The Effects of Estrogens and Phytoestrogens on the Metabolism and Oxidation of Plasma Lipoproteins

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Abstract

The aim of this thesis was to examine the effects of estrogens and phytoestrogens, on plasma lipoprotein levels and other risk factors for cardiovascular disease, including the oxidisability of low density lipoprotein (LDL).

Hepatic LDL receptor activity plays an important role in the regulation of plasma LDL levels. *In vitro* studies presented here in the human hepatoma cell line HepG2 revealed that of the three major human estrogens, only estradiol was able to upregulate LDL receptor activity at 50μM. The phytoestrogens, daidzein, biochanin A, formononetin and coumestrol were all able to upregulate LDL receptor activity *in vitro*. Comparison of the effects of the two lignan phytoestrogens suggested that estrogenicity is an important determinant of their LDL receptor activity regulation.

In an *in vivo* study of hormone replacement therapy (HRT) in postmenopausal women, it was found that estrogen and progestin did not cause significant upregulation of mononuclear cell LDL receptor activity, despite there being noted a reduction in plasma LDL cholesterol. A reduction in the level of the atherogenic lipoprotein(a) was found following three months of HRT, however there was no significant effect of HRT on the parameters of *ex vivo* LDL oxidation.

Ground flaxseed (10g/d), which is rich in phytoestrogenic lignans and α-linolenic acid, was found to increase the levels of Lp(a) in men, while there was no effect noted in

women. This appears unlikely to be due to the fatty acid component of the flaxseed as we found no effect of either n-3 fatty acids or a high polyunsaturated fat diet on Lp(a) in other studies. While statistically significant, the reduction in Lp(a) was small and is unlikely to provide significant cardiovascular benefit. Further dose response studies are required to clarify the role of flaxseed in this reduction and to determine whether the Lp(a) lowering effect is maintained with longer-term supplementation. Further *in vivo* studies with isoflavonoid phytoestrogens found no effect of these compounds on Lp(a). Despite the previously demonstrated ability of daidzein and genistein to protect LDL from oxidation *in vitro*, we found no effect of isoflavone supplementation on *ex vivo* LDL oxidation. Isolated soy-derived isoflavones did not elicit a cholesterol-lowering effect in post-menopausal women, at either moderate (75mg isoflavones/d) or high doses (150mg isoflavones/d). As whole soy protein consumption has been reported to have a cholesterol lowering effect at lower isoflavone levels, this suggests that there may be an additional component of soy which facilitates this effect.

Thus, while estrogen was shown to upregulate LDL receptor activity in vitro, it was not possible to demonstrate this effect *in vivo,* suggesting that postmenopausal hormone replacement may be affecting LDL receptor independent catabolism of LDL. Phytoestrogens can upregulate *in vitro* LDL receptor activity, but when isolated soy isoflavones were given to postmenopausal women, no LDL cholesterol-lowering effect was noted. While animal studies suggest phytoestrogenic isoflavones play a role in the cholesterol-lowering effect of soy diets, it appears this effect is not independently mediated by the phytoestrogens.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference has been made in the text.

I give consent for this copy of my thesis, when deposited in the University library, being made available for loan and photocopying.

Signed

Date

22/03/01

Alice Owen

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Abbreviations

ANOVA	analysis of variance
Apo	apolipoprotein
BMI	body mass index
BSA	bovine serum albumin
CVD	cardiovascular disease
DMEM	Dulbecco's modified Eagles medium
E1	estrone
E2	17β-estradiol
E3	estriol
EDTA	ethylenediaminetetra acetic acid -disodium salt
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FBS	foetal bovine serum
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
PBS	phosphate buffered saline
PUFA	polyunsaturated fatty acid
SEM	standard error of the mean
TBARS	thiobarbituric acid reactive substance
UV	ultraviolet
VLDL	very low density lipoprotein

CHAPTER 1

Introduction

Overview of Introduction

Women have lower rates of cardiovascular disease than age-matched men, a difference which diminishes with age (Castelli 1988), and has been attributed to estrogen (Barrett-Connor and Bush 1991). The introduction to this thesis will commence with a general discussion of lipoproteins, including their metabolism and relation to cardiovascular disease. It will then discuss the possible mechanisms by which estrogen may be acting to protect against the development of cardiovascular disease, and lead into a final discussion of phytoestrogens, dietary compounds which display estrogenic activity, and their potential as cardioprotectants.

1.1 Cardiovascular Disease and Lipids

Cardiovascular disease (CVD) is the leading cause of death in most Western societies. One of the major pathologies of CVD is atherosclerosis, which is characterised by the development of fatty plaques in the artery wall. These space-occupying, cholesterol-laden plaques can result in a reduction in blood flow through the artery, leading to peripheral vascular disease or myocardial infarction. There are a number of well-defined risk factors for the development of CVD, and these include smoking, hypertension and obesity. Additionally, the plasma lipid profile has been found to be a major influence on CVD risk (Martin et al. 1986).

The major lipids found in plasma are cholesterol (mostly in the form of cholesterol ester), triglyceride and phospholipid. With the exception of free fatty acids, these hydrophobic molecules are transported in plasma in the form of lipoproteins. Lipoproteins are comprised of proteins called apolipoproteins which interact with phospholipid and free cholesterol to form the polar outer surface of the lipoprotein encompassing an inner core of cholesterol ester and triglyceride. Lipoproteins differ in the their apolipoprotein content, and these proteins regulate the interaction of the lipoproteins with receptors and enzymes. However, the most commonly used nomenclature with which lipoprotein types are distinguished, relates to their density. (Harwood et al. 1994)

The largest, least dense lipoprotein particle in plasma is the intestinally derived chylomicron. Chylomicrons are responsible for the delivery of dietary triglyceride into

the circulation and are a post-prandial lipoprotein with a short half-life, normally undetectable after an overnight fast. Very low density lipoprotein (VLDL) is the major carrier of triglyceride in the plasma in the fasting state and is mostly produced in the liver. VLDL and chylomicrons differ in that the triglyceride carried by VLDL is mostly endogenously produced. In addition, VLDL is smaller and has slightly more cholesterol, phospholipid and protein. VLDL is metabolised by lipoprotein lipase in the endothelium of capillaries supplying skeletal muscle and adipose tissue (Harwood et al. 1994). The hydrolysis of the triglyceride within the VLDL particle by lipoprotein lipase, leads to the production of intermediate density lipoproteins (IDL). IDL is further metabolised by hepatic lipases to form the relatively triglyceride poor/cholesterol rich low density lipoprotein (LDL) (Thompson 1990).

LDL is the major carrier of cholesterol in the plasma. Derived from the metabolism of VLDL, LDL has much less triglyceride and its protein content is composed almost entirely of apolipoprotein B ($apoB_{100}$ in humans). LDL is responsible for the majority of the delivery of cholesterol to peripheral tissues. Smaller amounts of cholesterol are carried in the heterogenous high density lipoprotein (HDL) population. Most often divided into HDL_2 and HDL_3 subclasses according to density, HDL also differ in their apolipoprotein content and can contain either apoAI, apoAII or both apoA variants (Harwood et al. 1994). It appears that HDL is responsible for the removal of cholesterol from tissues and the return of this cholesterol to the liver, a process that has been named 'reverse cholesterol transport' (Barter 1993).

Data obtained from the Framingham study indicated that elevated levels of total plasma cholesterol were associated with an increased risk for development of CVD (Kannel et al 1981, Dawber 1990). Associations between plasma cholesterol and CVD risk are to a large degree attributable to plasma LDL levels (Kannel et al. 1981), and elevated LDL cholesterol has been shown to be a clearly defined CVD risk factor. Elevated plasma triglyceride levels have also been shown to be associated with increased CVD risk (Castelli 1986). Plasma high density lipoprotein (HDL) appears to be cardioprotective and thus low levels of HDL have been associated with an increased risk of developing CVD (Castelli et al. 1986).

1.2 LDL Metabolism

Removal of LDL from the circulation occurs mostly in the liver (Dietschy et al. 1993), and most of this occurs as a result of uptake by the LDL receptor (Pittman et al. 1979), which was discovered by Goldstein and Brown (1977). The main role of the LDL receptor is to provide all cells of the body with a mechanism for constant delivery of cholesterol for membrane synthesis. In addition, the LDL receptor provides a lipoprotein uptake mechanism for cells which use cholesterol as a substrate for production of bioactive compounds including sex steroids, bile acids and corticosteroids. Being the primary regulator of LDL levels, the liver has a high number of LDL receptors (Brown and Goldstein 1986).

The LDL receptor is a cell surface receptor which is subject to feedback regulation by intracellular cholesterol concentration (Brown and Goldstein 1986). The LDL receptor recognises apoB_{100} and apoE and binds these ligands in a high affinity saturable mechanism, resulting in LDL binding to LDL receptors in coated pits, which become endocytosed. Subsequently the LDL receptor dissociates from the LDL particle and is recycled to the surface of the cell. The LDL within the endosome undergoes lysosomal digestion, and the cholesterol esters released, undergo hydrolysis. The free cholesterol then acts to down regulate the cellular production of cholesterol by the enzyme HMG-CoA reductase (Brown and Goldstein 1986).

An increase in cellular unesterified cholesterol (or an oxygenated derivative) acts not only to downregulate cellular cholesterol production by suppression of transcription of the HMG CoA reductase gene, but also promotes cholesterol esterification by the enzyme acyl CoA: cholesterol acyltransferase (ACAT) to the metabolically inactive cholesterol ester. In concert with these two effects, the increase in unesterified cholesterol in the cell also results in suppression of cellular production of LDL receptors (Brown and Goldstein 1986).

Regulation of LDL receptor expression by sterols involves a complex interaction between DNA binding proteins and the promoter region of the LDL receptor gene. The LDL receptor promoter region contains sequences named Sp1 that bind transcription activating proteins, and also a region called the sterol response element (SRE-1) which binds sterol response element binding proteins (SREBP's) (Yokoyama et al 1993). Precursors to

SREBP are cleaved to generate a soluble SREBP which translocates to the nucleus where it is able to bind SRE-1 and promote transcription of the LDL receptor gene. Sterol regulation of LDL receptor appears to occur through regulation of SREBP proteolysis. The presence of sterols in the cell inhibits the cleavage of SREBP from its precursor, decreasing the amount of SREBP, and therefore reducing transcription of the LDL receptor gene (Wang et al. 1994b). This series of mechanisms allows cells to regulate their uptake of LDL, which aids in the regulation of cellular cholesterol content.

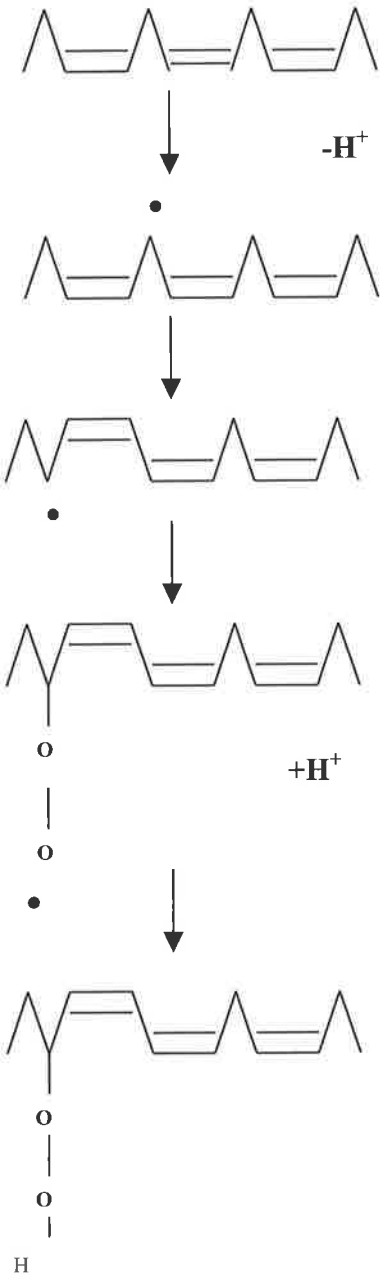
1.3 The Oxidation Hypothesis of Atherogenesis

1.3.1 LDL oxidation

Studies in animal models have revealed that atherosclerotic plaques are initiated by the formation of fatty streaks beneath the endothelium of the artery (Fagiotto et al. 1984). These fatty streaks are comprised of foam cells, cells laden with cholesterol ester. Foam cells are mostly of macrophage origin arising from circulating monocytes, but can also be derived from smooth muscle cells (Aqel et al. 1984, Steinberg et al. 1989). As it is tightly regulated by negative feedback, the LDL receptor is not thought to be responsible for massive accumulation of cholesterol in foam cells. In addition, in homozygous Familial Hypercholesterolaemia (FH), a hereditary condition in which mutations of the LDL receptor gene result in the translation of non-functional LDL receptors, the development of atherosclerosis occurs at a young age (Schaefer et al. 1994). A currently favoured hypothesis for the uptake of LDL into foam cells is through the scavenger receptor pathway.

LDL which has undergone oxidative modification has been shown to be taken up by macrophages at a greatly enhanced rate (Steinbrecher et al. 1989, Henriksen et al. 1981). As a result of the abstraction of H^+ from double bonds in the polyunsaturated fatty acids (PUFA) within the LDL, molecular rearrangement of the PUFA takes place and conjugated dienes are formed (Jialal and Deveraj 1996) which can be detected spectrophotometrically at 234nm. The conjugated dienes react further to form hydroperoxides, which degrade into aldehydes (*Figure 1.1*).

Figure 1.1 *Schematic diagram of oxidation of polyunsaturated fatty acids in LDL*



Hydrogen abstraction from a polyunsaturated fatty acid

Molecular rearrangement leads to formation of a conjugated diene

Reaction with oxygen to give peroxyl radical

Abstraction of H^+ from neighbouring PUFAs initiates an autocatalytic chain reaction of lipid peroxidation

Hydroperoxide formed which fragments into aldehydes and other decomposition products

The major aldehydes produced include malondialdehyde (MDA) and 4-hydroxynonenal (HNE), and these products can react with lysine residues on apo B (the major protein component of LDL). This results in a decrease in the net positive charge, and fragmentation of apo B, such that it can no longer be recognised by the LDL receptor. Unregulated uptake of the oxidised LDL can then occur by an alternate receptor that does not recognise unmodified LDL, leading to the production of lipid laden foam cells (Henriksen et al. 1981, Hoff et al. 1992). *In vitro*, LDL can be oxidised by incubation with transition metal ions such as copper (Heinecke et al. 1984). In addition, it has been shown that incubation of LDL with a variety of different cultured cells including endothelial cells, smooth muscle cells and macrophages can result in LDL oxidation (Morel et al 1984, Berliner and Heinecke 1996, Parthasarathy et al. 1986). The proposed mechanisms by which cells oxidise LDL include superoxide anion (Hiramatsu et al. 1987), myeloperoxidase (Savenkova et al. 1994) and thiol production (Sparrow and Olszewski 1993).

Oxidised LDL has been implicated in the development of atherosclerosis by a number of findings. Immunocytochemical techniques have been utilised to show that components of oxidised LDL are present in arterial atherosclerotic lesions, but not in normal arteries (Haberland et al. 1988, Yla Herttuala et al. 1994). LDL isolated from atherosclerotic plaques has also been shown to possess physiological properties similar to that of oxidised LDL (Yla-Herttuala et al. 1989). Palinski et al. (1995) found that immunisation

of Watanabe Heritable Hyperlipidaemic (WHHL) rabbits with MDA modified LDL (a form of oxidised LDL) resulted in a reduction in the extent of atherosclerotic lesions, but how this immunisation might be protective in this LDL receptor-deficient, atherosclerosis prone rabbit model is not known.

1.3.2 Antioxidants and LDL Oxidation, and their relation to CVD

As LDL oxidation appears to play a significant role in atherogenesis, there has been a great deal of interest in compounds that might prevent oxidation of LDL, that is antioxidants.

In vitro monitoring of LDL oxidation can be performed by incubation with Cu^{2+} coupled with spectrophotometric detection of conjugated dienes at 234nm (Esterbauer et al. 1989). In this model, three major phases of oxidation can be detected. The first is the lag phase, during which the PUFA within the LDL are protected from oxidation. This if followed by the propagation phase, where rapid oxidation of PUFAs to hydroperoxides occurs. The final decomposition phase is characterised by a shift in the balance between conjugated diene production (and therefore production of lipid peroxides), and breakdown of the lipid peroxides (to products which include reactive aldehydes). The concentration and type of antioxidants within the LDL particle or included in the incubation medium play a role in determining the length of the lag phase.

One of the major antioxidants in the LDL particle is α-tocopherol (Stocker, 1993). Early evidence suggested that oxidation of the LDL particle did not occur until all the

endogenous α-tocopherol was consumed (Jessup et al. 1990, Esterbauer et al. 1991). However later studies have suggested that α-tocopherol has the ability to act not only as an antioxidant, but also as a pro-oxidant through generation of the highly reactive tocopheroxyl radical (Bowry et al. 1992). The antioxidant activity of α-tocopherol appears dependent upon co-antioxidants such as ubiquinol-10 being present in the LDL particle (Thomas et al 1996). The resistance of the LDL particle to oxidation (which can be determined by the lag time) has been reported to be related to its α-tocopherol and ubiquinol-10 content (Esterbauer et al. 1991, Abbey et al. 1993b, Tribble et al. 1994). Addition of a number of different antioxidants to isolated LDL, enhances its resistance to oxidation (reviewed in Jialal and Scaccini, 1992), and following dietary supplementation with antioxidant vitamins, the *ex vivo* oxidisability of LDL is reduced (Abbey et al. 1993, Stocker 1993).

The ability of antioxidants to protect against *in vitro* LDL oxidation is well established, but how well this translates to a protective effect against atherosclerosis *in vivo* is still unclear. Probucol is a cholesterol-lowering drug which has potent antioxidant activity and inhibits the *in vitro* oxidation of LDL (Parthasarathy et al. 1986). In rabbits, probucol inhibits the development of atherosclerosis, however whether the effect is related to its cholesterol lowering properties or antioxidant properties cannot be ascribed (Mao et al. 1991). The study by Mao and co-workers also examined a probucol analogue which did not display the hypocholesterolaemic effects. The analogue slightly decreased lesion area in this cholesterol fed rabbit model, but this effect was not statistically significant (Mao et al. 1991).

Vitamin E is a racemic mixture of tocopherols, with α-tocopherol being the most potent antioxidant. It has been shown that supplementation with vitamin E causes an increase in plasma α-tocopherol concentration (Abbey et al. 1993b, Esterbauer et al 1991, Tribble et al. 1994). However while α-tocopherol contributes toward protection of LDL from oxidation, the ability of the vitamin E to inhibit atherogenesis has not been consistently shown. Vitamin E has been shown by some to reduce lesion area in WHHL rabbits (Williams et al. 1992), however others have found no effect (Kleinveld et al. 1995). In addition, very high doses of vitamin E have been found to be pro-atherogenic (Godfried et al. 1989). These *in vivo* effects may be a reflection of the ability of α-tocopherol to act as a pro-oxidant as well as an antioxidant *in vitro* (Bowry et al. 1992).

Treatment of LDL receptor-null mice with an antioxidant supplement containing vitamin E, β-carotene and the water soluble antioxidant vitamin C, resulted in a reduction in atherosclerotic lesion size and protection against *ex vivo* LDL oxidation, however the correlation between these two effects was not strong (Crawford et al. 1998).

Two large prospective epidemiological studies in humans have found that high vitamin E intake was associated with a reduction in CVD risk (Stampfer et al. 1993, Rimm et al. 1993). In these studies, vitamin E intake was assessed by a single questionnaire and it is difficult to rule out the possibility that consumers of large amounts of vitamin E may differ in other important variables.

Clinical trials have much more power with which to examine antioxidant supplementation and CVD, but two recent large trials examining α-tocopherol supplementation have not made consistent findings. The α-Tocopherol, β-Carotene Cancer Prevention Group (1994) found no effect of vitamin E on heart disease mortality. However the Cambridge Heart Antioxidant Study, found evidence to suggest that vitamin E may reduce the incidence of non-fatal myocardial ischaemia (Stephens et al. 1996).

In summary, while there is evidence implicating oxidised LDL in atherogenesis, and LDL oxidation can be inhibited *in vitro* and *ex vivo* by antioxidants, whether LDL oxidation can be related directly to the development of atherosclerosis has yet to be conclusively shown.

1.4 The Pathogenesis of Atherosclerosis

Our knowledge of the intricate processes of atherogenesis remains incomplete, and research in this area continues to illuminate a wide range of complex mechanisms by which cholesterol may be deposited in the artery wall. These appear to involve multiple interactions between cells, matrix components of the arterial wall, circulating monocytes and lipoproteins.

In vitro studies of cell-mediated oxidation of LDL have mostly been found to require the presence of trace amounts of transition metal ions such as Fe^{2+} and Cu^{2+} (Hiramatsu et al. 1987, Sparrow and Olszewski 1993). *In vivo*, transition metal ions are found bound to

plasma proteins rather than as free ions, and the most abundant plasma protein albumin, has been found to inhibit Cu^{2+} induced oxidation of LDL (Thomas 1992). Additionally, plasma contains water soluble antioxidants such as ascorbate which have been shown to protect LDL from oxidation *in vitro* (Jialal et al. 1990). Current theory suggests that extensive LDL oxidation does not occur in plasma, but rather in the intima of the artery sequestered from plasma antioxidants (Berliner and Heinecke 1996), where retention of LDL may provide opportunity for oxidation and degradation (Nielsen 1999).

In brief, it is thought that the monocytes respond to chemotactic and adhesive cell signals, leading them to differentiate into macrophages within the intima (Ross 1993). The macrophages can become loaded with cholesterol ester through the unregulated uptake of oxidised LDL, and possibly uptake of other lipoproteins via alternate receptors, this eventually results in foam cell formation and subsequent accumulation of lipid beneath the endothelium (Steinberg et al. 1989). In addition, proliferation of smooth muscle cells in the vessel wall forms a fibrous connective tissue matrix which contributes to the space-occupying fibro-fatty lesion (Ross 1993). The process feeds back into further inflammatory responses that continue a cycle of monocyte recruitment, cell proliferation and differentiation, and damage to the vessel wall.

In addition to being taken up at an enhanced rate by cells, oxidised LDL has been shown to have a number of effects that may contribute to the formation of the atherosclerotic lesion. Oxidised LDL is cytotoxic (Thorne et al. 1996), and has been found to enhance the production of chemokines, including the monocyte chemotactic protein MCP-1 in

endothelial cells (Cushing et al. 1990, Terkeltaub et al. 1998) which is thought to aid the entry of monocytes into the arterial wall.

1.5 Lipoprotein(a)

1.5.1 Structure of lipoprotein(a)

Lipoprotein(a) [Lp(a)] is an LDL-like lipoprotein first described by Berg in 1963 which is found almost exclusively in humans and higher primates. Like LDL, Lp(a) is cholesterol ester rich and contains apolipoprotein B_{100} (apoB). However Lp(a) contains the unique apolipoprotein(a) [apo(a)] linked by disulphide bonds to apoB (Gaubatz 1983). Evidence suggests that each Lp(a) contains one molecule of apoB and one or two molecules of apo(a) (Albers et al. 1996, Fless et al. 1994).

Apo(a) is a large glycoprotein which confers considerable heterogeneity to Lp(a), and can range in size from approximately 300-800 kDa. Apo(a) size is determined by the number of multiple protein subunit repeats which are structurally similar to kringle IV repeats of plasminogen, bearing approximately 80% homology (Marcovina and Morrisett 1995). Apo(a) comprised of between 12 to 51 kringle IV repeats have been identified (Lackner et al. 1993). It appears that most individuals are heterozygous, ie display a double band apo(a) phenotype (Klezovitch and Scanu 1995), although a null apo(a) allele has been identified which results in little or no detectable level of Lp(a) (Rees et al. 1990).

1.5.2 Metabolism of Lp(a)

Despite its structural similarity to LDL, levels of Lp(a) do not appear to be primarily regulated by the LDL receptor. An early study by Hoffmann et al. (1990) in a transgenic mouse model which overexpressed LDL receptors found accelerated clearance of injected human Lp(a), compared to normal mice. However a great deal of evidence since presented, suggests that LDL receptors do not play a major role in the metabolism of Lp(a). *In vitro*, interactions of Lp(a) with the LDL receptor have shown Lp(a) to be a much poorer ligand than LDL (Armstrong et al. 1990). LDL receptor simulating drugs such as HMG-CoA reductase inhibitors do not decrease Lp(a), in fact they have been shown to increase Lp(a) levels (Berglund, 1995). In addition, studies comparing normal and FH subjects have found that the absence of functional LDL receptors does not result in any difference in Lp(a) catabolic rate (Knight 1994, Rader et al. 1995).

Apo(a) is produced by the liver, but unlike LDL, Lp(a) is not thought to be a direct metabolic product of VLDL (Sandholzer et al. 1992). Lp(a) is thought to be formed by association of apo(a) with an apoB-containing lipoprotein particle outside the liver (Frank et al. 1996). In normolipdaemic subjects, it is the rate of synthesis rather than the rate of catabolism that determines Lp(a) levels (Rader et al. 1993).

In hyperlipidaemic states, there may be alterations in Lp(a) metabolism. In hypertriglyceridaemia, enhanced association of apo(a) with triglyceride-rich lipoproteins may allow additional clearance through the VLDL receptor (McTigue Argraves et al. 1997). Bartens et al. (1994) found hypertriglyceridaemia to be associated with a lower

plasma Lp(a) level, and in this subject group Lp(a) levels were inversely correlated to triglyceride levels. Conversely, in hypercholesterolaemic patients Lp(a) levels have been found to be elevated (Ritter et al. 1994). It has yet to be determined whether these hyperlipidaemic states are associated with changes in synthesis or degradation of Lp(a) or a combination of both.

While not significantly altered by a number of commonly used hypolipidaemic drugs, Lp(a) levels have been shown to be altered by hormones. Both androgens and estrogens have been shown to lower Lp(a) levels (Berglund 1995). Lp(a) has also been shown to be reduced following thyroxine treatment for hypothyroidism (Klausen et al. 1992). In the apo(a) transgenic mouse it has been found that estrogen and tamoxifen but not progesterone decrease the expression of apo(a) mRNA in the liver (Zysow et al. 1997), suggesting that these hormones modulate Lp(a) levels at least in part, at the level of synthesis.

1.5.3 Evidence for an association between Lp(a) and CVD

Lp(a) levels in Caucasian populations are markedly skewed toward levels less than 25mg/dl. Elevated levels of Lp(a) have been shown to be an independent risk factor for atherosclerotic disorders including myocardial infarction (Sandcamp et al. 1990, Rosengren et al. 1990), peripheral atherosclerosis (Sutton-Tyrell et al. 1996), and ischaemic heart disease (Craig et al. 1998), and have been shown to be associated with the severity of coronary artery disease (Labeur et al. 1992). However, an association between Lp(a) concentration and CVD has not been found by all investigators. Carmena

et al. (1996) found no association between Lp(a) levels and CVD in heterozygous familial hypercholesterolaemia (FH) patients, but the impact of the defect in LDL-receptor regulation (which confers hypercholesterolaemia to these patients) on Lp(a) is still unclear. Additionally, Moliterno et al. (1995) found no relationship between Lp(a) and coronary atherosclerosis in African-Americans. As a population, African-Americans have higher mean Lp(a) levels which is not associated with an increase in CVD occurrence, suggesting that the atherogenicity of Lp(a) may differ in this population (Guyton et al. 1995).

Lp(a) levels have been shown to be inversely correlated with apo(a) size by a number of researchers (Azrolan et al. 1991, Hegele et al. 1997, Utermann et al. 1987, Abe and Noma 1992, Gaubatz et al. 1990), but apo(a) phenotype has been estimated to account for only 35-70% of the variation in Lp(a) levels. The same phenotype can be associated with a 200 fold difference in Lp(a) levels (Perombelon et al. 1994).

Although Apo(a) size has been shown to be inversely related to Lp(a) levels, the relationship between apo(a) size and CVD has been harder to demonstrate. Farrer et al. (1994) found that elevated Lp(a) levels were associated with coronary artery disease independent of apo(a) isoform size. In addition, Brown and others (1993) found that apo(a) phenotype was not a significant predictor of carotid atherosclerosis whereas Lp(a) levels were an independent predictor of disease state. Conversely, a large multi-national study examining six different populations found that smaller apo(a) isoforms were more frequent in coronary heart disease patients than controls (Sandholzer et al. 1992). A

significant association between small apo(a) isoforms (low kringle IV number) and coronary heart disease was also noted by Kraft et al. (1996), and apo(a) size was found to be associated with degree of atherosclerosis in Japanese coronary heart disease patients (Amemiya et al. 1996).

1.5.4 The atherogenicity of Lp(a)

Despite the conflicting findings regarding a direct relationship between apo(a) size and CVD risk, apo(a) does appear to play a significant role in the pathogenicity of Lp(a). Following the development of a mouse model expressing human apo(a), Lawn et al. (1992) reported that in these cholesterol-fed transgenic mice expressing human apo(a), susceptibility to the development of atherosclerosis was increased. Apo(a) was immunodetected at the site of lesion in the aorta but not non-lesioned sections of aorta in this mouse model (Lawn et al. 1992). Immunostaining techniques have also been employed to examine atherosclerotic lesions in humans, and have shown that apo(a) is present in lesions in forms resembling whole oxidised Lp(a), as Lp(a) complexed to other plaque components or lipoproteins, and as degraded Lp(a) (Hoff et al 1993). Enhanced uptake of human Lp(a) into atherosclerotic lesions has also been noted in rabbits (Nielsen et al. 1996).

There is mounting evidence to indicate that Lp(a) is a risk factor for CVD, and further studies suggest that apo(a), partly by virtue of its marked homology to plasminogen, plays a major role in the pathogenicity of this lipoprotein. Plasminogen is the inactive version of the protease plasmin. Binding of plasminogen to fibrin or other binding sites

on the vessel wall allows subsequent activation to plasmin, which plays an important role in the prevention of thrombus formation. The similarity of apo(a) to plasminogen is thought to promote the association of Lp(a) with the fibrin-rich clots in arteries. This could result in interference with fibrinolysis, a hypothesis supported by the ability of Lp(a) to inhibit fibrin-mediated generation of plasmin from plasminogen (Hajjar et al. 1989). Inhibition of plasmin generation by Lp(a) may also inhibit the plasmin-mediated activation of transforming growth factor-beta (TGF-β). *In vitro*, the inhibition of TGF-β activation has been shown to enhance proliferation of smooth muscle cells (Grainger et al. 1993), and this may further enhance lesion formation.

It has been suggested that the Lp(a)-fibrin association can also result in retention, and consequently increased uptake, of Lp(a) into the arterial wall (Harris 1997). In the apo(a) transgenic mouse model, modification of the major lysine binding site on apo(a) (which is thought to be responsible for fibrin/apo(a) binding), results in a reduction of atherosclerotic lesion development compared to wild type apo(a) transgenic mice (Boonmark et al. 1997). Different apo(a) isoforms appear to display differences in the affinity for lysine binding to human monocytes (Kang et al. 1997) and fibrin (Hervio et al. 1993) which may contribute toward an explanation for the associations noted between apo(a) isoform size and CVD.

Cholesterol deposition in the artery is thought to occur through the transfer of lipoproteins into the intima of the artery, and subsequent uptake into macrophages and smooth muscle cells. In addition to fibrin, Lp(a) has been shown to avidly bind

glycosaminoglycans and other matrix components which are typically found in atherosclerotic plaques (Bihari-Varga et al. 1988, Harpel et al. 1989, Pillarsetti et al 1997, Dahlen et al 1978). Retention of lipoproteins within the intima of the artery is hypothesised to be an important feature of the development of the fatty streak, and aggregation of lipoproteins may contribute significantly to their retention. Lp(a) has been shown to readily complex with itself, LDL and VLDL in the presence of Ca^{2+} to form insoluble complexes (Yashiro et al. 1993). Interestingly, not only did HDL fail to complex with Lp(a), but it also prevented Lp(a) self aggregation, which illustrates another potential anti-atherogenic effect of HDL. The Ca^{2+} induced self-aggregation of Lp(a) has also been shown to enhance its uptake into macrophages compared with native Lp(a) (Tanaka et al. 1998).

Like LDL, malondialdehyde modification of Lp(a) enhances its scavenger receptor uptake by macrophages (Haberland et al. 1992, Naruszewicz 1992), however there is evidence to suggest that Lp(a) can also be taken up by the macrophages though other receptors or in association with other lipoproteins. Cholesterol loading of macrophages results in the induction of a novel cell surface receptor which recognises apo(a) (Keesler et al. 1994). Lp(a) has been shown to associate with triglyceride rich lipoproteins (Scanu et al. 1994, Bersot et al. 1986), which may provide an additional uptake route into cells (Gianturco et al. 1994, Bersot et al. 1986, Beisiegel 1995).

In summary, there is an increasing amount of evidence linking elevated levels of Lp(a) to CVD, and *in vitro* studies have revealed unique characteristics of this lipoprotein which

may explain its atherogenicity. These include the ability to associate with the vessel wall and other lipoproteins to enhance retention and uptake of this cholesterol rich lipoprotein.

1.6 The Cardioprotective Effects of Estrogen

1.6.1 Estrogen and Cardiovascular Disease

Epidemiological evidence suggests that in terms of prevalence, women develop CVD approximately 6-10 years later than men. However after menopause, the incidence of CVD rises in women, and in later years women have rates of CVD equal to that of age matched men (Castelli 1988). A number of studies have found that post-menopausal estrogen use is associated with a lower risk of developing CVD (reviewed in Barrett-Connor and Bush 1991). However others have not noted any reduction in risk associated with post-menopausal hormone replacement (Hemminki and McPherson 1997). A study examining the relationship between estrogen replacement therapy and CVD found that while current estrogen use was associated with reduced cardiovascular mortality, it did not reduce morbidity (Sourander et al. 1998). The HERS study, a large, randomised, blinded trial of estrogen/progestin replacement in women with established coronary disease has recently been completed, and the findings of this study suggest that estrogen/progestin hormone replacement does not provide any benefit in secondary prevention of CVD events (Hulley et al. 1998).

While there is debate as to whether the cardioprotective effect of estrogen can be shown epidemiologically before and after menopause (Tunstall-Pedoe 1998), there is evidence

to suggest estrogen is significantly involved in cardiovascular health. In both rabbit and primate models, estrogen supplementation after ovariectomy protects against the development of dietary induced atherosclerosis (Sulistiyani et al. 1995, Adams et al. 1990).

1.6.2 Estrogen, lipids and CVD

A great number of studies have been performed to examine the effects of female hormones on lipid levels and these have not always provided consistent results. At least part of the variability is due to the influence of route of administration, and differences in the types of estrogens and progestins used in the studies.

Transdermal postmenopausal estrogen administration appears to have minimal, if any, effects on plasma lipids (Basdevant et al. 1991, de Lignieres et al. 1986, Tilly-Kiesi et al. 1997, Walsh et al. 1991, Walsh et al. 1994, Lahdenpera et al. 1996 Taskinen et al. 1996). A great number of studies have been performed to examine the effect of oral estrogen on plasma lipids and the effects have not always been consistent.

Treatment of postmenopausal women with oral conjugated equine estrogens (CEE), oral 17β-estradiol or oral ethinyl estradiol alone, has been found to increase triglyceride levels (Muesing et al. 1992, Sacks et al. 1994, Walsh et al. 1991, Bruschi et al. 1996, Colvin et al. 1990, Walsh et al. 1991, Applebaum–Bowden et al. 1989, Tuck et al. 1997). While others have found no significant effect of these oral estrogens on triglyceride, in some cases there was a non-significant increase noted (Rajman et al. 1996, Kim et al. 1994,

Shewmon et al. 1994, de Lignieres et al. 1986, Basdevant et al. 1991, Brinton 1996). Interestingly, oral 17β-estradiol treatment has been shown to decrease triglyceride concentrations in men (Giri et al. 1998).

Oral estrogens alone (CEE and estradiol), have mostly been found to cause an increase in HDL cholesterol (Bruschi et al. 1996, Basdevant et al. 1991, Shewmon et al. 1994, Muesing et al. 1992, Sacks et al. 1994, Rajman et al. 1996, Colvin et al. 1990, Walsh et al. 1991, Haines et al. 1996, Kim et al. 1996), although de Lignieres et al. 1986 found no effect of either micronised 17b-estradiol or estradiol valerate on HDL levels. HDL cholesterol concentration has been shown to be inversely related to body mass index (BMI) (Steyn et al. 1989), and the decrease in HDL noted by Taskinen et al. (1996) following oral HRT may have been influenced by the significant increase in weight in that group of women. Where HDL subfractions were reported, it was mostly changes in HDL_2 that were seen (Colvin et al. 1990, Haines et al. 1996, Muesing et al. 1992), although decreases in HDL_3 have also been found (Basdevant et al. 1991).

The effect of oral estrogens alone on LDL cholesterol concentration is less consistent that that seen with HDL. Three studies published in 1996 examined the effect of bilateral ovariectomy followed by estrogen replacement therapy (consisting of CEE alone) on plasma lipids. Bruschi et al. (1996) and Kim et al. (1996) found that following ovariectomy, LDL cholesterol concentrations increased and subsequent estrogen replacement therapy decreased LDL. However, Rajman et al. (1996) noted no significant effect of either ovariectomy or subsequent estrogen replacement therapy on LDL,

although there was a slight non-significant decrease in LDL noted with estrogen therapy. Other studies have found either decreases (Sacks et al. 1994, Walsh et al. 1991, Basdevant et al 1991, Museing et al. 1992, Shewmon et al. 1994, Tuck et al. 1997, Haines et al. 1990) or no change (De Lignieres et al. 1986, Colvin et al. 1990) in LDL cholesterol with oral estradiol or CEE.

Estrogen replacement therapy has also been found to decrease Lp(a) levels (Haines et al. 1996, Tuck et al. 1997, Kim et al. 1996, Taskinen et al. 1996, Bruschi et al. 1996). Oral estrogen therapy appears to be more effective than transdermal estrogen in reducing Lp(a) levels (Taskinen et al. 1996). This may be due to the first pass effect of orally administered hormones as the changes to Lp(a) concentrations are thought to be a result of decreased production rather than increased rate of catabolism (Rader et al. 1994). In the apo(a) transgenic mouse, treatment with ethinyl estradiol and tamoxifen supressed apo(a) gene expression in the liver and therefore decreased circulating levels of apo(a). Progesterone did not have any effect on apo(a) in this model (Zysow et al. 1997).

Similar lipid lowering effects to those seen with oral estradiol have been found following treatment with oral estrone (Colvin et al. 1990), and tamoxifen has been found to lower LDL and Lp(a) to an extent comparable with oral estradiol (Shewmon et al. 1994).

Concomitant administration of a strongly androgenic progestin with estrogen therapy tends to antagonise the lipid effects of the estrogen. However the more modern progestins tend to be less androgenic, and a number of recent studies examining combination

estrogen + progestin hormone replacement therapy have demonstrated potentially cardioprotective effects of combination therapy. Ulloa et al. (1998) examined the effect of CEE and medroxyprogesterone acetate (MPA), a 21 carbon progestin with less androgenic activity than the 19-nor progestins, on plasma lipids. The MPA appeared to counteract the triglyceride-elevating effect of CEE, while the decrease in LDL cholesterol and increase in HDL cholesterol were preserved. In a study reported by Haines et al. (1996) examining oral estradiol and MPA, hormone treatment had no effect on triglycerides or HDL, but decreased LDL and Lp(a) levels. Most studies investigating oral combination hormone replacement therapy have found decreases in LDL (van der Mooren et al. 1994, Heikkinen et al. 1999, Kim et al. 1994, Wolfe et al. 1995), but the effects on HDL and triglyceride in the aforementioned studies have been less consistent. Transdermal estrogen replacement in combination with oral MPA has been found to decrease LDL and triglyceride levels (Mattsson et al. 1993), but another study noted only the hypotriglyceridaemic effect (Tilly-Kiesi et al. 1997).

The Postmenopausal Estrogen/Progestin Intervention (PEPI) study was a large, placebo-controlled, randomised, double-blinded clinical trial designed to compare CEE alone and CEE with either MPA or micronised progesterone (The Writing Group for the PEPI trial, 1995). The significant increase in endometrial hyperplasia in those women treated with unopposed estrogen highlighted the ethical difficulties in conducting such a study, and confirmed the need for restriction of unopposed CEE to those women without a uterus. The PEPI study reported increases in HDL cholesterol with active hormone treatment

which were greatest in the CEE alone group. All active treatments were associated with a decrease in LDL cholesterol and an increase in triglyceride.

In summary, oral estrogen replacement therapy has been found to be associated with a reduction in LDL cholesterol and Lp(a) and an increase in HDL cholesterol, all potentially cardioprotective effects. In addition, estrogen treatment can cause an increase in triglyceride, but in light of the increase in HDL, this probably does not contribute to a greatly enhanced CVD risk. The route of delivery of the estrogen appears to have a significant impact upon the effects seen, and transdermal estrogen administration has a lesser effect on plasma lipids. Co-administration of a progestin with estrogen therapy may negate some of the cardioprotective effects, but this is to some extent determined by the type and dose of progesterone used. Less androgenic progestins appear to have less adverse effects on plasma lipids.

1.6.3 Estrogenic regulation of LDL receptor activity

There is a great deal of evidence to suggest that estrogen decreases LDL cholesterol. This is thought to occur at least partly through increased hepatic LDL receptor activity, resulting in enhanced clearance of LDL. The effect of estrogen on hepatic LDL receptor activity in humans has not been extensively reported, most likely due to the difficultly associated with obtaining liver biopsies. In human derived HepG2 cells, Semenkovich and Ostlund (1987) found that incubation with 37μM 17β-estradiol significantly increased cell surface LDL receptor activity. Hepatic LDL receptor binding activity has also been found to be significantly increased in two male patients receiving estrogen

treatment (the type of estrogen was not specified) for treatment of prostatic cancer (Angelin et al. 1992).

Changes in LDL receptor activity mostly reflect changes in the amount of receptor expressed by cells. As LDL receptor degradation appears to occur at a rate independent of receptor expression, it is therefore changes in the rate of synthesis that are primarily responsible for regulating the amount of LDL receptors expressed by the cell (Soutar and Knight 1990). This is illustrated by treatment of rabbits with 17α-ethinylestradiol which results in an increase in LDL receptor number, which is correlated with an increase in LDL receptor mRNA (Ma et al. 1986).

The hormones insulin and estradiol, have been shown to increase LDL receptor activity *in vivo* (Krone et al 1988, Ma et al 1986). In an *in vitro* cell model it was shown that LDL receptor regulation by estradiol and insulin was dependent upon an intact SRE-1 sequence in the LDL receptor gene. However in the case of insulin, it was SREBP dependent, while estradiol exhibited SREBP independent effects (Streicher et al. 1995). Therefore, while insulin may be regulating LDL receptor levels via cellular SREBP content, it appears that estrogens are interacting with SRE-1 via another mechanism. The existence of SRE-dependent, but sterol- and SREBP-independent regulation of LDL receptor transcription, is supported by a finding by Makar et al. (1998). In the human Jurkat cell line (a human T cell line) it was found that mitogenic stimulation of LDL receptor transcription was independent of both cellular sterol content and SREBP's (Makar et al. 1998).

Whether the LDL receptor stimulating effect of estrogen is mediated through the estrogen receptor is still not known. Parini et al. (1997) examined the effect of an estrogen and anti-estrogen on hepatic LDL receptor activity in rats. It was found that treatment with 17α-ethinylestradiol increased the expression of LDL receptor mRNA, an effect which was abolished by co-administration of the anti-estrogen tamoxifen, while tamoxifen alone had no effect on either cholesterol levels or LDL receptor mRNA. However as very high doses of both hormones were used in this rat study, it may not represent a good physiological model for LDL receptor regulation in humans. Interestingly, tamoxifen has been shown to have estrogenic effects on the liver at therapeutic doses in vivo (Barkhem et al. 1997, Cassidy 1999), and results in a reduction of plasma LDL cholesterol in postmenopausal women (Guetta et al. 1995, Wiseman 1995).

Classically, estrogen receptor (ER) regulation of gene transcription involves estrogen binding to the ER, followed by binding of the ER to DNA at estrogen response elements in the promoter region of the gene, an effect which is antagonised by anti-estrogens (Beato 1989). However the LDL receptor promoter does not contain the currently defined estrogen response element (Streicher et al. 1996). Recently is has been discovered that two major classes of ER exist- ERα and ERβ. Their distribution in tissue has been well characterised in the rat (Kuiper et al. 1997), and it was found that ERα was the predominant ER type in the rat liver. A recent study in human tissue and human-derived cell lines has revealed that both the liver and HepG2 cell lines possess the $ER\beta_5$ isoform (Moore et al. 1998). The ER subtypes have differing binding affinity for estrogens, anti-

estrogens and other compounds with estrogenic activity, in addition to eliciting different responses at the level of gene transcription (Zou et al.1999, Cassidy 1999, Kuiper et al. 1997). Barkhem et al. (1997) examined the ability of the traditional anti-estrogen tamoxifen, to display 'estrogenic' activity in regulating transcription of sex hormone binding globulin (SHBG) genes in a transformed HepG2 cell line. Perhaps not surprisingly, it was found that tamoxifen did not have its effect through the classical pathway of ERα binding to estrogen response elements associated with the gene. Therefore, it may be either a non-classical ER pathway (possibly through ERβ), or an ER-independent mechanism which governs regulation of 'estrogenic' gene transcription in the liver.

In addition to more direct effects on the LDL receptor, estrogen may also be regulating hepatic cholesterol homeostasis through other mechanisms. Post-menopausal estrogen replacement has been found to increase hepatic biliary cholesterol secretion, but decrease bile acid secretion in women (Everson et al. 1991). The effects of estrogen and progesterone on the regulation of liver cholesterol have yet to be clarified, but may also include effects on 7α-hydroxylase (Colvin et al. 1998), and sterol 27-hydroxylase (Kushwaha et al. 1996) both of which are enzymes involved in bile acid synthesis.

Estrogens and other compounds displaying estrogenic or anti-estrogenic activity which decrease LDL cholesterol levels *in vivo*, appear to do so at least in part by regulating hepatic LDL receptor expression. The mechanism by which this occurs is not yet known, but may involve interaction with the sterol response element (SRE-1) region of the LDL

receptor promoter. Whether this interaction involves the estrogen receptor (either ERα or ERβ), again has yet to be conclusively shown. But in the case of the 'estrogenic' action of tamoxifen on hepatic gene expression, this may occur through ERβ rather than ERα (Zou et al. 1999).

1.6.4 Estrogens and LDL oxidation in vitro

Changes to plasma cholesterol concentrations have been estimated to account for approximately 25-50% of the cardioprotective effect of estrogens (Barrett-Connor and Bush 1991). The remaining 50-75% of the cardioprotective effect has not been apportioned, but part of this effect may be due to estrogens providing LDL with protection from oxidation. Studies in a cholesterol-fed rabbit model and an oviarectomised primate model have shown that 17-β-estradiol retards the development of arterial lesions independent of any reduction in cholesterol level (Hough and Zilversmit 1986, Adams et al. 1990). In ovariectomised monkeys fed a moderately atherogenic diet, Wagner et al. (1991) found that estrogen (17β-estradiol) and progesterone replacement therapy reduced the accumulation of LDL and LDL degradation products in the coronary arteries, while no significant changes in plasma lipid or lipoprotein concentrations were observed. Recently, it was found that both transdermal and oral estradiol were able to decrease lesion formation in an ovariectomised rabbit model independent of total cholesterol concentration (Haines et al. 1999).

Yagi and Komura (1986) hypothesised that estrogens might act as antioxidants due to the presence of their phenolic hydroxyl group. The ability of 17β-estradiol, estrone and

estriol to inhibit the peroxidation of methyl linoleate by UV irradiation was examined, and it was found that at 5mM concentrations, these hormones displayed antioxidant properties. Sugioka et al (1987) demonstrated that the concentrations of estradiol and estrone required to achieve 50% inhibition in Fe^{3+}-induced oxidation of phospholipid microsomes was about 4 and 6 times that of α-tocopherol. In the same system, Nakano et al. (1987) showed that the metabolite catechol estrogens were even more potent antioxidants than α-tocopherol, and Mukai et al. (1990) demonstrated the ability of these catechol estrogens to regenerate tocopherol from the tocopheroxyl radical. It appears that the addition of the second hydroxyl group on the phenolic ring of the estrogen confers greater strength with respect to the ability to scavenge oxygen radicals and terminate free radical chain oxidation of lipids.

Huber et al (1990) demonstrated that 17β-estradiol at concentrations ranging from 0.25-7.5μM, inhibited the oxidation of LDL *in vitro* by 10μM $Cu2^{+}$. Further to this, when cultured P388D.1 macrophages were incubated with LDL and either 10μM 17-β-estradiol or testosterone for 18 hours, the cellular cholesteryl ester content was reduced by 17-β-estradiol but not testosterone. The oxidation and uptake of LDL by cells was dependent upon the presence of Cu^{2+} in the medium. The authors concluded that 17-β-estradiol may have acted to reduce the accumulation of cellular cholesteryl ester by inhibiting LDL oxidation, and therefore subsequent scavenger receptor uptake (Huber at al. 1990).

Mazière et al (1991) reported upon the ability of the three major human estrogens; estradiol, estrone and estriol, to inhibit Cu^{2+} and cell induced LDL oxidation. In the 5-

10μM range, both estradiol and estriol significantly inhibited Cu^{2+} induced LDL oxidation with estradiol being the more potent. At 10μM, all three estrogens inhibited LDL oxidation by both endothelial cells and monocytes, with estradiol again being the most potent inhibitor. In addition, pre-treatment of monocytes with estrogens resulted in inhibition of oxidation when the monocytes were subsequently incubated with LDL. It was also noted that macrophages displayed a reduced ability to degrade ^{125}I-labelled LDL when co-incubated with estrogens. Therefore estrogens could be shown to act as antioxidants in the presence of cultured cells thought to oxidise LDL *in vivo*, and to reduce the LDL degradation by these cells in culture (Mazière et al. 1991). Recently Schwaery et al. (1998) also examined the *in vitro* antioxidant effects of estradiol, estrone and estriol, but found that only estradiol was able to afford protection against oxidation to LDL. Interestingly, the 4 hour pre-incubation of LDL with estradiol needed to occur in the presence of plasma rather than saline, highlighting the possibility that some component of plasma is mediating estradiol association with the LDL particle, or modifying the estradiol in some way to have a more potent effect. The authors suggested that formation of more hydrophobic estradiol- fatty esters in the LDL particle may be mediating the enhanced protection against LDL oxidation (Schwaery et al. 1998). However, the formation of catechol estrogens which have a greater ability to protect LDL against oxidation than estrogens (Taniguchi et al. 1994), may also be a possible mechanism.

Estradiol has been found to inhibit LDL oxidation by human mononuclear cells at a concentration of 1μM (Rifici and Kachadurian 1992) and to protect aortic endothelial

cells from cytotoxicity associated with oxidised LDL at concentrations of 0.5μM (Negre-Salvayre et al. 1993). The progestins levonorgestrel, progesterone and medroxyprogesterone acetate alone, or in combination with estradiol, have been found not to affect *in vitro* LDL oxidation (Schröder et al. 1996, Arteaga et al. 1998).

1.6.5 Estrogens and LDL oxidation ex vivo

It has been estimated that approximately 2-3% of plasma estrogen is found in the LDL lipid fraction (Leszczynski and Schafer 1989). As 17-β-estradiol has been shown to act as an antioxidant at concentrations as low as 0.25μM in an *in vitro* system (Huber et al. 1990), whether this *in vitro* activity could be translated to an effect seen *ex vivo* has been examined by a number of researchers. Keaney et al. (1994) used a miniature swine model to investigate the effect of ovariectomy and estrogen replacement therapy on plasma lipids and endothelial function. LDL from sham-operated animals and from animals that had undergone ovariectomy in association with an estradiol implant, was significantly more resistant to oxidation (demonstrated by a longer lag time), when compared to LDL from animals that had undergone an ovariectomy and received a placebo implant. However in ovariectomised monkeys, Schwenke et al. (1999) found no significant effect of either conjugated equine estrogens (CEE) alone, CEE + medroxyprogesterone acetate (MPA), MPA alone or tamoxifen on the lag time or rate of Cu^{2+}-mediated LDL oxidation. Moreover, in both of the estrogen treated groups (± MPA), lag time was inversely associated with plasma estradiol concentration.

Sacks et al. (1994) examined the effect of both transdermally delivered 17-β-estradiol (Estraderm 0.1 patch) and an acute arterial infusion of 17-β-estradiol, on the Cu^{2+}-mediated oxidation of LDL. Shortly following intra-arterial infusion of 17-β-estradiol the plasma level of estradiol reached 1.6nM which is comparable to the level that could be achieved by a pre-menopausal woman mid-cycle (Becker 1990). This resulted in a significant 36% increase in the lag time of LDL oxidation. Following three weeks of transdermal estradiol administration, estradiol levels were significantly elevated to 0.46 nM and lag time was significantly prolonged by 42%. Guetta et al. (1995) noted a less prominent effect of transdermal estradiol (Estraderm 0.1 patches) on the lag time of LDL oxidation. The study aimed to determine the contributions of Vitamin E and estradiol to lag time. Individually, Vitamin E and estradiol showed non-significant increases in lag time. When the two compounds were administered as a combined therapy, the effect on lag time reached significance, but no significant synergism was noted with both therapies combined.

Nenseter and colleagues (1996) were the first to report on the effect of combined estrogen/progesterone therapy in post-menopausal women on LDL oxidation. The study involved the administration of 17β-estradiol and norethisterone acetate for 12 weeks in 13 post-menopausal women. Using Cu^{2+}-initiated oxidation (final Cu^{2+} concentration 1.67μM) it was found that the combined HRT had no effect on the lag time or rate of oxidation. However in the study by Nenseter et al., LDL was dialysed extensively against an EDTA-containing PBS before being stored. This then required that the LDL be further extensively dialysed against EDTA-free PBS before the LDL could undergo oxidation.

Scheek et al (1995) have shown that dialysis of LDL results in a loss of lipophillic antioxidants, and it is therefore possible that the extensive dialysis performed by Nenseter et al. may have resulted in a loss of estrogen and other antioxidants from the LDL particle.

Schröder et al. (1996) found that estrogen replacement therapy protected against *ex vivo* Cu^{2+} mediated LDL oxidation, and this was not affected by co-administration of a progestin. Conversely Santanam et al. (1998) found no protective effect of estrogen replacement therapy on *ex vivo* LDL oxidation in postmenopausal women, nor any effect from cyclical changes in endogenous estrogen in pre-menopausal women. However in women hyperstimulated with estrogen during IVF, protection against LDL oxidation was noted.

In a recent study, estrogen replacement therapy did not affect the lag time of LDL oxidation, but did decrease levels of antibodies against oxidised LDL (Hoogerbrugge et al. 1998). Clearly the effect of postmenopausal estrogen treatment on LDL oxidation has yet to be clarified. This is hampered by the methods used for measuring LDL oxidation. Isolation of LDL for *ex vivo* oxidation has a number of limitations, including the fact that it can not take into account the effect of antioxidants not directly associated with the LDL particle.

1.6.6 Other potential cardioprotective effects of estrogen

The amino acid homocysteine has been found by some to be strongly correlated to CVD mortality (Alfthan et al. 1997, Nygard et al. 1997), however this effect has not been consistently noted (Evans et al. 1997). Estrogen therapy given to male → female transsexuals has been found to lower homocysteine levels (Giltay et al. 1998), as has low dose estrogen treatment in elderly males (Giri et al. 1998), and estrogen treatment in rats (Kim et al. 1997). In two groups of elderly women, one taking and the other not taking hormone replacement, no significant difference in serum homocysteine was noted (Carmel et al. 1996), however in post-menopausal women treated with tamoxifen for breast cancer, plasma homocysteine concentrations were found to decrease (Lien et al. 1997). The influence of postmenopausal estrogen replacement on homocysteine has yet to be clarified. A study by van der Mooren et al. (1997) found that changes in homocysteine concentration occurred only in women with high baseline levels.

Estrogen appears to have a great number of effects at the vessel wall that may be cardioprotective (Nasr and Breckwoldt 1998). Arterial smooth muscle cells have been shown to have estrogen receptors (Bayard et al. 1995), and estrogen has been shown to enhance the release of the vascular relaxation mediator, nitric oxide (Imthurm et al. 1997). In a cross sectional study comparing postmenopausal HRT users with post-menopausal women who had never used HRT, it was found that both estrogen replacement therapy and estrogen + progesterone replacement therapy was associated with improved flow-mediated dilatation determined by response to reactive hyperaemia in the forearm (McCrohon et al. 1996).

1.7 Phytoestrogens and their potential as cardioprotectants

Phytoestrogens are plant-derived compounds displaying estrogenic or anti-estrogenic activity. The ability of these weakly estrogenic compounds to have significant effects *in vivo* was demonstrated by early observations of infertility in sheep fed on subterranean clover pastures (Schinkel 1948). In the human diet, the polyphenolic phytoestogens can be sub-classified into groups which include the isoflavones, lignans and coumestans.

Major dietary isoflavones include genistein, daidzein and formononetin which can be found in high quantities in soy based foods. The major human lignans identified in human biological fluid, enterolactone and enterodiol, are derived from secoisolariciresinol and matairesinol through conversion by gastrointestinal bacteria. (Setchell and Adlercreutz 1988). Flaxseed (linseed) is the most commonly eaten lignan-rich food. Coumestans include the compound coumestrol which is strongly estrogenic (Cassidy 1999), and can be found in alfalfa sprouts.

As is characteristic of weak estrogens, these compounds also display anti-estrogenic activity (Collins et al. 1997). Whether phytoestrogens display estrogenic or anti-estrogenic activity is likely to depend upon the amount of other steroid or phytoestrogen present (Mousavi and Adlercreutz 1992) and the type of estrogen receptor present (Paige et al 1999).

In addition to possessing estrogenic activity, conferred by the presence of a phenolic hydroxyl group, a number of these compounds also display antioxidant acitivity (Rice Evans et al. 1996). This has been widely shown, and includes the ability to protect LDL from oxidation *in vitro*, which has been noted for genistein (Kerry and Abbey 1998) and daidzein (Hodgson et al. 1996). Consumption of isoflavone-containing soy bars was found by Tikkanen et al. (1998) to provide protection to LDL against oxidation *ex vivo*.

Asian populations are high consumers of soy derived foods and have lower rates of CVD, but they also consume less saturated fat which is an important dietary factor in relation to cholesterol levels and subsequently CVD risk (Tham et al. 1998). However there is some evidence that polyphenolic phytoestrogens lower cholesterol levels. Coumestrol has been shown to have a hypocholesterolaemic effect in an ovariaectomised rat model (Dodge et al. 1996). Additionally, work by Prasad et al. 1998 suggests that the lignans present in flaxseed may be involved in lowering LDL cholesterol levels, and may account for a significant proportion of the anti-atherogenic effect. Crouse et al. (1999) have demonstrated that increasing amounts of isoflavones consumed in a set amount of soy protein showed a dose-response effect in lowering of LDL and total cholesterol levels.

A study by Anthony et al. (1997) in a primate model noted a reduction in atherosclerotic lesion area with a soy protein diet with intact isoflavone content, compared with a soy diet from which the isoflavones had been removed. Recently Kirk and co-workers (1998) have provided further evidence that soy isoflavones may reduce plasma cholesterol and the development of atherosclerosis, and have also given insight into possible mechanisms

for these effects. Comparing two species of mouse which display accelerated atherogenesis; C57BL/6 mice which have low HDL cholesterol, and mice deficient in LDL receptors, they examined two soy based diets. One soy based diet was rich in isoflavones (soy^+), whereas in the other soy based diet the isoflavones had been extracted (soy^-). The soy^+ diet lowered cholesterol in the C57BL/6 mice whereas the soy^- diet did not. In addition, atherosclerotic lesion area was significantly reduced on the soy^+ diet in these mice. Meanwhile in the LDL receptor deficient mice, the cholesterol levels and extent of atherosclerosis was the same for both diets, suggesting that the LDL receptor played an important role in the mechanism of cholesterol lowering by isoflavones. This is supported by a study published by Baum et al. (1998) who found increased mononuclear cell LDL receptor mRNA following consumption of an isoflavone-rich soy protein diet in hypercholesterolaemic postmenopausal women. While there is evidence to suggest that phytoestrogens may lower cholesterol and protect against LDL oxidation, the evidence for this is by no means conclusive.

1.8 Overall Aims

The broad aims of this research are to examine the effects of estrogens and other compounds with estrogenic activity on lipids and other risk factors for cardiovascular disease.

Specifically, this will be examined through a number of research avenues:

- *In vitro* studies examining the ability of estrogens and phytoestrogens to regulate hepatic LDL receptor activity, and regulate uptake of LDL and Lp(a) into macrophages.

- A longitudinal study of hormone replacement therapy in postmenopausal women to examine the influence of female steroid hormones on lipids and on *ex vivo* LDL oxidation.

- The effects of phytoestrogens of the lignan and isoflavone classes on the hormonally sensitive lipoprotein Lp(a), and the effect of soy isoflavones on LDL oxidation and lipids.

As postmenopausal hormone and phytoestrogen therapies are increasingly being marketed as cardioprotective agents, it is hoped that this research will further our understanding of the effects of estrogens and phytoestrogens on lipoprotein metabolism.

CHAPTER 2

General Methods

This chapter contains general methods that are common to a number of the further chapters of this thesis. More specific methods will be given in the following chapters where appropriate.

2.1 Materials

General chemicals were supplied by Sigma-Aldrich (Castle Hill, VIC, Australia), Ajax (Auburn, NSW, Australia) and BDH (Kilsyth, VIC, Australia). Solvents were all HPLC or analytical grade and were supplied by BDH. Water was purified using the Milli-Q™ system (Millipore, MA, USA).

2.2 Plasma Isolation

Whole blood was collected into evacuated tubes containing 1mg/ml ethylenediaminetetra-acetic acid disodium salt (EDTA). Plasma was isolated from blood by centrifugation at 3000 rpm (1500 x g) for 10 minutes in a Beckman GS-6R centrifuge (Beckman Instruments, California, USA), and then removed and stored at -80°C until use, unless otherwise specified. Once thawed, the plasma was centrifuged at 3000 rpm for 4-5 minutes to pellet fibrin before the plasma sample was used.

2.3 Lowry Protein Assay

Protein was measured using a modification of the Lowry et al. (1951) method, on a Cobas Bio automated analyser (F. Hoffman- La Roche, Basel, Switzerland) as described by Clifton et al. (1988), utilising BSA standards and measuring absorbance at 750nm. HepG2 and mononuclear cell protein concentrations were measured using the Lowry method on either a Molecular Devices Spectramax 250 Microplate reader with SoftMax Pro software (Molecular Devices, California, USA), or a Beckman DU-65 Spectrophotometer (Beckman Instruments, California, USA).

2.4 Measurement of Plasma Lipids

Measurement of plasma cholesterol and triglyceride was performed on a Cobas-Bio automated analyser (F. Hoffman- La Roche, Basel, Switzerland), using Roche Unimate cholesterol and triglyceride kits (F. Hoffman- La Roche, Basel, Switzerland). Quality control serum (Ciba-Corning, Australian Diagnostics) was included with every test run.

Total HDL and HDL_3 cholesterol was determined using Dextralip 50 (Sochibo, France). Dextralip-MgCl2 solutions were used to precipitate Apo-B containing lipoprotein from the plasma samples and then cholesterol in the supernatent measured as described for plasma cholesterol. HDL_2 cholesterol was obtained by calculating the difference between total HDL and HDL_3. LDL cholesterol was calculated using the Friedewald equation (DeLong et al. 1986).

2.5 Lipoprotein (a) ELISA

Anti-human Lp(a) antibody from mouse (Boehringer Mannheim GmbH, Germany) was diluted 1/750 in phosphate buffered saline (PBS) (containing 137 mM NaCl, 101 mM KCl, 3 mM Na_2HPO_4, 2 mM KH_2PO_4), and a volume of 110µl was added to each well of a Nunc-Immuno Maxisorp C96 well microplate (Nunc, Australia). The plate was then incubated overnight at room temperature on a microplate shaker (DPC Micromix4, BioMediQ,UK),

Following the overnight incubation, the antibody was discarded and any unbound surface in the wells blocked with 150µl of PBS containing 0.5g/L RIA grade Bovine Albumin (BSA) for a period of one hour. The plate was then washed five times with wash buffer (consisting of PBS plus 5g/L Tween-20) using a Nunc-Immuno Wash 8 (Nunc, Australia), and this same wash procedure was followed throughout the assay. Samples, standards and controls (Lp(a) standards and controls from Behringwerke AG, Germany) diluted 1/100 in PBS/BSA/TWEEN (PBS containing 5g/L Tween-20, 0.5g/L BSA) were then added to the wells and incubated on the microplate shaker for 30 minutes. Following this, the wells were washed and 100µL of anti-Human Lp(a) antibody from goat diluted 1/1250 in PBS/BSA/TWEEN, was added to each well. Following a further two hour incubation, the plate was again washed and diluted peroxidase-linked anti-goat IgG antibody from rabbit was added and incubated for 90 minutes (Goat anti-Human Lp(a) and rabbit anti-goat IgG antibodies from Incstar, Minnesota, USA).

The plate was washed, and colour developed by incubation with an *o*-phenylenediamine solution (18mM *o*-phenylenediamine, 3.2mM $NaHPO_4$, 1.2mM citric acid, 0.06% H_2O_2) for 10 minutes, after which the reaction was stopped with 2N sulphuric acid. The plate was read at 492nm using a Molecular Devices Spectramax 250 Microplate reader and Lp(a) concentrations calculated using SoftMax Pro software (Molecular Devices, California, USA).

2.6 Isolation of LDL

In order to isolate LDL, 1.35ml of a solution with a density of 1.006 g / ml containing 0.15M NaCl and 0.1% EDTA, was placed into Beckman 11x32 mm PA-Quickseal centrifuge tubes. Plasma was adjusted to d1.21 by the addition of 0.3242 g KBr per ml of plasma. 0.65ml of the d1.21 plasma was then underlayed into each Quickseal tube and the tubes were heat sealed. The tubes were placed in a TLA 120.2 rotor and spun at 100,000 rpm (435,680 x g) for 30 minutes at 4°C in a Beckman benchtop Optima TLX-Ultracentrifuge (Beckman Instruments, California, USA) with an acceleration and decceleration time of 4 minutes. This rapid isolation technique as described by Chung et al (1986) results in the formation of a discrete LDL band approximately one third from the top of the tube.

The LDL band was removed from the Quickseal tube using a 25 gauge needle and a 1 ml syringe. A 25 gauge needle was using to puncture the top of the tube to release the

vacuum within the tube. The needle and syringe were inserted directly into the side of the tube beneath the LDL band. Approximately 200 µl of LDL was then removed from the tube and transferred into a 1.5 ml microtube, and stored immediately on ice.

LDL was dialysed against 3 changes of phosphate buffered saline (PBS) to remove KBr and EDTA. A maximum of 5 ml of LDL per 1 L of PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) was used. PBS was deoxygenated before use by purging with dry nitrogen. LDL was dialysed in a closed container for 2 hours, and then the PBS changed and deoxygenated and further dialysed overnight. The following morning, the PBS was again changed and deoxygenated for a further 2 hours of dialysis. Upon removal from dialysis tubing the LDL was placed in an microtube and stored on ice while not in use.

LDL samples in duplicate, were diluted 1/3 using the final dialysate, and LDL protein concentration was determined using the Lowry Protein Assay on a Cobas-Bio automated analyser (see 2.2).

2.7 LDL oxidation

LDL was oxidised at 37°C in the presence of 0.005mM $CuSO_4$ at a concentration of 100µg LDL/ml, using the final dialysate to dilute the LDL sample. It has previously been shown that the use of LDL isolated from frozen as opposed to fresh plasma does not affect LDL oxidation parameters. Oxidation was monitored using a Beckman DU-65

Spectrophotometer with a Kinetics softpac module (Beckman Instruments, California, USA) at 234nm with measurements taken at 120 second intervals for a total of 150 minutes. At 234nm the formation of conjugated dienes can be determined by an increase in absorbance (Esterbauer et al, 1989). The oxidation reaction was terminated with the addition of 32µl of 4mM EDTA, and 6µl of 8.5mM butylated hydroxytoluene (BHT) giving final concentrations of 0.107mM EDTA and 0.0425mM BHT. From the absorbance changes over time, lag time, rate of oxidation and maximum absorbance of dienes could be determined. Rate of oxidation and maximum diene concentration were calculated using the extinction coeffiecient for conjugated dienes at 234 nm (ε = 29,500 L. $mol^{-1}.cm^{-1}$) (Beuge and Aust 1978).

2.8 Thiobarbituric acid reactive substances determination in LDL

The measurement of malondialdehyde (MDA) in LDL was performed on a Cobas-Bio automated analyser using a method as described in Abbey et al (1993). This method measures the thiobarbituric acid reactive substances (TBARS) content of the sample. MDA concentration was calculated using the extinction coefficient for MDA at 535nm (ε=1.56 x 10^5 $L.mol^{-1}.cm^{-1}$) (Beuge and Aust 1978).

2.9 Cell Culture

J774 Macrophages and HepG2 cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% heat inactivated Fetal Bovine Serum (FBS), 200 mM

L-glutamine, 20 mM HEPES, 16 μg/ml gentamycin and 50 000 U/ml penicillin at 37°C in 5% CO_2 in air. All media and cell culture chemicals were purchased from GIBCO (Life Technologies, Australia) and Sigma (Sigma-Aldrich, Australia). FBS was sourced from Trace Biosciences (Australia).

Cells were subcultured following two washes with sterile PBS, by incubation with 0.1% trypsin/EDTA for 5 minutes. The cells were then resuspended in DMEM + 10% FBS as described above.

For experimental incubations, phenol red-free DMEM and lipoprotein-deficient (LPD), charcoal-stripped FBS was used in place of normal DMEM and FBS. Lipoprotein deficient FBS was prepared by ultracentrifugation. Briefly, FBS was adjusted to d1.215 g/ml with KBr and subjected to centrifugation for 40 hours at 38 000 rpm in a Beckman 50.2Ti rotor at 10°C to isolate serum lipoproteins. Following removal of the lipoproteins, the FBS was dialysed for 72 hours against five changes of 150mM NaCl. Charcoal stripping was performed by slowly mixing the FBS with DextranT75-coated charcoal for 24 hours at 4°C, after which the majority of the charcoal was removed by centrifugation at 1600g for 20 mins. The stripped LPD-FBS was then filtered with a 0.8μm filter to remove any remaining charcoal particles, and subsequently filter sterilised with a 0.45 μm filter (Millipore, Bedford, MA, USA).

2.10 Preparation of LDL-Gold conjugates

Whole blood provided by the Red Cross (South Australia), was centrifuged to isolate plasma. The plasma was subjected to ultracentrifugation to isolate LDL d1.025<LDL<d1.050. Prior to conjugation the LDL was diluted to 1mg LDL protein/ml and then dialysed overnight against 2 L of 50 mM Na_2EDTA, pH 8.

Colloidal Gold was prepared in siliconised glassware using the method of Frens (1973). 402 ml of a 0.1% solution of choloroauric acid ($HAuCl_4$) was brought to the boil under reflux. When the solution commenced boiling, 11.2 ml of 1% trisodium citrate solution was added and the mixture was boiled continuously until a red colour developed and stabilised (approximately 10-15 minutes). The colloidal gold solution was allowed to cool and stored at 4°C when not in use.

For conjugation, 150μg of LDL was diluted to 0.5ml using Milli-Q water. The LDL was then vortexed as 5 ml of colloidal gold solution was added. The mixture was centrifuged at 12,000 rpm, in a fixed angle rotor for 20 minutes to pellet the conjugates. The LDL-gold conjugates were collected and pooled, and 0.2g/ml solid sucrose added. The LDL-gold conjugates were frozen at -80°C.

2.11 Cellular LDL-receptor activity

Cellular LDL receptor activity was measure by the method of Roach et al. (1993). The protein content of the cells was determined using the Lowry method (see chapter 2.3). For total binding, a volume of cells equivalent to 100μg cell protein was incubated at room temperature for 1 hour with LDL-gold conjugates (20μg LDL protein/ml) and 2mM $Ca(NO_3)_2$ in incubation buffer (60mM Tris containing 20mg/ml BSA, pH 8), with a total volume of 300μl. For non-specific binding, 100μg cell protein was incubated with LDL-gold conjugates and incubation buffer as described above, with 0.02 M EDTA replacing the $Ca(NO_3)_2$. Both assays were done in duplicate. Following the incubation, cells were pelleted by centrifugation at 400 x g, 20°C for 10 minutes in a Beckman GS-6R centrifuge (Beckman Instruments, California, USA), and the supernatent removed.

For total binding the cells were resuspended in 500 μL of 2mM $Ca(NO_3)_2$, and for non-specific binding the cells were resuspended in 20mM EDTA. The cells were pelleted by centrifugation at 400 x g for 10 minutes, and the supernatent removed. The cells were resuspended in 120 μl of 4% gum arabic and transferred to sample cups. The amount of cell bound LDL-gold was determined using an IntenSE™BL silver enhancement kit (Amersham, UK) on a Cobas-Bio automated analyser (F. Hoffman- La Roche, Basel, Switzerland). LDL-gold conjugates diluted in 4% gum arabic were used as standards. The coefficient of variation for this assay is <10%.

CHAPTER 3

The effect of estrogens and phytoestrogens on regulation of LDL receptor activity *in vitro*

3.1 Introduction

Early studies demonstrated that pharmacological doses of 17α-ethinyl estradiol administered to male rats (Chao et al. 1979) and rabbits (Ma et al. 1986) resulted in a significant reduction in LDL cholesterol levels as a result of an increase in hepatic LDL receptor expression. However, Srivastava et al. (1993) highlighted a significant species-dependent effect, when it was shown that the regulation of LDL receptor levels differ in the rat and the mouse. In addition, Ferreri and Naito (1978) reported that the effect of estrogens on rat serum cholesterol was dependent not only on the dose, but also on the length of treatment and the type of estrogen. A biphasic response was noted with respect to dose, with lower doses of estrogens increasing serum cholesterol concentrations, whereas higher doses decreased serum cholesterol levels.

The case of tamoxifen treatment further illustrates some deficiencies of these animal models. In postmenopausal women, tamoxifen treatment lowers LDL cholesterol (Guetta et al. 1995a, Wiseman 1995), whereas in a rat model, tamoxifen has been found to have no effect on either total cholesterol or LDL receptor expression (Parini et al. 1997).

In humans, the response of hepatic LDL receptor activity to estrogens has been less well documented. Increases in LDL receptor activity following estradiol treatment of the HepG2 cell line *in vitro* (Semenkovich and Ostlund 1987), and in liver biopsies from estrogen-treated males *in vivo* (Angelin et al. 1992) have been noted. In a previous cross sectional study we found that postmenopausal women had significantly less mononuclear cell LDL receptor activity compared to premenopausal women, and

in postmenopausal women using HRT this difference was negated (Abbey et al. 1999).

While it appears that estrogen can regulate LDL receptor activity, how this occurs is not fully understood. This is unlikely to be occurring through the classical pathway of ERα regulation of gene transcription (Beato, 1989), as the promotor region of the LDL receptor gene does not contain the currently defined estrogen responsive element sequence (Streicher et al 1996). However this does not preclude estrogen and ERα involvement above the level of direct DNA binding, or regulation could involve one of the ERβ subtypes or be independent of any ER.

Phytoestrogens may also have LDL cholesterol-lowering properties. The hypocholesterolaemic effect of a soy-rich diet has been suggested to be due in part to a hepatic LDL receptor stimulating effect (Potter, 1995), which may be due the phytoestrogenic isoflavones (Kirk et al. 1998). One of the major soy derived isoflavones genistein, has been shown to down-regulate *in vitro* LDL receptor activity by virtue of its ability to inhibit tyrosine kinase (Chan et al. 1997, Graham and Russell 1994). The other major soy isoflavone, daidzein does not appear to have the same tyrosine kinase effects (Akiyama et al. 1987), and the effect on LDL receptor activity has not been reported. The isoflavones biochanin A and formononetin (*Figure 3.1*) isolated from chick peas (*cicer arietinum*) have also been found to have a hypolipidaemic effect in rats (Siddiqui and Siddiqi 1976).

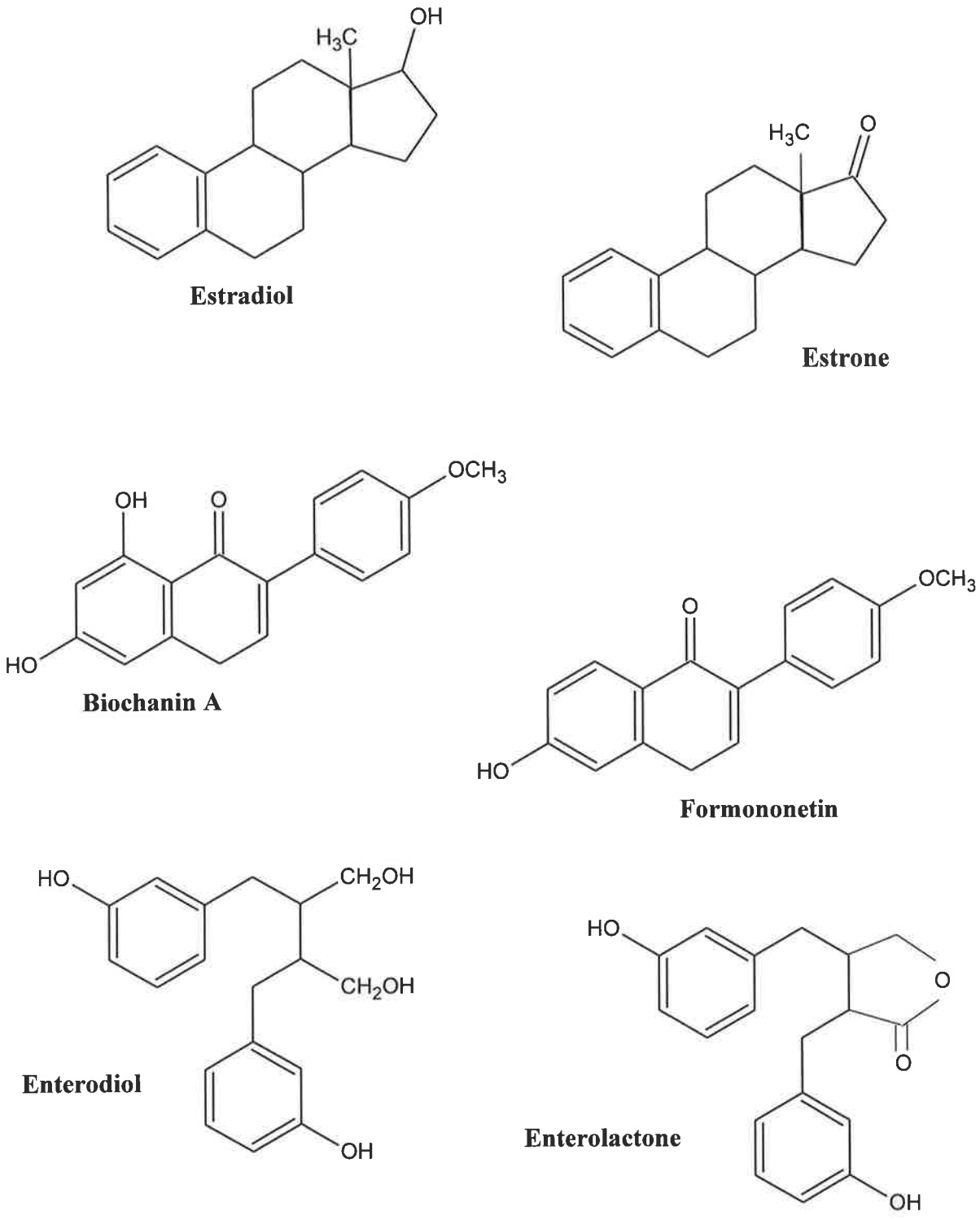


Figure 3.1 The structure of selected estrogens and phytoestrogens

Coumestrol, found in clover and alfalfa, displays strong estrogenic activity (Cassidy 1999) and has also been shown to have a hypocholesterolemic effect in the ovariectomised rat model (Dodge et al. 1996). Dietary supplementation of flaxseed, which has a high α-linolenic acid content in addition to being one of the richest sources of phytoestrogenic lignans, has been found to reduce the progression of atherosclerosis in a hypercholesterolaemic rabbit model (Prasad 1997a). A further study by Prasad et al. (1998) found that a flaxseed variant with a low n-3 fatty acid content but rich lignan content, was able to reduce total and LDL cholesterol in a rabbit model. This suggests that the lignans have the ability to lower LDL levels, which may account for a significant proportion of the anti-atherogenic effect of flaxseed.

Hepatic LDL receptor expression is subject to regulation by antioxidants (Pal et al. 1999). As differing estrogens and phytoestrogens have not only differences in their estrogenicity, but also in their antioxidant activity, any effects on LDL receptor regulation could be a function of either or both of these mechanisms.

The extent to which different estrogens and phytoestrogens can regulate LDL receptor activity *in vitro* has yet to be reported. The aim of this study was to examine the effects of estrogens and phytoestrogens on the stimulation of LDL receptor activity in the human hepatoma cell line HepG2 in light of their different levels of reported antioxidant activity and estrogenicity.

3.2 Methods

3.2.1 Cell culture

HepG2 cell cultures were maintained as described in section 2.9. Cells were trypsinised and split into 6-well culture plates (Nunc, Nalge Nunc, IL, USA), and cultured until approximately 80% confluency was achieved. The cells were then washed in sterile PBS before treatment for 24 hours with the estrogen or phytoestrogen to be tested. Phenol red-free DMEM containing charcoal-stripped, lipoprotein deficient FBS was used during the treatment period to minimise the presence of any estrogenic compounds. Following the 24 hour incubation, the medium was discarded and the cell monolayers were washed with sterile PBS. The cells were then removed from the cell culture plate by scraping, and gently resuspended in 0.5 mL of PBS.

3.2.2 Compounds to be tested

Estrone, 17β-estradiol, estriol, α-tocopherol, biochanin A, genistein and daidzein were supplied by Sigma (St Louis, MO, USA). Enterolactone and enterodiol were a gift from Dr Peter Baghurst. Coumestrol and formononetin were supplied by Eastman Kodak (Rochester, NY) and Indofine (Somerville, NJ) respectively.

3.2.3 Determining the effect of estrogens and phytoestrogens on LDL receptor activity

Cultured HepG2 cells were incubated with the estrogen or phytoestrogen to be tested for 24 hours in the presence of colloidal gold-labelled LDL. Cellular uptake of colloidal gold-labelled LDL (prepared as described in section 2.10) was then used to

determine cellular LDL receptor activity. The protein concentration of the cell suspension was determined using the method of Lowry (section 2.2), following which cellular LDL receptor activity was determined as described in section 2.11.

3.2.4 Estimation of antioxidant activity as measured by LDL oxidation

LDL isolation and oxidation was performed as described in chapter 2.6 and 2.7, however the LDL was oxidised in the presence of 1μM of the phytoestrogen to be tested. As the stock phytoestrogen solutions were made in ethanol, the control oxidation was performed in the presence of an equivalent amount of ethanol (<10μl).

3.2.5 Determination of 17-β-hydroxysteroid dehydrogenase activity

An experiment was designed to examine the interactions of estrogens and phytoestrogens with the enzyme responsible for the interconversion of estrone and estradiol, 17β-hydroxysteroid dehydrogenase (17β-HSD). HepG2 cells were incubated in phenol red-free DMEM with lipoprotein deficient stripped FBS containing 3nM ^{3}H-estradiol and 30nM ^{14}C-estrone (Amersham Life Science, UK and NEN Life Science Products, MA, USA), and were treated with 100μM estrone, estradiol, biochanin A or formononetin for 24 hours.

The media was then collected and cold-spiked with 5 μg of estrone and estradiol. The steroids were extracted 3 times with ethyl acetate, the extract combined and dried under a stream of nitrogen and re-dissolved in 500μL of ethanol.

Estradiol and estrone were separated by high performance liquid chromatography using a modification of the method of Parinaud et al (1988). Utilising a mobile phase

of 42.5/57.5 acetonitrile/H_2O at a flow rate of 1ml/min, the estrogens were passed through a 30 cm Supelco 5μm spherical C18 column (Supelco, Sigma-Aldrich, Castle Hill, VIC, Australia), with UV detection at 210nm. The isolated estrone and estradiol peaks were collected and specific activity of each steroid peak determined.

3.2.6 Statistical Analysis

Due to inter-assay variability in the control level of LDL receptor activity, in some cases results are presented as a percentage of the control. In all assays an ethanol control was also included but this did not differ from the control. Data is presented as mean ± SEM. Statistical analysis was performed with SPSS for Windows statistical software (SPSS, Chicago, USA) using students t-tests, Mann-Whitney U t-tests, and one way ANOVA with Tukey's post-hoc analysis.

3.3 Results

3.3.1 Antioxidant regulation of LDL receptor activity

Antioxidant regulation of LDL receptor activity was confirmed by incubation of HepG2 cells with α-tocopherol at concentrations ranging from 0-300μM. Significant increases in LDL receptor activity were noted at concentrations of 100, 150 and 300μM α denotes $p<0.01$, β denotes $p<0.005$, (*Figure 3.2*).

3.3.2 Estrogens and the regulation of HepG2 cell LDL receptor activity

Of the three human estrogens tested, at concentrations of 50μM only 17β-estradiol was able to cause significant upregulation of LDL receptor activity in HepG2 cells (*Figure 3.3*).

Further examination of the two major human estrogens, 17β-estradiol and estrone, revealed that at high concentrations (100μM) estrone exerted a slight but significant upregulatory effect on LDL receptor activity (*Figure 3.4*), but at lower concentrations had did not upregulate LDL receptor activity, and even appeared to slightly depress LDL receptor activity at concentrations of 0.5μM and 5μM (*Figure 3.4*). Incubation of HepG2 cells with increasing concentrations of 17β-estradiol resulted in increases in LDL receptor activity which achieved significance at concentrations above 5μM. (*Figure 3.5*).

3.3.3 Phytoestrogens and LDL receptor activity

At concentrations of 50μM, the phytoestrogens coumestrol and enterolactone caused significant upregulation of LDL receptor activity ($p<0.05$). While the lignan enterodiol had no significant effects *(Figure 3.6).* The effect of coumestrol on LDL receptor activity was of a magnitude comparable to that of 17β-estradiol, and was even slightly but not significantly greater than that of 17β-estradiol.

The isoflavones daidzein, formononetin and biochanin A all caused significant upregulation of LDL receptor activity at 40μM (*Figure 3.7*).

3.2.4 The antioxidant activity of phytoestrogens

At concentrations of 1μM, the isoflavones biochanin A and formononetin did not protect isolated LDL from oxidation. The two major human lignans, enterolactone and enterodiol did show differences in their antioxidant activity with enterodiol being a significantly stronger antioxidant at 1μM. Coumestrol was the most potent antioxidant of the phytoestrogens tested displaying a greater than 100% increase in lag time of LDL oxidation (*Table 3.1*).

3.2.5 17β-hydroxysteroid dehydrogenase activity

In the presence of 100μM 17β-estradiol, the percentage conversion of estrone to estradiol (E1→E2, reductive direction) was 20.4%, and in the oxidative direction (E2→E1) was 27.1%. In the presence of 100μM estrone, the reductive and oxidative conversions were 20.9 and 2.6% respectively (n=3). Unfortunately the 17β-HSD activity in the control and phytoestrogen incubations could not be determined due to low recovery of steroid from the media, suggesting significant metabolism to hydrophillic metabolites.

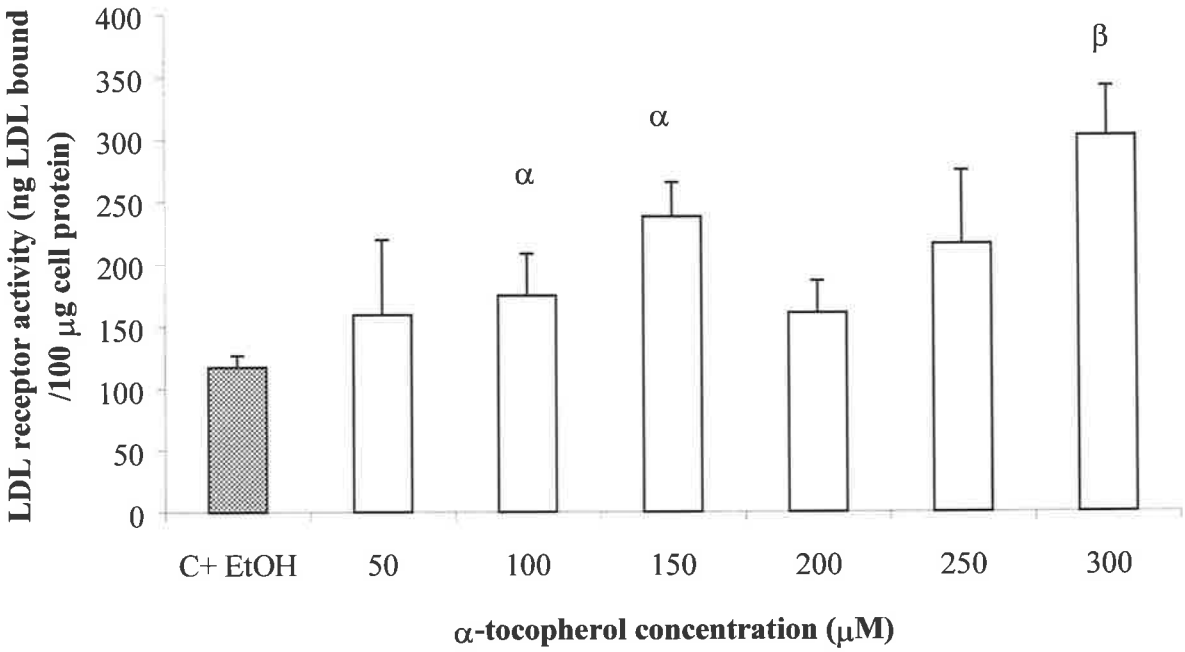


Figure 3.2 *The antioxidant regulation of LDL receptor activity in HepG2 cells by α-tocopherol.*

Hep G2 cells were incubated for 24 hours with α-tocopherol in phenol red-free DMEM + 10% charcoal stripped, lipoprotein deficient FBS. Values are presented as Mean ± SEM, n=4. Symbols denote significantly different to control; $p<0.01$ (α), $p<0.005$ (β).

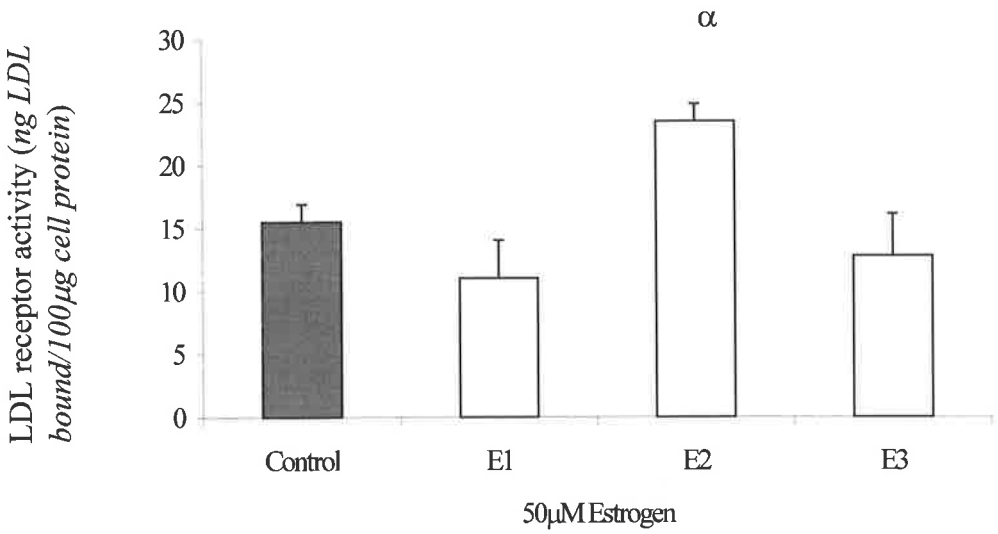


Figure 3.3 *The effect of different estrogens on LDL receptor activity in vitro*

HepG2 cells were incubated for 24 hours in phenol red free DMEM + 10% charcoal stripped, lipoprotein deficient FBS with 50μM concentrations of estrone (E1), 17β-17β-estradiol (E2), or estriol (E3). Values given as Mean ± SEM (n=5). Symbol (α) denotes significantly different to control $p<0.005$.

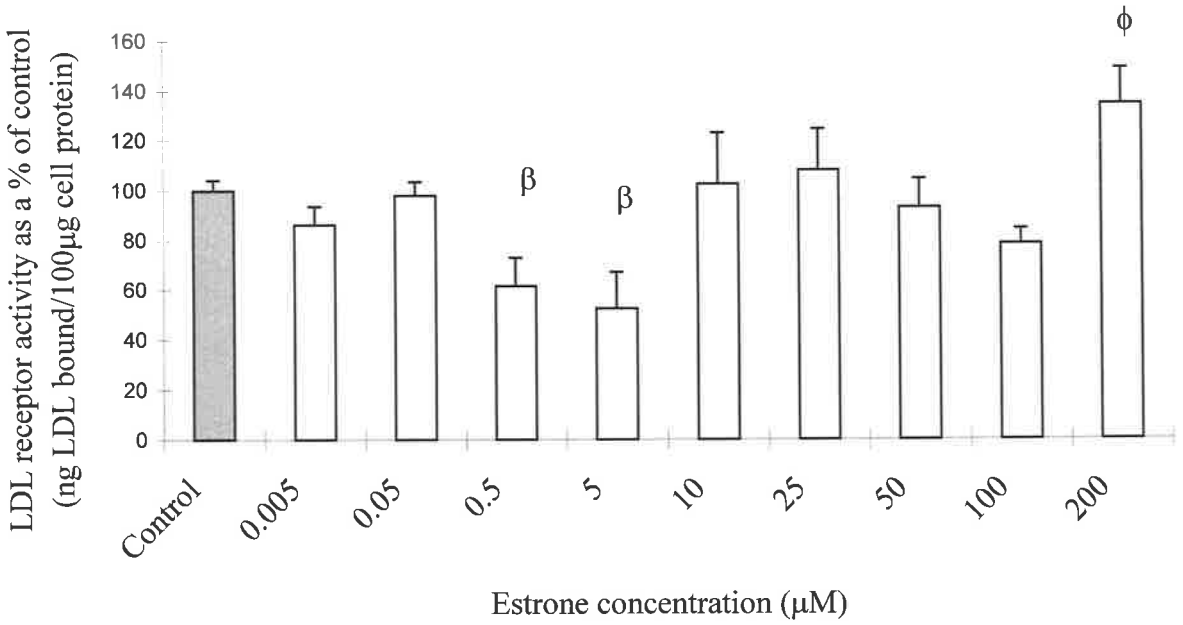


Figure 3.4 *The effect of estrone on LDL receptor activity in HepG2 cells*

HepG2 cells were incubated for 24 hours in phenol red-free DMEM + 10% charcoal stripped, lipoprotein-free FBS with increasing concentrations of estrone in the medium. Results are expressed as percentage of control due to significant inter-assay variability. Values given as Mean ± SEM, n>4 (range: 4-14) for all treatment groups. Statistical analysis was performed using Mann-Whitney U, non-parametric t-tests. Symbols denote control group significantly different to control; $p<0.005$ (β), $p<0.05$ (ϕ).

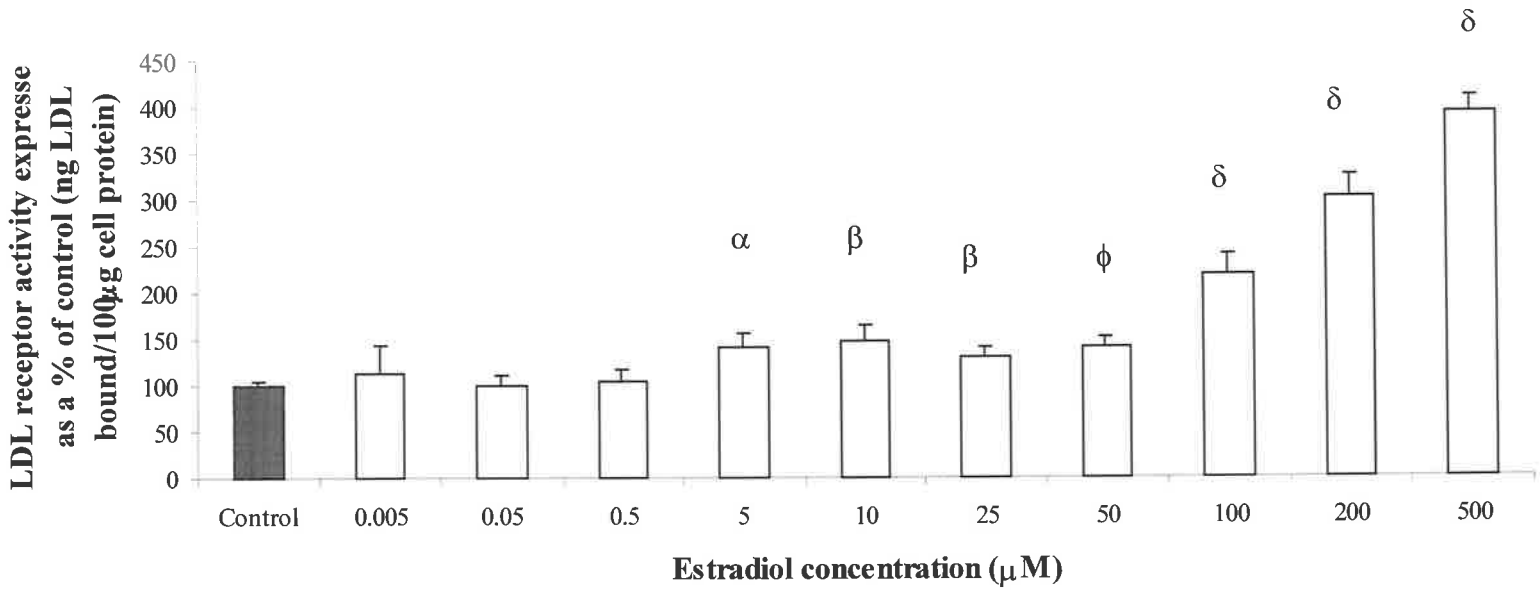


Figure 3.5 *The effect of 17β-estradiol on LDL receptor activity in HepG2 cells*

HepG2 cells were incubated for 24 hours in phenol red-free DMEM + 10% charcoal stripped, lipoprotein deficient FBS with increasing concentrations of 17β-Estradiol in the medium. Results are expressed as a percentage of control due to inter-assay variability. Values given as Mean ± SEM, n>4 (range: 4-19) for all treatment groups. Statistical analysis was performed using Mann-Whitney U, non-parametric T-tests. Symbols denote treatment group significantly different to control at levels of $p<0.01$ (α), $p<0.02$ (β), $p<0.005$ (ϕ), $p<0.0001$ (δ).

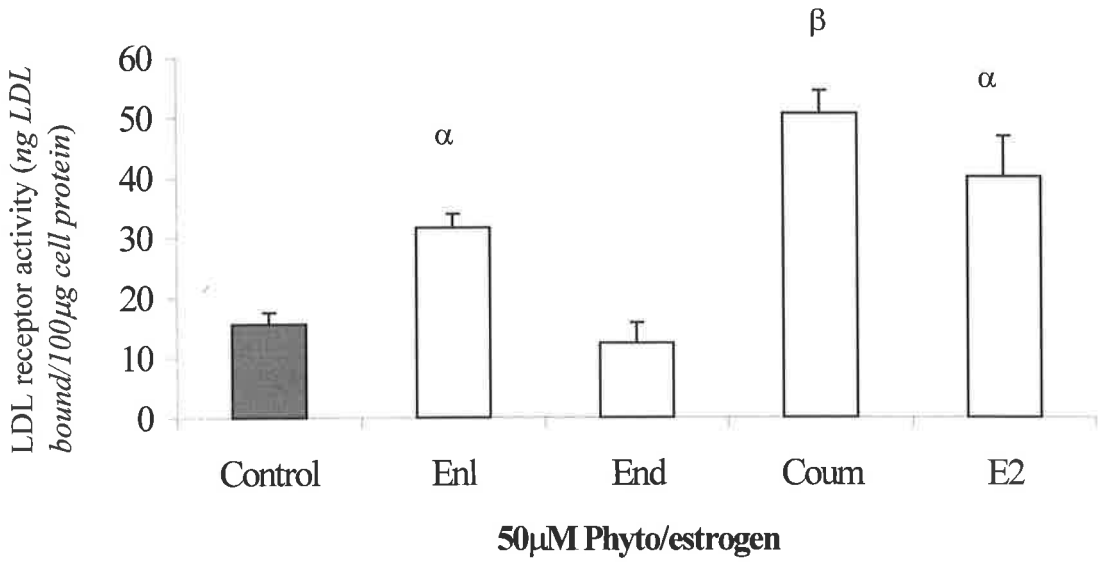


Figure 3.6 *The effect of 50μM enterolactone (Enl), enterodiol (End), coumestrol (Coum) and 17β-estradiol (E2) on LDL receptor activity in HepG2 cells*

HepG2 cells were incubated for 24 hours in phenol red-free DMEM + lipoprotein deficient, charcoal treated FBS with the phytoestrogen to be tested. Values are given as Mean ± SEM, n=3. Statistical analysis was performed using Students t-tests. Symbols denote significant differences compared to control at levels of $p<0.005$ (α) and $p<0.001$ (β).

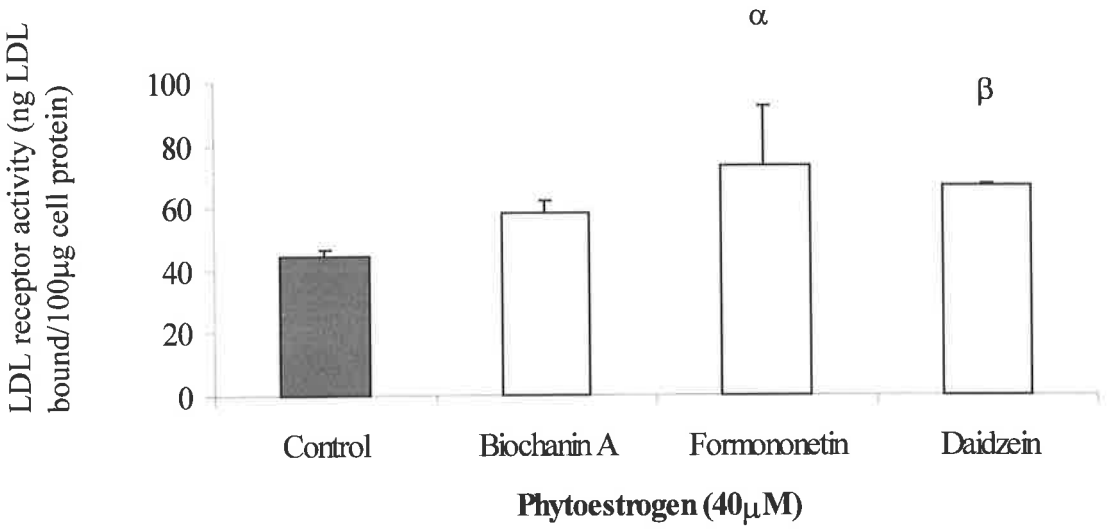


Figure 3.7 *The effect of isoflavonoid phytoestrogens on LDL receptor activity* <u>*in vitro*</u>

HepG2 cells were incubated for 24 hours with either 40μM of the isoflavone to be tested in phenol red free DMEM with 10% charcoal stripped, lipoprotein deficient FBS. Values are Mean ± SEM, n=4. Symbols denote significantly different to control at levels of $p<0.01(\alpha)$ and $p<0.005(\beta)$

Phytoestrogen (1μM)	Change in lag time of LDL oxidation *(% of control) ± SEM*
Biochanin A	-4.4 ± 0.1
Formononetin	-1.3 ± 0.5
Coumestrol	$107.6 \pm 3.6^{\alpha}$
Enterolactone	18.6± 4.1
Enterodiol	$30.7 \pm 2.6^{\alpha}$

Table 3.1 *The antioxidant activity of selected phytoestrogens*

Antioxidant activity was determined by the ability to inhibit Cu^{2+} inititated oxidation of isolated LDL (as described in *Chapter 2.6*). Values given are mean percentage change in lag time of oxidation ± SEM, n=3, α denotes $p<0.05$.

3.4 Discussion

Antioxidant regulation of LDL receptor activity was illustrated in this study by demonstration of the upregulatory effect of α-tocopherol. Pal et al. (1999) reported that the LDL receptor activity of HepG2 cells could be upregulated by the antioxidant vitamins, α-tocopherol, β-carotene, retinol and ascorbate, as well as a green tea extract containing epigallocatechin gallate, and red wine extract. These antioxidants have different mechanisms of antioxidant action, however Pal (1996) hypothesised that the LDL regulation could be due to a common effect of antioxidants on inhibition of the formation of oxysterols within the cell which are known to downregulate LDL receptor expression (Steinberg et al. 1989). A recent study in rat liver microsomes has found that α-tocopherol at a concentration of 500μM, has the ability to reduce the activities of HMG-CoA reductase and 7α-hydroxylase (Brunet et al 2000). The net result of both of these actions would be a reduction in cellular cholesterol, which is a potent stimulus for an increase in LDL receptor activity. In the current study the effect of α-tocopherol on LDL receptor activity achieved significance at concentrations above 100μM, which is approximately 5-10 fold higher than would commonly be seen in plasma. This finding was different to that of Pal (1996) who found that α-tocopherol caused significant upregulation of LDL receptor activity at concentrations of 50μM, with a return to control levels of LDL receptor activity at 100μM. The bimodal dose-response may be related to toxicity, which would not be expected to appear at levels of 100μM (Chan and Tran 1990) but may be seen at higher concentrations.

The results of the current study indicate that the increase in LDL receptor activity at the cell surface is stimulated to a greater degree by 17β-estradiol than estrone, and at lower concentrations, it appeared that estrone may even have an inhibitory effect on LDL receptor activity. The other major human estrogen, estriol, had little effect upon LDL receptor activity in this cell model. This parallels the ability of these human estrogens to inhibit LDL oxidation (Shwaery et al. 1998), however in addition to being the stronger antioxidant, 17β-estradiol also has a greater level of estrogenicity.

Estrogenicity can be measured using a variety of *in vitro* systems. In an *in vitro* pituitary cell culture system which determined estrogen-stimulated prolactin synthesis, Jordan and Koch (1989) found 17β-estradiol to have a level of estrogenicity 20 times higher than that of estrone. Fishman and Martucci (1980) examined the relative binding affinities of estrone and 17β-estradiol for rat uterine cytosol estrogen receptors and found that estrone had an affinity that was 11% of that of 17β-estradiol. The estrogen receptor is unique among other steroid hormone receptors in that it has the ability to interact with a variety of non-steroidal compounds. It is commonly thought to act as a dimer, and recent crystallography studies support this (Tannebaum et al, 1998). The hydrogen bond interactions between amino acids within the receptor structure and the phenolic hydroxyl group present on 17β-estradiol play a vital role in the structure and function of the receptor (Brzozowski et al. 1997). It has been hypothesised that the estrogen receptor can form a dimer pair with both an estrogen and anti-estrogen interacting with the receptor (Tannenbaum et al. 1998, Wang et al. 1995).

In vitro, when examining lipid peroxidation in the absence of cells, estrone is a significantly weaker antioxidant than 17β-estradiol, with IC_{50}s at least 2-3 fold higher in transition metal ion and peroxyl radical-generating systems (Subbiah et al. 1993, Lacort et al. 1995). As can be seen in *figure 4.1*, both 17β-estradiol and estrone share the characteristic phenolic hydroxyl group at the carbon 3 position, however estradiol has an additional hydroxyl group at the 17 carbon. This 17-OH is most likely to account for the greater antioxidant capacity of 17β-estradiol. As estrone has both lesser antioxidant activity and lesser affinity for the estrogen receptor, the role of estrogenic and antioxidant activities in the regulation of HepG2 cell LDL-receptor activity is difficult to distinguish. Incubation of fetal liver cells with estradiol (in a μM range) results in a 3-4 fold increase in cholesterol synthesis, and this is maintained for at least 48 hours of incubation (Carr and Simpson 1984). If the cholesterol regulation in human fetal hepatocytes is similar to a HepG2 cell model, then this would suggest that a reduction in HMG CoA reductase is not the stimulus for the increase in LDL receptor activity seen in the current study. The concentrations of estrogens used in both the present study and in those discussed here are supraphysiological, with normal plasma levels residing in the pmol-nmol range.

Of the phytoestrogens tested, coumestrol appeared to have the greatest effect on LDL receptor activity, showing marked upregulation at a concentration of 50μM of an equal magnitude to that displayed by 17β-estradiol. Interestingly, coumestrol is strongly estrogenic, with an estrogenicity 100 times greater than that of biochanin A (Collins et al. 1997). It is also a more potent ligand for both ERα and ERβ than estrone and estriol, and a more potent ligand than 17β-estradiol for the ERβ subtype (Kuiper et al. 1998).

The ERα subtype is the predominant subtype in the rat liver (Kuiper et al. 1997), but a recent study has found that HepG2 cells do not express ERα (Harnish et al. 1998). The HepG2 cell line has in the past been shown to possess estrogen receptors (Tam et al. 1986, Edmunds et al. 1990), and it is possible that genetic drift in the cell line could explain the lack of ERα (which is thought to be the major human hepatic ER) noted by Harnish et al. (1998). However, a recent study of human tissues and human cell lines has revealed that both human liver and HepG2 cells possess a newly discovered isoform of human ERβ named $ER\beta_5$ (Moore et al 1998).

In a study in which ER's were transfected into HepG2 cells to examine activation of the retinoic acid promoter α-1, it was found that the tamoxifen and raloxifene acted as agonists through ERβ, while estradiol alone had no effect, and blocked the agonist effects of tamoxifen and raloxifene. Conversely, estradiol acted as an agonist through ERα. In both cases, it appeared that the effect was not mediated by the classical receptor/DNA binding mechanism (Zou et al. 1999). Such findings add a new level of complexity to the issue of hepatic regulation of gene expression. The different binding affinities and tissue distributions of ER subtypes may help in explaining the effects of selective estrogen receptor modulators such as tamoxifen and phytoestrogens.

In addition to being strongly estrogenic, coumestrol was found in the present study to be a markedly stronger antioxidant than the isoflavones, biochanin A and formononetin, and the lignans, enterolactone and enterodiol, as determined by its ability to inhibit copper-

initiated LDL oxidation. As for 17β-estradiol, the relative contributions of the estrogenic and antioxidant activities of coumestrol to its upregulation of the HepG2 cell LDL receptor are not clear.

Of the two main lignans present in the human biological fluid, enterolactone appears to have stronger estrogenic activity than enterodiol (Mousavi and Adlercreutz 1992, Wang et al 1994a, Martin et al 1996). The dietary precursor of mammalian lignans, secoisolariciresinol-diglycoside, which undergoes conversion by intestinal flora (Setchell et al 1988), has previously been shown to possess *in vitro* antioxidant activity (Prasad 1997b). Recently both enterolactone and enterodiol have also been found to act as antioxidants at concentration of 100μM in an *in vitro* linoleic acid peroxidation system (Kitts et al 1999). When the ability of enterolactone and enterodiol to inhibit LDL oxidation was examined in this study, enterodiol appeared to be the more potent antioxidant of the two lignans. In the current study, in an estrogen-free medium, enterolactone significantly increased LDL receptor activity whereas enterodiol did not. This suggests that in the case of the lignans, LDL-receptor stimulating activity may be more influenced by estrogenic rather than antioxidant activity.

Both biochanin A and formononetin caused similar increases in LDL receptor activity at 40μM. This is consistent with biochanin A and formononetin being shown to have a similar level of estrogenicity at 1μM (Miksicek 1995). Although both share the phenolic hydroxyl that has been shown to be a key determinant of estrogen receptor binding (Grese et al. 1997), they differ structurally by the presence of an additional hydroxyl

group on the phenolic ring of biochanin A (*Figure 3.1*). This did not appear to confer enhanced antioxidant capacity to biochanin A when the ability to inhibit LDL oxidation was examined in this study. The 4-OH group is the only structural difference between biochanin A and formononetin, it is possible that this slight difference might influence the interaction of these compounds with the estrogen receptor (Collins et al. 1997), however this has yet to be determined by direct comparison. *In vivo*, biochanin A has a slightly, but not significantly, greater hypolipidaemic effect in triton-treated albino rats (Siddiqui and Siddiqi 1976), but in the current study there was no difference between the effects of these isoflavones on *in vitro* LDL receptor activity.

In this study, the isoflavone daidzein was shown to significantly increase LDL receptor activity. This is in contrast to the previously reported findings for the other major soy-derived isoflavone, genistein, which has been shown to decrease LDL receptor activity in HepG2 cells (Chan et al. 1997). Genistein has greater estrogenic and antioxidant activities than daidzein (Kuiper et al. 1997, Wang and Goodman 1999) but its downregulatory effect on the LDL receptor is thought to be due to its ability to inhibit tyrosine kinase (Chan et al. 1997), a property not shared by daidzein (Akiyama et al. 1987) and to a much lesser extent by its metabolic precursor biochanin A (Gaudette et al 1990) which was found to increase LDL receptor activity in the current study.

The isoflavones diadzein, biochanin A and formononetin, were found to upregulate LDL receptor activity in the current study, and this provides further evidence to support the hypothesis that the hypocholesterolaemic effect of soy is mediated partly by isoflavones

through LDL receptor regulation (Kirk et al. 1998, Anthony et al. 1997). However, Baum et al. (1998) found that while soy supplementation of both moderate and high isoflavone content increased mononuclear cell LDL receptor mRNA in postmenopausal women, there was no significant difference between the results seen for the two different isoflavone contents. While the phytoestrogen concentrations used in the current study may be supraphysiological, little is known of the tissue disposition of phytoestrogens. Levels of genistein in the 2μM range have been reported in plasma (Setchell 1998), while plasma levels of enterolactone and enterodiol in Australian postmenopausal women have been reported to be up to 1μM (Morton et al 1994) following linseed ingestion. It appears that genistein undergoes enterohepatic circulation (Barnes et al 1996), but the levels achieved in hepatic or intestinal tissues have yet to be reported. It has been estimated that in a person consuming 50g flaxseed, the lignan content of the colonic contents may reach 666μM, but again the concentrations reached at the hepatic level are not known (Kitts et al 1999).

The previously demonstrated ability of 17β-estradiol to bring about an increase in LDL receptor activity of HepG2 cells was confirmed in the current study. However, estrone was not shown to cause upregulation of LDL receptor activity, even though its estrogenicity is greater than many of the phytoestrogens examined. Tchernof et al. (1995) found that estrone levels were approximately 3-fold higher than estradiol levels in men, and that estrone was higher in obese compared to lean men. Estrogens can be modified in peripheral tissues by 17-β-hydroxysteroid dehydrogenase (17β-HSD) also known as 17-β-hydroxysteroid oxido-reductase, which converts estrone to estradiol and vice-versa

(Labrie et al. 1997) allowing estrogen sensitive tissues to alter the hormones at an intracellular level. In this study, the formation of estradiol from estrone was prominent, and persisted even in the presence of large amounts of estradiol. It is possible that HepG2 cells are modifying their estrogenic environment such that the level of bioactivity is enhanced. This is similar to the effect noted by Bayard et al. (1995) in cultured rat aortic smooth muscle cells.

In the current study, the HepG2 cells were examined in a medium in which the presence of estrogenic compounds was minimised to isolate the effects of the compound to be tested. However *in vivo*, there is likely to be significant interaction between phytoestrogens and estrogens. The administration of grapefruit juice which contains high levels of the phytoestrogen naringenin to ovariectomised women has shown that the metabolism of estrogens can be altered (Schubert et al. 1994). Phytoestrogens have been reported to influence the activity of 17-βHSD (Mäkelä et al. 1995) as well as the enzyme aromatase which converts androgens to estrogens (Wang et al. 1994a), and these effects could significantly alter the amounts of estrone and 17β-estradiol *in vivo*. In addition, physical interactions of endogenous estrogens with the estrogen receptor may be affected by the presence of phytoestrogens (Collins et al. 1997).

Thus, although there is a great deal of evidence to implicate estrogenic compounds in hepatic regulation of LDL receptor expression, whether this involves the binding to hepatic estrogen receptors (either ERα or ERβ), or via another mechanism (possibly involving antioxidant activity) has yet to be determined. However evidence is emerging

that ERβ may be involved in the hepatic 'estrogenic' activity of tamoxifen (Zou et al 1999), and both human liver and HepG2 cells have been shown to possess the $ER\beta_5$ isoform. As new studies are elucidating the behaviour of estrogens and selective estrogen receptor modulators in their interactions with different ER types (Paige et al. 1999), there should be an opportunity to gain further insight into the relative influence of estrogenic activity in regulating LDL receptor activity.

Differences in binding affinity of estrogens and selective estrogen modulators such as phytoestrogens for ERβ, bears similarity to the results seen in the current study. In this study coumestrol was able to upregulate LDL receptor activity to an equal (or even slightly greater) extent compared with 17β-estradiol and others have shown that coumestrol is significantly estrogenic having greater affinity for ERβ than 17β-estradiol itself. However as demonstrated in this study, coumestrol is also a potent antioxidant and the contribution of these effects to the upregulation of LDL receptor activity cannot be determined. Daidzein also has the ability to upregulate LDL receptor activity which may partly account for the hypocholesterolaemic effect of soy (Anderson et al. 1995). Comparison of the effects of enterolactone and enterodiol, suggests that estrogenicity plays a role in the *in vitro* upregulation of the LDL receptor activity by the two lignans.

The mechanisms by which estrogenic compounds regulate LDL receptor activity is still not known, but is unlikely to be via the classical steroid receptor pathway as the LDL receptor gene promotor does not possess the estrogen response element (Croston et al. 1997, Streicher et al. 1996). Similarly, the mechanism by which antioxidants regulate

LDL receptor activity has not been elucidated (Pal et al. 1996), but the compounds which increased LDL receptor activity in the current study tended to display both estrogenic and antioxidant activities. This study highlights the complex issues relating to the relative contributions of estrogenicity and antioxidant activity in regulation of LDL receptor activity.

CHAPTER 4

The effect of Hormone Replacement Therapy on Plasma Lipids, Lipoproteins and LDL oxidation

4.1 Introduction

Post menopausal hormone replacement is often associated with a reduction in total and LDL cholesterol and an elevation in HDL cholesterol, and in the case of oral unopposed estrogen also an increase in triglyceride (*Chapter 1.6.2*). There is evidence to suggest that the elevation in HDL is a result of an increase in production of apo A-I (Walsh et al. 1994, Brinton 1996). The decrease in LDL is thought to be a result of enhanced LDL catabolism, at least partly through an increase in LDL receptor activity (Angelin et al. 1992). However Colvin (1996) also found that LDL receptor independent catabolism was enhanced following estradiol treatment in rabbits. While progesterone is an inhibitor of cholesterol esterification through acyl : cholesterol acyltransferase (ACAT) and can therefore modulate free cholesterol in the cell, a study of LDL metabolism in a human monocyte cell line found no change in receptor mediated LDL binding following treatment with progesterone (Huang et al. 1997).

Levels of mononuclear cell LDL receptor activity and mRNA have been shown to be parallel to liver LDL receptor activity and mRNA levels (Roach et al. 1993, Powell and Kroon 1994), supporting the use of mononuclear cells as an indicator of hepatic LDL receptor activity. In a previous cross-sectional study, we found that postmenopausal women not using HRT had lower mononuclear cell LDL receptor activity than postmenopausal women using HRT or premenopausal women (Abbey et al 1999). The direct effect of HRT on LDL receptor activity has not been reported.

Changes to plasma LDL and HDL cholesterol levels have been estimated to account for 25-50% of the cardioprotective effect of estrogen (Barrett-Connor and Bush 1991). The

remaining 50-75% of this cardioprotective effect has yet to be apportioned, but may include the ability to protect LDL from oxidation.

LDL oxidation is considered to be an important underlying event in atherogenesis. While estrogen has a well-established ability to act as an antioxidant *in vitro* and has been shown to regenerate tocopherol from the tocopheroxyl radical (Mukai et al. 1990), and the effect of HRT on LDL oxidation *ex vivo* is inconsistent. Some researchers have found HRT to afford protection to LDL against oxidation (Wakasuki et al. 1998), while others have not seen any protective effect (McManus et al. 1997).

Oxidisability of LDL has been shown to be influenced by LDL particle size, with smaller denser LDL demonstrating shorter lag times (Chait et al. 1993). Smaller LDL particles have been found to be more prevalent in postmenopausal women compared to premenopausal women (Campos et al. 1988), however postmenopausal HRT has also been shown to result in a shift toward smaller LDL particle size (van der Mooren et al. 1994). Small LDL particles may also have reduced affinity for the LDL receptor (Nigon et al. 1991, Rajman et al. 1994). The increased residence time as a result of lower uptake by the LDL receptor may enhance oxidative susceptibility by increasing the age (i.e. time in circulation) of the lipoproteins (Walzem et al. 1995) in addition to any increases in oxidative susceptibility to due increased lipoprotein density. These hypothesised mechanisms for the increased atherogenicity of small LDL particles are supported by evidence suggesting associations between small LDL size and coronary artery disease (Campos et al. 1992).

The current study was designed to examine the effect of HRT on plasma lipoproteins, LDL oxidation and other CVD risk factors. Our previous cross-sectional study was the first to indicate that HRT might influence LDL receptor activity. In this study we aimed to further examine this effect with the power conferred by the longitudinal nature of the study design. In light of the conflicting findings regarding HRT and LDL oxidation, this study also aimed to include an examination of confounding factors which might mask the effect of HRT on LDL oxidation.

4.2 Methods

4.2.1 Study Subjects and Design

Ethical approval for the study was gained from the Ethics Committees of CSIRO Human Nutrition, the University of Adelaide, and the Royal Adelaide Hospital. Informed consent was gained from all subjects prior to their participation in the study.

Subjects for the study were recruited by public advertisement and through a gynaecologist at the Women's Health Centre of the Royal Adelaide Hospital. Women who had been post-menopausal for a period of at least one year, determined by an absence of menstrual bleeding for one year or a follicle stimulating hormone level above 30 IU/l, were eligible for the study. Subjects were excluded if they were taking any medication that was known to influence lipid metabolism, including lipid lowering drugs and thyroid hormones. Study subjects were asked to cease taking antioxidant and oil supplements for 4 weeks prior to and during the course of the study.

For those women not using HRT for the duration of the study, two blood samples were taken separated by a period of three months designated as Visit 1 (V1) and Visit 2 (V2) in chronological order. Those commencing HRT (either oral conjugated equine estrogen or transdermal estrogen, and oral progestin) as prescribed by their doctor, had a blood sample taken immediately prior to commencing HRT, this sample was termed Visit 1 (V1). The second sample, termed Visit 2 (V2), was then taken 3 months later during the period of expected lowest progestin concentration. Subject characteristics are shown in *Table 4.1*, BMI was calculated as weight in kg / (height in m)^2.

	AGE (year)	**BMI** (kg/m^2)
Control	56.7 ± 1.5	27.5 ± 1.3
HRT	53.22 ± 1.9	26.2 ± 1.5
Oral HRT	55.2 ± 3.0	26.9 ± 2.6
Transdermal HRT	50.75 ± 1.7	25.4 ± 1.6

Table 4.1 *Subject characteristics*

Two groups of postmenopausal women were recruited for the study. The control group (those not using HRT throughout the study, n=12) did not differ significantly in age or BMI to the group of women who commenced HRT (n=12). Women in the HRT group could be divided into those starting oral HRT (n=7) and those starting transdermal HRT (n=5), and when this was done transdermal HRT users were slightly, but not quite significantly, younger than controls (p=0.051).

4.2.2 Mononuclear cell sample preparation

Whole blood was collected into four 9ml evacuated tubes containing 1mg/ml EDTA. Plasma was immediately isolated as described in Chapter 2.2, aliquoted into microtubes and frozen at -80°C. Mononuclear cells were then isolated from the sample. The buffy coat was removed from all four tubes in the smallest volume possible and combined. It was then made up to 10ml with PBS containing EDTA (137mM NaCl, 101mM KCl, 3mM Na_2HPO_4, 2mM KH_2PO_4 with the addition of 1mM EDTA) and 5 ml of the diluted buffy coat was then layered onto 5 ml of lymphocyte separation media (Ficoll-Paque, Pharmacia, Uppsala, Sweden), and centrifuged at 400 x g for one hour. This resulted in collection of the mononuclear cells at the supernatant / Ficoll-Paque interface. The supernatant was removed down to the cell layer, and the cell layer was collected. The cells were pooled and washed twice in EDTA-containing PBS, (the washing procedure consisted of resuspension of the cells in 10ml of EDTA-PBS followed by pelleting of the cells by centrifugation for 10 minutes at 400 x g and removal of the supernatant). The cells were fixed by incubation for 10 minutes with 10ml of 1% paraformadehyde in PBS and pelleted by centrifugation at 200 x g. The cells were then washed twice in normal PBS, before being resuspended in 0.5ml of PBS and stored at -80°C.

4.2.3 Measurement of Plasma Lipids

Plasma samples stored at -80°C were thawed, mixed by gentle inversion and the fibrin pelleted by centrifugation as described in Chapter 2.2. The concentration of plasma total cholesterol, HDL cholesterol, triglyceride and LDL cholesterol were determined as described in Chapter 2.4. Lp(a) concentration was measured as described in Chapter 2.5.

4.2.4 LDL Oxidation and TBARS measurement

LDL isolation and oxidation was performed as described in Chapter 2.6 and oxidised in the presence of $Cu^{2+}$with continuous monitoring at 234nm for a period of 3 hours. Oxidations were performed in duplicate with both samples for each subject in the same assay and a blank (LDL without the addition of Cu^{2+}) included for each sample. TBARS was measured in un-oxidised LDL and also in the diluted LDL samples after oxidation (Chapter 2.7).

4.2.5 Determination of Plasma Antioxidants by HPLC

The plasma concentration of α-tocopherol, β-carotene and retinol was determined using a method as described by Yang and Lee (1987).

α-Tocopherol acetate was used as an internal standard. Standards (Retinol, α-tocopherol and trans β-carotene) were prepared in glass tubes then dried under a stream of nitrogen, before storage at -80°C. Before use, the standards were reconstituted in mobile phase, which consisted of 22% methanol, 55% acetonitrile, 11.5% dichloromethane, 11.5% hexane and 0.01% ammonium acetate.

Throughout the procedure, thawed plasma samples were stored on ice while not in use, and all work was performed in dim light. Antioxidants were extracted from plasma by vortexing the plasma sample with ethanol, internal standard and hexane. Following precipitation of plasma protein by centrifugation at 3000 rpm, the hexane layer was removed and evaporated to dryness under nitrogen. This was redissolved in mobile phase before injection. The assay utilised a Supelcosil LC-18, 25cm x 4mm column,

and a UV/VIS detector. Detection wavelengths were 325nm for retinol, 292nm for α-tocopherol and 450nm for β-carotene.

4.2.6 Determination of LDL Particle Size

Total lipoproteins were isolated from plasma by ultracentrifugation. Thawed plasma (0.5ml) was adjusted to d1.21 g/ml with KBr and overlayed with a density solution of d1.21 g/ml in Beckman 11x32 mm PA-Quickseal centrifuge tubes. These were then spun in a 120.2 rotor using a Beckman benchtop Optima TLX-Ultracentrifuge (Beckman Instruments, California, USA) at 120 K rpm for a period of 6.5 hours at 4°C. The lipoprotein-rich fraction contained within the top third of the tube was collected.

The lipoprotein fractions were then subject to polyacrylamide gel electrophoresis in 2-16% gradient gels (Supplied by Dr Kerry-Anne Rye, Royal Adelaide Hospital, SA, Australia). Gels were run in a Tris-Borate buffer (80mM Boric Acid, 3 mM EDTA, 90mM Tris, 3mM NaN_3) at 200mV for period of 20 hours. Latex beads were run to test gel concavity, and size was determined from high molecular weight standards (Pharmacia). The gels were fixed in 10% sulphosalicylic acid, stained with Coomassie Blue stain (0.4% w/v Coomassie Blue G and 3.5% Perchloric Acid), and then destained using 5% acetic acid.

The gels were scanned with an Ultroscan XL Laser Densitometer (LKB, Sweden).

4.2.7 Plasma Homocysteine Determination by HPLC

Plasma homocysteine was measured based on the methods described by Vester and Rasmussen (1991), and Dudman et al (1996), using mercaptopropionyl glycine as an

internal standard. Briefly, homocysteine standards added to pooled plasma and the plasma samples to be measured, were derivatized by the addition of tri-n-butylphosphine (TBP) in dimethylformamide (DMF). Protein was removed from the plasma following incubation with perchloric acid and centrifugation. The supernatant was then incubated at 60°C for 1 hour with ammonium 7-fluorobenzo-2 oxa-1,3-iazole-4-sulphonic acid (SBD-F) to allow formation of a fluorescent homocysteine-SBD derivative which was passed through a 25cm Spherisorb C18 column and measured with fluorescence detection (emission at 515nm and excitation at 385nm).

4.2.8 Plasma Fatty Acid Measurement

Plasma fatty acid composition was determined by gas chromatography (GC) using 0.5mg/ml triheptadecanoate as an internal standard. Lipids were extracted from a 100µl plasma sample in 1ml methanol and 2ml chloroform by being vigorously shaken for 30 seconds. Protein was pelleted from the samples by mixing with 0.1M HCl and then centrifugation, and the lipid-containing chloroform layer was then removed. The chloroform layer was then evaporated under vacuum and methyl esters of the fatty acids were prepared by incubation with 1.5 ml of 1% H_2SO_4 (in dry methanol) at 100°C for 45 minutes.

The methyl esters were thrice extracted in 5ml of petroleum spirit, evaporated to dryness under a stream of nitrogen and then redissolved in 1.5 ml of hexane. The hexane passed over columns containing Florisil (Sigma, Australia). Methyl esters were eluted with 10% diethyl ether in hexane and then evaporated to dryness under nitrogen. The methyl esters were dissolved in 50 µl of isooctane and then injected onto the GC.

The GC was a Hewlett-Packard 5711, using a 30m x 0.53mm bonded phase vitreous silica column, carrier gas hydrogen, make-up gas nitrogen, and a temperature program incrementing from 130°C to 230°C at 8°C/min. The ratio of peak area of fatty acids: internal standard was used to calculate the fatty acid content of plasma, expressed as percentage of total fatty acids.

4.2.9 LDL-Receptor Activity in Isolated Mononuclear Cells

Mononuclear cells were thawed by incubation in a water bath at 37°C. The measurement of cellular LDL-receptor activity was performed as described in Chapter 2.10. Briefly, cellular protein concentration was determined and then the cells were incubated with colloidal gold-labelled LDL. Both non-specific and total LDL-gold binding was measured and the specific LDL-receptor activity calculated.

4.2.10 Data Analysis

Unless otherwise stated, data is presented as Mean ± SEM. Statistical analysis was performed using the SPSS for windows statistical package (SPSS, Chicago, USA). Data was analysed utilising paired and unpaired t-tests for comparisons within and between groups respectively, except in the case of Lp(a). Lp(a) data was examined using Mann Whitney U tests. Multiple regression analysis with stepwise introduction of variables was used to examine the relationship between lag time of LDL oxidation and plasma antioxidant concentrations. Analysis of covariance (ANCOVA) was used to determine the independent effect of HRT on LDL oxidation lag time.

4.3 Results

4.3.1 The effect of hormone replacement therapy on plasma lipoproteins

Baseline subject characteristics are shown in *Table 4.1*. During the study there were no significant changes in BMI in either the control or HRT groups (data not shown), which may have influenced the lipid results. In the control group, there were no significant changes in total, LDL or HDL cholesterol or triglyceride over the period of three months (*Tables 4.2, Table 4.3*). Three months of combined HRT (estrogen plus progestin) resulted in a 9% reduction in LDL cholesterol which was also reflected in a 6% reduction in total cholesterol (*Table 4.2*). When the HRT group was subdivided into those using oral and transdermal therapy, the significance of the reductions in total and LDL cholesterol (6% and 7% respectively) was lost for the oral group, while being maintained in the transdermal group (7% and 13% reductions respectively). There were no significant changes in HDL cholesterol (or the HDL_3 and HDL_2 subfractions) or triglyceride concentrations noted with HRT (*Table 4.3*).

Women who did not take HRT for the duration of the study (control group) had slightly higher Lp(a) levels at baseline than those taking HRT but this difference was not significant. Hormone replacement therapy for a period of three months significantly reduced Lp(a) levels in postmenopausal women, while in the control group there was no change (*Table 4.4*).

LDL particle size was not significantly altered by HRT, although a slight but not significant reduction was noted in those using oral HRT ($p=0.093$) (*Figure 4.1*). In the current study LDL size was not significantly correlated with triglyceride, but was

significantly positively correlated with total HDL cholesterol, (r^2=0.33, p=0.02) for all subjects combined. When one woman who had an HDL cholesterol level four standard deviations higher than the mean was excluded from the correlation, the strength of the correlation increased (r^2=0.43, p<0.003).

Hormone replacement therapy had no effect on mononuclear LDL receptor activity as measured by binding of colloidal gold labelled LDL (*Figure 4.2*). In addition there was no relationship between the change in LDL cholesterol associated with HRT, and the change in LDL receptor activity (*Figure 4.3*).

	Total cholesterol (mmol/l)		*Total triglyceride (mmol/l)*		*LDL cholesterol (mmol/l)*	
	V1	**V2**	**V1**	**V2**	**V1**	**V2**
Control	5.84 ± 0.37	5.79 ± 0.30	1.47 ± 0.16	1.34 ± 0.13	3.53 ± 0.41	3.56 ± 0.30
HRT	5.92 ± 0.25	5.54 ± 0.20 $^{\alpha}$	1.68 ± 0.22	1.65 ± 0.18	3.54 ± 0.23	3.22 ± 0.21 $^{\alpha}$
Oral HRT	6.17 ± 0.35	5.81 ± 0.28	1.98 ± 0.34	1.80 ± 0.27	3.93 ± 0.24	3.66 ± 0.17
Transdermal HRT	5.57 ± 0.32	5.17 ± 0.22 $^{\alpha}$	1.26 ± 0.09	1.46 ± 0.18	2.99 ± 0.33	2.59 ± 0.25 $^{\alpha}$

Table 4.2 *The effect of hormone replacement on plasma cholesterol, triglyceride and LDL cholesterol.*

Blood samples were taken immediately before (V1) and 3 months after (V2), the commencement of hormone replacement therapy. In the case of controls, blood samples were taken separated by a period of 3 months. For both the control and HRT groups, n=12, (however the HRT group could be separated into oral HRT (n=7), and transdermal HRT (n=5)). Values are Mean ± SEM. $^{\alpha}$ denotes significantly different to V1, $p<0.05$.

	HDL_T cholesterol (mmol/l)		*HDL_2 cholesterol (mmol/l)*		*HDL_3 cholesterol (mmol/l)*	
	V1	**V2**	**V1**	**V2**	**V1**	**V2**
Control	1.64 ± 0.15	1.63 ± 0.12	0.46 ± 0.07	0.41 ± 0.05	1.19 ± 0.09	1.23 ± 0.07
HRT	1.63 ± 0.16	1.58 ± 0.17	0.48 ± 0.09	0.48 ± 0.11	1.15 ± 0.08	1.10 ± 0.08
Oral HRT	1.35 ± 0.13	1.34 ± 0.13	0.30 ± 0.05	0.27 ± 0.04	1.05 ± 0.10	1.06 ± 0.12
Transdermal HRT	2.02 ± 0.26	1.93 ± 0.31	0.73 ± 0.16	0.77 ± 0.19	1.29 ± 0.12	1.16 ± 0.12

Table 4.3 *The effect of hormone replacement therapy on HDL cholesterol levels.*

Blood samples were taken immediately prior to, and 3 months after commencement of HRT. In the case of controls not starting HRT, samples were taken 3 months apart. The numbers in the control and HRT groups were n=12, the HRT group could be subdivided into those using oral HRT (n=7) and transdermal HRT (n=5). HDL_T (total HDL) and HDL_3 cholesterol was measured following Dextralip-MgCl2 precipitation on an automated analyser. Values are Mean ± SEM.

	Lipoprotein(a) mg/dl	
	V1	***V2***
Control	25.7 (0-89)	29.3 (0-110)
HRT	9.1 (0-53.8)	4.1 [a] (0-54.3)
Oral HRT	9.5 (0-34)	4 (0-37.8)
Transdermal HRT	8.7 (0-53.8)	4.2 (0-37.8)

Table 4.4 *The effect of hormone replacement on Lp(a)*

Plasma Lp(a) levels were measured by an ELISA in women immediately before and 3 months after commencement of hormone replacement therapy (n=12), and in controls from whom samples were taken separated by a period of 3 months (n=12). The HRT group could be subdivided into oral and transdermal therapies (n=7 and n=5 respectively). Lp(a) displays a skewed distribution the population and values are therefore given as median and (range).

[α] denotes significant difference between V1 and V2 plasma Lp(a).

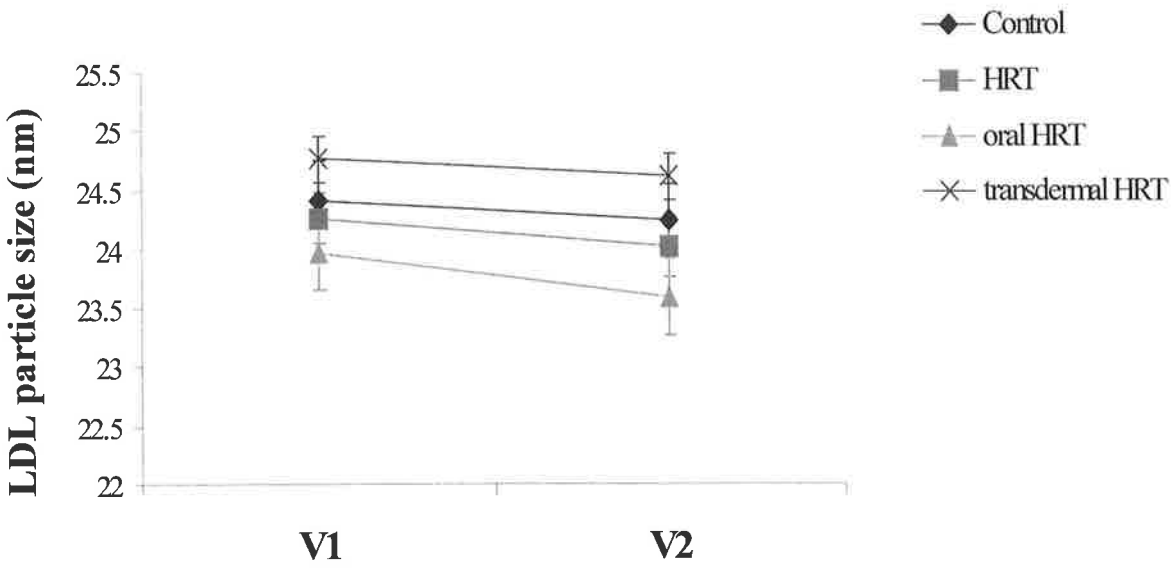


Figure 4.1 *The effect of hormone replacement on LDL particle size.*

LDL particle size was determined following lipoprotein isolation and gel electrophoresis from samples taken just prior to and 3 months after commencement of hormone replacement therapy. Control samples were taken three months apart in women who did not start HRT. Numbers in the control and HRT groups were n=12 for both groups, however when the HRT groups was subdivided into those taking oral hormone replacement and those using transdermal HRT, numbers were n=7 and n=5 respectively. Values are Mean ± SEM.

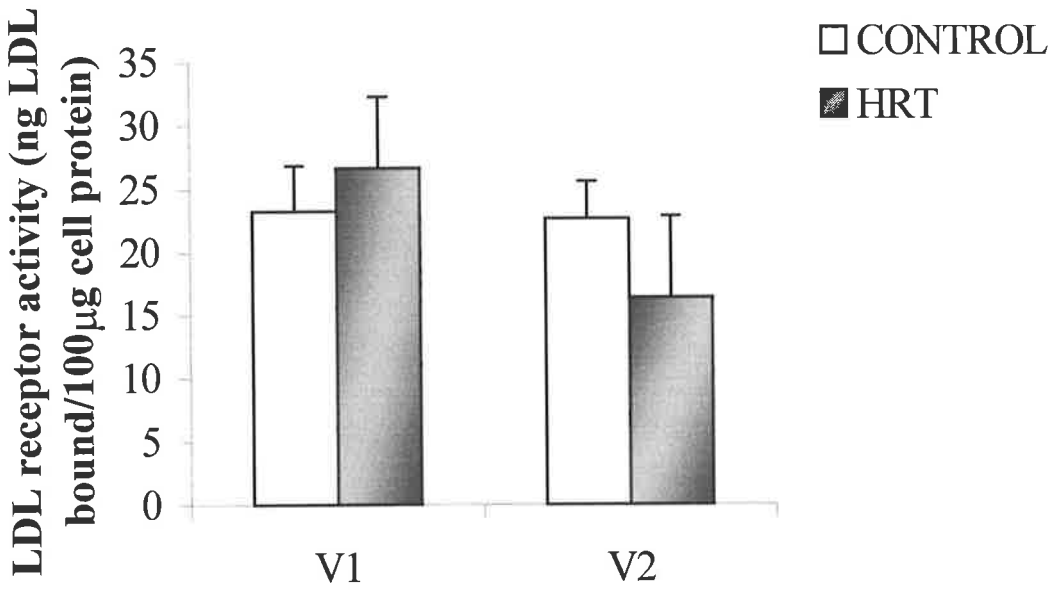


Figure 4.2 *The effect of hormone replacement therapy on mononuclear cell LDL receptor activity in postmenopausal women*

LDL receptor activity was determined by the binding of colloidal gold-labelled LDL to mononuclear cells. The cells were isolated from blood samples taken immediately prior to, and three months after, the commencement of hormone replacement therapy (HRT), and over the same time period in a control group of women who did not take HRT. No significant difference in LDL receptor activity was detected following three months of HRT.

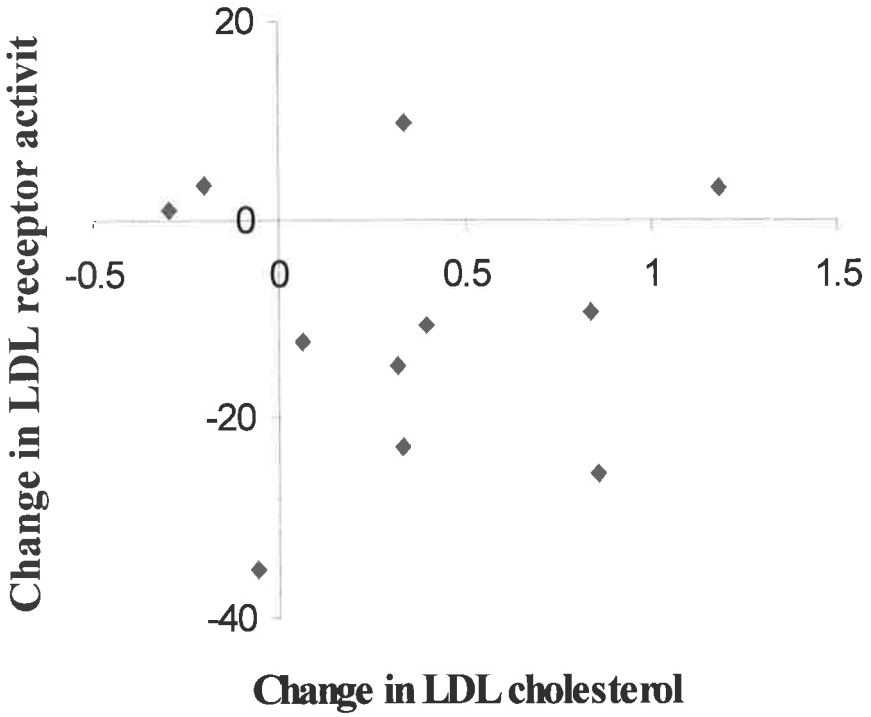


Figure 4.3 *The relationship between change in LDL cholesterol and LDL receptor activity with HRT*

LDL cholesterol was calculated using the Friedewald equation, and the LDL receptor activity of isolated mononuclear cells was determined by measuring the cellular binding of colloidal gold labelled LDL. The change in LDL cholesterol associated with three months of HRT was not related to LDL receptor activity.

4.3.2 The effect of hormone replacement therapy on LDL oxidisability

In both the control and HRT groups there were no significant changes in lag time, rate, or maximum diene concentration of LDL oxidation over the 3 month period, when isolated LDL was subject to Cu^{2+} mediated oxidation (*Table 4.5*).

There were no significant changes in the concentrations of the lipid soluble plasma antioxidants α-tocopherol, β-carotene and retinol in either the control or HRT groups during the course of the study (*Table 4.6*). Nor were there any differences in plasma fatty acid content (*Table 4.7*). The lack of any difference between groups, or over time, in the plasma antioxidants and fatty acids was reflected in the lack of any difference in lag time or rate of LDL oxidation. Lag time of LDL oxidation was significantly correlated with α-tocopherol ($p<0.001$) and β-carotene ($p<0.05$) concentrations, however multiple regression analysis revealed that only α-tocopherol was independently correlated to lag ($p<0.001$) (*Figure 4.4*). LDL oxidation rate was significantly correlated to α-tocopherol ($p<0.05$) and retinol ($p<0.05$) by not β-carotene. There was no correlation between LDL size and either lag or rate of oxidation noted in this study.

The LDL TBARS concentration after oxidation (an estimate of malondialdehyde concentration) was also unaffected by HRT use (*Figure 4.5*). The level of TBARS in unoxidised LDL was not altered by HRT, with mean levels of 0.16 ± 0.03 and 0.18 ± 0.03 nmol/mg LDL protein, before and 3 months after the commencement of HRT respectively.

	Lag time (*min*)		*Rate of oxidation* (*nmol diene/mg LDL/min*)		*Maximum diene concentration* (*nmol diene/mg LDL protein*)	
	V1	**V2**	**V1**	**V2**	**V1**	**V2**
Control	57.9 ± 3.69	59.01 ± 5.43	10.55 ± 0.5	10.79 ± 0.37	360.62 ± 8.19	345.34 ± 14.99
HRT	58.25 ± 3.70	56.33 ± 3.03	10.54 ± 0.63	10.6 ± 0.60	349.34 ± 12.42	334.11 ± 13.61
Oral HRT	57.88 ± 3.53	58.88 ± 4.67	10.07 ± 0.86	9.87 ± 0.87	325.25 ± 15.94	304.92 ± 13.49
Transdermal HRT	58.69 ± 7.52	53.28 ± 3.67	11.10 ± 0.97	11.49 ± 0.70	378.24 ± 9.18	367.15 ± 13.55

Table 4.5 *The effect of hormone replacement on LDL oxidation parameters*

LDL was isolated from plasma samples taken immediately prior to and 3 months after HRT had been commenced (n=11), and from a control group of women not taking hormone replacement (n=12). The isolated LDL was dialysed and then oxidised by that addition of 5μM Cu^{2+}. The Cu^{2+} mediated LDL oxidation was monitored using spectrophotometric detection at 234nm.

	Retinol (μg/ml)		*α-Tocopherol (μg/ml)*		*β-Carotene (μg/ml)*	
	V1	**V2**	**V1**	**V2**	**V1**	**V2**
Control	0.704 ± 0.021	0.757 ± 0.052	17.772 ± 1.337	18.886 ± 2.220	0.605 ± 0.136	0.721 ± 0.148
HRT	0.690 ± 0.035	0.673 ± 0.047	18.700 ± 1.990	15.940 ± 1.302	0.808 ± 0.243	0.770 ± 0.189
Oral HRT	0.721 ± 0.055	0.716 ± 0.080	18.486 ± 2.528	16.960 ± 2.045	0.796 ± 0.445	0.787 ± 0.339
Transdermal HRT	0.654 ± 0.038	0.623 ± 0.032	18.957 ± 3.457	14.716 ± 1.537	0.822 ± 0.167	0.750 ± 0.153

Table 4.6 ***Plasma concentrations of the lipid soluble antioxidants α-tocopherol, β-carotene and retinol***

Plasma antioxidants were measured by HPLC using the method of Yang and Lee (1987). Concentrations of retinol, α-tocopherol and β-carotene did not differ significantly in either the control or HRT groups during the study.

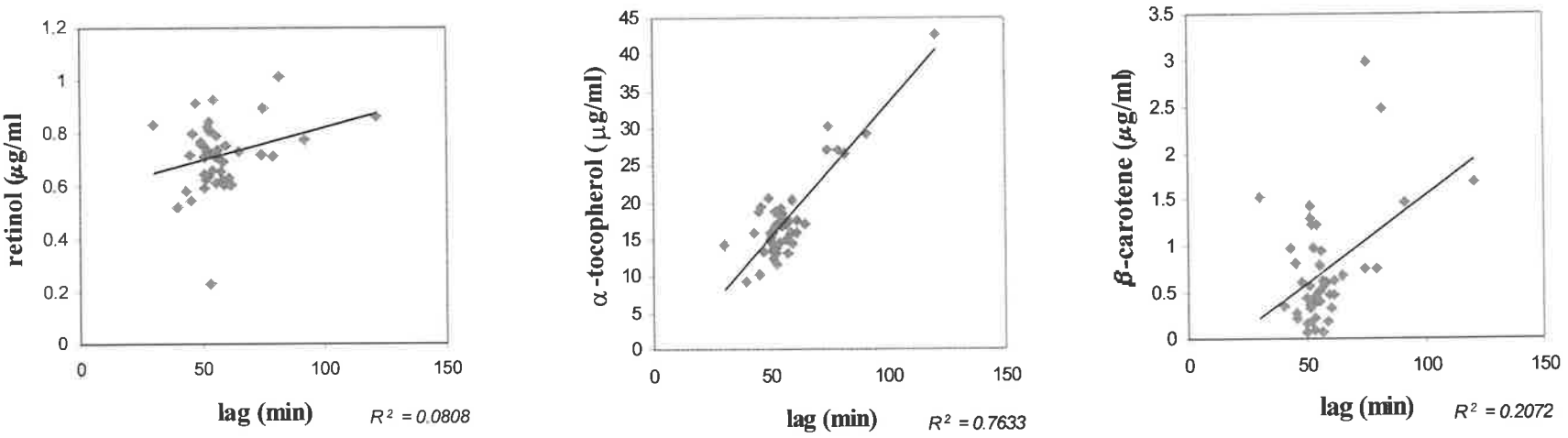


Figure 4.4 ***The relationships between plasma antioxidants and lag time of LDL oxidation***

The plasma antioxidants α-tocopherol, β-carotene and retinol, were measured by HPLC using the method of Yang and Lee (1987). Cu^{2+}-mediated oxidation of isolated LDL was monitored spectrophotometrically by detection of conjugated dienes at 234nm. Multiple regression analysis revealed that of the lipid soluble antioxidants, only α-tocopherol was independently correlated to lag time of LDL oxidation ($p<0.0001$).

	14:0	16:0	16:1 n-9	18:0	18:1	18:2 n-6	18:3 n-3	20:3 n-6	20:4	20-22:n-3
Control										
V1	0.92 ±	23.11 ±	2.38 ±	12.35 ±	26.99 ±	28.65 ±	0.12 ±	1.06 ±	4.28 ±	0.15 ±
	0.10	0.41	0.21	0.65	4.92	1.26	0.12	0.16	0.82	0.15
V2	0.89 ±	23.35 ±	2.28 ±	12.27 ±	25.63 ±	28.78 ±	0*	0.97 ±	4.66 ±	1.17 ±
	0.08	0.57	0.42	0.93	5.32	2.04		0.13	0.91	1.03
HRT										
V1	1.62 ±	26.62 ±	3.27 ±	13.11 ±	27.56 ±	22.77 ±	0.26 ±	1.07 ±	3.39 ±	0.18 ±
	0.09	0.76	0.42	0.70	0.29	1.21	0.11	0.17	0.53	0.132
V2	1.33 ±	25.7 ±	3.42 ±	11.82.±	30.77 ±	21.72 ±	0.25 ±	1.03 ±	3.71 ±	0.25 ±
	0.13	1.02	0.39	0.82	2.19	1.43	0.1	0.20	0.24	0.10

Table 4.7 *Plasma fatty acid content*

Plasma fatty acids were determined using gas chromatography. Peak areas of fatty acids were converted to a ratio relative to internal standard and then expressed as a % of total plasma fatty acids. There were no significant changes in plasma fatty acids in any group.

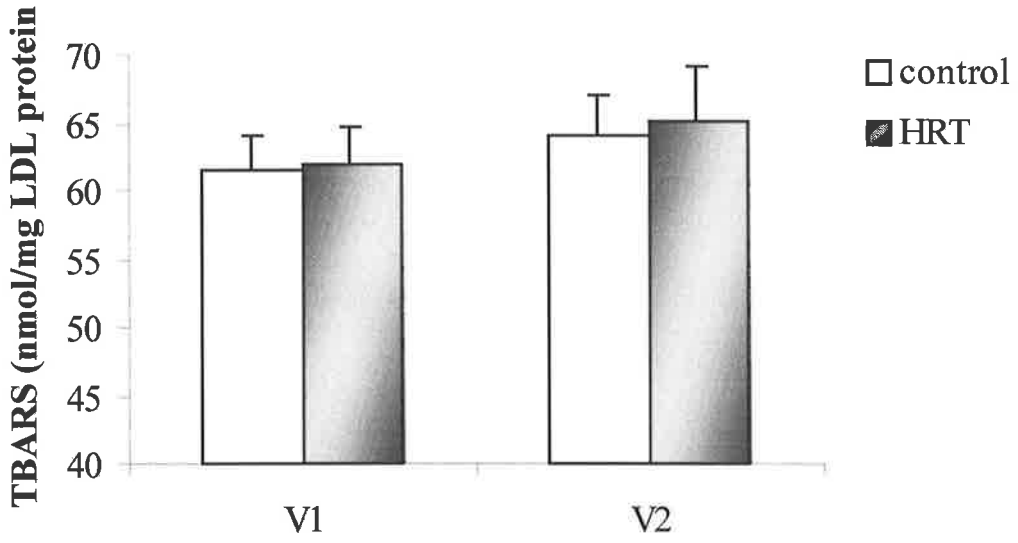


Figure 4.5 *The effect of HRT on LDL TBARS content after Cu^{2+} mediated LDL oxidation*

Thiobarbituric acid reactive substances (TBARS) were measured in isolated LDL before and after being subjected to Cu^{2+} mediated oxidation. TBARS did not differ significantly after treatment for 3 months with HRT in postmenopausal women. Values given as Mean ± SEM, n=10 for control group and n=11 for women treated with HRT.

4.3.3 The effect of hormone replacement therapy on plasma homocysteine

Baseline levels of plasma homocysteine were 7.08 ± 0.53μmol/l for the control group and 7.67 ± 0.57μmol/l for the group of women commencing HRT. These levels did not change significantly in either group over the three month study period (*Figure 4.6*).

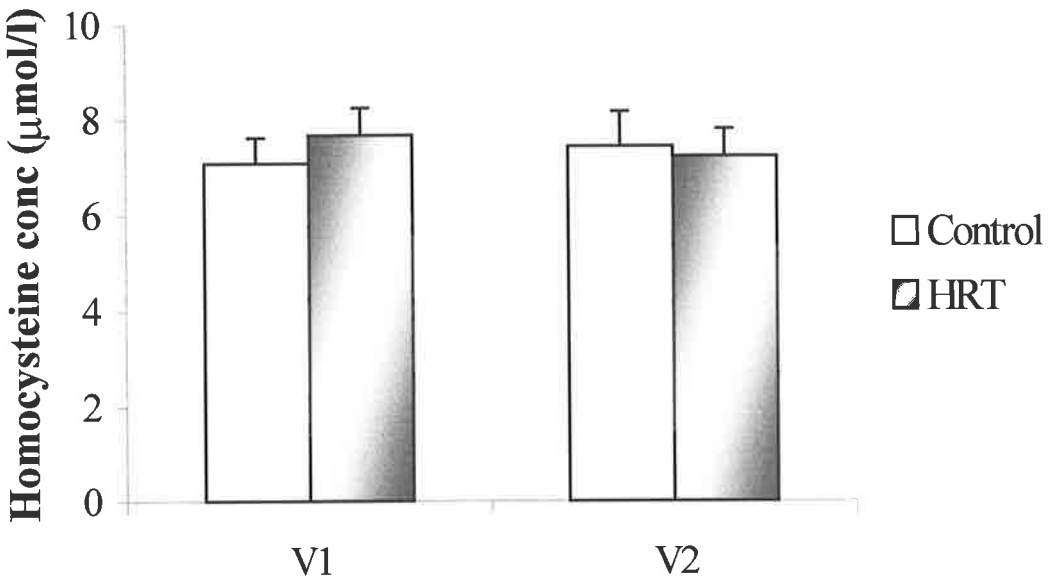


Figure 4.6 *The effect of hormone replacement therapy on homocysteine concentration in postmenopausal women*

Plasma homocysteine was measured in samples taken before and 3 months after commencement of hormone therapy (n=13), and in a control group who did not commence hormone therapy (n=13). The HRT group consisted of women using both transdermal and oral therapies, but these did not differ from the overall HRT values and are not presented. Values are given as mean ± SEM.

4.4 Discussion

The findings of the current study support those of previous studies in finding a reduction in LDL cholesterol with estrogen + progestin HRT (Ulloa et al 1998, Taskinen et al 1996, Mattson et al 1993), also noted in the large multi-centre, placebo-controlled PEPI Trial in 1995 (The Writing Group for the PEPI Trial). This reduction in LDL cholesterol was reflected in a similar reduction in total cholesterol in this group of mildly hypercholesterolemic postmenopausal women, however no change was noted in mononuclear cell LDL receptor activity. Due to the first-pass effect seen with oral estrogen, the effects of oral and transdermal HRT on hepatic LDL metabolism may be slightly different, but due to difficulties with recruitment the numbers in the current study were insufficient for such discrimination.

High dose ethinyl estradiol therapy in animal models has been shown to result in a reduction in plasma cholesterol concomitant with an increase in hepatic LDL receptor levels (Ma et al 1986). In addition, exposure to relatively high concentrations of estradiol (37μM) has been found to increase LDL receptor activity *in vitro* in the hepatocarcinoma-derived cell line, HepG2 (Semenkovich and Ostlund 1987). *In vivo*, in two men being treated with estrogen (of unreported type or dose) for prostate cancer, a higher hepatic LDL receptor activity was noted compared to prostate cancer-free age-matched controls (Angelin et al 1992). However in that study, the effect of disease state on hepatic LDL receptor activity cannot be accounted for. In an earlier cross-sectional study performed by this laboratory, it was found that postmenopausal women not using HRT had a lower level of LDL receptor activity than those using HRT (Abbey et al 1999). To our knowledge the current study is the first to directly examine the effect of HRT on LDL receptor activity in postmenopausal women.

Despite the lack of change in LDL receptor activity in the current study, there was still a reduction in LDL cholesterol noted in the HRT group. In a recent study examining the fractional catabolic rate of native and methylated LDL in a rabbit model, it was found that estrogen treatment promoted LDL receptor-independent catabolism of LDL (Colvin 1996). The exact pathways of LDL receptor independent catabolism are as yet not known, but may include pinocytosis and absorptive endocytosis (Shepard and Packard 1986). In addition, how and if they are regulated by estrogen is not known. In a study examining oral estrogen and progestogen therapy in postmenopausal women, it was noted that there was both a decrease in the cholesterol : apolipoprotein B ratio of LDL and an increase in the fractional catabolic rate of LDL apolipoprotein B (Wolfe and Huff 1995), suggesting that female hormones are having multiple effects on the regulation of LDL metabolism. In HepG2 cell lines into which the LDL receptor promoter and various combinations of estrogen receptor, androgen receptor and progesterone receptor had been transfected, progestins were found to antagonise the upregulatory effects of estrogen through their ability to interact with the androgen receptor, while having no effect on the LDL receptor transcription alone (Croston et al 1997). Although there are obviously many shortcomings in applying this co-transfected cell model to an *in vivo* situation, it raises the possibility that the progestin component of HRT could be antagonising the LDL receptor-stimulating effect of the estrogenic HRT component. The current findings also raise questions as to whether mononuclear cell LDL receptor status will reflect hepatic LDL receptor status with estrogen treatment, as has been shown for HMG CoA reductase inhibitor treatment (Roach et al 1993). This question cannot be answered directly, as estrogen receptor function and tissue distribution is not fully understood. Mononuclear cells are estrogen sensitive

(Hayashi et al 1998), but whether the effect of estrogen treatment on LDL receptor expression is similar for both cell types has yet to be established.

Another possible explanation for the reduction of LDL cholesterol without a corresponding increase in LDL receptor activity is that there may have been a decrease in the amount of cholesterol carried in LDL without any change in LDL particle number. If this were the case it might be expected to manifest as a change in LDL particle size, which was not noted in the current study. Smaller LDL particles are thought to be more atherogenic due to their increased oxidisability (Chait et al 1993), altered affinity for the LDL receptor (Nigor et al 1991, Rajman et al 1994) and enhanced ability to cross the arterial intima (Björnheden et al 1996) and there has previously been some suggestion that both oral and transdermal HRT decreases LDL size (van der Mooren et al 1994, Tilly-Kiesi et al 1996). While the findings of the current study provide further evidence that HRT impacts minimally upon LDL size (Vadlamudi et al 1998), it should be noted that baseline LDL size in this study was already in the lower range as proposed by Musliner and Krauss (1988). Surprisingly, there was no significant relationship between total plasma triglyceride and LDL size, a finding which is difficult to account for. A lack of correlation between LDL size and triglyceride has been noted in patients with Familial Combined Hyperlipidaemia treated with gemfibrozil (Hokanson et al 1993), but the subjects in the current study did not exhibit such characteristics.

In the present study, no change in HDL (total) or HDL2 or HDL3 cholesterol subfractions were noted following HRT. This lack of effect of estrogen + progestin HRT on HDL has been found by some (Kim et al 1994) but others have noted

reductions (Tilly-Kiesi et al 1997) or increases (van der Mooren et al 1997) in HDL. The PEPI trial (1995) reported a significant increase in HDL cholesterol with both estrogen alone and estrogen +progestin treatment, although inclusion of a progestin in the HRT regimen significantly diminished the HDL-elevating effect. Possibly the influence of the progestin in some subjects, combined with the small numbers led to the lack of effect seen on HDL cholesterol in the current study. In an ovariectomised baboon model, it was noted that while both estrogen and progesterone increased apo AI production, progesterone also increased the fractional catabolic rate, negating the effect of increased synthesis (Kushwaha et al 1990). The individual effects of progestins on HDL metabolism in humans are not clear, but in rats a reduction in HDL cholesterol has been noted with progestin treatment (Tkocz et al 1985), suggesting that progestins may counteract the estrogen induced increase in HDL. Levels of the atherogenic lipoprotein Lp(a) decreased significantly with hormone replacement in agreement with previous studies (Kim et al 1994, van der Mooren et al 1994). Lp(a) levels in the population are markedly skewed, and with the small numbers in the current study, baseline levels of Lp(a) were slightly, but not significantly lower in the HRT group compared to the control group. Despite the levels in the HRT group being low, there remained a significant reduction with HRT treatment. However, this reduction of Lp(a) is unlikely to be of significant cardiovascular benefit unless the patient also has elevated levels of LDL cholesterol.

Whether HRT has a measurable impact upon LDL oxidation has yet to be conclusively demonstrated. While some have noted an antioxidant effect of HRT (Schröder et al 1996, Wander et al 1996, Santanam et al 1998), the findings of the current study suggest that after accounting for important determinants of LDL oxidisability, namely

fat soluble antioxidant and fatty acid content, there is no measurable effect of HRT on *ex vivo* LDL oxidation. This finding is supported by those of others (McManus et al 1997, Santanam et al 1998). It has been estimated that 2-3% of estrogen is carried in the LDL fraction of plasma (Leeszczynski and Schafer 1989), thus *in vivo* there may be additional estrogen exposure which cannot be accounted for in the *ex vivo* LDL oxidation assay. Recently the plasma level of antibodies against oxidised LDL was found to be decreased following HRT treatment in postmenopausal hypercholesterolemic women, while the *ex vivo* oxidisability of LDL was unchanged (Hoogerbrugge et al 1998), illustrating a need for more sensitive measures of antioxidant potential.

Elevated levels of the amino acid homocysteine have been found to be strongly associated with mortality in cardiovascular disease patients (Nygård et al 1997) and a risk factor for CVD in postmenopausal women (Ridker et al 1999). Whether reducing homocysteine levels confers a reduction in cardiovascular disease risk has yet to be shown, which supports the need for large scale clinical trials for treatment of hyperhomocysteinemia. Homocysteine levels have been found to fluctuate throughout the menstrual cycle (Tallova et al 1999), and are lower in premenopausal women than in postmenopausal women (Wouters et al 1995). In addition, estrogen has been found to lower plasma homocysteine levels in male rats (Kim et al 1997) and in male to female transsexuals (Giltay et al 1998). The current study found no change in plasma homocysteine levels after three months of HRT in a subject group with mean homocysteine levels that fall within the normal range. The impact the progestogens might be having on homocysteine levels is not currently known.

The current study suggests that HRT is able to bring about favourable changes to plasma LDL cholesterol and Lp(a) without adversely affecting LDL size. Further to this, the study provides evidence to suggest that HRT-induced changes in LDL cholesterol may be mediated, at least in part, by a pathway independent of the LDL receptor. The hepatic LDL receptor is thought to play a major role in regulation of circulating levels of LDL, but other pathways of metabolism are thought to exist. HRT was found to have no effect on the parameters of *ex vivo* LDL oxidation or circulating plasma homocysteine levels in this study.

CHAPTER 5

The Influence of Dietary Factors on Lipoprotein(a)

5.1 Introduction

Despite its structural similarity to LDL, lipoprotein (a) [Lp(a)] appears remarkably resistant to the dietary and pharmaceutical modifications that are often used to bring about changes in LDL cholesterol levels. The most commonly used hypolipidaemic drugs, the statins and fibrates do not appear to lower Lp(a) levels (Berglund 1995, Angelin 1997).

Apo(a), the unique protein component of Lp(a) displays considerable heterogeneity, with isoforms ranging in size from approximately 280-700 KDa. Apo(a) has been found to bear marked homology to plasminogen, which is involved in wound repair in blood vessels and Lp(a) has been shown to be preferentially taken up into damaged vessels when compared to LDL, when corrected for plasma levels (Lui and Lawn 1994). Isoform size has been found to correlate inversely with Lp(a) concentration (Kamboh et al. 1995), and smaller apo(a) isoforms have been found to be more atherogenic (Sandholzer et al. 1992). However the difference in apo(a) size does not fully account for differences in Lp(a) levels in the population (Azrolan et al. 1991).

One of the few consistent modulators of plasma Lp(a) levels are sex hormones. Testosterone and anabolic steroids have been shown to lower Lp(a) levels (Soma et al. 1994). In addition, hormone replacement in postmenopausal women, and estrogen given to males for the treatment of prostatic cancer have been shown to reduce Lp(a) levels (Chapter 4, *Table 4.4*, Angelin et al. 1992). The anti-estrogen tamoxifen (which also has estrogen-agonist activities), has also been shown to reduce Lp(a) levels (Wiseman 1995). The sensitivity of Lp(a) to hormonal influence has been demonstrated by the fluctuation in Lp(a) levels during the menstrual cycle of

premenopausal women (Tonolo et al. 1995), and the ability of oral estrogen therapy to lower Lp(a) levels within one week of treatment (Tuck et al. 1997, Zysow et al. 1997).

Flaxseed (linseed) has a high polyunsaturated fatty acid content, particularly the n-3 fatty acid, α-linolenic acid (C18:3 n-3), and also contains a significant amount of phytoestrogenic lignans. The two main lignans derived from dietary flaxseed, enterolactone and enterodiol, have been shown to have both weak estrogenic and anti-estrogenic properties in addition to possessing antioxidant activity (Setchell and Adlercreutz 1988, Chapter 3, *Table 3.1*). The effect of flaxseed supplementation on lipid levels has been found to result in changes in total cholesterol, LDL and TG levels which are consistent with changes seen following supplementation with other polyunsaturated and monounsaturated fatty acids (Chan et al. 1991). However, polyunsaturated fatty acids have not consistently been shown to affect Lp(a) levels (Brown et al. 1991). To date, only one study has examined the effect of flaxseed on Lp(a). Arjmandi et al. (1998) found that flaxseed supplementation resulted in a slight decrease in Lp(a) in hypercholesterolaemic postmenopausal women.

Changing the dietary fatty acid intake is a well established and often first-line therapeutic mechanism for altering LDL cholesterol levels (Zöllner et al. 1992), however Lp(a) appears to be less susceptible than LDL to changes to dietary fatty acid intake (Brown et al. 1991). *Trans*-fatty acids have been found by some to raise Lp(a) (Nestel et al.1992, Mensink et al. 1992), however others have found no effect of a high trans diet on Lp(a) (Clevidence et al. 1997). It has also been suggested that a

high intake of saturated fatty acids (particularly short chain saturated fatty acids) is associated with lower Lp(a) levels (Tholstrup et al. 1995, Clevidence et al. 1997).

To date, the effect of n-3 polyunsaturated fatty acids on Lp(a) has not been conclusively shown. The suggestion that fish oil-derived n-3 fatty acids might affect Lp(a) levels was made by Pauletto et al. (1996) following their observations in native Tanzanians of lower Lp(a) levels in a population consuming a fish-rich diet compared to a population consuming a mainly vegetarian diet. Fish oil-derived n-3 fatty acids have been reported to alter Lp(a) levels in hyperlipidaemic and coronary artery disease patients (Beil et al. 1991, Hermann et al. 1995), but this effect is not seen in normolipidaemic subjects (Markmann et al. 1997, Malle et al. 1991).

As the lignans derived from flaxseed have estrogenic activity, and hormones are one of the few consistent modulators of Lp(a), the aim of this study was to examine the effect of flaxseed on Lp(a) levels. However, flaxseed is also rich in n-3 polyunsaturated fatty acids and the influence of dietary fatty acid intake on Lp(a) was also examined to attempt to account for any non-lignan effects of flaxseed on Lp(a).

5.2 Methods

5.2.1 Study subjects and design

The influence of dietary factors on Lp(a) concentration was examined in studies conducted at CSIRO Human Nutrition and the Royal Adelaide Hospital for which ethical approval had been given by the Human Ethics Committee of CSIRO Human Nutrition, and the Royal Adelaide Hospital Ethics Committee respectively

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5.2.1.1 Flaxseed supplementation study

The effect of flaxseed supplementation on Lp(a) concentration was determined in a group of men (n=50) and a group of pre-menopausal women (n=31) who consumed 10g of ground flaxseed daily for a period of one week. The flaxseed sample contained of approximately 4g of fat (of which 2.4g was the n-3 polyunsaturate, α-linolenic acid). Lp(a) levels were measured in fasted serum samples taken before and after the one week supplementation period. Additionally apo(a) size was determined in the male group.

5.2.1.2 n-3 fatty acid supplementation study

The effect of dietary n-3 fatty acid supplementation on Lp(a) concentration was examined in healthy male subjects. Subjects were randomly divided into two groups supplemented with increasing amounts of either eicosapentanoic acid (EPA) (n=14) or docosahexanoic acid (DHA) (n=16) for a period of 4 weeks. During week 1, no supplement was consumed. In week 2, a dose of 1g/day of EPA or DHA was taken. In week 3 subjects consumed a dose of 2g/day and this was increased to 3g/day in week 4. Fasted plasma samples were obtained at the end of each week of supplementation.

5.2.1.3 Dietary fat study

The influence of changes to dietary fatty acid intake on Lp(a) concentration was determined in a group of overweight, non insulin-dependent diabetics consisting of 31 women and 8 men. Subjects were divided randomly into three groups consuming one of three different calorie-restricted diets. Diet 1 was relatively high in saturated fat (SATURATED FAT), Diet 2 had a relatively high poly/monounsaturated fat intake (POLY) and the third diet was a low fat diet (LOW FAT) with significantly lower amounts of saturated, polyunsaturated and monounsaturated fat than either of the other two diets. Lp(a) was determined in plasma samples from immediately prior to commencement of the diet, and at the end of the 12 weeks experimental diet period.

5.2.2 Measurement of Lipoprotein(a) levels

Lp(a) levels in plasma and serum samples were determined by an ELISA as described in chapter 2.5.

5.2.3 Isolation of Lipoprotein(a) for determination of apo(a) size

Blood was collected into tubes containing 1mg / ml EDTA and spun at 3000 rpm to separate plasma. The fresh plasma was removed and adjusted to d1.21 using 0.3242 g of KBr / ml of plasma. Plasma (d1.21) was placed in Beckman 16 x 76 mm PA-Quickseal tubes (Beckman Instruments, California, USA) and spun at 277816 x g in a 70.1 Ti rotor to isolate the total lipoprotein fraction of the plasma. This was removed and then dialysed against benzamidine-phosphate (-PO_4) buffer (0.0015M NaN_3, 0.0003M Na_2EDTA, 0.02M KH_2PO_4, 0.08M Na_2HPO_4, 0.001M benzamidine, pH 7.4) overnight. The total lipoprotein fraction was passed over a Lysine-Sepharose column

(XK 16/40 Pharmacia column [Amrad Pharmacia Biotech, VIC, Australia] packed with Lysine-linked to Sepharose 4B, [Sigma-Aldrich, Castle Hill VIC, Australia]) equilibrated in a benzamidine-PO_4 buffer, at a rate of 1ml / min. The column was washed with one column volume of benzamidine-carbonate-saline (0.001M benzamidine, 0.5M NaCl, 0.1M $NaHCO_3$, pH 8.3). Lp(a) was eluted by passing one column volume of 200 mM ε-aminocaproic acid (in benzamidine -PO_4 buffer) over the column, which was collected in 0.5 ml fractions using a Pharmacia FRAC-100 fraction collector (AMRAD Pharmacia Biotech, VIC, Australia).

Aliquots (70uL) of the fractions collected were transferred to sample cups and the protein content determined using the Lowry Protein assay (see chapter 2.3). The Lp(a) content of candidate fractions with high protein content (which usually eluted in fractions collected after 10-15 mls had passed through the column) was then determined using the Lp(a) ELISA method (see chapter 2.5). Following identification of Lp(a) fractions, these were pooled and concentrated using CF50A Centriflo membrane cones (Amicon, Millipore).

5.2.4 Preparation of Apolipoprotein(a) size standards

In order to produce apo(a) size standards, a number of Lp(a) isolations from different plasma samples were performed. Blood was obtained from volunteers at the CSIRO Human Nutrition Clinic, and plasma isolated by centrifugation at 3000 rpm.

Following isolation of Lp(a) as described in chapter 4.2.3, Lp(a) samples were incubated for 5 min at 100 °C with sample buffer (containing 2% β-mercaptoethanol, 1% SDS, 0.1% bromophenol blue and 5% glycerol) and then apo(a) size measured by

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Apo(a) size was calculated by determining electrophoretic mobility relative to cross-linked phosphorylase-B SDS molecular weight markers (Sigma-Aldrich). Additional apo(a) size standards in the high molecular weight range were kindly provided by Dr Joel Morrisett (Waco, TX, USA).

5.2.5 Direct Determination of Apolipoprotein(a) size from plasma

Apo(a) size was determined by a western blotting technique utilising agarose / polyacrylamide gel electrophoresis. Standards and plasma/serum samples (10-40 µl depending on Lp(a) concentration) were made up to 250 µl volume with sample buffer containing bromophenol blue as a tracking dye (see chapter 4.2.4) and incubated for 5 min in a 100°C water bath. The samples and standards were run in a GNA-100 Pharmacia gel system (Amrad Pharmacia Biotech, VIC, Australia) on a slab gel composed of 1% agarose and 4% polyacrylamide, at 30mA for a period of 3 hours. The gels were transferred to nitrocellulose overnight at 30V in buffer comprised of 0.2M glycine, 0.025M TRIS-base with 20% methanol. After transfer the nitrocellulose was blotted with skim milk powder and the apo(a) bands detected following incubation with a goat anti-human apo(a) antibody, followed by a rabbit anti-goat antibody linked with peroxidase (Incstar, MIN,USA), and then chemiluminescent detection using an ECL™ detection kit and exposure onto Hyperfilm™ (Amersham, UK).

5.2.6 Measurement of urinary lignan excretion

Lignans were measured by gas chromotography using a modification of the method of Fotsis et al. (1982). Please see Appendix B for further detail.

5.2.7 Statistical Analysis

For the flaxseed and dietary composition studies, Lp(a) levels before and after supplementation were compared directly using the Wilcoxon Matched-Pairs Signed Ranks Test for non-parametric data. Given the non-parametric distribution of both lignan and Lp(a) levels, correlations performed on data from the flaxseed study were done following log-transformation to normalise the data.

Analysis of the data from the n-3 fatty acid supplementation study was performed using a Friedman Two-Way ANOVA for non-parametric data.

5.3 Results

5.3.1 The effect of flaxseed supplementation

Daily consumption of 10g of ground flaxseed for a period of one week had no significant effect on Lp(a) concentration in a group of healthy pre-menopausal women (n=31). However in healthy males (n=50), a slight but significant increase in Lp(a) was noted after flaxseed supplementation ($p<0.05$), as shown in *Figure 5.1*. The significant increase in Lp(a) seen in males was not correlated with apo(a) isoform size (*Figure 5.2*).

Flaxseed supplementation resulted in a significant increase in the urinary excretion of the lignans enterolactone and enterodiol (*See Appendix B*). The change in Lp(a) in males was significantly correlated with the change in enterolactone excretion ($p<0.05$), but not the change in enterodiol excretion (*Figure 5.3*). Interestingly the correlation between change in Lp(a) and enterolactone excretion indicated that greater enterolactone excretion was associated with a lesser effect on Lp(a).

5.3.2 The effect of n-3 fatty acid supplementation on Lp(a) levels

In normolipidaemic subjects, treatment with increasing amounts of n-3 fatty acids EPA and DHA for a period of 4 weeks had no significant effect on Lp(a) levels. *Figure 5.4* illustrates the mean Lp(a) concentration over the treatment period. In the present study, Lp(a) was unable to be detected in six subjects and if these subjects were omitted from the data analysis, the results remain consistent. *Table 5.1* shows the median Lp(a) in subjects with detectable levels of Lp(a). Lp(a) concentrations

were in the range 0-63 mg/dl for the DHA group (n=14), and 0-122 mg/dl for the EPA group(n=10).

5.3.3 The effect of dietary fat intake on Lp(a)

In three groups of overweight, non-insulin dependent diabetic patients fed a calorie-restricted diet for a period of 12 weeks, Lp(a) did not change significantly on either the high saturated fatty acid diet (SAT) or high poly/monounsaturated fatty acid diets (POLY). However, Lp(a) concentration increased slightly, but significantly, on the low total fat diet (LOW FAT) with the mean levels rising by 48% (25% increase in median level) ($p<0.05$) (*Figure 5.5*).

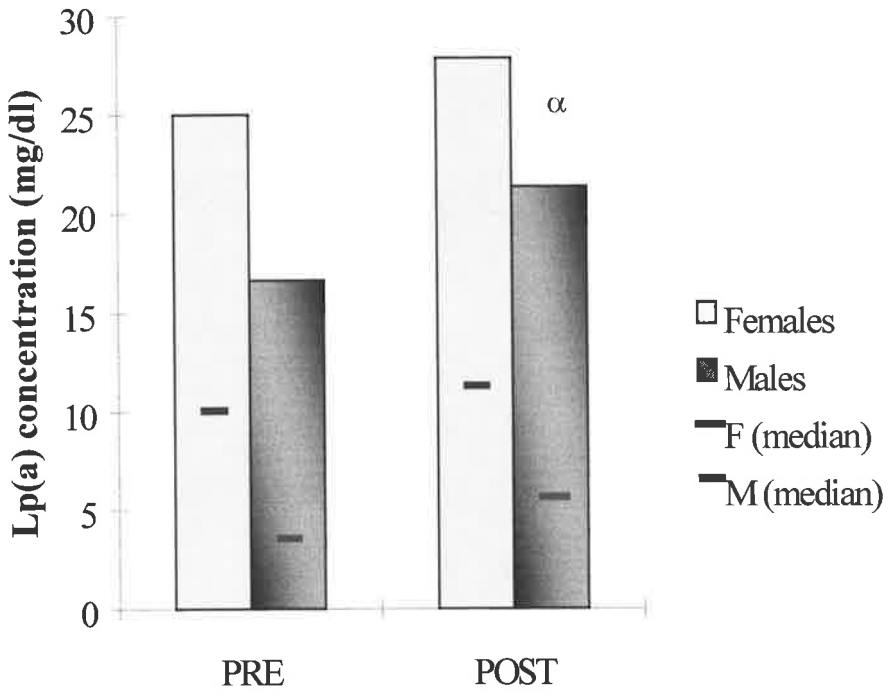


Figure 5.1 *The effect of one week of flaxseed supplementation on Lp(a) levels in men and pre-menopausal women.*

Serum Lp(a) levels were determined in males (n=50) and pre-menopausal females (n=31), before (PRE) and one week after (POST) dietary supplementation with 10g ground flaxseed/day.

The data presented in bars is mean Lp(a) (mg/dl), the data represented by the symbol (**–**) is the median Lp(a) levels for each group.

α denotes significantly different to pre-flax values ($p<0.05$).

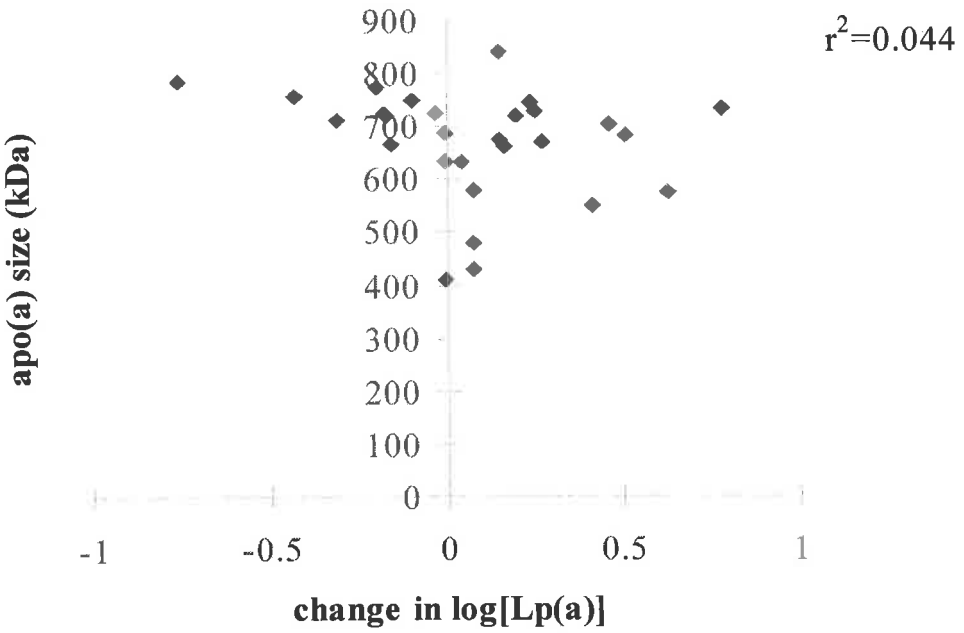


Figure 5.2 *Apo(a) isoform size is not related to the change in Lp(a) levels in response to flaxseed supplementation in healthy males.*

Apo(a) isoform size was determined by agarose /polyacrylamide gel electrophoresis followed by western blotting and detection using an enhanced chemiluminescence kit (ECL™, Amersham). Lp(a) levels were determined by a sandwich ELISA.

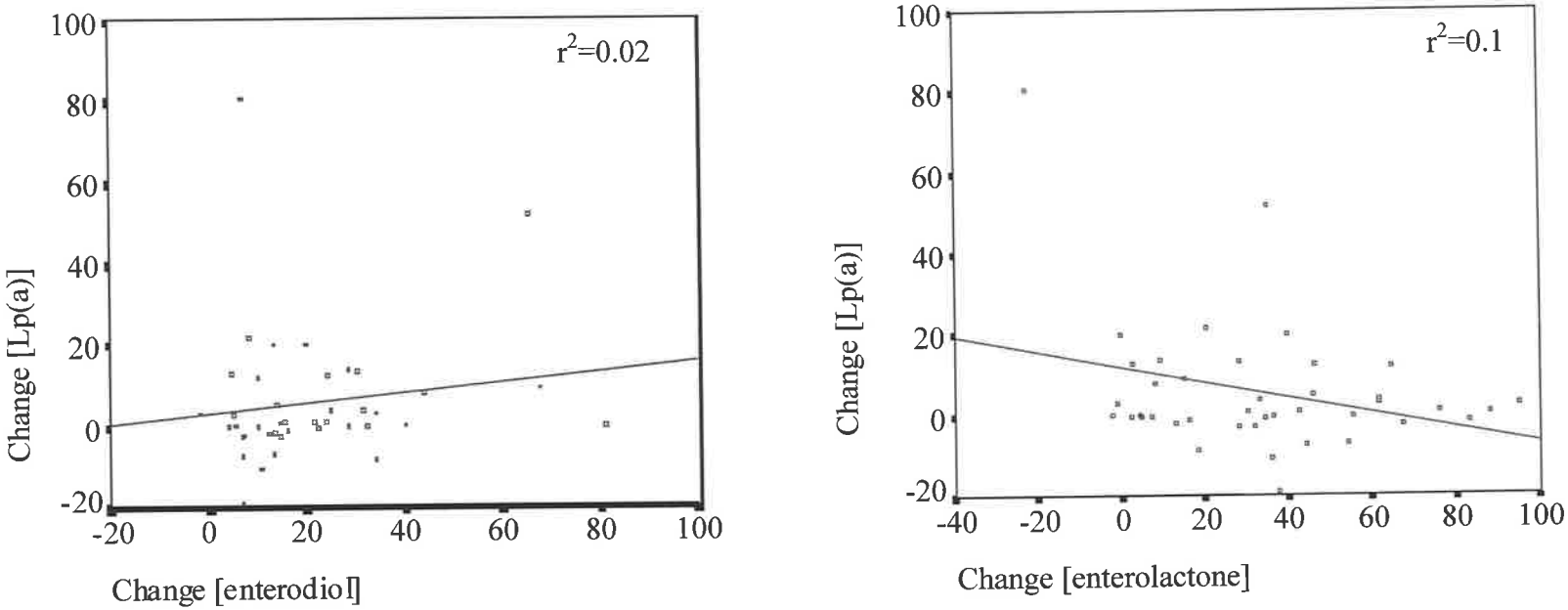


Figure 5.3 *Correlations between change in Lp(a) and urinary lignan excretion*

The change in Lp(a) with flaxseed supplementation in males was significantly correlated to the change in urinary excretion of the lignan enterolactone ($p<0.05$), but not enterodiol.

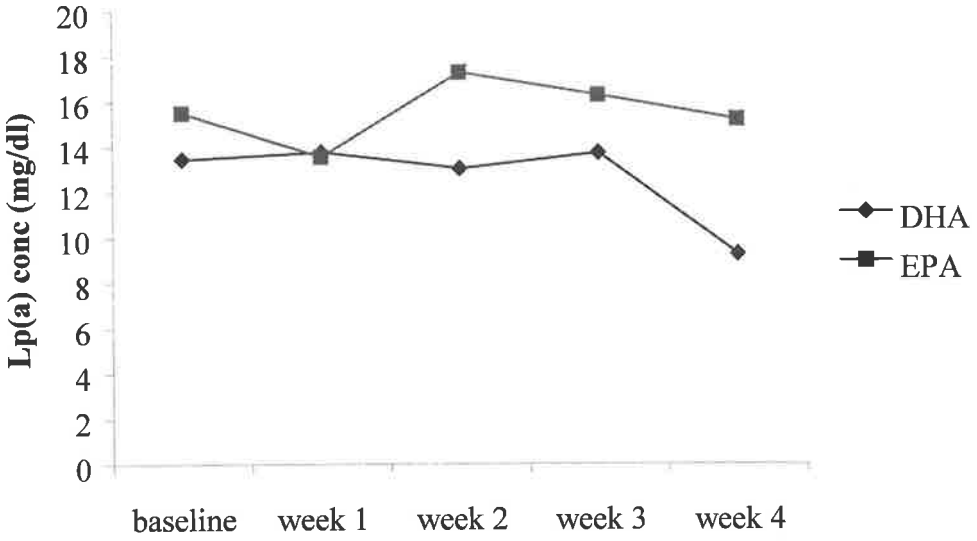


Figure 5.4 *The effect of n-3 fatty acid supplementation on Lp(a) levels.*

Plasma Lp(a) levels were measured by a sandwich ELISA after supplementation with increasing doses of either EPA or DHA over a period of 4 weeks. Week 1 was commenced with no fatty acid supplementation. During week 2, a daily dose of 1g of either DHA (n=16) or EPA (n=14) was taken. This increased to 2g/day in week 3 and then 3g/d in week 4.

Values are mean Lp(a) in mg/dl.

Lp(a): Median (range) [mg/dl]	**Baseline**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
DHA	10.6 (0 - 63.2)	9.7 (0 - 61.4)	10.7 (0 - 47.6)	12.2 (0 - 44.5)	6.9 (0 - 36.9)
EPA	9.2 (0 - 87.2)	8.2 (0 - 89.2)	10.6 (0 - 122.5)	13.0 (0 - 88.4)	9.6 (0 - 84.7)

Table 5.1 *The effect of incremental doses of EPA and DHA on median Lp(a) levels in healthy males*

Lp(a) was measured using a sandwich ELISA technique in plasma from healthy males who consumed increasing doses of either DHA or EPA over 4 weeks. During week 1 no n-3 fatty acid was taken. In week 2, subjects were supplemented with a dose of 1g/day. During weeks 3 and 4 doses of 2g/day and 3g/day respectively were taken.

The data presented is the median Lp(a) and concentration ranges of subjects with detectable levels of Lp(a) during at least one week of the study.

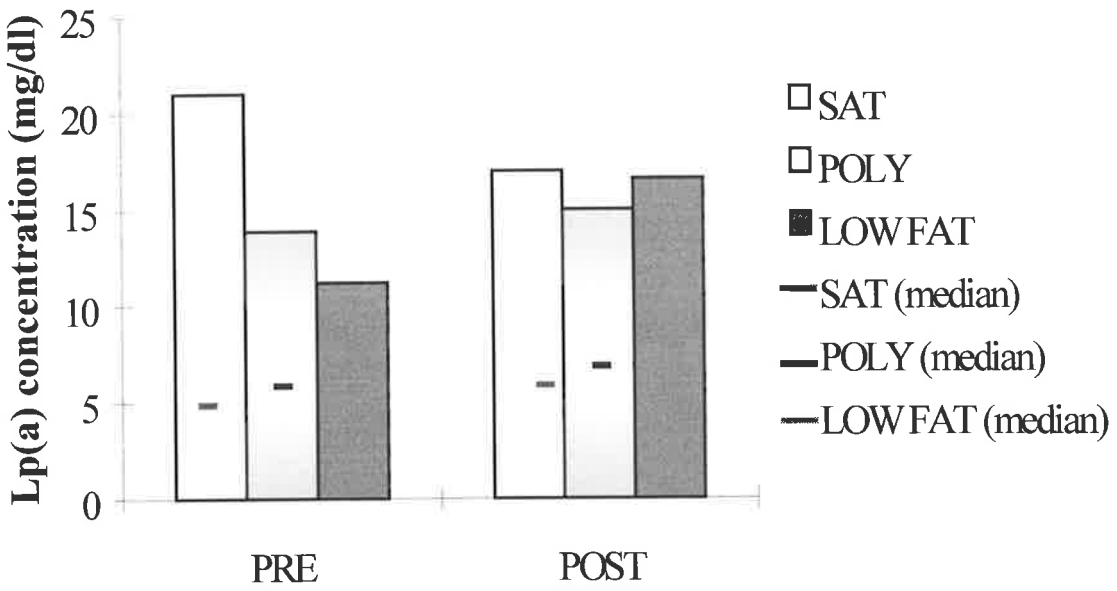


Figure 5.5 *The effect of dietary fatty acid intake on Lp(a) levels during a calorie restricted diet*

Plasma Lp(a) levels were determined in 3 groups of overweight diabetic patients before (PRE) and 12 weeks after (POST) commencement of a calorie-restricted diet rich in either saturated fatty acids (SAT), poly- and monounsaturated fatty acids (POLY) or low in total fat (LOW FAT).

The data presented in bars is mean Lp(a) (mg/dl), median Lp(a) levels are represented on the graph by lines (–).

α denotes significantly different to pre-diet values.

5.4 Discussion

The current study found that supplementation of 10g/day of ground flaxseed for one week significantly increased mean Lp(a) levels in men by 28% (equivalent to 4.7mg/dl), but did not affect Lp(a) levels in premenopausal females. Flaxseed, in addition to being a rich source of the n-3 fatty acid α-linolenic acid, is also rich in phytoestrogenic lignans which have been found to have both estrogenic and anti-estrogenic effects (Mousavi and Adlercreutz, 1992), and display antioxidant activity (*Chapter 3, Table 3.1*).

Plasma Lp(a) levels have been shown to display an inverse correlation to apo(a) isoform size (Amemiya et al. 1996, Azrolan et al. 1991), however Lp(a) phenotype has been shown not to influence the response to saturated fatty acids (Tsai et al. 1998). In the current study, the change in Lp(a) with flaxseed supplementation was also found to be unrelated to apo(a) isoform size. Thus, despite apo(a) isoform size being a significant predictor of Lp(a) levels, it appears that it is not related to the change in Lp(a) levels when Lp(a) levels are altered by dietary means.

It remains to be determined whether the slight Lp(a)-elevating effect seen with flaxseed supplementation may be attributed to the fat or lignans in flaxseed. To further examine dietary effects on Lp(a), Lp(a) was measured in two studies which had altered fat intake in the short to medium term. The possibility that the effect of flaxseed is due to its high α-linolenic acid content seems less likely as the current n-3 supplementation study found that fish oil-derived n-3 fatty acids have no significant effect on Lp(a) in

normolipidaemic men, a finding supported by those of Markmann et al. (1997), Nelson et al.(1997) and Malle et al. (1991). There was no significant difference between DHA and EPA treatments in terms of Lp(a) levels, even though these long chain n-3 fatty acids are thought to affect plasma lipids differently. It should be noted however, that there were no other changes in cholesterol or triglyceride found in the subjects in the current study following the n-3 supplementation for 4 weeks (Dr M James, personal communication). A study in normolipidaemic subjects which did find an Lp(a) lowering effect of n-3 fatty acids noted this effect in subjects with levels of Lp(a) above 20mg/dl (Schmidt et al. 1991). While other investigators have found that n-3 fatty acids reduce Lp(a) levels, this was mostly in hyperlipidaemic or CAD patients (Beil et al. 1991, Shinozaki et al. 1996, Herrmann et al. 1995). In hyperlipidaemic states, lower Lp(a) levels have been found to be associated with hypertriglyceridaemia whereas higher Lp(a) levels are associated with hypercholesterolaemia and the extent to which altering these states influences Lp(a) levels has yet to be conclusively shown (Bartens et al. 1995, Ritter et al. 1994). In the comparative study by Pauletto et al. (1996**a**), which concluded that fish oil consumption was associated with lower levels of Lp(a), the fish consuming population differed from the vegetarian population in a number of aspects. Vegetarians had significantly higher plasma cholesterol and triglyceride, and consumed significantly more alcohol (Pauletto et al. 1996**b**), although the effect of alcohol consumption on Lp(a) has yet to be clarified (Valimaki et al. 1991, Simons and Simons 1998).

The results of the study examining dietary fat intake and Lp(a) suggest that the increase in total polyunsaturated fatty acid (PUFA) intake associated with the flaxseed

consumption is also unlikely to affect Lp(a) levels. In the dietary fat study examined, PUFA intake was significantly higher on the POLY diet compared to the SAT rich diet (Heilbronn et al. 1999), and no significant difference in Lp(a) was observed following change in diet in these two groups. While the influence of dietary fatty acid content on Lp(a) remains unclear, it does appear that Lp(a) levels may be higher on a *trans* fatty acid rich diet (Nestel et al. 1992, Mensink et al. 1992) and lower on a saturated fatty acid rich diet (Clevidence et al. 1997, Aro et al. 1997). In the present dietary fatty acid study, a high saturated fat diet did not have any effect on Lp(a) levels. However the study did not control for previous dietary intake and although the SFA experimental diet contained a high proportion of saturated fat, it was calorie restricted and the total amount of saturated fat was similar to that which is seen in the average Australian diet (National Nutrition Survey 1995). While the baseline diet of the subjects in this study was not known, there was no significant change in LDL levels suggesting that the SAT diet was unlikely to have resulted in a large increase in saturated fat intake (Heilbronn et al. 1999). The diet high in PUFA, which was also higher in monounsaturated fatty acids, had no significant effect on Lp(a) levels, a finding that is supported by prior studies (Mensink et al. 1992).

Interestingly, those subjects consuming the LOW FAT diet showed a significant increase in Lp(a) levels. This diet was high in carbohydrate and low in total fat content while remaining isocaloric with the other two fat rich diets. The increase in Lp(a) seems unlikely to be due to the higher amount of carbohydrate in this diet compared to both the POLY or SAT diets, as previous studies suggest that carbohydrate has no effect on Lp(a) levels (Mehrabian et al. 1990). The dietary composition of these diets has been reported

elsewhere (Heilbronn et al. 1999), and the total fat content was significantly lower on the LOW FAT diet compared to either the POLY or SAT diets (10%, 32.9% and 32% of total energy derived from fat respectively). Accordingly the LOW FAT diet also had a significantly lower proportion of saturated fat than either of the two fatty acid-rich diets (the saturated fat content of the LOW FAT diet being 3.6% of total calories compared to 6.9% and 16.9% for the POLY and SAT diets). It is possible that the reduction in saturated fat intake on the LOW FAT diet is responsible for the increase in Lp(a). This suggestion is supported by findings from a study by Ginsberg et al (1998) which noted stepwise increases in Lp(a) as dietary saturated fatty acid intake was reduced. As Lp(a) is a very efficient vehicle for delivery of cholesterol to the periphery, it could be speculated that this response to lowering of saturated fat is a trait inherited from times when seasonal variation markedly affected nutrient intake. The atherosclerosis risk associated with the small increase in Lp(a) seen with the LOW FAT diet in the present study is likely to be more than compensated for by a reduction in total/LDL cholesterol.

It appears unlikely that the Lp(a)-elevating effect of flaxseed would be due to the antioxidant activity of flaxseed-derived lignans, as previous studies have found no effect of antioxidants on Lp(a) levels. Treatment with ascorbate in healthy adults and in patients with premature CVD was found to have no effect on Lp(a) levels (Muñoz et al. 1994, Bostom et al. 1995), nor has the antioxidant LDL-lowering drug probucol been found to affect Lp(a) levels (Naruszewicz et al. 1992).

As the effect of flaxseed on Lp(a) levels in men appears less likely to be due to fatty acid content or lignan antioxidant activity, it may be mediated by the phytoestrogenic attributes of the lignans. Lp(a) levels can be modified in the short-term (7days) hormonally, as demonstrated by adminstration of growth hormone resulting in an increase in Lp(a) (Hansen et al 1995). Two week treatment with estrogen has been found to reduce plasma Lp(a) and hepatic apo(a) mRNA in the apo(a) transgenic mouse model (Zysow et al 1997). Of the two major mammalian lignans, enterolactone appears to be a more potent phytoestrogen than enterodiol *in vitro* (Wang et al. 1994). Ingestion of 10g/day of flaxseed powder has been found to influence the menstrual cycle of pre-menopausal women, lengthening luteal phase and increasing the luteal phase progesterone:estradiol ratio (Phipps et al. 1993). Lp(a) levels have been shown to change during the menstrual cycle of premenopausal women (Tonolo et al. 1995), and the women in the current flaxseed study were not all at the same stage in their monthly cycle during the one week supplementation period, making it difficult to draw conclusions from the Lp(a) data of this group. However, the weak association noted between urinary excretion of enterolactone and change in Lp(a) in men in the present study was an inverse correlation. The relationship between plasma levels and urinary excretion of lignans has yet to be fully elucidated and appears to display wide inter-individual variability (Nesbitt et al 1999). As phytoestrogens may not have linear dose-response lipid lowering activities (Balmir et al. 1996, Dodge et al. 1996) and can behave differently in the presence or absence of endogenous estrogen (Collins et al 1997), it is clear that further studies examining different doses of lignans in populations with stable levels of endogenous estrogens are required to clarify this issue.

The only study to report the effect of flaxseed on Lp(a) was performed by Arjmandi et al. (1998) in a group of hypercholesterolaemic post-menopausal women. In this study, women fed 38g/day of flaxseed in the form of bread or muffins had significantly lower Lp(a) at the end of the six week experimental period. Past studies with fish oil n-3 fatty acids suggest that hyperlipidaemia may significantly impact the response of Lp(a) levels to treatment, and the dose of flaxseed used by Arjmandi et al. (1998) was considerably higher than was consumed in the present study. As phytoestrogens have been noted to have biphasic estrogenic effects in response to increasing dose (Anderson et al. 1998a), this may possibly account for the differences between the findings of Arjmandi et al. (1998) and that of the current study.

In conclusion, this study suggests that dietary flaxseed increases Lp(a) in men but not pre-menopausal women. As it was also found that n-3 fatty acids appear to have little effect on Lp(a) levels in normocholesterolaemic individuals, this effect of flaxseed in men appears unlikely to be due to its α-linolenic acid content. Changes to the PUFA/saturated fat content of the diet had little effect on Lp(a) as demonstrated by the dietary fat study, and so the contribution of the extra 4g/d PUFA contained in the flaxseed should not have affected Lp(a). The change in Lp(a) in men consuming flaxseed was not related to apo(a) isoform size, despite this having been found to be a significant predictor of Lp(a) levels by others (Amemiya et al. 1996, Azrolan et al. 1991). Whether phytoestrogenic properties of lignans ingested with the flaxseed resulted in a lowering of

Lp(a) needs to be examined further as both the dose of flaxseed and the magnitude of change of Lp(a) were small.

CHAPTER 6

The effect of isoflavonoid phytoestrogens on plasma lipoproteins and LDL oxidation

6.1 Introduction

The biological activity of phytoestrogens, plant derived compounds with estrogenic activity, was documented as early as 1948 by Schinckel, who observed infertility in sheep fed on subterranean clover pastures. Postmenopausal estrogen use is associated with a reduction in CVD risk, which is in part mediated by its influence on lipid metabolism (Barrett-Connor and Bush 1991). The focus of the current studies was to examine whether these estrogenic effects can be seen with phytoestrogen supplementation.

Isoflavones are a class of phytoestrogen displaying a characteristic diphenolic structure, which are found in high quantities in soy-based foods (Tham et al. 1998). A meta-analysis examining the effect of soy consumption on serum lipids by Anderson et al. (1995) concluded that soy has a hypocholesterolaemic effect, although some studies have found no effect of soy on either total or LDL cholesterol (Grundy and Abrams 1983, Sacks et al. 1983). It has been hypothesized that the isoflavones present in soy may be at least partly responsible for the hypocholesterolaemic effect (Potter 1995), and it has been shown in this thesis that the isoflavone daidzein can upregulate LDL receptor activity *in vitro* (Chapter 3). However it has also been suggested that it may be a protein component of the soy which mediates the cholesterol lowering effect (Sirtori et al. 1997).

Anthony et al. (1997) performed a study in a non-human primate model which compared diets with either intact soy, soy from which most of the isoflavones had been removed, or casein, as the source of dietary protein. It was found that the intact

soy (containing isoflavones) had the greatest effect on coronary artery lesion size with significant reductions seen. Animals fed the intact soy also had lower LDL and total cholesterol levels than animals fed either the casein or isoflavone-depleted soy diets. The hypothesis that isoflavones are involved in the cholesterol lowering effect of soy was also supported by a study from Kirk et al. (1998) in mice, in which it was found that the presence of isoflavones enhanced cholesterol-lowering in mice with functional LDL-receptors. However as alcohol extraction can also remove other components of soy such as saponins, it cannot be determined from these studies whether it is the isoflavones acting independently, interacting with another component of soy to produce the hypocholesterolaemic effects, or having no role in the cholesterol-lowering effect of soy.

In addition to their estrogenic effects, isoflavones have also been shown to have antioxidant activity, displaying the ability to inhibit *in vitro* LDL oxidation (Hodgson et al 1996, Kerry and Abbey 1998). In rats fed a high-genistein soy protein diet, it has been found that the lag time of VLDL-LDL oxidation was significantly prolonged (Anderson et al. 1998b). Supplementation of healthy volunteers (3 male, 3 premenopausal females) with soy food bars containing approximately 57mg/d of isoflavones was found by Tikkanen et al. (1998) to provide protection to LDL from oxidation, but again whether this is due purely to the isoflavones in the soy protein-containing bar cannot be determined.

As Lp(a) is sensitive to hormonal regulation (Berglund 1995), there has been interest in the effect of phytoestrogens on Lp(a) levels. Nilausen and Meinertz (1999) found that Lp(a) levels were slightly higher on soy compared to a casein rich diet. In a cross-

sectional study, Blankenship et al (1996) found that consumption of powdered soy milk in combination with a vegan diet was associated with higher Lp(a) levels than consumption of a vegan diet alone.

In the following studies the effects of isolated soy isoflavones on LDL oxidation, Lp(a) and plasma lipids will be examined, with the aim of providing insight into the ability of soy isoflavones to act independently to affect plasma lipoproteins.

6.2 Methods

6.2.1 Study designs

Analyses were performed on two studies examining isoflavone supplementation in postmenopausal women, from this point on referred to as *Studies A* and *B*.

Study A was a study co-ordinated by Professor Paul Nestel from the Baker Medical Research Institute which examined the effects of an 80mg/d soy isoflavone supplement consisting of 45mg genistein, 34mg daidzein and a small amount of glycetein. The isoflavones were aglycones i.e. hydrolysed conjugates. The study was run as a placebo-controlled cross over design, subjects were randomly assigned to either isoflavone or placebo to commence the study. Each supplement was taken for a period of 5-10 weeks and plasma samples were taken at the end of each supplementation period. The subject sample consisted of peri- and postmenopausal women (n=15) who had experienced recent menopausal symptoms and were not taking any form of hormone replacement therapy. The study was granted ethical approval by the Alfred Hospital Human Ethics Committee.

Study B was co-ordinated by Drs Richard Burnett and Peter Baghurst of the Royal Adelaide, and Women's and Children's Hospitals respectively. This study was of a double-blinded, placebo-controlled longitudinal design. Postmenopausal women who had not taken any form of hormone replacement therapy for at least three months prior to the study were eligible to participate. Postmenopausal status was defined as an absence of menstruation for a period of one year or at least 2 years since the onset

of menopausal symptoms. Subject exclusion criteria included a known sensitivity to estrogen or phytoestrogens, a history of breast cancer, endometrial cancer or venous thrombosis and recent use of isoflavone supplements. The study was granted ethical approval by the Royal Adelaide Hospital Human Ethics Committee. Serum samples were taken before and three months after the commencement of the supplement.

The subject sample consisted of 49 postmenopausal women who were randomly allocated to take either the placebo or one of two doses of isoflavone supplement (75mg or 150mg of soy-derived isoflavones) daily for a period of 3 months. The supplement consisted of approximately 41% daidzein, 51% genistein and 7% glycetein mainly in glycosidic form (ie daidzin, genistin and glycetin), the placebo consisted of anhydrous glucose. No dietary assessment or monitoring was performed as part of the study.

6.2.2 Laboratory Measurements

In *Study A*, LDL oxidation and plasma Lp(a) was measured. Isolation of LDL from plasma and subsequent Cu^{2+} mediated LDL oxidation, was performed as described in Chapter 2.6. LDL TBARS measurement was performed using the method described in Chapter 2.7.

In *Study B*, serum total cholesterol and triglyceride were measured as described in Chapter 2.4 using a Cobas Bio automated analyser and Roche Unimate cholesterol and triglyceride kits (F. Hoffman- La Roche, Basel, Switzerland). HDL cholesterol and LDL cholesterol were determined as described in Chapter 2.4. Serum Lp(a) levels

were measured using a WAKO turbidimetric Lp(a) kit (Wako Pure Chemical, Osaka, Japan) on a Cobas Bio automated analyser.

Staff at the Royal Adelaide Hospital, who were directly in contact with the study subjects, collected subject anthropomorphic data. Body mass index and age data was kindly provided by Dr Peter Baghurst. Serum levels of daidzein and genistein were measured by Ms Caroline Bignell (CSIRO Health Sciences and Nutrition) by HPLC using the method as described in King and Bursill (1998).

6.2.3 Statistical analysis

Unless otherwise stated, data is given as Mean ± SEM. Statistical analysis was performed using the SPSS for windows package (SPSS, Chicago, USA) and Microsoft Excel. Data was analysed using student's t-tests, except in the case of Lp(a) and serum isoflavones for which non-parametric tests were used on un-transformed data (Mann Whitney U tests, Wilcoxin Signed Rank tests). Multiple regression analysis was used to examine the relationship between initial (baseline) cholesterol levels and response following treatment, and ANCOVA was used to examine the influence of BMI on cholesterol levels.

6.3 Results

6.3.1 Study A

Supplementation with 80mg of soy isoflavones for a period of 5-10 weeks in 15 women who were either post- or perimenopausal, did not have any significant effect on the parameters of LDL oxidation; oxidation rate, lag time and maximum diene concentration (*Table 6.1*). Nor were there any differences in the concentration of LDL TBARS either before or after Cu^{2+} induced oxidation (*Table 6.2*).

6.3.2 Study B

The groups did not differ significantly in age or BMI at baseline, and there was no significant change in BMI for the duration of the study (*Table 6.3*). At baseline there were no significant differences in lipid levels or Lp(a) between the three groups, although mean total cholesterol levels were slightly (8%), but not significantly, lower in the 75mg isoflavone/d group compared to the 150mg/d group (*Table 6.4*).

In women treated with the placebo consisting of anhydrous glucose (n=16), there were slight, but significant, reductions in total and LDL cholesterol noted after the three-month treatment period (3% and 6% respectively), which could not be accounted for by BMI. There were no significant changes in HDL cholesterol noted in the placebo group (*Table 6.5*), and the ratio of total/HDL cholesterol did not change.

Following three months of treatment with isoflavones there were no significant changes in serum total cholesterol, LDL cholesterol, HDL cholesterol, total triglyceride or Lp(a) in either the 75mg/d and 150mg/d groups (*Tables 6.4 and 6.5*).

The meta-analysis by Anderson et al (1995) suggested that cholesterol-lowering effect of soy might only be seen in a hypercholesterolaemic population. When hypercholesterolaemic subjects (defined as those with baseline cholesterol levels greater than 5.5mmol/l) in the present study were examined separately, there remained no significant effect of either dose of isoflavones on any serum cholesterol (*Table 6.6*). In the placebo group, only the reduction in LDL cholesterol was significant (p=0.01) (*Table 6.6*).

At baseline, there were no significant differences in serum isoflavone levels between groups (*Table 6.7*). Following three months of supplementation with placebo, there was no change in either serum daidzein or genistein. Following three months of supplementation with 75mg or 150mg isoflavones/d, there were significant increases in the serum levels of both genistein and daidzein, $p<0.01$ (*Table 6.7*), suggesting study compliance was good. Serum levels of genistein and daidzein were 28% and 12% higher respectively in the 150mg isoflavone/d group compared to the 75mg isoflavone/d at study endpoint, although the differences between these two treatment groups were not significant (*Table 6.7*).

Regression analysis revealed that there was a significant relationship between the initial total cholesterol levels and the changes seen in both total and LDL cholesterol in all study participants ($r^2=0.19$, $p<0.01$ and $r^2=0.34$, $P<0.0005$ respectively, *Figure 6.1*). Power analysis (with $\alpha=0.05$ and $\beta=0.1$) using the magnitude of change attributed to soy protein from the meta-analysis by Anderson et al. (1995), suggested that n=30 would have been a more appropriate sample size in this study. The study had originally intended to recruit n=25 for each group (total study n=75), however the

death of a study investigator lead to difficulty with recruitment and the study was terminated before these numbers were reached.

	Oxidation Rate (*nmol diene/mg LDL protein/min*)	Lag time (*min*)	Maximum diene concentration (*nmol/diene/mg LDL protein*)
Placebo	14.4 ± 0.6	53.5 ± 1.5	409.0 ± 11.5
Isoflavone	14.2 ± 0.5	53.2 ± 1.4	406.0 ± 10.6

Table 6.1 *The effect of soy isoflavone supplementation on the parameters of ex vivo LDL oxidation*

In a cross-over design study, post- and peri-menopausal women (n=15) consumed a supplement consisting of either 80mg isoflavones or placebo for a period of 5-10 weeks. LDL was isolated from plasma samples taken after each supplementation period and subjected to Cu^{2+} mediated oxidation which was monitored spectrophotometrically at 234nm. Values are given as Mean ± SEM.

	LDL TBARS concentration before oxidation (*nM MDA/mg LDL protein*)	LDL TBARS concentration after oxidation (*nM MDA/mg LDL protein*)
Placebo	0.08 ± 0.02	70.14 ± 4.16
Isoflavone	0.11 ± 0.02	71.56 ± 3.53

Table 6.2 *The effect of soy isoflavone supplementation on LDL TBARS concentration before and after oxidation*

Post- and perimenopausal women (n=15) were supplemented with either placebo or 80mg isoflavones for a period of 5-10 weeks in a cross-over design study. The concentration of thiobarbituric acid reactive substances (TBARS) in isolated LDL was determined following each supplementation period by a spectrophotometric assay, and malondialdehyde (MDA) concentration estimated using the extinction coefficient for MDA at 535nm ($\varepsilon = 1.56 \times 10^5$ $L.mol^{-1}.cm^{-1}$) (Beuge and Aust 1978). Values given as Mean ± SEM.

	Age (*years*)	Baseline Body Mass Index (kg/m^2)	Endpoint Body Mass Index (kg/m^2)
Placebo	56.6 ± 1.9	27.4 ± 1.5	27.4 ± 1.5
75mg/d Isoflavone	53.5 ± 1.3	26.8 ± 1.4	27.0 ± 1.4
150mg/d Isoflavone	56.0 ± 1.8	27.2 ± 1.0	27.3 ± 0.9

Table 6.3 *Subject characteristics, Study B*

Values are given as Mean ± SEM. The numbers in the placebo, 75mg/d and 150mg/d groups were n=15, 18 and 16 respectively. There were no significant differences between groups or within groups at study endpoint.

	Total Cholesterol (*mmol/l*)		HDL cholesterol (*mmol/l*)		Total/HDL cholesterol	
	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint
Placebo	6.11 ± 0.36	5.92 ± 0.36 $^{\alpha}$	1.7 ± 0.17	1.73 ± 0.17	4.15 ± 0.47	3.86 ± 0.40
75mg/d Isoflavone	5.74 ± 0.22	5.70 ± 0.20	1.57 ± 0.11	1.8 ± 0.15	4.00 ± 0.33	3.54 ± 0.31
150mg/d Isoflavone	6.23 ± 0.29	6.02 ± 0.25	1.45 ± 0.15	1.48 ± 0.12	4.90 ± 0.47	4.53 ± 0.42

Table 6.4 *The effect of soy isoflavone supplementation on serum lipids in postmenopausal women*

Postmenopausal women were randomly allocated to one of three treatments; placebo (n=15), 75mg isoflavones daily (n=18) or 150mg isoflavones daily (n=16), for a period of three months. Serum samples were collected before (baseline) and after (endpoint) treatment.

$^{\alpha}$ denotes significantly different to baseline value within group, $p<0.05$.

	Triglyceride (*mmol/l*)		LDL cholesterol (*mmol/l*)		Lipoprotein(a) (*mg/dl*)	
	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint
Placebo	1.51± 0.18	1.54 ± 0.25	3.73 ± 0.35	3.50 ± 0.30$^{\alpha}$	17.5 (0-79.8)	18.9 (0-72.2)
Isoflavone (75mg/d)	1.55 ± 0.19	1.50 ± 0.17	3.35 ± 0.28	3.35 ± 0.24	16.1 (0-62.8)	16.4 (0-72)
Isoflavone (150mg/d)	1.65 ± 0.16	1.58 ± 0.15	4.05 ± 0.31	3.83 ± 0.25	14.0 (8.2-66.4)	16.1 (6.4-64.5)

Table 6.5 *The effect of isoflavone supplementation on serum levels of Triglycerides, LDL cholesterol and Lipoprotein(a)*

In a longitudinal study, postmenopausal women received either 75mg/d (n=18) or 150mg/d (n=16) of isolated isoflavones or placebo capsules (n=15) for a period of three months. Values given as Mean ± SEM for triglyceride and LDL cholesterol and Median (Range) for Lipoprotein(a). $^{\alpha}$ denotes significantly different to baseline value, $p<0.05$.

	Total Cholesterol (*mmol/l*)		LDL cholesterol (*mmol/l*)		HDL cholesterol (*mmol/l*)	
	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint
Placebo	6.77 ± 0.39	6.52 ± 0.41	4.17 ± 0.46	3.86 ± 0.40[α]	1.87 ± 0.23	1.88 ± 0.21
75mg/d Isoflavone	6.35 ± 0.18	6.26 ± 0.11	4.03 ± 0.23	3.87 ± 0.14	1.49 ± 0.09	1.58 ± 0.09
150mg/d Isoflavone	6.67 ± 0.28	6.34 ± 0.25	4.46 ± 0.32	4.15 ± 0.25	1.42 ± 0.18	1.44 ± 0.15

Table 6.6 *The effect of isoflavone supplementation on serum lipids of hypercholesterolaemic postmenopausal women from Study B (baseline cholesterol > 5.5 mmol/l).*

In those women with baseline cholesterol levels >5.5mmol/l, there was no effect of isoflavones on serum lipids following three months of supplementation. Numbers in these placebo, 75mg/d and 150mg/d subgroups were n=10, 11 and 12 respectively. Values give as Mean ± SEM. [a] denotes significantly different to baseline, $p<0.05$.

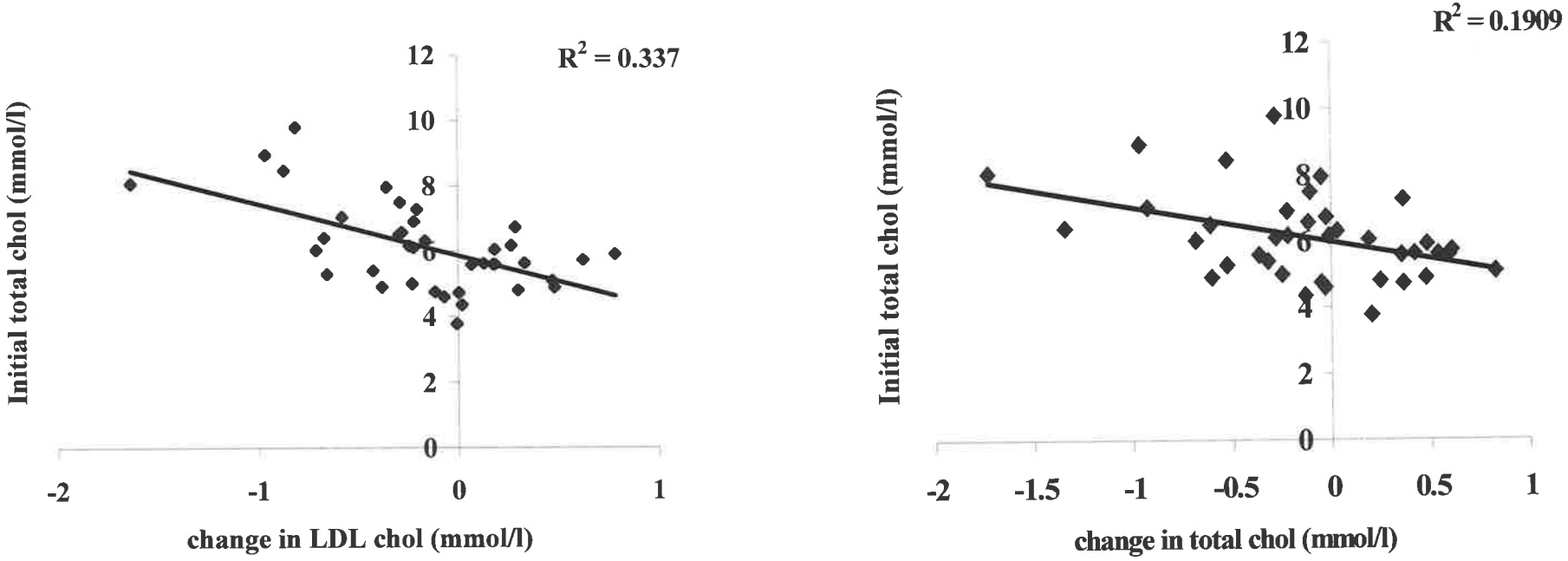


Figure 6.1 *The relationship between initial (baseline) cholesterol levels and the change in LDL and total cholesterol upon completion of the study*

Data is shown for all study subjects combined, and represents change in LDL and total cholesterol (visit2-visit1) versus initial (baseline) cholesterol levels.

	Serum Daidzein (μM)		Serum Genistein (μM)	
	Baseline	Endpoint	Baseline	Endpoint
Placebo	0.15 (0.07)	0.06 (0.00)	0.07 (0.04)	0.04 (0.00)
75 mg/d Isoflavone	0.06 (0.00)	0.43 (0.24)$^{\alpha}$	0.09 (0.00)	0.74 (0.45)$^{\alpha}$
150mg/d Isoflavone	0.22 (0.13)	0.48 (0.45)$^{\alpha}$	0.21 (0.10)	0.95 (0.80)$^{\alpha}$

Table 6.7 *Serum levels of daidzein and genistein following three months of isoflavone supplementation*

Values are given as Mean (Median). For placebo, 75mg/d Isoflavone and 150mg/d Isoflavone groups, n=15, 18 and 16 respectively.

$^{\alpha}$ denotes significantly different to baseline within the group, $p<0.01$.

6.4 Discussion

The study examining the effect of isoflavones on LDL oxidation (*Study A*) found that supplementation with 80mg of soy isoflavones in post- and perimenopausal women for a period of 5-10 weeks did not confer any significant protection to isolated LDL. An effect on systemic arterial compliance was seen in *Study A* of similar magnitude to that noted with conventional hormone replacement therapy (Nestel et al. 1997), indicating that the isoflavones were biologically active. While the model of LDL oxidation used in our study has deficiencies, notwithstanding that it does not allow measurement of the contribution of aqueous antioxidants present *in vivo*, this oxidation model is similar to that used by Tikkanen et al (1998) in their study of soy supplementation and LDL oxidation in healthy adults aged 20-30 years of age. Tikkanen et al. (1998) found that consumption of soy bars (containing soy protein that provided 57mg/d of isoflavones) resulted in protection against LDL oxidation as indicated by an increase in lag time, after only 2 weeks. In a study conducted by Anderson et al (1998) in a rat model it was found that feeding with a soy protein isolate rich in genistein resulted in a significant increase (49%) in lag time of VLDL-LDL oxidation. In addition, a soy protein isolate low in genistein was examined and this treatment also increased lag time (43%). This raises the possibility that there may be an additional component of soy that either facilitates the antioxidant activity of the isoflavones, or is solely responsible for the antioxidant effect seen by Tikkanen et al. (1998) and Anderson et al. (1998b).

Soybean saponins, like isoflavones, are ethanol extractable components of soy. However while they have been shown to have the ability to protect cells against

hydrogen peroxide-mediated cell damage (Yoshikoshi et al. 1996), in the presence of a peroxyl radical generator, soyasaponin I was not able to display antioxidant activity (Tsujino et al. 1994). While the possibility exists that there may be an as yet undiscovered antioxidant component of soy, there is evidence to support a role for isoflavones as antioxidants. Previous studies in this laboratory and studies by others have shown that the isoflavones genistein and daidzein can protect LDL from oxidation *in vitro* (Hodgson et al. 1996, Kerry and Abbey 1998). However it appears that the amounts of genistein incorporated into the LDL particle when directly incubated with genistein *in vitro,* are not sufficient to protect the particle against subsequent oxidation (Kerry and Abbey 1998). Recently it has been suggested that the formation of isoflavone fatty acid esters may enhance the incorporation into LDL particle to allow protection against oxidation (Meng et al. 1999). Estrogen fatty acid esters are formed both in the blood and liver and retain potent estrogenicity (Pahuja and Hochberg 1995), and estradiol fatty acid esters have been found to be the major form of estradiol associated with the LDL particle (Shwaery et al. 1997). Estradiol esters enhance resistance of LDL to oxidation, suggesting that estradiol also retains antioxidant activity when esterified (Shwaery et al. 1997). There is currently little known regarding the existence or activity of isoflavone-fatty acid esters. Meng et al. (1999) found that selected isoflavone-oleic acid esters enhanced the resistance of LDL particles to oxidation, however there was no direct correlation between the concentration of the fatty acid esters and the lag time of oxidation, suggesting that they may not be acting by directly scavenging lipid peroxyl radicals (Meng et al. 1999). The incorporation of the isoflavone-oleic acid esters was also quite variable between LDL from different individuals, suggesting that some other factor such as LDL lipid or antioxidant content may impact upon the level of incorporation (Meng et

al. 1999). In our study (*Study A*) subjects were asked to refrain from taking supplements such as vitamin E or evening primrose oil, and avoid soy-based foods (Nestel et al. 1997). In the study by Tikkanen et al. (1998) in addition to reducing soy intake and vitamin supplements, subjects also reduced the intake of polyunsaturated fat by minimising vegetable oil consumption and eliminating nuts and seeds from the diet. During the soy bar consumption period of the study, there would have been a 7.5g increase in daily fat intake, however detailed dietary information was not reported, so what impact this may have had upon oxidation parameters cannot be assessed (Tikkanen et al. 1998).

Animal studies suggest that isoflavones contribute significantly to the hypocholesterolaemic effects of soy (Anthony et al. 1997, Kirk et al. 1998), although these studies are also limited by the fact that extraction of isoflavones may result in the removal of other components of soy. An isoflavone-containing ethanol/acetone extract of soy has been shown to have a plateau effect in terms of cholesterol lowering in rats and hamsters (Balmir et al. 1996), and in ovariectomised rats, the cholesterol lowering effects of genistein plateaued over a dose range of 0.1-10mg/kg and only resulted in a further reduction in cholesterol at doses greater than 10mg/kg (Dodge et al. 1996).

In the meta-analysis of soy protein consumption performed by Anderson et al. (1995) it was proposed that the cholesterol lowering effect of soy was mostly due to the isoflavones, however human trials of isolated isoflavones have yet to provide strong evidence for this. In a study by Hodgson et al. (1998) in healthy men and postmenopausal women, no change in lipid levels were noted following 8 weeks of

supplementation with a 55mg/d dose of isolated clover-derived isoflavones. A similar finding was made by Nestel et al (1999) in mildly hypercholesterolaemic postmenopausal women supplemented with 80mg/d of clover-derived isoflavones. The findings of these human trials of isolated isoflavones suggest that isoflavones are not wholly and directly responsible for the hypocholesterolaemic effect of soy. This is further supported by a recent study in a cynomolgus monkey model by Greaves et al. (1999) which compared intact soy protein to casein supplemented with an isoflavone rich extract. It was found that the intact soy diet resulted in lower total and LDL cholesterol than the casein + isoflavone diet, despite identical levels of isoflavones in each diet (Greaves et al. 1999).

The current study (*Study B*) found that when examining the groups of postmenopausal women as a whole, there was no cholesterol lowering effect of either 75mg/d or 150mg/d of isolated soy-derived isoflavones. These findings provide support for those of Nestel et al (1997) and Hodgson et al (1998) who found no hypocholesterolaemic effect of isoflavones at a dose of 80mg/d. Further to this, the current study demonstrates that the lack of effect of such isoflavone supplementation alone may not be able to be ascribed to insufficient dose. Results from measurement of serum isoflavones revealed that the doubling of dose of isoflavone intake from 75 to 150mg/d only resulted in a 12-28% increase in serum isoflavone levels, suggesting that the limit of absorption may have been reached at these high levels of supplementation. The serum isoflavone levels achieved in the present study at a dose of 150mg/d were similar to those seen in a study by Gooderham et al (1996) who supplemented with soy protein containing 130mg isoflavones. This would suggest that compliance in the current study was good and thus cannot account for the lack of

effect in the treatment group. While glycosides (as used in the current study B) are reportedly absorbed less effectively than the aglycone form (Izumi et al 2000), we are unable to compare this to studies using aglycone supplements, which have not examined plasma isoflavone levels post-supplementation (Hodgson et al 1998, Nestel et al 1997, Nestel et al 1999).

Initial cholesterol levels have been found to have a powerful effect on changes seen with soy supplementation, and it was suggested by Anderson et al. (1995) that the hypocholesterolaemic effect of soy might only be seen in those with elevated cholesterol levels. In the current study, in those defined as hypercholesterolaemic (baseline cholesterol >5.5mmol/l), there remained no effect of either dose of isoflavones on serum lipids.

In Study B there was a slight lowering of cholesterol noted in the placebo group which is difficult to explain. Whether attitudes toward health and diet changed with study participation was not assessed, and should be considered for future studies to allow for further examination of such a placebo effect. We noted a significant relationship between the lipid changes at endpoint and the baseline cholesterol levels, and as the placebo group had the highest cholesterol levels upon commencement of the study, this placebo effect might be explained by regression toward the mean in the data set. There was no significant change in the total/HDL cholesterol ratio in the placebo group, which suggests this slight cholesterol lowering was of little clinical relevance.

Baum et al (1998) examined the effect of soy protein containing one of two doses of isoflavones, such that the postmenopausal hypercholesterolaemic subjects (defined by total cholesterol between 6.2mmol/l and 7.8 mmol/l) were consuming either 55mg/d or 90mg/d of isoflavones in equal amounts of soy protein. Interestingly the control group in the study by Baum et al (1998), had initial plasma 'non-HDL' cholesterol levels that were lower than either of the treatment groups, and these levels were lowered by 6% after 18 weeks of control treatment compared with a 7% lowering in the highest isoflavone treatment group at the same time. However after 24 weeks, at both doses of isoflavones there were significant reductions in non-HDL cholesterol (primarily LDL+VLDL cholesterol) with no difference between the two isoflavone doses. This may be due to a plateau effect of the isoflavones, as has been shown in animal models (Dodge et al. 1996, Balmir et al. 1996), or may indicate that the hypocholesterolaemic effect of soy is isoflavone independent.

Crouse et al. (1999) investigated the effects of differing amounts of isoflavones contained within a fixed amount of soy protein, but found that only the highest dose of isoflavones (62mg) in 25g of soy protein, was able to elicit a significant cholesterol-lowering effect in the moderately hypercholesterolaemic subjects. This does not support the postulation by Sirtori et al. (1997) that a protein component of soy is entirely responsible for the cholesterol lowering effect. Clearly studies which manipulate not only the isoflavone level, but also the protein level are required to examine any potential interaction between soy protein and isoflavones and determine whether either of these components of intact soy protein can be manipulated to maximise its cholesterol lowering effect.

Baum et al. (1998) found that a daily intake of soy protein containing either 56 mg or 90mg of isoflavones/day increased HDL cholesterol in hypercholesterolaemic postmenopausal women with no difference in the magnitude of the increase between the isoflavone doses. However a study by Gooderham et al. (1996) found no effect of soy protein on HDL cholesterol concentration in normocholesterolaemic men, and a similar finding was made by Crouse et al. (1999) in a large sample of mildly hypercholesterolaemic men and women. Thus while it appears that the effect of soy protein on HDL is not consistent, the current study (*Study B*) provides further evidence to support the studies suggesting that isolated isoflavones have little impact upon HDL levels (Nestel et al. 1999, Hodgson et al. 1998).

Studies in postmenopausal women have suggested that isoflavones can have estrogenic effects *in vivo* (Albertazzi et al. 1998, Baird et al. 1995, Nestel et al. 1997). As estrogens are known to regulate Lp(a) levels (Berglund 1995, Tonolo et al. 1995), the effect of the 75 and 150mg/d doses of soy isoflavones on Lp(a) levels were examined in *Study B*. Average Lp(a) levels were a little higher than is often reported (Nilausen and Meinertz 1999), which would perhaps enhance the likelihood of showing a change, however no significant effect of either of the isoflavone doses on Lp(a) levels was noted in the current study. In a study by Nilausen and Meinertz (1999) it was found that Lp(a) increased 20% after the male subjects changed from a self-selected diet to a soy protein diet ($p=0.065$), however this study may well have been influenced by a change in saturated fat intake when change to the soy protein diet was made. Anthony et al. (1996) in a rhesus monkey model, found that a soy protein isolate decreased Lp(a) in female monkeys, with no significant change noted in male monkeys. The results of the present study suggest that soy-derived

phytoestrogens do not affect Lp(a) levels in postmenopausal women, and provide evidence in support of the findings from the well-controlled study by Hodgson et al. (1998) in which isolated clover derived isoflavones (consisting mostly of genistein) had no effect on Lp(a) in a predominantly male subject group.

The current studies provide evidence to suggest that isoflavone supplementation alone is not able to provide protection to LDL against oxidation *ex vivo,* unlike that reported for whole soy supplementation (Tikkanen et al. 1998). However the LDL oxidation model used in this study is not able to account for the contribution of isoflavones carried in the plasma compartment which may provide additional protection against oxidation *in vivo*. Furthermore, the results of the studies presented in this thesis suggest that isoflavones alone are unlikely to exhibit a hypocholesterolaemic effect in post-menopausal women, with both moderate and high dose isoflavone supplementation having no effect of plasma lipids. The dose of 150mg isoflavones/d is equivalent to almost 4 times the reported average dietary intake for Japanese women (Kimira et al. 1998). Currently, the long-term effects of such high dose isoflavone supplementation are not known, this warrant further investigation if isoflavone supplements are to be marketed therapeutically.

CHAPTER 7

General Discussion

7.1 Estrogens, Phytoestrogens and LDL Cholesterol

It has been estimated that 25-50% of the reduction in CVD risk associated with postmenopausal estrogen use can be attributed to beneficial changes in the plasma lipid profile, and an estrogen mediated reduction in LDL cholesterol is thought to be a significant component of these changes (Barrett-Connor and Bush 1991). When the present investigation examined the effect of the three major human estrogens; estradiol, estrone and estriol, on LDL receptor activity *in vitro,* it was found that estradiol displayed the greatest ability to enhance LDL receptor activity (*Chapter 3*). LDL receptor activity was measured by the association and uptake of gold-labelled LDL particles into the human hepatoma cell line, HepG2. This method was chosen as it is safer and cheaper than comparable LDL labeling methods (e.g. I^{125}-LDL), and has a CV of <10% (Roach et al 1993). While measurement of LDL receptor mRNA could also have been used, previous studies have shown that a direct correlation between LDL receptor activity and LDL receptor mRNA does not always exist, and without another measure of transcription, this could not be confidently used as a measure of LDL receptor activity. Ideally LDL receptor activity, LDL receptor mass and LDL receptor mRNA should be used in conjunction to get a comprehensive picture of LDL metabolism in the cell model. In the studies presented here, the ability of estrogens to increase LDL receptor activity parallels both the estrogenic and antioxidant activities of estradiol relative to estrone and estriol (Jordan and Koch 1989, Shwaery et al. 1998), and thus the relative contribution of the estrogenic and antioxidant activities toward upregulation of LDL receptor activity cannot be distinguished from the current study. Further work examining estradiol-mediated LDL receptor regulation in HepG2 cells in the presence of a free radical generator, such as

2,2 azobis (2-amidinopropane) dihydrochloride, may help in determining the relative contributions of estrogenic and antioxidant activities to this effect. For whatever reason, it is of interest to note that the abilities of estradiol and estrone to regulate LDL receptor activity *in vitro* were found to be markedly different in the current study. This may potentially have an impact in situations where there may be differences in the balance of estradiol:estrone, such as is seen in lean versus obese men (Tchernof et al. 1995), and where the activities of the sex hormone modifying enzymes aromatase and 17β-hydroxysteroid oxidoreductase may influence local estrogen levels.

In a previous cross-sectional study we found that postmenopausal women not using HRT had lower LDL receptor activity than those using HRT (Abbey et al 1999). The present longitudinal investigation of HRT in postmenopausal women noted a decrease in LDL cholesterol levels, but this was not reflected in an increase in mononuclear cell LDL receptor activity (*Chapter 4*). This provides evidence to support the finding by Colvin (1996) of an estrogen-mediated increase in LDL receptor independent catabolism of LDL, although the aforementioned catabolic pathways have yet to be fully elucidated. Caution should also be used in examining these results as it has yet to be conclusively shown that both mononuclear cells and the liver display the same type of estrogen receptor, and so will have the same co-ordinate responses to estrogens.

In the current investigations a number of phytoestrogens were found to increase LDL receptor activity *in vitro* (*Chapter 3*). Again, as with the estrogens, what proportions of the LDL receptor activity upregulation can be attributed to antioxidant and estrogenic activities is difficult to determine, as for example, the most estrogenic of

the phytoestrogens, coumestrol, was also the most potent antioxidant. The isoflavones daidzein, biochanin A and formononetin all exhibited the ability to increase LDL receptor activity. This finding is of interest when examining the literature relating to the hypocholesterolaemic effects of dietary soy protein. There is currently much debate as to which component of soy is mediating the cholesterol lowering effect (Potter 1995), for which the main candidates appear to be either the isoflavones and a protein component of soy. It is interesting to note that in the study by Baum et al. (1998), which looked at two different doses of isoflavones in a standard amount of soy protein, no difference in either cholesterol lowering or mononuclear cell LDL receptor activity was noted between the two isoflavone doses, whereas studies using varying amounts of soy isoflavones in a fixed soy protein mass suggested a dose response effect on cholesterol levels (Crouse et al 1999). The present investigations of isolated isoflavones at two different moderate-high doses found no significant effect upon cholesterol levels. However a significant placebo effect was noted which might be manifestation of regression toward the mean in the data set, but clearly reinforces the importance of the placebo group in such studies (*Chapter 6*).

Animal studies have demonstrated the importance of isoflavones and functional LDL receptors in the cholesterol-lowering effect of soy, but as demonstrated in the current thesis, isoflavones alone are not able to mediate this hypocholesterolaemic effect. This suggests at least two possibilities; 1) that there may be an interaction of the isoflavones with some other component of soy, or 2) that an as yet undiscovered bioactive compound is present in soy that is extractable in exactly the same manner as isoflavones. Studies able to manipulate the isoflavone content of soy by adding

purified isoflavones should be most useful in elucidating the mechanisms mediating the cholesterol-lowering effect of soy.

7.2 Estrogens, Phytoestrogens and LDL oxidation

There is considerable disparity between studies examining the effect of HRT on LDL oxidation, which does not appear to be able to be accounted for by differences in dose or type of HRT (*Chapter 4.4*). The present investigations found no evidence that postmenopausal hormone replacement therapy has an effect on '*ex vivo*' LDL oxidation (*Chapter 4*). This method of examining LDL oxidation is limited by the fact that is not able to take into account the contribution of non LDL-associated estrogen. As only 2-3% of plasma estrogen has been estimated to be carried in LDL (Leszczynski and Schafer 1989), this obviously reduces the sensitivity of the *ex vivo* LDL oxidation method. However as some have found effects of exogenous estrogen on LDL oxidisability using these methods (Schroder et al. 1996, Sack et al. 1994, Wakatsuki et al. 1998), this suggests that work is needed to examine what effect small changes in LDL-estrogen concentrations *in vivo* may have upon LDL oxidisability. Recently a study by Hoogerbrugge et al. (1998) found that while estrogen replacement therapy did not alter the *ex vivo* oxidisability of LDL, there was a significant reduction in the concentration of plasma antibodies against an epitope of oxidised LDL. New biomarkers of LDL oxidation such as that examined by Hoogerbrugge et al. (1998), and isoprostanes (Witztum and Berliner 1998) provide promise for increased sensitivity in examining LDL oxidation.

In addition to the lack of effect of HRT on LDL oxidisability, the current studies also found no effect of isolated isoflavones on *ex vivo* LDL oxidation (*Chapter 6*). These findings are in contrast to those of Tikkanen et al. (1998) who found that soy supplementation, with a slightly lower isoflavone level to that of the present study, decreased LDL oxidisability as measured using a similar *ex vivo* LDL oxidation method. It has recently been suggested that the presence of isoflavone fatty acid esters within the LDL particle may be mediating the antioxidant effect (Wang et al. 1999). Whether the whole soy consumption (as opposed to the isolated isoflavone supplementation used in the present study) can enhance the production of isoflavone fatty acid esters has yet to be established. There is also the possibility that there may be a novel antioxidant contained within whole soy that can account for the difference between the present study and that of Tikkanen et al. (1998).

7.3 Estrogens, Phytoestrogens and Lp(a)

The present investigation found that postmenopausal HRT reduced levels of the atherogenic Lp(a) (*Chapter 4*). The effect of phytoestrogens on Lp(a) is less clearly defined, and given the sensitivity of Lp(a) to hormonal regulation (Zysow et al. 1997), is likely to be confounded by the relative estrogenicity/antiestrogenicity of the isoflavones. This in turn is likely to be influenced by the levels and types of endogenous steroids in the population being examined. In postmenopausal women, the current studies found no effect of isolated soy isoflavones on Lp(a) (*Chapter 6*), however it appeared that flaxseed, containing significant amounts of phytoestrogenic lignans, may influence Lp(a) levels in men (*Chapter 5*). However further studies with isolated lignan supplementation would need to be undertaken to determine whether

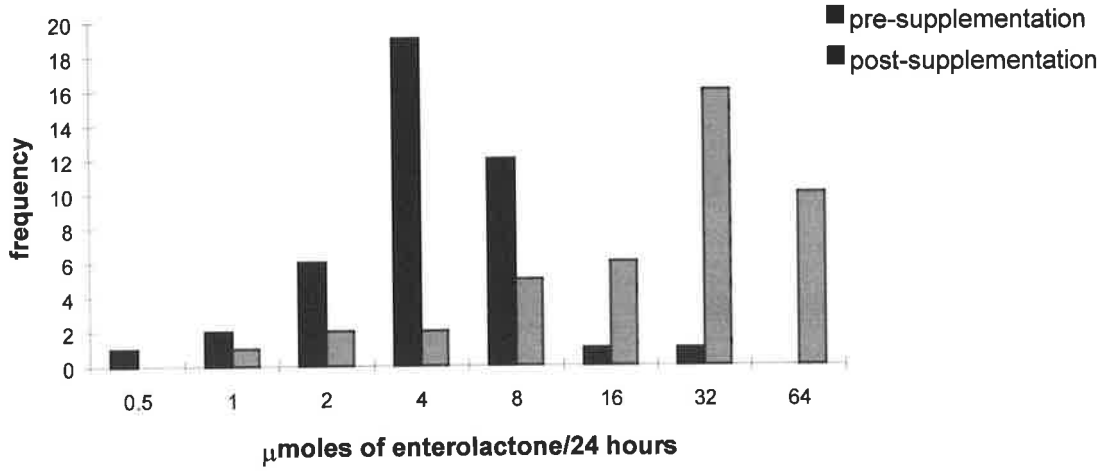
these lignans were indeed mediating the Lp(a) elevating effect. Such studies seem warranted given the apparent rise in popularity of phytoestrogen-containing foods and supplements.

Appendix

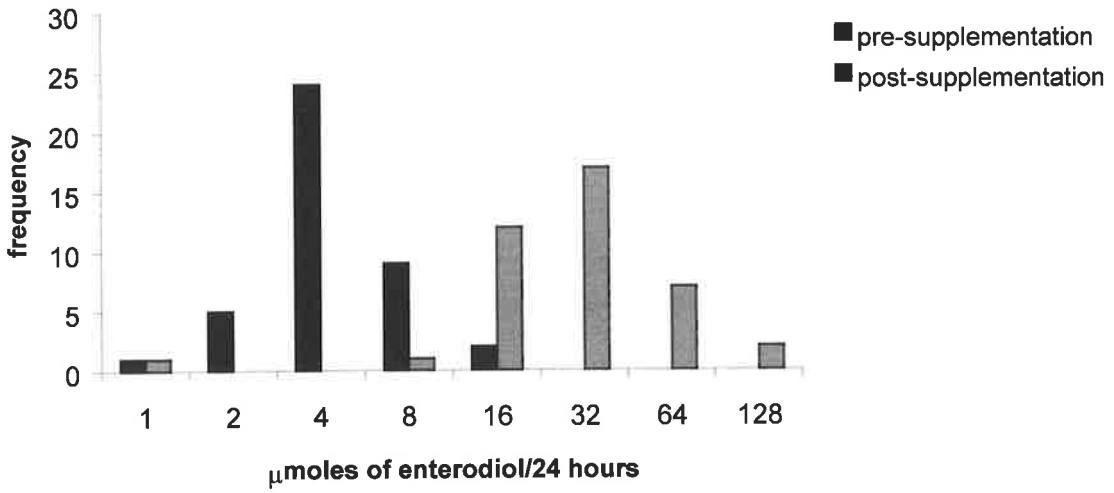
The enterodiol and enterolactone levels in urine were measured by Dr Wayne Luderer (CSIRO Human Nutrition) using a method based on that of Fotsis et al. (1982). Briefly, 10ml aliquots were taken from a 24 hour urine collection (to which ascorbic acid had been added). Lignans were extracted from the samples through C18 solid phase cartridges and Sephadex columns and then the extracted sample was hydrolysed, re-extracted using C18 cartridges and derivatised. The enterodiol and enterolactone concentrations of the sample extracts were determined by gas chromatography, using a Hewlett-Packard 5711 with a 0.53mm x 15m bonded phase vitreous silica column, hydrogen carrier gas at a flow rate of 5.5ml/min, and a temperature program incrementing from 200 °C to 300°C a rate of 8°C/min. The method utilised 5α-cholestane as an internal standard.

Urinary excretion of enterolactone and enterodiol was expressed as μmoles excreted/24 hours. Both enterolactone and enterodiol excretion increased markedly with flaxseed supplementation, as shown in the frequency distributions presented on page 177.

Urinary excretion of enterolactone following 1 week of 10g/day supplementation of flaxseed



Urinary excretion of enterodiol following 1 week of 10g/d supplementation of flaxseed



Bibliography

Abbey M, Belling BG, Noakes M, Hirata F, Nestel PJ. Oxidation of low density lipoproteins: intraindividual variability and the effect of dietary linoleate supplementation. *American Journal of Clinical Nutrition*. 1993;**57**:391-398.

Abbey M, Nestel PJ, Baghurst PA. Antioxidant vitamins and low-density lipoprotein oxidation. *American Journal of Clinical Nutrition*. 1993;**58**:525-532.

Abbey M, Owen A, Suzukawa M, Roch P, Nestel PJ. Effects of menopause and hormone replacement therapy on plasma lipids, lipoproteins and LDL receptor activity. *Maturitas*. 1999;**33**:259-269.

Abe A, Noma A. Studies on apolipoprotein(a) phenotypes. Part 1. Phenotype frequencies in a healthy Japanese population. *Atherosclerosis*. 1992;**96**:1-8.

Adams MR, Kaplan JR, Manuck SB, Koritnik DR, Parks JS, Wolfe MS, Clarkson TB. Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys. *Arteriosclerosis*. 1990;**10**:1051-1057.

Akiyama T, Ishida J, Nakagawaa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry*. 1987;**262**:5592-5595.

Albers JJ, Kennedy H, Marcovina SM. Evidence that Lp(a) contains on molecule of apo(a) and one molecule of apoB: evaluation of amino acid analysis data. *Journal of Lipid Research*. 1996;**37**:192-196.

Alftan G, Aro A, Gey KF. Plasma homocysteine and cardiovascular disease mortality. *Lancet*. 1997;**349**:397.

Amemiya H, Arinami T, Kikuchi S, Yamakawa-Kobayashi K, Li L, Fujiwara H, Hiroe M, Marumo F, Hamaguchi H. Apolipoprotein(a) size and pentanucleotide

repeat polymorphisms are associated with the degree of atherosclerosis in coronary heart disease. *Atherosclerosis*. 1996;**123**:181-191.

Anderson JJ, Ambrose WW, Garner SC. Biphasic effects of genistein on bone tissue in the ovariectomized lactating rat model. *Proceedings of the Society of Experimental Biology and Medicine.* 1998a;**217**:345-50.

Anderson JW, Diwadkar VA, Bridges SR. Selective effects of different antioxidants on oxidation of lipoproteins from rats. *Proceedings of the Society for Experimental Biology and Medicine*. 1998b;**218**:376-381.

Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein on serum lipids. *New England Journal of Medicine*. 1995;**333**:276-82.

Angelin B. Therapy for lowering lipoprotein(a) levels. *Current Opinion in Lipidology*. 1997;**8**:337-341.

Angelin B, Olivecrona H, Reihnér E, Rudling M, Ståhlberg D, Eriksson M, Ewerth S, Henriksson P, Einarsson K. Hepatic cholesterol metabolism in estrogen treated men. *Gastroenterology*. 1992;**103**:1657-1663

Anthony MS, Clarkson TB, Bullock BC, Wagner JD. Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1997;**17**:2524-2531.

Anthony MS, Clarkson TB, Hughes CL, Morgan TM, Burke GL. Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys. *Journal of Nutrition*. 1996;**126**:43-50.

Applebaum-Bowden D, McLean P, Steinmetz A, Fontana D, Mattys C, Warnick GR, Cheung M, Albers JJ, Hazzard WR. Lipoprotein, apolipoprotein and lipolytic enzyme changes following estrogen administration in postmenopausal women. *Journal of Lipid Research*. 1989;**30**:1895-1906.

Aqel NM, Ball RY, Waldman H, Mitchison MJ. Monocytic origin of foam cells in human atherosclerotic plaques. *Atherosclerosis*. 1984;**53**:265-271.

Arjmandi BH, Khan DA, Juman S, Drum ML, Venkatesh S, Sohn E, Wei L, Derman R. Whole flaxseed consumption lowers serum cholesterol and lipoprotein(a) concentrations in post-menopausal women. *Nutrition Research*. 1998;**18**:1203-1214.

Armstrong VW, Harrach B, Robenek H, Helmhold M, Walli AK, Seidel D. Heterogeneity of human lipoprotein Lp(a): cytochemical and biochemical studies on the interaction of two Lp(a) species with the LDL receptor. *Journal of Lipid Research*. 1990;**31**:429-441.

Aro A, Jauhainen M, Partanen R, Salminen I, Mutanen M. Stearic acid, *trans* fatty acids, and dairy fat: effect on serum and lipoprotein lipids, apolipoproteins, lipoprotein(a) and lipid transfer proteins in healthy subjects. *American Journal of Clinical Nutrition*. 1997;**65**:1419-1426.

Arteaga E, Rojas A, Villaseca P, Bianchi M, Arteage A, Duran D. In vitro effect of estradiol, progesterone, testosterone and of combined estradiol/progestins on low density lipoprotein oxidation in postmenopausal women. *Menopause*. 1998;**5**:16-23.

Azrolan N, Gavish D, Breslow JL. Plasma lipoprotein(a) concentration is controlled by apolipoprotein(a) (apo(a)) protein size and the abundance of hepatic apo(a) mRNA in a cynomolgus monkey model. *Journal of Biological Chemistry*. 1991;**266**:13866-13872.

Baird DD, Umbach DM, Lansdell L, Hughes CL, Setchell KD, Weinberg CR, Haney AF, Wilcox AJ, McLachlan JA. Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. *Journal of Clinical Endocrinology and Metabolism*. 1995;**80**:1685-1690.

Balmir F, Staack R, Jeffrey E, Berber Jiminez MD, Wang L, Potter SM. An extract of soy flour influences serum cholesterol and thyroid cancer in rats and hamsters. *Journal of Nutrition*. 1996;**126**:3046-3053.

Barkhem T, Andersson-Ross C, Hoglund M, Nilsson S. Characterisation of the estrogenicity of tamoxifen and raloxifene in HepG2 cells: regulation of gene expression from an ERE controlled reporter versus regulation of the endogenous SHBG and PS2 genes. *Journal of Steroid Biochemistry and Molecular Biology*. 1997;**62**:53-64.

Barnes S, Sfakianos J, Coward L, Kirk M. Soy isoflavonoids and cancer prevention. Underlying biochemical and pharmacological issues. *Advances in Experimental and Medical Biology*. 1996;**401**:87-100.

Barrett-Connor E, Bush TL. Estrogen and coronary heart disease in women. *Journal of the American Medical Association*. 1991;**265**:1861-1867.

Bartens W, Rader DJ, Talley G, Brewer HB Jr. Lipoprotein(a) in patients with hyperlipidemia. *European Journal of Clinical Investigation*. 1995;**25**:647-653.

Barter PJ. High density lipoprotein and reverse cholesterol transport. *Current Opinion in Lipidology*. 1993;**4**:210-217.

Basdevant A, Blauche D, de Lignieres B, Ponsin G, Simon P, Guy-Grand B. Hepatic lipase activity during oral and parenteral 17β-estradiol replacement therapy: high density lipoprotein increase may not be antiatherogenic. *Fertility and Sterility*. 1991;**55**:1112-1117.

Baum JA, Teng H, Erdman JW, Weigl RM, Klein BP, Persky VW, Freels S, Surya PM, Bakhit RM, Ramos E, Shay NF, Potter SM. Long term intake of soy protein improves blood lipid profiles and increases mononuclear cell low density lipoprotein receptor messenger RNA in hypercholesterolemic postmenopausal women. *American Journal of Clinical Nutrition*. 1998;**68**:545-551.

Bayard F, Clamens S, Meggetto F, Blaes N, Delsol G, Faye J-C. Estrogen synthesis, estrogen metabolism and functional estrogen receptors in rat arterial smooth muscle cells in culture. *Endocrinology*. 1995;**136**:1523-1529.

Beato M. Gene regulation by steroid hormones. *Cell*. 1989;**56**:335-344.

Becker KL (Ed). Endocrine drugs and values. *in*: Principles and Practice of Endocrinology and Metabolism. 1990. Lippincott, Philadephia USA. p.1742

Beil FU, Terres W, Orgass M, Greten H. Dietary fish oil lowers lipoprotein(a) in primary hypertriglyceridemia. *Atherosclerosis*. 1991;**90**:95-97.

Berg K. A new serum type system in man: the Lp system. *Acta Pathologica*. 1963;**59**:369-382.

Berg Schmidt E, Klausen IC, Kristensen SD, Lervang H-H, Faegerman O, Dyerberg J. The effect of n-3 polyunsaturated fatty acids on Lp(a). *Clinica Chimica Acta*. 1991;**198**:271-278.

Berglund L. Diet and drug therapy for lipoprotein(a). *Current Opinion in Lipidology*. 1995;**6**:48-56.

Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. *Free Radical Biology and Medicine*. 1996;**20**:707-727.

Bersot TP, Innerarity TL, Pitas RE, Rall SC, Weisgraber KH, Mahley RW. Fat feeding in humans induces lipoproteins of density less than 1.006 that are enriched in apolipoprotein(a) and that cause lipid accumulation in macrophages. *Journal of Clinical Investigation*. 1986;**77**:622-630.

Beuge JA, Aust SD. Microsomal lipid peroxidation. *Methods in Enzymology*. 1978;**52**:302-310.

Bihari-Varga M, Gruber E, Rotherneder M, Zechner R, Kostner GM. Interaction of lipoprotein Lp(a) and low density lipoprotein with glycosaminoglycans from human aorta. *Arteriosclerosis*. 1988;**8**:851-857.

Björnheden T, Babyl A, Bondjers G, Wiklund O. Accumulation of lipoprotein fractions and subfractions in the arterial wall determined in an in vitro perfusion system. *Atherosclerosis*. 1996;**1-2**:43-56.

Blankenship J, Crane M, Gregory R, Lukens R, Sample C. Lipoprotein(a) increased on vegan-type diet and powdered soy milk. Vegetarian Congress Research Presentations. 1996. Section IV: Biochemical/Clinical Outcome. #P32.

Blum A, Cannon RO. Effects of oestrogens and selective oestrogen receptor modulators on serum lipoproteins and vascular function. *Current Opinion in Lipidology*. 1998;**9**:575-586.

Boonmark NW, LouXJ, Yang ZJ, Schwartz K, Zhang JL, Rubin EM, Lawn RM. Modification of apolipoprotein(a) lysine binding site reduces atherosclerosis in transgenic mice. *Journal of Clinical Investigation*. 1997;**100**:558-564.

Bostom AG, Hume AL, Eaton CB, Laurino JP, Yanek LR, Regan MS, McQuade WH, Craig WY, Perrrone G, Jaques PF. The effect of high dose ascorbate supplementation on plasma lipoprotein(a) levels in patients with premature coronary heart disease. *Pharmacotherapy*. 1995;**15**:458-464.

Bowry VW, Ingold KU, Stocker R. Vitamin E in human low-density lipoprotein. *Biochemical Journal*. 1992;**288**:341-344.

Brinton EA. Oral estrogen replacement therapy in postmenopausal women selectively raises levels and production rates of lipoprotein A-I and lowers hepatic lipase activity without lowering the fractional catabolic rate. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1996;**16**:431-440.

Brown MS, Goldstein JL. A receptor mediated pathway for cholesterol homeostasis. *Science*. 1986;**232**:34-47.

Brown SA, Morrisett JD, Boerwinkle EE, Hutchison R, Patsch W. The relation of lipoprotein(a) concentrations and apolipoprotein(a) phenotypes with asymptomatic atherosclerosis in subjects of the Atherosclerosis Risk in Communities (ARIC) study. *Arteriosclerosis and Thrombosis*. 1993;**13**:1558-1566.

Brown SA, Morrisett J, Patsch JR, Reeves R, Gotto AM Jr, Patsch W. Influence of short term dietary cholesterol and fat on human plasma Lp(a) and LDL levels. *Journal of Lipid Research*. 1991;**32**:1281-1289.

Brunet S, Thibault L, Lepage G, Seidman EG, Dube N, Levy E. Modulation of endoplasmic reticulum bound cholesterol regulatory enzymes by iron/ascorbate mediated lipid peroxidation. *Free Radical Biology and Medicine*. 2000;**28**:46-54.

Bruschi F, Meschia M, Soma M, Perotti D, Paoletti R, Crosignani PG. Lipoprotein(a) and other lipids after oophorectomy and estrogen replacement therapy. *Obstetrics and Gynecology*. 1996;**88**:950-954.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Öhman L, Greene G, Gustafsson J-A, Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*. 1997;**389**:753-758 .

Campos H, Arnold KS, Balestra ME, Innerarity TL, Krauss RM. Differences in receptor binding of LDL subfractions. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1996;**16**:794-801.

Campos H, Genest JJ, Blijlevens E, McNamara JR, Jenner JL, Ordovas JM, Wilson PWF, Schaefer EJ. Low density lipoprotein particle size and coronary heart disease. *Arteriosclerosis and Thrombosis*. 1992;**12**:187-195.

Campos H, McNamara JR, Wilson PW, Ordovas JM, Schaefer EJ. Differences in low density lipoprotein subfractions and apolipoproteins in premenopausal and

postmenopausal women. *Journal of Clinical Endocrinology and Metabolism.* 1988;**67**:30-35.

Campos H, Sacks FM, Walsh BW, Chiff I, O'Hanesian MA, Krauss RM. Differential effects of estrogen on low-density lipoprotein subclasses in healthy postmenopausal women. *Metabolism.* 1993;**9**:1153-1158.

Carmel R, Howard JM, Green R, Jacobsen DW, Azen C. Hormone replacement therapy and cobalamin status in elderly women. *American Journal of Clinical Nutrition.* 1996;**64**:856-859.

Carmena R, Lussier-Cacan S, Roy M, Minnich A, Lingenhel A, Kronenberg F, Davignon J. Lp(a) levels and atherosclerotic vascular disease in a sample of patients with familial hypercholesterolemia sharing the same gene defect. *Arteriosclerosis, Thrombosis and Vascular Biology.* 1996;**16**:129-136.

Carr BR, Thompson ER. Cholesterol synthesis by human fetal hepatocytes: effects of hormones. *Journal of Clinical Endocrinology and Metabolism.* 1984;**58**:1111-1116.

Cassidy A. Potential tissue selectivity of dietary phytoestrogens and estrogens. *Current Opinion in Lipidology.* 1999;**10**:47-52.

Castelli WP. The triglyceride issue: a view from Framingham. *American Heart Journal.* 1986;**112**:432-437.

Castelli WP. Cardiovascular disease in women. *American Journal of Obstetrics and Gynaecology.* 1988;**158**:1533-1560

Castelli WP, Garrison RJ, Wilson PWF, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. *Journal of the American Medical Association.* 1986;**256**:2835-2838.

Chait A, Brazg RL, Tribble DL, Krauss RM. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *American Journal of Medicine*. 1993;**94**:350-356.

Chan AC, Tran K. Uptake of (RRR)alpha-tocopherol by human endothelial cells in culture. *Lipids*. 1990;**25**:17-21.

Chan JK, Bruce VM, McDonald BE. Dietary alpha-linolenic acid is as effective as oleic acid and linoleic acid in lowering blood cholesterol in normolipidemic men. *American Journal of Clinical Nutrition*. 1991;**53**:1230-1234.

Chan P-C, Lafrenière R, Parsons HG. Lovastatin increases surface low density lipoprotein receptor expression by retarding the receptor internalisation rate in proliferating lymphocytes. *Biochemical and Biophysical Research Communications*. 1997;**235**:117-122.

Chao Y, Windler EH, Chen GC, Havel RJ. Hepatic catabolism of rat and human lipoproteins in rats treated with 17α-ethinyl estradiol. *Journal of Biological Chemistry*. 1979;**254**:11360-11366.

Chen FP, Lee N, Soong YK. Changes in the lipoprotein profile in postmenopausal women receiving hormone replacement therapy. Effects of natural and synthetic progesterone. *Journal of Reproductive Medicine*. 1998;**43**:568-574.

Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, Beaudrie K, Cone JT. Single vertical spin gradient ultracentrifugation. *Methods in Enzymology*. 1986;**128**:181-209.

Clevidence BA, Judd JT, Schaefer EJ, Jenner JL, Lichtenstein AH, Muesing RA, Wittes J, Sunkin ME. Plasma lipoprotein(a) levels in men and women consuming diets enriched in saturated, *cis*- or *trans*-monounsaturated fatty acids. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1997;**17**:1657-1661.

Clifton PM, Chang L, Mackinnon AM. Development of an automated Lowry protein assay for the Cobas-Bio centrifugal analyzer. *Analytical Biochemistry*. 1988;**172**:165-168.

Collins BM, McLachlan JA, Arnold SF. The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids*. 1997;**62**:365-372.

Colvin PL Jr. Estrogen increases low density lipoprotein receptor-independent catabolism of apolipoprotein B in hyperlipidemic rabbits. *Metabolism*. 1996;**45**:889-896.

Colvin PL Jr, Auerbach BJ, Koritnik DR, Hazzard WR, Applebaum-Bowden D. Differential effects of oral estrone versus 17β-estradiol on lipoproteins in postmenopausal women. *Journal of Clinical Endocrinology and Metabolism*. 1990;**70**:1568-1573.

Colvin PL Jr, Wagner JD, Adams MR, Sorci Thomas MG. Sex steroids increase cholesterol 7alpha-hydroxylase mRNA in nonhuman primates. *Metabolism*. 1988;**47**:391-395.

Craig WY, Neveux LM, Palomaki GE, Cleveland MM, Haddow JE. Lipoprotein(a) as a risk factor for ischemic heart disease: metaanalysis of prospective studies. *Clinical Chemistry*. 1998;**44**:2301-2306.

Crawford RS, Kirk EA, Roesenfeld ME, LeBoeuf RC, Chait A. Dietary antioxidants inhibit the development of fatty streak lesions in the LDL receptor deficient mouse. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1998;**18**:1506-1513.

Croston GE, Milan LB, Marschke KB, Reichman M, Briggs MR. Androgen receptor-mediated antagonism of estrogen-dependent low density lipoprotein receptor transcription in cultured hepatocytes. *Endocrinology*. 1997;**138**:3779-3786.

Crouse JR, Morgan T, Terry JG, Ellis J, Vitolins M, Burke GL. A randomised trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins. *Archives of Internal Medicine*. 1999;**159**:2070-2076.

Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Scwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proceedings of National Academy of Sciences USA*.1990;**87**:5134-5138.

Dahlen G, Ericson C, Berg K. In vitro studies of the interaction of isolated Lp(a) lipoprotein and other serum lipoproteins with glycosaminoglycans. *Clinical Genetics*. 1978;**14**:36-42.

Dawber TR. The Framingham study: the epidemiology of atherosclerotic disease. Harvard University Press, 1980, Cambridge, Massachusetts.

DeLignieres B, Basdevant A, Thomas G, Thalabard J-C, Mercier-Bodard C, Conard J, Guyene T-T, Mairon N, Corvol P, Guy-Grand B, Mauvais-Jarvis P, Sitruk-Ware R. Biological effects of estradiol-17β in postmenopausal women: oral versus percutaneous administration. *Journal of Clinical Endocrinology and Metabolism*. 1986;**62**:536-541.

DeLong DM, DeLong ER, Wood PD, Lippel K, Rifkind BM. A comparison of methods for the estimation of plasma low and very low density lipoprotein. *Journal of the American Medical Assocation*. 1986;**256**:2372-2377.

Demacker PNM, Staels B, Stalenhoef AFH, Auwerx J. Increased removal of β-very low density lipoproteins after ethinyl estradiol is associated with increased mRNA levels for hepatic lipase, lipoprotein lipase, and the low density lipoprotein receptor in the Watanabe Heritable Hyperlipidemic rabbit. *Arteriosclerosis and Thrombosis*. 1991;**11**:1652-1659.

Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and lowe density lipoprotein homeostasis in different animal species, including humans. *Journal of Lipid Research.* 1993;**34**:1637-1659.

Dodge JA, Glasebrook AL, Maggee DE, Phillips DL, Sato M, Short LL, Bryant HU. Environmental estrogens: effects on cholesterol lowering and bone in the ovariectomized rat. *Journal of Steroid Biochemistry and Molecular Biology.* 1996;**59**:155-161.

Dudman NP, Guo XW, Crooks R, Xie L, Silberberg JS. Assay of plasma homocysteine: light sensitivity of the fluorescent 7-benzo-2oxa-1,3-diazole-4-sulfonic acid derivative, and use of appropriate calibrators. *Clinical Chemistry.* 1996;**42**:2028-2032.

Edmunds SEJ, Stubbs AP, Santos AA, Wilkinson ML. Estrogen and androgen regulation of sex hormone binding globulin secretion by a human liver cell line. *Journal of Steroid Biochemistry and Molecular Biology.* 1990;**37**:733-739.

Esterbauer H, Dieber-Rotheneder M, Streigl G, Waeg G. Role of vitamin E in preventing the oxidation of low-density lipoprotein. *American Journal of Clinical Nutrition.* 1991;**53**:314S-324S.

Esterbauer H, Streigl G, Puhl H, Rotheneder M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Research Communications.* 1989;**6**:67-75.

Evans RW, Shaten BJ, Hempel JD, Cutler JA, Kuller LH. Homocysteine and risk of cardiovascular disease in the multiple risk factor intervention trial. *Arteriosclerosis, Thrombosis and Vascular Biology.* 1997;**17**:1947-1953.

Everson GT, McKinley C, Kern F. Mechanisms of gallstone formation in women. Effects of exogenous estrogen (Premarin) and dietary cholesterol on hepatic cholesterol metabolism. *Journal of Clinical Investigation.* 1991;**87**:237-246.

Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhumna primate 1 Changes that lead to fatty streak formation. *Arteriosclerosis*. 1984;**4**:323-340.

Farrer M, Game FL, Albers CJ, Neil HA, Winocour PH, Laker MF, Adams PC, Alberti KG. Coronary artery disease is associated with increased lipoprotein(a) concentrations independent of the size of circulating apolipoprotein(a) isoforms. *Arteriosclerosis and Thrombosis*. 1994;**14**:1272-1283.

Ferreri F, Naito HK. Effect of estrogens on rat serum cholesterol concentrations: consideration of dose, type of estrogen and treatment duration. *Endocrinology*. 1978;**102**:1621-1627.

Fielding CJ. Lipoprotein receptors, plasma cholesterol metabolism and the regulation of cellular free cholesterol concentration. *FASEB Journal*. 1992;**6**:3162-3168.

Fishman J, Martucci C. Biological properties of 16α-hydroxyestrone: implications in estrogen physiology and pathophysiology. *Journal of Clinical Endocrinology and Metabolism*. 1980;**51**:611-615.

Fless GM, Snyder ML, Furbec JW, Hedo MTG, Mora R. Subunit composition of lipoprotein(a) protein. *Biochemistry*. 1994;**33**:13492-12501.

Fotsis T, Heikkinen R, Adlercreutz H, Axelson M, Setchell KD. Capillary gas chromatographic method for the analysis of lignans in human urine. Clinica Chimica Acta. 1982;121:361-371.

Frank S, Durovic S, Kostner GM. The assembly of lipoprotein(a). *European Journal of Clinical Investigation*. 1996;**26**:109-114.

Gaubatz JW, Ghanem KI, Guevera J, Nava ML, Patsch W, Morrisett JD. Polymorphic forms of human apolipoprotein(a): inheritance and relationship of their molecular weights to plasma levels of lipoprotein(a). *Journal of Lipid Research*. 1990;**31**:603-613.

Gaubatz JW, Heideman C, Gotto AM Jr, Morrisett JD, Dahlen GH. Human plasma lipoprotein(a). *Journal of Biological Chemistry*. 1983;**258**:4582-4589.

Gautdette DC, Holub BJ. Effect of genistein , a tyrosine kinase inhibitor, on U46619-induced phosphoinositide phosphorylation in human platelets. *Biochemical and Biophysical Research Communications*. 1990;**170**:238-242.

Gehm BD, McAndrews JM, Chien P-Y, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proceedings of the National Academy of Sciences*.1997;**94**:14138-14143.

Gianturco SH, Ramprasad MP, Lin AH, Song R, Bradley WA. Cellular binding site and membrane binding proteins in human monocyte-macrophages and THP-1 monocytic cells. *Journal of Lipid Research*. 1994;**35**:1674-1687.

Giltay EJ, Hoogeveen EK, Elbers JM, Gooren LJ, Asscheman H, Stehouwer CD. Effects of sex steroids on plasma total homocysteine levels: a study in transsexual males and females. *Journal of Clinical Endocrinology and Metabolism*. 1998;**83**:550-553.

Ginsberg HN, Kris-Etherton P, Dennis B, Elmer PJ, Ershow A, Lefevre M, Pearson T, Roheim P, Ramakrishnan R, Reed R, Stewart K, Stewart P, Phillips K, Anderson N, for the DELTA Research group. Effects of reducing dietary saturated fatty acids on plasma lipids and lipoproteins in healthy subjects. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1998;**18**:441-449.

Giri S, Thompson PD, Taxel P, Contois JH, Otvos J, Allen R, End G, Wu AHB, Waters DD. Oral estrogen improves serum lipid, homocysteine and fibrinolysis in elderly men. *Atherosclerosis*. 1998;**137**:359-366.

Goldstein JL, Brown MS. The low density lipoprotein pathway and its relation to atherosclerosis. *Annual Reviews of Biochemistry*. 1977;**46**:897-930.

Gooderham MJ, Adlercreutz H, Ojala ST, Wahala K, Holub BJ. A soy protein isolate rich in genistein and daidzein and its effects on plasma isoflavone concentrations, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipid in normal men. *Journal of Nutrition.* 1996;**126**:2000-2006.

Grainger DJ, Kirschenlohr, Metcalfe JC, Weissberg PL, Wade DP, Lawn RM. Proliferation of human smooth muscle cells promoted by lipoprotein(a). *Science.* 1993;**260**:1655-1658.

Graham A, Russell LJ. Stimulation of low-density lipoprotein uptake in HepG2 cells by epidermal growth factor via a tyrosine kinase-dependent , but protein kinase C-independent mechanism. *Biochemical Journal.* 1994;**298**:579-584.

Greaves KA, Parks JS, Williams JK, Wagner JD. Intact dietary soy protein, but not adding an isoflavone rich soy extract to casein, improves plasma lipids in ovariectomised cynomolgus monkeys. *Journal of Nutrition.* 1999;**129**:1585-1592.

Grese TA, Sluka JP, Bryant HU, Cullinan GJ, Glasebrood AL, Jones CD, Matsumoto K, Palkowitz AD, Sato M, Termine JD, Winter MA, Yang NN, Dodge JA. Molecular determinants of tissue selectivity in estrogen receptor modulators. *Proceedings of the National Academy of Sciences USA.* 1997;**94**:14105-14110.

Grundy SM, Abrams JJ. Comparison of the effects of soy protein and casein on the metabolism of plasma lipoproteins and cholesterol in humans. *American Journal of Clinical Nutrition.* 1983;**38**:245-252.

Guetta V, Lush RM, Figg WD, Waclawiw MA, Cannon RO III. Effects of the antiestrogen tamoxifen on low density lipoprotein concentrations and oxidation in postmenopausal women. *The American Journal of Cardiology.* 1995a;**76**:1072-1073.

Guetta V, Panza JA, Waclawiw MA, Cannon RO III. Effects of combined 17beta estradiol and vitamin E on low density lipoprotein oxidation in postmenopausal women. *The American Journal of Cardiology.* 1995b;**75**:1274-1276.

Guyton JR, Dahlen GH, Patsch W, Kautz JA, Gotto AM. Relationship of plasma lipoprotein Lp(a) levels to race and to apolipoprotein B. *Arteriosclerosis*. 1985;**5**:265-272.

Haberland ME, Fless GM, Scanu AM, Fogelman AM. Malondialdehyde modification of lipoprotein(a) produces avid uptake by human monocyte macrophages. *Journal of Biological Chemistry*. 1992;**267**:4143-4151.

Haberland ME, Fong D, Cheng L. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science*. 1988;**241**:215-218.

Haines CJJ, Chung TKH, Chang A, Masarei JRL, Tomlinson B, Wong E. Effect of oral estradiol on Lp(a) and other lipoproteins in postmenopausal women. *Archives of Internal Medicine*. 1996;**156**:866-872.

Haines CJJ, Chung TKH, Masarei JRL, Tomlinson B, Lau JTF. An examination of the effect of combined cyclical hormone replacement therapy on lipoprotein(a) and other lipoproteins. *Atherosclerosis*. 1996;**119**:215-222.

Haines CJJ, James AE, Panesar NS, Ngai TJ, Sahota DS, Jones RL, Chang AMZ. The effect of percutaneous oestradiol on atheroma formation in ovariectomized cholesterol-fed rabbits. *Atherosclerosis*. 1999;**143**:369-375.

Hajjar KA, Gavish D, Breslow JL, Nachman RL. Lipoprotein(a) modulation of endothelial cell surface fibrinolysis and its potential role in atherosclerosis. *Nature*. 1989;**339**:303-5.

Hansen PS, Kassem M, Brixen K, Klausen IC, Mosekilde L, Faergeman O. Effect of short term treatment with recombinant human growth hormone on lipids and lipoproteins in women and men without growth hormone disturbances. *Metabolism*. 1995;**44**:725-729.

Harnish DC, Evans MJ, Scicchitano MS, Bhat RA, Karathanasis SK. Estrogen regulation of the apolipoprotein AI gene promoter through transcription cofactor sharing. *Journal of Biological Chemistry*. 1998;**273**:9270-9278.

Harpel PC, Gordon BR, Parker TS. Plasmin catalyses binding of lipoprotein(a) to immobilised fibrinogen and fibrin. *Proceedings of the National Academy of Sciences USA*. 1989:**86**:3847-3851.

Harris ED. Lipoprotein(a): a predictor of atherosclerotic disease. *Nutrition Reviews*. 1997;**55**:61-64.

Harwood JL, Cryer A, Gurr MI, Dodds P. Medical and agricultural aspects of lipids. *in* The Lipid Handbook, 2nd Edition.1994.pp 665-707. Gunstone FD, Harwood JL, Padley TB (Eds), Chapman and Hall, London, UK.

Hayashi T, Yamada K, Esaki T, Muto E, Chaudhuri G, Iguchi A. Physiological concentrations of 17beta-estradiol inhibit the synthesis of nitric oxide synthase in macrophages via a receptor-mediated system. *Journal of Cardiovascular Pharmacology*. 1998;**2**:292-298.

Heilbronn LK, Noakes M, Clifton PM. Effect of energy restriction, weight loss, and diet composition on plasma lipids and glucose in patients with type 2 diabetes. *Diabetes Care*. 1999;**22**:889-895.

Heinecke JW, Rosen H, Chait A. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *Journal of Clinical Investigation*. 1984;**74**:1890-1894.

Hemminki E, McPherson K. Impact of postmenopausal hormone replacement therapy on cardiovascular events and cancer: pooled data from clinical trials. *British Medical Journal*. 1997;**315**:149-153.

Henriksen T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition

by the receptor for acetylated low density lipoproteins. *Proceedings of the National Academy of Sciences USA*. 1981;**78**:6499-6503.

Hermann W, Biermann J, Kostner GM. Comparison of effects of n-3 to n-6 fatty acids on serum level of lipoprotein(a) in patients with coronary artery disease. *American Journal of Cardiology*. 1995;**76**:459-462.

Hervio L, Chapman MJ, Thillet J, Loyau S, Angels-Cano E. Does apolipoprotein(a) heterogeneity influence lipoprotein(a) effects on fibrinolysis. *Blood*. 1993;**82**:392-397.

Hiramatsu K, Rosen H, Heinecke JW, Wolfbauer G, Chait A. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis*. 1987;**7**:55-60.

Hochberg RB, Pahuja SL, Zielinski JE, Larner JM. Steroidal fatty acid esters. *Journal of Steroid Biochemistry and Molecular Biology*. 1991;**40**:577-585.

Hodgson JM, Croft KD, Puddey IB, Mori T, Beilin LJ. Soybean isoflavonoids and their metabolic products inhibit in vitro lipoprotein oxidation in serum. *Journal of Nutritional Biochemistry*. 1996;**7**:664-669.

Hodgson JM, Puddey IB, Beilin LJ, Mori T, Croft KD. Supplementation with isoflavonoid phytoestrogens does not alter serum lipid concentrations: A randomised controlled trial in humans. *Journal of Nutrition*. 1998;**128**:728-732.

Hoff HF, O'Neil J, Yashiro A. Partial characterisation of lipoproteins containing apo(a) in human atherosclerotic lesions. *Journal of Lipid Research*. 1993;**34**:789-798.

Hoff HF, Whitaker TE, O'Neil J. Oxidation of low density lipoprotein leads to particle aggregation and altered macrophage recognition. *Journal of Biological Chemistry*. 1992;**267**:602-609.

Hoffman SL, Eaton DL, Brown MS, McConathy WJ, Goldstein JL, Hammer RE. Overexpression of human low density lipoprotein receptors leads to accelerated catabolism of Lp(a) lipoprotein in transgenic mice. *Journal of Clinical Investigation.* 1990;**85**:1542-1547.

Hokanson JE, Austin MA, Zambon A, Brunzell JD. Plasma triglyceride and LDL heterogeneity in familial combined hyperlipidemia. Atherosclerosis and Thrombosis. 1993;13:427-434.

Hoogerbrugge N, Zillikens MC, Jansen H, Meeter K, Deckers JW, Birkenhäger JC. Estrogen replacement decreases the levels of antibodies against oxidized low density lipoprotein in postmenopausal women with coronary heart disease. *Metabolism.* 1998;**47**:675-680.

Hough JL and Zilversmit DB. Effect of 17 beta estradiol on aortic cholesterol content and metabolism in cholesterol fed rabbits. *Arteriosclerosis*. 1986;**6**:57-63.

Huang Y, Ghosh MJ, Lopes-Virella MF. Transciptional and post-transcriptional regulation of LDL receptor gene expression in PMA treated THP-1 cells by LDL containing immune complexes. *Journal of Lipid Research*. 1997;**38**:110-120.

Huber LA, Scheffler E, Poll T, Zeigler R, Dresel HA. 17 beta estradiol inhibits LDL oxidation and cholesteryl ester formation in cultured macrophages. *Free Radical Research Communications*. 1990;**8**:167-173.

Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E. Randomised trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *Journal of the American Medical Association.* 1998;**280**:605-613.

Imthurn B, Rosselli M, Jaeger AW, Keller PJ, Dubey RK. Differential effects of hormone replacement therapy on endogenous nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17 beta estradiol valerate and cyproterone

acetate or medroxyprogesterone acetate. *Journal of Clinical Endocrinology and Metabolism*. 1997;**82**:388-394.

Jessup W, Rankin SM, DeWhalley CV, Hoult JRS, Scott J, Leake DS. α-Tocopherol consumption during low density lipoprotein oxidation. *Biochemical Journal*. 1990;**265**:399-405.

Jialal I, Devaraj S. Low density lipoprotein oxidation, antioxidants, and atherosclerosis: a clinical biochemistry perspective. *Clinical Chemistry*. 1996;**42**:498-506.

Jialal I, Scaccini C. Antioxidants and atherosclerosis. *Current Opinion in Lipidology*. 1992;**3**:324-328.

Jialal I, Vega G, Grundy SM. Physiologic levels of ascorbate inhibit the oxidative modification of LDL. *Atherosclerosis*. 1990;**82**:185-191.

Jordan C, Koch R. Regulation of prolactin synthesis *in vitro* by estrogenic and antiestrogenic derivatives of estradiol and estrone. *Endocrinology*. 1989;**124**:1717-1726.

Kamboh MI, Evans RW, Aston CE. Genetic effect of apolipoprotein(a) and apolipoprotein E polymorphisms on plasma quantitative risk factors for coronary heart disease in American Black women. *Atherosclerosis*. 1995;**117**:73-81.

Kang C, Durlach V, Soulat T, Fournier C, Anglés-Cano E. Lipoprotein(a) isoforms display differences in affinity for plasminogen-like binding to human mononuclear cells. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1997;**17**:2036-2043.

Kannel WB, Gordon T, Castelli WP. Role of lipids and lipoprotein fractions in atherogenesis: the Framingham Study. *Progress in Lipid Research*. 1981;**20**:339-348.

Keaney JF Jr, Schwaery GT, Xu A, Nicolosi RJ, Loscalzo J, Foxall TL, Vita JA. 17β-estradiol preserves endothelial vasodilator function and limits low density lipoprotein oxidation in hypercholesterolemic swine. *Circulation*. 1994;**89**:2251-2259.

Keesler GA, Li Y, Skiba PJ, Fless GM, Tabas I. Macrophage foam cell lipoprotein(a)/apolipoprotein(a) receptor. *Arteriosclerosis and Thrombosis*. 1994;**14**:1337-1345.

Kerry N, Abbey M. The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation in vitro. *Atherosclerosis*. 1998;**140**:341-347.

Kim CJ, Jang HK, Cho DH, Min YK. Effects of hormone replacement therapy on lipoprotein(a) and lipids in postmenopausal women. *Arteriosclerosis and Thrombosis*. 1994;**14**:275-281.

Kim CJ, Ryu WS, Kwak JW, Park CT, Ryoo UH. Changes in Lp(a) lipoprotein and lipid levels after cessation of female sex hormone production and estrogen replacement therapy. *Archives of Internal Medicine*. 1996;**156**:500-504.

Kim MH, Kim E, Passen EL, Meyer J, Kang S-S. Cortisol and estradiol: nongenetic factors for hyperhomocyst(e)inemia. *Metabolism*. 1997;**46**:247-249.

Kimira M, Arai Y, Shimoi K, Watanabe S. Japanese intake of flavonoids and isoflavonoids from food. *Journal of Epidemiology*. 1998;**8**:168-175

King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal. *American Journal of Clinical Nutrition*. 1998;**67**:867-872

Kirk EA, Sutherland P, Wang SA, Chait A, LeBoeuf RC. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *Journal of Nutrition*. 1998;**128**:954-959.

Kitts DD, Yuan YV, Wijewickreme AN, Thompson LU. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Molecular and Cellular Biochemistry*. 1999;**202**:91-100.

Klausen IC, Nielsen FE, Hegedus L, Gerdes LU, Charles P, Faergeman O. Treatment of hyperthyroidism reduces low density lipoproteins but not lipoprotein(a). *Metabolism*. 1992;**41**:911-914.

Kleinveld HA, Hak-Lemmers HLM, Hectors HPC, de Fouw NJ, Demacker PNM, Stalenhoef AFH. Vitamin E and fatty acid intervention does not attenuate the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1995;**15**:290-297.

Klezovitch O, Scanu AM. Heterogeneity of lipoprotein(a): growing complexities. *Current Opinion in Lipidology*. 1995;**6**:223-228.

Knight BL. Lp(a) catabolism in hypercholesterolaemic individuals. *Chemistry and Physics of Lipids*. 1994;**67-68**:233-239.

Knopp RH, Zhu X, Bonet B. Effects of estrogens on lipoprotein metabolism and cardiovascular disease in women. *Atherosclerosis*.1994;**110**:S83-S91.

Kraft HG, Lingehel A, Kochl S, Hoppichler F, Kronenberg F, Abe A, Muhleberger V, Schonistzer D, Utermann G. Apolipoprotein(a) kringle IV repeat number predicts risk for coronary heart disease. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1996;**16**:713-719.

Krone W, Naegele H, Behnke B, Greten H. Opposite effects of insulin and catecholamines on LDL receptor activity in human mononuclear leukocytes. *Diabetes*. 1988;**37**:1386-1391.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson J-A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology*. 1997;**138**:863-870.

Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology*. 1998;**139**:4252-4263.

Kushwaha RS, Foster DM, Murthy VN, Carey KD, Bernard MG. Metabolic regulation of apoproteins of high density lipoproteins by estrogen and progesterone in the baboon (Papio sp). *Metabolism*. 1990;**39**:544-552.

Kushwaha RS, Guntupalli B, Jackson EM, McGill HC Jr. Effect of estrogen and progesterone on the expressin of hepatic and extrahepatic sterol 27-hydroxylase in baboons (Papio sp). *Arteriosclerosis, Thrombosis and Vascular Biology*. 1996;**16**:1088-1094.

Labeur C, De Bacquer D, De Backer G Vincke J, Mulyldermans L, Vanderkerckhove Y, Van der Stichele E, Rosseneu M. Plasma lipoprotein(a) values and the severity of coronary artery disease in a large population of patients undergoing coronary angiography. *Clinical Chemistry*. 1992;**38**:2261-2267.

Labrie F, Luu-The V, Lin S-X, Labrie C, Simard J, Breton R, Bélanger A. The key role of 17β-hydroxysteroid dehydrogenases in sex steroid biology. *Steroids*. 1997;**62**:148-158.

Lackner C, Cohen JC, Hobbs HH. Molecular definition of the extreme size polymorphism in apolipoprotein(a). *Human Molecular Genetics*. 1993;**2**:933-940.

Lacort M, Leal AM, Liza M, Martin C, Martinez R, Ruiz-Larrea MB. Protective effect of estrogens and catecholestrogens against peroxidative membrane damage *in vitro*. *Lipids*. 1995;**30**:141-146.

Lahdenperä S, Puolakka J, Pyörälä T, Luotola H, Taskinen M-R. Effects of postmenopausal estrogen/progestin replacement therapy on LDL particles; comparison of transdermal and oral treatment regimens. *Atherosclerosis*. 1996;**122**:153-162.

Lawn RM, Wade DP, Hammer RE, Chiesa G, Verstuyft JG, Rubin EM. Atherogenesis in human transgenic mice expressing human apolipoprotein(a). *Nature*. 1992;**360**:670-672.

Leszczynski DE, Schafer RM. Characterisation of steroid hormone association with lipoproteins. *Steroids*. 1989;**54**:1110-1114.

Lien EA, Anker G, Lonning PE, Refsum H, Ueland PM. Effects of hormones on the plasma levels of the atherogenic amino acid homocysteine. *Biochemical Society Transactions*. 1997;**25**:33-35.

Liu AC, Lawn RM. Vascular interactions of lipoprotein(a). *Current Opinion in Lipidology*. 1994;**5**:269-273.

Lovati MR, Manzoni C, Corsini A, Granata A, Frattini R, Fumagalli R, Sirtori CR. Low density lipoprotein receptor activity is modulated by soybean globulins in cell culture. *Journal of Nutrition*. 1992;**122**:1971-1978.

Lowry OH, Rosenburg NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. *Journal of Biological Chemistry*. 1951;**193**:265-275.

Ma PTS, Yamamoto T, Goldstein JL, Brown MS. Increased mRNA for low density lipoprotein receptor in livers of rabbits treated with 17α-ethinyl estradiol. *Proceedings of the National Academy of Sciences USA*. 1986;**83**:792-796.

Makar RSJ, Lipsky PE, Cuthbert JA. Sterol independent, sterol response element dependent regulation of low density lipoprotein receptor gene expression. *Journal of Lipid Research*. 1998;**39**:1647-1654.

Mäkelä S, Poutanen M, Lehtimaki J, Kostian M-L, Santti R, Vihko R. Estrogen-specific 17β-hydroxysteroid oxidoreductase type 1 (E.C. 1.1.1.62) as a possible target for the action of phytoestrogens. *Proceedings of the Society for Experimental Biology and Medicine*. 1995;**208**:51-59.

Malle E, Sattler W, Prenner E, Leis HJ, Hermetter A, Gries A, Kostner GM. Effects of dietary fish oil supplementation on platelet aggregability and platelet membrane fluidity in normolipidemic subjects with and without high plasma Lp(a) concentrations. *Atherosclerosis*. 1991;**88**:193-201.

Manning JM, Campos G, Edwards IJ, Wagner WD, Wagner JD, Adams MR, Parks JS. Effects of hormone replacement modalities on low density lipoprotein composition and distribution in ovariectomised cynomolgus monkeys. *Atherosclerosis*. 1996;**121**:217-229.

Mao SJT, Yates MT, Parker RA, Chi EM, Jackson RL. Attenuation of atherosclerosis in a modified strain of hypercholesterolemic Watanabe rabbits with use of a probucol analogue (MDL 29,311) that does not lower serum cholesterol. *Arteriosclerosis and Thrombosis*. 1991;**11**:1266-1275.

Marckmann P, Bladbjerg EM, Jespersen J. Dietary fish oil (4g daily) and cardiovascular risk markers in healthy men. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1997;**17**:3384-3391.

Marcovina SM, Morrisett JD. Structure and metabolism of lipoprotein(a). *Current Opinion in Lipidology*. 1995;**6**:136-145.

Marsh MM, Walker VR, Curtiss LK, Banka CL. Protection against atherosclerosis by estrogen is independent of plasma cholesterol levels in LDL receptor deficient mice. *Journal of Lipid Research*. 1999;**40**:893-900.

Martin ME, Haourigui M, Pelisseo C, Benassayag C, Nunez EA. Interactions between phytoestrogens and human sex steroid binding protein. *Life Sciences*. 1996;**58**:429-436.

Martin MJ, Hulley SB, Browner WS, Kuller LH, Wentworth D. Serum cholesterol, blood pressure and mortality: implications from a cohort of 361 622 men. *Lancet*. 1986;**25**:933-936.

Mattsson LA, Samsoie G, von Schuoultz B, Uvebrant M, Wiklund I. Transdermally administered oestradiol combined with oral medroxyprogesterone acetate: the effects on lipoprotein metabolism in postmenopausal women. *British Journal of Obstetrics and Gynaecology*. 1993;**100**:450-453.

Mazière C, Auclair M, Ronveaux M-F, Salmon S, Santus R, Mazière J-C. Estrogens inhibit copper and cell-mediated modification of low density lipoprotein. *Atherosclerosis*. 1991;**89**:175-182

McCrohon JA, Adams MR, McCredie RJ, Robinson J, Pike A, Abbey M, Keech AC, Celermajer DS. Hormone replacement therapy is associated with improved arterial physiology in healthy postmenopausal women. *Clinical Endocrinology*. 1996;**45**:435-441.

McManus J, McEneny, Thompson W, Young IS. The effect of hormone replacement therapy on the oxidation of low density lipoprotein in post-menopausal women. *Atherosclerosis*. 1997;**135**:73-81.

McTeague Argraves K, Kozarsky KF, Fallon JT, Harpel PC, Strickland DK. The atherogenic lipoprotein Lp(a) is internalized and degraded in a process mediated by the VLDL receptor. Jour*nal of Clinical Investigation*. 1997;**100**:2170-2181.

Mehrabian M, Peter JB, Barnard RJ, Lusis AJ. Dietary regulation of fibrinolytic factors. *Atherosclerosis*. 1990;**84**:25-32.

Meng Q-H, Lewis P, Wahala K, Adlercreutz H, Tikkanen MJ. Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. *Biochimica et Biophysica Acta*. 1999;**1438**:369-376.

Mensink RP, Zock PL, Katan MB, Hornstra G. Effect of dietary *cis* and *trans* fatty acids on serum lipoprotein(a) levels in humans. *Journal of Lipid Research*. 1992;**33**:1493-1501.

Miksicek RJ. Estrogenic flavonoids: structural requirements for biological activity. *Proceedings of the Society for Experimental Biology and Medicine.* 1995;**208:**44-50.

Moliterno DJ, Jokinen EV, Miserez AR, Lange RA, Willard JE, Beoerwinkle E, Hillis LD, Hobbs HH. No association between plasma lipoprotein(a) concentrations and the presence or absence of coronary atherosclerosis in African Americans. *Arteriosclerosis, Thrombosis and Vascular Biology.* 1995;**15**:850-855.

Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su J-L, Kliewer SA, Lehmann JM, Willson TM. Cloning and characterisation of human estrogen receptor β isoforms. *Biochemical and Biophysical Research Communications.* 1998;**247**:75-78.

Morel DW, de la Llera-Moya M, Friday KE. Treatment of cholesterol fed rabbits with dietart vitamins E and C inhibits lipoprotein oxidation but not development of atherosclerosis. *Journal of Nutrition.* 1994;**124**:2123-2130.

Morton MS, Wilcox G, Wahlqvist ML, Griffiths K. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *Journal of Endocrinology.* 1994;**142**:251-259.

Mousavi Y, Adlercreutz H. Enterolactone and estradiol inhibit each other's proliferative effects on MCF-7 breast cancer cells in culture. *Journal of Steroid Biochemistry and Molecular Biology.* 1992;**41**:615-619.

Muesing RA, Miller VT, LaRosa JC, Stoy DB, Phillips EA. Effects of unopposed conjugated equine estrogen on lipoprotein composition and apolipoprotein E distribution. *Journal of Clinical Endocrinology and Metabolism.* 1992;**75**:1250-1254.

Mukai K, Daifuku K, Yokoyama S, Nakano M. Stopped flow investigation of antioxidant activity of estrogens in solution. *Biochimica et Biophysica Acta.* 1990;**1035**:348-352.

Muñoz JA, Garcia C, Quilez J-L, Andugar M-A. Effect of vitamin C on lipoproteins in healthy adults. *Annales Medecine Interne*. 1994;**145**:13-19.

Musliner TA, Krauss RM. Lipoprotein subspecies and risk of coronary disease. *Clinical Chemistry*. 1988;**34**:B78-B83.

Nakano M, Sugioka K, Naito I, Takekoshi S, Niki E. Novel and potent biological antioxidants on membrane phospholipid peroxidation: 2-hydroxy estrone and 2-hydroxy estradiol. *Biochemical and Biophysical Research Communications*. 1987;**142**:919-924.

Naruszewicz M, Selinger E, Davignon J. Oxidative modification of lipoprotein(a) and the effect of b-carotene. *Metabolism*. 1992;**41**:1215-1224.

Naruszewicz M, Selinger E, Dufour R, Davignon J. Probucol protects lipoprotein(a) against oxidative modification. *Metabolism*. 1992;**41**:1225-1228.

Nasr A, Breckwoldt M. Estrogen replacement therapy and cardiovascular protection: lipid mechanisms are the tip of an iceberg. *Gynecological Endocrinology*. 1998;**12**:43-59.

National Nutrition Survey for 1995. Australian Bureau of Statistics. Commonwealth of Australia 1999.

Negre-Salvayre A, Pieraggi M-T, Mabile L, Salvayre R. Protective effect of 17β-estradiol against the cytotoxicity of minimally oxidized LDL to cultured bovine aortic endothelial cells. *Atherosclerosis*. 1993;**99**:207-217.

Nelson GJ, Schmidt PC, Bartolini GL, Kelley DS, Kyle D. The effect of dietary docosahexanoic acid on plasma lipoproteins and tissue fatty acid composition in humans. *Lipids*. 1997;**32**:1137-1146.

Nenseter MS, Volden V, Berg T, Drevon CA, Ose L, Tonstad S. Effect of hormone replacement therapy on the susceptibility of low density lipoprotein to oxidation

among postmenopausal hypercholesterolaemic women. *European Journal of Clinical Investigation.* 1996;**26**:1062-1068.

Nesbitt PD, Lam Y, Thompson LU. Human metabolism of mammalian lignan precursors in raw and processed flaxseed. *American Journal of Clinical Nutrition.* 1999;**69**:549-555.

Nestel PJ, Noakes M, Belling B, McArthur R, Clifton P, Janus E, Abbey E. Plasma lipoprotein lipid and Lp(a) changes with substitution of elaidic acid for oleic acid in the diet. *Journal of Lipid Research.* 1992;**33**:1029-1036.

Nestel PJ, Pomeroy S, Kay S, Komesaroff P, Behrsing J, Cameron JD, West L. Isoflavones from red clover improve systemic arterial compliance but not plasma lipids in menopausal women. *Journal of Clinical Endocrinology.* 1999;**84**:895-898.

Nestel PJ, Yamashita T, Sasahara T, Pomeroy S, Dart A, Komesaroff P, Owen A, Abbey M. Soy isoflavones improve systemic arterial compliance but not plasma lipids in menopausal and perimenopausal women. *Arteriosclerosis, Thrombosis and Vascular Biology.* 1997;**17**:3392-3398.

Nielsen LB. Atherogenecity of lipoprotein(a) and oxidized low density lipoprotein: insight from in vivo studies of arterial wall influx, degradation and efflux. *Atherosclerosis.* 1999;**143**:229-243.

Nielsen LB, Juul K, Nordestgaard BG. Increased degradation of lipoprotein(a) in atherosclerotic compared with nonlesioned aortic intima-inner media of rabbits. *Arteriosclerosis, Thrombosis and Vascular Biology.* 1998;**18**:641-649.

Nielsen LB, Stender S, Jauhianen M, Nordestgaard BG. Preferential influx and decreased fractional loss of lipoprotein(a) in atherosclerotic compared with non-lesioned rabbit aorta. *Journal of Clinical Investigation.* 1996;**98**:563-571.

Nigon F, Lesnik P, Rouis M, Chapman MJ. Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor. *Journal of Lipid Research*. 1991;**32**:1741-1753.

Nilausen K, Meinertz H. Lipoprotein(a) and dietary proteins: casein lowers lipoprotein(a) concentrations as compared to soy protein. *American Journal of Clinical Nutrition*. 1999;**69**:419-425.

Nygård O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE. Plasma homocysteine and mortality in patients with coronary artery disease. *New England Journal of Medicine*. 1997;**337**:230-236.

Pahuja SL, Hochberg RB. A comparison of the esterification of steroids by rat lecithin:cholesterol acyltransferase and acyl coenzyme A:cholesterol acyl transferase. *Endocrinology*. 1994;**136**:180-186.

Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Chang C, Ballas LM, Hamilton PT, McDonnell DP, Fowlkes DM. Estrogen receptor (ER) modulators each induce distinct conformational changes in ERα and ERβ. *Proceedings of the National Academy of Sciences*. 1999;**96**: 3999-4004.

Pal S. LDL receptor regulation in human liver cells by dietary fatty acids and antioxidants. PhD thesis, University of Adelaide, Australia. 1996.

Pal S, Bursill C, Bottema C, Roach PD. Regulation of the low density lipoprotein receptor by antioxidants. In: Antioxidants in human health and disease. 1999. Basu TK, Temple NJ, Garg M (Eds). CABI, Oxford, UK.

Palinski W, Miller E, Witztum JL. Immunisation of low density lipoprotein (LDL) receptor deficient rabbits with homologous malondialdehyde-modified LDL reduces atherosclerosis. *Proceedings of the National Academy of Sciences USA*. 1995;**92**:821-825.

Parini P, Angelin B, Rudling M. Importance of estrogen receptors in hepatic LDL receptor regulation. *Arteriosclerosis Thrombosis and Vascular Biology.* 1997;**17**:1800-1805.

Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D. Macrophage oxidation of low density lipoprotein generates a modified form recognised by the scavenger receptor. *Arteriosclerosis.* 1986;**6**:505-510.

Parthasarathy S, Young SG, Witztum JL, Pittman RC, Steinberg D. Probucol inhibits oxidative modification of low density lipoprotein. *Journal of Clinical Investigation.* 1986;**77**:641-644.

Pauletto P, Puato M, Angeli MT, Pessina AC, Munhambo A, Bittolo-Bon G, Galli C. Blood pressure, serum lipids and fatty acids in populations on a lake-fish diet or a vegetarian diet in Tanzania. *Lipids.* 1996;**31**:S-309-S-312.

Perombelon YFN, Soutar AK, Knight BL. Variation in lipoprotein(a) concentration associated with different apolipoprotein(a) alleles. *Journal of Clinical Investigation.* 1994;**93**:1481-1492.

Perrone G, Stefanutti C, Galoppi P, Anelli G, Cpari O, Lucani G, Vivenzio A, Mazzarella B, Zichella L. Effect of oral and transdermal hormone replacement therapy on lipid profile and Lp(a) level in menopausal women with hypercholesterolemia. *International Journal of Fertility and Menopausal Studies.* 1996;**41**:509-515.

Phipps WR, Martini MC, Lampe LW, Slavin JL, Kurzer MS. Effect of flax seed ingestion of the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism.* 1993;**77**:1215-1219.

Pillarisetti S, Paka L, Obunike JC, Berglund L, Goldberg IJ. Subendothelial retention of lipoprotein(a) evidence that reduced heparan sulfate promotes lipoprotein binding to the subendothelial matrix. *Journal of Clinical Investigation.* 1997;**100**:867-874.

Pittman RC, Attie AD, Carew TE, Steinberg D. Tissue sites of degradation of low density lipoprotein: application of a method of determining the fate of plasma proteins. *Proceedings of the National Academy of Sciences USA*. 1979;**76**:5345-5349.

Potter SM. Overview of proposed mechanisms for the hypocholesterolemic effect of soy. *Journal of Nutrition*. 1995;**25**;606S-611S.

Potter SM. Soy protein and serum lipids. *Current Opinion in Lipidology*. 1996;**7**:260-264.

Potter SM, Baum JA, Teng H, Stillman RJ, Shay NF, Erdman JW. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *American Journal of Clinical Nutrition*. 1998;**68**:1375S-1379S.

Powell EE, Kroon PA. Low density lipoprotein receptor and 3-hydroxy-3-methocyglutaryl co-enzyme A reductase gene expression in human mononuclear leukocytes is regulated co-ordinatley and parallels gene expression in the human liver. *Journal of Clinical Investigation*. 1994;**93**:2168-2174.

Prasad K. Dietary flax seed in prevention of hypercholesterolemic atherosclerosis. *Atherosclerosis*. 1997a;**132**:69-76.

Prasad K. Hydroxyl radical scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flaxseed. *Molecular and Cellular Biochemistry*. 1997b;**168**:117-123.

Prasad K, Mantha SV, Muir AD, Westcott ND. Reduction of hypercholesterolemic atherosclerosis by CDC-flaxseed with very low alpha-linolenic acid. *Atherosclerosis*. 1998;**136**:367-375.

Rader DJ, Cain W, Ikewaki K, Talley G, Zech LA, Usher D, Brewer HB. The inverse association of plasma lipoprotein(a) concentrations with apo(a) isoform size is not due to differences in catabolism but to differences in production rate. *Journal of Clinical Investigation*. 1994;**93**:2758-2763.

Rader DJ, Mann WA, Cain W, Kraft H-G, Usher D, Zech LA, Hoeg JM, Davignon J, Lupien P, Grossman M, Wilson JM, Brewer HB Jr. The low density lipoprotein receptor is not required fro normal catabolism of Lp(a) in humans. *Journal of Clinical Investigation*. 1995;**95**:1403-1408.

Rainwater DL, Ludwig MJ, Haffner SM, VandeBerg JL. Lipid and lipoprotein factors associated with variation in Lp(a) density. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1995;**15**:313-319.

Rajman I, Lip GY, Grmab R, Maxwell SRJ, Zarifis MJ, Beevers DG, Kendall MJ. Adverse changes in low density lipoprotein subfractions profile with oestrogen only hormone replacement. *Quarterly Journal of Medicine*. 1996;**89**:771-778.

Rajman I, Maxwell S, Cramb R, Kendall M. Particle size: the key to the atherogenic lipoprotein? *Quarterly Journal of Medicine*. 1994;**87**:709-720.

Rees A, Bishop A, Morgan R. The apo(a) gene: structure/function relationships and the possible link with thrombotic atheromatous disease. *British Medical Bulletin*. 1990;**46**:873-890.

Rice-Evans CA, Miller NJ, Paganga G. Structure-Antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*. 1996;**20**:933-956.

Ridker PM, Manson JE, Buring JE, Shih J, Matias M, Hennekens CH. Homocysteine and risk of cardiovascular disease among postmenopausal women. *Journal of the American Medical Association*. 1999;**281**:1817-1821

Rifici VA, Khachadurian AK. The inhibition of low density lipoprotein oxidation by 17β-estradiol. *Metabolism*. 1992;**41**:1110-1114.

Rimm EB, Stampfer MJ, Ascherio A, Giovanucci E, Colditz GA, Willet WC. Vitamin E and coronary heart disease in men. *New England Journal of Medicine*. 1993;**328**:1450-1456.

Ritter MM, Geiss HC, Richter WO, Schwandt P. Lipoprotein(a) concentrations and phenotypes in controls and patients with hypercholesterolemia or hypertriglyceridemia. *Metabolism*. 1994;**43**:572-578.

Roach PD, Hosking J, Clifton PM, Bais R, Kusenic B, Coyle P, Wight MB, Thomas DW, Nestel PJ. The effects of hypercholesterolaemia, simvastatin and dietary fat on the low density lipoprotein receptor of unstimulated mononuclear cells. *Atherosclerosis*. 1993;**103**:245-254.

Rosengren A, Wilhelmsen L, Eriksson, Risberg B, Wedel H. Lipoprotein(a) and coronary heart disease: a prospective case-control study in a general population sample of middle aged men. *British Medical Journal*. 1990;**301**:1248-1251.

Ross R. The pathogenesis of atherosclerosis: a prospective for the 1990s. *Nature*. 1993;**362**:801-809.

Sack MN, Rader DJ, Cannon RO. Oestrogen and inhibition of oxidation of low density lipoproteins in postmenopausal women. *Lancet*. 1994;**343**:269-270.

Sacks FM, Breslow JL, Wood PG, Kass EH. Lack of an effect of dairy protein (casein) and soy protein on plasma cholesterol of strict vegetarians. An experiment and a critical review. *Journal of Lipid Research*. 1983;**24**:1012-1020.

Sacks FM, Walsh BW. Sex hormones and lipoprotein metabolism. *Current Opinion in Lipidology*. 1994;**5**:236-240.

Sandholzer C, Saha N, Kark JD, Rees A, Jaross W, Dieplinger H, Hopplichler F, Boerwinkle E, Utermann G. Apo(a) isoforms predict risk for coronary heart disease. *Arteriosclerosis and Thrombosis*. 1992;**12**:1214-1226.

Sandkamp M, Funke H, Schulte H, Kohler E, Assmann G. Lipoprotein(a) is an independent risk factor for myocardial infarction at a young age. *Clinical Chemistry*. 1990;**36**:20-23.

Santanam N, Shern-Brewer R, McClatchy R, Castellano PZ, Murphy AA, Voelkel S, Parthasarathy S. Estradiol as an antioxidant: incompatible with its physiological concentrations and function. *Journal of Lipid Research*. 1998;**39**:2111-2118.

Savenkova MI, Mueller DM, Heinecke JW. Tyrosyl radical generated by myeloperoxidase: a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. *Journal of Biological Chemistry*. 1994;**269**:20394-20400.

Scanu AM, Pfaffinger D, Edelstein C. Postprandial Lp(a): identification of a triglyceride rich particle containing apo E. *Chemistry and Physics of Lipids*. 1994;**67-68**:193-198.

Schaefer EJ, Foster DM, Zech LA, Lindgren FT, Brewer B, Levy RI. The effect of estrogen administration on plasma lipoprotein metabolism in postmenopausal females. *Journal of Clinical Endocrinology and Metabolism*. 1983;**57**:262-267.

Schaefer EJ, Genest JJ, Ordovas JM, Salem DN, Wilson PWF. Familial lipoprotein disorders and premature coronary artery disease. *Current Opinion in Lipidology*. 1993;**4**: 288-298.

Scheek LM, Wiseman SA, Tijburg LBM, van Tol A. Dialysis of isolated low density lipoprotein induces a loss of lipophilic antioxidants and increases the susceptibility to oxidation in vitro. *Atherosclerosis*. 1995;**117**:139-144.

Schinckel PG. Infertility in sheep grazing on subterranean clover pastures. Observations on breeding behaviour following transfer to 'sound' country. *Australian Veterinary Journal*. 1948;**24**:289-294.

Schröder J, Dören M, Schneider B, Oettel M. Are the antioxidative effects of 17β-estradiol modified by concomitant administration of a progestin. *Maturitas*. 1996;**25**:133-139.

Schubert W, Cullberg G, Edgar B, Hedner T. Inhibition of 17β-estradiol metabolism by grapefruit juice in ovariectomised women. *Maturitas*. 1995;**20**:155-163.

Schwenke DC, Wagner JD, Adams MR. In vitro lipid peroxidation of LDL from postmenopausal cynolmolgus macaques treated with female hormones. *Journal of Lipid Research*. 1999;**40**:235-244.

Semenkovich CF, Ostlund RE Jr. Estrogens induce low density lipoprotein receptor activity and decrease intracellular cholesterol in human hepatoma cell line HepG2. *Biochemistry*. 1987;**26**:4987-4992.

Setchell KDR. Phytoestrogens: the biochemistry, physiology and implications for human health of soy isoflavones. *American Journal of Clinical Nutrition*. 1998;**68**:1333S-1246S.

Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens recent studies on their formation, metabolism and biological role in health and disease. In: Role of the Gut Flora in Toxicity and Cancer. 1988. Roland IR (Ed). Academic Press, UK.

Sharpe PC, McGrath LT, McLean E, Young IS, Archbold GPR. Effect of red wine consumption on lipoprotein(a) and other risk factors for atherosclerosis. *Quarterly Journal of Medicine*. 1995;**88**:101-108.

Shepherd J, Packard CJ. Receptor independent low density lipoprotein catabolism. *Methods in Enzymology*. 1986;**129**:566-590.

Shewmon DA, Stock JL, Rosen CJ, Heiniluoma KM, Hogue MM, Morrison A, Doyle EM, Ukena T, Weale V. Baker S. Tamoxifen and estrogen lower circulating lipoprotein(a) concentrations in healthy postmenopausal women. *Arteriosclerosis and Thrombosis*. 1994;**14**:1586-1593.

Shinozaki K, Kambayashi J, Kawasaki T, Uemura Y, Sakon M, Shiba E, Shibuya T, Nakamura T, Mori T. The long term effects of eicosapentaenoic acid on serum levels

of lipoprotein(a) and lipids in patients with vascular disease. *Journal of Atherosclerosis and Thrombosis*. 1996;**2**:107-109.

Shwaery GT, Vita JA, Keaney JF Jr. Antioxidant protection of LDL by physiologic concentrations of estrogens is specific for 17-beta-estradiol. *Atherosclerosis*. 1998;**138**:255-262.

Siddiqui MT, Siddiqi M. Hypolipidemic principles of cicer arietinum: biochanin A and formononetin. *Lipids*.1976;**11**:243-246.

Simons LA, Simons J. Effect of moderate alcohol consumption on Lp(a) lipoprotein concentration: no effect seen in Australian drinkers. *British Medical Journal*. 1998;**316**:1675.

Sirtori CR, Gianazza E, Manzoni C, Lovati MR, Murphy PA. Role of isoflavones in the cholesterol reduction by soy proteins in the clinic. *American Journal of Clinical Nutrition*. 1997;**65**:166-167.

Soma MR, Meschia M, Brischi F, Morrisett JD, Paoletti R, Fumagalli R, Crosignani PG. Hormonal agents used in lowering lipoprotein(a). *Chemistry and Physics of Lipids*. 1994;**67/68**:345-350.

Sourander L, Rajala T, Raiha I, Makinen J, Erkkola R, Helenius H. Cardiovascular and cancer morbidity and mortality and sudden cardiac death in postmenopausal women on oestrogen replacement therapy (ERT). *Lancet*. 1998;**352**:1965-1969.

Soutar AK, Knight BL. Structure and regulation of the LDL receptor and its gene. *British Medical Bulletin*. 1990;**46**:891-916.

Sparrow CP, Olszewski J. Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions. *Journal of Lipid Research*. 1993;**34**:1219-1228.

Srivastava RA, Baumann D, Sconfeld G. In vivo regulation of low density lipoprotein receptors by estrogen differs at the post-transcriptional level in rat and mouse. *European Journal of Biochemistry*. 1993;**216**:527-538.

Stampfer MJ, Kennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC. Vitamin E consumption and the risk of coronary heart disease in women. *New England Journal of Medicine*. 1993;**328**:1444-1449.

Steinberg D, Parthasarathy S, Carew T, Knoo JC, Witztum JL. Beyond cholesterol modifications of low density lipoprotein that increase its atherogenicity. *New England Journal of Medicine*. 1989;**320**:915-924.

Steinbrecher UP, Lougheed M, Kwan WC, Dirks M. Recognition of oxidised low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *Journal of Biological Chemistry*. 1989;**264**:15216-15223.

Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study. *Lancet*. 1996;**347**:781-786.

Stocker R. Natural antioxidants and atherosclerosis. *Asia Pacific Journal of Clinical Nutrition*. 1993;**2**:15-20.

Streicher R, Kotzka J, Müller-Weiland D, Siemeister G, Munck M, Avci H, Krone W. SREBP-1 mediates activation of the low density lipoprotein receptor promoter by insulin and insulin-like growth factor-I. *Journal of Biological Chemistry*. 1996;**271**:7128-7133.

Subbiah MTR, Kessel B, Agrawal M, Rajan R, Abplanalp W, Rymaszewski Z. Antioxidant potential of specific estrogens on lipid peroxidation. *Journal of Clinical Endocrinology and Metabolism*. 1993;**77**:1095-1097.

Sugioka K, Shimosegawa Y, Nakano M. Estrogens as natural antioxidants of membrane phospholipid peroxidation. *FEBS Letters*. 1987;**210**:37-39.

Sulistiyani, Adelman SJ, Chandrasekaran A, Jayo J, St Clair RW. Effect of 17α-dihydroequlin sulfate, a conjugated equine estrogen, and ethynylestradiol on atherosclerosis in cholesterol-fed rabbits. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1995;**15**:837-846.

Sutton-Tyrell K, Evans RW, Meilahn E, Alcorn HG. Lipoprotein(a) and peripheral atherosclerosis in older adults. *Atherosclerosis*. 1996;**122**:11-19.

Tallova J, Tomandl J, Bicikova M, Hill M, Changes of plasma homocysteine levels during the menstrual cycle. *European Journal of Clinical Investigation*. 1999;**29**:1041-1044.

Tam S-P, Archer TK, Deeley RG. Effects of estrogen on apolipoprotein secretion by the human hepatocarcinoma cell line, HepG2. *Journal of Biological Chemistry*. 1985;**260**:1670-1675.

Tanaka S, Yashiro A, Tasaki H, nakashima Y. Enhanced macrophage uptake of lipoprotein(a) after Ca2+ induced aggregate formation. *Lipids*. 1998;**33**:385-392.

Tanenbaum DM, Wang Y, Williams SP, Sigler PB. Crystallographic comparison of the estrogen and progesterone receptor's ligand binding sites. *Proceedings of the National Academy of Sciences USA*. 1998;**95**:5998-6003.

Taniguchi S, Yanase T, Kobayashi K, Takayanagi R, Haji M, Umeda F, Nawata H. Catechol estrogens are more potent antioxidants than estrogens for the Cu(2+) catalysed oxidation of low or high density lipoprotein: antioxidative effects of steroids on lipoproteins. *Endocrinology Journal*. 1994;**41**:605-611.

Taskinen M-R, Puolakka J, Pyörälä T, Luotola H, Björn M, Kääriäinen J, Lahdenperä S, Ehnholm C. Hormone replacement therapy lowers plasma Lp(a) concentrations. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1996;**16**:1215-1221.

Tchernof A, Despres JP, Belanger A, Dupont A, Prud'Homme D, Moorjani S, Lupien PJ, Labrie F. Reduced testosterone and adrenal c19 steroid levels in obese men. *Metabolism*. 1995;**44**:513-519.

Terkeltaub R, Boisvert WA, Curtiss LK. Chemokines and atherosclerosis. *Current Opinion in Lipidology*. 1998;**9**:397-405.

Tham DM, Gardner CD, Haskell WL. Potential health benefits of dietary phytoestrogens: a review of the clinical epidemiological and mechanistic evidence. *Journal of Clinical Endocrinology and Metabolism*. 1998;**83**:2223-2235.

The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine*. 1994;**330**:1029-1035.

The Writing Group for the PEPI trial. Effects of estrogen or estrogen/progestin regimens on heart disease risk factors in postmenopausal women. The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. *Journal of the American Medical Association*. 1995;**273**:199-208.

Tholstrup T, Marckmann P, Vessby B, Sandström B. Effect of fats high in individual saturated fatty acids on plasma lipoprotein(a) levels in young healthy men. *Journal of Lipid Research*. 1995;**36**:1447-1452.

Thomas CE. The influence of medium components on Cu(2+) –dependent oxidation of low density lipoproteins and its sensitivity to superoxide dismutase. *Biochimica Biophysica Acta*. 1992;**1128**:50-57.

Thomas SR, Neuzil J, Stocker R. Co-supplementation with coenzyme Q prevents the pro-oxidant effect of alpha-tocopherol and increases the resistance of LDL to transition metal dependent oxidation initiation. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1996;**16**:687-696.

Thompson GR. A handbook of hyperlipidaemia. 1990, pp23-41. Current Science, London, UK.

Thorne SA, Abbot SE, Winyard PG, Blake DR, Mills PG. Extent of oxidative modification of low density lipoprotein determines the cytotoxicity to human coronary artery cells. *Heart*. 1996;**75**:11-16.

Tikkanen MJ, Wähälä K, Ojala S, Vihma V, Adlercreutz H. Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. *Proceedings of the National Academy of Sciences*. 1998;**95**:3106-3110.

Tilly-Kiesi M, Kahri J, Pyörälä T, Puolakka J, Luotola H, Lappi M, Lahdenpera S, Taskinen M-R. Respnses of HDL subclasses Lp(A-I) and Lp(A-I:A-II) levels and lipolytic enzyme activities to continuous oral estrogen-progestin and transdermal estogen with cyclic progestin regimens in postmenopausal women. *Atherosclerosis*. 1997;**129**:249-259.

Tilly-Kiesi M, Lappi M, Puolakka J, Luotola H, Pyörälä T, Taskinen M-R. Different effects of continuous oestrogen-progestin and transdermal oestrogen with cyclic progestin regimens on low density lipoprotein subclasses. *European Journal of Clinical Investigation.* 1996;**26**:1125-1133.

Tkocz R, Schmidt G, Hillesheim HG. Interactions between progestins and estradiol on serum lipids in the rat. *Experimental and Clinical Endocrinology*. 1985;**86**:237-240.

Tonolo G, Ciccarese M, Brizzi P, Milia S, Dessole S, Puddu L, Secchi G, Maioli M. Cyclical variation of plasma lipids, apolipoproteins and lipoprotein(a) during the menstrual cycle of normal women. *American Journal of Physiology*. 1995;**269**:E1101-E1105.

Tribble DL, van den Berg JJ, Motchnik PA, Ames BN, Lewis DM, Chait A, Krauss RM. Oxidative susceptibility of low density lipoprotein subfractions is related to their ubiqunol-10 and α-tocopherol content. *Proceedings of the National Academy of Sciences*. 1994;**91**:1183-1187.

Tsai YH, Park S, Snook JT. Interactions among Lp(a) phenotypes, Lp(a) concentrations and lipoprotein response to fat modified diets. *Journal of Nutritional Biochemistry*. 1998;**9**:106-113.

Tsujino Y, Tsurumi S, Yoshida Y, Niki E. Antioxidative effects of dihydor-gamma-pryonyl-triterpenoid saponin (chromosaponin I). *Bioscience, Biotechnology and Biochemistry*. 1994;**58**:1731-1732.

Tuck CH, Holleran S, Berglund L. Hormonal regulation of lipoprotein(a) levels: effects of estrogen replacement therapy on lipoprotein(a) and acute phase reactants in postmenopausal women. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1997;**17**:1822-1829.

Tunstall-Pedoe H. Myth and paradox of coronary risk and the menopause. *Lancet*. 1998;**351**:1425-1427.

Ulloa N, Verdugo C, Rios M, Sepulveda J, Sepulveda S, Navaes R, Calvo C. Increased activity of lecithin: cholesterol acyltransferase during short term oral estrogen progestin replacement therapy in a group of postmenopausal women. *Metabolism*. 1998;**47**:297-300.

Utermann G, Menzel HJ, Kraft HG, Duba HC, Kemmler HG, Seitz C. Lp(a) glycoprotein phenotypes. Inheritance and relation to Lp(a) lipoprotein concentrations in plasma. *Journal of Clinical Investigation*. 1987;**80**:458-465.

Vadlamudi S, MacLean P, Israel RG, Marks RH, Hickey M, Otvos J, Barakat H. Effects of oral combined hormone replacement therapy on plasma lipids and lipoproteins. *Metabolism*. 1998;**47**:1222-1226.

Valimaki M, Laitinen K, Ylikahri R, Enholm C, Jauhiainen M, Bard JM, Fruchart JC, Taskinen MR. The effect of moderate alcohol intake on serum apolipoprotein A-I containing lipoproteins and lipoprotein(a). *Metabolism*. 1991;**40**:1168-1172.

van der Mooren MJ, de Graaf J, Demacker PNM, de Haan AFJ, Rolland R. Changes in the low density lipoprotein profile during 17β-estradiol-dydrogesterone therapy in postmenopausal women. *Metabolism*. 1994;**43**:799-802.

van der Mooren MJ, de Rijke YB, Demacker PNM, Rolland R, Blom HJ. The effect of sequential three-monthly hormone replacement therapy on several cardiovascular risk estimators in postmenopausal women. *Fertility and Sterility*. 1997;**67**:67-73.

Vester B, Rasmussen K. High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. *European Journal of Clinical Chemistry and Clinical Biochemistry*. 1991;**29**:549-554.

Wagner JD, Clarkson TB, St Clair RW, Schwenke DC, Shively CA, Adams MR. Estrogen and progesterone replacement therapy reduces low density lipoprotein accumulation in the coronary arteries of surgically postmenopausal cynomolgus monkeys. *Journal of Clinical Investigation*. 1991;**88**:1995-2002.

Wakatsuki A, Ikenoue N, Sagara Y. Effects of estrogen on susceptibility to oxidation of low density and high density lipoprotein in postmenopausal women. *Maturitas*. 1998;**28**:229-234.

Walsh BW, Li H, Sacks FM. Effects of postmenopausal hormone replacement with oral and transdermal estrogen on high density lipoprotein metabolism. *Journal of Lipid Research*. 1994;**35**:2083-2093.

Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnikar V, Sacks FM. Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *New England Journal of Medicine*. 1991;**325**:1196-1204.

Walzem RL, Watkins S, Frankel EN, Hansen RJ, German JB. Older plasma lipoproteins are more susceptible to oxidation: a linking mechanism for the lipid and oxidation theories of atherosclerotic cardiovascular disease. *Proceedings of the National Academy of Sciences USA*. 1995;**92**:7460-7464.

Wander RC, Du S-H, Ketchum SO, Rowe KE. Effects of interaction of RRR-a-tocopheryl acetate and fish oil on low density lipoprotein oxidation in postmenopausal women with and without hormone replacement therapy. *American Journal of Clinical Nutrition*. 1996;**63**:184-193.

Wang C, Mäkelä T, Hase T, Adlercreutz H, Kurzer MS. Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. *Journal of Steroid Biochemistry and Molecular Biology*. 1994a;**50**:205-212.

Wang H, Peters GA, Zeng X, Tang M, Ip W, Khan SA. Yeast two-hybrid system demonstrates that estrogen receptor dimerization is ligand-dependent *in vivo*. *Journal of Biological Chemistry*. 1995;**270**:23322-23329.

Wang W, Goodman MT. Antioxidant property of dietary phenolic agents in a human LDL oxidation ex vivo model: interaction of protein binding activity. *Nutrition Research*. 1999;**19**:191-202.

Wang X, Sato R, Brown MS, Hua X, Goldstein JL. SREBP-1, a membrane bound transcription factor released by sterol regulated proteolysis. *Cell*. 1994b;**77**:53-62.

Wen Y, Doyle MCT, Harrison RF, Feely J. The effect of hormone replacement therapy on vitamin E status in postmenopausal women. *Maturitas*. 1997;**26**:121-124.

Williams RJ, Motteram JM, Sharp CH, Gallagher PJ. Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. *Atherosclerosis*. 1992;**94**:153-159.

Wiseman H. Tamoxifen as an antioxidant and cardioprotectant. *Biochemical Society Symposia*. 1995;**61**:209-219

Witztum JL, berliner JA. Oxidized phospholipids and isoprostanes in atherosclerosis. *Current Opinion in Lipidology*. 1998;**9**:441-448.

Wolfe BM, Huff MW. Effects of continuous low-dosage hormonal replacement therapy on lipoprotein metabolism in postmenopausal women. *Metabolism.* 1995;**44**:410-417.

Wouters MG, Moorrees MT, van der Mooren MJ, Blom HJ, Boers GH, Schellekens LA, Thomas CM, Eskes TK. Plasma homocysteine and menopausal status. *European Journal of Clinical Investigation.* 1995;**25**:801-805.

Yagi K, Komura S. Inhibitory effect of female hormones on lipid peroxidation. *Biochemistry International.* 1986;**13**:1051-1055.

Yang CS, Lee M-J. Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies. *Journal of Nutrition, Growth and Cancer.* 1987;**4**:19-27.

Yashiro A, O'Neil J, Hoff HF. Insoluble complex formation of lipoprotein(a) with low density lipoprotein in the presence of calcium ions. *Journal of Biological Chemistry.* 1993;**268**:4709-4715.

Ylä-Herttuala S, Butler S, Picard S, Palinski W, Steinberg D, Witztum JL. Rabbit and human atherosclerotic plaques contain IgG that recognises epitopes of oxidized LDL. *Arteriosclerosis and Thrombosis.* 1994;**14**:32-40.

Ylä-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *Journal of Clinical Investigation.* 1989;**84**:1086-1095.

Yokoyama C, Wang X, Briggs MR, Admon A, Wu J, Hua X, Goldstein JS, Brown MS. SREBP-1, a basic helix loop helix leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell.* 1993;**75**:187-197.

Yoshikoshi M, Yoshiki Y, Okubo K, Seto J, Sasaki Y. Prevention of hydrogen peroxide damage by soybean saponins to mouse fibroblasts. *Planta Medica.* 1996;**62**:252-255.

Zöllner N, Tatò F. Fatty acid composition of the diet: impact on serum lipids and atherosclerosis. *Clinical Investigator*. 1992;70:968-1009.

Zou A, Marschke KB, Arnold KE, Berger EM, Fitzgerald P, Mais DE, Allegretto EA. Estrogen receptor β activates the human retinoic acid receptor α-1 promoter in response to tamoxifen and other estrogen antagonists, but not in response to estrogen. *Molecular Endocrinology*. 1999;**13**;418-430.

Zysow BR, Kauser K, Lawn RM, Rubanyi GM. Effect of estrus cycle, ovariectomy, and treatment with estrogen, tamoxifen andprogesterone on apolipoprotein(a) gene expression in transgenic mice. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1997;**17**:1741-1745.