The Effect of Oligomeric Procyanidins on Endothelial Function and Cholesterol Homeostasis

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Declaration

I confirm that this is my own work and the uses of all material from other sources have been properly and fully acknowledged.

Gurvinder Kaur Rull
Abstract
Oligomeric procyanidins (OPC) are naturally occurring dietary substances, which studies suggest can protect against cardiovascular disease. To explore this further a double-blind randomised controlled crossover trial, EPICURE (Evaluation of high procyanidin intervention with dark chocolate on underlying age-related elements of cardiovascular risk) examined the effects of high procyanidin dark chocolate (HPDC) versus low procyanidin dark chocolate (LPDC) on various cardiovascular parameters in subjects with early hypertension. This was complemented by in vitro studies of OPC on cultured bovine aortic endothelial cells (BAEC) to determine the effect on cellular cholesterol levels.

HPDC reduced 24 h blood pressure (-2/0.6 mmHg) and heart rate (-3.3 bpm), but only the latter reached statistical significance (p < 0.001). Augmentation pressure, augmentation index, plasma lipid levels, apolipoproteins, HDL subclasses, and hsCRP were not different amongst the groups. Retrospective analysis suggests that the study was underpowered and there were only 21 subjects with completed data due to technical issues. Future clinical studies should be designed with sufficient statistical power to detect changes in vascular function. In addition, compliance should be checked, technical errors detected earlier and FMD rather than PWA should be used to measure endothelial vascular function.

OPC in vitro increased expression of the oxysterol cholesterol 25-hydroxylase (peaking at 1 h; p < 0.001) which suppressed the mRNA levels of HMGCR as previously reported (at 6 h; p < 0.01). In addition the mRNA for ABCG1 was increased (at 24 h p < 0.01), which has not been described to date in the literature. This implicates that OPC favour overall reduced cellular cholesterol via increased efflux and reduced synthesis. But when BAEC were loaded with LDL-C, OPC reduced both cell media and solubilised cell extract cholesterol levels, the former being greater, suggesting no enhancement in cholesterol efflux. Also there was poor reproducibility of the ABCG1 mRNA expression which was likely to be due to variable transcription of the reference gene. Despite this the in vitro results support a potential role for OPC in reducing cellular cholesterol levels and future studies should employ radioactive methods to measure cholesterol efflux.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>25-OHC</td>
<td>25-hydroxycholesterol</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette G1</td>
</tr>
<tr>
<td>ABCG4</td>
<td>ATP-binding cassette G4</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl coenzyme A:cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AI</td>
<td>Augmentation index</td>
</tr>
<tr>
<td>AP4</td>
<td>Apple procyanidin tetramer</td>
</tr>
<tr>
<td>AP3</td>
<td>Apple procyanidin trimer</td>
</tr>
<tr>
<td>AP5</td>
<td>Apple procyanidin pentamer</td>
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<td>Apo AI</td>
<td>Apolipoprotein AI</td>
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<td>Apolipoprotein J</td>
</tr>
<tr>
<td>Apo L</td>
<td>Apolipoprotein L</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine aortic endothelial cells</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl ester</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transport protein</td>
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<td>CH25OH</td>
<td>Cholesterol 25-hydroxylase</td>
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<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicrons</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EDCF</td>
<td>Endothelial derived contracting factors</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
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<td>FBF</td>
<td>Forearm blood flow</td>
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<td>Free cholesterol</td>
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<td>Flow mediated dilatation</td>
</tr>
<tr>
<td>GSE</td>
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<tr>
<td>GTN</td>
<td>Glyceryl trinitrate</td>
</tr>
<tr>
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<td>Human aortic endothelial cells</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoproteins</td>
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<td>HDL-C</td>
<td>High-density lipoprotein-cholesterol</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase</td>
</tr>
<tr>
<td>hsCRP</td>
<td>High sensitivity CRP</td>
</tr>
<tr>
<td>HTGL</td>
<td>Hepatic triglyceride lipase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoproteins</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NOS</td>
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<tr>
<td>INSIG</td>
<td>Insulin induced gene</td>
</tr>
<tr>
<td>KLF2</td>
<td>Kruppel-like factor 2</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin cholesteryl acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoproteins</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
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<tr>
<td>LDL-R</td>
<td>Low-density lipoprotein-receptor</td>
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<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
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<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>MnTMPyP</td>
<td>Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligomeric procyanidins</td>
</tr>
<tr>
<td>OSBP</td>
<td>Oxysterol binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisomes proliferator-activated receptor</td>
</tr>
<tr>
<td>PWA</td>
<td>Pulse wave analysis</td>
</tr>
<tr>
<td>PWV</td>
<td>Pulse wave velocity</td>
</tr>
<tr>
<td>q-RT PCR</td>
<td>Quantitative real time-polymerase chain reaction</td>
</tr>
<tr>
<td>RCT</td>
<td>Reverse cholesterol transport</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP2</td>
<td>RNA polymerase 2</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage activating-protein</td>
</tr>
<tr>
<td>SR-B1</td>
<td>Scavenger receptor B1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>STAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoproteins</td>
</tr>
<tr>
<td>WAE</td>
<td>Whole apple extract</td>
</tr>
</tbody>
</table>
Acknowledgements

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Chapter 1: Flavonoids and cardiovascular disease
1.1 Introduction

1.1.1 Burden of cardiovascular disease

CVD is the number one cause of death globally. The WHO estimates that 17.5 million people died from CVD in 2005, of which approximately 80% were due to coronary heart disease (CHD) and stroke (WHO, 2007a). This figure is projected to increase to 20 million by the year 2015, largely due to the burden of disease in non-developed countries. CHD is currently the leading cause of death in the United Kingdom (European Cardiovascular Disease Statistics, 2008). This not only influences population morbidity and mortality but also the economy from the combined result of direct health care costs, direct non-health service and productivity loss (Liu et al., 2002). Given that CVD develops because of a combination of risk factors, most of which are modifiable and that these risk factors are present decades before the disease manifests, then it can be concluded that risk factor control from an early age must be the target to achieve primary prevention on a global scale.

Although levels of CVD incidence, mortality, and case fatality are declining, further progress is needed (Superko & King, 2008). Increased understanding of CVD pathophysiology has led to improved and optimised therapy through randomised controlled trials. However despite this there are areas which still remain obscure and warrant further examination; a prime example of this is the role high density lipoprotein cholesterol (HDL-C) plays in CVD (Rosenson, 2005; Superko et al., 2009). HDL-C has long been described to be protective being inversely correlated to CVD, a finding based largely on epidemiological studies (Gordon et al., 1977; Sweetnam et al., 1994; Bakogianni et al., 2001; Asztalos et al., 2005). It is purported that these beneficial effects of HDL-C are through the process of reverse cholesterol transport, whereby cholesterol can be removed from the tissues and taken to the liver for excretion (Fredenrich & Bayer, 2003; Rosenson, 2005; Cuchel & Rader, 2006; Fu, 2010). There is accumulating evidence however, that HDL-C is not simply involved in reverse cholesterol transport, but rather it exerts beneficial effects through other unidentified mechanisms (Kontush & Chapman, 2006; Hausenloy & Yellon, 2008; Tall et al., 2008; Duffy & Rader, 2009).

Elements of the diet have long been suspected to influence the development of CVD, either directly or indirectly through other well known CVD risk factors (de Lorgeril et al., 1994; Hu & Willett, 2002). In support of this there are a growing number of observational and interventional studies, which have identified various food constituents as affording protection against CVD (de Lorgeril et al., 1994; Ness & Powles, 1997). This might explain why certain populations appear
to be more protected from CVD than other age-matched counterparts (Hollenberg et al., 1997; Hollenberg, 2006; Hollenberg et al., 2009). When this is considered in addition to our increasing knowledge regarding the role of diet in health, it is unsurprising that government agendas across the globe are promoting “healthy living”. In the WHO’s publication “Prevention of Cardiovascular disease: Guidelines for assessment and management of cardiovascular risk”, (2007b) a summary of observational evidence suggesting necessary dietary constituents for health is given with the following recommendation:

“Applying these principles to develop diets that match individual preferences and local customs, and demonstrating their effectiveness in reducing cardiovascular risk, are important priorities for research”.

Polyphenols are one of the many naturally occurring chemical compounds found in abundance in fruit, vegetables, nuts, seeds, and whole grains which fulfil a variety of functions including production of pigment in plants e.g. flowers and berries and a role in plant defence systems (Scalbert & Williamson, 2000; Rimbach et al., 2009; Crozier et al., 2010). Flavonoids are by far the most commonly cited polyphenols in the literature relating to health benefits. The positive relationship between flavonoids and health was also in part spurred by the French Paradox, i.e. the observation that individuals in France had lower CVD mortality despite levels of saturated fat intake comparable to their counterparts in neighbouring countries (Renaud & de Lorgeril, 1992). Exploration of this finding led to the proposal that there were beneficial properties of the diet, which conferred this CVD protection (Corder, 2002; Corder et al., 2006). These health benefits appear to relate to the flavon-3-ol subclass of flavonoids which contain oligomeric procyanidin (OPC) molecules and are found in abundance in apples, grapes, and cocoa (Hertog et al., 1993; Bravo, 1998; Arts et al., 2001a; Manach et al., 2004; Engler & Engler, 2006; D’Archivio et al., 2007; Chun et al., 2008; Corder et al., 2006; Caton et al., 2010; Pérez-Jiménez et al., 2010).

Red wine has been consistently associated with a greater reduction in cardiovascular risk compared to other alcohol-based drinks (Grønbaek et al., 1995; Criqui & Ringel, 1994; Klatsky et al., 1997; Di Castelnuovo et al., 2002), through a number of effects, such as, improved blood pressure (Ferrières, 2004; López-Sepúlveda et al., 2008), endothelial function (Cishek et al., 1997; Soleas et al., 1997; Flesch et al., 2001; Guarda et al., 2005), anti-platelet effects (Demrow et al., 1995; Rosenkranz et al., 2002; de Lange et al., 2003; de Curtis et al., 2001) and anti-
inflammatory effects (e.g. Estruch et al., 2004). There has been much debate as to whether it is the total polyphenol content of red wine that exerts the cardioprotective affect or whether one particular component is more important (Howard et al., 2002). The observation that almost identical effects to red wine are seen with isolated OPC rich, grape seed extracts (GSE) supports the notion that OPC mediate this cardioprotection (Corder, 2002; Corder et al., 2006). Additional evidence for this is based upon a number of studies using cocoa, which are also rich in OPC and have also been associated with reduced risk of CVD through similar mechanisms to red wine (e.g. Taubert et al., 2003; Grassi et al., 2005a; Vlachopoulos et al., 2005; Schroeter et al., 2006; Heiss et al., 2010). These features make OPC a potential ally in the prevention and treatment of cardiovascular disease.

1.1.2 Thesis hypothesis
This work follows on from previous studies in our laboratory using microarray analysis of gene expression in human aortic endothelial cells have revealed that OPC extracts from cranberry, red wine and grape seed suppressed the microarray signal for a number of enzymes involved in cholesterol homeostasis (Pothecary, 2007). This includes molecules involved in cholesterol synthesis including the rate limiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and low-density lipoprotein receptor (LDL-R) mRNA (Pothecary, 2007; Pothecary et al., 2007). This was simultaneously associated with increased mRNA of substances involved in cellular cholesterol efflux e.g. phospholipids transfer protein, ABCG1. These changes are identical to those induced in endothelial cells incubated with 25-hydroxycholesterol (25-OHC), which has a negative feedback on cholesterol synthesis (Kandutsch & Chen, 1974; Brown & Goldstein, 1974). Consistent with this, the microarray signal for cholesterol-25 hydroxylase (CH25OH) was increased two-fold by cranberry, red wine and grape seed OPC, suggesting that this response played a pivotal role in endothelial responses to OPC. Volunteer investigations have also reported that chocolate or cocoa may influence HDL-cholesterol levels (Wan et al., 2001; Mursu et al., 2004; Baba et al., 2007; Mursu et al., 2008), which suggests effects of OPC on endothelial function and altered cholesterol efflux might be linked to observed changes in circulating HDL-C.

The objective of this thesis was to test the following hypothesis:

“Dietary oligomeric procyanidins lead to a global reduction in CVD risk through reduced blood pressure, improved endothelial function, quantitative
and qualitative improvements in HDL, and lowered cellular cholesterol levels. The latter is through a combination of reduced de-novo cholesterol synthesis and enhanced cholesterol efflux, processes modulated by the enzyme, cholesterol-25-hydroxylase.”

The evidence presented towards this hypothesis consists of in vitro experiments on bovine aortic endothelial cells (BAEC) and translation of these findings to a clinical trial of human subjects. This thesis has helped to provide further insights into the effects of OPC on endothelial function, blood pressure, and HDL-C providing groundwork on which to perform further, larger clinical studies.
1.2 Polyphenols and flavonoids

A phenol compound consists of a six-membered aromatic ring with a directly attached hydroxyl group (Crozier et al., 2009). The basic structure of polyphenols consists of more than one phenolic group and to date more than 8,000 different substances have been described (Bravo, 1998; Zern & Fernandez, 2005). Not surprisingly polyphenols are consumed by almost all of us on a daily basis with average consumption levels being estimated at 1g per day (Manach et al., 2005; Scalbert et al., 2005), but the amount in foodstuffs varies widely between and within food types (Pérez-Jiménez et al., 2010). As our knowledge about polyphenols is increasing various observations have been reported regarding the role external influences play on the level of polyphenols in food, for example, growing and storage conditions can markedly reduce the levels (Corder, 2002).

1.2.1 Structure of flavonoids

The classification of polyphenols is complex, in part due to various methods of naming. The two commonest used depend upon either naming the skeleton of the chemical compound or relating it to the reactions of the compounds (D’Archivio et al., 2007). Polyphenols can be divided into several groups based on their skeleton, the most important of which are: hydroxybenzoic acids, hydroxycinnamic acids, stilbenes, lignans, phenolic alcohols, and flavonoids (Manach et al., 2004). The majority of purported health benefits relate to the flavonoid group (figure 1.1) which are water soluble and can be further subdivided into six major groups (figure 1.2). The functions of flavonoids in plants have only been recently investigated and include the following (Iwashina, 2003): ultraviolet filter (flavonol glycosides), pollinator attractants (anthocyanins, flavones, flavonols), feeding attractants and deterrents for insects (quercetin, isoflavonoids, flavonols), and as phytoalexins to ward of infection (anthocyanins and flavanones).

Flavonoids form the largest group of polyphenols and consist of two or more aromatic rings each containing a minimum of one aromatic hydroxyl connected by a three carbon bridge (Beecher, 2003). The bridge produces a third ring by combining with oxygen and two carbons of one of the aromatic rings (Beecher, 2003). Thus the basic skeleton comprises three rings based on the C₆-C₃-C₆ skeleton attached to a chroman ring, which has an aromatic ring at one of three places: C-2, C-3 or C-4 (Crozier et al., 2009). This group of polyphenols is subdivided into six subclasses characterised by the oxidation state of the central pyran ring i.e. based on the connection of the B and C ring (see figure 1.1). But the classification of flavonoids is not as simple as this and also takes into account other attributes such as oxidation state, hydroxylation
and conjugation patterns of the B ring, and conjugation patterns of hydroxyls on the A and C rings (Beecher, 2003).

![Figure 1.1 Skeleton of flavan-3-ol molecules](adapted from Aron & Kennedy, 2008).

![Figure 1.2 The various subclasses of flavonoids](Flavan-3-ols are the most ubiquitous of flavonoids; with procyanidins potentially underlying their cardioprotective effects.)
The commonest group of flavonoids in the diet are the flavan-3-ols or flavanols (Aron & Kennedy, 2008; Crozier et al., 2009). They are the most ubiquitous of flavonoids and are found in various foods e.g. apple, tea, red wine and cocoa (Crozier et al., 2009). The biosynthesis of flavanols depends on light and thus they tend to accumulate in outer and aerial tissue of fruits (Beecher, 2003). In nature most flavonoids are present as glycosides or other conjugates, and in foods they can be polymerised into large molecules (the exception being flavanols; Beecher, 2003).

![Flavan-3-ol structure of procyanidins](image)

**Figure 1.3 Flavan-3-ol structure of procyanidins.** When R=H and the OH group is projecting below the plane the molecule is epicatechin (a) and if the OH group is projecting above the plane the molecule is catechin (b).

Considering the flavan-3-ol structure further, when R=H and the OH at position 3 projects below the plane it is called epicatechin and when the OH projects above the plane the molecule is called catechin (Aron & Kennedy, 2008; see figure 1.3). Polymers of epicatechins are called procyanidins which can be of various lengths e.g. monomers, dimers, trimers, tetramers etc. It is the oligomers (up to 6-8 units) which are thought to exert beneficial effects (Beecher, 2003).

Flavanol polymers consist of monomeric units connected by carbon-carbon and ether links and a number of these “proanthocyanidins” have been discovered, of which three are prominent in foods (Aron & Kennedy, 2008):

- Procyanidins - the subunit of which is (epi)catechin;
- Propelargonidins – the subunits of which are (-)-epifzelechin and (+)-afzelechin;
- Prodelphinidins – the subunit of which is (epi)gallocatechin.
Procyanidins are more prevalent in food than propelargonidins and prodelphinidins (Crozier *et al.*, 2009). The monomeric units can be described as B linkages where units are mainly linked by carbon-carbon bonds (4→6 or 4→8) or as A linkages which contain both carbon-carbon (4→8) linkages and also ether bonds (2→7; Beecher, 2003). These polymers can range from dimers to large numbers and it is the oligomer procyanidins which are known to be present in red wine, GSE and cocoa, and which are the molecules of interest in this thesis (Corder *et al.*, 2001; Corder *et al.*, 2004 and 2006).
1.3 Flavonoids and their relation to health
1.3.1 Observational studies of flavonoids

Several observational studies have examined the levels of intake of dietary flavonoids and CVD. Many of these have reported an inverse association amongst flavonoid intake and the incidence of CHD (Hertog et al., 1993; Hertog et al., 1995; Knekt et al., 1996; Yochum et al., 1999; Hirvonen et al., 2001; Buijsse et al., 2006; Mink et al., 2007) and cerebrovascular disease (Keli et al., 1996; Mursu et al., 2008). The major sources of flavonoids in these studies vary with tea, onions, and apples being most commonly cited, although observational evidence suggests that flavonoids in apples and red wine were the most preventative (Arts et al., 2001b). Despite this, there are a number of studies which have not detected any beneficial properties of flavonoids with either CVD (Rimm et al., 1996; Sesso et al., 2003; Mursu et al., 2008) or cerebrovascular disease.

Many of these observational studies report on groups of polyphenols rather than individual foodstuffs (Arts & Hollman, 2005). For example, Djoussé et al., (2010) focused on cocoa in the NHLBI Family Heart Study and used a semi-quantitative food frequency questionnaire in over 4,000 individuals. They calculated the odds ratio of CHD as 1.01 (0.76 - 1.37), 0.74 (0.56 - 0.98), and 0.43 (0.28 - 0.67) for subjects consuming the following amounts of chocolate: 1 - 3 times/month, 1 - 4 times/week, and 5+ times/week, after adjusting for the common confounding factors. This is a significant reduction in CHD with chocolate but the main difficulty is that the term chocolate covers a whole range of products, which may or may not necessarily have high OPC content. These effects have been linked with reductions in blood pressure as reported by Buijsse et al., (2010). They also observed a relationship between the lowest intakes of chocolate consumption and incidence of myocardial infarctions and strokes in middle-aged healthy subjects in Germany. Those subjects with the highest intake of chocolate had a combined relative risk of MI and stroke of 0.61 associated with a 1 mmHg reduction in both systolic and diastolic blood pressure. Lewis et al., (2010) have also correlated high chocolate intake (daily intake compared with weekly intake) with the presence of less atherosclerotic events and less carotid artery atherosclerotic plaque burden, suggesting a link between chocolate and reduction of atherosclerosis.
There are a number of difficulties with the observational studies of flavonoids, which include:

- **Confounding factors** – subjects with the lowest intakes of flavonoids are often those with unhealthier lifestyles which in itself increases the risk of CVD e.g. smokers or lowest levels of physical activity (e.g. Hertog *et al.*, 1993; Knekt *et al.*, 1996; Mink *et al.*, 2007).

- **Questionnaires** – these can be associated with inaccurate recall and bias as subjects may be more likely to divulge information about “healthier” lifestyles (Grassi *et al.*, 2009; Heiss & Kelm, 2010).

- **Focus on the whole group of flavonoids** – leading to uncertainty as to whether each flavonoid subgroup carries the same weight in terms of health benefits (e.g. Keli *et al.*, 1996; Knekt *et al.*, 1996; Rimm *et al.*, 1996; Hertog *et al.*, 1997).

- **Large variations in flavonoid intake** – for example levels as high as 139.3 mg/day in Finnish men (Mursu *et al.*, 2008) and 25.4 mg/day in post-menopausal women (Arts *et al.*, 2001b) have been quoted. It is difficult to say whether these are true differences, and if they are why the risk reductions are also not more at variance.

- **Accuracy of measurement of food flavonoid content** – the intake of flavonoids is estimated by cross reference to pre-determined tables but the methodology to calculate the flavonoid content of foods is currently not agreed upon and far from being standardized (Mursu *et al.*, 2008).

Table 1.1 summarises a number of observational studies relating lowered CVD incidence and mortality associated with increased flavonoid intake. One meta-analysis based on seven of these cohort studies provided a significant protective association between flavonoid intake and CHD mortality, with relative risk for CHD mortality of 0.81 (95% CI 0.71 - 0.92; Ding *et al.*, 2006).
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>N</th>
<th>F/U/ yrs</th>
<th>Flavonoid</th>
<th>CHD/MI incidence</th>
<th>CHD/MI mortality</th>
<th>Stroke mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hertog et al., 1993</td>
<td>1993</td>
<td>552 – 806 men, Dutch</td>
<td>5</td>
<td>Total flavonoids (TF)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Keli et al., 1996</td>
<td>1996</td>
<td>552 – 806 men, Dutch</td>
<td>10</td>
<td>TF</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Knekt et al., 1996</td>
<td>1996</td>
<td>5133 m + w, Finland</td>
<td>26</td>
<td>TF</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rimm et al., 1996</td>
<td>1996</td>
<td>34789, men, USA</td>
<td>6</td>
<td>TF</td>
<td>Null</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Hertog et al., 1997</td>
<td>1997</td>
<td>1900, men, UK</td>
<td>14</td>
<td>TF</td>
<td>Null</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Yochum et al., 1999</td>
<td>1999</td>
<td>34492, women, Iowa</td>
<td>10</td>
<td>TF</td>
<td>↓</td>
<td>Null</td>
<td></td>
</tr>
<tr>
<td>Hirvonen et al., 2000</td>
<td>2000</td>
<td>25,372 men, Finland</td>
<td>6.1</td>
<td>TF</td>
<td>↓ MI</td>
<td>↓ (NS)</td>
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<td>2001</td>
<td>26,593 Men, Finland</td>
<td>6.1</td>
<td>TF</td>
<td></td>
<td>Null</td>
<td></td>
</tr>
<tr>
<td>Arts et al., 2001a</td>
<td>2001</td>
<td>806 men, Dutch</td>
<td>10</td>
<td>Catechins</td>
<td>↓</td>
<td>Null</td>
<td></td>
</tr>
<tr>
<td>Arts et al., 2001b</td>
<td>2001</td>
<td>34492 women, Iowa</td>
<td>13</td>
<td>Catechins</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geleijnse et al., 2002</td>
<td>2002</td>
<td>4807 m+w, Dutch</td>
<td>5.6</td>
<td>Total tea flavonoids</td>
<td>Null</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Knekt et al., 2002</td>
<td>2002</td>
<td>10054 m+w, Finland</td>
<td>28</td>
<td>Specific flavonoids</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Sesso et al., 2003</td>
<td>2003</td>
<td>38445 women, USA</td>
<td>6.9</td>
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<td>Null</td>
<td>Null</td>
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<tr>
<td>Mursu et al., 2008</td>
<td>2008</td>
<td>1950 Men, Finland</td>
<td>15.2</td>
<td>Flavonol and flavan-3-ol</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 Prospective studies of flavonoids and cardiovascular outcomes (adapted from Ding et al., 2006).
1.4 Cocoa

1.4.1 Kuna Indians

Hypertension is rare in certain communities, one of which is the Kuna Indians native to the San Blas Islands off the Caribbean coast of Panama (Kean, 1944). The initial theories as to why these communities are protected were thought to be genetic, but to everyone’s surprise the search for this genetic factor actually pointed towards environmental factors (Hollenberg, 2006). This was supported by the observation that migration to urban environments resulted in loss of this protection. Page in 1976 concluded that it was the low levels of salt intake in these communities, which accounted for the lack of an age-related rise in blood pressure (BP), and incidence of hypertension. Hollenberg and colleagues examined this theory further (1997) and compared island dwelling Kuna Indians to those who had migrated and were now living in Panama City. They confirmed a rise in BP (both systolic and diastolic) with age, but only in the residents of the city. This did not necessarily relate to salt intake as the Kuna Indians still living in the Islands have partially acculturated and their salt intake was similar to levels of the United States (possibly even more, see McCullough et al., 2006). This suggested the role of an alternative environmental factor underlying the lack of hypertension in this community.

Lifestyle factors especially diet, were the next parameter to be examined in the Kuna Indians. Further analysis of the diet of the island dwelling Kuna Indians revealed high intake of three main elements: fruit, fish and cocoa. The latter was largely in the form of beverages and the average intake amounted to a staggering 35 cups per week (Hollenberg, 2006). It became apparent that this level of cocoa beverage intake related to the hot and humid climate of the San Blas Islands and that many drank cocoa rather than water for fluid replacement. Cocoa is now known to contain significant levels of flavanols especially OPC thus it is postulated that these compounds provide health effects which mean that BP remains within the normal range despite advancing age, possibly through enhanced nitric oxide levels (Hollenberg et al., 2009).

These observations provide an interesting hypothesis but they are limited by a number of factors for example:

- It is difficult to exclude the role of confounding factors such as, stress, exercise and the use of herbal remedies (Hollenberg et al., 1997).
• The Kuna Indian studies are based on patient recall, which is not always accurate and liable to bias.

• The number of patients in the studies who went on to complete dietary and lifestyle questionnaires was in one study approximately one fifth of patients who had BP measurements taken (McCullough et al., 2006).

• Concerning salt intake, the Kuna Indians may well have only recently become acculturated to its use in cooking but it maybe that the hypertensive affect of salt is not apparent yet as it takes time for this to develop (Hollenberg et al., 2009).

1.4.2 Human studies of cocoa

For many people cocoa or dark chocolate are the main sources of procyanidin intake in Western nations at present, accounting for up to 20% of the total OPC intake (Corder, 2008). The primary flavonoids in cocoa are the flavan-3-ols, in the form of catechins, epicatechins, and procyanidins (Steinberg et al., 2003) and the procyanidin content of cocoa is similar to that of red wine (Corder, 2008). However, not all cocoa products have the same content of flavanols with dark chocolate containing a greater amount of flavanols per weight, the reason for the bitter taste. Once cocoa beans are harvested, they can undergo a number of processing procedures that may reduce the total flavanol content for example, fermentation, alkalization, and roasting (Corder et al., 2001; Corder et al., 2004). Better processing methods, which preserve the flavanol content of cocoa, are now available, the result of which is dark chocolate with higher OPC levels. Studies of the beneficial effects of cocoa largely came to the forefront in the last 10 – 15 years and a number of effects have been described and are discussed next.

Cocoa effects on lipids

Cocoa and flavonoids from other sources have variable effects on lipids in humans. For example, Baba and colleagues (2007) performed a large study on 160 subjects and examined the effects of a high polyphenolic diet on lipids. They reported lower plasma LDL-C, oxidised LDL and apo B concentrations, and raised HDL-C in those subjects who consumed the higher polyphenol diet. Other researchers have also reported improved HDL-C levels with dark chocolate (Wan et al., 2001; Mursu et al., 2004; Mellor et al., 2010). The major criticisms of these studies is that they were unblinded and of insufficient duration. Grassi and colleagues (2008) tested the effects of flavanol rich dark chocolate over a longer time period, 15 days in
hypertensive patients with impaired glucose tolerance, and reported both reduced TC and LDL-C, and improved insulin sensitivity. Other authors have failed to detect any significant changes in lipids (e.g. Taubert et al., 2007).

**Blood pressure lowering effects**

Much of the interest in cocoa flavanols has arisen from its potential effects on reducing blood pressure. Initial studies of the affects of polyphenol rich chocolate on blood pressure were very promising with large falls in blood pressure being observed. For example, Taubert and colleagues (2003) detected a fall in blood pressure of 5/1.8 mmHg in systolic/diastolic in thirteen patients with stage I hypertension following 10 days of eating polyphenol rich chocolate. In another study, 24 h ambulatory blood pressure monitoring in 20 subjects detected a fall in blood pressure of -11.9/-8.5 mmHg (Grassi et al., 2005a) which in a subsequent study was also associated with increased insulin sensitivity (Grassi et al., 2005b). These results are even greater than that of some of today’s commonly used anti-hypertensive medications and supported by meta-analyses, although a more modest reduction in blood pressure is reported such as, -4.5/2.5mmHg and -3/2mmHg (Desch et al., 2010; Ried et al., 2010). Again, these studies can be criticised as they were not properly blinded (white chocolate often being used as the control) and often the OPC or flavanol content were not defined.

**Cocoa effects on endothelial-dependent vasodilatation**

The effects on endothelial dysfunction have been scrutinized in both healthy volunteers and patients with pre-existing CVD and/or presence of CVD risk factors. Improvements in floe mediated dilatation (FMD – an index of endothelial function) in healthy volunteers were reported by Vlachopoulos et al., (2005) one hour after consumption of flavonoid-rich dark chocolate in a randomised single blind, sham procedure-controlled and crossover design. They found increased FMD and significantly decreased augmentation index (AI – an index of arterial stiffness) with chocolate, which were not associated with changes in plasma oxidant status. This study was only performed over three hours and it is likely that for full benefits longer duration studies are needed (which also need to be double blinded). Engler and colleagues (2004), Schroeter et al., (2006) and Westphal and Luley (2010) have also reported improved endothelial dysfunction in healthy subjects following flavanol rich cocoa or dark chocolate. Engler et al., (2004) correlated their findings with increased plasma levels of epicatechin although Schroeter et al., (2006) could only attribute one-third of the effects to epicatechins, suggesting that other oligomers or metabolites were also involved.
Cocoa also improves endothelium-dependent vasodilatation in patients with pre-existing CVD or risk factors, including diabetics, hypertensive’s, hypercholesterolaemia and those with CAD (Heiss et al., 2003; Heiss et al., 2005; Wang-Polagruto et al., 2006; Flammer et al., 2007; Balzer et al., 2008; Grassi et al., 2008; Heiss et al., 2010). Heiss et al., (2005) assessed FMD in 11 smokers after 2 h following ingestion of flavanol containing cocoa. FMD improved by 2.4% in the smokers, a change that was accompanied by increased NO in the plasma and correlated with flavanol metabolites. Balzer et al., (2008) assessed 41 medicated diabetic patients with flavanol-rich cocoa taken three times daily (321 mg flavanols per dose vs. 25 mg flavonols per dose). They not only reported acute increases in FMD in diabetics (at 2h) but a sustained increase in FMD, which was apparent throughout the study. This was supported by measures of free plasma flavanols and their conjugated metabolites, although as bioavailability of flavonoids is currently unclear it is difficult to determine if this was a causal relationship (Campia & Panza, 2008).

Heiss et al., in a subsequent study (2010) used flavanol rich cocoa and reported lowered systolic blood pressure and improved FMD response in patients with known CAD and who were also taking ACEI/ARB, statins, beta blockers and aspirin. This supports the potential role of cocoa in addition to currently used medications for secondary prevention of CVD. Flammer et al., (2007) also detected improvement in endothelium-dependent coronary vasodilatation and platelet function in 22 heart transplant recipients as did Faridi et al. (2008) in overweight individuals, who were otherwise healthy. In the latter study, there was also an improvement in blood pressure.

**Cocoa and biomarkers of endothelial function**

CRP has been reported as a sensitive biomarker for both chronic inflammation and risk for cardiovascular disease. Giuseppe et al., (2008) interestingly reported a J-shaped curve between dark chocolate consumption and serum CRP, with those having 20 g of DC every 3 days having lower CRP levels than non-consumers. Chun et al., (2008) also tested the association between flavonoid intake and serum CRP concentrations among U.S. adults. Total flavonoid and individual flavanol, anthocyanidin, and isoflavone intakes were all inversely associated with serum CRP. The food sources best for these were apples and other vegetables. In comparison, Grassi et al., (2008) did not identify any changes in CRP despite improvements in blood pressure, endothelial function, and dyslipidaemia. The subjects in this trial had impaired glucose
tolerance, which one would expect to be associated with an inflammatory state and thus a raised CRP.

At present from the available studies, it is difficult to determine the amount of flavanols necessary to result in benefit and whether a concentration-dependent effect exists. One study experimented with acute doses of polyphenol rich dark chocolate and found that 500 mg dose of polyphenol cocoa led to similar changes in blood pressure as 1000 mg. The authors concluded that a saturation effect might occur (Almoosawi et al., 2010). Davison et al., (2010) also examined the effect of differing doses of cocoa flavanols (33, 372, 712 and 1052 mg per day) consumed over 6 weeks on 24 h ABPM in men and women with mild hypertension, and reported reductions in systolic and diastolic blood pressure (~5.3/3 mmHg), but only at the highest dose. This could explain the lack of blood pressure reduction in a number of studies.

Studies of the causality of flavonoids and better cardiovascular health are fraught with difficulties despite the reported positive associations. Products that are currently tested in studies are not standardized and thus the concentration of flavanols can vary considerably (Schroeter et al., 2006). Moreover, as mentioned above controls in previous studies are not properly matched and blinding has not always been achieved (Taubert et al., 2007). There are also a large number of other substances present in chocolate bars which may have physiological consequences and these too need to be controlled for (Almoosawi et al., 2010). Another difficulty is that the endpoints and study designs are variable and comparison with other trials is made almost impossible (Balzer et al., 2008). It also remains unclear whether one OPC rich product e.g. cocoa augments another e.g. red wine. Many of the studies of polyphenols and OPC are in vitro and often in vivo results do not concur, leading to much criticism (Taubert et al., 2007). This may reflect the fact that procyanidins are poorly absorbed from the gastrointestinal tract and a high intake may be required to achieve high blood concentrations (Manach et al., 2005). A number of researchers are trying to overcome some of these difficulties (e.g. Heiss et al., 2005 and 2010).
1.5 Bioavailability of flavonoids

An understanding of the fate of flavonoids following ingestion is necessary to make the link between flavonoids and their effect on health. The word “bioavailability” originates from pharmacology and one definition is “… [the] rate and extent to which a drug reaches its site of action” (D’Archivio et al., 2007). When applying this to polyphenols the objective is to determine the amount of nutrients available to exert its action at the target sites. The target sites have yet to be elucidated, but bioavailability of flavonoids and indeed all polyphenols depends on their liberation, absorption, distribution, metabolism, and excretion. Numerous research groups are participating in studies aiming to elucidate the bioavailability of flavonoids. This section will review the current information available on this topic focusing on flavonoids in general and flavan-3-ols.

Structural diversities make it difficult to accurately determine the content of polyphenols in food. Values also differ within foods for example, apple flavonoid content can contrast four fold. Manach and colleagues (2005) reviewed 97 studies on polyphenol bioavailability and estimated daily intake of catechins at 18 - 50 mg per day with the majority being from tea, cocoa, apples and red wine. This study has subsequently been criticised as flavonoids were hydrolysed and the free aglycones measured which ignores flavonoid metabolites which are now known to form a large proportion of the excreted molecules. Daily intakes are also affected by the lack of standardized analytical methods (Scalbert & Williamson, 2000). The commonest techniques used include high performance liquid chromatography (HPLC) with detection by UV absorption or fluorescence often coupled to mass spectrometry identification or reduction of Folin-Ciocalteu reagent (Hellström et al., 2009). Both of these have disadvantages e.g. unknown metabolites may not be detected and there are substances, which can interfere with Folin’s reagent e.g. ascorbic acid. Immunochemical methods using monoclonal antibodies are under investigation (Kawai et al., 2008).

1.5.1 Flavonoids in general

Flavonoids, except flavan-3-ols and proanthocyanidins, exist in plants as glycoside conjugates (Crozier et al., 2010). Absorption is determined more by the structure of the polyphenol rather than the starting concentration. Flavonols, isoflavones, flavones and anthocyanins are usually glycosylated, the sugar is often glucose, rhamnose, galactose, arabinose and xylose to name a few (Scalbert & Williamson, 2000). The number of sugars ranges from 1 - 3 occupying a variety of possible positions (Crozier et al., 2009). The removal of this hydrophilic moiety is necessary
for passive diffusion across the small intestine brush border, of which there are two possible routes:

1. **Lactase phloridzin hydrolase** – this is present in the epithelial cells of the brush border, and results in the release of the aglycones, which can then enter epithelial cells (Donovan *et al.*, 2006).

2. **Cytosolic β-glucosidase** – this occurs within the epithelial cells necessitating the transport of polar glucosides into the cell via an as of yet unknown transporter but possible contenders include the active symporter sodium dependent glucose transporter SGLT1 and facilitated glucose transporter 1 (Gee *et al.*, 2000; Del Rio *et al.*, 2010).

Data suggest that glycosylation of polyphenols also changes depending on the species, for example, humans are unable to hydrolyse rhamnosides where as rats can (Appledoorn *et al.*, 2009). This was supported by human volunteer studies by Hollman *et al.*, (1999) where quercetin-3-O-rhamnoglucoside was absorbed slowly if at all in humans. Manach and colleagues (2005) have found that gallic acid is the best absorbed of all the polyphenols based on almost 100 previous published studies. Similarly, polyphenols from various berries also have high absorption rates (Koli *et al.*, 2010).

Following absorption polyphenols are frequently conjugated and this takes place in either the intestinal cells or the liver (Singh *et al.*, 2008). The metabolites can undergo any of the following:

- **Sulphation** – via the action of sulphotransferases.

- **Glucuronidation** - via the action of uridine-5’-diphosphate glucuronosyltransferases.

- **Methylation** – via Catechol-O-methyltransferases, which leads to the transfer of a methyl group to polyphenols like quercetin and catechins.
These steps increase solubility, which enable biliary and urinary elimination (D’Archivio et al., 2007). Conjugation is thought to be a highly efficient process so that virtually no free aglycones are present in the plasma. This is contentious however, as work in rats suggest that procyanidin dimers are absorbed without conjugation (Appledoorn et al., 2009). Another important exception is green tea catechins whose aglycones account for up to 75% in the plasma (D’Archivio et al., 2007). Not all the metabolites are absorbed into the circulation; some will be released back into the lumen of the small intestine, possibly via transporters from the ABC family (Crozier et al., 2010). Interestingly, it has been suggested that giving higher doses of aglycones will lead to some free aglycones being absorbed as conjugation is rapidly saturated, but this remains debatable (Hu, 2007). Metabolites entering the portal circulation will reach the liver and undergo further phase II metabolism and enterohepatic recirculation resulting in recycling back to the small intestine through bile excretion.

Significant amounts of flavonoids reach the colon via the small intestine. Here deconjugation takes place by the colonic microflora e.g. β-glucosidases resulting in the production of aglycones. These themselves will undergo ring fission producing smaller molecules such as, phenolic acids and hydroxycinnamates (Crozier et al., 2009; Marks et al., 2009). Data suggests that high colon polyphenol concentrations are important for local affects but do not necessarily impact any systemic effects (Scalbert & Williamson, 2002; Aron & Kennedy, 2008). Nevertheless, it is increasingly recognised that following absorption the phenolic acids undergo further phase II metabolism in the liver and that the quantities of flavonoid metabolites resulting from this process far outweigh those via the small intestine (Del Rio et al., 2010). Polyphenols that are not absorbed are excreted in the faeces or undergo enterohepatic circulation and elimination in the bile. Other polyphenol metabolites will be eliminated in the urine (see figure 1.4), especially those that undergo extensive metabolism. Excretion in the urine correlates with maximum plasma levels and the percentage of urine excretion is higher for flavanones from citrus fruits and for isoflavones, but amounts vary hugely from approximately 4 - 66% (D’Archivio et al., 2007).

The plasma concentration of polyphenols differs according to the source and type of polyphenol. Typically, maximum concentrations are reached 1 - 2 h following ingestion (D’Archivio et al., 2007). Transport of polyphenol metabolites in the plasma usually occurs by binding to proteins, the most predominant of which is albumin. The affinity of polyphenols for albumin varies and binding most likely affects clearance and delivery to cells but this still remains unclear. Most
important, it is difficult to say whether free polyphenols can exert effects meaning that in vitro studies may not necessarily reflect what takes place in vivo, indeed studies are being undertaken with flavonoid metabolites to see whether these are active in vitro (D’Archivio et al., 2007).

At present little is known about tissue uptake of polyphenols, which is difficult to determine. It is well known that polyphenols can be taken up by the liver where they are then metabolised. Data on the uptake of polyphenols in other tissues is limited, for example, there are a few studies measuring prostate concentrations of tea polyphenols, breast and colorectal tissue (see D’Archivio et al., 2007). Rats sacrificed 1 - 6 h after a single dose of radio-labelled polyphenols for example, have shown radioactivity at various sites such as, the blood and digestive system (Aron & Kennedy, 2008). HPLC analysis suggests uptake in other tissues e.g. brain, endothelium, heart, kidney, spleen, testes, bladder, bone and skin (Datla et al., 2001; Youdim et al., 2000; Siganuma et al., 1998; Kim et al., 2000; Chang et al., 2000; Coldham & Sauer, 2000).

Another critical factor in flavonoid bioavailability is membrane transport. The available data confirms that both processes of cellular influx and efflux occur in vivo. Various transporters have been linked with transport in the liver and gastrointestinal tract, but much information is still lacking (Hu, 2007). Following hydrolysis, it has been suggested that the free aglycones may enter epithelial cells purely by close proximity to the cellular membrane and the process of passive diffusion (Del Rio et al., 2010), but given the high molecular weights of some flavonoids this seems likely to play a minor role. Influx of flavonoids into the epithelial cells of the gastrointestinal tract has been linked to the active symporter sodium dependent glucose transporter SGLT1 and facilitated glucose transporter 1 (Gee et al., 2000; Del Rio et al., 2010). SGLT1 is present only on the apical surface of epithelial cells whilst the facilitated glucose transporter 1 is present on both sides of epithelial cells (Passamonti et al., 2009). Studies of various flavonoids and their metabolites suggest that these are universal transporters for flavonoids, indeed some of the tested molecules cause inhibition of these transporters (Passamonti et al., 2009).

Relatively more is known about efflux than influx of flavonoids. Some of the flavonoid metabolites are effluxed back into the lumen of the small intestine, and several molecules have been implicated in this including: the ATP-binding cassette transporters e.g. ABCG2 and P-glycoprotein (ABCB1), and the Multidrug Resistance Proteins MRP1 and MRP2 (Del Rio et al.,
The ABC transporters are present on both sides of the polarized cells. Yet there is evidence that bilitranslocase which is a mammalian liver bilirubin transporter, participates in flavonoid membrane transport, which is supported by the presence of its homologue in plants where flavonoid biosynthesis is plentiful (Passamonti et al., 2009). Bilitranslocase has been identified not only in liver cells but also in cells of the kidney and gastrointestinal tract. However, the data suggest that bilitranslocase mediates the uptake of anthocyanins in rat liver cells but similar data for flavan-3-ols is lacking (Passamonti et al., 2009). No flavonoid receptor has been identified to date, although the characteristics of active OPC molecules effecting downstream signalling mechanisms leading to changes in gene expression, are consistent with a receptor-mediated response (Catton et al., 2010).

Figure 1.4 Cartoon representation of the metabolic fate of polyphenols. This is based on the current evidence and many aspects remain obscure.
1.5.2 Flavan-3-ols

The cell studies and the EPICURE trial have used products which are rich in flavan-3-ols, predominantly containing OPC but also containing monomers, except were purified procyanidins were used. The bioavailability of these compounds is even more difficult to determine as there are very few reference compounds which can distinguish between epicatechin and catechin metabolites and presently it is not possible to differentiate between enantiomers (Crozier et al., 2010). Stalmach and colleagues (2009) detected 12 metabolites in the form of O-methylated, sulphated and glucuronide conjugates of (epi)catechin and (epi)gallocatechin, in 10 healthy subjects following 500 mL ingestion of green tea (containing 648 µmol of flavan-3-ols). It took 30 min after ingestion for the compounds to be first detected and were only detectable in trace quantities at 8 h and undetectable at 24 h. The predominant metabolite was (epi)gallocatechin-O-glucuronide with a $C_{\text{max}}$ of 29nmol/L and $T_{\text{max}}$ of 1.7 h. The timeframe of detectability and $T_{\text{max}}$ (which ranged from 1.6 to 2.3 h) confirms small intestine absorption, which was supported by a subsequent study by the same group in humans with ileostomies (Stalmach et al., 2010).

Urine was also collected and analysed in these studies. Human subjects with a functioning colon had a very similar profile of metabolites in the urine and overall ~8% of the total flavan-3-ol intake was excreted. Excretion of epicatechin metabolites was 28.5% of the (-)-epicatechin and (+)-catechin intake suggesting a higher bioavailability of these compounds compared to (epi)gallocatechins, a feature which has also been reported with cocoa (Baba et al., 2000; Auger et al., 2008). Interestingly in subjects with ileostomies over two thirds of the flavan-3-ol intake was recovered in ileal fluid as both native compounds and metabolites implying that colonic metabolism is an important process in flavan-3-ol bioavailability. The colonic microflora are an important source of flavan-3-ol ring fission products that lead to the detection of hippuric acid in the urine, but other pathways for this process exist as ring fission also occurs in ileostomy subjects (Roowi et al., 2010). In conclusion flavan-3-ol monomers, especially (epi)catechin and catechins have a very high bioavailability and the colon plays a major role by providing colon-derived catabolites (Crozier et al., 2010).

The bioavailability of oligomeric forms of flavan-3-ols including OPC, are important to understand in order for OPC to have health benefits. Procyanidins differ from other polyphenols, as they are polymeric and often considered as high molecular weight compared to monomers. However, it should be noted that OPC pentamer is roughly similar in size to vitamin B12, which
has a specific mechanism of absorption and plasma transport. Whether OPC absorption could involve a similarly complex pathway should not be excluded without more detailed investigation (Crozier et al., 2010).

Studies to date show phenolic acids from colonic catabolism of OPC are absorbed into the circulation and can be detected in the urine. Initial data by Spencer and colleagues (2000) suggested that procyanidins were hydrolysed to monomers and dimers in the stomach, which facilitated their absorption. Rios et al., (2002) analysed gastric juice from human volunteers after ingestion of OPC rich juice and confirmed that procyanidins are not degraded in the stomach although the dimer B2 was discovered in the plasma two hours after ingestion suggesting absorption of some B2 dimers. Two further human studies using GSE and cocoa also suggested the breakdown of OPC into much smaller molecules resulting in their absorption (Sano et al., 2003; Holt et al., 2002). However, these were small studies and the levels detected were low, in addition, subsequent studies have not supported these findings (e.g. Tsang et al., 2005).

It is now generally thought that OPC are not absorbed but rather pass to the colon unaltered where they are catabolised to produce phenolic acids e.g. 3-hydroxyphenylhydraacrylic acid and 4-O-methyl-gallic acid (Ward et al., 2004; Crozier et al., 2009). This is supported by in vitro experiments which show that oligomers larger than trimers are unable to cross the small intestine in their native form (Depréz et al., 2001; Pascaul-Teresa et al., 2010). Radioactively labelled procyanidin B2 has provided further information on the fate of OPC in the gastrointestinal tract. Stoupi and colleagues (2010) detected 60% radioactivity after 96 h in the urine following oral loading. Suggesting a much longer metabolic route and furthermore based on total clearance and volume of distribution, the forms are very different to the dosed OPC. For example, scission of the interflavan bond is one route but the more dominant is the production of phenolic acids and 1 or 2 phenolic hydroxyls and between 1-5 aliphatic carbons in the side chain (Appledoorn et al., 2009). It remains contentious at present as to whether it is these phenolic acids or other as of yet unknown metabolites, of procyanidins that lead to the health benefits reported from observational, in vitro and in vivo studies.

It is important to appreciate that widely used methods of extraction have not been optimised for OPC recovery and this could be an important flaw in the conclusions from studies so far (Corder, 2008). In vitro studies also highlight that procyanidin B2 yields a number of “dimeric”
catabolites with a mass greater than the constituent monomer. These account for 20% of the substrate, are produced early on, and more than likely retain the interflavan bond (Stoupi et al., 2010). Interestingly, Yamakoshi and colleagues (2002) reported that procyanidin oligomers greater than pentamer were more effective at preventing cataracts in hereditary cataractous rats than dimer to tetramer fractions, suggesting unexplained aspects of OPC bioavailability.

Cocoa deserves a special mention as dark chocolate was used in the clinical trials presented in this thesis. Cocoa beans contain 6 - 8% polyphenols by dry weight (Grassi et al., 2008). This consists mainly of catechins, flavonol glycosides, anthocyanins and procyanidins - the latter up to decamer oligomers (Rimbach et al., 2009). Several human studies have measured the bioavailability of cocoa products and generally the peak flavanol levels occur 2 – 3 h after ingestion and the resulting plasma concentrations are in the nanomolar range (Richelle et al., 1999; Wang et al., 2000; Baba et al., 2000; Schramm et al., 2001; Holt et al., 2002; Steinberg et al., 2002). Milk does not appear to affect the bioavailability of cocoa and nor does lipid or protein rich meals (Rimbach et al., 2009), but this is controversial (see Mullen et al., 2009) and perhaps incompletely understood.

Mullen and colleagues (2009) detected two flavan-3-ol metabolites in the plasma of human subjects who consumed a hot cocoa drink (containing procyanidins, catechin and epicatechins): O-methyl-(epi)catechin-O-sulphate and (epi)catechin-O-sulphate. For both the C\textsubscript{max} (< 100nM) and T\textsubscript{max} (<1.5 h) indicated that absorption was occurring in the small rather than large intestine. Urine samples from the same subjects contained both the sulphated metabolites and also (epi)catechin-O-glucuronide and (epi)catechin-O-sulphate. Thirty percent of the flavan-3-ols was excreted in the urine which is high, taken along with the fairly low C\textsubscript{max} values for the two plasma metabolites suggests that there is a rapid turnover of cocoa flavan-3-ols and its metabolites and low volume of distribution.

The conclusions of bioavailability studies are often limited by the lack of methods to measure the full range of metabolites (Mullen et al., 2009). Indeed many studies on flavonoid bioavailability have measured plasma and urine levels following treatment with glucuronidases or sulphatases, providing measures of released aglycones but no indication regarding the metabolites, which are usually well in excess of the aglycones (Crozier et al., 2010). The use of HPLC-MS without initial hydrolysis of samples has improved our understanding; but is still limited without reference compounds to completely delineate isomers and structural differences.
The HPLC-MS/MS systems can identify and provide structural information regarding flavonoids and their metabolites, quantification can be determined by consecutive reaction monitoring or selected ion monitoring. The drawbacks are that both of these methods require a calibration curve based on a related molecule which may not be available. Those that are available can be obtained commercially, but as the molecules are not identical, there will be an error in any subsequent defined quantity.

This section has reviewed the bioavailability of polyphenols based on the limited data currently available. It is important to realise that everyday processes affect the bioavailability of polyphenols, a factor that may not be apparent in studies that use relatively pure compounds. Factors can range from cooking methods to storage methods and also interaction with other plasma proteins (D’Archivio et al., 2010). Polyphenols may exert their effects on cells after deconjugation for example, UDP glucuronosyltransferases are found in the endoplasmic reticulum but more research in this area is necessary (Del Rio et al., 2010). Further studies are needed to try and resolve the bioavailability of flavonoids and in relation to cocoa the following studies are needed:

- A comparison of blood pressure and FMD vascular responses to a standard cocoa, compared to an epicatechin-rich extract of this cocoa and an epicatechin-depleted extract would provide considerable insight into what components are most important in the biological effects of cocoa.

- The availability of radio-labelled epicatechin and specific procyanidins would enable specific studies of the biological fate of these molecules, and help determine the main metabolites, their localisation in the body, and extent to which these molecules cross biological membranes.

- Understanding the fate of cocoa products in humans with determination of metabolites in urine and faeces while considering the impact of other substances e.g. milk, sugar etc., would start to highlight key factors affecting bioavailability.

- Testing cocoa metabolites in vitro and in vivo and comparing these to the free aglycones could potentially identify metabolites, which have a critical role in the vascular response.
1.6 Properties of flavonoids from \textit{in vitro} and animal studies

1.6.1 Isolated cell and system studies

Although there are only a few observational studies which have specifically examined the effects of individual flavonoids on CVD, there are a number of experimental investigations using animal models, isolated tissues, or cultured cells that have described various properties of flavonoids. These include:

- **Anti-atherosclerotic actions of procyanidins** – Klurfeld and Kritchevsky (1981) were one of the first to report significantly reduced aortic atherosclerosis in rabbits whose diet was supplemented with red wine in comparison to other alcoholic beverages (white wine, alcohol-water, whiskey and beer). Similar findings have been reported by other researchers (Da Luz \textit{et al.}, 1999; Kurosawa \textit{et al.}, 2005).

- **Alterations in blood lipid profiles** – favorable changes in total cholesterol, LDL-C and HDL-C have all been postulated as the cause of reduced atherosclerotic lesion sizes (Klurfeld & Kritchevsky, 1981; Tebib \textit{et al.}, 1994; Hayek \textit{et al.}, 1997; Matsumoto \textit{et al.}, 1998; Yamakoshi \textit{et al.}, 1999; Frémonet \textit{et al.}, 2000; Kurosawa \textit{et al.}, 2005; Del Bas \textit{et al.}, 2005; Rizzo & Berneis, 2008; Quesada \textit{et al.}, 2009; Osakabe & Yamagishi, 2009). However, reduced plasma cholesterol levels have not been reported in all studies (Hayek \textit{et al.}, 1997; Bladé \textit{et al.}, 2010). In fact some studies have shown significant increases in total cholesterol levels in rabbits fed with cocoa polyphenols despite still seeing reduced levels of aortic atherosclerosis (Yamakoshi \textit{et al.}, 1999; Kurosawa \textit{et al.}, 2005; Fuhrman \textit{et al.}, 2005).

- **Cellular cholesterol regulation** – flavonoids have also been shown to influence cellular cholesterol regulation (Wegrowski \textit{et al.}, 1984; Yamakoshi \textit{et al.}, 1999; Chang \textit{et al.}, 2001; Lee \textit{et al.}, 2008; Lam \textit{et al.}, 2008). For example, apple procyanidins increase HDL-C and reduce non-HDL-C with suppression of CETP activity in a dose-dependent manner (Lam \textit{et al.}, 2008). Furthermore application of various tannin derived polyphenols to Vero cells (derived from monkey kidneys) can result in effective HMGCR inhibition (Chang \textit{et al.}, 2001), of all the compounds tested the most effective was the proanthocyanidins and gallotannins.

- **Endothelial dysfunction** – polyphenols from a variety of sources have been reported to improve endothelial function in animals possibly through NO-mediated mechanisms.
including eNOS, cGMP and calcium (Fitzpatrick et al., 1993; Andriambeloson et al., 1997, 1998 and 1999; Stoclet et al., 1999; Flesch et al., 1998; Karim et al., 2000; Diebolt et al., 2001; Fitzpatrick et al., 2002; de Moura et al., 2004; Boban et al., 2006; López-Sepúlveda et al., 2008; Schmitt & Dirsch, 2009). The total daily production of NO is difficult to clarify due to complexities of endogenous production and exogenous sources of its precursors (Hord et al., 2009). But there is accumulating evidence that higher levels of NO are associated with improved endothelial function (Vanhoutte, 2009).

- **Inhibition of endothelin-1 (ET-1) synthesis** – flavonoid induced vasodilatation may also be the result of reduced vasoconstrictor substances. ET-1 is a potent vasoconstrictor and stimulates vascular smooth muscle cell proliferation and hypertrophy leading to reduced vessel wall distensibility, vascular and myocardial hypertrophy, all of which are risk factors for cardiovascular morbidity and mortality (Ito et al., 1991; Corder et al., 2001). There is increasing data showing that OPC-rich extracts, regardless of their source (red wine, cranberry, apple, cocoa, grape seed and hawthorn), reduce ET-1 synthesis in human and bovine cells (Corder et al., 2001; Corder et al., 2004 and 2006; Jiménez et al., 2007; García-Conesa et al., 2009; Caton et al., 2010).

- **Reduction in blood pressure** – vasorelaxation induced by red wine has also been associated with a reduction in systolic BP (Diebolt et al., 2001; Pechánová et al., 2004; López-Sepúlveda et al., 2008) and has also been seen with intragastric administration of red wine polyphenols in rats (Diebolt et al., 2001).

- **Reduced heart rate and heart rate variability** - Akita et al., (2008) examined the effects of cocoa polyphenol diet in rats and reported both reduced heart rate and heart rate variability resulting from preservation of parasympathetic function. Baroreflex receptor activity was also higher as was blood pressure in rabbits fed the standard diet, which may have contributed to the larger sized atherosclerotic lesions in the thoracic and abdominal aortas of this group.

- **Anti-platelet effects** – such as, interfering with platelet-derived growth factor (PDGF), platelet aggregation with both red wine and cocoa (Shanmuganayagam et al., 2007; Rosenkranz et al., 2002). But the effects are short-lived (hours) unlike the commonly used anti-platelet agent aspirin, making the risk of GI haemorrhage unlikely.
Studies of *in vitro* and isolated systems are difficult to correlate to actions in humans but there are also a number of other major criticisms of these studies such as, the possibility of other constituents of red wine accounting for these findings (including alcohol) and the lack of reproducibility across these studies (Osakabe & Yamagishi, 2009). Until the mechanism of effect is not clarified it will prove difficult to understand not only the significance of positive results but also the explanation of negative findings.

### 1.6.2 Structure activity relationships

Much of the research on flavonoids has shown that products and extracts rich in the flavan-3-ol family of molecules have the greatest effects on vascular function in experimental studies and clinical trials. An interesting question is whether the effects of flavan-3-ols vary according to the degree of polymerization of these molecules. The whole extract from a given source consists of a mixture of monomers, dimers, trimers etc. Fitzpatrick *et al.*, (2000 & 2002) tried to elucidate which fractions of GSE are the most potent inducers of endothelium-dependent vasodilator responses. Mass spectrometry characterisation of purified fractions revealed that of all the flavan-3-ol compounds it was the trimers, tetrarsers and pentamers, which had the greatest vasorelaxant effects on rat aortic rings (Corder *et al.*, 2004; Corder *et al.*, 2006). Similar findings have been obtained studying endothelium-dependent vasodilator responses by others using GSE (Aldini *et al.*, 2003) and purified cocoa OPC (Karim *et al.*, 2000). Moreover, inhibition of ET-1 synthesis mirrors this structure activity relationship (Corder *et al.*, 2006, Caton *et al.*, 2010), suggesting a single mechanism triggers these endothelial cell responses. These observations are further supported by microarray studies using an OPC-rich apple extract with an average degree of polymerisation of 3.9, which initiated multiple changes in gene expression in human umbilical vein endothelial cells, whereas (-)-epicatechin and procyanidin dimer B2 had no effect (García-Conesa *et al.*, 2009).

The contribution of OPC in the vascular response to high flavanol cocoa drink has been questioned by Schroeter *et al.*, (2006), who attributed the effects to (-)-epicatechin and its metabolites. In this study the effect of a mixture of flavonols/metabolites detected in the plasma of healthy subjects with improved flow mediated dilatation (FMD) after ingestion of flavanol rich cocoa, were tested on rabbit aortic rings *in vitro*. This showed relaxation of aortic rings, which was most likely through increases in NO resulting from epicatechin and its metabolite fractions. Based on these observations Schroeter and colleagues concluded that the improvement in FMD response after the high flavanol cocoa drink was likely due to epicatechin. However, the
biggest flaw in this argument, and surprisingly overlooked, was that purified epicatechin could not improve FMD response to the same extent as the high flavanol cocoa drink. Epicatechin at a dose of 2 mg/kg (presumably 140 – 160 mg, depending on the weight of the individuals) only increased FMD responses by ~30% of that achieved by the high flavanol cocoa drink (containing 170 mg epicatechin). Therefore, further investigation of the optimal mix of cocoa flavanols is needed using epicatechin-depleted and OPC-depleted products to determine whether observations in isolated systems using purified OPC translate into greater improvements in FMD in vivo.

1.6.3 Underlying mechanism of action of flavonoids

There is an ongoing debate as to the underlying mechanism of action of flavonoids. In vitro studies provide ample evidence to suggest the capability of flavanols to act as antioxidants (e.g. Yamokishi et al., 1999; Kurosawa et al., 2005; Vinson et al., 2006; López-Sepúlveda et al., 2008; Fraga et al., 2011), but antioxidant activity of flavonoids is not a consistent finding in human studies (Mursu et al., 2004; Taubert et al., 2007; Scheid et al., 2010). In fact the intracellular generation of superoxide has been implicated in polyphenol and red wine induced relaxation in porcine coronary artery rings (Diebolt et al., 2001; Ndiaye et al., 2003) and in promoting calcium signalling for red wine polyphenol induced vasodilatation (Duarte et al., 2004). There have been various other postulations as to the underlying mechanism of polyphenol cardioprotection including changes in cell receptors, intracellular signalling pathway proteins (Williams et al., 2004; Ghosh & Scheepens, 2009), and alterations in gene expression (Bagchi et al., 2003; Del Bas et al., 2005; García-Conesa et al., 2009).

Changes in gene expression are likely an important mechanism of action of polyphenols. The expression of Nuclear factor-κB (NFκB), a major transcription factor that controls transcription of DNA, was down-regulated in hepatic tissues of mice with type 2 diabetes mellitus exposed to proanthocyanidins (Lee et al., 2008). Furthermore, suppression of ET-1 synthesis with purified apple OPC correlates with a concomitant increase in the expression of Kruppel-like factor 2 (KLF2; Caton et al., 2010), which is a key regulator of the endothelial response to laminar shear stress (Parmar et al., 2006). These effects of OPC-rich extracts on ET-1 synthesis and endothelium-dependent vasodilatation, has many similarities to those achieved by subjecting the endothelium to physiological laminar shear stress (Brooks et al., 2000), suggesting that procyanidins mimic laminar shear stress leading to increases in NO through direct signalling mechanisms as well as by alterations in gene expression (Corder et al., 2004).
1.7 Atherosclerosis

Atherosclerosis does not affect all vessels uniformly it prefers the outer edges of blood vessel bifurcations where the blood flow becomes turbulent resulting from reduced flow and a change in direction. This change in velocity results in reduced laminar shear stress. Shear stress can be defined as “......force per unit area created when a tangential force (blood flow) acts on a surface (endothelium) ........” (Davies, 2009). Normally in straight areas of arterial blood vessels, the shear stress is around 15 - 20 dyne/cm$^2$. Originally high shear stress (>400 dyne/cm$^2$) was thought to lead to atherosclerosis but research over the last 30 years has confirmed that it is actually sustained low shear stress (<4 dyne/cm$^2$) which promotes the development of atherosclerosis (Malek et al., 1999). When low shear stress occurs at atherosclerotic prone areas of the circulation, the following processes occur leading to atherosclerosis (Malek et al., 1999; Libby et al., 2002; Davignon & Ganz, 2004):

- Endothelial cell loss through apoptosis
- Altered cell morphology and shape
- Increased monocyte attachment and migration
- Increased endothelial surface expression of vascular cell adhesion molecule-1 (VCAM-1) and production of cytokines and chemokines
- Impaired vasorelaxation

This section will begin by a discussion on endothelial dysfunction and then explore in greater detail cholesterol regulation in cells. Excessive cellular cholesterol along with endothelial dysfunction are core processes involved in the development of atherosclerosis. OPC have been reported to have beneficial effects on both.

1.7.1 Endothelial dysfunction

The endothelium is a single layer of cells that forms an inner lining of the circulatory system. It provides an interface between the plasma and the subendothelial tissues. The endothelium was once thought to be an inert barrier, but the last few decades have seen an increase in knowledge regarding the properties of the endothelium (Malek et al., 1999). The endothelium is now recognised as a crucial organ in its own right with both paracrine and endocrine functions (Davignon & Ganz, 2004). Endothelial dysfunction is a term, which describes any form of abnormal endothelium activity. It is increasingly recognised as one of the earliest features in the
chain of events, which lead to atherosclerosis (Werns et al., 1989). This can range from expression of proinflammatory molecules to abnormalities in vessel relaxation.

### 1.7.2 The endothelium and modulation of vessel contractility

The endothelium is involved in many functions the most important of which (in terms of endothelial dysfunction) is often considered to be modulation of vessel contractility. Until 30 years ago, blood vessels were thought to constrict and dilate in response to circulating substances or via neurotransmitters acting at nerve endings. Acetylcholine (ACh) has long been established as a powerful vasodilator and the endothelial response to ACh was established by Furchgott and Zawadzki (1980), who observed that ACh did not always lead to the anticipated vasodilatation in vitro. They realised that this anomaly was actually the result of accidental loss of the endothelial cell layer during preparation of the vessels. Thus, they concluded that the endothelium played a necessary role in vessel relaxation and postulated that the endothelium produced an “endothelium-derived relaxing factor” (EDRF) when rabbit aortic rings were exposed to ACh. This finding was subsequently reproduced in several species including rabbits, monkeys, and humans (Verbeuren et al., 1986; Freiman et al., 1986). It is now known that the endothelium does not only produce EDRF, but also other factors, which have an effect on vascular smooth muscle cells, leading to either vasoconstriction or relaxation. These factors can be broadly categorised into the following three groups (Malek et al., 1999):

- **EDRF** - the most important of which is NO
- **Endothelium-derived hyperpolarizing factors (EDHF)**
- **Endothelium-derived contracting factors (EDCF)**

A healthy endothelium has a fine-tuned balance of both vasodilators and vasoconstrictors. Of all the vasodilators, NO is probably the most important and by far the most researched at present. Vasorelaxation is mediated through increased NO release (see Davignon & Ganz, 2004). The role of NO has been confirmed by molecular studies which have detected elevated NO and endothelial nitric oxide synthase (eNOS) mRNA (Malek et al., 1999; Davignon & Ganz, 2004). Shear stress, which is the physical force created by the movement of blood across the vascular walls is the most important regulator of basal NO production. In fact, the observation that atheromatous plaques have a predilection to form at areas of abnormal levels of shear stress
provides another clue to the link between atherosclerosis and endothelial dysfunction (Malek et al., 1999). Laminar flow induced shear stress leads to NO and prostacyclin release which produces vasorelaxation and is antithrombotic, where as chronic exposure of blood vessels to turbulent flow and non-laminar shear stress leads to endothelial dysfunction with increased expression of pro-inflammatory and pro-atherosclerotic genes (Vanhoutte, 2009).

In a healthy vascular tree, and especially the coronary and cerebral circulation, exposure of the endothelium to either thrombin or serotonin (5-HT) from aggregating platelets results in the release of NO and subsequent vessel relaxation (Libby et al., 2002). This relaxation of the vessel provides increased blood flow allowing the thrombin and platelet aggregate to be in essence “flushed away” from the healthy endothelium. Coupling of the endothelial 5-HT and thrombin receptors to eNOS has now been well characterised (Vanhoutte, 2009). NO also protects the endothelium by inhibiting platelet activation, reducing smooth muscle cell proliferation, and preventing neointima formation (Vapaatalo & Mervaala, 2001). Any absence of the endothelium or malfunction leads to the vasoconstrictor effects of 5-HT and thromboxane A2 unopposed by endothelial NO and prostacyclin (PGI2) which permits the sequence of events leading to haemostasis (Lüscher et al., 1993).

Of the vasoconstrictors, ET-1 is the most prevalent of the endothelin family of peptides in the cardiovascular system. ET-1 is synthesized in response to various stimuli including, cytokines, hypoxia, and low shear stress. Physiological laminar shear stress, NO and PGI2 suppress ET-1 gene expression (Malek et al., 1999). Loss of these stimuli causes up-regulation of the gene with production of pre-proET1 mRNA, which is translated into proET-1 and eventually the mature ET-1 peptide (requiring endothelin converting enzyme). ET-1 then binds to endothelin receptors, of which there are two types, ET_A and ET_B, both of which can lead to vasoconstriction (Davignon & Ganz, 2004). The relative importance of ET_A and ET_B receptors varies between vascular beds with ET_A generally playing the greatest role in vasoconstrictor responses. The endothelium also expresses ET_B, activation of which leads to increased endothelial calcium and activation of endothelial derived relaxing factors; it also acts as a clearance receptor. Thus, there is an innate system, which counteracts ET-1 dependent vasoconstriction. These protective mechanisms maybe reduced once endothelium dysfunction occurs.

Cardiovascular risk factors are known to precede CVD by several decades. It is becoming increasingly clear that these risk factors all lead to endothelial dysfunction (Vanhoutte, 2009).
For example, hypertension is associated with impaired vascular relaxation, a finding which can be corrected early on with anti-hypertensive therapy. Hypercholesterolemia, diabetes mellitus, and obesity states all induce impaired vascular relaxation (Quyyumi & Patel, 2010). These changes have been associated with increased levels of EDCF and ROS. Furthermore, reduction of LDL-C in hypercholesterolaemia improves endothelial dysfunction (Libby et al., 2002). But these are not the only mechanisms through which risk factors lead to endothelial dysfunction.

Risk factors for cardiovascular disease are also associated with both oxidative stress and increased EC turnover. EC turnover can be as long as twelve months in areas which are resistant to atherosclerosis but in areas prone to atherosclerosis EC turnover is much faster and in the order of weeks (and even shorter as humans age), (Xu, n.d.). Reasons why EC turnover is increased may relate to changes in haemodynamic shear stress and EC turnover is hastened in the presence of dysfunction. These neo-EC can take days to weeks mature and during this period various harmful substances can enter the blood vessel intima including blood mononuclear cells, LDL-C and smooth muscle progenitors (Xu, n.d.).

Often the new EC also fail to respond appropriately to certain stimuli, such as the presence of aggregating platelets, 5-HT, and thrombin (Vanhoutte & Shimokawa, 1989). These regenerated cells also have the ability to take up more modified LDL-C and to generate oxidised LDL, in addition to producing more oxygen-derived free radicals and reduced activity and expression of eNOS (Libby et al., 2002). Despite these advances in knowledge it remains unclear why it takes decades for risk factors to result in the appearance of atherosclerotic lesions even though endothelial dysfunction may be detected at a much earlier time (Chambers et al., 1999). Perhaps it is the loss of innate protective homeostatic mechanisms, which would normally restore endothelial function, for instance in the post prandial phase, that ultimately lead to the changes in vascular function that result in atherosclerosis.

1.7.3 Biomarkers of vascular dysfunction: high sensitivity C-reactive protein

C-reactive protein (CRP) was first discovered in patients with *streptococcus pneumonia* in 1929 by Tillet and Francis (as cited by Calabró et al., 2009). It is a member of the pentraxin family and is a protein synthesized in the liver that activates the complement system by binding to phosphocholine, which is expressed on dead or dying cells and some bacteria. Its level in plasma rises in inflammation making it an acute phase protein largely arising from activated leukocytes and also from fibroblasts, endothelial cells and adipose tissue (Elks & Francis, 2010). It is
regulated by interleukin 6, but other inflammatory cytokines may also affect its synthesis (Nordestgaard & Zacho, 2009).

In acute inflammation and certain infections, CRP rises within 6 h and can increase 50,000 fold. It peaks around 48 h and its half-life is constant (19 h) and depends on the rate of production. CRP in plasma can be measured by various methods including ELISA, immunodiffusion, and visual agglutination and in health baseline levels are around 0.8 mg/L (Ford et al., 2003). Originally, CRP levels were measured by assays with insufficient sensitivity to detect the “normal” range but these have been largely superseded by the use of high sensitivity CRP (hsCRP) assays based on enhanced ELISA methods capable of detecting very low basal levels (Miller et al., 2007).

Inflammation is prominent in atherosclerosis, a process probably initiated by the invasion of cholesterol into the arterial wall. The resulting cytokines lead to the up-regulation of CRP synthesis by the liver. High levels of CRP have been correlated to risk of future cardiovascular events, with levels greater than 3 mg/L indicating high risk (Calabró et al., 2009). The predictive value of CRP in CVD has been reported for both CAD and cerebrovascular disease in a number of studies (e.g. US Physicians’ Health Study; MRFIT study and a study by general practitioners in Mendall et al., 1996; Ridker et al., 1998a; Ridker et al., 1998b; Ford et al., 2000; Ridker et al., 2000; Rost et al., 2001; Curb et al., 2003; Danesh et al., 2004; May & Wang, 2007; Calabró et al., 2009). Thus, CRP may be helpful in risk stratification, even refining current risk assessment tools (Kanda, 2001; Sellmayer et al., 2003; May & Wang, 2007).

Support for the casual effect of CRP in atherosclerosis has come from intervention studies such as the JUPITER study (Ridker et al., 2008). Here the use of rosuvastatin, a HMGCR inhibitor, in over 17,000 patients with normal levels of LDL-C (< 3.4 mmol/L) and elevated CRP (> 2 mg/L) was associated with marked reductions in both CVD events (by 44%) and mortality (by 20%) leading the study to be cut short. These changes were associated with reductions not only in LDL-C (50% reduction) but also CRP (by 37%). The best treatment benefits were observed in those with greatest reductions of both LDL-C and CRP. CRP levels are also influenced by a large number of diseases e.g. degree of glycaemic control in diabetes mellitus (Xu & Whitmer, 2006) and obesity (Yudkin et al., 1999), both of which cause low-grade inflammation. It also correlates with other risk factors. This limits use of CRP in CVD and casts doubt over its prognostic value (Napoli et al., 2005).
1.8 Regulation of cholesterol synthesis

A number of studies have reported reduction in the size of atherosclerotic lesions and some have even reported lowered LDL-C and/or increased HDL-C after periods of polyphenol consumption suggesting that altered cholesterol metabolism could be a component of the protective cardiovascular response (Wan et al., 2001; Mursu et al., 2004; Mellor et al., 2010). This section will review cholesterol metabolism, lipoproteins and apolipoproteins in order to define how dietary polyphenols and OPC in particular might interact with these processes. It will also discuss oxysterols as it is hypothesized that the OPC effects on cholesterol might be mediated by the oxysterol synthesis.

1.8.1 Cholesterol

Cholesterol is a subgroup of steroids, called sterols and contains a hydroxyl group at the 3-position of the A ring (Rifai et al., 2000). Inherently insoluble in water, cholesterol consists of four fused rings which are carbon loaded (Soccio & Breslow, 2004; figure 1.5). Cholesterol is present in all mammalian cells and can exist as free cholesterol (FC), accounting for a third of the total body cholesterol content or as cholesteryl esters (CE). Exactly how much is free in plasma membranes is not known (Duffy & Rader, 2006). Esterified cholesterol is even more insoluble than FC and thus it needs to be shepherded around the plasma by lipoproteins, which serve to solubilise cholesterol allowing it to transfer between tissues via the plasma (Rifai et al., 2000).

![Chemical structure of cholesterol](image)

Figure 1.5 Chemical structure of cholesterol. Note the hydroxyl group at the 3-position of the A ring and the extent of carbon loading.
On average the body produces 875 – 1000 mg cholesterol each day and we consume between 200 – 500 mg per day mainly in meat, eggs and dairy products, of which 30 – 60% is absorbed (Rifai et al., 2000). Of the cholesterol produced by the body 400 mg makes bile acids to replace those lost in the faeces and 50 mg is used to make steroid hormones (Rifai et al., 2000). All cells have the ability to produce cholesterol but twenty-five percent of all cholesterol synthesis occurs in the liver and intestine (10 and 15% respectively; Soccio & Breslow, 2004). Cholesterol synthesis is a tightly regulated process as cholesterol is cytotoxic in excess, due to changes in the endoplasmic reticulum initiated by excessive FC, which lead to activation the cell death effector CHOP (Feng et al., 2003).

The liver has a bile acid synthesis pathway which will help prevent cholesterol toxicity, but the majority of mammalian cells lack this pathway (Rifai et al., 2000). Non-hepatic cells must therefore have other pathways of regulating cholesterol as cholesterol homeostasis is tightly controlled (Soccio & Breslow, 2004). Endothelial cells are a good example of this last point, in some individuals they can be exposed to very high levels of plasma LDL-C but do not become overload compared with other cells e.g. macrophages (Duffy & Rader, 2006). Processes in non-hepatic cells which regulate cholesterol may include regulation of lipid biosynthesis enzymes (discussed further below), presence of acyl-coA:chol cholesterol acyltransferase (which converts FC from cell membranes to CE i.e. for storage) and activation of cholesterol efflux pathways (Yvan-Charvet et al., 2010). Despite the widespread presence and importance of cholesterol, little is known about the regulation of cholesterol in non-hepatic and non-endocrine cells but there is increasing work looking at cholesterol metabolism in various tissues including adipose, cerebral and endothelial cells (Superko et al., 2009).

De novo synthesis of cholesterol occurs either within the cytosol or at the endoplasmic reticulum (Rifai et al., 2000). There is some data to suggest that peroxisomes and the plasma membrane may also be involved (Soccio & Breslow, 2004). Cholesterol synthesis begins with the two-carbon acetate group, acetyl CoA and requires a number of enzymes and co-factors which eventually result in cholesterol. There are also a number of intermediate compounds which are produced and the rate-limiting step in the process involves HMGCR the target of statins (Brown & Goldstein, 1986).
1.8.2 Intracellular cholesterol transport

Cholesterol is an essential part of mammalian cells with 35 – 45% being located within the plasma membrane (Soccio & Breslow, 2004). In order to reach the plasma membrane, cholesterol has to move within the cytoplasm from its various sources such as the ER where de novo synthesis occurs, from cytosolic lipid droplets where cholesterol esters (CE) are stored, and following receptor-mediated endocytosis (as occurs with LDL-C binding to the LDL-R). Transport of cholesterol is also important for the effects of cholesterol on transcription for example, cholesterol has to pass to the ER to regulate SREBPs and oxysterols (oxygenated derivatives of cholesterol) and must travel to the mitochondria to regulate liver X receptors (LXR; Cavelier et al., 2006). Cholesterol concentrations are asymmetrical among membranes a process that is incompletely understood and cholesterol can move both up and down concentration gradients (Soccio & Breslow, 2004). Cholesterol movement up a concentration gradient is likely to occur via carrier proteins which have hydrophobic and hydrophilic centres, but this has yet to be clarified (Soccio & Breslow, 2004).

There are two main ways that cholesterol moves within cells: these can be broadly described as vesicular and non-vesicular (Soccio & Breslow, 2004). Intracellular vesicles contain cholesterol in their membranes which is one way cholesterol mobilises around the cell. For vesicular transport to take place an energy source is imperative, usually in the form of ATP, and the cell must also have an intact cytoskeleton. Non-vesicular mechanisms also exist as evidenced by the finding that blocking vesicular pathways does not hinder the transport of cholesterol around the cell (Soccio & Breslow, 2004). It is likely that these non-vesicular methods are mediated by diffusible carrier proteins such as steroidogenic acute regulatory protein (StAR) which typically has hydrophobic cavities that bind cholesterol and transport it across the cytosol and activates mitochondrial conversion of cholesterol to steroids (Ning et al., 2006). Non-vesicular mechanisms are not just restricted to diffusible carrier proteins and spontaneous desorption of cholesterol from one membrane to another closely applied membrane may also occur (Yvan-Charvet et al., 2010).

**Sterol regulatory element-binding proteins**

Free cholesterol is toxic to cells which has been identified both in vitro and in vivo (Kandutsch & Chen, 1974; Brown & Goldstein, 1974; Tabas, 2002). The mechanism of toxicity is unclear but cholesterol-induced macrophage death has been proposed to be due to plasma membrane...
loading with FC resulting in disruption of related enzymes and transporters (Kellner-Weibel et al., 1999) or abnormal endoplasmic reticulum function (Feng et al., 2003). Excessive cellular cholesterol negatively feeds back on cholesterologenic enzymes and the LDL-R. This is performed by certain transcription factors called sterol regulatory element-binding protein (SREBP). There are 3 proteins: SREBP 2, SREBP1a and SREBP 1c (Weber et al., 2004a; Sato, 2010). SREBP 2 activates genes in cholesterol synthesis and the other two activate genes in fatty acid synthesis. SREBP 1a and -2 also regulate transcription of the gene encoding the LDL-R and thus have been under considerable interest as potential therapeutic targets (Horton et al., 1999).

High cell cholesterol levels also lead to the activation of acyl coenzyme A:cholesterol acyltransferase (ACAT), an ER located enzyme that forms CE. CE are stored along with triglycerides (TG) in cytosolic lipid droplets, which probably form as an extension of the ER (Soccio & Breslow, 2004). Cholesterol esters can then be broken down usually by neutral cholesterol ester hydrolase (nCEH), an enzyme whose activity and activators varies according to the tissue (Okazaki et al., 2008; Igarashi et al., 2010). The “cholesterol ester cycle” is the term used to describe the role of synthesis and hydrolysis by ACAT and nCEH and both have been identified in the process of macrophage foam cell formation (von Eckardstein, 1996). However, esters are generally less toxic than FC so esterification affords partial protection. It is important to remember that it is the FC that is available for cellular efflux and following nCEH activation the resulting FC and FFA are transported out of the lipid droplet (Igarashi et al., 2010).

1.8.3 Cholesterol efflux
Cholesterol efflux is dependent on the cell type and the acceptor molecule. Cholesterol efflux takes place in the extravasal space and acceptor molecules, which usually consist of hydrophilic and hydrophobic portions, need to pass through capillaries to get to the cell surface, a process which is not understood at present (Yvan-Charvet et al., 2010). Cholesterol efflux can occur in a number of ways: the first method is desorption of cholesterol from plasma membranes, which is taken up by nearby acceptor particles. This can occur at any cell and requires the movement of cholesterol down a concentration gradient, a process which is not fully understood but is a process that requires high-energy substrates (Soccio & Breslow, 2004). Cholesterol efflux either via desorption or via acceptor molecules (discussed further below) may be initiated by the collection of pools of cholesterol in discrete areas of the plasma membrane in response to some as yet, unknown mediator (Rothblat et al., 1999).
**ATP-binding cassette transporters**

The most important cholesterol transporters are the ATP-binding cassette transporters ABCA1 and ABCG1 and the scavenger receptor B1 (SR-B1). The ATP-binding cassette transporters undertake the majority of cholesterol export, with ABCA1 accounting for approximately 50% and ABCG1 contributing 20% *in vitro* (Adorni *et al.*, 2007). There are various subtypes of ABC transporters and transcription is controlled by activation of the LXR, generally ABCA1 effluxes lipids to apo AI and ABCG1 effluxes lipids to HDL$_2$, a subclass of HDL (Cavelier *et al.*, 2006).

ABCA1 was discovered as part of the work searching for the cause of Tangier’s disease. Patients with Tangier’s disease are characterised by having virtually no plasma HDL and are unable to export cholesterol and phospholipids to apolipoprotein A I (apo AI) resulting from defective ABCA1 gene. The ATP binding cassette transporters require active diffusion and generally efflux cholesterol to lipid poor or lipid deplete apolipoproteins. The apolipoproteins may interact directly with the plasma membrane or lead to activation of another process further along the membrane. Active efflux using this method is known to increase in the presence of excessive cellular cholesterol, especially in endothelial cells (Hassan *et al.*, 2006).

ABCA1 effluxes cholesterol to apo AI can occur at the plasma membrane through binding of apo AI to phospholipid and cholesterol, which ABCA1 in turn promotes (Yokoyama, 1998). Alternatively apo AI may bind directly to ABCA1 and be internalised into the cell to pick up lipids, which will then be packed, and exocytosed (Yvan-Charvet *et al.*, 2010). The function of ABCA1 is necessary in the formation of high-density lipoprotein (HDL) particles. Interestingly, atorvastatin inhibits ABCA1 expression and cholesterol efflux in macrophages (Qiu & Hill, 2008), an effect which exhibited a dose-dependent increase and was abolished with LDL-C pre-treatment of cells.

In comparison, ABCG1 tends to efflux cholesterol mainly to HDL especially the more mature HDL, although it can efflux to other acceptors too e.g. LDL (Fu *et al.*, 2010). It also leads to an increase in plasma membrane FC without directly binding HDL (Smith, 2006). ABCG1 can also promote the efflux of oxysterols such as 7-ketocholesterol (Yvan-Charvet *et al.*, 2010). HDL particles have an outer hydrophilic layer and in order for cholesterol to pass through this to the inner lipid rich core, high energy requirements are necessary which is likely to be derived from the ABC transporters. The presence of ABCG1, like ABCA1, is also up regulated in the presence of excessive cellular cholesterol (Smith, 2006). ABCG1 has also been hypothesized to act as a phospholipid floppase, which leads to changes in the plasma membrane phospholipids.
allowing sterols to diffuse to the outer leaflet (Yvan-Charvet et al., 2010). Interestingly, inactivation of ABCG1 does not alter circulating HDL levels but results in accumulation of lipids (Marcel et al., 2008). There is synergy between ABCA1 and ABCG1, the former leads to the production of HDL, which can then interact with the latter to acquire lipids. It remains unknown as to whether this is a process which occurs in vivo on the same cell or nearby cells. Cell cholesterol efflux can also occur via the SR-B1 receptor, which is only expressed on certain cells for example, hepatocytes. SR-B1 mainly leads to re-organisation of membrane lipids, but can also bind acceptors such as HDL (Yancey et al., 2003).

Much work on cholesterol efflux is based on in vitro studies, in which cells are usually loaded with cholesterol and then challenged in the presence of various acceptors (Yvan-Charvet et al., 2010). Radioactively labelled cholesterol is most often used and efflux is measured before and after a stimulus. The commonest acceptors utilized include cyclodextrins, which are water-soluble cyclic oligosaccharides, which contain a hydrophobic cavity and phospholipid vesicles in unilamellar or multilamellar forms (Smith, 2006). Cyclodextrins are extremely efficient in solubilising cholesterol leading to very high removal rates of cholesterol in vitro. These molecules usually lead to unidirectional movement of cholesterol, although if they are coupled with FC bi-directional flux can occur (Rothblat et al., 1999). These acceptors have now been superseded by the use of lipid free or lipid-poor apolipoproteins in an attempt to mirror what occurs in vivo. The use of whole serum in experiments is the ideal way to replicate the in vivo process and provides numerous advantages compared with the use of isolated acceptor molecules (Smith, 2006). Yet the use of whole serum is not ideal as it prevents identification of the individual processes involved in cholesterol efflux. Furthermore, the effects of cell culture techniques on the expression of key molecules involved in cholesterol efflux may influence the results from in vitro studies (Jessup & Kritharides, 2008).

**Cholesterol efflux in endothelial cells**

Endothelial cells require a special mention as they are exposed to large amounts of cholesterol in the plasma yet do not seem to accumulate cholesterol as other cells such as, macrophages. O’Connell et al., (2004) tried to elucidate the mechanisms that govern endothelial cell cholesterol efflux. They performed microarray analyses of HUVEC following treatment with cholesterol for 24 h and discovered up-regulation of ABCG1 rather than ABCA1 and down-regulation of enzymes involved in cholesterol biosynthesis. Interestingly further analysis revealed induction of ABCG1 expression in HUVEC and HAEC when exposed to cholesterol or
22(R)-hydroxycholesterol, a potent LXR agonist. This was supported by the lack of stimulation of ABCA1 with its known enhancer of expression, 8-bromo-cAMP confirming little if any role of ABCA1 in endothelial cholesterol efflux. A reversed picture was seen in human fibroblasts with predominance of the ABCA1 transporter rather than ABCG1. Endothelial cells did not directly efflux cholesterol to HDL₃, which contrasts with previous work performed in macrophages, suggesting the involvement of other, as of yet unknown proteins. The authors concluded that endothelial cells efflux cholesterol to HDL₃ but by processes not involving those described in macrophages. O’Connell and colleagues (2004) also reported oxysterol formation in endothelial cells, and this may be the predominant method of cell cholesterol efflux in endothelial cells. Alternatively, SR-B1 may play a key role especially as it stimulates NO synthesis in endothelial cells (Yuhanna et al., 2001).

### 1.8.4 Oxysterols and cell cholesterol homeostasis

In vitro, oxysterols are both potent suppressors of de novo cholesterol synthesis and promoters of cholesterol efflux by up-regulation of various molecules involved in this process (Kandutsch & Chen, 1974; Brown & Goldstein, 1974). This is similar to the findings from the microarray study which was the stimulus for this thesis (Pothecary, 2007). In the microarray study the signal for the oxysterol 25-OHC was also enhanced which suggested the possibility that OPC rich extracts stimulated this oxysterol leading to reduced endothelial cell cholesterol levels. This section will discuss the oxysterols in further depth and the role they play in cholesterol homeostasis.

Oxidised derivatives of cholesterol made in the mitochondrion called oxysterols, are generated in the presence of excessive cellular cholesterol (Soccio & Breslow, 2004). Oxysterols have a very short half-life and thus they are found in very low concentrations in the plasma. Oxysterols result from the hydroxylation of the side chain of cholesterol and are mostly produced by the action of enzymes of the cytochrome P450 family (Fuchs, 2003). All nucleated cells have the ability to oxygenate cholesterol but some organs have a higher capacity than others do e.g. liver and endocrine organs. The liver manufactures oxysterols into water-soluble bile acids, which are then excreted in the bile. Although bile acid synthesis itself in the liver can also lead to one of three oxysterols: 24, 25, and 27-hydroxycholesterol (Fuchs, 2003). Oxysterols have various functions which appear to be regulatory in nature e.g. cholesterol homeostasis, calcium uptake, cell differentiation (Yvan-Charvet et al., 2010). In terms of cholesterol homeostasis, oxysterols regulate genes involved in cholesterol synthesis and efflux (Gill et al., 2008).
Oxysterols are the natural ligands for LXR, and binding up-regulates the ABC transporters amongst other molecules including apolipoproteins (Edwards et al., 2002). There are two isoforms of LXR: LXRα and LXRβ, the former appears to be restricted to certain tissues with highest levels in the liver whilst the latter is more ubiquitous (Morello, 2009). LXR activations lowers cellular cholesterol levels by increasing the expression of genes involved in cholesterol efflux, converting them to bile acids or influencing intestinal cholesterol absorption (Fuchs, 2003). Other properties of LXR include anti-inflammatory and improved glucose metabolism, which probably adds to its atheroprotective effects and may have therapeutic implications (Duffy & Rader, 2009).

**25-hydroxycholesterol and cellular cholesterol**

The oxysterol 25-OHC is the most powerful of oxysterols when it comes to suppressing cholesterol synthesis in vitro. For example, 25-OHC is a potent inhibitor of the activity of HMGCR activity in vitro (Kandutsch & Chen, 1974; Brown & Goldstein, 1974), and 25-OHC also down-regulates the number of LDL-R and enhances cholesterol esterification by ACAT (Brown et al., 1975; Zhang et al., 1990). This oxysterol is produced from the action of the enzyme CH25OH and is unlike other oxysterols in that its production does not involve the cytochrome P450 family of enzymes (Kandutsch et al., 1977; Bjorkhem, 2002; Ning et al., 2006). CH25OH is present in most tissues, located at the ER and Golgi apparatus (Fuchs, 2003). There is also increasing evidence suggesting that 25-OHC plays a role in inflammation (Diczfalusy et al., 2009 and Morello et al., 2009).

As discussed above excessive cellular cholesterol negatively feeds back on cholesterologenic enzymes and the LDL-R by the transcription factors called SREBP. The oxysterols 25-OHC and 27-hydroxycholesterol are also potent repressors of SREBP processing and activate cholesterol esterification (figure 1.6; Kandutsch & Chen, 1974; Brown & Goldstein, 1974). This may be through the activation of SCAP and ACAT, which are also both directly activated by cholesterol (Soccio & Breslow, 2004). Oxysterols also lead to activation of LXR transcription factors and in particular 25-OHC in hepatocytes leads to increased SREBP-1 protein levels (Xu et al., 2010). LXR activation leads to transcription of the ABC transporters, which act as gates at the plasma membrane allowing the efflux of cholesterol and lipids (Edwards et al., 2002).
It is postulated that like excessive cellular cholesterol, 25-OHC may regulate the expression of genes vital to cholesterol and lipid metabolism (Kilsdonk et al., 1995; Adams et al., 2004; Nishimura et al., 2005). Adams and colleagues (2004) reported that both cholesterol and 25-OHC resulted in inhibition of cleavage of the SREBP/SCAP complex (figure 1.6). However, unlike cholesterol, which produces a conformational change in SCAP leading it to bind to INSIG, 25-OHC did not do this. Thus they concluded that 25-OHC binds to some unknown intermediate molecule, which in turn bound to SCAP. Earlier work had discovered that 25-OHC binds to an unknown molecule, which is probably a protein (Kandutsch et al., 1977). In 1985, Taylor and Kandutsch discovered a protein that bound 25-OHC and this was termed oxysterol-binding protein (OSBP). Several OSBPs have now been characterised and it is thought that OSBP1 is implicated in the actions of 25-OHC (Shibata & Glass, 2010). However, changes in the expression of OSBP1 failed to alter cell responses to 25-OHC, suggesting OSBP1 may not be critical to the function of 25-OHC (Adams et al., 2004).

To examine the role of OSBP further, Nishimura et al., (2005) assessed the response of 25-OHC on cholesterol biosynthesis in Chinese hamster ovary cells and discovered that 25-OHC
promotes translocation of OSBP to the Golgi apparatus with subsequent reduction in cholesterol levels. The enzymes that were down regulated were those controlled by SREBP e.g. HMGCR and squalene epoxidase. Furthermore, when levels of OSBP were reduced to 10% of normal, addition of 25-OHC still suppressed cholesterol biosynthesis confirming that OSBP is not necessary for the actions of 25-OHC (Nishimura et al., 2005). It may be that oxysterols, including 25-OHC, bind directly to INSIG (Radhakrishnan et al., 2007). Alternatively Ning and colleagues (2006) reported that 25-OHC might regulate steroidogenic acute regulatory (StAR) protein. This protein is found in the liver, adrenal glands and also in endothelial cells. StAR is involved in the intracellular transport of cholesterol from outside of mitochondria to the inside. Hence, cholesterol can then be converted to steroids or bile acids depending on the location of the cell.

Little is known about the specific regulation of the enzyme CH25OH and its end product, 25-OHC. Cholesterol-25 hydroxylase (CH25OH) is a member of enzymes that use diiron cofactors (Lund et al., 1998). Expression of ABCA1 transporter on human embryonic cells leads to increased efflux of 25-OHC and reduction in the mRNA levels of genes involved in cholesterol synthesis (Tam et al., 2006). The formation of oxysterols and subsequent down-regulation of genes involved in cholesterol synthesis may protect cells from the toxicity of cholesterol. Indeed it may also protect from the toxicity of oxysterols, as there has been some evidence that oxysterols can lead to cell apoptosis probably mediated by calcium-dependent mechanisms. Oxysterol cell toxicity appears to be selective to some oxysterols, and has not been demonstrated for 25-OHC in human cells (Lemaire-Ewing et al., 2005). However Perales et al., (2009) and Liu et al., (2009) have demonstrated apoptosis with 25-OHC but only in non-human cells.

1.8.5 Lipoproteins
Lipoproteins facilitate the transport of lipids, which owing to their hydrophobic nature would not otherwise be able to circulate in the plasma. Lipoproteins consist of a variety of molecules whose basic structure consists of an outer hydrophilic surface and an inner core, which can carry immiscible hydrophobic lipids e.g. CE and TG (Rifai et al., 2000). The surface layer consists of a phospholipid bilayer, non-esterified cholesterol, and apolipoproteins, which have various functions such as, cofactors, receptor ligands, and transfer proteins. There are five main classes of lipoproteins based on ultracentrifugation methodology which separates the groups according to density: Chylomicrons (CM), Very-low density lipoproteins (VLDL), Intermediate density
lipoproteins (IDL), Low-density lipoproteins (LDL), and High-density lipoproteins (HDL). Passing from CM to HDL particles, the density increases and particle size decreases (see table 1.2; Socco & Breslow, 2004). Each class possesses a number of subclasses and the clinical significance of these subclasses is only recently being recognised for example, particular LDL and HDL particles have a lesser or greater impact on CVD risk.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (kg/L)</th>
<th>Particle diameter (nm)</th>
<th>Electrophoretic mobility</th>
<th>Primary apolipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.95</td>
<td>80-1200</td>
<td>Origin</td>
<td>Apo B48</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95-1.006</td>
<td>30-80</td>
<td>Pre-beta</td>
<td>Apo B100</td>
</tr>
<tr>
<td>Very low density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>23-35</td>
<td>Broad beta</td>
<td>Apo B100</td>
</tr>
<tr>
<td>Intermediate density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>18-25</td>
<td>Beta</td>
<td>Apo B100</td>
</tr>
<tr>
<td>Low density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>5-12</td>
<td>Alpha</td>
<td>Apo AI, Apo AII, Apo AIV</td>
</tr>
<tr>
<td>High density lipoproteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The various lipoprotein classes and characteristic features of each group. The last column lists the primary apolipoproteins (APO) found on the lipoprotein classes.
1.8.6 Apolipoproteins
Apolipoproteins are proteins expressed on lipoproteins that help transport lipoproteins to appropriate tissues and form the necessary cell-apolipoprotein interactions, which result in the lipoprotein contents being transferred to tissues (Rifai et al., 2000). Apolipoproteins exist as both lipid poor and lipid rich, and are mostly produced by the liver and the gastrointestinal tract. There are numerous classes and subclasses of apolipoproteins with new ones being recognised daily and yet very little is known regarding the majority, both in terms of metabolism and clinical significance (Duffy & Rader, 2006). In LDL, VLDL and CM apo B forms the largest group of apolipoproteins whereas, HDL particles especially of HDL₃-C and HDL₂-C subclasses contain mainly apo AI and also significant amounts of apo E (Rifai et al., 2000).

Apo AI is the most abundant of apolipoproteins in both plasma and tissue, and has a half-life of 5-6 days (Qiu & Hill, 2009). Apo AI has various isoforms and genetic mutations have been described such as, apo AI Tangier, apo AI Milano and apo AI Marburg (Walldius & Jungner, 2007). Apo AI begins as a precursor peptide called preproapo A which following cleavage of the N-terminal peptide results in proapo AI, which is secreted into the circulation especially after meals (Ajees et al., 2006). Subsequent to this the mature apo AI is formed by removal of the six amino acid N-terminal propeptide (by a calcium-dependent protease). Apo AI has an N-terminal four-helix bundle and two C-terminal helices containing positively and negatively charged areas which assist in its interaction with cell-surface receptors crucial for the RCT pathway (Ajees et al., 2006).

Apo AI like most other apolipoproteins, has a detergent like quality with non-polar groups being directed towards hydrophobic core lipids and polar groups, which interact with water molecules facing the plasma (Rifai et al., 2000). Apo AI not only has a structural role in accepting and promoting cholesterol transfer from tissues to HDL particles by binding with ABCA1 and ABCG1, it also activates the enzyme LCAT leading to esterification of FC producing an enlarged cholesterol filled HDL particle (Shao et al., 2008). These functions of apo AI are thought to underlie its anti-atherosclerotic properties. The antioxidant, anti-inflammatory and antithrombotic activities ascribed to HDL may also be mediated by apo AI (Walldius & Jungner, 2007).

Apo B on the other hand, is present in CM, VLDL, and LDL and is central to receptor binding of these particles (Rifai et al., 2000). Apo B is most abundant in LDL (90%) with a serum
concentrations around 50 - 180 mg/dl, whilst the remaining 10% is within VLDL particles (Richardson et al., 2005). Apo B is increased when LDL-C is also raised and premature atherosclerosis has been noted in patients with elevated apo B levels (Soccio & Breslow, 2004). Unlike apo AI, apo B is not exchanged between particles meaning that isotope-labelling studies are unhelpful. In addition, Apo B studies are hindered by its bulky size, insolubility, and tendency to aggregate (Rifai et al., 2000).

There are two main isoforms of apo B, apo B48 is made in the small intestine and consists of 48% of the sequence of apo B100 which is manufactured in the liver (Walldius & Jungner, 2007). Apo B48 and apo B100 thus share the same N-terminal but apo B48 lacks the C-terminal LDL-R binding region. They are coded for by the same gene and factors leading to the production of either isoform are thought to depend on either a trans-acting tissue-specific splicing gene or a cis-acting element upstream (Richardson et al., 2005). The apo B gene is marked by one of the longest RNA sequences known to date and this leads to a large molecule, with apo B100 estimated to have a MW of 500,000 Da (Soccio & Breslow, 2004). It is likely at this size, that there is only one molecule of apo B per LDL particle and it follows then that there is only one receptor-binding site. When VLDL is converted into LDL and transferring its lipid core, apo B undergoes a conformational change, which exposes the receptor site and allows binding to the LDL-R to take place (Rifai et al., 2000). The conformation changes vary amongst the different subclasses of LDL particles such that small dense LDL binds less to the LDL-R (Holejwin et al., 2010).

Cardiovascular risk predictors commonly focus on total cholesterol or LDL-C levels, but lipid ratios such as, LDL-C/HDL-C, may yield better risk prediction for a given patient (Walldius and Jungner, 2007). Similarly, apolipoprotein levels and ratios may also help determine CVD risk prediction, and they may be superior to using absolute LDL-C, TC and HDL-C levels. Apo AI levels are negatively correlated with CVD (e.g. Durrington et al., 2006; Qureshi et al., 2002; Möller et al., 2006; Walldius et al., 2001), and apo B positively correlated and an even better risk predictor than LDL-C (Lamarche et al., 1996; Holejwin et al., 2010). Even better risk prediction can be determined using the apo B/apo AI ratio (Walldius et al., 2001; Corsetti et al., 2004; Walldius et al., 2004; Holejwin et al., 2010).

There are several other apolipoproteins (see table 1.3), although very little is known about some of them at present.

65
<table>
<thead>
<tr>
<th>Apo</th>
<th>Tissue</th>
<th>Associated lipoprotein</th>
<th>Function</th>
<th>Other notes</th>
<th>References</th>
</tr>
</thead>
</table>
| L   | Pancreas, Liver, Kidney, Brain | HDL | Unknown | · Not found in free form in plasma  
· Higher in ↑TC, ↑TG, CETP deficiency, and diabetes mellitus | Duchateau et al., 1997 |
| CIII | Intestine, Liver | Apo B-containing lipoproteins and HDL | Inhibits the hepatic uptake of TG rich lipoproteins | · Levels correlate with TG and reduced LPL activity and presence of metabolic syndrome  
· May predict risk of CHD | Ooi et al., 2008  
Gangabadage et al., 2008 |
| H   | Intestine, Liver, Brain, Heart, Kidney | TG rich lipoproteins (No association with HDL or LDL) | Enhances procoagulation TG rich lipoprotein metabolism via LPL | · Associated with DM and metabolic syndrome and antiphospholipid syndrome | Polz & Kotsner, 1979  
Nimpf et al., 1985  
Crook, 2010 |
| E   | Liver, CNS, Adipose | TG rich lipoproteins | Hepatic uptake of TG rich lipoproteins | · Added once TG rich lipoproteins are hydrolysed by LPL  
· Binds to LDL-R more efficiently  
· Various alleles | Heeren et al., 2006  
Lahoz et al., 2001 |
| J   | Liver, Aorta, CNS, Eye | HDL subclasses | Lipid transport  
Complement system  
Cell death | · Higher levels in CHD | De Silva et al., 1990  
Burkey et al., 1992  
Poulakou et al., 2008 |
| AII | Liver, GIT | HDL | Metabolism of TG rich lipoproteins | · Overexpression leads to a proatherogenic state | Castellani et al., 1997  
Castellani et al., 2008 |

Table 1.3 The various apolipoproteins and their characteristics.
1.8.7 Cholesterol metabolism
There are three main pathways of cholesterol metabolism (Rifai et al., 2000):

- **Exogenous pathway** – the transport of dietary lipids to the liver.
- **Endogenous pathway** – transport of lipids synthesized in the liver to the peripheral tissues.
- **Reverse cholesterol transport (RCT)** – transport of cholesterol from the peripheral tissues to the liver.

**Exogenous pathway**
There are several dietary sources of cholesterol and the more well known include eggs, meat and dairy products (Rifai et al., 2000). Dietary consumption consists largely of TG with an average consumption of cholesterol around 0.5 g compared with 100 g of TG (Brody, 1999). Lipids from the diet are mostly absorbed at the small intestine (30 - 60%), the majority being in the form of TG, the rest being lost in the faeces (Soccio & Breslow, 2004). Cholesterol is absorbed as CE, which is hydrolysed by cholesterol esterase producing FC and fatty acids. Dietary TG undergo hydrolysis by lipases (from the pancreas and intestine) producing FFA and monoglycerides (Rifai et al., 2000). These lipids are packaged into CM and are taken to the liver where they undergo metabolism. The resulting cholesterol is then integrated into VLDL and released in the circulation along with some CM.

In the circulation VLDL and CM undergo remodelling (figure 1.7), largely by interacting with lipoprotein lipase (LPL) on the endothelium leading to partial hydrolysis of the lipid content (producing either monoglycerides or glycerol and three non-esterified fatty acids) and producing VLDL remnants (IDL; Duffy & Rader, 2006). VLDL and CM remnants are finally taken up by hepatocytes via the apolipoprotein B/E receptor (better known as the LDL-R). Another route involving binding to a LDL-R-related protein may take up a few CM remnant particles. Approximately 20% of VLDL does not pass to the liver but undergoes further remodelling by for example, hepatic triglyceride lipase (HTGL) leading to one source of LDL particles (Brody, 1999). These LDL particles are depleted of TG but rich in cholesterol and are internalised by cells through the LDL-R. Thus, both VLDL and LDL are means of delivering cholesterol to peripheral tissues. Internalisation of LDL-C allows CE to be liberated and consequently the
amount of cholesterol entering cells is dependent on the expression of LDL-R (Rifai et al., 2000).

The LDL-receptor (LDL-R) is a glycoprotein made at the ER, which sits at the cell surface and consists of two asparagines-linked oligosaccharide chains (Yvan-Charvet et al., 2010). It is found on all cultured mammalian cells and in vivo high concentrations are present in the liver, adrenal cortex and ovarian corpus luteum. Levels are increased in the presence of atheroma. The LDL-R participates in receptor-mediated endocytosis and can bind two proteins (Brody, 1999):

- Apolipoprotein B (apo B) 100 the predominant apolipoprotein on LDL particles.
- Apolipoprotein E (apo E) which is found in IDL and a subclass of HDL (multiple copies of apo E bind with higher affinity to LDL-R than a single apo B).

It takes 45 min following LDL-R synthesis for it to appear at the cell surface where LDL-C binds to LDL-R (through apolipoproteins) and is then internalized by clathrin-coated pits (Soccio & Breslow, 2004). The coated pits will promptly invaginate forming endocytic vesicles containing LDL-C bound to LDL-R. These vesicles will shed their coats and merge with endosomes, where the relatively acidic environment leads to dissociation of LDL-C from LDL-R and results in hydrolysis of the CE yielding FC (Soccio & Breslow, 2004). This is followed by recycling of the LDL-R and associated proteins, with eventual return of the LDL-R to the plasma membrane.

The LDL-R expresses a high affinity for binding LDL-C and this leads to its ability to cycle multiple times in and out of the cell, each round-trip taking approximately 10 min (Brody, 1999). The net result of this is that large amounts of cholesterol can be taken up by cells in a relatively short period (Brown & Goldstein, 1986). High LDL-R expression is necessary in the liver as impairment of hepatic LDL-R promotes the development of premature atherosclerosis. Interestingly, a high level of LDL-R expression also occurs on the vascular endothelium e.g. aortic, cerebral and hepatic, and rapid uptake of LDL-C is seen in endothelial cells in culture, yet it remains unclear as to how this is regulated in vivo or what role it serves (Cuchel & Rader,
Endothelial cells take-up LDL-C via the endocytosis process described above, but it is not clear whether other intimal cells can also do the same.

Figure 1.7 Interactions of the various lipoproteins.

**Endogenous pathway**

The endogenous pathway results from endogenous production of lipids within the liver. Lipids produced by the liver undergo 3 fates (Brody, 1999): firstly cholesterol and TG can be exported in VLDL particles as described above in the exogenous pathway. Secondly, lipids maybe stored within the hepatocytes if excess is produced and lastly, cholesterol can be eliminated in bile either unchanged or once oxidised into bile acids. Cholesterol levels are under tight regulation and it is likely that increased activity in the exogenous pathway leads to down-regulation of activity of the endogenous pathway.
**Reverse cholesterol transport**

Glomset first introduced the idea of reverse cholesterol transport (RCT) in 1968 and the following is one definition:

“…[RCT]…describe(s) the process by which extrahepatic (peripheral) cholesterol is returned to the liver for excretion in the bile and ultimately the faeces”, (cited from Cuchel & Rader, 2006).

This process is necessary for non-hepatic cells, as excessive cellular cholesterol is toxic to cells (Soccio & Breslow, 2004). Furthermore, these non-hepatic cells are unable to catabolise cholesterol thus they need a method for removal of cholesterol to protect them. Movement of cholesterol from peripheral tissues requires energy and the availability of a lipid-poor receptor (Rifai *et al.*, 2000). This process tends to occur in extracellular spaces, such as, the intima of blood vessels (Duffy & Rader, 2006).

HDL particles begin as lipid free or lipid poor apo AI particles which are made in the liver and the small intestine and are discoidal in shape – thus the terms nascent HDL, discoidal HDL and pre-beta HDL are all used interchangeably (Rifai *et al.*, 2000). These particles are thought to be largely non-functional and immature. Pre-beta HDL is derived from three sources (Rifai *et al.*, 2000; Soccio & Breslow, 2004):

1. *De novo* synthesis within the liver and intestine.
2. Small intestine via the hydrolysis of TG rich particles (CM).
3. From HDL₂ and HDL₃ in the circulation following contact with cholesteryl ester transport protein (CETP), HTGL or phospholipid transfer protein (PLTP; which transfers phospholipids from TG rich lipoproteins to HDL).

Pre-beta HDL is unstable and will readily take up cholesterol and phospholipids from cells. This uptake occurs with the assistance of enzymes such as, LCAT and by utilising the ATP-binding cassette transporters (Cuchel & Rader, 2006). The liver is the most important organ for lipidation of lipid-poor apo AI via ABCA1. Plasma HDL does not necessarily depend on the ABC group of transporters and there is evidence of the existence of other pathways for cholesterol efflux to HDL as a lack of ABCG1 does not completely abolish efflux of cholesterol to HDL (Cavelier *et al.*, 2006). For example, macrophage deficiency of ABCA1 has little effect
on plasma HDL-C but increases atherosclerosis is seen in mice. On the other hand, liver
deficiency of the same transporter is associated with reduced plasma HDL-C (Schmitz &
Langmann, 2001).

Once HDL is released into the circulation, it undergoes maturation and remodelling (Rifai et al.,
2000). HDL takes up further cholesterol that is esterified by LCAT leading to large spherical
shaped HDL$_2$. This consists of a neutral lipid core of CE and TG. This large HDL$_2$ can transfer
CE and TG to apo B-containing lipoproteins namely, LDL, IDL, and VLDL (all pro-
atherogenic) which requires CETP. CETP circulates in the plasma bound to lipoproteins and is
manufactured in the liver and adipose tissue and its actions make HDL$_2$ particle into the smaller
HDL$_3$ particle (Soccio & Breslow, 2004).

HDL$_3$ is also produced by uptake of CE by the liver and steroidogenic organs via the interaction
with SR-B1 and the enzyme HTGL (Duffy & Rader, 2006). Furthermore, endothelial lipase can
also cause hydrolysis of the TG rich core of HDL$_2$ leading to HDL$_3$. Endothelial lipase was first
described in 1999 being synthesized by endothelial cells of the liver, lung, kidney and placenta
(Jaye et al., 1999). It primarily hydrolyzes HDL-phospholipids leading to non-esterified fatty
acid and a consequent reduction in HDL particle size and also modulates the elimination of
HDL-C (Qiu & Hill, 2009). Eventually HDL reaches the liver where it combines with apo B
containing lipoproteins or with SR-B1 (Fredenrich & Bayer, 2003; Lewis & Rader, 2005;
Cuchel & Rader, 2006). HDL can thus be cleared in two ways:

- Via selective removal of cholesterol and other lipids (selective cholesterol uptake) or
- Endocytic uptake and degradation of the whole particle along with the apolipoproteins
  (termed holoparticle HDL uptake).

The latter takes place mainly in the liver and kidney (Meuwese et al., 2006). Cholesterol in the
liver is excreted into the bile of which 50 – 80% is then reabsorbed at the terminal ileum. This
cholesterol can then be repackaged into CM or effluxed by ABCA1 to lipid-free apo A1
(Cavelier et al., 2006). There is considerable interest in the mechanisms underlying this
reabsorption to aid discovery of methods to block this recycling process (Soumian et al., 2005).
**HDL subclasses**

The two predominant HDL subclasses are: HDL\(_2\) and HDL\(_3\), which can be further subdivided into HDL\(_{2a}\), HDL\(_{2b}\), HDL\(_{3a}\), HDL\(_{3b}\) and HDL\(_{3c}\) – the latter being the smallest in size (Walldius & Jungner, 2007). Clinically the most relevant division is into HDL\(_2\) and HDL\(_3\), but this may change as the functions of the subdivisions are discovered (Walldius & Jungner, 2007). HDL\(_3\)-C is associated with increased levels of atherosclerosis, for example HDL\(_3\)-C levels are strongly associated with angiographically defined CAD (Wallentin & Sundin, 1986), whilst HDL\(_2\)-C is associated with both protection against CAD (Salonen et al., 1991; Bakogianni et al., 2001) and improved risk assessment in patients at increased risk (Fellin et al., 1985). Levels of HDL\(_2\)-C are also reported to be higher in pre-menopausal women, which might account for the protection pre-menopausal women have from CVD (Meilahn et al., 1991; Ushiroyama et al., 2005) and are also lowered by cigarette smoking (Nesje & Mjøs, 1985). Furthermore, HDL\(_2\)-C levels correlate with lipoprotein lipase and can be converted from HDL\(_3\)-C (Taskinen & Nikkilä, 1981). However both subclasses have been positively correlated with CVD (Sweetnam et al., 1994), which may be the result of differing methods for measuring the individual subfractions.

**Atherosclerosis and lipoproteins**

There is a strong relationship between CAD and LDL-C levels and the risk is continuous with rising LDL-C (Davignon & Ganz, 2004). Aggressive lowering of LDL-C is a major therapeutic strategy currently used in both primary and secondary prevention of cardiovascular disease with HMGCR inhibitors (statins) reducing both morbidity and mortality in a number of trials (e.g. the Air Force/Texas Coronary Prevention Study, the West of Scotland Coronary Prevention study, the Scandinavian Simvastatin Survival Study and the Heart Protection Study). But statins only reduce cardiovascular events by 20-40% (Tall et al., 2008), with the greatest benefit in patients when treatment is combined with agents to increase HDL-C. Furthermore, patients with the lowest HDL-C levels have the highest event rates despite statin treatment (Superko & King, 2008). This has produced interest in developing new drugs and drug targets to reduce cardiovascular risk such as, the elevation of HDL-C.

However, it may not necessarily be quantitative defects in HDL-C, which are important in CVD, but rather qualitative defects (Duffy & Rader, 2006; Kontush & Chapman, 2006). This may explain some of the disappointing and varied results from trials that have so far been undertaken to boost HDL-C levels. HDL-C undergoes alterations in its structure and function in the
presence of inflammation. This leads to altered activity of the HDL particle called “dysfunctional HDL”, the presence of which may actually be pro-atherogenic (Superko et al., 2009). Strategies to enhance HDL-C levels may help prevent CVD in addition to therapies to reduce LDL-C. This is made even more important as some patients will fail to achieve target LDL-C levels with statins and some tolerate statins poorly (Superko & King, 2008).
Chapter 2: Experimental studies to evaluate the actions of flavonoids on endothelial cells
2.1 Background
This section describes studies undertaken to determine whether OPC have any effect on endothelial cell cholesterol metabolism. Initial studies used an OPC-rich GSE which contains approximately 80% OPC but the proportion of the various oligomer sizes has not been described. Thus to avoid variability in endothelial responses that may result from a mixture of OPC, subsequent experiments were conducted with purified apple procyanidins of defined size mainly trimer and tetramer and pentamer fractions. The results are presented here in the order in which they were undertaken i.e. GSE experiments followed by those using apple derived OPC.

BAEC were used for the studies described in this chapter because of their highly reproducible response to dietary polyphenols including OPC (Corder et al., 2001; Corder et al., 2004; Corder et al., 2006; Pothecary, 2007; Caton et al., 2010). In addition the responses obtained with BAEC bear a close similarity to those obtained with HAEC (Corder et al; 2007; Pothecary, 2007), whereas HUVEC have a reduced and more variable response to OPC (García-Conesa et al., 2009; and unpublished observations), which limits their suitability for investigating mechanisms linked to atherosclerosis. BAEC are also suitable for studying cholesterol gene regulation (O’Connell et al., 2004). Nevertheless, when conducting these investigations the limitations in using BAEC and other cells in culture have to be recognised. The most important of which are significant changes in channel, calcium handling and receptor function in cultured cells compared with the intact system and species related differences in terms of cell regulation and response to stimuli (Sandow & Grayson, 2009).

The following cellular experiments were performed:

- Changes in the relative levels of CH25OH mRNA expression after exposure to GSE over short and long-time periods. This consisted of three sets:
  - GSE at 10 µg/ml in 24 well plates incubated at 37°C for 15, 30, 60 and 150 min compared with medium alone.
  - GSE at 5 µg/ml in 12 well plates incubated at 37°C for 0, 30, 60, 120 and 240 min compared with medium alone.
  - GSE at 10 µg/ml in 12 well plates incubated at 37°C for 2.5, 6 or 24 h compared
with medium alone.

- Changes in the level of HMGCR mRNA expression (using three different sets of primers for HMGCR), when BAEC were exposed to GSE at two different concentrations (5 µg/ml and 10 µg/ml) over short-time periods (15 – 150 min and 30 – 240 min).

- Changes in the level of ABCG1 mRNA expression when BAEC are exposed to GSE (10 µg/ml) over 30 – 240 min compared with basal levels of mRNA at each time point.

- Changes in the levels of CH25OH mRNA expression after an initial 30 min incubation with either DMEM alone or MnTMPyP (10µM), a superoxide dismutase mimetic. This was followed by the addition of either DMEM or GSE (10 µg/ml) and incubated at 37ºC for a further 1 h.

- Changes in the levels of both HMGCR and ABCG1 mRNA expression after exposure to 25-OHC at three concentrations (1, 3 and 10µM) over 0, 2, 6 and 24 h.

- Changes in the levels of HMGCR, ABCG1 and CH25OH mRNA expression with varying degrees of polymerization of apple OPC (5 µg/ml) over 6 and 24 h. Oligomer sizes consisted of: trimers, tetramers, pentamers and whole apple extract (mixture of procyanidins ranging in size from monomers to decamers).

- Changes in the levels of HMGCR and ABCG1 mRNA expression in the presence of apple OPC tetramer or pentamer (both at 5 µg/ml) alone or with 25-OHC (3µM), incubated over 6 and 24 h.

Also in some experiments the cell media was collected and retained for subsequent ET-1 measurement. Further experiments were conducted to try and determine the effects of apple OPC on in vitro cellular cholesterol efflux. BAEC were pre-loaded with LDL-C and in some HDL-C was included to act as an acceptor of cholesterol. Again the mRNA expression of ABCG1, CH25OH and HMGCR was determined along with the collections of cell media for ET-1 and cholesterol measurements. The following sets of experiments were undertaken:
• Pre-treatment of BAEC with LDL-C (1 mg/ml final concentration) for 24 h and subsequent exposure to
  
  o Apple procyanidin pentamer (AP5; 5 µg/ml), 25-OHC (3 µM), a combination or no treatment.

  o HDL-C (20 µg/ml final concentration), AP5(5 µg/ml), 25-OHC (3 µM), a combination or no treatment.

In a separate experiment BAEC were pre-treated with atorvastatin (0.3 µM), a HMGCR inhibitor for 24 h and subsequently exposed to AP5 (5 µg/ml), 25-OHC (3 µM), a combination or no treatment.
2.2 Methodology

Materials

APS, acrylamide/bis, bromophenol blue, BSA, butan-2-ol, cholesterol, cholesterol esterase, cholesterol oxidase from Streptomyces species, cholic acid, chymostatin, DMEM, DTT, di-sodium hydrogen orthophosphate 12-hydrate, EDTA, glycerol, glycine, horseradish peroxidase, leupeptin, N-ethylmaleimide, NP-40, PBS, pepstatin A, phenazine methosulfate, phenol red, Polypep, potassium dihydrogen phosphate, phosphatase inhibitor cocktail 2, potassium bromide, SDS, sodium azide, sodium bicarbonate, sodium carbonate, sodium chloride, sodium fluoride, sodium deoxycholate, sodium dihydrogen orthophosphate dehydrate, surfactant solution, TEMED, Tris-HCl, Triton X-100, Tween-20 were all purchased from Sigma. 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red®) was purchased from Cambridge Biosciences (manufactured by Biotium Inc). Atorvastatin was a gift from Pfizer, milk protein was purchased from a local supermarket (Marvel®, Cadbury, UK), Rainbow marker from GE healthcare, methanol from vWR, primary antibody ABCG1 from AbCam (Cambridge), and goat anti-rabbit antibody from Jackson Immuno Research. QPCR master mix with ROX dye (5- or 6-carboxy-X-rhodamine) was from Thermo Scientific, Neutravidin-HRP was purchased from Perbio scientific, Ribogreen was from Invitrogen and the Absolute® RNA microprep kit was from Stratagene.

Tris EDTA buffer, nuclease free water, and reverse transcriptase system were all from Promega. GSE is manufactured in powder form and was obtained from Polyphenolics, a division of Constellation Wines (Madera, CA). GSE was >97% pure polyphenols consisting of 10% catechin monomers, 78% procyanidin oligomers and 12% polymers. It was prepared as 10 mg/ml stock solution (in 20 mM ascorbic acid), and stored at -80°C. Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) was purchased from Axxora, LLC, San Diego. 25-OHC was purchased from Sigma-Aldrich and stored at -80°C as a 10mM stock solution in 100% ethanol. Purified apple procyanidins as single oligomer sizes (trimers, tetramers, and pentamers) were isolated from unripe apples and purity confirmed by HPLC analysis (Malus pumila cv. Fuji) and provided by Asahi breweries, Japan (Shoji et al., 2006). Where not specified, materials and reagents were purchased from Sigma.
2.2.1 Cell culture
Clonal cell lines from primary cultures of BAEC were derived by single-cell culture methods (Corder & Barker, 1999). BAEC were cultured in Dulbecco's modified Eagle's medium (DMEM) in 6, 12 or 24 well plates (Multiwell), containing 10% (v/v) foetal bovine serum and 100 IU/ml penicillin-streptomycin. Cell cultures were sustained in a humidified incubator at 37°C with 5% CO₂ and were sub-cultured every 3 – 4 days, with experiments being undertaken on cells up to passage nine. Cells were incubated and grown to 80 - 85% confluency, which was confirmed by visual inspection using a binocular microscope prior to each experiment. BAEC were washed with DMEM after aspiration of the growth medium and the various treatments applied. Following the appropriate incubation period cells were re-examined microscopically, and again washed with DMEM before lysis buffer was added. Cells were then frozen at -80°C until RNA extraction was performed.

At the end of the respective incubation periods, the conditioned media was aspirated and lysis buffer added to the plates. The medium from some experiments was retained for ET-1 and cholesterol assays. Both the medium and plates were stored at -80°C for analysis later. The control vehicle for the majority of studies consisted of DMEM with ascorbic acid (which is required as a preservative for OPC) and in studies where 25-OHC was applied ethanol was also added to the control treatments (as 25-OHC is stored in ethanol).

2.2.2 Quantitative real time-polymerase chain reaction
Quantitative real time-polymerase chain reaction (q-RT PCR) was used to determine gene and/or transcript numbers in samples as it is a highly sensitive and reproducible method (Smith & Osborn, 2009).

RNA extraction
Total RNA from each culture dish was extracted using Absolute RNA® Microprep Kit (Stratagene) according to the manufacturer’s instructions. On the day of RNA extraction plates were removed from the freezer and kept on ice. Once thawed the lysate in each well was mixed by gentle pipetting, and then transferred into eppendorfs. This was followed by the addition of an equal volume of 70% ethanol. Samples were then transferred into a spin cup and RNA was subsequently extracted by various washes with high and low salt buffers with centrifugation, following the manufacturer’s instructions. Each sample was treated with DNase solution and
incubated for 15 min at 37°C. Samples were washed further with high and low salt buffers, until finally elution buffer was added and the resultant filtrates collected and frozen at -80°C.

**RNA quantification and cDNA**

Extracted total RNA was quantified with ribogreen at two different dilutions (1:75 and 1:150). RNA was measured by fluorescence using a CytoFluor™ II Fluorescence Multi-well plate reader (PerSeptive Biosystems). The results were then quantified using a RNA standard curve (0 – 1000 ng/ml). cDNA was prepared from the RNA primed with oligo-dT primer, using the reverse transcription system (Promega) following the manufacturer’s instructions.

**Primer design**

Primers and probes (if needed), were designed using Primer Express Software version 2.0 by myself. Primers were based on a PubMed search for published bovine DNA sequences of genes of interest i.e. ABCG1, HMGCR, CH25OH, and RP2 (table 2.1). All primers met the following criteria: length between 15-20 base pairs; melting temperature 69 – 70°C for TaqMan probe and 58 – 60°C for SYBR green; GC content between 20 – 70%; GC; avoidance of runs of identical nucleotides especially of guanine; and avoidance of guanine at the 5´ end. In addition when a TaqMan probe was used it was kept as short as possible, with T_m 10°C greater than primers and a G-C content of more than 50%. The probe was identified first and then the primers were chosen to be as close to the probe as possible without overlapping it.

For HMGCR three sets of primers were designed and used (table 2.1), and HMGCR primer set 2 consisted of primers which were extreme 3´ at positions 515F and 579R. After selecting the best possible sequences, a BLAST search was performed for all genes and no sequence homology with other known genes in the bovine sequence database were detected. All primers and probes were optimised before use.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Probe sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH25OH</td>
<td>CACAAGATGCAC</td>
<td>CCCCGACGCTCATGTACTG</td>
<td>TCCCCGTTCGCGCTGTCCA</td>
<td>XM_608003</td>
</tr>
<tr>
<td>HMGCR set 1</td>
<td>TACAGACAAGA</td>
<td>GACTTTTCCTCGTCCTCTATCCA</td>
<td></td>
<td>NC_007308</td>
</tr>
<tr>
<td>HMGCR set 2</td>
<td>AGCCAAGTTTCGC</td>
<td>CCAATCCCACGAGCCTCTCA</td>
<td></td>
<td>NC_007308</td>
</tr>
<tr>
<td>HMGCR set 3</td>
<td>TGGCTTGTGCTGTGTTG</td>
<td>ATTCATGCGGTGCCTTCAC</td>
<td></td>
<td>NC_007308</td>
</tr>
<tr>
<td>ABCG1</td>
<td>AGATGAGCG GTGTTCTCTCA</td>
<td>CTCAGGCTTTGTTGCTTCAC</td>
<td></td>
<td>NC_007299</td>
</tr>
<tr>
<td>RNA polymerase 2</td>
<td>CGATAAAGAAGG</td>
<td>TCGTGAGAATGC</td>
<td></td>
<td>XM_596010.1</td>
</tr>
<tr>
<td></td>
<td>CCAAGCAGG</td>
<td>GGTTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 CH25OH, HMGCR, ABCG1, and RP2 primer (and probe) sequences and accession numbers.

**Primer and cDNA optimisation**

Primer concentrations were optimized by varying forward and reverse primer concentrations (range 50 – 900 nM). qRT-PCR was performed on these samples and concentrations providing optimal assay performance determined. This was achieved by selecting primer concentrations that provided the lowest $C_t$ (threshold cycle) and highest $Rn$ (the reporter signal normalized to the fluorescence signal of ROX) for a fixed amount of target template (figure 2.1a and 2.1b). The response to varying concentrations of cDNA was linear and thus concentrations of 1 – 10 ng/µl of cDNA were used for all qRT-PCR. For CH25OH measurements TaqMan based methodology was used and the probe was also optimised using varying concentrations (range 125 – 1125 nM).
Figure 2.1 Example of data plot for primer optimisation using qRT-PCR. Logarithmic data plot used to optimise cDNA during qRT-PCR (a) and resultant standard curve when optimising HMGCR cDNA (b). Plot shows amplification curves for varying cDNA concentrations (samples were run in triplicate).

**Quantitative Real Time-PCR**

qRT-PCR was performed in triplicate for each cDNA sample. TaqMan based methodology (composed of a 5´ fluorogenic probe and 3´ quencher) was used for 25-OHC. TaqMan methodology uses a fluorogenic probe and binds only to DNA sequences between the two primers. For HMGCR and RP2, the SYBR green I dye detection system was used. SYBR green, unlike the TaqMan methodology, binds to any double-stranded DNA so results were compared...
with no template controls (NTC) to minimize non-specific product formation using the dissociation curve. The specificity of this experiment was based on primer design, as discussed above. The coefficient of variation for SYBR green was 5 – 7%, whilst that for TaqMan was 3 – 5%.

Absolute QPCR mix (Thermo Scientific) was used with an internal reference dye (ROX; 5- or 6-carboxy-X-rhodamine) to normalize for non-PCR related fluctuations in fluorescence between wells. To expand on this the fluorescence intensity can vary due to the position of the well in question, for example, the outer wells tend to provide a lower signal. The ROX dye is described as passive as it does not partake in the actual PCR process but it provides a baseline fluorescence which allows normalization for signal intensity. Thus the generated signal is determined by the ratio of the fluorescence of the reporter dye to the passive reference dye producing $R_n$. This can be further normalized by calculating the difference between the baseline and template containing sample producing the delta ($\Delta R_n$). The following cycling parameters were used: 2 min at 50ºC, 15 min at 95ºC, 15 sec at 95ºC and 60ºC at 1 min. PCR reactions consisting of 40 cycles, were performed on an ABI 5700 Sequence Detection System (Applied Biosystems).

A standard curve was prepared allowing absolute quantification of transcript, which correlates with total RNA concentration (Bustin, 2000). The standard curve was generated by three serial dilutions and each sample was assayed in triplicate. A line of best fit was generated for the standard curve with a correlation coefficient of greater than 0.90. If this was not achieved then the experiment was repeated. Absolute quantification was performed quantifying the unknowns of interest against the already defined standard curve. Specificity was maximised by good primer design but non-specific amplification is still an issue with SYBR green. Incorporating dissociation curves in the PCR runs did allow determination of the extent of non-specific fluorescent signals.

RNA polymerase 2 (RP2) was used as the reference gene in the experiments described here. This was based on a previous evaluation of different housekeeping genes in endothelial cells treated with polyphenol extracts and procyanidins, which concluded that RP2 was the most stable reference mRNA (Pothecary, 2007). This is also consistent with previous recommendations for its use in qRT-PCR (Radonic et al., 2004). All results were expressed relative to the reference gene and corrected for the mean of the control samples (Bustin, 2000).
Limitations, reproducibility and sensitivity

The accuracy of this methodology relies on good primer design. When problems with PCR were encountered, the design of the primer was reviewed and new sets were ordered if needed. Most primers were designed by the author but also reviewed by the supervisor. Another major problem documented is variation in the expression of the reference gene mRNA, which can lead to masking of real changes or even lead to artificial changes. This is a real problem and one that was also encountered during this work. Wherever possible experiments were repeated to try to resolve the extent of variation in expression of the reference gene mRNA.

An example of primer difficulty was encountered with HMGCR. The first two sets of primers failed to reveal adequate results. The HMGCR gene is highly regulated and is over 24.8 kilobases resulting in an 888-residue protein. A number of years ago there was some evidence suggesting the possibility of a second human HMGCR gene localised on peroxisomes (Breitling and Krisans, 2002). Subsequent data has refuted this and concluded that humans only have one HMGCR gene on chromosome 5, unlike other plants and organisms (Friesen & Rodwell, 2004). However, the potential for differential splicing of mRNA transcripts has not been fully excluded and to-date it is still unknown as to whether this applies to bovine cells too. If differential splicing occurs then quantification of variants would be difficult and require the use of separate sets of primers and standards which are mutually comparable.

Standard curve construction is also crucial to the detectability of the mRNA expression. In this series of experiments, if problems were encountered with the standard curve the experiment was repeated. Repeated freeze thawing of cDNA used for standard curve formation was also avoided. Bustin (2000) had reported earlier the reduced sensitivity of one-step q-RT PCR reactions and thus this approach was circumvented such that the RT reaction was carried out first followed by a separate PCR reaction. However the sensitivity and efficiency of the technique depends on several factors including some of which were not observed here:

- Obtaining high quality RNA extracts – the quality was not tested in the experiments here and in retrospect simple use of agarose gel electrophoresis with spectrophotometry would have helped.
- Reverse transcriptase quality – in the experiments here a robust quality reverse
transcriptase was used which did not have RNaseH activity, which would enhance the quality of any subsequent cDNA produced.

- Reference gene – this should be chosen based on the species and tested. This was not undertaken here and may have led to some spurious results. Also test and reference genes should be ran in the same experiment thus controlling for environmental factors. In some of the experiments this was not always possible as there was not enough space to run both.

Significant variation and non-reproducibility has been reported with the more traditional PCR methodology (Keilholz et al., 1998). Reproducibility is effected by both the efficiency of the reverse transcriptase and the use of two sequential enzymatic steps rather than one. The yield of the amplified product depends on thermal cycling conditions and reagents. All forms of PCR depend on an exponential nature and small quantities of target molecules which means they are prone to large errors when trivial variations in these occur. But real time RT-PCR is less variable than conventional RT-CR procedures with low coefficient of variation reported for \( C_t \) data (< 2% compared with reports of 14% for conventional RT-PCR (Heid et al., 1996; Gerard et al., 1998). The coefficient of variation also varies with the machine used. Other factors influencing reproducibility include distribution statistics (Poisson’s Law; de Vries et al., 1999), low copy numbers and longer amplicons, the latter two reduce reproducibility. In the experiments reported short amplicons were used. Although all RT-PCR runs were performed by the author determination of the coefficient of variation at regular intervals would have helped.
2.2.3 Endothelin-1 immunoassay

ET-1 release is suppressed by OPC, a characteristic that can be used to assess the effectiveness of OPC in experimental settings. For example, a reduction in HMGCR may not be seen which may represent a true result or the fact that the experiment was not correctly performed due to some unforeseen factor. In this setting, it is useful to assess levels of ET-1 production to monitor cell responses. ET-1 was measured using a double-recognition site sandwich ELISA with intra- and inter-assay coefficients of variation of <2% and <5% respectively, with a limit of detection of 1.9 fmol/ml (Corder, 2002).

Methodology

The ET-1 ELISA is described briefly here. The capture antibody was sheep anti-ET_{16-21} IgG and the detection antibody was rabbit anti-ET-1_{1-15} IgG, both developed “in-house” (Corder, 2002). The following buffers were made fresh and stored at 4°C: Capture antibody plate coating buffer - 16 mM sodium carbonate, 34 mM sodium bicarbonate; blocking buffer - 0.5% (w/v) bovine serum albumin, and 0.05% (w/v) Polypep coating buffer. Wash buffer - phosphate buffered saline (PBS); consisting of 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride with 0.05% tween-20. Assay buffer was PBS containing 0.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) IgG (bovine gamma globulin), 0.2% (w/v) Polypep, 0.05% (v/v) Triton X100, and 0.025% (w/v) sodium azide. This was sterile filtered and stored at -20°C. The sample neutralising buffer was also stored at -20°C and consisted of 25 mM potassium dihydrogen phosphate, 10mM EDTA, 0.05% (v/v) Triton X100, and phenol red.

Coating of plates with capture antibody

Black clear bottom Costar 96-well plates were coated with capture antibody, sheep anti ET_{16-21} IgG, in coating buffer overnight at 4°C. Two wells were used for testing non-specific binding (NSB) by pipetting coating buffer alone. To avoid the plate drying out during the procedure the plate was sealed. The following day the contents of the plate were decanted and blocking buffer was incubated for 2 h at room temperature. Once the blocking step was completed the plate was washed three times with wash buffer prior to use for ELISA measurements.

Sample measurement

Detection antibody, biotinylated ET-1_{1-15} IgG, was added to all wells at a 1 in 1200 dilution in sandwich assay buffer followed by ET-1 standard or test samples into appropriate wells. ET-1 standards were prepared in sandwich assay buffer in polypropylene tubes via 1 in 4 serial
dilutions (concentration range from 1.9 – 2000 fmol/ml ET-1). Samples consisted of the medium collected from cell experiments to which sample neutralising buffer was added. For the NSB and 0 fmol/ml wells, sandwich assay buffer alone was pipetted. Standards and NSB were run in duplicate and the plate was sealed and left overnight at 4°C.

**End-point detection**

The plate was washed three times with wash buffer. Neutravidin-HRP diluted in PBS containing 0.5% tween and 0.5 % (w/v) BSA, was added to all wells and the plate covered and incubated for 1 h at room temperature. The plate was then washed four times and substrate (Supersignal ELISA Pico chemiluminescence substrate at a 50/50 mix) added to each well. The plate was covered and mixed for 1 min on the plate mixer. The plate was read within a few minutes of adding the substrate in the scintillation/chemiluminescence counter, Microbeta Trilux plate reader (Wallac, Turku, Finland).

**2.2.4 Studies with LDL-C or atorvastatin pre-treatment**

Cells were pre-treated with either LDL-C or atorvastatin the day before cells reached confluence and incubated at 37°C. 24 h later cell confluence was checked and the various treatment conditions applied and plates incubated for 6 or 24 h. Pre- and post-treatment media was collected and lysis buffer added, before cells were frozen at -80°C until RNA extraction. RNA extraction, quantification, and cDNA were prepared as described above and the levels of HMGCR, ABCG1, and CH25OH mRNA, as appropriate were determined using q-RT PCR. Experiments were also undertaken in 6-well plates to prepare samples for western blotting in some cases.

**Isolation of LDL-C from human plasma**

In order to mimic the situation endothelial cells might face in vivo BAEC were pre-treated with LDL-C isolated from human plasma was based on methodology described by Vieira and colleagues (1996).

After an overnight fast, 60 ml of blood was collected from the volunteer into vacutainer tubes containing lithium heparin. Samples were centrifuged at 3,000 g for 15 min in a refrigerated centrifuge at 15°C. The resulting plasma was removed and adjusted to a density of 1.21 g/ml by adding solid KBr (0.236 g KBr/ml), based on Rifai et al., (2000) in order for lipoproteins with density less than HDL to float to the top i.e. apo B containing lipoproteins. EDTA was added to
give a final concentration of 1 mM and samples gently stirred. 2.8 ml plasma was pipetted into polycarbonate centrifuge tubes (Beckman Coulter, Inc.) and gently overlaid with 6.6 ml phosphate-buffered saline with 1mM EDTA. Tubes were balanced and subjected to ultracentrifugation in a Beckman L80 with a 70.1 Ti fixed angle rotor, at 65,000 rpm at 15°C for 3 h with slow acceleration and deceleration. Once the centrifugation was complete, tubes were removed carefully and placed vertically. The LDL-C fraction, which was a low-orange layer in the upper half of the tube, was carefully removed using a long-stem Pasteur pipette ensuring the VLDL layer at the meniscus remained undisturbed.

Once the LDL-C was collected, it was dialysed overnight at 4°C against 20 mM pH 7.4 containing 130 mM NaCl. The next day the sample was concentrated by ultrafiltration (Amicon® ultra filter centrifugal concentrator, cut-off range 100,000 kD) by centrifugation at 3,000 rpm, at 15°C for 15-20 min. The concentrated LDL-C was then transferred into a falcon tube (VWR International) and sterile filtered using a 0.22 µm pore-size filter (Appleton Woods Ltd). The LDL-C was then aliquoted into cryo-sterile tubes (Appleton Woods Ltd). The protein concentration in the LDL-C samples was determined colorimetrically using the BioRad protein assay. A standard curve was set up with BSA (0 - 12.5 µg/ml) and samples were diluted 1:4 to which BioRad dye reagent was added. The plate was then read at 595nm on a plate reader (Anthos-Hill HTLIII, Labtech Instruments). In order to maintain sterility of the fluid, a small volume of DMEM containing 100 IU/ml penicillin and 100 µg/ml streptomycin was added before storing at 4°C. These are standard antibiotic concentrations used in cell culture experiments with BAEC.

### 2.2.5 Western blotting

Western blotting experiments were performed for ABCG1 protein. BAEC were grown in six well plates and pre-treated with atorvastatin or human LDL-C the day before they became confluent and the various treatments applied, as above. The media was aspirated for ET-1 and cholesterol measurements. Cells were then lysed in ice-cold lysis buffer (consisting of 10mM Tris-HCl, pH 7.4, 1% (v/v) NP-40, 0.25% sodium deoxycholate (w/v), 1 mM EDTA, 5 mM N-ethylamide, 10 µm pepstatin A, 100 µM leupeptin, 100 µM chymostatin, phosphatase inhibitor cocktail 2 (Sigma-Aldrich), 1 mM sodium fluoride, 1 mM phenazine methosulfate) and scraped. Lysates were pipetted into 1.5 ml tubes and sonicated for 5 seconds (Jencons ultrasonic probe, UK) followed by centrifugation at 12,000 rpm (13,845g) for 15 min in a 4°C environment (Sigma 4K10). The supernatant was retained and the pellet discarded.
Protein estimation

Samples were incubated at room temperature for 15 min and protein concentrations determined colorimetrically using the detergent compatible BioRad protein assay system (BioRad, UK; based on the method of Lowry et al., 1951). Standards were prepared from BSA in lysis buffer at concentrations 0.1 – 2 mg/ml. 5µl sample was added to 25µl reagent A (made by adding 25 µl of Reagent S, sodium dodecyl sulphate to 1 ml Reagent A, sodium hydroxide) in a 96-well plate. This was followed by 200 µl Reagent B (Folin reagent) and absorbance was measured at 650 nm on an optical plate reader (Anthos-Hill HTLIII, Labtech Instruments).

SDS-PAGE gel

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (Laemmli, 1970). On the day of the experiment 7.5% resolving gel was prepared from 40% (v/v) acrylamide/bis (Bio-Rad), 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 10 µl N,N,N’,N’-tetra-methylethylenediamine (TEMED, Bio-Rad), and 0.5% (w/v) APS. This was topped with a thin layer of butan-2-ol and left to set over 60 min. The butan-2-ol was removed and 4% stacking gel inserted (consisting of 4% acrylamide/bis, 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.5% (w/v) APS and 10 µL TEMED in 10 ml) along with a 1.5 mm comb. 50 µl of sample protein was added with an equal volume of sample buffer consisting of 62.5 mM Tris-HCL, pH 6.8; 2% (w/v) SDS, 40% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 10 mM dithiothreitol. These samples were boiled for 3 min and then loaded onto the gel alongside a full range rainbow marker. The gel was electrophoresed in running buffer (0.05 M Tris base, 0.384 M glycine, 0.1% (w/v) SDS) initially at 100 V for 20 min to stack the samples, followed by 150 V for 45 min or until the bromophenol blue had run to the bottom of the gel.

Protein transfer

Gels were equilibrated in transfer buffer for 30 min containing 25 mM Tris(hydroxymethyl)methylamine (Tris-base), 0.19 mM glycine, and 15% (v/v) methanol. Proteins were then transferred from SDS-PAGE gel to nitrocellulose Hybond membrane (GE Healthcare, UK) after soaking the membrane for 10 min in transfer buffer, between two sheets of thick blotting paper and sponges in a sandwich cassette, as illustrated by Towbin (Towbin et al., 1979). These were placed in the transfer tank with membranes facing the cathode and ran at 200 mA for 2 h with cooling to 4°C.
**Immunodetection**

The protein transfer was followed by washing in TNT buffer (consisting of 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween-20) for 5 min. This was followed by a 1 h blocking step to prevent non-specific hybridisation to the membrane by gentle mixing in 3% milk protein with TNT buffer. After two vigorous 5 min TNT washes, the primary antibody anti-ABCG1 (AbCam, Cambridge) was applied in a concentration of 1:1000 in a solution of 3% (w/v) milk in TNT buffer and left overnight at 4°C. This concentration of ABCG1 antibody was determined in an initial experiment where concentrations of 1:1000 and 1:2000 were evaluated. After this, membranes were washed 3 times for 10 min each in TNT buffer. The secondary antibody, goat-anti-rabbit (GAR-HRP; Jackson Immuno Research) was applied in a concentration of 1:7500 again in 3% (w/v) milk protein with TNT buffer. This was slowly mixed for one hour, followed by three further 10 min washes with TNT buffer, and rinsed five times with deionised water.

**Development and visualisation**

Chemiluminescent reagent (Supersignal West Pico chemiluminescent substrate; Pierce, USA) was added to the membranes and gently mixed for 5 min at room temperature. The nitrocellulose membranes were exposed to Hyperfilm ECL (GE Healthcare, UK) for visualisation of the proteins. Initially membranes were exposed for 2, 10, 20, and 30 min and an exposure time of 20 min was selected for the best visualisation of proteins. The Hyperfilm ECL was then developed in Kodak D-19 developing solution and Ilford Hypam fixing solution for 90 sec (Agar Scientific Ltd, UK).

**2.2.6 Cholesterol assay**

This assay is based on fluorescence using the reaction scheme shown in figure 2.2. Blood borne lipoproteins contain both cholesterol and CE, making a hydrolysis step essential. This is achieved by cholesterol esterase, the efficiency of which has been reported to be over 99% (Warnick *et al.*, 1982; Khuchareontaworn *et al.*, 2010). This results in FC that is converted to cholest-4-ene-3-one ketone by cholesterol oxidase, yielding hydrogen peroxide as a by-product. Hydrogen peroxide then combines with Amplex red® producing resorufin which is highly fluorescent.
Figure 2.2 Enzymatic reactions for cholesterol quantitation.

Procedure
Five times concentrated reaction buffer was prepared consisting of a phosphate buffer (0.5 M, pH 7.4) prepared from sodium dihydrogen orthophosphate dehydrate (NaH$_2$PO$_4$.2H$_2$O) and disodium hydrogen orthophosphate 12-hydrate (Na$_2$HPO$_4$.12H$_2$O) in deionised water. This was thoroughly mixed and slightly warmed to 50°C for 30 sec to achieve better solubility. Once the buffer had cooled back to room temperature NaCl (0.25 M), 0.5% (v/v) Triton X-100 and finally cholic acid (25 mM) were added. The solution was thoroughly mixed and the volume made up to 50 ml with deionised water. This was stored at room temperature and used within one month. Reaction buffer was used at a 1x concentration for the experiments by diluting with deionised water.

A standard curve of cholesterol solutions was prepared from a 10 mM solution of cholesterol made in 100% ethanol in culture tubes. A further 200 µM solution of cholesterol was prepared by dilution with 1x reaction buffer and doubling dilutions were performed to set up a standard curve (range 0 – 20 µM). Fluorometric cholesterol assay reagent was prepared fresh consisting of 2 U/ml horseradish peroxidise, 2 U/ml cholesterol oxidase from Streptomyces sp., 0.2 U/ml cholesterol esterase and 300 µM 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red®) and protected from light by the use of aluminium foil.

Standards were aliquoted in triplicate and samples in duplicate in 96-well plates and 50 µl of the fluorometric assay reagent was added. The plate was protected in aluminium foil as the Amplex
red® reagent is sensitive to light. The volume of sample to aliquot was determined by varying sample volumes with the same concentration of cholesterol assay enzymes (figure 2.3). It was concluded that 100 µl sample provided the best linear regression plot (based on $r^2$ value). Samples were gently mixed for a few minutes and then incubated at 37°C for 30 min. Fluorescence was measured using CytoFluor™ II Fluorescence Multi-well plate reader (PerSeptive Biosystems; excitation 560/emission 590 and gain 50). Measurement of intra and inter-assay coefficient variation could also be performed on pooled samples with high, medium and low concentrations of cholesterol. In addition diluting the samples to see if the measurements had similar linear regression plots in terms of linearity/correlation and to show that the slope of the line for readings from measurements of the dilutions of each sample were the same as the standards, would have been helpful. Unfortunately this had not been considered at the time of the experiment but would be useful in future experiments.

![Figure 2.3 Levels of fluorescence using standard cholesterol concentrations. Three different volumes of sample were evaluated.](image)

**Cell solubilisation for cholesterol assay**  
Cell extracts from 12-well plates used for some of the above experiments were solubilised in order to determine total cell cholesterol levels. The procedure was as follows: cells were stood on ice and washed twice with 1 ml/well of ice-cold PBS. Cells were then scraped in 300 µl of extraction buffer consisting of 320 mM sucrose, 1 Mm EDTA, 1 mg/ml BSA, 10 mM Tris-HCl,
pH 7.1 and 1% (v/v) Triton X100. The samples were then centrifuged at 1,000 g for 10 min in a
refrigerated centrifuge at 4°C. Samples were cautiously transferred from the centrifuge and the
supernatant was carefully removed and inserted into fresh tubes. The remaining pellet was then
resuspended in a further 300 µl of extraction buffer and vortexted well. Cholesterol levels in both
the initial supernatant and the resuspended solution were then measured using the cholesterol
assay. The results were summated for each sample and expressed as absolute values and
percentage change from baseline.

2.2.7 Statistical analyses
mRNA levels were expressed after correction for the reference gene, relative to the control
levels. Cholesterol levels were compared as absolute values and relative to baseline. All data
was analysed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and by one-way
analysis of variance (ANOVA) using Statview (SAS Institute Inc., Cary, NC). This was
followed by post hoc analysis using Fisher’s least significance difference test.
2.3 Results

2.3.1 Effects of OPC on mRNA levels for CH25OH over short and long time periods

Initial experiments were performed to examine the changes in CH25OH mRNA levels from BAEC incubated with GSE for short time periods (<3h) and long time periods (<24 h). Two sets of short-time course experiments were undertaken with different concentrations of GSE:

- 10 µg/ml of GSE incubated over 0, 15, 30, 60, and 150 min (figure 2.4).
- 5 µg/ml of GSE incubated over 0, 30, 60, 120, and 240 min (figure 2.5).

CH25OH mRNA levels increased ~6 – 8 fold by 60 min (p < 0.001), before returning to baseline by 120 – 150 min in both sets of experiments. These changes were consistently seen with both 5 and 10 µg/ml of GSE (p < 0.001). The second short time-course experiment was extended to include a 240 min time point, and revealed that levels of CH25OH mRNA were not different from baseline levels (p < 0.001).

![Figure 2.4 Effect of GSE on relative mRNA levels of CH25OH over short time periods](image)

Figure 2.4 Effect of GSE on relative mRNA levels of CH25OH over short time periods. BAEC were incubated with GSE (10 µg/ml) for the times indicated (n=6, ***p< 0.001 vs. 0 min; ### p< 0.001 vs. 30 and 60 min; figures are expressed as mean ± SEM).
Figure 2.5 Effect of GSE on relative mRNA levels of CH25OH over short time periods (extended to include 240 min). BAEC were incubated with GSE (5 µg/ml) for the times indicated (n=6, ***p< 0.001 vs. 0 min; ###p<0.001 vs. 30 and 60 min; figures are expressed as mean ± SEM).

From the short-time course results it was difficult to anticipate what changes might occur over longer time periods. Therefore, BAEC were incubated with GSE (10 µg/ml) for longer durations and samples examined at 0, 2.5, 6, and 24 h (figure 2.6). The data suggested there might be a second phase of increased CH25OH mRNA levels at 6 h, although these changes did not reach statistical significance.

Figure 2.6 Effect of GSE on relative mRNA levels of CH25OH over long time periods. BAEC were incubated with GSE (10 µg/ml) for the times indicated (n=6; figures are expressed as mean ± SEM).
Taking the results of both the short-time course and long-time course experiments, it appeared that changes in the levels for CH25OH mRNA were mainly within the first 30 - 60 min after exposure to GSE with a return to baseline levels around 120 - 150 min. There was also a trend for a second rise in CH25OH mRNA levels around 6 h based on the longer time course (figure 2.6). But on closer analysis of this data, especially in conjunction with the data from figure 2.4 and 2.5 three points become apparent:

- If all these time points are put together then the increase in mRNA expression at 6 h is small compared with the increase at 30 or 60 min time points.

- The mRNA expression at 150 min in figure 2.4 suggests no change in the level where as in figure 2.6 there is a small increase, but again this is less than two-fold.

- Between the short and long time course experiments the duration of the response varies.
2.3.2 Effects of OPC on the levels of ABCG1 and HMGCR mRNA

As described earlier previous studies have shown that 25-OHC reduces the levels of various enzymes and substrates involved in lipid synthesis including levels of HMGCR mRNA and activity. As the short-time course experiments revealed an increase in CH25OH mRNA expression at 30 – 60 min the next step was to examine if this was associated with changes in HMGCR mRNA levels. Samples from both the short-time course experiments (GSE concentration of 5 µg/ml and 10 µg/ml) were thus used to measure changes in HMGCR mRNA levels (figure 2.7 and 2.8). Given that HMGCR might be processed into different isoforms, with separate roles in cholesterol and fatty acids synthesis for protein methylation, three primer sets were designed for HMGCR measurements (Breitling & Krisans, 2002). One of these was targeted to the extreme 3’ of HMGCR, however because satisfactory optimisation of the PCR conditions could not be achieved this set was not used for sample analysis.

An increase in HMGCR mRNA levels over 15 – 30 min was seen using the other sets of optimised primers (p < 0.05; figures 2.7 and 2.8). This is contrary to reduced HMGCR mRNA levels, which would have been expected with an increase in mRNA levels for CH25OH. The inferences from this could be that GSE does not have the same effects on BAEC as it does in HAEC – this could relate to differences in the cell media or differences in cholesterol regulation in bovine cells. These results would however, be consistent with changes in mRNA levels preceding protein synthesis and then production of 25-OHC. Indeed, at 120 and 150 min the levels of HMGCR mRNA were reduced compared with baseline (p < 0.05; figure 2.7 and 2.8) and increased again at 240 min (p < 0.001 compared with 120 min). This increase is likely to represent other feedback mechanisms that come into play in the regulation of HMGCR (figure 2.8). Based on these results Primer set 2 was used for subsequent experiments because the RNA standard plots consistently provided better r-values.
Figure 2.7 Effect of GSE (10 µg/ml) on relative mRNA levels of HMGCR over short time periods. Two sets of optimized primers for HMGCR were used (primer 1 left panel and primer 2 right panel). BAEC were incubated with GSE (10 µg/ml) for the times indicated (n=6, *p<0.05 and **p<0.01 vs. time 0; figures are expressed as mean ± SEM).

Figure 2.8 Effect of GSE (5 µg/ml) on relative mRNA levels of HMGCR over short time periods. HMGCR primer set 2 was used and BAEC were incubated with GSE (5 µg/ml) for the times indicated (n=6, *p<0.05 vs. 0 min; ###p< 0.001 vs. 120 min; figures are expressed as mean ± SEM).

The next step was to determine if under the same experimental conditions using the same BAEC whether there are any detectable changes in the mRNA expression of ABCG1. If the results from the HMGCR experiments were due to primer problems or qRT-PCR than it would be expected that BAEC incubated with GSE, just like in HAEC results in an increase in ABCG1 mRNA expression. GSE (10 µg/ml) resulted in a significant reduction in the levels of ABCG1 mRNA in BAEC from 15 min onwards (p < 0.001), and this persisted for the duration of the experiment (p < 0.01 at 30, 60 and 150 min; figure 2.9).
Figure 2.9 Effect of GSE on relative mRNA levels of ABCG1 over short-time periods. BAEC were incubated with GSE (10 µg/ml) for the times indicated (n=6, **p< 0.01 and ***p< 0.001 vs. time 0; figures are expressed as mean ± SEM).
2.3.3 Effect of superoxide dismutase mimetic on BAEC response to GSE

The underlying mechanism of action of OPC remains unknown. Studies of the vasodilator activity of other polyphenol extracts have shown responses to be inhibited by the superoxide dismutase mimetic MnTMPyP (see Diebolt et al., 2001), implicating a role for ROS in these endothelial responses (reference). To evaluate the role of ROS in changes in CH25OH mRNA levels BAEC were treated with GSE alone (10 µg/ml) or after MnTMPyP pre-treatment (10 µM). GSE resulted in an increase in the mRNA levels of CH25OH after 60 min as expected from figure 2.4 and 2.5 (p < 0.001 compared with control and pre-treatment with MnTMPyP; figure 2.10). This was abolished significantly in the presence of pre-treatment with MnTMPyP such that levels were almost back to control levels (p<0.01 compared with GSE; figure 2.10). This data suggest that the mechanism underlying increases in CH25OH mRNA levels associated with OPC of GSE origin are dependent upon signalling via ROS. It also suggests that the mechanisms triggering NO-dependent vasodilatation are similar to those involved in the CH25OH response.

Figure 2.10 Relative changes in mRNA levels of CH25OH following GSE with and without pre-treatment with MnTMPyP. BAEC were incubated with GSE (10 µg/ml) alone or after pre-treatment with MnTMPyP (10µM; labelled Mn; n=9, ***,p< 0.001 vs. control; ###p<0.001 vs. Mn and ++p< 0.01 vs. GSE; figures are expressed as mean ± SEM).
2.3.4 Effects of differing concentrations of 25-hydroxycholesterol on the levels of ABCG1 and HMGCR mRNA

Three differing concentrations of 25-OHC (1µM, 3µM and 10µM) were applied to BAEC cells over 0, 2, 6 and 24 h and the levels of HMGCR and ABCG1 mRNA were analyzed. The 25-OHC has to be stored in ethanol and thus the control vehicle consisted of DMEM with ethanol at a final concentration <0.001%, which was the same across all test substances. A different clone of BAEC was used in these experiments compared with earlier experiments discussed above, in case this may have impacted the results (figures 2.4 - 2.10). In addition experiments were performed over 24 h rather than shorter time courses to ensure that changes in mRNA levels which might occur several hours following treatment were not missed. Figure 2.11 shows that 25-OHC at all three concentrations led to significantly reduced mRNA levels for HMGCR (p < 0.001 with 3 µM concentration) and also increased levels of ABCG1 mRNA (p < 0.05 with 3 µM concentration).

HMGCR mRNA levels were suppressed early on and significantly with all concentrations at 6 h, on the other hand increased levels of ABCG1 mRNA took longer, being most notable at 24 h. Although the changes in HMGCR mRNA expression were repeatable those for ABCG1 were not. On the other hand the effects on ABCG1 mRNA levels were more variable, with ~100 fold increase in mRNA levels at 1 µM, ~10 fold increase in mRNA levels at 3 µM and ~300 fold increase at 10 µM concentrations (p < 0.05, p < 0.05 and p < 0.01 respectively; figure 2.11). Based on these results 25-OHC was used at a concentration of 3µM in subsequent experiments.
Figure 2.11 Effect of varying concentrations of 25-OHC on relative mRNA levels of ABCG1 and HMGCR. BAEC were treated with three differing concentrations of 25-OHC: (a) 1µM, (b) 3µM and (c) 10µM and mRNA levels of ABCG1 (left panel) and HMGCR (right panel) over 0, 2, 6 and 24 h measured (n=6, *p<0.05, **p< 0.01 and ***p< 0.001 vs. time 0; figures are expressed as mean ± SEM).
2.3.5 Procyanidin oligomer size and mRNA levels for ABCG1, HMGCR, and CH25OH

Trimer (AP3), tetramer (AP4), pentamer (AP5) apple OPC, and whole apple extract (WAE), which consist of a mixture of oligomer sizes were tested next. AP3 did not increase CH25OH mRNA levels and caused no appreciable changes in HMGCR and ABCG1 mRNA levels, whilst AP4 produced a small non-significant increase (almost 2-fold) in CH25OH mRNA levels at 24 h which was associated with a decrease in HMGCR mRNA levels (~50%; p < 0.05) at 6 h but no change in ABCG1 mRNA levels (figure 2.12 and 2.13).

AP5 was the most effective leading to an increase in CH25OH mRNA levels associated with reduced HMGCR and increased ABCG1 mRNA levels. A time difference was noticed in that HMGCR mRNA levels were reduced to less than 50% (p < 0.01) by 6 h with a non-significant rise in ABCG1 mRNA levels (over 2-fold increase) and no appreciable changes in CH25OH (figure 2.12). Nevertheless, by 24 h, CH25OH mRNA levels had increased (over 3-fold; p < 0.05) as were ABCG1 mRNA levels (5-fold; p < 0.01), but now HMGCR mRNA levels had risen 2.5-fold (p < 0.01; figure 2.13). In comparison, ABCG1 mRNA levels increase being highest at 24 h. A similar pattern of response was seen with WAE, which may be due to it containing a relatively high proportion of procyanidin pentamers (figure 2.12 and 2.13).
Figure 2.12 Effect of various apple OPC molecules on relative mRNA levels for HMGCR and ABCG1 over 6 h. BAEC were treated with various OPC molecules over 6 h (5 µg/ml of AP3, AP4, AP5 and a mixture called whole apple extract, WAE) and mRNA levels for HMGCR and ABCG1 (top panel) and CH25OH (bottom panel) measured (n=6, *p<0.05 and **p< 0.01; figures are expressed as mean ± SEM).
Figure 2.13 Effect of various apple OPC molecules on relative mRNA levels for HMGCR, ABCG1 and CH25OH over 24 h. BAEC were treated with various OPC molecules (5 µg/ml of AP3, AP4, AP5 and a mixture called whole apple extract, WAE) over 24 h and mRNA levels for HMGCR and ABCG1 (top panel) and CH25OH (bottom panel) measured (n=6, *p<0.05 and **p<0.01; figures are expressed as mean ± SEM).
Cell media ET-1 levels was suppressed by both AP5 and WAE, but not AP3 and AP4 (figure 2.14). ET-1 levels were suppressed to ~50% with AP5 (p < 0.05) and WAE (non-significant), compared with basal levels, changes that were most apparent at 6 h, and had disappeared by 24 h (figure 2.14). In fact at 24 h there was an increase in ET-1 levels (30 – 50% from basal) with both AP4 (p < 0.05) and AP5 (non-significant; figure 2.14).

![Figure 2.14 Percentage change in cell media ET-1 levels from basal with various apple OPC molecules.](image)

*Cell media ET-1 levels (fmol/ml) are expressed as percentage change from basal from the BAEC experiments depicted in figures 2.12 and 2.13 examining the effects of various apple OPC molecules on BAEC (5 µg/ml of AP3, AP4, AP5 and whole apple extract, WAE) over 6 h (left panel) and 24 h (right panel; n=6, *p<0.05 vs. control; figures are expressed as mean ± SEM)*.
2.3.6 Interaction between procyanidin tetramer or pentamer with 25-hydroxycholesterol

To further analyse the effects of the purified procyanidins AP4 and AP5, they were applied to BAEC (5 µg/ml) with and without 25-OHC (3µM) and the levels of HMGCR and ABCG1 mRNA measured. ABCG1 mRNA levels increased with AP4 and AP5 alone (~3-fold; non-significant), but changes with 25-OHC alone were much higher (1,000 - 30,000 fold; figures 2.15 - 2.17). There was an even greater increase when the combination of procyanidin and 25-OHC were tested (figures 2.15 - 2.17). These increases were greater than the summation of the AP4/AP5 and 25-OHC effects, especially for AP5 (1,000-5,000 fold change; p < 0.01 at 6 h and p < 0.001 at 24 h) suggesting synergism between the procyanidin and 25-OHC (figures 2.16 and 2.17). The pattern of changes in ABCG1 mRNA levels with both AP4 and AP5 with and without 25-OHC were similar at both 6 and 24 h, but more profound at 24 h (figure 2.15 – 2.17). The lack of reproducibility in the mRNA expression of ABCG1 is also manifest in these experiments as in figure 2.11 and must be taken into consideration and is discussed further below.

On the other hand, HMGCR mRNA levels were significantly reduced by AP4 and 25-OHC alone at 6 h (p < 0.001 for both; figure 2.15). At 24 h the suppression caused by 25-OHC was still present and even greater but did not reach statistical significance, whereas HMGCR mRNA levels with AP4 had increased to levels above control (p < 0.05). This was not seen with the combination of AP4 with 25-OHC and indeed HMGCR mRNA levels remain suppressed at both 6 and 24 h, although the latter did not reach statistical significance (figure 2.15). AP5 led to a significant reduction in HMGCR mRNA levels at 6 h (p < 0.05) but not 24 h (figure 2.16 and 2.17). Whereas 25-OHC alone led to greater suppression of HMGCR mRNA levels, than with AP5 and changes persisted at 24 h (p<0.001; figure 2.17). Interestingly the addition of the two compounds together did not lead to further suppression at either 6 or 24 h, rather HMGCR mRNA levels were slightly higher than those seen with 25-OHC alone (figure 2.16 and 2.17).

AP5 led to a reduction in cell media ET-1 levels by more than 50% at 6 h (p < 0.001; figure 2.16), an effect not seen at 24 h and which is consistent with earlier findings (figure 2.17). Interestingly, 25-OHC led to an increase in ET-1 levels at 24 h of which the cause remains unclear (p < 0.01; figure 2.17).
Figure 2.15 Effect of tetramer apple OPC on relative mRNA levels of HMGCR and ABCG1 with and without 25-OHC. BAEC were treated with tetramer apple OPC molecule (AP4; 5 µg/ml) with and without 25-OHC (3µM) over (a) 6 h and (b) 24 h and mRNA levels of HMGCR and ABCG1 measured (n=6, *p<0.05, **p< 0.01 and ***p< 0.001 vs. control; #p<0.05, ##p< 0.01 and ###p< 0.001 vs. AP4; figures are expressed as mean ± SEM).
Figure 2.16 Relative changes in mRNA levels for HMGCR and ABCG1 and percentage change in ET-1 release from basal with the pentamer apple OPC molecule over 6 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without 25-OHC (3µM) over 6 h and mRNA levels for HMGCR and ABCG1 (top panel) and percentage change from basal ET-1 release measured (fmol/ml; bottom panel; n=6, *p<0.05, **p< 0.01 and ***p< 0.001 vs. control; #p<0.05 vs. AP4; +p<0.05 vs. 25-OHC; figures are expressed as mean ± SEM).
Figure 2.17 Relative changes in mRNA levels for HMGCR and ABCG1 and percentage change in ET-1 release from basal with the pentamer apple OPC molecule over 24 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without 25-OHC (3µM) over 24 h and mRNA levels for HMGCR and ABCG1 (top panel) and percentage change from basal ET-1 release measured (fmol/ml; bottom panel; n=6, *p<0.05, **p< 0.01 and ***p< 0.001 vs. control; #p<0.05 and ##p< 0.01 vs. AP4; ++p< 0.01 vs. 25-OHC; figures are expressed as mean ± SEM).
2.3.7 Effect of pentameric OPC molecules on mRNA levels of HMGCR and ABCG1 with and without the presence of 25-OHC and in the presence or absence of HMGCR inhibition

Atorvastatin is a potent HMGCR enzyme inhibitor and affects enzyme activity rather than mRNA levels of HMGCR. In order to investigate the effects of HMGCR inhibition on the levels of ABCG1 and HMGCR mRNA, BAEC cells were initially incubated with atorvastatin for 24 h (0.3 µM) followed by the addition of AP5 (5 µg/ml) for either 6 or 24 h. Atorvastatin did not have any appreciable effects on the mRNA levels of ABCG1, but levels increased with 25-OHC (non-significant) and were again potentiated by AP5 (p < 0.05; figure 2.18). This latter effect was apparent at both 6 h and 24 h but more marked at 24 h with a rise in levels of over 2000 fold (p < 0.05; figure 2.18).

AP5 and 25-OHC in combination reduced HMGCR mRNA levels down to 20% of the initial levels at both 6 h and 24 h, although this failed to reach statistical significance (figure 2.19). Interestingly atorvastatin was associated with a significant increase in the levels of HMGCR mRNA at both 6 h and 24 h (p < 0.001 and p < 0.05 respectively), an affect that was lessened at 6 and 24 h with the addition of AP5 (p < 0.01 and p < 0.05 compared with atorvastatin alone respectively; figure 2.19). These findings imply that reduced HMGCR activity in the presence of atorvastatin increases mRNA levels probably through interference with a negative feedback process resulting from suppression of cholesterol synthesis. Furthermore, 25-OHC resulted in suppression of HMGCR mRNA levels with and without AP5 at both 6 and 24 h, which was also significant compared with atorvastatin (p < 0.001 and p < 0.05 respectively; figure 2.19).

Western blotting revealed increased ABCG1 protein at 24 h with 25-OHC alone and 25-OHC in combination with AP5 (figure 2.19). This implies that the increases in mRNA levels of ABCG1 are associated with an increase in protein levels. Further studies are needed to elaborate these effects and should include effects on mRNA levels of ABCG1 and HMGCR with a combination of AP5, 25-OHC and atorvastatin and measures of HMGCR enzyme activity.
Figure 2.18 Relative changes in mRNA levels of ABCG1 with and without 25-OHC and atorvastatin. BAEC were treated with the pentamer apple OPC molecule (AP5; 5 µg/ml) with and without 25-OHC (3µM) and atorvastatin (0.3µM) over 6 h (top panel) and 24 h (bottom panel) and mRNA levels of ABCG1 measured (n=6, *p<0.05 vs. control; #p<0.05 vs. atorvastatin; +p< 0.05 vs. AP5; @ p<0.05 vs. atorva and AP5; $ p<0.05 vs. 25-OHC; figures are expressed as mean ± SEM). The diagram includes western blotting result for ABCG1 protein after 24 h of treatment.
Figure 2.19 Relative changes in mRNA levels of HMGCR with and without 25-OHC and atorvastatin. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without 25-OHC (3µM) and atorvastatin (0.3µM) over 6 h (top panel) and 24 h (bottom panel) and mRNA levels of HMGCR measured (n=6, *p<0.05 and ***p< 0.001 vs. control; #p<0.05, ##p<0.01 and ###p<0.001 vs. atorvastatin; +p< 0.05 vs. AP5; figures are expressed as mean ± SEM).
2.3.8 Effect of pentameric OPC molecules on mRNA levels of HMGCR and ABCG1 with and without the presence of 25-OHC and in the presence or absence of pre-loading with LDL-C

In these experiments BAEC were initially incubated with LDL-C (1 mg/ml final concentration) extracted from human plasma intending to load BAEC with LDL-C to potentially up-regulate CH25OH physiologically before treatment with the AP5 (5 µg/ml) with or without 25-OHC (3 µM). For cells that were not pre-treated with LDL-C, treatment with AP5 alone increased mRNA levels for ABCG1 (p < 0.01 at 24 h; figure 2.20) and decreased HMGCR mRNA levels at 6 h (not significant; figure 2.21), findings similar to those described earlier. Also the addition of 25-OHC led to a further increase in the mRNA levels of ABCG1 at 6 and 24 h (p < 0.01; figure 2.20) and increases in HMGCR mRNA levels with AP5 and with AP5 in combination with 25-OHC at 24 h (p < 0.05 and p < 0.01 respectively; figure 2.21), again these findings are similar to those described above.

Loading the cells with LDL-C led to a more marked increase in levels of ABCG1 in the presence of AP5 which was most apparent at 6 h (p < 0.001) and similar levels were seen with the addition of 25-OHC (p < 0.001; figure 2.20). On the other hand, mRNA levels of HMGCR when cells were pre-incubated with LDL-C were reduced at 6 and 24 h, by approximately 50% at 24 h, although this only reached statistical significance at 6 h when the combination of 25-OHC and AP5 were tested (p < 0.05; figure 2.21).

Pre-incubation of cells with LDL-C resulted in the suggestion of an increase in ABCG1 protein on western blotting with all treatments (figure 2.20). This is in disagreement with the mRNA levels of ABCG1 seen at 24 h (figure 2.20). Nevertheless, these results are interesting and suggest that cells which face a high LDL-C may benefit the most from OPC and that the effect is greatest on mRNA levels of the cholesterol transporter ABCG1 than the cholesterol synthesizing enzyme HMGCR, the net result being that BAEC are switched towards cholesterol efflux. These results need to be viewed with caution however, as 25-OHC failed to cause suppression of HMGCR, which has been seen in earlier, experiments, the reason for which remains unclear.
Figure 2.20 Relative changes in mRNA levels of ABCG1 with and without 25-OHC after cell loading with LDL-C. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without 25-OHC (3 µM) following pre-incubation of cells with LDL-C (1 mg/ml) over 6 h (top panel) and 24 hr (bottom panel) and mRNA levels of ABCG1 measured (n=6, *p<0.05 and **p<0.01 vs. control; figures are expressed as mean ± SEM). The diagram includes western blotting result for ABCG1 protein after 24 h of treatment.
Figure 2.21 Relative change in mRNA levels of HMGCR with and without 25-OHC after cell loading with LDL-C. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without 25-OHC (3 µM) following pre-incubation of cells with LDL-C (1 mg/ml) over 6 h (top panel) and 24 hr (bottom panel) and mRNA levels of HMGCR measured (n=6, *p<0.05 and **p< 0.01 vs. control; figures are expressed as mean ± SEM).
2.3.9 Effect of pentameric OPC on mRNA levels of CH25OH, ABCG1 and HMGCR with and without HDL-C following pre-incubation of cells with LDL-C

In trying to determine changes in cell cholesterol levels with OPC that can then provide some insight into what may occur in vivo, BAEC were incubated with LDL-C (1 mg/ml final concentration) so that cells were fully loaded with cholesterol. HDL-C (20 µg/ml) and AP5 (5 µg/ml) alone and in combination, was added to the cells for 6 h. AP5 alone led to reduction in ABCG1, HMGCR (reduced by ~70%) and CH25OH mRNA levels (all p < 0.05; figure 2.22). This was dissimilar to previous findings where ABCG1 and CH25OH mRNA levels are increased with AP5. AP5 also led to a reduction in HMGCR mRNA levels in the presence of HDL-C and LDL-C in comparison to control (p < 0.05 for both), LDL-C alone and HDL-C alone (figure 2.22). In the presence of both LDL-C and HDL-C AP5 was observed to reduce the mRNA levels of ABCG1, HMGCR, and CH25OH (p < 0.01 only for ABCG1; figure 2.22).

The effects on ABCG1 and CH25OH mRNA levels are at variance from those described earlier. This may represent complex interactions between OPC in the presence of LDL-C and HDL-C, which are difficult to tease apart from this single experiment, or may relate to experimental conditions. Evaluating the changes of AP5 on ET-1 levels in this experiment revealed that an appropriate OPC stimulus was applied by the virtue of a greater than 50% reduction in ET-1 levels at 6 h present with AP5 alone (p < 0.001), with LDL-C (p < 0.001), HDL-C (p < 0.001) and with both LDL-C and HDL-C (P < 0.01; figure 2.23).
Figure 2.22 Changes in relative mRNA levels of CH25OH, HMGCR and ABCG1 with and without HDL-C and loading with LDL-C for 6 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1mg/ml) for 6 h and mRNA levels of CH25OH, HMGCR and ABCG1 measured (n=6, *p<0.05 and **p< 0.01 vs. control; figures are expressed as mean ± SEM).
Figure 2.23 Percentage change in ET-1 release from basal with the pentamer apple OPC molecule with and without HDL-C and loading with LDL-C for 6 h. ET-1 release (fmol/ml) from BAEC following treatment with the pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 6 h (n=6, **p<0.01 and ***p< 0.001 vs. control; figures are expressed as mean ± SEM).
2.3.10 Effect of pentameric OPC on cell media and solubilised cell cholesterol levels with and without HDL-C following pre-incubation of cells with LDL-C

Cholesterol levels in the cell media and solubilised cell extracts from the experiment in section 2.3.9, with the various treatments was measured using the previously described fluorescent cholesterol assay. As can be seen in figure 2.24 and 2.27, the levels of cholesterol in the cell media after 24 h treatment with LDL-C was almost doubled compared with the levels pre-treatment. This is followed by a striking reduction in the measurable levels following the various treatments (comparison of post LDL-C loading with control see figure 2.24 and 2.27). It is unclear what has resulted in this reduction and it may represent either that media cholesterol has moved into the cells or has been metabolised into molecules that were not measurable in this experiment.

Absolute cholesterol levels in the cell media after the various treatments over 6 h and 24 h is shown in figures 2.25 and 2.28 respectively. At 6 h AP5 reduced cell media cholesterol levels (p < 0.01; figure 2.25) and a similar reduction was seen in the presence of loading with LDL-C and AP5 (p < 0.01). There was also a reduction in the cell media levels at 6 h with AP5 and HDL-C and also a combination of AP5, LDL-C, and HDL-C, but these were non-significant. Examination of the results over 24 h showed similar effects of AP5 alone and in the presence of LDL-C loading (p < 0.05 and p < 0.01 respectively; figure 2.28), this was without any further reductions in the cell media cholesterol levels. When the cell media cholesterol levels were analysed in comparison to basal levels it is clear that AP5 with and without LDL-C loading results in an approximately 25 – 30% reduction at both 6 h and 24 h (figures 2.26 and 2.29). Interestingly, this reduction was reduced in the presence of HDL-C, more so at 24 h than 6 h. It is difficult to try to interpret the reasons for this on the basis of these results and further experiments are needed. Even so AP5 in the presence of HDL-C alone or HDL-C and LDL-C leads to significant reductions in cell media cholesterol at 6 h (p< 0.05 compared with AP5 alone and control; figure 2.26).
Figure 2.24 Effects of the pentamer apple OPC molecule on absolute levels of cell media cholesterol with and without HDL-C and loading with LDL-C for 6 h, including pre- and post-loading values. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 6 h on absolute levels of cell media cholesterol (µmol/ml; n=6, +++p<0.001 vs. Control and @@p<0.01 vs. post LDL-C loading). The first bar represents levels in cell media before pre-LDL-C loading and the second bar depicts the levels post LDL-C loading (1mg/ml) and the filled bars show cholesterol levels after the various treatments (this is expanded in the next figure). (Figures are expressed as mean ± SEM).
Figure 2.25 Effects of the pentamer apple OPC molecule on absolute levels of cell media cholesterol with and without HDL-C and LDL-C for 6 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) on absolute levels of cell media cholesterol (µmol/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 6 h (*p < 0.05 and **p < 0.01 vs. control; figures are shown as mean ± SEM).
Figure 2.26 Percentage change in cell media cholesterol levels from baseline in the presence of the pentamer apple OPC molecule, with and without HDL-C and loading with LDL-C for 6 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 6 h and percentage change in cell media cholesterol levels (µmol/ml) from baseline measured (n=6, *p<0.05 and ***p< 0.001 vs. control and ###p< 0.001 vs. AP5; figures are shown as mean ± SEM).
Figure 2.27 Effects of the pentamer apple OPC molecule on absolute levels of cell media cholesterol with and without HDL-C and loading with LDL-C for 24 h, including pre- and post-loading values. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 24 h and absolute levels of cell media cholesterol (µmol/ml) measured (n=6, +++p<0.001 vs. Control and @@ @p< 0.01 vs. post LDL-C loading). The first bar represents levels in cell media before pre-LDL-C loading and the second bar depicts the levels post LDL-C loading (1mg/m); and the filled bars show cholesterol levels after the various treatments (this is expanded in the next figure). (Figures are shown as mean ± SEM).
Figure 2.28 Effects of the pentamer apple OPC molecule on absolute levels of cell media cholesterol with and without HDL-C and LDL-C for 24 h. Effects of the pentamer apple OPC molecule (AP5; 5 µg/ml) on absolute levels of BAEC cell media cholesterol (µmol/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 24 h (*p < 0.05, **p < 0.01, ***p < 0.001 vs. control; #p < 0.05, ##p < 0.01 vs. AP5 alone; figures are shown as mean ± SEM; figures are shown as mean ± SEM).
Figure 2.29 Percentage change in cell media cholesterol levels from baseline in the presence of the pentamer apple OPC molecule, with and without HDL-C and loading with LDL-C for 24 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 24 h and percentage change in cell media cholesterol levels (µmol/ml) from baseline measured (n=6, ***p< 0.001 vs. control and #p<0.05 and ###p< 0.001 vs. AP5; figures are shown as mean ± SEM).
Cell extracts at 24 h were solubilised and the cholesterol levels measured after the various experiments. The absolute cell extract cholesterol levels appeared to increase slightly with LDL-C, HDL-C, and AP5 but the changes are small and non-significant (figure 2.30). Further analysis of the data reveals that AP5 reduces solubilised cholesterol levels by approximately 10% from baseline (p < 0.05; figure 2.31). Similar to cell media cholesterol, the extract levels were reduced in the presence of AP5 with HDL and AP5 with HDL and LDL but the reduction was in the order of ~5 – 8% (p < 0.05 for AP5, LDL, HDL compared with control; figure 2.31).

**Figure 2.30** Solubilised cell cholesterol levels after pentamer apple OPC, with and without HDL-C and loading with LDL-C for 24 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 24 h and solubilised cell cholesterol levels (µmol/well) measured (figures are shown as mean ± SEM).
Figure 2.31 Percentage change in solubilised cell cholesterol levels from baseline in the presence of the pentamer apple OPC molecule, with and without HDL-C and loading with LDL-C for 24 h. BAEC were treated with pentamer apple OPC molecule (AP5; 5 µg/ml) with and without HDL-C (20 µg/ml) following pre-incubation of cells with LDL-C (1 mg/ml) for 24 h and percentage change in solubilised cell extract cholesterol levels (µmol/well) from baseline measured (n=6, *p< 0.05 vs. control; figures are shown as mean ± SEM).
2.4 Discussion
Consumption of flavonoid foods and drinks that have a high OPC content have long been thought to play a protective role from CVD. The evidence for this postulation is based on a number of historical accounts, observational and in vitro studies and clinical trials (including Hertog et al., 1993; Fitzpatrick et al., 1993; Knekt et al., 1996; Cishek et al., 1997; Stoclet et al., 1999; Yochum et al., 1999; Taubert et al., 2003; Grassi et al., 2005a; Grassi et al., 2005b; Mursu et al., 2008; Allen et al., 2008). Although there are to date a fair number of reports, the results have been mixed and often conclusions are unclear and not definitive, being marred by the presence of multiple confounding factors and problems in study design. This chapter has attempted to discover properties exerted by OPC on BAEC focusing on the potential effects they might have on bovine cellular cholesterol regulation. The aim of these experiments was to test whether OPC led to reduced cellular cholesterol levels through both enhanced cholesterol efflux and decreased de novo cholesterol synthesis, mediated by the oxysterol 25-OHC. The main findings from this chapter are as follows:

- CH25OH rises in response to GSE reaching maximum levels by 30-60 min and then rapidly returns to baseline.
- The rise in CH25OH in response to GSE is inhibited in the presence of MnTMPyP, suggesting a role of ROS in this mechanism.
- Direct application of 25-OHC leads to an increase in ABCG1 mRNA expression greatest at 24 h and a decrease in HMGCR, apparent by 2-6 h, but the former showed poor reproducibility.
- AP5 especially in combination with 25-OHC also produced an increase in ABCG1 mRNA expression greatest at 24 h and a decrease in HMGCR, apparent by 6 h.
- AP5 reduced cell media cholesterol levels alone and in the presence of LDL-C and reduced solubilised cell cholesterol levels alone or in the presence of HDL-C or HDL-C in combination with LDL-C.
- OPC reduce the levels of the vasoconstrictor ET-1.
CH25OH rises in response to GSE reaching maximum levels by 30-60 min and then rapidly returns to baseline

Little is known about the oxysterol CH25OH for example, the physiological concentration has not been described (Kandutsch et al., 1977). The data presented here revealed that GSE incubation with BAEC leads to a rapid rise in CH25OH mRNA expression, which promptly returns to baseline, suggesting that CH25OH is regulated by a negative feedback system. These are novel findings, which have not been described to date. As described in chapter 1 an increase in CH25OH should be associated with a reduction in HMGCR and an increase in ABCG1. However, contrary to this the changes in CH25OH with GSE were accompanied by a small increase in the mRNA expression of HMGCR and a reduction in the mRNA expression of ABCG1 (figures 2.8 - 2.10). These variable responses are unlikely to result from incubation or experimental problems as the same procedures were used in subsequent experiments. Rather the problem is likely to relate to the BAEC as those tested in these initial experiments were of a lower passage number and therefore less mature than those used in subsequent experiments. Repeating these experiments with higher passage number BAEC may help to resolve this issue.

It could be argued that the GSE may have caused the discordant ABCG1 and HMGCR mRNA expression results. GSE is a relatively pure product of OPC, but approximately 60% of the OPC have a degree of polymerisation larger than pentamers. In hindsight further experiments to try to resolve this issue should have been undertaken, in particular determining the contribution of different GSE components on the expression of CH25OH, HMGCR, and ABCG1 mRNA. Other factors which may have influenced the results include days in culture with FCS, which contains growth factors, level of basal cholesterol at the time of the experiment, which may depend on whether cultures were confluent and quiescent or just reaching confluence. It is practically difficult to perform experiments which control for all these factors but future studies should endeavour to measure these.

The rise in CH25OH in response to GSE is inhibited in the presence of MnTMPyP, suggesting a role of ROS in this mechanism

ROS have generally been ascribed a pathophysiologival status and in the cardiovascular system have been associated with increased shear stress, inflammation, thrombin, impaired endothelial function and the development of atherosclerosis (Hishikawa & Lüscher, 1997; Weber et al., 1994; Patterson et al., 1999). The changes in CH25OH mRNA in BAEC treated with GSE reported here were inhibited by pre-treatment with MnTMPyP, a superoxide dismutase implying...
that signalling via reactive oxygen species (ROS) is an important factor in the regulation of CH25OH (figure 2.10). This is consistent with the work of other researchers who have also reported that OPC effects are mediated by ROS (Diebolt et al., 2001; Ndiaye et al., 2003; Duarte et al., 2004).

This data supports the increasing evidence that not all ROS are damaging such as Nox4 an endothelial cell ROS, which has been implicated in the physiological regulation of endothelium-dependent vasodilation both in vitro and in vivo (Ago et al., 2004; Van Buul et al., 2005; Ray et al., 2007; Larsen et al., 2009; Zhuang et al., 2010; Ray et al., 2011). It is possible that Nox4 activation is upstream of signalling pathways regulating CH25OH expression. To date there are no known inhibitors specifically directed towards CH25OH, which would help determine whether these changes were indeed mediated through CH25OH, and help further understand the regulation of this enzyme. SiRNA may be a viable next step in further evaluating this underlying mechanism of action.

**Direct application of 25-OHC leads to an increase in ABCG1 mRNA expression and a decrease in HMGCR, but the former showed poor reproducibility**

At this point a number of studies had reported differing effects of flavan-3-ols according to the degree of polymerization of these molecules (Fitzpatrick et al., 2000; Fitzpatrick et al., 2002; Aldini et al., 2003; Corder et al., 2006; Caton et al., 2010). Before testing purified fractions of apple a study looking at the direct application of 25-OHC on BAEC was tested. This also meant that it was possible to confirm whether BAEC respond to 25-OHC as has been described before in human aortic endothelial cells (Kandutsch & Chen, 1974; Brown & Goldstein, 1974; Pothecary, 2007). Indeed 25-OHC resulted not only in suppression of HMGCR mRNA as expected, but also increases in ABCG1, a finding which has not been reported in the literature to date (figure 2.11). It is possible that these effects are saturated, as changes in 25-OHC concentrations did not make much difference, especially when comparing HMGCR effects.

The effects on HMGCR and ABCG1 were also both time-dependent, in that ABCG1 mRNA levels increased over the 24 h period where as HMGCR mRNA levels were suppressed rapidly with maximal suppression occurring by 2 h. One possible mechanism that fits in with these findings is as follows: OPC lead to increases in 25-OHC (via CH25OH) within 30-60 min, this oxysterol prevents the SREBP from releasing terminal fragments and thus suppresses de novo cholesterol synthesis (one aspect of which is a reduction in HMGCR mRNA production).
ABCG1 mRNA expression begins to rise as HMGCR mRNA expression falls but continues to rise over 24 h which may be the result of CH25OH functions not yet described or the production of other oxysterols which lead to LXR stimulation or PPARγ stimulation, such as 22(R)-hydroxycholesterol, a potent LXR agonist (O’Connell et al., 2004). Cholesterol assays at the various time-points of both the cell media and solubilised cells might help clarify some of these issues as would the measurement of the levels of oxysterols other than 25-OHC.

**AP5 especially in combination with 25-OHC also produced an increase in ABCG1 mRNA expression greatest at 24 h and a decrease in HMGCR, apparent by 6 h**

There is also much growing interest as to the effects of different OPC oligomer sizes and sources of OPC. The current limitations to work in this area relates to inadequate techniques to extract OPC and its various fractions (Guyot et al., 2001). Indeed when various apple OPC oligomer sizes were experimented with, the pentamer and tetramer OPC molecules were relatively more potent than trimeric molecules (figure 2.12 and 2.13). Interestingly the addition of 25-OHC enhanced the effects of the pentamer OPC on the levels of ABCG1 markedly with minimal further effects on HMGCR mRNA levels (figure 2.15 and 2.16). Although pentamer molecules were more potent than tetramers this difference is likely to be attributable to experimental design or error rather than represent a real difference. This data adds to the research that oligomeric structures are the most potent (Teissedre et al., 1996; Fitzpatrick et al., 2000; Fitzpatrick et al., 2002; Aldini et al., 2003; Corder et al., 2006, Caton et al., 2010).

Another important issue which has been highlighted by the data is the lack of reproducibility of replicates especially for the mRNA expression of ABCG1. For example, examining the 24 h results there is a 100-fold increase with 1 µM 25-OHC, 15-fold increase with 3 µM and a 300-fold increase with 10 µM (figure 2.11). This poor reproducibility was also encountered in subsequent experiments (see figures 2.15, 2.16, 2.18 and 2.20). Although the results show poor reproducibility they none-the-less do support the theory, that 25-OHC leads to increased mRNA expression of the transporter ABCG1. All experiments were repeated at least twice but still there are differences in the results obtained. These results are unlikely to represent natural variation as some represent 1,000-fold changes. The potential causes of these differences may relate to:

- Low copy numbers of mRNA relating to inadequate RNA extraction. This is a problem which is always encountered in PCR experiments and one which was a problem in earlier experiments but not later ones as the technique of RNA extraction improved. But
it is still feasible that in a plate of 12 wells one or two are not extracted as well as others and this would affect the final results as samples were run in triplicates. These errors from the variation in the amount of starting material between samples can be theoretically reduced not only by good RNA extraction technique but also by having an internal reference i.e. housekeeping gene.

- Instability of the reference gene. There were a number of occasions where the mRNA expression of the reference gene RNA polymerase 2 was noted to provide disparate results between samples, which is likely to be due to the method used. When this occurred the RT-PCR run was repeated with the reference gene and a different result, but one which was more expected was obtained. It is possible that there were differences in the results within a plate such that, when the mRNA expression of the gene of interest is then corrected for the reference gene by division this error is multiplied in the fold change in the expression. Once this was apparent more care was taken in analyzing the results from the reference gene and newer reagents were also ordered and experiments repeated.

AP5 reduced cell media cholesterol levels alone and in the presence of LDL-C and reduced solubilised cell cholesterol levels alone or in the presence of HDL-C or HDL-C in combination with LDL-C

In an attempt to re-create the in vivo process of cholesterol efflux cells were initially loaded with LDL-C and HDL-C was added in some studies to act as an acceptor of cholesterol. The LDL-C and HDL-C loading studies revealed discordant results when AP5 was incubated with BAEC for 6 h (figure 2.22). In this study AP5 suppressed the mRNA expression of ABCG1, HMGCR and CH25OH. This has occurred despite a marked reduction in ET-1 levels (figure 2.23). The reduction in ET-1 synthesis, which denotes a healthier endothelial cell is what was expected with AP5 and indirectly suggests that an appropriate OPC stimulus was applied to the cells. The experiment also revealed the following:

- Loading cells with LDL-C results in a marked reduction in the mRNA expression of ABCG1, HMGCR and CH25OH. Based on what is known about cellular cholesterol regulation when cells are loaded with LDL-C it would be anticipated that the cells will take up excessive cholesterol and switch-off de novo cholesterol synthesis and activate
measures to remove cellular cholesterol. Therefore it would be expected that ABCG1 mRNA expression increases, HMGCR mRNA expression decreases and CH25OH mRNA expression increases. Although HMGCR mRNA expression did reduce the other molecules did not change in the expected direction.

• Applying HDL-C to cells without any pre-loading with LDL-C led to a similar reduction in the mRNA expression of ABCG1 and HMGCR with very little change in CH25OH. If the cells are in a steady state then adding HDL-C may not necessarily lead to any changes in cell cholesterol management. If the cells are loaded with LDL-C then the addition of HDL-C should lead to increased ABCG1 mRNA expression, decreased HMGCR mRNA expression and increased CH25OH mRNA expression. It can be argued that the mRNA expression of ABCG1 would be higher than that seen with LDL-C alone as HDL-C will facilitate the movement of cholesterol out of the cell. It is possible that ABCG1 expression does not occur at 6 h and actually, the same experiment at 24 h would reveal a different picture. Alternatively, it is possible that the presence of HDL-C negates the need for ABCG1 for cholesterol efflux in this scenario.

Some of the reasons for the discrepant results could include the presence of interfering substances which prevented the detection of mRNA, or that the in-house purified LDL-C contains other proteins and thus is not representative of LDL-C or that loading cells with LDL-C has lead to toxicity and cellular death. The physiological concentration of LDL-C is in the order of 2 - 4 mM and a similar concentration was tested in the cell experiments (0.8 – 1.6 mg/ml). Indeed this would be one reason why the control cholesterol levels without LDL-C are much higher than all other treatment arms where LDL-C is present. Although the cells looked healthy under the microscope formal methods of cell staining to detect cell death should have been undertaken. Agarose gel electrophoresis would have been a simple method to help determine whether the molecules obtained were likely to be lipoproteins. It should be noted that LDL-C and HDL-C produced almost similar effects on the percentage change in cell media cholesterol in combination with AP5 at 6 h (figure 2.26), supporting the idea that LDL-C was not a pure compound. Also CH25OH may not have been stimulated by AP5 in this particular experiment or some other experimental error occurred for example, the concentration of HDL-C might have been too low.
In order to try and delve deeper into this an initial set of experiments is needed which test LDL-C loading of cells over varying time periods e.g. 1 h, 2 h, 6 h and 24 h and changes in the expression of HMGCR, CH25OH and ABCG1. Ideally this should be performed using purified LDL-C concentrates and with a variety of loading concentrations and cell staining to detect features of toxicity. It should also be remembered that measuring cholesterol efflux in vitro and in vivo is a challenge due to various influencing factors, which cannot be controlled. The present gold standard for in vitro measures relies on radioactive based experiments. Unfortunately, due to health and safety requirements, this was not a viable option but should be considered for further studies.

**Media and cell cholesterol levels**

Cell media cholesterol levels at 6 h were not significantly changed but at 24 h the cholesterol levels in LDL-C loaded cells in the presence of both AP5 and HDL-C did lead to a slight increase (~7%) in cell media cholesterol levels (figure 2.29). On the other hand the cholesterol levels in solubilised cell extract levels revealed a ~10% reduction in cholesterol levels from baseline with AP5 alone which mirrors changes in HMGCR mRNA levels (figure 2.31). This was also seen in the presence of HDL-C and both HDL-C and LDL-C, but was only in the order of ~5%. These changes suggest there is increased cholesterol efflux. The cholesterol detection method detects cholesterol which is free and also that present in cholesterol esters, by aid of a hydrolysis step. In turn this implies that any other cholesterol containing molecules will not be detected. For example, if any cholesterol is oxygenated to oxysterols this would not be detected. Although these findings did not reach significance, they are in the directions anticipated and possible reasons for the lack of robust findings in this experiment may include:

- AP5 did not lead to an increase in cellular cholesterol efflux – in this particular experiment although the mRNA expression of HMGCR was reduced there was no change in the expression of the transporter molecule ABCG1. Unfortunately cell media for cholesterol had not been collected for the earlier studies where mRNA expression of HMGCR reduced and that for ABCG1 increased in response to OPC.
- Cholesterol was effluxed from the cell but the HDL-C concentration not high enough to influence this.
• Cholesterol was effluxed from the cell but metabolized into another undetectable molecule in the cell media.

• Testing the cells beyond 24 h may be necessary to see the full effects.

• The cholesterol concentrations measured are very small (nanomolar) and this will inherently increase the chances of error.

**OPC reduce the levels of the vasoconstrictor ET-1**

The levels of the vasoconstrictor ET-1 levels were also reduced by OPC. The effects were greatest with the pentameric molecules and lowest at 6 h. This is similar to findings from other researchers (Corder et al., 2001; Jiménez et al., 2007; Caton et al., 2010). At present it is not thought that this effect is mediated through CH25OH, but further experiments are warranted especially as ET-1 is increasingly recognised as important in CVD and ageing. ET-1 was also reduced with AP5 when cells were loaded with LDL-C and in the presence of HDL-C (figure 2.23), although the reduction was greatest with AP5 alone. The changes in ET-1, HMGCR, and ABCG1 all point towards OPC providing overall reduction in CVD. These effects on different factors suggest that the initiating factor is likely to be one or more regulatory gene and it would be interesting to measure the effects on the mRNA levels of KLF2, NFkB, and peroxisomes proliferator-activated receptor (PPAR).

This *in vitro* work adds support to the hypothesis that OPC have a beneficial action on cell cholesterol levels by enhancing cellular cholesterol levels through cholesterol efflux. This is in agreement with the work of other researchers, (Wegrowski et al., 1984; Yamakoshi et al., 1999; Chang et al., 2001; Lee et al., 2008; Lam et al., 2008), but also provides the novel information that flavonoids can influence cholesterol efflux through changes in ABCG1. In addition application of a HMGCR inhibitor resulted in an increase in HMGCR mRNA levels, which were suppressed partially by AP5 at 6 h (figure 2.19). This provides two interesting observations firstly, that although HMGCR inhibitors reduce cholesterol they also lead to an up-regulation in the mRNA levels of HMGCR, probably via a feedback mechanism. Secondly, statins are associated with high HMGCR mRNA levels, which are likely to be through up-regulation. Although changes in the levels of mRNA were detected, it would be useful to observe whether there are changes in activity of HMGCR and ABCG1. HMGCR and ABCG1 were used as genes
of interest to test each of these aspects respectively but there are a number of other enzymes and molecules, which could have been, investigated e.g. apolipoproteins.

**How do BAEC observations correspond to the original microarray in HAEC**

The original microarray study was performed on HAEC (unpublished; Pothecary, 2007; Pothecary *et al.*, 2007), where responses were detected at 6 h, not at 1h. It is difficult to know whether changes in mRNA for HMGCR and ABCG1 occur at different times, or over a more extended time period in HAEC without detailed experiments on these cells. Even so the studies performed in BAEC do suggest similarities to the original findings from the microarray study in HAEC. In addition, the influence of culture conditions need to be evaluated as the growth media for HAEC is substantially different to that used for BAEC. This work has also highlighted a number of other areas, which need to be further researched. For example, initial studies did not show any major difference in the level of increase in CH25OH mRNA levels with either 5µg/ml or 10 µg/ml of GSE and it would be interesting to see if there is a concentration-response effect especially at lower concentrations.

The main learning points from this chapter are as follows:

- Be vigilant to how reproducible the data acquired is and not to ignore this but to try and determine why a discrepancy is occurring.

- Although it is interesting and advisable to try new methodology at the same time it is important to ensure there is appropriate expertise and experience available so that any problems arising can be discussed and resolved.

- Continue to ask questions regarding the mechanism of the effects being seen to improve our understanding rather than moving on to new avenues of research.

In future studies a good design is crucial and should help clarify some of the above issues e.g. poor reproducibility. Although all experiments were done by the author, some methods were performed more frequently than others and it is inevitable that there is a learning curve. It is feasible that with more time and experience the reliability of some of the tests would increase. Some of the experiments above should be repeated especially those with an uncertain outcome to try and resolve the issues underlying the changes seen. Testing one OPC at a time may be
useful as although GSE contains OPC it is not easy to compare results from GSE to those obtained from apple procyanidins, unless data regarding the constituents of these preparations is known. In addition to the suggested experiments, western blotting of both ABCG1 and HMGCR will help determine if any changes in mRNA expression are indeed associated with increased levels of the enzyme or transporter.
Chapter 3: The effect of high procyanidin content dark chocolate on cardiovascular risk in humans with borderline or stage I untreated hypertension
3.1 Introduction

3.1.1 The EPICURE study

A number of studies of the effects of dark chocolate on blood pressure have been reported, but the majority have not used a suitable placebo chocolate for comparison. This chapter describes a clinical trial that was undertaken called the “Evaluation of high Procyanidin Intervention with dark Chocolate on Underlying age-Related Elements of cardiovascular risk” (EPICURE). This was a double blind placebo controlled study with crossover design comparing a high procyanidin dark chocolate with a low flavanol dark chocolate of similar taste and composition. The principal research question was to determine whether regular twice daily consumption of dark chocolate containing a high content of procyanidins can lower blood pressure in those with raised normal blood pressure (i.e. pre-hypertension) or mild hypertension. The secondary objectives were to measure vascular function in the form of pulse wave analysis (PWA) and various blood biomarkers including endothelin-1, platelet function, high sensitivity C-reactive protein, and blood lipids and lipoproteins.

3.2 Measuring vascular dysfunction and its relationship to blood pressure

In order to understand the relationship between blood pressure and changes in vascular tone or vascular stiffness a number of methods have been developed. These can be broadly divided into evaluation of endothelial vasomotor function, biomarker measures of endothelial function and pulse wave analyses or pulse wave velocity.

3.2.1 Measurement of endothelial dysfunction

The tests used to measure endothelial dysfunction are summarised in table 3.1. Many of these tests are only able to assess endothelial vasomotor function, and not the other components of endothelial dysfunction, such as increased inflammation or presence of a prothrombotic state (O’Rourke, 2002). Other limitations include the absence of well-define normal ranges for these tests and whether these measurements predict the risk of developing CVD, the extent of atherosclerosis in particular subjects (Vapaatalo & Mervaala, 2001), or whether abnormalities are a precursor to hypertension.
Measures of endothelial dysfunction

- Markers of NO production and/or metabolism
- Biomarkers of endothelial function
- Endothelin-1
- Von Willebrand factor
- High sensitivity C-reactive protein
- Adhesion molecules e.g. vascular cell adhesion molecule-1
- Functional measures of endothelium-dependent vasodilatation (invasive, iontophoresis)
- Forearm blood flow measures in response to ischaemia: reactive hyperaemia (invasive)
- Flow mediated dilatation using ultrasonography (non-invasive)

Table 3.1 Various measures that indicate the presence of endothelial dysfunction (adapted from Vapaatalo & Mervaala, 2001).

3.2.2 Measuring endothelium dependent vasodilatation

Initial work measured coronary artery responses using intracoronary ACh infusion combined with quantitative angiography. The presence of an intact and healthy endothelium leads to dilation in response to ACh through receptor-mediated increase in NO, whilst in the presence of endothelial dysfunction there is paradoxical coronary artery vasoconstriction (Tousoulis et al., 2005). This is the result of ACh directly stimulating the coronary artery smooth muscle cells. The results are usually compared to those resulting from intracoronary administration of glycercyl trinitrate, which provides an exogenous source of NO thus causing endothelium independent dilatation (O’Rourke & Gallagher, 1996). These studies are invasive and expensive thus research has looked for other non-invasive or less invasive methods, which might be more practical especially in the context of screening for endothelial dysfunction (Tousoulis et al., 2005).

Pulse wave analysis

Pulse wave analysis is a non-invasive method of measuring peripheral endothelial function through determination of the arterial pressure waveform (O’Rourke & Gallagher, 1996). It uses a pen-like tonometer at the radial artery to pick up the arterial waveform from which the central artery blood pressures can be derived. Information regarding arterial stiffness can be deduced by
observing the following three parameters (Vlachopoulos et al., 2005; Tousoulis et al., 2005):

1) Pulse wave velocity (simultaneous assessment of arterial waveforms at two locations, velocity being calculated by distance/time),

2) Arterial pulse contour analysis and

3) Direct measures of arterial geometry. Wave reflection indices are also helpful and are important in assessing arterial elasticity. Augmentation index (AI) of the central pressure provides a measure of wave reflections and can be defined as:

“...augmented pressure divided by pulse pressure and expressed as a percentage.” (Vlachopoulos et al., 2005)

A typical arterial pressure waveform and its relationship to pressure in the arterial system are shown in figure 3.1. The arterial system is essentially of low resistance, which terminates in vessels of much higher resistance. The result is that mean arterial pressure falls by only 1 - 2 mmHg in the long arterial conduits but falls much more abruptly in the arterioles beyond (O’Rourke, 2002). Furthermore, it is at this arterial/arteriolar junction that wave reflection (consisting of up to 90% of the incident wave) occurs leading to the relatively larger pressure fluctuation in this part of the arterial system (Tousoulis et al., 2005; Wang et al., 2008). This backward going wave can reinforce a forward moving wave giving the concept of wave reflection (Anderson, 2006). The overall effect is that the shape of the pulse waveform at the proximal aorta differs markedly for both pressure and flow compared with that at the arteriolar bed (O’Rourke, 2002). Thus in healthy young subjects AI is usually negative whereas in those with CVD risk factors or increasing ageing (associated with increased stiffness) AI becomes increasingly positive (Vlachopoulos et al., 2005; Tousoulis et al., 2005; Anderson, 2006; Wang et al., 2008).

The arterial system can be considered as a Windkessel type reservoir, i.e. an elastic system where the arteries have a recoiling ability (Tousoulis et al., 2005; Anderson, 2006). Thus, the system has a reservoir nature where the level of the reservoir is determined by the peripheral resistance superimposed by the aortic pressure as a result of ventricular ejection. Thus, in a “normal” system the aortic pressure waveform is a summation of this reservoir pressure and effects of the forward and backward flowing waves (O’Rourke, 2002; Vlachopoulos et al., 2005; Anderson, 2006). In the presence of arterial stiffness, the backward travelling wave returns earlier, usually at the end of systole leading to increased systolic pressure (Vlachopoulos et al., 2005; Wang et al., 2008). This not only increases cardiac after load, but also reduces diastolic
coronary artery filling, both of which are detrimental to health (Tousoulis et al., 2005; Quyyumi & Patel, 2010).

\[
\text{Augmentation Index (AI)} = \frac{P_s - P_i}{P_s - P_d} \times 100 = \frac{\Delta P}{PP} \times 100
\]

**Figure 3.1** A central aortic arterial pressure or flow wave and its relationship to systolic, inflection and diastolic pressures. The augmentation index is calculated as the difference between PS and PI (\(\Delta P\)) and is expressed as a percentage of the pulse pressure (PP). (Adapted from Wang et al., 2008).

The pictorial waveform generated (such as in figure 3.2) is the summation of forward and reflected pressure waves at a single point (Anderson, 2006). It follows that the amplitude of the measured pulse pressure wave depends on the timing of the two preceding waves at that particular site of the arterial tree where the tonometer is placed. As an example in figure 3.2 the amplitude of the radial artery waveform is greater than that of the aortic waveform. This is because placing the tonometer at the radial artery means it is close to the reflection site and the reflection occurs almost immediately resulting in the additive effect seen (O’Rourke & Gallagher, 1996; Tousoulis et al., 2005). In comparison at this single point of time the reflected wave will not have reached the central arteries (i.e. the aorta in figure 3.2) resulting in a waveform with a lower amplitude. Rather it will reach in diastole, which is advantageous as this enhances coronary perfusion without increasing cardiac after load (O’Rourke, 2002; Vlachopoulos et al., 2005; Anderson, 2006). The presence of arterial stiffness changes this
timing so that the reflected wave returns earlier making the forward and reflected waves synchronised even when measuring at the radial artery (Wang et al., 2008).

PWA is measured in the supine position as this allows better detection of the radial waveform and recording of 10 sequential waveforms of good quality. The supine position is also less likely to be effected observer error (Patvardhan et al., 2010). Validation of the use of PWA to determine AI and of the transfer function used to derive aortic pressures has been tested by the simultaneous recording of arterial waveforms using both invasive cardiac catheterisation and applanation tonometry (e.g. Chen et al., 1996 and Pauca et al., 2001). But in total the study numbers have been small and there is lack of prospective validation studies (Tousoulis et al., 2005). PWA provides valuable information regarding arterial stiffness, which has been shown to have prognostic value e.g. mortality in patients with end-stage renal disease (Blacher et al., 1999a) and CAD (Blacher et al., 1999b; Boutouyrie et al., 2002; Laurent et al., 2001 and 2003; Weber et al., 2004b). In the presence of aortic valve disease it is assumed that PWA results are unreliable due to reduced radial pulse volume (as in the case of aortic stenosis) and technical difficulties (obtaining multiple successive waveforms of good quality). But little has been documented regarding the use of PWA in patients with aortic valve disease and in fact manufacturers of PWA systems do not recommend its use in aortic stenosis. Factors other than age which may possibly influence AI are heart rate and height, both of which have been cited as being inversely correlated, although heart rate is corrected for (Yasmin & Brown, 1999; Tousoulis et al., 2005).
Figure 3.2 Example report from SphygmoCor® pulse wave analysis system from a healthy individual. Radial artery tonometry is obtained and by use of a transfer factor the aortic pressure and waveform is obtained (based on technique developed and validated by O’Rourke & Gallagher, 1996). Note that the radial artery pressure waveform is of greater amplitude than that of the aorta owing to amplification as the waveform travels to the periphery.

Flow mediated dilatation studies

PWA has been criticised as not providing direct information about vessel function in organs, which are affected by CVD such as, the heart (Tousoulis et al., 2005). In order to overcome this, some studies have reported a correlation between coronary artery endothelial dysfunction and that of the brachial artery (Jadhav et al., 2003; Kitta et al., 2005). Brachial artery endothelial function can be measure by forearm blood flow (FBF) studies, often combined with vasoactive
drugs to obtain measures of endothelial dependent and independent function. However, more commonly flow mediated dilatation (FMD) is measured. This can be done non-invasively using ultrasonography where a transducer is placed over the brachial artery just above the antecubital fossa in order to measure the FBF in response to hyperaemia. Hyperaemia is achieved by the use of a blood pressure cuff at the wrist, or on the upper arm, which is inflated above systolic pressure (Jadhav et al., 2003). Deflation of the cuff leads to increased shear stress in the brachial artery, resulting from increased blood flow in the peripheral arteries. NO is the main mediator of reactive hyperaemia as exhibited by the abolishment of this process when the NOS inhibitor L-NMMA is infused (Jadhav et al., 2003; Tousoulis et al., 2005; Kitta et al., 2005; Rambaran et al., 2008). FMD with ultrasonography is again user and subject dependent but shows good reproducibility in both the short and long term (Jadhav et al., 2003; 2005; Kitta et al., 2005; Rambaran et al., 2008). Moreover, brachial FMD has been shown to be predictive for cardiovascular events in the Multi-Ethnic Study of atherosclerosis (Yeboah et al., 2009). More novel methods for endothelial function are coming to the forefront and table 3.2 provides a short summary of the variety of methods presently available.

There are few studies comparing PWA with FMD. Although the numbers of subjects investigated are low studies suggest that PWA with β₂-adrenoceptor agonist correlates with FMD and has high sensitivity and specificity in detecting abnormal endothelial function (Rambaran et al., 2008). But the same authors have concluded that where endothelial interventions are being studied FMD is the investigation of choice as there is a high within-subject variation (up to 10%) in measuring AI (Rambaran et al., 2007). In summary, although there is overlap between arterial stiffness and endothelial dysfunction with drugs that improve endothelial function also reducing arterial stiffness, in order for PWA to be used as an index of endothelial function it needs to be coupled with pharmacological manipulation i.e. the use of β₂-agonist salbutamol to provide information regarding endothelium dependent function (i.e. NO mediated) and glyceryl trinitrate (GTN, a direct nitrovasodilator) to assess endothelium independent function.
<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coronary circulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery endothelial function testing</td>
<td>Coronary artery catheterisation with ACh infusion and Doppler to measure artery diameter</td>
<td>Direct visualisation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Brachial artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachial artery reactivity test</td>
<td>US measure of brachial artery diameter followed by 5 min suprasystolic pressure leading to reactive hyperaemia. FMD is the ratio of post-test diameter to pre-test diameter.</td>
<td>Non-invasive</td>
</tr>
<tr>
<td></td>
<td>Can be used with venous occlusion plethysmography</td>
<td></td>
</tr>
</tbody>
</table>
| Venous occlusion plethysmography               | Venous return is occluded at the arm and wrist. Forearm volume changes are measured using strain-gauge plethysmography. Intra-arterial drugs given e.g. ACh and L-NMMA. | Can be used with strain gauge plethysmography or ultrasonography | Invasive  
Lengthy procedure  
User dependent |
|                                                |                                                                           |                                              |                                              |
| **Radial artery**                               |                                                                           |                                              |
| Pulse wave analysis                             | Peripheral artery waveforms recorded and changes with GTN and inhaled β2-agonist can be determined | Non-invasive                                 | Operator dependent  
Adverse effects to GTN and inhaled β2-agonist possible |
|                                                | Provides measure of AI which gives measure of global endothelial function |                                              |                                              |
| **Fingers**                                     |                                                                           |                                              |
| Pulse contour analysis                          | Photo-plethysmography using IR on index finger                           | Non invasive                                 | Low reproducibility  
Easily influenced by autonomic tone |
|                                                |                                                                           |                                              |                                              |
| Peripheral artery tonometry                     | Index finger probe which detects pulse wave amplitude using plethysmography. Measures taken at baseline and following reactive hyperaemia. | Non invasive, easy to use. Correction against recording from contralateral control hand reduces variability. | Easily influenced by autonomic tone and environment |
|                                                |                                                                           |                                              |                                              |
| Digital thermal monitoring                      | Fingertip temperature measured at baseline and following reactive hyperaemia. | Non invasive                                 | Relatively expensive  
Easily influenced by autonomic tone and environment |

Table 3.2 Various methods for measuring arterial stiffness and endothelial dysfunction. Some methods have been shown to be predictive of CVD in a variety of populations (adapted from Patvardhan et al., 2010).
3.3 Methodology

3.3.1 EPICURE study design

The study was a double blind, randomised clinical trial with a two-phase crossover study of subjects with pre-hypertension (systolic BP 130-139 mmHg; diastolic BP 85-89 mmHg) or mild untreated hypertension (systolic BP 140-159 mmHg; diastolic BP 90-99 mmHg). Subjects were randomly assigned to receive either high or low procyanidin dark chocolate (herein called HPDC and LPDC respectively) for 6 weeks and then swapped over without a washout period (figure 3.3.). A washout period was considered unnecessary as the effect of dark chocolate on blood pressure is largely back to baseline within 2-3 days after stopping consumption (Taubert et al., 2003) and blood lipids reach a steady state after 3-4 weeks of a change in diet.

The amount of chocolate was 50 g daily and this was divided into two portions each consumed in the morning and evening. The mean values of polyphenol content based on four extracts of the LPDC and HPDC were 1.9 and 21.9 mg/g, corresponding to daily doses of 95 and 1,094 mg/50 g respectively (analysed by the principal investigator). Levels of theobromine and caffeine were similar for LPDC and HPDC. Further analysis of LPDC and HPDC by Brunswick laboratories, MA, measured the content of various flavan-3-ol oligomers and is shown in table 3.3.
Study Design
(two phase crossover clinical trial)

Screening of eligible recruits (n = 32)

Baseline Measurements

Randomisation
Block design - groups of 4
(i.e. 2 high and 2 low procyanidin chocolate)

phase 1

6 weeks
high procyanidin chocolate

end of phase 1 measurements

crossover

6 weeks
low procyanidin chocolate

phase 2

6 weeks
low procyanidin chocolate

end of phase 2 measurements

6 weeks
high procyanidin chocolate

Figure 3.3 Flow diagram showing the study design for the EPICURE study.
<table>
<thead>
<tr>
<th>Flavan-3-ol</th>
<th>HPDC</th>
<th>LPDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mers</td>
<td>7.39</td>
<td>0.74</td>
</tr>
<tr>
<td>2mers</td>
<td>6.38</td>
<td>0.76</td>
</tr>
<tr>
<td>3mers</td>
<td>4.27</td>
<td>0.45</td>
</tr>
<tr>
<td>4mers</td>
<td>3.69</td>
<td>0.20</td>
</tr>
<tr>
<td>5mers</td>
<td>3.67</td>
<td>0.14</td>
</tr>
<tr>
<td>6mers</td>
<td>2.80</td>
<td>0.12</td>
</tr>
<tr>
<td>7mers</td>
<td>1.57</td>
<td>0.10</td>
</tr>
<tr>
<td>8mers</td>
<td>2.04</td>
<td>0.13</td>
</tr>
<tr>
<td>9mers</td>
<td>2.24</td>
<td>0.18</td>
</tr>
<tr>
<td>10mers</td>
<td>1.25</td>
<td>0.00</td>
</tr>
<tr>
<td>&gt;10mers</td>
<td>17.12</td>
<td>0.66</td>
</tr>
<tr>
<td>Total (excluding &gt;10mers)</td>
<td>35.3</td>
<td>2.82</td>
</tr>
<tr>
<td>Total 1mers to 10mers per 50g bar (assuming fat content of 35%)</td>
<td>1147.25</td>
<td>91.65</td>
</tr>
<tr>
<td>Total</td>
<td>52.42</td>
<td>3.48</td>
</tr>
<tr>
<td>Total per 50g bar (assuming fat content of 35%)</td>
<td>1703.65</td>
<td>113.1</td>
</tr>
</tbody>
</table>

Table 3.3 Brunswick analyses of flavan-3-ol content (mg/g defatted weight) of HPDC and LPDC.

The study began in the summer of 2007 after local ethics committee approval and continued until 32 subjects were recruited who satisfied the inclusion and exclusion criteria. It should be noted that the basic methodology for the EPICURE study had already been determined at the point of the authors involvement and as the ethics process had almost been completed it was not possible for any changes in the design or methodology could be made. Potential subjects were identified by approaching local GP practices and after gaining primary care trust consent (Newham, Tower Hamlets, and Hackney Primary Care Trust’s). Potential subjects were invited to attend an initial pre-screening visit where the study was discussed. If patients were interested in participating a screening visit was arranged. The study continued for approximately 18 months to achieve the desired number of participants. Subjects attended the department on 10 occasions as listed in table 3.4.

The effects on blood pressure were determined by both clinic measurements and 24 h ambulatory measures at baseline, 6 and 12 weeks. It was important that the 24 h blood pressure
monitor device was begun on the penultimate day of taking chocolate for the particular arm of the study to ensure measurements were made while the respective chocolate was being consumed. At randomisation, 6 and 12 weeks PWA was performed to obtain measures of endothelial function. Blood was also withdrawn for measures of lipids, platelet function, and various biomarkers of vascular function including ET-1 and high sensitivity CRP. The study was powered to 80% to detect a 0.5 standard deviation change in blood pressure, which was based on changes reported by other studies (e.g. Grassi et al., 2005a; Grassi et al., 2005b; Taubert et al., 2003).

<table>
<thead>
<tr>
<th>Visit</th>
<th>Objective</th>
<th>Parameters measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pre-screen</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>Screen</td>
<td>Sign consent form</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Full history and examination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMI, waist/hip circumference</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clinic BP measurement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline 12 lead ECG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Screening bloods e.g. renal function, liver function tests, fasting lipids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Attach 24 h ABPM</td>
</tr>
<tr>
<td>3</td>
<td>Return 24 h ABPM</td>
<td>Nil</td>
</tr>
<tr>
<td>4</td>
<td>Randomisation (if meet inclusion/exclusion criteria as determined at visit 3)</td>
<td>Physical examination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fasting bloods for lipids, platelet function, biomarkers, vascular function</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pulse wave analysis with clinic BP measurement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Start treatment</td>
</tr>
<tr>
<td>5</td>
<td>3 week follow-up</td>
<td>Fasting bloods for lipids and vascular biomarkers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clinic BP measurement</td>
</tr>
<tr>
<td>6</td>
<td>6 week follow-up and end of phase I of treatment</td>
<td>Fasting bloods for lipids, platelet function, biomarkers, vascular function, plasma polyphenols</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pulse wave analysis with clinic BP measurement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h ABPM</td>
</tr>
<tr>
<td>7</td>
<td>Return 24 h ABPM</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Provide chocolate for phase II of study</td>
</tr>
<tr>
<td>8</td>
<td>9 week follow-up</td>
<td>Fasting bloods for lipids, vascular biomarkers and plasma polyphenol levels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clinic BP measurement</td>
</tr>
<tr>
<td>9</td>
<td>12 week follow-up and end of phase II of treatment</td>
<td>Fasting bloods for lipids, platelet function, biomarkers, vascular function, plasma polyphenols</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pulse wave analysis with clinic BP measurement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h ABPM</td>
</tr>
<tr>
<td>10</td>
<td>Return 24 h ABPM</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Table 3.4 Subject visits and procedures in the EPICURE study.
For the purpose of this study, female subjects were excluded to prevent potential confounding by gender variation and the also because of the lower prevalence of pre-hypertension or mild hypertension in this group which may have effected recruitment. The study recruited 32 men although this took longer than the anticipated 3 months. There are several reasons that may explain this lack of interest, lack of number of male patients with untreated hypertension, too cumbersome visit periods and low incentives are just a few. Despite these problems, the study recruited the appropriate numbers of subjects. The various inclusion and exclusion criteria were set out at the beginning of the study and are listed in table 3.5.

### Inclusion criteria for the EPICURE STUDY

- Male gender
- Pre-hypertension (systolic BP 130-139 mmHg; diastolic BP 85-89 mmHg) or mild hypertension (systolic BP 140-159 mmHg; diastolic BP 90-99 mmHg)
- Not on any regular medications
- Otherwise fit and well

### Exclusion criteria for the EPICURE STUDY

- Age <45 years or >70 years
- Diabetes mellitus or raised fasting glucose
- Total cholesterol > 7 mmol/L
- BMI > 30kg/m$^2$
- History of cardiac arrhythmias or ECG abnormalities at baseline
- History of psychiatric or psychological illness
- History of hypo- or hyperthyroidism
- Participation in another trial – whether active or in follow-up
- Abnormal liver function tests or other routine blood chemistry
- Excessive alcohol consumption
- Regular medicines for any condition
- Regular use of herbal medicines or other alternative remedies

Table 3.5 Inclusion and exclusion criteria for the EPICURE study.
In addition to this, subjects had to agree to abstain from red wine and chocolate (other than study chocolate) consumption throughout the study period. Subjects were also asked to refrain from taking aspirin and non-steroidal anti-inflammatory drugs (which could potentially influence vascular and platelet function results), for at least 4 weeks before randomisation and for the duration of the study. Paracetamol was advised for simple analgesia as an alternative to non-steroidal anti-inflammatory drugs and subjects were asked to inform the study team of any other medications they may have needed to take during the period of evaluation e.g. antibiotics. Subjects were also asked to maintain a diary of their chocolate consumption, which was used as a method for determining adherence.

3.3.2 Pulse wave analysis and clinic blood pressures
Blood pressure and heart rate was measured at every visit and as part of PWA. These measurements were taken from the upper arm devoid of clothing with calibrated electronic sphygmomanometers. The subjects were sitting at rest (for a minimum of 5-10 min) and their arm supported at heart level. Arm circumference was measured on the initial visit to ensure the correctly sized cuff was used. Three readings were taken in total with at least 2-3 minutes in between and the average of the last two taken as the measure.

PWA was recorded using a Millar tonometer (see figure 3.4), which is a pen like probe in conjunction with SphygmoCor software analysis (AtCor Medical Ltd). This is a fairly simple and non-invasive procedure and involves placing the tonometer over the radial artery and recording the pulse waveform for 1 - 2 min (see figure 3.4). Salbutamol (400 µg) was also administered in conjunction with PWA to obtain information regarding endothelium dependent vasodilatation (EDV). Three basal PWA measures were taken at 5 min intervals and then salbutamol was administered by inhalation. PWA measures were then taken at 5 min intervals for 20 min.
Figure 3.4 SphygmoCor pulse wave analysis system. This depicts (a) pen-like probe (tonometer) used in PWA and (b) example placement of tonometer over radial artery.
3.3.3 24 h ABPM

Twenty-four hour ambulatory blood pressure monitoring (24 h ABPM) is a non-invasive method to measure BP over 24 h. A cuff is placed around the non-dominant arm and is connected to a small box that will capture and store BP data. The cuff will inflate every 20 min during the daytime and every 30 min over night. After 24 h, the monitor is taken off and data downloaded to a computer, which will not only provide each of the individual BP readings but also the calculated day time, night time and overall averages. The advantages of 24 h ABPM relate to it representing a truer reflection of an individual’s BP as it provides readings for 24 h and readings are taken in the individuals own environment avoiding any “white coat” hypertension effects. There is also increasing data that BP measured over a 24-hour period is superior to clinic blood pressures in predicting future cardiovascular events and target organ damage e.g. left ventricular hypertrophy (McGrath, 2002; Clement et al., 2003; White, 2007; Padiyar & Rahman, 2007; Wexler, 2010).

The disadvantages of 24 h ABPM relate mostly to its not being widely available leading to reduced experience in its use and comprehension. Furthermore, although it is non-invasive some patients do find inflation of the cuff unbearable, especially at night time resulting in sleep disturbance and even bruising where the cuff is located. The test can also be affected by background noise (less of a problem with oscillometric methods) and arrhythmias may cause poor readings (O’Brien et al., 2001). The test also requires patients/subjects co-operation, as they need to be educated how to put the cuff back on and restarting the system if needed. For the EPICURE study 24 h ABPM was performed using the Spacelabs device (Spacelabs 90207, Spacelabs Healthcare, Hertford, UK). It was measured at baseline, 6 weeks, and 12 weeks. Subjects tolerated the 24 h ABPM well with no adverse effects being reported during the study. There were some machine errors resulting in only 21 patients with completed data sets for 24 h ABPM.
3.3.4 HsCRP measurements

Materials
A commercial hsCRP enzyme immunoassay test kit was purchased from MP Biomedical, NY. Where not specified, materials and reagents were purchased from Sigma.

Procedure
HsCRP was measured using an enzyme immunoassay kit according to the instructions. EPICURE serum samples were diluted 100 fold prior to assay and standards were also made up from the CRP reference standard set made available in the kit (by adding deionised water and subsequently storing at 2 - 8°C). The ELISA was based on a solid phase enzyme-linked immunosorbent assay using a mouse monoclonal antibody. This antibody was directed towards CRP in the solid phase immobilisation (using pre-coated microtiter wells) followed by a goat anti-CRP antibody in the antibody-enzyme conjugate solution (conjugated to horseradish peroxidase). Ten microlitres of the diluted serum samples and controls and standards were pipetted into the wells, being run in duplicate. This was followed by 100 µl of the CRP enzyme conjugate reagent.

The use of two antibodies directed towards the CRP allows the molecule to become sandwiched in-between. This was followed by a 45 min incubation period at room temperature. After this, the incubation mixture was discarded and the wells rinsed five times with deionised water to remove any unbound antibodies, taking care to remove all residual water from the plates. Tetramethylbenzidine reagent (100 µl) was added and after mixing the samples were incubated for a further 20 min at room temperature resulting in a colour change in the samples and standards. At this point 100 µl dilute hydrochloric acid solution was added which stopped any further colour change and resulted in a yellow discoloration and the CRP concentration was measured within 15 min using spectrophotometry at 450 nm, with levels being proportional to the intensity of the final colour change. The concentration of CRP in the test serum samples was then calculated by reading off the standard curve and multiplying by the dilution factor.

Limitations of the methodology
The minimum detectable concentration of hsCRP was 0.1 mg/l with an intra-assay coefficient of variation of 7.5 % and inter-assay coefficient of variation of 4.1%. For reproducible hsCRP measurements multiple freeze-thaw cycles of serum samples and long duration of storage were avoided by assaying the complete set of samples as soon as the study was completed. For
reliable measurements samples could not be lipaemic, haemolysed or turbid. One factor, which could not be controlled for, is the biological variation in CRP, which has been reported to range between 138% to 759% across differing ages, and factors contributing to this include leukocyte count and smoking, which were not controlled for (Chenillot et al., 2000).

3.3.5 Statistical analysis
Graphical representation of hsCRP is shown relative to basal (pre-treatment) levels, whilst PWA data are shown compared to time 0. Data were analysed using both MiniTab software (MiniTab Inc, Pennsylvania) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The general linear model was used and is an extension of multiple linear regression for a single dependent variable, which quantifies the relationship between several predictor variables. This regression assumes parametric distributions, which were informally determined by observing frequency distribution histograms in the MiniTab software, although the use of Kolmogorov-Smirnov test was not used. Statistical analyses, including general linear model analysis of variance (GLM ANOVA) was undertaken with MiniTab software, this was followed by post hoc analysis using Dunnett’s test where appropriate.
3.4 Results

Table 3.6 depicts the baseline characteristics of the subjects in the EPICURE study. The mean weight gain at the end of the study was 0.34 kg (SEM ± 0.39 kg), which was not statistically significant. In general, although trends were seen in many interesting areas there were no results that were clinically significant apart from heart rate both on the 24 h ABPM and PWA.

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.4 ± 1.5</td>
</tr>
<tr>
<td>Wt (kg)</td>
<td>80.4 ± 3.0</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.7 ± 0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood pressure in mmHg</th>
<th>Systolic / Diastolic (Heart Rate in bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinic</td>
<td>144.8 ± 2.1 / 87.1 ± 1.3 (69.3 ± 2.0)</td>
</tr>
<tr>
<td>24h ABPM (Overall)</td>
<td>131.4 ± 1.5 / 82.6 ± 1.2 (72.6 ± 1.6)</td>
</tr>
<tr>
<td>24h ABPM (daytime)</td>
<td>135.6 ± 1.7 / 86.0 ± 1.3 (74.5 ± 1.7)</td>
</tr>
<tr>
<td>24h ABPM (night time)</td>
<td>127.0 ± 1.8 / 78.4 ± 1.4 (69.9 ± 1.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid levels (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
</tr>
<tr>
<td>Triglycerides</td>
</tr>
</tbody>
</table>

Table 3.6 Baseline characteristics of subjects in the EPICURE study.  (Data is expressed as mean ± SEM).
3.4.1 24 hour ambulatory blood pressure monitoring

Completed ambulatory blood pressure data were available for only 21 subjects which was due to a combination of technical errors (n = 4) and excluded patients (n = 7, due to poor adherence and non-attendance). Mean systolic blood pressure overall (SBPO) was 131.7 mmHg after LPDC and slightly lower after HPDC (-2 mmHg; table 3.7a and figure 3.5). This did not reach statistical significance (General Linear Model (GLM) ANOVA p=0.217). The raw individual data (figure 3.5b) suggests that some subjects had a greater response than others did. A further sub-analysis did not relate this to factors such as increasing age or smoking status. On the other hand diastolic blood pressure overall (DBPO) was largely unchanged between LPDC and HPDC (-0.62 mmHg in the HPDC group; GLM ANOVA p= 0.608, see table 3.7a and figure 3.6).

Night time and daytime blood pressures were also analysed revealing that systolic blood pressure in the daytime (SBPD; table 3.7b and figure 3.7) and diastolic blood pressure in the daytime (DBPD; table 3.7b and figure 3.8) showed a non-significant trend to be lower with HPDC (SBPD -2.76 mmHg; GLM ANOVA p= 0.123 and DBPD -0.52 mmHg; GLM ANOVA p= 0.253). Systolic blood pressure at night (SBPN; table 3.7c and figure 3.9) and diastolic blood pressure at night (DBPN; table 3.7c and figure 3.10) were not significantly changed after HPDC (-0.43 mmHg with GLM ANOVA p= 0.838 and +0.67 mmHg with GLM ANOVA p= 0.668 respectively).
Table 3.7 24 h ABPM derived blood pressure results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM), differences, and p-value’s of blood pressures (mmHg) between 50 g of HPDC and LPDC in 21 subjects in the EPICURE study. Results are displayed as (a) overall (24 h) (b) daytime and (c) night-time comparisons.
Figure 3.5 Overall systolic blood pressure (SBPO; mmHg) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline and (b) Individual subject mean values.
Figure 3.6 Overall diastolic blood pressure (DBPO; mmHg) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
Figure 3.7 Overall daytime systolic blood pressure (SBPD; mmHg) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
Figure 3.8 Daytime diastolic blood pressure (DBPD; mmHg) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
Figure 3.9 Night time systolic blood pressure (SBPN; mmHg) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
Figure 3.10 Night time diastolic blood pressure (DBPN; mmHg) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
3.4.2 Heart rate

Overall heart rate was significantly reduced by HPDC (-3.28 bpm; GLM ANOVA p=0.024; table 3.8 and figure 3.11). This was also seen in the daytime and night time measurements (-4.05 bpm; GLM ANOVA p=0.001 and -3.66 bpm GLM ANOVA p=0.033 respectively; table 3.8 and figures 3.12 and 3.13).

<table>
<thead>
<tr>
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<th>Heart Rate</th>
<th>Heart Rate</th>
<th>Heart Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>SEM</td>
</tr>
<tr>
<td>High</td>
<td>73.24</td>
<td>10.09</td>
<td>2.20</td>
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<tr>
<td>Low</td>
<td>76.52</td>
<td>10.66</td>
<td>2.33</td>
</tr>
<tr>
<td>Difference</td>
<td>-3.29</td>
<td>6.14</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Table 3.8 24 h ABPM derived heart rate results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM), differences, and p-value’s of 24 h ABPM heart rate (bpm) between 50 g of HPDC and LPDC in 21 subjects in the EPICURE study. Results are displayed as overall, daytime, and night time comparisons.
Figure 3.11 Overall heart rate (HRO; bpm) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
Figure 3.12 Daytime heart rate (HRD; bpm) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
Figure 3.13 Night time heart rate (HRN; bpm) differences between 50 g HPDC and LPDC chocolate in the EPICURE study. Data are shown as (a) Box plot depicting sample minimum and maximum, lower and upper quartile’s and median from baseline (star represents outliers = mean ± 2SD) and (b) Individual subject mean values.
3.4.3 Pulse Wave Analysis

Mean brachial systolic pressure (B SP; figure 3.14) was significantly lower with salbutamol (-2.8 mmHg; table 3.9) after HPDC (GLM ANOVA p = 0.046) as was mean brachial pulse pressure (B PP; figure 3.15 - 2.807 mmHg; GLM ANOVA p= 0.016). Mean brachial diastolic pressure (B DP; table 3.9 and figure 3.14) was not different between LPDC and HPDC (GLM ANOVA p=0.41). Mean aortic systolic pressure (A SP; table 3.10 and figure 3.16), mean aortic diastolic pressure (A DP; table 3.10 and figure 3.16; GLM ANOVA p=0.659) and mean aortic pulse pressure (A PP; table 3.10 and figure 3.17; GLM ANOVA p= 0.202) were also not significantly different between the two treatment groups although there was a small non significant decrease in mean A SP after HPDC (-0.6198 mmHg, GLM ANOVA p= 0.4542; see table 3.10). The mean PP amplification ratio was significantly different being -0.03375 lower with HPDC (see table 3.11 and figure 3.18; GLM ANOVA p= 0.007).

Mean arterial pressure (MAP) was lower with HPDC but this did not reach statistical significance and was of a very small magnitude (-0.4770 mmHg, GLM ANOVA p = 0.5079; see table 3.12 and figure 3.19). Again mean heart rate on the pulse wave analysis was lower in subjects taking the HPDC (-2.88 bpm) a finding which was also statistically significant (see table 3.13 and figure 3.20; GLM ANOVA p= 0.0036). Mean augmentation pressure (AP) and mean augmentation index (AI) were not different between either treatment arms (see table 3.14 and figure 3.21; GLM ANOVA p= 0.1323 and p= 0.9508).
### a) Brachial systolic pressure comparison (N = 27)

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Baseline</th>
<th>Salbutamol 10 min</th>
<th>Salbutamol 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>SEM</td>
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<tr>
<td>HPDC</td>
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<td>LPDC</td>
<td>134.62</td>
<td>10.17</td>
<td>1.96</td>
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</table>

General linear model ANOVA for B SP versus PC content \( p = 0.046 \)

### b) Brachial diastolic pressure comparison (N = 27)

<table>
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</tr>
</thead>
<tbody>
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<td>LPDC</td>
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</table>

General linear model ANOVA for B DP versus PC content \( p = 0.411 \)

### c) Brachial pulse pressure comparison (N = 27)

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</thead>
<tbody>
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<td>SEM</td>
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<td>LPDC</td>
<td>53.97</td>
<td>10.92</td>
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General linear model ANOVA for B PP versus PC content \( p = 0.016 \)

**Table 3.9** PWA derived brachial pressure results from the EPICURE study. *Mean, standard deviation (SD), standard error of the mean (SEM) and p-value’s of PWA derived brachial pressures (mmHg) following salbutamol inhalation between 50 g of HPDC and LPDC in 27 subjects in the EPICURE study. Results are displayed as (a) systolic (b) diastolic and (c) pulse pressure comparisons.*
Figure 3.14 PWA derived brachial systolic and diastolic pressure in the EPICURE study. Changes in PWA derived mean brachial systolic pressure (B SP in mmHg; top panel) and mean brachial diastolic pressure (B DP in mmHg; bottom panel) following salbutamol inhalation over 15 min between 50 g of HPDC and LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Figure 3.15 PWA derived brachial pulse pressure in the EPICURE study. Changes in PWA derived mean brachial pulse pressure (B PP in mmHg) following salbutamol inhalation over 15 min between 50 g of HPDC and LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
### a) Aortic systolic pressure comparison (N = 27)

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Baseline</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>HPDC</td>
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<td>LPDC</td>
<td>121.99</td>
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General linear model ANOVA for A SP versus PC content p = 0.4542

### b) Aortic diastolic pressure comparison (N = 27)

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<tbody>
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<tr>
<td>HPDC</td>
<td>83.15</td>
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<td>1.52</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>LPDC</td>
<td>82.15</td>
<td>6.68</td>
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</table>

General linear model ANOVA for A DP versus PC content p = 0.659

### c) Aortic pulse pressure comparison (N = 27)

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<tr>
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</table>

General linear model ANOVA for A PP versus PC content p = 0.202

Table 3.10 PWA derived aortic pressure results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value’s of PWA derived aortic pressures (mmHg) following inhalation of salbutamol between 50 g of HPDC and LPDC in 27 subjects in the EPICURE study. Results are displayed as (a) systolic (b) diastolic and (c) pulse pressure comparisons.
Figure 3.16 PWA derived aortic systolic and diastolic pressure in the EPICURE study. Changes in PWA derived mean aortic systolic pressure (A SP in mmHg; top panel), mean aortic diastolic pressure (A DP in mmHg; bottom panel) following salbutamol inhalation between 50 g of HPDC and LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Figure 3.17 PWA derived aortic pulse pressure in the EPICURE study. Changes in PWA derived mean aortic pulse pressure (A PP in mmHg) following salbutamol inhalation between 50 g of HPDC and LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Pulse pressure amplification ratio comparison (N = 27)

<table>
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<tr>
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<td>SEM</td>
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<td>HPDC</td>
<td>1.3093</td>
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<td>0.02594</td>
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<tr>
<td>LPDC</td>
<td>1.3634</td>
<td>0.1436</td>
<td>0.0276</td>
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General linear model ANOVA for PP amplification versus PC content p = 0.007

Table 3.11 PWA derived pulse pressure amplification results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PWA derived pulse pressure amplification ratio after salbutamol inhalation between 50 g of HPDC and LPDC in 27 subjects in the EPICURE study.

![Pulse pressure amplification ratio](image)

Figure 3.18 PWA derived pulse pressure amplification in the EPICURE study. Changes in PWA derived mean pulse pressure amplification ratio following salbutamol inhalation between 50 g of HPDC and LPDC in the EPICURE study (GLM ANOVA p = 0.007). (Figures are expressed as mean ± SEM).
Mean arterial pressure comparison (N = 27)

<table>
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<th>Chocolate</th>
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<th>Salbutamol 15 min</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Mean</td>
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<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>HPDC</td>
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<td>1.80</td>
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<td>LPDC</td>
<td>99.80</td>
<td>6.53</td>
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<td>98.17</td>
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General linear model ANOVA for MAP versus PC content p = 0.5079

Table 3.12 PWA derived mean arterial pressure results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PWA derived mean arterial pressure (mmHg) after salbutamol inhalation between 50 g of HPDC and LPDC in 27 subjects in the EPICURE study.

Figure 3.19 PWA derived mean arterial pressure in the EPICURE study. Changes in PWA derived mean arterial pressure (mmHg) following salbutamol inhalation between 50 g of HPDC and LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Table 3.13 PWA derived heart rate results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PWA derived heart rate (bpm) after salbutamol inhalation between 50 g of HPDC and LPDC in 27 subjects in the EPICURE study.

Figure 3.20 PWA derived heart rate in the EPICURE study. Changes in PWA derived heart rate (bpm) following salbutamol inhalation between 50 g of HPDC and LPDC in the EPICURE study (GLM ANOVA p = 0.0036). (Figures are expressed as mean ± SEM).
a) Mean augmentation pressure comparison (N = 27)

<table>
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<tr>
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<th>Salbutamol 15 min</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>SEM</td>
</tr>
<tr>
<td>HPDC</td>
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<tr>
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<td>4.306</td>
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General linear model ANOVA for HR versus PC content p = 0.1323

b) Mean augmentation index comparison (N = 27)

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<th>Baseline</th>
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<th>Salbutamol 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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</tr>
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<td>HPDC</td>
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<td>LPDC</td>
<td>21.19</td>
<td>9.16</td>
<td>1.76</td>
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General linear model ANOVA for HR versus PC content p = 0.9508

Table 3.14 PWA derived augmentation pressure and augmentation index results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value’s of PWA derived a) augmentation pressure (mmHg) and b) augmentation index (%) following salbutamol inhalation between 50 g of HPDC and LPDC in 27 subjects in the EPICURE study.
Figure 3.21 PWA derived augmentation pressure and augmentation index in the EPICURE study. Changes in PWA derived augmentation pressure (mmHg; top panel) and augmentation index (%; bottom panel) following salbutamol inhalation between 50 g of HPDC and LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
### 3.4.4 High sensitivity C-reactive protein

There were no differences in hsCRP between LPDC and HPDC, even when mean values at 3 weeks consumption point were analysed (see table 3.15 and figure 3.22; GLM ANOVA p = 0.467; N=27).

<table>
<thead>
<tr>
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<th>Hs C-reactive protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Basal</td>
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</tr>
<tr>
<td>LPDCWk3</td>
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</tr>
<tr>
<td>LPDC</td>
<td>2.618</td>
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<tr>
<td>HPDCWk3</td>
<td>2.648</td>
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<td>HPDC</td>
<td>3.479</td>
</tr>
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</table>

General linear model ANOVA for hsCRP versus PC content p = 0.467

**Table 3.15 hsCRP results in the EPICURE study.** Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of hs C-reactive protein (mg/l) between 50 g of HPDC and LPDC in the EPICURE study at basal levels (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparisons with basal, pre-treatment level.
Figure 3.22 Changes in hsCRP levels in the EPICURE study. Changes in hs C-reactive protein (mg/L) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values, but for GLM ANOVA basal values are used as only one comparator. (Figures are expressed as mean ± SEM).
3.5 Discussion

Several human trials have shown that chocolate and chocolate products can lower blood pressure in humans (Grassi et al., 2005a and 2005b; Taubert et al., 2003; Taubert et al., 2007), improve endothelial dysfunction (Allen et al., 2008; Balzer et al., 2008; Engler et al., 2004; Farouque et al., 2006; Flammer et al., 2007; Heiss et al., 2005; Schroeter et al., 2006; Vlachopoulos et al., 2005; Wang-Polagruto et al., 2006) or both (Faridi et al., 2008; Heiss et al., 2010). Many of these studies have been criticised due to lack of blinding and other methodological issues, such as dose of OPC. The EPICURE trial was undertaken to address some of these issues. In particular, the EPICURE study was double blinded and used an appropriately matched placebo. In summary, HPDC reduced 24 h blood pressure and heart rate, but only the latter reached statistical significance (p < 0.001). Augmentation pressure, augmentation index, and hsCRP were not different amongst the groups. This study highlighted some of the problems in performing trials with cocoa/chocolate products and the importance of good study design. The main findings that are to be discussed here include:

- Reduction in 24 h blood pressure with HPDC and the heterogeneous response noted.
- Reduction in heart rate on both 24 h ABPM and PWA with HPDC.
- Lowered pulse pressure amplification with HPDC.
- Lack of change in augmentation index with HPDC.

It is likely that the lack of statistically significant results related to the study being underpowered, a suggestion that is supported by the trend to lower systolic BP in the HPDC group. Hooper and colleagues (2008) performed a meta-analysis on cardiovascular risk and flavonoids and up to the published date could only find 4 out of 133 eligible randomised controlled trials, which reported significant reductions in systolic and diastolic BP. The mean fall in BP was -5.88/-3.30 mmHg with chocolate. The authors concluded that a major shortcoming was the absence of studies powered to assess effects of flavonoids on CVD risk. This suggests that a real difference probably does exist even though this was not demonstrated in this study, and this is probably the result of lack of power. Similarly, the meta-analysis revealed that chocolate flavonoids also increased FMD after both acute and chronic intake (Hooper et al., 2008).
Initial power calculations were based on the available literature at the time. Large reductions in BP were initially reported (up to 10 mmHg in systolic blood pressure; for example, Taubert et al., 2003; Grassi et al., 2005a; Grassi et al., 2005b) but since then it is apparent that the BP changes are widely disparate. It should also be noted that although statistical significance for systolic BP was not achieved a reduction by 2 mmHg is clinically significant but not as much as current antihypertensive medications (e.g. ACEI are associated with a reduction in systolic blood pressure of 5 mmHg, and thiazide diuretics a reduction of 4 mmHg; Bahiru & Kloner, 2008). But the reported change in systolic blood pressure is not as much as has been reported with salt reduction (a reduction of 5 g per day is associated with a fall in blood pressure of 7/3 mmHg; Cappuccio et al., 1997) and even meditation appears more effective (with a reduction in systolic blood pressure by 4.7 mmHg (Bahiru & Kloner, 2008).

It was also interesting to see that some subjects had a greater response to HPDC but it remains unclear as to why this was the case and this warrants further exploration as it is likely to impact the selection of subjects for any further studies, and also recommendations as to which individuals may benefit most from increasing flavanol consumption. Possible reasons for this heterogeneous response include:

- Observer errors.
- Subject errors – this includes biological variation, smoking, age differences, electrolyte intake especially salt.

Preliminary studies exploring both the precision of the test, including inter- and intra-test coefficient of variations and determining whether a correlation exist between age, smoking and electrolyte intakes (e.g. urinary salt excretion) would help in understanding the influencing factors determining the heterogeneity of blood pressure responses (Cappuccio et al., 1997). HPDC also appeared to have a greater effect on BP in the daytime than at night. There could be a variety of reasons for this, including the effects of OPC when taken by mouth last about 8 h, thus by night time the effects of OPC had worn off and three times daily dosing may be more productive than the twice daily dosing used in the study (Davison et al., 2010).

The lack of statistically significant results may also be the result of errors in making these measurements. For example, it is well known that BP and PWA testing should be conducted in a
quiet environment but due to the nature of the study unit this was not always possible (Tousoulis et al., 2005; Anderson, 2006; Wang et al., 2008). In addition, although 32 subjects were recruited, completed 24 h ABPM data was only available for 21 subjects. This was due to a number of factors such as, malfunction of equipment and software and poor-adherence by subjects. Compliance check is an important factor and the method of diary monitoring is not conclusive. Further studies need to incorporate methods to provide an index of compliance. For example, incorporation of non-therapeutic substances which can subsequently be detected in urine. In addition, the average blood pressure at the beginning of the study may not have been high enough (mean 131.4 / 82.6 mmHg). Although the systolic level is towards the bottom end of prehypertension levels, the diastolic blood pressure is classified as “normal”, and a reduction in blood pressure may have been more readily detected with participants having higher blood pressures.

One statistical significant finding was the reduction of heart rate with HPDC which was seen in both the daytime and night time on the 24 h ABPM and with the clinic measurements conducted at the time of PWA. Reduced heart rate is known to reduce shear stress across vessels (reference required) and thus is likely to reduce endothelial dysfunction, which may be present in patients with early hypertension. It is difficult to truly know the benefits from HPDC induced lowered heart rate. BP is known to be a function of heart rate, stroke volume, and total peripheral resistance and thus a reduction in heart rate will help prevent an increase in BP. Under normal circumstances, the baroreceptors in the carotid sinus should sense any increase in BP and activate the parasympathetic nervous system to reduce BP (Davies, 2009; Malek et al., 2009). It is difficult to say without further work, whether the decreased heart rate represents better function of the baroreceptor reflex, which can be dysfunctional in people with hypertension. These findings have been explored in rabbits by Akita et al., (2008) and measures of heart rate variability would have been helpful.

PWA of subjects in the EPICURE study detected trends in favour of HPDC e.g. central blood pressure measures, but these did not reach statistical significance. Brachial systolic pressure was lower in the HPDC group especially at 15 min following salbutamol. Unfortunately, the study did not include measurements with GTN, which would have provided valuable evidence about the effect of HPDC on endothelium independent function (O’Rourke, 2002; Rimbach et al., 2008). Another criticism is that many of the PWA results are derived indices and it can be argued that as these are not independent variables the error of multiple testing enhances the
statistical error (Anderson, 2006). Perhaps a more stringent alpha level would be more appropriate when examining this data, but it is likely this issue will remain unresolved. Furthermore a large majority of the published evidence involving PWA has not reportedly taken into account the statistical error associated with multiple testing (reference examples), nonetheless this is an important factor which should be taken into consideration in any further studies using PWA (Vlachopoulos et al., 2005; Anderson, 2006; Wang et al., 2008).

The EPICURE study also reported lower PP amplification ratio, which is the ratio of aortic pulse pressure to the brachial pulse pressure (i.e. the central to peripheral pulse pressure (Anderson, 2006). Central pressures are more likely to determine the development of target organ damage and lower pulse pressure amplification is associated with all cause and CV mortality (Safar et al., 2002; Hashimoto et al., 2007; Benetos et al., 2010). Nevertheless, pulse pressure amplification measures are currently not standardised and can be affected by ageing (McEniery et al., 2008; Papaioannou et al., 2009; Segers et al., 2009). More work is needed in this field before any real conclusions are made regarding the importance of these findings. Again pulse pressure amplification is a derived index and can be prone to statistical error resulting from multiple testing, which was not taken into account. In order for this result to be taken as a true reflection it would have been expected that the augmentation index would be higher and as can be seen figure 3.20 this was not borne out. It is difficult to know whether the use of a differing measure of vasomotor function to detect endothelial dysfunction would have been better (Rimbach et al., 2008; Patvardhan et al., 2010).

Some authors have argued that it is not necessarily the OPC content of chocolate, which accounts for the positive effects, rather other constituents such as theobromine may also be vasoactive. Van den Bogaard and colleagues (2010) have recently examined peripheral and central systolic blood pressure in patients with hypertension with cocoa with either natural theobromine levels or theobromine enriched cocoa. They found that natural levels of theobromine did not affect central or peripheral blood pressures but cocoa enriched with theobromine led to significant improvements in both peripheral and central systolic blood pressure. In the EPICURE study, the theobromine levels of both chocolates were similar so this is unlikely to be the cause of the differences identified.

Greenwood (2006) nicely illustrated the criteria that need to be fulfilled in order to determine the impact of cocoa flavanols:
1. Metabolite needs to reach the supposed target tissue

2. Pharmacokinetics need to support these observations

3. The food should mimic the pure compound

4. Inhibition should work and withholding the compound should reverse the effects

These criteria were not fulfilled in the EPICURE study, like other cocoa/chocolate product trials and it may well be the above need to be taken into account when designing further studies. One of the main reasons these criteria are not used is that they rely on the preconceived idea of what the active metabolite is going to be which in turn depends on the development of reliable and reproducible methods to detect OPC in plasma. However as discussed in chapter 1 understanding the bioavailability of flavonoids is a complex area and thus a reasonable first step in understanding the cardiovascular effects of OPC would be to begin by conducting placebo controlled studies to determine what dose lowers blood pressure. Then it can be determined whether a purified extract has the same effect, which requires formulation studies and measures of a pharmacodynamic response (e.g. change in BP or improved FMD), and pharmacokinetic studies. This can then be followed by identification of the active compounds and metabolites. Perhaps further studies should be designed keeping both this approach and Greenwood’s criteria in mind.

The main learning points from this chapter are that study design and accurate power calculations are necessary for success. Design of the study needs to include robust methodology in order to obtain appropriate levels of results which means that firm conclusions can be made once the data is analysed. In summary, the EPICURE trial provides interesting new data although the anticipated significant decreases in blood pressure were not obtained. This is likely to be due to the insufficient power of the study and also technical difficulties in obtaining blood pressure and performing pulse wave analysis. HPDC reduced heart rate and BP and this data taken along with other published studies does suggest a role of HPDC as an aid to the prevention of CVD. Further studies are required with larger numbers of patients and perhaps a higher content of PC chocolate. Less-user dependent measures of endothelial dysfunction would also be helpful. The next logical step as a follow on from this study would be to design a study incorporating Greenwood’s criteria, which examines the effects of HPDC on patients with established
hypertension on one or two antihypertensive medications who have not yet reached their target blood pressure. Additionally measures of flow-mediated dilatation and heart rate variability would also be of interest.
Chapter 4: The effect of high procyanidin content dark chocolate on lipid levels, HDL subclasses and apolipoproteins in humans
4.1 Introduction

It is increasingly clear that the relationship between cholesterol and CVD is complex and consists of multiple parameters (e.g. Gordon et al., 1977; 4S study, 1994; Lamarche et al., 1996; Hu & Willett, 2002; Lahoz et al., 2001; Danesh et al., 2004). Measuring HDL subclasses and the various apolipoproteins may provide additional information regarding the risk of CVD and may help to elucidate and tackle the remaining morbidity and mortality associated with the ensuing diseases (Fellin et al., 1985; Salonen et al., 1991; Sweetnam et al., 1994; Corsetti et al., 2004; Asztalos et al., 2005; Durrington et al., 2006; Heeren et al., 2006; Walldius et al., 2006; Walldius & Jungner, 2007; Davidson, 2009; Duffy & Rader, 2009; Parish et al., 2009; Holejwin et al., 2010). This section consists of two aspects: 1) Validation of precipitation methodology to measure HDL subclasses and apolipoproteins and 2) Subsequent application of the methodology to plasma samples from the EPICURE study.

4.1.1 Measuring HDL subclasses

HDL subclasses can be measured by three general methods (Rifai et al., 2000):

- Ultracentrifugation by density which reveals two main subclasses: HDL$_2$ and HDL$_3$.

- Nuclear magnetic resonance which separates HDL according to particle size into small, medium and large.

- Electrophoresis by charge and particle size which leads to 12 discrete particles.

Lipoprotein molecules vary in density and ultracentrifugation methods are based on this principle. Ultracentrifugation is by far the most common method used and is considered to be the “gold standard” (Rifai et al., 2000). Ultracentrifugation is an excellent method to isolate and then quantify lipoproteins, but it is time consuming and impractical for determining lipoproteins especially in multiple samples (Warnick et al., 1982). The ideal method for measuring lipoproteins in multiple samples needs to be rapid, practical, and reliable. At present ultracentrifugation is used as a comparator for other, newer methods such as, precipitation methods (Dias et al., 1988). Other methods such as electrophoresis have also been used but again can be labour-intensive (Patsch et al., 1989).

There has been extensive research on other more rapid and simpler methods, which resulted in the discovery of double precipitation methods (Gidez et al., 1982; Warnick et al., 1982;
Lundberg et al., 1984; Warnick et al., 1985; Dias et al., 1988; Patsch et al., 1989). Sulphated polysaccharides form insoluble complexes when mixed with lipoproteins, a property first noted back in 1955 by the investigator Bernfeld (see Cornwell & Kruger, 1961) and also by Burstein and Samaille (1959). This formation requires metal ions and occurs at neutral pH and makes precipitation methods both convenient and reproducible (Patsch et al., 1989). Increasing either the ionic strength or adding chelating agents leads to dissociation (Warnick et al., 1982). To isolate HDL subclasses there are two steps (Warnick et al., 1982): firstly, precipitation of apo B-containing lipoproteins, in the presence of sulphated polysaccharides with divalent cations, leaving HDL-C in the remaining solution. Secondly, a further precipitation step needs to be performed which precipitates HDL₂-C, leaving HDL₃-C in the supernatant. Cholesterol levels can then be determined using enzymatic methods e.g. fluorescence based, as described in chapter 2, and HDL₂-C is calculated by subtracting HDL₃-C from the total HDL-C.

Interestingly the exact mechanism as to how and why lipoproteins become precipitated following the addition of specific reagents has yet to be described (Lundberg et al., 1984; Rifai et al., 2000). It is thought that an interaction between negatively charged groups on the polyanions and positively charged groups on the protein surface of lipoproteins is a crucial aspect (Warnick et al., 1982). The divalent metal ions bind to the negatively charged groups on lipoproteins leading to the formation of insoluble complexes and thus it follows that the larger lipoproteins such as VLDL and LDL will form complexes more readily than smaller, more protein rich lipoproteins like HDL (Patsch et al., 1989). Centrifugation after complex formation allows the insoluble molecules to sediment (based on the fact that their density is greater than that of the solution), a process that occurs less readily in hypertriglyceridaemic samples (Warnick et al., 1985).

There are various precipitation techniques that have been described and assessment of their effectiveness in quantifying HDL-C has been the subject of a number of papers (for example, Warnick et al., 1985; Wiebe & Smith, 1985; Dias et al., 1988; Patsch et al., 1989). Examples of the various methods include the following: dextran sulphate (DS), magnesium ions and heparin manganese or phosphotungstate magnesium and polyethylene glycol. The precision of apo B precipitation is within acceptable limits regardless of which method is used but can be incomplete (Warnick et al., 1982; Wiebe & Smith, 1985). Other difficulties encountered are problems precipitating HDL₂-C and interference between cations and common enzymic-based cholesterol measuring methods (Warnick et al., 1982).
4.1.2 Measuring apolipoproteins

Immunonephelometry or immunoturbidimetry are the most frequent assays employed to measure apolipoproteins and can be automated meaning large numbers of samples can be analysed with ease (Rifai et al., 2000). Both of these assays are affected by lipaemia and unstable complexes but the biggest problem with these methods is local standardization of the procedure, although international standards for apo A and apo B exist (Walldius & Jungner, 2007). Other advantages of measuring apolipoproteins include that they can be measured in non-fasting states and have no reliance on calculation (Walldius et al., 2006). The reliability and reproducibility of apo B and apo AI assays are also well documented and comparable to non-calculated HDL-C (Davidson, 2009).

Apo A group proteins are best measured in whole plasma using monoclonal antibodies or polyclonal antisera (Rifai et al., 2000). The potential antigenic sites can sometimes be masked by lipoproteins and so samples may require pre-treatment to expose these sites e.g. by delipidation, addition of denaturing agents or detergents (Walldius & Jungner, 2007). The standards employed in these experiments need to consist of purified apolipoproteins, which are usually derived by flotation ultracentrifugation (Walldius et al., 2006). This is then followed by ion-exchange chromatography on a DEAE Sepharose column (Rifai et al., 2000). Quantification of apo A can be performed by various methods, which have varying advantages and disadvantages for example (Rifai et al., 2000):

- Radioimmunoassay – high sensitivity, high specificity but needs high dilution (which increases experimental errors) and radioactive isotopes.

- ELISA – high sensitivity, high specificity, no need for isotopes but does need high dilution. This is probably the best method as it requires minimal amounts of antiserum and is not affected by turbidity of the samples. ELISAs are also easy to automate and are reproducible (Dupont et al., 2005).

- Radial immunodiffusion – easy, low dilution, no isotope needed but requires 48h for incubation and not easily automable.

Measuring apo B is usually based on immunoassays, which use antibodies that recognize apo B100 (Dupont et al., 2005). Baca and Warnick (2008) described a method whereby
ultracentrifugation can be used to isolate the infranate containing LDL and IDL and then apo B measured. The main source of interference is turbidity and crossreactivity with apo B48 on chylomicrons (Baca & Warnick, 2008).

Measuring each individual apolipoprotein would prove to be time consuming and likely require large volumes of sample. Thus, it is unsurprising that automated systems have been designed to overcome these issues, for example, the Luminex 100 system which was used for samples from the EPICURE study (Luminex Corporation, Texas). The Luminex 100 system is a compact laboratory analysis system which uses xMAP® technology and is based on flow cell fluorometry so that nearly 100 analytes can be assayed using very small sample volumes.

Various types of methods are used e.g. nucleic acid assays, receptor-ligand assays, immunoassays and enzymatic assays (Cordorean et al., 2010). The test is based on microspheres to which thousands of probes are bound targeted to the molecules under investigation. This is then mixed with the samples of interest and standards, resulting in the microspheres binding to the appropriate molecules if they are present. This reaction is then tagged by fluorescently labelled tags, which bind to the bound molecule (Dupont et al., 2005). The microspheres are then aligned in single file and pass by lasers one at a time, which excites the molecular tags leading to an increase in fluorescence, the intensity of which is measured in real time. Simultaneously, a second laser will also excite the microspheres and yield fluorescence, which aids the determination of the reaction. This allows recognition of each analyte under investigation as each microsphere will have a unique colour code (Cordorean et al., 2010).
4.2 Methodology

Lipid levels and apolipoproteins from the EPICURE study were measured after the study had finished. For details of the EPICURE study design and the procyanidin levels of HPDC and LPDC see chapter 3.

Materials

Manganese chloride, MgCl$_2$.6H$_2$O, sodium chloride, Heparin sodium salt, glycine, polyethylene glycol (MW 8,000), DS (MW 15,000, MW 50,000 called Dextralip 50® and MW 500,000), and sodium hydroxide were all purchased from Sigma. Where not specified, materials and reagents were purchased from Sigma.

4.2.1 Lipid levels from the hospital laboratory

EPICURE samples were sent to the laboratory on the same day and tested within 24 h. The hospital laboratory measured total cholesterol, HDL-C and TG using an enzymatic test for direct quantitative determination on Roche clinical chemistry analysers (Roche Diagnostics). The LDL-C was then calculated using Friedewald’s formula (Friedewald et al., 1972):

$$\text{LDL-C (mmol/L)} = \text{TC} - \text{HDL-C} - \frac{\text{TG}}{2.2}$$

4.2.2 Research laboratory methodology for measuring HDL subclasses

Precipitation method 1: Heparin and MnCl$_2$ / Dextran Sulphate 15,000 MW

One of the very first precipitation methods described involved the use of sodium heparin and MnCl$_2$ to precipitate apo B lipoproteins (Burstein & Samaille, 1959). Sodium heparin (final concentration of 1.2 - 2.0 mg/ml) followed by MnCl$_2$ (final concentration 0.046 M) was added sequentially to 3.0 ml of plasma in respective ratios of 1:25 and 1:20. Samples were then allowed to stand for 30 min at 4°C and finally centrifuged at 1,500 g for 30 min. This results in formation of insoluble complexes, which precipitate at the bottom of the vial and a clear supernatant above containing HDL-C. Cholesterol can then be measured in both the original sample (giving a measure of TC) and the supernatant containing total HDL-C.

A subsequent study by Srinivasan and colleagues (1975) suggested that this method also precipitated some of the HDL-C leading to underestimation of HDL-C. Warnick and Albers (1978) investigated this further and discovered that the heparin and MnCl$_2$ could be added simultaneously and that samples could then be stood at room temperature rather than colder
environments. Also using MnCl$_2$ at a concentration of 0.092 M led to a reduced number of samples with turbid supernates (suggestive of incomplete apo B precipitation). At this level, apo B lipoproteins were optimally precipitated without excessive HDL precipitation. Warnick and Albers (1978) postulated that the apo B lipoproteins that did not precipitate with the lower molarity of MnCl$_2$ represent the smaller lipoprotein classes of LDL, which need higher divalent cation levels to precipitate. The following is a summary of the altered technique and was the one tested in the laboratory:

- Heparin – manganese was combined as follows 0.6 ml sodium heparin (40,000 USP units/ml, 280 mg/ml) to 10.0 ml of 1.06 M MnCl$_2$.

- This combined solution was added to 2.0 ml of plasma in the following ratio 1:10 (final concentration of heparin 1.4 mg/ml and MnCl$_2$ 0.092 M).

- 10 min incubation at room temperature.

- Centrifugation at 1,500 g for 30 min – if samples remained turbid they underwent a further centrifugation step at 12,000 g for 10 min.

In order to determine HDL$_2$-C and HDL$_3$-C levels a second precipitation step using DS was performed. DS of MW 15,000 was added to 0.15 mol/L NaCl and added to 0.5 ml of the supernates derived from the initial heparin/manganese step. Samples were incubated for 20 min at room temperature and then underwent centrifugation at 1,500 g for 30 min at 4°C. Cholesterol was then measured in the resulting supernants consisting of HDL$_3$-C, and HDL$_2$-C calculated by subtraction.

Srinivasan et al., (1975) reported that plasma proteins, including albumin are also complexed by heparin-manganese. Warnick and Albers (1978) examined this further but did not find this to be a significant problem during HDL-C precipitation from plasma and serum with heparin-manganese. Similarly, protein precipitation has not been identified to be a major problem with other precipitation methods, such as the DS-magnesium method (Warnick et al., 1985). Other concerns with this methodology are possible interference between manganese ions and the commonly used, enzymatic methods for cholesterol assay but again this has not been borne out in further analyses (Warnick et al., 1982).
Experience with this methodology in the laboratory proved very disappointing. Incomplete precipitation of apo B associated lipoproteins was a common problem resulting in turbid samples. In practice, incomplete sedimentation is most notable in hypertriglyceridaemic samples and leads to the overestimation of HDL-C (Warnick & Albers, 1978; Warnick et al., 1985; Wiebe & Smith, 1985; Harris et al., 1996). There have been several suggestions as to how best to deal with turbid samples, ultrafiltration through filters allows clear filtrates to be produced which can be analysed, but this procedure can be time consuming and care has to be taken to ensure no HDL particles are also filtered (Warnick et al., 1985).

Warnick and Albers (1985) also noted previously that in hypertriglyceridaemic samples, there was a tendency for lipoproteins to aggregate above the subnatant solution and furthermore the subnatant was often cloudy representing inefficient precipitation of apo B lipoproteins. A useful tip provided by the authors was that clear subnatant can be aspirated using a fine tip Pasteur pipette. Others have suggested that addition of 0.15M sodium chloride (and proportionate amount of additional reagent) to samples before precipitating reagents are added might prevent turbid samples by reducing solution density and increasing the relative reagent concentration helping sedimentation of insoluble lipoprotein complexes (Burstein & Samaille, 1959; Warnick & Albers, 1978). This was not recommended by the authors as it is associated with imprecise results and increases HDL precipitation although further work has shown that addition of sodium chloride was unlikely to lead to physiologically significant changes in HDL-C (Warnick & Albers, 1978).

In all procedures supernatants that were turbid following centrifugation were treated as follows (Warnick et al., 1982): 1 ml of 0.15 M NaCl solution and another 0.1 ml combined precipitant (if initial sample was 1 ml) followed by thoroughly mixing and then centrifuging in the same environment and speed as the original step. The resulting cholesterol concentration was corrected for this extra dilution. Despite correcting for this factor and several repeated attempts, the HDL\(_2\)-C precipitation step still failed to work.

**Precipitation method 2: Double DS 15,000 MW / magnesium**

DS is a synthetic analogue of heparin and shows good correlation with the heparin/manganese precipitation methods (Warnick et al., 1982). DS is manufactured in a variety of molecular sizes and lipoprotein precipitation is a function of the molecular weight, being greater with larger molecules. The commonest molecular weights used in practice are 15,000, 50,000 and 500,000.
The 500,000 MW is associated with underestimation of HDL-C when compared to heparin/manganese and ultracentrifugation techniques, whilst DS of molecular weight 15,000 fails to completely precipitate apo B lipoproteins (Warnick et al., 1982). Warnick and colleagues (1982) discovered that optimal precipitation is achieved with a combination of DS of 50,000 daltons and magnesium. Magnesium was used as it does not exhibit interference with cholesterol enzymic assays and has good correlation with heparin/manganese methods.

The HDL$_2$-C precipitation step is performed by taking 0.5 ml supernatants and adding 0.05 ml of 10 g/L DS 15,000 MW in 1.5 mol/L MgCl$_2$ (it should be noted that it is only the concentration of magnesium which has altered in the second step). Mixtures were then incubated for 10 min at room temperature and this was followed by centrifugation for 30 min at 1,500 g at 4°C. Patsch et al., (1989) adapted the methodology and centrifuged only for 15 min to precipitate the apo B lipoproteins and for only 20 min at 1,500 g in a 4°C environment to precipitate HDL$_2$-C. This latter centrifugation technique was employed when this method was undertaken. Again, there were notable problems mostly relating to the lack of precipitation of HDL$_2$-C, which may have been related to the use of small sample volumes, which was unavoidable.

Rifai et al., (2000) suggested using DS of molecular weight 50,000 in both steps. This was also attempted to see if better results could be achieved and in summary the following was undertaken: DS (MW 50,000; Dextralip 50®) was added to deionised water with MgCl$_2$.6H$_2$O and made up to 100 ml. 0.1ml of this precipitant was added to 1 ml of sample followed by thorough mixing (final concentration of DS 10 mg/ml and 0.5 mol/L MgCl$_2$). Samples were allowed to stand for 5 - 30 min at room temperature followed by centrifugation at 1,500 g for 30 min at 4°C. Precipitation of HDL$_2$-C was achieved by transferring 0.5 ml of the supernatant following the precipitation of apo B lipoproteins and adding 0.05 ml of a precipitating reagent consisting of DS (MW 50,000; Dextralip 50®; final concentration 19.1 mg/ml) and MgCl$_2$ (final concentration 1.95 mol/L). Samples were allowed to stand for 5 – 30 min at room temperature followed by centrifugation at 12,000 g, for 5 min at 4°C or 1,500 g for 30 min. Again this did not yield to be an easy procedure when trying to precipitate HDL$_2$-C.
A number of experiments were undertaken to determine why the DS method failed to work:

i. **Altering the pH**

Warnick *et al.*, (1982) noted that with the DS /Mg$^{2+}$ method the pH had a significant impact on lipoprotein precipitation. Lower pH was associated with less lipoprotein precipitation but the actual values were only +/- 5 mg/L when changing the pH from 6.0 to 8.0. These changes were mostly in hypertriglyceridaemic samples and not apparent with normolipidaemic or hypercholesterolaemic samples.

ii. **Ionic strength of precipitating reagent solution**

Precipitation reagents were initially made up with regular deionised water. Warnick *et al.*, (1982) noticed that the ionic strength of precipitating reagents effects lipoprotein precipitation, with higher ionic strength the tendency for lipoprotein precipitation was reduced – this was noted when reagents were made up with 0.15 M NaCl (unlike the heparin/Mn procedure). Thus, it was advised to make solutions up in high-quality water e.g. doubly distilled water. The ultrapure water system in the laboratory has ion-exchange resins and activated charcoal columns to remove all charged and uncharged molecules producing high quality water, making this an unlikely cause of the lack of precipitation of HDL$_2$-C.

iii. **Temperature at which samples were stood and centrifuged**

It has been argued that the ambient temperature effects lipoprotein precipitation but some researchers have not found this to be a greatly significant factor (Warnick *et al.*, 1982). Samples can be incubated at room temperature and centrifuged at either room temperature or 4°C – although the authors recommend centrifugation in a 4°C environment (Warnick *et al.*, 1982). The latter was performed for the all the precipitation procedures described.

The other pertinent issue is that incomplete precipitation has been noted to be more of a problem with DS-magnesium than any other methods (Harris *et al.*, 1996). But the data is confusing for example, Wiebe and Smith (1985) compared various methods and reported good precision between heparin-manganese and DS-magnesium (MW 50,000).
Precipitation method 3: Glycine/PEG and DS 15,000 MW

As there were difficulties in precipitating HDL$_2$-C with the above methods, a third method for HDL subclass precipitation was tested. This method was based on glycine and polyethylene glycol to initially precipitate the apo B lipoproteins, which was followed, by glycine and DS (MW 15,000) to precipitate HDL$_2$-C. This method is not favourable with all researchers and indeed Warnick and colleagues (1982) rejected this method as the concentration of PEG required to precipitate apo B lipoproteins was very similar to that needed to precipitate HDL$_2$-C. However, glycine/PEG and DS has been shown to correlate well with density gradient ultracentrifugation (Dias et al., 1988).

The first step is to precipitate the apo B lipoproteins by adding 0.5 ml of buffered PEG (MW 8,000; note Lundberg et al., in 1984 used PEG-6000) in 0.2 mol/L glycine buffer adjusted to pH 10 with 1 M sodium hydroxide. The mixture was thoroughly mixed and then centrifuged at 9,500 g for 5 min at a temperature of 4°C. The subsequent supernatant was then sampled for measurement of total HDL-C. Following precipitation of apo B lipoproteins, a 0.36 ml aliquot was taken of the clear supernatant and 0.04 ml of a precipitation reagent containing: 0.72 g DS and 50 mmol/L MgCl$_2$ in 0.2 mol/L glycine buffer adjusted to pH 10 with 1 mol/L sodium hydroxide added. Samples were stood at room temperature for 30 min after mixing and then centrifuged at 9,500 g for 5 min at a temperature of 4°C, leaving a HDL$_3$-C containing supernatant.

Effect of sample storage on precipitation methods

It has been questioned whether storage of specimens effects the efficiency of double precipitation methods (Gidez et al., 1982). This was particularly pertinent to the EPICURE samples – some of which had been stored at -80°C for over 1 year (but less than 18 months) before analysis. Bausserman et al., (1994) reported that storage of serum at -70°C did not lead to any significant changes in HDL-C, but by 18 months HDL$_3$-C was reduced by 13%. Patsch et al., (1989) had also noticed reduced HDL-C values for samples stored at -70°C for two weeks, changes were most noticeable with the double DS method whereas heparin/manganese and DS was more stable (for up to one year). Storage may therefore have accounted for some of the difficulties in processing samples and obtaining adequate levels of precipitation of HDL$_3$-C. In retrospect, samples should have been analysed within 6 months of being obtained although Warnick et al., (1982) reported that samples could be frozen at -60°C for up to 2 years without any major changes in lipoproteins.
4.2.3 Final methodology used for measuring HDL subclasses in the EPICURE study

Samples were taken from individuals following a 12 - 14 h fast. It is important to appreciate that the role in fasting for cholesterol measurements especially HDL-C, has not been completely established, but it is well documented that LDL-C and TG change post-prandially especially if the meal was high in fat (Langsted et al., 2008; Mora et al., 2008). Samples were drawn into EDTA tubes using vacutainers, EDTA was preferred as lipoprotein stability is greater and avoids heparin and citrate which can lead to interference with lipoprotein precipitation. Samples were then centrifuged and plasma aspirated carefully and stored at -80˚C until analysis. The method that was finally used for analysing the samples from the EPICURE study was the glycine/PEG and DS MW 15,000 as described above. This method worked well and seemed to be relatively easier than the other methods used.

Before analysing the EPICURE samples, the optimal amount of sample necessary was determined. Three different samples volumes were tested: 400 µl, 300 µl and 200 µl. Ten samples were analysed from the same plasma sample and the coefficient of variance was determined – the analysis of the results are shown in table 4.1. Based on these results 300 µl was deemed to be the ideal volume as the coefficient of variation was consistently less than 10% between samples and close for the precipitation of the various lipoprotein fractions.
Table 4.1 Depicting the various coefficients of variation for the three sample volumes tested, 400µl, 300µl and 200µl. (SD = standard deviation, SEM = standard error of the mean, CI = confidence interval).
4.2.4 Cholesterol assay

Whichever method was used to precipitate cholesterol an aliquot was taken to measure total cholesterol, total HDL-C (following precipitation of apo B lipoproteins) and HDL$_{3-}$C (following precipitation of HDL$_{2-}$C). HDL$_{2-}$C was then calculated by subtracting HDL$_{3-}$cholesterol from total HDL-C. LDL-C was also calculated by subtracting the total HDL-C from TC.

The cholesterol fluorometric assay described in chapter 2 was undertaken to determine cholesterol levels. Samples were initially diluted 1:200 – 1:400 so that the resulting fluorescence levels were likely to fall within the standard curve generated, generally 1:400 dilutions were used. Cholesterol levels were calculated after correcting for background fluorescence and multiplying by the appropriate dilution factor.

4.2.5 Luminex method for measuring apolipoproteins

For apolipoproteins a pre-mixed multiplex bead kit (WideScreen®, Human CVD panel 1-plex panel, EMD Chemicals, Inc.) was used to detect the following human apolipoproteins simultaneously: apo AI, apo AII, apo B, apo CIII, apo E, apo H and apo J. This consisted of two types of immunoassays: for apo B, apo E and apo J these were conventional, non-competitive, sandwich-based immunoassays and for apo AI, apo AII, apo CIII and apo H these were competitive assays. For the competitive assays, biotinylated antigen is present in the blocking buffer and is competed off the beads by analyte, resulting in a decrease in the fluorescent signal. Concentrations of apolipoproteins in samples were quantified by reading off a standard curve, which was provided in the kit (varied for each apolipoprotein). The intra-assay and inter-assay coefficient of variations for detecting apolipoproteins has been reported as 1-11% and 7-26% respectively (WideScreen®, Human CVD panel 1-plex panel datasheet, EMD Chemicals, Inc).

To date there are no comparative studies of apolipoprotein measurement versus single point ELISA measurements. Yet there are some studies which have tried to compare the Luminex system with ELISA for detecting cytokines. For example, Richens and colleagues (2010) found that the Luminex system highly reproducible and reliable for cytokine detection, with good correlation between Luminex and ELISA ($R^2 >0.982$). The authors concluded that reliable results from multiplex systems can be achieved in comparison to ELISA, but crucial to this was standard curve generation. Similarly other researchers have also reported good correlation between Luminex and single ELISA measurements for cytokines (e.g. Dupont et al., 2005). Codorean et al., (2010) recommended that the multiplex assay is validated and compared to
ELISA before being used on samples of interest, despite reporting good correlation between the two. In the experiments presented here standard curves were generated and optimised, but due to time and financial constraints a comparison between the multiplex system and ELISA was not achieved.

4.2.6 Statistical analysis

The data reported were analysed using both MiniTab software (MiniTab Inc, Pennsylvania) and GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analyses, including GLM ANOVA were undertaken with MiniTab software. Correlation between the laboratory values and measured values was determined using GraphPad Prism. Graphical representation of some data is shown relative to baseline, but for GLM ANOVA analyses of these variables the baseline values were used as only one comparator.
4.3 EPICURE study results

4.3.1 Lipid levels measured in the hospital laboratory

Plasma lipids were analysed for 24 subjects in total both at 6 weeks of HPDC and LPDC and also at the midway point (called HPDCWk3 and LPDCWk3). There was no statistically significant changes in TC levels throughout the study (table 4.2 and figure 4.1; GLM ANOVA \(p=0.387\)). Other lipid parameters were also not statistically significant including the TG levels (table 4.3 and figure 4.2; GLM ANOVA \(p=0.346\)), HDL-C levels (table 4.4 and figure 4.3; GLM ANOVA \(p=0.369\)) and LDL-C levels (table 4.5 and figure 4.4; GLM ANOVA \(p=0.667\)).
Table 4.2 Hospital laboratory measured total cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of hospital laboratory measured total cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Hospital laboratory measured total cholesterol</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Basal</td>
<td>5.704</td>
<td>1.022</td>
</tr>
<tr>
<td>LPDCWk3</td>
<td>5.621</td>
<td>0.741</td>
</tr>
<tr>
<td>LPDC</td>
<td>5.641</td>
<td>1.057</td>
</tr>
<tr>
<td>HPDCWk3</td>
<td>5.648</td>
<td>0.863</td>
</tr>
<tr>
<td>HPDC</td>
<td>5.426</td>
<td>0.765</td>
</tr>
</tbody>
</table>

General linear model ANOVA for TC versus PC content p = 0.387

Figure 4.1 Changes in hospital laboratory measured total cholesterol levels in the EPICURE study. Hospital laboratory measured total cholesterol (TC) levels over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
Chocolate & Hospital laboratory measured triglycerides & & \\
& Mean & SD & SEM & P-Value* \\
Basal & 1.366 & 0.559 & 0.112 & \\
LPDCWk3 & 1.484 & 0.515 & 0.105 & 0.922 \\
LPDC & 1.612 & 0.933 & 0.195 & 0.305 \\
HPDCWk3 & 1.340 & 0.705 & 0.154 & 0.989 \\
HPDC & 1.470 & 0.715 & 0.149 & 0.805 \\

General linear model ANOVA for TG versus PC content \( p = 0.346 \)

**Table 4.3** Hospital laboratory measured triglyceride results from the EPICURE study. *Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of hospital laboratory measured triglycerides (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett's simultaneous tests, comparison with basal, pre-treatment level.*

![Hospital measured triglycerides (TG)](image)

**Figure 4.2** Changes in hospital laboratory measured triglyceride levels in the EPICURE study. Hospital laboratory measured triglycerides levels over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
Table 4.4 Hospital laboratory measured HDL cholesterol results from the EPICURE study.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Hospital laboratory measured HDL cholesterol</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td>1.584</td>
<td>0.316</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>LPDCWk3</td>
<td></td>
<td>1.541</td>
<td>0.302</td>
<td>0.062</td>
<td>0.992</td>
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<tr>
<td>LPDC</td>
<td></td>
<td>1.563</td>
<td>0.354</td>
<td>0.074</td>
<td>0.831</td>
</tr>
<tr>
<td>HPDCWk3</td>
<td></td>
<td>1.583</td>
<td>0.299</td>
<td>0.065</td>
<td>1.000</td>
</tr>
<tr>
<td>HPDC</td>
<td></td>
<td>1.500</td>
<td>0.290</td>
<td>0.061</td>
<td>0.234</td>
</tr>
</tbody>
</table>

General linear model ANOVA for HDL-C versus PC content p = 0.369

Table 4.4 Hospital laboratory measured HDL cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of hospital laboratory measured HDL cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

Figure 4.3 Changes in hospital laboratory measured HDL cholesterol levels in the EPICURE study. Hospital laboratory measured HDL cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
Table 4.5 Hospital laboratory measured LDL cholesterol results from the EPICURE study.
Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of hospital laboratory measured LDL cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Hospital laboratory measured LDL cholesterol</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>SEM</td>
<td>P-Value*</td>
</tr>
<tr>
<td>Basal</td>
<td>3.504</td>
<td>0.826</td>
<td>0.165</td>
<td></td>
</tr>
<tr>
<td>LPDCWk3</td>
<td>3.417</td>
<td>0.633</td>
<td>0.129</td>
<td>0.946</td>
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<tr>
<td>LPDC</td>
<td>3.413</td>
<td>0.956</td>
<td>0.199</td>
<td>0.740</td>
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<tr>
<td>HPDCWk3</td>
<td>3.462</td>
<td>0.821</td>
<td>0.179</td>
<td>0.493</td>
</tr>
<tr>
<td>HPDC</td>
<td>3.322</td>
<td>0.822</td>
<td>0.171</td>
<td>0.573</td>
</tr>
</tbody>
</table>

General linear model ANOVA for LDL-C versus PC content p = 0.667

Figure 4.4 Changes in hospital laboratory measured LDL cholesterol levels in the EPICURE study. Hospital laboratory measured LDL cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
4.3.2 Lipid levels from the PEG/glycine/DS precipitation methodology

Employing the PEG/glycine/DS precipitation methods the plasma lipids were analysed looking at TC, LDL-C, total HDL-C, HDL\(_{2}\)-C, and HDL\(_{3}\)-C. Again, this was examined at both 6 weeks of the particular chocolate and the midway point. There was no statistically significant changes in TC levels using the precipitation methodology throughout the study (table 4.6 and figure 4.5; GLM ANOVA p= 0.768), and unlike the laboratory values no trend was seen in the data (figure 4.5). As can be seen in figures 4.6 – 4.8 there was also no appreciable differences in the total HDL-C levels (table 4.7 and figure 4.6; GLM ANOVA p=0.858), HDL\(_{3}\)-C (table 4.8 and figure 4.7; GLM ANOVA p= 0.096) and HDL\(_{2}\)-C (table 4.9 and figure 4.8; GLM ANOVA p= 0.789) levels between the study arms. The calculated LDL-C levels based on the precipitation method was also not statistically significant between HPDC and LPDC (table 4.10 and figure 4.9; GLM ANOVA p=0.884).
<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Total cholesterol measured by PEG/glycine/DS precipitation</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td>4.594</td>
<td>1.088</td>
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<tr>
<td>LPDCWk3</td>
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<td>0.998</td>
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<tr>
<td>LPDC</td>
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<td>4.310</td>
<td>1.077</td>
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<tr>
<td>HPDCWk3</td>
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<td>4.538</td>
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<td>0.225</td>
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<tr>
<td>HPDC</td>
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<td>4.478</td>
<td>1.176</td>
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<td>0.962</td>
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</table>

General linear model ANOVA for TC versus PC content p = 0.768

Table 4.6 PEG/glycine/DS precipitation measured total cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PEG/glycine/DS precipitation measured total cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

Figure 4.5 Changes in PEG/glycine/DS measured total cholesterol levels in the EPICURE study. PEG/glycine/DS precipitation measured total cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
Table 4.7 PEG/glycine/DS precipitation measured total HDL cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PEG/glycine/DS precipitation measured total HDL cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Total HDL cholesterol measured by PEG/glycine/DS precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Basal</td>
<td>1.527</td>
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<td>LPDCWk3</td>
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<tr>
<td>LPDC</td>
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<td>HPDCWk3</td>
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<tr>
<td>HPDC</td>
<td>1.496</td>
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</table>

General linear model ANOVA for HDL-C versus PC content p = 0.858

Figure 4.6 Changes in PEG/glycine/DS measured total HDL cholesterol levels in the EPICURE study. PEG/glycine/DS precipitation measured total HDL cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
<table>
<thead>
<tr>
<th>Chocolate</th>
<th>HDL₃ cholesterol measured by PEG/glycine/ DS precipitation</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Basal</td>
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<tr>
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<td>LPDC</td>
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<tr>
<td>HPDCWk3</td>
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<td>HPDC</td>
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</table>

General linear model ANOVA for HDL₃-C versus PC content p = 0.096

Table 4.8 PEG/glycine/DS precipitation measured HDL₃ cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PEG/glycine/DS precipitation measured HDL₃ cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

Figure 4.7 Changes in PEG/glycine/DS measured HDL₃ cholesterol levels in the EPICURE study. PEG/glycine/DS precipitation measured HDL₃ cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
<table>
<thead>
<tr>
<th>Chocolate</th>
<th>HDL(_2) cholesterol measured by PEG/glycine/DS precipitation</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.156</td>
<td>0.388</td>
<td>0.076</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPDCWk3</td>
<td>1.208</td>
<td>0.410</td>
<td>0.085</td>
<td>0.653</td>
<td></td>
</tr>
<tr>
<td>LPDC</td>
<td>1.170</td>
<td>0.403</td>
<td>0.080</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>HPDCWk3</td>
<td>1.230</td>
<td>0.361</td>
<td>0.077</td>
<td>0.936</td>
<td></td>
</tr>
<tr>
<td>HPDC</td>
<td>1.158</td>
<td>0.301</td>
<td>0.059</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

General linear model ANOVA for HDL\(_2\)-C versus PC content \(p = 0.789\)

Table 4.9 PEG/glycine/DS precipitation measured HDL\(_2\) cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PEG/glycine/DS precipitation measured HDL\(_2\) cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

![HDL\(_2\) cholesterol based on PEG/ glycine/ DS precipitation](image)

Figure 4.8 Changes in PEG/glycine/DS measured HDL\(_2\) cholesterol levels in the EPICURE study. PEG/glycine/DS precipitation measured HDL\(_2\) cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
<table>
<thead>
<tr>
<th>Chocolate</th>
<th>LDL cholesterol based on PEG/glycine/DS precipitation method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Basal</td>
<td>3.067</td>
</tr>
<tr>
<td>LPDCWk3</td>
<td>2.911</td>
</tr>
<tr>
<td>LPDC</td>
<td>2.836</td>
</tr>
<tr>
<td>HPDCWk3</td>
<td>2.972</td>
</tr>
<tr>
<td>HPDC</td>
<td>2.982</td>
</tr>
</tbody>
</table>

General linear model ANOVA for LDL-C versus PC content p = 0.884

Table 4.10 PEG/glycine/DS precipitation measured LDL cholesterol results from the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of PEG/glycine/DS precipitation measured LDL cholesterol (mmol/L) between 50 g of HPDC and LPDC in the EPICURE study at basal level (pre-treatment), week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with basal, pre-treatment level.

Figure 4.9 Changes in PEG/glycine/DS measured LDL cholesterol levels in the EPICURE study. PEG/glycine/DS precipitation measured LDL cholesterol over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. The data in each treatment arm are shown relative to pre-treatment basal values (but for GLM ANOVA basal values are used as only one comparator). (Figures are expressed as mean ± SEM).
4.3.3 Correlation of EPICURE study lipid levels from the hospital laboratory and PEG/glycine/DS precipitation methodology

The precipitation method did not reveal any differences in HDL subclasses after either HPDC or LPDC. To assess whether the methodology was consistent with the hospital laboratory, values from both were correlated. The TC (figure 4.10), LDL-C (figure 4.11), and HDL-C (figure 4.12) measures did not correlate well, although correlation did appear to be better for HDL-C than the other parameters (figure 4.12).

![Figure 4.10 Correlation of the laboratory total cholesterol and the measured total cholesterol concentration from the PEG/glycine/DS precipitation method.](image-url)
Figure 4.11 Correlation of the laboratory LDL cholesterol and the measured LDL cholesterol concentration from the PEG/glycine/DS precipitation method.

Figure 4.12 Correlation of the laboratory HDL-cholesterol and the measured HDL-cholesterol concentration from the PEG/glycine/DS precipitation method.
4.3.4 Apolipoprotein levels from the EPICURE study

Apolipoprotein levels for the EPICURE subjects were measured using the Luminex 100 system. The raw data for each subject was corrected for baseline levels and then converted to percentages from which percentage change from basal was calculated. The following apolipoproteins were measured: apo AI, apo AII, apo B, apo CIII, apo E, apo H and apo J. There were no statistically significant changes in any of the measured apolipoproteins: apo AI (table 4.11 and figure 4.13; GLM ANOVA p= 0.134), apo AII (table 4.12 and figure 4.14; GLM ANOVA p= 0.168), apo B (table 4.13 and figure 4.15; GLM ANOVA p= 0.163), apo CIII (table 4.14 and figure 4.16; GLM ANOVA p= 0.365), apo E (table 4.15 and figure 4.17; GLM ANOVA p= 0.384), apo H (table 4.16 and figure 4.18; GLM ANOVA p= 0.113) and apo J (table 4.17 and figure 4.19; GLM ANOVA p= 0.804).
Table 4.11 Percentage change in apo A-I in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of percentage change from basal in plasma apo A-I (mg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with LPDC.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Percentage change in Apo A-I</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>SEM</td>
<td>P-Value*</td>
</tr>
<tr>
<td>LPDCWk3</td>
<td>0.168</td>
<td>0.456</td>
<td>0.097</td>
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<td></td>
</tr>
<tr>
<td>LPDC</td>
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<td>0.052</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPDCWk3</td>
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<td>0.054</td>
<td></td>
<td>0.749</td>
</tr>
<tr>
<td>HPDC</td>
<td>0.067</td>
<td>0.315</td>
<td>0.064</td>
<td></td>
<td>0.984</td>
</tr>
</tbody>
</table>

General linear model ANOVA for Apo A-I versus PC content p = 0.134

Figure 4.13 Mean percentage change in apo A-I in the EPICURE study. Changes in the mean percentage change from basal in apo A-I (mg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Table 4.12 Percentage change in apo AII in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of percentage change from basal in plasma apo AII (µg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with LPDC.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Percentage change in Apo AII</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPDCWk3</td>
<td></td>
<td>0.221</td>
<td>0.529</td>
<td>0.113</td>
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</tr>
<tr>
<td>LPDC</td>
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<td>0.267</td>
<td>0.055</td>
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</tr>
<tr>
<td>HPDCWk3</td>
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<td>0.014</td>
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<tr>
<td>HPDC</td>
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<td>0.181</td>
<td>0.432</td>
<td>0.088</td>
<td>0.708</td>
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</table>

General linear model ANOVA for Apo A-II versus PC content p = 0.168

Figure 4.14 Mean percentage change in apo AII in the EPICURE study. Changes in the mean percentage change from basal in apo AII (µg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Table 4.13 Percentage change in apo B in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM), and p-value of percentage change from basal in plasma apo B (µg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with LPDC.

<table>
<thead>
<tr>
<th>Chocolate</th>
<th>Percentage change in Apo B</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>P-Value*</th>
</tr>
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General linear model ANOVA for Apo B versus PC content p = 0.163

Figure 4.15 Mean percentage change in apo B in the EPICURE study. Changes in the mean percentage change from basal in apo B (µg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Table 4.14 Percentage change in apo CIII in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of percentage change from basal in plasma apo CIII (µg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with LPDC.

<table>
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General linear model ANOVA for Apo CIII versus PC content p = 0.365

Figure 4.16 Mean percentage change in apo CIII in the EPICURE study. Changes in the mean percentage change from basal in apo CIII (µg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Table 4.15 Percentage change in apo E in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of percentage change from basal in plasma apo E (µg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett's simultaneous tests, comparison with LPDC.

<table>
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<td>0.182</td>
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General linear model ANOVA for Apo E versus PC content p = 0.384

Figure 4.17 Mean percentage change in apo E in the EPICURE study. Changes in the mean percentage change from basal in apo E (µg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
<table>
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General linear model ANOVA for Apo H versus PC content p = 0.113

Table 4.16 Percentage change in apo H in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of percentage change from basal in plasma apo H (µg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with LPDC.

Figure 4.18 Mean percentage change in apo H in the EPICURE study. Changes in the mean percentage change from basal in apo H (µg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
Table 4.17 Percentage change in apo J in the EPICURE study. Mean, standard deviation (SD), standard error of the mean (SEM) and p-value of percentage change from basal in plasma apo J (µg/mL) between 50 g of HPDC and LPDC in the EPICURE study at week 3 of low procyanidin dark chocolate and high procyanidin dark chocolate consumption (LPDCWk3 and HPDCWk3 respectively) and at the end of chocolate consumption (LPDC and HPDC). *Post-hoc analysis using Dunnett’s simultaneous tests, comparison with LPDC.

![Percentage change in apo J in the EPICURE study](image)

**Figure 4.19 Mean percentage change in apo J in the EPICURE study.** Changes in the mean percentage change from basal in apo J (µg/mL) over 6 weeks consumption of 50 g of either HPDC or LPDC in the EPICURE study. (Figures are expressed as mean ± SEM).
### 4.4 Discussion

LDL-C is positively associated with risk and development of CVD and has heralded the widespread use of statins which has successfully abated a significant amount of CVD morbidity and mortality (Shepherd et al., 1995; Sacks et al., 1996; Lemaitre et al., 2002; Shepherd et al., 2002). In many patients CVD still occurs despite adequate reductions in LDL-C and the key to effective management of the residual disease may well lie in other lipid fractions and apolipoproteins (Tall et al., 2008; Superko & King, 2008). HDL-C has long been accepted as being negatively correlated with CVD through the mechanism of RCT (Fredenrich & Bayer, 2003; Lewis & Rader, 2005; Cuchel & Rader, 2006). In the EPICURE study of male subjects with prehypertension or stage I hypertension with no previous history of any illness and not on any regular medications, there were no discernible differences in the various lipoprotein fractions (LDL-C, HDL-C and TG) after HPDC compared to LPDC. The HDL\textsubscript{2}-C subclass has been associated with greater protection from CVD and is probably a better predictor of CHD than HDL-C or HDL\textsubscript{3}-C (Asztalos et al., 2005; Superko et al., 2009). In this study, the use of a precipitation method to detect the two HDL subclasses failed to yield any significant differences between HPDC and LPDC.

A growing number of apolipoproteins have been identified, but the function of many remains obscure. Several seem to have a role in lipid metabolism and inflammatory and coagulation pathways (Castellani et al., 1997; Rubin et al., 1999; Crook, 2010). Some of these proteins (apo AI, apo AII and apo B) may help in predicting the risk of CVD and clinically may provide better information in certain subgroups e.g. diabetic patients (Walldius et al., 2001; Sniderman et al., 2003; Corsetti et al., 2004; Walldius et al., 2004; Walldius et al., 2006; Sharobeem et al., 2007; Parish et al., 2009). The consumption of HPDC versus LPDC in the EPICURE study did not lead to any differences in any of the apolipoproteins measured (apo AI, apo AII, apo B, apo CIII, apo E, apo H and apo J).

The lack of significant results in both lipoproteins and apolipoproteins may relate to the experimental methods undertaken. Precipitation methods for measuring HDL subclasses are generally time consuming and are prone to being affected by small variations due to various factors e.g. storage of samples, ambient temperature, and concentrations of precipitation reagents (Warnick & Albers, 1978; Warnick et al., 1985; Wiebe & Smith, 1985). Indeed it is likely that some of these problems were encountered during the experimental analyses. This necessitated the evaluation of several different experimental procedures using different reagents.
to precipitate the various lipoprotein fractions. The technique was not employed until it had been perfected in the laboratory, but without a comparative analysis following separation by ultracentrifugation, it is difficult to be sure that the precipitation procedure worked with all samples. Obviously when measuring any parameter it is good practice to have a “gold standard” to make comparisons. Most papers use the ultracentrifugation method as the gold standard but this was not practical to use with the large number of samples from the EPICURE study (Rifai et al., 2000). It may have been better to compare two optimised precipitation methods. Despite this the fact that laboratory measurements of the various lipids also did not yield any differences between HPDC and LPDC supports the likelihood that the PEG/ glycine/DS precipitation did work.

Measurement of apo AI, apo AII and apo B in samples treated for precipitation would have provided an idea of how well the precipitation step had occurred although it is likely that the levels of apolipoproteins in the post-precipitation solution are too low to lead to accurate results. In comparison, methods of measuring apolipoproteins have been standardised but at present, these are largely restricted to research studies. The EPICURE study employed a new microsphere based system to measure apolipoproteins and it may well be that this is not the most reliable method of measuring apolipoproteins, although it is rapid and only requires a small amount of sample (Cordorean et al., 2010; Dupont et al., 2005; Richens et al., 2010).

Another possibility for the lack of significant differences with either HPDC or LPDC may be that OPC do not actually exert any effects on lipoproteins and apolipoproteins. Several studies of polyphenols have reported reduced levels of cholesterol in animals (Hayek et al., 1997; Matsumoto et al., 1998; Frémont et al., 2000; Augur et al., 2005; Bas et al., 2005; Rizzo & Berneis, 2008; Quesada et al., 2009; Osakabe & Yamagishi, 2009). But these findings have not been consistent and there are many animal studies where cholesterol levels are not affected or even adversely affected by polyphenols (Hayek et al., 1997; Matsumoto et al., 1998; Fuhrman et al., 2005). Even so some of these studies still detected a reduction in the size of atherosclerotic lesions in animals with polyphenols and/or procyanidins. This suggests that changes other than those relating to numbers of lipid particles occur with procyanidins leading to reduced atherosclerosis (Da Luz et al., 1999; Yamokishi et al., 1999). This may well relate to cellular cholesterol efflux and/or the activity of HDL and its apolipoproteins and/or enzymes, rather than levels.
Studies in humans have also resulted in a mixed picture with some studies of OPC (whether from GSE, cocoa or other sources) resulting in a more favourable lipid profile (Wan et al., 2001; Zern et al., 2003; Mursu et al., 2004; Baba et al., 2007; Grassi et al., 2008; Mellor et al., 2010), while others have not reported significant changes (Engler et al., 2004; Taubert et al., 2007). As has already been discussed these results need to be treated with caution as some of these earlier studies were not randomized and some were not blinded. Also the doses used are very different and it is unclear as to which dose range needs to be used and over what time period, for significant (both clinical and statistical) changes in lipids and apolipoproteins to occur. Ideally, a straightforward method of measuring the levels of cholesterol efflux and RCT in humans would be the best way to test the effectiveness of OPC.

The EPICURE trial is the first to report the effects of cocoa on HDL subclasses and apolipoproteins. Changes in lipids and apolipoproteins was a secondary outcome for the EPICURE trial and it is more than likely that the study was not powered to detect changes in these parameters. The potential effects of OPC from cocoa on lipids and their subclasses and apolipoproteins needs to be explored further initially through in vitro studies to define the appropriate parameters to measure, before undertaking randomized controlled studies. The former are necessary to better understand the function of the ever-growing number of lipids and apolipoproteins. It should be remembered that the hospital laboratory cholesterol measures also failed to detect any trends with HPDC thus supporting that no quantitative changes occurred in the study with OPC. It remains possible that cholesterol turnover has altered amongst other qualitative changes which the study was unable to detect. In future studies HDL cholesterol assays should be optimised with “model” samples containing high, medium and low HDL levels. In addition the use of protein electrophoresis may help determine whether any HDL related proteins had altered providing some evidence of qualitative changes in HDL.

Despite the lack of positive findings the main learning points from this chapter are the process of assay development, optimisation and validation of new techniques. This has been invaluable for the author and in future help from a laboratory where there is expertise in this technique would allow the author to further develop their knowledge.
Chapter 5
General Discussion
5.1 General discussion
Over the past twenty years an increasing level of evidence supports that, daily consumption of flavonoids can decrease the risk of CVD (Grassi et al., 2005a and 2005b; Taubert et al., 2003; Taubert et al., 2007; Allen et al., 2008; Balzer et al., 2008; Engler et al., 2004; Farouque et al., 2006; Flammer et al., 2007; Heiss et al., 2005; Schroeter et al., 2006; Vlachopoulos et al., 2005; Wang-Polagruto et al., 2006). During this time, research has switched from the hypothesis that flavonoids exert a general, all pervading antioxidant effect which prevents or cures all age-related diseases, to a more subtle approach that investigates detailed biological actions of specific flavonoids. Some of the strongest evidence for the health benefits of flavonoids is linked to consumption of products rich in the flavan-3-ol group of polyphenols (Corder, 2002; Corder et al., 2006; Caton et al., 2010). Although chemically similar, the flavan-3-ol family exists in nature primarily as a group of molecules ranging in size from monomers such as epicatechin and catechin, through to procyanidin oligomers of decamer length. Major criticisms of OPC research are that studies reporting dietary consumption and its association with disease do not distinguish between these molecules and other polyphenols and there has been no clinical trial comparing a product rich in monomers and depleted in oligomers, with one depleted of monomers and enriched in oligomers to demonstrate whether the vascular effects differ. Therefore, neither the optimal product, nor the recommended daily amounts for conferring a health benefit have yet been defined (Almoosawi et al., 2010; Davison et al., 2010).

OPC have been reported to have multiple health benefits which thus far failed to be reproducible in different studies, the most important of which are the anti-atherosclerotic actions. The reduction in CVD is multifactorial and of all the effects observed with purified OPC and OPC-rich products the most consistent finding is improved vascular function through endothelium-dependent vasodilatation mediated by NO (Fitzpatrick et al., 1993 and 2002; Cishek et al., 1997; Andriambeloson et al., 1997, 1998 and 1999; Flesch et al., 1998; Stoclet et al., 1999; Karim et al., 2000; Diebolt et al., 2001; de Moura et al., 2004; Boban et al., 2006; López-Sepúlveda et al., 2008; Schmitt & Dirsch, 2009), and also reduced ET-1 production (Corder et al., 2001 and 2006; Jiménez et al., 2007; García-Conesa et al., 2009; Caton et al., 2010). Other findings include reduction in blood pressure (Diebolt et al., 2001; Pechánová et al., 2004; López-Sepúlveda et al., 2008), improvements in dyslipidaemia (Tebib et al., 1994; Hayek et al., 1997; Matsumoto et al., 1998; Frémont et al., 2000; Auger et al., 2005; Del Bas et al., 2005; Rizzo & Berneis, 2008; Matsumoto et al., 1998; Osada et al., 2006; Yasuda et al., 2008; Quesada et al., 2009; Osakabe & Yamagishi, 2009; Klurfeld & Kritchevsky, 1981; Yamakoshi et al., 1999;
Kurosawa et al., 2005), and anti-platelet activity (Xia et al., 1998; Iijima et al., 2000, 2002a and 2002b; de Curtis et al., 2004; Rosenkranz et al., 2002; Shanmuganayagam et al., 2007). This thesis set out in order to try to clarify the effects of OPC from cocoa and apples on endothelial cell cholesterol levels and translate these observations into parameters that could be measured in humans by measuring the effects on various cardiovascular parameters in a clinical trial. The intended advantages of the EPICURE study were the longer duration, the use of an appropriate matched control and higher polyphenol content of the chocolate.

Cellular studies provide novel evidence that OPC promotes a reduction in cellular cholesterol levels through suppression of HMGCR mRNA levels and increased ABCG1 mRNA levels in vitro. This was associated with early increases in CH25OH mRNA levels by 1 h, supporting the idea that the oxysterol 25-OHC, is a key endogenous factor that regulates endothelial cell cholesterol homeostasis (figure 5.1). The effects seen persisted over 24 h, which in view of the low doses is promising. This is similar to other studies, which have shown that OPC affect cellular cholesterol regulation (figure 5.1; Wegrowski et al., 1984; Osman et al., 1998; Yamakoshi et al., 1999; Chang et al., 2001; Lee et al., 2008; Lam et al., 2008). Co-incubation of apple OPC in the presence of 25-OHC had a synergistic action. HMGCR inhibition with atorvastatin did not have any appreciable effects on the levels of ABCG1 mRNA but was associated with increased HMGCR mRNA. It is possible that increases in HMGCR mRNA leads to increased transcription of the enzyme, leading to re-bound cholesterol production with less potent statins or those with a shorter half-life. This may be one factor that contributes to the residual significant mortality from CVD despite the widespread use of statins. This data needs to be investigated further before any firm conclusions are made, to begin with the effect of statins on HMGCR mRNA expression, HMGCR activity and lipid synthesis must be determined.

Of all the oligomers evaluated, the pentamers were most potent in producing these effects, which were enhanced by the addition of 25-OHC and future work should focus on these molecules. This is in concordance with the findings of a number of previous publications describing the actions of purified OPC on endothelial cells (Fitzpatrick et al., 2000 and 2002; Aldini et al., 2003; Corder et al., 2006, García-Conesa et al., 2009; Caton et al., 2010). Interestingly, close scrutiny of microarray studies performed on human umbilical vein endothelial cells also show reduced HMGCR signal after apple OPC treatment (García-Conesa et al., 2009).
The mRNA expression changes with ABCG1 were all generally increased but the data shows poor reproducibility, with changes in expression varying from 2 - 1,000 fold. This is likely to be the result of methodological issues relating to variable expression of the housekeeping gene, RNA polymerase 2. In retrospect validation of RNA polymerase 2 in BAEC to identify the coefficient of variation should have been performed. Alternatively the use of a TaqMan probe may have resulted in an improved primer set.

Figure 5.1 Diagrammatic representation of the effects of OPC on cellular cholesterol levels. It remains unknown as to how OPC cross membranes and how the increase in CH25OH occurs. Furthermore, it remains to be confirmed if CH25OH changes are mediated through the inhibition of the SCAP-SREBP-INSIG complex.

Enhancement of cholesterol efflux was not seen in BAEC loaded with LDL-C and incubated with OPC and HDL-C. Increased cholesterol efflux would have been expected if OPC are changing the expression of ABCG1 mRNA and HMGCR mRNA as described above. Indeed both cell media and solubilised cell extract cholesterol levels were reduced, which was more notable in the former. It could be concluded that OPC lead to changes in the mRNA expression but these are not associated with changes in activity and thus there is no effect on cell
cholesterol levels. Rather it is likely that either these experiments have not worked as previous experiments, especially as the changes in mRNA expression of ABCG1 was not seen as anticipated. On the other hand, it is possible there were detrimental effects on cells from the plasma derived LDL-C, which is supported by the marked reductions in cholesterol levels seen following cell loading.

The in vitro effects of OPC on CH25OH were inhibited by the superoxide dismutase mimetic MnTMPyP, supporting the necessary role of ROS in this process in agreement with previous research (Diebolt et al., 2001). This challenges the hypothesis that antioxidant effects underlie the mechanism of action of OPC, a concept that has been frequently put forward by others as the explanation for the effects of flavonoids (Miyagi et al., 1997; Yamokishi et al., 1999; Vinson et al., 2006; López-Sepúlveda et al., 2008; Fraga et al., 2010). Indeed, the action of MnTMPyP supports the increasing evidence that some ROS are beneficial to health (Ago et al., 2004; Van Buul et al., 2005; Ray et al., 2007; Larsen et al., 2009; Zhuang et al., 2010; Ray et al., 2011). Recently, NOX4 was shown to increase endothelium-dependent vasodilatation via $\text{H}_2\text{O}_2$ production (Ray et al., 2011). These results imply that NOX4 could be involved in the signalling mechanisms mediating the actions of OPC on endothelial cells, which could be further investigated using siRNA. The changes in gene expression are likely to be mediated by specific transcription factors, such as alterations in NFkB or KLF2 as described by others (Spencer et al., 2001; Calabró et al., 2009; Caton et al., 2010). This is supported by the reduction in ET-1 levels noted with OPC in several of the experiments, which were greatest at 6 h.

The EPICURE trial described the effects of high and low procyanidin dark chocolate on healthy male subjects, with stage I (systolic BP 140-159 mmHg; diastolic BP 90-99mmHg) or prehypertension (systolic BP 130-139 mmHg; diastolic BP 85-89 mmHg). The main findings were as follows:

- No significant changes in 24 h ambulatory blood pressure which is similar to findings reported in other clinical trials (Farouque et al., 2006; Muniyappa et al., 2008; Reid et al., 2009).

- No improvement in pulse wave analysis parameters and in particular no improvement in augmentation index suggesting no changes in arterial stiffness, although the
significantly increased response to salbutamol does suggest increased endothelium-dependent vasodilation.

- Significantly reduced heart rate both on 24 h ambulatory blood pressure monitoring and pulse wave analysis with HPDC.
- Significant reduction in pulse pressure amplification with HPDC.
- No significant changes in various apolipoproteins, total cholesterol and the various cholesterol fractions with HPDC.

Based on studies of high flavanol cocoa it is unlikely that the dose of procyanidins or bioavailability issues are reasons why a significant change was not observed. Measuring blood pressure an index of vascular function, may be too insensitive to detect changes, and the parameters measured by pulse wave analysis may not reveal any change in vascular stiffness after six weeks of high procyanidin chocolate. In addition, whilst the EPICURE study was being undertaken it was becoming clear that it would have been valuable to have measures of PWA with both salbutamol and GTN along with PWV. Indeed retrospectively, it may have been far better to have used FMD with brachial artery ultrasonography, giving a more direct measure of endothelial function and more importantly one which meant that the EPICURE study could be compared with other cocoa trials. The study can also be criticised as the statistical analysis of the EPICURE study did not look for an order effect. It seems most appropriate to conclude that the study was underpowered, as a trend to lower systolic blood pressure (~2 mmHg) with HPDC was seen. The initial power calculations were based on studies where larger magnitude changes in blood pressure were seen of 5 mmHg (e.g. Taubert et al., 2003; Grassi et al., 2005a; Grassi et al., 2005b; Allen et al., 2008), which have not been replicated in the majority of subsequent studies (e.g. Taubert et al., 2007; Grassi et al., 2008; Heiss et al., 2010). Indeed a retrospective power analysis calculates that the number of subjects needed to detect a 2 mmHg change in systolic blood pressure is likely to be closer to 130.

Interestingly heart rate was significantly lower with HPDC on both the 24 h ABPM and clinic measurements prior to pulse wave analysis. Heart rate is influenced by the balance between the sympathetic and parasympathetic nervous system, the actions of circulating epinephrine and intrinsic activity in the sinus node. This is the first report of such effects in humans with OPC although Akita and colleagues (2008), reported similar findings in animals and ascribed these
effects to preserved parasympathetic function (Akita et al., 2008). How OPC cause these effects in humans is unknown, but as beta-blockers reduce myocardial demand for oxygen through lowered heart rate and are established to have prognostic benefit in the secondary prevention of CVD, OPC may provide additional protection from CVD through this mechanism. A reduction in heart rate with HPDC has not been reported before and more work is needed in this field before any real conclusions are made and the relationship if any to reducing CVD risk.

The reduction in pulse pressure amplification i.e. the ratio of peripheral to central pressure, seen with HPDC in the EPICURE study is also a novel finding and reflects both the lower peripheral and central pulse pressures. Central pressures are more likely to be important in the development of target organ damage and there is increasing evidence, albeit from small studies suggesting that lower pulse pressure amplification is associated with all cause and CV mortality (Safar et al., 2002; Hashimoto et al., 2007; Hashimoto & Ito, 2010; Benetos et al., 2010). However, pulse pressure amplification measures are currently not standardised and can be affected by ageing (McEniery et al., 2008; Papaioannou et al., 2010; Segers et al., 2009).

Total cholesterol, lipoproteins, and apolipoproteins were also measured in the EPICURE study. A precipitation method was used to further analyse HDL-C subclasses, providing a measure of qualitative changes in HDL. TC, LDL-C, and TG were non-significantly lower after 6 wk consumption of HPDC when measured in the hospital laboratory, but the PEG/glycine/DS precipitation method detected higher levels of both TC and LDL-C with HPDC. There was no discernible difference in total HDL-C measured by either the hospital laboratory or the precipitation method. Based on the PEG/glycine/DS precipitation, HDL-C and HDL-C levels were not different between the two types of chocolate, although a trend towards higher HDL-C levels was seen with the consumption of HPDC. The various apolipoproteins measured in subjects in the EPICURE study were also not significantly different between the two groups. The lack of changes in lipids, apolipoproteins, total HDL, and HDL subclasses may relate to the methodology used, storage time of samples and the lack of a reference method. Alternatively, these results may reflect that OPC do not lead to measurable changes in these parameters, despite changes in the in vitro studies of cellular cholesterol regulation or measuring steady state HDL levels is not the best way of obtaining information regarding RCT, which is a dynamic process. This is similar to research which has shown a reduction in atherosclerosis burden with OPC without changes in lipids (Hayek et al., 1997; Yamakoshi et al., 1999; Kurosawa et al., 2005; Fuhrman et al., 2005; Bladé et al., 2010).
The research goal of this thesis set out to test the following hypothesis:

“Dietary oligomeric procyanidins lead to a global reduction in CVD risk through reduced blood pressure, improved endothelial function, and quantitative and qualitative improvements in HDL. The latter results in decreased endothelial cell cholesterol levels through a combination of reduced de-novo cholesterol synthesis and enhanced cholesterol efflux, a process modulated by the enzyme, cholesterol-25-hydroxylase.”

The approach taken consisted of undertaking an experiment and then analysing the data and after further discussion of the results with the supervisor the next laboratory experiment was decided upon. The hypothesis can be argued to be very specific and consists of both realistic and testable elements (blood pressure, endothelial function and qualitative improvements in HDL), as well as qualitative changes in HDL and cholesterol synthesis and efflux studies which are not easily testable. If research hypotheses only considered testable effects than the element of intuition is disregarded. Indeed intuition forms the backbone of a number of research studies and often provides the stimulating challenges for research groups and allows the development of new research techniques. Furthermore there was evidence of a change in HDL-C before the studies were planned by other researchers, but these studies had been of short duration and small numbers of people (Wan et al., 2001; Mursu et al., 2004; Baba et al., 2007; Grassi et al., 2008; Mellor et al., 2010). As HDL-C could go up or down if efflux and reverse cholesterol transport were both increased, depending on how closely linked the processes are. Hence the objective of clinical study was to confirm changes in HDL-C plus get insights that might link this to altered endothelial function, the cell studies were to confirm changes in gene expression were linked to increased efflux. Although the results may not have occurred as anticipated this suggest that the work is unfinished rather than the approach is wrong.

Although evidence of cholesterol-25-hydroxylase modulated reductions in endothelial cell cholesterol levels in vitro was seen, improvements in blood pressure, endothelial function and HDL in human subjects was not. In hindsight, the study was probably not powered to detect the cardiovascular parameters listed. The lack of accurate bioavailability data and standardized methods of measuring OPC are further limitations and are critical in determining an effective
dose range and to make comparative analyses with existing studies. This information would also help to obtain a refined form of OPC negating the confounding role of other excipients.

The main learning points from this thesis are:

- A clear and testable hypothesis.
- Validating and optimising methodology for the purposes they are going to be used for.
- Reviewing results not only in terms of statistical significance but also looking at reproducibility and validity.
- Asking questions when the data does not appear consistent and trying to work out the difficulties.
- Importance of good clinical study design, including power calculations, compliance checks and reviewing all possible methods of obtaining data before deciding on which particular method.
- Trying new methodology but seeking expertise to enhance one’s own learning.

On the basis of the results presented here if a new PhD student were to continue this work I would suggest a clinical trial of high OPC cocoa with measures of atherosclerosis by MRI scanning of the carotids looking for intima-media thickness along with measures of lipid profile and brachial artery FMD in older aged men and women. The aim should be to eventually perform larger, randomised, placebo-controlled trials in patients with cardiovascular risk factors with purified oligomeric procyanidins in the form of a capsule are needed. These studies should include FMD, bioavailability measures, and cardiovascular imaging to observe if there are any changes in atherosclerotic burden over time. In addition BAEC studies with radioactively labelled cholesterol should be performed to test whether OPC do affect cholesterol efflux, using purified LDL-C and HDL-C of high quality.

Complete elucidation of the mechanism of action of OPC on endothelial cells is likely to identify biomarkers of the response that can be used to monitor better vascular responses in clinical investigations. Ultimately, such biomarkers could be used to identify individuals who could benefit most from increased OPC consumption. This in turn could be used to define a recommended daily amount for optimal vascular health.
References and Bibliography
References

Journals


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Meuwese, M.C., Franssen, R., Stroes, E.S. and Kastelein, J.J. (2006) and then there were acyl coenzyme A:cholesterol acyl transferase inhibitors. *Curr. Opin. Lipidol.* **17**: 426-430.


Yuhanna, I.S., Zhu, Y., Cox, B.E., Hahner, L.D., Osborne-Lawrence, S., Lu, P., Marcel, Y.L.,


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