LDL-C and VLDLC Low levels of HDL-C Elevated lipoprotein (a) Hypertension Type 2 Diabetes Elevated levels of homocysteine Elevated levels of haemostatic factors e.g. fibrinogen Metabolic syndrome Insulin Resistance Visceral Obesity C-reactive protein
Environmental factors	Smoking Lack of physical activity High fat diets Infectious agents
LDL-C- low density lipoprotein cholesterol; VLDL-C – very low density lipoprotein cholesterol; HDL-C – High density lipoprotein cholesterol	

Of these factors, some are modifiable, including smoking, elevated low density lipoprotein cholesterol (LDL-C), hypertension, physical inactivity, obesity and type 2 diabetes. Most of these have a relationship to nutrition or diet and several studies support the fact that dietary patterns are inextricably linked to CHD risk (Mann, 2002; De Caterina et al, 2006; Hu and Willet, 2002). These patterns are often adopted by individuals in their childhood and atheromatous plaque development has been identified as beginning in children as young as three years of

age (Berenson et al, 1998; McGill et al, 2000). Modifications to these detrimental lifestyle choices are now seen as one of the most effective ways of reducing the risk of atheroma development (Mann, 2002; De Caterina et al, 2006; Hu and Willet, 2002). Notably elevated serum low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) is singled out as the risk factor with immense clinical importance, being unique in that it is capable of driving the development of CHD even in the absence of other risk factors. Its importance was first recognised in animal studies in the early 20th century and has since been confirmed through numerous epidemiological and clinical studies (Mann, 2002; De Caterina et al, 2006; Hu and Willet, 2002; Ginsberg and Karmally, 2000; Shepherd, 2004).

2.2 The Pathophysiology of Coronary Heart Disease

Coronary heart disease is underpinned by the atherosclerotic disease process, characterised the formation of the atheromatous plaque in the coronary artery occluding the vessel and restricting blood flow to the heart muscle (Falk, 2006). The exact mechanism of plaque formation is unclear but it is fuelled by inflammatory responses to the deposition of lipids and involves endothelial cells, macrophages, leucocytes, fibroblasts and intimal smooth muscle cells. This disease process causes two major problems: Firstly, plaque formation that eventually leads to artery stenosis and occlusion reducing blood flow to the heart. This may manifest itself as Angina pectoris – temporary pain and pressure that radiates out from in and around the chest and occurs with exertion or stress. Chronic and cumulative plaque formation however will eventually lead to the second complication that is plaque rupture causing thrombosis and total obstruction of the blood flow. This leads to infarction and tissue death more commonly referred to as a heart attack. It may also cause arterial insufficiency of the arteries supplying other major organs such as the brain, intestines and kidneys confirming atherosclerosis as a body wide process (Mann, 2002). Two major proliferative mechanisms are thought to explain the pathogenesis of atherosclerosis – the chronic endothelial cell injury hypothesis and the lipid hypothesis (Carter and Jones, 2006).

2.2.1 Endothelial cell dysfunction

The endothelium is an active and dynamic tissue involved in maintaining vascular homeostasis in both healthy and diseased states (Esper et al, 2006). This thin mono-cellular layer lining the inner surface of the blood vessels plays an essential role in the haemostatic processes of cell adhesion and migration, thrombosis and fibrinolysis. Through the expression of adhesion molecules (P-selectin, E-selectin, intracellular adhesion molecule 1, vascular cell adhesion molecule 1) on the cell surface and plasma proteins (von Willebrand factor), endothelial cells are involved in leukocyte recruitment and platelet adhesion during thrombosis and inflammation. When exposed to inflammatory stimuli such as oxidised LDL, free radical species and lipopolysaccharide, the endothelium up-

regulates the expression of the adhesion molecules and releases cytokines. This endothelial activation is a transient process under normal physiologic conditions, maintaining vascular integrity (Brown and Hu, 2001). Additionally, the endothelial layer maintains vascular smooth muscle cell function and vascular tone through vasoconstriction and vasodilation (Cooke, 2000). Through the balanced synthesis of vasoconstrictors (thromboxane A_2, prostaglandin H_2 and endothelin 1) and the vasodilators (nitric oxide, endothelium derived hyperpolarizing factor and prostacyclin), vascular tone is maintained (Brown and Hu, 2001). When vascular homeostasis is disrupted through either mechanical or physiological processes, endothelial cell damage ensues , impairing the production and/or function of the many vasoprotective mediators that maintain vascular health, reducing the availability of nitric oxide and increasing vasoconstriction through the production of vasoconstrictors (Yang and Ming, 2006). Endothelial cell dysfunction then leads to release of chemo attractants and activation of endothelial-leukocyte adhesion molecules attracting leukocytes, monocytes and T-lymphocytes to the endothelial cell surface (Carter and Jones, 2006). Migration of the leukocytes into the sub-endothelial space then leads to their aggregation within the intima. Further damage is caused through the presence of oxidised LDL-C which forms the basis of the conversion of monocytes to lipid laden macrophages known as foam cells that injure the vascular wall and form the major part of the atherosclerotic lesion. The "fatty streak" development is completed by lymphocyte and smooth muscle cell accumulation and eventually the deposition of collagen in the vessel wall and subsequent calcification contribute to the loss of elasticity and hardening of the artery (Carter and Jones, 2006; Heinecke, 2006; Falk, 2006). Repeated injury to the endothelium leading to eventual dysfunction of the cells may be caused through many factors including hypercholesterolaemia, diabetes, infectious disease, hypertension and smoking and may be reversible if lifestyle changes are adopted (Carter and Jones, 2006).

2.2.2 The Lipid Hypothesis

This hypothesis has been postulated for several decades and originated from experiments showing that rabbits subjected to a high cholesterol diet developed arterial lesions characteristic of atherosclerosis (Falk, 2006). Simply explained,

excess dietary lipid leads to elevated small dense LDL-C particles that are thought to infiltrate the arterial wall causing an accumulation of lipid. LDL-C particles are prone to oxidation and are then more readily taken up by macrophages; in addition, LDL-C particles are chemotactic to monocytes. Cell hyperplasia and migration of smooth muscle cells and monocytes into the sub-intima and intima forms the early stages of the fatty streak and promotes the transformation of monocytes into lipid laden macrophages and foam cells that leads to plaque formation (Heinecke, 2006; Carter and Jones, 2006; Ginsberg and Karmally, 2000). The presence of excessive LDL-C particles is now recognised as a key initiator in atherogenesis (National Heart, Blood and Lung Institute (NHBLI, 2002; Heinecke, 2006). The molecular mechanisms responsible for LDL oxidation are unknown but theories include the effects of metal ions, the enzymatic activity of nitric oxide synthase, the effects of potent oxidants from lipo-oxygenase and the myloperoxidase pathway and the effect of nitric oxide released from endothelial cells (Heinecke, 1998; Navab et al, 2004). Additionally the site of oxidation is not known, it may occur locally in the artery walls or accumulate in atherosclerotic lesions following the uptake of oxidised lipoprotein form the circulation (Carter and Jones, 2006; Staprans et al, 2005). Some evidence however suggests that at least some of the oxidised lipids are derived from dietary sources supporting the opinion that a diet high in oxidised fat contributes to the increased occurrence of arterial atherosclerosis in our society (Staprans et al, 2005). Although a pathogenic role for oxidised LDL has been suggested for over 20 years, a majority of the controlled, prospective trials examining the effects of anti-oxidants on clinical end points of CHD have not shown any beneficial effects and thus the theory is not yet proven. The contribution of oxidised LDL to the development of atherosclerosis remains controversial (Carmena et al, 2004; Staprans et al, 2005).

2.3 Inflammation and coronary heart disease

Both endothelial cell dysfunction and lipid deposition within the arterial vessels are linked to CHD. The exact molecular and physiological mechanism underlying their involvement remains to be elucidated but two theories are proposed (Falk, 2006). Firstly, a pro-inflammatory environment influences the development of an intact but leaky, activated and dysfunctional endothelium. Plasma molecules

and lipoproteins pass through the tight junctions of the endothelial cell into the sub-endothelial space where they are retained and modified becoming cytotoxic, pro-inflammatory, chemotaxic and pro-atherogenic. Modification may be due to oxidation caused by myeloperoxidase, 15-lipooxygenase and/or nitric oxide synthase in macrophages. Secondly, inflammatory mediators influence the tight junctions of endothelial cells so that transit of lipid related substances across the vessel wall occurs at rates greater than usual, overwhelming the mechanisms of lipid removal and resulting in greater lipid deposition (Falk, 2006). An essential part of either mechanism is the contribution made by the inflammatory response which has been identified as playing a key role in the mechanisms underlying the initiation and development of CHD. It is increasingly recognised as participating in all stages of the disease from initiation, through progression to the thrombotic complications of the disease. This inflammatory process is mediated by a system of cytokines and chemokines released in response to a variety of factors (Libby, 2006). The expression of adhesion molecules by endothelial cells is prompted through factors such as consuming a high saturated fat diet, smoking, hyperglycaemia, hypertension, obesity and insulin resistance, supporting the attachment of leucocytes to the arterial wall. Vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule (ICAM-1) have been shown in both animal and human studies to be a major factor in lesion development through the ability to bind monocytes, leucocytes and T-lymphocytes (Libby, 2006; Libby et al, 2002; Kressel et al, 2008). These molecules are highly up regulated by increased levels of atherogenic lipoproteins (oxidised LDL and lipoprotein (a)) through a pathway mediated by nuclear factor κβ (NFκβ) and the pro-inflammatory cytokines including interleukin-1β (IL-1β) and tissue necrosis factor-α (TNF-α) (Libby, 2006; Fan and Watanabe, 2003; Hansson et al, 2006). Development of the lesion tends to be specific to certain arterial sites, particularly at branch points within the vessel where blood flow is disturbed. Laminar blood flow through the vessel creates shear stress which provides some atherogenic protection in the form of increased production of the vasodilator nitric oxide synthase (NOS), in addition to the antioxidant enzyme superoxide dismutase. NOS inhibits the activation of nuclear factor κβ and limits VCAM-1 expression, preventing cell adhesion. However, when blood flow is disturbed, NOS production is reduced and the atherogenic potential within the vessel is increased (Libby 2006). Additionally, disturbed blood flow may also increase the production of

proteoglycans by arterial smooth muscle cells which can bind and retain lipoprotein molecules, which then undergo oxidative modification sustaining the inflammatory response (Libby et al, 2002).

Adhesion of leucocytes to the endothelium is followed by the penetration into the intima which is facilitated by chemo attractant molecules. Monocyte chemo attractant protein-1 (MCP-1), and a number of other chemo attractants (oxidised LDL, Lp (a), ILβ-1, TNF-α) are thought to support the direct migration of monocytes into the intima at the sites of lesion formation where these inflammatory cells maintain a local inflammatory response. Scavenger receptors for modified lipoproteins then support the ingestion of lipids leading to the development of foam cells under the influence of factors such as macrophage colony stimulating factor (M-CSF) and inflammatory cytokines such as γ-interferon and lymphotoxin (Fan and Watanabe, 2003; Libby et al, 2002). As the sustained inflammatory response continues, the release of fibrogenic mediators promotes smooth muscle cell production leading to the advanced atherosclerotic lesion (Libby et al, 2002).

Plaque rupture and the subsequent thrombotic complications are the major issues of atherosclerosis (Libby, 2006). Two types of common atherosclerotic plaque develop: the stable plaque (a small lipid core with a thick fibromuscular cap of smooth muscle cells and extra cellular matrix) and the unstable or vulnerable plaque (a large lipid core with a thin cap and large numbers of inflammatory cells). Whilst the stable plaques are responsible for stenosis or occlusion, the vulnerable plaques are the prone to disruption, leading to thrombus formation and sudden expansion of the lesion with complete blockage of the artery (Fan and Watanabe, 2003). The mechanism underlying plaque rupture is also inflammatory in origin. The strength and stability of the fibrous cap is due to interstitial collagen. Inflammatory mediators affect the integrity of this collagen by blocking the creation of new collagen and destroying the existing collagen through matrix metalloproteases whose production is mediated by cytokines such as γ –interferon, TNF-α and ILβ-1 (Fan and Watanabe, 2003). T-lymphoctyes also contribute to the thrombogenicity through the expression of the CD40 ligand on macrophages which stimulates macrophage production of tissue factor, a pro-coagulant that initiates the coagulation

cascade upon exposure to factor VII in the blood, boosting the thrombogencity of the lipid core (Libby 2006).

It is clear that atherosclerosis is a chronic inflammatory process which also underlies several other metabolic abnormalities which are inextricably linked to CHD development. Insulin resistance, abnormal glucose tolerance, diabetes, hypertension, dyslipidaemia and abdominal obesity may cluster together in the same person where they are collectively defined as the metabolic syndrome (MetS). All are triggers for inflammation and major risk factors for CHD (Kressel et al, 2008). The definition of MetS is not yet consolidated but the most recent version is that of the International Diabetes Federation which is shown in table 2.2. This syndrome is an increasing public health concern as its prevalence is estimated to be at up to 25% of the adult population in Australia and the United States of America and increasing worldwide (Paoletti et al, 2006).

Table 2.2

International Diabetes Federation definition of the metabolic syndrome (taken from Reaven, 2006)

In order for a person to have a diagnosis of metabolic syndrome, he or she must have Central adiposity (defined as a waist circumference ≥ 94cm for European men and ≥ 80cm for European women, with ethnicity-specific values for other groups Plus any 2 of the following factors: • High triacylglycerol concentration: ≥ 150mg/dL (1.7mmol/L), or specific treatment for this abnormality • Low HDL-cholesterol concentration: < 40mg/dL (1.03 mmol/L) in males and < 50mg/dL (1.29 mmol/L) in females or specific treatment for this lipid abnormality • High blood pressure (BP): systolic BP ≥130mm Hg or diastolic BP ≥85mm Hg, or treatment for previously diagnosed hypertension • High fasting plasma glucose (FPG) concentration ≥ 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes. If FPG is above the values stated above, an oral glucose tolerance test is strongly recommended but is not necessary to define the presence of the syndrome

2.4 Aspects of metabolic syndrome and their association with inflammation

2.4.1 Visceral Obesity

Obesity is not only a major cardio metabolic risk factor per se; it has the potential to give rise to other emerging risk factors and can predispose to insulin resistance, hypertension and diabetes as well as contributing to dyslipidaemia. The inflammatory aspect of obesity relates to the adipose tissue which is active as a source of several active molecules (leptin, TNF-α, IL-6, plasminogen activator inhibitor 1 ((PAI-1)), adiponectin) which are collectively known as adipokines and may influence the function of endothelial cells and the process of atherosclerosis directly (Paoletti et al, 2006; Chudek and Wiecek, 2006). Table 2.3 shows a list of the most commonly indicated adipokines and their role in the pathogenesis of atherosclerosis.

Table 2.3

Adipokines and their role in atherogenesis (Chudek and Wiedek, 2006).

ADIPOKINE	ENDOTHELIAL CELL DYSFUNCTION	ATHEROSCLEROSIS
Leptin	Increased NO-induced vasorelaxation	Stimulation of thrombus formation
Adiponectin	Decreased expression of adhesion molecules Stimulation of NO- induced vasorelaxation	Inhibition of transformation of macrophages to foam cells Stabilisation of atherosclerotic plaques
IL-6	Stimulation of CRP synthesis in the liver which decreases NO production in endothelial cells	Stimulation of monocytes/macrophages infiltration of atherosclerotic plaque Destabilisation of atherosclerotic plaque (matrix metalloproteinase) Reduction of lipid accumulation in atherosclerotic plaque
TNF-α	Increased expression on adhesion molecules Increased expression of MCP-1 and M-CSF	Stimulation of oxidised LDL-C uptake by macrophages (scavenger receptors) Stimulation of pro-inflammatory cytokines release by monocytes/macrophages De-stabilisation of atherosclerotic plaque (matrix metalloproteinase)
Plasminogen activator inhibitor - 1	-	Inhibition of fibrin clot breakdown
Angiotensin ll	Vasoconstriction Increased expression of adhesion molecules, MCP-1 and M-CSF	Stimulation of intimal infiltration by monocytes Stimulation of migration and proliferation of smooth muscle cells

IL-6, interleukin-6; TNF-α, tissue necrosis factor alpha; NO, nitric oxide; CRP, C-reactive protein; MCP, monocytes chemo-attractant protein-1; M-CSF, macrophage colony stimulating factor; LDL-C, low density lipoprotein cholesterol.

2.4.2 Type 2 Diabetes

This is characterised by insulin resistance (IR), hyperglycaemia and relative insulin deficiency. Insulin resistance exists when insulin levels are higher than expected relative to the level of glucose. Thus, insulin resistance is by definition tethered to hyperinsulinaemia (Shanik et al, 2008). Stimuli such as over nutrition, physical inactivity and aging all contribute to IR along with chronic inflammation. Cytokines postulated to be involved in the development of IR include IL-6 which has been shown to be circulating at high levels in men and obese individuals with IR. It also demonstrates a strong linear relationship with the production of TNF-α which has been shown to induce insulin resistance through two mechanisms: its ability to produce serine phosphorylation of insulin receptor substrate 1, decreasing the tyrosine kinase activity of the insulin receptor and hence intracellular insulin signalling (Esposito and Giugliano, 2004) and decreasing the expression of the glucose transporter GLUT4 (Barnett, 2008). Decreased sensitivity to insulin also decreases the anti-inflammatory response it generally provides through increased levels of acute phase proteins such as C- reactive protein (hsCRP) and fibrinogen resulting in persistent low grade inflammation (Esposito and Giugliano, 2004).

Type 2 Diabetes is also primarily a disorder of post prandial glucose regulation (PPG) resulting in chronic hyperglycaemia (Ciriello et al, 2006). Studies have confirmed that PPG is an independent risk factor for CHD in type 2 diabetes in clinical settings (Cavalot et al, 2006) and intervention trials such as the STOP-NIDDM trial has shown that reducing PPG is associated with a 36% reduction in the risk of progression to diabetes, a 34% reduction in the development of hypertension and 49% reduction in the risk of cardiovascular events (Ciriello et al, 2006). PPG is thought to exert its effect through oxidative stress and the development of advanced glycation end products (AGE's) formed mainly by protein and lipid glucosylation. AGE's are capable of cross linking with the vascular endothelium and basic membrane and matrix proteins which leads to differentiation of their structure and function. The AGE's bind irreversibly with collagen, intracellular protein, phospholipid, cellular membranes, DNA, and lipoproteins initiating atherosclerosis (Xanthis et al, 2007; Ciriello et al, 2008). The actions of AGE's is through a specific cell surface receptor RAGE (receptor of AGE's) which activates intracellular reactions including the activation of NFκβ and the pro-inflammatory cytokines

leading to increased oxidative stress and the production of inflammatory cytokines such as endothelin-1, plasminogen activator inhibitor -1, Il-6 and TNF-α. It also leads to increased production of VCAM-1 from endothelial cells promoting monocytes and platelet accumulation in the arterial wall (Xanthis et al, 2007).

2.4.3 Hypertension

The underlying mechanisms of hypertension in Met S are complicated. Several factors contribute (Yanai et al, 2008). Insulin resistance plays a role through the fact that insulin has an anti-natriuretic effect, stimulating renal sodium re-absorption which is increased in individuals with IR possibly resulting in salt-sensitivity and hypertension (Strazzullo et al, 2006). IR may also stimulate the production of endothelin-1, a powerful vasoconstrictor produced from endothelial cells which is postulated to be involved with hypertension development in animal studies (Sarafidis and Bakris, 2007). Oxidative stress and endothelial cell dysfunction may also be involved. Animal studies have shown that rats fed a high fat, high refined sugar diet to induce MetS have greater levels of oxidative stress leading to hypertension with inactivation of nitric oxide, down regulation of nitric oxide synthase isoforms and endothelial nitric oxide synthase activator (Yanai et al, 2008). Systemic oxidative stress has also been linked with insulin resistance and the subsequent impairment of phasphatidylinositol 3-kinase (PI3K) –dependant signalling resulting in imbalance between nitric oxide production and endothelin-1 production (Kim et al, 2006).
A further mechanism may be through the rennin-angiotensin system (RAS) which affects renal function and modulates vascular tone. Obesity is thought to lead to increased production of angiotensin II (AII) in adipocytes (Yanai et al, 2008). Angiotensin II elicits the production of superoxide anion from endothelial cells and smooth muscle cells which act as a reactive oxygen species causing oxidative damage. It also increases the expression of pro-inflammatory cytokines such as IL-6, TNF-α and MCP-1 as well as adhesion molecules such as VCAM-1 (Libby et al, 2002).

2.4.4 Dyslipidaemia

Oxidative modification of lipoproteins activates the inflammatory actions of endothelial cells and is a part of MetS that have long been identified with CHD risk (Fan and Wanatabe, 2003). A detailed description of lipids/ lipoprotein function and

roles and how they relate to the development of atherosclerosis is discussed in greater detail from section 2.6. However table 2.4 summarizes the roles of the pro-atherosclerotic effects of oxidised LDL-C and anti-atherogenic effect of HDL-C and the underlying inflammatory mechanisms.

Table 2.4

Pro-atherosclerotic effects of oxidised LDL-C and anti-atherogenic effects of HDL-C (Fan and Wanatabe, 2003).

PRO-ATHEROGENIC EFFECTS OF OXIDISED LDL-C			**ANTI-ATHEROGENIC ROLE OF HDL-C**	
Effects	**Mechanisms**		**Effects**	**Mechanisms**
↑ Adhesion of monocytes to endothelial cells	↑ expression of adhesion molecules on endothelial cells		↓ Inhibition of lesion formation	Reverse cholesterol transport
↑ Monocyte and T lymphocyte chemotaxis	Induction of MCP-1 production and direct chemo attractant effect		↓ adhesion of monocytes to endothelial cells	Inhibition of adhesion molecules and decreased cytokine production
↑ scavenger receptor A and CD36	Activation of AP-1 and transcription factors		↓ thrombosis	Modulation of endothelial anti-thrombotic and pro-fibrinolytic properties
↑ foam cell formation	Enhanced uptake of ox LDL mediated by scavenger receptor		↑ antioxidant effects	Modulation of paraoxonase
Induction of pro-inflammatory genes	Activation of NFκβ and AP-1 and increased cAMP		↓ cellular death	Inhibition of apoptosis
↑ cellular death	Activation of apoptosis and formation of cholesterol crystals		↓ cellular proliferation	Inhibition of smooth muscle cell proliferation
↑ thrombosis	Induction of tissue factor, increased platelet aggregation			
Impaired vascular function	Dysfunction of endothelin -1 and nitric oxide			
↑ plaque rupture	Increased MMPs production			

MCP-1, monocytes chemo attractant protein -1; NFκβ, nuclear factor kappa beta; MMP, matiz metalloproteins ; AP-1, activator protein – 1; cAMP, cyclic adenosine monophopshate

2.5 Cellular markers of inflammation

2.5.1 C-reactive protein (hsCRP)

HsCRP is a recognised marker of inflammation and whilst the clinical significance of inflammation to atherosclerosis is still being elucidated, there is growing evidence strengthening the theory and it is thought to be the most promising inflammatory biomarker for CHD (Sirtori and Fumagalli, 2006; Libby et al, 2002). HsCRP is a classical acute phase marker and a member of the pentraxin family of innate immune response proteins (Libby et al, 2002). It is produced mainly in the liver in response to IL-6 secretion but has been found in the endothelium of atherosclerotic plaques, in smooth muscle cells, macrophages and adipocytes. Its actions include the stimulating the expression of adhesion molecules, tissue factor, MCP-1 and PAI-1. It activates leucocytes and the complement system and reduces bioavailability of nitric oxide (Kressel et al, 2008). Over 20 prospective studies have shown that elevated basal hsCRP levels confer greater risk for cardiovascular disease (including stroke, myocardial infarction and peripheral vascular disease), diabetes and hypertension (Morrow and Ridker, 2000; Ridker, 2007). In many studies the relative impact of hsCRP is at least as large as that individually of LDL-C, HDL-C, hypertension or smoking (Ridker, 2007). HsCRP may become a valuable serum marker for CHD as it uses a standardised methodology and the analyte is stable over time. Treatments for CHD, such as statin therapy, also reduce hsCRP concentrations and clinical trials evaluating hsCRP as a primary target for risk reduction are underway (de Ferranti and Rifai, 2007).

2.5.2 Haemostatic factors

These include platelets, fibrinogen, activated factor VII, PAI-1, tissue plasminogen activator, von Willibrand factor, factor V Leiden, protein C and antithrombin III which have been implicated in elevated CHD risk but the strength of the association is not yet established and it is still unknown whether modification of these markers will have any effect on reducing cardiovascular morbidity and mortality. Further clinical studies are required (NHLBI, 2002).

2.5.3 Plasminogen activator inhibitor (PAI-1)

The plasminogen activator system controls the formation of plasmin and subsequently has a key role in modulating haemostasis, thrombosis and other biological processes (Fay et al, 2007). Plasmin formation is closely regulated by plasminogen activator inhibitors (PAI) particularly PAI-1 and levels of PAI-1 are stimulated by IL-6, IL-1β, NFκβ, adipocytes and TNF-α produced in the pro-inflammatory state. PAI-1 inhibits tissue type plasminogen activator (t-PA) and increased levels lead to impaired fibrinolysis and enhanced progression of thrombosis. Individuals with obesity or atherosclerosis associated with MetS have been shown to have elevated PAI-1 levels and high levels of plasma PAI-1 have been shown to be an independent predictor of coronary heart disease (Kressel et al. 2008; Chudek and Wiecek, 2006).

2.5.4 Acute phase protein fibrinogen

Fibrinogen plays a role in platelet aggregation, plasma viscosity and fibrin formation that is elevated in inflammatory states and is stimulated by the cytokine IL-6 (Kressel et al, 2008). Research undertaken in the Framingham Study confirmed that this factor shows a significant linear trend towards being a risk factor for CHD for age, body mass index, smoking, diabetes mellitus, total cholesterol, HDL-C and TG in both sexes (Kannel, 2005). Levels of fibrinogen rise in the presence of other CHD risk factors including hypertension, dyslipidaemia, smoking, obesity, stress and type 2 diabetes (Kannel, 2005; Kressel et al, 2008).

2.5.5 Homocysteine

Homocysteine is a breakdown product in the metabolism of methionine that can be further degraded to cysteine via vitamin B_6 dependant reactions or re-methylated into methionine which requires a methyl group from 5-methyltetrahydrofolate. High homocysteine levels result when there is an imbalance between the production and metabolism of homocysteine (Mangoni 2006). Although not well understood, a link between elevated homocysteine and development of CHD has been highlighted. Several studies and epidemiological evidence suggest that homocysteine is a strong, independent risk factor for

atherothrombosis and occlusive vascular disease, possibly through its ability to increase inflammation (Youssef et al, 2007). Also fuelling the link between CHD and homocysteine is the fact that individuals with the inherited form of severe homocysteinuria have premature vascular injury and atherosclerosis which is ameliorated with supplementation of folic acid and vitamin B_6 or B_{12} (NHLBI, 2002; Clarke and Lewington, 2002). However significant research is still required to determine the exact relationship of homocysteine to CHD and presently routine measurement is not recommended as a treatment therapy (NHLBI, 2002). Definitive trials are lacking. A recent study using stroke patients showed that folic acid supplementation reduced plasma homocysteine but did not affect recurrent vascular events (Mangoni, 2006). Observational studies have shown that 25% lower homocysteine is associated with 10% less CHD and 20% less stroke incidence (B Vitamin Treatment Trialists' Collaboration, 2006) and retrospective studies indicate that CHD or stroke patients have higher homocysteine levels than age matched controls (Clarke and Lewington, 2002). Dietary intervention in the form of folic acid supplementation has potential as a treatment strategy for the pro-inflammatory state that is associated with homocysteine and CHD. Imbalance in homocysteine levels are caused through deficiencies of vitamin B_{12}, folic acid and vitamin B_6 as these are essential in the homocysteine metabolic pathway (Mangoni, 2006). Studies have shown inverse relationships between serum levels of homocysteine and folic acid, vitamin B_6, and vitamin B_{12} and further work has established that at 25% reduction in homocysteine levels can be achieved with 0.5-5.7mg of folic acid daily (Mangoni, 2006). Further work also hypothesises that folic acid supplementation may exert further beneficial effects through its ability to enhance endothelial cell function through the interaction with NOS, the enzyme that transforms L-arginine into nitric oxide and citrilline thus ameliorating the effects of endothelial cell dysfunction apparent in the pro-inflammatory state of CHD (Mangoni, 2006).

2.5.6 Impaired Fasting Glucose

Whilst some studies see impaired fasting glucose (a pre-diabetic state identified by the American Diabetes Association as a fasting glucose level of 100-

125mg/dL (5.6 to 6.9 mmol/L) as an independent risk factor for CHD it is not established (Meigs et al, 2000; Levitzky et al, 2008). The relationship between this factor and other risk factors of the metabolic syndrome (hypertriglyceridaemia, abdominal obesity, hypertension) that can also lead to CHD (discussed in earlier sections) confounds the issue and prevents it from being classified as such (Haffner, 1997; NHLBI, 2002).

2.6 Dietary Lipids

The heterogeneous mixture of fats that constitute lipids are primarily made up of the principal dietary lipid triglyceride but also include phospholipids, glycolipids and sterols that all share the common characteristic of being insoluble in water and soluble in organic solvents (Eastwood, 2003). Lipids have important, diverse roles in nutrition and health including enhancing the sensory aspect of food through improved flavour and palatability, providing a major energy source and having involvement in other metabolic and physiological processes. They also provide the structural and functional integrity of all cell membranes in addition to providing fat soluble vitamins, corticosteroid hormones and mediators of electron transport such as coenzyme Q. Triglyceride and sterols (in the form of cholesterol) are the main lipids associated with cardiovascular disease risk (Mann and Skeaff, 2000; Small, 2000).

2.6.1 Triglyceride

Triglyceride (TG) also known as triacylglycerol, is the predominant form of fat in food and accounts for 95% of all dietary lipid. It is characterised by a glycerol backbone esterified to three fatty acid molecules that may be all the same type or mixed. The fatty acid molecule composed of even numbered carbon-hydrogen units have a methyl group at one end and a carboxylic acid group at the other. Physical and biological properties are determined by the constituent fatty acids (saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA)) depending upon the degree of saturation indicated by the presence of double bonds. Unsaturated fatty acids have either a single double bond (MUFA) or two or more double bonds (PUFA) between the carbon hydrogen units which lower the melting point and

render them liquid at room temperature. SFA have no double bonds making them a straight tightly packed molecule and usually solid at room temperature (Mann and Skeaff, 2000; Dupont, 2006). The influence exerted by the different kinds of fatty acids on cardiovascular disease risk has been extensively studied and largely focuses on the effect that each one plays on serum levels of cholesterol as discussed in a later section (Fernandez, 2005; Hu et al, 2001).

2.6.2 Cholesterol

Cholesterol is the principal sterol of animal tissues found in egg yolk, organ meat, shellfish, whole-fat dairy products, and red meat. Mixed dishes containing cheese, butter or fatty meat are also usually high in dietary cholesterol. It is essential for maintaining cell membrane integrity and fluidity, in addition to being a precursor for steroid sex based hormones (progesterone, oestrogen and testosterone), bile acids, adrenocortical hormones (cortisol and aldosterone) and vitamin D (Mann and Skeaff, 2000; Dupont, 2006; Goodridge and Sui, 2000). Cholesterol may be present in the free form (as in cell membranes) or it can be esterified to a fatty acid becoming a cholesterol ester, making it more hydrophobic thus allowing it to combine with triglyceride and facilitate transport in the blood. Cholesterol esters constitute about two thirds of the cholesterol in human plasma and it is the accumulation of these molecules in the arterial intima that is a characteristic feature of atherosclerosis. Cholesterol and cholesterol esters are characterised by a four ring structure known as the steroidal structure that is demonstrated in figure 2.1 (Wood, 2006).

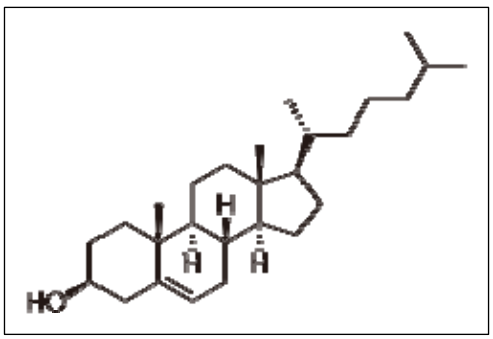


Figure 2.1: Cholesterol (taken from Wood, 2006).

As well as being obtained from the primary dietary sources of red meat, dairy and egg yolk (about 0.6g/day) cholesterol is also synthesized by the body. Up to 25% (~1g/day) of synthesis occurs in the liver and other sites of high synthesis include the adrenal glands, intestines and reproductive organs. Homeostasis is tightly balanced between cholesterol intake, absorption/excretion and synthesis (Gylling, 2004).

2.6.3 Cholesterol intake

Cholesterol intake amongst the general NZ population is variable with average dietary intake being 359mg/d for males and 243mg/d for females (Russell, et al 1999). The New Zealand Adult National Nutrition Survey conducted in 1997 reported the mean serum total cholesterol levels amongst New Zealanders at 5.7 mmol/L for both genders. Other studies (Gentles et al, 2007) have also demonstrated mean levels to be at 5.52 mmol/L amongst Europeans, 5.45 mmol/L for Maori and 5.44 mmol/L for Pacific Island populations. However, levels at less than 4 mmol/L are considered optimal for maintaining a low risk of CHD (NHLBI, 2002; Russell et al, 1999). Table 2.5 shows the reference levels recommended for lipids in New Zealand.

Table 2.5: New Zealand Reference values for lipids *(New Zealand Guidelines Group, 2003)*

CHOLESTEROL	RECOMMENDATION
Total Cholesterol	Less than 4 mmol/L
LDL cholesterol	Less than 2 mmol/L
HDL Cholesterol	Equal to/greater than 1 mmol/L
Total Cholesterol:HDL ratio	Less than 4 mmol/L
Triglycerides	Less than 1.7 mmol/L

Although high dietary intakes of cholesterol have been highlighted over recent years as having a major effect of levels of serum cholesterol and thus contributing to the development of CHD, the scientific evidence does not support this relationship. Meta – analyses of cholesterol feeding studies have shown dietary cholesterol to have a small but statistically significant effect on total plasma cholesterol concentration (0.05-0.07mmol/L per 100 mg dietary cholesterol) by increasing both LDL-C (0.05mmol/L per 100mg dietary cholesterol) and HDL-C cholesterol (0.01mmol/L per 100mg dietary cholesterol) as well as Apo B concentrations. The effect is highly variable in both size and effect between individuals and studies show that this has limited clinical significance on CHD risk (Clark et al, 1997; McNamara, 1997; Howell et al, 1997; Lichtenstein, 2006). The spotlight is now more focussed towards the role played by individual lipid sub fractions such as LDL-C, HDL-C and triglyceride and the effect of high dietary saturated fat intake. It has been demonstrated that cholesterol intakes of greater than 300mg/day have a more marked effect on LDL-C and total cholesterol when combined with diets high in saturated fat (Lee and Griffin, 2006; McNamara, 2000).

2.6.4 Cholesterol Absorption

Absorption of dietary (one fourth) and biliary (three fourths) cholesterol is largely controlled via the small intestine and is a multi-step process regulated by multiple genes. Cholesterol enters the lumen of the small intestine from three sources: diet (approx. 300-500mg/d), bile (800-1200mg/d) and intestinal epithelial sloughing (300mg/d) (Lammert and Wang, 2005). During the process of digestion, food is mixed with bile from the liver and gall bladder containing bile acids that emulsify cholesterol and other lipids forming lipid micelles. These micelles are transported to the brush border membrane of the enterocyte where it passes through the unstirred water layer and surface mucous coat via both active and passive processes (Kruit et al, 2006). Receptors from the scavenger receptor class B type 1 (SR-B1) known to mediate cellular uptake of cholesterol, triglyceride and other lipid nutrients are thought to be involved in the uptake process along with other transporters including the Niemann-Pick type C1 like 1 protein that is critical for both cholesterol and plant sterol absorption. However, the exact mechanism is not yet fully elucidated (Lairon et al, 2007; Davis et al, 2004; Gylling, 2004; Ostlund,

2002). Once inside the enterocyte, cholesterol is either transported back to the intestinal lumen by ABC transporters (ABCG5 and ABCG8) or is esterified by acyl-CoA-cholesterol acyltransferase and packed into chylomicrons with triglyceride before secretion into the lymph and entry to the circulatory system (Kruit et al, 2006). Triglyceride is then rapidly hydrolysed and free fatty acids are taken up by the peripheral tissue before chylomicron remnants are cleared by the liver. Cholesterol from the small intestine, is mixed with the cholesterol already present in the liver and used to form bile acids, excreted into bile or incorporated into very low density lipoproteins (Plotsch et al, 2005; Lairon et al, 2007; Lammert and Wang, 2005; Gylling, 2004; Wang, 2006).

The efficiency of intestinal cholesterol absorption in humans is highly variable ranging from 15% to 85% in healthy individuals (Kruit et al, 2006). The mechanisms are not yet fully understood but intestinal cholesterol absorption is subject to the influence of multiple factors including dietary, pharmacologic, biliary, cellular and luminal factors. Table 2.6 shows a list of some of the possible factors influencing intestinal cholesterol absorption.

Table 2.6: Some factors affecting intestinal cholesterol absorption (Lammert and Wang 2005).

FACTORS	EFFECTS ON % CHOLESTEROL ABSORPTION	TYPE OF STUDY
Increased cholesterol intake	Decrease in dietary cholesterol absorption	Animal/human
Increased monounsaturated fat	Decrease in dietary cholesterol absorption	Animal/human
Increased polyunsaturated fat	Decrease in dietary cholesterol absorption	Animal/human
Increased fibre	Decrease in dietary cholesterol absorption	Animal/human
Increased phytosterol intake	Decrease in dietary cholesterol absorption	Animal/human
Increased hydrophilic bile salts	Decrease in dietary cholesterol absorption	Animal/human
Cholesterol absorption inhibitors – Ezetimibe	Decrease in dietary cholesterol absorption	Animal/human
Decreased biliary bile salt output	Decreased cholesterol 7α-hydroxylase	Animal
Decreased size of bile salt pool	Decreased cholesterol 7α-hydroxylase	Animal
Increased biliary cholesterol output	Increase in cholesterol absorption	Animal
Increased cholesterol content of bile	Increase in cholesterol absorption	Animal
Increased hydrogen ion content of bile salt pool	Increase in cholesterol absorption	Animal
Decreased HMG-CoA reductase	Increase in cholesterol absorption	Animal/human

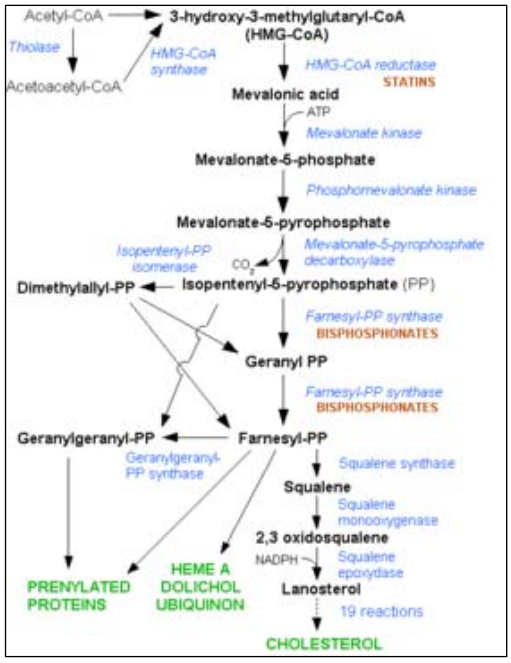
2.6.5 Cholesterol synthesis

De novo synthesis of cholesterol occurs in the endoplasmic reticulum of all nucleated cells and is regulated by the availability of cholesterol in the blood occurring through a multi step process originating from acetyl coenzyme A (acetyl Co A), the final end product of glucose metabolism and fatty acid oxidation. Acetyl Co A goes through a series of steps to become HMG-CoA which is converted to the cholesterol pre-cursor mevalonate by HMG-Co A reductase, a rate limiting enzyme which determines the rate of cholesterol synthesis. At least 26 steps are known to be involved in this pathway which can be thought of as occurring in three stages:

1. A cytoplasmic sequence by which HMG-CoA is formed from 3 mol of acetyl CoA
2. The conversion of HMG CoA to squalene, including the important rate limiting step of cholesterol synthesis in which HMG-CoA is reduced to mevolonic acid by HMG-CoA reductase. It is the inhibition at this step that is vital for the action of statin drugs used in treatment of hypercholesterolaemia.
3. The formation of cholesterol from squalene (Groff and Gropper, 2000).

The HMG CoA pathway is demonstrated in figure 2.2 (Goodridge and Sui, 2000).

Figure 2.2 HMG CoA pathway (Goodridge and Sui, 2000).



Cholesterol synthesis is highly sensitive and tightly governed to cope with fluctuating dietary intakes as well as cholesterol turnover. This is achieved by a feedback mechanism regulated by a unique family of membrane bound transcription factors known as sterol regulatory element binding proteins 1 and 2 (SREBP's) in the endoplasmic reticulum (Kruit et al, 2006). When cholesterol intake from food is high, there is a net decrease in endogenous production whilst the opposite is achieved from a lower dietary cholesterol intake. SREBP binds to two other proteins in the presence of cholesterol: SREBP-cleavage activating protein (SCAP) and Insulin induced gene (Insig-1). When cholesterol falls, Insig -1 dissociates allowing the SREBP-SCAP complex to migrate to the Golgi apparatus where it is cleaved by site 1 and site 2 proteases to its active form. Figure 2.3 shows a diagrammatic representation of the interactions between SREBP, SCAP and Insig-1 in the membrane of the endoplasmic reticulum (King, 2008).

Figure 2.3: Interactions between SREBP, SCAP and Insig-1 in the membrane of the endoplasmic reticulum when sterols are high.
When sterols are low, SCAP does not interact with Insig and the SREBP-SCAP complex migrates to the Golgi where the proteases, S1P and S2P reside.
Taken from King, 2008.

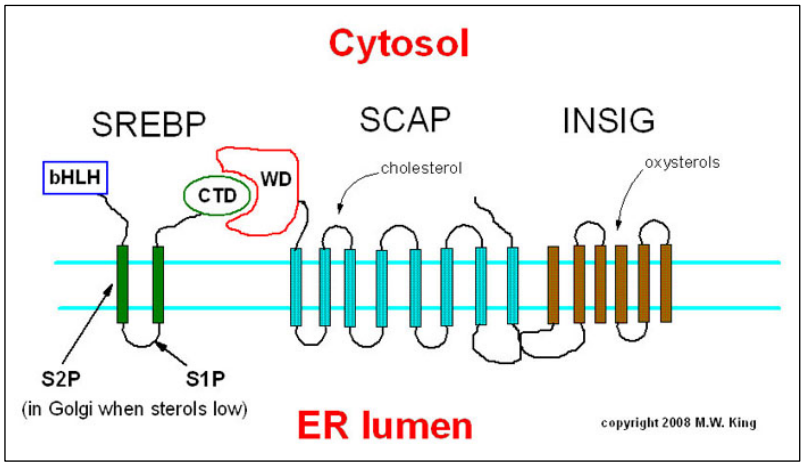


Figure 2.3: SREBP, sterol regulatory element binding proteins; SCAP, SREBP cleavage activating protein; Insig-1, insulin induced gene 1; bHLH, basic helix-loop-helix; CTD, C-terminal domain; S1P, site-1 protease; S2P, site-2 protease; WD = WD40 domain

The SREBP complex then travels to the nucleus and binds to the sterol regulatory element of several genes including those genes which regulate the synthesis of the LDL-C receptor and HMG-CoA reductase where it activates transcription that is regulated in accordance to the changing cholesterol levels. HMG-CoA reductase leads to increased cholesterol synthesis via the HMG-CoA pathway. LDL-C receptors play an important role in scavenging circulating low density lipoprotein from the blood stream and allowing entry of cholesterol into cells, thus when intracellular cholesterol is low, LDL-C receptor activity will increase to bring more cholesterol in from the blood. Accumulation of sterols in the endoplasmic reticulum membrane leads to cholesterol binding to the sterol –sensing domain of SCAP causing a change in conformation that leads to the re-association of SCAP to insig-1. Further SCAP-SREBP complexes are then prevented from reaching the Golgi apparatus for activation (Gyliing, 2004; Espenshade, 2006, Kruit et al, 2006). Newly synthesised cholesterol is released to the circulation as very low density lipoproteins (VLDL-C). In addition to removal via the LDL-C receptors, the triglyceride component of VLDL-C may also be hydrolysed by lipoprotein lipase to leave VLDL-C remnants known as intermediate density lipoprotein (IDL) that are taken up by receptors in the liver or converted to low density lipoproteins (LDL-C) (Gylling, 2004).

Hepatic lipoprotein receptors play a major role in the removal of lipoprotein remnants and LDL-C thus regulating human plasma cholesterol concentration (Fielding, 2000). The discovery of the LDL-C receptor was made when work into familial hypercholesterolaemia (FH), characterised by extreme high levels of cholesterol in the blood and early development of CHD, showed the disease to be a product of defects in the gene encoding for the LDL-C receptor, disrupting the receptor mediated feedback control of normal cholesterol metabolism (Brown and Goldstein, 1986). A variety of mutations were identified affecting synthesis, processing, binding or clustering of the receptor on the cell surface. Heterozygosity for an LDL-C receptor defect occurring in 1/500th of the general population results in double LDL-C levels whilst homozygote's for LDL-C receptor defects (1/1000000th general population) can present with levels six or more times higher than normal (Fielding, 2000; Breslow, 2000). In healthy individuals, regulation of the LDL-C

receptor is thought to be affected by several dietary factors. Down regulation of LDL-C receptors occurred in animal models consuming high saturated fat diets, suppressing receptor dependent clearance of LDL-C from the circulation whereas diets high in polyunsaturated fatty acids have been shown to increase LDL-C receptor function. There is also some evidence to suggest that increased dietary intake of soy protein may have an effect on LDL-C receptor activity (Ginsberg and Karmally, 2000).

2.6.6 Cholesterol excretion

Excess cholesterol is excreted via the liver accounting for approximately 50% of cholesterol elimination. Excess cholesterol in the blood is removed via hepatic receptors (LDL-C receptor related protein and SR-BI) that bind lipoproteins. Cholesterol is eliminated in the form of bile acids via the liver or transported to the intestine for excretion as faecal bile acids or conversion to LDL-C and triglyceride that is used for energy by the extra hepatic tissues (Mann and Skeaff, 2000; Gylling, 2004).

Bile acids are a major product of the hepatic degradation of cholesterol. Regulation of this pathway is principally through the rate limiting 7α-hydroxylation (a microsomal enzyme reaction) of cholesterol where cholesterol is metabolised to cholic acid, 7, 12-α-trihydroxycholanoic acid, chenodeoxycholic acid and 3, 7-α-dihydroxycholanoic acid through a series of oxidation steps in the mitochondria of the liver hepatocytes. These lipid soluble bile acids (bile salts) are amphiphilic molecules which are conjugated to taurine or glycine to become water soluble primary conjugated bile acids. These travel to the lower small intestine and colon where the activity of anaerobic bacteria results in the formation of secondary bile acids through the deconjugation and conversion of cholic acid to deoxycholic acid and chenodeoxycholic acid to lithocholic acid. Along the proximal and distal ileum, the primary bile acids and some of the secondary bile acids are actively reabsorbed into the hepatic portal circulation. They are extracted from the blood circulation by the liver, unconjugated and secreted back into bile. Remaining conjungated bile

acids are passively absorbed. The ileal venous blood travels through the portal vein and into the liver sinusoids where hepatocytes extract bile acids, leaving little to be found in the systemic circulation. During this process of enterohepatic circulation, each bile salt is used multiple times during a single digestive phase. Ninety eight percent of bile salts are re-absorbed by the intestine with the excess 2% being lost through faeces (Eastwood, 2003; Charlton-Menys and Durrington, 2007; Redinger, 2003).

The regulation of cholesterol homeostasis is still being elucidated and recently discovered transcription factors in the hepatic cells have been found to play important roles. The livers X receptors (LXra and LXRh) have the ability to alter the expression of many genes in cholesterol homeostasis, down regulating SREBP-2 and other cholesterogenic genes. LXRs up-regulate the expression of the ABC transporters and stimulate the production of lipoprotein lipase thus having a vital role in the control of cholesterol absorption and metabolism. They may also play a role in cholesterol excretion via their role in the up-regulation of ileal bile acid-binding protein (I-BABP) expression, a transporter involved in the movement of bile acids from enterocytes to the ileal lumen (Gylling, 2004; Lammert and Wang, 2005).

2.7 Lipoproteins

Cholesterol solubility in water is low and transport of triglyceride and cholesterol in the plasma is achieved through their association with apolipoproteins which stabilise the lipid within a coat of amphiphilic compounds of phospholipid and protein. The resulting lipoproteins are globular, high molecular weight particles which differ in the ratio of lipid to protein and have different proportions of lipids, triglyceride, free and esterified cholesterol and phospholipids. They are a heterogeneous group, divided into five major classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density proteins (IDL), low density lipoproteins (LDL) and high density lipoprotein (HDL). Each has a different biological function depending on the density, associated apolipoprotein, particle size

and chemical composition (Eastwood, 2003). The differential lipoprotein characteristics are shown in table 2.7.

Table 2.7: **Differential lipoprotein characteristics** (Tulenko and Sumner, 2000)

LIPOPROTEIN CLASS	DENSITY (G/ML)	SIZE (NM)	MAJOR LIPIDS	MAJOR APO - LIPOPROTEINS
Chylomicron	<0.93	100-500	Dietary TGs	B-48, C-II, E
VLDL-C	0.93-1.006	30-80	Endogenous TGs	B-100, C-II, E
IDL	1.006-1.019	25-50	CEs and TGs	B-100, E
LDL-C	1.019-1.063	18-28	CEs	B-100
HDL-C	1.063-1.210	5-15	CEs	A,C-II, E
Lp(a)	1.040 -1.090	25-30	CEs	B-100 and glycoprotein
TG, Triglyceride; CE, cholesterol ester; LP(a), lipoprotein little 'a" , LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; IDL, intermediate density lipoprotein; VLDL-C, very low density lipoprotein cholesterol				

Associated apolipoproteins are also categorised into groups based on their broad functionality which, in addition to providing stability for the lipoproteins, includes conferring specificity on the lipoprotein complexes, allowing recognition by specific receptors on cell surfaces and acting as co-factors in the activation of enzymes involved in lipoprotein modification (Groff and Groper, 1999). There are six classes, each with several subclasses. Table 2.8 highlights some of the characteristics of the apolipoproteins and their physiological function.

Interest has intensified on the potential value of measuring circulating concentrations of apolipoproteins as a marker of CHD risk (Walldius and Jungner, 2006). Apo B is a potential marker for all atherogenic lipoproteins. It is the sum of total VLDL, IDL and LDL, chylomicrons, and Lp (a) particles in plasma. Apo A-I is carried in HDL-C and acts as the major anti-atherogenic protein. Low Apo A-I levels indicate low HDL-C levels which is associated with increased CHD risk. Thus the ratio of Apo B: Apo A-I represents the balance of pro-atherogenic and anti-atherogenic lipoproteins and some strong evidence suggests that this index may be better at predicting the likelihood of vascular events than the cholesterol indices (Sniderman and Kiss, 2007; Thompson and Danesh, 2006; Walldius and Jungner, 2006; Walldius et al, 2004; Barter and Rye, 2006).

Table 2.8: **Characteristics and functions of apolipoproteins** (adapted from Groff and Gropper (1999) and Davis and Wagganer (2006).

APOLIPOPROTEIN	LIPOPROTEIN	SYNTHESIS	ACTION
A-I	Chylomicron, HDL-C	Liver, Intestines	Accepts cholesterol from peripheral cells through ABCA1. Stabilises structure of the lipoprotein. Co factor for LCAT, facilitates lipid uptake through SR-BI. Mediates reverse cholesterol transport and has anti-inflammatory and antioxidant properties
A-II	Chylomicrons HDL-C	Liver	Facilitates lipid uptake through SR-BI; displaces Apo A-1 from HDL-C Possible inhibitor of LCAT
A-IV	Secreted with chylomicrons but transfers to HDL-C	Liver	Associated with the formation of triacylglycerol – rich lipoproteins Function unknown
B-48	Chylomicron Chylomicron remnants	Intestine	Structural component
B-100	LDL, VLDL, IDL	Liver	Facilitates lipid uptake through LDL receptor
C-I	Chylomicron, VLDL, HDL-C	Liver, lung, skin, testes	Inhibits HL activity, Activates LPL activity, Inhibits Apo-E mediated lipid uptake by LDL –receptor and LRP, possible activator of LCAT
C-II	Chylomicron, VLDL, HDL-C	Liver, Intestine	Cofactor for extra hepatic LPL
C-III	Chylomicron, VLDL, HDL-C	Liver, Intestine	Several polymorphic forms. Inhibits LPL and HL activity. May stimulate CETP activity
D	Sub fraction of HDL-C		Function unknown
E	Chylomicron, chylomicron remnants, VLDL, HDL-C	Liver, brain, skin, testes, spleen	Ligand for chylomicron remnant receptor in the liver and LDL receptor.
(a)	Lp(a)	Liver	Thought to inhibit fibrinolysis through competing with plasminogen for binding with fibrin

HDL-C , high density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; ABCA1, adenosine triphosphate-binding cassette-A1; LCAT, lecithin:cholesterol acyltransferase; SR-BI, scavenger receptor BI; HL, hepatic lipase; LPL lipoprotein lipase; LRP, LDL receptor related protein; CETP; cholesterol ester transfer protein

A major advantage in the Apo B: Apo A-1 ratio is that the measurement accounts for all pro-atherogenic and anti-atherogenic lipoprotein particles in the estimate whereas measuring LDL-C has some methodological limitations. Firstly, it is not equivalent to measuring the number of LDL-C particles which vary in composition with some being larger than others. Additionally LDL-C is usually calculated from fasting total cholesterol, triglyceride and HDL-C measurements which may introduce inaccuracy (Sniderman and Cianflone, 1999). However, further work is required in this area to support the superiority of the Apo B: Apo A-1 ratio over LDL-C in risk prediction (NHLBI, 2002)

2.7.1 Lipoprotein Metabolism

The metabolism of lipoprotein is achieved via both exogenous and endogenous pathways. The exogenous pathway transports dietary fat via chylomicrons synthesized in the intestinal mucosal cells from dietary fat which renders them triglyceride rich with small amounts of phospholipids, cholesterol and apolipoprotein B48 (Apo B48). After secretion into the lymphatic system they enter the circulation via the thoracic duct where they acquire both Apo E and Apo C-II from other lipoproteins that facilitate chylomicron catabolism. Adipose lipoprotein lipase, an enzyme bound to the capillary endothelium which is activated by Apo C-II, hydrolyses approximately 90% of the triglyceride to free fatty acids (FFA) which are then used for energy by the muscle or heart or are stored in the adipose tissue. The remnant chylomicron particle returns to the liver where it is taken into the hepatocytes via a receptor mediated process with Apo E serving as a ligand for the receptor. Here the remaining triglyceride, cholesterol esters, phospholipids and apolipoproteins are released before the hepatocyte re-assembles them with endogenous triglyceride and cholesterol esters into very low density lipoproteins (VLDL-C) for release into the circulation (Tulenko and Sumner, 2002; Eastwood, 2003; Fielding, 2000).

VLDL-C is responsible for transport of triacylglycerol of endogenous origin from the liver and intestine, acting as a delivery vehicle for fatty acids to the heart, muscles and adipose tissue (Mann, 2000). These smaller, triglyceride rich particles

enter the circulation and pick up cholesterol esters, Apo C-II and Apo E donated from high density lipoprotein to become mature VLDL-C. The major apolipoprotein is Apo B-100, the physiologic ligand for the LDL-C receptor. Lipoprotein lipase activated by Apo C-II reduces the VLDL-C particle removing triglyceride, and Apo C (which is transferred back to HDL-C) leaving a smaller, more dense particle rich in cholesterol esters transferred from HDL-C in exchange for phospholipids and triglyceride via cholesterol ester transfer protein. Progressively, the molecule changes composition to become intermediate-density lipoprotein (IDL) of which around 50% is removed from the liver by receptors recognising the ApoB-100 or Apo E content. The other 50% are hydrolysed by hepatic triglyceride lipase to produce cholesterol rich low density lipoprotein with the primary apolipoprotein of ApoB-100 (Eastwood, 2003; Tulenko and Sumner, 2002). The presence of this apolipoprotein in chylomicron remnants, VLDL-C, IDL and LDL-C is significant because of its potential atherogenic properties (Tulenko and Sumner, 2002; St Pierre et al, 2006).

Levels of triglycerides and remnant lipoproteins from chylomicrons, IDL and VLDL-C which are apparent postprandially, have all been shown to be associated with increased CHD risk (Berry, 2005; Nakamura and Kugiyama, 2006; Karpe, 1999). Humans exist in the post prandial state for a large majority of each 24 hour period, eating between 4-6 times daily. Consumption of high fat meals will result in a large influx of TG (90-98%) and the frequently changing levels are postulated to be involved with the atherogenic potential of these lipids (Berry, 2005; Wilhelm and Cooper, 2003). A positive relationship between elevated serum triglycerides and CHD has been frequently reported (NHLBI, 2002; Cullen, 2000) but the role of TG is not fully elucidated since elevated TG levels can exist with other factors known to influence CHD making it difficult to determine an independent causal role of these lipids. Large prospective studies examining the post prandial lipid response with long term follow up to monitor CHD incidence are still lacking (Zock, 2007). Elevated triglyceride levels are classified as over 150mg/dl (3.87 mmol/L). Primary hypertriglyceridaemia results from genetic aberrations leading to disordered triglyceride metabolism (including familial chylomiconaemia, primary mixed hyperlipidaemia and familial hypertriglyceridaemia), whereas secondary hypertriglyceridaemia is multi-factorial with obesity, metabolic syndrome,

type 2 diabetes, low HDL-C lipoprotein, lifestyle activities (smoking, low physical activity , excess alcohol), genetics, certain medications and other diseases (hypothyroidism, renal failure, nephrotic syndrome) all having influence (Yuan et al, 2007; Pejic and Lee, 2006). Studies have examined the role of elevated TG as an independent risk factor of CHD. Assmann et al (1998) demonstrated that in the Prospective Cardiovascular Münster Study (PROCAM), triglyceride levels $\geq$ 5.17mmol/L doubled the coronary heart disease risk in patients with elevated ratios (> 5.0) of low- to high-density lipoprotein cholesterol (LDL-C /HDL-C). Most patients with LDL-C /HDL-C values > 5.0 had low HDL-C levels. These data suggest that individuals with hypertriglyceridaemia and reduced HDL-C levels are at particularly high risk of CHD. Similarly, Austin et al (1998) conducted a meta-analysis of 17 studies and showed that a 2.28mmol/L increase in plasma triglyceride levels increased the relative risk of CHD by approximately 14% in males and 37% in females after adjustment for HDL-C and other CHD risk factors. More recent work in the Asian and Pacific populations (The Asia-Pacific Cohort Studies Collaboration APCSC) has shown that increased fasting triglyceride levels are significantly associated with fatal and non-fatal CHD and cerebral infarction independently of any other major risk factors (Koba and Sasaki, 2006).

The relationship between TG and CHD is attributed to its associated atherogenic lipoprotein profile including the presence of non HDL-C including chylomicron remnants, increased VLDL, elevated Apo C-lll and increased small dense LDL particles (Cullen, 2000).

2.7.2 Chylomicron remnants

Chylomicron remnants (CMRs) carrying dietary derived fats play both a direct and indirect role in atherosclerosis and strong evidence exists to support their atherogenic nature (Wilhelm and Cooper, 2003). CMRs are directly involved in the initiation of the atherosclerotic lesion during their transport to the liver. Evidence has been presented showing that, despite their size, CMRs transit the endothelial cell wall and are anchored there by proteoglycan binding, leading to retention of atherogenic fatty material that may be the initiating step in fatty streak formation (Proctor and Mamo, 1998) and further validated by work showing that human CMRs

are capable of inducing the adhesion of monocytes to the endothelial cell wall. This work however remains controversial as it is more likely that the CMRs are taken up by monocytes which then in turn produce the adhesion molecules facilitating cell adhesion (Botham and Wheeler -Jones, 2007). Additionally CMRs are thought to mediate endothelial cell dysfunction through their ability to activate leucocytes. This results in increased expression of leukocyte adhesion molecules such as selectins, integrins, VCAM-1 and ICAM-1 and assists with the migration and adhesion of inflammatory cells into the sub-endothelial space. Furthermore they act in an inflammatory role and have an ability to activate the complement system – most notably the C3 component which is associated with obesity, coronary stenosis and the metabolic syndrome (Alipour et al, 2007). The mechanisms through which the CMRs exert their effects are not fully elucidated but are thought to involve the activation of intracellular signalling pathways including nuclear factor-κβ and mitogen-activated protein kinases that mediate gene expression (Wheeler-Jones, 2007). The role of CMRs in atherosclerosis also includes their uptake by macrophages and induction of triacylglycerol and cholesterol as part of the formation of foam cells. It is well documented that foam cell accumulation is the initial step in the development of the fatty streak that is characteristic to atherosclerosis and that oxidised LDL plays a major role in its formation (Falk, 2006). However, CMRs have been shown capable of producing the same damage to the arterial wall without the need for oxidation which actually appeared to inhibit their uptake and the induction of lipid accumulation (Botham et al, 2007; Bravo et al, 2007). This finding may explain why a majority of trials looking at the protective role of anti-oxidants in CHD development have not been successful (Botham and Wheeler-Jones, 2007). The effect of CMRs on foam cell formation appears to be modulated by several dietary factors including extra cellular lipolysis and the type of dietary fat present (Botham et al, 2007).

The indirect role of CMRs in atherosclerosis relates to their ability to alter the metabolism of the other lipoprotein classes. The evidence is limited but it appears that CMRs may be involved in changing the distribution of the circulating LDL population towards small dense LDL particles which in turn are related to endothelium dependant vasodilatation and blood vessel integrity. HDL-C metabolism may also be affected. Transfer of lipid between HDL-C and triglyceride

rich particles such as CMRs is a constant process. Cholesterol ester transfer protein mediates the exchange of cholesterol esters from HDL-C and triglycerides (TG) from triglyceride rich particles which results in triglyceride rich HDL-C particles that are a better substrate for hepatic lipase that subsequently removes the TG from HDL-C producing small HDL-C particles that are metabolised faster and can lead to lower HDL-C levels, a recognised risk factor for CHD development (Wilhelm and Cooper, 2003).

2.7.3 Very Low Density Lipoproteins

The involvement of small remnant VLDL in atherosclerosis is widely accepted and these molecules have been shown to be an independent risk factor for the presence, severity and progression of atherosclerosis (Hodis, 1999; Lui et al, 2006; Krauss, 1998). Although the mechanisms are not fully understood, the evidence thus far has supported the kinetic theory for the atherogenicity of VLDL – that the inhibition of triglyceride rich lipoprotein catabolism by ApoC-lll increases the residence time in the vascular walls, increasing exposure to high concentrations to atherogenic particles and increasing the risk of CHD (Zheng et al, 2007).

VLDL are cholesterol rich particles produced as a result of partial lipolysis from lipoprotein lipase activity on large TG rich particles and represent the level of TG in the fasting state. There are six subclasses of VLDL (V1 – V6) with V6 being the largest and most triglyceride laden (Davis and Wagganer, 2006). In addition to altering the metabolism of LDL (increasing its atherogenic potential) and HDL-C (decreasing its cardio protective potential) VLDL have been shown to be capable of penetrating the artery wall where they are taken up by macrophages and smooth muscle cells in the progression of the atherosclerotic lesion and may also be associated with altering blood flow by affecting both procoagulant and prothrombotic factors in the blood (Cullen, 2000; Hamsten, 1990). Studies have also shown that they can promote cholesteryl ester deposition in macrophages (Karpe, 1999). Numerous animal studies have also provided support to show VLDL is strongly associated with CHD (Karpe, 1999; Breslow, 1996). The presence of Apo C-lll in VLDL is associated with smaller, denser VLDL particles which have a

strong association with atherosclerosis (Carmena et al, 2004). In vitro studies show that ApoC-lll can inhibit lipoprotein lipase and hepatic lipase and slow the clearance of VLDL by interfering with the binding of ApoB-100 or ApoE to hepatic receptors (Zheng et al, 2007). Further work with transgenic mice shows that over expression of ApoC-lll causes hypertriglyceridaemia whereas ApoC-lll deficiency protects against it. Additionally, impaired particle clearance via the hepatic LDL-C receptors can produce reduced binding affinity to cell surface proteoglycans and inhibition of lipolysis with over production of VLDL (Zheng et al, 2007). Human studies have also shown that disturbance in the metabolism of triglyceride rich lipoproteins leads to lower secretion rates and higher concentrations of ApoC-lll levels in the plasma which are correlated to increased VLDL triglyceride. This disturbance also affects HDL-C-Apo A-l metabolism. Increased lipid exchange between VLDL and HDL-C generates triglyceride rich HDL-C, the preferred substrate for hepatic lipase that accelerates the catabolism of these particles. An excess of ApoC-lll, acting as an inhibitor of the hydrolysis of triglycerides, will favour the formation of these triglyceride rich HDL-C molecules and increase the catabolism of HDL-C apoA-l and thus lead to low plasma HDL-C (Chan et al, 2008). Several clinical studies have established plasma ApoC-lll as a predictor of cardiovascular outcomes. These are summarized in table 2.9 (Chen et al, 2008).

VLDL also has ApoE as a major constituent which is an important modulator of many stages in lipoprotein metabolism (Jofre-Monseny et al, 2008). It acts as a co-factor in VLDL-C synthesis and the hydrolysis of VLDL remnants to produce LDL and also has an important role as a high-affinity ligand for cellular lipoprotein uptake (Minhane et al, 2007).

Table 2.9: ApoC-lll as a predictor of CHD (Chen et al, 2008)

		RISK RATIO (95% CI)			
Study	No of participants	ApoC-lll in TRL	ApoC-lll in HDL-C	Total ApoC-lll	Triglycerides
Cholesterol and recurrent events (CARE) Sacks et al, 2000	418 CAD patients	**2.25** (1.4-3.6)	**ns**	**n/a**	**1.58** (1-2.5)
Turkish Adult Risk Factor Survey Onat et al, 2003	393 men	**8.87** (2.64-29.8)	**2.70** (1.01-7.25)	**3.88** (1.32-11.4)	**n/a**
	463 women	**3.22** (1.29-8.01)	**2.57** (0.94-7.01)	**2.54** (1.00-6.42)	**n/a**
Monitored Atherosclerosis regression study Hodis et al, 1994	220 CAD patients				
	Male	**2.3** (1.1-4.7)	**n/s**	**2.8** (1.1-6.9)	**1.7** (1.0-2.8)
	Female	**5.0** (1.4-17.1)	**n/s**	**n/s**	**ns**
Cholesterol Lowering Atherosclerosis Study Blankenhorn et al, 1990	162 CAD men	**n/s**	**0.6** (0.4-0.9)	**n/s**	**n/s**

Further pro-inflammatory atherosclerotic mechanisms of VLDL remnants include their ability to induce the activity of adhesion molecules in vascular endothelial cells leading to the recruitment of circulating monocytes and the activation of nuclear factor κβ which may stimulate a diverse pro-inflammatory response through monocyte activation (Kawakami et al, 2006; Kawakami et al, 2007).

Intermediate density lipoproteins (IDL) are a transient intermediate in the breakdown cascade of VLDL to LDL and as such they are sometimes referred to as VLDL remnants and have a relatively small concentration in the plasma. Evidence supporting an independent role in CHD development is lacking and in clinical practice IDL is included in the LDL fraction (NHLBI, 2002)

2.7.4 Lipoprotein (a) - (Lp (a))

Lp (a) is a modified LDL particle found only in primates and the European hedgehog consisting of a LDL moiety with a cholesterol rich core and one molecule of Apo B-100 and Apo(a) in a 1:1 molar ratio (Berglund and Ramakrishnan, 2004). Apo (a) is a unique protein, structurally different from other apolipoproteins, which influences the metabolic and physiochemical properties of Lp (a) which are similar to those of LDL. Levels of Lp (a) are largely genetically determined with varying levels found amongst different populations; African Americans generally have much higher levels than Caucasians and Asians (Carmena, 2004; Hilpert et al, 2006; Boffa et al, 2004). Dietary effects on Lp(a) are exerted through a high dietary intake of saturated fatty acids and trans fats which are both capable of decreasing Lp (a) concentrations, however the decrease in Lp(a) is offset by the other detrimental effects of these fatty acids (Nestel et al, 1992; Clevidence et al, 1997). Polyunsaturated fats and other dietary factors have not been shown to alter levels (Hilpert et al, 2006). Elevated levels of Lp (a) are now recognised as a major independent risk factor of CHD in several retrospective case control studies and some prospective studies although not all the evidence is conclusive Problems relating to measurement of Lp (a) is a major issue for studies as standardized methods are unavailable making accurate quantification of plasma levels difficult (Carmena et al, 2004; NHLBI, 2002; Berglund and Ramakrishnan, 2004). The mechanism of action of Lp (a) is not fully understood; some data supports a link between Lp (a) and IL-6 and an involvement in the inflammatory response whilst some evidence exists for interactions between Lp (a) and LDL-C, HDL-C and homocysteine. Furthermore Lp (a) is thought to be retained in the vessel wall where it attenuates fibrinolysis and promotes coagulation which is due to the structural similarity of Apo (a) to plasminogen (Berglund and Ramakrishnan, 2004). Presently, Lp (a) is resistant to therapeutic treatments (NHLBI, 2002).

2.7.5 Low Density Lipoprotein

LDL transports cholesterol to the tissues and consists of a lipid core that is essentially cholesterol with the polyunsaturated fatty acid, linoleate and a surface of unesterified cholesterol, phospholipids and single copy of Apo B-100. It is a derivative of VLDL after removal of triaglycerol and the amount produced relies

upon the amount of VLDL produced or removed by the liver through LDL receptors which dictate the LDL concentration and removal from the circulation. LDL is divided into three subclasses, L1-L3 with up to six further subclasses (Davis and Wagganer, 2006). LDL enters the cells through specific extra hepatic LDL receptors and the distribution of LDL to the tissues depends on the number of cell surface receptors available and the rate of transcapillary transport which is regulated by regulatory proteins and genes according to the amount of cholesterol in the cell. This occurs in all nucleated cells but mainly in the liver which removes up to 70% of LDL. LDL receptors, carrying a recognition site for both ApoB and ApoE, are to be found in the clathrin –coated pits representing 2% of the cells surface which form vesicles that are endocytosed into the cell where LDL is degraded by lysosomal enzymes. Cholesterol esters are hydrolysed by cholesterol ester hydrolase and the incorporation of cholesterol into the endoplasmic reticulum of the cell inhibits HMG-CoA reductase. Further cholesterol may be incorporated into cell membranes or exported to the plasma (Eastwood, 2003).

The evidence linking LDL-C to coronary heart disease is vast, with elevated LDL-C being associated to CHD in all studied populations and significant reduction in clinical events being achieved by treatment therapies reducing plasma LDL-C (Sirtori et al, 2006). Its atherogenic potential is linked to both size, density and oxidative state of the molecule (Carmena, 2004). Epidemiological studies such as The Framingham Heart Study, the Multiple Risk Factor Intervention Trial and the Lipid Research Clinics trial all presented evidence to show a direct link to elevated LDL-C levels and rates of CHD and numerous animal and human studies have been performed to verify the involvement of this lipoprotein in atherosclerosis (NHILB, 2002). The levels of LDL-C required to post a risk for CHD are variable. Levels below 2.6mmol/L are optimal but even marginal increases above this level are shown to be associated with elevated risk thus only in populations that maintain very low levels of LDL-C indefinitely, is CHD risk reduced (NHLBI, 2002).

Increasingly, evidence is now also revealing that the heterogenicity of LDL plays a vital role in its atherogenic potential (Davis and Wagganer, 2006). The concentration and size of the LDL particle, rather than the amount of cholesterol it contains is strongly related to the extent of atherosclerotic progression (Carmena,

2004; Davis and Wagganer, 2006). Small, dense LDL particles (LDL-III) have diameters of 25.5 to 24.2 nm and a density of 1.040 and 1.060 g/ml with the B phenotype. These small dense LDL-C particles are more atherogenic than large buoyant LDL-C particles and are often termed the atherogenic lipoprotein phenotype (ALP) (Sartipy et al, 1999).Animal studies using genetically modified mice have demonstrated that in two strains of mice with identical levels of plasma cholesterol, the ones with smaller LDL-C particles had a greater level of atherosclerosis (Veniant et al 2008). Several human prospective, nested case control studies have also shown that the presence of small dense LDL-C is associated with a 3 fold risk in CHD risk (Carmena et al, 2004). The Quebec Cardiovascular Study presented evidence that men exhibiting a higher proportion of smaller LDL particles had a significant 2.2 fold increase in the prevalence of CHD than men with larger LDL particle sizes. This was independent of LDL, triglyceride, HDL-C, Apo B and total cholesterol: HDL-Cholesterol ratio (Lamarche et al, 2001). Conversely, the Physician's Health Study showed that mean LDL particle size was not a significant risk indicator for myocardial infarction (Stampfer, 1996). The small dense LDL particle phenotype usually exists concurrently with other risk factors for CHD such as hypertriglyceridaemia, reduced HDL-C, obesity, insulin resistance, impaired endothelial cell function and increased susceptibility to thrombosis and as such the extent to which they predict CHD risk is not elucidated. The susceptibility to develop ALP is apparently associated with genes in chromosome 19, 11 and 16 with heredity controlling 50% of its expression. Other metabolic reasons for the excess of small dense LDL particles are still being investigated with one theory being that overproduction of large triglyceride rich very low density lipoproteins causes an increase in the exchange of triglyceride for cholesterol esters between this particle and LDL resulting in a momentarily greater level of triglyceride in LDL. This becomes a good substrate for hepatic lipoprotein lipase producing small dense particles with a reduced content of core cholesterol ester and surface components of phospholipids and unesterified cholesterol (Sartipy et al, 1999). The increased atherogenicity of the small dense LDL particles is attributed to two possible mechanisms; one is its preferential entry and retention in the arterial wall at sites of lesion development and the other is the possibility that these particles are more susceptible to oxidative and hydrolytic modification than the larger more buoyant LDL particles (Sartipy et al 1999). At present measurement of LDL particle size is

not recommended on a routine basis as methodologies for measurement are not standardized and LDL cholesterol remains the primary treatment target (NHLBI, 2002).

2.7.6 High Density Lipoprotein cholesterol

High density lipoprotein is a complex macromolecule consisting of a core of hydrophobic lipids (cholesterol esters and triglycerides), an envelope of phospholipids, some unesterified cholesterol and the apolipoproteins, ApoA1 and ApoA2. The surface coat also contains some ApoC, ApoE and ApoD (Genest, 2008; Eastwood, 2003). Elevated levels of HDL-C have been shown in many studies to have cardioprotective effects with incidences of CHD decreasing as HDL-C levels increase to the point where it has been suggested that for every 1% increase in HDL-C observed, a 2-3% reduction in CHD is achieved (Choi et al, 2006; Ascasco et al, 2004). There appears to be numerous mechanisms by which HDL-C exerts this effect which include: the prevention of LDL oxidation through the presence of paraoxonase, an enzyme that catalyses the degradation of oxidized LDL phospholipids; prevention of vascular wall inflammation by the inhibition of sphingosine kinase, an enzyme produced by endothelial cells that decreases the action of sphingosine 1-phosphate, a key molecule in mediating the inflammatory process mediated by tissue necrosis factor; inhibition of thrombosis through the actions of ApoA-I and stabilization of prostacyclin that protects against CHD through vasodilatation, inhibition of platelet aggregation and inhibition of endothelin-1 synthesis; preservation of endothelial cell function through decreased adhesion molecule expression; prevention of macrophage and endothelial cell apoptosis and an increase in endothelial cell progenitor cells (Genest, 2008; Davis and Wagganer, 2006; Choi, 2006). However the major mechanism of HDL-C is the promotion of reverse cholesterol transport (RCT) that facilitates the movement of cholesterol from the arteries and arterial plaque to the liver for subsequent catabolism or excretion into the bile. Macrophage reverse cholesterol transport is the term used to describe the specific process of movement of cholesterol from the macrophage foam cells (Tall, 2008).

2.7.7 Reverse Cholesterol Transport

Lipid poor apolipoprotein A-I is synthesized by the liver and intestine and interacts with adenosine triphosphate-binding cassette transporter A1 (ABCA1) located on the arterial macrophages which promotes efflux of cholesterol and phospholipids to extra cellular lipid poor HDL generating nascent (pre-β) HDL. Lecithin: cholesterol acyltransferase (LCAT) esterifies cholesterol within the nascent HDL particle to produce mature α-HDL particles, small dense HDL_3 and larger less dense HDL_2, which take up free cholesterol via the macrophage adenosine triphosphate-binding cassette transporter G1. This mature HDL then enters one of two pathways: the direct pathway where cholesteryl esters contained in HDL undergo selective uptake by hepatocytes and steroid hormone-producing cells via the scavenger receptor type B1 and consequent excretion into the bile and the indirect pathway where the action of cholesteryl ester transfer protein (CETP) promotes the exchange of cholesterol esters in HDL for triglycerides in the apolipoprotein B-rich particles LDL and VLDL. This LDL may then be subsequently returned to the liver via LDL receptors (LDLr) and LDL-related protein receptors (LRPr) which may be responsible for up to 50% of RCT. Triglyceride rich HDL then undergoes hydrolysis by hepatic lipase and endothelial lipase to form small HDL-C for transport (Singh et al, 2007; Tall, 2008; Gillotte et al, 1998). Figure 2.4 shows a diagrammatic representation of reverse cholesterol transport (Mooradian et al, 2006).

HDL-C levels vary considerably in the population with around 50% of that variation attributable to genetics and the rest being affected by lifestyle behaviours such as smoking, physical inactivity, obesity, high carbohydrate intakes (>60%) and interactions with pharmaceuticals (NHLBI, 2002). Low HDL-C levels have been linked to increase morbidity from CHD for three decades and its role as an independent risk factor has been supported by strong epidemiological evidence (Castelli et al, 1986; Sharret et al, 2001; Tall, 2008). Low HDL-C levels also correlate strongly with high triglyceride levels, remnant lipoproteins and small dense LDL particles (termed “the lipid triad”) and has association to insulin resistance and the metabolic syndrome (NHLBI, 2002).

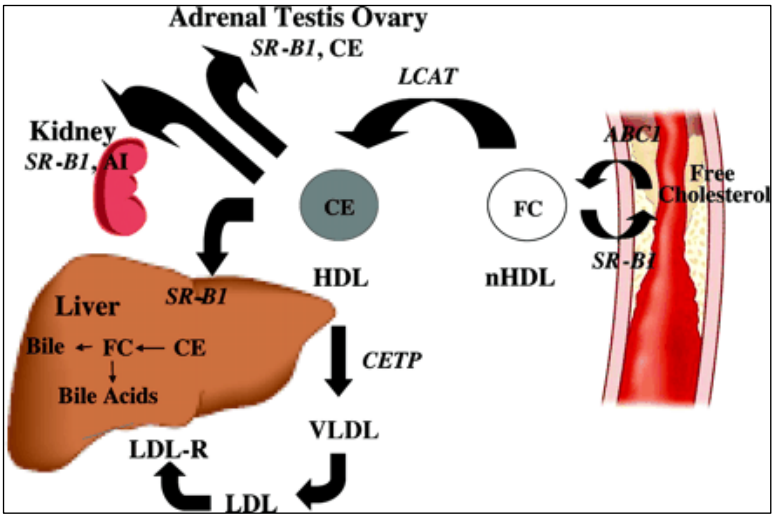


FIG. 2.4. Schematic diagram summarizing some of the key steps in Reverse Cholesterol Transport (Mooradian et al, 2006).

Free cholesterol (FC) from the periphery is transported to the nascent HDL (nHDL) particle through the activity of the ATP binding cassette transporter 1 (ABC1) transporter. Free-cholesterol associated with the cofactors apo A-I and apo A-IV is a substrate for LCAT, which esterifies the cholesterol to form cholesterol esters. The HDL particle circulates in the plasma where it can be modified by CETP to form VLDL, or the cholesterol esters (CE) can be off-loaded by the scavenger receptor class B1 transporter protein (SR-B1) located in the liver (where it is converted to free cholesterol as well as bile and bile acids), kidney, and the steroid hormone synthesizing adrenal gland and ovary or testes.

The relationship of HDL-C to CHD have triggered interest in this lipoprotein as a potential target for therapy, however simply raising HDL-C levels in individuals has not yet been proven to prevent CHD and has provided some conflicting evidence (NHLBI, 2002). The functionality of HDL-C is also affected by the size, density and composition of the HDL molecules. Inflammation can cause modification of apoA-I and render HDL-C dysfunctional with pro-inflammatory and atherogenetic properties. Individuals with CHD appear to have greater levels of pro-inflammatory HDL-C (Singh et al, 2007). In other studies, presence of the large α-1 HDL-C particle was shown to be a significant risk factor for recurrent CHD events in the Veteran's Affairs High Density Lipoprotein Cholesterol Intervention Trial (Asztalos et al, 2005). Van der Steeg et al, 2008 have also shown that elevated levels of HDL-C are not cardio protective and may confer additional risk once corrected for ApoA-I and ApoB levels.

Despite these contradictory findings, low HDL-C levels are considered a categorical cardiovascular risk factor for CHD in the National Cholesterol Education Program guidelines which define a low HDL-C level as <40mg/dL (1mmol/L) but do not identify a specific HDL-C goal level to reach. Lipid modifying drugs (statins, nicotinic acid, fibrates) are all successful at raising HDL-C

to varying degrees whilst lowering LDL-C and use of these drugs that modify multiple inter-related lipid risk factors appear to be successful in reducing CHD risk (NHLBI, 2002). HDL-C levels can also be beneficially modified through the incorporation of regular aerobic activity into the habitual lifestyle (Varady and Jones, 2005). Studies have reported a 20% increase in HDL-C levels over a ten week period in elderly hypercholesterolaemic women through the inclusion of moderate aerobic exercise with out changes to body weight or dietary intake (Fahlman et al, 2002). Other studies have reported a 10% rise in HDL-C after including a moderate level of jogging (16km per week) into trial participant's weekly programme (Sagiv and Goldbourt, 1994). Halle et al, 1999 also demonstrated that healthy male individuals with a moderate level of physical fitness displayed higher levels of HDL-C and lower levels of small dense LDL-C than men with lower levels of physical fitness. The level of benefit gained from physical activity with respect to raising HDL-C levels is dependant on the amount and level of intensity of exercise, smaller gains are observed with lower levels of activity (Tavintharan et al, 2005). However the combination of dietary therapy and physical activity can be significantly more advantageous due to their complementary effects on lipid profiles. Low saturated fat diets combined with exercise have been shown to lower total cholesterol, LDL-C and triglyceride concentrations between 7-18% whilst increasing HDL-C between 5-14% (Varady and Jones, 2005). Alternatively nutritional supplementation with fish oil, oat bran or plant sterols, combined with exercise has demonstrated reductions of total cholesterol, LDL-C and triglyceride concentrations between 8-39% and increased HDL-C levels between 2-8% (Varady and Jones, 2005). The combination of lifestyle therapies is thus a successful means of improving serum lipoprotein profiles in individuals with dyslipidaemia although the link to this decreasing cardiovascular disease risk has yet to be firmly established (Varady and Jones, 2005).

2.7.8 Genetic variation in lipoproteins

Although environmental factors such as diet, smoking and physical activity have a major influence on determining lipid profiles in individuals, about half of the variation in these traits can be attributed to genetic variation (Breslow, 2000). Evidence exists to support the fact that variation in the genes for apolipoprotein A-I,

apo A-IV, apo B and apo E all contribute to the heterogeneity in the lipid response to dietary intervention, with apo E being the most widely studied in relation to CHD (Masson et al, 2003; Minihane et al, 2007). Apo E is a multifunctional protein that plays a key role in cholesterol and triglyceride metabolism with roles as a cofactor for VLDL synthesis, the in the hydrolysis of VLDL remnants to produce LDL and as a high affinity ligand for the receptor mediated cellular removal of lipoprotein remnants (Bennet et al, 2007; Minihane et al, 2007). Variation in the apo E gene results in three alleles, ε2, ε3 and ε4 which can produce 3 homozygous (E2/2, E3/3 and E4/4) and three heterozygous (E2/3, E2/4,E3/4) genotypes (Masson et al, 2003). Linear relationships have been demonstrated between these apo E genotypes with both LDL-C levels and CHD risk however, the ε2 genotype is thought to have the lowest risk (20% lower risk) and ε4 carriers the highest risk (40% higher) when compared to the ε3 genotype (Minihane et al, 2007; Bennet et al, 2007). Throughout white populations, these genotypes are distributed with a frequency of ε2; 0.08, ε3; 0.77 and ε4; 0.15 (Masson et al, 2003). The impact of apoE on CHD risk has been largely attributed to the higher circulating cholesterol and triglyceride levels it produces however studies are now showing that the impact is lipoprotein independent with effects been shown on macrophage, vascular smooth muscle cell, endothelial cell and platelet function (Minihane et al, 2007). Furthermore, apoE genotype has been shown to alter the way an individual responds to the total fat content and fatty acid composition of the diet. A review by Masson et al, 2003 examined 46 studies that involved altering the dietary fat content of the diet. Eight studies showed a significantly different response to total cholesterol whilst eleven showed greater LDL-C responses; ε4 carriers showed the greatest response. Similarly, Schaefer et al, 1997 also showed that a lower fat diet resulted in a significant reduction of LDL-C levels in study participants with ε4 being associated with the greater reduction. Although the evidence suggests that background dietary fat composition may be involved in the relationship between apoE genotype and CHD risk, there remains an opportunity for larger scale studies to be performed to confirm such findings. Small sample sizes used in the a majority of the reviewed cases are liable have insufficient power to detect possible inter-genotype difference in response and be therefore subject to bias (Minihane et al, 2007). Table 2.10 lists some of the other identified genetic variation associated with different lipoprotein types and their influences on CHD.

Table 2.10 Genetic variations of different lipoprotein types and influences on CHD (Breslow 2000)

LIPOPROTEIN	GENETIC VARIATION	OUTCOME	EFFECT ON CHD
Low density lipoprotein	LDL-c receptor gene (chromosome 19p13.2)	Familial hypercholesterolaemia ↑LDL-C	↑ CHD
	LDL-C receptor binding region of apoB100 (chromosome 2p23-p24)	Familial defective apoB100 ↑LDL-C	↑ CHD
	apoB gene mutations	Heterozygous hypobetalipoproteinaemia ↓LDL-C	↓CHD
	cholesterol 7α-hydroxylase (CYP7) gene (chromosome 8q11-q12)	Rate limiting enzyme in bile acid formation. Affects LDL-C receptor synthesis in liver and plasma LDL-C ↑LDL-C	↑ CHD
	chromosome 1p34-p32	Autosomal dominant hypercholesterolaemia ↑LDL-C	↑ CHD
	Chromosome 15q25-q26	Precocious hypercholesterolaemia ↑LDL-C	↑ CHD
	ApoE (chromosome 19q13.2)	variation- 3 alleles resulting in 6 phenotypes E3/3, E4/3, E3/2, E4/4, E4/2 and E2/2 ↑LDL-C	E4/3 = ↑ CHD
		E2/2 – familial type III hyperlipoproteinaemia (1/5000th of population but only overtly manifested in 1/50th of these individuals) ↑LDL-C and chylomicron remnants	↑ CHD
Lipoprotein (a) levels	Chromosome 6q2.6-q2.7	Possible inhibition of plasminogen activation interfering with fibrinolysis and increasing thrombogenic risk ↑ Lp(a)	Not fully elucidated
High density lipoprotein	Apo A1 gene mutations (chromosome 11q23-q24)	Plasma apo A-1 deficiency ↓ HDL-C	↑ CHD
	Defect of ABC1 transporter gene (chromosome 9q31)	affects cholesterol excretion to free apolipoproteins resulting in Tangier Disease ↓ HDL-C	↑ CHD
	Mis-sense mutations in apo A-1 gene	Apo A-1 milano (Arg173Cys mutation) ↓HDL level	No apparent effect on CHD
Triglyceride	Homozygous mutations in gene for LPL (chromosome 8p22) or for its cofactor apo CII (chromosome 19q13.2)	↑ triglyceride	Not fully elucidated
	Mis-sense mutations in Asp9Asn and Asn291Ser	Decreased LPL activity, ↑ triglyceride ↓ HDL-C	Not fully elucidated. Some evidence of ↑ CHD in women

2.8 Diet and Cholesterol

2.8.1 Saturated fatty acids

Dietary fatty acids have a significant effect on plasma cholesterol concentration and therefore affect the risk for coronary heart disease (Schaefer, 2002). Early studies have all demonstrated a positive relationship between saturated fat intake and CHD risk through the elevation of total and LDL-C cholesterol. It is indicated that SFA are possibly the most damaging dietary factors in terms of the fact that when compared to carbohydrate on an energy equivalent basis, SFA raise total and LDL-C plasma cholesterol concentration despite their ability to raise HDL-C cholesterol levels (Hu et al, 2001; Keys; 1966; Hegsted, 1965; Mensink and Katan, 1992). However, not all saturated fatty acids have the same effect. SFA with 8-10 carbon atoms, caprylic and capric acid, appear to have little influence on cholesterol levels. Myristic acid (C14:0) and palmitic acid (C16:0) found in dairy products and meat are hypercholesterolaemic, raising total and LDL-C cholesterol whilst having a moderate effect on HDL-C relative to carbohydrate. Lauric acid (C12:0) found in tropical oils is more potent in its ability to raise total and LDL-C cholesterol than myristic and palmitic acid but actually raises HDL-C cholesterol proportionally higher than LDL-C thus decreasing the total: HDL-C ratio relative to carbohydrate (Hu et al, 2001; Fernandez and West, 2005; Kris-Etherton and Yu, 1997; Caggiula and Mustad, 1997; Mensink et al, 2003). In comparison to other long chain SFA, stearic acid (C18:0) has little effect on total and LDL-C cholesterol concentrations when compared to carbohydrate and is rapidly converted to oleic acid, a monounsaturated fatty acid in the human body (Schaefer, 2002). It may however, reduce HDL-C levels when compared to monounsaturated and polyunsaturated fatty acids as well as increasing Lp(a) and negatively affecting factors involved in blood clotting (Hu et al, 2001; Tholstrup, 2005). The regulatory mechanisms controlling the effect of dietary fatty acids on plasma LDL-C cholesterol are not fully elucidated but are thought to be through decreasing LDL-C receptor mediated catabolism, reducing protein and mRNA abundance and thus affecting membrane fluidity and causing less receptor cycling across the cell membranes (Schaefer, 2002; Fernandez and West, 2005). SFA intake in the Western diet is prevalent and currently is estimated to contribute levels of around 15% energy

to the diet although dietary recommendations suggest that levels of SFA should contribute no more than 12% to the total energy intake (WHO, 2003).

2.8.2 Monounsaturated fatty acids

In the human diet, oleic acid is the major cis-monounsaturated fatty acid derived from sources such as olive oil, canola oil and nuts. A majority of studies indicate that this type of fatty acid has a favourable effect on plasma lipids and mortality rates for CHD (Hu et al, 2001; Willet, 2006a). Studies reporting an increased risk of CHD with increasing MUFA offer a conflicting opinion but have been criticized for their failure to adjust for confounding factors such as the strong correlation between MUFA,SFA and trans fats intake in common food sources (Wahrburg, 2004; Hu et al, 2001). When compared with carbohydrate, MUFA has been shown to increase HDL-C concentrations and decrease plasma triglycerides without affecting LDL-C concentrations (Kris-Etherton and Yu, 1997) and similarly when substituted for SFA, monounsaturated fat has been shown to demonstrate a hypercholesterolaemic response, significantly lowering total and LDL-C cholesterol (Kris-Etherton, 1997; Gardner and Kraemer, 1995) however it is still debated as to whether this effect is as significant as that achieved with polyunsaturated fat (Schaefer, 2002; Hu et al, 2001; Lada and Rudel, 2003). Additionally, a further postulated benefit of the substitution of MUFA for saturated fat is that MUFA have been recently shown to offer some protection against the oxidative damage caused to LDL-C that is an important factor in the development of atherosclerosis (Wahrburg, 2004).

2.8.3 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids are divided into two major sub-classes, n-6 and n-3 which differ in the location of the first double bond in the carbon chain. These fatty acids are of major biological importance but are not synthesized by the body thus they are obtained through dietary intake and are termed essential fatty acids (Schaefer, 2002). Linoleic acid (18:2) is the most predominant n-6 polyunsaturated fat in the diet obtained from vegetables and vegetable oils (corn, safflower, soybean and sunflower). When compared with both carbohydrate and SFA (replacing around 8% total energy) this fatty acid demonstrates a significant lowering effect on both

total and LDL-C cholesterol. Additionally n-6 PUFA above 10% of total energy intake lowers HDL-C concentrations but this appears to be less pronounced than the LDL-C reducing capacity thus resulting in a lower total: HDL-C ratio. The effect of increased dietary levels of PUFA on serum triglycerides is yet to be elucidated but is thought to be negligible (Wahrburg, 2006; Kris-Etherton, 1997; Lichtenstein, 2006; Hu et al, 2001; Lada and Rudel, 2003).

N-3 polyunsaturated fatty acids include eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) found in oily fish. It also includes α-linolenic acid (ALA) from plant sources such as flaxseed, walnuts, canola and soybean oil which is converted in low levels to EPA through the de-saturation-chain via the elongation pathway (Lichtenstein, 2006). Studies have shown that increasing intake of n-3 fatty acids from dietary sources of fish or through fish oil supplementation appears to elicit no effect on total cholesterol levels and a small increase in HDL-C levels seen in individuals whose HDL-C levels may otherwise decrease with time (Balk et al, 2006; Wahlberg, 2006). They also produce a significant reduction in serum triglycerides via the ability of DHA and EPA to inhibit hepatic VLDL-C-triglyceride synthesis, thus whilst VLDL-C particle number stays the same, the triglyceride content is reduced. This effect is much more pronounced in individuals with hyperlipidaemia before addition of n-3 PUFA to the diet but an increase of 1g/day fish oil is associated with a decrease of triglyceride of approximately 8mg/dl (0.2mmol/L) (Balk et al, 2006). Some studies have shown that n-3 supplementation may increase LDL-C concentrations in people with underlying hyperlipidaemia when dosages exceeded 10g per day, these results are not yet explained but it has been postulated that it may be due to an increased conversion rate of VLDL-C to LDL-C (Lichtenstein, 2006; Wahrburg, 2006, Balk et al, 2006). Less research is available on the effects of ALA. The demonstrated effects of ALA on lipid profiles are limited and effects of less than 2mg/dl (0.05mmol/L) net change in total, LDL-C and HDL-C levels have been reported (Balk et al, 2006). However there may be other beneficial effects of ALA in terms of other triggers for the development of CHD. Increased ALA consumption may decrease platelet aggregation and reduce the formation of the pro-aggregatory thromboxane A_2. Additionally several epidemiological studies have shown that higher intakes of ALA are associated with reduced CHD risk (Hu et al, 2001). Levels of ALA and its conversion to long chain

n-3 fatty acids are influenced by linoleic acid levels as they compete for the desaturation and elongation pathway and the optimal balance between the two polyunsaturated acids may be important in the prevention of thrombosis and atherosclerosis. ALA and its metabolite EPA have the ability to reduce thromboxane A_2 production due to their inhibitory action on the conversion from linoleic acid to arachidonic acid. This occurs through the activity of the enzyme cyclooxygenase and it is thought that the greater the ratio of ALA to linoleic acid, the greater the reduction in thrombotic tendency. However the optimal ratio has not yet been elucidated and it is important to recognise that n-6PUFA have other beneficial effects including reducing LDL-C and the improvement of insulin sensitivity which need to be taken into consideration when considering how to define the most favourable ratio (Hu et al, 2001).

n-3PUFA may also modulate endothelial cell function and may be another mechanism by which they may reduce CHD risk. Several studies have shown that supplementation with n-3PUFA can result in improved vasodilation, decreased expression of endothelial cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin and also decreased soluble thrombomodulin and von Willibrand factor (Brown and Hu, 2001). The mechanism for the effects is still unknown but it is evident that the n-3PUFA must be incorporated into the cellular phospholipids to be effective. This results in a simultaneous reduction in n-6PUFA supporting the hypotheses that the ratio of n-3 to n-6PUFA may be important (Brown and Hu, 2001).

2.8.4 Trans Fatty Acids

Unsaturated fatty acids (both mono and polyunsaturated) exist in the natural *cis*-transfiguration, having two hydrogen atoms attached to the carbons on the same side of the double bond where the molecule bends. Upon hydrogenation, the hydrogen atoms are placed on opposite sides of the double bond and the molecule stays straight resulting in the *trans* configuration which is a more linear molecule similar to SFA (Lichtenstein, 2006). *Trans* fats in the diet occur largely from partially hydrogenated fats found in many baked products and fried foods, although a

lesser amount are present in ruminant animal fats such as meat and dairy. They are estimated to contribute 2-3% of energy to the Western diet (Willet, 2006b; Lichtenstein, 2006; Hu and Willet, 2002). Although little information is available on the specific effect of individual *trans* fatty acids, multiple studies have demonstrated adverse effects on blood lipid profiles (Asherio, 2006; Willet 2006b). Relative to *cis* unsaturated fatty acids, increased levels of *trans* fats raise LDL-C cholesterol and lower HDL-C cholesterol. Additionally the increase in the total: HDL-C cholesterol ratio is approximately twice the response seen with saturated fatty acids – a response that may occur with only a small amount of *trans* fat in the diet. A recent study has shown that less than 1% energy from *trans* alpha linoleic acid, a product found in many refined liquid vegetable oils, cause an 8.1% increase in the total:HDL-C ratio (Vermunt et al , 2001; Ascherio, 2006). Considering the relationship between the total cholesterol: HDL-C ratio and increased CHD risk, this would have the potential to cause a greater number of the population being put at excess risk (Willet, 2006b). The deleterious effects of *trans* fatty acids also extend to their ability to increase Lp(a) and triglycerides when substituted for saturated fat (Ascherio, 2006; Hu and Willet, 2002; Lichtenstein, 2006; Hu et al, 2001). Mauger et al (2003) also reported that *trans* fatty acids may increase the numbers of small dense LDL-C particles, whilst other research has demonstrated some effects on endothelial cell function and essential fatty acid metabolism but the consequences of these changes are not yet understood (Ascherio, 2006).

2.8.5 Dietary Cholesterol

The exact contribution of increased dietary cholesterol intake to dyslipidaemia and development of CHD remains under debate. Since only one third of cholesterol absorbed is from dietary sources, the endogenous source of cholesterol is the major contributor to circulating cholesterol (Huff, 2003). Animal studies have shown some evidence of a positive relationship but also point out that there is a large variation between species as to the amount of cholesterol needed to induce hypercholesterolaemia and data from these studies is difficult to extrapolate to humans because of the vast difference in lipoprotein profiles (McNamara, 2000). Currently, some studies have shown that there is a positive linear relationship between dietary cholesterol intake, LDL-C and triglyceride levels with each 100mg

dietary cholesterol resulting in 0.5-1.0 mmol/L increase in triglyceride due largely to LDL-C increases (National Academy of Sciences, 2002). Additionally a recent meta-analysis has also shown the dietary cholesterol raises the total cholesterol: HDL-C ratio, a known risk factor for CHD (Weggemans et al, 2001). However the consensus is that the effect of dietary cholesterol is highly variable with up to 80% of the population being hypo-responders and 20% being hyper-responders that leads to a small but significant effect on plasma cholesterol concentration (2.2-2.7 mg/dl or 0.05 – 0.07mmol/L per mg of dietary cholesterol) which overall has little relevance to CHD risk (McNamara, 2000). It is recommended that reduction in saturated fatty acids is more important as a dietary intervention for the prevention of hyperlipidaemia and CHD except for those who are obese, have large amounts of abdominal fat and are physically inactive who should reduce all fat intake whilst maintaining some sources of polyunsaturated fats (Tim, 2003).

2.8.6 Dietary Carbohydrate

Dietary carbohydrate (CHO) is categorised into three classes: sugars (glucose, fructose, sucrose, and lactose), oligosaccharides (maltodextrins, raffinose, fructo-oligosaccharides) and polysaccharides (starch and non-starch polysaccharides) which differ in chemical composition and physical structure and exert varied physiological effects (Brand-Miller, 2002). These carbohydrates are further categorised in terms of the blood glucose and insulin responses they produce following digestion leading to their classification as glycaemic and non-glycaemic carbohydrate (Englyst and Englyst, 2005). Glycaemic CHO (simple sugars, oligosaccharides and some starch) has the ability to raise blood glucose levels after digestion and absorption whereas non-glycaemic CHO (non-starch polysaccharides and resistant starch) pass through the small intestine largely unchanged and are fermented by bacteria to various degrees in the large intestine, contributing little change to blood glucose levels (Brand-Millar, 2002; Englyst and Englyst, 2005). Currently the Western diet consists of a high proportion of highly processed, calorie dense, nutrient depleted foods, high in glycaemic CHO which upon consumption, lead to elevated post-prandial spikes of blood glucose and lipids (O'Keefe and Bell, 2007). Prolonged intake of glucose leads to increased lipogenesis, following the saturation of muscle and liver glycogen stores. Glucose is converted to pyruvate via

glycolysis which is converted to Acetyl Co A, the starting a material for fatty acid synthesis which are either metabolised to carbon dioxide or stored as triglyceride (Brand-Millar, 2002). Additionally, high glucose intake results in elevated and prolonged post prandial hyperglycaemia resulting in impaired glucose intolerance leading to insulin resistance, key initiators in the development of metabolic syndrome, type 2 diabetes and cardiovascular disease (Cordain et al, 2005).

The glycaemic response of carbohydrates is expressed through the glycaemic index (GI) (area under the 2 hour glycaemic curve after consumption of a food containing 50g carbohydrate divided by the area under a curve for a standard food containing 50g carbohydrate, usually glucose or white bread) or glycaemic load (GL) (which also takes into account the amount of carbohydrate in the food: GI*available carbohydrate) and may be a more relevant way to determine the effects of carbohydrate rich foods on lipid parameters (Hilpert et al, 2006). Observational studies have shown that consumption of low GI products can result in reduced postprandial rises in glucose and insulin, improving insulin sensitivity and reducing hepatic synthesis and secretion of triglycerides that leads to lower concentrations of triglyceride rich lipoproteins and increased plasma HDL-C (Hilpert et al, 2006; Poli et al, 2008; Venn and Green, 2007). Conversely, a meta-analysis of intervention trials has shown only limited, weak evidence of an inverse relationship between GI and total cholesterol with no effect on LDL, HDL-C, triglycerides, fasting glucose or fasting insulin (Venn and Green, 2007). Whilst the relationship of GI and GL to dyslipidaemia is not fully understood, the presence of excessive postprandial hyperglycaemia and thus hyperinsulinaemia is linked to all-cause and CHD mortality, increased carotid intima media thickness and impaired endothelial cell function and thus consumption of a lower GI/GL diet may be an additional non-pharmacological treatment option for improving plasma lipid profiles and reducing CHD risk (Hilpert et al, 2006).

There is little uncertainty that substituting saturated and *trans* fatty acids with carbohydrate can lead to a reduction in total and plasma LDL but this is coupled with the fact that this can also lead to the deleterious effect of increasing plasma triglycerides due to an alteration in lipoprotein secretion and clearance that results in VLDL and chylomicron remnant accumulation (Mann, 2008; Lopez-Miranda et al,

2007). Additionally clinical studies have demonstrated that this substitution is also associated with a decrease in plasma HDL-C increasing CHD risk (Berneis and Krauss, 2002). The type of carbohydrate plays a critical role in determining the effect elicited on the plasma lipoprotein profile. Simple sugars, sucrose and fructose, are associated with increases in plasma triglyceride particularly when consumed as part of a diet high in carbohydrate and saturated fatty acids. High triglyceride levels resulting from sucrose intake may also lower HDL-C as triglyceride enriched HDL-C particles are more readily catabolised (Mann, 2008; Poli et al, 2008). The mechanism involved in the association of dietary sucrose and HDL-C is uncertain however some studies have suggested that dietary sucrose may increase plasma cholesterol in the VLDL and LDL fractions leading to a concomitant reduction in HDL-C (Archer et al, 1998). Mono and disaccharides do not appear to significantly affect plasma cholesterol profiles (Poli et al, 2008).

Dietary fibre is the term given to a variety of plant substances that are resistant to digestion and absorption in the human small intestine with complete or partial digestion in the large intestine. It can be classified as water-soluble (natural gel forming or viscous fibres including β-Glucan, pectin, gum, glucomannans, psyllium) or as insoluble (structural or non-viscous fibres including lignins, cellulose, some hemicelluloses) (Theuwissen and Mensink, 2008). Dietary fibre has a significant effect on plasma lipids and lipoproteins which is more notable for soluble/gel forming fibre from cereal and plant sources (Poli et al, 2008). These fibre sources lower total and LDL cholesterol between 5 to 10g/day which appears to be due to reduced ileal bile acid absorption and increased faecal excretion (Mann, 2008). Brown et al, 1999 demonstrated that each gram of soluble fibre reduces total plasma cholesterol concentration by 0.05mmol/L and plasma LDL by 0.06mmol/L. Soluble fibre does not induce significant effects on plasma triglycerides and HDL-C (Poli et al, 2008). To date, insoluble dietary fibre appears to have no effect on lipid profiles (Mann, 2008). Randomized controlled trials assessing the effects of wholegrain cereals on lipid profiles as a risk factor for CHD were analysed in a Cochrane Review (Brunner et al, 2005). From the ten studies included, eight assessed the effects of wholegrain oats and its derivatives showing them to have no effect on triglyceride or HDL-C cholesterol but to be highly capable of reducing

plasma LDL cholesterol by around 0.2mmol/L when compared to a control diet (Brunner et al, 2005).

The mechanism for the hypocholesterolaemic effect of water soluble fibres is not fully understood but evidence suggests that some water soluble fibres may form a thick unstirred water layer in the intestinal lumen and binds to bile salts decreasing the re-absorption of cholesterol and bile acids. This results in increased faecal output and the increase of hepatic conversion of cholesterol into bile acids. Hepatic pools of free cholesterol decrease and endogenous cholesterol synthesis increases. Additionally, hepatic LCL-C receptors are up-regulated to re-establish hepatic free cholesterol stores and these processes will ultimately lead to decreased serum LDL-C concentrations (Theuwissen and Mensink, 2008). Furthermore, the lipid altering ability of diets high in soluble fibre and carbohydrate may be due to the enhanced satiety associated with these diets that are higher in bulk and less energy dense. Whilst the research continues into the exact mechanism responsible for the hypocholesterolaemic effect, regular consumption of these foods may encourage lower caloric intake and subsequent weight loss will ultimately contribute to the less atherogenic lipid profiles exhibited (Mann, 2007; Poli et al, 2008).

2.9 LDL-C reduction and pharmacological treatment therapies for CHD prevention

The strong direct relationship between levels of LDL-C and CHD in populations is supported by extensive evidence gained from animal, genetic, epidemiologic and clinical studies. The relationship is linear and is present over a wide range of LDL-C levels (Navare and Thompson, 2006). Thus reduction of LDL-C has become a primary target for decreasing risk and the prevention of CHD (NHLBI, 2002). The National Education Program (NCEP) Adult Treatment Panel, published in 2002 provides guidelines for the management of dyslipidaemia based on the evidence supplied by large, randomized, controlled clinical trials that examine the efficacy of available pharmacological therapies and also includes a focus on primary prevention of CHD using a public health approach such as the adoption of a diet low in saturated fat, maintenance of a healthy weight and regular physical activity (NHLBI, 2002). The intensity of the intervention recommended relates to

the degree of CHD risk determined by the number of major risk factors present. A 10 year CHD risk is then calculated (Navare and Thompson, 2006). Using risk categories provided by the Framingham Heart Study, treatment goals are characterized into three levels of risk shown in table 2.11.

Table 2.11 Classification of CHD risk and LDL-C goals (Navare and Thompson, 2006)

RISK CATEGORY	CRITERIA	LDL-C GOAL (mg/dl)	NON-HDL-C GOAL (mg/dl)	LDL-C LEVEL TO INITIATE TLC (mg/dl)	LDL LEVEL TO INITIATE DRUG THERAPY (mg/dl)
High	Existing CHD CHD equivalents ≥2 risk factors (10 year risk >20%)	< 2.59 (<0.06mmol/L)	<3.36 (<0.09mmol/L)	≥ 2.59 (<0.06mmol/L)	> 2.59 (<0.06mmol/L)
Intermediate	≥2 risk factors 10 year risk 10-20% 10 year risk <10%	< 3.36 < 3.36 < 3.36 (<0.09mmol/L)	< 4.14 < 4.14 < 4.14 (<0.11mmol/L)	 ≥ 3.36 ≥ 3.36 (≤0.09mmol/L)	 ≥ 3.36 ≥ 4.14 (<0.11mmol/L)
Low	0-1 risk factors	< 4.14 (<0.11mmol/L)	< 190 <4.91mmol/L)	≥ 4.14 (<0.11mmol/L)	≥ 4.91 (<0.13mmo/L)

- Reduction in dietary saturated fat intake to <7% of total calories and cholesterol intake to <200mg/day. This may reduce LDL-C by 6-10%
- The use of viscous (formerly called soluble) fibre and plant stanols/sterols. A viscous fibre intake of 5-10g reduces LDL-C by 5% while 2-3g plant sterols/stanols daily reduce LDL-C by 6-15%.
- Weight reduction
- Increase in physical activity

Pharmacological therapy is generally required for individuals with high or intermediate risk rating of which there are five major classes that can be used in isolation or in combinations.

2.9.1 Statins

Statins are the most powerful and consistent LDL-C reducers shown to reduce the risk of all atherosclerotic clinical events (NHLBI, 2002). These drugs include simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin and rouvastatin. They work through competitive inhibition of the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG Co A) reductase that catalyses the conversion of HMG to

mevalonate, the rate limiting step in cholesterol synthesis. Meta-analyses from primary and secondary intervention studies show that these drugs significantly reduce mortality and morbidity from CHD although inter-individual variation exists (Puccetti et al, 2007; Stein, 2003). Overall statins reduce LDL-C by 18-63%, increase HDL-C by 5-10% and lower TG concentrations by 7-30% although there are variations amongst the different statins and the effect on LDL-C reduction is dose –dependent (Navare and Thompson, 2006). Currently all statins are regarded as safe although side effects can include non-specific myalgias, gastrointestinal problems and myocytis (Stein, 2002).

2.9.2 Bile acid sequestrants

These bind bile acids in the gut, interrupting their enterohepatic circulation, sequestering them and preventing their reabsorption. They include the drugs, cholestyramine, colesevelam and colestipan which are moderately effective in lowering LDL-C by 15-25% over their dose range and are often used as adjuncts to other therapies to increase the LDL-C lowering effect. They are considered safe but have a range of side effects including gas, bloating, constipation and nausea and individuals can have issues with palatability as they are administered as powders (Stein, 2002). These drugs have the ability to interfere with other drugs such as warfarin, thyroxine or oral hypoglycaemic agents and can bind the fat-soluble vitamins, D, E, K, A so patients may need vitamin supplementation (Stein, 2002; Navare and Thompson, 2006).

2.9.3 Niacin

Is also known as nicotinic acid and vitamin B3 and has been shown in numerous studies to have an LDL-C reducing ability achieving between 5-25% reduction (Navare and Thompson, 2006). This compound has many actions but mainly inhibits lipolysis in peripheral tissues reducing free fatty acid availability for synthesis of TG in the liver that ultimately reduces hepatic secretion of Apo-B and VLDL. Niacin also raises HDL-C significantly, achieving between 5-40% increases and reduces triglycerides between 20-35%. Unfortunately this drug has side effects which reduces patient compliance, mainly flushing and irritation of the skin and can

interfere with glucose tolerance, gout and peptic ulcers. Further, more serious, side effects include hepatotoxicity, jaundice and hepatitis. Again this treatment is more effective at lowering LDL-C when used in combination with statins (Navare and Thompson, 2006; Stein, 2002).

2.9.4 Fibrates

Derivatives of fibric acid, these drugs are effective in lowering TG's by 30-35% in normal subjects and 55% in hypertriglyceridaemic individuals. They are also capable of increasing HDL-C by 10-15% but have variable success on LDL-C with reductions of 10-20% reported. The mechanism of action is activation of nuclear transcription factor, peroxisome proliferator activated receptor (PPAR)-alpha in the muscle, liver and other tissues. This results in increased fatty acid oxidation in the liver and muscle reducing VLDL-TG synthesis, increasing lipoprotein lipase (LPL) activity and decreasing expression of the LPL inhibitor Apo C-III which increases VLDL and other remnant particles. It also increases HDL-C synthesis through greater synthesis of Apo A-I and Apo A-II (Navare and Thompson, 2006)

2.9.5 Cholesterol absorption inhibitors

Ezetimibe is a selective inhibitor of cholesterol and phytosterol absorption which has been shown to produce LDL-C reductions of 18% alone or 38-52% when combined with statin therapy (Navare and Thompson, 2006). Its site of action is the brush border membrane of the small intestine where it binds to the Niemann-Pick C1-Like 1 protein on the epithelial cell and hepatocytes, inhibiting dietary and biliary cholesterol. Decreased cholesterol absorption leads to an increase in LDL-C uptake in cells and decreasing levels in the plasma. No significant side effects have yet been identified (Stein, 2002; Navare and Thompson, 2006).

2.9.6 Fish oils

Inclusion of fish oils rich in n-3 (omega) fatty acids such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) in the diet can reduce elevated triglycerides by up to 50% but may also increase LDL-C levels. Most evidence suggests that n-3 fatty acids reduce the synthesis and secretion of VLDL and

increase removal of TG from these remnant particles through the up regulation of enzymes such as LPL but the exact mechanism of action is unknown. This therapy is best used in complement to statin or other drug therapy and further research is required to elucidate their cardiovascular effects (Bays et al, 2008; Navare and Thompson, 2006)

2.10 Therapeutic options for lowering LDL-C

Pharmacological agents used to lower LDL-C demonstrate significant clinical benefit but other primary prevention treatment strategies are now being recommended incorporating public health initiatives aimed at reducing LDL-C levels in the general population and thus lower CHD risk (Talbert, 2002; NHLBI, 2002). The National Cholesterol Education Treatment Program (NCETP) Adult Treatment Panel lll have now included features into their recommendations which focuses on therapeutic lifestyle changes aimed at CHD prevention in individuals with multiple risk factors for the disease, as well as adding a complementary therapy for those already receiving clinical treatment for established CHD. It is envisaged that this population based approach will provide the greatest impact on reducing the extent of CHD within society (NHLBI 2002). This public health approach includes smoking cessation, the adoption of healthy dietary practices such as low saturated fat, low cholesterol, maintenance of a healthy weight and regular physical activity. Additionally it has recognised the role of therapeutic dietary options that have been demonstrated to augment LDL lowering such soluble fibre, soy protein and phytosterols (NHLBI, 2002). The role of phytosterols in this area has been of interest since the early 1950's and is now gathering a wealth of evidence demonstrating their beneficial effects on LDL-C (Katan, 2003). Their role in dyslipidaemia and CHD is discussed in detail in the following chapter.

Chapter 3

Phytosterols and their role reducing CHD risk

3.1 Phytosterol structure and function

Phytosterols is the collective term for plant sterols and stanols (the less abundant hydrogenated form of plant sterols) that are natural components of edible vegetable oils, legumes, nuts, and grains. These compounds are an essential constituent of cell membranes, influencing cell permeability and fluid exchange that are structurally and functionally similar to animal derived cholesterol, with a Δ5 double bond and a 3β-hydroxyl group, differing only in their side chain configuration (Sudhop et al, 2005; Ostlund, 2002b). Figure 3.1 shows the structure of common plant sterols.

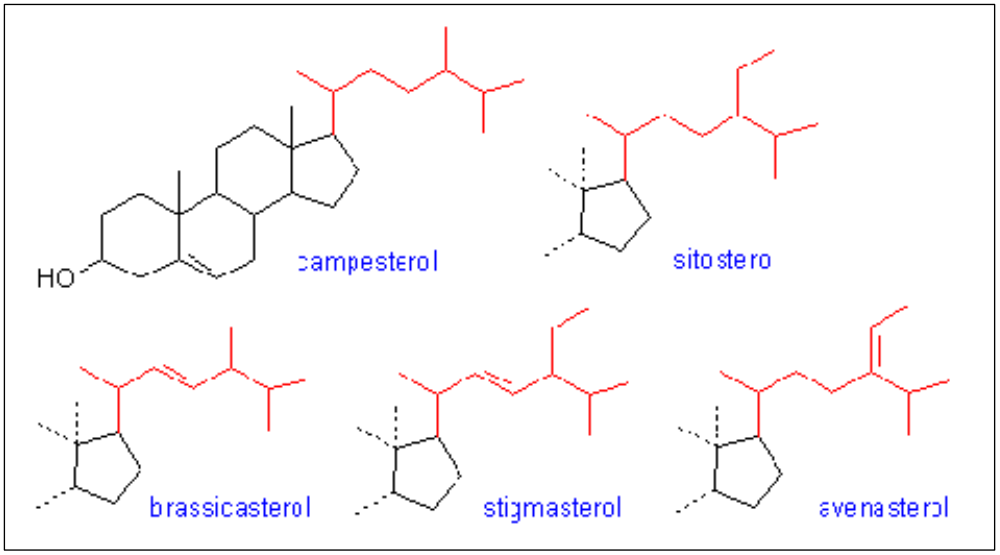


Figure 3.1: plant sterol structure (Sudhop et al, 2005)

Over 40 plant sterols have been identified which fall into three categories: 4-desmethylsterols (no methyl groups); 4-monomethylsterols (one methyl group) and 4, 4-dimethylsterols (two methyl groups). The three compounds that account for most of the plant sterols found in food are β-sitosterol, campesterol and stigmasterol which belong to the 4-desmethylsterols (Ostlund, 2002b; Ling and Jones, 1995).

The average daily intake of plant sterols in Western countries varies between 167-437mg/day of which little is absorbed by the human intestine. Whereas 50% of cholesterol is absorbed, levels for campesterol are around 10-15%, and sitosterol around 4 to 7% with plasma plant sterol levels usually less than 1mg/dl (Lichtenstein and Deckelbaum, 2001; Katan et al, 2003). Studies have shown that although intestinal sterol uptake is a very rapid process, they are rapidly re-secreted into the intestinal lumen and later excreted (Sudhop et al, 2005). The adenosine triphosphate binding cassette (ABC) subfamily G transporters, ABCG5 and ABCG8 transporter proteins work as efflux pumps to transport free sterols out of the cells and back into the intestinal lumen or into the bile, regulating the absorption and biliary secretion of cholesterol and plant sterols (Katan et al, 2003; Sudhop, 2005). The poor absorption of plant sterols from the gut contributes to their being declared Generally Recognised As Safe (GRAS) by the US Food and Drug Administration and these compounds have repeatedly been proven to be non-toxic in a variety of studies at levels 150 times higher than the recommended dose for human consumption (Sanders et al, 2000; Chan et al, 2006; Lea and Hepburn, 2006; Katan et al, 2003; St Onge and Jones, 2003). However, in the rare autosomal recessive inherited genetic disorder of Sitosterolemia, individuals have mutations in the intestinal ABCG5 and ABCG8 transporters and present with significantly increased plasma plant sterol levels, xanthomas and premature onset of atherosclerosis in the absence of hypercholesterolaemia (Sudhop et al, 2005). This finding suggests that high levels of plasma plant sterols may possibly have some correlation to CHD. A study by Fransen et al, 2007 has highlighted that using plant sterol enriched products can elevate serum plant sterol concentrations in humans that may be a potential health risk and therefore negative effect that requires extensive further investigation (Fransen et al, 2007).

Plant sterols are thought to have several metabolic effects, most notably their ability to lower serum total and LDL-C concentrations in humans. The mechanism of action is not fully elucidated but is based on the inhibition of absorption of dietary and endogenously produced cholesterol relating to the similarity in physical structure borne by plant sterols (Ostlund, 2007). This similarity allows plant sterols to compete with cholesterol for space in the mixed micelle, which has limited capacity, resulting in less cholesterol being absorbed. They may also reduce the esterification

rate of cholesterol in the enterocyte and thus the amount of cholesterol excreted via the chylomicrons or limit trans-membrane transport by their presence in the unstirred water layer or other mucosal barriers (Lichtenstein and Deckelbaum, 2001; de Jong et al, 2003; Ostlund, 2002b).

The evidence supporting the ability of phytosterols to reduce blood cholesterol has been available since the 1950's when large doses of up to 25g/day were fed to participants (Pollack, 1953). The large bulk and taste of these products reduced the practicality of introducing this strategy as a standard therapy. The problem was that the inherently hydrophobic plant sterols in their free form are largely insoluble and must be solubilised before they become bioavailable. This may be achieved by emulsification such as sitostanol emulsified with lecithin or mixed phytosterols with sucrose esters. With more recent technologies, hydrogenation of plant sterols to their corresponding stanols followed by esterification with fatty acids has produced esters that are readily dissolvable in fat and allows for their incorporation into products such as margarine and mayonnaise, increasing dispersion in the intestine and improving efficacy at much lower doses. Fat based spreads have been shown to be a very successful vehicle for delivery of plant sterols, readily accepted by consumers and efficacious in reducing cholesterol levels (Katan, 2003). Furthermore, specific health claims relating to the inclusion of plant sterol enriched spreads and mayonnaise in the reduction of heart disease risk has been approved by the FDA since 2000 (FDA, 2000). As interest in plant sterols/stanol esters increases, more formulations are becoming available and products now include yoghurt, cream cheese and milk (Katan et al, 2003; Clifton, 2002). The evidence produced from several studies have shown that the hypocholesterolaemic properties of these new functional food products are apparent even in the context of the habitual background diet of individuals, although the effect may be more pronounced if used in conjunction with a diet lower in saturated fat and higher in whole grain and dietary fibres (Maki et al, 2001).

Extensive evidence supports the role of phytosterols in total and LDL cholesterol lowering. It appears that both free and saturated plant sterols and their ester derivatives are capable of reducing cholesterol absorption and that stanols and sterols are equally effective (Clifton, 2002; Vanstone et al, 2002; Hallikainen et al,

2000; Westrate and Meijer, 1998). Berger et al, 2004 examined a variety of studies dating from 1990 to 2004 looking at factors affecting the safety and efficacy of phytosterols. Conclusions from this review included the finding that a minimum dose of 0.8 to 1g daily of free sterol or free sterol equivalents will reduce LDL cholesterol by 5% or more which will correlate to an approximate 6 to 10% reduction in CHD risk at age 70 years. Furthermore the dosage of plant sterols can be delivered in a variety of food vehicles. Other studies have supported this finding with 0.8 to 1.0g/day being shown to be the lowest dose that could produce a clinically significant reduction in LDL-C of 5% (Pellitier et al, 1995; Hallikainen et al, 2000; Hendricks et al, 1999). Another study by Law et al, 2000 combined the findings of 18 controlled clinical trials involving the consumption of plant sterol or stanols. They concluded that an average intake of 2g/day lowered serum cholesterol by up to 14% compared to levels prior to plant sterol consumption whilst a further detailed meta-analysis by Katan et al, 2003 compared a total of 50 studies (23 clinical trials of plant sterol enriched foods and 27 clinical trials of plant stanol enriched foods) and demonstrated that a reduction of up to 10% in LDL-C concentrations can be achieved with doses of 2g/day compared to levels prior to plant sterol consumption. Greater levels of intake did not lead to any greater reduction. More recently a meta analysis by Chen et al, 2005 examined 23 studies and found an average reduction of 11% in LDL-C with a daily intake of 3.4g of plant sterols and stanols compared to levels prior to plant sterol consumption. Generally, the participants in these studies were healthy individuals with mild hypercholesterolaemia , however, Mouruisi et al, 2006 recently conducted a meta-analysis of 6 studies examining the effects of plant sterols in adults and children suffering from familial hypercholesterolaemia (FH), characterised by very high concentrations in LDL-C and premature coronary heart disease. The authors found that similarly a reduction of 10-15% in LDL-C was observed with a daily dosage of 2.3g plant sterols/stanols without any adverse effects suggesting that this therapy is appropriate for the treatment of this disorder, moreover it also was effective in children which may be of benefit since long term safety, poor compliance and cost effectiveness of drug treatment is a complication of treating FH patients (Mouruisi et al, 2006).

A selection of some of the studies supporting the effects of phytosterols in enriched margarines and food products are included in table 3.1

3.2 Plant sterols in the natural diet

Clinical studies of LDL lowering by phytosterols are performed with respect to the natural baseline diet (containing an average of 150-450mg/d) and not a diet free from phytosterols and therefore do not address the potential role of basal levels of dietary phytosterols in cholesterol lowering. Recently, Andersson et al, 2004, examined the relationship between dietary intake of natural plant sterols and serum lipid concentrations as part of the European Prospective Investigation into Cancer (EPIC) study. This study of 22,256 men and women aged between 39-79 years in a free living population showed that serum total and LDL cholesterol concentrations were inversely related to dietary phytosterol intake after adjustment for age, body mass index and total energy intake suggesting that dietary sterols may have a role in decreasing cholesterol absorption. This work was validated by a second study conducted in Sweden also showing a significant inverse relationship between naturally occurring plant sterols and serum cholesterol (Klingberg et al, 2008). Fuentes et al, 2008, have recently shown that basal levels of dietary sitosterol in hyperlipidaemic subjects can predict the changes in LDL cholesterol with changes being greater in individuals with higher basal levels than lower levels. Conversely, other work by Houweling et al, 2007 has shown that baseline plasma plant sterol concentrations are not associated with or predictive of changes in serum cholesterol. Thus the subject of basal levels of phytosterols in the habitual diet requires more research to evaluate their impact on serum cholesterol parameters.

Table 3.1: studies evaluating efficacy of plant sterol enriched food products

Author	Trial	Finding
Fransen et al, 2007	Examining changes in serum cholesterol, plant sterol and plant sterol concentrations after long-term use of enriched margarines. Comparison of plant sterol (n=67) and plant stanol(n=13) based margarines with matched non-users (n=81)	1.1±0.6g/d plant sterols produced a significant 4% decrease in serum total cholesterol (p=<0.05) from non-users. Serum sitosterol and campesterol increased by 22% (p=0.0001) and 103% (p=<0.01) respectively in plant sterol users. Serum sitostanol and campestanol increased by 197% (P=0.02) and 196% (p=0.01) respectively in plant stanol users
Westrate and Meijer, 1998	Randomised double blind placebo controlled trial comparing the effects of margarine enriched with vegetable oil sterols or sitostanol ester with non-enriched control. n=100. Five treatments for 3.5 weeks.	1.5-3.3g/d sterol intake produced a significant reduction in plasma total (8%) and LDL cholesterol (13%) compared to a control spread of similar fatty acid content and composition. HDL-C cholesterol was not affected
De Jong et al, 2007	Community intervention study investigating exposure and effectiveness of plant sterol/stanol enriched margarines post launch onto the Dutch market over six years. n=2379 subjects were classified into four groups: (1) phytosterol/stanol enriched margarines; (2) cholesterol lowering drugs; (3) combination; (4) control – neither therapy	Mean daily intake of phytosterol/stanol margarine was 14±9g. Total serum cholesterol concentration changed significantly amongst four groups. Combination group showed a decrease of 29%; Cholesterol lowering drug group showed a decrease of 17%; the enriched margarine group showed a decrease of 4% and non users showed an increase of 2%.
Madsen et al, 2007	Double blind randomised placebo controlled cross over study evaluating the effect of low fat products (spread and milk) enriched with plant sterols in addition to the NCEP step 1 diet on serum lipids and lipoproteins. n=46. Two intervention periods of 4 weeks each. 2.3g plant sterols per day	Compared to placebo, plant sterol enriched products significantly reduced serum total cholesterol and LDL-C by 5.5% (p=<0.001) and 7.7% (p=0.001) respectively. Apo B was significantly reduced by 4.6% (p=<0.05) and apoB:apoA-1 ratio was reduced by 3.4% (p=<0.05)
Cleghorn et al, 2003	To determine the effect of plant sterol enriched spread on plasma cholesterol concentrations when replacing butter or standard polyunsaturated spread in a diet of 30% fat in mildly hypercholesterolaemic individuals. n=50. Parallel butter phase followed by randomised double blind cross over polyunsaturated spread phase. 2g plant sterols daily	When compared to butter, standard polyunsaturated spread reduced mean plasma total cholesterol by 4.6% (P=<0.01) and LDL-C by 5.5% (<P=0.05). Polyunsaturated spread containing plant sterols reduced total plasma cholesterol by 8.9% (P=<0.01) and LDL-C by 12.3% (P=<0.01). HDL-C was unaffected
Hallikainen et al, 2000	Randomised double blind repeated measure design investigating the cholesterol lowering effects of plant sterol and plant stanol enriched margarines as part of a low fat diet. n=34 mean sterol/stanol intake 2g/day. 3 intervention periods of 4 weeks each	Compared to control, serum total cholesterol was reduced by 9.2% with plant stanols and by 7.3% with plant sterols (P=<0.001). LDL-C was reduced by 12.7% with stanols and 10.4% with sterols (P=<0.001). No significant changes were found in fat soluble vitamin and carotenoid concentrations when related to serum cholesterol concentrations
Hendriks et al, 1999	Randomised double blind placebo controlled Latin square design using five spreads (butter, polyunsaturated spread, and three plant sterol enriched spreads at 0.83, 1.61, 3.24g/d) over four periods of 3.5 weeks. n=100 healthy and mildly hypercholesterolaemic individuals	Low doses of plant sterols of 0.83, 1.61 and 3.24g/day produced a reduction in total cholesterol of 0.26, 0.31 and 0.35 mmol/L respectively. For LDL-C the reduction was 0.20, 0.26 and 0.30 mmol/L respectively and the LDL/HDL-C ratio was 0.13, 0.16 and 0.16 respectively. Differences in reductions between the plant sterol doses consumed were not significant. Plasma Vit K1 and Vit D, lycopene and α-tocopherol were not affected by plant sterols but plasma alpha and beta carotene were decreased by 11% and 19% by daily consumption of 0.83 and 3.24g plant sterol spread.
Maki et al, 2001	Randomised double blind 3 group parallel controlled study. Effect of esterified plant sterols on serum lipid concentrations in a mildly hypercholesterolaemic population. NCEP step 1 diet with 4 week run in period using 50% fat spread followed by 3 types of reduced fat spread for 5 week intervention period. Control spread (n=92), plant sterol enriched spread at 1.1g/day (low sterol group, n=92) or plant sterol enriched spread at 2.2g/day (high sterol group n=40)	Total cholesterol was reduced by 5.2% and 6.6% for low and high plant sterol group respectively. LDL-C was reduced by 7.7% and 8.1%. Apo B values were 6.2% and 8.4% lower and total: HDL-C cholesterol were 5.9% and 8.1% lower (P=<0.001 for all values). Triaglycerol concentrations decreased by 10.4% in high sterol group and soluble vitamins and carotenoids were not significantly affected.

3.3 The relationship of lowering cholesterol using plant sterols to a reduction in risk for CHD

It has been suggested that a 1% reduction in LDL-C can result in a 2% reduction in CHD risk and as such, the average 10% reduction facilitated by the inclusion of 2g plant sterols in the daily diet could result in a significant 20% reduction in CHD risk, although this remains to be fully established over a long-term period (NHLBI, 2002). From the research previously discussed however, it is evident that these plant components are effective in cholesterol reduction and by combining this therapy with a healthy diet, low in saturated fat and cholesterol and high in vegetables, fruit and whole grains, there may be a cumulative effect and a substantial CHD risk reduction (Maki et al, 2001; NHLBI, 2002). Jenkins et al, 2005 showed that by combining foods with cholesterol lowering properties (soy protein, soluble fibre, plant sterols), serum LDL-C could, on average, be reduced up to 30% thus conferring considerable reduction in CHD risk.

Animal studies have also provided evidence supporting the anti-atherogenic activity of plant sterols beyond that of LDL-C reduction. These are summarized in table 3.2:

Table 3.2: Animal studies supporting the activity of plant sterols beyond LDL-C reduction (Berger et al, 2004).

AUTHOR	FINDING
Awad et al, 2001	Reduction of the hyper proliferation of vascular smooth muscle cells and regulation of prostacyclin synthesis in rats
Ntanios et al, 1998	Decreased plaque accumulation in coronary arteries after addition of sitostanol in rabbits
Moghadasian et al, 1997	Decreased platelet counts, reduction of red cell haemolysis and deceased plasma fibrinogen in Apo-E deficient mice
Moghadasian et al, 1997; Moghadasian et al, 1999; Ntanios, 2003	Decreased formation of atherosclerotic lesions in Apo-E deficient mice

3.4 Further Biological effects of plant sterols

Further effects on lipids and lipoproteins include increased cholesterol synthesis in response to the reduced absorption and increased expression of mRNA and protein expression of the LDL receptor escalating clearance of LDL and IDL.

Triacylglycerol and HDL-C cholesterol are not affected in healthy individuals (de Jong et al, 2003).

Other metabolic effects of plant sterols are less well verified but the effect on other fat soluble vitamins has been a focus of this area of research. Daily consumption of 3 to 4g of plant sterols has been shown to lower serum concentrations of carotenoids such as α-carotene, β-carotene and lycopene in parallel with cholesterol (Plat and Mensink, 2001; de Jong et al 2003; Richelle et al, 2004). In some studies vitamin E (alpha –tocopherol) has been shown to decrease, possibly due to the reduction in LDL which acts as its carrier protein (Katan et al, 2003). Vitamin A, D, and K (retinol and cholecalciferol) do not appear to be affected. Adjusting dietary intake and increasing the amount of fruit and vegetables in the daily diet is thought to be sufficient to counter this negative effect (Noakes et al, 2002).

There is also a suggestion that high intakes of plant sterols may suppress tumour cell growth and be effective in reducing the risk of certain cancers including cancers of the colon, breast and prostate (Awad and Fink, 2000). Observational studies have shown that individuals from Asian countries have lower incidences of the aforementioned cancers which have been in part attributed to their dietary intake of predominantly plant based foods. When these individuals relocate to western countries and dietary practices change to include a high consumption of animal products, the rates of cancer increase significantly suggesting that it may be the high levels of phytosterols that are providing some protection against cancer development (Bradford and Awad, 2007). Case control studies conducted in Uruguay have also provided some strong epidemiological evidence for the role of phytosterols in risk reduction for lung, breast, stomach and oesophageal cancer. In a comparison of up to 500 individuals matched for age, gender and socioeconomic status, it was found that dietary plant sterol intakes were lower amongst people diagnosed with stomach, oesophageal, lung or breast cancer than in cancer free individuals (Bradford and Awad, 2007; De Stefani et al, 2000; Mendilaharsu et al, 1998; Ronco, et al, 1999). Further work conducted in the US has also shown that dietary phytosterol intake is lower in women diagnosed with breast or endometrial cancer (McCann et al, 2000) whilst other researchers (Normen et al, 2001; Strom et al, 1999) have used case

control studies to demonstrate that higher intakes of plant sterols were not associated with the increased incidence of colorectal or prostrate cancer.

A wealth of animal models has been used to demonstrate the anti-cancer properties of phytosterols, some of which are summarized in table 3.3.

Table 3.3: Anti-cancer properties of phytosterols

AUTHOR	FINDING
Raicht et al, 1980	Found that the growth of methylntirosourea-induced tumours in rats fed 0.2% β-sitosterol for 28 weeks was reduced. The number of rats developing tumours was reduced by 39% with a 60% reduction in the number of tumours per rat.
Janezic and Rao, 1992 Awad et al, 1997	Reduction in the hyper proliferation of colonic mucosal cells in mice and rats fed 1-2% plant sterol mixture of β-sitosterol, campesterol, stigmasterol and dihydrobrassicasterol.
Awad et al, 2000	40% reduction in serum cholesterol and a 33% reduction in tumour size in female SCID mice injected with MDA-MB-231 oestrogen receptor-negative human breast cancer cells and fed diets supplemented with 2% phytosterols for 8weeks. A reduction in the number of tumours that had metastasized was also demonstrated.
Ju et al, 2004	32-42% reduction in tumour size observed in ovariectomized athymic mice fed diets enriched with β-sitosterol after injection with MCF-7 oestrogen receptor positive human breast cancer cells
Awad et al, 2001	40-43% reduction in tumour size plus a 50% reduction in the rate of tumour metastasis in male SCID mice injected with PC-3 human prostate cancer cells after 8 weeks on a diet of enriched with 2% phytosterols

The mechanism of action for phytosterols on tumour development is not known but several theories exist. These include alteration of the cell cycle progression and kinetics; induction of cellular apoptosis; alteration of membrane structure, integrity and fluidity; interfering in the signal transduction pathways or activity of membrane bound enzymes; stimulation of immune function and alterations to tissue estrogenic properties. Finally, a role influencing the activity of colonic bacteria and levels of faecal sterols that may affect cholesterol absorption has been proposed (Awad and Fink, 2000; Bradford and Awad, 2007; de Jong et al, 2003).

It is clear that although there is a lot of evidence suggesting a protective role for phytosterols in cancer prevention, the results are far from conclusive and longer term studies are required (Awad and Fink, 2000; Bradford and Awad, 2007).

Other reported effects of plant sterols include an anti-oxidant role with evidence to show that sitosterol and sitosterol glucoside can reduce lipid peroxidation in platelet membranes and have an ability to protect LDL particles from oxidation (Homma et al, 3003; van Rensburg et al, 2000); an anti-inflammatory role in various disease processes such as pulmonary tuberculosis, rheumatoid arthritis and HIV (Bouic, 2001); anti-ulcer activity in models of ethanol induced and cysteamine induced ulceration (Paul Jayaraj et al, 2003) and anti-fungal activity in some mushroom species (Smania et al, 2003).

3.5 Interactions with drug therapies

In the recent meta-analysis, Katan, 2003 reviewed several clinical studies looking at the effects of plant sterols in combination with cholesterol lowering drug therapies. Addition of plant sterols to statin therapy increased LDL-C reduction by 10-20% which is significantly more than doubling the statin dose which elicits an additional 6% lowering of LDL-C.

3.6 Rice Bran Oil

Rice bran and its oil contain large concentrations of compounds that include unsaturated fatty acids, triterpene alcohols, phytosterols, tocotrienols and α-tocopherol which have the potential to prevent chronic disease through their demonstrated ability to favourably alter lipid profiles (Cicero and Gaddi, 2001). Traditionally, consumption of rice bran oil (RBO) has been limited due to problems with rancidity caused by high lipase activity in the bran which affects the stability of the oil (Rukmini, 1988, de Deckere and Korver, 1996). However methods of extraction have evolved to increase stability and in Asian countries such as Japan, China, Korea, Pakistan, Taiwan and Thailand, RBO is extracted for edible purposes and is in demand as a “healthy oil” (Rukimini, 1988; Sugano and Tsuji, 1997). Rice bran oil has many favourable characteristics including a mild taste and high smoke point making it suitable for most culinary uses including frying. Rice bran is produced as a by product of the rice milling industry obtained during polishing when the bran is separated from between the paddy husk and endosperm (Lee et al, 2005). Rice bran contains 10-25% oil consisting of saturated fatty acids (palmitic acid 17%)

and oleic acid (40%). It also contains linoleic acid (40%) and a detectable amount of α-linolenic acid (~2%), along with negligible amounts of water-soluble β glucans and larger amounts of insoluble dietary fibre (Sugano and Tsuji, 1997). Of greater interest is the large amount of unsaponifiable material (4.2% in crude oil and up to 2.6% in refined oil) which is much higher than that found in other vegetable oils and is characterised by several compounds including gamma oryzanol and phytosterols (Lee et al, 2005).

3.6.1 Phytosterols

Three groups of phytosterols are present in RBO; 4,4'-dimethylsterols, 4-desmethylsterols (campesterol, stigmasterol, β-sitosterol) and also 4 monomethylsterols (e.g. citrostadienol) which have an ethylidene side chain that may contribute to its oxidative stability for heating at higher temperatures. The proportions of these individual steryl ferulates is highly variable and is dependent upon the environmental conditions in which the rice is grown. However the phytosterols contribute to a significant proportion of the unsaponifiable material with reported with ranges of 68%-76% for 4, 4'-dimethylsterols and 24-32% for 4-desmethylsterols (Miller and Engel, 2006; Sugano and Tsuji, 1997). The characteristics of phytosterols have been discussed previously and it is this component which contributes to the hypercholesterolaemic potential of RBO (de Decekere and Korver, 1995; Rao, 2000).

3.6.2 Gamma oryzanol (γ oryzanol)

Gamma oryzanol is a ferrulate ester of triterpene alcohols- a mixture of ferulic acid esters (4-hydroxy-3-methoxycinnamic acid) with sterols (campesterol, β-sitosterol) and triterpene alcohols (cycloarteno, 24-methylene-cycloartanol and cyclobranol) (Rong et al, 1997). The composition of γ oryzanol depends on the rice variety ranging from 115-780ppm depending on the degree and method of processing, and this compound is credited with many diverse pharmacological effects including growth promotion, gonadotrophic action, stimulation of the hypothalamus, anti-inflammatory action and most notably a hypolipidaemic effect caused through the mechanism of decreasing cholesterol absorption in the intestine

and increasing cholesterol excretion in the faeces (Sugano and Tsuji, 1997; Chen and Cheng, 2006). The ferulic acid fraction of γ oryzanol is partly split from the molecule in the small intestine by the action of cholesterol esterase and is reported to have anti-oxidant activity and possible anti-cancer activity (de Deckere and Korver, 1996).

Several animal feeding trials have provided support for the hypolipidaemic effects of γ oryzanol. Rong et al, 1997 showed that in hypercholesterolaemic hamsters, a diet supplemented with 1% γ-oryzanol resulted in a significant reduction in plasma total cholesterol by 28% ($P < 0.01$) when compared to a control group. The sum of non-HDL-C was also reduced by 34% ($P<0.01$). Further studies by this group with 0.5% γ- oryzanol also showed significant reduction in total plasma cholesterol (44%, $P=<0.001$) and non HDL-C (57%, $P=0.01$) compared to the control. Additionally both levels of γ- oryzanol reduced the accumulation of foam cells in the aorta by a level of 67% ($P=<0.01$). Further data obtained from the work of Wilson et al, 2007 demonstrates a reduction in plasma lipid and lipoprotein cholesterol concentrations and aortic cholesterol ester accumulation in hamsters fed diets supplemented with rice bran oil, γ oryzanol or ferulic acid. Forty eight hamsters were fed a high cholesterol diet then split into groups of 12. Group 1 acted as the control, group 2 were fed the high cholesterol diet supplemented with rice bran oil, group 2 consumed the high cholesterol diet plus 0.5% ferulic acid and group 4 the high cholesterol diet with 0.5% γ oryzanol. After ten weeks total plasma cholesterol and non-high density lipoprotein were significantly lower in hamsters fed the rice bran oil (64% and 70% respectively), the ferulic acid (22% and 24% respectively) and the γ oryzanol (70% and 77% respectively). Plasma HDL levels were significantly higher in the RBO group ($P<0.5$) and γ oryzanol group and plasma triglyceride concentrations were also significantly lower in these groups when compared to the control group. Lower aortic cholesterol ester accumulation was also evident in hamsters consuming the RBO and γ oryzanol. However, vitamin E levels were significantly lower in the RBO and γ oryzanol groups than the control or ferulic acid groups. These results suggest that at equal dietary levels, γ oryzanol has a greater effect on lowering non HDL cholesterol and raising HDL cholesterol than ferulic acid (the other major unsaponifiable material in RBO). This may be due to its ability to raise faecal excretion of cholesterol and its metabolites. The ability

of ferulic acid to maintain vitamin E concentrations suggests that this component may have a greater role in the area of anti-oxidant activity than γ oryzanol which is an additional mechanism for exerting anti-atherogenic properties.

Although animal studies have provided a useful indication that γ-oryzanol, has potential as a lipid lowering agent, animals do not provide an ideal model for cholesterol metabolism in humans. A study by Berger et al, 2005 examined the effects of feeding varying levels of γ-oryzanol to mild hypercholesterolaemic men on plasma cholesterol concentration. Thirty free living males were split into two groups and fed 50g/d rice bran oil supplemented with either low (0.05g/d) or high (0.8g/g) γ-oryzanol for 4 weeks in a randomised controlled parallel design clinical trial. Subjects otherwise consumed their habitual diet. The diets resulted in an overall reduction of total plasma cholesterol (6.3%), LDL-C (10.5%) and LDL-C: HDL-C ratio (18.9%) although no greater benefit was observed with the higher level of γ-oryzanol when compared to a control. This suggests that there may be compensatory mechanisms (such as increased cholesterol synthesis) working to prevent the γ-oryzanol for reducing the lipid parameters further. This study was small in the number of participants and did not elaborate on any possible effects on plasma lipid levels elicited by the habitual diet of the participants. Further evidence is required from human studies to examine the particular effect of γ-oryzanol as an isolated component of rice bran oil in the area of lowering plasma cholesterol concentration.

3.6.3 Tocotrienols and tocopherols

RBO is rich in vitamin E isomers (tocopherols and tocotrienols) with the major constituents being β and γ-tocotrienols possessing antioxidant and vitamin E activity (Rao, 2000). Gamma tocotrienol is the most abundant and is thought to be involved in the regulation of cholesterol exerting hypocholesterolaemic properties through the inhibition of HMG-CoA reductase activity, decreasing liver cholesterol levels, total cholesterol and LDL-C. Tocopherols are present at a much lower level and contribute to the anti-oxidant potency of RBO (de Deckere and Korver, 1996; Chen and Chang, 2006; Sugano and Tsuji, 1997).

The evidence supporting the hypolipidaemic properties of tocotrienols is limited but some studies have shown positive effects, Qureshi et al, 1991(a), examined the effects of supplementing standard pig diets with 50 micrograms of the tocotrienol rich fraction (TRF) of palm oil in an 8 week feeding trial of hypercholesterolaemic pigs compared to a standard control diet. The TRF fraction showed decreases in total serum cholesterol (44%), LDL-C (60%) and apolipoprotein B (26%). Markers of endothelial function and platelet aggregation (thromboxane B2 and platelet factor 4) were also reduced significantly. Yu et al, 2006 also examined the effects of diets supplemented with 50, 100, 250, 500, 1000 or 2000 rpm of TRF from palm oil, alpha tocopherol, alpha tocotrienol or 6-tocotrienol against a control diet in chickens fed over 4 weeks. A dose response effect of TRF on lowering serum total (22%) and LDL –C (52%) was observed. Alpha tocotrienol at levels up to 500ppm produced a dose response lowering of total (17%) and LDL (33%) cholesterol. HDL-C levels were minimally impacted by any of the supplements and alpha tocopherol did not affect any measured lipid parameters. In human models, Qureshi et al, 1991(b) conducted a double blind cross over study to examine the effects of TRF from palm oil (200mg/d) against those from corn oil (300mg/d) in mildly hypercholesterolaemic subjects. Significant decreases were observed in total cholesterol (15%), LDL-C (8%), ApoB (10%), thromboxane (25%), platelet factor 4 (16%) and glucose (12%) in those subjects consuming the TRF fraction from palm oil which is rich in γ-tocotrienol. Similarly, Qureshi et al, 2002 also conducted a randomised cross over trial where ninety subjects following the American Heart Association (AHA) step -1 diet were fed TRF from rice bran oil at concentrations of 25, 50, 100 and 200 mg/d for periods of thirty five days each. Doses of 100mg/d produced maximum decreases in serum total cholesterol (20%), LDL-C (25%), ApoB (14%) and triglycerides (12%) compared with baseline values. In type 2 diabetic subjects with hyperlipidaemia, Baliarsingh et al, 2005, conducted a randomised, double blind, placebo controlled trial with 19 subjects. Subjects were randomly assigned to one of two groups. In group A, each subject received two capsules daily for 60 days, each containing a dose of dietary TRF based on 3 mg TRF/kg body weight. At the completion of first phase (60 days), participants received colour matched placebo capsules containing 100 mg of TRF-free RBO for a period of 60 days. In group B, in the first phase participants received two placebo capsules daily for 60 days. In the second phase, these subjects were

given two capsules of TRF (3 mg/kg body weight) daily for 60 days. Administration of TRF was associated with a significant decline in serum total lipids, TC, and LDL-C levels of type 2 diabetic patients. The decrease in total lipids, TC, and LDL-C was 21%, 28%, and 38%, respectively, when compared to zero time values. On withdrawal of TRF after 60 days of treatment and subsequent switch over to placebo for another 60 days, total lipids, TC, and LDL-C levels were increased to a level similar to zero time control values.

Although the literature is not vast, the positive results demonstrated indicate that supplementation with tocotrienols may be useful in the prevention and treatment of hyperlipidaemia.

3.6.4 Minor components

Phytonutrients from RBO that may also have promising disease-preventing and health related benefits include squalene, phenolic compounds, methyl ferulate, coenzyme Q and lipoic acid. These still require further investigation (Rao, 2000; Jariwalla, 2001).

3.6.5 Health Benefits of RBO

Considerable evidence now exists to support the pharmacological properties of RBO, some of which are outlined below.

3.6.6 Plasma Cholesterol Reduction

The hypocholesterolaemic property of RBO is well documented and attributed largely to the unsaponifiable fraction (de Deckere and Korver, 1996). Numerous animal and human studies have been conducted (Cicero and Gaddi, 2001). Studies in rats demonstrated that the unsaponifiable material in RBO can lower the total cholesterol and LDL-C whilst raising HDL-C due to the association with increased faecal secretion of neutral steroids and bile acids (Wilson et al, 2007; Sugani and Tsuji, 1997; Ha et al, 2005). Minhajuddin et al, 2005 also showed that feeding the tocotrienol rich fraction (TRF) of RBO to rats with induced

hyperlipidaemia, decreased plasma triglyceride, total cholesterol and LDL-C in a dose dependant manner with an optimum effect at 8mg TRF/kg/d. Additionally markers of oxidation were also reduced. Work with hamsters (Kahlon et al, 1996) demonstrated that the addition of the unsaponifiable material to control diets reduced plasma cholesterol, VLDL-C and liver cholesterol whilst not affecting LDL-C and HDL-C, whilst Ausman et al, 2005, showed that both total cholesterol and LDL-C were both significantly reduced. Further more, Rong et al, 1997 showed that by the addition of γ oryzanol, a reduction in plasma total cholesterol, non-HDL-C and triglyceride concentration could be achieved as well as a significant reduction in the formation of the aortic fatty streak. These alterations in hamsters were also attributed to the increased excretion of fat and neutral steroids (Wilson et al, 2007). Extrapolation of data obtained from rodent studies to human subjects is difficult as lipid metabolism is considerably different, however human studies have independently supported the hypocholesterolaemic effect of RBO and some of the more recent evidence is presented in table 3.4.

Table 3.4: Hypocholesterolaemic properties of rice bran and rice bran oil

Author	Subjects	Treatment	Finding
Gerhart and Gallo, 1998	52 moderately hypercholesterolaemic men and women	6wk randomised double blind trial comparing hypocholesterolaemic properties of full fat rice bran, oat bran and rice starch as an addition to normal diets	Rice bran and oat bran produced a significant reduction in serum cholesterol with rice bran reducing LDL-C by 13% and oat bran reducing LDL-C by 17.1%. Compared to baseline values. No change to HDL-C or triglycerides reported
Vissers et al, 2000	28 men and 32 women. Healthy normolipaemic volunteers	3 wk randomised double blind crossover trial looking at the effects of plant sterols from rice bran oil and triterpene alcohols from shea nut oil on lipid profiles	2.1g plant sterols from RBO lowered serum cholesterol by 5% and LDL-C by 9% Triterpene alcohols from shea nut oil did not affect lipoprotein concentrations

Qureshi et al, 2002	90 hypercholesterolaemic men and women	Randomised cross over trial of 3 phases each for 35 days evaluating the effects of different doses of a tocotrienol rich fraction (TRF25) of stabilized and heat treated rice bran on cholesterol parameters. Dosages of 25, 50, 100 and 200 mg/d were administered to participants following the restricted AHA step 1 diet.	Dosages of 100mg/d of TRF25 produced a 20% decrease in total cholesterol, 25% decrease in LDL-C, 14% decrease in Apolipoprotein B and 12% reduction in triglycerides compared to baseline values.
Berger et al 2005	30 mildly hypercholesterolaemic men	Evaluation of RBO with high (0.8g.d) and low (0.05g/d) γ-oryzanol compared to peanut oil in a randomised controlled parallel design study	Low and high γ-oryzanol RBO lowered total plasma cholesterol by 6.3%, LDL-C by 10.5% and the LDL-C: HDL-C ratio by18.9% and were not significantly different from each other. Peanut oil with a similar FA profile did not alter lipid parameters
Most et al, 2005	27 healthy men and women.	Randomised cross-over 10 week feeding study to assess the effects of defatted rice bran and rice bran oil in an average American diet on blood lipids in mildly hypercholesterolaemic individuals.	RBO did not alter lipid concentrations compared with a low fibre control diet but RBO decreased LDL-C by 7% without affecting HDL-C when compared to a diet of similar fat content.

RBO lowered cholesterol despite having a large component of saturated fatty acids which are known to be detrimental to cholesterol profiles (Most et al, 2005) which supports the theory of the unsaponifiable fraction being the active component of RBO. Whilst these studies show rice bran and RBO to be effective at lowering cholesterol, an earlier study by Lichtenstein, 1994, showed that RBO was no more effective than other commonly consumed oils. Fifteen moderately hypercholesterolaemic individuals were studied in a randomised double blind trial comparing canola oil, olive oil, corn oil and RBO on plasma lipid concentrations for

a period of 32 days. Plasma total cholesterol and LDL-C concentrations were similar and statistically indistinguishable between all treatments. However the diets were not matched for fatty acid composition which could have been a confounding factor in this study and overwhelmingly the evidence available supports the hypocholesterolaemic properties of RBO (de Deckere and Korver, 1996; Ciero and Gaddi, 2001).

The ability of RBO to lower serum cholesterol levels is one of the proposed health benefits providing some protection against cardiovascular disease. Other possible mechanisms contributing to the positive effect on cardiovascular health include the antioxidant activity relating to γ-oryzanol. Oxidative damage, particularly to LDL-C, thought to have a pivotal role in the development of plaque formation, an initiating event in atherosclerosis (Lee et al, 2005).

3.6.7 Anti-cancer benefits

These benefits relate to the presence of plant sterols which has been previously discussed and also to the presence of ferulic acid and high levels of vitamin E isomers. Vitamin E has been shown to prevent or cause regression of certain cancers in animal models and *in vitro* systems and to a lesser extent in limited large scale intervention trials. Conversely other studies have observed no beneficial effect of vitamin E supplementation in populations of cancer patients (Iqbal et al, 2004; Bramley et al, 2000). Both tocotrienols and tocopherols are thought to have some anti-cancer activity although tocotrienols are thought to be the more effective (Wada et al, 2005; Sen et al, 2007). Kline et al, 2004 have demonstrated that synthetic vitamin E analogues are capable of inducing apoptosis through restoration of pro-death signalling pathways in breast, ovarian, and prostate cancer cells and Galli et al, 2004 showed that tocopherol and tocotrienol metabolites were also capable of inhibiting prostate cancer cell growth in tissue culture. Furthermore Wada et al, 2005 showed that oral supplementation of tocotrienol-rich vitamin E prevented lung and liver carcinogenesis in mice and inhibited proliferation of human liver cancer cells in vitro.

Ferulic acid has also been investigated for its chemo protective attributes. Mori et al, 1999 showed that the incidence of oral cancer was lower in rats fed ferulic acid at levels of 0.5g/kg for 5 weeks. Colon and rectal cancer lesions were also reduced in rat studies following ferulic acid supplementation and topical application of ferulic acid in mice protected against skin tumours (Ou and Kwok, 2004).

3.6.8 Immune Modulation

Limited evidence is also available to support an immunomodulatory effect for RBO. Sierra et al, 2005, conducted a study in mice, feeding them RBO or sunflower oil enriched diets for four weeks and demonstrated that the mice fed RBO had increased stimulation of the immune system with proliferation of the T and B lymphocytes and an enhanced primary macrophage response. Additionally, the mice demonstrated an increase in the stimulation of TH1-type cytokines (IL-2 and TNF-α) whilst having a decrease in the TH-2 cytokine, IL-4 and IgE suggesting a possible anti-allergenic role for RBO. Furthermore, this study also fed refined RBO and γ-oryzanol enriched sunflower oil to the mice and found that although the mice demonstrated an increase in TH1 lymphocyte response and reduction inTH2 lymphocyte response, there was no increase in lymphocyte proliferation or IL-4 expression. The authors concluded therefore that the immune modulation effect was due in part to the fatty acid composition rather than simply the unsaponifiable material of RBO. RBO is rich in linoleic acid, an n-6 polyunsaturated fatty acid and precursor to the eicosanoids which play an important role in the regulation of immune and inflammatory response (Calder, 2003). More research is required in this area to elucidate the effectiveness of RBO in immune modulation.

3.6.9 Anti-ulcerogenic properties

γ-oryzanol has been widely investigated for its anti-ulcer action. Early rat studies showed that a dose of 100mg/kg daily for 5 days showed significant inhibition of water immersion and fasting induced ulcers whilst reducing serum 11-hydroxycorticosterone levels due to action of the mono-aminergic neurone

system (Cicero and Gaddi, 2001). Further animal studies have also shown a reduction in serum gastrin levels following γ-oryzanol consumption. The mechanism of action is postulated to be through the high levels of polyunsaturated fatty acids acting as precursors to arachidonic acid and prostaglandin synthesis which acts as an inhibitor of gastric acid secretion (Cicero and Gaddi, 2001). Human studies are lacking in this area and further studies are required to establish the gastroenterological effect of RBO.

3.6.10 Neuroendocrinological effects

γ-oryzanol is thought to have a modulatory effect on anterior pituitary hormone secretion with results suggesting that it can inhibit luteinising hormone, thyroid stimulating hormone, prolactin releasing hormone and growth hormone. Limited evidence supplied from early animal studies provide some support but human studies are lacking and further research is required in this area (Cicero and Gaddi, 2001).

3.6.11 Osteoporosis

Godber et al, 2002 showed some evidence in rats that feeding a 7% RBO concentrate after an ovariectomy reduced the observed amount of bone loss, however other research in this area has not been presented and this effect remains unsubstantiated.

3.6.12 Safety and toxicity

Like plant sterols, RBO is considered safe for human consumption and work has found it to have no acute or chronic activity, no mutagenicity or carcinogenicity and no side effects (Ciecero and Gaddi, 2001).

3.7 Alfa One™Rice Bran Oil Spread

Alfa One™rice bran oil spread was developed in 2006 through collaboration between the New Zealand Institute for Crop and Food Research and Old Fashioned Foods Limited. Based on the Alfa One™ rice bran oil, the spread contains 1.5% plant sterols as a mixture of γ-oryzanol and campesterol, stigmasterol and sitosterol. The spread was launched onto the Australian market where two other brands of plant sterol containing margarines are available; Flora Pro-activ® (Unilever) containing 8% plant sterol esters and Meadow Lea Logicol® (Goodman Fielder) also containing 8% plant sterols. In order to evaluate Alfa One™rice bran oil spread, Old Fashioned Foods commissioned Crop and Food Research to conduct a clinical study comparing the cholesterol lowering properties of their product against that of a standard margarine and of the market leader, Flora Pro-activ®. The study involved eighty mildly hypercholesterolaemic individuals (total cholesterol ≥ 5 mmol/L and ≤ 7.5 mmol/L) recruited and randomised into two groups of forty. Participants were asked to continue with their normal dietary pattern but to replace any margarine/butter/fat consumption with the trial products. One group of 40 were then assigned to a margarine-only group and were randomised to consume 20 g (4 teaspoons) RBO margarine daily for 4 weeks, or 20 g Flora margarine daily for 4 weeks, or 20 g Flora proactiv® daily for 4 weeks. At the end of each 4-week period individuals changed to the next treatment. The second group of 40 were allocated to a margarine and oil group and consumed 20 g RBO margarine and 30 ml RBO daily for 4 weeks, or 20 g Flora margarine and 30 ml sunflower oil daily for 4 weeks, or 20 g RBO margarine daily for 4 weeks, changing treatment at the end of each 4-week period. Following each intervention, measurements were made of weight and blood pressure. Venous blood samples were collected for analysis of total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and total cholesterol: HDL-C, triglycerides and plasma phytosterols. Three-day diet records were also collected for analysis of normal dietary intake. This study forms the basis of this thesis.

Chapter 4

Methods

4.1 Candidate's contribution to the research

The project was initially conceived by the candidate's supervisor (Dr Alison Wallace) but the candidate was involved in planning of the experimental design and was responsible for the overall running of the trial including the application for ethics, recruitment, screening and all contact and management of the participants for the twelve week intervention period. She was also responsible for preparation of all dietary advice and participant information for the trial, collection of blood samples and sample preparation for the laboratory testing of cholesterol and phytosterol analysis. In addition the candidate carried out the data entry and analysis (with advice from Dr Chris Frampton, Statistician) and entry and analysis of the three day diet records.

4.2 Ethical approval

The study protocol was approved by the Canterbury Upper South A Ethical Committee (Reference number URA/07/02/016) and all participants gave their written informed consent before commencing the study.

4.3 Participants

Participants were recruited by advertisement and articles in local Christchurch newspapers. One hundred and fifty-four individuals were screened to assess baseline cholesterol levels. Of these 90 individuals were eligible to take part in the study. Of these, four individuals declined the offer to take part further in the study and 80 individuals were selected on a first come first served basis from the remaining 86 for inclusion into the trial. Seventy-six identified themselves as New Zealand European

ethnicity, three were of Indian ethnicity and one identified as Asian. Inclusion criteria required that participants were healthy and aged between 30 and 65 years of age with a body mass index (BMI) of less than 35 kg/m^2. Total cholesterol levels were required to be ≥ 5 mmol/L and ≤ 7.5 mmol/L with serum triglyceride levels below 4.5 mmol/L. Participants were required to have normal glucose tolerance with fasting blood glucose levels of ≤ 6.1 mmol/L and were not eligible if they were smokers, had a history of diabetes or were taking lipid-lowering medication or other medication likely to affect lipid metabolism. Other exclusion criteria included renal, hepatic, cardiovascular, endocrine, gastrointestinal or other systemic disease; any known blood-borne disease, untreated hypertension, pregnancy, history of substance abuse including alcohol abuse, extreme dietary habits or food allergies and extreme exercise regimes. 80 volunteers (53 females and 27 males) with a mean age of 52 years and a mean body mass index of 26 kg/m^2 were included in the original population for this study. Table 5.1 shown in the results section of this thesis demonstrates the baseline characteristics for the study population.

Seventy-five of the eighty individuals who initially agreed to participate completed the 12-week study. Two males and three females withdrew from the study. Three developed illness unrelated to the study products and two had an unforeseen change in circumstance. No adverse effects related to the treatment margarine were noted over the course of the study.

4.4 Experimental design

The study involved a randomised double blind cross-over trial of two dietary interventions based around consuming plant sterol-based margarine and oil products. Eighty individuals were randomly assigned to one of two groups of 40. The randomisation for all aspects of this trial was prepared in advance by an independent statistician using a randomisation procedure in Microsoft EXCEL.

One group were asked to substitute margarine only to their existing diet whilst group two was asked to include both margarine and the corresponding oil on which the margarine was based. All participants were asked to exclude any plant sterol-containing products from their diet for at least three weeks before the start of

the trial. This included avoidance of any commercially available plant sterol based margarine (Flora-proactiv®, Logicol®, Alfa One ™rice bran oil spread) plus rice bran oil and any products based on this oil. Participants were given one on one interviews at the point of screening to discuss the dietary implications of this requirement. Baseline measurements were taken prior to allocation of the first intervention. Participants were asked to complete an estimated three-day diet record of their usual dietary pattern and were asked to use three non-consecutive days including one weekend/non-working day. They were also given a colour picture booklet to help estimate food portion sizes. Fasting venous blood samples were collected on two occasions on non consecutive days. Three tubes of blood were collected, one into tubes containing heparin for lipid analysis, one containing fluoride/oxalate for glucose analysis and one into K_2EDTA tubes for phytosterol analysis. Blood was centrifuged at 3000 g for 10 minutes the serum and plasma removed and frozen at -20^{o}C for lipid and glucose analysis, and the plasma removed and frozen at -80^{o}C for phytosterol analysis. Samples were analysed for blood glucose, phytosterols, total cholesterol, LDL-C, HDL-C, TC: HDL and triglycerides. Height, weight and blood pressure measurements were also collected. Seated blood pressures were measured in the brachial artery with a standard mercury sphygmomanometer after a minimum five minute rest after arrival at the clinic. With the exception of blood glucose, individual measurements of lipid levels, blood pressure height and weight were repeated at the end of each intervention stage.

One group of 40 was then assigned to the margarine-only group and was randomised to consume either 20 g RBO margarine daily for 4 weeks, or 20 g Flora margarine daily for 4 weeks, or 20 g Flora Pro-activ daily for 4 weeks. At the end of each 4-week period individuals changed to the next treatment. Phytosterol levels delivered in these amounts were: RBO margarine: 118 mg phytosterol and 14 mg γ oryzanol; Flora Pro-activ: 1600 mg phytosterol, Flora 0mg phytosterol. The second group of 40 were allocated to the margarine and oil group and consumed 20 g RBO margarine and 30 ml RBO daily for 4 weeks, or 20 g Flora margarine and 30 ml sunflower oil daily for 4 weeks, or 20 g RBO margarine daily for 4 weeks. At the end of each 4-week period individuals changed to the next treatment. Phytosterol amounts delivered in these amounts were: RBO margarine: 118 mg phytosterol and

14 mg γ oryzanol; RBO 222mg mg phytosterol, 150 mg γ oryzanol. Figure 4.1 shows a diagrammatic representation of the trial design.

Participants were required to use the trial products as a replacement for any spread/margarine products already included in their everyday diet and were asked not to exceed the specified amounts. In all other aspects, individuals were required to maintain their normal dietary intake patterns and encouraged to keep their lifestyle, such as levels of physical activity, constant throughout the study. At each visit to the clinic, participants were given one on one interviews and asked to highlight any concerns relating to the study and any changes to their normal daily routines. Individuals were asked to report any sickness or commencement of new medication and changes to physical activity levels. Four individuals reported a bout of sickness that prevented them taking the trial products for one day of the trial which was reflected in the levels of compliance for this study. No individual reported changes to their levels of physical activity. Two participants withdrew from the study due to changes to medications for controlling newly developed hypertension.

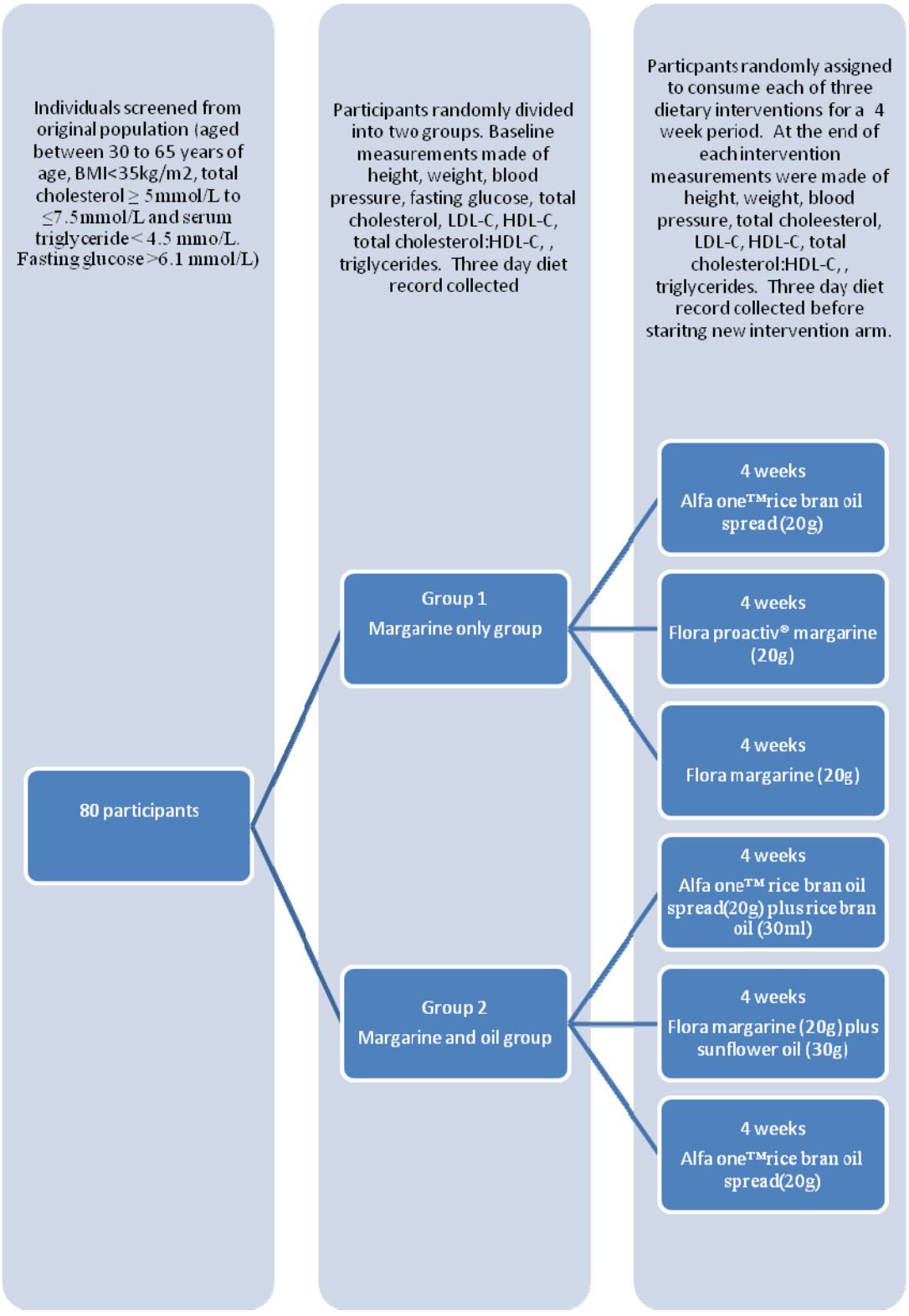


Figure 4.1 Showing a diagrammatic representation of trial design

Each participant was also given booklets (Appendix I) containing recipe ideas for using the products and general advice based on the New Zealand Food and Nutrition Guidelines on how to maintain a healthy eating pattern. These guidelines were given to participants to provide ideas for including the trial products into their daily food intake and to help maintain variety so as to encourage compliance for the margarines and oils. They were not intended to encourage a change in eating patterns and from analysis of the provided diet records did not appear to change the individuals' dietary habits. In all, four 3-day diet records and eight blood samples were collected from each individual. Blood pressure, height and weight measurements were collected on every clinic visit, following each intervention arm, totalling four measurements in all. There were no washout periods during the study. It was decided not to include a washout period between treatment arms as the length of each treatment arm was four weeks which provided enough time for the stabilisation of lipid levels even after a change in dietary intake. Previous studies (Bonanome and Grundy, 1988; Mensink and Katan, 1987; O' Dea et al, 1990) have shown that plasma lipid levels stabilise within 2-3 weeks after dietary changes are made. The inclusion of a washout period in this study would have increased the length of the study and may have led to less participant compliance and greater number of withdrawals from the study.

4.5 Dietary compliance

Individual compliance with consuming the trial products was assessed using self completed check sheets (Appendix II), which required individuals to record their intake on a daily basis and through analysis of plasma phytosterol levels.

4.6 Trial products

The trial products used were Alfa One™ rice bran oil (RBO) spread (Old Fashioned Foods Group, Auckland, New Zealand), Flora margarine and Flora Pro-activ® (Unilever, Australia). The oils used were Alfa One™ RBO (Old Fashioned Foods Group, Auckland, New Zealand) and Sunflower Oil (Tasti Products Limited,

Auckland, New Zealand). The information regarding composition of the trial products was obtained from the company producing them and is shown in tables 4.1−4.2. Trial products were distributed to participants at the beginning of each treatment arm. Participants were provided with enough product to allow for the required daily intake plus half as much again allowing for any unforeseen problems. Although participants were given the option to use the products in baking, the recipes supplied identified the amount of product contained in one serve of each baked item and it was stressed that individuals must not exceed the daily allowance for this study. This option was provided to allow individuals variety in their method of consumption to encourage people to continue with the study. Following the end of the treatment arm, participants were asked to either discard the remaining product or freeze for use after the conclusion of the study.

All products were packaged in identical unmarked tubs or oil bottles and given unique identifier codes by an independent individual to ensure blinding. The codes were revealed after the completion of the trial. At the end of each intervention period, participants were asked to fill out a questionnaire (Appendix II) regarding their general thoughts and comments regarding the taste, texture and usability of the products in order to gauge the feasibility of regular use of these products in everyday diets. This aspect of the trial was not the main focus of the work and thus the assessment of the sensory aspects of the products was carried out for the interest of the company marketing the margarines and oil. The questionnaires devised for assessing these aspects were devised by the candidate, based several criteria which included consideration of the following: What we needed to know and why; what aspects of the products we were measuring; the demographic of the population being surveyed (age, education, culture); the best and most appropriate method of data collection to use; how to best reduce any influence and bias and the willingness/likelihood of the respondents to complete the questionnaire. The questionnaires were based on a Likert Scale design and, although they were assessed by the marketing company for their appropriateness, they were not independently validated.

Table 4.1: Oil composition (Data supplied by Old fashioned Foods Ltd and Tasti products Ltd)

RBO	**Per 100 g**		**Sunflower oil**	**Per 100 g**
Energy	3700 kJ		Energy	3760 kJ
Protein	0.0 g		Protein	Trace
Fat, total	100 g		Fat, total	99 g
- saturated	19.9 g		- saturated	11.7 g
- polyunsaturated	32.1 g		polyunsaturated	61.9 g
- monounsaturated	40.6 g		monounsaturated	21.1 g
Cholesterol	0.0 g		Cholesterol	0 mg
Sodium	0.0 g		Sodium	Trace
Trans fatty acids	0.0 g			
Vitamin E	25 mg			
Alpha-tocopherol	5−20 mg			
Tocotrienols	20−50 mg			
Oryzanol	500 mg			
Phytosterol	740mg			

Table 4.2: Margarine composition (Data Supplied by Old Fashioned Foods Ltd and Unilever, Australia).

RBO spread	Per 100 g		**Flora standard margarine**	Per 100 g		**Flora Pro-activ**	Per 100 g
Energy	3010 kJ		Energy	2600 kJ		Energy	2380 kJ
Protein	0.5 g		Protein	<1 g		Protein	<1 g
Fat, total	81.0 g		Fat, total	70 g		Fat, total	64 g
saturated	23.0 g		saturated	18 g max		saturated	15 g max
polyunsaturated	24.8 g		polyunsaturated	30 g min		polyunsaturated	26 g min
monounsaturated	32.0 g		monounsaturated	17 g min		monounsaturated	19 g min
Trans fatty acids	0.2 g		Trans fatty acids	0.63 g		Trans fatty acids	0.6 g
Carbohydrate	0.0 g		Carbohydrate	<1 g		Carbohydrate	<1 g
Sugars	0.8 g		Sugars	<1 g		Sugars	<1 g
Sodium	580 mg		Sodium	590 mg		Sodium	360 mg
Oryzanol	1.5g		Omega-3 ALA	2g min		Plant sterols	8 g
Phytosterols	592mg		Omega -6	28g min		Potassium	28 mg
Vitamin E	432mg		Cholesterol	<3 mg			
			Potassium	20 mg			
			Vitamin D	10 µg			
			Vitamin E	16 mg			

4.7 Analyses

4.7.1 Blood glucose and lipid analysis

Following collection, blood samples were centrifuged at 3000 g for 10 minutes and serum samples were removed and frozen at -20^{o}C for subsequent analysis at Canterbury Health Laboratories. Total cholesterol was measured using enzymatic Cholesterol Oxidase and Abbott reagent on an Aeroset/c8000 analyser. HDL-C was analysed using enzymatic Roche reagent on an Aeroset/c8000 analyser. Triglyceride was measured using enzymatic hydrolysis of triglycerides with Abbott reagents on an Aeroset/c8000 analyser. LDL-C was calculated by difference from the above results using the formula presented below.

LDL cholesterol = Total cholesterol – HDL Cholesterol – Triglycerides/2.22) mmol/L

Calculation of LDL cholesterol is an approximation and is invalid in the presence of VLDL. Therefore LDL cholesterol is only calculated if the triglyceride measurement is less than 4.5 mmol/L (Canterbury Health Laboratories).

The results presented in this thesis for lipid analysis are the mean values of the two individual measurements taken 48 hours apart. Blood glucose measurements were obtained via an enzymatic method using glucose hexokinase and an Aeroset/c8000 analyser (Abbott Clinical Chemistry, Germany).

4.7.2 Plasma plant sterol concentrations

Plant sterols were extracted using a combination of two methods utilising GCMS adapted from Phillips et al. (2005) and Ahmida et al. (2006). Briefly plasma was collected from EDTA coated tubes and centrifuged for 10 minutes at 3000 g. Two hundred μL of plasma sample was added to 10 μL of 5α-cholestane (200 μM), the internal standard, along with 1 mL of 1 M potassium hydroxide and heated at 70°C in the dark for 1 hour. Nitrogen was added to minimise oxidation. Upon cooling, samples were diluted with 1 mL of water and extracted twice using a total of 1.5 mL of lipid extraction agent comprising hexane: ethanol at a 20:1 ratio with 12.5 mg/L butylated hydroxytolulene (BHT). The organic phase was then evaporated under nitrogen before derivatisation of the sample with 200 μL of freshly prepared pyridine: BSTFA with 1% TMCS (1:1, v/v) and incubation at 70°C for 1 hour. Following evaporation of the excess solvent under nitrogen the samples were reconstituted in 200 μL of hexane for analysis on the gas chromatograph.

4.7.3 Diet record analysis

Dietary intake was assessed by estimated diet records completed on three non-consecutive days (including one weekend/non-working day). Detailed instructions for filling out the record were given to each participant (Appendix ll). The records and photo assessment records were purchased from the Department of Human Nutrition, Otago University, Dunedin, New Zealand. (Records were completed at baseline and at the end of the first, second and third intervention periods. Nutrient intakes were analysed using the New Zealand database of Foodworks professional, version 4, 2005(Xyris Software, Australia) based on the 1999 New Zealand food composition data from the New Zealand Institute for Crop & Food Research Ltd.

4.8 Statistical analysis

Standard descriptive statistics, including means and standard deviations were used to summarise plasma and diet data for the entire cohort of 75 individuals and separately for the two groups of 39 (group 1) and 36 (group 2). A total sample of 40 in each group in this cross-over design enabled effect sizes of >0.45 to be detected as statistically significant (two tailed α=0.05) with 80% power. Effect sizes of this order and larger, have been demonstrated as clinically relevant for dietary interventions on lipid levels. Comparisons of the three cross-over interventions were undertaken within each of the two groups and utilised repeated measures ANOVA to test for statistical significance. Where significant treatment effects were detected, planned pairwise comparisons were undertaken between treatments (post-hoc analysis). The appropriateness of the parametric ANOVA models was confirmed by visual inspection of residual plots from the models to confirm normality. Order of treatment effects were explored but were not demonstrated in this trial. The trial encompassed a balanced complete latin square design for both groups encompassing all 6 possible sequences for the three treatments. All possible combinations were used with equal numbers of participants so as to not confound the comparison of treatments with the main dietary effect. Within the analyses, the sequence of one to six was entered as a between subjects factor, and additionally the interaction between

sequence and treatment was tested to confirm the consistency of the treatment effects irrespective of sequence. The numbers used in each sequence group (n=6) was small and did not provide a lot of statistical power to test the interaction effect.

Chapter 5

Results

5.1: Participants

A total of 75 participants completed the trial. Table 5.1: Demonstrates the baseline characteristics of the study population.

Table 5.1: Study participant's baseline characteristics.

Variable	Total study group (n=80)	Group 1 Margarine only (n=40)	Group 2 Margarine and oil (n=40)
Sex	53 females / 27males	30female / 10 male	23 female / 17 male
Age (years)	52.1 (7.9)	51.5 (8.1)	52.8 (7.6)
Weight (kg)	72.9 (14.8)	70.7 (13.9)	75.2 (15.6)
BMI (kg/m^2)	25.8 (3.9)	24.9 (3.5)	26.6 (4.0)
Total cholesterol (mmol/L)	5.9 (0.7)	5.8 (0.7)	5.9 (0.8)
HDL-Cholesterol (mmol/L)	1.5 (0.3)	1.5 (0.3)	1.4 (0.3)
LDL cholesterol (mmol/L)	3.8 (0.7)	3.8 (0.6)	3.8 (0.8)
Total cholesterol:HDLc ratio	4.2 (1.1)	4.0 (0.9)	4.4 (1.2)
Triglycerides (mmol/L)	1.3 (0.5)	1.2 (0.5)	1.3 (0.6)
Systolic blood pressure mmHg	128.5 (19.3)	128.2 (19.5)	128.8 (19.3)
Diastolic blood pressure mmHg	77.7 (10.5)	77.2 (10.9)	78.2 (10.0)

Values are shown as means ± standard deviation

Changes to BMI from baseline values over the course of the trial are shown in table 5.2

Table 5.2 showing changes to BMI over duration of trial

Treatment	Baseline	BMI(kg/m^2) after treatment	Difference	% difference	Significance
Flora margarine (n=39)	25.05 (3.62)	24.97 (3.50)	0.08	0.3	0.474
Flora proactiv® (n=39)	25.13 (3.54)	24.97 (3.50)	0.16	0.6	0.135
Alfa One ™ rice bran oil (n=39)	25.00 (3.47)	24.97 (3.50)	0.03	0.1	0.726
Flora margarine plus sunflower oil (n=36)	26.61 (4.11)	26.61 (4.07)	0	0	1
Alfa One™rice bran oil plus Alfa One™ rice bran oil spread (n=36)	26.83 (4.07)	26.61 (4.07)	0.22	0.8	0.04 (s)

Values shown as means ± standard deviation. P=<0.05 = significant difference.

No significant differences were detected in BMI throughout the trial with the exception of the treatment of Alfa One™ rice bran oil and Alfa One™ rice bran oil spread which reached a low level of significance.

No significant differences were detected in systolic or diastolic blood pressure throughout the study (data not shown).

No adverse effects were reported regarding the products although some participants found 20 g of margarine and 30 ml of oil difficult to consume daily for 4 weeks due to the perceived large volume of oil and overall increase in fat to their diet.

Compliance with consuming the trial products was reported to be greater than 90% (data not shown) for both trial arms and was to be verified by phytosterol analysis. All samples were processed for analysis and the initial chromatograms produced did indicate the presence of phytosterols in the samples but also showed a large amount of contamination in the samples. This method proved not to be satisfactory for these sample types as it was not possible to determine or confirm the individual plant sterol types. Presently the method for phytosterol analysis is still being refined in order to increase sensitivity and allow quantification of phytosterol levels in the serum samples and will therefore not be presented in this thesis.

5.2 Lipoprotein results

Changes in serum lipoprotein status were observed for participants in the margarine-only group when consuming Alpha One™ RBO spread and Flora Pro-activ® spread. Both products produced significant changes in total cholesterol, total cholesterol: HDL-C, and LDL-C levels. No changes were observed for HDL-C or triglycerides. Results are presented in Table 5.1.

Table 5.3: Margarine-only group: changes to lipid parameters

MARGARINE ONLY GROUP (n=39)				
CHOLESTEROL				
Treatment	**Means**	**Paired differences (adjusted for baseline)**	**% difference**	**Significance**
Flora margarine vs. Alfa One™Rice bran oil spread	5.78 (0.61) - 5.65 (0.55)	0.013	2.2	0.045 (s)
Flora margarine vs. Flora proactiv®	5.78(0.61) - 5.54(0.62)	0.24	4.1	0.001 (s)
Alfa One™Rice bran oil spread vs. Flora proactiv®margarine	5.65 (0.55) – 5.54(0.62)	0.11	1.9	0.095 (n.s)
TRIGLYCERIDES				
Treatment	**Means**	**Paired differences (adjusted for baseline)**	**% difference**	**Significance**
Flora margarine vs. Alfa One™Rice bran oil spread	1.21(0.47) – 1.32 (0.92)	-0.11	-0.08	0.405(n.s)
Flora margarine vs. Flora proactiv®	1.21 (0.47) – 1.22(0.54)	- 0.01	-0.82	0.781 (n.s)
Flora proactiv®margarine vs. Alfa One™Rice bran oil spread	1.22(0.54) – 1.32(0.92)	-0.1	-7.5	0.481 (n.s)
HDL -C				
Treatment	**Means**	**Paired differences (adjusted for baseline)**	**% difference**	**Significance**
Flora margarine vs. Alfa One™Rice bran oil spread	1.49(0.32) – 1.51(0.31)	-0.02	-1.32	0.45 (n.s)
Flora margarine vs. Flora proactiv®	1.49(0.32) – 1.49 (0.33)	0.00	0	0.76 (n.s)
Flora proactiv®margarine vs. Alfa One™Rice bran oil spread	1.49(0.33) – 1.51(0.31)	-0.02	-1.32	0.27 (n.s)
TOTAL CHOLESTEROL:HDL				
Treatment	**Means**	**Paired differences (adjusted for baseline)**	**% difference**	**Significance**
Flora margarine vs. Alfa One™Rice bran oil spread	4.06(1.07) – 3.9 (0.90)	0.17	4.2	0.005 (s)
Flora margarine vs. Flora proactiv®	4.06 (1.07) – 3.92 (1.08)	0.14	3.4	0.014 (s)
Flora proactiv®margarine vs. Alfa One™Rice bran oil spread	3.92(1.08) – 3.9 (0.90)	0.02	0.51	0.649 (n.s)
LDL-C				
Treatment	**Means**	**Paired differences (adjusted for baseline)**	**% difference**	**Significance**
Flora margarine vs. Alfa One™Rice bran oil spread	3.73(0.60) – 3.60 (0.55)	0.13	3.5	0.016 (s)
Flora margarine vs. Flora proactiv®	3.73(0.60) – 3.52 (0.60)	0.21	5.6	0.001 (s)
Flora proactiv®margarine vs. Alfa One™Rice bran oil spread	3.52(0.60) – 3.60(0.55)	-0.08	0.02	0.245 (n.s)

Values shown as means± standard deviation

($P<0.05$ = significant difference)

The results show that although Alfa One™ RBO spread reduced total cholesterol by 2.2% the reduction was not as great as that seen with the Flora Pro-activ® spread (4.1%). Alfa One™ RBO spread also produced a greater reduction in the TC: HDL ratio (4.1%) than the Flora Pro-activ® Spread (3.4%), in addition to reducing LDL cholesterol by 3.5% when compared to standard Flora margarine. The reduction was not as great as that seen with the Flora Pro-activ® spread (5.6%).

Alfa One™ RBO spread is therefore shown to be effective in reducing the levels of total and LDL cholesterol when consumed as part of normal dietary intake. In comparison to Flora Pro-activ® the reduction is not as great but it is to be noted that Flora Pro-activ® contains a much higher level of plant sterols (8% versus 1.5%). This factor contributes to the significant retail cost of the Flora Pro-activ® margarine thus Alfa One™RBO spread provides a cheaper option for consumers to enable individuals to incorporate this effective method of lowering LDL-C into the diet, which may result in some significant reductions in this lipid parameter.

In Group 2 of the trial the effect of consuming additional amounts of the corresponding base oil to the margarine was tested on the basis that increasing the intake of plant sterols would increase the reduction in cholesterol. However, in this group we did not observe any reductions in any cholesterol levels (Table 5.4)

Table 5.4: Margarine and oil group. Comparison of Alfa One™ RBO spread and RBO to Flora and sunflower oil and Alfa One™ RBO spread

Margarine and oil group (n=36)			
TOTAL CHOLESTEROL	**Means**	**Paired differences (adjusted for baseline)**	**Significance**
Alfa One™rice bran oil spread vs. Alfa One™rice bran oil spread plus Alfa One™rice bran oil	5.91 (0.76)-5.84(0.63)	0.07	0.323 (ns)
Alfa One™rice bran oil spread vs. Flora margarine plus sunflower oil	5.91 (0.76)-5.84 (0.81)	0.08	0.352 (ns)
Alfa One™rice bran oil spread plus Alfa One™rice bran oil vs. Flora margarine plus sunflower oil	5.84 (0.81) – 5.83 (0.81)	0.01	0.973 (ns)
TRIGLYCERIDES	**Means**	**Paired differences (adjusted for baseline)**	**Significance**
Alfa One™rice bran oil spread vs. Alfa One™rice bran oil spread plus Alfa One™rice bran oil	1.44 (0.70) - 1.36(0.61)	0.08	0.21 (ns)
Alfa One™rice bran oil spread vs. Flora margarine plus sunflower oil	1.44 (0.70) – 1.36 (0.64)	0.08	0.13 (ns)
Alfa One™rice bran oil spread plus Alfa One™rice bran oil vs. Flora margarine plus sunflower oil	1.36(0.61) – 1.36 (0.64)	0	0.99 (ns)
HDL -C	**Means**	**Paired differences (adjusted for baseline)**	**Significance**
Alfa One™rice bran oil spread vs. Alfa One™rice bran oil spread plus Alfa One™rice bran oil	1.45 (0.34) – 1.46 (0.39)	-0.01	0.69 (ns)
Alfa One™rice bran oil spread vs. Flora margarine plus sunflower oil	1.45 (0.34) – 1.47 (0.34)	-0.02	0.35 (ns)
Alfa One™rice bran oil spread plus Alfa One™rice bran oil vs. Flora margarine plus sunflower oil	1.46 (0.38) – 1.47 (0.34)	-0.01	0.66 (ns)
TOTAL CHOLESTEROL:HDL	**Means**	**Paired differences (adjusted for baseline)**	**Significance**
Alfa One™rice bran oil spread vs. Alfa One™rice bran oil spread plus Alfa One™rice bran oil	4.29(1.18) – 4.27(1.23)	0.02	0.69 (ns)
Alfa One™rice bran oil spread vs. Flora margarine plus sunflower oil	4.29 (1.18) – 4.19 (1.12)	0.10	0.09 (ns)
Alfa One™rice bran oil spread plus Alfa One™rice bran oil vs. Flora margarine plus sunflower oil	4.27 9(1.23) – 4.19 (1.12)	0.08	0.32 (ns)
LDL-C	**Means**	**Paired differences (adjusted for baseline)**	**Significance**
Alfa One™rice bran oil spread vs. Alfa One™rice bran oil spread plus Alfa One™rice bran oil	3.80 (0.65) – 3.83 (0.78)	-0.03	0.79 (ns)
Alfa One™rice bran oil spread vs. Flora margarine plus sunflower oil	3.80 (0.65) – 3.79 (0.69)	0.01	0.81 (ns)
Alfa One™rice bran oil spread plus Alfa One™rice bran oil vs. Flora margarine plus sunflower oil	3.83 (0.78) – 3.79 (0.69)	0.04	0.65 (ns)

Values shown as means ± standard deviations P=<0.05 = significant difference

5.3 Dietary Information

Participants were asked to complete 4 three-day diet records over the course of the trial, one at baseline and one after each treatment. Characteristics of the dietary variables are shown in Tables 5.5-5.6

Table 5.5: Energy and nutrient composition of dietary intake: Group 1 margarine-only.

	Baseline	**Alfa One™RBO spread**	**Flora Pro-activ®**	**Flora**
	n = 39	n = 39	n = 39	n = 39
Energy (kJ)	8735 (1724)	8330 (1909)	7927 (1409) a (↓)**	7861 (1741) a (↓)**
CHO (%TE)	49 (7)	47(7)	48 (10)	47 (7)
Protein (%TE)	17 (3)	18 (3)	18 (4)	18 (3)
Fat (%TE)	31 (7)	34 (7)	34 (9)	33 (8)
SFA (%TE)	12 (4)	11 (3)	12 (4)	12 (4)
MUFA (%TE)	11 (2)	11 (3)	13 (2) b(↑)***	11 (3)
PUFA (%TE)	5 (2)	7 (2) b(↑)***	7 (2) b(↑)***	8 (2) b(↑)***
Cholesterol (mg)	264 (103)	206 (93) a(↓)**	243 (110)	208 (111) a(↓)**

Results are presented as mean (sd) Percentage of total energy (%TE)

a (↓) indicates decrease b (↑) indicates increase

*p≤0.05 **p≤0.01 ***p≤0.001

Table 5.6: Energy and nutrient composition of dietary intake:

Group 2 margarine and oil group.

	Baseline	**Alfa One™RBO spread & oil**	**Flora & sunflower oil**	**Alfa One™RBO spread**
		n=36	n=36	n=36
Energy (kJ)	8189(2076)	8983 (1787) b(↑) *	8766 (1747)	8241 (1944)
CHO (%TE)	52 (8)	51 (13)	46 (7) a(↓) ***	51 (7)
Protein (%TE)	17 (3)	15 (3) a(↓) ***	15 (4) a(↓) **	16 (4)
Fat (%TE)	32 (9)	40(6) b(↑) ***	37(7) b(↑) **	34(6)
SFA (%TE)	13 (5)	12 (3)	12 (5)	12 (3)
MUFA (%TE)	11 (3)	15 (3) b(↑) ***	15 (8) b(↑) **	12 (2)
PUFA (%TE)	6 (2)	9 (2) b(↑) ***	9 (5) b(↑) ***	8 (3) b(↑) ***
Cholesterol (mg)	217 (104)	208 (92)	225 (86)	193 (84) a(↓) **

Results are presented as mean (sd) Percentage of total energy (%TE)

a (↓) indicates decrease b (↑) indicates increase

*p≤0.05 **p≤0.01 ***p≤0.001

5.4 Dietary intake: differences from baseline dietary intake and between treatments

Dietary records were analysed for an average of the total energy consumed over each three day period recorded in each of the three treatment phases in each arm of the trial. Average intakes of each macronutrient over the three day period were then calculated as a total of the percentage energy.

5.4.1 Total energy

There were no significant differences from baseline dietary intake (defined as dietary intake before beginning the dietary intervention) detected in total energy intake for participants when consuming Alfa One™ RBO spread ($P = 0.422$). However, when consuming Flora Pro-activ® and Flora margarine, total energy intake decreased significantly by 9.3% ($P = 0.008$) and 10% ($P = 0.003$) respectively. In Group 2 the effect of substituting habitual margarine intake with the trial margarine products and the corresponding oil showed varying responses. The treatment of Flora margarine and sunflower oil showed no significant changes from baseline ($P = 0.089$), but there was a significant increase of 20% ($P = < 0.001$) in total energy seen during the treatment of Alfa One™ RBO spread and RBO.

During the intervention stages, there were no significant changes in total energy between any of the treatments except between the Alfa One™ RBO spread treatment arm and the Alfa One™ RBO spread & RBO treatment arm, which showed a significant increase in total energy of 8.2% ($P = 0.004$).

5.4.2 Carbohydrate as a percentage of total energy

No significant changes were seen from baseline in carbohydrate intake during the Alfa One™ RBO spread treatment ($P = 0.284$), Flora Pro-activ® treatment ($P = 0.795$) Flora treatment ($P = 0.162$) or Alfa One™ rice RBO bran oil spread & RBO treatment ($P = 0.685$). A significant decrease in carbohydrate intake as % energy of 11.5% ($P = < 0.001$) from baseline was observed during the treatment with Flora margarine and sunflower oil.

There were no significant differences detected in carbohydrate intake between the treatment arms except for between the Alfa One™RBO spread & RBO treatment and the flora margarine & sunflower oil treatment, which showed a decrease in carbohydrate intake of 9.8% (P=0.04).

5.4.3 Protein as a percentage of total energy

No significant changes were seen from baseline during the Alfa One™ RBO spread treatment ($P = 0.93$), Flora pro-activ® treatment (p=0.287) or, Flora treatment (p=0.405). Significant decreases of protein intake as % energy of 11.0 % ($P = 0.008$) were seen in the Flora & sunflower oil and of 14.6% ($P = < 0.001$) in the Alfa One™ RBO spread & RBO treatment.

Protein intake remained constant between all treatments except between Alfa One™ RBO spread and Alfa One™ RBO spread & RBO treatment where a decrease of 10.6% (P=0.01) was demonstrated

5.4.4 Total fat intake as a percentage of total energy

Significant changes from baseline were not seen during any of the treatment phases in the margarine only group although a trend towards an increase in total fat was demonstrated (Alfa One ™ rice bran oil $P = 0.07$, Flora proactiv® $P = 0.07$ and Flora margarine P= 0.08).For all other treatments fat intake as % of energy increased significantly: Flora & sunflower oil of 13.6% ($P = <0.001$) and Alfa One™ RBO spread & RBO treatment of 20% ($P = <0.001$).

Between treatments, an increase of 14.3% (P=0.002) was seen between the Alfa One™ RBO spread and the Alfa One™RBO spread & RBO treatment. No significant differences were detected between any of the other treatments.

5.4.5 Saturated fat intake as a percentage of total energy

Alfa One™ RBO spread , Flora Pro-activ® and Flora treatments did not show any significant changes in saturated fat intake as % energy from baseline ($P = 0.24$, $P = 0.0.87$ and P=0.73 respectively). Additionally in group 2, no significant differences from baseline were observed (Alfa One™RBO& RBO P= 0.37, Flora margarine & sunflower oil P=0.36, Alfa One™RBO P= 0.34)

The saturated fat content of the diets also remained constant between all treatments.

5.4.6 Monounsaturated fat intake as a percentage of total energy

Treatment with Flora pro-activ® resulted in a significant increase of 15% in monounsaturated fat (*P*= <0.001). Similarly rises of 15% were shown with Alfa One™RBO spread & RBO (*P*= < 0.001) and Flora margarine & sunflower oil (*P*= <0.01). Between treatment arms a significant increase of 20 % (P = <0.001) was seen during the Alfa One™RBO spread & RBO and Flora margarine & sunflower oil phases when compared to the Alfa One™RBO spread only

5.4.7 Polyunsaturated fat intake as a percentage of total energy

Significant increases from baseline were observed in all treatments for polyunsaturated fat intake as a % of total energy: Alfa One™ RBO spread of 27% (*P* ≤ 0.001); Flora Pro-activ® of 21% (*P*= <0.001); Flora margarine of 28% (*P*= < 0.001); Flora & sunflower oil of 39% (*P*= <0.001) and Alfa One™ RBO spread & RBO treatment of 38% (*P*= <0.001).

Between treatments, no significant differences were observed.

5.4.8 Dietary cholesterol intake

No significant changes from baseline were seen in the Flora pro-activ® treatment (*P*=0.248), Flora & sunflower oil treatment (*P*=0.646) or Alfa One™ RBO spread & RBO treatment (*P*=0.644). Significant decreases in dietary cholesterol intake (mg/d) were seen in the Alfa One™ RBO spread treatment of 22% (*P*= <0.01) and Flora treatment of 21% (*P*= <0.01).

Between treatments, a significant decrease of 14.2% ($P = 0.04$) occurred between the Flora margarine and sunflower oil treatment and the Alfa One™ RBO spread. No other differences between treatments were detected.

5.5 Consumer opinion of trial products

Overall the trial products were liked by the consumer and compliance was reported to be high (>90%). The results for overall opinion, taste, texture, cooking methods and continuation of use are shown in the tables presented in Appendix III. This analysis was not intended to be the main focus of the thesis and was assessed for the interest of the company marketing the margarines and oil to gain a preliminary insight into the products. Whilst the results of the research indicated some differences between consumer opinions of the products, the questionnaires used in this part of the work were not independently validated and the results were therefore not analysed statistically. They are presented in this thesis to demonstrate the acceptability of the trial products and support the reported compliance rate amongst participants

Chapter 6

Discussion

The results of this study demonstrate that, compared to a standard margarine, Alfa One™ RBO spread containing 1.5% phytosterols reduces total cholesterol (2.2%), LDL cholesterol (3.5%) and the total cholesterol: HDL cholesterol ratio (4.1%) when consumed at a level of 20 g (4 teaspoons) per day as part of the daily diet over 4 weeks in mildly hypercholesterolaemic subjects. Neither margarine affected HDL or triglyceride concentrations. Daily consumption of 20 g Flora Pro-activ margarine containing plant sterols at a level of 8% demonstrated a greater reduction in total cholesterol (4.4%) and LDL-C (5.6%) but contains 81% more phytosterol than Alfa One™ RBO spread. This suggests that whilst consumption of Alfa One™ RBO spread results in less LDL-C lowering when consumed on a weight for weight basis compared with Flora pro-activ®, it still results in a significant reduction which may be of clinical relevance when it is considered that a 1% reduction in LDL-C can result in a 2 % reduction in CHD (Ostlund, 2007). When the relative amounts of plant sterols provided by Alfa One™ RBO spread is taken into consideration (RBO margarine: 118 mg phytosterol and 14 mg γ oryzanol; Flora Pro-activ: 1600 mg phytosterol), it appears that consuming the Alfa One™ RBO product results in a greater reduction than Flora pro-activ®. This may be due to the other fractions of the unsaponified fraction found only in rice bran oil. The presence of gamma oryzanol, tocotrienols and tocopherols and other minor constituents such as squalene and phenolic compounds, in addition to phytosterols have been shown to elicit strong hypercholesterolaemic properties in several studies (De Deckere and Korver, 1996) and it is possible that these extra components are having some effect on the reduction in serum cholesterol parameters observed in this arm of the trial.

To date the lowest effective daily dose of plant sterols demonstrated to be effective in lowering LDL-C levels has been to be between 700 to 1000mg/day. Plant sterols are more commonly supplemented at 1500- to 3300mg/day (Katan et al,

2003). This study showed that effective reductions can be achieved with levels as low as 118mg/day which is similar to doses already consumed by individuals living in Western populations as part of their typical daily intake, suggesting that products derived from rice bran oil may provide an option for lowering the levels of supplementation from the 2g/day advised by the National Cholesterol Education Council whilst still providing an effective reductions in serum cholesterol parameters. This may prove to significantly cost effective if using this intervention strategy for a wider population, not only since rice bran oil is produced as a by-product in the rice milling industry and is therefore relatively lower in cost to produce but also because phytosterol supplementation as a therapy for reduction in CHD events is significantly less expensive than traditional pharmacological treatments (Gerber et al, 2006; Wiboonsirikul et al, 2008). The addition of higher levels of phytosterol in Flora Pro-activ® affects its purchase price on the market with an average price of $5.88/250g. Alfa One™ RBO spread, with its natural plant sterols, provides the consumer with an effective, lower cost alternative (average price $4.39/250g) that can provide a long-term non-pharmacological treatment strategy for the reduction of cholesterol when included in daily food intake. Additionally vitamin E isomers and gamma oryzanol also present in the Alfa One™ RBO spread may confer additional health benefits, particularly anti-oxidant activity, that has been credited to these compounds in several studies (Sugano and Tsuji, 1997; Godber et al, 2002; Jariwalla, 2001; De Deckere and Korver, 1996). Further research to identify other health benefits of Alfa One™ RBO spread would be beneficial. For example, studies examining the possible anti-inflammatory action of γ oryzanol on markers of CHD risk and possible anti-oxidant properties exerted by the vitamin E isomers present in the unsaponified fraction.

The results of this trial are consistent with those shown by other studies investigating the effect of plant sterols on cholesterol. Numerous human intervention trials have reported that daily consumption of 1.5−3 g of phytosterol/stanols can reduce total cholesterol by 8−15% (Hallikanien et al. 2000; Neil 2001; Westrate & Meijer 1998; Naumann et al. 2003; Nestel et al. 2001; Madsen et al. 2007; Fransen et al. 2007; Katan et al. 2003). As previously mentioned, the mechanism of action of plant sterols in products such as Alfa One™ RBO spread and Flora proactiv® is not fully elucidated but is based on the inhibition of absorption of dietary and

endogenously produced cholesterol due to the similarity in physical structure borne by plant sterols (Ostlund, 2007). This similarity allows plant sterols to compete with cholesterol for space in the mixed micelle, which has limited capacity, resulting in less cholesterol being absorbed. They may also reduce the esterification rate of cholesterol in the enterocyte and thus the amount of cholesterol excreted via the chylomicrons or limit trans-membrane transport by their presence in the unstirred water layer or other mucosal barriers (Lichtenstein and Deckelbaum, 2001; de Jong et al, 2003; Ostlund, 2002b).

The safety of consuming plant sterol-enriched spreads has also been studied and no adverse side effects or detrimental changes in clinical parameters have been reported (Hendricks et al. 2003).

The cholesterol-lowering effects seen in this study were independent of the diet normally consumed by the participants as demonstrated in previous studies, (Westrate and Meijer, 1998; Hendricks et al, 1999) suggesting that the effects of these products may be greater if consumed as part of a healthy diet, low in saturated fat and cholesterol and high in vegetables, fruits and wholegrains (Maki et al. 2001).

In addition to the group of participants consuming margarine only, the second arm of this trial included a group of individuals consuming Alfa One™ RBO spread along with Alfa One™ RBO. It was hypothesised that the increase in intake of plant sterols (268 mg/day as opposed to 118 mg/day) may further increase the cholesterol-lowering effect. Participants in this group consumed 20 g/day of the Alfa One™ RBO spread with 30 ml/day Alfa One™ RBO each day and the effects were compared with the consumption of 20 g Flora margarine and 30 ml sunflower oil. Also included was a treatment arm of 20 g Alfa One™ RBO spread alone. The results presented here demonstrate that there were no significant changes in any serum lipid parameters for any of the participants in this part of the study. The Alfa One™ RBO spread alone did not replicate the reduction in cholesterol parameters that was demonstrated in the margarine only arm of the trial. The explanation for this aspect of the second arm of the trial has yet to be elucidated.

One of the reasons that may explain why the addition of RBO to the Alfa One™RBO spread may not have been effective is the higher level of dietary fat provided by the addition of oil to the diet. It is well documented that high levels of

saturated fatty acids in the diet can increase serum total and LDL cholesterol (Hu et al. 2001; Mensink & Katan. 1992). Examination of the dietary information generated from participants does not support this theory for these trial results. The dietary intake data indicate that during the study participants total dietary fat intakes were around 34% for the margarine only group and slightly higher (37%) for the margarine plus oil group with only a significant increase being seen during the treatment arms with Alfa One™ RBO spread and the Alfa One™ RBO spread & Alfa One™ RBO. However, levels of saturated fat intake decreased when in comparison to baseline values taken before intervention and the rise in total fat was attributable to a significant increase in monounsaturated and polyunsaturated fat, both of which are reported to have favourable effects on LDL-C profiles (Hu et al. 2001). Additionally, although there were some significant differences between macronutrient intakes in some of the different intervention arms, there was no apparent trend for a particular trial product that would infer that the nutrient content of the background diet was responsible for the lack of hypercholesterolaemic effect. There is a possibility that the dietary records were not accurate. High variation in the pattern of food consumption amongst individuals make it difficult to accurately estimate usual dietary intake, despite individuals being asked to report non-consecutive and weekend days in an effort to reduce this. Social desirability also introduces a large element of recall bias into reporting food intake as participants tend to report food intake habits that are consistent with accepted perceptions of health. Under-reporting of food intake is common amongst individuals and may have affected the final estimations of nutrient intake in this study (Gibson 2005). Compliance of the participants to consume the products in this part of the study may also have been under estimated as the volume of margarine and oil expected to be consumed was reported to be much more than individuals would normally eat. The reliance on self reporting for a measure of compliance is one of the major limitations of this study. The measurement of serum phytosterol content was initially included to help support the compliance data however the method adopted for this parameter proved to be unsuitable for the study samples and was unable to provide supporting evidence for the consumption of phytosterols. Further method development using liquid chromatography/mass spectrometry for this aspect of the study will be undertaken but will not be completed within the time frame for this thesis.

The method of use for the oil products may also be a contributing factor to the lack of hypercholesterolaemic effect. Overall, although some individuals reported consuming the oil as a standalone product, a majority used the oil for cooking purposes. Studies have shown that although heat treatment may only confer a modest reduction in the levels of phytosterol components, the level of phytosterol oxides increases (Soupas et al, 2004). These oxidative products may interact with lipids, carbohydrates and proteins present in food matrices that may mask small changes in lipid parameters, having a subsequent effect on phytosterol stability, absorption and health attributes. Further research in this area is required to evaluate the effects of phytosterol oxides (Soupas et al, 2004; Moreau et al, 1999).

Participants in this study were randomly assigned by the statistician using a simple randomisation technique to either the margarine only group or the margarine and oil group. Whilst it appears from the data presented in table 5.1 that the treatment groups were similar in regards to all relevant participant characteristics (particularly lipoprotein profiles), this study may have benefited from using a stratified randomisation to ensure that there was no possibility of any characteristic influencing response to treatment. Furthermore this study may have benefited from the inclusion of a washout period between each treatment. Washout periods are included to remove any possible carry over effect from the first treatment period into the second treatment. In this study it was decided not to include a washout period between treatment arms as the length of each treatment arm was four weeks which provided enough time for the stabilisation of lipid levels even after a change in dietary intake. Previous studies (Bonanome and Grundy, 1988; Mensink and Katan, 1987; O' Dea et al, 1990) have shown that plasma lipid levels stabilise within 2-3 weeks after dietary changes are made. The inclusion of a washout period in this study would have increased the length of the study and may have led to less participant compliance and greater number of withdrawals from the study.

Finally, a further limitation of this study may relate to the variation in blood cholesterol concentrations that occurs daily within individuals ranging from 5 to 10% as a consequence of several factors including dietary intake, alcohol consumption, menstrual cycle fluctuations, hydration status, illness and physical activity (Grandjean and Alhassan, 2006). Whilst every effort was made to standardise the collection of blood samples and account for these factors (taking two

fasting samples with at least 24 hours in between at the same time of day and monitoring dietary intake through dietary intake records and changes in activity levels through face to face interviews during clinic visits), the participants in this study were free living individuals and their exact behaviours cannot be predicted. Natural day to day variation in blood lipid parameters may have had an impact when examining small changes.

Chapter 7

Conclusion

Non pharmacological therapies for lowering serum, low density lipoprotein cholesterol levels are an essential part of reducing the risk of coronary heart disease, which remains a leading cause of morbidity and mortality worldwide. The addition of foods fortified with plant sterols into the daily diet can significantly reduce cholesterol levels independently of background dietary intakes and the introduction of these foods has offered another dimension to the dietary management of cholesterol. Consumption of Alfa One™ RBO spread significantly reduced total cholesterol, LDL cholesterol and total: HDL cholesterol compared with standard Flora margarine. Flora pro activ® produced a greater reduction in the cholesterol parameters measured but contains around five times the level of plant sterols. Alfa One™ RBO spread has therefore been shown to be an alternative effective method for reducing lipid parameters, presenting the consumer with a choice to purchase a natural plant sterol based margarine which is beneficial in the control of cholesterol levels and lower in price. Additionally this product may have further as yet unidentified health benefits arising from the other bioactive compounds (γ-oryzanol, tocotrienol and tocopherols) found in the unsaponified fraction which is an area that requires more investigation.

Further research is required to explain the lack of hypercholesterolaemic effect when Alfa One™ rice bran oil spread is consumed along with the rice bran oil. The higher level of plant sterols consumed by individuals when combining test products did not produce any impact upon cholesterol parameters suggesting that there may be some effects elicited by the method of cooking or unreported compliance issues that need addressing. However, the results of this study, supported by other investigations into the cholesterol lowering effect of plant sterol and stanol products, provide a significant amount of evidence that these compounds are an effective and safe adjunct to diet controlled therapy that can produce clinically significant reductions in LDLcholesterol in persons with mild to moderate hypercholesterolaemia.

Appendices

Appendix I: Participant information booklets

Comparison of a rice bran oil margarine with Flora margarine and Flora Pro-activ® margarine for lowering cholesterol

Date:

Group:

Name:

Welcome to the study
Thank you once again for taking part

You have been randomised to group _____ which means that you will be consuming the margarine only. You will be using margarine _____ First, followed by _____ and finally _____

The study consists of three parts:

The first test period will begin on _____ and will end on _____ it will last for a period of four weeks. During this time you will add_____ to your diet on a daily basis as part of your everyday diet instead of the usual margarine/spread you usually use.

At the end of this period you will come back to the hospital for a second set of blood tests and fill in another three day diet record. You will then start on the second product. During this time you will add _____to your diet on a daily basis as part of your everyday diet. This period will begin on _____ and end on _____. It will last for a period of four weeks.

At the end of this period you will come back to the hospital for a third set of blood tests and fill in another three day diet record. You will then start on the third product. During this time you will add _____to your diet on a daily basis as part of your everyday diet. This period will begin on _____ and end on _____. It will last for a period of four weeks.

You will then be asked to come to the hospital to have the final set of blood tests and fill out the last dietary record.

You will be given the test products required for your diet at the beginning of each four week intervention. You should continue with usual diet during the whole study but just substitute the test products for your usual margarine.

It is important to recognise that the margarine that you are using in this trial as to be used as a REPLACEMENT for the ones you are currently using NOT IN ADDITION to the ones already in your diet. This will mean that there should not be any changes to your current overall daily energy intake. Your daily intake of these products should be enough to cover all your daily requirements for margarine/oil, please try not to exceed the amounts specified. So, if you normally have cereal for breakfast you may like to change to having two pieces of toast with margarine and jam. If you normally have pasta salad for lunch then replace this with a cheese salad sandwich using wholegrain bread and the study margarine. 5g of margarine is equal to one teaspoon of margarine, about what you would spread on one piece of toast, so overall you are being asked to eat four teaspoons of margarine a day. 30g of oil is equal to two tablespoons of oil a day which can be used in a variety of ways such as stir frying or baking. There are a selection of recipes and suggestions for using the margarines and oils in this booklet. Rice bran margarine and oil can be used for most baking applications with the exception of pastry. Simply substitute the butter/margarine/oil content for the rice bran products. Calculate the amount each serve contains by dividing the total amount of fat added by the number of serves the recipe makes.

Suggested daily meal plans

Day One	Day Two	Day Three
Breakfast 2 slices multigrain toast 2 tsp margarine 2 tsp berry jam 125 ml trim milk 20g Milo 1 small bowl peaches in natural juice	Breakfast 50g Natural Muesli 125ml skim milk 1 slice multigrain toast 1tsp margarine 1 tsp vegemite 1 cup of tea 20ml skim milk	Breakfast 2 slices wholemeal toast 2 tsp margarine 1 egg scrambled 1 250ml orange juice 1 cup tea 20 ml trim milk
Morning Tea 1 cup trim cappuccino	Morning tea 1 cup coffee 1 plain scone with 2 tsp margarine 30ml skim milk	Morning tea 1 cup tea 20ml trim milk 1 medium apple
Lunch 2 slices wholemeal bread 2 tsp margarine 25g edam cheese 1 small tomato 20g lettuce 1 tsp low fat mayonnaise 1 125 ml low fat fruit yoghurt 1 medium apple 1 cup of tea 20 ml trim milk	Lunch 1 cup cooked pasta 150g tuna 1 small tomato ½ cup chopped red pepper 2 spring onions 1 tbsp oil mixed with 1 tbsp lemon juice, salt pepper and 1 tsp fresh basil 1 small blueberry muffin with 1 tsp margarine 1 trim latte	Lunch 1 bowl minestrone soup 2 brown bread rolls 2 tsp margarine 1 large kiwifruit 1 medium orange 1 cup trim latte

Afternoon tea 1 cup coffee 30ml trim milk 1 small banana	Afternoon tea 1 cup herb tea	Afternoon tea 1 cup tea 20ml trim milk 1 small muesli bar
Dinner 180g fillet steak Stir fried vegetables with spices 1 medium baked potato 2tsp light sour cream 1 glass white wine 1 cup canned fruit salad in natural juice 100g vanilla ice cream	Dinner 179g chicken pieces ½ cup carrots ½ cup peas 1 small onion ½ cup broccoli 1 cup tomato based sauce with garlic 1 cup brown rice boiled	Dinner 500g pork strips ½ cup red pepper 1 cup broccoli florets 2 tsp fresh ginger ½ cup mushrooms sliced 1 small onion 1 300g packet Hokien noodles 1 cup black bean stir fry dressing ½ cup reduced fat yogurt 1 cup sliced strawberries 1 glass white wine
After Dinner 1 cup coffee 30ml trim milk	After dinner 125 ml trim milk 20g Milo	After dinner 1 cup coffee 30ml milk

Recipes

Treat foods such as cakes, muffins, biscuits and muesli bars can be high in fat and sugar content so it is recommended that theses foods are only eaten occasionally.

Chocolate Chip Muffins (12 muffins)
2 Cups flour
2 tsp. baking powder
1/2 tsp. baking soda
1/2 tsp. salt
2/3 Cup brown sugar
2 large eggs
1 cup milk
1/2 Cup oil or 125g margarine melted

1 tsp. vanilla extract
1/2 Cup mini-chocolate chips

Preheat oven to 425. Grease 12 muffin cups. Combine dry ingredients. Combine sugar, eggs, oil, milk and vanilla and stir into dry ingredients. Stir in chips. Spoon into cups. Bake 18-20 minutes. Each muffin will contain 10 g (2 tsp) of oil which is a third of your daily allowance

Blueberry Muffins (Makes 12 muffins)

Cream together
120g melted margarine or oil
3/4 cup sugar
1 extra-large egg
Add:
1 tsp vanilla
1 cup milk

Mix together and add:
2 1/2 cups all-purpose flour
1/2 tsp salt
1 tbsp baking powder
1 1/2 cups fresh blueberries with a metal spoon. Fill the cups 2/3rds full and sprinkle with cinnamon-sugar mix.

Instructions:
Bake for 20-25 minutes at 350 degrees until lightly brown.

Each muffin contains 10 g of the margarine which is half your daily allowance or 10ml of oil which is 1/3 of your daily allowance

Scones

3 cups plain flour

6 teaspoons baking powder

¼ teaspoon salt

75 g margarine

1 to 1 ½ cups of milk

1. Sift flour, baking powder and salt into a bowl.
2. Cut margarine in until it resembles fine breadcrumbs.
3. Add milk and mix quickly with a knife to a soft dough.
4. Knead a few times.
5. Lightly dust an oven tray with flour.
6. Press scone dough out onto this.
7. Cut into 12 even-sized pieces.
8. Leave a 2 cm space between scones.
9. Brush tops with milk.
10. Bake at 220^{0}C for 10 minutes or until golden brown

Each scone will contain 6.25 g margarine which is approximately 1/3 of your daily allowance

Fruity Rice Salad

Serves 6

Dressing

¼ cup orange juice
2 tablespoons oil
1 clove garlic crushed
1 teaspoon ginger grated
2 teaspoons honey
1 teaspoon lemon juice

½ cup pinenuts or pumpkin seeds
½ cup sultanas
1 stick celery
4 spring onions
1 tablespoon chopped ginger
2 dried apricots chopped
1 cup cooked rice

Combine all the dressing ingredients in a screw top jar and shake well to combine

Combine all the ingredients in a salad bowl and toss well with dressing to combine. Cover and refrigerate for several hours before serving. The flavour improves the longer you leave it.

Each serving contains 5ml (5 g) of the oil which is 1/6 of your daily allowance.

French Salad Dressing Recipe

- 1/2 cup fresh lemon juice or vinegar
- 1 1/4 cups oil
- 2 teaspoons salt
- 1/4 teaspoon pepper
- 1 teaspoon dry mustard
- dash cayenne

Whisk together all ingredients in a mixing bowl or shake in a jar. Makes 2 cups (500 ml) of French dressing. Each 50 ml serve contains 31 g (6 teaspoons or 30 ml) of oil which is your daily allowance of oil.

Banana & Raspberry Muffins (Serves 12)

1½ cups white self-raising flour
½ cup wholemeal self-raising flour
1 cup castor sugar
1 teaspoon cinnamon
1 cup raspberries, fresh or frozen and defrosted
2 bananas, peeled, chopped and sprinkled with lemon juice
1 teaspoon grated lemon rind
¼ cup oil or 62.5 g melted margarine

¾ cup un-homogenised full-cream milk

2 eggs or 3 egg-whites, beaten well with a fork

Preheat the oven to 180^{0}C and lightly wipe some oil around a non-stick 12-piece muffin tray or 4x12 mini muffins trays.
Sift the top four dry ingredients into a bowl.
Thoroughly mix the bottom three wet ingredients.
Toss the bananas and berries on top of the dry ingredients.
Fold the wet ingredients into the dry ingredients using a hand whisk (not a beater).
Fold and lift the whisk through gently only a half dozen times until roughly mixed – it doesn't matter that there are patches of flour showing. If you mix too thoroughly, the muffins may not be soft and fluffy.
Spoon into the muffin tray and bake for 20-25 minutes or until golden brown.
Each muffin will contain 5 ml (1 tsp) oil which is 1/6th of your daily allowance or roughly 5 g of margarine (1/4 of your daily allowance)

Banana Smoothie

This is a quick easy way to get all the required oil in one hit and it tastes good too.

1 ripe banana

30 ml oil

2 heaped table spoons low fat yoghurt (Fresh and Fruity lemon and honey is great but natural yoghurt also works well)

Low fat milk to make up to desired consistency (about 100 ml)

Blend all ingredients together until smooth. Drink immediately.

Muesli Bars

75 ml/5 tbsp oil or 75 g margarine melted
½ cup brown sugar
¼ cup honey
¼ cup of sesame seeds
1 ½ cups wholegrain oats
½ cup sultanas
1/3 cup walnuts chopped
½ cup wholemeal flour
½ tsp baking powder

1. heat oil, honey and sugar over a low heat until melted or microwave on high for 2-3 minutes
2. mix well and add oats, sultanas, chopped walnuts and sesame seeds
3. sift together flour and baking powder, then stir together into mixture to form a stiff dough
4. Press into an 18 cm*22 cm roll tin or microwave slice dish. Smooth top
5. bake at 300°F /175°C for 20-30 minutes, or until golden brown or cook on 70% microwave power for 7-10 minutes
6. mark out in 10 even squares while still warm
7. cut into squares and lift out of container when cold

1 bar = 7.5 ml oil (which is ¼ or your daily allowance) or 7.5 g margarine (which is roughly 1/3 of your allowance).

Tipsheet

Choosing Healthy Foods (adapted from the Heart Foundation of New Zealand)

Recommendations for all people include:

1. Maintain a healthy body weight (BMI 20-25 kg/m^2)
2. Keep physically active
3. Eat a varied diet
4. Enjoy three meals a day, selecting from dishes that encourage you to eat plant foods and fish, with little or no dairy fat, meat fat or deep fried foods
5. Choose fruits and/or vegetables at every meal and for most snacks
6. Select whole grains, whole grain breads or high fibre breakfast cereals in place of white bread and low fibre varieties at most meals and snacks
7. Include fish or dried peas (e.g. chickpeas), beans (e.g. red kidney beans) and soy products or a small serving of lean meat or skinned poultry at one or two meals a day
8. Drink plenty of fluids each day, particularly water and limit sugar sweetened drinks and alcohol
9. Use small amounts of sugar and salt when cooking and preparing meals, snacks or drinks. Choose ready –prepared foods low in these ingredients
10. Mostly avoid butter, deep fried and fatty foods and only occasionally choose sweet bakery products

Incorporating margarine into your daily diet

Some ideas for using the 20 g/day

Breakfast:

2 pieces of bread or toast with 2 teaspoons of spread and/or:

Lunch

Either a sandwich, roll or wrap with 2 teaspoons of spread and/or:

Dinner

Use 2 teaspoons of spread as a topping on vegetables or in mashed potatoes and sauces and/or:

Snacks

Incorporate the spread into one of the snack recipes provided

The following table describes the quality and quantity of food to support a healthy dietary pattern. Following the guidelines can help with maintaining a healthy weight and reduce individual risk for developing nutritional related diseases such as cardiovascular disease and type 2 diabetes

Healthy Foods

Healthy Food Groups	Daily Servings	How much is a serving
Fruit and coloured vegetables	Aim to eat at least 5 servings per day	1 piece of fruit, ½ cup canned fruit in natural juice, 1 cup raw leafy or salad vegetables, ½ cup cooked vegetables, 1 tomato, 1 carrot
Grains and starchy vegetables Choose a variety of grain products (at least half of these should be whole grain products)	Aim to eat at least 6 or more grains and starchy vegetables	1 medium sliced bread, ½ cup pasta or porridge, 2/3 cup whole wheat cereal, 1 small potato, ½ kumara, 3 whole grain crisp breads
Fish and seafood, dried peas and beans, lean meat and skinned poultry	Eat between 1-3 depending on kilojoules requirements	½ cup tuna, 2 small or 1 large fillet of cooked fish, ½ cup lean mince or casserole, 1 cup dried beans
Milk and milk products	Eat 2-3 depending on kilojoules requirements	1 cup trim or very low fat milk, 1/3 cup cottage cheese, 1 pottle low fat yoghurt, 3 tsp grated cheddar cheese
Non alcoholic drinks	Between 6-8 non alcoholic drinks	1 glass water, 1 cup of tea, coffee or diet soft drink

What Makes a Healthy Snack?

If you are hungry in between meals try and fill up on healthy and nutritious snacks. Here are some of our suggestions:

- Fresh fruit, tinned or dried
- Unsalted nuts
- Low-fat yoghurt or ice cream with fruit
- Fruit bread with margarine
- Wholegrain crisp bread with low-fat cheese/tomato
- Pumpkin or fruit scone with margarine
- Cut up vegetables (e.g. Celery and carrot sticks) or pita bread with salsa or hummus
- Air-popped popcorn or plain pretzels

Try and limit your consumption of these foods:

- Butter, full fat dairy products. Choose low fat milk, cheese and yogurts instead
- Fatty meats, including sausages and delicatessen meats such as salami
- Take-away foods including pies, pizza, hamburgers and creamy pasta dishes
- Foods cooked in butter
- Cakes, sweet biscuits, pastries and chocolate
- Snack foods such as potato crisps

Hints for coping with eating out, travelling & entertaining

Adapted from www.eatwell.gov.uk and www.mypyramid.gov

During the study period it is probable that you will be eating out, travelling or entertaining. There are a few simple things that you can do to make your meal healthier.

1. As a beverage choice, ask for water or order fat-free or low-fat milk, unsweetened tea, or other drinks without added sugars.

2. Ask for whole wheat/ whole grain bread for sandwiches.

3. In a restaurant, start your meal with a salad packed with veggies, to help control hunger and feel satisfied sooner. Try not to have lots of bread or other nibbles before your meal arrives.

4. Ask for salad dressing to be served on the side. Then use only as much as you want.

5. Choose main dishes that include vegetables, such as stir fries, kebabs, or pasta with a tomato sauce. Avoid cream based sauces.

6. Order steamed or grilled dishes instead of those that are fried or sautéed.

7. Choose a "small" or "medium" portion. This includes main dishes, side dishes, and beverages.

8. Order an item from the menu instead heading for the "all-you-can-eat" buffet

9. If main portions at a restaurant are larger than you want, try one of these strategies to keep from overeating:

- Order an appetizer or side dish instead of an entrée.
- Share a main dish with a friend.
- If you can chill the extra food right away, take leftovers home in a "doggy bag."
- When your food is delivered set aside or pack half of it to go immediately.
- Resign from the "clean your plate club" – when you've eaten enough, leave the rest.

10. To keep your meal moderate in calories, fat, and sugars:

- Ask for salad dressing to be served "on the side" so you can add only as much as you want.
- Order foods that do not have creamy sauces or gravies
- Add little or no butter to your food.
- Choose fruits for dessert most often.

11. On long commutes or shopping trips, pack some fresh fruit, cut-up vegetables, low-fat string cheese sticks, or a handful of unsalted nuts to help you avoid stopping for sweet or fatty snacks.

5+ A Day is the fun and easy way to remind you to eat lots of fruit and vegetables: Taken from www.vegfed.co.nz and www.5+aday.org

How many servings should I eat?
New Zealand guidelines recommend you eat five or more servings of fruit and vegetables every day. Specifically, three or more servings of vegetables and two or more servings of fruit. The recommendation of five servings per day is seen as a minimum requirement for good health.

What is a serving?
It is about a handful and everyone uses their own hand.

Why should I "eat my colours"?
By eating your colours every day you will stay fit and healthy. Colourful fruit and vegetables contain many of the vitamins, minerals and phytochemicals (fight-o-chemicals) your body needs to maintain good health and energy. They also protect against the effects of aging. Many of the phytochemicals and other compounds that make fruit and vegetables such healthy foods also give them their colour. There are many different phytochemicals and compounds associated with the colour - so to ensure you get a wide range of them you need to try to eat fruit and vegetables from each of the colour groups every day.

What happens if I eat 5+ a day everyday but don't always cover all the colours?
For good health it is essential to eat at least 5 servings a day and ideal if you can eat from each colour group. That is why we say eat 5+ A Day and try to eat the colour way! By eating the colour way you will give your body even better protection.

What are the colour groups?
There are five groups and the main aim of eating the colour way is to encourage variety. You have to use your common sense, for example a cucumber is mainly in the white group - but by eating the skin you get the benefits of the green group too. This list is far from comprehensive but gives you the idea…

RED – tomatoes, red peppers etc; ORANGE/YELLOW – carrots, pumpkins etc; BROWN/WHITE – cauliflower, potatoes etc; GREEN – beans, broccoli etc; BLUE/PURPLE – egg plant, beetroot etc

- Toss fruit into your green salad for extra flavour, variety, colour, and crunch.
- Expand your plate and your palette with green, red, orange, yellow, and purple peppers.
- Think frozen! Frozen fruits and vegetables are just as healthy as fresh, and they're ready when you need them.
- Save time with pre-cut vegetables and salad mixes.
- Add apples, raisins, or pineapple chunks to deli salads like chicken, tuna, or pasta.
- Add frozen mixed vegetables to canned or dried soups.
- Make a quick smoothie using frozen fruit.

Appendix II: Participants check sheets and questionnaires

Comparison of a rice bran oil margarine with Flora margarine and Flora Pro-activ margarine for lowering cholesterol

Participant Number: _____

Group Number: _____

Date:	Day	Margarine consumed
	1	
	2	
	3	
	4	
	5	
	6	
	7	
	8	
	9	
	10	
	11	
	12	
	13	
	14	
	15	
	16	
	17	
	18	
	19	
	20	
	21	
	22	
	23	
	24	
	25	
	26	
	27	
	28	
Swap to treatment #2		

INSTRUCTIONS FOR COMPLETING A THREE DAY DIET RECORD

1. Choose three days prior to your appointment which includes one weekend day (or your equivalent of if you work on weekends). Our eating patterns can vary considerably between week days and weekends and we want to get a snapshot of your total dietary habits.

2. Please record EVERYTHING YOU EAT AND DRINK on these days as accurately as possible. It is great if you can write things down straight after you have eaten as things are fresh in your mind but if this is not possible do it as soon as you can.

3. Please try to write down the type of foods and the amounts you are eating. For example if you have vegetable soup please list the different vegetables it contains and estimate how many was in your serve size. If it is a homemade soup please try to give us the recipe or if it is a shop bought soup, please give us the label with the list of ingredients. If you have fruit or foods such as beans please give us the variety e.g. kidney beans or baked beans. Braeburn or Royal Gala apples. Please try to estimate the size – if you have an apple, tell us whether that is a small, medium or large and estimate its weight. If you have tea or coffee, please note if you add milk or sugar and please tell us the type. We need to know as much as possible about the foods and beverages you are consuming to get an accurate picture.

4. It is helpful to us if you can give us the trade or brand names of foods e.g. Watties baked beans or Sanitarium muesli

5. Please don't get too intimidated by the form – it can look like you are eating a lot when you are listing every single ingredient that is in something. We just need to get some idea of your normal dietary habits to help us with interpreting what effects our study products may be having.

Margarine rating questionnaire

Participant Identification Number: _____

Intervention Arm: _____

1: Please choose the phrase that best describes your overall opinion of the product:

□ Like extremely
□ Like very much
□ Like moderately
□ Neither like nor dislike
□ Dislike Moderately
□ Dislike very much
□ Dislike extremely

2: Please choose the phrase that best describes your overall opinion of the TEXTURE of the product

□ Like extremely
□ Like very much
□ Like moderately
□ Neither like nor dislike
□ Dislike moderately
□ Dislike very much
□ Dislike extremely

Please elaborate on what you did or did not like about the texture of the product _____

_____

3: Please choose the phrase that best describes your overall opinion of the TASTE of the product

□ Like extremely
□ Like very much
□ Like moderately
□ Neither like nor dislike
□ Dislike moderately
□ Dislike very much
□ Dislike extremely

Please elaborate on what you did or did not like about the taste of the product _____

_____

4: Please indicate the methods in which you used the product (you may tick more than one box)

□ as a spread
□ in baking
□ in cooking/frying

Was there any of these methods that were not suitable for the product in your opinion? _____

If not, why not? _____

_____

5: How likely are you to continue to want to use the product?

□ Very likely
□ Likely
□ Neither likely nor unlikely
□ Not likely
□ Definitely not going to use the product again

Please specify your reasons for your answer to question 5: _____

_____

Appendix III Consumer opinion results

Taste

	Flora margarine (% participants)	Flora-proactiv® margarine (% participants)	Alfa One™ Rice bran oil spread (% participants)
Like	**48.1**	**57.1**	**49.6**
Neutral	**41.8**	**33.3**	**47**
Dislike	**10.1**	**9.5**	**3.4**

	Flora margarine	Flora-proactiv® margarine	Alfa One™ Rice bran oil spread
Comments	**Mild, neutral , light** **buttery** **Too salty** **Plasticy, definite** **margarine taste**	**Neutral, creamy** **Salty** **Plasticy** **No aftertaste**	**Creamy, smooth** **Very little taste** **Oily taste** **Like butter** **Enhanced other** **flavours** **Pleasant**

Texture

	Flora margarine (% participants)	Flora-proactiv® margarine (% participants)	Alfa One™ Rice bran oil spread (% participants)
Like	**70.9**	**78.6**	**41.9**
Neutral	**22.8**	**11.9**	**21.4**
Dislike	**6.3**	**9.5**	**36.8**

	Flora margarine	Flora-proactiv® margarine	Alfa One™ Rice bran oil spread
Comments	**Easily spread** **Smooth** **Bit greasy** **Too soft** **Heavy and oily**	**Creamy** **Firm** **Clumpy** **Easy to spread but not too runny**	**Too soft even with fridge storage** **Creamy and smooth** **Sloppy** **Too thin** **Slightly grainy** **Easily spread**

Cooking Methods

	Flora margarine (% participants)	Flora-proactiv®margarine (% participants)	Alfa One™ Ricebran oil spread (% participants)	Alfa One™ Ricebran oil	Sunflower Oil
As a spread	75.3	86.5	88	n/a	n/a
In baking	18.9	8.4	3.3	3.6	4.3
Cooking/frying	5.8	5.1	8.7	96.4	95.7

	Flora margarine	Flora-proactiv® margarine	Alfa One™ Rice bran oil spread	Alfa One™ Ricebran oil	Sunflower Oil
Comments	Was Ok in baking Just substituted for fat in recipe, worked well Prefer to use oil for cooking Burnt too easy in frying	Gave products awful texture Made baking 'heavy' Tried it once but preferred to use it as spread	Too runny Very oily Muffins were too dense Preferred taste as spread	Great for stir fry Neutral taste Made great salad dressing Worked well in muffins	Was Ok for cooking but did not like taste for salad dressing Quite smoky when used in frying

Continuing Use

	Flora margarine (% participants)	Flora-proactiv® margarine (% participants)	Alfa One™ Rice bran oil spread (% participants)
Like	**48.6**	**48.7**	**39.3**
Neutral	**31.9**	**38.5**	**34.8**
Dislike	**19.4**	**12.8**	**25.9**

	Flora margarine	Flora-proactiv® margarine	Alfa One™ Rice bran oil spread
Comments	**Depends on study results and its effect on cholesterol levels** **Depends on price** **Still too many chemical additives** **Would need further information on nutritional qualities**	**Depends on study results and its effect on cholesterol levels** **Depends on price** **Still too many chemical additives** **Would need further information on nutritional qualities**	**Depends on study results and its effect on cholesterol levels** **Would depend on price – softer texture meant double the amount of product** **Would prefer reduced fat variety** **Would depend on chemical additives**

Overall Opinion

	Flora margarine (% participants)	Flora-proactiv® margarine (% participants)	Alfa One™ Rice bran oil spread (% participants)
Like	**59.5**	**47.6**	**46.2**
Neutral	**35.4**	**40.5**	**39.3**
Dislike	**5.1**	**11.9**	**14.5**

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