Diet, kiwifruit and genotypes: their influence on lipid profiles and CVD-related metabolic markers in hypercholesterolaemic men

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Abstract

In New Zealand, cardiovascular disease (CVD) is a major public health concern and a leading cause of death. Lifestyle modification, including dietary change, is a crucial element in the prevention of CVD, with fruit and vegetables key elements of a cardioprotective diet. Kiwifruit is a commonly-consumed, nutrient-dense fruit that contains many components individually shown to have positive effects on CVD risk factors. This study investigated the effects on plasma lipid profiles and other CVD-related metabolic markers of consuming kiwifruit daily in conjunction with a healthy diet. Further, plasma lipid responses were examined according to inflammatory status, *APOE* genotype, and additional high-density lipoprotein cholesterol (HDL-C)-related gene polymorphisms.

Eighty-five hypercholesterolaemic men (low-density cholesterol (LDL-C) >3.0 mmol/L and triglycerides (TG) <3 mmol/L) completed a 4-week healthy diet run-in before randomisation to a controlled cross-over study of two 4-week interventions of two green kiwifruit/day plus healthy diet (intervention), or healthy diet alone (control). Anthropometric measures, blood pressure (BP), heart rate (HR), stroke volume (SV), cardiac output (CO), total peripheral resistance (TPR) and fasting blood samples (lipid profile, insulin, glucose, high-sensitivity C-reactive protein (hs-CRP), interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and IL-10) were taken at baseline, 4 and 8 weeks. An additional blood sample was taken for genetic testing: *APOE, CETP Taq1B*; *APOA1* -75G/A; *LIPC*-514C→T; *LIPG* I24582. Subjects were divided into low and medium inflammatory groups, using pre-intervention hs-CRP concentrations (hs-CRP <1 and 1-3 mg/L, respectively). Repeated measures ANOVA was used to examine genotype/inflammatory status x treatment interactions.

After the kiwifruit intervention, plasma HDL-C concentrations were significantly higher (mean difference 0.04 [95% CI: 0.01, 0.07] mmol/L [*P*=0.004]) and the total cholesterol (TC)/HDL-C ratio was significantly lower (-0.15 [-0.24, -0.05] mmol/L [*P*=0.002]), compared to control. For the total group, there were no significant differences between the interventions for TC, LDL-C, insulin, glucose, hs-CRP, BP, HR, SV, CO and TPR. Lipid responses were modulated according to inflammatory status and *APOE* and *CETP Taq1B* genotypes. The

medium inflammatory group had a significant improvement in CRP with kiwifruit compared to control. Significant inflammatory group x intervention interactions were seen for TC/HDL-C (*P*=0.02), TG/HDL-C (*P*=0.05), and plasma IL-6 (*P*=0.04). Examined by genotype, *APOE4* carriers had significantly decreased TG (-0.18 [-0.34, -0.02] mmol/L [*P*=0.03]) with kiwifruit compared to control. A significant *CETP Taq1B* genotype x intervention interaction was seen for the TG/HDL-C ratio (*P*=0.03), and *B1/B1* homozygotes had a significantly lower TG/HDL-C (-0.23±0.58 mmol/L, *P*=0.03) ratio after the kiwifruit intervention, compared to control. Lipid response was not affected by other polymorphisms.

In conclusion, kiwifruit independently of a healthy diet containing fruit improved dyslipidaemic profiles, significantly improving mean HDL-C concentration and TC/HDL-C ratio compared with the control intervention. Effects were even more pronounced in men with phenotypes and genotypes which placed them at higher risk of CVD, with more sizeable improvements in HDL-C and TC/HDL-C ratio, and significant decreases in TG concentrations and TG/HDL-C ratio. Although modest, the improvements suggest that regular inclusion of green kiwifruit as part of a healthy diet could translate into a significant reduction of CVD risk in men with high cholesterol concentrations.

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List of Abbreviations

2-MG	2-monoacylglycerol	DASH	Dietary Approaches to Stop Hypertension
AAMI	Association for the Advancement of Medical Instrumentation	DBP	diastolic blood pressure
ABC	adenosine triphosphate-binding cassette	DDAH	dimethylarginine dimethylaminohydrolase
ACE	angiotensin converting enzyme	DGAT	acyl-coenzyme A: diacylglycerol acyltransferase
ADMA	asymmetrical dimethylarginine	DHAH	Diabetes, Heart and Health Survey
AHA	American Heart Association	DNA	deoxyribonucleic acid
Ang	angiotensin	eNOS	endothelial nitric oxide synthase
ANOVA	analysis of variance	ESS	endothelial shear stress
ANZCTR	Australian New Zealand Clinical Trial Registry	FA	fatty acid
Apo	apolipoprotein	FABP	fatty acid-binding protein
APOE	apolipoprotein E	FAO	Food and Agriculture Organization
AT1	angiotensin II type 1-receptor	FFA	free fatty acid
ATP	Adult Treatment Panel	FW	fresh weight
AUC	area under the curve	GAE	gallic acid equivalents
BF	body fat	HDL	high-density lipoprotein
BH_4	tetrahydrobiopterin	HDL-C	high-density lipoprotein cholesterol
BMI	body mass index	HL	hepatic lipase
BODPOD	air displacement plethysmography	HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
BP	blood pressure	HOMA	homeostasis model assessment
CD36	type B scavenger receptor	HOMA2-IR	homeostasis model assessment 2 computer model for insulin resistance
CDC	Centers for Disease Control and Prevention	HR	heart rate
CE	cholesterol ester	hs-CRP	high-sensitivity C-reactive protein
CETP	cholesteryl ester transfer protein	HSPG	heparin sulphate proteoglycans
cGMP	cyclic guanosine monophosphate	IANZ	International Accreditation New Zealand
CHD	coronary heart disease	ICAM-1	intracellular adhesion molecules 1
CI	confidence interval	IDL	intermediate-density lipoprotein
CM	chylomicron	IHD	ischaemic heart disease
CO	cardiac output	IKK	inhibitor of κ kinase
CRP	C-reactive protein	IL	interleukin
CT	computer topography	IR	insulin resistance
CtE	catechin equivalents	IRS-1	insulin receptor substrate 1
CV	coefficient of variation	ISAK	International Society for the Advancement of Kinanthropometry
CVD	cardiovascular disease	ISH	isolated systolic hypertension
DALY	disability-adjusted life year	JNK	c-jun N-terminal kinase

LCAT	lecithin cholesterol acyltransferase	PLTP	phospholipid transfer protein
LDL	low-density lipoprotein	PON1	paraoxonase/arylesterase 1
LDL-C	low-density lipoprotein cholesterol	PR	peripheral resistance
LDLR	LDL receptor	PUFA	polyunsaturated fatty acids
LMIC	low- and middle-income countries	PWV	pulse wave velocity
LOX-1	oxidised LDL lecithin-like receptor-1	RAS	renin-angiotensin system
LPL	lipoprotein lipase	RCT	reverse cholesterol transport
LRP	LDL receptor-related protein	ROS	reactive oxygen species
LV	left ventricle	RPAQ	recent physical activity questionnaire
LXR	nuclear receptor liver X receptor	SBP	systolic blood pressure
MANOVA	multivariate analysis of variance	SD	standard deviation
MAP	mean arterial pressure	SE	standard error of the difference between means
MGAT	acyl-coenzyme A: monoacylglycerol acyltransferase	SFA	saturated fatty acids
MHO	metabolically healthy obese	sLDL	small-dense LDL
MONW	metabolically obese, normal weight	SMCs	smooth muscle cells
MTP	microsomal triglyceride transfer protein	SNP	single nucleotide polymorphism
MUFA	monounsaturated fatty acids	SR-A	type A scavenger receptor
MUHEC	Massey University Human Ethics Committee	SR-BI	scavenger receptor class BI
NADPH	nicotinamide adenine dinucleotide phosphate	SV	stroke volume
NCD	non-communicable diseases	TC	total cholesterol
NCEP	National Cholesterol Education Program	TG	triglycerides
NF-κβ	nuclear factor-kappa betta	TGF-β	transforming growth factor-β
NHANES	National Health and Nutrition Examination Survey	TGRL	TG-rich lipoproteins
NHLBI	National Heart, Lung and Blood Institute	T_H1	T helper 1 cells
NLRP3	nucleotide-binding domain leucine-rich repeat containing (NLR) family, pyrin domain containing 3	TLC	Therapeutic lifestyle changes
NO	nitric oxide	TNF-α	tumour necrosis factor alpha
NOX	NADH/NADPH oxidase	TPR	total peripheral resistance
NPC1L1	Niemann-Pick C1-like 1	T_{reg}	regulatory T cells
NZ	New Zealand	UK	United Kingdom
NZEO	NZ European and other	US	United States
O_2^-	superoxide anion	VCAM-1	vascular cell adhesion molecules 1
$ONOO^-$	peroxynitrite	VLDL	very low-density lipoprotein
OECD	Organisation for Economic Co-operation and Development	WC	waist circumference
oxLDL	oxidised LDL	WHO	World Health Organisation
PKR	protein kinase R	WHR	waist/hip ratio

Chapter 1: Introduction

1.1 Introduction

The Framingham Heart Study celebrated 65 years of epidemiological research into cardiovascular disease (CVD) on the 29th of September, 2013. As the longest-running study investigating CVD, it has made significant contributions to our understanding of behavioural and physiological risk factors of CVD over those years, such as identifying in 1977 an inverse association between high-density lipoprotein cholesterol (HDL-C) concentrations and coronary heart disease (CHD) incidence (Mahmood et al., 2013).

Cardiovascular disease is the leading cause of death globally (World Health Organization, 2011). In the developed world, chronic diseases such as CVD replaced infectious diseases as the main cause of death midway through the last century. Now, at the beginning of the 21st century, non-communicable diseases (NCDs) are the most common cause of death in nearly every country (Arsenault et al., 2011, Mendis et al., 2011). These changes have been paralleled by the on-going progression of industrialisation and globalisation around the world, which has seen developing countries sharing with developed countries the benefits of improvements in sanitation and medical advances, but also the increased availability of high-calorie food, and decreased physical activity. (Arsenault et al., 2011, World Health Organisation, 2003).

However, although many consider CVD a modern-day lifestyle disease, atherosclerotic plaques were recently identified in computed tomography (CT) scans of mummies from four pre-industrial populations. The mummies were from four different geographical regions, with different climates and disparate diets. The findings suggest that the presence of atherosclerosis was common even then, and raises the possibility of an inherent component to its development that is independent of diet or lifestyle (Thompson et al., 2013).

In developed countries, such as New Zealand (NZ), prevention and treatment programmes, including ones aimed at decreasing cholesterol levels, blood pressure (BP) and tobacco use, have led to a decline in CVD mortality over the past few decades, but these gains are now threatened by the 'obesity epidemic' (Mendis et al., 2011, Ministry of Health, 2012, Ordovas and Smith, 2010). In NZ, CVD remains one of the leading causes of death and a major public health

concern (Ministry of Health, 2012). In a 2008 Organisation for Economic Co-operation and Development (OECD) Health data report, NZ was ranked first amongst OECD countries for deaths from acute myocardial infarction (Anderson and Markovich, 2010). In response to the report, the New Zealand Heart Foundation medical director, Professor Norman Sharpe, said many of the deaths are 'premature and preventable' and New Zealanders are 'not effective at prevention', with unhealthy lifestyles, obesity and diabetes contributing to this unenviable statistic (Todd, 2010). Subsequently, in a 2011 OCED report, NZ adults were ranked third-highest for obesity rates (OECD, 2011).

As a chronic, complex and multi-factorial disease, CVD due to atherosclerosis takes decades to develop and is influenced by both genetic and environmental factors (Ordovas, 2009). Although our genetics are a non-modifiable risk factor, other identified risk factors, such as elevated serum cholesterol, raised BP, diabetes mellitus and obesity, are modifiable (Mendis et al., 2011, Arsenault et al., 2011). In the most recent cross-sectional study measuring CVD risk factors in Aucklanders, more than 60% of participants, classified as predominantly Europeans, had elevated low-density lipoprotein cholesterol (LDL-C) concentrations (≥3.0 mmol/L), and over 20% had raised BP (systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg) or were on BP-lowering medication (Gentles et al., 2006, Gentles et al., 2007).

Elevated LDL-C is a well-established risk factor, being recognised as a key contributor to the initiation and progression of atherosclerosis (Badimon and Vilahur, 2012, National Cholesterol Education Program (NCEP) Expert Panel, 2002, Dzau et al., 2006). It is also a recognised target for risk reduction in the general population, with statins currently the first-line pharmacological treatment (National Cholesterol Education Program (NCEP) Expert Panel, 2002, New Zealand Guidelines Group, 2012). They have been shown to reduce CVD risk by 20-30%, predominantly through their ability to lower LDL-C, but, even with aggressive use, a residual degree of risk remains. Obesity, physical inactivity and the development of an atherogenic dyslipidaemia have been shown to exacerbate this residual CVD risk (Arsenault et al., 2011, Fruchart et al., 2008).

In contrast to LDL-C, HDL-C is considered to be atheroprotective through its ability to promote cholesterol efflux from cholesterol-laden macrophages. However, under hypertriglyceridaemic conditions, as is commonly associated with obesity, the increased concentration of large very low-density lipoprotein (VLDL) promotes triglyceride (TG) enrichment of both HDL and LDL by cholesteryl ester transfer protein (CETP). This TG enrichment results in increased remodelling of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) into smaller particles by hepatic lipase (HL). This consequently leads to a decrease in plasma HDL-C and an increase in the number of atherogenic small-dense LDL (sLDL) particles (Chapman et al., 2010, Charles and Kane, 2012). To better capture the interactions between various lipid fractions, different ratios have been proposed to improve the accuracy of CVD risk assessment, including total cholesterol (TC)/HDL-C and TG/HDL-C ratios (Millan et al., 2009, Miller et al., 2011).

The recognition of the importance of HDL-C in CVD prevention has seen much research focussed in recent years on strategies to increase HDL-C. However, although the newly emerging CETP inhibitors have been shown to increase HDL-C concentrations, CVD outcomes have not improved, and there have been some deleterious side effects. Therefore, non-pharmacological measures to increase HDL-C concentrations are still the recommended treatment (Badimon and Vilahur, 2012, Fruchart et al., 2008, Wright, 2013).

Lifestyle modification including dietary change is recognised as crucial elements in the prevention of CVD. Fruit and vegetables have long been identified as key elements of a healthy diet (Lichtenstein et al., 2006). Interestingly, there is a distinct lack of evidence to justify a causal relationship between fruit and vegetable intake and CVD prevention, with most evidence derived from observational studies (Bhupathiraju and Tucker, 2011, Dauchet et al., 2009). Despite this, in the 2003 World Health Organisation (WHO) report, fruit and vegetables were rated, along with a few other lifestyle factors, as having the strongest strength of evidence for decreasing CVD risk (World Health Organisation, 2003).

Another approach is to investigate the benefits of fruit and vegetables on cardiovascular risk factors. A number of interventions have shown that increased fruit and vegetable consumption is associated with decreases in BP (Appel et al., 1997, John et al., 2002, Smith-Warner et al., 2000). However, for LDL-C, Dauchet et al. (2009, p605) cite in their review 'the lack of appropriately designed studies has led to a situation where there is little evidence for an effect of fruit and vegetable consumption on LDL-cholesterol levels'. Lastly, although few studies have been conducted, there is some evidence from individual whole fruit studies, with fruit such as berries and grapefruit, showing favourable effects on dyslipidaemia and/or BP (Erlund et al., 2008, Gorinstein et al., 2006).

Fruit are chemically complex foods that contain a range of components, all of which may contribute independently or jointly to cardiovascular health (Badimon et al., 2010, Voutilainen et al., 2006, World Health Organisation, 2003). Many of these components have been investigated individually, including fibre, minerals such as potassium, anti-oxidant vitamins such as vitamin C and E, and some polyphenols, with many showing positive benefits on some CVD risk factors. Although for some components, such as fibre and potassium, the benefits are supported by robust evidence, in most cases further studies are needed to validate the findings and to elucidate mechanisms (Bhupathiraju and Tucker, 2011, Chong et al., 2010, Dauchet et al., 2009).

In NZ, kiwifruit is a commonly consumed, nutrient-dense fruit. Green kiwifruit are notable for containing significant levels of soluble fibre (Schakel et al., 2001), one of the highest concentrations of vitamin C of any readily available fruit, significant amounts of vitamin E, which is more commonly associated with green leafy vegetables than fruit (other than avocados), potassium, folate, carotenoids and polyphenols (Ferguson and Ferguson, 2003, Hunter et al., 2011).

Two previous studies have investigated the effects of kiwifruit on plasma lipids (Chang and Liu, 2009, Duttaroy and Jorgensen, 2004). Neither of the studies included control groups, and they reported conflicting results. Duttaroy & Jorgensen (2004) showed that the consumption of 2 or 3 kiwifruit (cultivar not

specified) per day for 28 days, in healthy subjects, significantly reduced serum plasma TG by 15% compared to baseline values, without affecting cholesterol levels. On the other hand, Chang & Liu (2009) showed that consumption of two green kiwifruit per day for 8 weeks, in hyperlipidaemic subjects, significantly increased HDL-C, leading to associated decreases in the LDL-C/HDL-C and TC/HDL-C ratios.

That many of the studies conducted in this area to date have not been well designed, often with small group sizes, of short intervention duration, and uncontrolled, is a recurring theme in the literature (Chong et al., 2010, Dauchet et al., 2009, Hooper et al., 2008, Wright, 2013). In relation to kiwifruit studies, in addition to being uncontrolled, in both previous studies the kiwifruit were added to the subject's usual diet, and so the specific effects of kiwifruit could not be tested independently of the effects of a healthy diet containing fruit (Chong et al., 2010, Duttaroy and Jorgensen, 2004).

To date, dietary recommendations have been generalised for populations, but it is recognised that there are often large inter-individual variations in response to interventions. A range of potential contributing factors, including genetic (e.g. single nucleotide polymorphisms (SNPs)), physiological (e.g. inflammatory status, gender), environmental (e.g. exercise), and pathological factors (e.g. obesity) having been being identified (Masson et al., 2003, Rideout, 2011).

In the area of lipid and lipoprotein metabolism, over 250 SNPs in about 30 genes encoding key proteins have been identified (Lairon et al., 2009). The most widely researched SNP in this area is apolipoprotein E (*APOE)* (Minihane et al., 2007). Carriers of the *APOE4* genotype are considered to be at increased risk of CVD, however, more recent larger meta-analyses indicate that the increased risk may only be present in some subgroups, such as smokers (Minihane, 2013). Numerous studies have shown that *E4* carriers appear to be the most responsive to reduced saturated fat, total fat, cholesterol and long chain *n*-3 polyunsaturated fatty acid (PUFA) intake (Jofre-Monseny et al., 2008, Minihane et al., 2007). However, there appear to be no published studies that have investigated possible associations between the *APOE* polymorphism and plasma lipid response to a fruit intervention.

It is proposed that obesity initiates the development of a more chronic pro-inflammatory environment, which can be measured by an increase in acute-phase reactants, such as C-reactive protein (CRP) (Gregor and Hotamisligil, 2011, Mathieu et al., 2009). It has been suggested that inflammation (as measured by high-sensitivity C-reactive protein (hs-CRP)), may also affect responsiveness to interventions. This was highlighted in a recent review, where a number of studies that used hs-CRP as a marker of inflammatory status showed that plasma lipid response to dietary saturated fatty acids (SFA) and cholesterol modifications was less in obese individuals than in lean individuals (Flock et al., 2011). No published studies have investigated inter-individual variability in responsiveness to a fruit intervention as a result of differences in inflammation status.

Reduction in CVD is an important public health issue in NZ. The regular inclusion of kiwifruit to a healthy diet has the potential to improve lipid profiles and other CVD-related metabolic risk factors. Although two previous studies have reported favourable responses on plasma lipids, as the kiwifruit was added to the subject's normal diet, it cannot be ruled out that the effects were due to the addition of fruit to the diet, rather than kiwifruit specifically. Conducting a randomised controlled cross-over study, where subjects replace two of their normal fruit servings with two green kiwifruit, will effectively test if kiwifruit have additional benefits to a healthy diet containing fruit. Whilst the goal of personalised dietary recommendations is still somewhere in the future, investigating diet-gene and inflammatory status interactions that may contribute to the inter-individual variations in response seen in interventions may identify groups that would particularly benefit, should kiwifruit consumption be shown to improve plasma lipid profiles.

1.2 Aims and objectives

The aim of this research is to investigate the effects of consuming green kiwifruit as part of a healthy diet on plasma lipids in a group of men with hypercholesterolaemia.

1.2.1 Primary objective

To investigate the effects of consuming two green kiwifruit per day as part of a healthy diet compared to a healthy diet without green kiwifruit for four weeks on lipid profiles including plasma TC, LDL-C, TG, HDL-C, TC/HDL-C ratio, sLDL, serum apolipoprotein (apo)A1 and apoB in hypercholesterolaemic men.

1.2.2 Secondary objectives

To investigate:

1. The effects of the kiwifruit intervention on other CVD-related metabolic markers namely glucose, insulin, and CRP.

2. Whether the effects of the kiwifruit intervention on lipid profiles is modulated by *APOE* genotype by comparing the effects of the intervention between *APOE4* carriers and non-*E4* carriers.

3. Possible interactions between inflammatory status, as measured by hs-CRP and the impact of treatment on plasma lipid profiles and markers of inflammatory status (hs-CRP, interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and IL-10).

4. If selected gene polymorphisms related to lipid metabolism (based on the primary outcome results) modulate the plasma lipid response to intervention.

5. The effects of the kiwifruit intervention on BP and markers of cardiovascular function (using automated oscillometric and finometer devices).

1.3 Hypotheses

Primary objective hypothesis

Hypothesis: The replacement of two fruit servings in a healthy diet with two green kiwifruit will have favourable effects on plasma lipids.

Secondary objective hypotheses

Hypothesis 1: The replacement of two fruit servings in a healthy diet with two green kiwifruit will have favourable effects on other CVD-related markers of risk.

Hypothesis 2: Carriers of different *APOE* genotypes will show differences in response to the intervention.

Hypothesis 3: Subjects with different inflammatory statuses will show differences in response to the intervention.

Hypothesis 4: Carriers of the selected lipid-related polymorphisms (based on the primary results) will show differences in response to the intervention.

Hypothesis 5: The replacement of two fruit servings in a healthy diet with two green kiwifruit will have favourable effects on BP and other markers of cardiovascular function.

1.4 Structure

This thesis begins with a review of the literature (Chapter 2), starting with our current understanding of the pathophysiology of CVD, due to atherosclerosis. A discussion follows on the extent of the problem, both globally and in NZ, before the role of relevant risk factors are discussed. Finally, the role of diet in CVD prevention are investigated and causes of inter-individual variability to interventions considered.

Next, Chapter 3 summarises the overall methodology of the study, followed by four manuscripts presenting the results of this research. Each manuscript is presented in a form suitable for publication, although to avoid repetition as much as practical, all general methodology has been removed.

The primary results from the randomised controlled trial, showing the effect of consuming two green kiwifruit daily in conjunction with a healthy diet on plasma lipids and other metabolic markers, and whether the response was modulated according to *APOE* genotype, are reported in Chapter 4. Chapter 5 contains the results of one of the predefined secondary objectives of the randomised kiwifruit trial, which investigated if inflammatory status, as measured by CRP, would modulate the effect of consuming two green kiwifruit daily on plasma lipids and markers of inflammation. This is followed by Chapter 6, where, based on the primary results, other selected gene polymorphisms were investigated to see if they also modulated response to the intervention. Finally, in Chapter 7 the results of the trial on BP and other markers of cardiovascular function, as measured by the Finometer MIDI® is reported

The thesis is concluded in Chapter 8 with a discussion which brings together the main results observed, including their significance and relevance, and methodological strengths and limitations. Final conclusions are drawn, bringing the thesis to a close.

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Chapter 2: Literature Review

2.1 Introduction

Cardiovascular disease first emerged as a major public health issue in the 1940s. Despite the decline in CVD mortality in many countries, including NZ, over the past 40 to 50 years, the disease, which primarily arises from atherosclerosis, remains a leading cause of death in many developed countries and is the overall leading cause of death globally (Ministry of Health, 2012b, Ordovas and Smith, 2010, World Health Organization, 2011a). The burden of CVD, both in the global and NZ contexts, and how the current rise in obesity rates is threatening the gains that have been made will be examined in this literature review.

Firstly, our current understanding of the causes and pathophysiology of atherosclerosis will be investigated. Cardiovascular disease due to atherosclerosis is recognised as a chronic, complex and multi-factorial disease, which takes decades to develop and is influenced by both genetic and environmental factors. Three modifiable CVD risk factors, dyslipidaemia, raised BP, and obesity will be examined, along with some of the inter-relationships between the three.

Once considered a male-related disease, CVD in fact affects as many women as men. The main gender difference is in age of presentation; CVD generally presents 7 to 10 years later in women than in men, and as a result fewer years of life are lost (Mendis et al., 2011). Certainly, differences in lipid kinetics between the genders have been demonstrated; pre-menopausal women, for example, have a less pro-atherogenic lipid profile than men. Although the mechanisms to explain these differences remain unclear, they likely involve multiple factors, including the influence of sex hormones (Ordovas and Smith, 2010, Wang et al., 2011). To minimise the influence of such compounding factors, men were chosen as the subject group for this study, and this review will focus primarily on the impact of CVD on men.

Although an individual's genetic profile is a non-modifiable risk factor, an understanding of the interactions between genes and the environment may in future lead to more personalised dietary recommendations that could reduce risk in susceptible individuals. A selection of SNPs, whose resultant genotypes,

have been shown to have differential effects on plasma lipids will therefore be considered. The second part of the review will examine how diet can reduce CVD risk, by improving lipid profiles and reducing BP and inflammation, but also how carrying different alleles may explain some of the variability that is observed between individuals consuming various dietary components.

Diet modification is recognised as a crucial element in the prevention of CVD, with fruit and vegetables identified as key elements of a healthy diet. The evidence supporting the position of fruit in a cardioprotective diet will be reviewed, before examining green kiwifruit's unique composition and the results from intervention studies to date.

Finally, current dietary recommendations have been generalised for populations, but it is recognised that there is often large inter-individual variability in response to interventions. Besides genetic variation, other factors have also been shown to contribute, such as inflammatory status, which may be of particular relevance in today's obesogenic environment, and will also be explored.

2.2 Cardiovascular disease

Cardiovascular disease refers to a range of diseases of the heart and blood vessels, and to vascular diseases of the brain. Most commonly it refers to diseases that result from atherosclerosis, which include ischaemic heart disease (IHD) or coronary artery disease, cerebrovascular disease and diseases of the aorta and arteries, such as peripheral vascular disease and hypertension. Sometimes the term may refer to diseases from other causes, such as congenital heart disease, cardiac arrhythmias, rheumatic heart disease and cardiomyopathies (Mendis et al., 2011). Unless otherwise specified, only CVD due to atherosclerosis will be discussed in this review.

At the beginning of the 20th century, infectious diseases were the main public health concern. During that century, as mortality rates decreased with improvements in sanitation and medical advances, such as the introduction of penicillin, non-infectious (chronic) diseases took over as the principle cause of death in developed countries (Arsenault et al., 2011, National Heart Lung and Blood Institute and Boston University, 2012). In the United States (US), heart disease was identified as becoming one of the leading causes of death as early as 1924 (Heffron, 1924), but it wasn't until after World War II that the Public Health Service made it one of its central focuses, with the establishment of the National Heart Institute (now National Heart, Lung and Blood Institute (NHLBI)) (Oppenheimer et al., 2012). Little was known about its epidemiology or pathophysiology; it appeared to mainly affect men and was considered a consequence of aging (Arsenault et al., 2011). In the 1930s, it had been identified that very high levels of serum cholesterol, as seen in patients with xanthomatosis, was a likely contributor to coronary artery disease, but for those with lower levels it was largely dismissed (Stamford and Moffatt, 2006). Joseph Mountin, the founder of the Communicable Disease Center (later the Centers for Disease Control and Prevention (CDC)), advocated applying the same programs that had been developed for infectious diseases, including an epidemiological component, to chronic diseases. This culminated in the initiation of one of the premier longitudinal studies of our time, the Framingham Heart Study (National Heart Lung and Blood Institute and Boston University,

2012, Oppenheimer et al., 2012). This study was key to raising serum cholesterol status to that of a CVD risk factor (Stamford and Moffatt, 2006).

The original Framingham Heart Study cohort was a random sample of two-thirds of the adult population of Framingham, Massachusetts, consisting of 5,209 respondents, 30 to 62 years of age, who were recruited between 1948 and 1952. The study continues today with the enrolment of the grandchildren of the original cohort (3rd generation) (National Heart Lung and Blood Institute and Boston University, 2012). Over the years, in addition to identifying elevated serum cholesterol as a risk factor, a number of other risk factors, including male sex, age, hypertension, smoking status and diabetes mellitus have been identified through this study. These risk factors have formed the basis of various risk assessment algorithms which are still used today (Arsenault et al., 2011, National Heart Lung and Blood Institute and Boston University, 2012).

Before further examining the contribution of some of these risk factors to CVD and detailing the extent of the global problem of CVD, the consequences and pathogenesis of atherosclerosis will first be discussed.

2.2.1 Atherosclerosis

Atherosclerosis is a disease characterised by the thickening of arterial walls. Once believed to result from the passive accumulation of cholesterol in vessel walls, it is now considered to be a chronic inflammatory disease with a far more complex pathogenesis, which appears to involve dysregulation of both lipid metabolism and immune responses (Weber and Noels, 2011, Hansson and Hermansson, 2011).

A slowly progressing disorder (developing over decades), it is primarily seen in the large and medium-sized muscular elastic arteries, like the aorta, carotid, femoral and coronary arteries, at sites of disturbed laminar flow, such as arterial tree branch points and inner areas of curvature. These areas tend to have low or oscillatory shear stress (Radar and Hobbs, 2010, Vohnout et al., 2011, Weber and Noels, 2011). The disease process involves the formation of atherosclerotic plaques (Figure 2.1), and can result in a range of clinical manifestations.

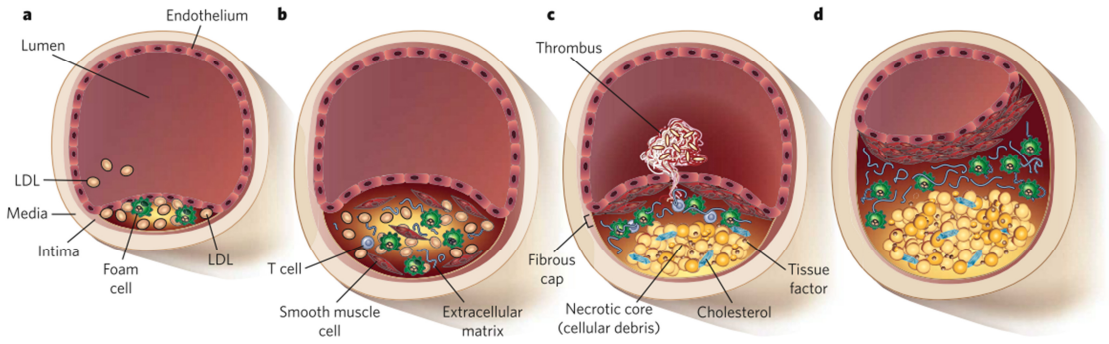

Figure 2.1: Atherosclerosis progression: from atherosclerotic plaque formation through to significant obstruction of the lumen
LDL: low-density lipoprotein
Reprinted from Nature, 451 (7181), Rader, D. J. & Daugherty, A., Translating molecular discoveries into new therapies for atherosclerosis, 904-13 © (2008) with permission Macmillan Publishers Ltd.

As a result of luminal narrowing, blood flow can be reduced, contributing to localised ischaemia, with the heart and brain the most commonly affected organs. In coronary arteries this can manifest as angina pectoris. Alternatively, a plaque can rupture and the resultant thrombus can block blood flow, and cause a myocardial infarction or, if in the brain, a stroke (Hansson and Hermansson, 2011, Rader and Daugherty, 2008).

2.2.1.1 Pathogenesis

Despite marked advances in our understanding of the pathophysiology of atherosclerosis, gaps remain and many aspects remain poorly understood. Two recent reviews highlight the complexity of the pathogenesis of atherosclerosis (Libby et al., 2011, Rader and Daugherty, 2008). Libby et al. (2011, p317) even asserts that 'we still lack definitive evidence to show that processes such as lipoprotein oxidation, inflammation and immunity have a crucial involvement in human atherosclerosis' (Libby et al., 2011). However, according to our current understanding, the steps involved in the initiation and progress of atherosclerotic lesions is presented in Figure 2.2.

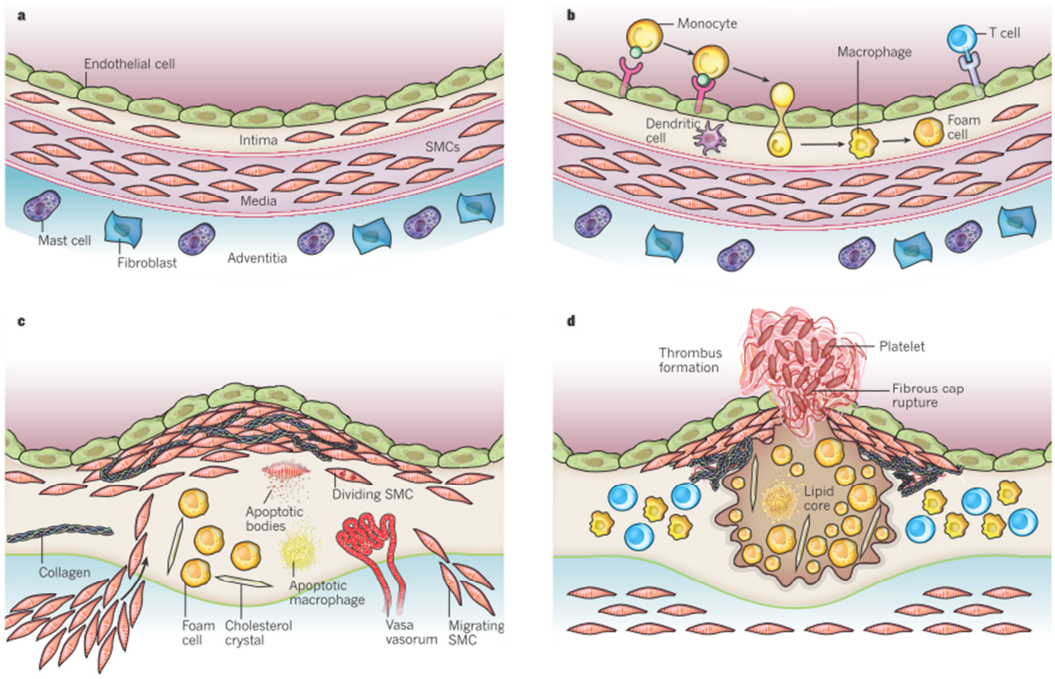

Figure 2.2: Stages in the development of atherosclerotic lesions
SMC: smooth muscle cell
Reprinted from Nature, 473 (7347), Libby, P., Ridker, P. M. & Hansson, G. K., Progress and challenges in translating the biology of atherosclerosis, 317-25 © (2011) with permission Macmillan Publishers Ltd.

a) The normal 'healthy' muscular artery contains three layers. The inner layer, the tunica intima, is covered with a continuous single-cell lining called the endothelium. The main function of this layer had initially been thought to simply be a wall that separated the vessel wall from the inside cavity. However, today it is recognised as the largest endocrine organ in the body, with roles in maintaining vascular tone and structure, regulating growth, inflammation, vasodilation, coagulation, thrombosis and oxidative status. The other two layers are the tunica media (middle layer), which contains layers of elastin and smooth muscle cells (SMCs), and the adventia (outer layer), which contains microvessels, nerve endings and mast cells (Higashi et al., 2012, Libby et al., 2011, Vohnout et al., 2011).

b) The disruption to the endothelium's functions (endothelial dysfunction) and the recruitment of inflammatory cells into the intima are key steps in the initiation of atherosclerosis. Healthy endothelial cells normally resist attachment of leukocytes when they flow past. However, when repeatedly exposed to injuring stimuli, such as hypertension, dyslipidaemia or pro-inflammatory mediators, adhesion molecules that capture leukocytes are expressed.

Selectins P & E initially tether monocytes in a low affinity interaction, then vascular cell adhesion molecules (VCAM)-1 and intracellular adhesion molecules (ICAM)-1 mediate a firmer attachment. Once attached, monocytes migrate by diapedesis into the sub-endothelial space, where they differentiate into macrophages. At the same time, changes in endothelial permeability promote the entry and retention of atherogenic lipoproteins, such as LDL, in the intima. These particles are prone to oxidative modifications, making them more susceptible to phagocytosis by macrophages, which lead to the generation of lipid laden foam cells. These cells produce reactive oxygen species (ROS), pro-inflammatory cytokines, including IL-1 and TNF-α, and other inflammatory mediators that amplify the local inflammation. Following antigen-specific activation, T cells can also enter the intima. The T helper 1 (T_H1) cells, help sustain the inflammatory response, while regulatory T (T_{reg}) cells produce two cytokines, with anti-inflammatory actions, namely transforming growth factor-β (TGF-β) and IL-10. Fatty streaks, an accumulation of foam cells and other immune cells, such as T cells, are considered an initial lesion stage that leads to the development of more advanced lesions (Hansson and Hermansson, 2011, Libby et al., 2011, Rader and Daugherty, 2008, Vohnout et al., 2011).

c) Lesion progression involves the recruitment of SMCs from the tunica media (middle layer of the artery wall) to the intima. In the intima SMCs proliferate and produce extracellular matrix macromolecules, such as collagen and elastin, leading to the development of a fibrous cap. Over time a lipid or necrotic core forms underneath the cap, made up of dead and dying cells, extracellular lipid and cholesterol derived from apoptotic macrophages, and other cellular debris. Other morphological changes include new microvessel formation and compensatory remodelling of the vessel (Figure 2.1 and Figure 2.2), to preserve the size of the lumen (Libby et al., 2011, Rader and Daugherty, 2008, Vohnout et al., 2011).

d) Finally, the plaque can rupture or the endothelium can erode, exposing thrombogenic material in the core, to coagulation proteins in the blood, triggering thrombus formation. The thrombus may either extend into the lumen and impede blood flow, or detach to become an embolus. Plaque ruptures tend to occur at the shoulder region, typically in lesions with thin, collagen-poor

fibrous caps, which contain few SMCs but lots of macrophages. Infiltrating inflammatory cells are implicated in plaque disruption, by producing pro-inflammatory mediators and enzymes that induce SMC death (reducing collagen synthesis) and cause collagen and matrix degradation (Libby et al., 2011, Rader and Daugherty, 2008, Vohnout et al., 2011).

Recent *in vitro* and animal model studies propose that minute cholesterol crystals, which have been identified as being present in early lesions, induce NLRP3 (nucleotide-binding domain leucine-rich repeat containing (NLR) family, pyrin domain containing 3) inflammasome activation in macrophages, leading to secretion of IL-1β, a potent pro-atherogenic cytokine. There is a suggestion that this may represent a critical link between cholesterol metabolism and inflammation in the development of atherosclerotic lesions (Duewell et al., 2010, Rajamaki et al., 2010).

2.2.1.2 Risk factors

In addition to the risk factors already mentioned, a number of other risk factors have been identified that promote the process of atherosclerosis. These can be grouped as metabolic risk factors, which act directly; behavioural risk factors, which tend (except for smoking) to act more indirectly by leading to the development of metabolic risk factors; and other risk factors, which have the potential, either directly or indirectly, to amplify or reduce risk. These include the non-modifiable risk factors including age, gender and genetics; and other influencing factors, such as poverty and psychological factors that, although more removed from the process, can have a significant impact on other risk factors (Figure 2.3) (Mendis et al., 2011).

An individual's total risk is increased by the co-existence of a number of risk factors, as many act synergistically. The WHO estimates that eight risk factors (high BP, elevated cholesterol, high blood glucose, alcohol use, tobacco use, high body mass index (BMI), low fruit and vegetable intake and physical inactivity) account for more than 75% of deaths from ischaemic and hypertensive heart disease globally (World Health Organisation, 2009).

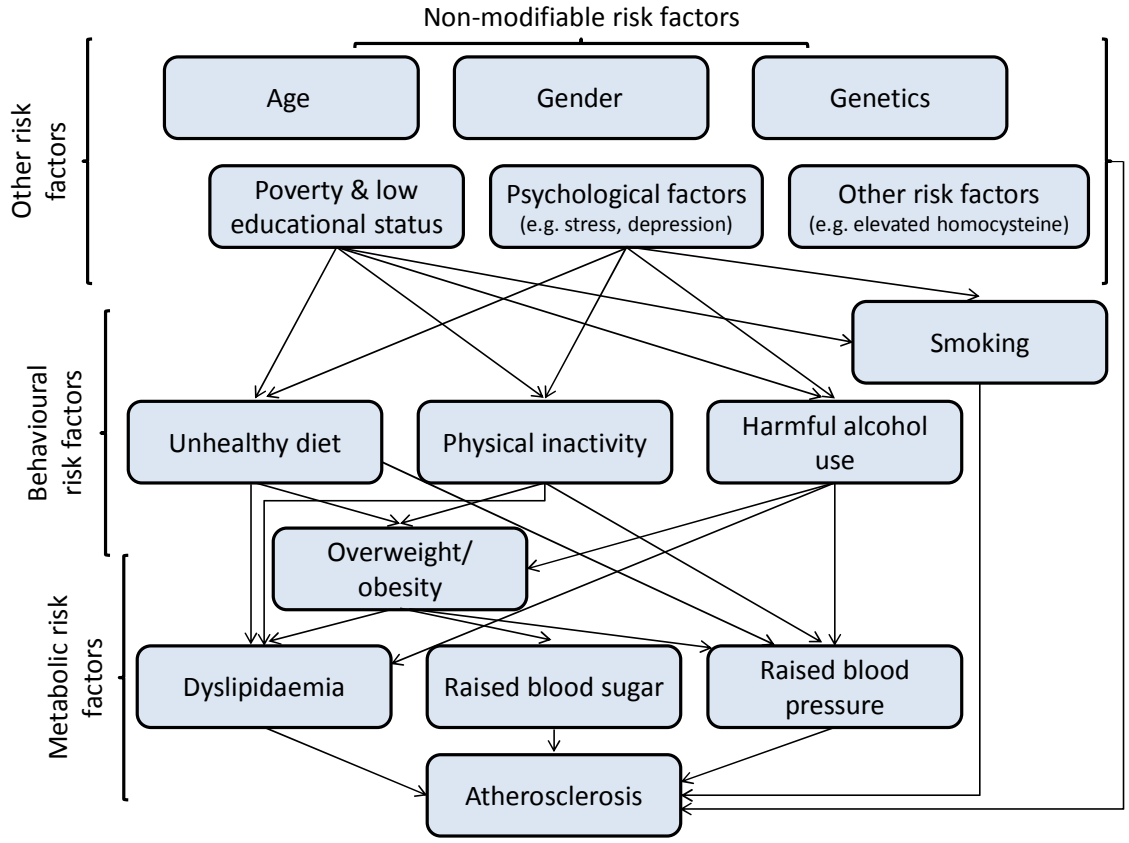

Figure 2.3: Risk factors for atherosclerosis
Figure created with data from Mendis et al. (2011).

2.2.1.3 Role of endothelial shear stress

Despite the whole vasculature being exposed to the atherogenic effects of the risk factors, certain regions (sites of disturbed laminar flow) are at increased risk of lesion development as a result of local hemodynamic forces. Chatzizisis et al. (2007) in their review illustrate the proposed role of low endothelial shear stress (ESS; the frictional force exerted on the vascular wall) on the stages of atherogenesis (Figure 2.4). Mechanoreceptors trigger intracellular pathways that modulate gene expression, upregulating pro-atherogenic genes (many via nuclear factor-kappa betta (NF-κβ) activation) and suppressing atheroprotective genes in areas of ESS, shifting endothelial structure and function towards an atherosclerotic phenotype. The resultant increased production of ROS and the decreased levels of nitric oxide (NO) appear to be fundamental to the development of endothelial dysfunction.

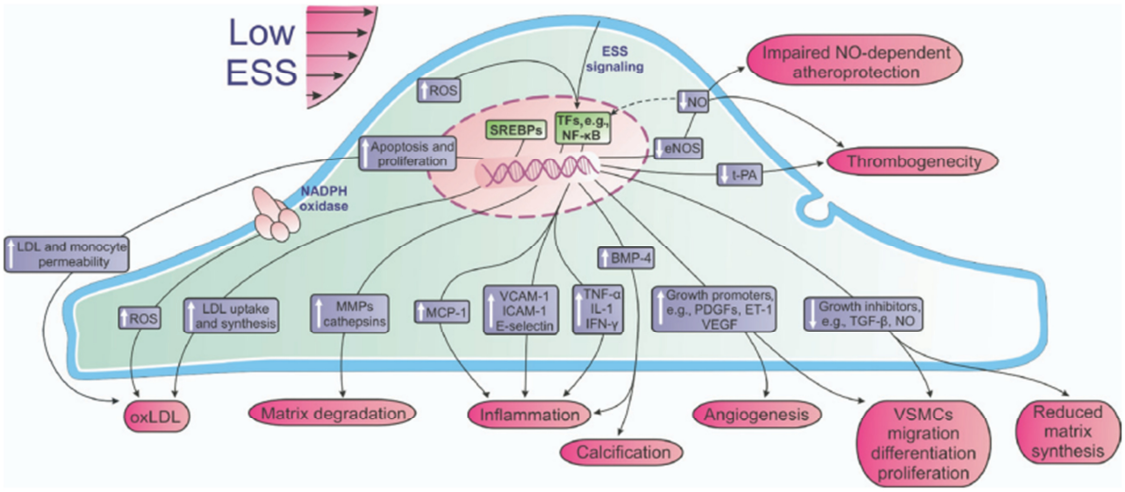

Figure 2.4: Proposed role of low shear stress in atherosclerosis
BMP: bone morphogenic protein; eNOS: endothelial nitric oxide synthase; ESS: endothelial shear stress; ET: endothelin; ICAM-1: intercellular adhesion molecule 1; IFN: interferon; IL: interleukin; LDL: low-density lipoprotein cholesterol; MCP: monocyte chemoattractant protein; MMP: matrix metalloproteinase; NADPH: nicotinamide adenine dinucleotide phosphate; NF-κβ: nuclear factor-kappa beta; NO nitric oxide; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; SREBP: sterol regulatory elements binding protein; TF: transcription factor; TGF: transforming growth factor; TNF: tumor necrosis factor; t-PA: tissue plasminogen activator; VCAM-1: vascular cell adhesion molecule 1; VEGF: vascular endothelial growth factor; VSMC: vascular smooth muscle cell
Reprinted from J Am Coll Cardiol, 49 (25), Chatzizisis, Y. S., Coskun, A. U., Jonas, M., Edelman, E. R., Feldman, C. L. & Stone, P. H., Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behaviour, 2379-93, © (2007) with permission from Elsevier.

2.2.1.4 Nitric oxide, oxidative stress and endothelial dysfunction

Oxidative stress and endothelial dysfunction are recognised as common features of all of the metabolic risk factors such as dyslipidaemia, obesity, elevated BP and blood sugar, and other factors such as cigarette smoking and age. Oxidative stress develops when the generation of ROS (such as free oxygen radicals, oxygen ions, and peroxides), exceeds the capability of the anti-oxidant defence systems to deal with them (Dzau et al., 2006, Forstermann, 2008, Higashi et al., 2012, Vohnout et al., 2011). As the main regulator of vascular homeostasis, endothelial cells secrete a range of vasoactive substances, of which possibly the most important is the vasoprotective molecule, NO. A consequence of oxidative stress is a reduction in NO bioavailability; this appears to be a crucial element in the development of endothelial dysfunction (Dzau et al., 2006, Forstermann, 2010, Napoli and Ignarro, 2011).

Many mechanisms have been proposed, both for the production of ROS and in the reduction of NO, but most are based only on experimental animal and cell

studies, and may not translate to humans. A selection, primarily relevant to hypercholesterolaemia, hypertension and inflammation, will now be discussed.

Endothelium derived NO is a paracrine factor, produced by the endothelial enzyme nitric oxide synthase (eNOS). In addition to its role in BP homeostasis, and regulating vascular tone, NO mediates the prevention of leukocyte adhesion, limits oxidative modification of LDL-C, reduces vascular smooth muscle proliferation, inhibits platelet aggregation and adhesion, and decreases the expression of pro-inflammatory genes in the vasculature (Forstermann, 2010, Sudano et al., 2011).

The arterial vasculature normally exists in a vasoconstricted state, referred to as 'vascular tone'. In response to shear stress the healthy endothelium initiates NO production by causing endothelial calcium channels to open, promoting the calcium dependent activation of endothelial eNOS, which catalyses the production of NO from L-arginine. In the maintenance of normal BP, NO then diffuses to the underlying vascular smooth muscle cells, where it activates the enzyme guanylate cyclase, and increases intracellular cyclic guanosine monophosphate (cGMP) concentrations, leading to relaxation of vascular tone (Giles et al., 2012, Higashi et al., 2012, Sudano et al., 2011).

Where NO exerts vasoprotective effects on vasculature, angiotensin (Ang) II (better known for its role in regulation of salt and water homeostasis) exerts vasoconstrictive effects, via the Ang II type 1 (AT_1)-receptor. The metabolic pathways of the two are closely interlinked, with the balance between them appearing to be crucial to the homeostasis of vascular tone (Schulman et al., 2006). Like NO, the final site of Ang II synthesis is the endothelium, where angiotensin converting enzyme (ACE) converts Ang I to Ang II. Both can inhibit the production of the other, with Ang II regulating the expression of eNOS and NO inhibiting ACE activity and down regulating the AT_1-receptor (Schulman et al., 2006). Angiotensin II, via the AT_1-receptor is also one of the main sources of ROS, stimulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate superoxide anions (O_2^-**)** in the vessel's endothelium, smooth muscle cells, and adventitia (Higashi et al., 2012, Schulman et al., 2006, Sudano et al., 2011).

Experimental work has demonstrated interactions between hypercholesterolaemia and the renin-angiotensin system (RAS). Hypercholesterolaemia has been shown to increase AT_1-receptor density and functional responsiveness to Ang II, and to increase plasma angiotensinogen (Ang I precursor) and Ang peptide concentrations, predominantly Ang II (Daugherty et al., 2004, Sitia et al., 2010). A recent human study showed that the incidence of new onset hypertension was greater in subjects who had hypercholesterolaemia and high plasma renin activity (Borghi et al., 2007).

Other enzyme systems that have been implicated in the production of ROS are xanthine oxidase, enzymes in the mitochondrial respiratory chain and eNOS itself. There is evidence that NADPH oxidase derived ROS (O_2^-) combines with NO to form peroxynitrite ($ONOO^-$), which can then oxidize tetrahydrobiopterin (BH_4) (an essential co-factor of eNOS) and trigger eNOS uncoupling (oxygen reduction is uncoupled from NO synthesis). The outcome is the creation of a dysfunctional superoxide generating enzyme. Endothelial NO synthase uncoupling has been observed in smokers and subjects with endothelial dysfunction as a result of hypercholesterolaemia, hypertension and diabetes mellitus (Forstermann, 2008, Forstermann, 2010).

It has also been shown that ROS can inhibit NO production, by inhibiting eNOS mRNA expression (Higashi et al., 2012). Some cell studies have implicated a direct role for CRP in attenuating NO production, through its effects on eNOS expression (Chen et al., 2012, Verma et al., 2002).

Finally, asymmetrical dimethylarginine (ADMA), a naturally occurring L-arginine analogue, is an endogenous inhibitor of eNOS. Cell studies suggest that oxidised LDL (oxLDL) may inhibit the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) leading to increased ADMA concentrations, which further enhances oxidative stress by inhibiting NO synthesis and possibly also uncoupling eNOS. It has also been reported that ADMA may upregulate the expression of oxLDL lecithin-like receptor-1 (LOX-1, receptor for oxLDL in endothelial cells) (Forstermann, 2010, Siroen et al., 2006, Sitia et al., 2010). Asymmetrical dimethylarginine may activate RAS (Veresh et al., 2008).

2.2.1.5 Linking oxidative stress with atherogenesis

A review of the current knowledge of the pathophysiology of CVD (Dzau et al., 2006, p2851) describes a 'pathophysiological continuum', which includes 'oxidative stress, endothelial dysfunction, inflammatory processes, and vascular remodelling in the initiation and continuation of atherosclerotic disease'.

To recap, CVD risk factors activate ROS producing enzyme systems, via various pathways (for which many the mechanisms remain unclear). This increase is countered by the ROS detoxifying systems but, when an imbalance occurs in favour of ROS, vascular oxidative stress results.

The altered redox state activates redox-sensitive transcription factors (such as NF-κβ), which upregulate the expression of pro-inflammatory genes, resulting in increased levels of adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, and pro-inflammatory cytokines. Reactive oxygen species can also activate signalling cascades, such as mitogen-activated protein kinases, which stimulate SMC proliferation (Forstermann, 2008).

Interestingly, in his review, Forstermann (2008, p345) notes that 'despite major evidence in favor of the above hypothesis, the possibility remains that inflammation is the primary process of atherogenesis and oxidative stress the detrimental secondary response'.

In summary, atherosclerosis is a highly complex, slowly progressing, chronic inflammatory disease of the arterial walls. Disturbances of regulatory mechanisms involved in lipid metabolism and immune responses appear to be responsible for initiating the atherogenic process. Although many advances have been made in our understanding of its pathogenesis, a full understanding of the underlying mechanisms is still somewhere in the future. Many of the currently known mechanisms are based on cellular and animal models, which need to be translated and confirmed in humans.

In the next section, the extent of the disease burden on public health systems around the world will be discussed.

2.2.2 CVD as a public health issue and recent trends

The global burden of CVD will first be examined, and trends in incidence and the prevalence of various risk factors discussed, followed by an exploration of the current situation in NZ.

2.2.2.1 Global picture of CVD

In 2011 the WHO published the Global Atlas on cardiovascular disease prevention and control, based on statistics from 2008. Of the 17.3 million cardiovascular deaths, heart attacks and strokes reported, atherosclerotic disease was responsible for 78% of all CVD deaths (80% in males, Figure 2.5) (Mendis et al., 2011).

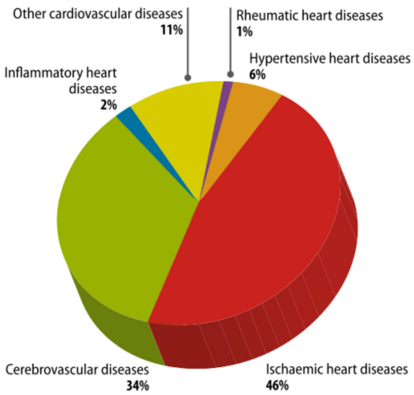

Figure 2.5: Distribution of CVD due to heart attacks, strokes and other cardiovascular diseases in males

Reprinted from Global Atlas on Cardiovascular Disease Prevention and Control, Mendis, S., Puska, P. & Norrving, B. (eds.), © (2011) with permission from World Health Organisation.

Another measure of disease burden is the disability-adjusted life year (DALY) which is the estimated number of years lost due to ill-health, disability or premature death (Mendis et al., 2011). In 2008, CHD and cerebrovascular disease accounted for 109,178 million DALYs (over 7% of the global disease burden) (Mendis et al., 2011).

In addition to being a major contributor to global morbidity and mortality, another concern is the increasing inequalities in the incidence and outcome of CVDs between countries (and population subgroups within countries). In countries classified as high-income by the WHO, mortality rates have declined over the past few decades through a combination of prevention and treatment strategies that have reduced incidence rates and/or improved survival rates

following a cardiovascular event. In contrast, low- and middle-income countries (LMIC) have seen an alarming increase in mortality rates from CVD. Non-communicable diseases are now the most common cause of death in nearly all countries, except in Africa. Over 80% of deaths from CVD and diabetes now occur in these countries (Mendis et al., 2011). Figure 2.6 shows the global distribution of IHD mortality rates.

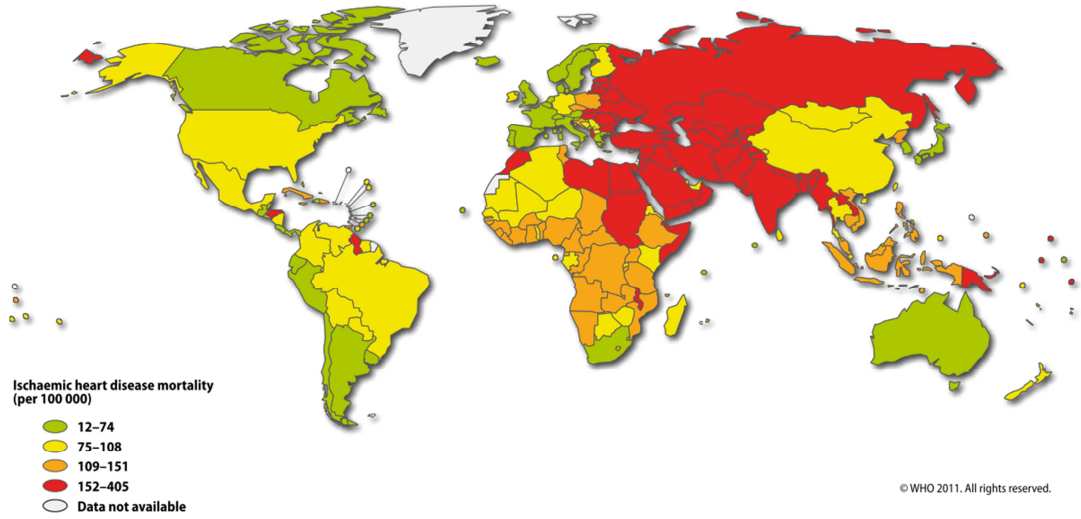

Figure 2.6: Global distribution of ischaemic heart disease mortality rates (age standardised, per 100,000)
Reprinted from Global Atlas on Cardiovascular Disease Prevention and Control, Mendis, S., Puska, P. & Norrving, B. (eds.), © (2011) with permission from World Health Organisation.

Figure 2.6 also illustrates the west-east gradient that occurs across Europe. Mortality rates for CVD have declined across most of Western Europe since 1980. The mortality rate in the United Kingdom (UK), for men between 1996 and 2006 fell by 38%, with only Ireland, Norway and Austria showing larger decreases. In contrast, between 1996 and 2006 Russia (LMIC European region, upper middle income country globally) and the Ukraine (LMIC), following the dissolution of the Soviet Union in 1991, had increases in CVD mortality rates (British Heart Foundation Statistics Database, 2010, Deckert et al., 2010, World Health Organisation, 2008). This has partly been attributed to the increase in stress seen following the political changes during this period, which resulted in considerable social, economic, and lifestyle changes. In addition, both have high rates of smoking (2008 estimated prevalence in males, 65.5% Russia, 58.8% Ukraine) and alcohol consumption (Deckert et al., 2010, World Health Organisation, 2011b).

In comparison, in the UK the estimated prevalence of smoking in males was only 18.5% in 2008 (World Health Organisation, 2011b). Reduction in smoking prevalence between 1981 (39%) to 2000 (28%) was identified as the biggest contributor to the 62% decline in CHD mortality that was seen between 1981 and 2000 in men in England and Wales (Unal et al., 2004, Unal et al., 2005). Using modelling to determine how much of the decline could be attributed to population risk factor reductions or individual medical and surgical treatments (such as the initial treatment of acute myocardial infarctions, and secondary prevention including pharmacological and surgical treatments). It was found that 58% could be attributed to changes in the major risk factors, with 48.1% from smoking reduction. Decreases in TC concentration (9.6%), BP (9.5%) and socioeconomic deprivation (3.4%) also contributed to the decline, though adverse trends for diabetes (-4.7%), physical activity (-4.3) and obesity (-3.4%) cancelled out some of the gains (Unal et al., 2004).

In many parts of the world, obesity is now considered to be reaching epidemic proportions, with the WHO describing obesity as one of today's most blatantly visible public health issues. In 2008 34% of adults (33.6% of men and 35% of women) were classified as overweight, with a BMI greater than or equal to 25 kg/m^2. Figure 2.7 shows the prevalence of overweight in men globally.

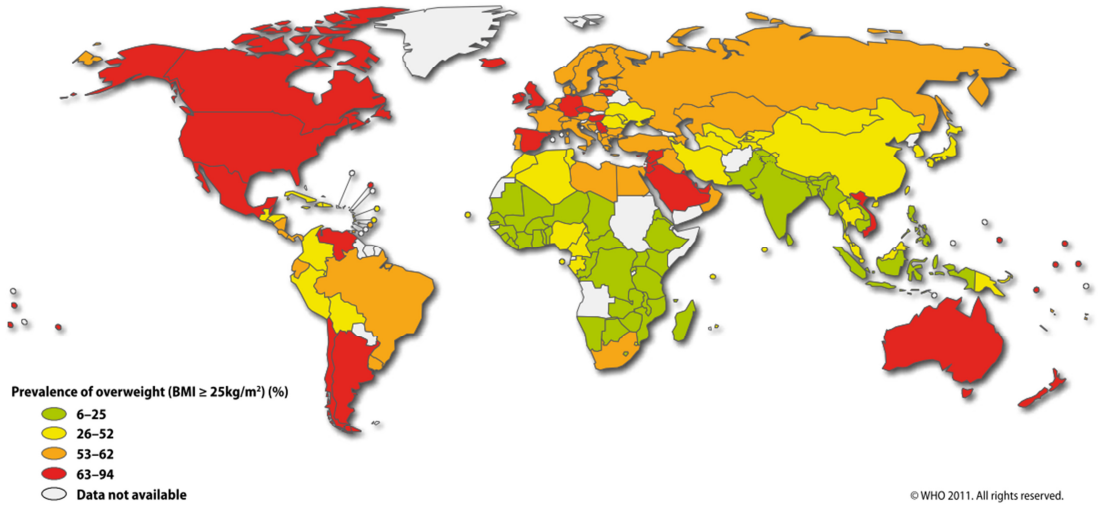

Figure 2.7: Global prevalence of overweight
(BMI≥25kg/m^2) in males (age 20+, age standardised)
Reprinted from Global Atlas on Cardiovascular Disease Prevention and Control, Mendis, S., Puska, P. & Norrving, B. (eds.), © (2011) with permission from World Health Organisation.

Between 1980 and 2008 the number of men that were classified as obese (BMI greater than or equal to 30 kg/m^2) rose from 4.8% to 9.8% (Mendis et al., 2011). Obesity is strongly related to all of the metabolic cardiovascular risk factors (raised BP, dyslipidaemia and raised blood glucose) (Perez Perez et al., 2007, Van Gaal et al., 2006).

The WHO groups countries by income and by region to compare CVD related statistics. Table 2.1 shows the statistics for men from some representative countries in 2008 (reported by WHO in 2011), with at least one country from each region represented. As a group, high-income and upper-middle-income countries have more than double the prevalence of overweight than low- and lower-middle-income countries (Mendis et al., 2011). The exception in Table 2.1 is Tonga, whose rates for both overweight and obesity far exceed that of any other country in the table. Interestingly, within high-income countries, such as the US and UK, there is an inverse relationship between socioeconomic status and obesity prevalence (Mendis et al., 2011).

Comparing the statistics for the US, UK and Australia, whom NZ most commonly compares itself with, NZ has the highest percentage of deaths from all causes of CVD. Although the prevalence of the metabolic factors, are all slightly higher (except elevated BP) in NZ males, compared to Australian males, the more marked differences appear to arise from differences with behavioural risk factors, with NZ males having significantly higher rates of smoking and physical inactivity. United States males have the lowest rates of elevated cholesterol and BP, but a higher rate of elevated blood glucose, which is a likely reflection of the higher overweight/obesity rates. Likewise, rates of elevated blood sugar are high in Tonga males, where nearly 50% of men can be classified as obese. Interestingly, Saudi Arabia, which has the highest death rate from CVD, also has the highest rates of elevated blood glucose, with nearly 20% of males having elevated blood glucose concentrations (World Health Organisation, 2011b).

Table 2.1: CVD related statistics for men from representative countries for 2008*

Country	NZ	Australia	UK	US	Russian Federat.	South Africa	China	Tonga	India	Saudi Arabia
Region	WPR	WPR	EUR	AMR	EUR	AFR	WPR	WPR	SEAR	EMR
Income Group	High	High	High	High	Upper middle	Upper middle	Lower middle	Lower middle	Lower middle	High
Estimated deaths from NCDs, %	91	90	88	87	82	29	83	74	53	71
Total deaths from CVD (all causes), %	37	35	34	35	62	11	38	38	24	42
Metabolic risk factors, % prevalence males										
Elevated cholesterol	57.5	55.9	65.6	53.3	47.8	31.3	31.8	52.5	25.8	35.4
Elevated blood pressure	40.8	41.1	46.4	34.8	46.6	43.1	40.1	42.1	33.2	35.2
Elevated blood glucose	NA	10.8	9.2	13.8	NA	10.3	9.5	15.8	10.0	18.1
Overweight	69.2	68.2	67.7	73.5	56.2	58.5	25.5	84.2	9.9	69.1
Obesity	27.3	26.4	26.0	31.1	18.6	21.0	4.7	46.6	1.3	28.6
Behavioural risk factors, % prevalence in males										
Daily tobacco smoking (current)	21.4	18.3	18.5	18.6	65.5	21.2	49.3	36.6	25.1	8.5
Physical inactivity†	45.9	38.0	61.1	35.5	22.9	46.4	29.3	30.6	10.8	60.7

AFR: African region; AMR: Region of Americas; CVD: cardiovascular disease; EMR: East Mediterranean Region; EUR: European Region; NCDs: Non-communicable disease; NZ: New Zealand; SEAR; South East Asia Region; WPR: Western Pacific Region; UK: United Kingdom; US: United States

*NCD Country Profiles (World Health Organisation, 2011b)

Definitions - elevated cholesterol: the percentage of the population aged 25 or older having a TC ≥5.0 mmol/L (190 mg/dl); elevated BP: SBP ≥140 mmHg and/or DBP ≥90mmHg or on medication to lower BP; raised blood sugar: the percentage of the population aged 25 or older having a fasting plasma glucose value ≥7.0 mmol/L (126 mg/dl) or on medication for raised blood glucose; overweight: the percentage of the population aged 20 or older having a BMI ≥25 kg/m^2; obesity: the percentage of the population aged 20 or older having a BMI ≥30 kg/m^2, current daily tobacco smoking: the percentage of the population aged 15 or older who smoke tobacco on a daily basis; physical activity: the percentage of the population aged 15 or older engaging in less than 30 minutes of moderate activity per week or less than 20 minutes of vigorous activity three times per week, or the equivalent.

2.2.2.2 United States

As with other high income countries, the death rate attributable to CVD in the US has declined over recent decades (by 30.6% between 1998 and 2008). As in the UK, the decrease is a result of a combination of the wider use of treatments in those with existing CVD, and downward shifts in population levels of smoking, cholesterol and BP (Lloyd-Jones et al., 2010, Roger et al., 2012). Offsetting the positive declines in these risk factors is the continuing increase in undesirable levels of fasting glucose and BMI, with some evidence that further reductions in mortality rates are slowing (Lloyd-Jones et al., 2010, Roger et al., 2012). In conjunction with the increase in prevalence of obesity and type 2 diabetes, mean TG levels have increased. Using data from National Health and Nutrition Examination Surveys (NHANES) 1999 to 2008, the overall prevalence of hypertriglyceridaemia (≥150 mg/dL, ≥1.70 mmol/L) in adults is estimated to be 31% (Miller et al., 2011, Talayero and Sacks, 2011).

Moving forward into the current decade, the American Heart Association (AHA) has recently created a new set of Impact Goals, specifically 'By 2020, to improve the cardiovascular health of all Americans by 20%, while reducing deaths from cardiovascular diseases and stroke by 20%.' In these goals, 'ideal' cardiovascular health is not only defined by the absence of disease, but includes the presence of seven measurable health behavioural goals (not smoking; being physically active; having normal TC levels, BP, blood glucose and weight, and eating a healthy diet). Each goal has specific cut-offs/targets to allow compliance to be categorised into poor, intermediate and ideal, to encompass the full spectrum of cardiovascular health (see Table 2.2) (Lloyd-Jones et al., 2010, Roger et al., 2012, Yang et al., 2012). In regards to the dietary metric, 5 goals were selected: (1) fruit and vegetables: ≥4.5 cups/day; (2) fish: ≥two 3.5-oz (99 g) servings/week (preferably oily fish); (3) fiber-rich whole grains (≥1.1 g of fiber per 10 g of carbohydrate): ≥three 1-oz (28 g) equivalent servings/day; (4) sodium: <1500 mg/day; (5) sugar-sweetened beverages: ≤450 kcal (36 oz, approximately 1 litre) per week. It is suggested that these should be achieved by following a dietary pattern that is consistent with a dietary Approaches to Stop Hypertension (DASH)-type eating plan, with

an energy intake appropriate to maintaining energy balance (Lloyd-Jones et al., 2010).

Table 2.2: Definitions of poor, intermediate and ideal cardiovascular health for each metric in the AHA 2020 goals
(for adults ≥20 years of age)*

	Level of Cardiovascular Health for Each Metric		
	Poor	**Intermediate**	**Ideal**
Current smoking	Yes	Former ≤12 months	Never or quit >12 months
BMI	≥30 kg/m^2	25-29.9 kg/m^2	<25 kg/m^2
Physical activity	None	1–149 min/wk moderate or 1–74 min/wk vigorous or a combination 1–149 min/wk moderate + 2x vigorous	≥150 min/wk moderate or ≥75 min/wk vigorous or a combination ≥150 min/wk moderate + 2x vigorous
Healthy diet score, no. of components†	0-1	2-3	4-5
Total Cholesterol,	≥240 mg/dL (6.21 mmol/L)	200-239 mg/dL or treated to goal (5.17-6.18 mmol/L)	<200 mg/dL (5.17 mmol/L)
Blood Pressure	SBP ≥140 or DBP ≥90 mmHg	SBP 120–139 or DBP 80–89 mmHg or treated to goal	<120/<80 mmHg
Fasting plasma glucose	≥126 mg/dL (6.99 mmol/L)	100–125 or treated to goal (5.55-6.94 mmol/L)	<100 mg/dL (5.55 mmol/L)

AHA: American Heart Foundation; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure
*Table modified from Heart disease and stroke statistics–2012 update: a report from the American Heart Association (Roger et al., 2012)
†Diet components: (1) Fruit and vegetables: ≥4.5 cups/day; (2) Fish: ≥two 3.5-oz servings/week (preferably oily fish); (3) Fiber-rich whole grains (≥1.1 g of fiber per 10 g of carbohydrate): ≥three 1-oz-equivalent servings/day; (4) Sodium: <1500 mg/day; (5) Sugar-sweetened beverages: ≤450 kcal (36 oz) per week

A recent study examining trends in these measures, using NHANES data, found that, while meeting a greater number of cardiovascular health metrics was associated with a lower risk of total and CVD mortality, the prevalence of meeting all seven cardiovascular health metrics was low; 2.0% (95% CI, 1.5%-2.5%) in 1988-1994 and 1.2% (0.8%-1.9%) in 2005-2010 (Yang et al., 2012).

Briefly, comparing differences between the two periods, the prevalence of smoking declined significantly (27.5% to 22.6%), however, so did the prevalence of consuming a healthy diet (≥2 healthy diet score components)

(33.1% versus 22.3%), as did having a desirable blood glucose level and BMI. The prevalence for desirable TC levels and BP remained largely unchanged (Yang et al., 2012).

2.2.2.3 New Zealand

In 2008, NCDs accounted for 91% of all deaths in NZ, with CVD from all causes accounting for 37% of deaths (Table 2.1, World Health Organisation, 2011b). Of those deaths, IHD accounted for 18.9%, and cerebrovascular disease for 8.9% (Ministry of Health, 2011).

The 2009 statistics (the latest available) remain relatively unchanged, with IHD accounting for 19% of deaths and cerebrovascular disease at 8.5%. Males accounted for 54.7% of deaths from IHD and had nearly twice the female age-standardised death rate. Table 2.3 shows the age-standardised rate of death (WHO World Standard Population), per 100,000 population, for males and females and Māori and non-Māori. The Māori male had the highest age-standardised rate for deaths from IHD, 83.3% higher than for non-Māori males, (Ministry of Health, 2012b). It is recognised that both socioeconomic and ethnic inequalities in CVD incidence and mortality rates exist in NZ (Riddell and North, 2003).

Table 2.3: Mortality rates for ischaemic heart disease, cerebrovascular and hypertensive disease in NZ in 2009*

	Total		**non-Māori**		**Māori**	
	Males	Females	Males	Females	Males	Females
Ischaemic heart disease	96.6	48.6	90.5	45.7	166.0	93.7
Cerebrovascular disease	29.0	30.7	28.2	29.4	36.8	45.9

Māori population includes everyone who was identified as Māori, non-Māori population includes everyone else

*Age-standardised rate of death (WHO World Standard Population), per 100,000 population (Ministry of Health, 2012b)

As with other countries classified as high income by the WHO, NZ's mortality rates from CVD have been steadily declining over the past few decades (with the greatest decline among those of higher socioeconomic status), although it

still remains one of the leading causes of death (Ministry of Health, 2012b, Riddell and North, 2003). Deaths from IHD peaked in the late 1960's (Figure 2.8), with the male age-standardised rate of death for IHD decreasing by 68.3% from 1980-2009. Likewise, the male age-standardised death rate from cerebrovascular disease decreased by 70.1% during this period.

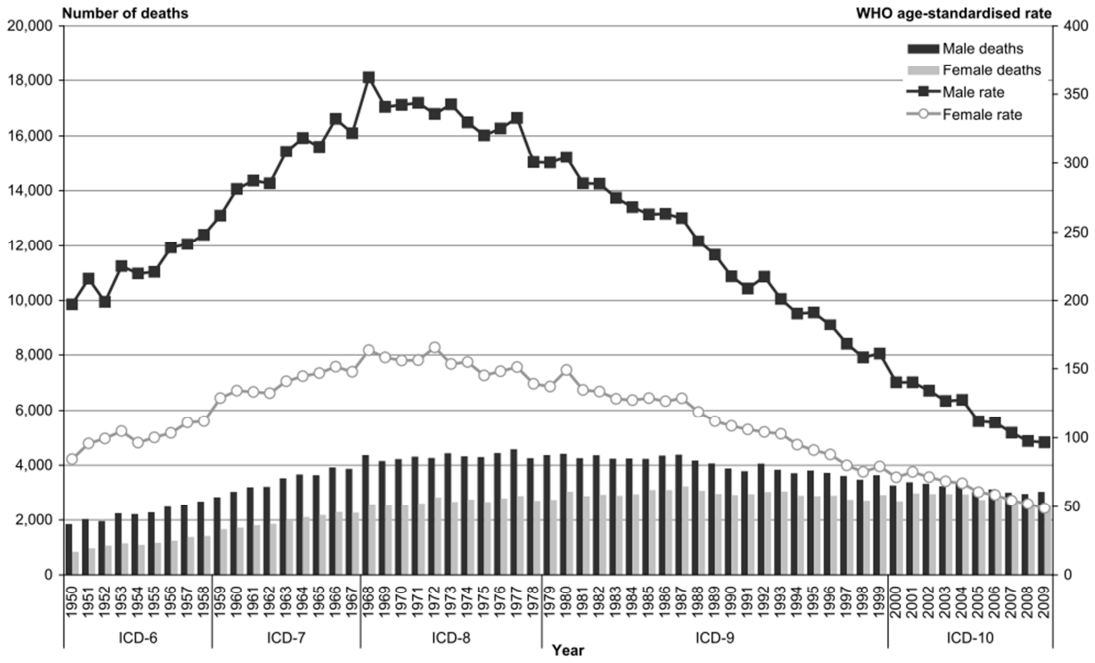

Figure 2.8: Death rates from ischaemic heart disease, by sex, 1950-2009*

*Rates per 100,000 population, age-standardised to WHO World Standard Population

In a 2005 study investigating a 25 year period between 1980 and 2004, it was calculated that approximately 73.4% of the observed decrease in CHD mortality in males could be attributed to favourable population trends in total blood cholesterol (25.9%, estimated contribution), SBP (39.5%) and smoking prevalence (27.6%) (Tobias et al., 2008).

In a study examining the trends in CVD risk factors between 1982 to 2002-2003 in Auckland, researchers reported that mean serum TC had declined from 6.18 mmol/L to 5.62 mmol/L in men, while HDL-C increased over the period. There was a steady decrease in SBP over this period, while DBP decreased between 1982–1993-4, but has then remained relatively unchanged. There was a downward trend in cigarette smoking between 1982 and 1993-4, but between

1993-4 and 2002-3 there was little change (Metcalf et al., 2006). In the Auckland, Diabetes, Heart and Health survey (DHAH) 2002-2003, whose data contributed to that analysis, men classified as 'others' (who were predominantly Europeans) were found to have the following mean lipid profile; TC 5.57 mmol/L, LDL-C 3.53 mmol/L, HDL-C 1.31 mmol/L, TG 1.52 mmol/L and TC/HDL-C ratio 4.50 mmol/L. Further, 66.3% had a LDL-concentration ≥3.0 mmol/L, higher than the recommended cut-off for CVD risk screening (Gentles et al., 2007).

The current screening and optimal lipid profile (for people with known CVD or CVD risk >15% or diabetes) values are shown in table Table 2.4.

Table 2.4: Screening and optimal lipid profile values

	Screening*	Optimal†
TC (mmol/L)	<5.0	<4.0
LDL-C (mmol/L)	<3.0	<2.0
HDL-C (mmol/L)	>1.0	≥1.0
TC/HDL-C ratio (mmol/L)	<4.5	<4.0
TG (mmol/L)	<2.0	<1.7

TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides
*Reference intervals lipids Diagnostic MedLab (Auckland, New Zealand)
†Optimal levels for people with known CVD or CVD risk >15% or diabetes (New Zealand Guidelines Group, 2012)

In NZ, a risk chart for those not at high risk is used to calculate an individual's 5-year absolute CVD risk, using an individual's age, gender, whether they are a smoker or not, have diabetes or not, and using their systolic BP and TC/HDL-C ratio. The overall goal is to reduce their 5-year risk to less than 15%, with all treatment decisions to be based on the risk result (New Zealand Guidelines Group, 2012).

As pharmacological interventions (in particular antihypertensives and statins) have really only made significant contributions to the CHD mortality decline since the 1990s, Tobias et al. (2008) suggest that most of the improvements seen between 1982 and 2002-2003 are likely due to changes in smoking prevalence and dietary behaviours. Although it has since slowed, smoking

prevalence decreased by nearly 20% overall in the 1980s (Tobias et al., 2008). Data from the 2011/12 NZ Health Survey shows that 17% of NZ adults smoke daily, down slightly from the 2006/7 survey (18%), but significantly decreased from the 1996/97 survey (25%). However, high rates of current smoking (smoke at least monthly) persist among Māori adults (41%) and Pacific adults (26%) (Ministry of Health, 2012a).

Tobias et al. (2008), in their analysis of dietary behaviours between 1980 and 2004, propose increased intake of fruit and vegetables, PUFA and monounsaturated fatty acids (MUFA), and possibly wholegrain cereal and fish, and decreased intake of SFA, trans fat, and salt were the main contributors to a healthier heart diet.

Table 2.5 shows the trend in fruit and vegetable consumption in males from 1997 to 2011/12. For vegetables there has been little change, (although there was a significant decrease in the 2006/07 Health survey), while fruit intake has steadily increased since 1997 (Ministry of Health, 2006, Ministry of Health, 2012a). More females than males met the fruit (≥2 serves/day) and vegetable (≥3 serves/day) guidelines, (Ministry of Health, 2012a).

Table 2.5: Vegetable and fruit intake in males
(Percentage consuming ≥3 vegetable and ≥2 fruit serves/day)

	1997	2002/3	2006/7	2011/12
Vegetables	60.8%	63.3%	56.1%	63.0%
Fruit	34.5%	43.3%	49.6%	52.1%

Rates are age-standardised to the WHO world population
Source 1997 National Nutrition Survey, New Zealand Health Surveys (2002/03, 2006/07, 2011/12) (Ministry of Health, 2006, Ministry of Health, 2012a)

The latest National Nutrition 2008/9 survey reported that 59.3% of males consumed the recommended servings of vegetables (≥3 serves/day) and 54.6% the recommended servings of fruit (≥2 serves/day) (University of Otago and Ministry of Health, 2011).

The results for the intake of some selected nutrients from the last two National Nutrition Surveys for men are reported in Table 2.6.

Table 2.6: National Nutrition Survey results for selected nutrients in men*

	Recommended Targets	1997	2008/9
SFA (%E)	<10%†	15.1%	13.1%
MUFA (%E)	10-20%‡	11.8%	12.4%
PUFA (%E)	6-10%‡	5.0%	4.8%
Cholesterol (median)	<300 mg§	359 mg	316 mg
Fibre (median)	SDT 38 g†	23 g	22.1 g

SFA: saturated fatty acid; %E: percentage energy of total energy; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid
*Russell et al., (1999), University of Otago and Ministry of Health, (2011)
†Recommendations to reduce chronic disease risk (Baghurst, 2005)
‡New Zealand Nutrition Taskforce recommendations (Department of Health, 1991)
§American Heart Association (Lichtenstein et al., 2006)

These results show decreases in the mean contribution of SFA to daily energy intake and cholesterol intake between 1997 and 2008/9, but no changes in MUFA, PUFA or fibre intake. However, while the decreases in SFA and cholesterol indicate that more NZ men are meeting the recommended targets for these nutrients, fibre intakes are still low. This is not unexpected, given fruit and vegetables are significant contributors to fibre intake and over a third of men are still not consuming the recommended 3 or more vegetable servings a day, and nearly half are not consuming the recommended 2 or more fruit servings a day.

Results for blood cholesterol collected from the last two National Nutrition Surveys for men are reported in Table 2.7. As the blood samples were non-fasting, neither TG or LDL-C were measured.

There was a significant decrease in TC from 1997 to 2008/9, which the authors of the report attribute both to dietary factors, such as the decreased intake of SFA, and also the increased prescribing of lipid lowering medication (University of Otago and Ministry of Health, 2011). Results from the 2011/12 NZ Health Survey (adults 15 years and over) show that over the last 10 years the

percentage of people taking medication for high cholesterol has steadily increased from 6% in 2002/03 to 8% in 2006/07 and 10% (men 12%, women 9%) in 2011/12 (Ministry of Health, 2012a).

Table 2.7: National Nutrition Survey mean blood cholesterol results in men

	1997*	2008/9†
Total cholesterol (mmol/L)	5.70 (0.05)	5.09 (5.01, 5.16)
HDL-C (mmol/L)	1.20 (0.01)	1.23 (1.21, 1.26)
TC/HDL-C ratio	NA	4.32 (4.22, 4.41)

HDL-C: high density lipoprotein cholesterol; NA: not assessed; TC/HDL-C ratio: total cholesterol/HDL-C ratio
*Mean (SEM) (Russell et al., 1999)
†Mean (95% CI) (University of Otago and Ministry of Health, 2011)

There has been no change in the percentage of people meeting physical activity guidelines since 2002/3, with only 54% of adults (57% men, 51% women) being physically active (at least 30 minutes of moderate-intensity (or equivalent) physical activity/day on five or more days of the last week) in 2011/12 (Ministry of Health, 2012a).

The Health Survey also found that the CVD and related risk factors were more common among adults living in more deprived areas. Adults in those areas had a higher incidence of IHD and stroke, and were more likely to be taking BP medication, be obese, have diabetes and smoke, and less likely to eat the recommended daily amount of vegetables and fruit or do any physical activity (Ministry of Health, 2012a).

In 2009, NZ adults were ranked third-highest for obesity rates in the OECD, behind the US (34%) and Mexico (30%) (OECD, 2011). Over the past 15 years, obesity rates (BMI ≥30) in adults have substantially increased from 19% in 1997 to 26% in 2006/07 and 28% (men 28%, women 29%) in 2011/12. However, while the obesity rates were still significantly higher for Māori (44%) and Pacific (62%) adults in 2011/12, they remained unchanged from 2006/7. Further, in 2011/12 another 35% of adults could be classified as overweight (BMI of 25.0–29.9), with the result that only 1 in 3 had a normal weight (18.5–24.9) (Ministry of Health, 2012a). In conjunction with the increase in obesity rates, there has

been a steady increase in prevalence rates of diagnosed diabetes in adults from 2.8% in 1996/97 to 5.5% (men 6%, women 5%) in 2011/12, with Pacific, Māori and Asian (male) adults having significantly higher rates than Europeans. Further it is estimated that for 3 people diagnosed with diabetes, another person remains undiagnosed (Ministry of Health, 2012a).

In summary, although the rates of death attributable to CVD have declined in many developed countries, such as NZ, CVD remains one of the leading causes of death and the burden of the disease remains high.

The decreased incidence seen in developed countries has been attributed both to advances in treatments and to prevention strategies that have decreased the prevalence of various risk factors, such as smoking, elevated cholesterol and BP. As with the UK, the US and many other countries, the increased prevalence of obesity and type 2 diabetes threatens to slow further declines in IHD mortality rates in NZ. These factors in conjunction with an increasing and also aging population mean that the actual number of people being diagnosed with CVD (possibly at younger ages) and the burden on the health system will likely only continue to increase, with some population groups such as Māori and Pacific appearing to be at greater risk (Lloyd-Jones et al., 2010, Tobias et al., 2006).

In the next three sections the pathogenesis, inter-relationships and impacts of plasma lipids, obesity and BP on CVD risk will be explored.

2.2.3 Lipid and lipoprotein metabolism in relation to CVD risk

In this section, the main plasma lipid components and their relationship with CVD risk will be examined. Further, key pathways involved in lipid metabolism and how these are altered under certain conditions, such as hypertriglyceridaemia, will be discussed.

2.2.3.1 Lipids, lipoproteins and apolipoproteins

Biochemically, lipids are classified as macromolecules, although they are smaller than the other true polymeric macromolecules. They consist of a diverse (both in form and function) group of biological molecules that all share one common trait, they have little or no affinity for water (Campbell and Reece, 2002). In relation to CVD, the most important lipids are plasma TG and cholesterol (Hegele, 2009).

Triglycerides are composed of three fatty acids (FAs) (hydrocarbon chains consisting of 2 to 20 or more carbons, with a carboxyl group at one end) esterified to a glycerol backbone. Their main function is as an energy source for use and storage (Campbell and Reece, 2002, Dupont, 2006). Cholesterol is classified as a sterol, with a rigid ring structure (consisting of four fused rings), a hydroxyl group and a hydrocarbon tail. It has a number of biological roles, including as a component of all cell membranes, and as a precursor for bile salts, vitamin D and steroid hormones (Campbell and Reece, 2002, Dupont, 2006, Hegele, 2009). Although dietary cholesterol contributes to the body's cholesterol pool, most is synthesised endogenously, mainly by the liver. In circulation most cholesterol is esterified, and excretion from the body is by hepatocytes or enterocytes (Dupont, 2006, Hegele, 2009, Radar and Hobbs, 2010). As both TGs and cholesterol are insoluble in bodily fluids (plasma, lymph and interstitial fluid), they must be transported within lipoproteins (Davis and Wagganer, 2006, Hegele, 2009, Radar and Hobbs, 2010).

Lipoproteins are complexes of lipids and proteins, which form large spheroidal macromolecules (Hegele, 2009, Radar and Hobbs, 2010). They consist of a hydrophilic monolayer shell whose amphipathic components, phospholipids, apolipoproteins and a small amount of free (unesterified) cholesterol surround a hydrophobic core containing electrically neutral lipid (cholesterol ester (CE) and

TG), and fat soluble antioxidants and vitamins (Davis and Wagganer, 2006, Hegele, 2009).

Lipoproteins are primarily classified according to their relative density, with density being determined by the amount of lipid in each lipoprotein (the more lipids the lower the density). Ultracentrifugation separation techniques first described in the 1950s resulted in the description of 5 major lipoprotein classes – chylomicrons (CM), VLDL, intermediate-density lipoprotein (IDL), LDL and HDL (Davis and Wagganer, 2006, Grandjean and Alhassan, 2006, Hegele, 2009, Radar and Hobbs, 2010). The size of each lipoprotein (as measured by gel electrophoresis) is inversely related to its density, and they can also be distinguished from each other by composition (including apolipoprotein content), electrophoretic mobility and function (see Table 2.8) (Davis and Wagganer, 2006, Grandjean and Alhassan, 2006, Hegele, 2009, Radar and Hobbs, 2010). Chylomicron and VLDL are the main transporters for TG, and LDL and HDL are the main transporters for CE (Hegele, 2009, Radar and Hobbs, 2010).

Apolipoproteins are the proteins associated with lipoproteins. They are the primary determinant of both its function and the composition of its lipid content. As well as serving as a structural component, different apolipoproteins (at least 13 have been identified) serve as cofactors for specific enzymes and as ligands for specific receptors. Changes in the lipid content of a lipoprotein can in turn affect the affinity for a particular enzyme or receptor, through changing the tertiary structure of an apolipoprotein (Davis and Wagganer, 2006, Hegele, 2009). Most apolipoproteins, with the exception of apoB, can freely exchange between other lipoproteins (Burnett and Barrett, 2002).

The major apolipoproteins and their recognised functions are shown in Table 2.9. The roles of some apolipoproteins are well established, but remain under investigation for others.

Table 2.8: Characteristics of main lipoprotein classes

Lipoprotein class	Density (g/mL)	Size (nm)	Composition (%)					Apolipoproteins	
			TG	Cholesterol ester	Free cholesterol	Phospholipid	Apolipoprotein	Major	Others
Chylomicron	<0.930	75-1200	86	3	2	7	2	apoB48	A1, A4, C1, C2, C3, E
CM remnants	0.930-1.006	30-80						apoB48	A1, A4, C1, C2, C3, E
VLDL	0.930-1.006	30-80	55	12	7	18	8	apoB100	A1, A2, A5, C1, C2, C3, A5
IDL	1.006-1.019	25-35	23	29	9	19	19	apoB100	C1, C2, C3, E
LDL	1.019-1.063	18-25	6	42	8	22	22	apoB100	
HDL_2	1.063-1.125	9-12	5	17	5	33	40	apoA1	A2, A4, E, C1, C2, C3
HDL_3	1.1.25-1.210	5-9	3	13	4	35	55	apoA1	A2, A4, E, C1, C2, C3

CM: chylomicron; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein VLDL: very low-density lipoprotein (Burnett and Barrett, 2002, Radar and Hobbs, 2010)

Table 2.9: Major apolipoproteins and their recognised functions

Apolipoprotein	Primary Source	Lipoprotein Association	Functions
ApoA1	Intestine, liver	HDL, CM	Structural protein HDL; cofactor for LCAT; accepts cholesterol from peripheral cells through ABCA1; facilitates lipid uptake through SR-BI
ApoA2	Liver	HDL, CM	Structural protein HDL; facilitates lipid uptake through SR-BI; displaces apoA1 from HDL
ApoA4	Intestine	HDL, CM	Possible role in modulating CM size and clearance in the periphery (Kohan et al., 2012)
ApoA5	Liver	VLDL, CM	Promotes LPL-mediated TG lipolysis
ApoB48	Intestine	CM	Structural protein for CM
ApoB100	Liver	VLDL, IDL, LDL	Structural protein for VLDL, LDL, IDL; ligand for binding to LDLR
ApoC1	Liver	CM, VLDL, HDL	Inhibits apoE-mediated lipid uptake by LDLR and LRP
ApoC2	Liver	CM, VLDL, HDL	Cofactor for LPL
ApoC3	Liver	CM, VLDL, HDL	Inhibits LPL activity, inhibits lipoprotein binding to receptors
ApoE	Liver	CM remnants, VLDL, IDL, HDL	Facilitates lipid uptake (ligand) through LDLR and LRP

ABCA1: adenosine triphosphate-binding cassette-A1; Apo: apolipoprotein; CM: chylomicron; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LCAT: lecithin cholesterol acyltransferase; LDL: low-density lipoprotein; LDLR: LDL receptor; LPL: lipoprotein lipase; LRP: LDL receptor-related protein; SR-BI: scavenger receptor BI; TG: triglycerides; VLDL: very low-density lipoprotein (Davis and Wagganer, 2006, Radar and Hobbs, 2010)

Apolipoprotein A1 is the most abundant apolipoprotein and the main structural protein of HDL, followed by apoA2, which comprises about 20% of the total protein mass. One molecule of apoB, which may be pictured as a belt around each particle, is present either as apoB48 in CM or apoB100 in VLDL, IDL and LDL lipoproteins. Apolipoprotein E and the apolipoproteins of the C series all participate in the metabolism of TG-rich lipoproteins (TGRL) (Davis and Wagganer, 2006, Radar and Hobbs, 2010, Segrest et al., 2001). Many of these apolipoproteins will be discussed further in later sections.

2.2.3.2 Lipoprotein metabolism

Lipoprotein metabolism (Figure 2.9) involves a complex set of biological pathways involving assembly, secretion, processing and catabolism. These pathways are often subdivided into those involved in the metabolism of lipoproteins carrying exogenous lipids, endogenous lipoprotein metabolism and reverse cholesterol transport (RCT) (Corella and Ordovas, 2005, Hegele, 2009).

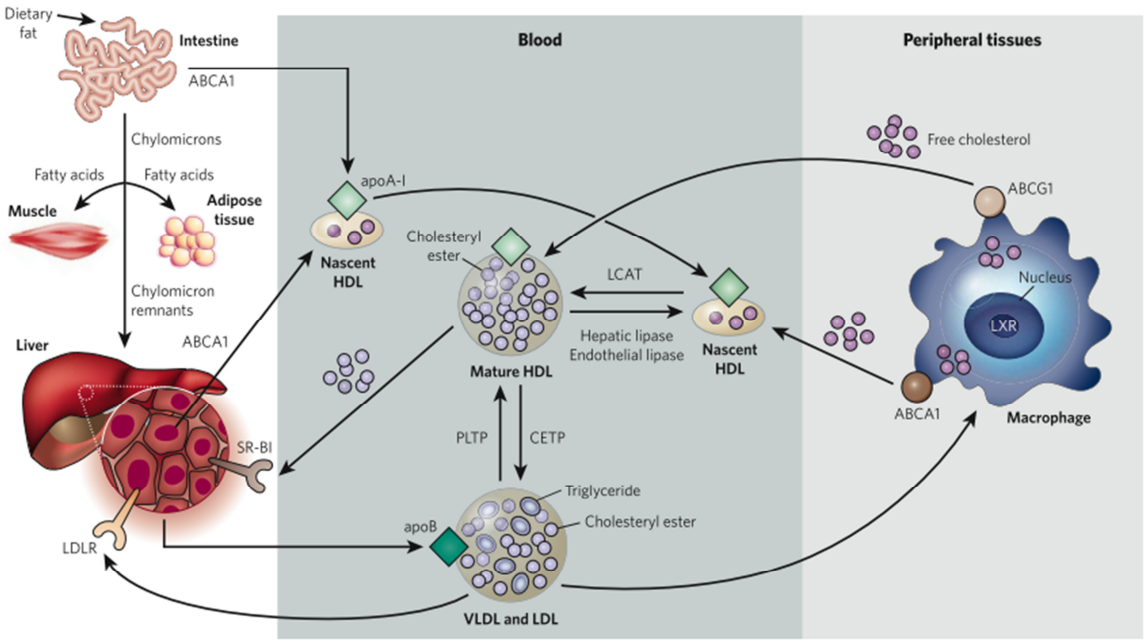

Figure 2.9: Lipoprotein metabolism

ABCA1: adenosine triphosphate binding cassette A1; ABCG1: adenosine triphosphate binding cassette sub-family G; apoA1: apolipoprotein A1; apoB: apolipoprotein B; CETP: cholesteryl ester transfer protein; CM: chylomicron; HDL: high-density lipoprotein; LCAT: lecithin cholesterol acyltransferase; LDL: low-density lipoprotein; LDLR: LDL receptor; LXR: liver X receptor nuclear receptor; PLTP: phospholipid transfer protein; SR-BI: scavenger receptor class BI; VLDL: very low-density lipoprotein

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Each of these pathways will be discussed in more detail. Similarities can be seen between the transport pathways for exogenous and endogenous lipids, with large lipid-laden lipoproteins that are secreted from the intestine or liver, and are hydrolysed by specific lipases into remnants or smaller lipoproteins (releasing FAs). These are then taken up into the liver and other tissues, via receptor mediated mechanisms (Davis and Wagganer, 2006, Hegele, 2009).

Achieving lipid homeostasis involves the coordinated action of a substantial number of nuclear factors, apolipoproteins, binding proteins, enzymes, and receptors, and therefore hundreds of genes. Lipid metabolism is also inextricably linked with energy metabolism, and so is additionally subjected to many hormonal controls (Corella and Ordovas, 2005, Ordovas, 2009).

Lipid transport: exogenous pathway

In a Western diet, dietary fat contributes 30-40% of energy intake, mostly in the form of TG. Following ingestion, TG digestion begins in the stomach with acid lipase, although most hydrolysis is carried out by pancreatic lipase in the intestinal lumen. The resultant products, predominantly 2-monoacylglycerol (2-MG) and 2 FAs are then emulsified with bile salts to form micelles, which enhance their uptake into the enterocytes (Corella and Ordovas, 2005, Kindel et al., 2010). Once absorbed, fatty acid-binding proteins (FABP) transport the FAs intracellularly to the endoplasmic reticulum, where 2-MG is reacylated by either acyl-coenzyme A: monoacylglycerol acyltransferase (MGAT) or acyl-coenzyme A: diacylglycerol acyltransferase (DGAT) into TG (Corella and Ordovas, 2005, Kindel et al., 2010). In the case of cholesterol, specific sterol carriers (Niemann-Pick C1-like 1 (NPC1L1)) and other proteins such as scavenger receptor class BI (SR-BI) and the adenosine triphosphate-binding cassette (ABC) transporters have been identified in cholesterol absorption and, once in the enterocyte, cholesterol is esterified to form CEs (Corella and Ordovas, 2005, Radar and Hobbs, 2010).

The TG and cholesterol from the diet, along with endogenously sourced cholesterol from bile and enterocyte turnover, are then packaged with apoB48, phospholipids, and fat soluble vitamins into CM. This process is mediated by microsomal triglyceride transfer protein (MTP). Apolipoprotein B48 is

synthesised by intestinal cells, and is derived from the same gene as apoB100, but mRNA editing means a stop codon is about halfway along, hence the intestinal apoB is 48% of the molecular weight of the full-length version produced by the liver (Davis and Wagganer, 2006, Kindel et al., 2010, Radar and Hobbs, 2010, Sacks, 2011). Other apolipoproteins incorporated at this stage include apoA1 and apoA4. Large TG-rich nascent CM are then secreted via the lymphatic system into the systemic circulation to start their journey to the liver (Davis and Wagganer, 2006, Radar and Hobbs, 2010).

Once in circulation, CM undergo continuous processing. On contact with HDL, further apolipoproteins C1, C2, C3 and E are transferred and incorporated and they become mature CM. Apolipoprotein C2 is an essential cofactor for lipoprotein lipase (LPL), which is found attached via proteoglycans to the luminal surface of capillary endothelial cells in adipose tissue, heart and skeletal muscle. Lipoprotein lipase hydrolyses TG (primarily at the sn-1 and sn-3 positions) releasing free fatty acids (FFAs), which are either taken up by adjacent adipocytes or myocytes, and utilised as an energy source or stored as re-esterified TGs, or are bound to albumin to be transported to other tissues, such as the liver (Corella and Ordovas, 2005, Davis and Wagganer, 2006, Radar and Hobbs, 2010, Burnett and Barrett, 2002). As the hydrophobic core is progressively hydrolysed, the CM shrinks and becomes a CM remnant through further transfer of surface hydrophilic lipids and apolipoproteins (most of apoA and some apoC) to HDL. These remnants are removed from circulation by hepatic receptors (LDL receptor (LDLR) or LDL receptor-related protein (LRP)), which require apoE as a ligand. The remnants are taken up by endocytosis into lysosomes, where the remaining components are catabolised. Hepatic receptors do not recognise apoB48 (Davis and Wagganer, 2006, Radar and Hobbs, 2010).

Experimental studies suggest that cell-surface heparin sulphate proteoglycans (HSPGs) may play an important role in remnant removal (CM and VLDL). It is thought that the remnants are ‘captured’ by HSPGs, for which apoE is a ligand, before undergoing further lipolytic processing by LPL or HL. The remnants are then handed on to LDLR or another unidentified receptor, or may even be internalised by HSPGs directly (Mahley and Huang, 2007).

Lipid transport: endogenous pathway

Endogenous lipoprotein metabolism follows a similar route to the exogenous pathway, except that the process starts in the liver, where VLDLs are assembled and secreted and where the TG and cholesterol which they transport are synthesised. Both CM and VLDL are large TGRL, although VLDL particles are smaller and have a higher ratio of cholesterol to TG. They also contain apoB100, instead of apoB48. The enzyme MTP again facilitates the packing of components to form the nascent lipoprotein, TG (predominantly derived from esterification of FAs in the liver), apoB100, some apoC and apoE, phospholipids and CEs. As with CMs after secretion, the nascent lipoprotein acquires additional apolipoproteins from HDL (more apoC and apoE), at which point it becomes a mature VLDL. Then, as TGs are hydrolysed by LPL, smaller, denser remnants are formed (IDL). These contain about equal amounts of TG and cholesterol (Burnett and Barrett, 2002, Davis and Wagganer, 2006, Radar and Hobbs, 2010).

The plasma concentration of IDL is relatively small and it may be directly removed from circulation by the liver (approx. 40 to 60%) via LDLR mediated endocytosis, facilitated by apoE. Alternatively, the lipoprotein may undergo further processing, with most of the remaining TG being hydrolysed by LPL and HL, and all apolipoproteins except apoB100 being transferred to other lipoproteins, resulting in the formation of LDL (Burnett and Barrett, 2002, Davis and Wagganer, 2006, Radar and Hobbs, 2010). It should be noted that the liver also secretes some LDL directly (Sacks, 2011). The main lipid component is now CE, and it is LDL's function to transport this to extra-hepatic tissues. In most individuals, the cholesterol in LDL accounts for more than half of plasma cholesterol. Although LDL does not contain apoE, apoB100 is also recognised by LDLRs. All nucleated cells express LDLRs, allowing LDLR mediated endocytosis of the lipoprotein, so that cholesterol is available for functions such as incorporation into cell membranes or synthesis of steroid hormones. The liver remains the main site of clearance, clearing approximately 70% of circulating LDL (Burnett and Barrett, 2002, Davis and Wagganer, 2006, Radar and Hobbs, 2010).

Very low-density lipoprotein exhibit considerable heterogeneity with regard to density and apolipoprotein content (Burnett and Barrett, 2002). For instance, several VLDL 'phenotypes' have been identified in relation to apoE and apoC3 content. These two apolipoproteins are closely linked to apoB metabolism, and some VLDL contain both, some just apoC3 and some neither (Sacks, 2011). Both apoE and apoB100 are ligands for LDLR, although apoE has the stronger affinity for the receptor and it can also bind to LRP (in the liver). It has been shown that apoC3 can block the binding of both the ligands to hepatic receptors, thereby delaying the clearance of VLDL from circulation and pushing it towards conversion to LDL. Conversely if VLDL contain apoE but do not contain apoC3, they are rapidly cleared by the liver, potentially affecting energy distribution to peripheral tissues, suggesting that a balance between the two is important (Sacks, 2011).

It can be seen that there are a number of factors that could influence plasma LDL concentrations, including VLDL synthesis and clearance rates, LDLR number (whose synthesis, for instance, is down-regulated when saturated dietary fat intake is high), LPL and HL activity and LDL secretion rate. Elevated plasma cholesterol carried by LDL (and other apoB containing lipoproteins) is associated with atherosclerosis (Burnett and Barrett, 2002, Davis and Wagganer, 2006, Sacks, 2011).

Lipid transport: reverse cholesterol transport

Cholesterol is transported back from peripheral cells to the liver, for the synthesis of bile salts and/or excretion via the process termed RCT (Davis and Wagganer, 2006, Franceschini et al., 2011, Radar and Hobbs, 2010). The process is facilitated by HDL (see section 2.2.3.4. for more details on HDL synthesis and subspecies) and represents an important pathway that is considered to be atheroprotective (protective against the development of atherosclerosis) (Franceschini et al., 2011, Radar and Hobbs, 2010). However, HDL metabolism is complex and interlinked with that of TGRL (Sacks, 2011). Cholesteryl ester transfer protein, which plays a central role in the indirect pathway of RCT, appears to exert both anti- and pro-atherogenic actions

depending on whether normotriglyceridaemic or hypertriglyceridaemic conditions exist (de Grooth et al., 2004, Vourvouhaki and Dedoussis, 2008).

Cholesterol efflux from peripheral tissues, including macrophages, can occur by simple aqueous diffusion from cell plasma membranes. However, cell studies indicate that the process is more regulated, with ABCA1 and ABCG1 transporters and SR-BI receptor, three types of membrane protein that can promote the net efflux from cells, identified to date (Franceschini et al., 2011, Linsel-Nitschke and Tall, 2005, Rader and Daugherty, 2008). Nascent HDL (preβ-HDL) are the favoured acceptors of ABCA1-mediated efflux, with the process involving a direct interaction between ABCA1, which transports cholesterol and phospholipids to cell surface, and apoA1 which accepts them. In contrast, mature HDL promotes cholesterol efflux via ABCG1 and SR-BI. Unlike the ABCA1 and ABCG1 mediated pathways which promote a net efflux to HDL and are energy consuming, SR-BI mediates a bidirectional exchange of cholesterol with the direction of movement dependent on the concentration gradient (Franceschini et al., 2011, Lund-Katz and Phillips, 2010, Rader and Daugherty, 2008). Gene expression of ABCA1 and ABCG1 is increased in cholesterol-loaded cells, such as foam cells, as a result of increased levels of oxysterols (oxidized derivatives of cholesterol) which activate the nuclear liver x receptor (LXR) (Linsel-Nitschke and Tall, 2005, Lund-Katz and Phillips, 2010, Rader and Daugherty, 2008).

In nascent HDL, the enzyme lecithin cholesterol acyltransferase (LCAT) esterifies the free cholesterol to CE, which is moved to the core of the particle as they are more hydrophobic. As HDL acquires more CEs, it matures and becomes more spherical (Linsel-Nitschke and Tall, 2005, Radar and Hobbs, 2010, Rader and Daugherty, 2008).

Two pathways transport CEs to the liver. The direct pathway involves SR-BI, which selectively uptakes (without degrading the lipoprotein) CE from the hydrophobic core of HDL, into hepatocytes (Davis and Wagganer, 2006, Franceschini et al., 2011, Rader and Daugherty, 2008). Current evidence suggests that SR-BI can bind to a range of ligands, including most lipoproteins, but has a high affinity for larger CE-rich HDL particles. Both apoA1 and apoA2

interact with SR-BI, but their roles in the process remain unclear (Davis and Wagganer, 2006, Lund-Katz and Phillips, 2010, Valacchi et al., 2011).

The indirect pathway accounts for delivery of about 70% of CE to the liver. Cholesteryl ester transfer protein mediates the transfer of neutral lipids, including CEs (mainly in HDL generated by the reaction catalysed by LCAT) and TG (found mainly in plasma as a component of TGRL) between plasma lipoproteins (Barter et al., 2003, Barter and Rye, 2012, Chapman et al., 2010).

Under normotriglyceridaemic conditions, the transfer of CE from HDL to LDL dominates, with only a small transfer to TGRL (Figure 2.10). The CE is subsequently delivered to the liver by LDL and VLDL, for removal by LDLR (and LRP) mediated endocytosis, as previously discussed. Under these circumstances, CETP may be considered anti-atherogenic (Chapman et al., 2010, de Grooth et al., 2004)

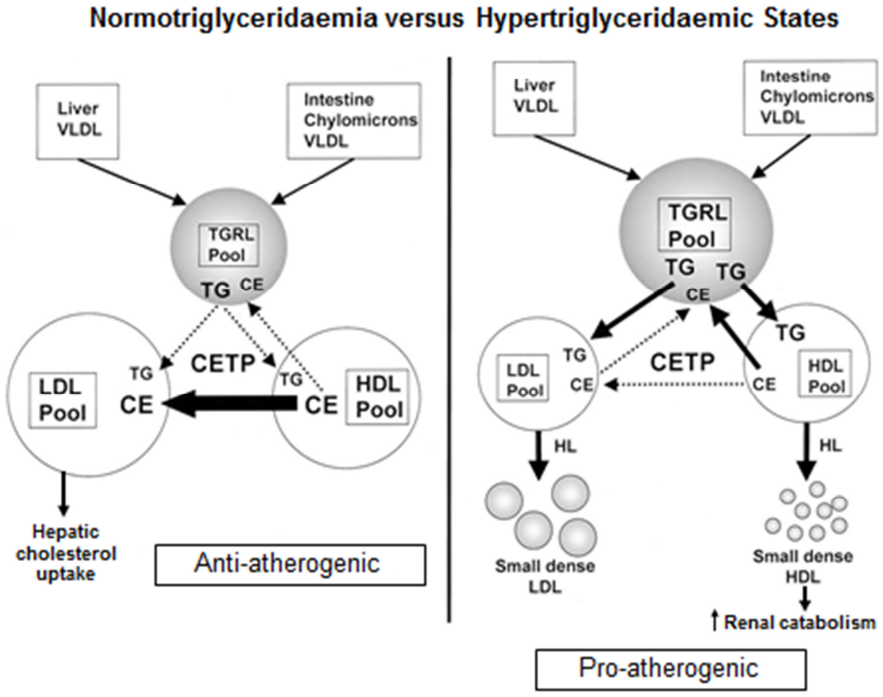

Figure 2.10: Comparison of CETP-mediated bidirectional transfer of CEs and TG pathways in normotriglyceridaemia and hypertriglyceridaemia

CE: cholesterol ester; CETP: cholesteryl ester transfer protein; HDL: high-density lipoprotein; HL: hepatic lipase; LDL: low-density lipoprotein; TG: triglycerides; TGRL: triglyceride-rich lipoproteins; VLDL: very low-density lipoprotein

Adapted from Arterioscler Thromb Vasc Biol, 23 (2), Barter, P. J., Brewer, H. B., Jr., Chapman, M. J., Hennekens, C. H., Rader, D. J. & Tall, A. R., Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis, 160-7, © (2003) with permission Lippincott Williams and Wilkins.

Under hypertriglyceridaemic conditions, such as might be seen in individuals with atherogenic dyslipidaemia (see section 2.2.3.3), transfer of CE to TGRLs is enhanced in the post-prandial phase. The increased concentration of TG-donor lipoproteins (particularly large VLDL) drives the enhanced exchange of CE from HDL to TGRL, and transfer of TG from TGRL to HDL and LDL (Barter et al., 2003, Chapman et al., 2010). This results in TG enrichment of both, making them good substrates for HL, which then remodels them into smaller particles. The formation of smaller particles consequently leads to a decrease in plasma HDL-C and apoA1 levels, as a result of increased renal catabolism and/or the removal by the hepatic holo receptor (HDL holoparticle endocytosis), and the generation of sLDL particles (Barter et al., 2003, Chapman et al., 2010, Charles and Kane, 2012, Franceschini et al., 2011). Cholesterol ester transfer under these conditions may be considered pro-atherogenic (Chapman et al., 2010, Dullaart and Sluiter, 2008).

2.2.3.3 Low-density lipoprotein

The principle atherogenic lipoprotein, LDL, measured clinically by its cholesterol concentration, is the primary lipid measure used in CVD risk assessment. Consisting of about 50% cholesterol (free and CE, Table 2.8), LDL carries the majority of serum cholesterol (typically 60 to 70% of TC) (National Cholesterol Education Program (NCEP) Expert Panel, 2002, Sacks, 2011). As separating LDL-C from VLDL is quite involved, a direct measurement is not normally made. Instead, an estimate is derived from other lipid measurements (LDL-C = TC - HDL-C - (TG/2.22) mmol/L). This estimate has been shown to be robust if subjects are fasted and/or subjects do not have hypertriglyceridaemia (ratio of total TG to VLDL is altered), which may result in an underestimation of LDL-C (Sacks, 2011).

The relationship between CVD risk and elevated serum LDL-C is well established, with multiple lines of evidence derived over the years from epidemiological studies, animal and laboratory investigations, genetic studies and intervention trials. As a result, reduction of LDL-C has been the primary target of cholesterol-lowering therapy (National Cholesterol Education Program (NCEP) Expert Panel, 2002). Currently, statins (inhibitors of 3-hydroxy-3-

methylglutaryl coenzyme A (HMG-CoA) reductase, required for cholesterol biosynthesis) with their proven efficacy and patient tolerability are used as first-line drug therapy to reduce LDL-C levels in most patients (Brautbar and Ballantyne, 2011, National Cholesterol Education Program (NCEP) Expert Panel, 2002). A 2005 meta-analysis, of data from 14 randomised statin trials in over 90,000 participants, showed that a 1 mmol/L reduction in LDL-C concentrations was associated with a 19% reduction in CHD mortality and a 12% decrease in all-cause mortality (Baigent et al., 2005).

Elevated LDL-C is recognised as a key contributor to the initiation and progression of atherosclerosis (Badimon and Vilahur, 2012). As discussed in section 2.2.1.4, hypercholesterolaemia has been shown to decrease NO bioavailability, which can lead to endothelial dysfunction, the preceding step to the entry of LDL into the arterial intima. Entry into the intima is thought to depend mainly on sustained plasma levels of LDL, and this is influenced by the lengthy time of LDL in the circulation (1.5 to 4 days) (Badimon and Vilahur, 2012, Sacks, 2011). Once in intima, proteoglycans (glycosylated proteins) interact with cholesterol-rich lipoproteins such as LDL, leading to their retention and the formation of aggregated LDL. Retained LDL particles can undergo modification, for example, oxLDL is generated in the presence of oxidants (Badimon and Vilahur, 2012). Oxidised LDL participates in several atherogenic cellular responses, contributing to vascular inflammation, further inhibiting NO, and two scavenger receptors (type A scavenger receptor (SR-A) and the type B scavenger receptor (CD36)) expressed on macrophages, have been shown to have a higher affinity for it than native LDL (Badimon and Vilahur, 2012, Davis and Wagganer, 2006).

There is growing evidence that not only the quantity of LDL (particle number), but also the quality (particle size), impacts CVD risk. Low-density lipoproteins are a heterogeneous class of lipoprotein particles which, depending on their classification, comprise three or four major subclasses, which vary in size, density and physiochemical composition, and display different metabolic behaviours and degrees of atherogenicity (Hirayama and Miida, 2012, Rizzo and Berneis, 2007). Increased levels of sLDL have been identified as being

associated with increased risk for CVD (National Cholesterol Education Program (NCEP) Expert Panel, 2002, Rizzo and Berneis, 2007).

The prevalence of sLDL is influenced by genetics, gender and age (30-35% in adult men), and environmental factors, for example, a low fat, high carbohydrate diet has been shown to increase the prevalence of sLDL. Low-density lipoprotein size has been shown to inversely correlate with TG levels and positively correlate with HDL-C concentrations (Lamarche et al., 1999, Rizzo and Berneis, 2007). The 'atherogenic lipoprotein phenotype' describes a form of dyslipidaemia characterised by the combination of elevated sLDL and TG and decreased HDL-C (National Cholesterol Education Program (NCEP) Expert Panel, 2002, Rizzo and Berneis, 2007). The phenotype is associated with obesity, particularly abdominal obesity, insulin resistance (IR) and physical inactivity and is a risk factor for metabolic syndrome (Lamarche et al., 1999, National Cholesterol Education Program (NCEP) Expert Panel, 2002).

Under hypertriglyceridaemic conditions, as discussed, the increased concentration of large VLDL, promotes TG enrichment of both HDL and LDL by CETP. Triglyceride-enriched LDL is remodelled by HL, which hydrolyses TG and phospholipids from the lipoprotein to form sLDL.

Based on experimental studies, a number of mechanisms to explain the increased atherogenicity of sLDL compared to larger LDL have been proposed. These include: that their size means that they can more readily penetrate arterial walls; they have a lower affinity for LDLR (decreasing their clearance from plasma); but a higher affinity for proteoglycans; and they are at increased risk of oxidation (the consequences of which are discussed above), as a result of altered properties of the surface lipid layer, including decreased anti-oxidants, such as vitamin E (Hirayama and Miida, 2012, Rizzo and Berneis, 2007).

2.2.3.4 High-Density Lipoprotein

High-density lipoprotein is the smallest of the lipoproteins, but in contrast to LDL, it is considered to be protective against atherosclerosis (Badimon and Vilahur, 2012). HDL carries 20-30% of total plasma cholesterol and HDL-C

levels have been shown to be inversely correlated with CHD risk (National Cholesterol Education Program (NCEP) Expert Panel, 2002). It is estimated that each 0.1 mmol increase in HDL reduces CHD risk by 8 to 15% (Gordon et al., 1989, Turner et al., 1998).

Considerable variability exists in HDL-C concentrations in the general population, with genetics accounting for between 40 to 60% of the variation, and a range of environmental and metabolic factors accounting for the rest (Peloso et al., 2010). In a cross-sectional study of nearly 15,000 dyslipidaemic (DL) subjects, 8 out of 16 clinical and biological factors measured were identified as showing a significant and independent association with HDL-C levels. Age and alcohol consumption had positive correlations, and male gender, TG level, waist circumference (WC), smoking, homeostasis model assessment (HOMA) index and hs-CRP had negative correlations, with three factors, degree of triglyceridaemia, age and sex accounting for 37% of the total variability (Hansel et al., 2006). Other factors associated with low HDL-C include physical inactivity and very high carbohydrate intakes (National Cholesterol Education Program (NCEP) Expert Panel, 2002). As discussed in the previous section 2.2.3.3, a low HDL-C may reflect the presence of an atherogenic phenotype, with elevated levels of TG and the associated increase in CETP activity the likely driver.

Structure and biosynthesis

As with VLDL and LDL particles, circulating HDL particles are heterogeneous, varying in shape, size, density, apolipoprotein composition and anti-atherogenic properties (Franceschini et al., 2011, Joy and Hegele, 2008). Table 2.8 shows the characteristics of the two major subpopulations based on density, HDL_2 (large, light, CE-rich) and HDL_3 (small, dense, protein-rich, CE-poor). In addition to a range of apolipoproteins, HDLs also contain several enzymes and transfer proteins, including CETP, LCAT and phospholipid transfer protein (PLTP), and anti-oxidant molecules, such as paraoxonase/arylesterase 1 (PON1). In contrast to LDL, where one apoB100 molecule remains integrated with the LDL particle, all HDLs apoliproteins are readily exchangeable, and apoA1 can even

leave to interact with the ABCA1 transport protein on its own (Franceschini et al., 2011, Joy and Hegele, 2008, Navab et al., 2011).

Figure 2.11 shows the main synthesis and metabolism pathways for HDL.

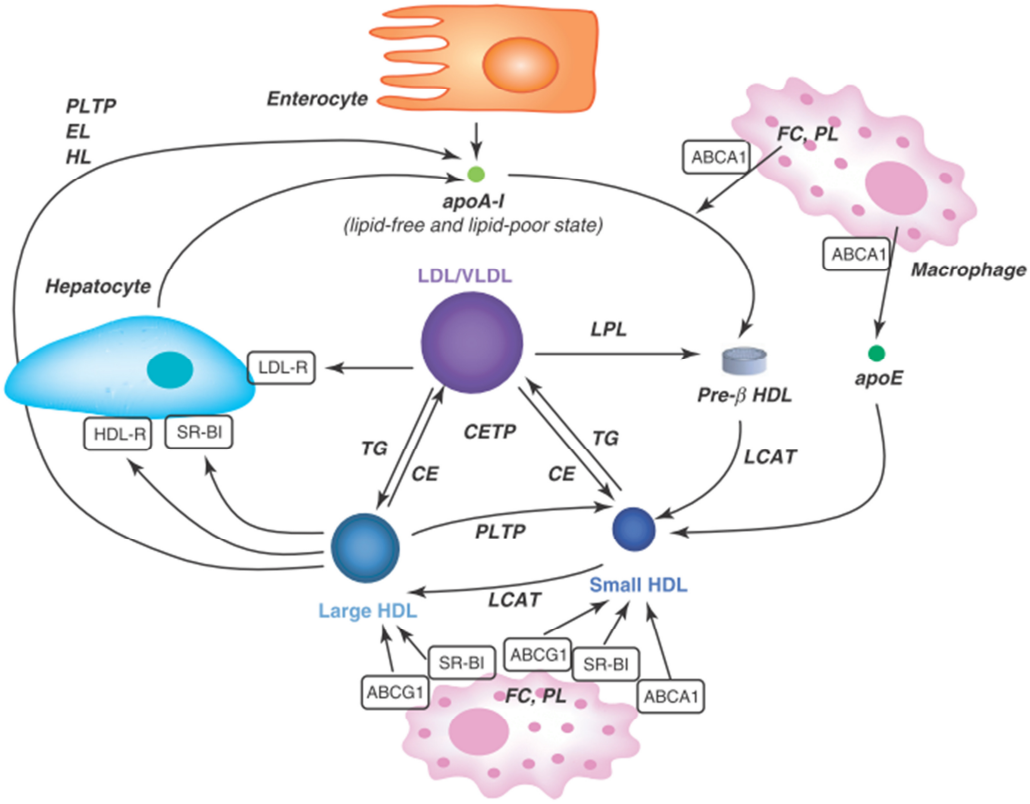

Figure 2.11: Main synthesis and metabolism pathways for HDL

ABCA1: adenosine triphosphate-binding cassette-A1; ABCG1: adenosine triphosphate binding cassette sub-family G; apoA-1: apolipoprotein A1; CE: cholesterol ester; CETP: cholesteryl ester transfer protein; EL: endothelial lipase; FC: free cholesterol; HDL: high-density lipoprotein; HDL-R: unidentified HDL holoparticle receptor; HL: hepatic lipase; LDL: low-density lipoprotein; LDL-R: LDL receptor; LPL: lipoprotein lipase; PL: phospholipid; PLTP: phospholipid transfer protein; SR-BI: scavenger receptor class BI; VLDL: very low-density lipoprotein.
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Apolipoprotein A1 is synthesized primarily by the liver, with a small amount also synthesized by the small intestine. It is then secreted either as lipid-free apoA1 (up to 5% of total plasma apoA1) or as a component of VLDL or CM. Lipid-free apoA1 can also be generated as a result of remodelling of spherical HDL by CETP, PLTP and HL. Lipid-free apoA1 acquires cholesterol and phospholipids by interacting with ABCA1 transporters present in various cells, including arterial macrophages, to form small, discoidal preβ-HDL. Alternatively, lipolysis of the TGRL by LPL causes dissociation of some surface components,

including apolipoproteins, phospholipids and cholesterol, which can combine to form preβ-HDL (Camont et al., 2011, Davis and Wagganer, 2006, Franceschini et al., 2011, Rye et al., 2009).

Acting on preβ-HDL, LCAT, for which apoA1 is a cofactor, converts cholesterol and lecithin into CEs and lysolecithin. The hydrophobic CEs split the bilayer and move to the core, which leads to the creation of a mature, spherical small HDL (HDL_3). The depletion of cholesterol at the surface of the lipoprotein creates a concentration gradient down which free cholesterol moves via cellular efflux mediated pathways or from other lipoproteins, ensuring a constant supply for LCAT to esterify. The continued acquisition of cholesterol means that small HDL are converted into less dense, large HDL (HDL_2) (Camont et al., 2011, Davis and Wagganer, 2006, Franceschini et al., 2011, Rye et al., 2009). High-density lipoprotein is constantly being remodelled, changing the size, shape, composition and surface charge, with components transferred, recycled and removed from circulation, with distinct HDL particles existing for maybe only a few seconds. The SR-BI mediated uptake of CE by hepatocytes, and the CETP mediated transfer of CE and TG between lipoproteins and consequent effects on HDL have already been discussed. Cholesteryl ester transfer protein can also transfer lipids between HDL subspecies. Additionally, the lipid content of HDL can be further altered by the actions of PLTP, and HL and endothelial lipases (Camont et al., 2011, Goldberg and Hegele, 2012, Rader and Daugherty, 2008, Navab et al., 2011, Rye et al., 2009).

Functions of HDL

The ability of HDL to promote cholesterol efflux from cholesterol-laden macrophages (foam cells) and deliver it to the liver for biliary excretion is considered to be the main mechanism by which it protects against atherosclerosis. A number of non-RCT functions have also been proposed, with differences in capacity observed between subspecies. The HDL_3 species appears to exert superior anti-oxidant, anti-inflammatory, and anti-thrombotic effects compared to the HDL_2 species, likely as a result of differences in apoliprotein and phospholipid content. However, the clinical relevance of many of these activities remains to be determined and, as will be discussed in the

next section, there is evidence that under certain conditions these functions become compromised (Badimon and Vilahur, 2012, Camont et al., 2011, Navab et al., 2011, Joy and Hegele, 2008).

There are a number of important non-RCT roles of HDL relating to areas covered in this literature review. High-density lipoprotein has been shown to be an effective anti-oxidant, including protecting LDL from oxidation and removing oxidised lipids from cell membranes and other lipoproteins. Its anti-oxidant activities are mediated by apolipoproteins, including apoA1, apoA2, apoA4 and apoE and enzymes such as PON1 and LCAT (Camont et al., 2011, Joy and Hegele, 2008, Navab et al., 2011).

By inhibiting the formation of lipid oxidation products, there is also a reduction in the associated inflammatory response. Experimental evidence suggests that HDL may also be directly anti-inflammatory, for example by inhibiting cytokine-induced endothelial VCAM-1 and ICAM-1 expression (Badimon and Vilahur, 2012, Camont et al., 2011, Navab et al., 2011).

Maintenance of endothelial and vascular function is also influenced by HDL. Although the effects are likely mainly due to its anti-inflammatory and anti-oxidant properties, independent mechanisms may also exist. HDL has been shown to influence NO levels, by both inhibiting NADPH oxidase activity through it anti-oxidative activity and upregulating NO production (Badimon and Vilahur, 2012, Camont et al., 2011, Navab et al., 2011).

Quantity versus quality of HDL

The strong epidemiological evidence that low HDL-C concentrations are associated with increased CHD mortality and morbidity prompted interest in research focussed on strategies to increase HDL-C, and also to address the residual risk observed in patients on statin therapy (National Cholesterol Education Program (NCEP) Expert Panel, 2002, Badimon and Vilahur, 2012). However, so far, drugs like CETP inhibitors, which elevate HDL levels, have been shown to be ineffective in regards to cardiac outcomes and even deleterious, with unacceptable side-effects (Wright, 2013). These results

suggest, along with other accumulating evidence that, as well as HDL quantity, HDL function and quality is also important.

In 1995, Lenten et al. demonstrated differences in HDLs anti-inflammatory capacity pre- and post- cardiac surgery, with HDL losing its protective effects during the post-surgery acute-phase response stage. In the acute phase, apoA1, PON1 and platelet-factor activating acetylhydrolase levels decreased, while HDL-associated acute phase reactants, serum amyloid and coeruloplasmin increased (Van Lenten et al., 1995). Further studies have supported the hypothesis that during inflammation HDL may lose its protective capacity and become dysfunctional (Badimon and Vilahur, 2012, Navab et al., 2011).

In a recent study, researchers identified a subgroup of subjects with high HDL-C levels, who showed increased risk for incident CVD. These subjects also had high levels of CRP. The researchers suggest this increased risk may be associated with impaired HDL remodelling as a result of inflammation (Corsetti et al., 2010).

There is increasing evidence that post-translational modification of apoA1, such as by oxidation or glycation (glycated apoA1 has been seen in diabetics), results in conformational changes that impair HDL production and function (Fisher et al., 2012). It has been suggested that the myeloperoxidase (part of the innate defence system), mediated pathway, might be an important source of oxidants (Fisher et al., 2012, Shao et al., 2012).

Experimental studies have demonstrated that oxidative modifications to lipid-free apoA1 results in inhibition of the ABCA1 cholesterol efflux pathway, thereby reducing the generation of preβ-HDL, while oxidation of lipid-associated apoA1, results in reduced LCAT activity, and impaired particle maturation. These consequences all impact on HDLs ability to remove cholesterol from macrophages and therefore to be atheroprotective (Shao et al., 2012, Navab et al., 2011, Fisher et al., 2012).

Future studies will no doubt expand our knowledge of the functionality or dysfunctionality of HDL and its subpopulations. However, the findings to date

suggest that, in assessing an individual's risk, measurement of function is also important in addition to measurement of HDL-C concentration (size of the HDL pool). Unfortunately, at present no routine diagnostic assays are available to test HDL functionality (Fisher et al., 2012).

2.2.3.5 Triglycerides

As hypertriglyceridaemia commonly occurs in conjunction with obesity and IR, the rates of hypertriglyceridaemia have also increased with the current increase in the prevalence of these conditions (Miller et al., 2011, Talayero and Sacks, 2011). Although an association between elevated TG levels and CVD risk has long been identified, there has been on-going debate as to whether the relationship is causal or whether elevated TG simply represents a biomarker of risk (Kannel and Vasan, 2009, Miller et al., 2011, Talayero and Sacks, 2011). After reviewing all the current evidence, the AHA in 2011 issued a scientific statement concluding that 'triglyceride is not directly atherogenic but represents an important biomarker of CVD risk because of its association with atherogenic remnant particles and apo CIII' (Miller et al., 2011, p2318).

Very low-density lipoprotein synthesis and secretion are increased in obese individuals, as a result of two main factors, increased FFA availability and impaired insulin action. In addition to the generation of sLDL and small HDL particles, CETP generates VLDL that are rich in cholesterol. These particles are then partially hydrolysed by LPL, resulting in the formation of TGRL remnants. These smaller cholesterol-enriched particles have been shown to be taken up into macrophages to form foam cells, in a similar manner to modified LDL (Miller et al., 2011, Talayero and Sacks, 2011).

A shift from an apoE to an apoC3 dominated system has been seen in hypertriglyceridaemic subjects (Zheng et al., 2010). Apolipoprotein C3 is implicated in the delayed catabolism and clearance of TGRL that is seen in hypertriglyceridaemia, by inhibiting endothelial-bound LPL, and apoB and apoE mediated binding of lipoproteins to hepatic receptors (Ooi et al., 2008, Talayero and Sacks, 2011).

In vitro studies have also shown that VLDL and LDL that contain apoC3 promote a range of pro-atherogenic responses, including activation of endothelial cells and monocytes to produce adhesion molecules, impairment of eNOS production and activation of pro-inflammatory transcription factors such as NF-κβ (Miller et al., 2011, Talayero and Sacks, 2011).

Causes identified as contributing to elevated TG include: genetic factors; excess adiposity, particularly visceral adiposity; lifestyle factors including physical inactivity, cigarette smoking, high alcohol intake and very high carbohydrate diets; and diseases such as type 2 diabetes (Miller et al., 2011, National Cholesterol Education Program (NCEP) Expert Panel, 2002).

2.2.3.6 Lipoprotein ratios

In an attempt to better capture the interactions between various lipid fractions; various ratios have been proposed over the years to improve the accuracy of CVD risk assessment (Millan et al., 2009).

The TC/HDL-C ratio is considered to be a measure of the ratio of pro-atherogenic to anti-atherogenic lipoproteins, and several studies have shown that it is a stronger predictor of risk than LDL-C alone. In one recent, large meta-analysis involving data from almost 900,000 adults, of all the simple indices investigated, TC/HDL-C ratio was found to be the strongest predictor of IHD mortality, and more than twice as informative than the least informative index, which was TC (Lewington et al., 2007). An elevated TC concentration indicates the presence of atherogenic lipoproteins, and a low HDL-C concentration correlates with various risk factors associated with the metabolic syndrome (Millan et al., 2009, National Cholesterol Education Program (NCEP) Expert Panel, 2002). A one unit change in the TC/HDL ratio (after adjustment for other factors including age, smoking, history of angina, hypertension, diabetes and assignment to aspirin group) has been found to be associated with a 53% change in myocardial infarction risk (Stampfer et al., 1991).

In 2002 the Adult Treatment Panel (ATP) III incorporated the TC/HDL-C ratio into its suggested risk assessment. However, it didn't define it as a treatment target, preferring that specific lipoprotein fractions remain the priority (National

Cholesterol Education Program (NCEP) Expert Panel, 2002). In NZ, the New Zealand Guidelines Group in 2003, defined the optimum TC/HDL-C ratio as <4.5 (New Zealand Guidelines Group, 2003) and the ratio was added to the assessments used in the 2008/9 National Nutrition Survey in NZ. It is also the single value used in the NZ Guidelines Group, Cardiovascular risk charts (New Zealand Guidelines Group, 2012).

Another index of pro-atherogenic and anti-atherogenic lipoproteins is the apoB/apoA1 ratio. Rather than measuring cholesterol, this ratio provides a means to quantify the concentration of lipoproteins. All atherogenic lipoproteins carry one apoB molecule, LDL, VLDL and IDL. Unlike LDL-C, which can underestimate the number of sLDL particles, individuals carrying an equivalent amount of cholesterol, but a greater number of sLDL, will have a higher apoB concentration. Thus apoB is considered a more accurate measure of risk of atherogenic particles, and most studies have shown that the apoB/A1 ratio is an even better discriminator of CVD than the TC/HDL-C ratio (Arsenault et al., 2011, Walldius and Jungner, 2006).

The association between apoA1 and HDL-C is not as clear, and it has been demonstrated that increasing levels of TG have a differential impact on HDL-C (greater reduction) and apoA1 (Tremblay et al., 2007). The number of apoA1 proteins present in HDL particles can also vary, therefore it is not possible to count HDL particles in the same way as with LDL. At present, the predictive value of apoA1 is considered to be equivalent to that of HDL-C (Arsenault et al., 2011).

The TG/HDL-C ratio can be considered a summary measure of elevated TGs, low HDL-C or both. Elevated TG and low HDL-C concentrations, as discussed, indicate the presence of atherogenic dyslipidaemia, which is associated with metabolic syndrome (Miller et al., 2011). As such, the ratio is considered by some researchers as a surrogate marker for IR, describing an altered lipid and carbohydrate metabolism (Cordero et al., 2009). However, the relationship with IR has been shown to differ with ethnicity. Although it has been shown to be a good predictor of IR in Europeans, Chinese, and Aboriginal Canadians, it was not for those of South Asian or African American origin (Gasevic et al., 2012,

Kim-Dorner et al., 2010, Miller et al., 2011). The ratio has also been linked to the presence of sLDL and CVD incidence, and some studies have shown that it has improved predictive power compared to LDL-C (Miller et al., 2011).

In summary, lipid metabolism involves a complex set of pathways engaging the co-ordinated action of an array of components, including lipoproteins, apolipoproteins, binding proteins, enzymes and receptors. The outcome of these interactions determines an individual's lipid profile, which in turn may influence their CVD risk, with increased levels of LDL-C considered to be atherogenic and increased levels of HDL-C considered to be atheroprotective. In determining CVD risk, there is increasing evidence that the balance between atherogenic and atheroprotective lipoproteins is more important than a simple increase in the number of atherogenic lipoproteins. Therefore, measuring ratios that reflect this balance gives a more complete measure of risk than measuring an individual lipoprotein subfraction. Further, these interactions can be modified by other risk factors, such as obesity. In the case of obesity, it may firstly lead to the development of the atherogenic lipoprotein phenotype and, secondly, the resultant increase in inflammation can impact on HDL function.

2.2.4 Obesity and inflammation

Proposed mechanisms through which increasing adiposity leads to the development of metabolic dysfunction and how this dysfunction can impact on CVD risk will be examined in this section. In addition, problems in the measurement of obesity, and markers used to measure inflammation will be discussed.

Framingham researchers were one of the first groups to identify obesity as an independent risk factor for CVD, reporting their findings in 1983 (Hubert et al., 1983). Additionally, obesity increases the prevalence of a number of other risk factors, including dyslipidaemia, type 2 diabetes, impaired glucose tolerance and hypertension (Perez Perez et al., 2007, Van Gaal et al., 2006). However, it was not until the early 1990s that interest in inflammation as a possible link between obesity and the development of obesity related disorders increased, when it was demonstrated that TNF-α levels were elevated in an obese rodent model, and that IR was improved by neutralising this cytokine (Hotamisligil et al., 1993, Rocha and Folco, 2011). Currently, a substantial amount of evidence supports obesity as a cause of a chronic-low grade inflammatory state that promotes systemic metabolic dysfunction, which contributes to the development of these metabolic disorders (Gregor and Hotamisligil, 2011, Mathieu et al., 2010, Ouchi et al., 2011)

Adipose tissue was viewed until relatively recently as a passive long-term storage reservoir for TG (energy). It is now recognised as an active endocrine and paracrine organ that secretes a wide range of cytokines (cell-signalling protein molecules) and other bioactive substances, collectively termed adipokines (Ouchi et al., 2011, Wang and Nakayama, 2010, Van Gaal et al., 2006). These adipokines are not only critical in maintaining adipose tissue homeostasis, but some have been shown to influence many of the CVD risk factors, including lipid levels, IR, atherosclerosis and body weight homeostasis (Ouchi et al., 2011, Van Gaal et al., 2006).

Adipose tissue is mainly located in subcutaneous and visceral depots, with other sites of accumulation including organs such as the heart and kidneys (Ouchi et al., 2011). Although total body fatness is a significant risk factor for

CVD, not every obese person is at high risk. Risk increases, however, if there is an excess of intra-abdominal or visceral fat (Tchernof and Despres, 2013, Van Gaal et al., 2006). Differences have been identified between the two main depots, with visceral adipocytes being shown to be more metabolically active, showing greater lipolytic and lipogenic behaviour and secreting a more pro-inflammatory cytokine profile. In comparison, subcutaneous adipocytes are less susceptible to lipolysis and have a different secretion profile, with better adiponectin (anti-inflammatory adipokine) secretory ability (Baglioni et al., 2012, Wronska and Kmiec, 2012). In their recent study, Baglioni et al. (2012) suggests that these differences originate from 'differences in the adipose stem cell' (Baglioni et al., 2012).

Adipose tissue comprises a number of cell types (Figure 2.12).These include adipocytes which are the main cellular component (energy storage and endocrine activity); vascular cells which give rise to blood vessels (delivery of oxygen and nutrients and distribution of adipokines); immune cells including macrophages and lymphocytes (immune status); fibroblasts (mechanical support) and precursor cells (e.g. pre-adipocytes) (Ouchi et al., 2011).

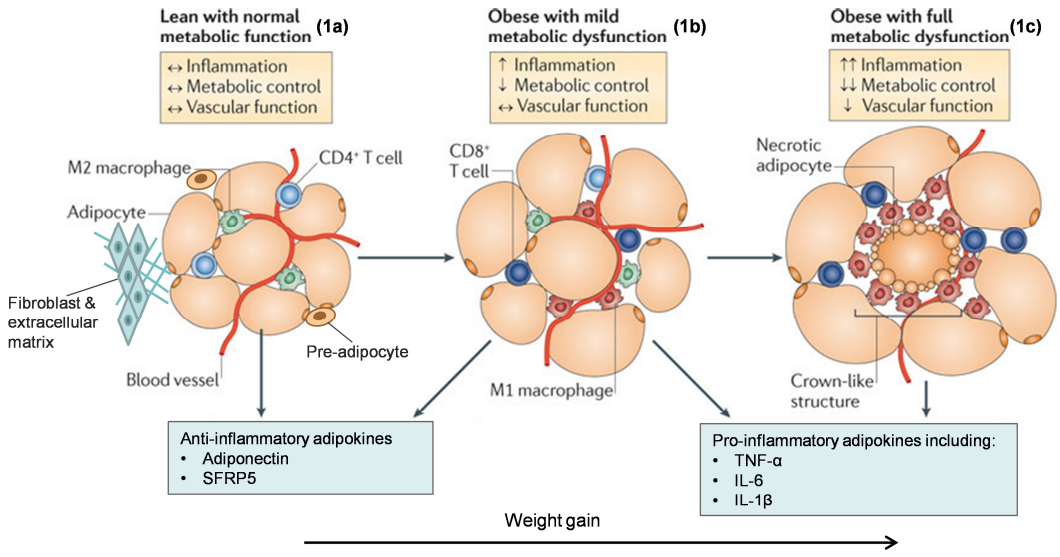

Figure 2.12: Changes in adipose tissue with increasing adiposity

SFRP5: Secreted frizzled-related protein 5; TNF-α: tumour necrosis factor alpha; IL-6: interleukin 6; IL-1β: interleukin 1 beta

Adapted from Nat Rev Immunol, 11 (2), Ouchi, N., Parker, J. L., Lugus, J. J. & Walsh, K., Adipokines in inflammation and metabolic disease, 85-97 © (2011) with permission from Macmillan Publishers Ltd.

The review by Ouchi et al. (2011, p90) suggests that 'adipose tissue can be described by at least three structural and functional classifications: lean with normal metabolic function; obese with mild metabolic dysfunction and obese with full metabolic dysfunction.' Expectedly, the recent global rise in excess body weight has seen a shift to more people having the latter two types.

Besides absolute fat quantity, it has become evident that the cellular composition and functionality of adipose tissue is also important in driving an individual towards a metabolically dysfunctional state. Under conditions of excess energy intake (and decreased energy expenditure), changes in the cellular composition of adipose tissue are initiated and a chronic inflammatory state is induced (Gregor and Hotamisligil, 2011, Ouchi et al., 2011). There is tissue expansion in the metabolically intermediate state (Figure 2.12, 1b), as adipocytes undergo hypertrophy from increased TG storage and a greater infiltration of macrophages. Other changes compared to the lean state include a recruitment of the pro-inflammatory M1 macrophages. In lean tissue, macrophages express markers of an M2 (or anti-inflammatory) activation state which downregulates the production of pro-inflammatory cytokines and upregulates the synthesis of the anti-inflammatory cytokine, IL-10. Additionally, CD8+ T cells (natural killer cells) and mast cell numbers also increase and may contribute to the inflammatory environment. There is a change from the preferential production of anti-inflammatory adipokines, such as adiponectin, to the generation of pro-inflammatory adipokines, cytokines such as TNF-α and IL-6 and (Gregor and Hotamisligil, 2011, Ouchi et al., 2011). This imbalance in expression of anti- and pro-inflammatory cytokines is thought to play an important part in the metabolic dysfunction which occurs with excess adipose tissue and the subsequent development of obesity-related complications (Ouchi et al., 2011).

Further expansion and infiltration ultimately leads to full metabolic dysfunction. In the third, fully dysfunctional, metabolic state, additional changes can be observed (Figure 2.12, 1c). Necrotic adipocytes and crown-like structures, aggregates of single or fused M1 macrophages around dead adipocytes, are present. There is a reduction in the density of capillaries (rarefaction) leading to localised hypoxia, which may further contribute to an inflammatory state. (Ouchi

et al., 2011). With weight gain, remodelling of the extracellular matrix is required to allow adipose tissue expansion. It has also been suggested that there is an upregulation of some extracellular matrix components in metabolically dysfunctional adipose tissue, which may further contribute to metabolic dysregulation, by interfering with adipose tissue expansion (Khan et al., 2009). Compared to the metabolically intermediate state, those in the fully dysfunctional state have worse metabolic parameters, increased inflammatory marker expression and decreased vascular function (Ouchi et al., 2011).

Current evidence suggests that this obesity-induced inflammation is initiated and orchestrated by metabolic cells, such as adipocytes, in response to nutrient overload. Although the actual stimulus (initiating factor) remains unclear, metabolic stress signals engage inflammatory intracellular signalling pathways, with possibly multiple signalling pathways activated. Three kinases, namely inhibitor of κ kinase (IKK), c-jun N-terminal kinase (JNK) and protein kinase R (PKR), have all been identified as important intracellular contributors, as members of pro-inflammatory cascades (Gregor and Hotamisligil, 2011, Rocha and Folco, 2011). The kinases can activate transcription factors, NF-κβ, interferon regulatory factor and activator protein-1, resulting in upregulation of inflammatory mediator gene expression. These pathways mediate a modest, low-level induction of inflammatory cytokines, including TNF-α, IL-6 and IL-1β. Other components of the innate immune system, including Toll-like receptors and the inflammasone (an immune sensor) have also been shown to be activated in obese tissues (Gregor and Hotamisligil, 2011).

Gregor and Hotamisligil (2011) propose that a ‘pulsatile inflammatory response’ occurs in metabolic cells during feeding/fasting cycles. In lean individuals, once the nutrients are metabolised, the inflammatory response resolves but, under conditions of excess feeding, the response becomes more frequent and intense and resolution less efficient. With time, these signals amplify and, once the level of inflammatory response reaches a certain threshold, immune cells such as macrophages and T cells are recruited and activated (Gregor and Hotamisligil, 2011). This infiltration further drives the tissue towards a pro-inflammatory environment, with cross-talk between macrophages and adipocytes, and the increase in pro-inflammatory adipokines, promoting positive feedback loops and

inhibition of normal metabolic cell function. The inflammatory state becomes chronic, with no apparent resolution. Adipokines can also spill-over into the circulation, leading to modest increases in systemic levels of cytokines or acute-phase reactants, such as CRP, which can promote effects elsewhere (Gregor and Hotamisligil, 2011, Mathieu et al., 2010, Rocha and Folco, 2011).

In addition to their involvement in mediating an inflammatory response, JNK, IKK and PKR are implicated in inhibiting insulin signalling. In response to inflammatory signals, (e.g. TNF-α or IL-6), or nutrient signals (e.g. FFAs) the activated kinases phosphorylate the serine residues of insulin receptor substrate 1 (IRS-1). This disrupts the insulin receptor cascade and provides a link between inflammation and IR (Gregor and Hotamisligil, 2011, Rocha and Folco, 2011, Mathieu et al., 2010).

There are many proposed mechanisms linking obesity and CVD, with increasing evidence that inflammation plays a central role in both conditions. Most of the mechanisms relating to both quantitative and qualitative changes to serum lipids, and the subsequent impact on atherosclerosis have previously been discussed in this review. These include how the increased flux of FFAs and impaired insulin action lead to increased synthesis and secretion of large VLDL, and the ensuing consequences. These include the shift of the normally anti-atherogenic CETP-mediated pathway to a more pro-atherogenic one, with the generation of sLDL and HDL, and cholesterol rich VLDL. Further, HDL may become dysfunctional through modifications and remodelling, affecting its atheroprotective abilities. It has been shown that adipose tissue can synthesise angiotensinogen, which provides a link between obesity and hypertension by contributing to RAS activation. Further, ANG II appears to interfere with the development of preadipocytes, thereby promoting an increase in large, dysfunctional adipocytes (Mathieu et al., 2009). Continued research will undoubtedly clarify our understanding of inflammation and its role in these conditions, and may lead to strategies that could counteract the effects.

2.2.4.1 Measuring obesity

Obesity is defined as an excess accumulation of body fat (BF). One of the most frequently used methods to assess obesity is the BMI (weight in kg divided by

the square of height in metres). Using this index, overweight is defined as a BMI ≥25 kg/m^2 and obese as a BMI ≥30 kg/m^2 (World Health Organization, 2000). A progressive increase in risk of conditions such as CVD, type 2 diabetes, dyslipidaemia and hypertension has been shown with increasing BMI. However, while easy to measure, it does not necessarily provide an accurate measure of body composition or BF distribution (Tchernof and Despres, 2013).

As discussed in the previous section, intra-abdominal or visceral fat has been identified as being particularly unsafe. Body mass index fails to identify metabolically obese, normal weight (MONW) individuals who have a low BMI, but high level of visceral fat, and metabolically healthy obese (MHO) individuals who have a high BMI, but low of level visceral fat (Tchernof and Despres, 2013).

A recent study highlights these issues. Taking measures of BMI and % BF (measured by BODPOD, air displacement plethysmography), researchers firstly assessed the degree of misclassification of obesity using BMI compared to % BF, and secondly evaluated CVD and metabolic risk in different groups. Briefly, they found 80% of subjects classified as overweight by BMI had a % BF in the obese range. Subjects classified as non-obese by BMI (<30 kg/m^2) but obese by % BF (≥25% men, ≥35% women) exhibited similarly high BP, glucose, insulin, CRP, TG and LDL-C to those in a % BF-matched group, classified as obese by both methods. In many cases the levels of these risk markers were above established cut-offs for increased CVD risk (Gomez-Ambrosi et al., 2012).

Two other anthropometric measures that give an indication of abdominal fat accumulation are WC (≥94 cm Caucasian men) and waist/hip ratio (WHR >1.0 in men). However, gender and ethnic differences in relative risk mean that ethnic and sex-specific cut-offs have had to be developed (World Health Organization, 2000, Alberti et al., 2009). Various studies have shown that WC and/or WHR are better predictors of CHD than BMI (Tchernof and Despres, 2013). Elevated WC is a criterion for the diagnosis of metabolic syndrome (Alberti et al., 2009). It is suggested that, as WC and BMI complement each other, in a clinical setting both should be used to identify overweight/obese

individuals at higher cardiometabolic risk. For example, a sedentary post-menopausal woman who has an increasing WC, without gaining weight, is likely to be gaining abdominal fat and losing lean body mass (Tchernof and Despres, 2013).

2.2.4.2 C-reactive protein

C-reactive protein is an acute phase protein, blood levels of which rise rapidly in response to infections and tissue injury. First described by Tillett and Francis in in the early part of the 20th century, it received its name as a result of its ability to precipitate the C-polysaccharide of *Streptococcus pneumonia*. C-reactive protein is a key component of the acute phase immune response, recognising pathogens and activating the complement system. In more recent times, in addition to its protective role, it has been implicated as a contributor to inflammatory diseases such as atherosclerosis (Abd et al., 2011, Ferri et al., 2007, Nakou et al., 2008).

C-reactive protein is primarily synthesised by the liver, with its expression mainly regulated at the transcriptional level, predominantly by IL-6, but also to a lesser degree by TNF-α and IL-1β (Ferri et al., 2007, Nakou et al., 2008). Following an acute stimulus, plasma levels rise rapidly (up to 100-fold or more) peaking within 48 hours, then return to baseline within 1 to 2 days after removal of the stimulus (Abd et al., 2011, Ferri et al., 2007, Nakou et al., 2008).

Diseases and conditions that are associated with increases in plasma CRP include acute and chronic viral or bacterial infections, chronic inflammatory conditions, such as rheumatoid arthritis, and Crohn's disease, necrosis (e.g. myocardial infarction) and malignant tumours. In these conditions, the CRP levels can be as high as 100 mg/L (Nakou et al., 2008, Yeh and Willerson, 2003). In addition, abdominal obesity and adiposity have been found to strongly correlate with increased CRP levels. Associations have also been seen with other CVD risk factors, including cigarette smoking, physical inactivity, elevated BP, high TG, low HDL-C, increased blood glucose and metabolic syndrome (Pearson et al., 2003, Vohnout et al., 2011). As discussed in the previous section, these increases are modest compared to the increases seen with an

acute stimulus, and prior to the development of the high-sensitivity CRP (hs-CRP) test, were regarded to be in the normal range (Yeh and Willerson, 2003).

In 2002, a workshop on inflammatory markers and CVD convened by the CDC and the AHA recommended hs-CRP as the analyte of choice where an inflammatory marker is to be used in clinical or public health practice. C-reactive protein has a long half-life, is stable during storage, and has been reported to show little diurnal or seasonal variation (Pearson et al., 2003, Yeh and Willerson, 2003). Three relative risk categories were proposed: low, <1 mg/L; average, 1.0 to 3.0 mg/L; and high, >3.0 mg/L, which correspond to the approximate tertile values from aggregated population studies in adults. Patients with unexplained, marked elevation (>10 mg/L) should be assessed for non-cardiovascular causes (Pearson et al., 2003).

In a large meta-analysis published in 2010, CRP concentrations were found to be associated with the risk of CHD, IHD, stroke and vascular mortality. However, after adjustment for several conventional risk factors, including smoking, SBP, diabetes, BMI, TG and HDL-C, the associations were considerably weakened (Kaptoge et al., 2010). Studies such as this, alongside genetic studies, are proposed arguments for a lack of a causal role for CRP in atherogenesis (Anand and Yusuf, 2010).

Meanwhile, other researchers argue that CRP is an active participant in the development of atherosclerosis. Both CRP mRNA and protein have been found in atherosclerotic lesions. Experimental evidence, mainly from cell-culture studies, suggests that CRP may exert an array of pro-atherogenic effects, including inducing the expression of endothelial adhesion molecules (ICAM-1, VCAM-1 and selectin E); upregulating LOX-1; stimulating monocytes to release pro-inflammatory cytokines, such as TNF-α and IL-1β, through the upregulation of NF-κβ, and decreasing eNOS expression and bioactivity (Bisoendial et al., 2010, Nakou et al., 2008, Vohnout et al., 2011).

So, despite CRP being the most extensively studied marker of inflammation and its acceptance as an inflammatory predictor of future cardiovascular risk, there is still much debate as to whether CRP is a mediator or just a bystander (marker) of CVD (Bisoendial et al., 2010, Vohnout et al., 2011).

2.2.4.3 Cytokines

Cytokines are the mediators of inflammation. As part of the innate immune response, responding to an infectious organism invasion for example, inflammation is beneficial. However, if the process becomes chronic, it may become detrimental and lead to the development of inflammatory diseases, such as atherosclerosis (Hohensinner et al., 2011).

Over 90 cytokines and cytokine receptors have been identified, with a number identified as possibly being related to atherosclerosis, including TNF-α and IL-6 (pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokine) which will now be discussed briefly (Hohensinner et al., 2011, Myers et al., 2004).

Advances in assay techniques have led to the development of multiplex assays which can measure an array of cytokines with reasonably acceptable within- and between-run precision, although at present they remain research tools. Analyte stability is a limitation in a clinical setting, with the requirement that samples are rapidly processed and frozen to at least -70°C to avoid degradation (Myers et al., 2004).

Tumour necrosis factor alpha

Tumour necrosis factor alpha is produced mainly by monocytes and macrophages. It is a key cytokine in the initiation of the acute-phase reaction, promoting the secretion of other pro-inflammatory cytokines, such as IL-6 and IL-1β (Ouchi et al., 2011, Siasos et al., 2011). Levels of TNF-α have been found to be increased in both plasma and adipose tissue in obese individuals. Weight reduction is associated with reduced TNF-α expression, and it is also positively correlated with markers of IR (Ouchi et al., 2011). In addition to its possible role in inhibiting insulin signalling, experimental studies indicate that TNF-α may reduce NO bioavailability and activate NF-κβ, which has been shown to, for example, induce the expression of endothelial adhesion molecules (Lau et al., 2005). Finally, it is suggested that inflammatory induction of TNF-α and IL-6, may induce serum amyloid expression, which displaces apoA1 in HDL and potentially leads to the formation of dysfunctional HDL (Badimon and Vilahur, 2012).

Interleukin-6

As with TNF-α, IL-6 levels have been shown to positively correlate with BMI and may also be involved in the development of obesity related IR. Increased levels of CRP and its inducer IL-6 have been shown to be predictive of type 2 diabetes (Ouchi et al., 2011). Further, moderate associations have been seen with other CVD risk factors, such as smoking and dyslipidaemia, and long-term (average) levels have been shown to be associated with CHD (Danesh et al., 2008, Vohnout et al., 2011). Although produced in various tissues, it is estimated that adipose tissue is responsible for producing approximately a third of all circulating IL-6 (Ouchi et al., 2011). In atherosclerotic lesions, IL-6 is secreted by macrophages and SMCs. Sometimes called the 'messenger' cytokine, IL-6, as mentioned previously, is the key driver of the production of CRP and other acute-phase proteins in the liver (Mathieu et al., 2010, Vohnout et al., 2011).

Interleukin-10

Interleukin-10 is a potent anti-inflammatory cytokine that can counteract the activity/production of pro-inflammatory cytokines. It is produced by a variety of immune cells, including regulatory T cells and macrophages. The expression of IL-10 can be upregulated in macrophages and leukocytes by adiponectin (Gregor and Hotamisligil, 2011, Libby and Theroux, 2005, Kleemann et al., 2008). As a result of its anti-inflammatory properties, IL-10 is thought to be atheroprotective, and data from some *in vivo* studies suggest that it may inhibit foam cell formation, with one study showing that it can up-regulate ABCA1 expression, thereby decreasing cholesterol accumulation (McLaren et al., 2011, Kleemann et al., 2008).

Obesity initiates the development of a more chronic pro-inflammatory environment. This can be measured by an increase in inflammatory cytokines and acute-phase reactants, such as CRP. Although it is still undecided whether CRP is an active participant in the process or merely a marker, cytokines such as TNF-α have been shown in experimental studies to contribute to metabolic dysfunction by a number of means, for example, by decreasing NO bioavailability. A decrease in NO appears to be a key factor in the development of endothelial dysfunction, and additionally affects BP homeostasis, as will be discussed further in the next section.

2.2.5 Blood pressure

Before discussing the pathogenesis of hypertension and its role as a CVD risk factor, it is important to understand the haemodynamics, terminology and classification of BP.

Systemic arterial blood pressure, which, for the purposes of this review will be referred to simply as BP, is the pressure (force per unit area) exerted by circulating blood upon the walls of blood vessels. It is expressed in millimetres of mercury (mmHg). The pumping action of the heart generates blood flow through the blood vessels, with the pressure gradient within the vascular system providing the force that continues the movement through the systemic circulation. Pressure results from the resistance which opposes the blood flow, with the most resistance seen in the peripheral circulation. The three main sources of resistance are vessel length, blood viscosity and vessel diameter. Generally, while the first two remain fairly constant, frequent changes can occur in vessel diameter, which may significantly alter peripheral resistance (PR) (Marieb, 2004).

Blood pressure is usually measured in the brachial artery in a person's upper arm, and normally expressed in terms of the SBP, which is the maximum pressure after the left ventricle (LV) contracts expelling blood into the aorta, over DBP, which is the minimum pressure, when the aortic semi-lunar valve closes and the walls of the aorta and elastic arteries recoil, as a result of reducing blood volume (Marieb, 2004). The pulse pressure, which is felt as a pulsation in the artery during systole, is the difference between the two. The primary determinants of pulse pressure are stroke volume (SV), i.e. the volume of blood pumped out of the LV with each beat, and arterial compliance (distensibility). Pulse pressure increases either with an increase in SV, e.g. during exercise, or through a decrease in arterial compliance, as seen with aging. In young people, the proximal pulse travels at around 4-5 m/sec, whereas in the elderly it travels at 10-15 m/sec. This faster pulse wave velocity (PWV) is due to the increasing arterial wall stiffness that occurs with aging. When the blood is ejected into the proximal elastic arteries, it must ‘create space’ either by expansion of the vessels or displacing the blood previously

occupying the space. With age, there is less distensibility of the walls and so more blood is forced to move forwards (Levick, 2010).

It is currently accepted that SBP reaches a plateau in early adulthood, and then rises progressively from around 35 years of age, whereas DBP increases steadily to about 50 years of age, then starts declining. These differences result in a steeper rise in pulse pressure with age. There is a significant positive association between pulse pressure and cardiovascular events above 60 years of age (O'Rourke et al., 2011).

Another expression used to describe BP is the mean arterial pressure (MAP), which is the average pressure in the arteries during a single cardiac cycle. In the aorta, MAP is approximately halfway between SBP and DBP. In the brachial artery, the time-averaged mean is shifted in the diastolic direction and the MAP is approximately the DBP plus 1/3 of the pulse pressure (SBP-DBP). The MAP is determined by cardiac output (CO) x total peripheral resistance (TPR), where CO is the amount of blood pumped out of the LV in a minute (CO = heart rate (HR) x SV (L/min)) and TPR is a measure of the overall resistance (friction) encountered by blood as it flows through blood vessels (Levick, 2010).

Blood pressure homeostasis is vital in maintaining optimal tissue perfusion through a range of physiological conditions, such as changes in physical activity level, body position and blood volume. In the short-term, the arterial baroreflex can stabilise BP within seconds-to-minutes, by adjusting CO and the PR (vascular tone). In response to an acute rise in BP, for example, stimulation of the baroreceptors leads to a rise in vagal parasympathetic activity and a fall in cardiac sympathetic activity, resulting in a reduction in HR and heart contractility, and a resultant decrease in CO. Simultaneously, TPR is reduced, with a decrease in sympathetic vasomotor activity causing peripheral vasodilation (Brashers, 2012, Marieb, 2004, Levick, 2010).

Long-term regulation involves more complex interactions between neural, hormonal, and renal mechanisms. Nutrition can influence some of these interactions.

2.2.5.1 Blood pressure classification

Hypertension is defined as the chronic elevation of systemic arterial blood pressure. Hypertension is virtually symptomless and so clinical criteria, based on risk, have been established over the years. Currently, clinical hypertension is defined as a resting brachial BP measurement of SBP ≥140 mmHg and /or DBP ≥90 mmHg (Brashers, 2012, Levick, 2010). Hypertension maybe further classified, firstly into 3 subtypes depending on which component is raised: isolated diastolic hypertension (DBP ≥90 mmHg, SPB <140 mmHg), isolated systolic hypertension (ISH) (SBP ≥140 mmHg, DBP of <90 mmHg), and systolic-diastolic hypertension (SBP ≥140 mmHg, DBP ≥90 mmHg). Isolated diastolic hypertension and systolic-diastolic hypertension are generally more prevalent in younger adults, while ISH is the dominant form of hypertension in older adults (>50 years) (Franklin et al., 2005). However, the prevalence of ISH has been increasing in all age groups. A recent study using the NHANES III (1988-1994) and NHANES 1999-2004 data, showed a near doubling in the prevalence of ISH in the decade between surveys, in adults 18 to 39 years. The survey identified smoking, obesity and low socioeconomic status as possible important contributing factors to ISH in this group. An increase in the prevalence of these risk factors has also been observed during this time period (Grebla et al., 2010).

Hypertension may also be classified as primary or secondary hypertension. Secondary hypertension accounts for only 5 to 8% of diagnosed hypertension and has an identifiable underlying cause, such as renal disease. Primary hypertension in contrast has no obvious organic cause. It is normally benign, with no symptoms, and arises from alterations within the walls of small and large arteries (Brashers, 2012, Levick, 2010). It is the pathophysiology of this form that is of interest in this study and will be discussed in more detail in this review.

The NHLBI Seventh Report of Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure was published in 2003. This report provides the basis of many of the current guidelines for

hypertension prevention and management (currently being updated for version 8). Table 2.10 shows the current classification of BP in adults, 18 years or older. In contrast to the previous report, two changes were made: a prehypertension category was added; and stages 2 and 3 hypertension were combined (Chobanian et al., 2003).

Table 2.10: Classification of BP in adults aged 18 years or older*

BP classification	Systolic (mmHg)		Diastolic (mmHg)
Normal	<120	and	<80
Prehypertension	120-139	or	80-89
Stage 1 hypertension	140-159	or	90-99
Stage 2 hypertension	≥160	or	≥100

BP: blood pressure
*Chobanian et al. (2003)

Management strategies and treatment algorithms have been established for each of the hypertension classifications, using both lifestyle modification and drug therapy. Both stages 1 and 2 are considered to require effective long-term drug therapy and lifestyle modifications, with a treatment target of <140/90 mmHg (or <130/80 mmHg for those with known diabetes or renal disease). Substantial evidence exists to show that pharmacologic treatment reduces the incidence of events and complications associated with hypertension. Despite the advances in treatment and increased awareness of the disorder, in the US, and likely other developed countries, many individuals are still receiving no treatment. A substantial number of those who are treated are not meeting the recommended treatment targets (Chobanian, 2009, Coffman, 2011). For individuals with prehypertension, lifestyle modification is the first choice of treatment, unless comorbidities exist (such as diabetes, CHD, chronic kidney disease). However, the use of drug treatment is also under debate (Pimenta and Oparil, 2010), as this group has been shown to be at twice the risk of developing hypertension compared to those with lower levels of BP (Chobanian et al., 2003).

2.2.5.2 Pathophysiology of hypertension

Despite years of research into the pathogenesis of hypertension, gaps still remain in the understanding of the basic cause(s) (Coffman, 2011). Certainly, hypertension is likely to be multifactorial, developing in genetically susceptible individuals as a result of disruption to one or more of the regulatory mechanisms. Whatever the initial trigger is, the process appears to become self-perpetuating once started (Levick, 2010).

The age-related rise in hypertension prevalence is largely due to the increase in PWV and alteration in wave reflection, which occurs with increased arterial stiffness in the elastic arteries ('the proximal thoracic aorta and proximal parts of the aortic arch branches to the head and upper limbs') (O'Rourke et al., 2011, p782). In young adults, these arteries are distensible (Figure 2.13) and the pulsations that are created by each ejection of blood from the LV are absorbed (first arrow thicker than second arrow), so that the outflow at the peripheral end is almost a steady flow. In the arterial tree there is reflection of the pulse wave at any point of discontinuity, predominantly where low resistance arteries terminate into high-resistance arterioles. In this group, their slower PWV means that the aortic valve is closed by the time the pulse wave is reflected back, which leads to an elevation of DBP and provides a boosting affect to coronary perfusion (O'Rourke et al., 2011, O'Rourke and Hashimoto, 2007, Pimenta and Oparil, 2012)

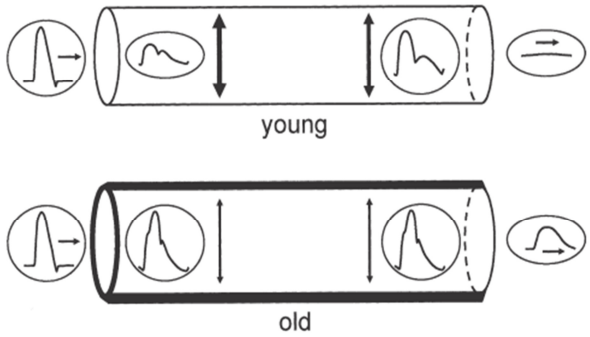

Figure 2.13: Differences in distensibility and pulse wave velocity between young and old individuals
Simple tubular models of the arterial system, connecting the heart (left) to the peripheral circulation (right).

Arterial aging involves the progressive stiffening and dilating of the elastic arteries with age. There is a gradual thickening of the intima-media. Most of this occurs in the non-loadbearing intima, as a result of hyperplasia. Although this has little impact on mechanical properties, it likely contributes to age-related impaired endothelial function, as it presents an increased barrier for NO diffusion between endothelial cells and muscle cells in the media (O'Rourke et al., 2011, O'Rourke and Hashimoto, 2007). Meanwhile, elastic fibres and laminae lose their orderly arrangement in the load-bearing media; elastin fibres split, fray, thin and fragment and are replaced by more rigid collagen fibres; and calcification occurs, all contributing to the stiffening process. As a result, in older adults (Figure 2.13) the arteries are less distensible, and so the pulsations that are created with each ejection are not absorbed (arrows same size), resulting in a more pulsatile flow into the peripheral micro-vessels (O'Rourke and Hashimoto, 2007, Pimenta and Oparil, 2012). In older adults, in addition to a faster PWV, which means wave reflection returns while the aortic valve is still open, increased PR enhances the degree of reflection (larger wave) and together these factors augment afterload (the pressure the ventricle must overcome to eject blood), increase pulse pressure and SBP, and decrease DBP (Levick, 2010, O'Rourke and Hashimoto, 2007, Pimenta and Oparil, 2012).

This phenomenon accounts for the increased prevalence of ISH that is commonly seen in the elderly, and explains many of the ensuing consequences (O'Rourke et al., 2011, Pimenta and Oparil, 2012). As well as increasing LV load, the increase in aortic and LV systolic pressure increases LV blood and oxygen demand and predisposes the LV to hypertrophy and heart failure. In the smallest, fragile arterial vessels, particularly those with high resting flow, such as the kidneys and brain, the endothelium can be damaged (shedding of endothelial cells) by the pulsations that are not absorbed upstream. This can lead to subsequent micro-infarction and thrombosis (O'Rourke et al., 2011).

The roles of NO and Ang II in the maintenance of vascular tone and the development of endothelial dysfunction have been covered in section 2.2.1.4. The endothelium also secretes other vasodilators, such as endothelium-derived hyperpolarizing factor and prostacyclin, and vasoconstrictors, such as endothelin-1, and thromboxane A2 (Higashi et al., 2012). These are released in

response to circulating hormones, autocoids, cytokines, drugs, changes in pH, and physical stimuli, such as changes in shear stress and pressure (Giles et al., 2012). As with the development of atherosclerosis, endothelial dysfunction is an important contributor to elevation of BP (Pimenta and Oparil, 2012). Hypertension is also considered to be an 'injuring stimuli' in the initiation of atherosclerosis. The question that remains is whether endothelial dysfunction causes, or is a consequence of, hypertension (Higashi et al., 2012).

2.2.5.3 Risk factors for hypertension

Hypertension is a chronic complex condition with both genetic and environmental factors contributing to its development (Brashers, 2012, Zhao et al., 2011). Many of the risk factors associated with hypertension are associated with other CVD disorders and, likewise, some are modifiable and some are not. They include family history, ethnicity, gender, advancing age (men>women before 55 years, women>men after 55 years), cigarette smoking, obesity, glucose intolerance and dietary factors, such as high dietary sodium intake, heavy alcohol consumption and low dietary intake of potassium, calcium and magnesium (Brashers, 2012).

2.2.5.4 Blood pressure as CVD risk factor

As early as 1914, raised BP or hypertension was reported as a health risk (Fisher, 1914). In spite of this knowledge, widespread hypertension exists worldwide, and, in terms of attributable deaths worldwide, it is the leading risk factor for CVD (Mendis et al., 2011, Pimenta and Oparil, 2012). In 2008, the overall prevalence of hypertension in adults (aged 25 and over) worldwide was around 40%, with an estimated 1 billion people with uncontrolled hypertension.

Figure 2.14 shows the prevalence of hypertension in males worldwide, with the WHO African Region having the highest prevalence (46%) and the Americas having the lowest (35%) (Mendis et al., 2011).

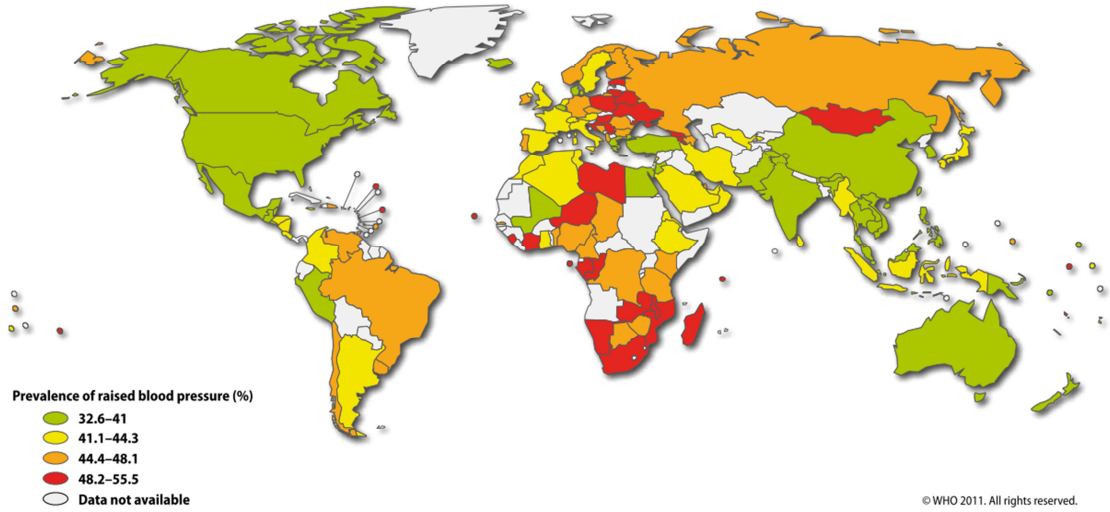

Figure 2.14: The prevalence of raised blood pressure in males (SBP ≥ 140 HHmg and /or DBP ≥90 HHmg) in males globally (ages 25+, age standardised)

Reprinted from Global Atlas on Cardiovascular Disease Prevention and Control, Mendis, S., Puska, P. & Norrving, B. (eds.), © (2011) with permission from World Health Organisation.

Hypertension is particularly prevalent among older adults (≥65 years). With the aging of the population and increase in adiposity in many regions of the world, the concern is that this prevalence rate will only continue to increase (Danaei et al., 2011, Pimenta and Oparil, 2012). Between 1980 and 2008, age-standardised mean SBP decreased by about 1 mmHg per decade (130·5 mmHg (127·3–134·0) to 128·1 mmHg (126·7–129·4) in men). It is suggested that if the mean the BMI had stayed at 1980s levels, this trend may have been more favourable. Between 1980 and 2008, mean BMI for men has increased 0·4 kg/m² per decade worldwide (Finucane et al., 2011). During this period the number of people with uncontrolled hypertension has increased by over 60%, as a result of population growth in general and the growth of the aging population (Danaei et al., 2011).

It has been shown that the relationship between the risk of CVD events and BP is independent of other risk factors, continuous, and consistent. A meta-analysis of 61 studies showed that, for each 20 mmHg incremental increase in SBP or 10 mmHg in DBP for individuals aged 40 to 70 years, there is a

doubling of CVD risk (Lewington et al., 2002). In addition to increasing the risk of CHD and cerebrovascular disease, uncontrolled BP also contributes to damage to retinal blood vessels, peripheral vascular disease, heart failure and renal impairment (Mendis et al., 2011)

New Zealand-specific data

Analysis of data from the 2008/9 National Nutrition Survey shows the age-standardised prevalence of hypertension (SBP ≥140 mmHg or DBP ≥90 mmHg or self-reported use of antihypertensive medications) amongst NZ adults (15 years and over) was 26.4% (men 29.3%). The mean SBP from the survey was 126 mmHg (men 130 mmHg) and DBP was 74 mmHg (men 75 mmHg). The age standardised prevalence across ethnic groups for men was: Māori 36%; Pacific 29% and NZ European and other (NZEO) 28%, with a significant difference in the prevalence between Māori men and NZEO men (McLean et al., 2013, University of Otago and Ministry of Health, 2011).

Table 2.11 shows the age-standardised rate of death (WHO World Standard Population) per 100,000 population for males and females and Māori and non-Māori. Again, ethnic disparities can be seen, with Māori having more than double the non-Māori age-standardised rate of hypertensive disease mortality than non-Māori (Ministry of Health, 2012b).

Table 2.11: Mortality rates for hypertensive disease in NZ in 2009*

	Total		**non-Māori**		**Māori**	
	Males	Females	Males	Females	Males	Females
Hypertensive disease	4.2	3.9	3.8	3.4	9.5	10.8

Māori population includes everyone who was identified as Māori; non-Māori population includes everyone else

*Age-standardised rate of death (WHO World Standard Population), per 100,000 population (Ministry of Health, 2012b)

As discussed in section 2.2.2.3, Metcalf et al. (2006) noted a steady decline in mean BP between 1982 and 2002. Based on comparisons with this previously published data, researchers analysing the National Nutrition Survey data showed that the average BP has increased over the last 10 years for NZEO aged between 35 and 54, reversing the previous downward trend. This

increased prevalence among younger age groups has also been observed in other countries, such as the US (section 2.2.5.1) (McLean et al., 2013).

Finally, data from the 2011/12 NZ Health Survey shows that antihypertensive medication use has increased from 14% in 2006/2007 to 16% (men 14%, women 17%) in 2011/12. About half of all adults over 65 years are now taking medication for high BP (Ministry of Health, 2012a).

2.2.5.5 Indirect measuring of BP and other vascular measures

For most of the 20th century, BP was measured indirectly (non-invasively) using a mercury sphygmomanometer and a stethoscope. An inflatable cuff placed around the upper arm is inflated until the brachial artery is occluded, as assessed by the loss of a palpable radial pulse. The stethoscope is placed over the brachial artery in the hollow of the elbow, and the pressure is slowly released using a screw-valve, until the first appearance of dull tapping noises arising from spurts of blood vibrating the artery wall (Korotkoff sound) is heard. The pressure at this point is accepted as the SBP. The cuff is further deflated until the point when the Korotkoff sound disappears; the pressure at this stage is the DBP (Bogert and van Lieshout, 2005, Levick, 2010). Automated oscillometric cuff devices are now available, which measure brachial SBP, DBP and MAP. These devices automatically inflate and deflate, with changes in arterial pulsations generating oscillations in the cuff pressure, which are measured by a transducer and translated electronically into BP measurements (Bogert and van Lieshout, 2005, Levick, 2010).

Measuring BP by either of these methods provides only a momentary value of BP, and so a method was sought to continuously measure BP. The Finapress® (FINger Arterial PRESSure) was introduced in the early 1980s. In their review Imholz et al.(1998, p605) state that the method offered 'for the first time a reliable measurement of the beat-to-beat BP signal in a noninvasive manner.'

Based on the Peñáz volume-clamp method, the arterial pressure in the finger is measured using a cuff, which contains an inflatable bladder. This keeps the cuff at a constant diameter (set-point), despite the changes in arterial pressure that occur during each heartbeat. A built-in plethysmograph, consisting of a light

source (infrared light-emitting diode) and a light detector (infrared photodiode) detects changes in diameter. A rapid pressure servo-controller system adjuster, which utilises the physiocal algorithm developed by Wesseling, then responds to maintain the set-point and make periodic adjustments if needed (Bogert and van Lieshout, 2005). The generated finger pressure waveform is then modelled to reconstruct a brachial artery pressure wave, from which SBP, DBP, MAP and HR are derived in a beat-to-beat mode (Finapres Medical Systems, 2002).

However, although the Finapress was found to be a useful tool to track changes in BP, it lacked diagnostic accuracy in measuring BP (Imholz et al., 1998). It was found to meet the accuracy requirements of the Association for the Advancement of Medical Instrumentation (AAMI) for the MAP and DBP, but not for SBP (Silke and McAuley, 1998). Its successor, the Finometer®, incorporated a number of further developments, including upgraded filtering software. Most recently, the Finometer PRO,which can measure the brachial pressure, using an additional upper-arm cuff and make corrections for the finger pressure (return-to-flow calibration), was introduced (Guelen et al., 2003, Guelen et al., 2008, Schutte et al., 2004, Schutte et al., 2003).

Both Schutte et al. (2004) and Guelen et al. (2008) have shown in validation studies that by using return-to-flow calibration in conjunction with the other improvements, the Finometer can accurately reconstruct brachial arterial pressure from finger arterial pressure. This reconstruction brings the accuracy of systolic, diastolic and mean pressure level measurements within AAMI requirements (Guelen et al., 2008, Schutte et al., 2004).

The Finometer Midi does not have the ability to perform a return-to-flow calibration, therefore should not be used for absolute measurements. However, it is still useful in studies where recording cardiovascular change is important (Schutte et al., 2003).

The Finometer also incorporates the Modelflow method, a computer based algorithm which can compute an aortic flow waveform. It does so by simulating the response of a non-linear three-element model (aortic characteristic impedance, arterial compliance and systemic peripheral resistance) of the aortic input impedance to arterial pressure (Wesseling et al., 1993). ‘Integrating

the computed aortic flow waveform per beat provides left ventricular SV' (Bogert and van Lieshout, 2005, p440), which then allows CO to be calculated (SV x HR). Total peripheral resistance is derived as 'the ratio of mean arterial pressure to cardiac output, assuming zero venous pressure' (Finapres Medical Systems, 2002, section 3.5). Optimal results for these factors are only achieved with the correct input of age and gender (Finapres Medical Systems, 2002)

Since the Framingham Heart Study identified elevated serum cholesterol and hypertension as important risk factors for developing CVD, research to improve our understanding of how these factors contribute to risk has expanded greatly, and strategies and treatments have been developed to mitigate risk. Improvements in serum cholesterol concentrations and incidence of hypertension have both contributed to the decline in CVD mortality seen in many developed countries. Obesity, which has been identified as an independent risk factor for CVD, also increases the prevalence of dyslipidaemia, independently of elevated LDL-C levels, and hypertension, and threatens to reverse the gains that have been made.

This completes the examination of the selected metabolic modifiable risk factors covered in this review; the next section will investigate the effects of fixed genetic variations on CVD risk, particularly their impact on lipid profile.

2.2.6 Selected genes and CVD risk

Lifestyle changes, including diet modification, are central elements of both CVD prevention strategies and treatment plans (National Cholesterol Education Program (NCEP) Expert Panel, 2002, New Zealand Guidelines Group, 2012). However, while the evidence behind general dietary recommendations can explain changes in lipids at a population level, they do not necessarily predict an individual's response (Garcia-Rios et al., 2012). The current large research focus in nutrigenetics aims to identify common gene variants which determine the physiological response to dietary change. It is hoped that in the future genetic information can be used to provide stratified dietary advice, at least to individuals identified as being at risk of disease. It is likely that such a 'personalised' approach can be used to maximise the benefit of the dietary changes afforded to the individual.

Nutrigenetics examines the impact of genetic variation (mainly SNPs) on the interaction between dietary components, physiological processes and health status (Corella and Ordovas, 2005, Minihane, 2009). Single-nucleotide polymorphisms have arisen in the evolutionary process as a result of interactions between the genome and the environment (Ordovas, 2006). They involve a single deoxyribonucleic acid (DNA) base change and, although they are estimated to account for about 90% of all DNA polymorphisms, most have little phenotypic impact, as they are found in non-functional regions of the genome (Cambien and Tiret, 2007, Corella and Ordovas, 2005). Other causes of genetic variation include insertions or deletions of various lengths, block substitutions, and inversions of DNA sequences. In contrast to SNPs, these involve changes in more than one DNA base pair (Cambien and Tiret, 2007, Frazer et al., 2009).

In the area of lipid and lipoprotein metabolism, over 250 relevant SNPs in about 30 genes encoding key proteins have been identified (Lairon et al., 2009). This review focuses on five selected genes related to CVD; *APOE,* the most widely researched SNP in this area (Minihane et al., 2007) and four genes related specifically to HDL metabolism. Diet-gene interactions for *APOE* and *CETP Taq1B* genotypes will be discussed in section 2.3.4.

2.2.6.1 Apolipoprotein E protein function and genotype

Apoliprotein E is a single-chain glycoprotein of 299 amino acids that was first identified as a constituent of TGRL in the 1970s (Dominiczak and Caslake, 2011, Mahley et al., 2009). Since then it has been found to be a constituent of most of the lipoprotein subclasses, including HDL, which accounts for approximately 60% of plasma apoE. Plasma apoE is synthesised primarily by the liver (>75%), but is also synthesised by a range of other tissues, such as the brain (the second most common site), monocyte-derived macrophages (including those present in atherosclerotic lesions), the kidney, and adipose tissue (Dominiczak and Caslake, 2011, Egert et al., 2012, Mahley et al., 2009).

Apoliprotein E is a multifunctional protein involved in several aspects of lipid metabolism. As discussed in section 2.2.3.2, apoE is a key ligand, binding to cell surface receptors, including LDLR and LRP, to mediate the clearance from plasma of TG- and cholesterol-rich lipoproteins (CM remnants, VLDL, IDL, and a subclass of HDL). Further, apoE can bind to HSPGs to facilitate removal of apoE-containing remnant lipoproteins (Egert et al., 2012, Mahley et al., 2009).

A role has also been suggested for apoE in VLDL synthesis and secretion. Plasma apoE levels explain 20 to 40% of the variability of TG concentrations. Overexpression of apoE appears to result in increased VLDL production and impairment of VLDL lipolysis, leading to hypertriglyceridaemia. Conversely, too little apoE can result in impaired clearance of TGRL (Huang, 2010). Apolipoprotein E has also been shown to facilitate RCT by allowing increased CE-rich core expansion compared to non-apoE containing HDL. These larger HDL-apoE containing particles are then able to deliver cholesterol via the LDL receptor directly to the liver (Mahley et al., 2006).

The gene coding apoE is located on chromosome 19 and is highly polymorphic. Most of the research has concentrated on the common *APOE* epsilon variant, involving two SNPs (rs429358C>T, rs7412C>T) in the coding region of exon 4, which results in 3 protein isoforms E2, E3, and E4. Table 2.12 shows the isoform differences at the transcript and protein level and the resultant changes in LDLR binding and lipoprotein preference (Egert et al., 2012, Minihane et al., 2007).

Table 2.12: Isoform differences of *APOE* at the transcript and protein level and the resultant changes in LDLR binding and lipoprotein preference

Protein isoform	Transcript variation		Protein variation		LDLR binding	Lipoprotein preference
	388	487	112	158		
E2	T	T	Arg	Arg	Low	HDL
E3	C	T	Cys	Arg	High	HDL
E4	C	C	Cys	Cys	High	VLDL, CM

Arg: arginine; Cys: cysteine; HDL: high-density lipoprotein; CM: chylomicron; VLDL: very low-density lipoprotein; LDLR: low-density lipoprotein receptor (Egert et al., 2012, Minihane et al., 2007)

There is considerable variation in allelic distribution globally, with higher frequencies of *E4* carriers seen in indigenous populations and those with darker skin pigmentation. The *E4* allele is considered to be the ancestral form, from which the other two alleles have evolved. It has been suggested that the *E4* isoform represents a 'lipid-thrifty' variant, which allows better intestinal absorption of cholesterol and lipids, including fat-soluble vitamins, which may have conferred advantages in less favourable environments, e.g. when food was scarce (Corbo and Scacchi, 1999, Gerdes, 2003, Huebbe et al., 2011). In Caucasian populations, the prevalence of the genotypes has been reported to be approximately 21% are *E4* carriers (*E3/E4* or *E4*/*E4*), 65% are homozygous *E3/E3* (wild-type form), 11% are *E2* carriers (*E2/E2* or *E2/E3*) and 4% heterozygous *E2*/*E4* (Eichner et al., 2002).

The two amino acid substitutions, although outside the receptor and lipid binding regions, bring about conformational changes that ultimately affect the protein's function. The protein contains two structural domains separated by a hinge region. The N-terminal domain (amino acids 1–191) incorporates the receptor binding region (amino acids 134–150 and Arg-172) and the C-terminal domain (amino acids ~225-299) which contains the major lipid binding region (amino acids ~244–272) (Mahley et al., 2009).

In apoE4, Arg-112 forms a salt bridge with Glu-109. This changes the orientation of the Arg-61 side chain, making it available to interact with Glu-255, which results in an interaction between the two domains, leading to it having a more compact conformation than apoE3. This results in it preferentially binding

to TGRL, rather than HDL (Mahley et al., 2009). Carriers of the *E4* allele typically have higher TC, LDL-C and apoB concentrations, and a tendency towards higher TG and lower HDL-C concentrations compared to *E3* homozygotes (Minihane et al., 2007, Wu et al., 2007, Bennet et al., 2007). A number of mechanisms have been suggested to explain the observed increase in LDL-C seen in *E4* carriers, including that the increased number of apoE containing TGRL would likely lead to increased competition for LDL receptors, delaying the clearance of LDL-C. Further, the faster clearance of VLDL and remnants (VLDL and CM) may result in a down-regulation of LDL receptors. An increased conversion of VLDL to LDL has also been observed (Mahley et al., 2009, Minihane et al., 2007).

In apoE2, the presence of cysteine at position 158 means the normal salt bridge between Arg-158 and Asp-154 is lost, causing Asp-154 to interact with Arg-150, which results in it being pulled out of position, altering the conformation and reducing the positive potential of the receptor binding domain. Compared to *E3* homozygotes, *E2* carriers have less than 2% LDLR binding activity, higher TG and lower LDL-C concentrations (Mahley et al., 2009). The defective receptor binding results in reduced clearance of VLDL and remnants, which leads to an upregulation of LDL receptors due to the reduced cholesterol load in the liver, which in turn results in increased apoB100-mediated LDL removal (Mahley et al., 2009, Minihane et al., 2007).

A 2004 meta-analysis showed that the risk of CHD was 40 to 50% higher in *E4* carriers compared to *E3* homozygotes (Song et al., 2004). A more recent meta-analysis suggests that at a population level the risk may not be as high as that. In the analysis of 17 studies comprising less than 500 cases each, the increase in risk was only 6%. Conversely, in the same analysis, CHD risk in *E2* carriers was shown to be 20% lower (Bennet et al., 2007).

It is accepted that the relatively modest effects on lipid profiles between carriers of different alleles only partly explains the differences in risk. Other suggested mechanisms include variations in anti-oxidant activity (apoE2 >E3 >E4) and modulation of inflammation, with a more pro-inflammatory state and higher

oxidative stress being shown to be associated with the *E4* allele (Jofre-Monseny et al., 2008).

In a re-analysis of data from the Framingham Offspring cohort, all smokers regardless of genotype group showed increased CVD risk compared to non-smokers, overall hazard ratio for smoking was 1.95 (1.52, 2.50). Compared to non-smoking-*E3* homozygotes, smoking-*E4* carriers had an increase in risk of 281%, with a significant interaction on risk observed between *E4* carriers and smoking (Talmud et al., 2005). In a more recent study, comparing ever- and never-smokers, smoking-*E4* carriers again showed the highest CHD risk, although a significant interaction was only observed in women. Physical inactivity was also shown to increase CHD risk in *E4* carriers compared to inactive *E3* homozygotes (Gustavsson et al., 2012). Collectively, these findings suggest that the higher risk that has been observed in *E4* carriers may only be present in certain subgroups, such as smokers. Gustavsson et al. (2012) noted that some of the factors, such as declining smoking rates and the increased use of cholesterol lowering medication, may have contributed to the weakening of the strong associations that were seen between *APOE* genotype and CHD risk in earlier meta-analyses (Gustavsson et al., 2012, Minihane, 2013). Together, these findings suggest that common lifestyle factors, such as smoking and physical activity, which may modify the degree of risk, need also to be considered when assessing an individual's genetic risk for CVD.

2.2.6.2 HDL related genes

Determinants of HDL-C concentrations, as already mentioned, have been shown to have a strong genetic component, with heritability estimates ranging from 40-60% (Boes et al., 2009, Heller et al., 1993). Given the complex nature of HDL metabolism, involving interactions with other lipoproteins, lipid transfer proteins, and enzymes and receptors, a large number of gene polymorphisms have been identified that may contribute to HDL-C variability. If SNPs associated with increased risk for a chronic disease, such as CVD, are carried by a significant proportion of the population, the potential impact on population health of dietary interventions to reduce risk is greater. The four SNPs chosen to be included in this review are common variants that, firstly, have been shown

to have minor-allele frequencies (MAF) of ≥20%, thus allowing for adequate sample sizes for statistical analysis in my research (Table 2.13). Secondly, these SNPs have previously been shown to impact on the responsiveness of HDL-C to dietary intervention. There is a substantial amount of evidence for an association between *CETP Taq1B* and HDL-C concentrations, but it is more limited for the other SNPs.

Table 2.13: MAF of selected HDL-related genes*

Gene	Carriers of minor allele	MAF (%)
CETP Taq1B	B2 carriers	41-44
APOA1 -75G/A	A carriers	16-20
LIPC 514C→T	T carriers	21-25
LIPG I24582 (T+2864C/In8)	C carriers	44

APOA1: apolipoprotein A1; *CETP:* cholesteryl ester transfer protein; *LIPC*: hepatic lipase; *LIPG*: endothelial lipase
* Boes et al. (2009)

$CETP\ Taq1B\ (rs708272)$

Cholesteryl ester transfer protein is a highly hydrophobic, curved glycoprotein of 476 amino acids which can be found in plasma, mainly bound to HDL (Dullaart and Sluiter, 2008, Joy and Hegele, 2008, Zhang et al., 2012). Although it is primarily secreted by the liver, it is also synthesised by a number of tissues, including adipose tissue and arterial wall macrophages (de Grooth et al., 2004, Dullaart and Sluiter, 2008, Joy and Hegele, 2008, Vourvouhaki and Dedoussis, 2008).

As discussed in the RCT section, CETP has a central role in cholesterol metabolism. It also facilitates the remodelling of HDL particles to create both smaller pre-β-HDL and larger HDL_2 from HDL_3, by modulating the transfer of CEs between HDL subspecies, (Goldberg and Hegele, 2012). However, despite advances in technology (e.g. x-ray crystallography) and the knowledge gained from drug trials of CETP-inhibitors, observations in people with genetic deficiencies, studies of individuals carrying different polymorphisms and animal studies, it's structure, mechanism of action and roles remain the topic of much discussion (Charles and Kane, 2012, Vourvouhaki and Dedoussis, 2008).

As previously mentioned, CETP appears to exert both anti- and pro-atherogenic actions, with its behaviour likely dependent on genetic, metabolic and environmental factors. For example, smoking is associated with elevated CETP activity and decreased HDL-C, whereas alcohol and physical activity are associated with decreased CETP concentrations and increased HDL-C. Concentrations of CETP have also been shown to be increased in various dyslipidaemias, such as hypercholesterolaemia and atherogenic dyslipidaemia, although it is still not clear if CETP has a causative role (de Grooth et al., 2004, Vourvouhaki and Dedoussis, 2008).

The CETP gene locus is highly polymorphic, and numerous polymorphisms have been characterised. Several, including the *Taq1B* polymorphism, which is probably the most widely studied, are relatively common in both Caucasian and non-Caucasian populations (de Grooth et al., 2004, Dullaart and Sluiter, 2008).

The *TaqIB* polymorphism is a silent mutation, which does not affect protein sequence, but produces a restriction site for the endonuclease TaqI. It arises from a single base change from G to A at position 277 in intron 1 of the gene. It is unclear how this base change results in the differences in CETP expression that are observed, but one theory is that it is by linkage disequilibrium with other variants that more directly affect expression. Homozygotes for the common CETP allele are labelled *B2B2* (absence of the TaqI restriction site), homozygotes for the polymorphism *B1B1* (presence of the TaqI restriction site), and heterozygotes *B1B2* (Boekholdt and Thompson, 2003, de Grooth et al., 2004, Vourvouhaki and Dedoussis, 2008).

Results from a recent meta-analysis showed that for each inherited *A* allele (B2), individuals had lower mean CETP mass (–9.7% (95% CI, –11.7% to –7.8%)) and activity (–8.6% (–13.0% to –4.1%)), higher mean HDL-C (0.059 (0.050 to 0.068) mmol/L) and apoA1 concentrations (0.033 (0.022 to 0.044) g/L), and lower mean TG (–0.029 (–0.010 to –0.047) mmol/L) than *B1B1* homozygotes. A weak, inverse association with coronary disease risk was also observed with *B2* carriers (OR 0.95 (0.92-0.99) per *A* allele) (Thompson et al., 2008). These findings are in-line with, but also extend, what has been reported

in previous meta-analyses (Boekholdt et al., 2005, Boekholdt and Thompson, 2003).

APOA1 -75G/A (rs670)

Apolipoprotein A1 is the main structural protein of HDL and serves as an important ligand for many of the proteins involved in HDL metabolism and RCT, including ABCA1, SR-BI and LCAT (Boes et al., 2009, Meurs et al., 2010).

The gene coding apoA1 is located in the APOA1/C3/A4 gene cluster on chromosome 11. It is highly polymorphic and the SNP *APOA1 -75G/A* results from a guanine to adenine transition, 75 base pairs upstream from the *APOA1* transcription start (Boes et al., 2009, Ordovas et al., 2002a). Located in the promoter region, it is suggested that gene expression could be influenced by this polymorphism (Souverein et al., 2005).

Conflicting results have been reported in the literature regarding the association of this SNP to HDL-C and apoA1 concentrations. In a meta-analysis in 3,000 healthy subjects, carriers of the *A* allele were shown to have higher apoA1, but other studies have shown no differences (Juo et al., 1999, Ordovas et al., 2002a). In a study investigating the effects of multiple SNPs on TG concentrations, *APOA1 -75G/A* was one of 2 SNPs that was shown to be independently related, with *AA* homozygotes having significantly higher TG levels (Souverein et al., 2005).

In an intervention in 75 healthy subjects who undertook a 6 month exercise programme, *A* allele carriers had significantly higher TG concentrations at baseline and had a greater decrease in TG after training. There was no significant change in overall HDL-C concentration as a result of the intervention. However, when looking at HDL subfractions, *A* allele carriers had a significant decrease in the amount of large HDL and an increase in the amount of small HDL, compared with *GG* homozygotes (Ruano et al., 2006).

In a mixed sample from the Framingham Offspring Study, Ordovas et al. (2002a) showed that PUFA intake appeared to modulate HDL-C in this SNP, but only in women. When PUFA intake was more than 8%, the HDL-C concentration was 13% higher in *A* allele carriers than *GG* homozygotes

(Ordovas et al., 2002a). As a result of their findings, the researchers suggest that interactions with environmental factors may modulate the effect of this genetic polymorphism on HDL-C and apoA1 concentrations and therefore explain some of the inconsistencies observed in the literature (Ordovas et al., 2002a).

LIPC -514C→T (Hepatic Lipase, rs1800588)

Hepatic lipase is primarily synthesised and secreted in hepatocytes, with a small amount also produced by macrophages. Hepatic lipase hydrolyses TG and phospholipids (TG lipase activity > phospholipase activity), from nearly all lipoproteins subclasses, resulting in the generation of smaller, denser particles. In the case of HDL, this also results in the generation of lipid-free apoA1. Additionally, HL mediates bridging between cell surface proteoglycans, to which it is anchored, and lipoproteins, promoting their receptor-mediated uptake (Annema and Tietge, 2011, Rye et al., 2009).

The HL gene is located on chromosome 15 and the SNP *LIPC -514C→T* describes a C to T substitution at position -541. Another study by Jose Ordovas and his research group examined whether dietary fat intake might modulate the effect of the polymorphism, again in a mixed sample of subjects from the Framingham Offspring study. Increased HDL-C and HDL_2-C concentrations and larger particle size were seen in carriers of the *T* allele and, in line with other studies, the *T* allele was associated with decreased HL activity. After stratification by total fat intake, the authors found a strong nutrient x gene interaction, with the higher HDL-C concentration only seen in *T* allele carriers who consumed <30% energy from total fat (Ordovas et al., 2002b).

LIPG I24582 (T+2864C/In8) (Endothelial lipase, rs6507931)

Endothelial lipase is primarily synthesised and secreted by endothelial cells. It is another member of the triglyceride lipase family but, in contrast to HL, it has low TG lipase and high phospholipase activity. The preferred substrate of EL is HDL, but in its remodelling of HDL it does not generate lipid-free apoA1. Endothelial lipase also mediates bridging between cell surface proteoglycans and lipoproteins (Annema and Tietge, 2011, Rye et al., 2009). Plasma EL concentration has been shown to positively correlate with pro-inflammatory

cytokines, such as CRP and IL-6 (Paradis et al., 2006). Up-regulation of EL results in the generation of small HDL and decreased plasma HDL-C concentrations, therefore the effect of inflammation on this enzyme may contribute to the low HDL-C levels seen in atherogenic dyslipidaemias (Yasuda et al., 2010).

The EL gene is located on chromosome 18 and SNP *LIPG I24582* describing an intronic T to C substitution. In a study investigating association between *LIPG* polymorphisms and HDL-C, 7 SNPs were examined in subjects with either high or low HDL-C. The T+2864C/In8 variant was identified as the single SNP contributing the largest effect to variation in HDL-C (Mank-Seymour et al., 2004).

In another study, associations between this SNP, HDL and screen time (TV viewing/computer use), used as a marker of physical inactivity, were examined. Differences were observed only in women, with *C* carriers having significantly lower sLDL than *TT* homozygotes, before stratification for screen time. After stratification, significant interactions were observed for HDL-C concentration, large HDL particle size and large LDL particles and particle size, with *C* carriers having higher levels of these variables than *TT* homozygotes. Examining screen time as a continuous variable, as screen time increased HDL-C significantly decreased in *TT* homozygotes, but not *C* carriers (Smith et al., 2009).

As well as potentially affecting physiological processes, such as lipid metabolism, resulting in variability in lipid profiles, carrying different alleles of some genes may explain some of the inter-individual variability in response that is seen in dietary interventions. This will be explored in the next section, along with additional non-genetic factors which may also contribute to inter-individual variability. Firstly, the impact of diet on CVD risk reduction and the potential benefits of fruit and vegetables as a central component of a heart healthy diet will be discussed.

2.3 Diet and CVD

The second half of the 20th century saw increased industrialisation, urbanisation, and globalisation of societies all around the world, resulting in dramatic changes in diet and lifestyle for many people. Although some aspects have been advantageous, such as increased food diversity and availability of labour-saving devices, it is also accepted that the changes have significantly contributed to the increased rates of NCDs (Arsenault et al., 2011, World Health Organisation, 2003). A joint WHO/Food and Agriculture Organization (FAO) Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases in 2002, identified unhealthy dietary practises, such as the increased consumption of energy-dense foods, the high consumption of saturated fats, refined carbohydrates and salt and low consumption of fruit and vegetables as important contributors to chronic disease risk (World Health Organisation, 2003).

Diet, as a modifiable risk factor, is therefore recognised to have an important role to play in CVD risk prevention. Given the chronic nature of the disease, with its development at least in part the consequence of previous exposure to unhealthy diets and other behavioural risk factors, like smoking and insufficient physical activity, it is recognised that a life-long dietary approach is required to achieve optimal health outcomes (World Health Organisation, 2003).

Initial nutritional epidemiological research tended to concentrate on the role of single nutrients, and in particular dietary fat, in the prevention of CVD. A seminal study, the Seven Countries Study led by Ancel Keys in the 1950s and 1960s, was a cross-population study that demonstrated for the first time the 3-way relationship between dietary SFA intake, serum cholesterol and CHD risk (Bhupathiraju and Tucker, 2011, Mancini and Stamler, 2004). Over the years, different nutrients have been identified that either increase or reduce CVD risk. However, as individuals do not consume single nutrients, there has also been a shift to look at dietary patterns and how these impact on risk (Bhupathiraju and Tucker, 2011).

In the next section some general dietary recommendations and dietary patterns will be discussed, before looking more specifically at the role of fruit in CVD risk

reduction, and examining some of the evidence for why fruit is such a key component.

2.3.1 General dietary recommendations for risk reduction

Lifestyle advice, including advice on a cardio-protective dietary pattern, physical activity, smoking cessation and weight management, are now central to CVD treatment and prevention recommendations (Lichtenstein et al., 2006, New Zealand Guidelines Group, 2012).

General dietary recommendations include balancing caloric intake with physical activity level with the aim of achieving and maintaining a healthy body weight; limiting SFA intake to <7% of energy, trans fat to <1% of energy (NZ <10% combined), and cholesterol to <300 mg/day; consuming a diet rich in fruit and vegetables (400-500 g/day, equivalent to 5 to 6 portions of about 80 g each); choosing whole-grain, high-fibre foods; consuming fish (particularly oily fish) at least twice a week; and minimising salt usage (<5 g/day salt as sodium chloride) (Lichtenstein et al., 2006, World Health Organisation, 2003, Baghurst, 2005).

Table 2.14 shows the cardioprotective dietary plan recommended in the NZ Primary Care Handbook 2012, and the NZ Heart Foundation's suggested eating pattern for a healthy heart, alongside two other examples of dietary eating patterns for heart health, namely the Therapeutic Lifestyle Changes (TLC) diet and the DASH diet. The latter three diets are based on a 2000 calorie or 8400 kilojoule/day energy intake. Although there are minor differences between all four examples, they all advocate a pattern of a diet rich in fruit and vegetables and whole grain products, which also includes fish, nuts and low-fat milk and milk products, but contains reduced amounts of red meat, sugar and salt.

Table 2.14: Examples of dietary patterns for heart health*

Eating Pattern	NZ Cardioprotective dietary plan†	NZ HF‡	TLC§	DASH Diet‖	Examples of Serving Size
Grains¶	At least 6 serves/day (incl. starchy veg)	8-10 serves/day (incl. starchy veg)	7 serves/day	6-8 servings/day	1 slice bread, ½ c cooked pasta, cereal or rice (⅓ c NZ)
Vegetables	At least 3-4 serves/day (include 1 at every meal)	4+ serves/day (coloured vegetables)	5 serves/day	4-5 serving/day	1 c raw leafy vegetables, ½ c cooked vegetables
Fruit	At least 3-4 serves/day	5 serves/day	4 serves/day	4-5 servings/day	1 medium fruit ½ c frozen or canned fruit
Fat-free or low-fat milk and milk products	2-3 serves/day or replace with soy products	3 serves/day	2-3 serves/day	2-3 servings/day	1 c milk, 1 c yoghurt
Lean meats, poultry and fish	Chicken/lean meat 1-1½ serves/day. Fish 1-2 serves & legumes 4-5 serves/week	2 serves/day	≤5 oz/day (142 g)	<6 oz servings/day (170 g)	NZ: 100-120 g lean cooked meat, NZ HF 150 g oily fish
Nuts, seeds and legumes	Eat regularly up to 30 g/day	4-6 serves/day oils, nuts, seeds (NB: HF includes legumes under meat)	Counted in veg servings	4-5 servings/week	⅓ c (1 dsp NZ) nuts, 2 Tbsp (1 Tbsp NZ) peanut butter or seeds
Fats and oils	3 or more servings		Amount depends on daily calorie level	2-3 servings/day	1 tsp oil, 1 tsp soft margarine
Sweets and added sugars	Up to 3 serves/day or 1 serve for weight control, TG & diabetes	Limit	No recommendation	5 or fewer servings/week	1 Tbsp sugar or jam

NZ: New Zealand; HF: Heart Foundation; TLC: Therapeutic Lifestyle Changes; DASH: Dietary Approaches to Stop Hypertension

*Based on 2000 calorie or 8400 kilojoule/day energy intake (except NZ cardioprotective dietary plan)

†New Zealand cardioprotective dietary pattern (New Zealand Guidelines Group, 2012)

‡ New Zealand Heart Foundation (Heart Foundation of New Zealand, 2012)

§Therapeutic Lifestyle Changes (U.S. Department of Health and Human Services et al., 2005)

‖Dietary Approaches to Stop Hypertension (U.S. Department of Health and Human Services et al., 2006)

¶Whole grain foods are recommended for most grain servings

The NZ Heart Foundation recommendations are part of a comprehensive guide to heart healthy eating based around their '9 steps for healthy heart eating' (Appendix 7). The guide is aimed at people at risk of heart attack, stroke or blood vessel disease and it is recommend that individuals work through the guide with a health professional (nurse, doctor or dietitian) (Heart Foundation of New Zealand, 2012). The NZ Primary Care Handbook 2012 brings together current best practice, and provides summary guidance for practitioners, for the complete management of patients with CVD (including CHD, stroke, heart failure, rheumatic fever) and/or diabetes, and includes risk assessment, pharmacological management and lifestyle advice. In addition to the cardioprotective diet, the Heart Foundation's '9 steps for healthy heart eating' are also incorporated into nutritional lifestyle advice (New Zealand Guidelines Group, 2012).

The TLC diet is part of the comprehensive TLC program, a US initiative, which is a 3-part programme aimed at reducing elevated blood cholesterol. On top of the dietary recommendations, the programme also incorporates recommendations for physical activity and weight management. In addition to the general dietary recommendations as outlined in Table 2.14, the diet offers two further options to lower LDL-C, firstly the addition of 2 g/day of plant stanols or sterols and secondly the addition of 10 to 25 g/day of soluble fibre. They suggest that by following the TLC program: decreasing SFA intake to <7% of energy; dietary cholesterol to <200 mg/day; decreasing weight by 10 pounds (4.5 kg) if overweight; adding 5 to 10 g/day soluble fibre and 2 g/day of plant stanols/sterols, it is possible to decrease LDL-C by 20 to 30%, which compares well with the reduction that could be expected by many cholesterol lowering drugs (U.S. Department of Health and Human Services et al., 2005).

The rationale behind the development of the DASH diet was epidemiological evidence that a dietary pattern high in certain minerals and fibre was associated with lower BP (Sacks et al., 1995). In the mid-1990s, a multicentre, randomised feeding study compared a combination diet, now known as the DASH diet (rich in fruit, vegetables, and low-fat dairy products, with reduced amounts SFA, total fat and cholesterol), with a diet rich in fruit and vegetables and a control diet (low in fruit, vegetables, and dairy products and with a fat content typical of the

average US diet). Body weight and sodium intake were held constant. Both the DASH diet and the diet rich in fruit and vegetables reduced BP, but the reduction was far greater with the DASH diet (Appel et al., 1997). Many studies have since confirmed the effectiveness of the DASH diet in reducing BP, and it has also been shown to be effective in reducing other CVD risk factors including CRP, TC and LDL-C, although HDL-C was also decreased (Azadbakht et al., 2011, Obarzanek et al., 2001).

The Mediterranean and Portfolio diets are two other CVD dietary patterns of note. The Seven Countries Study initiated interest in the Mediterranean diet, as it was identified that the lowest incidence of chronic diseases were seen in populations living near the Mediterranean Sea. The Mediterranean dietary pattern includes the daily consumption of 2 to 3 servings of unrefined cereals and cereal products, 2 to 3 servings of vegetables, 4 to 6 servings of fruit, 1 to 2 servings of dairy products, olive oil and 1 to 2 glasses of wine. Weekly consumption includes 4 to 5 serves of fish, 4 to 5 serves of potatoes, more than 4 servings of nuts, pulses and olives and 1 to 3 serves of eggs and sweets. Finally, consumption of red meat and meat products is limited to 4 to 5 serves per month. This dietary pattern has consistently been shown to be beneficial in reducing CVD risk, with evidence from both epidemiological and clinical trials (Bhupathiraju and Tucker, 2011).

The Prevención con Dieta Mediterránea (PREDIMED) study is a large, 3-group parallel, multicentre trial in Spain, which was designed to test the efficacy of the Mediterranean diet on primary cardiovascular prevention. Over 7400 participants who were diabetic or who had at least 3 risk factors for CVD, but no CVD at enrolment, were randomly assigned to a low-fat diet or a Mediterranean diet supplemented with either 1 litre a week of virgin olive oil or 30 g/day of nuts. After a median follow-up of 4.8 years, both Mediterranean diets were shown to reduce the incidence of major cardiovascular events by approximately 30%. As a result of these findings, the safety monitoring board recommended that the trial was stopped (Estruch et al., 2013). In a 3-month assessment of surrogate markers of CVD risk in a subset of participants within 6 months of recruitment, both Mediterranean diets were shown to have favourable effects on TC/HDL-C ratio and HDL-C, compared to control. The changes were greater

in the olive oil group; mean change (95% CI) -0.38 (-0.55, -0.22) and 0.08 (0.04, 0.10) mmol/L, respectively. There were also positive changes for SBP, DBP, HOMA index and IL-6 observed in both groups, while TC and TG were decreased in the nuts group and CRP decreased in the olive oil group (Estruch et al., 2006).

In 1999, David Jenkins first combined foods or food components to achieve better cholesterol reductions, initially combining vegetable protein and soluble fibre (Jenkins et al., 1999). This evolved into the current Portfolio diet, which incorporates four main components; plant sterols, viscous fibre, soy protein and whole almonds (Jenkins et al., 2007). In a randomised crossover study, 34 healthy hyperlipidaemic participants completed three 1-month treatments, comprising a very-low SFA diet (control diet), control diet plus statin (Lovastatin 20 mg/day), or a diet containing 1 gram of plant sterols, 10 g viscous fibre, 21.4 g of soy protein and 14 g of whole almonds per 1000 kilocalories of diet. Compared to the control diet, LDL-C was reduced by 29.1% on the portfolio diet and 34.5% on the statin-containing diet (Jenkins et al., 2005). In a longer study involving 66 participants under free-living real-world conditions, LDL-C was reduced by 12.8% at 1 year, with more than 20% reduction observed in 31.8% of participants. The diet was also associated with a 12.7% reduction in the TC/HDL-C ratio (mean change and SD, -0.73±0.01) and a small, but significant, decrease in TG and increase in HDL-C (Jenkins et al., 2006). Reductions in CRP and BP have also been reported in some studies incorporating this dietary pattern (Jenkins et al., 2007).

2.3.2 Fruit and vegetables and CVD risk reduction

Fruit and vegetables have long been identified as key elements of a healthy diet. In the 2003 WHO report, fruit and vegetables were rated, along with a few other lifestyle factors, as having the strongest strength of evidence for decreasing CVD risk (World Health Organisation, 2003). This is in spite of the limited evidence supporting a causal relationship (Bhupathiraju and Tucker, 2011, Dauchet et al., 2009). The relationship is largely based on observational epidemiological studies, such as a recently published, large prospective cohort study. In this study, more than 300,000 participants were followed for an average of 8.4 years, and fruit and vegetable intake was examined in relation to

risk of mortality from IHD. Participants who consumed at least 8 portions of fruit and vegetables a day had a 22% lower risk of dying of IHD, compared with those consuming less than 3 portions a day (Crowe et al., 2011).

Not even all cohort studies, considered the strongest observation study design, have been able to show a statistically significant inverse relationship between fruit and vegetables and CVD events. In the Dauchet et al. (2009) review only 4 out of 7 cohort studies reached significance, with the lack of comparability in how diets were assessed cited as one factor that was thought to partly explain the difficulty in detecting associations. Other factors which make fruit and vegetable studies difficult include the recognition that fruit and vegetable intake may be associated with other lifestyle characteristics, including higher levels of physical activity, lower rates of smoking and replacement of more harmful foods, that may confer protection, and which are often difficult to control for (Dauchet et al., 2009, Joshipura et al., 1999). Further, fruits are chemically complex foods that contain a range of nutrients and non-nutrients which may contribute independently or synergistically to cardiovascular health (Badimon et al., 2010, Voutilainen et al., 2006, World Health Organisation, 2003).

The review by Dauchet et al. (2009) also highlights some of the issues associated with investigating the effects of increased fruit and vegetable consumption on the incidence or recurrence of CHD in prevention trials. It notes that these studies are difficult to perform and rely heavily on the compliance of participants, who may need to participate for several years. In the few controlled, nutritional prevention trials that have been conducted, differences in intake between active and control groups have ended up often very modest. Not surprisingly, these studies have failed to provide conclusive evidence for a cardioprotective effect (Dauchet et al., 2009).

A potentially more feasible approach is to conduct studies of shorter duration, to investigate the effects of increased fruit and vegetable intake on established surrogate endpoints, such as plasma lipids and BP. However, results to date with these types of studies have also been inconclusive. In relation to plasma lipids, some studies have reported positive effects (decrease in LDL-C) (Djuric et al., 2006, Smith-Warner et al., 2000), while others, including a NZ

intervention, showed no or little effect (Broekmans et al., 2001, John et al., 2002, Zino et al., 1997).

Evidence from DASH intervention studies provides some support for the benefit of fruit and vegetable consumption on risk factors such as BP, with significant decreases also observed with the fruit and vegetable dietary pattern (Appel et al., 1997). In contrast, significant reductions in lipids were only observed on the DASH diet (Obarzanek et al., 2001).

In a very recent Cochrane review, only four studies of at least 3 months duration were identified that investigated the effects of increased fruit and vegetable intake through dietary advice (in the absence of other dietary or lifestyle modifications) on primary prevention of CVD (Hartley et al., 2013). For this group, there was some evidence of favourable effects on BP and LDL-C at 6 months. From the pooled analysis of two studies which examined the effects of fruit and vegetable intake on BP (891 subjects), SBP was found to be significantly reduced (mean difference (95% CI) -3.0 mmHg (-4.92 to -1.09)) (John et al., 2002, Smith-Warner et al., 2000). For the two studies that investigated effects of fruit and vegetable intake on LDL-C and HDL-C, the pooled analysis (251 subjects) showed a reduction in LDL-C (-0.17 mmol/L (-0.38 to 0.03)), although it did not reach statistical significance, but no effect on HDL-C (Djuric et al., 2006, Smith-Warner et al., 2000). Likewise, the pooled analysis of the four studies that measured TC (970 subjects) showed no effect (Djuric et al., 2006, John et al., 2002, Maskarinec et al., 1999, Smith-Warner et al., 2000). The Cochrane review concluded that additional studies will be needed to confirm these effects on LDL-C and SBP, and also notes the lack of randomised, controlled trials specifically designed to examine the effects of increased fruit and vegetable consumption for the primary prevention of CVD (Hartley et al., 2013).

A further approach to investigate the effects of fruit and vegetables on CVD risk markers is to conduct studies where a specific fruit or vegetable is provided to subjects for the duration of an intervention. Studies which have investigated the effects of specific fruit (other than kiwifruit) will be discussed in section 2.3.2.4 and kiwifruit interventions in section 2.3.3.2.

As previously mentioned, fruits contain a range of nutrients and non-nutrients, including fibre, potassium, vitamin C, carotenoids and other polyphenols. Many of these have also been studied individually in animal and *in vitro* models, epidemiological studies and randomised controlled trials, to try and elucidate possible mechanisms by which fruit and vegetables may exert their protective effects. For some components, for example, dietary fibre (cholesterol lowering) and potassium (BP lowering), ample evidence now exists (Brown et al., 1999, Savica et al., 2010). However, for yet other components, such as vitamin C, despite supporting evidence from experimental and observational studies, results from randomised controlled trials have been far from consistent (Dauchet et al., 2009, Honarbakhsh and Schachter, 2009). Some components of fruit will now be discussed briefly, in regard to their proven or proposed beneficial effects, in particular on lipids and other risk factors such as BP and inflammation.

2.3.2.1 Fibre

Dietary fibre intake is associated with reduced risk for developing CHD, stroke, hypertension, obesity and diabetes, with increased consumption being shown to improve serum lipid concentrations, blood glucose control, and lower BP and levels of inflammatory markers (Anderson et al., 2009, Erkkila and Lichtenstein, 2006, Salas-Salvado et al., 2006). Dietary fibre is commonly classified into insoluble and soluble dietary fibre, according to its solubility in water, with most naturally occurring fibre-rich foods containing a mixture of both. In general, insoluble fibre is more abundant in wholegrain cereals, which are also considered the richest source of dietary fibre, and soluble fibre such as gums, pectins and mucilages are more abundant in fruit and vegetables (Salas-Salvado et al., 2006, Anderson et al., 2009, Erkkila and Lichtenstein, 2006).

Soluble fibre has significant cholesterol lowering effects, decreasing total and LDL-cholesterol concentrations (Brown et al., 1999). The primary mechanism is through the binding of bile acids in the small intestine, reducing their absorption, thereby requiring the liver to increase de-novo synthesis of bile acids from intracellular cholesterol (Jenkins et al., 2000). Another proposed mechanism is via the production of the short-chain fatty acid, propionate, which is produced

as a result of colonic fermentation, and has been shown to attenuate cholesterol synthesis (Wright et al., 1990).

In the meta-analysis by Brown et al. (1999), 2 to 10 g of soluble fibre from pectin, oats, psyllium or guar gum was shown to significantly lower TC and LDL-C. There was a significant, but small HDL-C-lowering effect with psyllium or guar gum, but no effect on TG concentrations with any fibre (Brown et al., 1999).

2.3.2.2 Anti-oxidant vitamins - C and E

With oxidative stress implicated in the development of endothelial dysfunction, it has long been thought that anti-oxidant vitamins, such as vitamins E and C, are likely responsible for some of the protective benefits of fruit and vegetables, through their ability to neutralise ROS. Vitamin E is the main lipid-soluble vitamin found in LDL and plasma, and it has been shown to reduce LDL oxidation. Whereas vitamin C is a potent water-soluble anti-oxidant which, in addition to scavenging several reactive species, has been shown to work synergistically with vitamin E to regenerate it from an oxidised to an active state (Honarbakhsh and Schachter, 2009, Nunez-Cordoba and Martinez-Gonzalez, 2011). Further, a number of non-antioxidative properties which could affect disease progression have been suggested through *in vitro* studies. For example, both vitamins have demonstrated abilities to inhibit adhesion molecule gene expression, enhance NO synthesis (Honarbakhsh and Schachter, 2009), and inhibit activation of NF-κβ, a key regulator of pro-inflammatory signalling molecules, such as CRP, IL-6 and TNF-α (Bowie and O'Neill, 2000, Glauert, 2007, Karlsen et al., 2010).

In a meta-analysis investigating the effect of anti-oxidant supplementation on the progression of atherosclerosis, as measured by various imaging techniques, no evidence of a protective effect was found (Bleys et al., 2006). In a recent review that examined a number of meta-analyses of randomised controlled trials which had investigated the effects of vitamin C and E, alone or in combination, on cardiovascular outcomes (such as mortality, myocardial infarction, stroke). All failed to show any significant benefit from

supplementation, with some results even suggestive of an increase in risk (Nunez-Cordoba and Martinez-Gonzalez, 2011).

Results from two recent studies involving a polymorphism of the haptoglobin (Hp) gene are suggestive that some of the observed variance in effects may be due to genetic variation. The Hp 2-2 genotype has been found to be associated with increased CVD risk in diabetics. In a double-blinded placebo controlled trial, 1434 diabetics with Hp 2-2 genotype and taking statins were randomly assigned to receive 400 IU/day vitamin E or placebo. After 18 months, subjects on the dual therapy had significantly lower event rates, compared to statin treatment alone, which led to the early termination of the study (Milman et al., 2008). In a double-blind placebo controlled crossover study in 59 diabetics randomly assigned to receive vitamin E 400 IU/day or placebo for 3 months, HDL function, as measured by cholesterol efflux, significantly increased in Hp 2-2 individuals and decreased in Hp 2-1 individuals, compared to placebo (Farbstein et al., 2011).

The evidence for a beneficial effect of these vitamins alone and in combination on plasma lipids also remains unclear, with mixed findings reported (Honarbakhsh and Schachter, 2009). There is possibly more evidence for vitamin C, but the evidence is still far from conclusive. Guinea pig studies (used as a model of vitamin C deficiency) have shown that vitamin C deficiency can cause an increase in TC, LDL-C and VLDL and a decrease in HDL-C, with the effects more pronounced if animals were on an atherogenic diet (Frikke-Schmidt and Lykkesfeldt, 2009). In a meta-analysis of 13 studies, where subjects received at least 500 mg of vitamin C per day, 9 out of 11 had a trend towards a reduction in LDL-C, although only 4 were statistically significant. Likewise, 6 out of 12 showed a trend towards an increase in HDL-C, but only one was significant and 5 out of 10 showed a trend towards a reduction in TG, but only 2 were significant (McRae, 2008).

Most studies with vitamin E have used α-tocopherol, but there is some evidence that other forms may have properties that are important in CVD protection. For example, plasma levels of γ-tocopherol (the major form of vitamin E in plant seeds and nuts) have been shown to be inversely associated with CVD

morbidity and mortality (Jiang et al., 2001). Further, gamma and delta tocotrienols have demonstrated lipid lowering properties in cells, animal models and humans. In a recent intervention involving hypercholesterolaemic mice and humans, significant decreases in TC, LDL-C and TG were seen in mice, but only a significant decrease in TG in the humans (Zaiden et al., 2010).

A review examining the effect of nutritional doses of antioxidants on BP found no evidence that they were effective in preventing or treating high BP (Czernichow et al., 2004). In a randomised, double-blind, placebo-controlled study, 30 male hypertensives received either combined vitamin E (400 IU/day) and vitamin C (1000 mg/day) or placebo for 8 weeks, before crossing to the other treatment, after completing a 4 week washout period. Vitamin supplementation significantly improved flow-mediated dilation and decreased central PWV and markers of oxidative stress, compared to placebo. However, no changes were seen in BP (Plantinga et al., 2007).

2.3.2.3 Polyphenols

Epidemiological evidence suggests that the cardioprotective effects associated with increased fruit and vegetable intake are likely, in part, due to their polyphenol content (Chong et al., 2010). Polyphenols are a large, diverse group of compounds present in foods and drinks of plant origin, ranging from simple monomeric molecules to highly polymerized compounds. Their shared structural feature is the presence of one or more phenolic groups, which are capable of reducing ROS, a property to which many of their benefits have been ascribed (Galleano et al., 2010, Perez-Jimenez et al., 2010). In addition to their potential ability to decrease oxidative stress, other mechanisms by which polyphenols may exert cardioprotective effects have been postulated. Largely based on findings from *in vitro* studies, these include increasing NO levels, decreasing inflammation (through inhibition of transcription factors, such as NF-κβ), modulation of lipid metabolism (e.g. apoA1 mRNA expression was increased with cacao polyphenols, resulting in increased levels of apoA1) and a number of flavonoids have demonstrated ACE inhibitory activity (Badimon et al., 2010, Grassi et al., 2009, Guerrero et al., 2012, Yasuda et al., 2011).

Dietary polyphenols can be classified into 4 main groups: phenolic acids, lignans, stilbenes and flavonoids. Fruit are the main source of flavonoids in the diet and this group provide much of the fruit's colour and flavour. Flavonoids can be further subdivided into 6 main subclasses (see Table 2.15). This table also provides examples of major dietary sources of each subclass. However, it should be kept in mind that all plant foods contain a range of polyphenols (Grassi et al., 2009, Hooper et al., 2008, Perez-Jimenez et al., 2011).

Table 2.15 Flavonoid subclasses and food sources*

Flavonoid subclass	Synonyms	Example of compounds	Examples of major dietary sources
Flavonols		quercetin, kampferol	onions, broccoli, tea, various fruit
Flavones		apigenin, luteolin, tangeretin	herbs, celery, some citrus
Flavanones		naringenin, hesperidin	citrus fruit
Flavanols	Flavan-3-ols Catechins Polymeric forms- Proanthocyanidins, also known as condensed tannins	catechin, epicatechin	cocoa, apples, strawberries, grapes, tea
Anthocyanidins	Anthocyanins (glycosylated forms)	cyanidin, pelargonidin	coloured berries, cherries, black grapes
Isoflavones		daidzein, genistein	soy products

* Grassi et al. (2009); Hooper et al. (2008); Perez-Jimenez et al. (2011)

A number of epidemiological studies support an inverse association between regular consumption of flavonoid-rich foods and CVD risk (Grassi et al., 2009, Hooper et al., 2008). In one recent prospective study in nearly 35,000 post-menopausal women, dietary intakes of anthocyanidins and flavanones, but not total flavonoid intake, were associated with decreased CHD and CVD mortality. Specific fruit (apples, pears, grapefruit and strawberries) were identified as flavonoid-food that were associated with lower CVD mortality (Mink et al., 2007).

In 2008, a meta-analysis of 133 randomised controlled trials examined the effectiveness of flavonoid-rich foods and flavonoid subclasses on CVD risk factors, including BP and lipids. The main findings were that chronic intake of

cocoa and chocolate decreased SBP and DBP, soy protein isolates decreased DBP, and green tea and soy protein isolates decreased LDL-C. It noted that, for many of the flavonoids, conclusions about efficacy could not be drawn due to insufficient evidence. Further, many of the studies had shortcomings, including study design and lack of adequate power (Hooper et al., 2008).

Another review, this time of fruit polyphenols, based on the current, but limited evidence, suggested that fruit such as pomegranate, berries, and purple grapes rich in anthocyanins and procyaninidins appeared to be associated with beneficial effects on BP, while flavanone-rich fruit, such as oranges and grapefruit appeared to have favourable effects on plasma lipids. The authors also commented on the need for larger, longer, well designed and better powered studies, so that more conclusive conclusions can be made in the future (Chong et al., 2010). Table 2.16 in the next section summarises the results of some individual whole fruit, fruit extract and juice intervention studies, many of which were also included in the fruit polyphenol review by Chong et al. 2010). I would like to comment here that, while I found the fruit polyphenol review useful as it highlighted many of the current short-comings with studies, it was apparent that there were a number of reporting errors in relation to individual studies described.

2.3.2.4 Individual whole fruit or juice intervention studies

To support the observational findings that increased fruit and vegetable intake reduces CVD, human interventions testing the effects of consuming individual fruit on established CVD risk factors can be undertaken.

Table 2.16 summarises the findings of studies identified in the course of this literature review where the effects on lipids, BP and inflammatory markers of whole fruit, fruit extract and fruit juice interventions were investigated. There are limited studies involving whole fruit, with kiwifruit possibly being one of the most widely studied whole fruit, and CVD-related kiwifruit interventions are presented in the next section. The type of fruit studied may have been driven by the availability of funding by organisations such as the Canadian Cranberry Growers Coalition and Zespri International.

Table 2.16 Whole fruit, fruit extract and juice intervention studies investigating effects on lipids, BP and inflammatory markers.

Source	Intervention product	Study Design	No. of Subjects	Subject characteristics	Treatment	Outcomes	
						Effect on Lipids	Effect on BP and inflammatory markers
	Whole fruit						
Gorinstein et al. (2006)	Blond or red grapefruit	Randomised, parallel, controlled	57	Hyperlipidaemic (unmedicated), post bypass patients	Subjects consumed either 1 red or 1 blond grapefruit/day or were in control group for 30 days	↓ TC & LDL-C (both), ↓TG (red only) ↔ HDL-C, compared to control	↔ SPB or DBP
Erlund et al. (2008)	Mixed berries	Single-blind, randomised, controlled	71 (25M, 46F)	≥1 of: mild hypertension, elevated blood glucose, or TC or TG and low HDL, but unmedicated	8-week dietary intervention, 2 portions of berries or control products (to control ↑ energy from berries: either 200 ml sugar-water, 100 g sweet semolina porridge, 100 g sweet rice porridge, or 40 g marmalade sweets)	↑ HDL-C, ↔ TC or TG, compared to control	↓ SBP, subgroup analysis sig in highest baseline SBP tertile only
Larmo et al.(2008)	Sea buckthorn berries	Randomised, placebo-controlled, double-blind, parallel	233 (53M, 180F)	Healthy subjects	28 g of frozen sea buckthorn puree or placebo product for 90 days	NA	↓ CRP compared to placebo
Ravn-Haren et al. (2012)	Whole apples or apple products	Randomised, single-blinded, crossover study	23 (9M, 14F)	Healthy subjects (unmedicated)	5 x 4 weeks: 5 periods were restricted control diet (low in polyphenols and pectin), or control diet plus whole fresh apples (~550g/day), apple pomace 22mg/day), cloudy apple juice or clear apple juice (500ml/day).	TC almost sig ↓ whole apple compared to control. Sig ↓TC & LDL-C in whole apple & pomace compared to clear juice ↔TG, HDL-C or TC/HDL-C ratio with any treatments	↔ BP, CRP
	Capsules						
Naruszewicz et al. (2007)	Chokeberry flavonoid extract	Semi-randomised double-blind, placebo-controlled, parallel	44 (33M, 11F)	MI survivors, statins for at least 6 months (32% hypertensives, 54% on ACE inhibitors extract group)	3x 85 mg/d either chokeberry extract or placebo as capsules for 6 weeks	↔plasma lipids	↓ SBP & DBP ↓ CRP & IL-6, compared to placebo ↓ ACE activity in 7out of 10 subjects not on ACE inhibitors

ACE: angiotensin converting enzyme; AHA: American Heart Association; apoA1: apolipoprotein A1; BP: blood pressure; CHD: coronary heart disease; CRP: C-reactive protein; CVD: cardiovascular disease; DBP: diastolic BP; HC: hypercholesterolaemic; HDL-C: high-density lipoprotein cholesterol; IL-6: interleukin 6; LDL-C: low-density lipoprotein cholesterol; MI: myocardial infarction; NA: not applicable; NS: not significant; SBP: systolic BP; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor alpha; WC: waist circumference

Table 2.16: continued:

Source	Intervention product *Juice*	Study Design	No. of Subjects	Subject characteristics	Treatment	Outcomes	
						Effect on Lipids	Effect on BP and inflammatory markers
Kurowska et al. (2000)	Orange	Single arm, dose response	25 (16M, 9F)	Most moderate HC (no lipid lowering medication)	17-wk study AHA Step I diet, plus 1, 2, or 3 x 250 mL orange juice sequentially for 4 weeks each, followed by 5-wk washout	750 mL ↑ HDL-C, ↓ LDL/HDL ratio, ↑ TG, compared to baseline	NA
Aviram and Dornfeld (2001)	Pomegranate	Single arm	10 (7M, 3F)	Hypertensives	50 mL (contained 1.5 mmol of total polyphenols)/day for 14 days	NA	↓ SBP and ↓ ACE activity
Albers et al. (2004)	Grape	Randomised double blind placebo controlled crossover study	20 (17M, 3F)	Prev. diagnosed CHD, on std meds, 90% on statins	7 mL/kg/d for 14 days each separated by 2-wk washout	↑ HDL-C, ↓TG (P=0.05) grape juice vs placebo	↔ CRP
Aviram et al. (2004)	Pomegranate	Randomised, parallel, controlled	19 (14M, 5F)	Asymptomatic severe carotid artery stenosis, antihypertensives &/or statins	50 mL (contained 1.5 mmol of total polyphenols/day) for 1year or placebo (unspecified)	↑ TG from baseline (i.e. not compared to control)	↓ SBP from baseline, ↔ control
Ruel et al. (2005)	Cranberry	Single arm	21 (M)	Healthy (no lipid lowering medication)	7 mL/kg/d for 14 days	↔TC, LDL-C, HDL-C or TG	↔ SPB or DBP
Ruel et al. (2006)	Cranberry	Single arm, dose response	31 (M)	Moderate HC, WC >90cm, otherwise healthy (no lipid lowering medication)	4-wk run in on 500 mL placebo juice, followed by 125 mL, 250 mL and 500 mL cranberry for 4 wks each	↑ HDL-C 250 & 500 mL, ↓TG (P=0.05) ↑ apoA1 (P=0.08) across doses	NA
Sumner et al. (2005)	Pomegranate	Randomised, placebo-controlled, double-blind, parallel	45 (40M, 5F)	CHD & myocardial ischaemia, all subjects on statins, ~60% hypertensive	240 mL of pomegranate juice a day or placebo for 3 months	↔ plasma lipids	↔ SPB or DBP
Karlsen et al.(2010)	Bilberry	Randomised, controlled, parallel	63 (46M,17F)	Have at least 1 CVD risk factor, otherwise healthy (no lipid lowering medication)	3-wk washout, 1 litre bilberry juice or 1 litre water for 4 weeks	↔plasma lipids	↓ CRP & IL-6 ↑ TNF-α compared to water

ACE: angiotensin converting enzyme; AHA: American Heart Association; apoA1: apolipoprotein A1; BP: blood pressure; CHD: coronary heart disease; CRP: C-reactive protein; CVD: cardiovascular disease; DBP: diastolic BP; HC: hypercholesterolaemic; HDL-C: high-density lipoprotein cholesterol; IL-6: interleukin 6; LDL-C: low-density lipoprotein cholesterol; MI: myocardial infarction; NA: not applicable; NS: not significant; SBP: systolic BP; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor alpha; WC: waist circumference

The problems highlighted in the Chong et al. (2010) review around study design can be seen amongst the interventions reported in the table, with small group sizes, often short intervention duration, differences in recruitment (healthy versus diagnosed CVD), and uncontrolled studies, all factors which make inter-study comparisons difficult.

Three studies had favourable effects on plasma lipids; red or blond grapefruit decreased TC and LDL-C (Gorinstein et al., 2006), and berries (Erlund et al., 2008) and grape juice (Albers et al., 2004) increased HDL-C, compared to control. Further, two uncontrolled, dose response studies showed favourable effects on HDL-C, orange juice (Kurowska et al., 2000) and cranberry juice (Ruel et al., 2006). Of the five other studies which investigated, but did not observe any effects on plasma lipids, subjects were on statins in three of the studies, (Aviram et al., 2004, Naruszewicz et al., 2007, Sumner et al., 2005), although Albers et al. (2004) saw an increase in HDL in their subjects on statins (Albers et al., 2004), and in one study, subjects were healthy (Ruel et al., 2005). In addition to the suggestion by Chong et al. (2012) that flavanone-rich fruit may have a favourable impact on plasma lipids, these studies are suggestive that anthocyanidin-rich fruit may also be beneficial. All of the favourable responses were seen in hypercholesterolaemic subjects or subjects with risk factors or diagnosed CVD.

In the study with different types of apple products, there was a near-significant decrease in TC with whole apple, compared to control. Comparing periods where whole apple or apple pomace were consumed, there was a significant difference in TC compared to clear juice. Changes in TC for the 4 products were as follows: whole fruit -5.6%, apple pomace (by-product of juice production) -3.7%, cloudy apple juice -1.4% and clear apple juice +5%. The researchers suggest that the difference in response may be related to the loss of pectin and other cell wall components during processing (Ravn-Haren et al., 2012). This study does highlight how the unique properties of whole fruit may change as it is processed.

Two studies (which investigated BP) showed significant decreases in BP; mixed berries (SBP) (Erlund et al., 2008), and chokeberry flavonoid extract (SBP and

DBP) (Naruszewicz et al., 2007), compared to control. Two pomegranate juice studies also showed decreases in SBP: one which was uncontrolled, but also showed a decrease in ACE activity (Aviram and Dornfeld, 2001) and the other, although controlled, only reported the change from baseline (Aviram et al., 2004). Of the four other studies which reported BP, but in which no effects were seen, it is likely that this was because subjects were healthy and normotensive in two of the studies (Ravn-Haren et al., 2012, Ruel et al., 2005). A number of intervention studies have observed that antihypertensive effects were less marked in subjects who had lower baseline BPs (Desch et al., 2010, Erlund et al., 2008, Karlsen et al., 2013, McKay et al., 2010).

Finally, of the studies in the table that also investigated the effects of the intervention on inflammatory markers, 3 out of 5 showed decreases in CRP, compared to control: sea buckthorn berries (Larmo et al., 2008), chokeberry flavonoid extract (Naruszewicz et al., 2007) and bilberry juice (Karlsen et al., 2010). In the chokeberry intervention this was in addition to any decrease which may have occurred from being on a statin, which is known to also decrease CRP (Nakou et al., 2008). It is more difficult to speculate on why no difference was seen in the remaining two studies which measured CRP. However, in one of the studies, all patients were on statins and aspirin (both medications which are anti-inflammatory) (Albers et al., 2004).

Although the results from these studies are far from conclusive, they do add to the body of evidence, which may in the future be able to show conclusively a causal relationship between fruit and vegetable intake and CVD. They also emphasise the need for better, well-designed, studies, and draw attention to some of the difficulties with research in this area. In addition to previously noted issues, other factors, including whether subjects are medicated or not, and their BMI, which can impact on overall metabolic health (Tchernof and Despres, 2013), can potentially lead to differences in response and make inter-study comparisons difficult.

In the next section, the history of green kiwifruit in NZ will be briefly discussed, its unique composition and how green kiwifruit compares to other commonly consumed fruit will be examined, and interventions which have investigated the

effects of consuming kiwifruit on plasma lipids and other CVD-related markers will be reviewed.

2.3.3 Green kiwifruit (*Actinidia deliciosa* var Hayward)

Green kiwifruit are the edible berry of the woody vine *Actinidia deliciosa.* Originally native to southern China, where it grew wild, it was first introduced to NZ as seeds by missionaries in 1904. In China, it had a number of common names, including mihoutao' or monkey peach, as wild monkeys were known to enjoy eating the ripe fruit. In NZ it quickly became known as the Chinese gooseberry, as the fruit were perceived to have a gooseberry flavour and they were originally from China (Ferguson, 2004, Nishiyama, 2007). The cultivar 'Hayward', the mainstay of the kiwifruit industry, is a direct descendant of those first seeds. It was first developed by Hayward Wright in the 1920s in Avondale (Zespri, 2011), with exports starting to the United States in the 1950s (Ferguson, 2004). During this time, as a result of political connotations due to the Cold War, the name kiwifruit was proposed and adopted (Ferguson, 2004, Ministry for Culture and Heritage, 2011). Today, the kiwifruit industry is one of NZ's horticultural success stories.

Green kiwifruit (*Actinidia deliciosa* var Hayward*)* are a small oval-shaped fruit, about the size of a large hen's egg, covered with dull-brown, fuzzy skin. Inside, the edible flesh is a vibrant green, in contrast to a central white core surrounded by rows of tiny, black edible seeds. The flavour has been described as 'a tangy, sweet and sour combination of invigorating flavours' (Nishiyama, 2007, Zespri, 2011).

2.3.3.1 Nutrient composition

Green kiwifruit are considered a nutrient-dense fruit in that they contain relatively high concentrations of a range of nutrients. These include significant levels of dietary fibre, potassium, vitamin C, vitamin E, vitamin K, folate, carotenoids and other phytochemicals (Ferguson and Ferguson, 2003, Hunter et al., 2011).

One of the key qualities identified with this variety when the industry was first being developed was its remarkable storage life (Ferguson, 1999). However,

unlike a commercial supplement containing standardised concentrations of nutrients, the actual, as distinct to the nominal, composition of fruit is subject to variability because of a range of factors. Maturity at harvest and postharvest storage conditions, along with other factors, such as environmental conditions (climate and soil) and farming practises, can influence the concentrations of the compounds present (Hunter et al., 2011, Tavarini et al., 2008). For example, in one study where two lots of kiwifruit were harvested one week apart at brix level 8 versus brix level 10, the carotenoid content was significantly higher in the fruit harvested first. After 2 months of cool storage, the carotenoid content had increased in both lots of fruit, but after 6 months of cool storage they both showed significant losses, with a greater loss in the fruit harvested during the first time period, ultimately ending up with a lower concentration than the fruit harvested second (Tavarini et al., 2008).

In Table 2.17, the nutrient composition of green kiwifruit are compared with that of some other commonly consumed fruit – oranges, apples, bananas (the top 3 selling fruit in NZ (Statistics New Zealand, 2010)), as well as strawberries, blueberries and gold kiwifruit. Another factor that can influence fruit composition is genotype, which is why both green and gold kiwifruit are included in the table. The main differences that can be identified are that, relative to gold kiwifruit, green kiwifruit contain significantly more total fibre and vitamin K, but less total sugars and vitamin C. These differences in composition highlight why it is important, where possible, to identify varieties that are used in databases and studies.

Green kiwifruit has a higher total dietary and insoluble fibre content per 100 g of the edible portion than apples, oranges, bananas or blueberries. It also contains a significant amount of soluble fibre as a percentage of its total fibre content (24%). However, proportionally this is lower than oranges (58%) and is similar to apples (26%), bananas (25%) and strawberries (26%).

There are a few fruit richer in vitamin C than kiwifruit, such as guavas (228.3 mg/100g) and blackcurrants (181 mg/100g), but neither of these fruits are readily available in NZ. While citrus and strawberries have traditionally been considered a good source of vitamin C, on a fresh weight (FW) basis green

kiwifruit contain almost double the amount of vitamin C than an orange or strawberries (Ferguson and Ferguson, 2003, USDA, 2011).

A recent study using kiwifruit as a fruit source of vitamin C highlighted the potential advantages of deriving such nutrients from a whole food source. In the study, Gulo knockout mice (a model for vitamin C deficiency) were given either ascorbate (vitamin C) as a purified supplement in their drinking water or fed kiwifruit puree, in the form of a gel. When kiwifruit (either green or gold varieties) was used as the source of ascorbate, a significantly lower daily intake was needed to reach 50% of the maximum wild-type plateau concentrations for the median effective dose in serum and various tissues, including heart, kidney, and liver. In the liver for instance, there was a 5-fold difference in the dose of kiwifruit required compared to ascorbate (2.29±0.27 versus 11.2±3.51 mg ascorbate/day, respectively) (Vissers et al., 2011).

Interestingly, green kiwifruit also contain significant levels of two fat-soluble vitamins, namely vitamins E and K (as phylloquinone). These vitamins are more commonly associated with green leafy vegetables than fruit (other than avocados which contains 2.07 mg α-tocopherol per 100 g and 21 µg of vitamin K per 100 g) (Ferguson and Ferguson, 2003, USDA, 2011). There is some question as to how bioavailable these fat soluble vitamins may be, as they may be restricted to the seed (Ferguson and Ferguson, 2003). However, Fiorentino et al. (2009) showed that α-tocopherol, along with two other analogues of vitamin E, δ-tocopherol and δ-tocomonoenol are present in pulp. In one green kiwifruit intervention in which vitamin E concentrations were measured, plasma vitamin E concentrations significantly increased from 43.6 ± 19.3 µmol/L to 63.5 ± 43.7 µmol/L after consuming two kiwifruit (green 'Hayward') per day for 8 weeks (Chang and Liu, 2009).

Kiwifruit are nearly as good a source of potassium as bananas. Increasing potassium and decreasing sodium intakes are associated with improvements in BP (Savica et al., 2010).

Table 2.17: Comparison of the edible portion of kiwifruit and some other commonly consumed fruit*

Nutrient	Units	Kiwifruit green *Actinidia deliciosa*	Kiwifruit gold *Actinidia chinensis*	Orange naval Citrus sinensis	Apple inc. peel† *Malus domestica*	Banana *Musa acuminata Colla*	Strawberries *Fragaria X ananassa*	Blueberries *Vaccinium spp.*
Proximates (value per 100 g)								
Energy	kJ	255	251	207	218	371	136	240
Water	g	83.07	83.22	85.97	85.56	74.91	90.95	84.21
Protein	g	1.14	1.23	0.91	0.26	1.09	0.67	0.74
Total lipid (fat)	g	0.52	0.56	0.15	0.17	0.33	0.30	0.33
Ash	g	0.61	0.76	0.43	0.19	0.82	0.40	0.24
CHO (by diff.)	g	14.66	14.23	12.54	13.81	22.84	7.68	14.49
Fibre, total dietary‡	g	3.39	2.0	2.40	2.70	2.40	2.30	2.70
Insoluble DF		2.60	1.40	1.00	2.00	1.80	1.70	2.40
Soluble DF		0.80	0.50	1.40	0.70	0.60	0.60	0.30
Sugars, total	g	8.99	10.98	8.50	10.39	12.23	4.89	9.06
Sucrose	g	0.15	0.05	4.28	2.07	2.39	0.47	0.11
Glucose (dextrose)	g	4.11	5.20	1.97	2.43	4.98	1.99	4.88
Fructose	g	4.35	5.68	2.25	5.90	4.85	2.44	4.97
Maltose	g	0.19	0.05	0	0	0.01	0.00	0.00
Galactose	g	0.17	NA	0	0	0.00	0.00	0.00
Selected Vitamins and Minerals (value per 100 g)								
Vitamin C, total ascorbic acid	mg	92.7	105.4	59.1	4.6	8.7	58.8	9.7
Vitamin E (α-tocopherol)	mg	1.46	1.49	0.15	0.18	0.10	0.29	0.57
Vitamin K (phylloquinone)	µg	40.3	5.5	0	2.2	0.5	2.2	19.3
Folate (total)	µg	25	34	34	3	20	24	6
Beta carotene	µg	52	43	87	27	26	7	32
Lutein + zeaxanthin	µg	122	114	129	29	22	26	80
Potassium	mg	312	316	166	107	358	153	77

*Nutrient values for the edible portion of fruit (USDA, 2011)
†Based on analytical data for red delicious, golden delicious, gala, granny smith, and fuji varieties
‡Fibre values (Schakel et al., 2001), except for kiwifruit gold: total DF (USDA, 2011); insoluble and soluble DF (Personal communication with Drummond L, Zespri International Ltd)

Green kiwifruit contain a number of pigments, including carotenoids, β-carotene, lutein, zeaxanthin, 9'-cis-neoxanthin, violaxanthin, and chlorophylls *a* and *b* (Latocha et al., 2010, McGhie et al., 2002). Normally, as fruit ripen they change colour from green to colours such orange, yellow or red. This has been attributed to the degradation of chlorophyll and the synthesis/presence of carotenoids and anthocyanins. The unique colour of green kiwifruit is attributed to the retention of chlorophyll during ripening, which masks the yellow colour of the carotenoids. Comparing *A. deliciosa* 'Hayward' with *A. chinensis* 'Hort 16A', which are yellow fruit, both contain similar levels of carotenoids, but green kiwifruit contain about 1mg/100g of chlorophyll, whereas the yellow kiwifruit only contains traces (McGhie et al., 2002, Nishiyama, 2007).

In addition, *A. deliciosa* 'Hayward' also contains a range of other phytochemicals/polyphenols. A number of phytosterols have been characterised: β-sitosterol, stigmasterol, campesterol, stigmast-7-en-3β-ol, ergosterol, the peroxide derivative of ergosterol, and 5,7,14,22-ergostatetraen-3β-ol (Fiorentino et al., 2009). Polyphenols include; tannic acid, 2,5-dihydroxybenzoic acid, hydroxybenzoic acid, chlorogenic acid, benzoic acid derivative, caffeic acid, ursolic acid, quercetin, (L)-epicatechin, (+)-catechin, tricin, quercetin-3-O-β-D-glucopyranoside, kaempferol-3-O-β-rutinoside, rutin, epicatechin and gallocatechin (Fiorentino et al., 2009, Latocha et al., 2010).

Two studies (Du et al. (2009) and Latocha et al. (2010)) have recently reported the total phenol content of *A. deliciosa* var Hayward, reporting contents of 41.7 mg gallic acid equivalents (GAE)/100 g FW and 76.5 mg GAE/100 g FW, respectively. Of the selection of genotypes tested, both studies found that 'Hayward' had the lowest total content (Du et al., 2009, Latocha et al., 2010).

It is recognised that much work remains to determine both the full polyphenol composition of most foods and also their biological/physiological properties (Elmadfa and Meyer, 2010, Arranz et al., 2010). In response to this need, the Phenol-Explorer was set up as the 'first comprehensive database on polyphenol content in foods.' The database contains data for more than 400 foods, and includes 35,000 content values for 500 different polyphenols (Neveu et al., 2010). The total phenol content for 'kiwifruit' is given as 179.71±111.85

mg/100g FW. This figure was based on data from 4 papers, but no consideration appears to have been made for differences in how the total content was expressed, either as catechin equivalents (CtE/100 g) or GAE/100 g, and only one paper specified a varietal type (Chun et al., 2005, Cieslik et al., 2006, Imeh and Khokhar, 2002, Wu et al., 2004). These factors limit its usefullness at this time.

Another issue is that, until recently, non-extractable polyphenols have largely been ignored. Based on their solubility properties, dietary polyphenols can be classified as either extractable polyphenols, which are soluble in aqueous organic solvents, and non-extractable polyphenols, which aren't soluble and remain in the extraction residue (Arranz et al., 2010). Research over the last 30 years has almost exclusively looked at extractable polyphenols. In most berries and fruit, extractable proanthocyanidins tend to be the predominant form. However, in some fruit more than half of proanthocyanidins have been identified as un-extractable proanthocyanidins, and in the case of kiwifruit this is about 80% (Tarascou et al., 2010).

2.3.3.2 Kiwifruit intervention studies

Table 2.18 summarises the four kiwifruit intervention studies which have, as part of their study protocol, investigated the effects of consuming kiwifruit on plasma lipids and other CVD-related markers, including BP, inflammatory markers and blood glucose.

The studies varied in experimental study design, primary outcome measures, subject group, and the number of kiwifruit consumed, although in all cases the kiwifruit were added to either the subjects' normal diet or, in the case of Karlsen et al. (2013), a restricted diet where subjects were asked to restrict their consumption of certain foods (berries, nuts, pomegranates, tomatoes, kiwifruit, tea and coffee) to less than 3 cups/day.

Table 2.18: Kiwifruit intervention studies

Source	Study Design	No. of Subjects	Subject characteristics	Kiwifruit supplementation			Background diet, anthropometric measurements	Outcomes	
				Cultivar	Number KF/day	Duration		Effect on lipids	Other CVD-related markers
Duttaroy and Jorgensen (2004), Olso Norway Primary outcome measures plasma lipids & platelet aggregation	Randomised cross-over design No control group	30 (12M; 18F)	Healthy subjects 20-51 years; Mean BMI 22.43±0.52 kg/m^2 Plasma lipids (mmol/L): TC 5.14, LDL-C 3.28, HDL-C 1.55, TG 1.16	Not specified	2 or 3	28 days per period, with 2-wk washout	Added to normal diet ↔ BMI	↓ TG ↔TC, LDL-C, HDL-C	↑ plasma vit. C
Chang and Liu (2009), Taipai Taiwan Primary outcome measures plasma lipids & antioxidative status	Single arm	43 (13M; 30F)	Hyperlipidaemic 20-65 years; Mean BMI Men 26.0±3.8, Women 23.6±3.1 kg/m^2. Plasma lipids (mmol/L): TC 5.64, LDL-C 3.82, HDL-C 1.40, TG 1.26	*Actinidia deliciosa* Hayward	2	8 weeks, following 2-wk dietary stabilisation	Added to normal diet ↑ % BF men, no other wt. changes	↑ HDL-C ↓ LDL-C/HDL-C & TC/HDL-C ratios NS trend ↓ TG ↔ TC or LDL	↑ plasma vit. C & E
Brevik et al., (2011) Norway Primary outcome measures markers of oxidation damage & antioxidant protection	Randomised cross-over design No control group	24 (M & F)	Healthy subjects 20-57 years BMI 20-30 kg/m^2 Plasma lipids (mmol/L): TC 4.05, LDL-C 2.32-2.39, HDL-C 1.50-1.53, TG 0.88-0.86	*Actinidia chinensis* Hort 16	1 or 2	4-weeks per period, with 4-wk washout	Subjects selected who ate modest amounts of F& V Asked to add to normal diet No mention wt. monitoring	↓ TG ↔TC, LDL-C, HDL-C	NS ↑ plasma vit. C ↔CRP ↔glucose
Karlsen et al., (2013) Norway Primary outcome measures BP & platelet aggregation	Randomised, parallel design: Kiwifruit AO-rich diet Control	102 (all M)	Smokers 44-74 years; Mean BMI 24.7 kg/m^2 Plasma lipids (mmol/L): TC 5.9, LDL-C 3.9, HDL-C 1.5, TG 1.2	*Actinidia deliciosa*	3	8 weeks, following a 4-wk run-in	4-wk run in, limit certain foods (polyphenol rich) <3 cups/d, KF added. No mention wt. monitoring, but sig dif. energy intake KF vs control	↔ Lipids	↓ SBP & DBP ↓ ACE activity ↔ CRP, TNF-α, IL-6

% BF; percentage body fat; ACE: angiotensin converting enzyme; AO: anti-oxidant; BMI: body mass index; BP: blood pressure; CRP: C-reactive protein; CVD: cardiovascular disease; DBP: diastolic BP; F&V: fruit and vegetables; HDL-C: high-density lipoprotein cholesterol; IL-6: interleukin 6; KF: kiwifruit; LDL-C: low-density lipoprotein cholesterol; NS: non-significant; NZ: New Zealand; SBP: systolic BP; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor alpha; wt: weight

There have been conflicting results on the effect of kiwifruit consumption on plasma lipids. Duttaroy & Jorgensen (2004) showed that the consumption of 2 or 3 kiwifruit per day for 28 days significantly reduced serum plasma TG by 15%, compared to baseline values, without affecting cholesterol levels. Brevik et al. 2011 showed that consuming 1 or 2 kiwifruit per day for 4 weeks decreased TG by 13.3% with 1 kiwifruit per day and 7.4% with 2 kiwifruit per day, compared to baseline values. In contrast, Chang & Liu (2009) showed that consumption of 2 green kiwifruit per day for 8 weeks significantly increased HDL-C, leading to associated decreases in the LDL-C/HDL-C and TC/HDL-C ratios. None of these studies included control groups (Brevik et al., 2011, Chang and Liu, 2009, Duttaroy and Jorgensen, 2004). In the Karlsen et al. (2013) study, which included a control group, no change in plasma lipids was seen (Karlsen et al., 2013).

Karlsen et al. (2013) showed that consumption of 3 green kiwifruit per day for 8 weeks significantly reduced DBP and SBP in male smokers, compared to control treatment, with the greatest effects (-15 mmHg SBP, -13 mmHg DBP) seen amongst hypertensive subjects. Additionally, they observed a reduction in ACE activity (Karlsen et al., 2013). This effect has previously been observed *in vitro* with kiwifruit extracts (Jung et al., 2005).

With regard to other metabolic markers, neither of the two studies that measured CRP showed any effect of kiwifruit (Brevik et al., 2011, Karlsen et al., 2013). Brevik at al. (2011) saw no effects on plasma glucose concentrations (Brevik et al., 2011).

Three studies have also shown decreased collagen and/or adenosine diphosphate-induced *in vitro* platelet aggregation (Brevik et al., 2011, Duttaroy and Jorgensen, 2004, Karlsen et al., 2013). As this was not a planned investigation in my intervention, I have not included details in the table.

In addition to the variability in response that is frequently observed between studies, which is often attributed to differences in experimental design, considerable within-study variability can also exist. Factors which may contribute to this variability are discussed further in the next section.

2.3.4 Inter-individual diet responsiveness

Inter-individual differences in plasma lipid response to dietary exposure has been recognised for some time, with studies in the 1980s reporting hypo- and hyper-responders to dietary lipid and cholesterol interventions (Beynen et al., 1987, Katan et al., 1986). This phenomenon is also recognised to explain differences in response to medications, such as statins (Bouchard and Ordovas, 2012).

A substantial body of evidence derived from both observational studies and randomised controlled trials supports the theory that genetic factors account for much of this variability (Bouchard and Ordovas, 2012). However, a number of other factors broadly grouped under the headings: environmental, physiological, and pathological factors have also been identified as potentially contributing to inter-individual diet responsiveness. Figure 2.15 shows examples of these factors, grouped under these headings. In addition to influencing variability in response, these factors can also interact with each other to impact on response and overall health status (Minihane, 2013, Rideout, 2011).

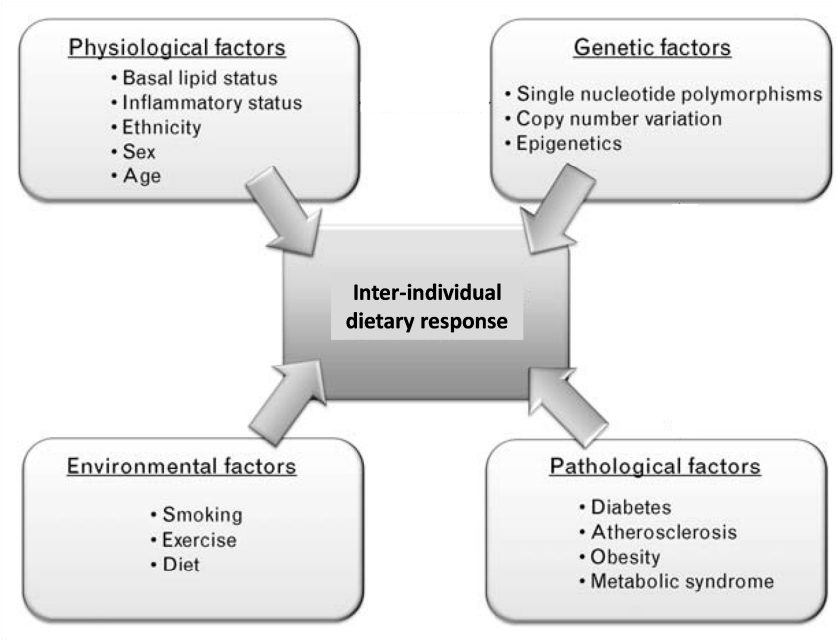

Figure 2.15: Potential genetic, environmental, physiological, and pathological factors influencing inter-individual dietary response

Adapted from Curr Opin Lipidol, 22 (1), Rideout, T. C., Getting personal: considering variable interindividual responsiveness to dietary lipid-lowering therapies, 37-42 © (2011) with permission Wolters Kluwer.

In the following sections the literature will be reviewed in regards to effects of two of these identified factors, inflammatory status (as measured by CRP) and selected SNPs (*APOE* and *CETP Taq1B* genotypes), on the plasma lipid response to dietary change.

One of the main criticisms of studies which have investigated the effect of selected SNPs on dietary response is that they were not specifically designed to examine diet-gene interactions. For randomised controlled trials, the majority of such studies have been of retrospective genotyping design. In such randomised controlled trials the study of nutrigenetic interactions is almost never the primary outcome and often not even considered in the study design stage. This retrospective assignment of individuals to subgroups means the groups are often not matched at baseline for key prognostic variables. In addition, low group numbers in the rarer allele group means the studies are often underpowered to draw explicit conclusions, which can result in the impact of potentially important variants being undetected or underestimated (Minihane et al., 2007, Ordovas, 2009). Further, studies investigating the impact of genetic variation on the inter-individual variation in response to interventions ideally should: use a randomised, crossover design, where subjects serve as their own control; should be adequately powered; and with prospective recruitment according to genotype, if possible and appropriate (Rideout, 2011).

2.3.4.1 APOE genotype and diet responsiveness

In discussing the impact of *APOE* genotype on the plasma lipid response to dietary change, Anne Marie Minihane (2013, p44) recently observed that 'overall, the evidence is suggestive that *APOE4* carriers are most responsive to the plasma cholesterol modulating impact of total fat, cholesterol, saturated fat intake and long chain *n*-3 PUFA, EPA and DHA intake'. She comments that often findings have been inconclusive, as a result of being retrospective and underpowered (Minihane, 2013). Her research group, using retrospective analysis, originally showed that LDL-C was raised in *E4* carriers in response to high-dose fish oil (Minihane et al., 2000). More recently, using prospective genotype recruiting, they have shown that it is likely that DHA rather than EPA

is responsible for the cholesterol raising effect seen in this group (Olano-Martin et al., 2010).

The increased responsiveness seen in *E4* carriers has generally been reflected in greater changes in TC and/or LDL-C, although a few studies have also observed changes in HDL-C (Masson et al., 2003). In contrast to their responsiveness to changes in dietary fat and cholesterol, *E4* carriers appear to be the least responsive to some other dietary components, including wheatbran and oatbran (Jenkins et al., 1993) and plant sterol esters (Sanchez-Muniz et al., 2009).

In a review by Egert et al. (2012), the authors considered if *APOE* genotype may also explain in part the heterogeneity seen in studies examining the potential health benefits of flavonoids (Egert et al., 2012). In a double-blinded, placebo-controlled, crossover trial in a group of overweight-obese participants with metabolic syndrome traits, participants received 150 mg/day quercetin or placebo for 6-weeks each. Significant differences in response were seen between *E3* homozygotes and *E4* carriers, with quercetin significantly decreasing HDL-C and apoA1 in *E4* carriers compared to placebo, with no changes in these variables in *E3* homozygotes. Conversely, quercetin significantly decreased SBP in *E3* homozygotes, but no change was seen in *E4* carriers. The authors also reported a 'significant decrease in ox-LDL levels and serum TNF-α in *APOE3* and *E4* subgroups', but in fact these were significant decreases from baseline and it was only in *E4* carriers that oxLDL significantly decreased with quercetin compared to placebo (Egert et al., 2010).

Finally, in a post-prandial investigation examining the effect of *APOE* genotype on post-prandial lipaemia, *E4* carriers were shown to have higher TG concentrations and an exaggerated postprandial TG response (22% greater area under the curve (AUC)), compared to *E3* homozygotes. When stratified by genotype and age (≤50 and >50 years), a significant post prandial response was only seen in participants >50 years, (30% AUC) (Carvalho-Wells et al., 2010). These findings highlight the complexity of investigating inter-individual variability, with other factors, in this case age, also impacting on response.

2.3.4.2 CETP Taq1B genotype and diet responsiveness

Table 2.19 summarises the five intervention studies that have investigated diet-gene interactions with respect to the *CETP Taq1B* polymorphism and plasma lipids.

Wallace et al. (2000) showed in hypercholesterolaemic subjects that *B1/B1* homozygotes had greater decreases in TC and LDL-C in response to a short term high PUFA diet, compared to a high SFA diet (Wallace et al., 2000). This result could not be repeated in a subsequent similar study (Aitken et al., 2006). In another study, no differences according to *CETP Taq1B* genotype in response to consuming a NCEP-I diet were seen (Carmena-Ramon et al., 2001, Carmena-Ramon et al., 1998). More recently, in a study in hypercholesterolaemic children, *B1/B1* homozygotes had greater increases in HDL-C following consumption of skim milk enriched with olive oil, compared to regular skim milk (Estevez-Gonzalez et al., 2010). The Du et al. (2010) study, although included in the table, was non-randomised and of inadequate duration to support the findings that they reported. The limited number of randomised controlled trials and inconsistencies in findings investigating the modulating effect of *CETP Taq1B* mean further studies will be needed before this genotype can be confirmed or discounted as a modulator of dietary response.

2.3.4.3 CRP and diet responsiveness

A number of studies, using CRP as a marker of inflammatory status, have shown that inflammatory status has modulated plasma lipid response to dietary interventions involving some sort of dietary fat manipulation (Table 2.20). Although the studies differed in how they divided their participants into low and high baseline CRP status and many had small group numbers, all saw differences in response between low and high CRP groups on various plasma lipid components.

Table 2.19: *CETP Taq1B* studies which have investigated diet-gene interactions

Source	Study Design	No. of Subjects	Subject characteristics	Treatment	Results - Effect on lipids by *CETP Taq1B* polymorphism
Wallace et al. (2000)	Randomised double crossover	55 (23M,32F)	Hypercholesterolaemic subjects	High SFA diet (21%E SFA, 3%E PUFA) versus high PUFA (11%E SFA, 10%E PUFA), 4 wk phases	*B1/B1* homozygotes - sig. greater ↓ TC & LDL-C, ↑ CETP activity (ns) compared to B2 carriers
Carmena-Ramon et al. (2001)	Non-randomised, single-arm	77 (Mixed)	Familial hypercholesterolaemia	4 wk run-in baseline diet (35% fat, 10% SFA & 300mg cholesterol), 3 months NCEP-I diet	No sig. gene x diet interactions *B2/B2* subjects had a less atherogenic profile and lower incidence of arcus cornealis, xanthomas, etc
Aitken et al. (2006)	Randomised single crossover	70 (22M, 48F)	Hypercholesterolaemic subjects	High SFA diet (23%E SFA, 3%E PUFA) versus high PUFA (10%E SFA, 15%E PUFA), 4 wk phases	No sig. gene x diet interactions. Results not supporting previous study
Estevez-Gonzalez et al. (2010)	Randomised crossover	36 (14M, 22F)	Pre-pubertal children with hypercholesterolaemia	Cow's skim milk enriched with virgin olive oil versus regular cow's skim milk, 6 weeks each	*B1/B1* homozygotes sig. ↑ HDL-C
Du et al. (2010)	Non-randomised	56 (27M, 29F)	University students	7-day washout (31% fat, 54% CHO) followed by 6-day intervention HC/LF (15% fat, 70% CHO)	Reported male *B1/B1* homozygotes ↑ HDL-C & apoA1, The short duration of the intervention makes these results unreliable.

apoA1: apolipoprotein A1; CHO: carbohydrate; HC: high carbohydrate; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; LF: low fat; MUFA: monounsaturated fatty acid; NCEP-1: National Cholesterol Education Program step 1; ns: not significant; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; TC: total cholesterol

Table 2.20: Studies which have observed modulation of dietary responsiveness to inflammatory status

Source	Study Design	No. of Subjects	Subject characteristics	Treatment	Results - Effect on lipids by CRP group	
					Low CRP group	High CRP group
Erlinger et al. (2003)	Randomised, controlled, parallel	100 (46M, 52F)	Healthy adults, SBP 120 to 159 mmHg & DBP 80 to 95 mmHg. Not on anti-hypertensives	DASH diet (27% E fat, 6% SFA, 13% MUFA, 8% PUFA, 151 mg/d cholesterol) or control diet (37% E from fat, 16% SFA, 13% MUFA, 8% PUFA, 300 mg/d chol.) for 12 wks	CRP <2.37 mg/L (median) ↓ TC, LDL-C, HDL-C, compared to control	CRP ≥2.37 mg/L ↑ TG, ↓ HDL-C, compared to control
					Sig. interaction diet x CRP: TC, LDL-C, TG (adjusted for baseline lipid level, age, race, sex, smoking and BMI); ↔ CRP (whole group); NB: groups sizes not indicated	
Zhao et al. (2004)	Randomised, controlled, crossover	23 (20M, 3F)	Moderate HC BMI between 25 and 35 kg/m^2.Not taking lipid lowering medication	2 diets – low in SFA and cholesterol and high in ALA or LA, control (AAD- average American diet- higher SFA) Each diet 6 wks with a ≤3-wk break in between	CRP <2 mg/L Greater ↓ LDL-C 2 high-PUFA diets (vs.AAD)	CRP ≥2 mg/L –
					P=0.068 for interaction; sig. reduction CRP ALA diet (whole gp) compared to ADD; NB: groups sizes not indicated	
Hilpert et al. (2005)	Randomised, crossover	32 (14M, 18F)	Healthy subjects, women post-menopausal	3-wk run in on NCEP step 1 diet, then step 1 plus soy protein isolate or milk protein isolate for 6 wks each, with 2 wk break in between	CRP <3.5 mg/L (median), n=16 ↓ LDL-C, LDL-C/HDL-C ratio, both proteins comp. to run-in diet	CRP ≥3.5 mg/L, n=16 ↑ LDL-C, LDL-C/HDL-C ratio, both proteins comp. to run-in diet
					Sig. interaction diet x CRP: LDL-C, LDL-C/HDL-C ratio, apoB, Lp (a) ↔ CRP both groups	
Desroches et al. (2006)	Randomised, parallel	61M	Healthy subjects, with a wide range of BMI (20-44 kg/m^2).	Ad libitum a moderately low-fat diet (25.8% E intake from fat) or a high-fat diet rich in MUFA (40.1% of energy intake from fat, 22.5% from MUFA) for 6–7 wk	CRP <1 mg/L (CDC/AHA classification) ↓ TG low fat (n=17), compared to baseline ↓ TG, VLDL-C MUFA (n=13), compared to baseline	CRP ≥ 1mg/L (med & high gps combined) ↑ TG low fat (n=15), compared to baseline ↓ TC, LDL-C MUFA (n=16), compared to baseline
					Sig interaction CRP x low fat diet VLDL-C, TG; MUFA diet TC, VLDL-C, LDL-C, TG; ↓ CRP MUFA diet from baseline	
St-Onge et al. (2009)	Randomised, controlled, crossover	45 (12M, 33F)	LDL-C 3.37-4.66 mmol/L, TG <3.96 mmol/L, glucose <6.99 mmol/L, BMI 20-35 kg/m^2	3 diets – LF control diet (30.8%E- NECP step 1), mod fat and SFA (37.9 and 11.4% E-western diet (WD)) and mod fat PUFA (36.3 and 9.7%E)	CRP <1 mg/L (CDC/AHA classification) Trend ↓ TG PUFA & LF, compared to WD Trend greater ↓ LDL LF	CRP 1–3 & CRP >3 mg/L Trend ↑ TG LF, compared to WD Trend ↓ TG PUFA, compared to WD
					Sig. interaction diet x CRP: TG; ↔ CRP; NB: group sizes not indicated	

AHA: American Heart Association; apoB: apolipoprotein B; BMI: body mass index; CDC: Centers for Disease Control; CRP: C-reactive protein; E: energy; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; HC: hypercholesterolaemic; LDL-C: low-density lipoprotein cholesterol; LF: low fat; lp(a): lipoprotein(a); MUFA: monounsaturated fatty acid; NC: no change; NCEP step 1: National Cholesterol Education Program step 1; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; SBP: systolic blood pressure; TG: triglycerides; VLDL-C: very low-density lipoprotein cholesterol

Three of the studies showed that subjects in the low CRP group had a more favourable response to the dietary modification. Erlinger et al. (2003) showed significant reductions in TC and LDL-C in participants consuming the DASH diet. Likewise, Hilbert et al. (2005) showed significant decreases in LDL-C and LDL-C/HDL-C ratio in response to consuming the NCEP step 1 diet plus either milk or soy isolate, and Zhao et al. (2004) showed greater reductions in LDL-C, in response to high PUFA diets. Further, three of the studies showed a worsening of TG concentrations on a low fat (higher carbohydrate) diet, in the higher CRP group (Desroches et al., 2006, Erlinger et al., 2003, St-Onge et al., 2009).

In contrast, in response to a high MUFA diet, the high CRP group showed significantly greater reductions in TC and LDL-C concentrations, compared to the low CRP group. On the MUFA diet, the high (≥1 mg/L) CRP group (n=17) also had a significant decrease in CRP from baseline (P<0.01) (Desroches et al., 2006). The Desroches et al. (2006) study was the only one to examine the effect of the diet on CRP concentrations by CRP group.

In addition to small group sizes (which were often not stated), another factor which could confound the results of these studies was changes in body weight. This was only addressed by two studies; both Hilbert et al. (2005) and Desroches et al. (2006) note that there were small decreases in body weight over the course of the intervention, but there were no significant differences between CRP groups (Hilpert et al., 2005, Desroches et al., 2006).

These results suggest that different strategies may be needed in individuals with elevated inflammation, given that recognised dietary modifications aimed at improving lipid profiles appear to be less effective in this group.

2.3.5 The potential role of personalised dietary recommendations

To date dietary recommendations have been generalised for populations, with some acknowledgement made for specific subgroups, such as different age groups or physiological status, for example pregnant women or diabetics. The nutrient reference values prescribed are based on these population group requirements. As discussed in the previous section, a range of factors can potentially modulate an individual's response, including some genotypes and inflammatory status. Therefore, a more personalised approach to dietary recommendations could be advantageous (de Roos, 2012).

The potential would be to use genetic information to detect disease risk, then tailor diet and lifestyle advice to reduce that risk (Minihane, 2013). However, integrating the complexities of gene-environment-physiological interactions, disease pathophysiology and the chemical complexity of whole foods represents a considerable challenge to nutrition researchers, making the goal of personalised dietary recommendations still some way in the future.

Would people embrace a personalised diet? De Roos (2013) reports results from a European survey showing that 66% of respondents would be willing to have genetic tests, but only 27% would follow a personalised diet (de Roos, 2012). Another question is what would be the cost be? Would the benefits derived from personalised dietary recommendations, be available only to those with money and education (Fenech et al., 2011).

Central to all heart-healthy eating patterns are fruit and vegetables. Surprisingly, despite the WHO rating them as having the strongest strength of evidence for decreasing CVD risk, little evidence supports a causal relationship between fruit and vegetable intake and CVD prevention. Most evidence for a protective effect comes from observational studies. Many of the studies that have investigated the effects of either individual fruit or fruit components, such as polyphenols, on surrogate endpoints, such as plasma lipids and BP, have been identified as having poor study design and/or being inadequately powered.

Good study design would reduce not only inter-study inconsistences, but allow within-study inter-individual responsiveness to be investigated. This area of research has now identified a range of factors that individually or interacting with each other help explain the variability in response that is often seen to a dietary intervention. These factors add another layer of complexity to this already difficult area of research.

2.4 Summary

Cardiovascular disease is a major contributor to global morbidity and mortality. Although research has given us a greater understanding of many aspects of the disease, including the atherosclerotic disease process, factors that contribute to CVD risk and the relationships between these factors, many questions still lack definitive answers. Many of the insights that have been gained have come from animal or cell studies, which have yet to be confirmed in humans. The rapidly developing 'omics' technologies will likely play a part in unravelling this. Instead of looking at single pathways, metabolomics, for example, offers the ability to investigate complex metabolic pathways (Rasmiena et al., 2013).

Even though CVD has a complex pathogenesis, it is to a large degree preventable, and a number of factors contributing to increased risk are modifiable. An unhealthy diet, as a behavioural risk factor, can directly lead to the development of metabolic risk factors, such as dyslipidaemia, raised BP, and obesity. Conversely, a healthy diet is a central feature of all prevention and treatment strategies, with fruit and vegetables a fundamental component of such a diet.

Although evidence exists that fruit and vegetable consumption is positively associated with decreases in BP, the evidence is less conclusive for other CVD risk factors. Well-designed whole fruit studies could add to the body of evidence, and not only support what has been shown in observational studies, but potentially also allow future recommendations about the quantity and mix of fruit required for optimal prevention.

As a particularly nutrient dense fruit, kiwifruit has the potential to offer benefits beyond those of some other commonly consumed fruit. However, conflicting results have been seen in the four studies conducted to date, particularly in regard to the effects of kiwifruit on lipids. The studies varied in experimental study design, in the primary outcomes measured, in subject groups and three studies were uncontrolled.

Another factor that may have contributed to the variability in responses is inter-individual heterogeneity. A wide range of factors which may contribute to inter-

individual variability in responsiveness have been identified, including genetic factors, such as SNPs, and physiological factors, such as inflammation, which currently are often studied in isolation. To integrate the influences of multiple factors into our understanding, thereby potentially allowing the development of personalised dietary recommendations, will require the implementation of more robust study design, together with further technological advances to explain the complex biological systems involved.

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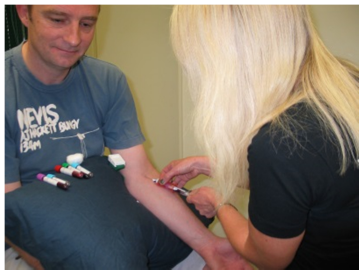
Chapter 3: Methodology

Checking diaries and discussing the next stage of the study

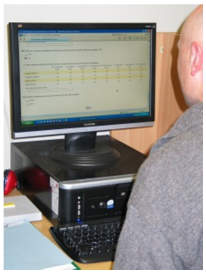
A subject in the BODPOD



Avril taking blood samples from a subject

Welma conducting a Finometer reading

Olivia, checking a subject's 24-hr food record

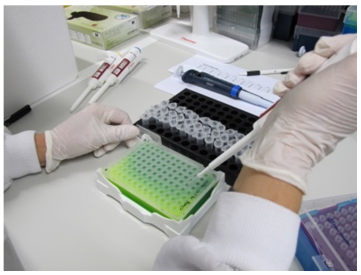


Completing on-line FFQ

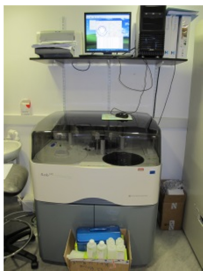
Retrieving kiwifruit from the cooler

Preparing kiwifruit for nutrient analysis

Vacuum-packed kiwifruit samples ready for freezing



Preparing a PCR plate for SNP genotyping assay



I-Lab running the sLDL analysis

3.1 Introduction

This chapter describes the methodology for the intervention study, which was conducted between May and September, 2010.

The trial was registered with the Australian New Zealand Clinical Trial Registry www.ANZCTR.org.au (ACTRN12610000213044), 16th March, 2010.

The primary outcome measures for the study were lipid profiles; plasma TC, LDL-C, TG, HDL-C, TC/HDL-C ratio, sLDL, and serum apoA1 and apoB.

The secondary outcome measures were markers of glucose metabolism (plasma glucose, serum insulin), BP and other markers of cardiovascular function (including HR, SV, CO and TPR), serum hs-CRP and plasma cytokines (IL-6, TNF-α, and IL-10).

3.2 Subjects

Men, aged 21 years or older, with a LDL-C concentration greater than 3.0 mmol/L and a plasma TG concentration below 3.0 mmol/L were recruited.

As differences in lipid kinetics have been identified between the genders (Wang et al., 2011), men were chosen as the subject group for this study to remove the possible confounding and interaction effects of having a mixed subject group.

Exclusion criteria included smoking, an allergy to kiwifruit, the use of any product that could affect lipid concentrations including lipid lowering medication (statins, fibrates and cholesterol absorption inhibitors), supplements such as nicotinic acid, fish oil capsules, fibre-containing laxatives, or functional foods such as sterol-enriched spreads, and diagnosed chronic disease such as CHD, diabetes, and cancer (for the complete list see appendix 4). Additionally, subjects were excluded if familial hypercholesterolaemia was suspected, as determined by using the diagnostic criteria for familial hypercholesterolaemia described in de Sauvage Nolting et al. (2002) subjects with an LDL-C of >8.5 mmol/L or with an LDL-C of >6.5-8.4 mmol/L in combination with a family history (first degree relative with CHD or LDL-C >95th percentile (5.5 mmol/L in NZ) (Gentles et al., 2007) or personal history of CHD).

As one of the objectives of the study was to compare the effects of the intervention between carriers of the *APOE4* allele and *APOE4* non-carriers, it was calculated that a minimum of 80 subjects would be required to ensure a sample size of 20 carrying the *APOE4* allele, based on an estimated prevalence of 25% in Caucasian populations (Jofre-Monseny et al., 2008). A sample size of 20 provided 80% power to detect a significant difference (α=0.05, two-tail) of 0.5 mmol/L (±7-10%) in TC and LDL-C between the experimental and control treatments. Power calculations were based on evidence that a reduction of 0.5 mmol/L is estimated to result in a 12-14% reduction in CHD mortality risk which is a clinically significant reduction (Gould et al., 2007). More subjects (n=87) were recruited to allow for drop-outs from the study. Based on a previous study conducted in Auckland, which found a 60% prevalence rate of LDL-C >3.0 mmol/L amongst adults, it was estimated that between 130 and 150 men would need to be screened (Gentles et al., 2007).

Men in and around Auckland were invited to participate in the study through a range of recruitment strategies. Emails were sent to individuals on the Massey Albany Nutrition Research volunteer database, employees of Massey University registered to the 'Albany All' database, colleagues and friends, and contacts at organisations such as the New Zealand Heart Foundation, Massey University Alumni, Auckland Nutrition Research Network and the South African club. Posters were distributed locally to medical practices, Diagnostic MedLab collection centres, gymnasiums and some local businesses in the Albany area, and a local area letter box flyer drop was organised. The email and poster advertisements were not only aimed at men, but also women and the men in their lives. There was also an advertisement in the 'Healthy Food Guide' magazine and a press release to local newspapers, resulting in articles in several local newspapers e.g. the North Shore Times and Rodney Times. A web-site and email address were set-up so that men could register their interest and/or seek further information. On registering interest, the men were then emailed an information sheet and a health-screening questionnaire (Appendix 4).

3.3 Screening

Interested subjects were screened in two stages. The first stage utilised the medical history and health-screening questionnaire, to identify any subjects who had conditions that would require them to be excluded (screening procedure and criteria Appendix 4). Subjects meeting the inclusion criteria were sent an email requesting them to have a fasting blood test (at least 8 hours with no food or beverage, excluding water) at a Diagnostic MedLab (Auckland, NZ; International Accreditation New Zealand (IANZ) accredited medical testing laboratory) collection centre. This served as the second screen, identifying those with an LDL-C >3.0 mmol/L and plasma TG <3.0 mmol/L.

3.4 Study design

The study was a 12-week randomised controlled cross-over intervention trial, conducted under free-living conditions. The study consisted of two 4-week treatment sequences of two green kiwifruit/day (replacing two of the subject's usual fruit servings) plus healthy diet (intervention) or healthy diet alone (control). Prior to commencement of the intervention, subjects followed a healthy diet for a 4-week run-in period. A per protocol analysis was used, excluding subjects, based on their diaries, if they did not consume 80% or more of their kiwifruit during the intervention. Figure 3.1 illustrates the study flow, from recruitment to completion, and the assessments made at each visit.

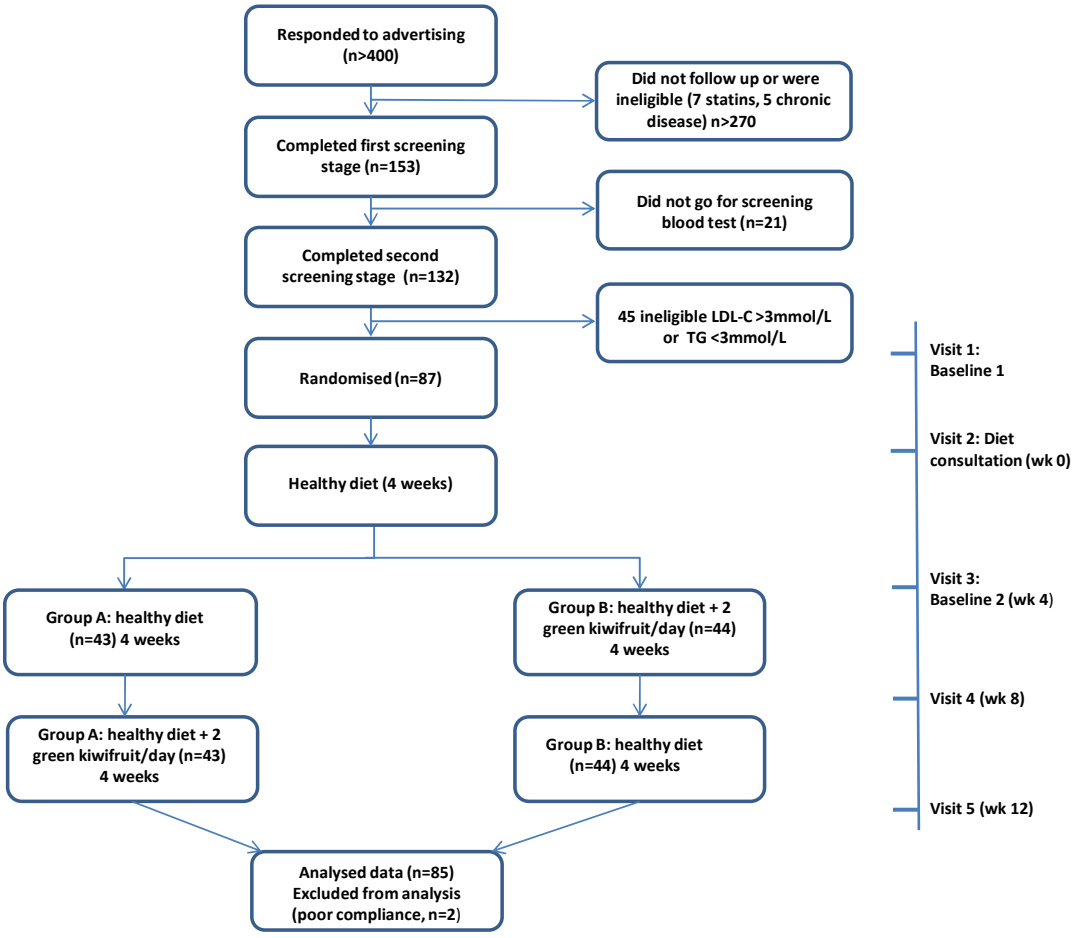
3.5 Ethics

Ethical approval was obtained from the Massey University Human Ethics Committee (MUHEC): Southern A 09/76. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all the subjects signed informed consent forms prior to participating in the study.

The *APOE* genotyping raised some additional ethical issues. The ethics committee determined that the individual results of the *APOE* genotype analysis had to be reported to the subjects if they wanted to know their results. In this regard MUHEC required the following: that subjects be provided with additional information regarding the *APOE* genotype and the possible implications of knowing/not knowing their results (*APOE* gene fact sheet - Appendix 5); the

analyses be completed by a laboratory with IANZ accreditation for medical testing; subjects signed an additional disclaimer form if they wanted their results; a medical practitioner informed and consulted with all *APOE4* carriers; genetic results had to be de-identified three months after statistical analysis was completed.

All subjects received nominal compensation for travel expenses.



% BF: percentage body fat, hs-CRP: high sensitivity C-reactive protein; sLDL: small-dense LDL; wt: weight

Visit 1
•Demographic questionnaire
•Anthropometric measures: height, wt waist, hip, % BF
•3-day food record
•Fasting blood sample: lipids

Visit 2
•Nutrition consultation

Visit 3
•Anthropometric measures: wt, waist, hip ,% BF
•Fasting blood sample: lipids, glucose, insulin, hs-CRP, sLDL, inflammatory cytokines
•Blood pressure: Omron & Finometer
•Dietary assessment: 24-hour food record, fruit frequency questionnaire

Visit 4
Same as for visit 3 plus:
•Tolerance questionnaire

Visit 5
Same as for visit 4 plus:
•Physical activity questionnaire
•Blood sample for genetic testing

Figure 3.1 Study flow

3.6 Setting

Human Nutrition Research Unit, Massey University, Albany

3.7 Procedure

If eligible, the men were invited to enrol (by email) and make an appointment for their first visit (baseline 1). Once an appointment was made, further information was emailed outlining the procedures during the first visit and the specific requirements for the fasting blood sample and BF measurements.

Visit 1: When subjects arrived for their first visit they were required to sign the consent form and to fill in a personal information sheet and a demographic questionnaire. Anthropometric measures and a fasting blood sample were taken. Subjects were given instructions on the completion of a 3-day food record and an appointment was made for the nutrition consultation (visit 2).

Visit 2: Subjects attended a group session, where the guidelines for the healthy (control) diet were outlined. In addition, each subject received an individual consultation based on their food record and recommendations on how to achieve the guidelines. These sessions were conducted by nutritionists in the research team, including the author. After a 4-week run-in period on the control diet, during which time subjects were asked to not consume kiwifruit, subjects returned to the research unit for visit 3 (baseline 2).

Visit 3: Anthropometric measures were repeated, BP and other markers of CVD function measured and fasting blood samples taken. Subjects were also required have an interview with a nutritionist, to discuss their 24-hour food record that they had been asked to complete of their previous days food intake, and complete a fruit-frequency questionnaire on-line.

The subjects were then randomly assigned, using computer generated random numbers (http://www.randomization.com/), to one of two groups (dietary treatment sequences): control diet or control diet plus two green kiwifruit per day for 4 weeks (where two fruit servings were replaced with two kiwifruit).

Visit 4: Anthropometric, dietary and clinical measures as per visit 3 were repeated. In addition, the men who had completed the kiwifruit interventions completed a tolerance questionnaire regarding their experience of consuming

two kiwifruit a day. Subjects crossed over to complete the other dietary treatment for a further four weeks. No washout period was needed since serum lipid levels are known to stabilise within three weeks (Kris-Etherton and Dietschy, 1997).

Visit 5: After completing the next 4-week intervention period, subjects returned for the anthropometric, dietary and clinical measures as per visit 4. Additionally, an on-line physical activity questionnaire was completed, and an additional blood sample taken for genetic testing.

The subjects were asked to maintain their normal daily routine (eating pattern, physical activity, and alcohol consumption) for the duration of the study. Compliance with these instructions, following the intervention, use of medication and the presence of illness during the study was monitored in a weekly self-completed diary (Appendix 6).

3.8 Interventions

3.8.1 Healthy diet

All subjects were required to attend a nutrition consultation (visit 2). These were held in the evenings (3 session times/evening), with groups of up to 4 men and their partners. Each subject received a brochure outlining the 9 'Healthy Heart' guidelines for the healthy diet (control diet) that they were requested to follow, and a laminated A4 card (and Zespri kiwifruit magnet) to put on their fridge as a reminder, with guidelines listed on one side and examples of portion sizes on the other (Appendix 7). The guidelines were based on the Ministry of Health's 'Eating for Healthy Adult New Zealanders' and the New Zealand Heart Foundation's '9 steps to Eating for a Healthy Heart' guidelines (www.heartfoundation.org.nz). The consultation consisted of a 30-minute group presentation by a nutritionist, giving an overview of the guidelines, including examples and tips on how to achieve them. This was followed by a 30-minute individual consultation with a nutritionist, where subjects received a personalised report that was produced (using the guidelines) based on their 3-day food record, identifying positive parts of their current diet and areas where

there is room for improvement. Any specific issues that were identified were discussed and questions answered.

3.8.2 Kiwifruit intervention

Kiwifruit were provided by Zespri, prior to each 4 week period of the intervention. These were stored a cooler at 2° C, prior to distribution.

Subjects in the kiwifruit intervention received two trays (15 fruit per tray) of Zespri green kiwifruit (*Actinidia deliciosa* var Hayward*)* and were requested to consume two kiwifruit per day.

The men were asked to continue to consume the healthy diet and replace two servings of fruit from their healthy diet with the kiwifruit. The subjects all received verbal instructions on how to store and ripen their kiwifruit, and these were also included in their weekly diary (Appendix 6). In addition, the diary had some suggestions on ways to consume the kiwifruit, such as sliced onto breakfast cereal, scooped out with the Zespri spoon as a snack, or chopped as an accompaniment with some low fat yoghurt as a dessert.

The men were asked to record in their weekly diary whether they consumed the required amount of kiwifruit.

3.9 Assessment methods

A summary of the methods used for anthropometric, clinical, dietary and other assessments are provided in Table 3.1

3.9.1 Demographics

The personal details questionnaire included full contact details and name of their general practitioner, while the demographic questionnaire (Appendix 8) included questions about ethnicity, country of birth and length of time in NZ based on the NZ Census demographic information.

3.9.2 Anthropometric measurements

Height (visit 1 only) and waist and hip circumferences were measured at visits 1 and 3 to 6, using the International Society for the Advancement of Kinanthropometry (ISAK) anthropometry methods using a stadiometer and

Lufkin tape. Weight and percentage BF were measured using the BODPOD. Body mass index and WHR ratio were then calculated.

Table 3.1: Summary of methods used for anthropometric, clinical, dietary and other assessments

Assessment	Method
Height, waist and hip circumference	ISAK anthropometry protocol and standards, using stadiometer, Lufkin tape.
Weight and percentage body fat	BODPOD (air displacement plethysmography, BODPOD 2007A, software version 4.5.1). (Life measurement, Inc (now COSMED US, Inc) California, US).
Brachial arterial blood pressure	Omron HEM-907 Digital Automatic Blood Pressure Monitor. (Omron Healthcare, Inc. Kyoto, Japan).
Finger arterial blood pressure, heart rate, and other cardiac markers	Finometer Midi (Model 2), BeatScope Easy software v01.02 (Finapres Medical Systems B.V, Amsterdam, The Netherlands)
Questionnaires	
Health-screening questionnaire	Age, medical and family history, medication and supplement use, and use of functional foods such as sterol enriched spreads (Appendix 4).
Demographic questionnaire	Teleform questionnaire based on questions from the NZ Census of Population and Dwellings (Appendix 8). (Autonomy Cardiff, California, US).
Dietary assessment (baseline)	3-day food record. Completed by subjects. Analysed using Foodworks 2009 (Xyris Software). (Xyris Software (Australia) Pty Ltd, Queensland, Australia)
Dietary assessment (visits 3-5)	24-hour food records. Completed by subjects. Analysed using Foodworks 2009 (Xyris Software). (Xyris Software (Australia) Pty Ltd, Queensland, Australia).
Fruit frequency questionnaire	On-line questionnaire (*www.surveymonkey.com*) completed by subjects assessing fruit intake over the previous week (Appendix 8). (SurveyMonkey, California, US)
Tolerance questionnaire	On-line questionnaire (*www.surveymonkey.com*) completed by subjects assessing tolerance to consuming 2 kiwifruit per day (Appendix 8). (SurveyMonkey, California, US)
Physical activity questionnaire	RPAQ validated questionnaire (Besson et al., 2010) converted into an on-line format and completed by subjects *(www.surveymonkey.com*) (Appendix 8). (SurveyMonkey, California, US)

ISAK: International Society for the Advancement of Kinanthropometry; RPAQ: recent physical activity questionnaire; US: United States

3.9.3 Dietary assessment

Subjects were given a 3-day food record booklet to complete. This included written instructions and examples of how to complete the food record, and how to record recipes. Verbal instructions to reinforce the written instructions were also given and the specified days on which to complete the record (3 sequential days, including 1 weekend day) were noted. In addition, subjects received a digital video disc (DVD) containing the instructions in a pictorial format that they were encouraged to watch at home (with partners), before starting their 3-day food records. The 3-day food record was checked for completeness during the individual nutrition consultation at the next visit.

Subjects received a one day version of the 3-day food record, to record their food intake as they consumed it, specifically for the day before their appointment for visits 3 to 5. During their visit the 24-hour food records were checked by a nutritionist for completeness and portion size accuracy, and a quality control checklist was completed based on their achievement of the 'healthy diet' guidelines including aspects such as alcohol consumption, type of margarine/butter, and number servings of fruit per day over the previous week.

An on-line fruit frequency questionnaire was completed during visits 3 to 5, to assess their fruit intake over the previous week (Appendix 8).

3.9.4 Nutrient analysis

Nutrient analysis for the 3-day food record and subsequent 24-hour food records was conducted using the computer programme FoodWorks Professional Edition Version 4.00 (Xyris Software, 2009, Queensland, Australia). The three 24-hour food records were combined to give an average dietary intake, which allowed comparison with the 3-day record (i.e. pre-nutrition consultation intake versus during the intervention intake). Additionally, the 24-hour food records were analysed for some nutrients and non-nutrients including fibre and vitamins C and E, separately for comparison of intake between intervention periods. It was expected that increases in the levels of these dietary components would be seen during the kiwifruit intervention.

3.9.5 Physical activity assessment

The validated Recent Physical Activity Questionnaire (RPAQ) (Besson et al., 2010) was converted into an on-line format (www.surveymonkey.com) and completed by all subjects during visit 5 (Appendix 8). The RPAQ assessed their physical activity at home, work and recreation, and over the last four weeks.

3.9.6 Blood samples, processing and analysis

Summaries of the blood samples taken and how they were processed and analysed are provided in Table 3.2 and Table 3.3.

Venous blood samples were taken by a registered phlebotomist, using a sterile vacutainer flashback needle and needle holder or a winged blood collection set. If a winged blood collection set was used, the first 4.0 ml of blood was discarded, to avoid 'dead space' in the tubing. Subjects fasted overnight (at least 8 hours with no food or beverage, excluding water which could be consumed up to 2 hours before the visit) and the sample was taken with minimal stasis between 7.00 am and 9.15 am (to avoid diurnal variation), preferably from the left arm, since the right arm was used for BP assessment.

Analysis was conducted by the Canterbury Health Laboratories, Christchurch, NZ (IANZ ISO 15 189) for all samples except the cytokines (IL-6, TNF-α and IL-10), which were analysed by the Nutrition Laboratory, Massey University, Palmerston North (IANZ ISO 17025), and sLDL which was analysed at the Biomedical Research Centre, University of East Anglia; Norwich, UK.

For the sLDL analysis, samples were defrosted and spun briefly, before 30μL was pipetted into sample cups for loading into the I-Lab 650 (automated chemistry analyser). The sLDL concentration was determined by a two-step assay, using the sLDL-EX "SEIKEN" 2-reagent kit and the designated calibrator and quality control solutions. Inter and intra days coefficient of variance (CV) for the analysis are reported in Table 3.3.

Table 3.2: Processing of blood samples

Order of drawing blood	Tube	Processing	Type of analysis
1	1 x 10 ml Serum tube (red top)	Serum tubes were stood at ambient temperature for 30 mins prior to processing to allow clotting. All other tubes were kept on ice and processed within 60 minutes of collection. Tubes were centrifuged for 15 min at 3000 rpm, 4°C Aliquots were stored at -80°C until the end of the study for analysis in one batch.	Serum ApoA1 Serum ApoB Serum hs-CRP
2	1 x 6 ml Heparin tube (green top)		Plasma TC, TG, HDL-C Plasma glucose
3	1 x 6 ml EDTA tube (lavender top)		Plasma insulin Plasma cytokines Plasma sLDL Buffy coat (white cells) transferred to LoBind DNA free eppendorf tubes (Eppendorf, Hamburg, Germany) for genomic DNA isolation

ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; DNA: Deoxyribonucleic acid; EDTA: ethylene diamine tetra acetic acid; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high sensitivity C-reactive protein; sLDL: small-dense LDL; TC: total cholesterol; TG: triglycerides

Genotyping (other than *APOE*) was conducted at the Biomedical Research Centre, University of East Anglia; Norwich, UK. Genomic DNA was isolated from the buffy coat samples using a QIAamp DNA Mini Blood Kit. Applied Biosystems 7500 Fast Real-Time PCR System (software version 2.0.5; Applied Biosystems, Warrington, UK) and TaqMan® SNP genotyping assays (Applied Biosystems, Warrington, UK) were used to determine the allelic discrimination of the selected gene variants (Real-time PCR SNP genotyping assay procedure including DNA isolation, Appendix 9).

The HOMA 2 model was used to calculate IR (HOMA2-IR), based on fasting insulin and glucose (Wallace et al., 2004).

Table 3.3: Biochemical analysis

Measure	Method	Coefficient of variance (%)
Place of analysis	*Canterbury Health Laboratory, Canterbury District Health Board, Christchurch, NZ*	
Plasma TC	Enzymatic cholesterol oxidase, Abbott reagent kit (Cat. No.7D62-21), Abbott C8000 analyser (Abbott Laboratories, Illinois, US)	2.5 mmol/L: 2.0% 4.5 mmol/L: 1.7% 6.8 mmol/L: 2.1%
Plasma TG	Enzymatic hydrolysis of triglycerides, Abbott reagent kit (Cat. No.7D74-21), Abbott C8000 analyser (Abbott Laboratories, Illinois, US)	0.9 mmol/L: 5.9% 1.5 mmol/L: 3.5% 2.3 mmol/L: 2.5%
Plasma HDL-C	Accelerated reaction using selective detergent, Abbott reagent kit (Cat. No.3K33-21), Abbott C8000 analyser (Abbott Laboratories, Illinois, US)	0.91 mmol/L: 10.1% 1.26 mmol/L: 4.6% 2.09 mmol/L: 3.7%
Plasma LDL-C	Calculated (LDL-C = TC - HDL-C - (TG/2.22) mmol/L)	NA
Serum ApoA1	Abbott Architect/Aeroset reagent kit (Cat. No. 9D92-20), Abbott C2000 biochemistry analyser (Abbott Laboratories, Illinois, US)	3%
Serum ApoB	Abbott Architect/Aeroset reagent kit (Cat. No. 9D93-20), Abbott C2000 biochemistry analyser (Abbott Laboratories, Illinois, US)	4%
Plasma glucose	Enzymatic assay, glucose hexokinase, Abbott reagent kit (Cat. No.3L82-41), Abbott C8000 analyser (Abbott Laboratories, Illinois, US)	3.29 mmol/L:1.72% 6.83 mmol/L: 2.49%
Plasma insulin	2-site sandwich after PEG extraction, Roche Elecsys 2010 Insulin kit (Cat. No. 12017547) (Roche, Mannheim, Germany)	5.90 mIU/L: 8.7% 61.91 mIU/L: 3.8%
Serum hs-CRP	Nephelometry assay, Siemens BNII, Siemens Reagent (Cat. No. 10446091), (Siemens Healthcare Diagnostic Products GmbH, Marburg ,Germany)	1.75 mg/L: 5.1% 17.75 mg/L: 4.5%
APOE genotype	In house polymerase chain reaction (PCR) (Hixson and Vernier, 1990)	NA
Place of analysis	*Nutrition Laboratory, Massey University, Palmerston North*	
Cytokines	High-sensitivity human cytokine Lincoplex kits (cat. No. Cat. HCYTO-60K) (Linco Reasearch, Inc., Missouri, US,)	
IL-6		intra-assay 3.51% inter-assay 4.48%
TNF-α		intra-assay 3.49% inter-assay 3.78%
IL-10		intra-assay 3.31% inter-assay 11.84%

ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; *APOE*: apolipoprotein E genotype; CETP: cholesteryl ester transfer protein; DNA: Deoxyribonucleic acid; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high sensitivity C-reactive protein; IL: interleukin; LDL-C: low density lipoprotein cholesterol; LIPC: hepatic lipase; LIPG: endothelial lipase; sLDL: small-dense LDL-C; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor-alpha

Table 3.3: Biochemical analysis continued:

Measure	Method	Coefficient of variance (%)
Place of analysis	*Biomedical Research Centre, University of East Anglia, Norwich, UK*	
Plasma sLDL	sLDL-EX "SEIKEN" 2-reagent kit (Cat. No. 56216), Randox Laboratories, UK. l-Lab 650 chemistry analyser (Instrumentation Laboratory, Massachusetts, US)	intra-assay 0·99% inter-assay 1·24%
DNA purification	QIAamp® DNA Mini kit (Cat. No. 51104) (QIAGEN Ltd, Crawley, UK)	NA
CETP Taq1B genotype	Human Pre-designed Assay, rs708272 (ID. C___9615318_10), Applied Biosytems, Life Technologies, California, US	NA
APOA1 -75G/A	Custom TaqMan® SNP Genotyping Assay, rs670, Applied Biosytems, Life Technologies, California, US	NA
LIPC -514C→T	Human Pre-designed Assay, rs1800588 (ID. C___8757333_30), Applied Biosytems, Life Technologies, California, US	NA
LIPG I24582 (T+2864C/In8)	Human Pre-designed Assay, rs6507931 (ID. C___8757333_30), Applied Biosytems, Life Technologies, California, US	NA

ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; *APOE*: apolipoprotein E genotype; CETP: cholesteryl ester transfer protein; DNA: Deoxyribonucleic acid; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high sensitivity C-reactive protein; IL: interleukin; LDL-C: low density lipoprotein cholesterol; LIPC: hepatic lipase; LIPG: endothelial lipase; sLDL: small-dense LDL-C; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor-alpha

3.9.7 Blood Pressure

3.9.7.1 Brachial arterial blood pressure

An Omron HEM-907 Digital Automatic Blood Pressure Monitor was used to measure brachial arterial BP and HR. The subjects were asked to lie down quietly on the bed with arms relaxed by their sides, covered with a blanket if cool, for about five minutes before any measurements were taken. The right arm was used for all measurements (the phlebotomists were requested to draw blood from the left arm). The Omron arm cuff was then attached, and two measurements taken consecutively, one minute apart, and the average calculated, using the auto function of the monitor.

3.9.7.2 Finger arterial blood pressure, heart rate and other cardiac markers

The Finometer MIDI® together with the accompanying BeatScope Easy software (v01.02) integrates the subject's gender, age, weight and height with the recordings and computes the following cardiovascular variables: SBP, DBP,

MAP, HR, TPR, CO and SV. After the oscillometric measurement of BP, the appropriately sized finometer cuff was attached to either the middle or ring fingers of the right hand, the pressure box secured to the wrist and gravity correction strap attached to the upper arm, about heart level. The subjects details (ID no, age, weight, height, and gender) were then entered into the computer, and the finometer started and a recording made for 7 minutes. Subjects were asked to relax and not to talk during the recording, and the lights were dimmed.

The finometer data was analysed by selecting the most stable minute (where variability was the lowest) between four and a half and six and a half minutes, with the beginning of each selected area being flagged on the readout. Where the finometer reading was more variable, two areas were selected. The data points from the selected area were then exported to an Excel file, where they were averaged and the standard deviation and % CV were calculated.

3.9.8 Tolerance to the kiwifruit intervention

After the 4-week period that subjects were on the kiwifruit intervention, an on-line tolerance questionnaire was completed. The questionnaire included questions such as rating how easy they found consuming two kiwifruit a day or if they had any unwanted side-effects (Appendix 8).

3.9.9 Preparation and nutrient analysis of kiwifruit samples

Twenty kiwifruit were randomly selected from each batch of kiwifruit, with one batch being delivered at the start of each 4-week intervention period. The fruit was allowed to ripen at room temperature to the same level of ripeness as recommended to the subjects for consumption. Batches of five kiwifruit were weighed, peeled, reweighed, then sliced, reweighed and vacuum-packed in a single layer in plastic bags. These were stored in the minus 20°C freezer, protected from light. The samples were freeze dried, and analysed for nutrient content by accredited laboratories: the Nutrition Laboratory, Massey University, Palmerston North (all nutrients except dietary fibre) and the Pilot Plant Laboratory, Massey University Palmerston North (dietary fibre). See Table 3.4 for details of analysis methods.

Table 3.4: Methods used for nutrient analysis of kiwifruit

Nutrient	Method of Analysis
Moisture	Convection oven 105° C, AOAC 930.15, 925.10
Protein	Leco, total combustion method. AOAC 968.06
Ash	Furnace 550° C, AOAC 942.05.
Fat	Cold extraction using chloroform/methanol. AOAC 969.24
Carbohydrate	By difference
Starch	α-amylase method. AOAC 996.11
Sugars	Phenol sulphuric, sub-contracted
Vitamin C	HPLC (Lee and Coates, 1987)
Dietary fibre	Megazyme total dietary fibre kit. Method types: AOAC 991.43, AOAC 985.29, AACC32-07 and AACC 32-05

AOAC: association of analytical chemists; HPLC: high performance liquid chromatography

3.10 Data handling and statistical analysis

All study data was entered into Microsoft Excel spreadsheets, with subjects only identified by their unique study number. Statistical analyses were performed using IBM SPSS statistics version 20 (IBM Corporation, New York, US). Power calculations were performed using G*Power version 3.1.5 (Faul et al., 2012).

The variables were tested for normality using the Kolmogorov-Smirnov, Shapiro-Wilk tests, and normality plots. Non-normally distributed data was transformed into approximately normal distributions, if possible, by logarithmic or square root transformations. Normally distributed data is summarised as the mean (standard deviation (SD)), or if more appropriate, mean and a 95% confidence interval (CI) for the mean, and when reporting inter-group differences, as standard error of the difference between means (SE). In cases where transformations improved normality, the summary quantities and tests were computed for the transformed data and then back transformed to the original scale. Non-normally distributed data was expressed as medians (25, 75 percentiles), and categorical data as frequencies.

The data was examined for any interaction effects due to the sequence of intervention (kiwifruit followed by control and vice versa) using 2-way analysis of variance (ANOVA). If no interaction was seen, the data from the two

intervention periods were combined. If an interaction was seen, only the data from the first four weeks of the intervention was analysed.

Descriptive statistics were used to describe the subject's characteristics at baseline. Changes in plasma lipids and anthropometric measures from baseline 1 to baseline 2 and comparisons between baseline 2 and end of the interventions (control and green kiwifruit interventions separately) and between control and kiwifruit interventions for changes in variables, were made using Dependent Student *t*-tests for normally distributed data or Wilcoxon Signed Ranked test for non-normally distributed data.

Repeated measures ANOVA was used to examine group and treatment interactions. Where data was stratified by group, within group comparisons were made using Dependent Student *t*-tests for normally distributed data or Wilcoxon Signed Ranked test for non-normally distributed data. Other between group comparisons were made using Independent Student *t*-tests, Mann-Whitney test for non-normally distributed data, or Pearson's chi-square (χ^2) test for categorical data. Differences were considered significant at $P<0.05$.

Supplementary statistical methodology specific to each investigation follows.

3.10.1 The effects of kiwifruit consumption on plasma lipids, and modulation of response by *APOE* genotype (Chapter 4)

Two-way ANOVA was used to examine *APOE* genotype and treatment interactions. However, since the assumptions of homogeneity of variance for ANOVA were not satisfied, lipid results for the two interventions were analysed stratified for *APOE4* carriers versus non-carriers.

See section 3.2 for details of the power calculation and the sample size required, to ensure a sample of at least 20 carriers of the *APOE4* allele.

3.10.2 The effects of inflammatory status on plasma lipid and inflammatory marker response (Chapter 5)

Additional subject exclusion criteria for this analysis included an elevated CRP concentration at any time point (>8 mg/L) or the taking of any medication known to affect inflammation. Subjects were divided into low and medium inflammatory

groups using their baseline 2 (pre-intervention) plasma hs-CRP measurements. This was done using the classification system described by the CDC and the AHA (low <1 mg/L, n=38; medium 1–3 mg/L, n=32) (Pearson et al., 2003). Due to the small group size, the 10 subjects in the high group (hs-CRP >3 mg/L) were excluded from analysis.

The sample size of n=38 and n=32 in the low and medium inflammatory groups, respectively, retrospectively provided 99% power in both groups to detect a mean difference of 0.29 mmol/L in the TC/HDL-C ratio, at an alpha level of 0.05. This difference equates to about a 15% reduction in CVD risk (Stampfer et al., 1991).

3.10.3 The effects of selected HDL-related genes on plasma lipid response (Chapter 6)

Multivariate analysis of variance (MANOVA) was used as an initial screen to investigate differences in multivariate patterns of lipid variables between genotype groups (major allele homozygotes versus minor allele carriers), to reduce the likelihood of chance findings from multiple comparisons.

Significant effects were seen for *CETP Taq1B* on the multivariate pattern of lipid responses and this conclusion was backed by an assessment of assumptions and univariate patterns. Therefore, further analysis of this genotype was conducted. Repeated measures ANOVA was used to examine genotype x treatment interactions. For ANOVA interactions where $P<0.05$, a stratified analysis was performed.

The sample size of n=51 and n=31 in the *Taq1B B2* carriers and *B1/B1* group, respectively, retrospectively provided 98% power in the *B2* carriers and 87% power in *B1/B1* group to detect a mean difference of 0.24 mmol/L in the TC/HDL-C ratio, at an alpha level of 0.05. This was based on the significant difference between kiwifruit and control treatments for TC/HDL-C ratio, in *B1/B1* homozygotes. This difference is related to about a 12% reduction in CVD risk (Stampfer et al., 1991). Additionally, the sample size provided 70% power to detect a genotype x treatment interaction for TC/HDL-C ratio.

3.10.4 The effects of the kiwifruit intervention on BP and other markers of cardiovascular function as measured by the Finometer (Chapter 7)

Baseline 2 (pre-intervention) BP measurements made by the Omron Digital Automatic Blood Pressure Monitor were used to divide subjects into BP groups using the classifications outlined in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) (Chobanian et al., 2003).

The sample size of 85 retrospectively provides 99.9% power to detect a mean difference of 10 mmHg, a clinically relevant reduction in SBP, at an alpha level of 0.05. A 10 mmHg reduction in SBP has been associated with a 38% reduction in risk of stroke and a 16% reduction in risk of CHD (Neal et al., 2000).

3.11 Provision of results to subjects

Subjects received two personal reports by email after completion of the interventions. The first report included their body composition, BP and blood results (lipids, insulin and glucose). The second was a dietary assessment report, comparing their intake before and after their nutrition consultation. This also included some further dietary recommendations for lowering blood cholesterol. If requested and the disclaimer form signed, subjects also received their *APOE* genotype result. In the case of *APOE4* carriers, a medical practitioner informed the subjects by telephone, discussed any concerns they might have and offered a consultation. A presentation was given summarising the main findings of the study.

3.12 Funding

Unrestricted grant from Zespri International Limited, New Zealand

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Chapter 4: Kiwifruit consumption favourably affects plasma lipids in a randomised controlled trial in hypercholesterolaemic men

This chapter presents the primary results from the randomised controlled trial. The overall aim of the study was to investigate the effect of consuming two green kiwifruit daily in conjunction with a healthy diet on plasma lipids and other CVD-related metabolic markers in hypercholesterolaemic men. Further, it was aimed to examine whether the response might be modulated according to APOE genotype.

Data published in: Gammon, C. S., Kruger, R., Minihane, A. M., Conlon, C. A., von Hurst, P. R. & Stonehouse, W. (2013) Kiwifruit consumption favourably affects plasma lipids in a randomised controlled trial in hypercholesterolaemic men. *British Journal of Nutrition,* 109 (12), 2208–2218

(Publications are listed in Appendix 1; related presentations in Appendix 2; contributions of authors in Appendix 3)

Abstract

The unique composition of green kiwifruit has the potential to benefit CVD risk. The aim of the present study was to investigate the effect of consuming two green kiwifruit daily in conjunction with a healthy diet on plasma lipids and other metabolic markers and to examine response according to *APOE* genotype in hypercholesterolaemic men. After undergoing a 4-week healthy diet, 85 hypercholesterolaemic men (LDL-C >3.0 mmol/L and TG <3.0 mmol/L) completed an eight week randomised controlled cross-over study of two 4-week intervention sequences of two green kiwifruit/day plus healthy diet (intervention) or healthy diet alone (control). Anthropometric measures, BP and fasting blood samples (plasma lipids, serum apoA1, apoB, insulin, glucose, and hs-CRP) were taken at baseline, 4 and 8 weeks. After the kiwifruit intervention plasma HDL-C concentrations were significantly higher (mean difference 0.04 [95% CI: 0.01, 0.07] mmol/L [*P*=0.004]) and the TC/HDL-C ratio was significantly lower (-0.15 [-0.24, -0.05] mmol/L [*P*=0.002]), compared to control. In carriers of *APOE4* allele TG decreased significantly (-0.18 [-0.34, -0.02] mmol/L [*P*=0.03]) with kiwifruit compared to control. There were no significant differences between the two interventions for plasma TC, LDL-C, insulin, glucose, hs-CRP and BP. The small but significant increase in HDL-C and decrease in TC/HDL-C ratio and TG (in *APOE4* carriers) suggests that the regular inclusion of green kiwifruit as part of a healthy diet may be beneficial in improving the lipid profiles of men with high cholesterol.

4.1 Introduction

In NZ, despite a decline of over 60% in CVD mortality rates since its peak in the late 1960s, CVD remains one of the leading causes of death, contributing to over 28% of overall mortality (Ministry of Health, 2011, Tobias et al., 2006). While pharmacological interventions have made a substantial contribution to the decline in incidence since the 1990s, it is speculated that changes in dietary consumption behaviours may have made the biggest contribution before then (Tobias et al., 2008).

One of the changes in dietary patterns has been a steady increase in fruit consumption, but 45% of NZ males still do not consume the recommended number of two fruit servings per day (University of Otago and Ministry of Health, 2011). Fruit are chemically complex foods that contain a range of nutrient and non-nutrient components which may contribute independently or synergistically to cardiovascular health (Badimon et al., 2010, Voutilainen et al., 2006, World Health Organisation, 2003). These include soluble fibre, antioxidant vitamins, folate and phytochemicals (Badimon et al., 2010, Brown et al., 1999, Erkkila and Lichtenstein, 2006, Salas-Salvado et al., 2006, Voutilainen et al., 2006).

In NZ kiwifruit is a commonly consumed, nutrient dense fruit. Green kiwifruit are unique in that they contain significant levels of soluble fibre (Schakel et al., 2001) and have one of the highest concentrations of vitamin C of any readily available fruit (Ferguson and Ferguson, 2003). Kiwifruit has been shown to be a significantly better delivery vehicle to replenish depleted vitamin C tissue levels than a supplement, in a mouse model (Vissers et al., 2011). Green kiwifruit also contains a significant amount of vitamin E, which is more commonly associated with green leafy vegetables than fruit (other than avocados), potassium, folate and other phytochemicals, including polyphenols and carotenoids (Ferguson and Ferguson, 2003, Hunter et al., 2011). Many of the phenolics and flavonoids are yet to be identified, as to date they have been unextractable (Tarascou et al., 2010). Few studies have investigated the effects of kiwifruit on CVD-related metabolic markers. Two previous studies investigating their effects on lipid profiles have shown conflicting results, and neither study included a control

group (Chang and Liu, 2009, Duttaroy and Jorgensen, 2004). A recent study has shown beneficial effects on BP (Karlsen et al., 2013).

Genetic variation is known not only to contribute to >45% of CVD risk (Elder et al., 2009), but also to explain a large component of the highly heterogeneous response to interventions (Corella and Ordovas, 2005, Masson et al., 2003). An example is the common *APOE* epsilon variant where, in a general population, up to a 50% higher risk of CVD is associated with the *APOE4* compared to the wild-type *APOE3/E3* genotype (Song et al., 2004). Although recent evidence is suggestive of a pro-inflammatory and pro-oxidant impact of the *APOE4* allele (Jofre-Monseny et al., 2008), the higher risk has been traditionally attributed to higher TC and LDL-C concentrations in this subgroup (Bennet et al., 2007). Furthermore, although the literature is inconsistent, carriers of the *APOE4* allele appear to show greater LDL-C lowering responses in dietary fat manipulation studies (Rimbach and Minihane, 2009).

We hypothesised that the replacement of two fruit servings in a healthy diet with two green kiwifruit would have favourable effects on plasma lipids and other CVD-related markers of risk and that carriers of different *APOE* genotypes may show differences in response to the intervention. We tested this hypothesis using a randomised controlled cross-over study design comparing a healthy diet including kiwifruit with a healthy diet with no kiwifruit. In addition to having a control group, unlike other dietary intervention studies of kiwifruit, this is the first to examine the beneficial impact of kiwifruit in addition to general recommended healthy eating guidelines. Thus the beneficial effects of kiwifruit beyond the effects of a healthy diet containing fruit could be investigated.

4.2 Subjects and methods

See Methodology Section (Chapter 3)

4.3 Results

4.3.1 Characteristics of subjects

All 87 men that met the inclusion criteria and were randomly assigned to the 2 intervention groups completed the 12 week intervention. However, based on their diaries kept during the intervention, two subjects were excluded from the analysis as a result of poor compliance (<80%) for the kiwifruit intervention.

The baseline characteristics of the 85 subjects who completed the study are shown in Table 4.1.

The men were healthy (no diagnosed chronic disease) and predominantly normotensive, but three-quarters were overweight or obese (BMI ≥25 kg/m^2) and more than 60% were carrying excess BF (% BF ≥25%) (Lee and Nieman, 2010, World Health Organisation, 1995). Analysis of the *APOE* genotype identified 23 men (27.4%) who were carriers of the *APOE4* allele (1 *E2/E4*, 21 *E3/E4* and 1 *E4/E4*) and 61 (72.6%) non-carriers (6 *E2/E3* and 55 *E3/E3*). One subject requested not to be included in any gene analysis.

4.3.2 Effects of the intervention on dietary intake, body weight and blood pressure

An assessment of the group's mean energy intake and that of selected other dietary components was made before the nutrition consultation, based on the average intake from a 3-day food record and during the intervention by using the average of three 24-hr records collected at visits 3 to 5 (Table 4.2). Significant differences were seen for all nutrients assessed (except % energy from PUFA) between the two periods, with decreases in total energy and cholesterol intake and the percentage of energy from fat and saturated fat.

Table 4.1: Baseline characteristics of subjects*

		Range
Age (yr)	48.0 (46.0, 50.0)	27.0 - 73.0
Weight (kg)	86.7 (83.3, 90.2)	60.0 - 161
Height (m)	1.78 (1.76, 1.79)	1.61 - 1.95
BMI (kg/m^2)	27.4 (26.5, 28.2)	19.8 - 42.4
Overweight/obese† ≥25 kg/m^2 (%)	75.3	
Waist circumference (cm)	93.4 (91.3, 95.6)	74.0 - 122
Central obesity†, WC ≥102 cm (%)	20.0	
Body fat (%)	27.4 (25.8, 29.0)	13.1 - 48.0
% BF‡ ≥25% (%)	60.0	
LDL-C (mmol/L) (n=82)§	4.07 (3.91, 4.24)	2.89 – 6.49
SBP (mmHg)¶	122 (120, 125)	102 - 143
DBP (mmHg)¶	70.9 (69.0, 72.8)	52 - 93
Hypertension† ≥140/90 (%)	2.35	
hs-CRP (mg/L)§, ¶	1.07 (0.89, 1.28)	0.15 -5.70
HOMA2-IR§, ¶	0.99 (0.86, 1.13)	0.2-5.5
% HOMA2-IR** >1.0 (%)	44.7	
APOE4 carriers (n, %) (n=84)††	23 (27.4)	
Ethnicity		
NZ European or European origin‡‡	91.8	
Maori	3.5	
Other§§	4.7	

% BF: percentage body fat; *APOE4*: apolipoprotein *E4* genotype; DBP: diastolic blood pressure; hs-CRP: high-sensitivity C-reactive protein; HOMA2-IR: homeostasis model assessment (HOMA) 2 model for insulin resistance; LDL-C: LDL cholesterol; SBP: systolic blood pressure; WC: waist circumference

*Values are means (95% CI) at Baseline 1, unless otherwise indicated, n=85

†Recognised cut-offs for WC, BMI and hypertension (National Cholesterol Education Program (NCEP) Expert Panel, 2002)

‡Recognised cut-off for excess body fat (Lee and Nieman, 2010, World Health Organisation, 1995)

§Geometric mean (95%CI)

¶Baseline 2 values

**Elevated IR (Wallace et al., 2004)

††One subject requested not to be included in any gene analysis

‡‡European origin: United Kingdom, South Africa, Europe and Australia

§§ Chinese, Philippines, Tonga and India

Table 4.2: Composition of the diet pre-nutrition consultation and during the intervention*

	Pre-consultation	During intervention	$P^{\dagger}$
Energy (kJ)	10623 (10040, 11206)	9094 (8694, 9494)	<0.0001
Protein (% energy)[‡]	17.8 (17.2, 18.4)	19.2 (18.5, 20.0)	<0.0001
Carbohydrate (% energy)	44.6 (43.0, 46.2)	49.6 (48.2, 51.0)	<0.0001
Fat (% energy)	32.4 (31.0, 33.7)	27.5 (26.3, 28.7)	<0.0001
SFA (% energy)	12.8 (12.0, 13.5)	9.74 (9.13, 10.4)	<0.0001
MUFA (% energy)	11.3 (10.8, 11.9)	9.92 (9.41, 10.4)	<0.0001
PUFA (% energy)[‡]	4.34 (4.04, 4.67)	4.41 (4.14, 4.69)	0.74
Cholesterol (mg)[§]	328 (230, 462)	239 (174, 363)	<0.0001
Fibre (g)[§]	25.2 (21.3, 32.9)	27.9 (23.0, 34.3)	0.03

kJ: kilojoules
*Values are means (95% CI) unless otherwise indicated, n=85. Pre-consultation intake: average of 3-day food record. During intervention intake: average three 24-hour food records from visits 3, 4 and 5
[†]Significant differences between pre-consultation and during intervention (P<0.05) (Dependent Student *t*-test), or Wilcoxon Signed-Ranks test for non-parametric data (P<0.05)
[‡]Values are geometric mean (95% CI)
[§]Values are medians (25^{th}, 75^{th} percentiles)

During the kiwifruit intervention, the men consumed half a fruit serving per day more (fresh, canned/stewed, dried and real fruit juice) compared to the control intervention. Significantly higher intakes were seen for vitamin C and vitamin E during the kiwifruit intervention compared to control intervention (Table 4.3).

During the 4-week healthy diet run-in period, small but significant decreases were observed for all anthropometric measures (body weight, BMI, WC, WHR ratio, and % BF). From the end of the run-in period (baseline 2) to the end of the 8-week intervention, there were no further changes in body weight, regardless of which intervention the subjects were receiving (green kiwifruit or control) (Table 4.4).

There were no significant changes in SBP or DBP (Table 4.4).

Table 4.3: Fruit servings, dietary fibre and vitamin C intakes at baseline 1 to baseline 2, and after the 2 intervention periods*

Variable	Baseline 1	Baseline 2	$P^{\dagger}$	End-Kiwifruit	End-Control	$P^{\ddagger}$
Fruit servings/day	1.33 (0.67, 2.67)	2.00 (1.00, 3.50)	<0.001	3.00 (3.00, 4.00)[§]	2.50 (2.00, 4.00)	<0.001
Fibre (g/day)	25.2 (21.3, 32.9)	27.2 (22.2, 33.7)	0.17	29.4 (22.3, 35.0)	27.6 (22.5, 33.6)	0.05
Vitamin C (mg/day)	111 (73.9, 164)	124 (77.9, 160)	0.93	282 (238, 335)[§]	236 (114, 313)[§]	<0.001
Vitamin E (mg/day)[¶]	10.0 (9.25, 10.9)	9.30 (8.48, 10.2)	0.12	13.0 (12.0,13.9)[§]	8.35 (7.60, 9.17)	<0.001

*Values are medians (25^{th}, 75th percentiles) unless otherwise indicated, n=85. Subjects followed a cross-over design protocol for the 2 treatment periods
[†]Significant differences from baseline 1 to baseline 2 (P<0.05) (Dependent Student *t*-test)
[‡]Significant differences between green kiwifruit *vs* control (P<0.05) (Dependent Student *t*-test)
[§]Significant differences from baseline 2 to end (P<0.05) (Dependent Student *t*-test)
[¶]Values are geometric mean (95% CI)

Table 4.4: Anthropometric and blood pressure assessments at baseline 1 to baseline 2, and after the 2 intervention periods*

Variable	Baseline 1	Baseline 2	$P^{\dagger}$	End-Kiwifruit	End-Control	$P^{\ddagger}$
Weight (kg)	86.7 (83.3, 90.2)	86.4 (83.0, 89.8)	0.03	86.7 (83.3, 90.0)	86.6 (83.2, 90.0)	0.86
BMI (kg/m^2)	27.4 (26.5, 28.2)	27.3 (26.5, 28.1)	0.03	27.3 (26.5, 28.2)	27.3 (26.5, 28.2)	0.87
Waist (cm)	93.4 (91.3, 95.6)	93.1 (90.9, 95.3)	0.03	92.8 (90.7, 94.9)	93.0 (90.9, 95.2)	0.31
Waist/hip ratio	0.91 (0.90, 0.92)	0.90 (0.89, 0.92)	0.001	0.90 (0.89, 0.91)	0.90 (0.89, 0.91)	0.39
Body fat (%)	27.4 (25.8, 29.0)	26.9 (25.2, 28.6)	0.002	26.9 (25.2, 28.6)	26.8 (25.2, 28.5)	0.53
SBP (mmHg)	NA	122 (120, 125)		124 (122, 127)	124 (122, 127)	0.90
DBP (mmHg)	NA	70.9 (69.0, 72.8)		72.3 (70.4, 74.3)	72.6 (70.4, 74.7)	0.80

Baseline 1 (before run-in period); Baseline 2 (after run-in period); DBP: diastolic blood pressure; NA: not assessed; SBP: systolic blood pressure
*Values are means (95% CI), n=85, subjects followed a cross-over design protocol for the 2 intervention periods
[†]Significant differences from baseline 1 to baseline 2 (P<0.05) (Dependent Student *t*-test)
[‡]Significant differences between green kiwifruit *vs* control (P<0.05) (Dependent Student *t*-test)

4.3.3 Tolerance to the kiwifruit intervention

The majority of subjects rated eating two green kiwifruit every day as easy or very easy at both the beginning (78.8%) and end (89.4%) of the intervention. Seventy-five subjects (88%) reported no unwanted side effects from eating two green kiwifruit a day. The most common reported unwanted side-effect was more frequent or looser bowel motions (3.5%).

4.3.4 Nutrient composition of the green kiwifruit

Two green kiwifruit, without their skin, weighed on average 167 g and provided on average 1.58 g of protein, 21.9 g CHO, 14.9 g sugars, 130 mg of vitamin C, 1.00 mg vitamin E, 4.11 g insoluble fibre and 1.42 g soluble fibre.

4.3.5 Effects on plasma lipid and apolipoprotein concentrations

Significant improvements were seen in lipid profiles following the 4-week run-in period (Table 4.5) with both plasma TC and LDL-C decreasing and HDL-C increasing significantly ($P<0.05$). There was also a tendency towards a decrease in plasma TG.

No significant differences between the two interventions were seen for plasma TC, LDL-C, TG and sLDL. There was a significant increase in HDL-C concentrations while consuming green kiwifruit compared to control (mean difference 0.04 (95%CI: 0.01, 0.07) mmol/L, $P=0.004$), with TC/HDL-C ratio significantly lower after the green kiwifruit period (mean difference 0.15 (95% CI: -0.24, -0.05) mmol/L, $P=0.002$). Serum apoA1 concentrations increased significantly from baseline 2 to end of the green kiwifruit intervention (0.03 (95% CI: 0.001, 0.06) g/L, $P=0.03$) resulting in a decrease in the apoB/apoA1 ratio with green kiwifruit consumption compared to control (although not significant, $P=0.05$).

Table 4.5: Plasma lipid and apolipoprotein concentrations at baseline 1 and 2, and after the 2 intervention periods*

	Baseline 1	Baseline 2	$P^{\dagger}$	End-Kiwifruit	End-Control	$P^{\ddagger}$
TC (mmol/L)	6.23 (6.04, 6.42)	6.04 (5.85, 6.23)	0.01	6.10 (5.93, 6.29)	6.11 (5.92, 6.30)	0.96
LDL-C (mmol/L)	4.07 (3.91, 4.24)	3.91 (3.76, 4.06)	0.008	3.92 (3.77, 4,08)	3.95 (3.80, 4.12)	0.50
HDL-C (mmol/L)	1.34 (1.28, 1.41)	1.38 (1.31, 1.44)	0.02	1.39 (1.33, 1.46)	1.35 (1.29, 1.41)	0.004
TG (mmol/L)	1.62 (1.47, 1.79)	1.52 (1.39, 1.65)	0.07	1.55 (1.42, 1.70)	1.58 (1.45, 1.72)	0.71
TC/HDL-C ratio[§]	4.75 (4.54, 4.97)	4.46 (4.29, 4.63)	<0.001	4.46 (4.28, 4.63)	4.60 (4.41, 4.79)[¶]	0.002
ApoA1 (g/L)	NA	1.36 (1.31, 1.41)	-	1.39 (1.34, 1.44)[¶]	1.37 (1.32, 1.42)	0.19
ApoB (g/L)[§]	NA	1.11 (1.07, 1.16)	-	1.12 (1.07, 1.16)	1.12 (1.08, 1.17)	0.60
ApoB/A1 ratio[§]	NA	0.83 (0.78, 0.87)	-	0.82 (0.77, 0.86)	0.83 (0.79, 0.88)	0.05
sLDL (mmol/L)[§]	NA	1.28 (1.17, 1.40)	-	1.29 (1.17, 1.40)	1.31 (1.21, 1.42)	0.51

Baseline 1 (before run-in period); Baseline 2 (after run-in period); ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; NA: not assessed; sLDL, small dense LDL-C; TC: total cholesterol; TG: triglycerides

*Values are geometric mean (95% CI) unless otherwise indicated, n=85. Subjects followed a cross-over design protocol for the 2 intervention periods. No interaction effects were seen between sequence of intervention and intervention (2-way ANOVA)

[†]Significant differences from baseline 1 to baseline 2 (P<0.05) (Dependent Student t-test)

[‡]Significant differences between green kiwifruit *vs* control (P<0.05) (Dependent Student t-test)

[§]Values are means (95% CI)

[¶]Significant differences from baseline 2 to end (P<0.05) (Dependent Student t-test)

Table 4.6: The impact of *APOE* genotype on lipid and apolipoprotein conc. at baseline 1 and 2, and after the 2 intervention periods*

		Baseline 1	Baseline 2	$P^{\dagger}$	End-Kiwifruit	End-Control	$P^{\ddagger}$
TC (mmol/L)	*APOE4* carriers	6.38 (5.95, 6.84)	6.18 (5.75, 6.65)	0.30	6.31 (5.89, 6.74)	6.33 (5.92, 6.77)	0.84
	Non-carriers	6.18 (5.97, 6.40)	6.00 (5.79, 6.22)	0.02	6.03 (5.84, 6.24)	6.02 (5.81, 6.25)	0.87
LDL-C (mmol/L)[§]	*APOE4* carriers	4.25 (3.84, 4.66)	4.11 (3.70, 4.52)	0.38	4.25 (3.90, 4.60)[¶]	4.24 (3.89, 4.59)	0.91
	Non-carriers	4.11 (3.91, 4.30)	3.92 (3.76, 4.09)	0.01	3.89 (3.71, 4.07)[¶]	3.95 (3.76, 4.14)	0.33
HDL-C (mmol/L)	*APOE4* carriers	1.41 (1.28, 1.57)	1.43 (1.28, 1.60)	0.58	1.48 (1.34, 1.64)	1.45 (1.30, 1.61)	0.29
	Non-carriers	1.32 (1.25, 1.39)	1.36 (1.29, 1.43)	0.02	1.37 (1.30, 1.44)	1.32 (1.25, 1.38)**	0.003
TG (mmol/L)	*APOE4* carriers[§]	1.69 (1.29, 2.09)	1.50 (1.21, 1.80)	0.14	1.34 (1.14, 1.54)[¶]	1.52 (1.31, 1.72)	0.03
	Non-carriers	1.66 (1.48, 1.86)	1.56 (1.42, 1.72)	0.19	1.66 (1.50, 1.85)[¶]	1.62 (1.46, 1.80)	0.61
TC/HDL-C ratio[§]	*APOE4* carriers	4.62 (4.17, 5.08)	4.37 (4.05, 4.70)	0.05	4.32 (3.99, 4.65)	4.44 (4.10, 4.77)	0.15
	Non-carriers	4.78 (4.53, 5.03)	4.48 (4.28, 4.68)	<0.001	4.49 (4.28, 4.71)	4.66 (4.43, 4.89)**	0.005
ApoA1 (g/L)	*APOE4* carriers	NA	1.41 (1.30, 1.53)	-	1.43 (1.32, 1.56)	1.44 (1.32, 1.57)	0.80
	Non-carriers	NA	1.35 (1.29, 1.40)	-	1.38 (1.33, 1.44)**	1.35 (1.30, 1.41)	0.05
ApoB (g/L)[§]	*APOE4* carriers	NA	1.10 (1.02, 1.19)	-	1.13 (1.03, 1.22)	1.14 (1.05, 1.22)	0.74
	Non-carriers	NA	1.11 (1.06, 1.67)	-	1.11 (1.06, 1.16)	1.12 (1.06, 1.17)	0.75
ApoB/apoA1 ratio[§]	*APOE4* carriers	NA	0.79 (0.71, 0.88)	-	0.80 (0.71, 0.88)	0.80 (0.72, 0.89)	0.80
	Non-carriers	NA	0.84 (0.78, 0.89)	-	0.82 (0.76, 0.87)	0.84 (0.78, 0.90)	0.03
sLDL (mmol/L)[§]	*APOE4* carriers	NA	1.22 (1.03, 1.40)		1.32 (1.08, 1.55)	1.36 (1.15, 1.58)	0.49
	Non-carriers		1.29 (1.15, 1.43)		1.26 (1.13, 1.39)	1.28 (1.16, 1.41)	0.71

Baseline 1 (before run-in period); Baseline 2 (after run-in period); ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; HDL-C: HDL cholesterol; NA: not assessed; LDL-C: LDL cholesterol; sLDL, small dense LDL-C; TC: total cholesterol; TG: triglycerides

*Values are geometric mean (95% CI) unless otherwise indicated, n=23 *APOE4* carriers and n=61 *APOE4* non-carriers. Subjects followed a cross-over design protocol for the 2 intervention periods. No interaction effects were seen between sequence of intervention and intervention (2-way ANOVA)

[†]Significant differences from baseline 1 to baseline 2 (P<0.05) (Dependent Student t-test)

[‡]Significant differences between green kiwifruit versus control (P<0.05) (Dependent Student *t*-test)

[§]Values are means (95% CI)

[¶]Significant differences between *APOE4* carriers and non-carriers (P<0.05) (Independent Student *t*-test)

**Significant differences from baseline 2 to end (P<0.05) (Dependent Student *t*-test)

4.3.6 *APOE* genotype-kiwifruit treatment interaction

There were no significant differences between *APOE4* carriers and non-carriers at baseline for any anthropometric measures, BP, hs-CRP, or HOMA2-IR (data not shown) or lipid concentrations (Table 4.6). Significant improvements in TC, LDL-C and HDL-C were seen in the non-carriers of *APOE4* in response to the 4-week healthy diet period ($P<0.05$). The same trends and magnitude of change were seen for the *APOE4* carriers but the changes were not significant, which is likely reflective of the smaller sample size in the *APOE4* carriers subgroup.

The most significant difference seen between *APOE4* carriers and non-carriers in response to the two interventions was the effects on TG concentrations. *APOE4* carriers showed a significant decrease in TG concentrations following the kiwifruit intervention compared to control, resulting in a significant difference between the two interventions (mean difference 0.18 (95% CI: -0.34, -0.02) mmol/L, $P=0.03$). This also resulted in a significant difference in TG concentrations between *APOE4* carriers and non-carriers for the kiwifruit intervention, 1.34 (95%CI: 1.14, 1.54) mmol/L versus 1.66 (1.50, 1.85) mmol/L, $P=0.01$.

In non-carriers, there was a significant difference between kiwifruit and control for plasma HDL-C and the TC/HDL-C ratio. This was largely due to the significant decrease in HDL-C and increase in the TC/HDL-C ratio for the control intervention from baseline 2. In contrast, while not significant, *APOE4* carriers had an increase in HDL-C from baseline 2 ($P=0.14$). Serum apoA1 concentration increased in the *APOE4* non-carriers during the green kiwifruit period which was reflected in the apoB/apoA1 ratio ($P=0.03$).

4.3.7 Other CVD-related markers

There were no significant changes in hs-CRP (Table 4.7), plasma insulin, glucose or HOMA2-IR (Table 4.8), between the two interventions.

Table 4.7: CRP at baseline 2 and after the 2 intervention periods*

Variable	Baseline 2	End-Kiwifruit	End-Control	$P^{\dagger}$
hs-CRP (mg/L)	1.08 (0.89, 1.30)	0.98 (0.81, 1.18)	1.06 (0.88, 1.27)	0.28

Baseline 2 (after run-in period); hs-CRP: high sensitivity C-reactive protein
*Values are geometric mean (95% CI), n=80 (5 subjects were excluded: 3 with elevated hs-CRP as a result of illness)
Subjects followed a cross-over design protocol for the 2 intervention periods
†Significant differences between green kiwifruit versus control (P<0.05) (Dependent Student *t*-test)

Table 4.8: Glucose, insulin and insulin resistance at baseline and after the 2 intervention periods*

	Green kiwifruit (n=43)		Control (n=42)		
Variable	Baseline 2	End	Baseline 2	End	$P^{\dagger}$
Glucose (mmol/L)	5.50 (5.20, 5.90)	5.60 (5.30, 5.90)	5.60 (5.40, 5.90)	5.70 (5.40, 6.00)	0.93
Insulin (mU/L)	6.37 (5.37, 7.56)	6.96 (5.75, 8.42)	8.65 (7.01, 10.7)	8.61 (6.86, 10.8)	0.79
HOMA2-IR	0.85 (0.71, 1.01)	0.93 (0.77, 1.12)	1.16 (0.94, 1.42)	1.15 (0.92, 1.45)	0.75

Baseline 2 (after run-in period); HOMA2-IR: Homeostasis 2 model assessment for insulin resistance
*Interaction effects were seen between sequence of intervention and intervention (2-way ANOVA), therefore only the data from the first four weeks of the intervention was analysed
Values are medians (25, 75 percentiles)
†Significant differences between change baseline to end green kiwifruit versus control (*P*<0.05) (Mann-Whitney test)

4.4 Discussion

In this study, consumption of two green kiwifruit a day for 4 weeks had favourable effects on plasma HDL-C and the TC/HDL-C ratio compared to a healthy control diet. The hypotriglyceridaemic impact of the intervention was only evident in *APOE4* carriers.

A large proportion (60%) of men did not meet the recommended 2 servings of fruit/day at the start of the study compared to 45% men from the latest (2008/2009) NZ Adult Nutrition survey, even when including dried fruit and real fruit juice (not included in the nutrition survey) (University of Otago and Ministry of Health, 2011). The median number of fruit servings per day increased from 1.3 (0.7 – 2.7) at baseline to 3.0 (3.0 – 4.0) during kiwifruit and 2.5 (2.0 – 4.0) during control interventions. Although the aim was to replace two fruit servings with two kiwifruit, half a serving more of fruit was consumed during the kiwifruit intervention compared to the control period. The 5 most commonly consumed other fruit were apples, bananas, oranges, pears and mandarins.

The 4-week run-in period on the healthy dietary recommendations resulted in significant improvements in all measured lipid parameters, except TG, which approached significance. This confirms the efficacy of these current NZ Heart Foundation's guidelines.

Two green kiwifruit a day did not affect LDL-C compared to the control diet. Brown et al. (1999) showed that for every 1 gram increase in soluble fibre, LDL-C decreased by 0.06 mmol/L (Brown et al., 1999). The 1.8 g/day difference in total fibre (including insoluble plus soluble fibre) intake between the kiwifruit and control interventions may not have been sufficient to affect LDL-C concentrations.

The green kiwifruit intervention resulted in a significantly improved mean HDL-C concentration and TC/HDL-C ratio compared with the control intervention. Adding strength to these findings, the green kiwifruit intervention significantly increased apoA1 (the main structural protein component of HDL) concentrations from baseline 2 and consequently contributed to the almost significantly lower apoB/A1 ratio (P=0.05) compared to control intervention. It has been suggested that for every 0.1 mmol/L increase in HDL-C would reduce coronary heart disease risk by between 8 to 15% (Gordon et al., 1989, Turner et al., 1998), therefore the difference seen in this study could be translated into a 3 to 6% reduction in risk. These findings are in line with that of Chang and Liu (2009), who showed that consumption of two green kiwifruit per day for 8 weeks significantly increased HDL-C concentration and decreased the TC/HDL-C ratio compared to baseline levels (Chang and Liu, 2009). In contrast, Duttaroy and Jorgensen (2004) found no effect on HDL-C (Duttaroy and Jorgensen, 2004). The small group size of their study (n=28 completed the study) may have meant that they were underpowered to detect a difference in HDL-C. There were also differences in subject groups, with the men in our study having a significantly higher mean BMI, than those in the Duttaroy and Jorgensen (2004) study (27.4±3.83 versus 22.4±0.52 kg/m^2).

Similarly to kiwifruit, berries contain vitamins C and E and folic acid, and are a good source of carotenoids and other polyphenols (Basu et al., 2010). An 8-week randomised, controlled berry intervention has also shown significant

improvements in HDL-C concentrations compared to the control products (Erlund et al., 2008).

The most likely constituent to exert the observed effects are polyphenols. Flavonoid–rich juices such as orange juice (Kurowska et al., 2000), grape juice (Albers et al., 2004) and cranberry juice (Ruel et al., 2006) have all been shown to increase plasma HDL-C. While components other than polyphenols, such as vitamin C in fruit and juices, cannot be discounted, polyphenols from cocoa have also shown HDL-C raising effects (Khan et al., 2012, Mellor et al., 2010). Various mechanisms have been proposed, but in one recent cell study it was shown that cocoa polyphenols increased apoA1 production (Yasuda et al., 2011).

Circulating TG is metabolically intimately linked with HDL; with elevated TG levels resulting in increased HL-mediated HDL hydrolysis and decreased HDL-C concentration (Lamarche et al., 1999). Lowering TG concentrations has been shown to increase HDL-C in both normo- and hypertriglyceridaemic subjects (Miller et al., 2007). Chang and Liu (2009) showed a trend towards a decrease in TG, as did some of the flavonoid rich juice studies mentioned above (Albers et al., 2004, Ruel et al., 2006). This study showed no significant change in TG concentrations for the complete group. However, in *APOE4* carriers, plasma TG concentrations were significantly lowered after consumption of green kiwifruit.

Duttaroy and Jorgensen (2004) showed plasma TG were significantly reduced by 15% compared to baseline values (Duttaroy and Jorgensen, 2004) following kiwifruit consumption, as did a more recent study examining the effects of kiwifruit on biomarkers of oxidative status (Brevik et al., 2011). Both publications offered little explanation beyond that such effects have been seen before with fruits and vegetables.

A recent placebo-controlled trial in subjects who received 120 mg gamma delta tocotrienols per day showed a 28% reduction in serum TG (Zaiden et al., 2010). Despite the dose being higher than is found naturally in fruit, it does hint at the possibility that a component such as this, working synergistically with other bioactive components may affect TG concentrations. Polyphenols could also explain the decrease, with studies with red grapefruit and tomatoes also

showing positive effects on plasma TG concentrations (Shen et al., 2007, Gorinstein et al., 2006). Interestingly, in the tomato study the positive effects were seen with tomato juice and whole fruit, but not the lycopene drink (Shen et al., 2007).

Apolipoprotein *E4* carriers appear to be the most responsive to reduced SFA, total fat and cholesterol intake, with reductions seen in TC and LDL-C (Masson et al., 2003). One study showed a greater responsiveness of *APOE4* carriers to the TG lowering effect of fish oils (Caslake et al., 2008). To the best of our knowledge this is the first study to show a different response to eating fruit on TG in *APOE4* carriers. It has been suggested that *APOE4* represents a 'lipid-thrifty' variant, which allows better intestinal absorption of lipids, including fat soluble vitamins (and polyphenols) (Corbo and Scacchi, 1999, Gerdes, 2003, Huebbe et al., 2011). Thus, it could be speculated that the absorption of vitamin E, carotenoids and polyphenols from kiwifruit may be enhanced in *APOE4* carriers resulting in a TG lowering effect.

Given the chemically complex composition of kiwifruit and the opportunity for both independent and/or synergistic contributions of constituents in biological processes, the complete elucidation of the mechanism to explain the observed increase in HDL-C and decrease in TG in *APOE4* carriers remains to be determined.

Considering that benefits on plasma TG and HDL-C were evident, which represent core components of the dyslipidaemia associated with obesity and loss of insulin sensitivity, further work to identify the bioactive constituents and the mechanisms underlying their observed benefits is merited.

An unavoidable limitation of the current study is that the use of a fruit intervention precludes the use of a double-blind study design. A further limitation is that the results cannot be extrapolated to women. Strengths include the randomised crossover design with the 4-week run in period, wide age and BMI ranges, the inclusion of *APOE4* subgroup analysis and a large sample size with sufficient statistical power for *APOE4* subgroup analysis.

In conclusion, we found that consuming two green kiwifruit a day showed improvement (although modest) in the CVD risk profile against an overall healthy diet background, with some indication that the *APOE4* (~25% of the population) subgroup may be most responsive.

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Chapter 5: Inflammatory status modulates plasma lipid and inflammatory marker responses to kiwifruit consumption in hypercholesterolaemic men

This chapter reports the results of one of the predefined secondary objectives of the randomised kiwifruit trial. The aim of this retrospective analysis was to investigate whether inflammatory state, as measured by CRP, would modulate the effect of consuming two green kiwifruit daily on plasma lipids and markers of inflammation.

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(Publications are listed in Appendix 1; related presentations in Appendix 2; contributions of authors in Appendix 3)

Abstract

Kiwifruit has the potential to improve markers of metabolic dysfunction, but the response may be influenced by inflammatory state. We aimed to investigate whether inflammatory state would modulate the effect of consuming two green kiwifruit daily on plasma lipids and markers of inflammation. Eighty-five hypercholesterolaemic men completed a 4-week healthy diet run-in, before randomisation to a controlled cross-over study of two 4-week interventions of two green kiwifruit/day plus healthy diet (intervention) or healthy diet alone (control). Anthropometric measures and fasting blood samples (plasma lipids, serum apoA1 and ApoB, hs-CRP, IL-6, TNF-α and IL-10) were taken at baseline, 4 and 8 weeks. Subjects were divided into low and medium inflammatory groups, using pre-intervention hs-CRP concentrations (hs-CRP <1 and 1-3 mg/L, respectively). In the medium inflammatory group the kiwifruit intervention resulted in significant improvements in plasma HDL-C (mean difference 0.08 [95% CI: 0.03, 0.12] mmol/L [*P*<0.001]), TC/HDL-C ratio (-0.29 [-0.45, -0.14] mmol/L [*P*=0.001]), plasma hs-CRP (-22.1 [-33.6, -4.97]% [*P*=0.01]) and IL-6 (-43.7 [-63.0, -14.1]% [*P*=0.01]) compared to control treatment. No effects were seen in the low inflammatory group. There were significant between inflammation group differences for TC/HDL-C (*P*=0.02), TG/HDL-C (*P*=0.05), and plasma IL-6 (*P*=0.04). Inflammatory state modulated responses to the kiwifruit intervention by improving inflammatory markers and lipid profiles in subjects with modestly elevated CRP, suggesting this group may particularly benefit from the regular consumption of green kiwifruit.

5.1 Introduction

Obesity, identified by Framingham researchers in 1983 as an independent risk factor for CVD, has been shown to increase the prevalence of a number of other CVD risk factors including dyslipidaemia, type 2 diabetes, impaired glucose tolerance and hypertension (Perez Perez et al., 2007). Metabolic syndrome describes the presence of a cluster of these factors: raised blood glucose; BP and TG; decreased HDL-C; and central obesity (Alberti et al., 2009). There is now a substantial amount of evidence that central obesity in particular initiates a chronic-low grade inflammatory state that promotes systemic metabolic dysfunction, which leads to the development of these disorders (Ouchi et al., 2011). An intermediate state with mild metabolic dysfunction where there is some increase in inflammation has also been identified (Ouchi et al., 2011).

With increasing adiposity, as well as tissue expansion, there is an increasing shift towards a pro-inflammatory environment, with the recruitment of immune cells (such as macrophages and T cells) and the upregulation of pro-inflammatory adipokines, such as TNF-α IL-6, and a down-regulation of anti-inflammatory cytokines, such as IL-10 (Ouchi et al., 2011). These adipokines can then spill-over into the circulation, leading to modest increases in systemic levels of cytokines or acute-phase reactants, such as CRP, which may then promote pro-atherogenic effects (Mathieu et al., 2010).

High-sensitivity-CRP is commonly used as a marker of inflammation, in studies which investigate the effect of dietary modification on inflammation (Galland, 2010). Various cross-sectional studies have shown an inverse association between plasma CRP and dietary intakes of fibre, fruit and vegetables, carotenoids, flavonoids, vitamin C, vitamin E (Galland, 2010, Nanri et al., 2007) and phylloquinone (vitamin K) (Pan and Jackson, 2009). Different fruit intervention studies have shown reductions in plasma CRP (Buscemi et al., 2012, Karlsen et al., 2010, Kelley et al., 2006, Watzl et al., 2005).

Kiwifruit is a good source of many of the dietary components that are associated with a beneficial effect on CRP. These include significant levels of fibre, vitamins E and K, folate, carotenoids, flavonoids and polyphenols, and

one of the highest concentrations of vitamin C of any commonly consumed fruit (Ferguson and Ferguson, 2003).

We recently conducted a randomised controlled trial, which assessed the impact of consuming two green kiwifruit a day alongside a healthy diet on plasma lipids, in a group of hypercholesterolaemic men. The primary results from this study have been published (Gammon et al., 2013), and showed that consuming two green kiwifruit a day for 4 weeks had favourable effects on plasma HDL-C and the TC/HDL-C ratio, compared to a healthy control diet, but no effect on CRP was seen. Likewise, no significant change in plasma CRP concentrations was seen in two other kiwifruit interventions (Brevik et al., 2011, Karlsen et al., 2013). Inflammatory status may modulate the responsiveness to dietary manipulation, as has been shown by a small number of studies. All these studies involved some sort of dietary fat manipulation and, although the studies often differed in the manner in which CRP is categorised and most had small group numbers, all found significant differences in response between low and high CRP groups on various plasma lipid components (Desroches et al., 2006, Erlinger et al., 2003, Hilpert et al., 2005, St-Onge et al., 2009, Zhao et al., 2004).

The present study, a predefined secondary analysis of our randomised kiwifruit trial, aimed to determine if there was a difference in plasma lipid and inflammatory response to the kiwifruit intervention, by stratifying subjects based on pre-intervention plasma CRP concentration into low and medium inflammatory groups.

5.2 Subjects and methods

See Methodology Section (Chapter 3)

5.3 Results

5.3.1 Baseline characteristics of subjects by inflammatory group

As previously reported 85 subjects completed the 12 week intervention (Gammon et al., 2013). Exclusions from stratification included 5 subjects, 3 with an elevated CRP concentration at one time point (>8 mg/L) and 2 subjects who

took a medication known to affect inflammation, and, as mentioned, the 10 subjects with a pre-intervention hs-CRP >3.0 mg/L.

The baseline characteristics of the 70 subjects stratified by inflammatory group (low CRP <1 mg/L, n=38; medium CRP 1–3 mg/L, n=32) are shown in Table 5.1**.** The medium inflammatory group had significantly higher values for all anthropometric measures (except height), baseline TG concentration and HOMA2-IR.

The baseline 2 values for the 3 cytokines measured and hs-CRP are also reported in Table 5.1. There were no significant differences between the two groups for any cytokine measures.

5.3.2 Physical activity measures of subjects by inflammatory group

No significant differences were seen between the two groups for measures of physical activity or energy expenditure (Table 5.1).

5.3.3 Effects of the intervention on dietary intake, body weight and BP by inflammatory group

Dietary intake according to inflammatory group is shown in Table 5.2. No significant differences in macronutrient intake were seen either within or between groups. In both groups significantly higher intakes were seen for vitamins C and E during the kiwifruit intervention compared with the control intervention.

There were small decreases observed for nearly all anthropometric measures during the 4-week healthy diet run-in period. From the end of this period (baseline 2) to the end of the 8-week intervention, there were no changes in body weight in either group regardless of which intervention the subjects were receiving (green kiwifruit or control) (Table 5.3). No effects were seen on blood pressure (data not shown).

Table 5.1: Baseline characteristics and physical activity measures of subjects by inflammatory group*

Variable	Inflammatory groups		
	Low (n=38)	Medium (n=32)	*P*†
Age (yr)	47.7 (44.8, 50.6)	47.6 (44.3, 50.9)	0.97
Height	1.77 (1.76, 1.79)	1.77 (1.74, 1.79)	0.71
Weight (kg)	81.7 (78.2, 85.2)	89.6 (84.0, 95.2)	0.02
BMI (kg/m^2)	25.9 (25.1, 26.7)	28.5 (27.2, 29.9)	0.002
Overweight/obese, ≥25 kg/m^2 (%)‡	65.8	84.4	0.08
Waist circumference (cm)	89.9 (87.3, 92.5)	95.7 (92.3, 99.1)	0.007
Elevated WC, ≥94 cm (%) per group‡	21.1	56.2	0.002
Body fat (%)	24.1 (22.3, 26.0)	29.7 (27.1, 32.4)	0.001
% BF, ≥25% (%) per group‡	47.4	71.9	0.04
SBP (mmHg)§	121 (118, 124)	123 (119, 126)	0.48
DBP (mmHg)§	70.3 (67.5, 73.0)	71.2 (67.5, 74.8)	0.69
Elevated BP, ≥130+/or ≥85 (%)‡	18.4	25.0	0.50
HOMA2-IR§,¶	0.77 (0.65, 0.92)	1.18 (0.92, 1.53)	0.005
HOMA2-IR ≥1.0 (%) per group‡	34.2	53.1	0.11
HDL-C (mmol/L)¶	1.36 (1.27, 1.45)	1.34 (1.22, 1.46)	0.75
TG (mmol/L)¶	1.43 (1.25, 1.64)	1.83 (1.53, 2.19)	0.03
Physical Activity (RPAQ)			
TEE (MET h/d)¶	37.8 (35.5, 40.3)	38.7 (36.5, 41.1)	0.61
Sedentary time, <1.5 METs (h/d)**	3.32 (2.45-4.23)	2.86 (2.04-4.52)	0.33
Light PA, 1.5 to 3 METs (h/d)**	6.88 (2.30-7.54)	7.03 (4.93-8.62)	0.33
Moderate, PA 3 to 6 METs (h/d)**	0.81 (0.31-2.52)	0.55 (0.23-1.22)	0.34
Vigorous PA, ≥6 METs (h/d)**	0.10 (0.02-0.31)	0.07 (0.01-0.52)	0.62
hs-CRP (mg/L)§, ††	0.57 (0.49, 0.65)	1.67 (1.47, 1.90)	<0.001
Cytokines‡‡			
IL-6 (pg/ml)¶	1.36 (0.85, 2.16)	1.03 (0.62, 1.69)	0.51
TNF-α (pg/ml)††	2.91 (2.19, 3.75)	3.53 (2.43, 4.84)	0.37
IL-10 (pg/ml)¶	20.6 (17.1, 24.8)	18.3 (14.2, 23.6)	0.43

% BF: percentage body fat; BP: blood pressure; hs-CRP: high-sensitivity C-reactive protein; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; HOMA2-IR: homeostasis model assessment (HOMA) 2 model for insulin resistance; IL: interleukin; MET: metabolic equivalents; PA: physical activity; RPAQ: recent physical activity questionnaire; SBP: systolic blood pressure; TG: triglycerides; TEE: total energy expenditure; TNF-α: tumour necrosis factor-alpha; WC: waist circumference

*Values are means (95% CI) at Baseline 1, unless otherwise indicated

†Significant differences between low (hs-CRP <1.0 mg/L) and medium (hs-CRP 1 to 3 mg/L) inflammatory groups (P<0.05) (Independent Student t-test, Pearson's chi- square)

‡Recognised cut-offs for BMI and excess body fat (Lee and Nieman, 2010), WC and elevated BP (Alberti et al., 2009), elevated IR (Wallace et al., 2004)

§Baseline 2 values

¶Mean (95% CI) for the log transformed data values, back transformed to the original scale

**Median (25th-75th quartiles)

††Mean (95% CI) for the square root transformed data values, back transformed to the original scale

‡‡Cytokines are Baseline 2 values, low (hs-CRP <1.0 mg/L), n=30 and medium (hs-CRP 1 to 3 mg/L), n=27 inflammatory groups

Table 5.2: Dietary intake changes for the 2 intervention periods by inflammatory group*

Variable	Baseline 2	End-Kiwifruit	End-Control	*P*†
Energy (kJ)				
hs-CRP <1.0 (mg/L)	8868±2023	9504±2425	8621±2099	0.05
hs-CRP 1 to 3 (mg/L)	9458±2932	9418±2721	9025±2377	0.34
Fat (% energy)				
hs-CRP <1.0 (mg/L)	26.4±6.16	28.3±8.16	26.8±7.75	0.25
hs-CRP 1 to 3 (mg/L)	27.5±7.54	29.6±6.88	27.8±7.95	0.34
Protein (% energy)				
hs-CRP <1.0 (mg/L)	18.4±4.40	18.8±4.10	20.1±4.65	0.15
hs-CRP 1 to 3 (mg/L)	20.0±6.12	19.7±4.64	19.5±4.87	0.80
CHO (% energy)				
hs-CRP <1.0 (mg/L)	50.4±7.68	49.2±9.44	48.7±10.0	0.72
hs-CRP 1 to 3 (mg/L)	49.3±10.9	47.6±6.82	50.3±8.51	0.15
Vitamin C (mg)‡				
hs-CRP <1.0 (mg/L)	135 (79.2-178)	297 (226-340)§	241 (119-320)§	0.007
hs-CRP 1 to 3 (mg/L)	122 (77.3-149)	283 (238-336)§	251 (117-323)§	0.01
Vitamin E‡				
hs-CRP <1.0 (mg/L)	8.57 (6.68-11.4)	12.1 (10.3-16.2)§	8.23 (6.72-10.9)	<0.001
hs-CRP 1 to 3 (mg/L)	10.6 (6.92-14.1)	14.7 (10.1-17.2)§	8.13 (5.88-10.3)§	<0.001

*Values are means±SD unless otherwise indicated, low (hs-CRP <1.0 mg/L), n=38 and medium (hs-CRP 1 to 3 mg/L), n=32 inflammatory groups
†Significant differences between green kiwifruit versus control ($P<0.05$) (Dependent Student *t*-test or Wilcoxon Signed Ranks Test)
‡Median (25^{th}-75^{th} quartiles)
§Significant differences from baseline 2 to end of intervention ($P<0.05$) (Dependent Student *t*-test or Wilcoxon Signed Ranks Test)

Table 5.3: Anthropometric assessments at baseline 1 and 2, and after the 2 intervention periods by inflammatory group*

Variable	Baseline 1	Baseline 2	*P*†	End-Kiwifruit	End-Control	*P*‡
Weight (kg)						
hs-CRP <1.0 (mg/L)	81.7±10.7	81.3±10.5	0.06	81.3±10.4	81.4±10.7	0.63
hs-CRP 1 to 3 (mg/L)	89.6±15.6	89.2±15.3	0.03	89.3±15.2	89.3±15.2	0.90
Waist (cm)						
hs-CRP <1.0 (mg/L)	89.9±7.88	89.3±7.80	0.02	89.1±7.21	89.4±7.81	0.20
hs-CRP 1 to 3 (mg/L)	95.7±9.46	95.4±9.58	0.20	95.2±9.37	95.1±9.21	0.83
BMI (kg/m^2)						
hs-CRP <1.0 (mg/L)	25.9±2.50	25.8±2.45	0.07	25.8±2.42	25.8±2.55	0.66
hs-CRP 1 to 3 (mg/L)	28.5±3.81	28.4±3.71	0.04	28.5±3.69	28.4±3.71	0.93
Body fat (%)						
hs-CRP <1.0 (mg/L)	24.1±5.67	23.1±6.01	<0.001	23.4±6.16	23.4±6.18	0.82
hs-CRP 1 to 3 (mg/L)	29.7±7.36	29.5±7.13	0.24	29.2±7.23	29.3±7.11	0.78

Baseline 1 (before run-in period); Baseline 2 (after run-in period); BMI: body mass index; hs-CRP: high-sensitivity C-reactive protein
*Values are means±SD, low (hs-CRP <1.0 mg/L), n=38 and medium (hs-CRP 1 to 3 mg/L), n=32 inflammatory groups
†Significant differences from baseline 1 to baseline 2 ($P<0.05$) (Dependent Student *t*-test)
‡Significant differences between green kiwifruit *vs* control ($P<0.05$) (Dependent Student *t*-test)

5.3.4 Effects of the intervention on lipids by inflammatory group

The effects of the intervention on plasma lipids by inflammatory group are shown in Table 5.4. In the medium inflammatory group, there was a significant increase in HDL-C (P=0.001) and decrease in TC/HDL-C ratio (P=0.003) from baseline during the kiwifruit intervention. This resulted in a significant difference between kiwifruit and control treatments for HDL-C, mean difference 0.08 (95% CI: 0.03, 0.12) mmol/L, P<0.001 and TC/HDL-C ratio -0.29 (-0.45, -0.14) mmol/L, P=0.001. Furthermore during the kiwifruit intervention, this group had a significant increase in apoA1 from baseline (P=0.006) and a decrease in apoB/A1 ratio (P=0.009), and during the control intervention they had a significant increase in TG/HDL-C ratio (P=0.04).

In the low inflammatory group there were no significant differences between kiwifruit and control interventions, although the TC/HDL-C ratio increased significantly from baseline for both treatments.

The difference in response seen in the medium inflammatory group during the kiwifruit intervention resulted in significant between-inflammatory group differences in the TC/HDL-C (P=0.02) and TG/HDL-C (P=0.05) ratios and a tendency towards a significant difference for HDL-C (P=0.07).

5.3.5 Effects of the intervention on inflammatory markers by inflammatory group

Figure 5.1 shows the difference in response by inflammatory group to the intervention. For all cytokines, marked variability in response can be seen. During the kiwifruit intervention, the two groups differed in direction of change for CRP and magnitude of change for IL-6, with significant differences between kiwifruit and control for CRP (-18.4% versus 1.8%) and IL-6 concentrations (-31.6% versus 21.3%), in the medium group. There was also a significant between inflammatory group effect for IL-6 (P=0.04).

Table 5.4: Plasma lipid and apolipoprotein concentration changes for the 2 intervention periods by inflammatory group*

	Low inflammatory group				Medium inflammatory group				
	Baseline 2	**Kiwifruit change**	**Control change**	**Within group KF vs C†**	**Baseline 2**	**Kiwifruit change**	**Control change**	**Within group KF vs C†**	Between Groups‡
TC (mmol/L)§	6.09 (5.83, 6.35)	0.09 (-0.17, 0.36)	0.11 (-0.14, 0.35)	0.90	5.94 (5.65, 6.24)	0.12 (-0.12, 0.35)	0.11 (-0.13, 0.35)	0.98	0.91
LDL-C (mmol/L)§	3.98 (3.76, 4.20)	0.09 (-0.09, 0.28)	0.13 (-0.07, 0.33)	0.65	3.79 (3.57, 4.02)	0.02 (-0.19, 0.22)	0.05 (-0.16, 0.25)	0.73	0.95
HDL-C(mmol/L)¶	1.42 (1.33, 1.51)	-0.02 (-0.08, 0.04)	-0.04 (-0.08, 0.00)	0.44	1.33 (1.21, 1.46)	0.08 (0.03, 0.12)**	0.00 (-0.06, 0.06)	<0.001	0.07
TG (mmol/L)¶	1.40 (1.26, 1.55)	0.09 (-0.16, 0.35)	0.01 (-0.15, 0.17)	0.55	1.54 (1.31, 1.80)	0.04 (-0.20, 0.30)	0.17 (-0.04, 0.37)**	0.28	0.22
TC/HDL-C ratio§	4.31 (4.09, 4.54)	0.14 (0.01, 0.27)**	0.18 (0.06, 0.31)[f]	0.54	4.51 (4.21, 4.80)	-0.19 (-0.31, -0.07)**	0.10 (-0.04, 0.24)	0.001	0.02
TG/HDL-C ratio¶	0.99 (0.85, 1.14)	0.10 (-0.11, 0.31)	0.03 (-0.08, 0.13)	0.68	1.16 (0.93, 1.44)	-0.05 (-0.26, 0.16)	0.16 (-0.03, 0.34)**	0.07	0.05
ApoA1 (g/L)§	1.39 (1.32, 1.47)	0.02 (-0.03, 0.07)	0.003 (-0.04, 0.05)	0.46	1.38 (1.27, 1.48)	0.06 (0.02, 0.10)**	0.03 (-0.01, 0.08)	0.31	0.79
ApoB (g/L)§	1.08 (1.03, 1.14)	0.03 (-0.02, 0.07)	0.03 (-0.02, 0.07)	0.99	1.11 (1.04, 1.19)	-0.001 (-0.04,0.04)	-0.004,(-0.04, 0.03)	0.91	0.93
ApoB/apoA1 ratio§	0.80 (0.74, 0.85)	0.009 (-0.01, 0.03)	0.02 (-0.004, 0.05)	0.38	0.84 (0.76, 0.92)	-0.04 (-0.06, -0.01)**	-0.02 (-0.04, 0.01)	0.20	0.74
sLDL§	1.20 (1.07, 1.34)	0.06 (-0.08, 0.21))	0.09 (-0.04, 0.22)	0.67	1.33 (1.13, 1.54)	-0.005 (-0.14, 0.13)	-0.02 (-0.17, 0.133)	0.86	0.68

ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; sLDL: small-dense LDL; TC: total cholesterol; TG: triglycerides

*n=38 low (hs-CRP <1.0 mg/L) and n=32 medium (hs-CRP 1 to 3 mg/L) inflammatory groups, subjects followed a cross-over design protocol for the 2 intervention periods.

†Significant differences between green kiwifruit *vs* control ($P<0.05$) (Dependent Student *t*-test).

‡Significant differences between inflammatory groups ($P<0.05$) (Repeated measures ANOVA)

§Values are means (95% CI)

¶Mean (95% CI) for the log transformed data values, back transformed to the original scale

**Significant differences from baseline 2 to end ($P<0.05$) (Dependent Student *t*-test)

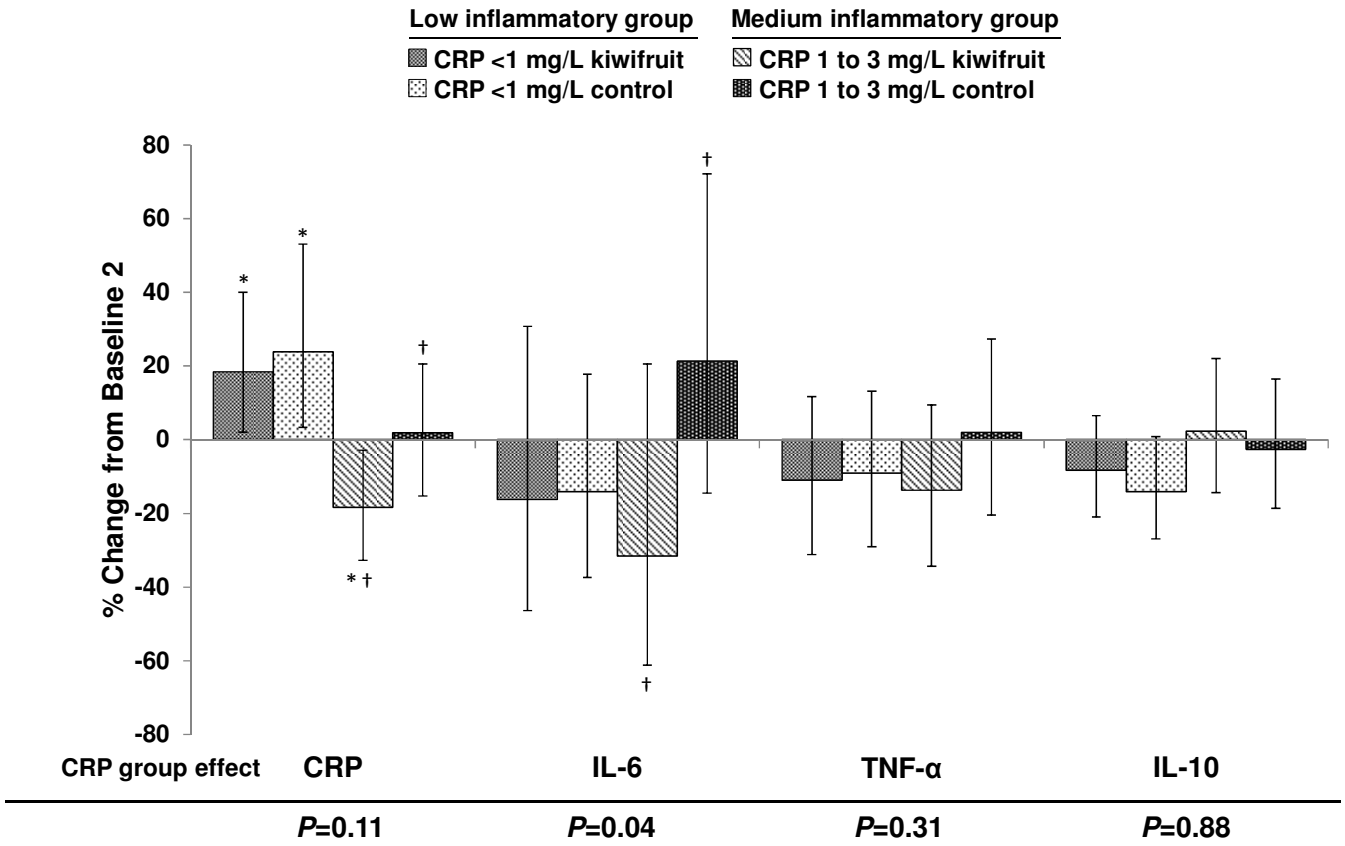

Figure 5.1: The percentage change of plasma inflammatory markers from baseline 2 in subjects with low (hs-CRP <1 mg/L) and medium (hs-CRP 1 to 3 mg/L) inflammatory levels.

The *P*-values define the effect of hs-CRP group interaction (CRP & TNF-α, data square root transformed, IL-6 & IL-10, data log transformed)

*Significant differences from baseline 2 to end of intervention ($P<0.05$) (Dependent Student *t*-test)

†Significant differences between green kiwifruit versus control ($P<0.05$) (Dependent Student *t*-test)

5.4 Discussion

To our knowledge, this is the first study to report that inflammatory status (as measured by CRP) prior to the intervention has modulated lipid and inflammatory responses to a fruit intervention. Favourable effects to consuming two green kiwifruit a day against the background of a healthy diet were seen on plasma HDL-C, TC/HDL-C and TG/HDL-C ratios and some markers of inflammation, but only for the medium inflammatory group.

Comparing the metabolic profile of the two groups, the between-group differences are certainly suggestive that the medium inflammatory group were at greater risk of metabolic dysfunction. In addition to having higher hs-CRP concentrations, nearly 85% of subjects in the medium inflammatory group were overweight/obese (BMI ≥25 kg/m^2), 72% carried excess BF (% BF ≥25%) (Lee and Nieman, 2010), and more than half had a WC ≥94cm (Alberti et al., 2009). Further, over half had an elevated risk for IR (HOMA2-IR ≥1.0) (Wallace et al., 2004) and plasma TG concentrations were significantly higher in the medium inflammatory group, compared to the low inflammatory group. Unexpectedly, we did not find a significant difference between the two groups for the inflammatory cytokines (IL-6, TNF-α, IL-10). A difference in one of these would have strengthened our classification into the inflammatory groups. Inflammatory cytokines, as with CRP, were measured pre-intervention, after the healthy diet run-in period. We speculate that the healthy diet during the run-in period may already have had some positive effects on these markers in subjects in the medium inflammatory group. The co-existence of a metabolic dysfunctional profile in the medium inflammatory group may indicate that this group was indeed at increased risk of chronic low-grade inflammation.

Inflammatory status has resulted in significant differences in lipid response to the intervention, with significant inflammatory group x intervention interactions for TC/HDL-C and TG/HDL-C ratios. These interactions were largely the result of the significant improvement in plasma HDL-C concentration during the kiwifruit intervention, compared with the control intervention in the medium inflammatory group. In contrast, no significant change in HDL-C was seen in the low inflammatory group.

One of the proposed mechanisms by which HDL-C concentrations are decreased in obesity is through the down-regulation of apoA1 (the main structural protein component of HDL) gene expression by inflammatory cytokines, such as IL-6 and TNF-α (Navarro et al., 2005). The kiwifruit intervention in the medium inflammatory group significantly reduced plasma hs-CRP and IL-6, compared to the control intervention, suggestive of a decrease in overall inflammation. Plasma apoA1 concentrations increased from baseline during the kiwifruit intervention, further supporting this as a means to explain the increase in HDL-C concentration that was seen in this group.

Kiwifruit is considered a very nutrient dense fruit (Ferguson and Ferguson, 2003), and their unique composition may contribute to a positive effect on CRP concentrations. Although previous studies (Brevik et al., 2011, Karlsen et al., 2013) found no effect of kiwifruit on CRP concentrations, the researchers have not reported stratifying their subjects into groups by CRP concentration, which appears from our study to modulate response. We can only speculate as to which bioactive component/s may be responsible for the decrease in inflammation seen in the medium inflammatory group, but a recent study does offer a possible mechanism (Karlsen et al., 2010). In a 4-week randomised, controlled trial, subjects who consumed bilberry juice had significant decreases in plasma concentrations of CRP and IL-6, and increases in plasma levels of quercetin and p-coumaric acid. The researchers, using a human monocytic cell line, then showed that quercetin (which is also present in kiwifruit) (Fiorentino et al., 2009), resveratrol and epicatechin, inhibited NF-κβ activation. The transcription factor, NF-κβ, is a key regulator of pro-inflammatory signalling molecules, such as CRP and IL-6 (Karlsen et al., 2010). Vitamins C and E, which kiwifruit contain high concentrations of, have similarly been shown to inhibit NF-κβ activation (Bowie and O'Neill, 2000, Glauert, 2007).

As modulation of the intervention response by CRP was not a primary objective of the study, this study has some limitations. Importantly hs-CRP and the other markers of inflammation were not measured at baseline 1. Therefore we do not know what effect, if any, the healthy diet may have had on inflammatory markers during the run-in period. Further, it would have been useful to measure biochemical markers of mechanistic components of interest, such as plasma

vitamins C and E. It will be important for future studies to confirm our findings and to explore mechanistic pathways, including whether kiwifruit and some of their known bioactive components, can inhibit NF-κβ activation.

These results are suggestive that those with moderately increased inflammation, as measured by hs-CRP, may particularly benefit from the regular daily consumption of green kiwifruit, with potential improvements in CRP, IL-6 and HDL-C concentrations, and the TC/HDL-C and TG/HDL-C ratios. These findings could partly explain inter-individual dietary responses to interventions and contribute to the limited, but growing, body of evidence regarding diet and inflammatory/metabolic state interactions. As the incidences of obesity and metabolic syndrome, both associated with an increased inflammatory state, continue to rise, strategies which mitigate any of the metabolic effects before full metabolic dysfunction occurs will be of increasing importance. Research in this field may lead in the future to personalised dietary recommendations for individuals with a specific metabolic/inflammatory state.

In conclusion, inflammatory status modulated the response to consuming two green kiwifruit a day against an overall healthy diet background, with improvements in inflammatory status and lipid profile in subjects with modestly elevated CRP concentrations. These effects could be expected to translate into a modest reduction of CVD risk.

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Chapter 6: *TaqIB* polymorphism in the *CETP* gene influences lipid responses to consuming kiwifruit in hypercholesterolaemic men

One of the secondary objectives of the study was, based on the primary results to select gene polymorphisms related to lipid metabolism, which could in part explain the heterogeneity in response observed. The aim of this study was to assess the effects of these selected HDL-C related polymorphisms on the plasma lipid response to the kiwifruit intervention.

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(Publications are listed in Appendix 1; related presentations in Appendix 2; contributions of authors in Appendix 3)

Abstract

Fruit and vegetables are key elements of a cardioprotective diet, but benefits on plasma lipids, especially HDL-C, are inconsistent both within and between studies. We investigated whether four selected HDL-C-related polymorphisms (*CETP Taq1B*; *APOA1* -75G/A; *LIPC* -514C→T; *LIPG* I24582) modulate the plasma lipid response to a kiwifruit intervention. This is a retrospective analysis of data from our 12-week randomised controlled cross-over trial. Eighty-five hypercholesterolaemic men completed a 4-week healthy diet run-in period before randomisation to one of two 4-week intervention sequences of two green kiwifruit/day plus healthy diet (intervention) or healthy diet alone (control). Anthropometric measures and fasting blood samples were taken at baseline 1, after run-in (baseline 2) and treatment periods. At baseline 2, *B1/B1* homozygotes of the *CETP Taq1B* gene had significantly higher TC/HDL-C, TG/HDL-C and apoB/apoA1 ratios and sLDL, compared to *B2* carriers. A significant *CETP Taq1B* genotype x intervention interaction was seen for the TG/HDL-C ratio (P=0.03). *B1/B1* homozygotes had a significantly lower TG/HDL-C (-0.23±0.58 mmol/L, P=0.03) ratio after the kiwifruit intervention, compared to control, whereas *B2* carriers were not affected. Lipid response was not affected by other gene polymorphisms. In conclusion, the significant decrease in the TG/HDL-C ratio in *B1/B1* homozygotes, suggest that regular inclusion of green kiwifruit as part of a healthy diet may improve the lipid profiles of hypercholesterolaemic men with this genotype.

6.1 Introduction

Cardiovascular disease is the leading cause of death globally (WHO, 2011). Plasma HDL-C concentrations are inversely associated with CVD risk, and should be considered alongside LDL-C in managing DL individuals (Vergeer et al., 2010, Hansel et al., 2006). Low HDL-C concentrations may reflect disturbances in triglyceride metabolism (Vergeer et al., 2010). In conjuction with increased levels of TG and sLDL particles, they form the lipid triad or 'atherogenic lipoprotein phenotype' which is associated with obesity and IR, the prevalence of and subsequent contribution to CVD risk of which is steadily increasing (Musunuru, 2010). Therefore, an understanding of the determinants of HDL-C concentration is timely. Considerable variability exists in HDL-C concentrations within population groups (Bove et al., 2007, Hansel et al., 2006). Heritability estimates for HDL-C concentrations range between 40 and 60%. In addition, a range of environmental and metabolic factors, including TG concentrations, WC, hs-CRP, IR, alcohol consumption, and smoking have all been shown to be associated with HDL-C concentrations (Bove et al., 2007, Hansel et al., 2006, Heller et al., 1993, Peloso et al., 2010).

Fruit and vegetables have long been identified as key elements of a cardioprotective diet. Yet, beyond the cholesterol lowering properties of dietary fibres, conclusive benefits in relation to blood lipids remain largely unresolved. There are inconsistent findings, for example, as to the beneficial effects of flavonoid rich fruits on HDL-C (Chong et al., 2010, Dauchet et al., 2009). Genetics, for example differences in the prevalence of key gene variants, are likely to contribute to some of the response variability between populations. To our knowledge, however, no studies have investigated the variability in responsiveness of blood lipids to an intervention involving fruit.

In 2010 we conducted a randomised controlled trial in hypercholesterolaemic men, in which we assessed the impact of consuming two green kiwifruit a day alongside a healthy diet on plasma lipids. The primary results from this study showed that consuming two green kiwifruit every day for 4 weeks had favourable effects on plasma HDL-C concentrations and the TC/HDL-C ratio compared to consuming a healthy control diet alone (Gammon et al., 2013).

Based on the primary results, one of the secondary objectives of the above-mentioned study was to select gene polymorphisms related to lipid metabolism, which could in part explain the heterogeneity in response observed. For this analysis we selected four SNPs related to HDL metabolism, and given our group size we focussed on common variants with minor-allele frequencies (MAF) of >20%. The SNPs chosen were the *CETP Taq1B*, *APOA1* -75G/A, *LIPC* -514C→T and *LIPG* I24582 (T+2864C/In8) variants.

CETP TaqIB is one of the most widely studied polymorphisms (de Grooth et al., 2004) and substantial evidence from meta-analyses shows an association with HDL-C levels (Boekholdt et al., 2005, Thompson et al., 2008). Evidence is more limited for the other three SNPs, but all have been reported either to be associated with HDL-C concentrations (Mank-Seymour et al., 2004, Nettleton et al., 2007, Ordovas et al., 2002b, Zhang et al., 2005), or related factors (Juo et al., 1999, Ruano et al., 2006, Souverein et al., 2005).

Therefore, the aim of this study was to assess the effects of these selected HDL-C related polymorphisms on the plasma lipid response to the kiwifruit intervention.

6.2 Subjects and methods

See Methodology Section (Chapter 3)

6.3 Results

As previously reported 85 subjects completed the 12 week intervention (Gammon et al., 2013). Genotyping was completed for 84 subjects, although the alleles for 2 subjects for three of the SNPs could not be determined.

Table 6.1 reports the position and genotypic distributions for the four selected SNPs. High-density lipoprotein cholesterol concentrations at baseline 1, by genotype grouping (minor allele carriers versus major allele homozygotes), are also reported in Table 6.1. There was no significant difference in HDL-C concentrations between minor allele carriers and major allele homozygotes for any SNP at baseline 1.

Table 6.1:Position, genotypic distributions and HDL-C concentration at baseline of selected SNPs

Gene	SNP ID	Chromosome	Location	Genotypes	n (%)	HDL-C (mmol/L)*, †	
CETP Taq1B	rs708272	16	Intron	AA (B2/B2)	16 (19.5)	*B2* carriers vs	1.41±0.32
				GA (B1/B2)	35 (42.7)	*B1/B1*	1.31±0.34
				GG (B1/B1)	31 (37.8)		*P*=0.18
APOA1 -75G/A‡	rs670	11	Promoter	AA	5 (6.1)	*A* carriers vs	1.29 (1.20, 1.38)
				GA	30 (36.6)	*G/G*	1.38 (1.29, 1.48)
				GG	47 (57.3)		*P*=0.15
LIPC 514C→T‡	rs1800588	15	Intergenic/unknown	CC	48 (58.5)	*C/C* vs	1.35 (1.27, 1.44)
				CT	27 (32.9)	*T* carriers	1.32 (1.22, 1.44)
				TT	7 (8.5)		*P*=0.65
LIPG I24582‡	rs6507931	18	Intron	CC	18 (21.4)	*C* carriers vs	1.33 (1.26, 1.40)
(T+2864C/In8)				CT	40 (47.6)	*T/T*	1.39 (1.25, 1.53)
				TT	26 (31.0)		*P*=0.41

*Values are means±SD at Baseline 1, unless otherwise indicated
†Significant differences between genotypic groups (minor allele carriers versus major allele homozygotes ($P<0.05$) (Independent Student *t*-test)
‡Geometric mean (95% CI)

Based on the results of the initial statistical screening using MANOVA, *CETP Taq1B* SNP affected the lipid profile at baseline 2, F (10, 71) = 3.561, *P*=0.001. No significant results were seen for any other SNP, therefore, only the *CETP Taq1B* SNP underwent further analysis.

6.3.1 Baseline characteristics of subjects by *CETP Taq1B* genotype group

The baseline characteristics stratified by *CETP Taq1B* genotype group are shown in Table 6.2. There were no significant differences between the two groups for any anthropometric measure, BP, HOMA2-IR or hs-CRP.

Table 6.2: Baseline characteristics of subjects by *CETP Taq1B* genotype*

	CETP Taq1B		
Variable	*B2* carriers (n=51)	*B1/B1* (n=31)	$P^{\dagger}$
Age (yr)	46.6±9.54	50.2±9.09	0.09
Height (m)	1.78±0.06	1.77±0.06	0.63
Weight (kg)	87.5±15.2	85.0±17.6	0.50
BMI (kg/m^2)	27.6±3.71	27.0±4.20	0.52
Waist circumference (cm)	94.0±10.4	92.3±9.85	0.46
% Body fat	27.3±7.34	28.0±8.19	0.71
SBP (mmHg)‡	123±9.94	122±10.1	0.76
DBP (mmHg)‡	71.3±9.18	70.5±8.94	0.70
HOMA2-IR$^{\ddagger, \S}$	1.03 (0.64-1.47)	0.96 (0.60-1.72)	0.76
hs-CRP (mg/L)$^{\ddagger, \S}$	0.98 (0.61-2.16)	1.06 (0.52-1.83)	0.70

hs-CRP: high-sensitivity C-reactive protein; DBP: diastolic blood pressure; HOMA2-IR: homeostasis model assessment 2 model for insulin resistance SBP: systolic blood pressure
*Values are means±SD at Baseline 1, unless otherwise indicated
†Significant differences between *B2* carriers and *B1/B1* group (*P*<0.05) (Independent Student *t*-test and Mann-Whitney test)
‡Baseline 2 values
§Median (25^{th}-75^{th} quartiles)

6.3.2 Dietary intakes during the intervention by *CETP Taq1B* genotype

Dietary intake according to genotype group is shown in Table 6.3. There was a small, but significant difference in energy intake between kiwifruit and control treatments, in both groups. No significant differences in macronutrient intake were seen between kiwifruit and control treatments for either group. In both groups significantly higher intakes were seen for vitamins C and E during the kiwifruit intervention compared with the control intervention.

When fruit servings per day were analysed by genotype, no significant differences were seen between the two groups for any period. At baseline 1 the median intake for both groups was 1.33 serves per day (below recommendations); this increased to 2 serves at baseline 2 and 3 serves for the two intervention periods.

Table 6.3: Dietary intake changes for the 2 intervention periods by *CETP Taq1B* group*

Variable	Baseline 2	End-Kiwifruit	End-Control	*P*[†]
Energy (kJ)				
B2 carriers	9261±2705	9759±2696	8987±2386	0.04
B1/B1	8774±2705	9226±1892	8211±1813	0.01
Fat (% energy)				
B2 carriers	27.7±7.00	28.3±8.34	26.9±7.70	0.32
B1/B1	24.3±5.91	29.6±5.88[†]	27.3±6.87	0.14
Protein (% energy)				
B2 carriers	19.9±4.98	19.5±4.94	19.9±4.50	0.61
B1/B1	19.2±5.95	20.0±4.17	19.9±4.84	0.92
CHO (% energy)				
B2 carriers	48.5±8.64	49.5±9.20	50.0±9.71	0.69
B1/B1	53.1±9.83	47.4±6.18[†]	49.6±8.43[†]	0.22
Vitamin E (mg)				
B2 carriers	9.58±3.51	13.4±4.29[‡]	9.81±4.24	<0.001
B1/B1	11.2±6.17	14.2±4.66[‡]	8.03±3.47[‡]	<0.001
Vitamin C[§]				
B2 carriers	122 (70-160)	296 (251-336)[‡]	251(130-321)[‡]	0.05
B1/B1	128 (80.7-152)	272(226-336)[‡]	182(89-282[‡]	<0.001

*Values are means±SD unless otherwise indicated, *B2* carriers and n=31 *B1/B1* homozygotes
[†]Significant differences between green kiwifruit versus control ($P<0.05$) (Dependent Student *t*-test or Wilcoxon Signed Ranks Test)
[‡]Significant differences from baseline 2 to end ($P<0.05$) (Dependent Student *t*-test or Wilcoxon Signed Ranks Test)
[§]Median (25^{th}-75^{th} quartiles)

6.3.3 Effects of diet run-in period and intervention on body weight by *CETP Taq1B* genotype group

There were small, but significant decreases during the 4-week healthy diet run-in period in *B2* carriers for weight and BMI, mean (±SD) difference -0.39±1.33 kg, *P*=0.04 and -0.12±0.41 kg/m^2, *P*=0.05, respectively, and % BF in *B1/B1* homozygotes -0.60±1.35, *P*=0.02. From the end of this period (baseline 2) to the end of the 8-week intervention, there were no changes in body weight in either group regardless of which intervention the subjects were receiving (green kiwifruit or control) (Table 6.4).

6.3.4 Effects of the intervention on lipids by *CETP Taq1B* genotype group

The effects of the intervention on plasma lipids by *CETP Taq1B* genotype group are shown in Table 6.5. At baseline 2, *B2* carriers had significantly lower TC/HDL-C ratio, mean (±SE) difference -0.39±0.19 mmol/L, *P*=0.05, TG/HDL-C ratio -0.31±0.15 mmol/L, *P*=0.05 and apoB1/apoA1 ratio -0.10±0.05 g/L, *P*=0.04 and sLDL -0.24±0.12 mmol/L, *P*=0.05, compared to *B1* homozygotes. The difference between the *B2* carriers and *B1* homozygotes for HDL-C and apoA1 were 0.14±0.07 mmol/L, *P*=0.07 and -0.11±0.05 g/L, *P*=0.06, respectively.

There was no genotype x treatment interaction for HDL-C. The kiwifruit treatment resulted in greater HDL-C concentrations compared to control in both genotype groups. The mean (±SD) difference between kiwifruit and control treatments for HDL-C was 0.06±0.10 mmol/L, *P*=0.002 for *B1/B1* homozygotes and 0.05±0.15 mmol/L, *P*=0.03 for *B2* carriers, with this group (B2 carriers) during the control intervention having a significant decrease from baseline -0.05±0.15 mmol/L, *P*=0.02.

There was a significant genotype x treatment interaction for the TG/HDL-C ratio (*P*=0.03). Stratifying for this variable, the two genotype groups differed in response to the intervention, with the *B1/B1* homozygotes favourably responding, with significant improvements with kiwifruit compared to control treatments for TG/HDL ratio-0.23±0.58 mmol/L, *P*=0.03. There were no significant treatment differences for *B2* carriers. Since significant (*P*< 0.05) genotype x treatment interactions were not seen for any of the other lipid variables, stratified analysis for these are not reported.

Table 6.4: Anthropometric assessments at baseline 1 and 2, and after the 2 intervention periods by *CETP Taq1B* group*

Variable	Baseline 1	Baseline 2	$P^{\dagger}$	End-Kiwifruit	End-Control	$P^{\ddagger}$
Weight (kg)						
B2 carriers	87.5±15.2	87.1±15.2	0.04	87.3±15.1	87.2±15.3	0.60
B1/B1	85.0±17.6	84.9±17.1	0.70	85.2±17.3	85.2±17.1	0.75
Waist circumference (cm)						
B2 carriers	94.0±10.4	93.6±10.5	0.06	93.3±10.2	93.5±10.5	0.56
B1/B1	92.3±9.85	92.0±9.83	0.34	91.9±9.36	92.2±9.41	0.23
BMI (kg/m^2)						
B2 carriers	27.6±3.71	27.5±3.74	0.05	27.5±3.74	27.5±3.78	0.59
B1/B1	27.0±4.20	27.0±4.04	0.71	27.1±4.08	27.1±4.04	0.72
Body fat (%)						
B2 carriers	27.3±7.33	26.9±7.61	0.10	26.9±7.54	26.7±7.54	0.35
B1/B1	28.0±8.19	27.4±8.33	0.02	27.5±8.18	27.6±8.03	0.86

Baseline 1 (before run-in period); Baseline 2 (after run-in period)
*Values are means±SD, n=51 *B2* carriers and n=31 *B1/B1* homozygotes
†Significant differences from baseline 1 to baseline 2 ($P<0.05$) (Dependent Student *t*-test)
‡Significant differences between green kiwifruit versus control ($P<0.05$) (Dependent Student *t*-test)

Table 6.5: Plasma lipid and apolipoprotein concentration changes for the 2 intervention periods by *CETP Taq1B* genotype*

	B2 carriers (n=51)				*B1/B1* (n=31)				Between groups
	Baseline 2	Kiwifruit change	Control change	$P^{\dagger}$	Baseline 2	Kiwifruit change	Control change	$P^{\dagger}$	$P^{\ddagger}$
TC (mmol/L)	6.17±0.97	0.05±0.76	0.008±0.78		5.99±0.84	0.06±0.69	0.09±0.59		0.59
LDL-C (mmol/L)	4.00±0.74	-0.002±0.59	0.03±0.65		3.88±0.73	0.07±0.54	0.09±0.48		0.93
HDL-C(mmol/L)	1.46±0.31	-0.006±0.18	-0.05±0.15[§]		1.33±0.35	0.05±0.16	-0.01±0.13		0.75
TG (mmol/L)	1.57±0.54	0.17±0.82	0.06±0.46		1.71±0.76	-0.10±0.60	0.07±0.59		0.08
TC/HDL-C ratio	4.31±0.60[¶]	0.07±0.40	0.17±0.37[§]		4.70±0.96[¶]	-0.12±0.39	0.12±0.44		0.14
TG/HDL-C ratio	1.12±0.44[¶]	0.15±0.66	0.08±0.35	0.43	1.43±0.79[¶]	-0.14±0.51	0.09±0.56	0.03	0.03
ApoA1 (g/L)	1.42±0.23	0.02±0.13	-0.003±0.12		1.31±0.26	0.05±0.14	0.03±0.13		0.85
ApoB (g/L)	1.10±0.21	0.005±0.13	0.001±0.14		1.13±0.22	0.01±0.13	0.03±0.11		0.52
ApoB/apoA1 ratio	0.79±0.16[¶]	-0.004±0.09	0.009±0.08		0.89±0.24[¶]	-0.02±0.07	0.004±0.08		0.42
sLDL (mmol/L)	1.18±0.50[¶]	0.04±0.38	0.03±0.37		1.41±0.55[¶]	-0.03±0.46	0.03±0.42		0.47

ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; sLDL: small-dense LDL; TC: total cholesterol; TG: triglycerides

*Values are means±SD, subjects followed a cross-over design protocol for the 2 intervention periods

[†]Significant differences between kiwifruit versus control (P<0.05) (Dependent Student t-test). Only P values for variables with an ANOVA interaction (P<0.05) are reported

[‡]Significant differences between genotype groups (P<0.05) (Repeated measures ANOVA)

[§]Significant differences from baseline 2 to end (P<0.05) (Dependent Student *t*-test)

[¶]Significant differences between *B2* carriers and *B1/B1* group (P<0.05) (Independent Student *t*-test)

6.4 Discussion

To our knowledge, this is the first study to report a possible association between a polymorphism in the *CETP* gene and plasma lipid response to a fruit intervention. Since the results are based on one SNP and a relatively small cohort the findings should be seen as explorative and will need to be confirmed in future research, in a larger sample.

The genotype frequency for all four SNPs investigated in the study were in line with other studies in the literature (Boekholdt et al., 2005, Do et al., 2009, Mank-Seymour et al., 2004, Nettleton et al., 2007, Ordovas et al., 2002a, Ordovas et al., 2002b, Ruano et al., 2006, Smith et al., 2009, Zhang et al., 2005). However, there was a tendency towards a greater percentage of minor allele carriers for the *APOA1* -75G/A (42.7% versus 28 to 29.6%) (Do et al., 2009, Ordovas et al., 2002a) in our subjects.

The *CETP Taq1B* genotype significantly modulated the TG/HDL-C ratio in response to the kiwifruit intervention. Favourable effects from consuming two green kiwifruit a day against the background of a healthy diet were seen on the plasma TG/HDL-C ratio, but only in *B1/B1* homozygotes of the *CETP Taq1B* genotype. High-density lipoprotein cholesterol response was not modulated by *CETP Taq1B* genotype, since improvements were seen in both genotype groups. Although no genotype interactions were observed for TC/HDL-C ratio or TG, the lack of observed effect may have been due to insufficient power (70% power to observe an interaction effect on TC/HDL-C) as there were trends of greater improvement in these variables in *B1/B1* homozygotes, while this was not observed in *B2* carriers.

The most established function of CETP is to mediate the transfer of neutral lipids, CEs (mainly from HDL-C) and TG (found in triglyceride-rich lipoproteins, VLDL and CM) between plasma lipoproteins (Barter et al., 2003, Chapman et al., 2010). Elevated CETP activity results in enhanced TG enrichment of both HDL and/or LDL, which are more readily remodelled by HL into smaller particles. This consequently leads to a decrease in plasma HDL-C and ApoA1 levels as a result of increased removal of these HDL remnants from circulation

by renal clearance and/or the hepatic holo-receptor, and an increase in the number of atherogenic sLDL particles (Chapman et al., 2010, Charles and Kane, 2012).

The *TaqIB* polymorphism of the *CETP* gene is due to a base change from G to A at position 277 in intron 1. It is not readily apparent how this base change can modulate CETP expression. However, it has been suggested that, while the SNP is not itself functional, it may act as a marker due to its linkage disequilibrium with a functional variant (Boekholdt and Thompson, 2003, de Grooth et al., 2004). Moderate inhibition of CETP activity, and higher HDL-C and apoA1 and lower TG concentrations have been seen in *B2* carriers compared to *B1/B1* homozygotes (Thompson et al., 2008). Conversely, *B1/B1* homozygotes tend to have a more atherogenic lipoprotein profile (Dullaart and Sluiter, 2008).

In our study, MAF for *CETP Taq1B* was 41%, which compares closely with the value of 42% calculated in healthy white individuals in a meta-analysis (Thompson et al., 2008). *B2* carriers tended to have higher concentrations of HDL-C and apoA1 and lower concentrations of TG, compared to *B1/B1* homozygotes at baseline 2, but the differences were not significant, possibly as a result of a lack of power. However, at baseline 2 TC/HDL-C, TG/HDL-C and apoB/apoA1 ratios and sLDL concentrations were significantly lower in *B2* carriers compared to *B1/B1* homozygotes. Overall this is suggestive of a better CVD risk profile for *B2* carriers, in-line with the literature (Thompson et al., 2008).

The current increase in obesity rates and associated deleterious effects on lipid and lipoprotein metabolism, independent of LDL-C concentrations, has seen much research focussed on strategies to address these other lipoprotein abnormalities. Recent research suggests that lipid and lipoprotein ratios have greater predictive value than isolated parameters, in regard to CVD risk, as they more accurately capture overall lipoprotein metabolism (Millan et al., 2009, Musunuru, 2010). The TG/HDL-C ratio has been suggested as a surrogate marker for IR, describing an altered lipid and carbohydrate metabolism (Cordero et al., 2009). Further, the ratio is linked to the presence of atherogenic

sLDL (Miller et al., 2011). With improvements seen in the TG/HDL-C ratio, the results from this study are suggestive that *B1/B1* homozygotes of the *CETP Taq1B* SNP (more than 30% of the population) could particularly benefit from the regular daily consumption of green kiwifruit. Although modest, the reduction in the TG/HDL-C ratio, if confirmed, could be expected to translate into CVD risk reduction in this group.

Although we did not measure CETP mass or activity we hypothesise that the variance in response to the kiwifruit intervention between *B1/B1* homozygotes and *B2* carriers observed in our study may be mediated in part by a differential impact of kiwifruit bioactives on CETP activity, with a greater inhibition in *B1/B1* homozygotes and the net effect of increasing HDL-C and decreasing TG concentrations in this group. In a 6-week study in hamsters the addition of apple polyphenols to a control diet lowered CETP activity and resulted in a higher HDL-C and lower TG concentrations. CETP inhibition by apple polyphenol was also confirmed *in vitro* (Lam et al., 2008). Some of these apple polyphenols are also found in kiwifruit, with kiwifruit also containing a high percentage of additional unextractable (non-identified) phenolics (Fiorentino et al., 2009, Latocha et al., 2010, Tarascou et al., 2010).

A strength of this study was the randomly controlled crossover design, as this is considered the most robust statistically to identify inter-individual variability in dietary response, as subjects serve as their own control (Rideout, 2011). The SNPs chosen were carefully considered with regard to factors, such as frequency of the alleles and their relationship to HDL metabolism.

As a retrospective analysis, which is therefore exploratory by nature, the study does have some limitations. Importantly, mechanistic pathways could not be explored, including whether kiwifruit can inhibit CETP activity. The study was also conducted in a specific population group. Therefore it will be important for future studies to not only confirm our findings, but to test the effects in other populations, including women.

In conclusion, we report that the *Taq1B* polymorphism of the *CETP* gene could modulate the plasma lipid response to the consumption of green kiwifruit against an overall healthy diet background in hypercholesterolaemic males,

with improvements in the TG/HLD-C ratio of *B1/B1* homozygotes, a group at higher risk of an atherogenic lipoprotein profile. Although moderate, if confirmed, these effects could be expected to translate into an overall reduction of CVD risk in *B1/B1* homozygotes and add to the accumulating evidence that this genotype may particularly benefit from targeted interventions.

These findings add to the body of evidence on diet-gene interactions, which may in future lead to a greater personalisation of dietary recommendations. However, this area of research is far more complex than first thought and the challenge is to integrate multiple genetic, physiological, and environmental interactions into stratified recommendations which will afford maximum benefit to the individual (Minihane, 2013, Rideout, 2011).

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Chapter 7: Effects of kiwifruit consumption on blood pressure and markers of cardiovascular function in men with hypercholesterolaemia

This chapter reports the results of another one of the predefined secondary objectives of the randomised kiwifruit trial. The aim of this study was to investigate the effects of the kiwifruit intervention on BP and other markers of cardiovascular function, as measured by the Finometer MIDI®.

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(Publications are listed in Appendix 1 ; contributions of authors in Appendix 3)

Abstract

Increasing fruit and vegetable consumption is a key lifestyle modification in the prevention and treatment of hypertension. Kiwifruit has previously been shown to have favourable effects on BP, likely through inhibiting ACE activity. We aimed to investigate the effects of consuming kiwifruit on BP and other markers of cardiovascular function, including HR, SV, CO and TPR. We hypothesised that consuming two green kiwifruit daily in conjunction with a healthy diet would significantly improve these markers. Using a controlled cross-over study design, 85 hypercholesterolaemic men completed a 4-week healthy diet run-in period before randomisation to one of two 4-week intervention sequences of consuming two green kiwifruit/day plus healthy diet (intervention) or healthy diet alone (control). Blood pressure and other measures of cardiovascular function (using a Finometer MIDI® and standard oscillometric device), and anthropometric measurements were taken at baseline 2 (after run-in), and at the end of the treatment periods. A physical activity questionnaire was completed during the last visit. Subjects were found to be predominantly normotensive (43.5%) or prehypertensive (50.6%) and quite physically active (>30 minutes of moderate to vigorous physical activity/day in >80% subjects). No significant differences were seen for BP or any of the other markers including HR, SV, CO and TPR. In conclusion, in this hypercholesterolaemic, non-hypertensive group, no beneficial effects on BP or other markers of cardiovascular function were seen from consuming two kiwifruit a day against the background of a healthy diet.

7.1 Introduction

In terms of attributable deaths worldwide, raised BP or hypertension is the leading risk factor for CVD (Mendis et al., 2011). It is also one of the most modifiable risk factors, with changes in lifestyle factors having central roles in both its prevention and management (Chobanian et al., 2003, Pimenta and Oparil, 2012). Important lifestyle modifications include weight loss, regular aerobic exercise and adopting a healthy dietary pattern. After reduction of dietary sodium, the next key component of a healthy diet is increasing the consumption of fruits and vegetables (Chobanian et al., 2003). Strategies that decrease BP by even modest amounts can result in significant reductions in CVD risk (Chobanian et al., 2003). The role of lifestyle strategies is potentially even greater in prehypertensives, a group that has been shown to be at increased CVD risk, but where for most individuals pharmacological therapy is not indicated (Pimenta and Oparil, 2010).

There is strong evidence for the antihypertensive effects of fruit and vegetables and specific dietary patterns rich in fruits and vegetables, such as the DASH diet (American Dietetic Association, 2013). Fruits contain a range of nutrients and non-nutrient components that may contribute independently or synergistically to lowering BP, including fibre, potassium, vitamin C and carotenoids (Bhupathiraju and Tucker, 2011, Juraschek et al., 2012, Savica et al., 2010). Further, there is evidence suggesting that some fruit polyphenols may have BP lowering effects (Chong et al., 2010).

Compared to most other commonly consumed fruit, kiwifruit are particularly good sources of vitamin C, carotenoids, potassium, polyphenols, and fibre (Ferguson and Ferguson, 2003, Hunter et al., 2011). Kiwifruit extract has been shown *in vitro* to inhibit ACE activity, a key regulator of BP through the RAS (Jung et al., 2005). Karlsen et al. (2013) conducted an 8-week randomised, parallel intervention, in which male smokers consumed three green kiwifruit daily, significant reductions were observed in DBP and SBP compared to control treatment (habitual diet), with the greatest effects (-15 mmHg SBP, -13 mmHg DBP) seen amongst hypertensive subjects. They also observed an 11%

reduction in serum ACE activity in subjects in the kiwifruit group (Karlsen et al., 2013).

The present study, a predefined secondary analysis of our randomised kiwifruit trial (Gammon et al., 2013), aimed to investigate the effects of the kiwifruit intervention on BP and other markers of cardiovascular function, as measured by the Finometer MIDI®. We hypothesised that consuming two green kiwifruit daily in conjunction with a healthy diet would significantly improve these markers of function including SV, CO and TPR in hypercholesterolaemic men. Our study design allowed us to investigate the specific effects of kiwifruit, independent of the effects of a healthy diet containing fruit.

7.2 Subjects and methods

See Methodology Section (Chapter 3)

7.3 Results

As previously reported, 85 subjects completed the 12 week intervention (Gammon et al., 2013). Baseline characteristics of subjects are presented in Table 7.1. Using the JNC 7 report classifications of BP, few (5.9%) of the men were hypertensive and could be classified as having stage 1 hypertension (140-159 or 90-99 mmHg), and half were prehypertensive (120-139 or 80-99 mmHg) (Chobanian et al., 2003). Based on the RPAQ more than 80% of subjects did more than 30 minutes of moderate to vigorous physical activity per day.

Table 7.1: Characteristics of subjects who completed the study*

Variable	
Age (yr)	48.0 (46.0, 50.0)
BMI (kg/m^2)	27.4 (26.5, 28.2)
Body fat (%)	27.4 (25.8, 29.0)
LDL-C (mmol/L)[†]	4.07 (3.91, 4.24)
TG (mmol/L)[†]	1.62 (1.47, 1.79)
SBP (mmHg)[‡]	122 (120, 125)
DBP (mmHg)[‡]	70.9 (69.0, 72.8)
Heart rate (bpm)[‡§]	55.6 (53.6, 57.6)
Prevalence of hypertension	
Normal <120/80 mmHg, n (%)[§]	37 (43.5)
Prehypertension 120-139 or 80-99 mmHg, n (%)[§]	43 (50.6)
Stage 1 hypertension 140-159 or 90-99 mmHg, n (%)[§]	5 (5.9)
Total energy expenditure (MET.h/d)[¶]	37.7 (33.8-41.5)
Moderate or vigorous PA, ≥3 METs (h/d)[¶]	1.01 (0.39-2.38)
>30 mins moderate or vigorous PA per day, n (%)	69 (81.2)

BMI: body mass index; DBP: diastolic blood pressure; LDL-C: low-density lipoprotein cholesterol; MET: metabolic equivalents; PA: physical activity; SBP: systolic blood pressure
*Values are means (95% CI) at Baseline 1, unless otherwise indicated; n=85
[†]Mean (95% CI) for the log transformed data values, back transformed to the original scale
[‡]Omron measurements, baseline 2 values
[§]Recognised cut-offs for hypertension stages (Chobanian et al., 2003)
[¶]Recent physical activity questionnaire data, median (25th-75th quartiles) (Besson et al., 2010)

Changes in dietary intakes during the intervention have previously been reported (Gammon et al., 2013). Briefly, the men's overall diets improved reflecting that of a healthy diet. There were significant decreases in total energy, cholesterol and percentage of energy from fat and saturated fat and an increase in fruit servings per day (median (25^{th}, 75^{th} percentiles), from 1.33 (0.67-2.67) serves/day at baseline 1, to 2.0 (1.00-3.50) serves/day at baseline 2, and 3.00 (3.00-4.00) serves/day during kiwifruit and 2.50 (2.00-4.00) serves/day during control interventions). Significantly higher intakes were seen for vitamin C and vitamin E during the kiwifruit intervention compared to control intervention.

Anthropometric data has also been previously reported (Gammon et al., 2013). Briefly, there were small decreases observed for nearly all anthropometric measures during the 4-week healthy diet run-in period. From the end of this period (baseline 2) to the end of the 8-week intervention there were no further changes in body weight, regardless of which intervention the subjects were receiving (green kiwifruit or control).

The results of the intervention on BP and other markers measured by the Finometer are shown in Table 7.2. There were no significant between-treatment differences for any variable. This parallels whole group findings for SBP or DBP when measured by standard oscillometric device, where no significant changes were observed (data previously presented in Gammon et al. (2013)). No significant BP group (prehypertensive versus normotensive) x treatment interactions were seen for any variable, whether measured by the Finometer or standard oscillometric device. Therefore, no further stratified analysis was conducted.

One subject was taking medication for BP (baseline BP 136/83 mmHg). As there was no change in the results when he was excluded, he has been included in the statistical analysis.

Table 7.2: Changes in BP and other markers of cardiovascular function measured by the finometer for the 2 intervention periods*

	Baseline 2	Δ Kiwifruit	Δ Control	$P^{\dagger}$
SBP (mmHg)	124 (121, 128)	1.29 (-2.13, 4.71)	-0.53 (-4.04, 2.98)	0.33
DBP (mmHg)	66.5 (64.4, 68.6)	-0.76 (-2.75, 1.22)	-1.48 (-3.49, 0.53)	0.51
MAP (mmHg)	85.1 (82.8, 87.4)	-0.73 (-2.88, 1.43)	-1.76 (-3.90, 0.39)	0.35
HR (bpm)	57.8 (55.9, 59.6)	-0.25 (-1.29, 0.78)	-0.22 (-1.29, 0.85)	0.94
SV (mL)	98.9 (94.3, 104)	3.20 (-0.37, 6.77)	3.01 (-1.37, 7.40)	0.93
CO (L/min)	5.67 (5.37, 5.97)	0.18 (-0.05, 0.41)	0.16 (-0.13, 0.46)	0.89
TPR (mmHg.s/mL)	959 (898, 1020)	-34.0 (-88.5, 20.5)	-17.8 (-83.3, 47.6)	0.65

CO; cardiac output; DBP: diastolic blood pressure; HR: heart rate; MAP: mean arterial pressure; SV: stroke volume; SBP: systolic blood pressure; TPR: total peripheral resistance
*Values are means (95% CI), subjects (n=85) followed a cross-over design protocol for the 2 intervention periods.
†Significant differences between kiwifruit versus control (P<0.05) (Dependent Student t-test)

7.4 Discussion

In the present study, consuming two green kiwifruit against the background of a healthy diet did not significantly change BP or the other markers of cardiovascular function as measured using the Finometer, in a group of hypercholesterolemic men. Therefore, rejecting our research hypothesis.

The Finometer provides a non-invasive, continuous measurement of BP and tracks changes in other markers of function, including SV, CO and TPR, compared to automated oscillometric devices which only provide a momentary value of BP (Bogert and van Lieshout, 2005).

Given kiwifruit's unique composition and the positive results seen in the *in vitro* study (Jung et al., 2005) and the other human intervention study (Karlsen et al., 2013), both having shown ACE inhibition by kiwifruit, we anticipated beneficial effects of kiwifruit on cardiovascular factors, such as TPR and BP, especially within the prehypertensive group. However, we saw no significant changes in these factors using the Finometer, or in BP using a standard oscillometric device, either for the whole group or by BP group (prehypertensive versus normotensive).

Total peripheral resistance is a measure of the overall resistance (friction) encountered by blood as it flows through blood vessels (Levick, 2010). Local, regional, and systemic neural, humoral, and renal factors regulate TPR, which is influenced by a range of factors, one of which is Ang II (Singh et al., 2010). ACE catalyzes the conversion of Ang I to Ang II in the endothelium, where it exerts vasoconstrictive effects, countervailing the vasoprotective effects of NO (Schulman et al., 2006).

ACE inhibitors are one of the most successful classes of antihypertensive drugs (O'Rourke et al., 2011). A number of plant extracts have now also been identified as natural ACE inhibitors, with most of the favourable effects being attributed to flavonoid molecules (Guerrero et al., 2012), several of which have been identified in kiwifruit, including rutin, epicatechin, and quercetin (Fiorentino et al., 2009, Latocha et al., 2010). Interestingly, in the Karlsen et al. (2013) study, in addition to kiwifruit and control groups, a third group consumed an anti-oxidant rich diet for 8 weeks. In this group a decrease in SBP was seen only in hypertensive subjects and there was no reduction in ACE activity. The authors suggested that, as the anti-oxidant rich diet was also rich in vitamin C and polyphenols, the favourable effects seen on ACE inhibition and BP in the kiwifruit were likely due to specific polyphenols not commonly found in other anti-oxidant rich foods (Karlsen et al., 2013). Certainly many of the phenolics and flavonoids in kiwifruit have yet to be identified, as to date they have been unextractable (Tarascou et al., 2010). Future studies will be needed to elucidate if compounds in kiwifruit are acting as natural ACE inhibitors.

A number of intervention studies have observed that antihypertensive effects were less marked in subjects who had lower baseline BPs, including the Karlsen et al. (2013) kiwifruit study (Desch et al., 2010, Erlund et al., 2008, Karlsen et al., 2013, McKay et al., 2010). This may explain why we did not observe any effects, as few of our subjects were hypertensive. However, we were also unable to show beneficial effects in the prehypertensive group. Further, unlike the Karlsen et al. (2013) study, none of our subjects were smokers. Two markers of endothelial dysfunction are decreased bioavailability of NO and an increased production of ROS, both of which are aggravated by smoking (Higashi et al., 2012, Tousoulis et al., 2011). There were also

differences in study design; in the Karlsen et al. (2013) study participants consumed three kiwifruit a day in addition to their habitual diet, for 8 weeks. Finally, our subjects were also quite physically active, with most doing more than 30 minutes of moderate to vigorous physical activity a day, a key lifestyle recommendation to prevent and manage hypertension (Chobanian et al., 2003). It may be that kiwifruit do not add additional benefits to a healthy diet, containing fruit. Future studies should test the antihypertensive effects of kiwifruit in other groups, including non-smoking hypertensive subjects and, ideally in studies designed to measure the benefits of kiwifruit, independent of the effects of other fruit.

A strength of this study was the randomly controlled crossover design, as this increases statistical power and eliminates inter-individual differences across treatment groups (Rideout, 2011). Although consistent positive effects have been shown on BP for flavanol-rich cocoa products (Desch et al., 2010, Hooper et al., 2008), and some berry fruit interventions have shown significant effects (Erlund et al., 2008, Naruszewicz et al., 2007), the evidence for other flavonoids and fruit polyphenols is more limited (Chong et al., 2010, Hooper et al., 2008). This has in part been attributed to the number of underpowered or poorly designed studies that have been conducted in this field (Chong et al., 2010, Hooper et al., 2008). To address these issues, future studies should be well designed, including being suitably controlled, well powered and of adequate duration.

In conclusion, this study in predominantly normotensive and prehypertensive hypercholesterolaemic men found no beneficial effects on BP or other markers of cardiovascular function when subjects consumed two green kiwifruit a day against an overall healthy diet background.

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Chapter 8: Discussion and conclusions, including recommendations for future research

8.1 Introduction

The randomised controlled trial reported in this thesis investigated the effects of consuming two green kiwifruit as part of a healthy diet on lipid profiles and other CVD-related metabolic markers in a group of men with hypercholesterolaemia. Further, it examined whether response was modulated by selected lipid-related genotypes and inflammatory status.

8.2 Summary of findings and outcomes of hypotheses

The aim of the randomised controlled trial was to investigate the effect of consuming two green kiwifruit daily in conjunction with a healthy diet on plasma lipids.

Primary objective

In addressing the primary objective for this study, the effect of the green kiwifruit consumption on plasma lipids, the kiwifruit intervention resulted in a significantly higher mean HDL-C concentration and lower TC/HDL-C ratio compared with the control intervention, in this group of hypercholesterolaemic men (reported in Chapter 4).

Thus, the primary hypothesis that the replacement of two fruit servings in a healthy diet with two green kiwifruit will have favourable effects on plasma lipids is accepted.

Secondary objectives

1. In investigating the effects of the kiwifruit intervention on other CVD-related markers, namely CRP, plasma glucose and insulin, no significant differences were observed between the two intervention periods for glucose and insulin, and for CRP in the total group (reported in Chapter 4). However, for CRP, when subjects were grouped into low (<1 mg/L) and medium (1-3 mg/L) inflammatory groups using their pre-intervention plasma hs-CRP measurements, there was a significant decrease in CRP for subjects in the medium inflammatory group (reported in Chapter 5).

Thus, secondary hypothesis 1 that the replacement of two fruit servings in a healthy diet with two green kiwifruit will have favourable effects on other CVD-

related markers of risk is rejected for glucose and insulin, and for CRP in the total group. For subjects in the medium inflammatory group the hypothesis is accepted for CRP.

2. Further, the study aimed to examine whether the plasma lipid response might be modulated according to *APOE* genotype. When subjects were stratified into *APOE4* carriers and non-*APOE4* carriers, *E4* carriers showed a significant decrease in TG concentrations following the kiwifruit intervention compared to control. No significant differences between kiwifruit and control interventions were observed for TG in non-*E4* carriers. This resulted in a significant difference in plasma lipid response for TG between *APOE4* carriers and non-*E4* carriers for the kiwifruit intervention (reported in Chapter 4).

Thus, secondary hypothesis 2 that carriers of different *APOE* genotypes will show differences in plasma lipid response to the intervention is accepted.

3. Another secondary objective was to investigate whether inflammatory state, as measured by hs-CRP, would modulate plasma lipid and inflammatory marker response to the intervention. Subjects were divided into low (<1 mg/L) and medium (1-3 mg/L) inflammatory groups using their pre-intervention plasma hs-CRP measurements.

In the medium inflammatory group, there was a significant increase in plasma HDL-C and decrease in TC/HDL-C ratio from baseline during the kiwifruit intervention. This resulted in a significant difference between kiwifruit and control intervention periods for HDL-C and TC/HDL-C ratio. No significant differences between kiwifruit and control interventions were observed in the low inflammatory group. Further, in the medium inflammatory group, the kiwifruit intervention resulted in significant improvements in plasma CRP and IL-6, compared to control intervention period. The differences in response observed between the two inflammatory groups resulted in significant inflammatory group x intervention interactions for the TC/HDL-C and TG/HDL-C ratios, and IL-6 (reported in Chapter 5).

Thus, secondary hypothesis 3 that subjects with different inflammatory statuses will show differences in plasma lipid and inflammatory marker response to the intervention is accepted.

4. Based on the results from the primary study, four SNPs related to HDL metabolism were selected, namely *CETP Taq1B*, *APOA1* -75G/A, *LIPC* -514C→T and *LIPG* I24582 (T+2864C/In8) variants, to investigate if any might modulate the plasma lipid response to the intervention.

As a result of statistical screening, further statistical analysis was only conducted on the *CETP Taq1B* SNP. At baseline 2, *B2* carriers had significantly lower TC/HDL-C, TG/HDL-C and apoB1/apoA1 ratios, and sLDL concentrations, compared to *B1* homozygotes. A significant genotype x treatment interaction was observed for the TG/HDL-C ratio. The two genotype groups differed in response to the intervention, with the *B1/B1* homozygotes having a significantly lower TG/HDL-C ratio after the kiwifruit intervention, compared to control, whereas there was no significant treatment difference for *B2* carriers (reported in Chapter 6).

Thus, secondary hypothesis 4 that carriers of the selected lipid-related polymorphisms (based on the primary results) will show differences in plasma lipid response to the intervention is accepted.

5. The final secondary objective was to investigate the effects of the kiwifruit intervention on BP and other markers of cardiovascular function, namely HR, SV, CO and TPR.

No significant between-treatment differences were observed for BP, measured by either standard oscillometric device or the Finometer, or for other markers of function, as measured by the Finometer (reported in Chapter 7).

Thus, secondary hypothesis 5 that the replacement of two fruit servings in a healthy diet with two green kiwifruit will have favourable effects on BP and other markers of cardiovascular function is rejected.

8.3 Discussion of the main findings

The main finding from this randomised controlled, crossover trial was that kiwifruit improved the dyslipidaemic profile of subjects, with significant improvements in mean HDL-C concentration and TC/HDL-C ratio compared with the control intervention. This was despite only a small number of subjects having a low (<1 mmol/L) HDL-C concentration. However, the effects were even more pronounced in men who had phenotypes which resulted in them having a higher inflammatory status and carriers of certain genotypes which put them at higher risk of CVD, with more sizeable improvements in HDL-C and TC/HDL-C ratio, and significant decreases in TG concentrations and the TG/HDL-C ratio, in these subjects. Further, the favourable effects of the kiwifruit intervention were independent of the effects of a healthy diet containing fruit.

It has been suggested that one unit change in the TC/HDL-C ratio is associated with a 53% change in risk of myocardial infarction (Stampfer et al., 1991). Therefore the improvement in the TC/HDL-C ratio for the total group could be translated into about an 8% reduction in risk. This increases to 15% reduction of risk in subjects in the medium inflammatory group, who showed the greatest improvement in TC/HDL-C ratio.

Two previous interventions had investigated the effects of kiwifruit on plasma lipids. In the Duttaroy and Jorgensen (2004) study, a significant reduction in plasma TG from baseline was observed. In contrast, the Chang and Liu (2009) study showed a significant increase in HDL-C and decreases in the LDL-C/HDL-C and TC/HDL-C ratios. Since we conducted our randomised controlled trial, the results from two further kiwifruit studies which reported the effects of consuming kiwifruit on plasma lipids had been published, although for neither study it was a primary outcome (Brevik et al., 2011, Karlsen et al., 2013). In the Brevik et al. (2011) study, which investigated the effects of consuming one or two gold kiwifruit per day for 4 weeks on biomarkers of oxidative stress, decreases in TG (13.3% with 1 kiwifruit/day and 7.4% with 2 kiwifruit/day), compared to baseline values were observed. However, Karlsen et al. (2013) showed no significant changes in plasma lipids in their study, which investigated the effects of consuming three green kiwifruit per day for 8 weeks

on BP and platelet aggregation. The only study to include a control group was Karlsen et al. (2013), and none of the studies had been designed to test the specific effects of kiwifruit, independent of the effects of a healthy diet containing fruit. In three of the studies the kiwifruit had been added to the subject's normal diet or, in the Karlsen et al. (2013) study, added to a diet where certain polyphenol-rich foods were restricted.

In the design of our own intervention we sought to address some of the design shortcomings of previous interventions then published. The findings from our study add to the body of evidence supporting favourable benefits on plasma lipids with kiwifruit consumption. However, our overall findings have not resolved the inconsistencies in results that have been observed in other studies. Although these inter-study differences in response may be explained by differences in study design, such as duration, dose and background diet (Rideout, 2011), some may also be explained by inter-individual variability in response, due to factors such as metabolic phenotype and genotype. With this in mind, we attempted to address this by examining the effect of selected genotypes and subjects' inflammatory status.

Our study showed differences in response to the intervention by *APOE* and *CETP Taq1B* genotypes, and inflammatory status. Although *APOE* genotype is now thought to have only a modest impact on CVD risk in the general population (with a much larger impact in smokers), *APOE4* allele carriers have relatively consistently been shown to be more responsive to dietary change (Minihane, 2013). Typically, carriers of the *E4* allele have higher TC, LDL-C and apoB concentrations, and a tendency towards higher TG and lower HDL-C concentrations, compared to *E3* homozygotes, and display a greater change to dietary fat manipulation in these plasma lipid CVD biomarkers (Minihane et al., 2007, Wu et al., 2007, Bennet et al., 2007). Our intervention resulted in significantly reduced TG concentrations in *APOE4* carriers after consumption of green kiwifruit, compared to control. This is in contrast to the total group, where no significant change in plasma TG concentration was observed. Further, for the total group of subjects, the improvement in HDL-C came not only from an increase in HDL-C during the kiwifruit period, but also from a decrease in HDL-C during the control period, whereas in *E4* carriers there was a trend towards a

greater increase in HDL- C during the kiwifruit period. Although a greater TG lowering response has been observed in individuals with higher TG concentrations (Miller et al., 2011) and *APOE4* carriers tend to have higher concentrations, in our subject group this was not the case. At this stage we can only speculate as to why *APOE4* carriers had the favourable TG lowering response to the kiwifruit intervention compared to non-*E4* carriers. This will be further discussed below.

As with *APOE4* carriers, *B1/B1* homozygotes of *CETP Taq1B* genotype also are considered to have a more atherogenic lipoprotein profile, with lower HDL-C and higher TG concentrations compared to *B2* carriers (Thompson et al., 2008), as was observed in our subject group. As a result of the intervention, *B1/B1* homozygotes had a significant decrease in TG/HDL-C ratio, and trends to greater improvement in TC/HDL-C ratio and TG concentration, which were not observed in *B2* carriers. In regard to HDL-C, for both genotype groups the kiwifruit intervention period resulted in greater concentrations in HDL-C compared to control. However, in *B2* carriers the significant difference was driven by a significant decrease in HDL-C from baseline during the control intervention. Moderate inhibition of CETP activity has been observed in *B2* carriers (Thompson et al., 2008). Further, in an animal study CETP inhibition was observed in response to consumption of apple polyphenols (Lam et al., 2008). In our study, there may have been a differential response to the kiwifruit intervention, with greater inhibition of CETP activity in the group (*B1/B1* homozygotes) not already exhibiting inhibited activity.

Finally, subjects grouped by their pre-intervention hs-CRP concentration into a medium inflammatory group (CRP 1-3 mg/L), showed a greater risk of metabolic dysfunction with higher values for anthropometric measures and significantly higher TG concentrations, compared to subjects in the low inflammatory group (CRP <1 mg/L). In response to the intervention, a significant increase in HDL-C was only observed in the medium inflammatory group, with the improvement all due to a significant increase in HDL-C during the kiwifruit intervention period, with no change from baseline during the control intervention period. There were also significant decreases in TC/HDL-C ratio,

hs-CRP and IL-6 in this group, and an almost significant decrease in TG/HDL-C ratio.

To our knowledge, no study had previously investigated the variability in responsiveness of blood lipids to an intervention involving fruit. Numerous studies have investigated the effects of *APOE* genotype on intervention response, and favourable responses on plasma lipids have been observed in *APOE4* carriers in response to a range of dietary fat or cholesterol interventions (Masson et al., 2003). For the *CETP Taq1B* genotype, four randomised controlled trials have investigated the modulating effect of this genotype to some sort of dietary fat manipulation (Aitken et al., 2006, Carmena-Ramon et al., 2001, Estevez-Gonzalez et al., 2010, Wallace et al., 2000), but no consensus has been reached on whether this genotype has a clinically meaningful nutrigenetic impact.

A small number of studies have shown that inflammatory status, as measured by CRP, may modulate responsiveness to dietary manipulation. In these studies, which involved some sort of dietary fat manipulation, the higher inflammatory group generally either had an unfavourable response, showing a worsening of TG concentrations on a low fat (higher carbohydrate) diet (Desroches et al., 2006, Erlinger et al., 2003, St-Onge et al., 2009) or a blunting of effect, compared to the improvements seen by the low inflammatory group in response to a healthy diet pattern (DASH or NCEP step 1) (Erlinger et al., 2003, Hilpert et al., 2005). In contrast, in the study by Desroches et al. (2006), the higher inflammatory group had significantly greater reductions in TC and LDL-C concentrations in response to a high MUFA diet, compared to the low inflammatory group. This group also had a significant decrease in CRP from baseline (Desroches et al., 2006). It was the only study to examine the effect of the diet on CRP concentrations stratified by CRP group.

In our study, the medium inflammatory group had a positive response to the kiwifruit intervention. We speculate that the improvement in lipid profile in the medium inflammatory group is due to the decrease in inflammation overall, as observed by reductions in CRP and IL-6. In the Desroches et al. (2006) study described above, where CRP decreased, subjects in the higher CRP group also

had the more positive lipid response. Cell studies have shown that apoA1 gene expression is down-regulated by the inflammatory cytokines IL-6 and TNF-α (Navarro et al., 2005). In concurrence with the decrease in inflammation, apoA1 concentrations significantly increased from baseline during the kiwifruit intervention, which was then reflected in the increase in HDL-C, thereby improving the dyslipidaemic profile in this moderately inflamed group. In groups with higher levels of CRP, such as might be seen in subjects with metabolic syndrome (Pearson et al., 2003), the positive effects could be even more pronounced.

The extent and mechanisms by which visceral adiposity alters normal lipid metabolism are still being uncovered. In their review, Flock et al. (2011, p271) suggest that 'to realize the maximal benefits [in lipid profile improvements] of dietary interventions low in SFA and cholesterol', weight loss should be recommended to normalise adipose tissue mass. Although this may be the ideal goal, a dietary plan which incorporates components such as kiwifruit to reduce inflammation may also have an important role in maximising the benefits of healthy dietary patterns.

When we conducted explorative analyses, subjects were also stratified by BMI (<25 kg/m^2, n=21 or ≥25 kg/m^2, n=64) or IR (≤1.0 HOMA2-IR, n=46 or >1.0 HOMA2-IR, n=38). In the higher groups, HDL-C concentrations were significantly lower and TG concentrations significantly higher, indicating a more atherogenic phenotype, as could be expected with increased adiposity or IR (Musunuru, 2010). In response to the intervention, all BMI or IR groups showed similar responses with significant or almost significant (in the BMI <25 kg/m^2 group) differences in HDL-C between kiwifruit and control interventions. The difference mainly arose from a decrease in HDL-C during the control intervention period. Thus, the significant increase in HDL-C that we observed in the medium inflammatory group appears to be due to inflammatory factors which were independent of BMI and/or IR.

Although hs-CRP has been commonly used as a marker of inflammation, in studies which investigate the effect of dietary modification on inflammation (Galland, 2010), there are limitations in using a single marker to classify

subjects. Future studies should look to use additional confirmatory markers to strengthen the classifications of subjects into these inflammatory/metabolic groups. The rapidly developing field of metabolomics also offers possibilities that are starting to be realised. In a very recently published paper, researchers showed greater responses to the consumption of sea buckthorn and bilberberries in subjects categorised into a higher cardiometabolic risk group using a metabolic clustering of factors at baseline, including plasma lipids and inflammatory markers (Larmo et al., 2013).

Interventions which could improve an atherogenic dyslipidaemia are of particular relevance given the current obesity epidemic (Miller et al., 2011, Musunuru, 2010). Circulating TG is metabolically intimately linked with HDL-C, with an inverse association between the two (Miller et al., 2007). As the incidence of obesity has increased, so have rates of hypertriglyceridaemia (Miller et al., 2011). Under hypertriglyceridaemic conditions, a number of changes to normal lipid metabolism have been observed, including a shift from an apoE to an apoC3 dominated system, which leads to the reduced clearance of TGRL (Zheng et al., 2010), a shift of the normally anti-atherogenic CETP-mediated pathway to a more pro-atherogenic one (Chapman et al., 2010), and increased concentrations of CETP (Vourvouhaki and Dedoussis, 2008). These changes ultimately lead to the generation of sLDL and small HDL, and a consequent decrease in HDL-C concentrations. Further, apoC3 has been shown to activate pro-inflammatory transcription factors such as NF-κβ (Talayero and Sacks, 2011). The technology associated with metabolomics lends itself to more readily identifying these types of shifts in lipoprotein and apolipoprotein profiles, with the ability to measure multiple analytes and create metabolic profiles (Rasmiena et al., 2013). This has the potential to allow a more comprehensive investigation of mechanistic pathways involved in responses seen with interventions such as ours.

Mechanisms as to how kiwifruit increase HDL-C or decrease TG will also need further investigation. Compared to other commonly consumed fruit, kiwifruit stand out for their nutrient density. Green kiwifruit contain significant levels of soluble fibre (Schakel et al., 2001), one of the highest concentrations of vitamin C, significant amounts of vitamins E and K, potassium, folate, carotenoids and

polyphenols (Ferguson and Ferguson, 2003, Hunter et al., 2011). However, unlike fruit such as apples and berries, many of the phenolics and flavonoids in kiwifruit are yet to be identified, as to date they have been unextractable (Tarascou et al., 2010). Many of these constituents, which are found in kiwifruit, have been shown to be associated with beneficial effects on cardiovascular health (Badimon et al., 2010, Brown et al., 1999, Erkkila and Lichtenstein, 2006, Salas-Salvado et al., 2006, Voutilainen et al., 2006) and CRP concentrations (Basu et al., 2010, Buscemi et al., 2012, Galland, 2010, Karlsen et al., 2010, Kelley et al., 2006, Nanri et al., 2007, Pan and Jackson, 2009, Watzl et al., 2005).

Given the chemically complex composition of fruit in general, and the opportunity for both independent and/or synergistic contributions of constituents in biological processes, the components that are responsible for the favourable effects seen on plasma lipids and inflammation remain to be determined. Based on evidence from the literature, the most likely constituents to exert the observed effects are polyphenols and the anti-oxidant vitamins C and E.

Flavonoid-rich juices such as orange juice (Kurowska et al., 2000), grape juice (Albers et al., 2004) and cranberry juice (Ruel et al., 2006) have all been shown to increase plasma HDL-C. Polyphenols could also explain the decrease in plasma TG concentrations in *APOE4* carriers, with studies conducted using red grapefruit and tomatoes also showing positive effects on plasma TG concentrations (Shen et al., 2007, Gorinstein et al., 2006). Bilberry juice has been shown to significantly reduce plasma concentrations of CRP and IL-6. Mechanistic investigations in the same study showed that quercetin (which is also present in kiwifruit) (Fiorentino et al., 2009), resveratrol and epicatechin all inhibited NF-κβ activation, a key regulator of pro-inflammatory signalling molecules (Karlsen et al., 2010). Finally, polyphenols found in apples (some of which are also found in kiwifruit) were shown to decrease CETP activity in hamsters, which resulted in increased HDL-C and decreased TG concentrations (Lam et al., 2008).

The contribution of components other than polyphenols, for example vitamin C and E, which have been shown to inhibit NF-κβ activation (Bowie and O'Neill,

2000, Glauert, 2007), cannot be discounted. It has been suggested that *APOE4* represents a 'lipid-thrifty' variant, which allows better intestinal absorption of lipids, including fat soluble vitamins and polyphenols (Corbo and Scacchi, 1999, Gerdes, 2003, Huebbe et al., 2011). In a recent placebo-controlled study, gamma and delta tocotrienols were shown to significantly reduce TG concentrations (Zaiden et al., 2010). Although the dose used was higher than is found naturally in fruit, there may still be a contribution to a synergistic effect with other bioactive components.

A number of plant extracts have been identified as natural ACE inhibitors (Guerrero et al., 2012), including kiwifruit extract, which has demonstrated activity *in vitro* (Jung et al., 2005). In their 8-week randomised, parallel intervention, Karlsen et al. (2013) observed an 11% reduction in serum ACE activity in a group of male smokers who consumed three green kiwifruit a day in addition to their habitual diet. Significant reductions were observed in DBP and SBP, compared to control treatment (habitual diet), with the greatest effects amongst hypertensive subjects. We observed no change in BP in our study to consuming two green kiwifruit against the background of a healthy diet. A number of intervention studies have observed that basal BP status may affect inter-individual dietary response, with less marked responses seen in subjects with lower BP (Desch et al., 2010, Erlund et al., 2008, Karlsen et al., 2013, McKay et al., 2010). As measuring lipids was our primary objective, our subjects were recruited based on their lipid profile. However, our study group included a lower than expected number of subjects who could be classified as hypertensive. Nevertheless our subject group did include a significant proportion of prehypertensive subjects, in whom some response to the intervention could have been expected. The differences in findings between the Karlsen et al. (2013) and our study may be either a result of different study groups (smokers versus non-smokers) or design factors (such as 3 kiwifruit versus 2). Alternatively, it may be that kiwifruit do not add additional BP benefits to a healthy diet containing fruit (Zhao et al., 2011). Future studies will be needed to confirm which of these theories is correct.

The literature review highlighted the limited randomised controlled trial evidence that supports the consumption of fruit and vegetables for CVD risk reduction,

and also the need for more whole fruit studies which investigate effects on CVD risk markers. This study adds to the body of evidence that further supports the benefits of fruit consumption on CVD risk markers and, more specifically, the evidence supporting the consumption of kiwifruit to improve dyslipidaemia. Further, the majority of men in our study (89.4%) rated eating two green kiwifruit every day as easy or very easy at the end of the intervention, with 88% of subjects reporting no unwanted side effects. In NZ, kiwifruit is a readily available fruit, inexpensive in season and so has the potential to be easily added to a heart healthy diet to improve dyslipidaemic profiles. Although the improvements were modest, it does suggest that kiwifruit could be incorporated into a dietary plan aimed at heart health, or used as an adjunct to statin therapy in individuals who are not reaching treatment goals with statins alone, with statins targeting LDL-C and kiwifruit targeting HDL-C and TG. This approach has been demonstrated with long-chain omega-3 fatty acids (Davidson et al., 2007). Further, the effects may be more significant in individuals who are more responsive, such as *APOE4* carriers, *B1/B1* homozygotes of *CETP Taq1B* genotype or those with higher CRP concentrations.

Our study also involved some pre-defined secondary analyses, which were therefore explorative in nature. The findings from these will need to be confirmed by further studies, and mechanistic pathways explored. However, the findings do add to the growing body of evidence demonstrating diet-inflammatory/metabolic state and diet-gene interactions, which may one day allow for personalised dietary recommendations.

The development of commercial arrays (SNP chips) has seen a greater utilisation of genome-wide scans to identify genetic variants that modulate the effect of dietary factors (Fenech et al., 2011, Manolio et al., 2009). This is in contrast to the targeted approach that we used in our study to identify HDL-related genes of interest. Both approaches have their own strengths, with candidate gene studies tending to have more statistical power, while genome-wide association studies have the ability to discover new genes of interest (Amos et al., 2011). Regardless of which approach is utilised, it has become ever more apparent that the interactions between genes, health and diet are far more complex than first anticipated (Fenech et al., 2011, Minihane, 2013). As

Anne Marie Minihane, Professor in nutrigenomics, (2013, p7) concludes in a recent review, ‘Most of the benefits of genetics in public health remain to be realised and we undoubtedly have a long way to go’. Despite the identification through genome-wide association studies of hundreds of genetic variants associated with chronic diseases, most identified common variants explain only a fraction of heritability (Manolio et al., 2009). Several hypotheses have been proposed to explain this missing heritability, including epigenetics. Epigenetic effects are yet another strand to be integrated into personalised nutrition recommendations (Baccarelli and Ghosh, 2012, Burdge et al., 2012, Cowley et al., 2012).

It is important that studies investigating genotype/phenotype interactions should be well-designed, with an appropriately sized group, of sufficient duration and controlled. The strengths and limitations of this study will now be discussed.

8.4 Strengths

The main strengths of our 12-week, randomised controlled cross-over study lie with its design. Randomised controlled trials allow cause and effect relationships to be established (Boushey et al., 2006). Further, a crossover design is considered the most robust statistically to identify inter-individual variability in dietary response, as subjects serve as their own control (Rideout, 2011). In regard to study duration, the recent European Food Safety Authority (ESFA) Panel guidelines recommend a study duration of 3 to 4 weeks as sufficient to test short-term effects on BP and lipids (EFSA Panel on Dietetic Products Nutrition and Allergies, 2011). Plasma lipid concentrations are known to stabilise within three weeks, and no washout period is needed between treatments (Kris-Etherton and Dietschy, 1997). In addition to measuring isolated lipid parameters, such as LDL-C, a range of ratios namely TC/HDL-C, TG/HDL-C and ApoB/A1 were included, which allowed a more informative representation of what was happening with overall lipoprotein metabolism.

Secondly, as previously discussed, the study was designed to investigate the specific effects of kiwifruit, independent of the effects of a healthy diet containing fruit. The 4-week run-in period on a healthy diet allowed all subject's to be starting the intervention from the same position, excluding variation due to some subjects having no fruit intake prior to their nutrition consultation.

A prospective power calculation ensured a large enough sample size to give sufficient statistical power for *APOE4* subgroup analysis. Regarding the HDL-related SNPs, although it was a retrospective analysis, the SNPs chosen were carefully considered with regard to factors such as frequency of the alleles and their relationship to HDL metabolism. Thus, we used a hypothesis-driven approach reducing the possibility of a chance finding. In addition, by employing a screening phase using multivariate analysis we have excluded the possibility of a type 1 error due to repeated testing.

8.5 Limitations

An unavoidable limitation was that the use of fruit as the intervention product precludes the use of a double-blind study design. Due to the nature of the intervention, subjects cannot be blinded, and although it might be theoretically possible to blind the main researcher, it would be challengingly logistically. Although no washout period was needed for plasma lipids, given an interaction effect due to the sequence of intervention was observed for insulin, a washout period between treatments could have been considered for other cardiovascular risk markers.

After their nutrition consultation, 24-hr food records (versus repeated 3-day food records) were chosen to assess subjects' compliance to the fruit intervention and to assess the pre- and post-nutrition consultation dietary intakes of the group. However, the limitations of this method of dietary assessment may mean that a subject's usual intake is not accurately captured.

The unequal group sizes between *APOE4* allele and *APOE4* non-carriers and a greater degree of variance among non-*E4* carriers meant that 2-way ANOVA (considered a more robust statistical test) could not be used to examine *APOE* genotype and treatment interactions, as homogeneity of variance was not satisfied. To avoid this, subjects would need to be recruited according to *APOE* genotype to give equal numbers in each group.

We also did not have the benefit of having confirmatory biomarkers of compliance, such as plasma vitamins C and E. These vitamins were also mechanistic components of interest.

There are a number of limitations associated with the retrospective and explorative nature of two of the sub-studies. In the inflammatory status investigation, CRP and the inflammatory cytokines were not measured at baseline 1. St-Onge et al. (2009) note as a limitation of their own study the 'lack of measurement of other inflammatory markers'. In our study we included some other markers, but as they were only measured at baseline 2, we don't know what effect the healthy diet run-in period might have had on these markers. No differences between the low and medium inflammatory groups were seen for

any of the cytokines at baseline 2, therefore we could not use them to add support to our classification of subjects into inflammatory groups

Despite carefully choosing the HDL-C SNPs, when a retrospective power calculation was conducted it only gave us 70% power to observe an interaction effect on TC/HDL-C. There were trends of greater improvement in TC/HDL-C ratio and TG in *B1/B1* homozygotes, which was not observed in *B2* carriers; a lack of statistical power may explain the lack of observed effect.

As the study was only conducted in men, its generalisability is limited.

8.6 Future research recommendations

1. Further studies which investigate the specific effects of kiwifruit, independent of the effects of a healthy diet containing fruit, need to be conducted to confirm the findings of the primary objective.
2. Dose response studies should also be conducted to provide further validity for the results, i.e. show that effects on plasma lipids change with intake, and to establish what the optimal daily intake might be.
3. The study was conducted in a specific population group (hypercholesterolaemic men). Therefore, it will be important for future studies to test the effects on plasma lipids and inflammatory markers in other populations, including women, and individuals with metabolic syndrome, where the effects could be even more pronounced due to their worse dyslipidaemic profile.
4. Although the improvements were modest, investigating the effects of kiwifruit in combination with statins in dyslipidaemic individuals should be investigated as a means to reduce the residual risk not addressed by statins.
5. Likewise, in subjects who have a metabolically unhealthy profile, the effects of an 'anti-inflammatory diet' which contains kiwifruit could be investigated to see if responses to healthy diet patterns, such as the DASH diet, can be further improved.
6. Further studies will also be required to confirm the findings of our two explorative analyses. Firstly, the modulation of lipid response to the intervention by *CETP Taq1B* genotype, which resulted in the significant genotype x treatment interaction for the TG/HDL-C ratio. Secondly the modulation of plasma lipids and inflammatory markers by inflammatory status, as measured by CRP, which resulted in significant between inflammation group differences for TC/HDL-C, TG/HDL-C, and plasma IL-6.
7. The inhibition of NF-κβ activation by kiwifruit should be investigated as a possible mechanistic pathway for the effects observed in the inflammatory status sub-study.

8. Apolipoprotein C3 is thought to be associated with a more atherogenic lipoprotein profile and activation of NF-κβ. As part of investigations into possible mechanistic pathways, the secretion patterns of VLDL (whether apoC3 dominant versus apoE dominant) could be measured and whether there is a change in patterns in response to kiwifruit consumption investigated.
9. In relation to the *CETP Taq1B* genotype sub-study, future studies should measure CETP mass and activity, and the inhibitory effect of kiwifruit on CETP activity to elucidate mechanistic pathways.
10. CETP inhibitors have been shown to elevate HDL-C concentrations, but also have deleterious effects. When a routine diagnostic assay to test HDL functionality becomes readily available, this should be considered to ensure that, as well as increasing HDL-C concentration, HDL quality is maintained.
11. Whether the beneficial effects on plasma lipids and inflammatory markers are maintained with a longer term intervention should be investigated.
12. It would be useful to determine how many men continued to meet the recommended two fruit servings/day six or 12 months after completion of the intervention, to assess how successful the intervention was at changing a dietary habit.

In future studies advantage should be taken of the rapidly developing 'omics' technologies to advance our understanding of mechanistic pathways.

8.7 Conclusions

This study adds to the limited number of whole fruit studies which have investigated the effects of consuming the fruit on lipids, BP and inflammatory markers. Further, it was well-designed and suitably controlled, the study was adequately powered, and the study was of adequate duration.

The consumption of two green kiwifruit a day for 4 weeks had favourable effects on plasma HDL-C and the TC/HDL-C ratio compared to a healthy control diet. The analysis of the results by *APOE* and *CETP Taq1B* genotypes and inflammatory status has explained some of the inter-individual variability in responses that were observed, with the most significant improvements in HDL-C and TC/HDL-C ratio in subjects in the medium inflammatory group and TG/HDL-C ratio in *B1/B1* homozygotes of the *CETP Taq1B* genotype (more than 30% of the population). A significant decrease in TG was only observed in *APOE4* carriers (about 25% of the population). These findings add to the body of evidence on diet-gene interactions and diet-inflammatory status interactions. Although the improvements were modest, they could still be expected to translate into a reduction in CVD risk, with improvements in TC/HDL-C ratio translating into between an 8 to 15% reduction in risk, depending which inflammatory phenotype or genotype was carried. Additionally, with the current increased incidence of obesity, the kiwifruit intervention appeared to be particularly beneficial for subjects identified as having a more atherogenic lipoprotein profile, subjects with a moderate inflammatory status, *APOE4* carriers and *B1/B1* homozygotes of the *CETP Taq1B* genotype.

In many ways, as the first fruit intervention to show interactions with *APOE* and *CETP Taq1B* genotypes and inflammatory status, this study highlights issues around the complexity of inter-individual variability in response and has generated more questions than it has answered. The development of meaningful personalised/stratified dietary recommendations to reduce cardiovascular risk will require an understanding of the contribution of multiple interrelated behavioural, phenotypic and genetic factors which influence response to intervention.

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Appendix 1: Papers (published or submitted)

Papers (published or submitted as part of thesis)

Gammon, C. S., Kruger, R., Minihane, A. M., Conlon, C. A., von Hurst, P. R. & Stonehouse, W. (2013). Kiwifruit consumption favourably affects plasma lipids in a randomised controlled trial in hypercholesterolaemic men. British Journal of Nutrition, 109 (12), 2208–2218.

Gammon, C. S., Kruger, R., Conlon, C. A., von Hurst, P. R., Jones, B., & Stonehouse, W. (2013). Inflammatory status modulates plasma lipid and inflammatory marker responses to kiwifruit consumption in hypercholesterolaemic men. (In press Nutrition Metabolism and Cardiovascular Disease)

Gammon, C. S., Minihane, A. M., Kruger, R., Conlon, C. A., von Hurst, P. R., Jones, B., & Stonehouse, W. (2013). *TaqIB* polymorphism in the *CETP* gene influences lipid responses to consuming kiwifruit in hypercholesterolaemic men. (In press British Journal of Nutrition)

Gammon, C. S., Kruger, R., Brown, S. J., Conlon, C. A., von Hurst, P.R., & Stonehouse, W. (2013). Effects of kiwifruit consumption on blood pressure and markers of cardiovascular function in men with hypercholesterolaemia. (Submitted for publication with Nutrition Research)

<u>Other papers</u>

Stonehouse, W., Gammon, C. S., Beck, K. L., Conlon, C. A., von Hurst P. R., & Kruger, R (2013). Kiwifruit: our daily prescription for health (Review). Canadian Journal of Physiology and Pharmacology, 91 (6), 442-7.

Appendix 2: Conference presentations and abstracts

Conference presentations and abstracts

2013

Gammon CS, Minihane AM, Kruger R, Conlon CA, von Hurst PR, Jones B, Stonehouse, W (2013). *TaqIB* polymorphism in the *CETP* gene influences lipid responses to consuming kiwifruit in hypercholesterolaemic men.
Oral presentation at the Nutrition Society of New Zealand and Nutrition Society of Australia 2013 Joint Annual Scientific Meeting Program, Brisbane, Australia.

2012

Gammon CS, Kruger R, Conlon CA, von Hurst PR, Jones, B, Stonehouse, W (2012). Baseline inflammatory status modulates plasma lipid and inflammatory marker responses to kiwifruit consumption in hypercholesterolaemic men: a randomised controlled trial.
Oral presentation at the Auckland Nutrition Research Network student presentations.

Gammon CS, Kruger R, Conlon CA, von Hurst PR, Jones, B, Stonehouse, W (2012). Baseline inflammatory status modulates plasma lipid and inflammatory marker responses to kiwifruit consumption in hypercholesterolaemic men: a randomised controlled trial.
Oral presentation at the Annual Scientific Meeting of the Nutrition Society of New Zealand, Auckland.

Gammon CS, Kruger R, Minihane AM, Conlon CA, von Hurst PR, Stonehouse, W. (2012). Green kiwifruit: effects on plasma lipids and *APOE* interactions.
Poster presented at the International Society for the Study of Fatty Acids and Lipids (ISSFAL), Vancouver, Canada.

2011

Gammon CS, Kruger R, Minihane AM, Conlon CA, von Hurst PR, Stonehouse, W. (2011). Green kiwifruit: effects on plasma lipids and *APOE* interactions.
Oral presentation at the Auckland Nutrition Research Network student presentations.

Gammon CS, Kruger R, Minihane AM, Conlon CA, von Hurst PR, Stonehouse, W. (2011). Green kiwifruit: effects on plasma lipids and *APOE* interactions.
Poster presented at the Joint Annual Scientific Meeting of the Nutrition Society of New Zealand and the Nutrition Society of Australia, Queenstown, NZ.
Winner of Best Student Poster (NZ).

Gammon CS, Kruger R, Minihane AM, Conlon C A, von Hurst PR, Stonehouse, W. (2011). Green kiwifruit consumption favourably affects plasma lipids in hypercholesterolaemic men. In *Ann Nutr Metab,* 58 Supplement (3)**,** 130.
Poster presented at the 11th European Nutrition Conference Fens, Madrid Spain.

2010

Gammon CS (2010) Kiwifruit and Metabolic Health Study.
Oral presentation at the Auckland Nutrition Research Network student presentations.

Appendix 3 Contribution of Authors
(including statements of contribution to doctoral thesis containing publications)

Contributions of Authors

Chapter	Author	Contribution to paper
Chapter 4: Plasma lipids and *APOE* paper	Cheryl Gammon	Led the research; was involved in the study design, including development of all of the questionnaires; recruited participants; conducted the research; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.
	Rozanne Kruger	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Anne Marie Minihane	Provided significant advice regarding the analysis and interpretation of genotypes data; revised and approved the manuscript.
	Cathryn Conlon	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Pamela von Hurst	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Welma Stonehouse	Study leader; designed research; recruited participants; conducted research; assisted with statistical analysis; revised and approved the manuscript.
Chapter 5: Inflammatory status paper	Cheryl Gammon	Led the research; designed research; recruited participants; conducted the research; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.
	Rozanne Kruger	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Cathryn Conlon	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Pamela von Hurst	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Beatrix Jones	Advised regarding the statistical analysis of data; revised and approved the manuscript.
	Welma Stonehouse	Study leader; designed research; recruited participants; conducted research; revised and approved the manuscript.

Contributions of Authors

Chapter	Author	Contribution to paper
Chapter 6: HDL-genes paper	Cheryl Gammon	Led the research; designed research; recruited participants; conducted the research; performed DNA extraction and SNP determination; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.
	Anne Marie Minihane	Provided significant advice regarding which SNPs to investigate; and the analysis and interpretation of genotypes data; revised and approved the manuscript.
	Rozanne Kruger	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Cathryn Conlon	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Pamela von Hurst	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Beatrix Jones	Advised regarding the statistical analysis of data; revised and approved the manuscript.
	Welma Stonehouse	Study leader; designed research; recruited participants; conducted research; revised and approved the manuscript.
Chapter 7: Finometer paper	Cheryl Gammon	Led the research; designed research; recruited participants; conducted the research; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.
	Rozanne Kruger	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Stephen Brown	Advised on conducting the Finometer research; conducted the research; revised and approved the manuscript.
	Cathryn Conlon	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Pamela von Hurst	Designed research; recruited participants; conducted research; revised and approved the manuscript.
	Welma Stonehouse	Study leader; designed research; recruited participants; conducted research; revised and approved the manuscript.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Cheryl Gammon

Name/Title of Principal Supervisor: Welma Stonehouse

Name of Published Research Output and full reference:

Gammon, C. S., Kruger, R., Minihane, A. M., Conlon, C. A., Von Hurst, P. R. & Stonehouse, W. (2013). Kiwifruit consumption favourably affects plasma lipids in a randomised controlled trial in hypercholesterolaemic men. British Journal of Nutrition, 109 (12), 2208–2218.

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Led the research; was involved in the study design, including development of all of the questionnaires; recruited participants; conducted the research; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.

Cheryl Gammon — Digitally signed by Cheryl Gammon DN: cn=Cheryl Gammon, o=Massey University, ou=IFNHH, email=C.Gammon@massey.ac.nz, c=NZ Date: 2013.10.28 19:51:41 +13'00'

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STATEMENT OF CONTRIBUTION
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Name/Title of Principal Supervisor: Welma Stonehouse

Name of Published Research Output and full reference:

Gammon, C. S., Kruger, R., Conlon, C. A., von Hurst, P. R., Jones, B., & Stonehouse, W. (2013). Inflammatory status modulates plasma lipid and inflammatory marker responses to kiwifruit consumption in hypercholesterolaemic men. In press Nutrition Metabolism and Cardiovascular Disease

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Led the research; designed research; recruited participants; conducted the research; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.

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STATEMENT OF CONTRIBUTION
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Name of Candidate: Cheryl Gammon

Name/Title of Principal Supervisor: Welma Stonehouse

Name of Published Research Output and full reference:

Gammon, C. S., Minihane, A. M., Kruger, R., Conlon, C. A., von Hurst, P. R., Jones, B., & Stonehouse, W. (2013). TaqIB polymorphism in the CETP gene influences lipid responses to consuming kiwifruit in hypercholesterolaemic men. In press British Journal of Nutrition

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Led the research; designed research; recruited participants; conducted the research; performed DNA extraction and SNP determination; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.

Cheryl Gammon — Digitally signed by Cheryl Gammon DN: cn=Cheryl Gammon, o=Massey University, ou=IFNHH, email=C.Gammon@massey.ac.nz, c=NZ Date: 2013.10.28 20:01:34 +13'00'

Candidate's Signature — 28/10/13 — Date

Welma Stonehouse — Digitally signed by Welma Stonehouse DN: cn=Welma Stonehouse, o=CSIRO, ou=CAFHS, email=welma.stonehouse@csiro.au, c=AU Date: 2013.10.29 10:57:15 +10'30'

Principal Supervisor's signature — 29/10/13 — Date

GRS Version 3– 16 September 2011

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Cheryl Gammon

Name/Title of Principal Supervisor: Welma Stonehouse

Name of Published Research Output and full reference:

Gammon, C. S., Kruger, R., Brown, S. J., Conlon, C. A., Von Hurst, P. R. & Stonehouse, W. Effects of kiwifruit consumption on blood pressure and markers of cardiovascular function in men with hypercholesterolaemia. Submitted for publication in Nutrition Research.

In which Chapter is the Published Work: Chapter 7

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Led the research; designed research; recruited participants; conducted the research; analysed data and performed statistical analysis; interpreted the results; main author of manuscript.

Cheryl Gammon — Digitally signed by Cheryl Gammon DN: cn=Cheryl Gammon, o=Massey University, ou=IFNHH, email=C.Gammon@massey.ac.nz, c=NZ Date: 2013.10.28 19:54:01 +13'00'

Candidate's Signature — 28/10/13 — Date

Welma Stonehouse — Digitally signed by Welma Stonehouse DN: cn=Welma Stonehouse, o=CSIRO, ou=CAFHS, email=welma.stonehouse@csiro.au, c=AU Date: 2013.10.29 10:56:53 +10'30'

Principal Supervisor's signature — 29/10/13 — Date

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Appendix 4: Screening documents

a) Health screening questionnaire

b) Screening procedure and criteria

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Men's "Healthy Heart" Study ~ 2010

Health Screening Questionnaire

Thank you for your interest in our research project. To ensure that you fit the inclusion criteria of the study, we would appreciate it if you could answer the questions below. If you have any queries or concerns about the form, please feel free to contact Cheryl Gammon during working hours on 414 0800 X41183 or send an email to healthyhearts@massey.ac.nz

When you have completed this form, please email the form to Cheryl Gammon at healthyhearts@massey.ac.nz.

Name: _____

Gender (Please make a cross): Male ☐ Female ☐

Date of birth: _____

Daytime telephone number: _____

Email address: _____

****

Have you been diagnosed with a cholesterol related genetic disorder?

e.g. Familial hypercholesterolaemia

Yes □ No □

If yes, please specify condition

_____

Do any close family members (mother, father or sibling) have a high cholesterol concentration (>5.5mmol/L)?

Yes □ No □ Don't know □

If yes, please give details

_____

Have any close family members (mother, father or sibling) suffered from coronary heart disease or vascular disease?
e.g. heart attack, angina, stroke

Yes □ No □ Don't know □

If yes, please give details

_____

Do you smoke tobacco?

Yes □ No □

Do you have an allergy to kiwifruit or dairy products?

Yes □ No □

Have you ever been diagnosed with any of the following:

	Indicate yes or no
Cancer	
Heart condition	
Gut disorder that interferes with the digestion and absorption of your food	
Diabetes or high blood sugar levels	
Endocrine disease (hormone trouble)	
Thyroid disease (e.g. goiter, myxoedema)	
Kidney or renal function problems	
Disorders of the liver	
Blood borne diseases (e.g. Hepatitis B)	

If yes, provide more details please:

_____

_____

_____

Are you currently suffering from any other illness not listed above that could affect the fat and cholesterol processing in your body? (Please provide details)_____

_____

_____

Are you taking any prescription medications for your cholesterol?

e.g. simvastatin (Lipex®, Arrow-Simva), simvastatin/ezetimibe (Vytorin®), atorvastatin (Lipitor®), bezafibrate (Bezalip® Retard, Fibalip)

Yes □ No □

If yes, please specify which tablet, dosage and how long you have been taking them

Tablet	Dosage & frequency	Number of months/years

Are you taking any natural health products for your cholesterol?

e.g. Thompsons cholesterol manager, Niacin/Nicotinic acid tablets, Fish oil capsules

Yes □ No □

If yes, please specify which products and how long you have been taking them

Product	Dosage & frequency	Number of months/years

Do you use any sterol enriched spreads to lower cholesterol?

E.g. Flora Pro-Activ®, Meadowlea Logicol®

Yes □ No □

If yes, please specify which one, how much you use daily and how long you have used it

Product	Amount & frequency	Number of months/years

Do you use any fibre containing laxatives?

E.g. Metamucil, Mucilax, Normacol, Benefiber, Phloe

Yes □ No □

If yes, please specify which product and how long you have used it

Product	Dosage & frequency	Number of months/years

Are you taking other medication, including traditional or homeopathic medicine?

Yes □ No □

Please specify the condition, the medication, the dosage and the length of time taken in the table provided.

Condition	Medication	Dosage & Frequency	Number of months/years

Are you taking any form of supplements, including tablets or drinks?

Yes □ No □

If yes, please specify the name, brand, dosage and length of time taken for the supplements you are taking in the table provided.

Supplement	Brand	Dosage & Frequency	Number of months/years

If you are unsuccessful in becoming a participant in this study, would you be interested in hearing about future research projects within the Institute of Food, Nutrition and Human Health

Yes □ No □

Screening Procedure:

1. Subject responds to advertisement and contacts us by email or phone. Interest from the website will be flagged via an email redirected to us.

2. Obtain all contact details (email + address + contact telephone) and preferred method of contact and enter into recruitment Access spreadsheet.
   Ask if they would like the information/screening pack sent via email or mail.

3. Email/mail information sheet and screening questionnaire (information/screening pack). Subject completes and emails/mails back. Print out and file.

Post (information/screening pack)	**Email** (information/screening pack)
Acknowledgement letter	Acknowledgement email
Information sheet	Information sheet
Screening questionnaire	Screening questionnaire
Consent form	
Reply paid envelope	

4. Check screening questionnaire against in-/exclusion criteria – see screening criteria list. Olivia to forward any screening questionnaires with 'queries' onto Welma or Cheryl.

5. If eligible – email or post a Diagnostic MedLab form. Include cover letter with instructions of where to go, etc. If ineligible - phone or email.

6. Diagnostic MedLab results will be posted c/- Welma

   a. If LDL-C >3 mmol/L and TG <3 mmol/L subject is eligible to participate in study. Notify them by email/phone call and make appointment for 1st visit (between 11 May – 4 June).
      Enter details on MHHS database.

   b. If LDL-C <3 mmol/L and TG <3 mmol/L – email/phone call that they are not eligible.

   c. If TG >3 mmol/L – check that subject did a fasting blood test. If test was fasting - not eligible - review by Dr John Birkbeck (medical doctor) and referral.

   d. If FH subjects suspected to have FH – not eligible - review by John Birkbeck and referral – this can be done by email with John.

   Confirmation of first appointment email, with attached information sheet for first visit.

Screening criteria:

Inclusion criteria

- Male
- Over 21
- Non-smoker

Exclusion criteria

- Using lipid lowering medication
- Using supplements or functional foods that will affect lipid concentrations (e.g. sterol enriched spreads)
- Chronic disease e.g. CHD, diabetes, cancer
- Allergy to kiwifruit
- Familial hypercholesterolaemia
    - Subjects with an LDL-C of >8.5 mmol/L
    - Subjects with an LDL-C of 6.5-8.4 mmol/L in combination with a family history (first degree relative with CHD or LDL-C >95th percentile (5.5 mmol/L in NZ) or personal history of CHD.

Exclude - if answer yes to any of the following questions in the screening questionnaire

Have you been diagnosed with a cholesterol related genetic disorder?
e.g. Familial hypercholesterolaemia

Do you smoke tobacco?

Do you have an allergy to kiwifruit?

Are you taking any prescription medications for your cholesterol?

Query if 'yes' to any of the following questions in the screening questionnaire

Do any close family members (mother, father or sibling) have a high cholesterol concentration (>5.5 mmol/L)?

Have any close family members (mother, father or sibling) suffered from coronary heart disease or vascular disease?

Have you ever been diagnosed with any of the following**:**

Cancer
Heart condition
Gut disorder that interferes with the digestion and absorption of your food
Diabetes or high blood sugar levels
Endocrine disease (hormone trouble)
Thyroid disease (e.g. goiter, myxoedema)
Kidney or renal function problems
Disorders of the liver
Blood borne diseases (e.g. Hepatitis B)

Are you currently suffering from any other illness not listed above that could affect the fat and cholesterol processing in your body?

Are you taking any natural health products for your cholesterol?

Do you use any sterol enriched spreads to lower cholesterol?

Do you use any fibre containing laxatives?

Are you taking other medication, including traditional or homeopathic medicine?

Are you taking any form of supplements, including tablets or drinks?

Appendix 5: *APOE* fact sheet

Originally written by Anne Marie Minihane

Modified by Cheryl Gammon and Welma Stonehouse

The *APOE* gene fact sheet

Genes contain the information to make all the proteins our body needs. Humans have around 25, 000 genes, 99.9% of which are exactly the same in all people. There is much interest in the genes that differ between people and how they impact our health. At Massey University, we are interested in how these variations affect people's response to foods. For this reason, in some of our studies we ask you to provide a blood sample that we use to determine if you have variations of a particular gene.

As a participant in the Men's "Healthy Heart" Study, your blood sample will be tested for variations in *APOE*, and this factsheet is designed to explain what apoE does in the body and what impact variations in the *APOE* gene may have on your health.

What is apoE, and what does it do?
The primary role of apoE, as a component of lipoproteins (carriers of fats in the body), is to assist with the transport and metabolism of fats, such as cholesterol and triglycerides, around the body. ApoE has recently been found to also be involved in processes other than fat metabolism, such as regulation of the immune system, and cognitive processes within the brain.

Does everyone have the same *APOE* gene?
The *APOE* code may differ from person to person. Three common versions of the gene exist, namely *E2*, *E3* and *E4*, which differ in 2 protein building blocks (amino acids) at position 112 and 158 in the protein. These differences alter the activity of the protein and which lipoproteins it is associated with.

Everyone inherits two *APOE* genes, one from each parent. Therefore it is possible for you to have one of the following six combinations, *E2*/*E2* (1%), *E2*/*E3* (11%), *E2*/*E4* (2%), *E3*/*E3* (61%), *E3*/*E4* (23%), *E4*/*E4* (2%), with the figures in brackets indicating approximately what proportion of the Caucasian population have that combination.

How does this affect me as an individual, and what can I do about it?
Research has shown that different gene combinations for *APOE* can influence an individual's predisposition towards future disease risk, in particular heart disease, memory loss, dementia and Alzheimer's disease.

Although the evidence is not fully consistent, it has been estimated that having the *E3*/*E4* or *E4*/*E4* combination is associated with on average a 30-40% increased risk of heart disease relative to the common *E3*/*E3* genotype. There is some evidence that having an *E2*/*E2* or *E2*/*E3* genotype may be associated with a lower heart disease risk, especially in women. The negative impact of the *E3*/*E4* or *E4*/*E4* combination appears to be most evident in smokers.

APOE genotype appears to have an even greater impact on risk of age-related memory loss, dementia and Alzheimer's disease (the most common form of dementia). Having an *E3*/*E4* or *E2*/*E4* combination will increase your risk of Alzheimer's disease and having an *E4*/*E4* genotype is associated with an even greater risk. In addition to increasing risk these genotype combinations also lower the average age of onset, with an average age of onset 15-20 years earlier in *E4*/*E4* relative to *E3*/*E3*. In contrast, having the *E2*/*E3* or *E2*/*E2* combination may reduce your risk of Alzheimer's disease.

The increased risk of disease in those who are *E4* carriers is thought to be due to elevated cholesterol levels, lower antioxidant levels and inflammation.

There is accumulating evidence to indicate that individuals who are *E4* carriers may be sensitive to total dietary fat, saturated fat and cholesterol, and would gain particular cholesterol-lowering benefit through restriction of these dietary components. Furthermore, *E4* individuals are sensitive to the impact of smoking on heart disease risk and stopping of smoking would significantly reduce disease risk in this genotype group. Furthermore as an *E4* genotype is associated with lower antioxidant levels, meeting the recommendations of five (80 g) portions of fruit and vegetables per day is especially important in this genotype group. Recent evidence suggest that flavonoid-rich plant derived foods such as teas, red wine (in moderation), berries, cocoa and citrus fruit may be effective in reducing age-related loss of memory and cognitive function.

If you have an *E4*/*E4* genotype you may wish to contact your GP. There is some evidence to suggest that statins (cholesterol lowering medication) may be a useful strategy to reduce both heart disease risk and Alzheimer's risk in *E4*/*E4* individuals. However, this decision will be taken by your GP, who holds full records of your medical history.

Keeping physically active and not being overweight are also going to help reduce everyone's risk of heart disease. The National Heart Foundation website http://www.nhf.org.nz has lots of tips on how to look after your heart.

Implications for health and life insurance
It is important that you understand before requesting your results that insurers may request that knowledge of any genetic test results be disclosed to the insurer for the purposes of risk classification. In the future, this may have implications for your insurance cover.

Why are researchers interested in this gene?
We are interested to further determine if individuals of different *APOE* genotype respond differently to dietary change. In the future, rather than providing everyone with general dietary advice, it may be that a more personalised approach is taken, providing advice to suit an individual's genetic make-up.

Sources of further information
It must be emphasised that genotyping is a relatively new area which is still in the research stage, with information in this area far from complete. If you would like to read more on this topic, you may find the following web-site of the National Health Committee useful, http://www.nhc.health.govt.nz

If you have any questions or would like further information please contact
Cheryl Gammon during working hours on (09) 414 0800 x 41183 or send an email to healthyhearts@massey.ac.nz.

Welma Stonehouse
Associate Professor in Human Nutrition
Institute of Food, Nutrition and Human Health
Massey University Auckland
w.stonehouse@massey.ac.nz

Appendix 6: Weekly compliance diary

Men's "Healthy Heart" Study ~ 2010

Your Diary

Name: _____

Study ID: _____

Dear Participant,

We very much appreciate your participation in this research project. By committing time and effort to this project we assume that it is just as important to you as it is to us that this project is a success. For this project to be successful we need reliable data which will also allow us to publish the findings of the study in a scientific journal.

In order to do this it is extremely important that you consume the kiwifruit that we will provide to you according to the guidelines given, and continue to consume a healthy diet, as directed in your nutrition consultation.

During the study, do not make major changes to your habitual daily routine for the duration of the study. Any changes can affect the results of the study and therefore the reliability of the results. We realise that changes are sometimes inevitable, for example illness.

Therefore, we would appreciate it if you could supply information about your kiwifruit intake when on the kiwifruit intervention and indicate any changes from your daily routine or with regard to the intake of the kiwifruit by completing this compliance diary. Please complete one sheet for each week of the study. It will only take you about 5-10 minutes to complete each week.

If you have any further questions please feel free to contact
Cheryl Gammon
Tel: 414 0800 ext 41183
or Welma Stonehouse
Tel: 414 0800 ext 41207

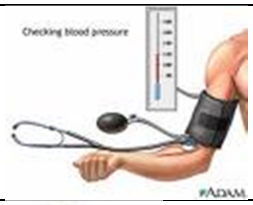
Email: healthyhearts@massey.ac.nz

Thank you again for taking part in this study.
Kind regards,
The Men's "Healthy Heart" Research Team.

Important dates to remember

Activity	Date
1st visit to the Massey Human Nutrition Research Unit	Completed
2nd visit to the Massey Human Nutrition Research Unit Healthy Diet Nutrition Consultation Follow healthy diet for 4 weeks 	Completed
3rd visit to the Massey Human Nutrition Research Unit     	Week 1: Start of intervention
Fill in your compliance diary every week. 	Weeks 1 to 8
Eat 2 kiwifruit a day for the 4 weeks specified 	Week ____ to ____
4th visit to the Massey Human Nutrition Research Unit     	Week 4:
5th visit to the Massey Human Nutrition Research Unit     	Week 8

Symbol Key:

	Questionnaire		Blood pressure
	Blood samples		Body composition
	24 hour food record		Healthy diet
	Diary		Kiwifruit

Remember not to consume kiwifruit unless we have provided it to you

Consuming your kiwifruit

On the 4 specified weeks please eat 2 kiwifruit a day

You can either

- Peel and remove the skin with a knife. Then once peeled, the kiwifruit can be sliced, diced, pureed or eaten whole.
- Cut the fruit in half with a sharp knife and scoop the contents out with the plastic spoon provided or a teaspoon.

Ways in which to use your kiwifruit

- Consume as a healthy snack during the day
- Slice or chop onto your cereal or porridge at breakfast
- Slice, chop or puree and have with low-fat yoghurt as a dessert with dinner

As part of the Men's "Healthy Heart" Study Dietary Guidelines you should eat at least 2 servings of fruit each day.
The 2 kiwifruit will replace 2 of your normal fruit servings each day for these 4 weeks.

Storing your kiwifruit

Unripe kiwifruit are best stored at room temperature, away from direct sunlight or exposure to heat.

A ripe kiwifruit will yield gently to your fingers when you press it, and can be eaten immediately or stored in the refrigerator for a further 2-3 weeks.

It normally takes between 3-5 days for a firm kiwifruit to ripen although this process can be hastened if placed in a paper bag with an apple or banana.

If you forget to consume your 2 kiwifruit for a day, please make a note in your weekly diary.

WEEKLY COMPLIANCE DIARY

Week 1 **Date:** _____

If you are currently on the kiwifruit intervention, did you consume your kiwifruit every day this week?

O Yes O No O Not applicable

If no, please provide more details: _____

_____

Were you ill this week?

O Yes O No

If yes, what was the nature of your illness?_____

_____

Did you consume any medication for the illness?

O Yes O No

If yes, please provide details of the medication used: _____

_____

Did you do anything different this week from your normal routine with regard to physical activity? e.g. went tramping, skiing, joined a gym, took part in a competition

O Yes O No

If yes, please provide details of the change to your physical activity: ___

_____

Have there been any changes in your normal daily routine this week, e.g. eating habits, sleeping habits, alcohol consumption, use of other nutritional supplements, etc?

O Yes O No

If yes, please provide more detail _____

_____

Do you have anything else you would like to report? _____

_____

Appendix 7: Dietary material

a) Healthy diet brochure
b) Fridge reminder

a) Healthy diet brochure

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Men's "Healthy Heart" Study ~ 2010

Dietary Guidelines

These are based on The Heart Foundation's 9 steps to eating for a healthy heart and the Ministry of Health Guidelines "Eating for Healthy Adult New Zealanders"

1. Enjoy three meals each day. Select dishes that include plant foods and fish. Limit dairy fat and meat fat, and avoid deep-fried foods

Tips: *Plan your meals for a healthy day*
Don't skip meals
Include healthy snacks

Meal	Example 1	Example 2
Breakfast ~ aim to have: •1-2 servings of whole grain cereals and/or bread •1 serving of low-fat dairy food •At least 1 serving of fruit or vegetables	½ cup rolled oats or muesli with 200-250ml low fat or trim milk and ½ cup fresh berries, OR ½ cup canned peaches (drained)	2 slices toasted wholegrain bread with 30g cheddar cheese 1 dsp peanut butter 1 kiwifruit
Lunch ~ aim to have •1-2 servings of whole grain bread, rice, pasta or noodles •1 serving of lean meat or alternative •1 or more servings of fruit or vegetables	2 slices wholegrain bread with 2 slices lean beef or ham ½ cup mixed salad (lettuce, tomato, cucumber, capsicum) 1 apple	¾ c cooked risoni pasta with 1 small can of salmon in spring water ½ cup mixed salad (spinach leaves, tomato, cucumber, capsicum) 1 banana
Dinner ~ aim to have •1 serving of grain or starchy vegetables •1 serving of lean meat or alternative •2 or more servings of different-coloured vegetables	1 cup rice, couscous or pasta 1 medium fillet of fish (grilled) ½ cup cooked mixed vegetables 1 piece cooked pumpkin ½ cup broccoli	1 medium potato or kumara with 1 grilled chicken leg ½ cup cooked peas or beans ½ medium carrot (cooked or raw) 4 grilled mushrooms
Snacks ~ use one or more: • 1 serving of fruit or vegetables • 1 serving of cereal • 1 serving of low-fat dairy • 1 serving of nuts • or a combination	1 pottle low fat yoghurt 1 banana or 1 dsp mixed nuts with 2 tbsp raisins	2 wholegrain crackers with 1 tbsp mashed avocado or ½ cup celery and/or carrot sticks

2. Choose fruits and/or vegetables at every meal & most snacks

Eat a 'rainbow' of colours (**red, green, blue, white, yellow/orange**) every day! **(At least 3 servings of vegetables and 2 servings of fruit)**

- **Different colours** in fruits and vegetables signal the presence of different vitamins and antioxidants.
- E.g. dark green leafy vegetables are rich in **vitamin A**, orange fruits are a good source of **vitamin C** & dark blue berries are packed with **anti-oxidants**

Tip: *Add another vegetable to a pasta dish / casserole eg. spinach or frozen peas*

3. 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 (At least 6 servings per day)

Choose a variety of grain products. Look for these words early in the ingredients list: barley, brown rice, granary, kibbled (grain), millet, mixed grain, multi-grain, oats, rye, seeded, stoneground (grain), whole grain, wholewheat

Bread look for

- more fibre: > 5g per 100g is good, > 7g per 100g is better
- less sodium: < 400mg per 100g is good, < 300mg per 100g is better

Cereal look for

- Sugar: < 15g per 100g, cereal with dried fruit—< 25g per 100g
- Sodium: < 400mg per 100g
- Fibre: > 5 grams per 100g

Tip: *Choose whole grain crackers over rice and water crackers for more fibre*

4. Include a small serving of fish, legumes (e.g. peas, beans, soy products), lean meat or skinned poultry, at 1 or 2 meals each day

- Have 1 to 2 servings of fish per week (eg.1 main, 1 lunch).
- Try to include some oily fish such as tuna, kahawai, salmon, dory, or mussels
- Choose lean meat (eg. pork, beef, lamb). Limit mutton to 1x week.
- Buy 125g to 150g raw meat per person.
- Always trim visible fat and remove skin from chicken.
- Use meat alternatives 2-3 times a week (chickpeas, lentils, tofu, eggs, dairy)
- Limit eggs to about 3 per week (3-4 including those used in cooking)

Tip: *Try adding a can of legumes such as kidney beans, lentils or chickpeas to your favourite recipe to reduce the amount of meat*

5. Choose low fat milk or products, or replace with soy products (at least 2 servings per day)

- Use **low-fat or trim milk**—aim for 1.5% total fat or less
- Choose **reduced fat yoghurts** – aim for less than 1% total fat
- Hard and semi-soft cheeses can be included up to 4 times weekly in small amounts (30 g portions)
- Cheeses vary widely in total fat content and there are now several lower fat varieties

Type of cheese	% fat	% saturated fat
Cheddar; colby	35	22
Blue vein	30	20
Edam	26	17
Reduced-fat cheddar	24	16
Camembert	22	14
Feta	20	13
Parmesan; block	20	14
Mozzarella	18	12
Light cream cheese	13	8
Ricotta	11	7
Low-fat cheese slices	7	5
Cottage cheese	4	2
Low-fat cottage cheese	<1	<1

Tip: *Use a low-fat cheese such as cottage cheese/ricotta when cooking; and add a small amount of a strong-flavoured cheese (e.g. parmesan/cheddar) to boost flavour*

6. Use small amounts of oil, margarine, nuts or seeds

Choose products made from sunflower, soya bean, olive, canola, linseed, safflower. Avoid coconut based products.

Liquid oils, unsaturated margarines and spreads		Nuts, seeds	
Have 3 to 5 healthy servings per day	• 1 tsp soft table margarine or oil • 2 tsp light margarine (50-60% fat) • 2 tsp mayonnaise or vinaigrette (50-60% fat) • 3 tbsp reduced-fat mayonnaise or dressing (10% fat or less) • 1 tbsp avocado	Eat regularly up to 30 g/day	•1 dsp nuts or pumpkin seeds •1 dsp peanut butter •1 tbsp sunflower or sesame seeds

Tip: *Dilute standard mayonnaise with low-fat yoghurt or milk*

7. Drink plenty of fluids each day, particularly water. Limit alcohol and sugar-sweetened drinks

- Drink 6 to 8 **non-alcoholic drinks** per day—1 glass of water or trim / low-fat milk (250ml), 1 glass of 'diet' soft drink (200ml), 1 cup of tea, coffee or cocoa
- Limit **alcoholic intake.** Men drink no more than 3 drinks on any given day; women drink no more than 2 drinks on any given day — 1 bottle of beer (330ml), 1 glass of wine (100ml), 1 measure of spirits (30ml), 1 glass fortified wine (60ml)
- Limit the consumption of fruit juice, cordial and fizzy drinks because of their high sugar content

8. When cooking or preparing meals, snacks or drinks, use only small amounts of fat, oil, sugar and salt. Choose ready-prepared foods low in these ingredients

Grill, bake, steam, boil or stir-fry your food

- Skim the fat off stews and gravies
- Use minimal salt in cooking and do not add salt to meals at the table
- Limit high salt foods to less than 4 servings per day:

30g lean ham/pastrami	50g canned products eg. sweetcorn
1 tsp vegemite/marmite	1 tsp soy sauce
1/2 c canned/packet soup	20 to 30g cheese

- Check labels for fat (total and saturated fat), sugar and salt content

Tip: *Use oil sprays for frying / browning*

9. Mostly avoid or rarely include butter, deep-fried and fatty foods, and only occasionally choose sweet bakery products

When eating out or having takeaways, order healthier options—

- Pizza – choose thin crust, vegetable-rich toppings, and minimal cheese instead of thick crust, extra cheese, lots of meat toppings
- Pasta – choose tomato-based sauces over cream-based sauces
- Grilled or roasted meat and fish over fried or deep-fried options
- Fruit-based desserts, gelato or sorbet over creamy puddings, pastry-based desserts and ice cream
- Try a chicken or ham salad, but ask for a low-fat dressing
- Choose yoghurt rather than cream with cake or dessert

Tip: *Limit fast foods to no more than once a week.*
Make these foods the exception rather that the rule.

Remember it is important that the changes you make to your diet are maintained for the duration of the study.

Phone or email us if you have any questions

Email: healthyhearts@massey.ac.nz
Phone: (09) 424 0800 ext 41183

We would also like to acknowledge the Healthy Food Guide. We would encourage you to visit their website for healthy recipe ideas and useful shopping tips
www.healthyfoodguide.co.nz

b) Fridge reminder

Did you achieve your healthy heart eating guidelines today?

- 3 Meals
- 5 portions of fruits / vegetables
- Whole grain or high fibre breads / cereals
- 1 or 2 portions fish, legumes, lean meat or skinned poultry
- 2 portions low fat milk & dairy products
- Using small amounts of margarine, oil, nuts or seeds
- Plenty of fluids – limit sugary drinks & alcohol
- Using small amounts of fats, oils, sugar and salt in cooking
- Rarely use butter, deep-fried / fatty foods, & bakery products

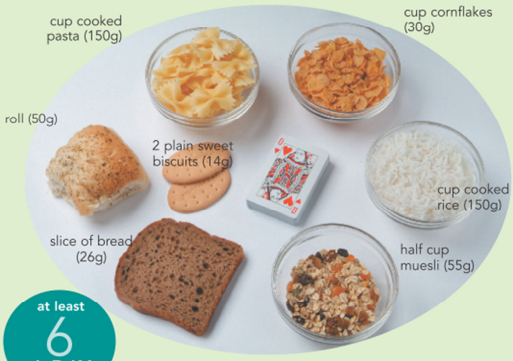
WHAT DOES A "SERVING" LOOK LIKE?

We see a lot of information about "servings" in nutrition advice and on food packaging. If you've ever wondered what a "serving" of a particular food looks like, here's a guide to help you. These are the recommendations from the Ministry of Health for the main food groups. The playing cards are there to give you an idea of the size of each item.

FRUIT

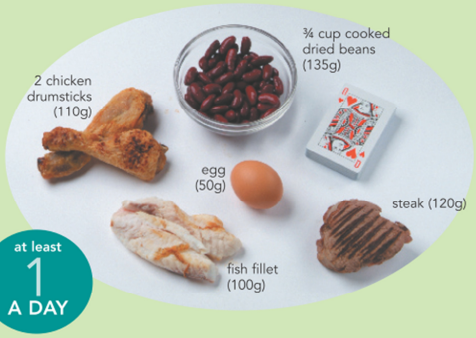
at least 2 A DAY

BREAD, CEREAL, RICE, PASTA, NOODLES



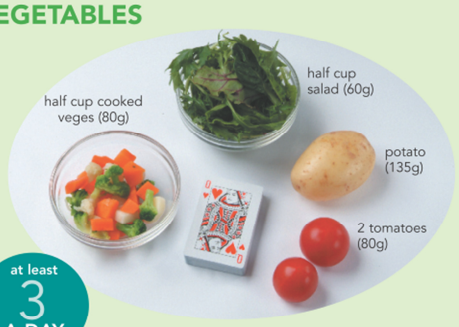
at least 6 A DAY

MEAT, POULTRY, FISH, EGGS, LEGUMES



at least 1 A DAY

VEGETABLES



at least 3 A DAY

MILK, YOGHURT, CHEESE

at least 2 A DAY

OILS AND FATS

WATER AND OTHER FLUIDS

6-8 A DAY

Published in *Healthy Food Guide* January 2006.
Healthy Food Guide is a monthly magazine available at supermarkets and bookstores for just $5.50. To subscribe, go to www.healthyfood.co.nz

NEW ZEALAND healthyfood GUIDE
IDEAS FOR REAL LIFE

Appendix 8: Teleform and on-line questionnaires

a) Demographics questionnaire
b) Fruit frequency questionnaire
c) Tolerance questionnaire
d) Recent physical activity questionnaire (RPAQ)

a) Teleform demographics questionnaire

Subject Number 1 7 0 0 | | |

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Men's "Healthy Heart" Study

General Information Questionnaire

Please complete the following questionnaire, a researcher is available to help if needed.

Please, Shade Circles Like This--> ●
Not Like This--> ⊗ ✓

Are you (please shade)

- O Male
- O Female

Which ethnic group do you belong to? Shade whichever applies to you (you may shade more than one box)

- O New Zealand European
- O Maori
- O Samoan
- O Cook Island Maori
- O Tongan
- O Niuean
- O Chinese
- O Indian
- O Other

Other...Please state which ethnicity_____

Which country were you born in?

- O New Zealand
- O Australia
- O England
- O Scotland
- O China (People's Republic of)
- O South Africa
- O Samoa
- O Cook Islands
- O Other

Other...Please state which country_____

If you live in New Zealand but were not born here, when did you first arrive to live in New Zealand?

☐☐ / ☐☐☐☐

month (eg. 02=February / Year (eg. 2000)

Please turn over

7142631475

Page 1 of 2

Subject Number 1700

What is your first language?

- ○ English
- ○ Other

Other...Please state_____

What is your highest secondary school qualification? (Single response)

- ○ None
- ○ NZ School Certificate in one or more subjects, or National Certificate Level 1
- ○ NZ Sixth Form Certificate in one or more subjects, or National Certificate L 2
- ○ NZ University Entrance before 1986 in one or more subjects
- ○ NZ Higher School Certificate, or Higher Leaving Certificate
- ○ University Entrance qualification from NZ University Bursary
- ○ NZ A or B Bursary, Scholarship, or National Certificate Level 3

- ○ Overseas secondary qualification
- ○ Other

Other NZ secondary school qualification.(Specify)_____

Apart from secondary school qualifications, do you have another qualification? Don't count incomplete qualifications or qualifications that take less than 3 months of full-time study to get. *(Single response)* Yes - *Please name it. Record the highest qualification (e.g. BSc, PhD, etc.)*

- ○ Bachelors degree, e.g. BA. BSc. LLB
- ○ Bachelors degree with honours
- ○ Masters degree, e.g. MA, MSc
- ○ PhD

- ○ Diploma (not Post Graduate)
- ○ Diploma - Post Graduate
- ○ Trade or technical certificate which took more than 3 months full time study
- ○ Professional qualifications like ACA, teachers, nurses

- ○ No qualification beyond secondary school
- ○ Other

Other...(Specify)_____

What is your current occupation (in the job you work the most hours in)?

- ○ Administrator/Manager
- ○ Professionals
- ○ Technicians & Associate Professionals
- ○ Clerks
- ○ Service & Sales Workers
- ○ Agriculture and Fishing
- ○ Trade Workers
- ○ Plant & Machinery Operators
- ○ Labourers/Unskilled Work
- ○ Armed Forces
- ○ Other

Other...(Specify)_____

b) On-line fruit frequency questionnaire

Fruit Frequency Questionnaire

1. Men's "Healthy Heart" Study

We would like you to tell us about the amount of fruit you have eaten this last week

*** 1. Please enter your subject identification number:**

*** 2. How many servings of fruit did you have on each day last week?**

	Number of servings
Monday	
Tuesday	
Wednesday	
Thursday	
Friday	
Saturday	
Sunday	

If you had more than 5 servings on any day, please note day and how many here

3. For each of the fruits (fresh) please indicate how many servings you consumed over the last week? (serving size in brackets)

	1 serving per week	2 servings per week	3-4 servings per week	5-6 servings per week	1 serving per day	2 servings per day	3+ servings per day
Apple (1 medium)	◯	◯	◯	◯	◯	◯	◯
Banana (1 medium)	◯	◯	◯	◯	◯	◯	◯
Pear (1 medium)	◯	◯	◯	◯	◯	◯	◯
Orange (1 medium)	◯	◯	◯	◯	◯	◯	◯
Grapefruit (1 medium)	◯	◯	◯	◯	◯	◯	◯
Mandarin (1 medium)	◯	◯	◯	◯	◯	◯	◯
Tangelo (1 medium)	◯	◯	◯	◯	◯	◯	◯
Lemon (1 medium)	◯	◯	◯	◯	◯	◯	◯
Kiwifruit (1 medium)	◯	◯	◯	◯	◯	◯	◯
Tamarillos (1 medium)	◯	◯	◯	◯	◯	◯	◯
Pineapple (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Grapes (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Berries e.g blueberries (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Other	◯	◯	◯	◯	◯	◯	◯

Other (please specify type)

Fruit Frequency Questionnaire

*** 4. Did you consume any canned, stewed or frozen fruit last week? (If you answer NO go to question 6)**

◯ Yes

◯ No

5. How many servings of stewed, canned or frozen fruit did you consume last week? (serving size in brackets)

	1 serving per week	2 servings per week	3-4 servings per week	5-6 servings per week	1 serving per day	2 servings per day	3+ servings per day
Peaches (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Pears (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Apricots (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Plums (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Apple (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Rhubarb (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Berries (1/2 cup)	◯	◯	◯	◯	◯	◯	◯
Other	◯	◯	◯	◯	◯	◯	◯

Other (please specify type)

*** 6. Did you consume any dried fruit last week? (if you answer NO go to question 8)**

◯ Yes

◯ No

7. How many servings of dried fruit did you consume last week? (serving size in brackets)

	1/2 serving per week	1 serving per week	2 servings per week	3-4 servings per week	5-6 servings per week	1 serving per day	2 servings per day	3+ servings per day
Apricots (2 halves)	◯	◯	◯	◯	◯	◯	◯	◯
Raisins (2 Tbsp)	◯	◯	◯	◯	◯	◯	◯	◯
Dates (3)	◯	◯	◯	◯	◯	◯	◯	◯
Prunes (3)	◯	◯	◯	◯	◯	◯	◯	◯
Sultanas (2 Tbsp)	◯	◯	◯	◯	◯	◯	◯	◯
Cranberries (2 Tbsp)	◯	◯	◯	◯	◯	◯	◯	◯
Pears (2 halves)	◯	◯	◯	◯	◯	◯	◯	◯
Peaches (2 halves)	◯	◯	◯	◯	◯	◯	◯	◯
Other	◯	◯	◯	◯	◯	◯	◯	◯

Other (please specify type)

Fruit Frequency Questionnaire

*** 8. Did you consume any glasses of fruit juice last week? (if you answer NO go to question 10)**

- ◯ Yes
- ◯ No

9. How many servings of fruit juice did you consume last week? (serving size in brackets)

	1/2 serving per week	1 serving per week	2 servings per week	3-4 servings per week	5-6 servings per week	1 serving per day	2 servings per day	3+ servings per day
Orange (250ml)	◯	◯	◯	◯	◯	◯	◯	◯
Grapefruit (250ml)	◯	◯	◯	◯	◯	◯	◯	◯
Apple (250ml)	◯	◯	◯	◯	◯	◯	◯	◯
Other (250ml)	◯	◯	◯	◯	◯	◯	◯	◯

Other (please specify type)

*** 10. Finally, did you find this survey easy to complete**

- ◯ Yes
- ◯ No
- ◯ Not sure

Any other comments

c) On-line tolerance questionnaire

Fruit Frequency Questionnaire 3

2. Kiwifruit intervention subjects

Finally, here a few quick questions about how you found eating 2 Green Kiwifruit a day.

*** 1. Please rate how easy you found eating 2 Green Kiwifruit a day**

	Very difficult	Difficult	Neutral	Easy	Very Easy
At the beginning of the 4 weeks	◯	◯	◯	◯	◯
At the end of the 4 weeks	◯	◯	◯	◯	◯
Overall	◯	◯	◯	◯	◯

Any further comments

*** 2. When did you generally consume your 2 Green Kiwifruit?**

	Kiwifruit 1	Kiwifruit 2
Weekdays		
Weekend		

*** 3. Did you experience any benefits from eating 2 Green Kiwifruit a day?**

◯ Yes

◯ No

If Yes, please specify

*** 4. Did you experience any unwanted side-effects from eating 2 Green Kiwifruit a day?**

◯ Yes

◯ No

If Yes, please specify

Fruit Frequency Questionnaire 3

*** 5. Which of the following would influence your consumption of Green Kiwifruit. Rate the following:**

	A real turn off	A slight turn off	Neutral	A slight attraction	A real attraction
Cost	◯	◯	◯	◯	◯
Health benefits related to cholesterol	◯	◯	◯	◯	◯
Preference of household members	◯	◯	◯	◯	◯
Taste	◯	◯	◯	◯	◯
Appearance	◯	◯	◯	◯	◯
Convenience	◯	◯	◯	◯	◯
Time to ripen	◯	◯	◯	◯	◯
Peeling	◯	◯	◯	◯	◯
Other health benefits	◯	◯	◯	◯	◯

*** 6. For the 12 months prior to this trial, would you say your consumption of Kiwifruit has changed?**

	Gone down significantly	Gone down slightly	About the same as ever	Gone up slightly	Gone up significantly
Quantity consumed	◯	◯	◯	◯	◯

*** 7. How strongly do you feel that Kiwifruit helps you to... (where 1 = Not at all and 5 = Very strongly)**

	1	2	3	4	5
meet your daily need for fibre	◯	◯	◯	◯	◯
feel 'alive'	◯	◯	◯	◯	◯
improve your digestion	◯	◯	◯	◯	◯
relieve bloating	◯	◯	◯	◯	◯
consume the vitamins and minerals you need	◯	◯	◯	◯	◯
meet your daily recommended healthy fruit intake	◯	◯	◯	◯	◯

*** 8. After completing this study, how likely is it that you will continue to consume Green Kiwifruit as part of a healthy diet to lower your choletserol?**

	0	1	2	3	4	5	6	7	8	9	10
Where 0 = Not at all likely and 10 = Absolutely likely	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯	◯

d) On-line recent physical activity questionnaire

Recent Physical Activity Questionnaire MHHS - RPAQ

1. Introduction

This questionnaire is designed to find out about your physical activity in your everyday life in the past 4 weeks.

The questionnaire is divided into 3 sections.

Please answer every question.

Section A asks about your physical activity patterns in and around the house.
Section B is about travel to work and your activity at work.
Section C asks about recreations that you may have engaged in during the last 4 weeks.

Your answer will be treated as strictly confidential and will be used only for medical research

*** 1. Please enter you ID number.**

*** 2. Please enter your date of birth.**

	DD	MM	YYYY
Date/Month/Year	/	/	

Recent Physical Activity Questionnaire MHHS - RPAQ

2. Section A - Home Activities

Getting about

*** 1. Which form of transport have you MOST OFTEN used in the last 4 weeks APART from your journey to and from work?**

- ◯ Car/motor vehicle
- ◯ Walk
- ◯ Public transport
- ◯ Cycle

TV, DVD or Video Viewing

*** 2. On average over the last 4 weeks how many hours of TV, DVD or video have you watched per day?**

	None	Less than 1 hour per day	1 to 2 hours per day	2 to 3 hours per day	3 to 4 hours per day	More than 4 hours per day
On a weekday before 6pm	◯	◯	◯	◯	◯	◯
On a weekday after 6pm	◯	◯	◯	◯	◯	◯
On a weekend day before 6pm	◯	◯	◯	◯	◯	◯
On a weekend day after 6pm	◯	◯	◯	◯	◯	◯

Computer use at HOME but not at work (e.g. internet, email, Playstation, Xbox, Gameboy, etc)

*** 3. On average over the last 4 weeks how many hours of home computer use have you had per day?**

	None	Less than 1 hour per day	1 to 2 hours per day	2 to 3 hours per day	3 to 4 hours per day	More than 4 hours per day
On a weekday before 6pm	◯	◯	◯	◯	◯	◯
On a weekday after 6pm	◯	◯	◯	◯	◯	◯
On a weekend day before 6pm	◯	◯	◯	◯	◯	◯
On a weekend day after 6pm	◯	◯	◯	◯	◯	◯

Stair Climbing at home

*** 4. On average over the last 4 weeks how many times have you climbed up a flight of stairs (approx. 10 steps) each day at home?**

	None	1 to 5 times per day	6 to 10 times per day	11 to 15 times per day	16 to 20 times per day	More than 20 times per day
On a weekday	◯	◯	◯	◯	◯	◯
On a weekend day	◯	◯	◯	◯	◯	◯

Recent Physical Activity Questionnaire MHHS - RPAQ

3. Section B - Activity at Work

Please answer this section to describe if you have been in paid employment at any time during the last 4 weeks or you have done regular, organised voluntary work.
If you answer 'no' - you will move onto the recreation section.

*** 1. Have you been in employment during the past 4 weeks?**

- ◯ Yes
- ◯ No

Recent Physical Activity Questionnaire MHHS - RPAQ

4. Section B Activity at Work continued:

*** 1. During the last 4 weeks how many TOTAL hours work did you do per week? (excluding travel)**

4 weeks ago
3 weeks ago
2 weeks ago
1 week ago

Type of work
We would like to know the type and amount of physical activity involved in your work.

*** 2. Please chose the option that BEST corresponds with your occupation(s) in the last 4 weeks from the following four possiblities:**

◯ Sedentary occupation: you spend most of your time sitting (such as in an office)

◯ Standing occupation: you spend most of your time standing or walking. However, your work does not require intense physical effort. (e.g. shop assistant, hairdresser, guard)

◯ Manual work: this involves some physical effort including handling heavy objects and use of tools (e.g. plumber, electrician, carpenter)

◯ Heavy manual work: this implies very vigorous physical activity including handling of very heavy objects (e.g. dock worker, miner, bricklayer, construction worker)

Travel to and from work in the last 4 weeks

*** 3. What is the approximate distance from your home to your work in kilometres? (e.g. 10.5)**

*** 4. How many times a week did you travel from home to your main work? (Count outward journeys only)**

*** 5. How did you normally travel to work?**

	Always	Usually	Occassionally	Never or rarely
By car/motor vehicle	◯	◯	◯	◯
By works or public transport	◯	◯	◯	◯
By bicycle	◯	◯	◯	◯
Walking	◯	◯	◯	◯

6. What is the postcode for your main place of work during the last 4 weeks? If you don't know your post code please go to question 8

Post code

Recent Physical Activity Questionnaire MHHS - RPAQ

7. Work address

Work address

*** 8. What is the postcode of your home address?**

Recent Physical Activity Questionnaire MHHS - RPAQ

5. Section C - Recreation

The following questions ask about how you spent your leisure time

Please indicate how often you did each activity on average over the last 4 weeks

Please indicate the average length of time that you spent doing the activity on each occasion

1. Please indicate the number of times you did each activity in the past 4 weeks and the average time spent each time (note: not time per week).

For example: if you go jogging for an hour and a half twice a week, you would click 'twice a week' for the number of times, then '1' under average time hours and '30'under minutes.
Please answer for EVERY activity - choose 'none' for the number of times, if you DID NOT do a activity.

	number of times	average time hours (per session)	minutes
Swimming-competitive			
Swimming leisurely			
Backpacking or mountain climbing			
Walking for pleasure (not as a means of transport)			
Racing or rough terrain cycling			
Cycling for pleasure (not as a means of transport)			
Mowing the lawn			
Watering the lawn or garden			
Digging, shovelling or chopping wood			
Weeding or pruning			
DIY e.g. carpentry, home or car maintenance			
High impact aerobics or step aerobics			
Other types of aerobics			
Exercise with weights			
Conditioning exercises e.g. using a bike or rowing machine			
Floor exercises e.g. stretching, bending, keep			

Recent Physical Activity Questionnaire MHHS - RPAQ

fit or yoga			
Dancing e.g. ballroom or disco			
Competitive running			
Jogging			
Bowling- indoor, lawn or 10 pin			
Tennis or badminton			
Squash			
Table tennis			
Golf			
Football, rugby or hockey			
Cricket			
Rowing			
Netball, volleyball or basketball			
Fishing			
Horse-riding			
Snooker, billiards or darts			
Musical instrument playing or singing			
Ice skating			
Sailing, wind-surfing or boating			
Martial arts, boxing or wrestling			

PLEASE call the researcher before clicking 'done' to finish the questionnaire.

Appendix 9: Real-time SNP genotyping assay procedure

UEA FOH – Department of Nutrition – Prof. Anne Marie Minihane's Research Group

Real-time PCR SNP genotyping assay – standard operating procedure

Authorised by: Professor Anne Marie Minihane

Date: September 2011

Materials

Blood sample collection

Potassium-EDTA vacutainer® Blood Collection Tubes, (Becton, Dickinson and Company))

DNA isolation

The QIAamp DNA Mini Blood kit are obtained from QIAGEN and can be purchased in small (50) and large (250) size. The QIAamp Mini spin columns and the Buffer AW1, Buffer AW2 and Buffer AL should be stored at room temperature, 15-25°C. The lyophilised QIAGEN protease should be stored at room temperature (for long term storage (>12 months) store dry at 4°C). The reconstituted QIAGEN Protease should be stores at 2-8°C up to two months. However, multiple aliquots of QIAGEN Protease should be made for storage at -20°C, as freezing and thawing should be avoided.

QIAamp DNA Mini Blood Kit (50), small size (50), Cat. No. 51104 (QIAGEN)
Ethanol (96-100%)
Water, molecular biology grade, RNase, DNase and Protease free Fisher BioReagents - Catalogue. No: BPE2819-1 (Fisher Scientific)
Universal or 15 ml centrifuge tube
Eppendorf tubes, sterile 1.5 mL/2.0 mL) (communal consumable, shelf)
Sterile pipettes 3 ml
Pipette tips with filter plugs P1000, P100, P10 (shelf)
Disposable gloves (nitrile) (communal consumable, shelf)

Real-time PCR SNP genotyping assay

The SNP genotyping kits are obtained from Applied Biosystems and ordered directly from this company or indirectly from the company Fisher Scientific. Please make sure to state clearly the Assay ID and dbSNP ID when ordering the SNP genotyping kits. Each kit comes with a CD containing an Assay Information File (AIF), which contains e.g. the primer sequences, and a vial of the primer and probes for the analysis of 2,500 samples (7500 Fast RT-PCR system with reaction volume 10 µL) or 1000 samples (7500 RT-PCR system with reaction volume 25 µL) for a medium size kit. (On the shelf and custom made kits can be purchased in 750 (small), 2,500 (medium) or 6,000 (large) reactions size, when using 7500 Fast RT-PCR system with reaction volume 10 µL). **Important:** If you plan to use the Applied Biosystems 7500 RT-PCR machine please see additional notes at *Laboratory equipment* section. The catalogue and lot number are written on the label on the primer/probe vials. Store the SNP genotyping kits at -20°C protected from light. The TaqMan Genotyping Mastermix (Applied Biosystems 4371355) must be purchased separately and should be stored at 4°C.

MicroAmp Optical Adhesive Film Applied Biosystems - Catalogue. No: 4311971 (Applied Biosystems)
MicroAmp Fast 96-well reaction plate, 0.1 mL Applied Biosystems - Catalogue. No: 4346907 (Applied Biosystems)
TaqMan® Genotyping Master Mix, 1-Pack (1 x 10 mL) Applied Biosystems - Catalogue. No: 4371355 (Applied Biosystems)
Water, molecular biology grade, RNase, DNase and Protease free Fisher BioReagents - Catalogue. No: BPE2819-1 (Fisher Scientific)
TE buffer, pH 8.0 Applied Biosystems - Catalogue. No: AM9849 (Applied Biosystems)
RNaseZap® RNase Decontamination Solution - Catalogue No: AM9782 (Applied Biosystems)
Ethanol (70%) (communal consumable, shelf)
Eppendorf tubes, sterile 1.5 mL/2.0 mL) (communal consumable, shelf)
Pipette tips with filter plugs (shelf)

Disposable gloves (nitrile) (communal consumable, shelf)

Laboratory equipment

Microcentrifuge

Centrifuge with adapter for 96-well plates

Vortexer

Pipettors, positive-displacement and preferably also a multichannel pipette

Water bath or heating block at 56°C

NanoDrop ND-1000 Spectrophotometer in BMRC general lab floor 02.

Applied Biosystems 7500 Fast RT-PCR machine (Edwards instrument, BIO) in BMRC lab floor 02.

Alternative one can use the RT-PCR machine Applied Biosystems 7500 RT-PCR machine (Graham Riley instrument, BIO) in BMRC lab floor 02. Please note that you will need different plates (MicroAmp 96-well Optical Reaction Plate Applied Biosystems - Catalogue. No: N8010560 (Applied Biosystems)) and need to use different protocol.

Preparation before starting Real-time PCR SNP genotyping assay

Dilute SNP Genotyping Assays.

- (Wear lab coat and nitrile gloves at all times.)
- Dilute 40× (or 80×) SNP Genotyping Assay to a 20×working stock with 1 × TE buffer. **Note:** The 1× TE buffer should be 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and made using DNase-free, sterile-filtered water.

  The 40× SNP Genotyping Assay has been aliquoted into tubes containing 25 µl 40× SNP Genotyping Assay (enough for one 96-well plate). Prior to use of this SNP Assay, the 40× SNP Genotyping Assay (25 µl) has to be diluted to a 20×working stock with 25 µl 1× TE buffer. The 50 µl 20× working stock of the SNP Genotyping Assay is enough for one 96-well plate (0.5 µl SNP Assay used per reaction, so in total 100 reactions).

- Vortex, then centrifuge the mixture.
- Cover the tube containing the 20×SNP Assay working stock with aluminium foil protected from light and store them on ice. Long term storage of the SNP Genotyping Assay 20× stock should be at –20 °C (range –15 to –25 °C). **Note**: SNP Genotyping Assay should preferably not have more than 3 freeze-thaw cycles and definitely not more than 10 freeze-thaw cycles. SNP genotyping Assays are stable for 3-5 years when properly stored and repeated freeze-thaw cycles are prevented.

Standard operating procedure for real-time PCR SNP genotyping assay including DNA isolation from whole blood

Genomic DNA isolation

Genomic DNA will be isolated from whole blood or buffy coat (leukocyte-enriched fraction of whole blood) using the QIAamp DNA Mini Blood Kit. (Note: QIAamp Mini spin columns will isolate both RNA and DNA from the sample.) The average total DNA yield is 6 µg DNA from 200 µl of whole human blood and up to 50 µg DNA from 200 µl of buffy coat. If you are dealing with previous isolated DNA samples proceed with Assessment of DNA quality and quantity.

Before starting make sure the following preparations are in place.

- Equilibrate samples to room temperature.
- Heat a water bath or heating block to 56°C for use in step 8 of *DNA isolation from blood or buffy coat*.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 15&16 of *DNA isolation from blood or buffy coat*.
- Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the following instructions.
- If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.

If you are using whole blood samples for DNA extraction, please proceed to *Genomic DNA isolation (step 5)*, although it is recommended to use buffy coat as this will yield 5-10 times more DNA than equivalent volume of whole blood.

Preparation of buffy coat.

1. Collect 10 ml of whole blood into a potassium-EDTA vacutainer.
2. Centrifuge at 3000rpm (1700 x g) for 10 minutes at room temperature. After centrifugation 3 different fractions are distinguishable, namely plasma (upper clear layer), buffy coat containing concentrated leukocytes (intermediate layer) and concentrated erythrocytes (bottom layer).
3. Remove plasma and pipette 1 mL of the intermediate white layer (buffy coat) using a 3 mL transfer pipette into an appropriately labelled 1.5 mL eppendorf tube.
4. Spin at 3000rpm (1700 x g) for 5 minute at room temperature.

Genomic DNA isolation

Follow the procedure as described in *QIAamp DNA Mini and Blood Mini Handbook*, in protocol 'DNA purification from Blood or Body Fluids (Spin Protocol)' on page 27-29, provided with the kit. (www.qiagen.com/literature/render.aspx?id=200373)

5. Pipette 20µl **QIAGEN Protease** (or Proteinase K) into the bottom of a 1.5 ml eppendorf tube.
6. Dispense 200µl of buffy coat or whole blood (sample) into the eppendorf tube.

   Note: As a result of defrosting in transit, the buffy coat samples were very viscous. On day 1 the 1st 20 samples, were difficult to pipette and gave low yields. On day 2 the protocol was modified – samples were briefly pulse-vortexed and then spun, before pipetting 100µl of buffy coat into the eppendorf tube and diluting with 100µl PBS (phosphate buffered saline).

7. Add 200µl **Buffer AL** to the sample. Mix by pulse-vortexing for 15 seconds.
8. Incubate at 56°C for 10 minutes.
9. Briefly pulse centrifuge (10s) the 1.5 ml eppendorf tube to remove drops from the inside of the lid.
10. Add 200µl **ethanol** (96-100%) to the sample and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge (10 s) the 1.5 ml eppendorf tube.
11. Carefully apply the mixture to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim, close the cap, and centrifuge at 6000 x g (8000rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.
12. Carefully open the QIAamp spin column and add 500µl **Buffer AW1** without wetting the rim. Close the cap and centrifuge at 6000 x g (8000rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.
13. Carefully open the QIAamp spin column and add 500µl **Buffer AW2** without wetting the rim. Close the cap and centrifuge at full speed 20,000 x g (14,000 rpm) for 3 minutes.

14. Place the QIAamp spin column in a clean 2 ml collection tube and discard the tube with the filtrate. Centrifuge at full speed 20,000 x g (14,000 rpm) for 1 minute.
15. Place the QIAamp spin column in a clean 1.5 ml eppendorf tube and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 200µl **Buffer AE**. Incubate at room temperature for 7 minutes, and then centrifuge at 6000 x g (8000 rpm) for 1 minute.
16. Using the same 1.5 ml eppendorf tube, carefully open the QIAamp spin column and add 200µl of fresh **Buffer AE**. Incubate at room temperature for 7 minutes, and then centrifuge at 6000 x g (8000rpm) for 1 minute.

Assessment of DNA quality and quantity

The concentration and the quality of the DNA samples will be checked using the NanoDrop ND-1000 Spectrophotometer.

The NanoDrop ND-1000 Spectrophotometer recommends a sample volume of 1.5-2.0 µl and can make accurate measurements of dsDNA up to concentration of 3700 ng/µl.

1. Start NanoDrop Software and select the 'Nucleic Acid' application module and select 'DNA-50' for double-stranded DNA (dsDNA) or 'ssDNA-33' for single-stranded DNA samples.
2. Clean the pedestals with MilliQ water and clean tissue (wipe away all fluid).
3. Make a Blank measurement, pipet 1.5 µl MilliQ water on the pedestal and click on Blank.
4. Measure your DNA samples, pipet 1.5 µl of well-homogenised sample on pedestal and click on Measure. (It is recommended to repeat a Blank measurement between every 10 samples.)

   The DNA samples are considered 'pure' with 260/280 ratio of >1.8 and 260/230 ratio of 2.2 (acceptable range 1.8-2.2). (A low 260/280 ratio may indicate presence if protein, phenol or other contaminants with absorbance at 280 nm. A low 260/230 ratio indicate the contamination of carbohydrates, peptides, phenols, and aromatic compounds, which absorb light at the

230nm wavelength.) The DNA concentration of the samples is based on absorbance at 260 nm and given in ng/µl.

Note: Due to the variability in DNA concentrations, samples were diluted to make all 5ng/µl in 50µl

Real-time PCR SNP genotyping assay

Important! The SNP Genotyping Assays have been aliquoted and stored as 25 µl aliquots of 40X SNP Genotyping Assays at -20°C. Before starting make sure that these aliquots have been diluted into 20× SNP Genotyping Assay working stock.

Preparation of the PCR plate

1. (Wear lab coat and nitrile gloves at all times.) Spray the work bench with RNAzap or 70% ethanol and wipe down with clean white towel paper. Check pipettes and spray with ethanol as necessary.
2. Defrost Master Mix (2 x pre-aliquoted 250 µl aliquots), primer/probes and DNA samples, placing them on ice and covering with tinfoil to protect from light.
3. Prepare the stock mixture of Master Mix and primer/probes in a sterile Eppendorf as follows: 10 µl reactions – 5.0 µl Master Mix, 0.5 µl Primer/probes. Multiply volumes by number of reactions (i.e. samples/no template controls (NTCs)) plus 3 (to compensate for reagent transfer loss).

   Important! The Primer/probes (20X working stock of SNP Genotyping Assay) needs to be prepared from a 40X aliquot using 1X TE buffer. 25 µl Aliquots of 40X SNP Genotyping Assay (stored at -20°C) need to be diluted to 20X solution by adding 25 µl 1X TE buffer, giving rise to 50 µl Primer/probes (20X working stock of SNP Genotyping Assay) that is enough for 100 (10µl) reactions (one RT-PCR 96-well plate). Any remaining 20X working stock of SNP Genotyping Assay can be frozen and stored at –20 °C (up to 3-5 years) for use in a next experiment, however more than 3 freeze-thaw cycles should be avoided.

Component	For a Fast 96-well plate using Wet DNA method (10 µL reaction volume)	For a 93 sample volume
TaqMan Genotyping Master Mix (2X)	5.0 µL	465 µL
Primer/probe (20X working stock of SNP Genotyping Assay)	0.5 µL	46.5 µL
RNase and DNase free water	0.5 µL	46.5 µL
Total PCR reaction volume	6 µL	
DNA sample or DNase free water (NTC)	4 µL	
Total Volume per Well	10 µL	

NB. 93 (sample volume) = 85 samples + 3 negative controls + 5 extras

Note: Gently swirl the bottle of TaqMan Genotyping Master Mix (2x) before use, to ensure it is well mixed. Pipette the required volumes in sterile Eppendorf, cap the tube and invert several times to mix and perform a short spin in the bench centrifuge to eliminate any air bubbles from the reaction mix. Place on ice. Any remaining stock can be frozen and stored for up to a year and pooled if necessary with freshly prepared stock.

4. Place the 96-well reaction plate (MicroAmp Fast 96-well reaction plate, Applied Biosystems 4346907) onto a chilled solid holder. Label plate with marker pen. Pipette 6.0 µL of stock reaction mix into each well in the 96-well reaction plate using a sterile pipette tip for each component, mixing well. Pipette 4.0 µL of DNA sample (containing 20 ng purified genomic DNA) into indicated well (prepare a word template with sample numbers to use as a guide) and 4.0 µL of RNase/DNase free water for the NTC. It is recommended to have 3 NTCs on each SNP genotyping reaction plate. **Important**! Be sure that no well to well cross-contamination occurs during pipetting.
5. Inspect all wells for uniformity of volume, and make note of any wells which do not appear to contain the proper volume.
6. Place a plastic disposable cover (MicroAmp Optical Adhesive Film, Applied Biosystems 4311971) onto the plate and use paddle to ensure that it is firmly pressed down on the plate. Transfer to a clean black plate holder and

vortex the plate to mix the wells and centrifuge the plate briefly at 1,000 rpm for 1-2 min to spin down the contents and eliminate any air bubbles. Transfer back onto chilled holder or keep the reaction plate on ice until loading in the 7500 Fast Real-Time PCR system.

Operating the software on the RT-PCR machine and performing the Pre-Read Run.

- Log on onto the computer attached to Real-Time PCR machine (Applied Biosystems 7500 fast RT-PCR machine (Edwards instrument, BIO)) in BMRC lab floor 02. Click on the "7500 Software v2.0.5" icon to start software, login by selecting User Name: GUEST and clicking OK and switch on the PCR machine with the button at the front of the machine.
- On the main menu click on Design Wizard icon and the New Experiment Wizard will take you through each stage of defining and setting up the experiment. Provide here details on Experiment Name, User Name and (optional) comments. Change the default Experiment Name to e.g. *CETP Genotyping Assay 'your name' 'today's date'*. Change User Name to *'your name' AMM lab*. Optional you can provide Additional Details, for example *SNP ID's*. Select the instrument you are using by selecting '7500 Fast (96 wells)'.* Select 'Genotyping' for the type of experiment you are setting up. Click on Next.
- After finishing the 'Experiment Properties', you will next define the 'Methods and Materials'. Check the following default settings are selected: 'TaqMan® Reagents'; 'Wet DNA (gDNA or cDNA)'; 'Standard (~2 hours to complete a run)'. **Important:** The TaqMan SNP Genotyping Assays are always run using Standard Run, as they are not compatible with the fast run option! Finally, ensure that the boxes for 'Pre-PCR Read', 'Amplification' and 'Post-PCR Read' are all ticked. Click Next.
- In the Set-up SNP Assays menu, you will select the SNP Assay(s) you will use in the experiment. Indicate how many SNP assay you will use in this experiment, for example if you perform an *APOE* genotyping experiment using both SNP assays rs7412 and rs423958, type '2' in the box. Select 'Yes (Select SNP Assay from Library), this requires that the Assay Information File (AIF) of the SNP Assay you are using is imported

into the SNP Assay Library. Click subsequently on ‘Select SNP Assay(s) from Library’ and select ‘C____904973_10’. (In this screen you can also import the SNP Assay from AIF.) Select the second SNP Assay ‘C___3084793_20’ in the same way. Click Next.

- In the Set Up: Samples and Replicates menu, you will define your samples, replicates and controls. Type in the corresponding boxes the number of samples and replicates you use in this experiment, e.g. 85 samples in singleton. Optional enter the Sample Name. If you want to run all previously selected SNP assays (e.g. SNP assays rs7412 and rs423958) on all samples, select ‘All Sample/SNP Assay Reactions’. If you want to run specific SNP Assays on specific samples select ‘Specific Sample/SNP Assay Reactions’. (**Note:** In the View plate Layout, you can add the assay colour to samples by clicking on ‘Show in wells’ and selecting ‘SNP Assay Colour’.) Select the number of negative controls, it is recommended to have at least 3 NTC on each SNP genotyping plate, and (optional) select the number of positive controls and specify the controls genotype. (In case you do not use any positive control enter ‘zero’.) Click Next. **Note:** In the Design Wizard menu you are not able to modify the plate layout manually, however in the analysis phase of your experiment you will be able to modify the plate setup to the one you used allowing correct analysis of experimental plate. However, you can manually set up the reaction plate setup if you use the Advanced Setup menu option (for details see Appendix I). e.g. move controls to bottom right
- In the Set Up Run Method you will define the run method of your experiment. Change the Reaction Volume per Well to 10 µL. And Click on ‘Open Run Method’ and select ‘TaqMan SNP Genotyping Assay_Pre-Read-Amplification-Post-Read_Run Method’. The Run Method details are as follows:

  Pre-PCR Read (Holding Stage): 1 min at 60 °C;

  Holding Stage: 10 min at 95°C;

  Cycling Stage: 40 cycles, 15 sec at 95°C and 1 min at 60°C;

  Post-PCR Read (Holding Stage): 1 min at 60 °C.

Click Finish Designing Experiment.

In the newly opened screen click on Save Experiment and save your experiment as *CETP Genotyping Assay 'your name' 'today's date',* to retain the name you assigned when you created the plate document. The file will close and you can now run your experiment, see next section 'Running the PCR experiment'

Running the PCR experiment

- Load the reaction plate into the thermal cycler (7500 Fast system), make sure the 'notched' A1 position is in the top-left side of the instrument tray. Then click on icon 'Run Experiment' in main menu and in the Open dialog box, navigate to the *experiments* folder and to the *Kenna* folder. Open the your previously saved experiment , e.g. CETP genotyping assay Cheryl

  Check the plate layout and when correct click on Start Run. If plate layout is not corresponding to your plate, click on Setup > Plate Setup in Experiment Menu on left side screen and adjust plate layout before returning to Run menu and starting the run.

  Note: Alternatively you can do a Quick Run with no plate setup information, using the 'Quick Start' icon underneath Run. You will then provide the details of the plate setup after the run, but before analysis of SNP Assay Experiment.

Analysis of the SNP Assay Experiment

- Immediately after a run, the 7500 software automatically analyses the data using default analysis settings (including 'Assign Calls Automatically' option) and displays the Allelic Discrimination Plot screen.

  Alternatively you can (re-)analyse your experiment at a later time. In that case open the 7500 Software v2.0.5 and click in the Main Menu on icon 'Analyse Experiment' and in the Open dialog box, navigate to the *experiments* folder and to the *Kenna* folder. Open the desired previously saved and run experiment, e.g. CETP genotyping assay Cheryl

- The 7500 Software plots the results of the SNP Genotyping Assay Run on an Allelic Discrimination scatter plot of Allele 1 versus Allele 2. Each well of the 96-well reaction plate is represented with a 'dot' on the plot. The clustering of the points can vary across the X-axis, Y-axis and Diagonal axis, please see legend below.

Symbol	**Are grouped along the...**	**The genotypes of the samples are…**
● (red)	X-axis of the plot	Homozygous for Allele 1 of the selected SNP assay.
● (blue)	Y-axis of the plot	Homozygous for Allele 2 of the selected SNP assay.
● (green)	Midway between the homozygote clusters	Heterozygous for both alleles of the selected SNP assay (Allele 1 and Allele 2).
■ (black)	Bottom-left corner of the plot	Negative controls.
✕ (black)	Anywhere on plot	Undetermined.

Select the Allelic Discrimination Plot. (If you are using pre-designed SNP Assays and the AIF are imported, the 7500 software uses the Assign Calls Automatically setting to automatically assign the genotype to the samples.) Highlight all of the wells which have no sample in them and/or wells you want to exclude from analysis, 'right-mouse' click on well and click Omit, the well will now have a red crossed box in the upper left, and click Reanalyse. (Wells that have generated a QC flag (orange triangle (▲)) and are omitted by the QC flag settings have a red open box displayed in corner of well.) Check to controls by Click-Drag a box around each cluster to select associated wells in plate layout and verify if the expected wells are selected, e.g. when selecting cluster bottom-left only NTC should be selected. If some of the samples have not been called or have a generated QC flag, check the Amplification Plot and the quality on the QC Summary. In addition, the NTC should be as close to zero as possible, if not, you may want to take out the NTC as a greater signal could indicate contamination of the NTC.

The Amplification Plots which display the amplification of all samples in the selected wells are consulted for correct baseline and threshold

values and to locate outliers. The ΔRn vs Cycle plot shows the difference in normalised fluorescence of reporter between Pre-PCR Read and Post-PCR Read (ΔRn) as function of cycle number. The Rn vs Cycle plot displays the fluorescence of reporter normalised to fluorescence of passive reference (Rn) against cycle number. The C_T vs Well plot displays the C_T against the well position and is used to locate outliers. (The Amplification Plot and Multicomponent Plot will be missing, if a run is performed without Amplification Phase.)

Additional option: Click on the Results section and select the Report tab. The report tab displays the results in table form, with the allele calls listed in the Call column as the name of the detectors you specified for the markers.

- When you are happy with the results, save the data and export the results to Excel.

- Dispose of the PCR plate by placing in the biohazard bag.

Interpretation of the data

- The software will automatically assign Allele 1 (VIC probe), Allele 2 (FAM probe) or both to the samples. (You can assign call manually by clicking on analysis settings and deselecting Assign Calls Automatically option by unticking 'Autocaller Enabled'

- Use the CD which comes with the SNP genotyping kit, which contains the AIF, to check the primer sequences and whether they are designed from the forward or reverse sequence (this is very important if the SNP is in the promoter region of a protein).

  For example, for FABP2, the SNP is A54T, the primers were designed using the reverse sequence and so Ala = AGC and Thr = AGT (if you need to translate this amino acid change you will need to consult a codon table and translate). Allele 1 is C = Ala (A) and Allele 2 is T = Thr (T). The forward sequence is:

  AAAACAACTTCAATGTTTCGAAAAG[C/T]GCTTGATTCTTTGACTGTGAA TTTA

  Therefore GCGCT – Alanine polymorphism at codon 54

  GCACT – Threonine polymorphism at codon 54

Trouble shooting

- See Manual *'Allelic Discrimination Getting Started Guide for 7300/7500/7500 Fast Systems' (2010),* Applied Biosystems. (Part Number 4347822 Rev. E 06/2010)

Safety information/Risk phrases

See relevant CoSSH forms - All persons undertaking this protocol must review relevant CoSSH forms before initial undertaking of protocol outlined above.

Procedure	**Hazard**	**Control**
Handling the QIAamp DNA Mini Blood Kit (Buffers)	Irritant to skin and eyes. Harmful when swallowed	Wear appropriate protective lab coat, gloves and safety glasses.
Handling the QIAamp DNA Mini Blood Kit (QIAGEN protease)	Irritant to skin, eyes and respiratory tract	Wear appropriate protective lab coat, gloves and safety glasses. Avoid breathing vapours, mist or gas.
Handling the Taqman Genotyping Master Mix	Irritant to skin, eyes and respiratory tract	Wear appropriate protective lab coat, gloves and safety glasses. Avoid breathing vapours, mist or gas.
Handling the SNP genotyping kits (primers and probes).	Irritant to skin, eyes and respiratory tract Toxic to the unborn child	Wear appropriate protective lab coat, gloves and safety glasses. Avoid breathing vapours, mist or gas. Ensure female members of staff and students are made aware of this potential hazard.

References

QIAamp® DNA Mini and Blood Mini Handbook, Third edition (April 2010), QIAGEN

NanoDrop 1000 Spectrophotometer User's Manual v3.8 (2010, Thermo Fisher Scientific Inc.

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