The effect of dietary fat, antioxidants, and alcohol on serum lipoprotein concentrations and aortic fatty streak formation in the C57BL/6 mouse model of atherosclerosis.

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"You scientists *think* too much," blurted Miss Pefko. She laughed idiotically. Dr Breed's friendliness had blown every fuse in her mental system.

A winded, defeated-looking woman in filthy coveralls trudged beside us, hearing what Miss Pefko said. She turned to examine Dr. Breed, looking at him with helpless reproach. She hated people who thought too much. The fat woman's expression implied that she would go crazy on the spot if anybody did any more thinking.

"I think you will find," said Dr. Breed, "that everybody does about the same amount of thinking. Scientists simply think about things one way, and other people think about things in others."

"Ech," gurgled Miss Pefko emptily. "I take dictation from Dr. Horvath and it's just like a foreign language. When I used to come home from school Mother used to ask me what happened that day, and I'd tell her. Now I come home from work and she asks me the same question, and all I can say is-" Miss Pefko shook her head and let her crimson lips flap slackly-

"I dunno, I dunno, I dunno."

"If there's something you don't understand," urged Dr. Breed, "ask Dr. Horvath to explain it."

He turned to me. "Dr. Hoenikker used to say that any scientist who couldn't explain to an eight-year-old what he was doing was a charlatan."

"Then I'm dumber than an eight-year-old," Miss Pefko mourned. "I don't even know what a charlatan is."

from 'Cat's Cradle' by Kurt Vonnegut Jr. Victor Gollancz Ltd. London. 1963

Abstract

In this research programme the effect of dietary fat, alcohol, and antioxidants on the serum lipoprotein profile and the development of atherosclerosis was studied in a series of experiments, primarily using the C57BL/6 mouse model of atherosclerosis. These mice, when fed a special diet, develop fatty streaks (thought to be the earliest lesion of atherosclerosis) in the intima of the aortic sinus within 15 weeks. Another mouse model of early atherogenesis, the human apoB-transgenic mouse model, was also used but was not found to possess any clear advantages over the non-transgenic C57BL/6 model. Pilot *in vitro* studies using isolated human low density lipoprotein (LDL) and macrophage cell cultures were also performed.

The effects of different dietary fats on serum lipoprotein concentrations are generally well known, however, their role in atherogenesis remains controversial. To provide further information on this, atherogenic diets containing different proportions of saturated and unsaturated fats supplied from a variety of commercial fats/oils were fed to C57BL/6 mice for 15 weeks. In contrast to the results of human studies, a high proportion of saturated fatty acids in the diet of these mice increased the ratio of serum high density lipoprotein (HDL) to total cholesterol. Although diets high in saturated fat also reduced atherogenesis in the mouse model, the literature suggests that dietary fats produce dissimilar changes in the lipoprotein profile of humans. Therefore, whether or not saturated fatty acids will have similar effects in humans remains unknown. Another group of dietary fats which have been suggested to reduce atherogenesis are the conjugated linoleic acids. However, despite producing a less atherogenic lipoprotein profile, the inclusion of conjugated linoleic acids in the experimental diet promoted fatty streak formation in C57BL/6 mice.

Epidemiological evidence suggests that dietary antioxidants may reduce atherogenesis. To investigate this experimentally, vitamin E and butylated hydroxytoluene (BHT) were examined using the C57BL/6 mouse model. Compared to controls, vitamin E lowered serum total cholesterol concentration but did not reduce fatty streak formation, while BHT lowered the ratio of serum HDL to total cholesterol and increased fatty streak formation. Because both these antioxidants were found to affect key enzymes involving lipid metabolism, it is impossible to use data from these studies to determine whether or not their

antioxidant properties influenced atherosclerosis.

The ability of dietary antioxidants to protect LDL particles from oxidation was investigated. Human subjects were given 6g of raw garlic, 2.4g of aged garlic extract, or 0.8g vitamin E each day for 7 days. Supplementation with vitamin E greatly increased the resistance of the isolated LDL to oxidation. Less, but still significant, protection was provided by aged garlic extract, but raw garlic was without effect.

There are reports in the literature that moderate consumption of alcohol by human subjects increases serum HDL cholesterol concentration and decreases atherosclerosis risk. To investigate the effect of alcohol in the mouse model, C57BL/6 mice were given water containing 3.1% alcohol in the form of either red or white wine. In contrast to humans, dietary alcohol lowered the proportion of serum cholesterol contained in the HDL fraction and promoted fatty streak formation in the C57BL/6 mouse model. While it is likely that some of the increased atherogenesis was attributable to lowered serum HDL cholesterol concentrations, data from this study suggested that dietary alcohol also influenced atherogenesis independently of the serum lipoprotein profile. No differences were observed between mice receiving alcohol from either red or white wine, suggesting that the greater quantity of antioxidants contained in red wine did not influence serum lipoprotein concentration or fatty streak formation in this model.

In conclusion, it is probable that differences in lipid metabolism between humans and C57BL/6 mice resulted in some of the dietary factors altering the serum lipoprotein concentration of mice in a way which would be considered unlikely to occur in humans. Changes in the serum lipoproteins probably then contributed to the seemingly anomalous increases in fatty streak formation that was observed in some experiments. These results illustrate some of the problems involved in investigating a disease which only occurs naturally in humans. Data derived from studies using animal and *in vitro* models may be able to provide useful information regarding human atherosclerosis. However, because it is unknown how accurately animal and *in vitro* models of atherosclerosis represent the human disease, the results of such studies must be interpreted with caution.

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Chapter 1

Review of the literature

Atherosclerosis is a vascular disease characterised by chronic inflammatory changes in the walls of arteries associated with the progressive accumulation of lipid. Many atherosclerotic lesions remain silent, but partial or complete occlusion of an arterial lumen by an expanding lesion or by thrombosis triggered by the rupture of an atheroma, can have a major effect on key organ systems. Some of these effects on various organ systems are illustrated in Figure 1.1.

The most frequent complication of atherosclerosis is ischaemic heart disease (IHD)[1], associated with atheroma development in the coronary arteries (coronary artery disease). At this site, rupture of an atheroma will result in thrombus formation, possibly occluding the arterial lumen and resulting in myocardial infarction[2]. Myocardial infarction is one of the most common causes of death in the Western world and in 1992 was the cause of 26.1% of all deaths in New Zealand[1,3].

Atheromas which involve the aorta, carotid or cerebral arteries can result in cerebrovascular disease secondary to either thrombosis or embolism[4,5]. Aortic atherosclerotic lesions, as well as damaging the aorta, can also predispose an individual to renal infarction[6,7]. Peripheral artery disease is also commonly caused by atherosclerosis[8].

Presented here is an overview of the pathogenesis of atherosclerosis along with some information on the risk factors for this disease. The first section covers lipid transport and the function and metabolism of the serum lipoproteins. This is followed by a review of the regulation of serum lipoprotein concentration. In the third section, the pathogenesis of atherosclerosis, including the oxidation of low density lipoprotein (LDL), is discussed. In part four, animal models used to examine the pathogenesis of atherosclerosis are reviewed, with emphasis on models utilised in the studies reported in this thesis. Section five examines the risk factors for atherosclerosis while section six reviews some of the factors which are believed to alter the risk an individual has for developing atherosclerotic disease.

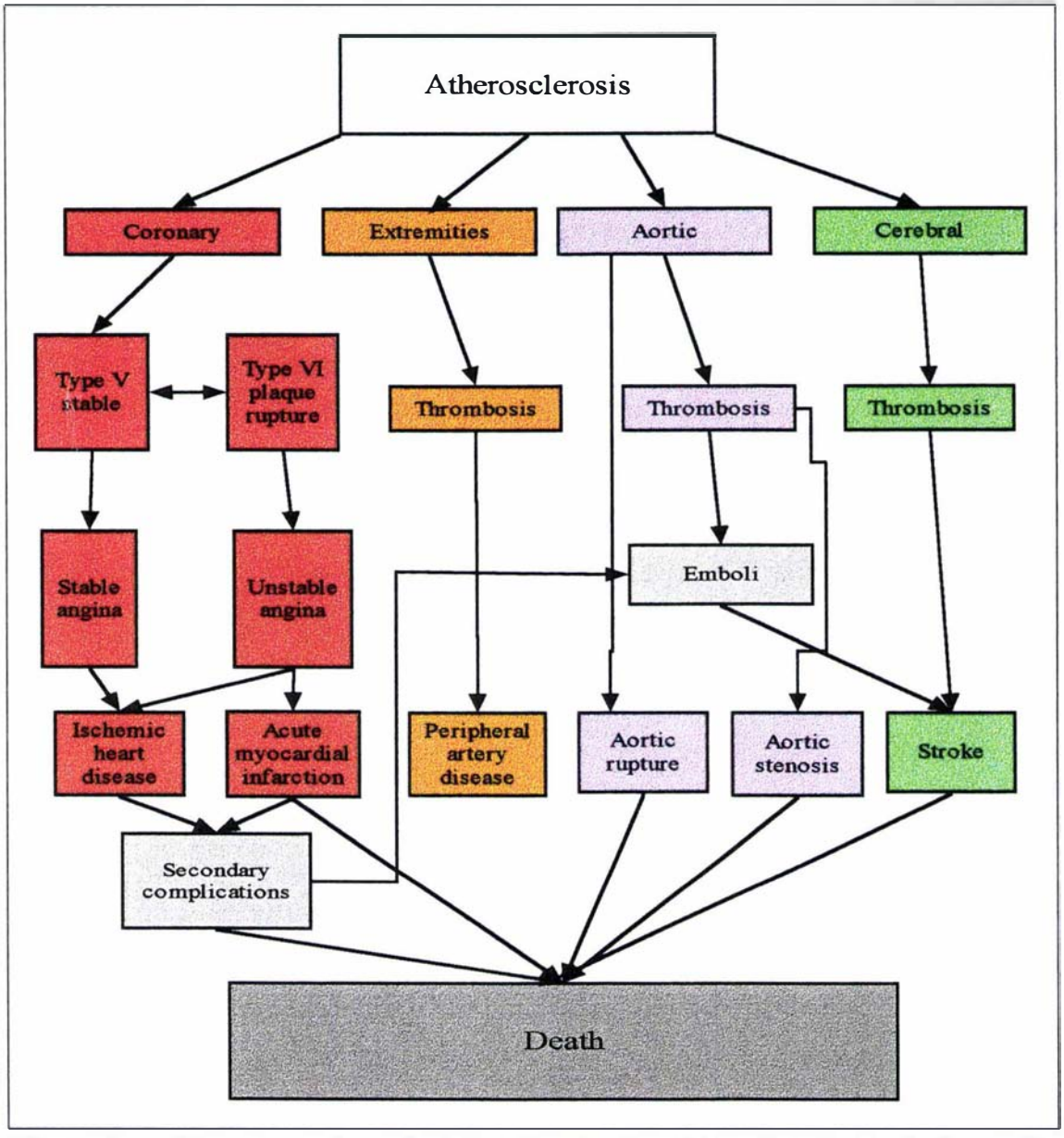


Figure 1.1. ***Clinical sequelae of atherosclerosis.*** The first row of boxes indicates the arterial system in which the atherosclerotic lesion is present.

1. Lipid metabolism

Human beings have evolved to eat a low fat diet consisting predominantly of grains and roots[9]. This differs markedly from the modern Western diet which derives approximately 40% of its calories from fat[10]. Excess dietary fat alters lipid metabolism, increasing the serum concentration of lipoproteins[10]. Lipoproteins form microemulsions in serum and are used by the body to transport long-chain hydrophobic molecules[11]. The solid elements of the emulsion are spherical particles consisting of a core of triglyceride and cholesteryl esters surrounded by a monolayer of phospholipid and unesterified cholesterol[11]. Associated with

the monolayer are apolipoproteins, which have structural and receptor recognition functions[11]. Some classes of lipoprotein particles are believed to play an important role in the pathogenesis of atherosclerosis[12,13].

When considering atherosclerosis, the two most important lipid classes are triglyceride and cholesterol. A triglyceride molecule consists of three fatty acids bonded to glycerol. Triglyceride comprises around 90% of the lipid in the typical Western diet and provides a major energy source for the body[14]. Cholesterol belongs to the steroid group of lipids and contains 27 carbon atoms, one hydroxyl group and a branched aliphatic side-chain[15]. Cholesterol is a major component of biological membranes and is a precursor for steroid hormone production[16]. It is also metabolised to bile acids, necessary for the absorption of fat[16].

Pre-hepatic lipid metabolism

In the intestine, dietary lipids are absorbed by enterocytes after combining with bile acids to form micelles[14]. Triglyceride that is absorbed from the small intestine is derived solely from the diet, however, this is not true of cholesterol which undergoes significant enterohepatic circulation[15,17].

The enterocyte distributes the absorbed lipid to the rest of the body using two mechanisms[18]. Fatty acids with a chain length less than 12 carbon atoms are transported in serum, bound to albumin[18] while longer-chain lipids, including cholesterol, which are too hydrophobic to remain in solution, are packaged into chylomicrons[15,16].

Chylomicrons

Chylomicrons have a density of 0.93g/ml and are the largest class of lipoprotein, typically containing 85% triglyceride, 7% cholesterol, 6% phospholipid and 2% protein[11]. Apolipoproteins (apo) B-48, C, and E are associated with chylomicron particles[11,19-22]. Chylomicrons are secreted by enterocytes into lymph and travel through the thoracic duct until they enter the systemic circulation at the cranial vena cava[19].

As chylomicron particles circulate, apoC activates endothelium-bound lipoprotein lipase[23,24]. This enzyme, which is found at high concentrations in the capillaries of adipose tissue, mammary gland, and striated muscle, removes the triglyceride allowing it to be utilised by tissues[11,25]. Once the triglyceride content of the chylomicron has been depleted, apoC dissociates and the resulting particle is classified as a chylomicron remnant[11]. These contain cholesterol and are endocytosed by hepatocytes after recognition of apoB-48 and apoE by the LDL-receptor-like protein[11]. Although this receptor binds to the same apolipoproteins as the LDL receptor, both receptors appear to be specific for the two different classes of lipoprotein[22,26].

Hepatic lipid metabolism

Triglyceride

Triglycerides, synthesised in the liver from free fatty acids, are used to meet the energy needs of the body and to supply substrates for storage in adipose tissue. Free fatty acids are manufactured in the liver from glucose, amino acids, and alcohol in a reaction which is rate-limited by acetyl CoA carboxylase[11]. The activity of this enzyme is regulated by insulin and glucagon[16,19].

Cholesterol

Hepatic cholesterol may either be derived from serum lipoproteins, removed from circulation by receptor-dependent endocytosis, or be synthesised by hepatocytes[11,15,27]. Cholesterol removed from serum is initially contained in lysosomes and in these, the cholesteryl esters are hydrolysed, enabling free cholesterol to pass through the lysosomal membrane into the cytoplasm of the cell[27]. The free cholesterol is then either re-esterified by acyl-CoA acyl transferase (ACAT) or retained as free cholesterol[28-30]. Hepatic cholesteryl esters are either stored within the hepatocyte or packaged into very low density lipoprotein (VLDL) particles and exported[11,29]. Free cholesterol can be used to meet cellular cholesterol requirements or excreted into the bile after hydroxylation by cholesterol 7α-hydroxylase[11]. The rate of hepatic cholesterol synthesis is regulated by the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.

Therefore, the metabolism of cholesterol within the liver depends on three key enzymes which, due to their importance, will be considered separately.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase

This is the rate-limiting enzyme in the mevalonate pathway. Mevalonate is not only a substrate for cholesterol synthesis, but is also crucial for the production of dolichol, ubiquinone, farnesyl pyrophosphate, and other essential non-sterols[31]. All nucleated cells possess this enzyme, but most cholesterol synthesis occurs in the liver and intestine[32].

Controlling several metabolic pathways with one enzyme requires a finely tuned mechanism. To prevent excess cholesterol production, while ensuring sufficient mevalonate is present within the cell, HMG-CoA reductase is regulated by both transcriptional and post-transcriptional controls[11,31]. Transcription of the enzyme gene is governed by a promoter region designated the sterol regulatory element (SRE)[11,31]. This region also controls LDL receptor activity and is discussed in more detail later in this review. Briefly, the SRE is activated by low cellular cholesterol concentrations and gene transcription proceeds only when the SRE is activated[33,34].

Once it has been produced, the degradation rate of HMG-CoA reductase is also closely controlled. When present in cells that have been deprived of mevalonic acid and sterols, HMG-CoA reductase has a long half life, however, when adequate concentrations are present, the rate of degradation is increased[31,35]. The enzyme is also reversibly inactivated by an ADP-stimulated protein kinase[36].

Acyl-CoA acyl transferase (ACAT)

This enzyme esterifies cholesterol using a fatty acyl CoA molecule[37]. Cholesterol in the liver is divided into two pools, the free cholesterol pool and the cholesteryl ester pool[11,37]. ACAT is activated by high intracellular concentrations of free cholesterol[38]. In effect, ACAT and HMG-CoA reductase have opposing actions, HMG-CoA reductase increasing free cholesterol within the hepatocyte and ACAT removing it.

Cholesterol 7α-hydroxylase

Cholesterol 7α-hydroxylase catalyses the insertion of a 7α-hydroxyl group into the nucleus of cholesterol[39,40] and is the rate-limiting enzyme in the production of primary bile acids (cholic acid or chenic acid) from cholesterol[39,40]. The production of bile acids by the liver is the only metabolic pathway available to excrete significant amounts of cholesterol[41]. Hepatic bile acids invariably inhibit cholesterol 7α-hydroxylase activity[39,40,42]. However, species variations exist regarding the response of the enzyme to high levels of hepatic cholesterol. In animals with efficient enterohepatic circulation of cholesterol, such as monkeys, an increase in hepatic cholesterol decreases cholesterol 7α-hydroxylase activity[41]. In animals such as rodents which reabsorb less cholesterol from the gut, high hepatic cholesterol concentrations increase the activity of this enzyme[42,43].

Post-hepatic lipid metabolism

As previously discussed, the liver produces, stores, and exports triglyceride and cholesterol. Lipid transport from the liver can be divided into two separate systems. The first, exports triglyceride to the body packaged in very low density lipoprotein (VLDL) particles. The other, transports cholesterol between the liver and tissues using LDL and high density lipoprotein (HDL) particles. However, this simple concept is complicated by the fact that LDL is a derivative of VLDL and both cholesterol and triglycerides are frequently exchanged among all three types of particles. A fourth lipoprotein, intermediate density lipoprotein (IDL), is a VLDL particle which has lost some triglyceride but still contains apolipoprotein E.

Very low density lipoproteins

VLDL particles have a density of between 0.93 and 1.006g/ml and typically contain 55% triglyceride, 19% cholesterol, 18% phospholipid, and 8% protein[11]. A VLDL particle contains apolipoproteins apoB-100, apoC, and apoE[44]. Although both apoE and apoB are present, binding to LDL receptors is prevented by the presence of apoC[11]. ApoC also activates endothelium-bound lipoprotein lipase which progressively removes triglyceride from the particle. When the majority of triglyceride has been depleted, structural changes within the lipoprotein result in the dissociation of apoC[11]. This particle is now classified as

an IDL particle with a density of between 1.006 and 1.019g/ml and containing approximately 23% triglyceride, 38% cholesterol, 19% phospholipid, and 19% protein[11].

Intermediate density lipoproteins

As IDL particles do not contain apoC, they can be removed from the circulation by LDL receptor-mediated endocytosis[27,45,46]. LDL receptors recognise both apoE and apoB, and IDL particles, which contain both, have a strong affinity for this receptor. IDL particles remaining in circulation are degraded further by hepatic lipase, which, unlike lipoprotein lipase, is inhibited by apoC and can only hydrolyse triglyceride contained in IDL or LDL particles[11,47]. As more triglyceride is lost, apoE dissociates resulting in the formation of an LDL particle[11,48]. The average time for a VLDL particle produced by the liver to be removed from circulation, or to be degraded to an LDL particle, is 45 minutes[25]. In normal humans, approximately half the apoB-containing particles produced by the liver are degraded to LDL[11].

Low density lipoproteins

An LDL particle has a density of between 1.019 and 1.063g/ml and contains approximately 6% triglyceride, 50% cholesterol, 22% phospholipid, and 22% protein[11]. ApoB-100 is the only apolipoprotein present in LDL particles, fulfilling both a structural role and acting as a ligand for LDL receptors[49]. As apoE is absent, LDL particles have low affinity for the LDL receptor and remain in circulation for an average of 2.5 days[11]. Seventy five percent of the LDL in serum is removed by LDL receptors[11,45,46,50] with hepatocyte LDL receptors responsible for approximately half of this[11].

High density lipoproteins

The fourth type of serum lipoprotein has a density of between 1.063 and 1.21g/ml and typically contains 4% triglyceride, 28% cholesterol, 34% phospholipid, and 45% protein[11]. HDL particles contain apolipoproteins A-I, A-II, C, D, and E[11,51,52]. As HDL primarily transports cholesterol from tissues to the liver, HDL cholesterol metabolism is often referred to as the 'reverse cholesterol transport pathway'[51].

Serum HDL particles are classified according to their size. Pre-β HDL particles are the

smallest, and are discoid or 'empty'[51]. Intermediate sized particles are called HDL_3, while the largest particles, which contain the highest concentration of lipids, are classified as HDL_2 particles[51,53]. HDL particles are then further classified according to the A class apolipoproteins present. This results in the classifications of Lp-I (apo A-I) and Lp-I/A-II (apoA-I and A-II)[51,53,54]. The A class apolipoproteins are important in determining the amount of cholesterol that the HDL particle is able to transport[54-56].

Cholesterol contained in HDL particles can be derived either from lipoproteins in the serum or from cholesterol contained in peripheral cells. In both cases, free cholesterol entering the HDL particle is esterified by the lethicin : cholesterol acyl transferase (LCAT) enzyme[51]. Esterification enables the storage of large quantities of cholesteryl esters within the core of the lipoprotein[51].

Pre-β HDL particles are the acceptors of cholesterol from tissues. ApoA-I anchors the HDL particle to peripheral cells enabling cholesterol to diffuse into the lipoprotein[56-58]. As cholesteryl esters accumulate, the pre-β HDL particle becomes rounded and apoA-I is no longer able to tether the particle. These larger HDL_3 particles derive most of their cholesterol from circulating VLDL particles and chylomicrons[57].

Cholesteryl esters contained in HDL particles can be metabolised in three ways. Firstly, they can be transferred directly to a variety of cell types, including hepatocytes[51], regenerating a pre-β HDL particle[59]. Secondly, since HDL particles contain apoE, LDL receptor-mediated endocytosis can remove the particle from circulation[11]. Thirdly, cholesteryl esters can be exchanged for a triglyceride from a VLDL particle in a reaction mediated by cholesteryl ester transfer protein (CETP)[51,60,61]. Once the VLDL particle has been degraded into IDL or LDL, the cholesterol is then taken up by the liver or tissues[11]. The exchanged triglyceride contained in the HDL particle is removed by hepatic lipase, resulting in the regeneration of a pre-β HDL particle[59].

Lipoprotein (a)

Lipoprotein (a) [Lp(a)] consists of an LDL particle enveloped by a unique apolipoprotein, apo(a)[62,63]. The function of this lipoprotein is unknown and individuals genetically deficient

in Lp(a) appear to suffer no adverse effects[62]. The serum concentration of Lp(a) is dependent on the synthesis of apo(a) by the liver[62], the rate of production being genetically determined[63,64]. The mechanism by which Lp(a) is removed from the blood is also largely unknown, although, involvement of LDL receptors is unlikely[62,65]. Lp(a) is believed to be a risk factor for atherosclerosis[62,65] and will be discussed in this context later.

2. Regulation of serum lipid concentrations

This section discusses how the body regulates the concentration of both triglyceride and cholesterol in each of the classes of lipoprotein circulating in serum.

Triglyceride

Chylomicrons

The serum concentration of chylomicron triglyceride is determined by the amount of fat absorbed from the gut and the rate at which it is removed from serum by tissues[16]. The rate of chylomicron particle production is constant and the large fluctuations in lipid export from enterocytes is managed by increasing the size of the chylomicrons[11]. The removal of chylomicron triglyceride from serum is dependent on the concentration and the activity of lipoprotein lipase in tissues[24,66].

Very low density lipoproteins

Only small increases in the number of VLDL particles produced by the liver are possible. Therefore, increases in hepatic triglyceride export are mainly achieved through increased VLDL particle size[11]. The time that VLDL triglyceride remains in circulation is dependent on the activity of lipoprotein lipase and hepatic lipase. Lipoprotein lipase activity is controlled both by hormones (insulin, thyroid hormone, and glucocorticoids) and by regulation of enzyme production and degradation[67-69]. Data on the regulation of hepatic lipase are scarce and contradictory. It has been reported that enzyme activity may either increase[70] or decrease[71] in association with a high cholesterol diet.

Cholesterol

Low density lipoproteins

The serum LDL cholesterol concentration is determined by the number of apoB-containing particles produced by the liver, the proportion of these particles that are degraded to LDL, and the rate that LDL is removed from serum by LDL receptors. As will be discussed later in this review, the concentration of serum LDL cholesterol is considered a significant risk factor for atherosclerosis and each determinant will be discussed separately.

Hepatic apoB particle production

The rate of apoB-100 secretion by the liver is genetically determined and only small increases in this rate occur in response to increased hepatic triglyceride[72] or cholesterol[73] concentration. Oestrogen[74] and glucocorticoids[75] also promote apoB-100 secretion, while insulin inhibits it[76].

Proportion of VLDL particles degraded to LDL

Due to their greater triglyceride content, larger VLDL particles spend more time as IDL and are less likely to be degraded to LDL[66]. Larger VLDL particles are also poorer substrates for hepatic lipase[46,77] and possess increased affinity for LDL receptors due to their greater content of apoE[78]. However, as will be discussed later, it is undesirable to have large VLDL particles because, although less LDL is produced from them, any LDL that is produced is smaller, denser, and more atherogenic[48,79].

LDL removal from circulation

The rate that IDL and LDL particles are removed from serum by LDL receptor-dependent endocytosis is the single most important determinant of serum cholesterol concentration[46,80]. It is determined by LDL receptor activity and by the affinity of both IDL and LDL particles for this receptor.

LDL receptors remove approximately 75% of the cholesterol in serum[11,45,46,50]. Hepatic LDL receptor activity is the biggest determinant of serum cholesterol concentrations because, although all cells can remove cholesterol from the circulation, only the liver can

efficiently excrete it from the body[46]. High LDL receptor activity reduces serum cholesterol concentration while low activity increases it[46].

LDL receptor activity is controlled by regulating the transcription of the LDL receptor gene[75]. Transcription is governed by three promoter regions[81]. While two of these are activated by the universal transcription factor Sp1[81], the third is different and is designated the sterol regulatory element (SRE)[11,31,81,82]. This region is activated when bound to the sterol regulatory element binding protein (SREBP)[81]. When the intracellular sterol concentration is low, protease enzymes release the SREBP from the endoplasmic reticulum[83]. Oxygenated derivatives of hepatic free cholesterol are most potent in regulating protease activity[31,33]. Therefore, low concentrations of free cholesterol result in protease activity, the release of SREBPs, and gene transcription. If the SRE has not been activated by SREBPs, no transcription of LDL receptors occurs[34,81,83,84] and LDL receptor number decreases within a few hours[75].

Therefore, LDL receptor activity and the subsequent concentration of serum cholesterol are determined by the concentration of free cholesterol in hepatocytes. This concentration is dependent on the activity of ACAT, cholesterol 7α-hydroxylase, and HMG-CoA reductase. ACAT transfers cholesterol from the free cholesterol pool (which determines SREBP release) to the cholesteryl ester pool. As esterified cholesterol does not regulate LDL receptor activity, high ACAT activity lowers free cholesterol and increases LDL receptor activity[85]. Cholesterol 7α-hydroxylase activity determines the amount of free cholesterol in the liver that is excreted in bile[42,43]. This enzyme also excretes the oxygenated derivatives of free cholesterol and so strongly influences receptor activity[42,43]. HMG-CoA reductase synthesises free cholesterol and high enzyme activity increases serum cholesterol concentration[11].

LDL receptor activity is also influenced by hormones. Thyroid hormone increases LDL receptor expression[75] and hypothyroid subjects develop high serum cholesterol concentrations[75]. Oestrogen, insulin and glucagon also stimulate the production of hepatic LDL receptors, whereas glucocorticoids reduce receptor production[75,86-88].

The affinity of an IDL particle for the LDL receptor is dependent on the structure of the apoE apolipoproteins associated with it[89]. In humans, three alleles (E_2, E_3, and E_4) have been identified[90]. IDL particles associated with $ApoE_2$ have the lowest affinity for the LDL receptor, $apoE_4$ the highest, while $apoE_3$ is intermediate[90]. Paradoxically, because IDL particles with low affinity for the LDL receptor decrease hepatic cholesterol concentration, due to increased LDL receptor activity, these particles actually decrease serum cholesterol concentrations[91].

It is estimated that differences in receptor affinity among apoB isoforms account for between 2 and 5% of the population variation in serum cholesterol concentration[92]. Receptor affinity is also determined by the size of the LDL particle[48], with both small and large LDL particles having lower affinity for the LDL receptor than those of intermediate size[24,48,93]. LDL particle size is dependent on the activity of CETP[94] which exchanges a triglyceride from a VLDL particle for a cholesteryl ester from LDL or HDL[60,61]. If CETP activity is high, more cholesteryl esters will be exchanged for triglyceride molecules from VLDL particles[95]. These triglycerides are removed by hepatic lipase, producing a small, dense LDL particle[60]. Unlike the relationship between IDL particle affinity and serum cholesterol concentration, a decreased affinity of LDL particles for the LDL receptor will increase serum cholesterol concentration[46].

High density lipoproteins

Serum HDL cholesterol concentration is regulated by the serum concentration of apoA-I, the main apolipoprotein present in an HDL particle[96]. However, it is unclear whether the fractional catabolic rate or the production rate of the apolipoprotein determines the concentration in serum[96-99]. The catabolic rate of apoA-I may be related to the size of the HDL particle, with small particles degraded at a faster rate than large ones[96,100]. However, contradictory evidence has recently suggested that a high content of saturated fat in the diet increases serum HDL cholesterol concentrations due to a reduced fractional catabolic rate of apoA-I without altering particle size[97,101,102].

3. Pathogenesis of atherosclerosis

Human atherosclerosis is a chronic inflammatory and degenerative process associated with the deposition of lipid in the walls of arteries. Lesions occur first, and most extensively, in the abdominal aorta and iliac arteries[103,104], but are also regularly seen in the coronary arteries[103,104]. They develop less commonly in the thoracic aorta, femoral, popliteal, internal carotid, vertebral, and basilar arteries and are only rarely seen in the renal, mesenteric, and pulmonary arteries[103-105].

The first recognisable lesion believed to be associated with atherosclerosis is the fatty streak which is ubiquitous in Western subjects by the age of 10 years[106]. While preatheroma lesions develop in normolipidaemic subjects around the time of puberty, it is not until the third decade of life that more advanced atheromas begin to develop[106].

The normal artery wall

An artery wall consists of three layers, the intima, which is closest to the lumen, the media, and the adventitia[107]. The lumen of the artery is lined by a continuous layer of endothelial cells attached to each other by junctional complexes[107]. These cells are anchored to a basement membrane which is supported by a proteoglycan matrix[6]. The junction between the intima and the media is demarcated by the internal elastic lamina[107]. The media consists of smooth muscle cells and is the muscular layer of the artery, maintaining arterial tone and blood pressure[107]. The abluminal border of the media is marked by the external elastic lamina. Outside this is the adventitia[6] containing small blood vessels (the vasa vasorum) and nerves[6,107].

Lipoprotein accumulation and oxidation

Early atherogenesis is dependent on two processes occurring within the intima of the artery. Firstly, lipoproteins must leave the circulation and accumulate in the intima, and secondly, to become atherogenic, these lipoproteins must then become oxidised.

There are currently two hypotheses regarding the mechanism by which lipoproteins accumulate within the intima of large arteries. The response-to-injury hypothesis proposes

that damage to endothelial cells increases the permeability of the endothelial barrier allowing increased influx and accumulation of lipoproteins[108-111]. The response-to-retention hypothesis suggests that in a normal artery, lipoproteins pass freely in and out of the intima and only accumulate if they become trapped within the subendothelial matrix[112]. According to this hypothesis, risk factors for atherosclerosis alter the subendothelial matrix so that increased amounts of lipoprotein are trapped and unable to return to the circulation[112].

It is still unclear which model more closely reflects the physiological process, although some recent evidence supports the retention hypothesis better than the injury hypothesis[113-116]. Perhaps the best model for lipoprotein deposition is a combination of both, whereby, in a normal artery a constant proportion of serum lipoproteins penetrate the endothelium, and a constant proportion of these become trapped. Therefore, either an increase in endothelial permeability or a change in the structure of the subendothelial matrix will result in greater lipid accumulation.

Regardless of the mechanism by which lipoproteins accumulate in the subendothelial matrix, their oxidation is the next crucial atherogenic process[117-120]. Significant lipoprotein oxidation does not occur in plasma because of the antioxidant properties of the plasma proteins[121,122]. However, once trapped in the subendothelial matrix, a microenvironment that excludes this protection is created[123] and the lipoprotein becomes exposed to the normal products of cellular metabolism including metal ions[124], thiols[124-126], superoxide[124], nitric oxide[124], hypochlorous acid, and tyrosyl radicals[127].

Oxidation of LDL has been extensively studied and only a brief summary of some of the key aspects will be reviewed. Although this review will be confined to oxidation of LDL, other lipoprotein classes have also been found to become trapped and oxidised by similar processes[128-130].

This review of the mechanism of lipid oxidation will focus on superoxide ($\cdot O_2^-$) and is summarised in Figure 1.2. Other prooxidants can initiate oxidation by producing other radical species (eg tyrosyl radicals), however, since these initiate lipid oxidation by a similar mechanism to superoxide[124], they will not be discussed separately. Once produced,

1) Production of superoxide from thiol breakdown and myeloperoxidases within the arterial cell.

2) Superoxide dismutases into hydrogen peroxide.

$$2\,O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2$$

3) Hydrogen peroxide reacts with metal ion to produce hydroxyl radical.

$$M^{(n+)} + H_2O_2 \rightarrow M^{(n+1)} + OH^- + {}^{\cdot}OH$$

4) Hydroxyl radical enters membrane and produces a carbon radical.

$$OH + R\text{-}(\text{-}CH_2\text{-}) \rightarrow H_2O + R\text{-}(\text{-}HC\text{-})$$

5) Carbon radical molecular rearrangement occurs so that a conjugated diene is formed.

$$R\text{-}(\text{-}HC\text{-}) \rightarrow =CH$$

6) Conjugated diene reacts with oxygen to form a peroxyl radical.

$$=CH + O_2 \rightarrow =CHO_2^{\cdot}$$

7) Chain reaction continues with peroxyl radical reacting with another polyunsaturated fatty acid.

$$=CHO_2^{\cdot} + R\text{-}(\text{-}CH_2\text{-}) \rightarrow =CHO_2H + R\text{-}(\text{-}HC\text{-})$$

Figure 1.2. ***Lipid oxidation within an LDL particle.***

superoxide is rapidly dismutased, either spontaneously or in a reaction catalysed by superoxide dismutase, producing hydrogen peroxide (H_2O_2)[131]. Hydrogen peroxide then reacts with a metal ion, most often iron or copper (M^{n+}) to produce ($M^{(n+1)}$), a hydroxide ion (OH^-) and a hydroxyl radical ($^{\cdot}OH$)[131]. The hydroxyl radical, and all other radical species capable of initiating lipoprotein oxidation, have two critical properties. They are able to enter the hydrophobic interior of lipoproteins and, once there, have sufficient reactivity to extract a hydrogen atom from a methylene ($-CH_2-$) group in a fatty acid[124,131]. A hydrogen atom from an unsaturated fatty acid is likely to be targeted as the C-H bond is weaker adjacent to a double bond[131]. The carbon radical formed undergoes molecular rearrangement producing a more stable conjugated diene[131], which reacts with an oxygen molecule, producing a peroxyl radical ($ROO^{\cdot}$)[131]. Antioxidants within the particle are able to scavenge these radicals, however, if no antioxidants are present, peroxyl radicals are able to extract another hydrogen atom from a fatty acid, thereby continuing the chain reaction of lipid peroxidation[132].

LDL oxidation can be divided into three phases, lag, propagation, and decomposition[133]. In the lag phase, peroxyl radicals produced in the LDL particle progressively deplete it of antioxidants[133]. Small amounts of oxidised lipid are present within the LDL particle during

the lag phase[133] and these particles are classified as mildly oxidised LDL (mLDL)[133,134]. If the oxidant stress continues, the antioxidant protection becomes exhausted and the LDL particle progresses from the lag into the propagation phase. In this phase, the autocatalytic reaction characterising lipid peroxidation can occur unimpeded and oxidised lipids form rapidly within the LDL particle[134]. The final phase, decomposition, involves the fragmentation of oxidised lipids into highly reactive aldehydes and ketones which damage the apoB protein[133-135]. Once apoB has been damaged so severely that it is no longer recognised by the LDL receptor, the particle is classified as oxidised LDL (oxLDL)[117].

When contemplating the changes that occur during LDL oxidation, it must be remembered that, while it is convenient to divide oxidation into phases, the process of lipid oxidation is continuous and best thought of as chaotic, rather than orderly[133,134]. The specific products of oxidation are also variable depending on both the initiator and the conditions in which the oxidation reaction occurs[133,136].

Formation of foam cells

The presence of mLDL in the intima stimulates endothelial cells to produce monocyte chemotactic protein, which attracts monocytes from the blood[123,137], and colony-stimulating factor, which prevents them from leaving[138]. Interestingly, as mLDL does not stimulate endothelial cells to produce endothelial-leucocyte adhesion molecule, neutrophils are not attracted[139,140]. Once monocytes enter the intima, they differentiate into macrophages and become activated[141]. Activated macrophages produce chemotactic factors for T lymphocytes[141], activate platelets, and stimulate endothelial cells, forming a self-perpetuating cycle[141]. The activated macrophages also leak lysosomal contents, increasing the oxidative stress on the trapped lipoprotein[117].

The presence of a damaged apoB protein in an oxLDL particle is critical for foam cell development. Macrophages possess LDL receptors which recognise apoB but, as in all cells, receptor activity is strictly regulated by the intracellular concentration of cholesterol[49]. Macrophages also express scavenger receptors which recognise damaged apoB and importantly, their activity is not regulated[142]. Therefore if sufficient oxLDL is present within

the intima, macrophages engulf large quantities of lipoprotein resulting in cells with abundant, lipid-filled cytoplasm[120]. These macrophages are classified as foam cells, and their accumulation in the intima signifies the development of a type I fatty streak, proposed to be the earliest lesion of atherosclerosis[2].

A type I lesion progresses to a type II lesion by the migration of smooth muscle cells from the media to the intima of the artery[2]. These cells are attracted to factors produced by activated macrophages, platelets, T lymphocytes, and endothelial cells[141], including platelet derived growth factor, interleukin-1, fibroblast growth factor, and endothelin[2,141,143]. LDL in this environment is quickly oxidised and engulfed by the increasing numbers of macrophages[143]. Oxidised LDL, due to its lysophosphotidylcholine content is also directly chemotactic for monocytes[144], smooth muscle cells[145], and T-lymphocytes[144]. The migrating smooth muscle cells engulf lipid and a type II lesion has been defined as an intimal accumulation of macrophages and smooth muscle cells containing lipid droplets[2].

Type II lesions may be further classified depending on their proximity to an intimal pad[146]. Intimal pads are normal thickened areas of the intima present near branch points of arteries as an adaption to physiological variations in shear and tensile forces[146]. These areas of the intima have an altered subendothelial matrix which possesses a greater affinity for lipoproteins than the normal artery wall[112,147]. Type IIa lesions occur around intimal pads[146] and are likely to progress to an advanced lesion, whereas type IIb lesions are found away from areas of intimal thickening and progression is unlikely[146].

HDL is important in regulating lesion progression and has two functions during the initial stages of fatty streak development[119]. Firstly, it removes lipids from cells in the intima as part of the 'reverse cholesterol transport pathway'[2] and secondly, it decreases LDL oxidation[148,149]. HDL acts as an antioxidant by producing platelet activating factor-acetylhydrolase[150] and paraoxanase[151], and by chelating transition metal ions[152]. However, HDL is also susceptible to oxidation, resulting in a loss of antioxidant action and, by becoming deposited in the intima, can contribute to lesion progression[128].

The type III lesion or preatheroma, is characterised by the presence of free lipid droplets in

the intima[106,146]. This lipid may either be from trapped serum lipoproteins or from dead foam cells[2]. Foam cells die because the increased concentrations of intracellular unesterified cholesterol result in membrane crystallisation[153,154]. Dead foam cells release enzymes, highly oxidised lipids and free radicals[2], causing injury to adjacent macrophages and endothelial cells[134]. However, an atheroma has not yet formed and, if the intimal environment changes then lesion regression is possible[146].

Development of an atheroma

While the development of the first three stages of an atherosclerotic lesion is a slow, insidious process, occurring over decades, once an atheroma has formed, lesion progression and the development of clinical signs can occur within weeks[2].

An atheroma, or type IV lesion, is distinguished from a type III lesion by the presence of a lipid core formed by the coalescence of extracellular lipid droplets[106]. The lipid in the core is believed to be mostly derived from ruptured macrophages and contains a high proportion of unesterified cholesterol[155]. Increased numbers of macrophages, smooth muscle cells, lymphocytes and mast cells are present in the area of the intima between the lipid core and the lumen[146]. A type IV lesion does not result in any significant reduction in the diameter of the arterial lumen or in clinical signs[146].

In an attempt to increase strength, collagen is produced between the lipid core and the lumen[146]. This is called a fibrous cap and once developed, the lesion is classified as a fibroatheroma or type V lesion[146]. Type V lesions are also characterised by the presence of lipid droplets within smooth muscle cells in the media[146]. Attracted to the lipid, monocytes and lymphocytes migrate from the vasa vasorum to adjacent areas of the adventitia[146]. Type V lesions are prone to disruption, leading to the formation of mural thrombi[146].

Development of a complicated lesion

Disruption of a type V lesion, exposing thrombogenic material within the plaque, promotes rapid lesion growth and the development of clinical syndromes[156]. A type V lesion with evidence of disruption is classified as a complicated atheroma or a type VI lesion[146]. Plaque

disruption is a common event and it is estimated that, at any time, 9% of 'normal' people have a disrupted plaque in their coronary arteries[157]. Plaque disruption may occur as either superficial fissures or deep ruptures exposing the lipid core.

Surface fissures are associated with damage to endothelial cells caused by oxidised lipids contained in the fibrous cap[124]. The resultant thrombi are usually small, remain attached to the plaque, and are clinically silent[158]. Repeated episodes of these mural thrombi result in a multilayered, stenotic, type V lesion which can encroach into the lumen of the artery[158]. With increasing stenosis, ischaemic syndromes may occur, however, due to the development of collateral circulation, these episodes are usually less severe than a sudden occlusive event[2].

Plaque rupture is a potentially catastrophic, but comparatively uncommon event[124]. The lipid core is extremely thrombogenic and a large, clinically significant thrombus is likely to develop once it becomes exposed[124]. Properties of the atheroma which promote rupture include the presence of a large, eccentrically-placed lipid core[159] and a thin fibrous cap[156,160]. Inflammatory cells, attracted to oxidised lipid can degrade the fibrous cap by releasing collagenase, gelatinase and stromelysin[161]. Increased shear stress on the plaque also increases the chance of rupture[2].

The outcome of plaque disruption depends on the size of the thrombus formed. This is dependent on the amount and character of the plaque contents exposed[162], the degree of flow disruption[136,163], and the thrombotic tendency of the blood[160,164]. The balance between thrombosis and fibrinolysis is a strong determinant in the rate of growth of the lesion and in the severity of clinical events[164]. Many factors affect this balance, but since the focus of this review is atherosclerosis, a detailed discussion of thrombosis is not included. Suffice to say, without atherosclerosis, coronary thrombosis would not occur and without thrombosis, atherosclerosis would not be a significant cause of clinical symptoms. This has led to the introduction of the term 'atherothrombogenesis' to illustrate the integration of both processes in the development of ischaemic heart disease[165].

4. Animal models of atherosclerosis

To study atherosclerosis, a wide variety of animal models have been developed. Although animal models of human disease invariably have limitations, they can provide valuable information which could not be gained from studies on human patients.

In an ideal animal model of atherosclerosis, the animal, when fed a Western type diet should consistently develop a serum lipoprotein profile and atherosclerotic lesions resembling those seen in humans. Furthermore, when factors believed to affect human atherogenesis are altered, reproducible changes in lesion formation should occur. The model should be cheap, easily handled, ethically justifiable, genetically homologous, obtainable in large numbers, and develop atherosclerotic lesions quickly. Unfortunately, no such perfect model exists, and the researcher must choose between models offering low cost and ease of handling, and those in which the physiology and pathogenesis more closely resembles that seen in human subjects.

Mouse models of atherosclerosis

The C57BL/6 mouse model

A survey of atherosclerosis susceptibility among various inbred strains of mice (*Mus musculus*) was conducted in 1976[166]. These findings were supported by a second survey in 1985 which found that, depending on the strain of mouse, serum cholesterol concentrations varied between 3.4 and 8.6 mmol/l and the average number of fatty streaks ranged from 0 to 1.8 per mouse[167]. In this second survey, the greatest lesion development was observed in C57BL/6 mice[167]. These lesions have since been found to histologically resemble fatty streak lesions in humans[168].

Recent studies have reported that the increase in cholesterol concentration observed in C57BL/6 mice fed an atherogenic diet is associated with a reduction in cholesterol 7α-hydroxylase activity and increased hepatic cholesterol concentration[169]. Interestingly, in BALB/c mice, which are resistant to atherosclerosis, reduced cholesterol 7α-hydroxylase activity does not result in either increased hepatic or serum cholesterol concentrations suggesting that resistant mice have an alternative mechanism to excrete cholesterol which

does not involve the formation of bile acids[169]. Another recently identified difference between susceptible and resistant strains is the production of large quantities of inflammatory mediators by C57BL/6 mice when fed an atherogenic diet[170]. This has led to the suggestion that the fatty streaks seen in C57BL/6 mice may be due more to chronic inflammation than to the atherogenic processes which occur in human subjects[170]. The value of the C57BL/6 model is also reduced by the absence of serum lipoprotein (a) and CETP[171], both of which have both been shown to play a significant role in human atherogenesis.

Unlike the situation in human populations, when fed an atherogenic diet, female C57BL/6 mice develop lower serum HDL cholesterol concentrations and more extensive aortic fatty streaks than males[172]. When female mice were treated with testosterone, the serum HDL cholesterol concentration increased, and lesion formation decreased to a level similar to that observed in males[172]. The greater susceptibility of female mice to atherosclerosis may therefore be related to their lower serum HDL cholesterol concentrations[172].

The effect of dietary fats on fatty streak formation in C57BL/6 mice has been studied previously[173]. Mean lesion size was greatest with saturated fats, least with monounsaturated fats, and intermediate with polyunsaturated fats[173]. Dietary alcohol has previously been found to reduce fatty streak formation and serum total cholesterol concentration in C57BL/6 mice[174].

Transgenic/knockout mice

By manipulating the genome, mice which either have a normal gene suppressed (knockout mice) or which express an abnormal gene (transgenic mice) can be produced[171,175,176]. These mice can then be used to study the role of different proteins in atherogenesis. Examples of genetically-modified mice which have been produced include:

- Mice expressing human LDL receptor genes. These have a promoter region regulated by transferrin, so that LDL receptor activity can be manipulated experimentally[177].
- LDL receptor-knockout mice are a model for human familial hypercholesterolaemia. These, like the WHHL rabbit model, have poorly functioning LDL receptors and develop high serum LDL cholesterol concentrations and atheromatous lesions[178].

- Transgenic mice expressing the human apoB gene were used during this project and a more detailed discussion of this model is contained in Chapter 8.
- Human apoA-I genes have been inserted into the mouse genome to investigate dietary regulation of serum HDL cholesterol concentrations[97].
- ApoA-I-knockout mice have low serum concentrations of HDL cholesterol, although, in these mice, this is not associated with increased atherogenesis[179].
- Insertion of a human apoA-II gene does not significantly affect the serum lipoprotein profile, supporting the hypothesis that apoA-II does not regulate serum HDL cholesterol concentration[180].
- Mice over-expressing human lipoprotein lipase have lowered serum triglyceride and increased serum HDL concentration[181].
- The expression of CETP lowers serum HDL cholesterol concentrations. ApoA-I/CETP-double-transgenic mice develop even lower serum HDL cholesterol concentrations, suggesting an interaction between ApoA-I, CETP, and HDL particle size[182].
- Over-expression of rat apoE in mice lowers serum LDL cholesterol concentration[183].
- ApoE-knockout mice develop very high cholesterol levels (around 50 mmol/l) and atheromatous lesions when fed a Western type diet (0.15% cholesterol, 20% fat, no cholic acid)[184]. Unlike the C57BL/6 model which only develops atherosclerotic lesions within the aortic sinus, ApoE-knockout mice develop widespread lesions throughout the arterial tree[184].
- Double transgenic mice expressing both human apoB and human apo(a) form Lp(a) in serum providing a model to investigate this lipoprotein[185].

To conclude, transgenic mice have been useful tools in determining the effect of altered expression of genes coding for proteins important in lipid metabolism. As many models have been developed but not yet fully evaluated, the information derived from these animals is likely to increase in the future.

Non-murine animal models of atherosclerosis

Rabbits

Rabbits (*Oryctolagus cuniculus*) were the first animals to be used in atherosclerosis research and are still the most commonly used animal model[186,187]. When fed diets containing 1-3% cholesterol and 6-8% fat, rabbits develop serum cholesterol concentrations of 26-70 mmol/l and cholesterol deposits in medium sized arteries, liver, spleen, bone marrow, adrenal gland, and eyes[188]. This distribution is suggestive of a cholesterol storage disease, raising doubts about the validity of comparisons between lesions seen in this model and human atherosclerosis[188,189]. Arterial fat deposition is also seen in rabbits after endothelial damage by physical (eg balloon catheter), chemical (eg viosterol), and immunological (eg graft rejection) means[187].

The Watanabe heritable hyperlipidaemic (WHHL) rabbit is a special strain useful in atherosclerosis research[190]. These rabbits are LDL receptor-deficient and, when fed normal rabbit food, develop atherosclerotic lesions similar to those seen in human homozygous familial hypercholesterolaemic patients[186,190].

Pigs

After being fed an atherogenic diet for three months, swine (*Sus scrofa*) develop significant atherosclerotic lesions in the aorta and branch arteries[186,188]. Pigs have many physiological and anatomical similarities to humans[186] and provide a popular model for atherosclerosis research[187]. Pure-bred lines are available and surgical interventions and measurements which are limited in smaller species are possible[187]. Swine deficient in von Willebrand factor have also been identified and have been used to investigate the role of thrombosis in atherogenesis[191]. Disadvantages of swine include handling difficulties and, due to their large size, the high cost of housing and feeding[187].

Non-human primates

Non-human primates develop hyperlipidaemia and arterial lesions in response to the same dietary modifications as human beings[188]. Disadvantages of non-human primates include cost, handling difficulties, and rarity of animals[186,188]. Old World monkeys are the non-

human primates used most often in atherosclerosis research[188]. Of these, the macaque species are the most popular, especially cynomolgus (*Macaca fascicularis*), rhesus (*Macaca mulatta*) and stumptails (*Macaca arctoides*)[186]. Different species of monkey are used to study different facets of atherogenesis. For example, to test diet-induced atherosclerosis, the rhesus monkey is recommended, while for the investigation of coronary artery lesions and their subsequent complications, cynomolgus monkeys are considered to be more appropriate[192]. Other non-human primates that have been used in atherosclerosis research include the African green monkey (*Cercopithecus aethiops*), the Celebes ape (*Macaca nigra*), and the squirrel monkey (*Saimiri sciureus*)[186].

Rats

Rats (*Rattus norvegicus*) are not commonly used in atherosclerosis research[186]. Rats fed a high cholesterol diet develop mild intimal and medial lipid deposits, but no atheromatous plaques[188]. An exception to this is the inbred JCR:LA-corpulent rat which develops hypertension, hyperlipidaemia, and fatty streaks[190]. Rat strains which have been used for investigations of serum lipoproteins include the LA/N-corpulent rat, the obese Zucker rat, and the spontaneously hyperlipidaemic Zucker rat[190].

Hamsters

Unlike other rodents, golden Syrian hamsters (*Mesocricetus auratus*) carry a significant proportion of their serum cholesterol in the LDL fraction[190]. The hamster also responds to hypocholesterolaemic drugs and, unlike mice, possesses serum CETP activity[190]. When fed a diet containing 10% fat and 0.12% cholesterol, hamsters develop serum total cholesterol concentrations around 15mmol/l and fatty streak lesions[193].

Birds

Birds, especially pigeons (*Columba livia*) and Japanese quail (*Coturnix coturnix*), are commonly used for atherosclerosis research[186]. White Carneau pigeons develop atherosclerosis when fed a normal diet[188]. These lesions resemble human atheromas histologically, but are usually found in small arteries[188]. Chickens (*Gallus gallus)*, bronze-breasted turkeys (*Meleagris gallopavo*), and ducks (*Anas platyrhynchos*) have also been used for atherosclerosis research, although recently they have been less popular[186].

5. Risk factors for atherosclerosis

A risk factor for a disease can be defined as any environmental, genetic, or phenotypic characteristic which predisposes an individual to that disease. There has been considerable effort to identify risk factors for atherosclerosis and the ones believed to be the most important are the subject of the next part of the review.

LDL cholesterol

The concentration of LDL cholesterol in serum is considered to be an important risk factor for atherosclerosis[194]. Evidence for the atherogenecity of LDL cholesterol is derived from animal research, epidemiological studies, histological examination of lesions, observations of familial dyslipidaemic syndromes, and clinical trials of cholesterol-lowering drugs[195]. However, the association between serum LDL cholesterol and atherosclerosis is not universally accepted and deficiencies in the 'lipid hypothesis' have been identified by some researchers[196,197].

LDL particles are suggested to contribute to atherogenesis after crossing the endothelial barrier and accumulating within the intima of the artery[112]. Since the endothelial barrier is a semi-permeable membrane, a rise in serum LDL concentration may increase the flux of LDL particles into the intima[198]. High serum LDL cholesterol concentrations may also decrease the rate at which the particles return to the circulation, prolonging the time spent by LDL in the intima, and increasing the chance of particles becoming trapped by the subendothelial matrix[112]. Once LDL has been trapped, it becomes oxidised and initiates or contributes to the atherogenic process[199,200].

Intrinsic properties of the subendothelial matrix, including the matrix proteoglycans[112], the activity of sphingomyelinase[201], and the activity of lipoprotein lipase[112], may also influence the number of LDL particles trapped within the intima. The hypothesis that properties of the subendothelial matrix can influence atherogenesis is comparatively new, however, these properties are believed by some to be among the most important risk factors for atherogenesis[112].

The size of an LDL particle is also believed to be an important determinant of atherogenicity[48,202]. Smaller particles are able to pass through the endothelial membrane at a higher rate than large ones[195] and, as they have greater affinity for the subendothelial matrix, they are more likely to become trapped[203]. Small LDL particles are also more susceptible to oxidation[203-206].

VLDL, IDL, and chylomicrons

Some authors suggest that triglyceride-rich lipoproteins (TGRLPs) are more important than either LDL or HDL in the development of atherosclerosis[207-209]. This hypothesis, the atherogenic remnant hypothesis, has been supported by studies which showed that, after the endothelium had been damaged, large amounts of TGRLPs are able to enter the intima[210]. A complementary hypothesis to this suggests that lipoprotein lipase on endothelial cells is able to tether the TGRLP, removing triglyceride until the particle is rich in cholesterol and small enough to pass into the intima[211,212].

HDL cholesterol

Epidemiological studies show a strong negative correlation between serum HDL cholesterol concentration and atherosclerosis[51], however, the mechanism by which HDL may provide protection is uncertain[51,212,213]. The 'causalist hypothesis' suggests that HDL particles transport cholesterol from the tissues to the liver. Therefore, high HDL cholesterol concentrations decrease atherosclerosis because more cholesterol is removed from tissues to be excreted by the liver[51,212]. The 'non-causalist' hypothesis is part of the atherogenic remnant hypothesis and suggests that both high HDL concentration and a low concentration of atherogenic TGRLPs are associated with an efficient lipoprotein metabolism[129,213]. The antioxidant properties of HDL are another suggested protective mechanism of this lipoprotein[51]. Regardless of the mechanism, the ratio of serum HDL to LDL cholesterol is strongly and inversely correlated to the likelihood of an individual developing atherosclerosis[194].

Lipoprotein (a)

Epidemiological evidence suggests that serum Lp(a) concentration is a risk factor for

atherosclerotic disease[214-217]. Lp(a) has a similar structure to LDL and like LDL, it can cross the endothelial barrier, become oxidised, and be engulfed by macrophages[130]. Lp(a) has a greater affinity for the subendothelial matrix than LDL[218] and, due to its lower antioxidant content, is more susceptible to oxidation[62]. Some authors suggest that, despite the much lower concentrations of Lp(a) than LDL in serum, much of the cholesterol contained in an atherosclerotic lesion is derived from Lp(a)[218].

Because of its structural similarity to plasminogen, Lp(a) also has prothrombotic properties[130]. Lp(a) competitively binds to plasminogen activator proteins, decreasing plasmin activation and fibrinolysis[219]. Because fibrin clots are important in atheroma growth, this disruption of fibrinolysis is believed to accelerate lesion development[219].

Triglyceride

A high serum triglyceride concentration results in an increase in the size of circulating VLDL particles. These large particles are good substrates for CETP, resulting in an increased exchange of VLDL triglyceride for HDL and LDL cholesterol[61,212]. This is undesirable for three reasons. Firstly, it produces LDL particles which contain a high concentration of triglyceride. The triglyceride is then removed by hepatic lipase producing small, dense, highly atherogenic LDL particles[48,202]. Secondly, it reduces the serum HDL cholesterol concentration[212,220]. Finally, TGRLPs containing a higher concentration of cholesterol are produced, which, if deposited within the intima, may accelerate atherogenesis[129]. Increased triglyceride levels also increase the coagulability of the blood, predisposing to thrombus formation and atheroma growth[221,222].

Thrombosis and fibrinolysis

Once an atheroma is disrupted, the size of the thrombus formed and the speed of fibrinolysis, are suggested to be critical factors in determining lesion progression and clinical outcome[2]. Increased coagulability or decreased fibrinolysis will result in the production of a larger, more persistent thrombus that is more likely to produce clinical symptoms, and, once remodelled, greater arterial stenosis[2].

Hypertension

Epidemiological studies have indicated increased blood pressure to be a strong independent risk factor for atherosclerosis[223]. Hypertension produces changes in the intimal proteoglycan matrix which have been suggested to increase lipoprotein affinity[112]. The renin-angiotensin system controls blood pressure within the body. One of the products of this, angiotensin II, may cause atherogenic changes to the intimal environment by activating endothelial cells[224-226]. In the later stages of the disease, hypertension may also increase the risk of disruption of an atheroma[2].

Genotype

Genetic makeup has been suggested to be the single most important determinant of an individual's risk for atherosclerotic disease[206]. It is estimated that 77% of coronary artery disease patients have an underlying genetically-linked defect in lipid metabolism. Of these people, 54% will have a first or second degree relative who will also suffer from atherosclerotic disease as a result of the same metabolic defect[206].

Diabetes

Both insulin-dependent and non-insulin-dependent diabetes have been shown to be strong risk factors for atherosclerosis. Diabetes increases serum triglyceride concentration, decreases serum HDL cholesterol concentration, and produces a preponderance of small, dense, glycosylated LDL particles[227]. Subjects with non-insulin-dependent diabetes also commonly have hypertension, abdominal obesity, and impaired fibrinolysis[227]. These changes are believed to result in 80% of North American diabetics dying as a result of atherosclerotic diseases[227].

6. Possible modifiers of risk factors for atherosclerosis

In this review, the risk factors investigated during the preparation of this thesis will be reviewed in detail while others will be discussed more superficially.

Dietary fat

Despite much research, the effect of individual fatty acids on atherogenesis remains

controversial. Evidence from epidemiological studies suggests that saturated fats are more atherogenic than unsaturated fats[228], however, the conclusions from this type of study are not universally accepted[196]. Animal studies examining the effects of dietary fat on atherogenesis are also difficult to interpret because of inconsistency in the results and questions regarding the validity of the experimental models used[173,188,189,229]. Therefore, studies examining the effect of fats on risk factors believed to influence atherogenesis have provided much of the evidence regarding the atherogenicity of dietary fats. A summary of the reported effects of some common dietary fatty acids is presented in Figure 1.3.

Saturated fatty acids

The effect of saturated fatty acids (SFAs) on the serum lipoprotein profile is dependant on the length of the carbon chain[230]. The medium chain SFAs, C12:0 (lauric acid), C14:0 (myristic acid), and C16:0 (palmitic acid) increase serum cholesterol concentrations, while shorter fatty acids and C18:0 (stearic acid) have little effect[230,231]. The quantity of dietary medium chain SFAs contained in a typical Western diet has a significant serum cholesterol-raising effect and it is estimated that replacing 50% of the SFAs in the diet with mono- or polyunsaturated fats would, on average, decrease the serum cholesterol concentration by 0.5mmol/l[232,233].

Palmitic acid (C16:0) is the second most common fatty acid in the Western diet, constituting 30% of total fatty acid intake in the United States[234]. It is the predominant fatty acid in palm oil, the most commonly consumed edible fat in the world, and is present in most plant oils and animal fats[234]. Palmitic acid has been shown to raise both serum LDL and HDL cholesterol concentrations when contained in a Western diet[235,236].

Dairy products are the major source of dietary myristic (C14:0) and lauric (C12:0) acids[235] and each constitutes between 3 and 10% of the fatty acids contained in a Western diet[235]. Early experiments indicated that serum cholesterol concentrations were increased four times as much by dietary myristic acid as by palmitic acid[237]. However, two recent experiments failed to confirm these findings, instead suggesting that dietary palmitic and myristic acid raised serum cholesterol concentrations approximately equally[234,238].

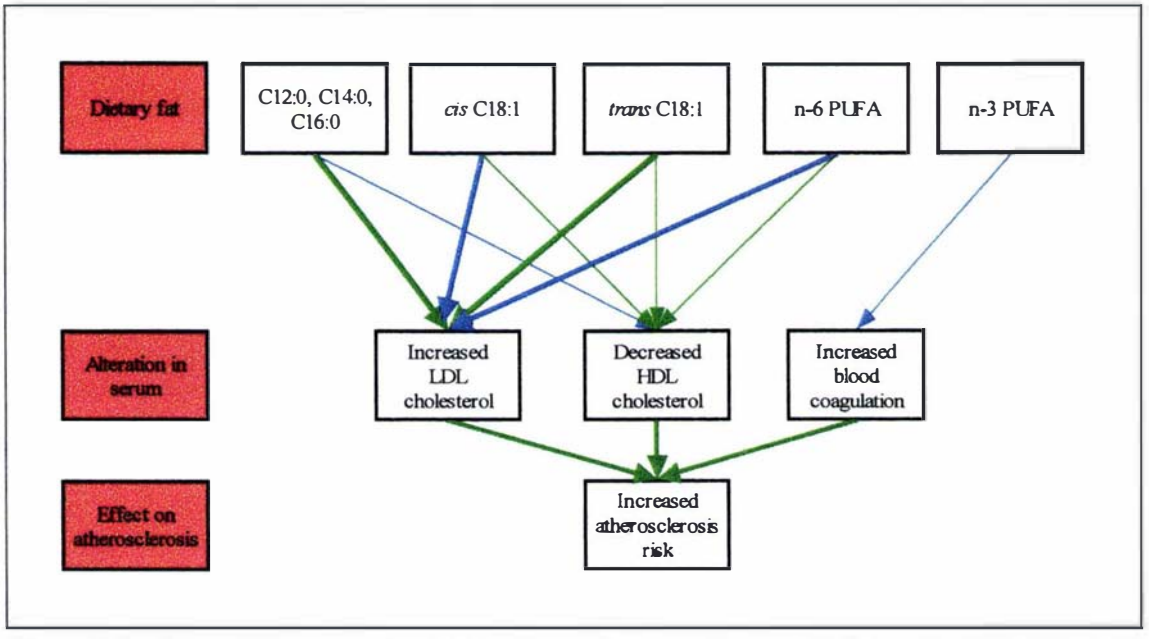


Figure 1.3. ***Dietary fats and atherosclerosis***. The green arrows indicate that the fatty acid promotes the risk factor in the box, the blue arrows show inhibitory effects. The thickness of the line shows the major effect of the fatty acid, thinner lines indicate a less significant or secondary action.

Of the medium chain SFAs, lauric acid appears to raise serum cholesterol concentration the least. In a study comparing the effects of high fat diets in human subjects, dietary palmitic acid resulted in the highest concentration of cholesterol, oleic acid (see monounsaturated fats) the least, with lauric acid intermediate[239].

In the body, stearic acid (C18:0) is rapidly metabolised into oleic acid and the effects on the lipoprotein profile of stearic and oleic acid are similar[18,230,240].

Serum LDL cholesterol concentrations are believed to be increased by medium chain SFAs because of reductions in the activity of hepatic LDL receptors[46]. These receptors are regulated by oxysterols which are produced from free cholesterol at a constant rate[33,85,169]. The amount of free cholesterol, and therefore oxysterols, present in the liver is dependent on the activity of ACAT[85] which esterifies hepatic cholesterol, removing it from the free cholesterol pool[15]. Unsaturated fatty acid acyl-CoA derivatives, especially oleoyl-CoA, are the preferred substrate for the ACAT enzyme[85,241,242]. Saturated fats appear to be poorer substrates, reducing cholesterol esterification, leading to increased hepatic oxysterol and serum cholesterol concentrations[85].

It is less clear how saturated fats raise serum HDL cholesterol concentration. It is thought that serum HDL cholesterol concentrations are determined by the serum concentration of apoA-I, which, in turn is determined by the production rate and the fractional catabolic rate of the apolipoprotein[96-98]. Some studies have shown that saturated fats increase serum HDL cholesterol concentrations by decreasing the catabolic rate of apoA-I[97,101]. However, others have failed to find any variation in the catabolic rate, but observed relationships between dietary saturated fat and the production rate of apoA-I[97-99]. Further work is needed to definitively establish how saturated fats affect HDL cholesterol concentrations.

Ingestion of all types of fat lowers serum triglyceride concentration when compared to dietary carbohydrate[243]. The extent of this effect is relatively constant regardless of the type of fat, the exception being the n-3 polyunsaturated fatty acids which result in significantly lower serum triglyceride concentrations when compared to other dietary fat classes[243].

Myristic, palmitic, and stearic acids are believed to be the most thrombogenic dietary fats[244,245].

Monounsaturated fatty acids

Dietary MUFAs are classified into *cis* and *trans* isomers according to the arrangement of the hydrogen atoms around the double bond. Due to their differing effects on serum lipoprotein concentrations, these isomers will be considered separately.

Oleic acid (*cis* C18:0) is the predominant source of *cis*-MUFA. It is found in both vegetable and animal fats[232] and is the most common fatty acid in the Western diet[246]. When either dietary SFAs or dietary carbohydrate are replaced with oleic acid, the ratio of serum HDL to LDL cholesterol increases[243,246]. Compared to SFAs, this increase is due mainly to a reduced concentration of serum LDL cholesterol, however, small reductions in HDL cholesterol are also seen[243,246].

Many different *trans* fatty acids are contained in Western diets. These are produced when unsaturated fat is hydrogenated either by bacteria or by an industrial chemical hardening process[247]. Early experiments showed that, compared to oleic acid, *trans*-fatty acids

increased LDL and decreased HDL cholesterol concentration[248]. More recent studies have shown that serum cholesterol concentration is increased more by the ingestion of *trans* fatty acids than by the ingestion of their parent polyunsaturated fat, however, *trans* fatty acids raised serum cholesterol concentrations significantly less than palmitic acid[85]. The effect of *trans*-fatty acids on serum HDL cholesterol concentration has been examined in six separate studies. In three of these, *trans*-fatty acids lowered HDL cholesterol concentrations, while no effect was observed in the others[85]. Overall, the evidence suggests that *trans*-fatty acids are more atherogenic than *cis*-monounsaturates, and are probably about as atherogenic as palmitic acid[85].

Polyunsaturated fatty acids

PUFAs can be subdivided into two functionally distinct groups according to the position of the first double bond. When the first double bond from the methyl terminal end involves the 6th carbon atom, the fatty acid is classified as n-6 PUFA, whereas when the first double bond involves the third carbon atom, the acid is classified as n-3 PUFA[19].

The content of n-6 PUFA in Western diets is highly variable, depending on the country, constituting between 6 and 15% of the fatty acid intake[246]. The most commonly consumed n-6 PUFA is linoleic acid (n-6 C18:2) which is found mainly in seed oils[246]. When dietary SFAs or carbohydrate are replaced with n-6 PUFA, the ratio between serum HDL and LDL cholesterol rises[18,233,243,246].

The n-3 PUFAs occur in Western diets at a level of around 2%[246]. These fatty acids are slowly metabolised into the longer chain eicosapentaenoic (EPA n-3 C20:5) and docosahexaenoic acids (DHA n-3 C22:6)[230], both of which are antithrombotic due to their promotion of the production of series 3 prostanoids[249,250]. High levels of EPA and DHA are found in fish oils and these fatty acids constitute a significant proportion of the diet in some populations. Epidemiological studies have revealed a relationship between high intake of EPA and DHA and low rates of atherosclerosis[251]. This is unlikely to be due to changes in serum lipoproteins as both EPA and DHA have been shown to increase serum LDL cholesterol concentration[252-254]. However, fish oils reduce serum triglyceride concentration[255] and whether the beneficial effects of high EPA and DHA intake are due to

lowered triglyceride concentrations, altered platelet function, or some other unidentified action is still unclear[246].

Cholesterol

The average Westerner's daily intake of cholesterol is around 350mg per day[256]. The effect of this cholesterol is genetically determined, with the serum cholesterol concentration of 75% of the population being unresponsive to dietary cholesterol[91,257]. Even in subjects who are sensitive to cholesterol in the diet, the effect on serum lipoproteins is small and it has been estimated that a reduction in dietary cholesterol of 100mg per day will only reduce serum cholesterol concentration by around 0.07mmol/l.

Conjugated linoleic acids

Conjugated linoleic acids (CLAs) are a special type of dietary fats which are currently the subject of intense research due to their possible antiatherogenic and anticarcinogenic properties. CLAs are all positional isomers of linoleic acid containing a conjugated (- C = C - C = C -) double bond[258].

These isomers are produced by bacteria and are found at highest concentrations in ruminant milk and meat products[258]. Meat from ruminants contains approximately 5.5mg CLAs per gram of fat, dairy products around 7mg CLAs per gram of fat. Fats from plants and non-ruminants contain only approximately 0.7mg per gram of fat[259].

The relationship between dietary CLAs and atherogenesis has been examined in two animal experiments. Feeding 0.5g CLAs per day to rabbits increased the ratio of serum HDL to total cholesterol when compared to controls[260]. However, despite developing a lipoprotein profile considered to be less atherogenic, no significant decreases in atherogenesis were observed[260]. When atherogenic diets were fed to hamsters, the inclusion of CLAs decreased serum total cholesterol concentrations but did not decrease fatty streak development when compared to controls fed a diet containing linoleic acid[193].

While the effects of CLAs on atherogenesis remain uncertain, there is good evidence to suggest that CLAs possess anticarcinogenic properties[261]. In studies using animal models,

dietary CLAs have been shown to reduce the development of mammary[262-264], epidermal[265], and forestomach[266] tumours. It was initially thought that the anticarcinogenic properties of CLAs were due to their ability to protect membranes from oxidation[264,266], however, no antioxidant properties of CLAs could be identified in a recent *in vitro* investigation[267]. Currently it is unclear how CLAs prevent tumour development[261].

The average intake of CLAs per Westerner is around 250mg per day[264]. Extrapolating from animal trials, it has been estimated that a human subject would need to consume at least 1.5g CLAs per day to produce significant anticarcinogenic effects[268]. Unfortunately, as the major dietary source of CLAs is ruminant fat, it is unlikely that the intake of CLAs can be increased using normal dietary constituents without also increasing the intake of SFAs[268]. To illustrate this point, Huang *et al* observed the effect of increased cheddar cheese consumption on serum CLA concentrations[269]. Associated with a 35% increase in plasma CLAs, cheddar cheese also increased total fat intake by 33% and SFA intake by 95%. This suggests that using cheddar cheese to increase the concentration of CLAs in the serum is likely to produce proatherogenic changes in the serum lipoprotein profile[269].

Therefore, if CLAs are going to promote longevity, much higher concentrations in food are required. Studies investigating modification of animals and their feed to enrich the fat with CLAs are being conducted and interest in CLAs appears likely to continue in the future.

Antioxidants

Oxidation of LDL leading to damage to the apoB protein moiety and particle recognition by macrophage scavenger receptors is undeniably an important process in foam cell development[139]. The use of antioxidants to prevent or delay this oxidation, thus ameliorating atherogenesis, is not only an area of intense research but also some controversy. Antioxidants, unlike other protective factors, do not alter any established risk factor for atherosclerosis. A diet high in antioxidants will result in LDL particles that are resistant to oxidation, however, there is no convincing evidence that this protects against atherosclerosis. Many antioxidant compounds have been evaluated for possible antiatherogenic properties, the major ones are reviewed here.

Vitamin E

Vitamin E contains 8 different tocopherol and tocotrienol isomers[270], with α-tocopherol showing the strongest *in vivo* antioxidant activity[271]. The main sources of dietary vitamin E are vegetables and seed oils, such as soybean, safflower and corn oil[270]. Vitamin E is transported in the serum in lipoproteins and vitamin E contained in LDL particles is able to scavenge peroxyl radicals, preventing the autocatalytic propagation phase of lipid peroxidation in the following reaction[133,272].

$=CHO_2^{\cdot}$ (peroxyl radical) + tocopherol ➜ $=CHO_2$ + tocopheroxyl radical$^{\cdot}$

Epidemiological studies examining the relationship between dietary factors and disease are of two types, observational and interventional. An observational study examines rates of disease in a population and then compares these rates with levels of the dietary factor in the diet or in the blood. One of the largest to investigate antioxidants was the Health Professionals Follow-up Study, which examined vitamin E intake and atherosclerosis in men[273]. This was followed by the Nurses Health Study, which examined the same factors in women[274]. Both studies demonstrated that, after adjustment for other risk factors, people who took supplements containing over 100IU vitamin E per day had a significantly lower risk of developing major coronary artery disease[273,274]. The Scottish Heart Health Study supported these findings with both men and women in the highest quintile of dietary vitamin E intake having a lower risk for undiagnosed coronary artery disease than those in the lowest quintile[275]. A study of men less than 65 years old in 19 Western European countries and 5 non-European countries showed a significant inverse association between α-tocopherol intake and coronary heart disease mortality[276]. In contrast, no significant effects of vitamin E supplementation were found in the Established Populations for Epidemiologic Studies of the Elderly[277] or when 4 000 people from the WHO MONICA Augsburg cohort were examined[278].

Interventional studies are the second type of epidemiological study. Here, two groups are formed, one receiving a treatment, while the other receives a placebo. Any differences in endpoint between the two populations can then be observed. One of the largest antioxidant intervention studies examined a population of normal subjects for five years in Linxian,

China. In this trial, no differences in the incidence of atherosclerotic diseases were noted between subjects who received either a supplement containing 30mg α-tocopherol, 15mg β-carotene, and 50μg selenium or a placebo[279]. In the Finnish α-tocopherol β-carotene Cancer Prevention Trial (ATBC), 50IU vitamin E had no significant effect on heart disease mortality in smokers[280]. In contrast, the Cambridge Heart Antioxidant Study (CHAOS) found that, although supplementing subjects with a history of previous myocardial infarction with either 400IU or 800IU vitamin E/day did not decrease total mortality rates, it did decrease the rate of non-fatal myocardial infarctions[281].

Measurement of LDL oxidation *in vitro* is another method for investigating possible mechanisms for an atherosclerosis-reducing effect of antioxidants. Here, the ability of LDL to resist oxidation is measured by exposing the lipoprotein to a pro-oxidant and measuring the time taken before the propagation phase of oxidation begins. Daily supplementation with vitamin E at doses as low as 200IU has been shown to protect isolated LDL from oxidation[282-284]. In a study using rabbits, dietary vitamin E dose-dependently decreased the susceptibility of isolated LDL to oxidation[285]. These, and other studies, have led some authors to conclude that if all other constituents of an LDL particle are equal, the content of α-tocopherol is strongly correlated with it's resistance to oxidation[270,286].

Animal models have been widely used to study the role of vitamin E in preventing atherogenesis. In a three-year trial using non-human primates, dietary vitamin E decreased the severity of atherosclerotic lesions in some sampled sites without significantly altering serum cholesterol concentrations[287]. Vitamin E also has antiatherosclerotic effects, independent of serum cholesterol concentration, in chickens[288]. However, 8 other studies have investigated vitamin E in animal models without observing a significant reduction in atherosclerosis without concurrent significant changes to the serum cholesterol concentration[289-297] These results also question the value of *in vitro* studies of LDL oxidation since dietary vitamin E supplementation increased *in vitro* oxidation time in every study it which it was measured[291-294,297].

Carotenoids

The carotenoids are the most widespread group of pigments in nature and more than 600

naturally-occurring compounds have been identified[298]. The most extensively studied of these is β-carotene, found predominantly in fruit and vegetables, and like α-tocopherol, transported in serum in LDL particles[271]. In an oxidatively stressed particle, β-carotene may prevent oxidation by deactivating singlet oxygen and quenching free radicals in the following reaction[132,299].

$=CHO_2^{\cdot}$ (peroxyl radical) + β-carotene ➜ $=CHO_2$ + β-carotene radical$^{\cdot}$

However, as recently reviewed by Rice-Evans *et al*[298], whether or not β-carotene has antioxidant properties remains controversial.

The Health Professionals Follow-up Study identified a statistically significant reduction in coronary artery disease risk in men with β-carotene intakes in the top quintile[273]. However, β-carotene did not appear to protect women as no relationship was observed in the Nurses Health Study[274]. These conflicting results were mirrored by the Scottish Heart Health Study, which observed a difference between rates of undiagnosed coronary artery disease only in men in the top and bottom quintiles of β-carotene intake[275]. In a Finnish study, β-carotene intake was not correlated with coronary mortality[300]. Furthermore, a cross-cultural study comparing plasma β-carotene concentrations in European populations revealed no relationship between β-carotene and coronary heart disease[301].

The Physician's Health Study did not observe any protective effect from supplementing 22 071 subjects with 50mg β-carotene for 12 years[302]. The Linxian study also showed no effect of β-carotene on cardiovascular disease when it was supplemented along with selenium and vitamin E[279]. The Carotene and Retinol Efficacy Trial (CARET) examined the effect of 30mg β-carotene and 25 000IU retinol per day in 18 300 smokers. Interestingly, this treatment resulted in a significant increase in lung cancer (17%), cardiovascular death (26%) and mortality (28%) resulting in the premature termination of the trial[303]. These results confirmed the findings of the ABTC study which also reported increased lung cancer and mortality association with 20mg/day β-carotene supplementation[280].

Beta-carotene has little or no effect on LDL oxidation *in vitro*[282,304,305].

Only one animal experiment has investigated the effect of β-carotene on atherogenesis. Here, intravenous β-carotene was administered twice-weekly to cholesterol-fed rabbits. After 8 weeks, the area of aortic atherosclerotic lesion formation was found to be smaller in rabbits given β-carotene than in the controls[306]. However, like vitamin E, β-carotene also lowers serum cholesterol concentrations making it difficult to determine whether the decrease in lesion size was due to an antioxidant effect or to a cholesterol-lowering action[306]. In this study, the LDL particles isolated from supplemented rabbits became highly enriched with β-carotene, however, this did not protect against *in vitro* oxidation[306].

Vitamin C

Vitamin C is a water-soluble antioxidant which protects against free radicals formed in the aqueous phase[307,308]. As indicated by the following reaction, vitamin C may also have a sparing action on vitamin E by reducing the tocopheroxyl radical to tocopherol at the water-lipid interface[309].

tocopheroxyl radical· + vitamin C ➜ tocopherol + vitamin C radical·

No epidemiological evidence for an antiatherogenic action of vitamin C was provided by the Health Professionals Follow-up Study[273] or in the Scottish Heart Health Study[275]. Vitamin C supplements also did not reduce coronary heart disease mortality in a population of older people[277]. However, in a Finnish population, a high intake of vitamin C contained in food was associated with a decreased risk of atherosclerotic disease[300]. This was supported by data from the US National Health and Nutrition Examination Survey (NHANES I) which revealed that vitamin C intake was inversely correlated with cardiovascular disease and mortality, especially in males[310]. In European populations, cross-cultural examination revealed no relationship between plasma vitamin C concentrations and coronary heart disease[301]. Vitamin C produced no change in cardiovascular disease or mortality rates when administered at 120mg/day in the Linxian intervention trial[279].

LDL is protected from oxidation by the addition of vitamin C to the oxidation reaction medium[311]. LDL isolated from subjects supplemented with 1g vitamin C/day was shown, in one study, to be more resistant to oxidation than LDL isolated from controls[312], however,

in a similar experiment, no differences in oxidation resistance were detected between LDL from controls and from subjects supplemented with 2g vitamin C/day[282].

Vitamin C has also produced contradictory results when examined in cholesterol-fed rabbits. In one study, there was no reduction in lesion formation due to 500mg vitamin C/rabbit/day[313], however in another, 150mg vitamin C/rabbit/day resulted in a significant reduction of lipid deposition within the wall of the aorta[314].

Selenium

Selenium is an essential component in the enzyme responsible for detoxifying hydrogen peroxide, glutathione peroxidase. This enzyme metabolises hydrogen peroxide in the following reaction[315].

$H_2O_2 + 2\ GSH$ ➜ (catalysed by glutathione peroxidase) ➜ $2\ H_2O + GSSG$

$GSSG + NADPH + H^+$ ➜ (catalysed by glutathione reductase) ➜ $2\ GSH + NADP^+$

where GSH is glutathione and GSSG is glutathione disulphide.

The results of epidemiological studies examining the relationship between selenium and atherosclerosis have been equivocal. Two studies have observed an inverse correlation between serum selenium concentration and incidence of cardiovascular disease[316,317]. These results were supported by the observation of an inverse correlation between plasma selenium and the extent of coronary atherosclerosis in patients undergoing coronary arteriography[318]. In contrast, three Finnish and two Dutch studies failed to demonstrate any significant relationship between the incidence of myocardial infarction and serum selenium concentrations[319-323] and no relationship was observed between serum selenium concentrations and the progression of carotid atherosclerosis[324].

The effect of selenium on atherosclerosis has been examined in the cholesterol-fed rabbit model[290]. Selenium significantly reduced the area of lipid deposition on the aortic sinus suggesting a protective effect, although whether this was attributable to an antioxidant action is unclear as dietary selenium also significantly lowered serum cholesterol concentration[290].

Polyphenols

Polyphenols are compounds containing an aromatic ring and two or more hydroxyl groups. They are divided into three classes, simple phenols and phenolic acids, hydroxycinnamic acid derivatives, and flavonoids[325]. Polyphenol compounds may sequester catalytic metal ions[326] as well as scavenging superoxide anions, hydroxyl radicals, and lipid peroxyl radicals[327]. Polyphenols are found in red wine, tea, vegetables, and fruit[328]. The studies of these antioxidant compounds can be divided into three groups, epidemiological studies examining the relationship between total polyphenol intake and heart disease, *in vitro* examination of the ability of isolated polyphenolic compounds to prevent lipid oxidation, and studies of antiatherosclerotic properties of foods containing polyphenols.

Epidemiological studies

Three large epidemiological studies have examined the relationship between total polyphenol intake and risk of coronary heart disease. In the Health Professionals Follow-up Study there was no significant relationship between the intake of polyphenols and the risk for coronary heart disease[328]. This was supported by a study of 1 900 Welsh men aged 45-59 years[329]. In contrast, the Zutphen Elderly Study revealed a significant inverse association between polyphenol intake and coronary heart disease in men aged between 65 and 84 years[330].

In vitro examination of individual flavonoid compounds

The flavonoids morin, fisetin, quercetin, gossypetin, and caffeic acid have all been shown to be powerful inhibitors of LDL oxidation when included in the oxidation medium[331,332]. However, others such as ferulic acid and p-coumaric acid, had little or no effect on LDL oxidisability at physiological concentrations[332].

Trials examining dietary components containing flavonoids

Both red wine and grape extract have been shown to contain phenolic substances which are able to protect LDL from *in vitro* oxidation[326,333]. Furthermore, the amount of protection provided by these compounds was greater on a molar basis than α-tocopherol[326]. LDL isolated from human subjects given red wine was significantly more resistant to oxidation than LDL from controls[334]. In a recent study using apoE-knockout mice, compared to alcohol-matched controls, an antiatherogenic action of red wine and red wine polyphenols

was observed[335]. However, in contrast, a study which compared fatty streak formation in rabbits receiving red wine, white wine, spirits, beer, and alcoholised water, observed no significant differences among the groups[336].

Tea also contains a high concentration of phenolic antioxidants and has been investigated for antiatherosclerotic effects. In the Scottish Heart Health Study no protective effect of tea consumption was observed[337]. However, LDL isolated from human subjects receiving tea was found to be less susceptible to copper-initiated oxidation than controls and oxidation of LDL is inhibited by the inclusion of flavonoids from tea in the oxidation medium[338,339].

Extra virgin olive oil contains high quantities of polyphenolic substances[340] and olive oil constituents have been shown to inhibit *in vitro* oxidation of LDL[341].

Other antioxidants

Many other antioxidant compounds have been examined for antiatherosclerotic properties. A report on the antioxidant properties of garlic and aged garlic extract is included within Chapter 9 of this thesis. Both butylated hydroxytoluene (BHT) and diphenyl-1,4-phenylenediamine (DPPD) are discussed in Chapter 5, however, as so many others exist and, as they all have similar properties to vitamin E, no further reviews of the numerous antioxidant chemicals investigated for antiatherosclerotic properties will be included.

Alcohol

There is evidence to suggest that the moderate intake of alcohol (two to four standard drinks per day, 22 - 44g alcohol) is associated with a reduced incidence of, and decreased mortality from, atherosclerotic diseases[342-344]. However, heavy drinking (greater than four standard drinks per day) has the opposite effect[345]. As a result, there is believed to be a 'J' shaped relationship between alcohol and atherosclerotic disease mortality, with both abstainers and heavy drinkers having higher disease incidence[344]. The combination of harmful and beneficial effects of alcohol is the subject of the next part of the review. Also discussed is the hypothesis that wine is more antiatherosclerotic than other types of alcoholic beverage.

The consumption of moderate quantities of alcohol produces predictable changes in the serum lipoprotein profile. The most important of these is believed to be a decrease in serum CETP activity which results in an increased serum HDL cholesterol concentration[346]. Lower serum CETP activity may also reduce atherogenesis by decreasing the production of small, dense, atherogenic LDL particles[48]. However, if alcohol intake becomes excessive, liver cirrhosis reduces the production of apoA-I, decreasing serum HDL cholesterol concentration[347].

The effect of moderate alcohol consumption on serum LDL cholesterol concentration is less predictable[344,348,349]. LDL may decrease due to a reduction in the amount of cholesterol carried in each LDL particle[350], although this effect is only consistent in heavy drinkers where a significant proportion of the total energy intake from dietary fat and cholesterol is replaced by energy from alcohol[344,346].

Moderate alcohol consumption has also been shown to decrease lipoprotein lipase activity, stimulate the release of free fatty acids from adipose tissue, and provide a substrate for hepatic fatty acid production[351]. This results in reduced chylomicron hydrolysis, increased quantities of fatty acids arriving at the liver, increased triglyceride export, and consequently, hypertriglyceridaemia[344]. This is transient and fasting triglyceride levels are not altered[344]. However, with alcohol abuse, the ability of the liver to remove chylomicrons and IDL from the circulation becomes impaired, resulting in severe, prolonged hypertriglyceridaemia[344].

Many epidemiological studies have examined the relationship between alcohol and atherosclerotic disease (recently reviewed by Rimm *et al*[352], Maclure[353] Kannel and Ellison[345], Steinberg *et al*[344], and Goldberg *et al*[343]). Most studies, including the Framingham Heart Study, Nurses Health Study, Health Professionals Follow-up Study, and the Established Populations for Epidemiology Study of the Elderly have found an inverse relationship between alcohol intake and coronary heart disease[352]. One notable exception to this trend was the NHAMES I study which did not[352].

Animal experiments examining the relationship between alcohol and atherosclerosis have been less conclusive. Two mouse studies, one using C57BL/6 mice and one using LDL

receptor-knockout mice, demonstrated an antiatherosclerotic effect in animals fed 36% of their calories as alcohol[174,354]. This protective effect of alcohol intake has also been observed in nonhuman primates[355] and atherosclerosis-prone JCR:LA-corpulent strain rats[356]. In contrast, cholesterol-fed rabbits receiving 20 or 30% of their calories as alcohol and rats fed 36% total calories as alcohol developed significantly higher serum cholesterol concentrations, and greater aortic atherosclerotic lesions than controls[357,358].

Whether or not different types of alcoholic beverage vary in atherogenecity is still unresolved. St. Leger *et al* revealed a strong inverse correlation between coronary mortality and wine consumption when mortality and alcoholic beverage consumption was studied in 18 countries[359]. A weaker trend was observed for spirits but no association was observed between beer consumption and mortality rates from coronary artery disease[359]. Since these initial observations, 11 further studies have reported similar findings[352]. This relationship led to the suggestion that the consumption of wine causes the 'French Paradox', whereby the high wine consumption in France results in a lower level of coronary heart disease than could be predicted from the dietary and smoking habits of the population[326,360]. Since red wine also contains polyphenolic compounds, the French Paradox has also been suggested to be due, in part, to the high antioxidant content of red wine[360]. However, in a survey of 13 000 people, it was observed that, while the lowest risk of coronary artery disease was seen in people who consumed wine as their most frequent alcoholic beverage, no differences between red and white wine drinkers were observed[361].

The data from other types of epidemiological studies have been less convincing. In a meta-analysis of 30 cohort studies, Maclure concluded that the relative risk of atherosclerotic disease for people consuming beer, wine, and spirits was almost identical[353]. In a recent review, Rimm *et al* reported that, of the cohort studies investigating different beverage consumption and atherosclerotic disease risk, only four studies reported a significant difference in risk among subjects consuming individual beverages[352]. Of these, two trials observed the lowest risk for wine drinkers[362,363], and two trials for those who preferred spirits[364,365]. However, different alcoholic beverages are associated with different lifestyle choices important in atherogenesis, for example smoking, heavy drinking, and obesity, making interpretation of epidemiological studies difficult[345,359,366].

Tobacco smoking

Tobacco smoking is well recognised as a strong risk factor for atherosclerosis[367]. Suggested mechanisms for this include endothelial damage, vasoactive neurotransmitter release, predisposing lipoproteins to oxidation, stimulating the immune system, and promoting thrombosis[367].

Obesity

There is no strong evidence for a causal link between obesity and atherosclerosis[368]. Obese people tend to have higher serum triglyceride concentrations and lower serum HDL cholesterol concentrations[232] however, the precise relationship between obesity and atherosclerosis is difficult to determine using population studies since obesity is positively associated with diabetes and hypertension, and negatively associated with smoking[368]. This results in a significant univariate association between obesity and atherosclerosis which becomes insignificant when multivariates are included in the model[368,369].

Physical exercise

Regular physical exercise reduces the risk of atherosclerosis by decreasing blood pressure, increasing fibrinolysis, and promoting efficient carbohydrate metabolism[370]. Exercise also increases HDL cholesterol concentrations by inhibition of CETP[61,371,372]. In an epidemiological study, patients who regularly performed a moderate level of exercise had 30% fewer fatal heart attacks than their less active cohorts[373].

Homocystinaemia

It is well recognised that people with congenital hyperhomocystinaemia develop premature atherosclerotic disease[368]. The mechanism for this is unknown, but both endothelial injury and increased thrombosis have been suggested[368,374]. Newer evidence suggests that mild increases in serum homocystine concentration may be a risk factor for heart disease[206,374]. Since serum homocystine concentration is decreased by folate, it has been suggested that increasing folate intake may decrease the risk of atherosclerotic disease[374].

Infectious agents

Antibodies to *Helicobacter pylori* and *Chlamydia pneumoniae* are found in atherosclerosis patients more frequently than in normal controls[375] and it has been suggested that chronic mild inflammation associated with these bacteria may predispose to atherosclerosis[375]. Herpes virus and cytomegalovirus have also been implicated in the pathogenesis of atherosclerosis[376,377]. Both viruses are capable of causing chronic inflammation, and may also promote atherogenesis by producing metaplasia in intimal smooth muscle cells[376,377].

Chapter 2

Materials and methods

Only methods common to more than one experiment are reported in this chapter. Any unique experimental procedures are detailed separately within the appropriate chapter. All *in vitro* experimental procedures are contained within Chapter 9.

Except when indicated otherwise, all chemicals used throughout this research programme were purchased from either Sigma Chemicals Co, MO, USA or BDH Chemicals New Zealand Ltd, New Zealand.

All procedures reported in this thesis were conducted under guidelines established by the appropriate Massey University Committee and were only performed after their prior approval.

Experimental animals

Inbred C57BL/6JIco (abbreviated to C57BL/6 or B6) mice were obtained from the Animal Breeding Station, Department of Laboratory Animal Sciences, University of Otago, Dunedin and housed at the Food Evaluation Unit, Crop & Food Research, Palmerston North. Mice were kept in solid-floor 'shoebox' cages containing sawdust throughout all experiments reported in this thesis. The animal room was maintained at 22±1 °C, humidity 60±5%, air exchange 12 times/hr on a 12-hr light-dark cycle. In all experiments, the mice were given free access to food and water. After arrival at Palmerston North, the animals were acclimatised for at least two weeks, during which time they were fed standard mouse food (Diet 86, Sharpes Grain & Seeds Ltd, New Zealand).

Experimental diets

The experimental diets given to mice throughout this thesis were based on those reported by Nishina *et al*[378]. During the research programme, the composition of the diet changed from being based on a 50/50 mixture of cornstarch and sucrose in the first trial, to being based solely on sucrose in later experiments. This change was made in an attempt to improve palatability, however, since similar numbers of mice refused to eat the diet

regardless of sucrose content, palatability may not have been significantly increased. The vitamin and mineral mixes used in the diets were formulated by staff at the Food Evaluation Unit, Crop & Food Research, Palmerston North according to guidelines established by the National Research Council[379].

In order to avoid the food wastage that was found to be associated with the use of powdered diets, the diets were given to mice in biscuit form. Biscuits were produced by adding water to the dry ingredients to form a dough which was then cut into strips and dried for 3 days at 29°C. Once dry, the biscuits were stored in a freezer at -20°C. Diet was removed from the freezer weekly and kept refrigerated at 4°C until fed. Mice were provided with fresh diet from the refrigerator each day of the study. Although feeding the diets in biscuit form reduced wastage, a significant proportion of the experimental diet was found to be left by mice on the floor of the cage, preventing accurate measurement of feed intake. To provide an indirect measurement of feed intake, the mice were weighed weekly throughout the experiments.

Sample collection and processing

Immediately after euthanasia, between 0.5 and 1 ml of blood was collected from each mouse by cardiac puncture. The blood was transferred into a test tube and serum was separated by centrifugation at 1 500 x g for 15 min. Serum cholesterol and triglyceride concentrations were measured using a Hitachi 704 autoanalyser (Boehringer Mannheim GmbH, Germany).

Serum HDL cholesterol concentration was determined after selective precipitation of apoB-containing lipoproteins using polyethylene glycol 6000[380]. In this technique, a 0.1 ml serum sample was incubated for 10 min at 25°C with 0.1 ml of a 200g/l PEG 6000 solution buffered at pH 10 (Appendix). After centrifugation for 20 min at 2 000 x g, the concentration of cholesterol contained in the HDL fraction could be measured in the supernatant.

Serum LDL cholesterol concentrations were not determined in these experiments. As the Friedewald formula[381] has been found to be inaccurate in mice[174], measuring serum LDL cholesterol concentration would require ultracentrifugation. Considering the small amount

of serum harvested from each mouse, this was not practicable.

Histological techniques

The area of fatty streaks in the aortic sinus was quantified using a method similar to that previously described[382]. After euthanasia, the heart was placed in 0.9% saline for 1hr and then in 0.9% saline containing 4% formalin for at least 24hr. The formalin-fixed hearts were stored for up to 7 days before further processing. The hearts were sectioned in a plane parallel to the base of the heart, caudal to the auricles. The orientation of this cut was essential to enable a true cross section of the aortic sinus and an accurate assessment of the size of the aortic fatty streaks. The cut hearts were then incubated at 37°C in a 5% gelatin solution for 3hr, followed by 3hr in a 10% gelatin solution, before a final incubation in a 25% gelatin solution for 12hr. After the final incubation, hearts were placed in 25ml of a 25% gelatin solution and cooled to 4°C. Once the gelatin had hardened, the block was trimmed and frozen in a cryostat. Sections (40μm thick) were cut and discarded until the aortic sinus was recognised by the appearance of aortic valves and the rounded appearance of the aortic wall. Twenty-four consecutive 10μm sections were then cut and mounted onto slides coated with a 10% gelatin solution. To ensure adherence of the sections, the slides were cooled to 4°C for at least 4hr prior to staining.

The slides were stained using Oil red O and Meyer's haematoxylin method with light green counterstaining (Appendix). The area of lipid deposition observed microscopically in the intima, which stained red with Oil red O, was quantified using the SigmaScan Scientific Measurement System (Jandel Scientific, CA, USA). The cross-sectional area of lesions in every second section was measured so that a total of 12 sections from a 240μm segment of aorta were examined per heart. Other authors commonly use sections taken from a 400μm segment of the aortic sinus to measure lesion formation[173,174]. However, detailed examinations of the distribution of lesions within the aortic sinus by Paigen *et al* have suggested that a 280μm segment provides the best estimate of lesion formation[382]. Therefore, as 40μm sections were taken to locate the beginning of the aortic sinus, taking 24 consecutive 10μm sections ensures that all the sections used to measure lesion formation were from this critical 280μm segment. The investigator who assessed the atherosclerotic

lesions was blind to the group of origin of the mice throughout all experiments.

Livers were examined histologically in most mouse experiments. The livers were fixed in neutral buffered formalin before being sectioned and stained with haematoxylin and eosin.

Statistical analysis

All statistical analyses reported in this thesis were performed using the SAS statistics package (SAS Institute Inc, NC, USA). Differences between means were examined using analysis of variance techniques incorporating both balanced and unbalanced designs. Relationships between variables were investigated using both linear and multiple regression techniques.

Chapter 3

Preliminary studies attempting to improve the palatability of atherogenic diets

1. Variation in the palatability of different cholic acid compounds

Atherogenic diets for C57BL/6 mice contain either cholic acid[172] or sodium cholate[383]. Both chemicals have an intense bitter taste which is believed to decrease palatability, reducing dietary intake and resulting in weight loss. After four weeks, mice receiving diets containing cholic acid have previously been shown to be 30% lighter than control mice receiving a similar diet without cholic acid[384]. In the mouse studies performed in this experimental programme, reluctance of the mice to consume the atherogenic diets resulted in the exclusion of around 15% of the mice due to chronic weight loss.

	Dietary Group	
Ingredient	Test diet	Control
	grams/kilogram, as fed	
Cornstarch	235.64	240.64
Sucrose	235	235
Anhydrous milk fat	150	150
Casein	200	200
Corn oil	10	10
Cellulose	50	50
Salt mix	50	50
Vitamin mix	50	50
dl-methionine	3	3
(dl)-α-tocopherol	1.36	1.36
Cholesterol	10	10
Cholate (acid, salt or ester)	5	0

Table 3.1. ***Composition of diets used in preliminary studies.***

It was hypothesised that the methyl ester of cholic acid, which dissolves to form a neutral solution[14], would not possess a bitter taste and so may be better accepted by the mice. Cholic acid methyl ester should possess similar physiological properties to other cholate compounds as cholic acid is liberated from it by hydrolase enzymes contained in the small intestine[14].

The purpose of this trial was to investigate whether or not differences in weight gain existed among mice fed cholic acid, sodium cholate or cholic acid methyl ester. If one compound resulted in greater weight gain than the others, it could then be incorporated into future atherogenic diets, therefore reducing the mouse

exclusion rate.

Materials and methods

Forty, six-week-old male C57BL/6 mice were randomly divided into 8 cages of five mice. After a six-week acclimatisation period, two cages were allocated to each of the four dietary groups. As illustrated in Table 3.1, three groups of mice were fed diets containing one of, cholic acid, sodium cholate, or cholic acid methyl ester, while the fourth group consumed a control diet which contained no cholate. The methods involved in producing and feeding the diets, and the conditions in which the mice were kept, are described in Chapter 2. The mice were fed *ad libitum* for four weeks and were weighed twice weekly. The data was analysed using a repeated measures design.

Results and discussion

As illustrated in Figure 3.1, no differences in weight gain were observed among mice consuming the three different cholic acid compounds during the four-week trial period.

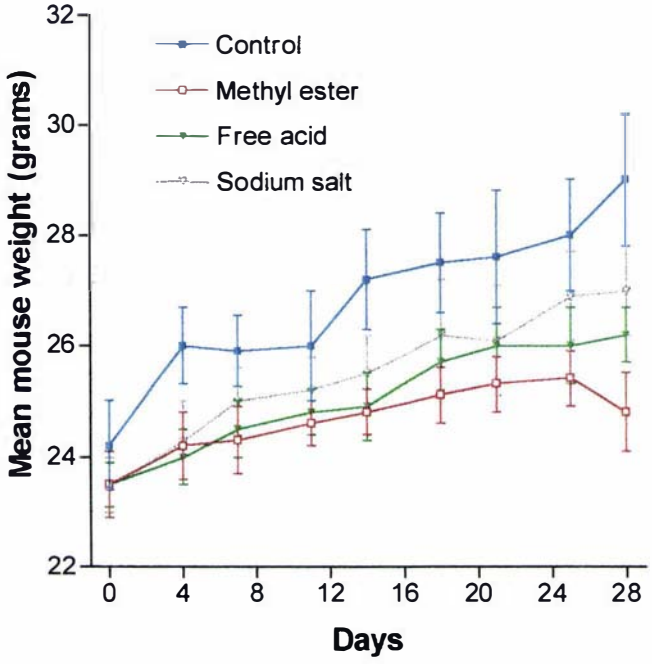


Figure 3.1. ***Mean body weight of mice fed diets containing one of three cholic acid compounds or a cholic acid-free control diet.*** Male C57BL/6 mice were fed diets contained 16% fat and 1% cholesterol. The 'Methyl ester' diet also contained 0.5% cholic acid methyl ester. The 'Free acid' diet contained 0.5% cholic acid. The 'Sodium salt' diet contained 0.5% sodium cholate. The 'Control' diet contained no cholate. The error bars indicate one standard error.

Mice in the control group were significantly heavier than mice in the cholic acid methyl ester group (p=0.04), but no different from mice in either the sodium cholate or cholic acid groups. These results were surprising as cholic acid methyl ester, which was believed to be tasteless, was expected to be more palatable than other cholic acid compounds. However, when a small amount was tasted by the author, this chemical was found to be bitter, suggesting it may also reduce diet palatability and mouse weight gain.

In this study, all three cholic acid compounds resulted in similar impairment to weight gain. Since no compound was considered to be more palatable than any other, cholic acid was included in atherogenic diets used in subsequent experiments.

2. Necessity of including cholic acid in atherogenic diets

The results of the first part of this study suggested that it was unlikely that acceptance of the atherogenic diets by mice could be improved by changing the cholic acid compound included in the diet. The next part of this preliminary study investigated whether or not cholic acid was essential for the development of fatty streaks in mice.

Materials and methods

Ten mice from each of the control and cholic acid groups of the previous trial were fed their respective diets for a further 11 weeks. The methods involved in the determination of serum lipid concentrations and aortic fatty streak quantification are described in Chapter 2. Differences between group means were analysed using a two-sample t-test.

Results and discussion

During the 15-week feeding period, two mice from the control group developed a severe dermatitis and were removed from the study. C57BL/6 mice are predisposed to an immune complex vasculitis and the gross lesions seen in these mice were consistent with those previously described for this disease[385].

The weight gain of the mice throughout the trial period is illustrated in Figure 3.2. At the completion of the trial, the fasting weight of the control mice (37.1g) was significantly

greater than that of mice fed cholic acid (28.7g) ($p<0.0001$).

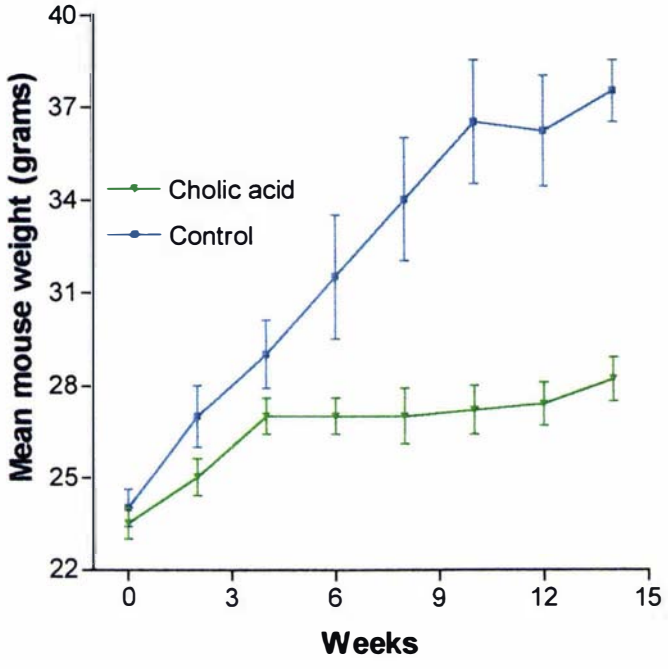


Figure 3.2. ***Mean body weight of mice fed diets with and without cholic acid.*** Male C57BL/6 mice were fed diets containing either 0.5% cholic acid (Cholic acid) or no form of cholate (Control). Error bars indicate one standard error.

The mean serum total cholesterol concentration observed in mice fed cholic acid (6.6mmol/l) was significantly higher ($P<0.0001$) than that in controls (4.5mmol/l). Unfortunately, due to an error in reagent formulation, no values for serum HDL cholesterol concentrations were obtained. A discussion of the mechanism by which dietary cholic acid alters serum cholesterol concentration is included in Chapter 5.

Fatty streaks in the aortic sinus were observed histologically in all mice receiving diets containing cholic acid. As illustrated in Figure 3.3, these lesions consisted of focal subendothelial aggregations of macrophages containing numerous lipid globules. These globules were also present extracellularly in areas of macrophage accumulation and in the underlying media. They were most common subendothelially at the origin of the coronary arteries and at the base of the aortic valves. Aortic sinus fatty streaks were present in five of the mice fed the control diet. However, these lesions were smaller and consisted of isolated, lipid-filled macrophages. The mean total fatty streak area observed in mice fed diets containing cholic acid ($0.373mm^2$) was significantly greater ($P<0.001$) than the area

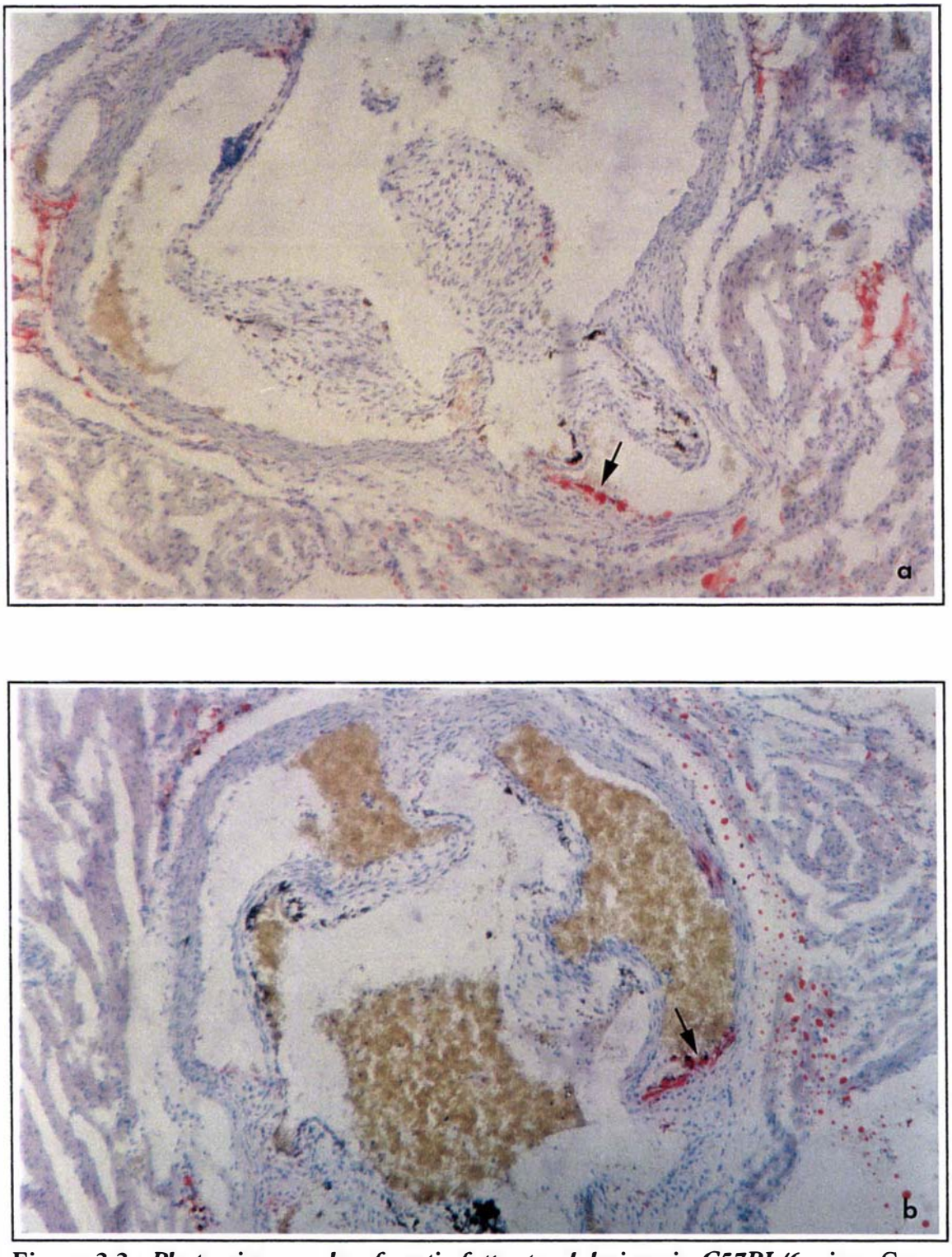
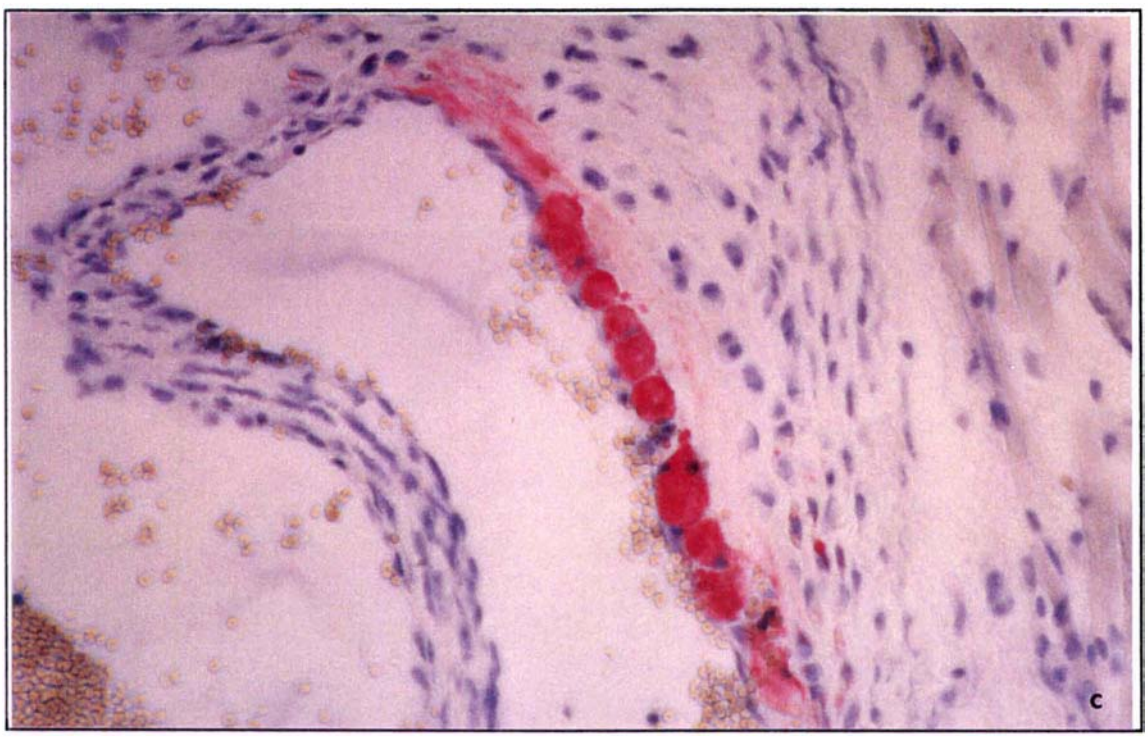


Figure 3.3. ***Photomicrographs of aortic fatty streak lesions in C57BL/6 mice.*** Cross-section of the aortic sinus from male C57BL/6 mice which had received a diet containing cholic acid for 15 weeks. Photomicrographs (a) and (b) show the position of the red-staining fatty streak lesions (arrows) in the aortic sinus. As can be seen from (c) these lesions consist of focal subendothelial aggregations of macrophages containing numerous lipid droplets. Although mainly intracellular, as shown in (d) these lipid droplets were also present extracellularly in areas of macrophage accumulation and in the underlying media. Magnification: (a) and (b) x 85, (c) x 320, (d) x 1285.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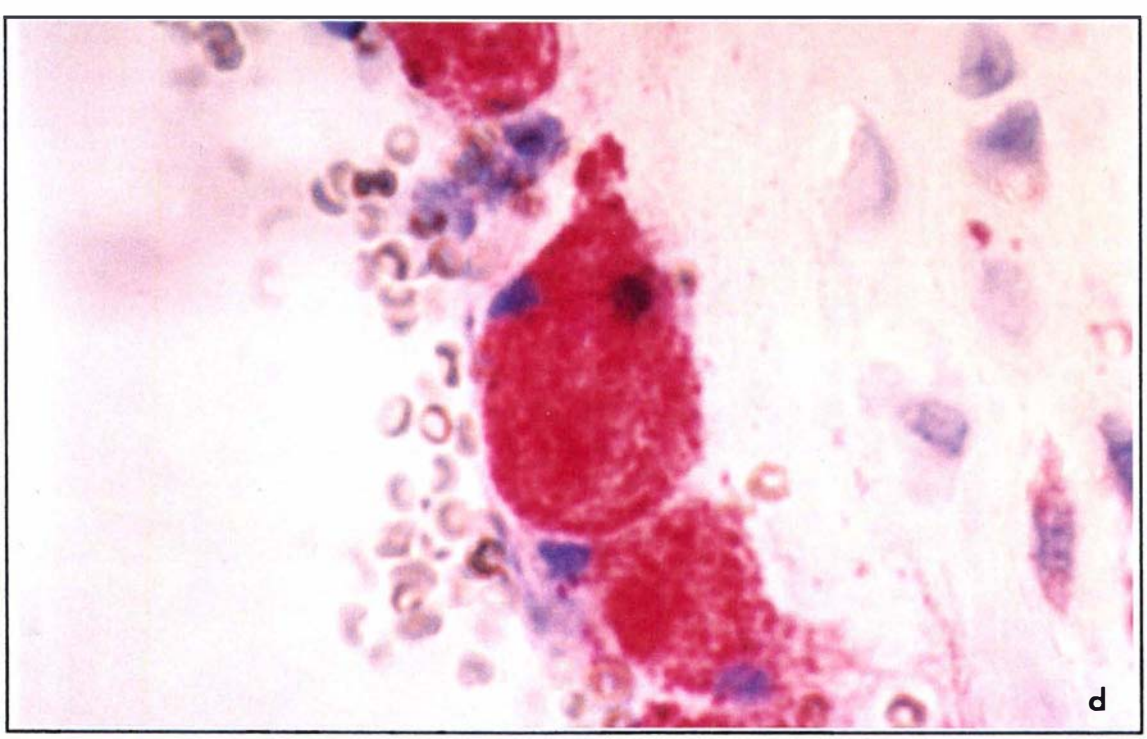
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observed in control mice (0.0125mm^2). As can be seen from Figure 3.4, the mean lesion area of fatty streaks observed in male C57BL/6 mice was correlated with total serum cholesterol concentrations (r^2=0.51 p=0.001, n=17).

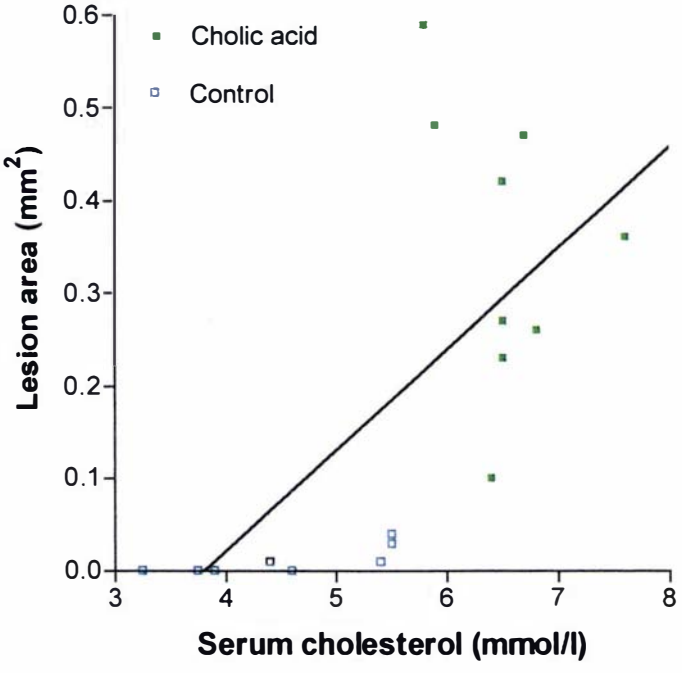


Figure 3.4. ***Relationship between fatty streak area and serum cholesterol concentration.*** Each symbol represents an individual mouse. Male C57BL/6 mice were fed a diet containing either 0.5% cholic acid (Cholic acid) or no form of cholate (Control) for 15 weeks. r^2=0.51, p=0.001, n=17.

Because they have been found to develop a smaller area of aortic fatty streaks than females, male C57BL/6 mice are less commonly used in atherosclerosis research[172]. In mice, fatty streak formation appears to be inversely correlated with serum HDL concentration[172]. The concentration of HDL falls sharply in female mice when fed an atherogenic diet, an effect that is ameliorated by testosterone in males[172]. Male mice were used in this experiment because they were mistakenly sent by the Animal Breeding Station.

Significantly (p<0.001) lower serum fasting triglyceride concentrations were observed in mice fed cholic acid (0.63mmol/l) than in control mice (1.05mmol/l).

Grossly, the livers from the control animals were large and pale tan. On histological examination there was diffuse hepatic lipidosis. Similar, but milder changes were present

in the livers from mice fed diets containing cholic acid. The gallbladders from these mice, but not from the controls, contained large quantities of fine, granular material.

In conclusion, this study showed that the addition of cholic acid to the diet reduces the weight gain of mice, possibly because of its unpalatability. This study also confirmed the results of others indicating that cholic acid is required to increase serum cholesterol concentrations and consistently produce fatty streak lesions in the aortic sinus of C57BL/6 mice[169,378].

Chapter 4

An evaluation of the human apoB-transgenic mouse model of atherosclerosis

The availability of a limited number of human apoB-transgenic mice presented the opportunity to observe the serum lipoprotein profile and aortic fatty streaks which develop in human apoB-transgenic mice when fed a diet high in fat and cholesterol for 15 weeks.

Introduction

The B group of apolipoproteins (apoB48 and apoB100) are critical structural components of all serum lipoproteins which are currently hypothesised to be atherogenic[11,386]. Non-transgenic mice, however, transport the majority of their cholesterol within HDL particles and have a low concentration of apoB in serum[170]. This reduces their suitability for investigations of this apolipoprotein[170,171]. In contrast, transgenic mice which express a human apoB gene develop much higher concentrations of serum apoB, providing a valuable model for examinations of the role of this apolipoprotein in atherogenesis[387-391].

The transgenic mice used in this experiment were C57BL/6 mice which had a 145kb section of human DNA added to their genome[392]. This DNA contains the human apoB gene and regions which promote both enteric and hepatic gene expression[387,392].

The aim of this experiment was to observe if dietary cholic acid was necessary to produce aortic fatty streaks in mice expressing the human apoB gene. If consistent fatty streaks were shown to be produced without dietary cholic acid in this model, apoB mice, rather than non-transgenic C57BL/6 mice, may have been used for studies investigating the effects of dietary factors on atherosclerosis.

Materials and methods

Experimental animals

Sixteen, six-month-old male apoB-transgenic mice were donated by the Department of Biochemistry, University of Otago, New Zealand. All were offspring of human apoB-transgenic mice produced at the Gladstone Institute of Cardiovascular Disease, CA, USA.

Prior to their arrival at Massey University, human apoB had been identified in the serum of all 16 mice. The mice were acclimatised for two weeks and caged individually in conditions described in Chapter 2.

Experimental design

The diets used in this experiment were the same as those given to male non-transgenic C57BL/6 mice as described in Chapter 3. The diet fed to 11 of the mice contained cholic acid, while five received the same diet without any form of cholate. Mice were fed diets *ad libitum* throughout the experimental period. The methods used to produce and feed the diets are described in Chapter 2.

Sample collection and processing

At the completion of the experiment, the mice were fasted for between 8 and 12 hr and killed by carbon dioxide overdose. Serum lipoproteins and aortic fatty streaks were measured using methods described in Chapter 2.

Statistical analysis

Differences between the two groups were investigated using analysis of variance techniques. As the groups contained an uneven number of animals, differences between means were analysed using an unbalanced design.

Results

During the trial, one mouse from each of the two dietary groups developed a severe exudative dermatitis which necessitated euthanasia. The data collected from the remaining mice at the completion of the trial are summarised in Table 4.1.

No significant differences in body weight were observed between the two groups of apoB mice. Serum total and HDL cholesterol concentrations were also not significantly altered by the inclusion of cholic acid in the experimental diet. The ratio of serum HDL to total cholesterol and serum triglyceride concentration, however, were significantly lowered by dietary cholic acid.

	Group	
	Acid n=10	CNT n=4
Total aortic fatty streak area (mm^2)	0.60 (0.32) a	0.11 (0.04) b
Serum total cholesterol (mmol/l)	7.37 (1.53) a	6.70 (1.21) a
Serum HDL cholesterol (mmol/l)	3.09 (1.14) a	4.11 (0.47) a
HDL cholesterol: total cholesterol	0.41 (0.10) a	0.62 (0.07) b
Serum triglyceride (mmol/l)	0.93 (0.26) a	1.59 (0.33) b
Final weight (grams)	38.9 (8.1) a	42.0 (6.1) a

Table 4.1. ***Results of trial investigating human apoB-transgenic mice.*** All measurements were made after 15 weeks of feeding one of two diets. 'Acid' indicates the diet contained cholic acid while 'CNT' indicates the diet did not. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($p<0.05$). HDL indicates high density lipoprotein.

After receiving the experimental diets for 15 weeks, all mice developed aortic fatty streaks similar to those described in Chapter 3. Photomicrographs of the aortic fatty streaks which developed in apoB mice during this experiment are shown in Figure 4.1. The mean area of fatty streaks which developed in mice receiving diets containing cholic acid was significantly greater than that which developed in mice receiving the control diet. As illustrated in Figure 4.2, fatty streak development in apoB mice was negatively correlated with the ratio of serum HDL to total cholesterol ($r^2=0.64$, $p=0.001$, $n=14$). The development of fatty streaks was also negatively correlated with body weight ($r^2=0.39$, $p=0.002$, $n=14$), serum HDL cholesterol concentration ($r^2=0.43$, $p=0.01$, $n=14$), and serum triglyceride concentration ($r^2=0.47$, $p=0.006$, $n=14$). However, fatty streak development was not correlated with the concentration of serum cholesterol ($r^2=0.01$, $p=0.74$, $n=14$).

Discussion

Dietary cholic acid significantly increased the development of aortic fatty streaks in mice expressing the human apoB gene. As reported in Chapter 3, this effect of cholic acid has also been observed in non-transgenic C57BL/6 mice[169,378]. When fatty streak development

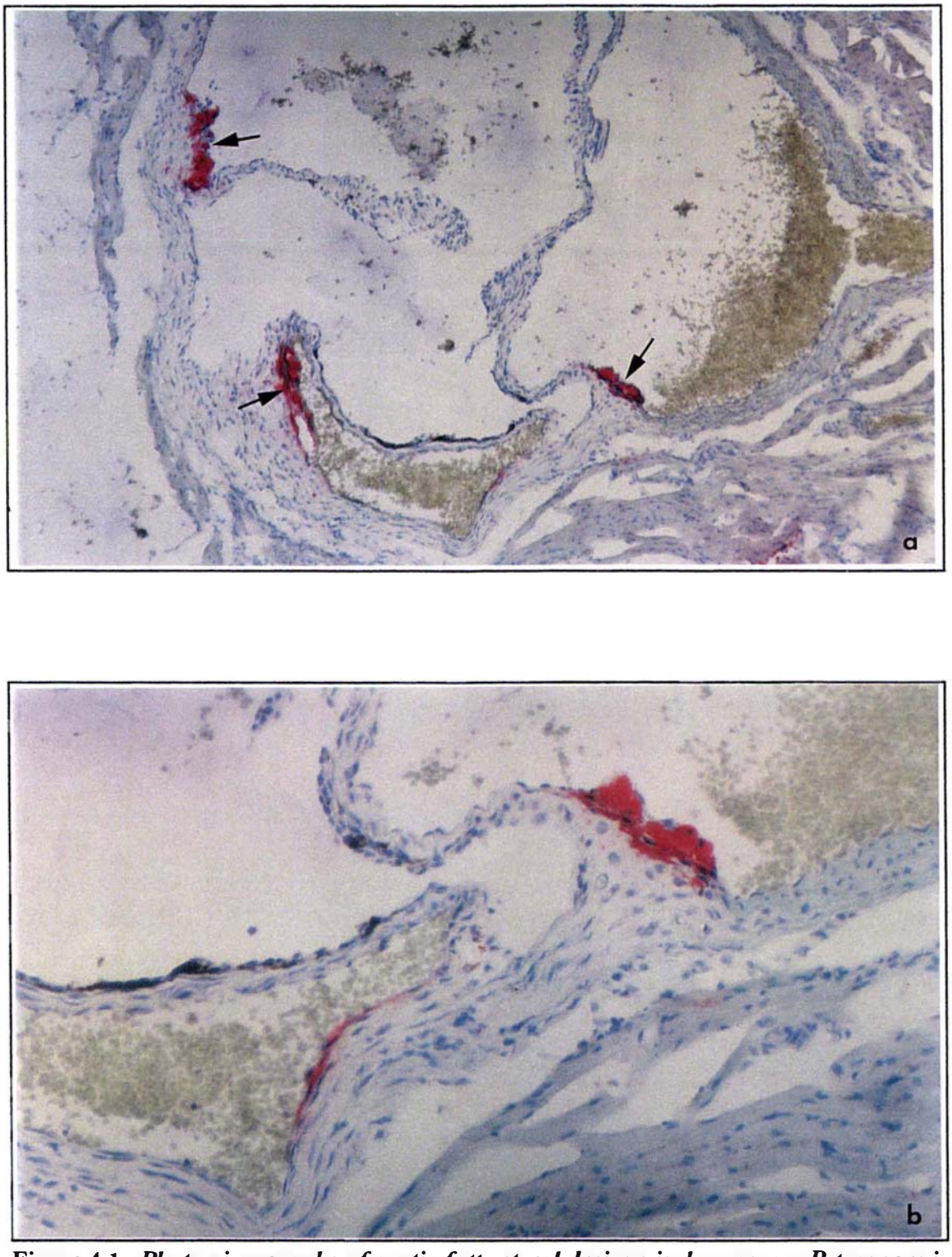
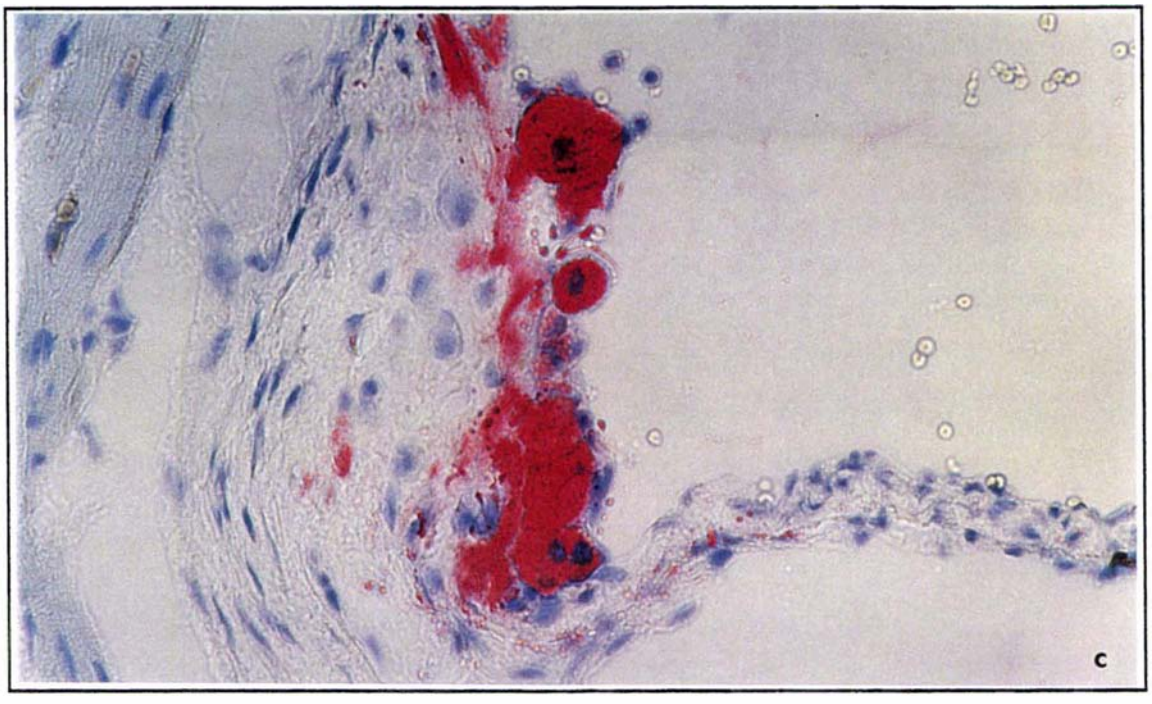


Figure 4.1. ***Photomicrographs of aortic fatty streak lesions in human apoB-transgenic mice.*** Cross-section of the aortic sinus from a transgenic mouse which had received a diet containing cholic acid for 15 weeks. Photomicrograph (a) shows the position of the red-staining fatty streak lesions (arrows) in the aortic sinus. As can be seen from (b) and (c) these lesions consist of focal subendothelial aggregations of macrophages containing numerous lipid droplets. Although mainly intracellular, as shown in (d) these lipid droplets were also present extracellularly in areas of macrophage accumulation and in the underlying media. Magnification: (a) x 85, (b) x 214, (c) x 428, (d) x 1285.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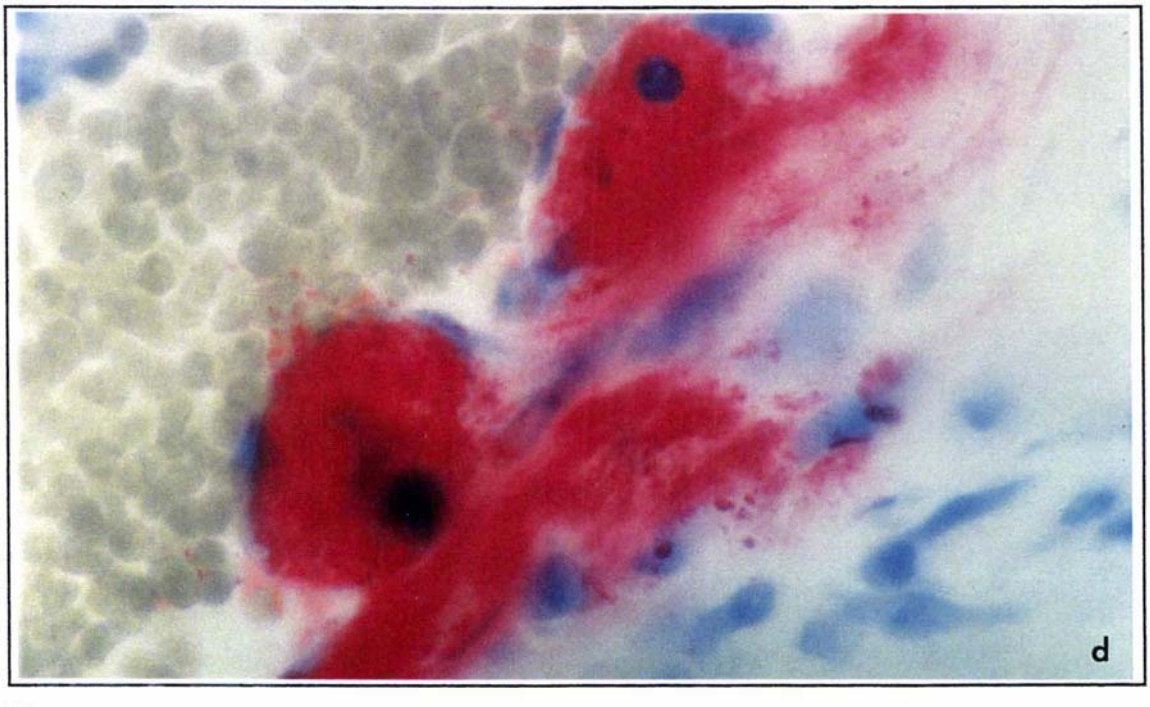
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in apoB and non-transgenic mouse models is compared, previous studies have observed greater lesion formation in apoB mice[393]. Although it is impossible to make direct comparisons between mouse models using results reported in this thesis, there is some evidence to support the suggestion that the presence of the apoB gene increases fatty streak development.

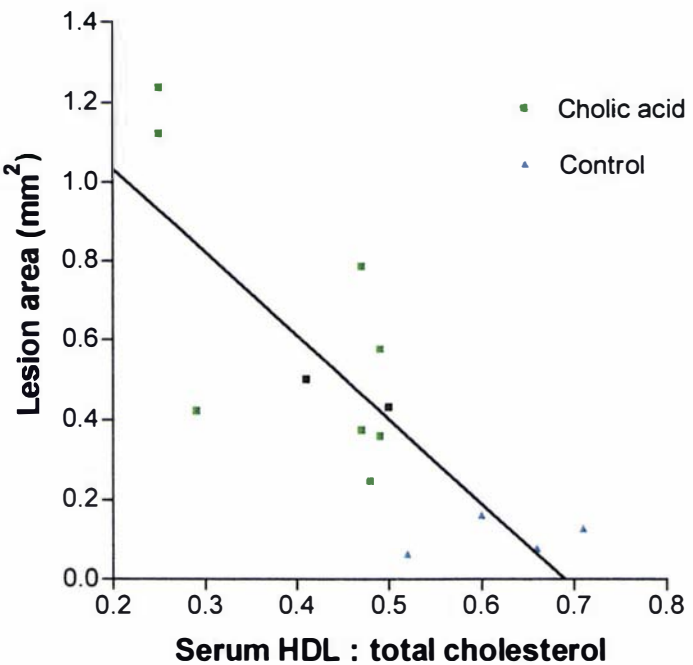

Figure 4.2. ***Relationship between fatty streak area and the ratio of serum HDL to total cholesterol in human apoB-transgenic mice.*** Each symbol represents an individual mouse. Mice in the 'Cholic acid' group received atherogenic diets containing 0.5% cholic acid for 15 weeks. Diets fed to mice in the 'Control' group contained no cholate. r^2=0.64, p=0.001, n=14. HDL is an abbreviation for high density lipoprotein.

No differences in serum cholesterol concentration were observed between the two groups of apoB mice. This is in contrast to the results of experiments using non-transgenic C57BL/6 mice which have shown that high-cholesterol diets do not increase serum cholesterol concentrations unless dietary cholic acid is also present[172]. As discussed in more detail in Chapter 5, cholic acid raises serum cholesterol concentrations in non-transgenic mice by inhibiting LDL receptor activity, preventing the removal of cholesterol from serum[169]. In apoB mice however, removal of cholesterol from serum is thought to be prevented by the low affinity of lipoproteins containing human apoB for the mouse LDL receptor[393]. Therefore, although a high cholesterol diet will increase the activity of LDL

receptors in both mouse models, in apoB mice this results in little LDL receptor-mediated removal of lipoproteins. Because apoB is the only apolipoprotein present in LDL particles, the increase in serum cholesterol concentration in apoB mice is contained mainly in this fraction[393]. Dietary cholic acid, as in non-transgenic C57BL/6 mice, will inhibit LDL receptor activity in apoB mice. However, because LDL receptors are only able to remove a small proportion of circulating lipoproteins, this results in minor changes to the serum concentration of cholesterol.

Serum HDL cholesterol concentrations were not significantly altered by the inclusion of cholic acid in the experimental diets. This was surprising as reduced HDL cholesterol concentrations have previously been associated with dietary cholic acid in apoB mice[393]. It is probable that statistically significant differences were not attained in the present experiment because of the small number of animals contained in the control group and the unexpectedly large variation in serum HDL cholesterol concentration observed in mice fed cholic acid.

The ratio of serum HDL to total cholesterol was significantly lower in apoB mice receiving cholic acid than in controls. When the two mouse models are compared, previous studies have shown that, when fed a similar diet, apoB mice develop a lower ratio of serum HDL to total cholesterol than non-transgenic C57BL/6 mice[393].

As can be seen from Figure 4.1, fatty streak development in apoB mice was correlated most strongly with the ratio of serum HDL to total cholesterol. This correlation has also been observed in human epidemiological studies and in other experiments using mouse models of atherosclerosis[12,13,172,173].

Surprisingly, the inclusion of cholic acid in the experimental diets did not significantly alter the body weight of apoB mice. This is in contrast to the study using male non-transgenic C57BL/6 mice which is reported in Chapter 3. The reason for this discrepancy is not clear but, as the apoB mice used in the present experiment were caged individually and were older than the male C57BL/6 mice, the results of the two experiments cannot be directly compared.

Although apoB mice fed a cholic acid-free diet developed high serum cholesterol concentrations, these mice did not consistently develop significant fatty streaks. This observation, and the lack of correlation between total cholesterol and lesion development, suggest that to produce significant fatty streaks in mice, it is necessary to reduce the ratio of serum HDL to total cholesterol. This ratio is lowered by cholic acid and it appears that to consistently develop fatty streaks, like non-transgenic mice, apoB mice also require this chemical in their diet.

In conclusion, the apoB-transgenic mouse model appears to offer no clear advantages over the non-transgenic C57BL/6 model for examining the effect of dietary factors on atherosclerosis. Therefore, considering the superior availability of non-transgenic C57BL/6 mice, it was decided to use this model throughout the mouse experiments reported in this thesis.

Chapter 5

The effect of dietary fat on fatty streak development in the C57BL/6 mouse model

The purpose of this study was to investigate the validity of the C57BL/6 mouse model for testing the atherogenicity of dietary fats. To do this, fats with known effects on human serum lipoprotein concentration were tested. If the C57BL/6 mouse model could be validated, it could then be used to screen a wide range of dietary lipids for antiatherogenic effects.

Introduction

The effect of common dietary fats on blood lipoprotein concentrations has been investigated in both human and animal studies[229,394-397]. As reviewed in Chapter 1, compared to dietary carbohydrate, ingestion by human beings of the medium chain saturated fatty acids (SFAs), lauric acid (12:0), myristic acid (14:0), and palmitic acid (16:0), increases both serum LDL and HDL cholesterol concentration. *Cis*-monounsaturated fatty acids (*cis*-MUFAs) lower LDL and raise HDL cholesterol, while polyunsaturated fatty acids (PUFAs) lower both serum LDL and HDL cholesterol concentration[243,246,398,399]. When dietary carbohydrate is replaced with *cis*-MUFA, the highest ratio of serum HDL to LDL cholesterol is produced, PUFAs result in an intermediate ratio, while SFAs produce the lowest ratio[243,246,398,399].

Data from both epidemiological studies and animal experiments have shown an association between a decreased ratio of serum HDL to LDL cholesterol and an increased risk for atherosclerosis[12,13,194,400]. Therefore, *cis*-MUFA are expected to be the least atherogenic, followed by PUFA, with SFA being the most atherogenic.

Because this study was designed to validate the C57BL/6 mouse model, fats with known effects on human serum lipoprotein concentration were examined. These were hydrogenated coconut oil, olive oil, fish oil and milk fat. To confirm that the atherosclerotic lesions were developing as a result of dietary fat, a low-fat control diet was also included.

Materials and methods

Experimental animals

One hundred, six-week-old female C57BL/6 mice were obtained and housed as described in Chapter 2. The mice were acclimatised for 14 weeks, during which time they were fed standard mouse food. At the start of the trial their mean weight was 24.9g.

Experimental design

Four cages of five mice were randomly allocated to each of the five experimental groups. The treatment diets contained 20% fat, consisting of 5% corn oil plus 15% of one of the following fats/oils, extra virgin olive oil, anhydrous milk fat, hydrogenated coconut oil, or MaxEPA fish oil (Seven Seas Health Care Ltd, United Kingdom). The control diet contained only 5% corn oil which was included to meet the nutrient requirements of mice[379]. In the control diet, the test fat/oil was replaced with equal quantities of cornstarch and sucrose. This resulted in a less energy-dense diet containing an estimated 13.4KJ/g compared to the 16.7KJ/g contained in the high fat diets[401]. All five diets contained 1% cholesterol and 0.5% cholic acid.

The mice were fed *ad libitum* for 15 weeks and weighed weekly. At the end of the trial period, the mice were fasted for between 8 and 12 hr and then killed by carbon dioxide inhalation.

Experimental diets

The composition of the five experimental diets is illustrated in Table 5.1. The

Ingredient	Dietary Group	
	Test diet (20% fat)	Control (5% fat)
	grams/kilogram, as fed	
Cornstarch	215.64	290.64
Sucrose	215	290
Test fat	150	0
Corn oil	50	50
Casein	200	200
Cellulose	50	50
Salt mix	50	50
Vitamin mix	50	50
dl-methionine	3	3
(dl)-α-tocopherol	1.36	1.36
Cholesterol	10	10
Cholic acid	5	5

Table 5.1. ***Composition of diets used to investigate dietary fats.*** Test fats consisted of one of, hydrogenated coconut oil, milk fat, fish oil or olive oil.

Fatty acid	Experimental diet*			
	AMF	HCO	Olive oil	CNT
	grams/kilogram of diet			
12:0	3.7	43.3	0.4	0
14:0	13.1	20.7	0.4	0
14:1	1.1	0	0	0
15:0	1.6	0	0	0
16:0	47.1	25.5	18.4	4.6
16:1	2.8	0	1.0	0
17:0	1.4	0	0	0
18:0	24.1	28.7	5.7	1.3
18:1	54.5	30.3	120	15
18:2	35.2	39.1	39.3	23.7
18:3	2.4	1	1.6	0.6
20:0	1.2	0.8	1.2	0
22:0	0.6	0	0	0
Total SFA	92.8	119	26.1	5.9
Total MUFA	58.4	30.3	121	15
Total PUFA	37.6	40.1	40.9	24.3
Unidentified	11.2	10.6	12	4.8

Table 5.2. ***Fatty acid composition of diets used to investigate dietary fats.*** SFA denotes saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids. AMF is an abbreviation for anhydrous milk fat. HCO is an abbreviation for hydrogenated coconut oil. CNT is an abbreviation for the control diet. *Because the group receiving diets containing fish oil (Max-EPA) was discontinued after 3 weeks of the experiment, fatty acid analysis of this diet was not performed.

techniques used to prepare the diets are described in Chapter 2.

To analyse the lipid content of the experimental diets, total lipid was extracted from sub-samples of diets using chloroform/methanol (2/1, v/v) (40ml) and 0.1mol/l KCl (8ml)[402]. Triglycerides in the lipid extracts of the diets (ca. 20mg) were then transesterified by incubation with 14% BF_3 in methanol (2.0ml) and benzene (0.5ml) at 100°C for 90min[403]. After cooling, water (4.0ml) and hexane (4.0ml) were added and the fatty acid methyl esters extracted into hexane and fractionated by gas-liquid chromatography using a Hewlett Packard Gas Chromatograph (Model 5890 A) equipped with a stainless steel column (25mm x 0.22mm id) packed with BPX70 and helium carrier gas, programmed over 140-210°C at 2%/min. The fatty acid composition of each diet is presented in Table 5.2.

Sample collection and processing

The methods used to measure serum lipid concentrations and aortic fatty streak development are contained in Chapter 2. As a marker of liver damage, the serum activity of alanine aminotransferase was determined using a Hitachi 704 autoanalyser (Boehringer Mannheim GmbH, Germany).

Statistical analysis

Differences among dietary groups were investigated using analysis of variance techniques. As no data was obtained from some experimental animals, the differences were analysed using an unbalanced design. Aortic fatty streak area, total serum cholesterol, and lipoprotein cholesterol concentrations were modelled using linear regression. Fatty streak area was also modelled using multiple regression techniques.

Results

As can be seen from Figure 5.1, the mean body weight of mice receiving diets containing fish oil decreased by 6g (24%) during the first three weeks of the trial. Mice in this group became emaciated and the group was discontinued due to welfare concerns. When the mice were returned to a diet of standard mouse food, their mean weight returned to the pre-trial level within 7 days, suggesting that reluctance to consume the diet, rather than disease, was the cause of the weight loss.

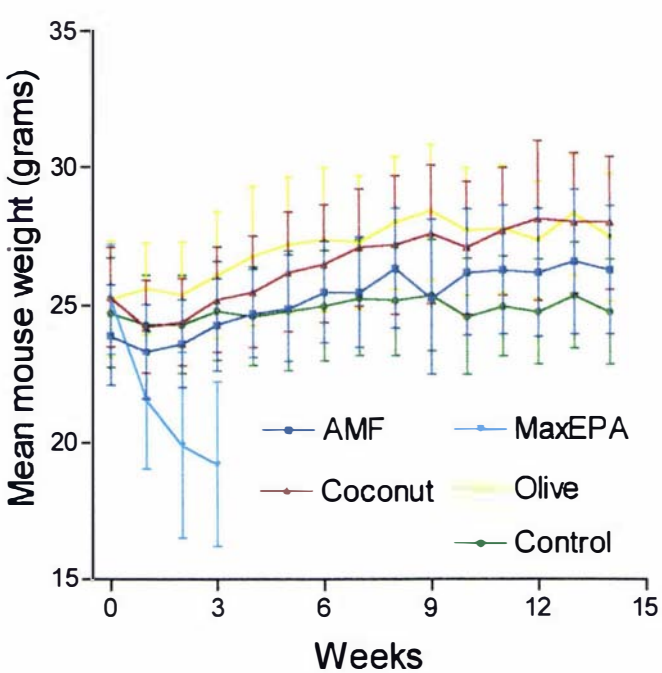

Figure 5.1. ***Mean body weight of mice fed diets containing different dietary fats.*** 'Control' indicates mice were fed a diet containing 5% corn oil. 'AMF', 'Coconut', 'MaxEPA', and 'Olive' indicates mice received a diet containing 5% corn oil plus 15% of one of, anhydrous milk fat, hydrogenated coconut oil, MaxEPA fish oil, or olive oil. The group receiving diet containing fish oil was discontinued after 3 weeks due to weight loss. The error bars indicate one standard deviation.

	Dietary Group			
	AMF n=17	HCO n=19	Olive oil n=19	CNT n=18
Total aortic fatty streak area (mm^2)	0.35 (0.26) a b	0.27 (0.19) b	0.50 (0.40) a c	0.59 (0.30) c
Serum total cholesterol (mmol/l)	4.42 (0.75) a	4.34 (0.58) a	4.61 (0.78) a b	5.05 (0.96) b
Serum HDL cholesterol (mmol/l)	1.51 (0.26) a	1.90 (0.48) b	1.14 (0.42) c	1.33 (0.26) a c
HDL cholesterol: total cholesterol	0.34 (0.05) a	0.44 (0.10) b	0.24 (0.09) c	0.27 (0.06) c
Serum triglyceride (mmol/l)	0.58 (0.07) a	0.82 (0.22) b	0.59 (0.14) a	0.68 (0.15) a
Serum ALT activity (IU/l)	203 (63) a	134 (54) b	158 (71) b	217 (86) a
Mouse weight gain (grams)	2.37 (0.35) a	2.73 (1.10) a	2.28 (0.75) a	0.04 (0.39) b

Table 5.3. ***Results of trial investigating dietary fats.*** Summary of the measurements made after 15 weeks of feeding an atherogenic diet containing 5% corn oil and one of, 15% anhydrous milk fat (AMF), 15% hydrogenated coconut oil (HCO), 15% olive oil, or no extra fat (control, CNT) to C57BL/6 mice. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($p<0.05$). HDL indicates high density lipoprotein, ALT indicates alanine aminotransferase.

A summary of the measurements made on the remaining four groups at the completion of the experiment are presented in Table 5.3. During the trial, two mice were removed from the milk fat group, and one from both the olive oil and control groups. This was due to weight loss, presumably after failing to adapt to the test diet. By the completion of the trial, mice fed the control diet had gained significantly less weight than mice in the other three groups. There were no significant differences in the mean weight gain among the three groups of mice receiving high fat diets.

The mean serum total cholesterol concentration was significantly higher in control mice than in mice receiving diets containing either milk fat or coconut oil. However, the mean serum total cholesterol concentration in mice fed a diet containing olive oil was not significantly different from those in any other group. The mean serum HDL cholesterol concentration was highest in mice fed the diet containing coconut oil as the test fat. Serum HDL cholesterol concentrations were higher in mice fed milk fat than in mice fed olive oil, but were not significantly different to those in mice fed the control diet. As illustrated in

Figure 5.2, the mean concentration of serum HDL cholesterol was significantly correlated with the proportion of saturated fat contained in the diet (r^2=0.94 p=0.03 n=4). The highest ratio of serum HDL cholesterol to total cholesterol was observed in mice fed coconut oil. This was significantly higher than the ratio observed in serum from mice fed milk fat, which was significantly higher than that which developed in mice fed either the olive oil or the control diets.

The mean serum triglyceride concentration was highest in mice receiving the diet containing coconut oil.

After 15 weeks, all mice developed lipid-containing lesions in the intima of the aortic sinus similar to those previously described[168]. The lesions were most common close to the origins of the coronary arteries and at the base of the aortic valves and consisted of sub-endothelial collections of macrophages containing numerous Oil red O-positive lipid globules. These globules were also found extracellularly around areas of macrophage accumulation and were

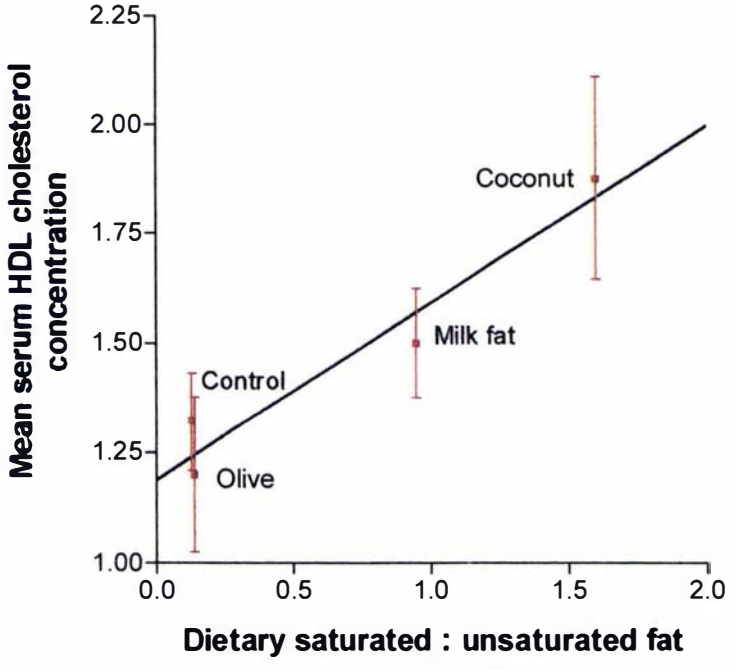

Figure 5.2. ***Relationship between the mean serum HDL cholesterol concentration and the ratio of saturated to unsaturated fat in the diet.*** 'Control' indicates mice were fed a diet containing 5% corn oil. 'Olive', 'Milk fat', and 'Coconut' indicate mice were fed a diet containing 5% corn oil plus 15% one of, olive oil, anhydrous milk fat, or hydrogenated coconut oil. The error bars indicate two standard errors. r^2=0.94 p=0.03 n=4. HDL is an abbreviation for high density lipoprotein.

present in the underlying media.

Aortic fatty streak development was greater in controls and olive oil-fed mice than in mice receiving diets containing coconut oil. However, only the control mice developed a mean area of fatty streaks which was significantly greater than in mice fed milk fat. As illustrated in Figure 5.3, mean fatty streak area was negatively correlated with the ratio of saturated to unsaturated fatty acids in the diet (r^2=0.90, p=0.049, n=4).

Figure 5.4 shows a weak inverse correlation between fatty streak area and the ratio of serum HDL to total cholesterol (r^2=0.094, p=0.011 n=73). When lesion size was modelled on both the ratio of serum HDL to total cholesterol and the dietary content of polyunsaturated fat, the relationship was strengthened (r^2=0.16, p=0.003 n=73).

The livers of mice from all four dietary groups were enlarged and pale tan in colour. Histological examination revealed diffuse hepatic lipidosis. Serum activities of alanine aminotransferase were significantly higher in mice fed the control and milk fat diets than in

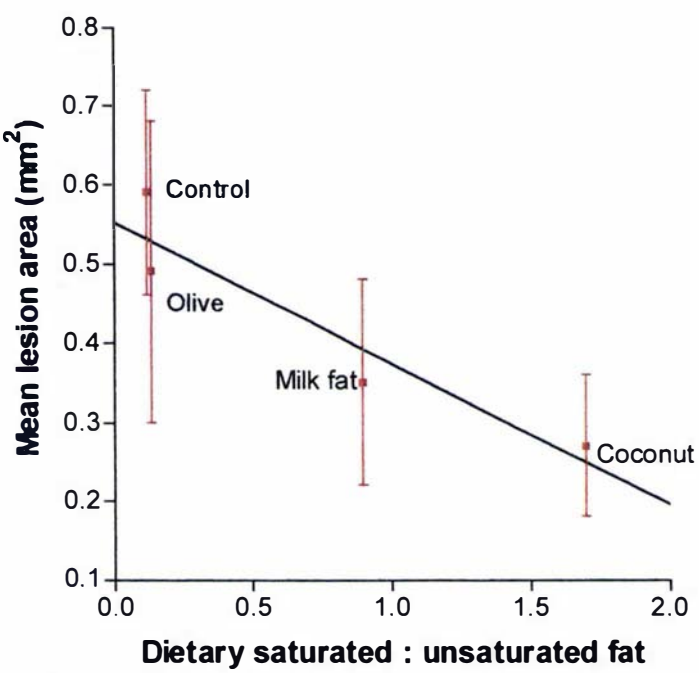

Figure 5.3. ***Relationship between mean fatty streak area and the ratio of saturated to unsaturated fatty acids in the diet.*** 'Control' indicates that the mice were fed a diet containing 5% corn oil. 'Olive', 'Milk fat', and 'Coconut' indicate the mice were fed a diet containing 5% corn oil plus 15% of one of, olive oil, anhydrous milk fat, or hydrogenated coconut oil. The error bars indicate two standard errors. r^2=0.90 p=0.049 n=4.

those receiving diets containing either olive or coconut oil, although, no differences in the severity of hepatic lipidosis were detected histologically among the dietary groups.

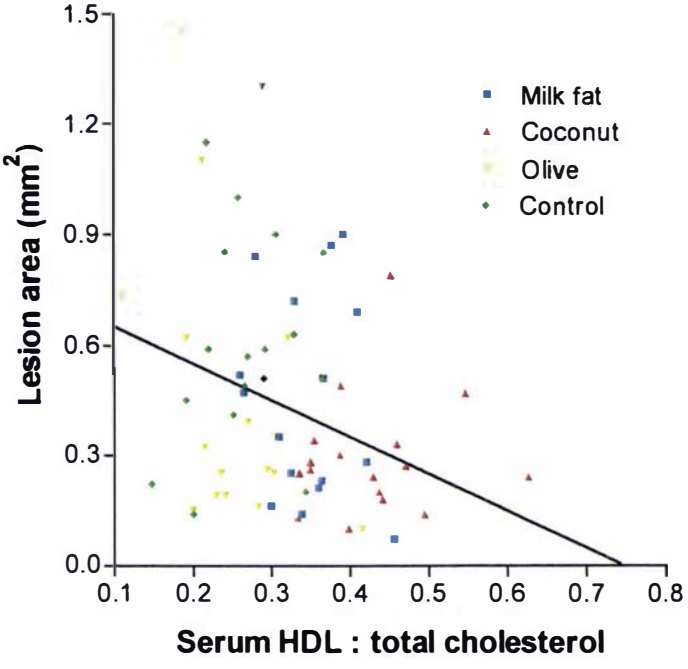

Figure 5.4. ***Relationship between fatty streak area and the ratio of serum HDL to total cholesterol in mice receiving different dietary fats***. Each symbol represents an individual mouse. Mice were fed diets containing 5% corn oil plus 15% of one of, anhydrous milk fat 'Milk fat', hydrogenated coconut oil 'Coconut', or olive oil 'Olive'. Mice in the 'control' group received diets containing only 5% corn oil. r^2=0.09 p=0.03 n=73. HDL is an abbreviation for high density lipoprotein.

Discussion

Aortic fatty streak formation in C57BL/6 mice was inversely correlated with the concentration of SFAs in the diet. This is contrary to the findings of previous animal and human studies which have suggested an association between increased dietary SFA and increased risk of atherosclerotic disease[229,394-397,404]. The results are also in contrast to those of a similar mouse trial performed by Nishina *et al* in which dietary SFA content was correlated with aortic fatty streak formation[173]. It is difficult to explain the discrepancy between our trial and that of Nishina *et al* as C57BL/6 mice were fed diets containing 1% cholesterol and 0.5% cholic acid in both. One difference between the two trials was the use of hydrogenated soy oil as the MUFA tested by Nishina *et al*. This contains a high proportion of *trans* fatty acids which are expected to behave differently to the predominantly

cis-MUFA-containing olive oil used in our trial, although whether or not this could account for the conflicting results is unclear.

The degree of saturation of the dietary fat did not significantly alter the total serum cholesterol concentration among the three groups receiving high-fat diets. Again, this is in contrast to the findings of previous human trials and epidemiological studies which have shown SFAs increase and PUFAs decrease total serum cholesterol concentration[233,243,246,390,391].

These unexpected effects of dietary fats on serum cholesterol concentration can be explained by reference to its mode of regulation. As reviewed in Chapter 1, serum cholesterol concentration is mainly determined by the activity of hepatic LDL receptors[46]. These receptors bind LDL and IDL particles and are responsible for the removal of around 40% of the circulating cholesterol[46]. Their activity is regulated by the concentration of oxygenated derivatives of free cholesterol present in hepatocytes[33,85,169]. Oxysterols are produced from free cholesterol at a constant rate and removed after conversion to bile acids by cholesterol 7α-hydroxylase[169]. Therefore, the activity of hepatic LDL receptors is determined by the amount of free cholesterol in the liver and the activity of cholesterol 7α-hydroxylase.

In human beings, the amount of hepatic free cholesterol is dependent on the activity of acyl-CoA acyl transferase (ACAT)[85]. This enzyme esterifies hepatic cholesterol, removing it from the cytoplasmic pool of free cholesterol[15]. The preferred substrate for ACAT appears to be oleic acid[85] and when the liver is enriched with this fatty acid, ACAT activity is high. However, SFAs appear to be a poorer substrate for this enzyme, resulting in decreased ACAT activity and an increased concentration of free cholesterol in hepatocytes[85]. Therefore SFAs decrease LDL receptor activity in human beings by inhibiting ACAT activity.

In the C57BL/6 mouse model, high levels of cholesterol (1%) and cholic acid (0.5%) are fed to induce fatty streak formation. Cholic acid inhibits cholesterol 7α-hydroxylase[405] and, as this enzyme is responsible for the excretion of cholesterol and oxysterols[39], its inhibition

produces a two-fold increase in hepatic free cholesterol and oxysterol concentration[169,173]. This results in a 70% decrease in hepatic LDL receptor activity[169].

Therefore, in human subjects, dietary SFAs raise serum cholesterol concentrations by slowing ACAT activity and thereby decreasing LDL receptor activity. In the C57BL/6 mouse atherosclerosis model, no effect of SFAs is expected because the LDL receptors are suppressed due to decreased oxysterol excretion[169], a process wholly mediated by cholic acid and independent of dietary fat. Furthermore, the hepatic free cholesterol pool is vastly increased, making it unlikely that subtle effects of SFAs on ACAT activity will produce any significant changes in receptor activity. Therefore, in this model, the total serum cholesterol concentration will depend largely on the amount of cholesterol and cholic acid consumed by the mouse, regardless of the principle fatty acids contained in the diet.

As illustrated in Figure 5.2, the concentration of serum HDL cholesterol was positively correlated with the proportion of dietary SFA. This finding supports the results of human and animal trials showing that SFAs raise serum HDL cholesterol concentration to a greater extent than PUFAs with intermediate effects being seen in response to dietary *cis*-MUFA[233,406]. This effect appears to be particularly marked with coconut oil, possibly due to its high trilaurin content[102]. It is believed that the proportion of cholesterol carried in HDL is dependent on the serum concentration of apoA-I[96-98]. Trials using non-human primates suggest that dietary SFA increases the concentration of this apolipoprotein in a process independent of hepatic lipid metabolism[101]. Therefore, in the present trial, SFAs increased serum HDL cholesterol concentrations as expected, but not IDL and LDL cholesterol concentration. HDL particles also contain apoE, and, in animals which do not possess serum CETP activity, such as mice, the major pathway for the removal of HDL from serum is the hepatic LDL receptor[100]. As this receptor was equally inhibited by cholic acid in all high-fat groups, it can be explained why little variation in total serum cholesterol concentration was observed among the three dietary groups.

The ratio of serum HDL to total cholesterol was significantly and inversely correlated with lesion size (Figure 5.4). This supports other experiments using this model[173] and findings of human population studies[12, 13,407]. The strength of this correlation was increased when the

PUFA content of the diet was included in the regression equation. From this it can be hypothesised that LDL may become enriched with polyunsaturated fatty acids predisposing it to oxidative damage. However, while the oxidation of LDL appears to be a critical process in atherogenesis[118,124,408], it is still unclear whether or not dietary factors can influence atherosclerosis by altering the oxidisability of LDL particles.

The group of mice fed hydrogenated coconut oil had significantly higher serum triglyceride concentrations than the other groups. Although this effect of coconut oil has been previously described in trials using rabbits[102], the three different types of dietary fat examined in this experiment are considered to have roughly equal effects on serum triglyceride concentration in humans[233].

Serum activities of alanine aminotransferase (ALT) were measured to provide an indication of hepatic damage[409]. The high levels of activity observed in this experiment were similar to those reported from another mouse atherosclerosis trial[174] and probably reflect hepatic lipidosis. Although there were significant differences in serum ALT activity among dietary groups, no differences in the severity of lipidosis was observed histologically.

In conclusion, the C57BL/6 mouse model is a popular model for atherosclerosis research. However, to produce fatty streaks quickly, cholic acid and cholesterol must be included in the experimental diet. This results in an inhibition of LDL receptor activity independent of dietary fatty acid type. The differences in fatty streak formation observed among the dietary groups in this trial were probably due to the effects of different dietary fats on HDL cholesterol metabolism. This resulted in dietary SFAs producing smaller fatty streaks than dietary unsaturated fatty acids, a finding which contrasts the results of most human epidemiological studies[394-397]. Because of differences in lipid metabolism between C57BL/6 mice and humans, the results of this trial should not be taken as evidence that SFAs are likely to decrease atherogenesis in humans. However, the results of the trial do provide some evidence that atherogenesis can be influenced by the predominant fatty acid type which is contained in the diet.

This trial suggests that the C57BL/6 mouse model is a poor model for investigating the role

of dietary fatty acid type in atherogenesis. This was exemplified by the significantly greater fatty streak formation in the low fat control group (which was included to observe minimal lesion formation) than in the coconut oil group (included to observe maximum lesion formation). Therefore, the findings of this study do not support the conclusion of Nishina *et al* that C57BL/6 mice provide an excellent model to study the effects of dietary fats on atherogenesis[173].

In this trial, the development of atherosclerotic lesions was found to be influenced by the concentration of total and HDL cholesterol in serum. This suggests that fatty streak formation in this model may be determined by similar serum changes to those believed to influence atherogenesis in humans. Therefore, the C57BL/6 mouse model may be useful for investigating dietary factors which do not influence atherogenesis by altering hepatic LDL receptor function. There was also some evidence that oxidation of LDL may be important. This, and the unsuitability of the model for testing dietary fats, changed the emphasis of the project towards investigating other dietary components which may influence atherosclerosis.

Chapter 6

The effect of dietary antioxidants on fatty streak development in the C57BL/6 mouse model

As discussed in the previous chapter, the C57BL/6 mouse model may not be an appropriate model for investigating the role of dietary fat in atherosclerosis. Therefore the emphasis of this project shifted to identifying other dietary components which may influence fatty streak formation in this model. Dietary antioxidants were the first of these to be examined and the results of this experiment have been published in *Arteriosclerosis, Thrombosis and Vascular Biology*[410].

Introduction

The earliest lesion of atherosclerosis is believed to be the fatty streak[117], which consists of a subendothelial collection of foam cells (macrophages containing lipid droplets), small amounts of extracellular lipid, and increased numbers of smooth muscle cells[2]. As reviewed in Chapter 1, oxidation of low density lipoprotein (LDL) is considered to be an important process in the development of fatty streaks[124]. Macrophages do not engulf native LDL rapidly enough to form foam cells[411], but oxidised LDL is recognised by macrophage scavenger receptors[411,412], leading to fast, unregulated LDL uptake and foam cell formation[112,117,124]. Oxidation of LDL only occurs once the LDL has become trapped in the subendothelial matrix, where it loses the protection of antioxidants contained in plasma[112,139]. Dietary antioxidants may, however, delay the oxidation of LDL[284,286,292,296], enabling the lipoprotein to leave the subendothelial space prior to oxidation[284]. This could reduce the incorporation of LDL into foam cells, thus ameliorating atheroma development and progression[284]. Low plasma antioxidant status has been suggested as a risk factor for atherosclerosis[413].

Studies of a possible cardioprotective effect of vitamin E began in 1949[414], but the results of this study, and of subsequent experiments have been inconclusive. Vitamin E was reported to decrease the severity of atherosclerosis in Dutch-belted rabbits[289,295] and in primates[287], but had no effect in Watanabe heritable hyperlipidaemic (WHHL) rabbits[291,292,294]. In a rabbit model in which atheroma formation was induced by damaging

the endothelium, vitamin E increased the severity of atherosclerosis[415]. As reviewed in Chapter 1, the results of epidemiological studies investigating the relationship between vitamin E and atherosclerosis have also been inconclusive.

The antioxidant butylated hydroxytoluene (BHT) has been shown to reduce fatty streak formation in WHHL rabbits[297]. This chemical is widely used at low levels in foods and cosmetics[416,417], although its chronic toxicity makes it is unlikely to be used as an antioxidant supplement in human diets[416]. Another synthetic antioxidant, diphenyl-1,4-phenylenediamine (DPPD) has also been shown to decrease atherosclerosis in rabbit[418] and mouse[419] models.

The C57BL/6 mouse model was used in the present study to examine the effect on subendothelial fatty streak formation of dietary supplementation with the antioxidants vitamin E and BHT.

Materials and methods

Experimental animals

Sixty, six-week-old female C57BL/6 mice were obtained and housed as described in Chapter 2. The mice were acclimatised for 20 weeks, during which time they were fed standard mouse food. At the start of the trial the mean weight of the mice was 26.3g.

Experimental design

Twenty mice were randomly allocated into one of the three dietary groups. Two treatment groups received diets supplemented with either 2% vitamin E (10IU/g diet) or 1% BHT by weight. The control group received the same diet but without supplementary antioxidant. The mice were fed *ad libitum* for 15 weeks. They were then fasted for between 8 and 12hr, and killed by carbon dioxide inhalation.

Experimental diets

The composition of the experimental diets is shown in Table 6.1. Dry vitamin E 50%, type CWS/F containing 500IU of vitamin E per gram in the form of dl-α-tocopherol acetate was obtained from Roche Products (NZ) Ltd, New Zealand. The production and feeding of the

diets is discussed in detail in Chapter 2.

Sample collection and processing

The serum lipoprotein measurements, routine liver section preparation, and aortic lesion quantification were all done using methods previously described in Chapter 2.

Serum total antioxidant status was measured using a commercial kit (Randox Laboratories Ltd, United Kingdom) run on a Hitachi 704 autoanalyser. The kit contains a reagent which is oxidised at a known rate by a peroxidase. The ability of the test sample to inhibit this reaction is determined by measuring the formation of oxidation products of the reagent.

	Dietary Group		
Ingredient	BHT	Vit E	Control
	grams/kilogram, as fed		
Sucrose	477	472	482
Casein	200	200	200
Corn oil	50	50	50
Olive oil	50	50	50
Anhydrous milk fat	50	50	50
Cellulose	50	50	50
Salt mix	50	50	50
Vitamin mix	50	50	50
dl-methionine	3	3	3
BHT	5	0	0
Vitamin E acetate	0	10	0
Cholesterol	10	10	10
Cholic acid	5	5	5

Table 6.1. ***Composition of diets used to investigate antioxidants and alcohol.*** 'BHT' is an abbreviation for butylated hydroxytoluene. 'Vit E' is an abbreviation for vitamin E.

Statistical analysis

Differences among dietary groups were investigated using analysis of variance techniques. At the completion of the trial the groups contained uneven numbers of animals and the differences between means were analysed using an unbalanced design. Lesion size, total serum cholesterol, total antioxidant status, triglyceride and lipoprotein cholesterol concentrations were modelled using linear regression.

Results

During the trial, three mice from both the control and vitamin E groups, and two from the BHT group, were removed from the study after losing weight presumably as a result of failure to adapt to the trial diet. A summary of the measurements made on the remaining mice at the completion of the trial is presented in Table 6.2.

	Dietary Group		
	BHT n=18	Vit E n=17	CNT n=17
Total aortic fatty streak area (mm^2)	0.48 (0.26) a	0.30 (0.16) b	0.31 (0.11) b
Serum total cholesterol (mmol/l)	6.13 (0.81) a *	4.68 (0.85) b *	5.53 (1.54) a
Serum HDL cholesterol (mmol/l)	2.00 (0.42) a	1.96 (0.51) a	2.70 (0.59) b
HDL cholesterol: total cholesterol	0.33 (0.08) a *	0.42 (0.10) b	0.50 (0.08) c *
Serum triglyceride (mmol/l)	0.48 (0.24) a	0.58 (0.17) ab	0.82 (0.69) b
Serum total antioxidant status (mmol/l)	1.40 (0.22) a	1.43 (0.29) a	1.22 (0.26) b
Final weight (grams)	24.21 (2.21) a *	25.62 (1.93) ab	26.35 (2.52) b *

Table 6.2. ***Results of trial investigating antioxidants.*** All measurements were made after C57BL/6 mice had been fed an atherogenic diet for 15 weeks. The 'Vit E' group received a diet containing 2% vitamin E acetate, the 'BHT' diet contained 1% butylated hydroxytoluene. The 'CNT' group was fed a diet containing no antioxidants. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($p<0.05$). '*' indicates that the differences between the means are significant at a $p<0.01$ level. HDL indicates high density lipoprotein.

At the completion of the trial, mice in the control group were significantly heavier than those in the BHT group. The mean weight of mice receiving vitamin E was not significantly different to the mean weight of mice in either of the other groups.

The mean total cholesterol concentration of mice supplemented with vitamin E was significantly lower than that of the other groups. The mean serum HDL cholesterol concentration of mice in the control group was significantly higher than that in the BHT and vitamin E groups. The highest mean ratio of serum HDL cholesterol to total cholesterol was

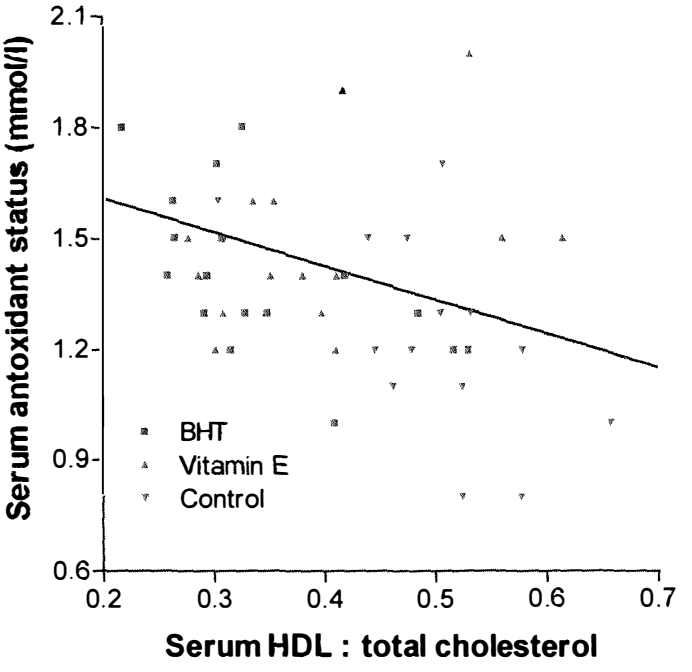

Figure 6.1. ***Relationship between serum total antioxidant status and the ratio of serum HDL to total cholesterol.*** Each symbol represents an individual mouse. 'BHT' and 'vitamin E' indicates mice were fed an atherogenic diet containing 1% butylated hydroxytoluene or 2% vitamin E acetate. 'Control' mice received a diet containing no additional antioxidants. r^2=0.16, p=0.005, n=52. HDL indicates high density lipoprotein.

observed in the control group, while the BHT group had a significantly lower ratio than the other groups. Serum total antioxidant status was significantly higher in mice fed diets containing antioxidants than from control mice and, as illustrated in Figure 6.1, negatively correlated to the ratio of serum HDL cholesterol to total cholesterol (r^2=0.16 p=0.005 n=52). Mean serum triglyceride concentrations in mice from the BHT group were higher than in mice from the control group.

After 15 weeks, all mice developed lipid-containing lesions in the intima of the aortic sinus similar to those previously described. The mean fatty streak area in mice receiving a diet containing BHT was significantly greater than in mice in either the control or vitamin E groups. The ratio of HDL to total cholesterol was not significantly correlated with mean fatty streak area for individual mice (r^2=0.07, p=0.07, n=52) or, as shown in Figure 6.2, when mean values of the dietary groups were considered (r^2=0.75 p=0.33 n=3).

The livers of mice from all groups were enlarged and pale tan in colour. Histological

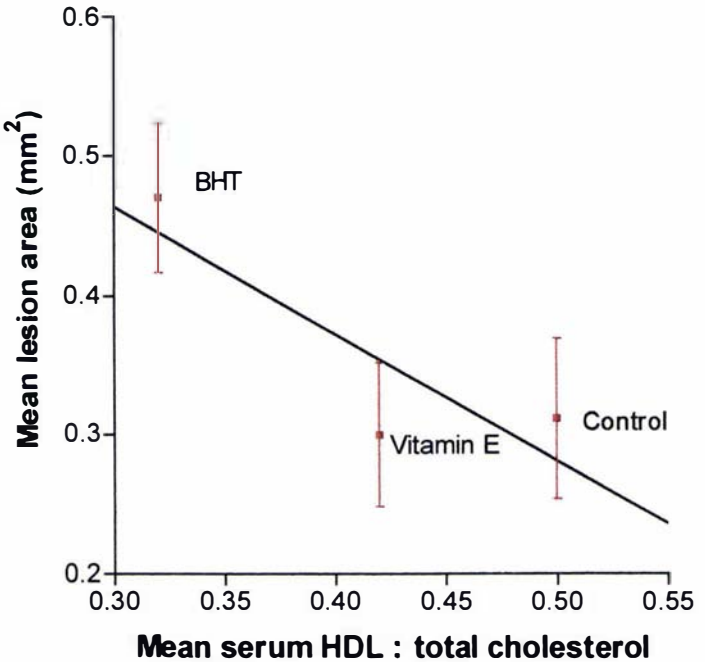

Figure 6.2. ***Relationship between mean fatty streak area and mean ratio of serum HDL to total cholesterol in mice receiving dietary antioxidants.*** 'BHT' indicates mice were fed diets containing 1% butylated hydroxytoluene, mice in the 'Vitamin E' group were fed diets containing 2% vitamin E acetate. Mice in the control group received diets containing no additional antioxidants. Error bars indicate one standard error. r^2=0.75, p=0.33, n=3. HDL indicates high density lipoprotein.

examination revealed diffuse hepatic lipidosis which did not differ in severity among groups on the basis of subjective assessment.

Discussion

The addition of either vitamin E or BHT to an atherogenic ration fed to C57BL/6 mice did not reduce the severity of fatty streak formation in the aortic sinus. However, both antioxidants significantly changed the serum lipoprotein profile.

The failure to observe diminished aortic fatty streak development in mice receiving diets containing vitamin E is in agreement with previous studies using cholesterol-fed[290,293] and WHHL rabbits[291,292,294]. In contrast, two studies where a low-cholesterol diet was fed to rabbits for ten months did report an antiatherogenic affect of vitamin E[289,295]. In these last two studies, the vitamin E supplemented diet was associated with lower serum cholesterol

concentrations, and the observed beneficial effect of vitamin E may have reflected exposure to this, rather than an antioxidant effect[289,295]. In a further study, the addition of vitamin E to an atherogenic diet fed to non-human primates resulted in significant reductions in arterial stenosis at some sample sites, although no significant differences were observed in the aorta[287].

In the present study, mice receiving the diet supplemented with vitamin E had a significantly lower mean total serum cholesterol concentration than controls. A serum cholesterol-lowering effect of vitamin E has been previously reported in rats[420] and rabbits[289,294-296,421]. Significant changes in serum cholesterol concentrations have not been reported in human subjects receiving vitamin E supplements[422,423], although the doses of vitamin E used in these studies (600-800mg/day per person) were far lower than those given to experimental animals. Vitamin E probably lowers serum cholesterol concentration by increasing the activity of cholesterol 7α-hydroxylase[421,424], the enzyme responsible for controlling the conversion rate of hepatic cholesterol to bile acids[15].

Mean serum HDL cholesterol concentrations in mice supplemented with either vitamin E or BHT were significantly lower than in controls. This is consistent with a previous experiment in which vitamin E was shown to reduce serum HDL cholesterol concentrations in rabbits[293]. In another rabbit study, a non-significant trend towards lowered serum HDL cholesterol concentration was observed in animals receiving BHT[297]. In the present study, the ratio of HDL to total cholesterol was negatively correlated with serum total antioxidant status, suggesting that the proportion of cholesterol carried in the HDL fraction may be reduced by dietary antioxidants.

The addition of 1% BHT to the atherogenic diets of C57BL/6 mice resulted in significantly greater aortic fatty streak development than in controls. This is in contrast to previous trials using rabbits which reported either a reduction in atherosclerosis[297], or no change[295] in animals given BHT. Furthermore, total serum cholesterol concentrations in the C57BL/6 mice fed BHT were not significantly different from controls. Again, this is in contrast to previous results from rabbit[297], rat[416], and mouse[425] studies where BHT increased serum cholesterol concentrations, possibly due to inhibition of ACAT[425]. Due to the presence of

cholic acid in the atherogenic diets, ACAT has little influence in determining serum cholesterol concentrations in the C57BL/6 mouse model[169]. This may explain why BHT did not increase serum cholesterol concentrations in the present experiment.

The inhibition of ACAT activity by BHT may also reduce foam cell production. Foam cells develop after cholesterol contained in modified LDL is phagocytosed by macrophages[142]. The excretion of cholesterol from macrophages is most rapid when ACAT activity is low[426], suggesting that BHT may reduce foam cell formation by inhibiting ACAT rather than through an antioxidant action. Macrophages from C57BL/6 mice, when compared to macrophages from atherosclerosis resistant mice, posses high ACAT activities when challenged with a high cholesterol diet[426]. BHT may not depress ACAT activity sufficiently in this model to cause the reduction in foam cell formation that was observed in rabbits.

The mean serum triglyceride concentration was significantly lower in mice consuming a diet containing BHT than in controls. This does not support findings of previous rabbit trials where dietary BHT increased serum triglyceride concentrations[295,297]. However, in our trial the mice fed diets supplemented with BHT for 15 weeks weighed less than those fed the control diet. As body weight is known to effect serum triglyceride concentrations[212], this may explain why lower concentrations were observed in this study and not in the previous rabbit experiments where no weight differences were observed[295,297].

As reported in Chapter 5, in a previous trial where the C57BL/6 mouse atherosclerosis model was used to examine the effect of dietary fats on fatty streak formation, the aortic fatty streak area was correlated with the ratio of serum HDL to total cholesterol (r^2=0.1, p=0.01, n=73). This relationship was not observed in the present trial, (r^2=0.07, p=0.07, n=52), despite the fact that all the mice were fed essentially the same diet. As illustrated in Figure 5.2, despite having a significantly lower HDL to total cholesterol ratio, which would be expected to result in greater fatty streak formation, mice fed vitamin E had a similar mean lesion area to controls. However, although vitamin E appeared to influence fatty streak formation independently of the serum lipoprotein profile, considering the effect of vitamin E on cholesterol 7α-hydroxylase, a key enzyme in lipid metabolism, it is impossible to attribute this influence to an antioxidant action.

To my knowledge, no published study examining the effect of dietary vitamin E supplementation on atherosclerosis has demonstrated a decrease in atherosclerotic lesion formation without a concurrent decrease in serum cholesterol[287,289-295]. This is despite LDL particles from animals fed vitamin E becoming more resistant to *in vitro* oxidation[285,291-294].

Due to the apparent effects on lipid metabolism of the antioxidants investigated in this trial, our results cannot be used to support the hypothesis that antioxidants confer protection from atherosclerosis. Since most antioxidants appear to have an effect on blood lipoprotein profiles[295,297,418] and/or body weight[419] it is difficult to evaluate the benefits of antioxidants from animal feeding trials. *In vitro* investigations into the oxidisability of LDL have been performed[282-284,286,292,293,333,334] but convincing data on a causal relationship between LDL oxidisability and atherosclerosis development is lacking[292,293]. Results of epidemiological studies examining the relationship between antioxidants and atherosclerosis are also difficult to interpret due to inconsistency of results and the presence of confounding factors[273,274,277,300,321,322,328,427]. Therefore, until more conclusive proof of a protective action of antioxidants is produced, discretion is needed before dietary antioxidant supplementation can be recommended to reduce the risk of atheroma-associated coronary heart disease.

Chapter 7

The effect of dietary alcohol on fatty streak development in the C57BL/6 mouse model

This trial used the C57BL/6 mouse model to investigate the effect of alcohol on atherosclerosis. Because of the inconclusive results of the previous trial investigating antioxidants (Chapter 6), this trial was also used to investigate whether or not there were any differences in fatty streak development observed in response to supplementation with red wine, which contains phenolic antioxidants, compared to white wine which contains a much lower antioxidant concentration.

Introduction

There is considerable epidemiological evidence to suggest that the consumption of alcohol at a moderate level (two to four standard drinks [22 - 44g alcohol] per day) is associated with a decreased risk of atherosclerotic disease[342-344]. Heavy drinking (greater than four drinks per day), however, results in an increased risk[345]. This produces a 'J' shaped relationship between alcohol and atherosclerosis, with both abstainers and heavy drinkers suffering higher rates of atherosclerotic disease[344].

Increases in serum HDL cholesterol concentration are believed to be responsible for most of the antiatherogenic effect of moderate alcohol consumption[346]. However, if alcohol intake becomes excessive, liver damage reduces the production of apoA-I resulting in a decrease in serum HDL cholesterol concentration[347]. The effect of moderate alcohol intake on serum LDL cholesterol concentration is less predictable[344,348,349]. Some studies have shown reductions in LDL[350], although this effect appears to be consistent only when a significant proportion of calories from dietary fat and cholesterol are replaced with calories from alcohol[344,346].

Although strong evidence for an antiatherogenic effect of alcohol has been derived from epidemiological studies[343-345,352], evidence from animal trials has been less conclusive. In experiments using C57BL/6 mouse[174], LDL receptor-knockout mouse[354], JCR:LA corpulent rat[356], and non-human primate[355] models, alcohol feeding has been reported to decrease

atherogenesis. However, increased atherogenesis was observed when alcohol was administered to cholesterol-fed rabbits[357] and rats[358].

Whether or not red wine provides greater protection from atherosclerosis than other alcoholic beverages is still controversial. Red wine contains antioxidant polyphenolic compounds[326,333,334] and the consumption of red wine has been shown to decrease the susceptibility of isolated LDL to oxidation *in vitro*[334]. LDL oxidation is considered to be an important process in the development of atherosclerosis[124], and delaying it has been suggested to reduce atherogenesis[413]. In a study using apoE-deficient mice, fatty streak formation was reduced in mice receiving red wine and red wine polyphenolics when compared to alcohol-matched controls[335]. In contrast, no differences in fatty streak formation were observed among rabbits fed a variety of alcoholic beverages including red and white wine[336]. In an epidemiological study of 13 000 people, no differences in the risk of atherosclerotic disease were observed between red and white wine drinkers[361].

In this trial, C57BL/6 mice were used to test whether or not a level of alcohol intake comparable to moderate intake in humans would affect atherogenesis. The alcohol was provided as either red or white wine so that the effects of these two beverages could be compared.

Materials and methods

Experimental animals

Sixty, six-week-old female C57BL/6 mice were obtained and housed in conditions described in Chapter 2. The mice were acclimatised for 20 weeks during which time they were fed standard mouse food. At the start of the trial their mean weight was 26.5g.

Experimental design

Twenty mice were randomly allocated into each of the three treatment groups. All three groups were fed identical diets *ad libitum* for 15 weeks. Two groups received water containing either 25% red wine or 26% white wine while the control group received plain water. At the end of the trial period, the mice were fasted for between 8 and 12 hr, then

killed by carbon dioxide inhalation

Experimental diets

The atherogenic diet fed to mice in this study had the same composition as the 'control' diet illustrated in Table 6.1. The methods used to prepare the experimental diets have been described in Chapter 2.

The drinking water/wine mixtures were formulated twice weekly. The red wine was a 1994 Cabernet Sauvignon Merlot from Longridge of Hawkes Bay, New Zealand with an alcohol content of 12.5% by volume. The white wine was a 1995 Sauvignon Blanc from Vidals of Hawkes Bay, New Zealand with an alcohol content of 12% by volume. The alcohol content of the wine was analysed by the wine makers. The total alcohol content for both wine/water mixtures was 3.1% by volume. The wine was kept refrigerated to minimise any loss of antioxidant content over the 15 week trial period. Water bottles were weighed twice-weekly to provide information on fluid intake during the trial. It is conceded, however, that this method does not account for differences among the intakes of individual mice or for fluid spilled on the floor of the cage.

Analysis of phenolic content of wine

Total oxidisable phenols was determined using Folin and Ciocalteu's phenol reagent, measuring absorbance at 765nm (Appendix). A standard curve was prepared using gallic acid (3,4,5-trihydroxybenzoic acid)[428]. The phenolic content of the white wine was 260 gallic acid meq/l while the red wine contained 1 960meq/l.

Sample collection and processing

The methods used to measure serum lipoprotein and triglyceride concentrations have been described in Chapter 2. The measurement of serum total antioxidant status was described in Chapter 6. A commercial diagnostic laboratory used gas chromatography to measure the alcohol concentration in serum pooled from each of the three treatment groups.

The area of fatty streaks in the aortic sinus was quantified using methods described in Chapter 2.

The liver from one mouse in each cage was fixed in neutral buffered formalin, embedded in paraffin, sectioned and then stained with haematoxylin and eosin for routine histological examination.

Statistical analysis

Differences among dietary groups were examined using analysis of variance techniques. Since no data were obtained from some experimental animals, the differences were analysed using an unbalanced design. Aortic fatty streak area, total serum cholesterol, total antioxidant status, triglyceride, and serum lipoprotein cholesterol concentrations were modelled using linear regression.

Results

During the trial, three mice were removed from both the control and white wine groups, while four were removed from the red wine group. In all instances, removal was due to weight loss, presumably as a result of reluctance to consume the experimental diet.

When the fluid intake per cage was averaged among the number of mice in each cage, no differences existed among the three groups. The mean fluid intake per mouse was 4.5 ml per day.

A summary of the measurements made at the completion of the trial is presented in Table 7.1. Two sets of comparisons are illustrated in this table. One shows differences among mice in the red wine, white wine and control groups. The other is a summary of differences between control mice and alcohol-fed mice, i.e. data combined from both the red wine and white wine groups.

No significant differences among groups were observed in mean body weight, serum total cholesterol concentration, or serum triglyceride concentration. When data from the two groups of mice fed alcohol were combined, the mean serum HDL cholesterol concentration in mice fed alcohol was significantly lower than that in control mice. When the two alcohol-

	Dietary Group			
	Control n=17	Red wine n=16	White wine n=17	Alcohol n=33
Total aortic fatty streak area (mm^2)	0.31 (0.11) a	0.43 (0.17) ab	0.48 (0.26) b	0.46 (0.22) *
Serum total cholesterol (mmol/l)	5.53 (1.54) a	5.86 (0.91) a	6.25 (1.32) a	6.06 (1.14)
Serum HDL cholesterol (mmol/l)	2.70 (0.59) b	2.25 (0.54) a	2.42 (0.32) ab	2.34 (0.44) *
HDL cholesterol: total cholesterol	0.50 (0.08) b	0.39 (0.08) a	0.42 (0.08) a	0.40 (0.09) *
Serum triglyceride (mmol/l)	0.82 (0.69) a	0.64 (0.32) a	0.56 (0.33) a	0.60 (0.33)
Serum total antioxidant status (mmol/l)	1.22 (0.26) a	1.27 (0.25) a	1.33 (0.23) a	1.30 (0.24)
Final weight (grams)	26.35 (2.52) a	27.0 (2.30) a	27.0 (1.92) a	27.0 (2.07)
Pooled serum alcohol (mmol/l)	None	2.9	2.6	2.75

Table 7.1. ***Results of trial investigating alcohol.*** All measurements were made after 15 weeks of feeding C57BL/6 mice an atherogenic diet. Mice in the red wine group were given drinking water containing 25% red wine, mice in the white wine group were given water containing 26% white wine. The control group was given drinking water without any alcoholic beverage. The combined alcohol-fed group comprises mice from both the red and white wine groups. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($p<0.05$). * indicates that the combined alcohol-fed group was significantly ($p<0.05$) different from the control group. HDL indicates high density lipoprotein.

fed groups were considered separately, mean serum HDL concentration was significantly lower in mice receiving red wine, but not white wine, than in controls. The mean ratio of serum HDL cholesterol to total cholesterol observed in mice fed either red or white wine was lower than controls. No significant differences in mean serum total antioxidant status were observed among the groups. Pooled serum samples from the red wine group contained alcohol concentrations of 2.9mmol/l, serum from the white wine group contained 2.6mmol/l, while no alcohol was detected in serum from the control group.

After being fed an atherogenic diet for 15 weeks, all mice developed lipid-containing lesions in the intima of the aortic

sinus similar to those described in Chapter 3. The mean area of these fatty streaks was greater in mice receiving alcohol than in control mice. When mice from the two alcohol-fed groups were considered separately, the lesion area in mice receiving white wine was significantly greater than that in controls. Fatty streak area in mice receiving red wine was not significantly different from that in any other group. No significant correlations between fatty streak area, serum lipids or body weight were observed. The relationship between fatty streak development and the ratio of HDL to total cholesterol is illustrated in Figure 7.1.

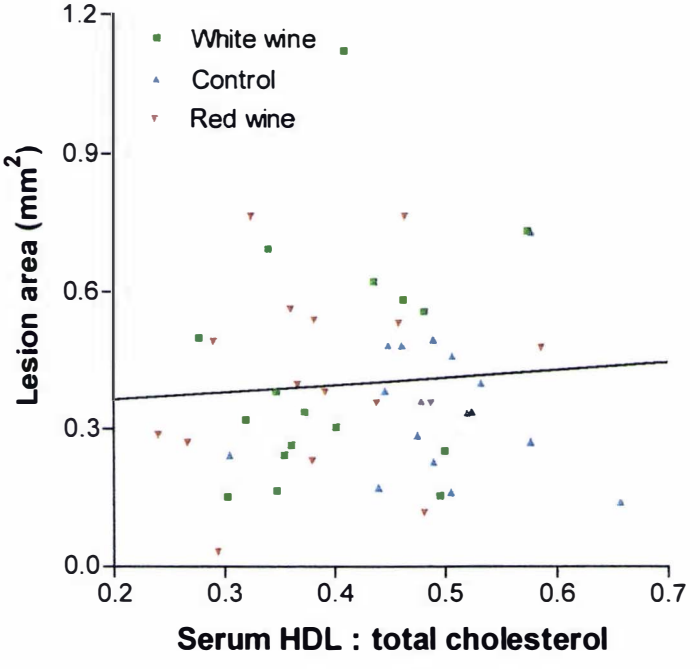

Figure 7.1. ***Relationship between fatty streak area and the ratio of serum HDL to total cholesterol in mice receiving dietary alcohol.*** Each symbol represents an individual mouse. Mice in the 'Red wine' group received drinking water containing 25% red wine. Drinking water in the 'White wine' groups contained 26% white wine. Control mice drank water with no additives. r^2=0.01, p=0.6, n=49. HDL is an abbreviation for high density lipoprotein.

All the livers examined were enlarged and pale tan in colour. Histological examination revealed diffuse hepatic lipidosis which did not differ in severity among groups on the basis of subjective assessment.

Discussion

The addition of 3.1% alcohol to the drinking water of mice significantly increased the development of fatty streaks. This supports the observation that feeding alcohol to cholesterol-fed rabbits promotes fatty streak formation[357]. However, it is in contrast to two mouse studies, one using C57BL/6 mice, the other using LDL receptor-knockout mice, both of which demonstrated an antiatherosclerotic effect of feeding 36% calories as alcohol[174,354]. Similar levels of alcohol intake also provide a protective effect to non-human primates[355] and atherosclerosis-prone JCR:LA-corpulent strain rats[356]. The findings of the present experiment are also in contrast to the evidence from human epidemiological studies which suggest that a moderate intake of alcohol protects against atherosclerotic disease[345,352].

The mice in the present study received a lower level of alcohol than that used in other reported animal trials. This level was designed to approximate the alcohol intake of a moderate drinker. It has been estimated that, due to differences in alcohol metabolism, mice require 15 times more dietary alcohol to enable comparisons on a weight to weight basis with humans[429]. Therefore, a 27g mouse consuming 4.5ml/day of a solution containing 3.1% alcohol should be comparable to a human consuming 2.5 standard drinks per day. Evidence that levels comparable to those seen in a moderate drinker were achieved was provided by the detection of alcohol in the pooled serum samples. This serum was taken from mice between three and six hours after the commencement of the light cycle. As mice rarely drink in the light[174,429] and have a fast clearance rate of alcohol[429], it appears likely that the desired serum alcohol levels would have been present during parts of the dark cycle.

As has been shown previously[174], moderate alcohol consumption significantly lowered the concentration of serum HDL cholesterol in C57BL/6 mice. The mechanism of this effect is unclear. In humans, the intake of moderate amounts of alcohol increases serum HDL cholesterol concentration due to inhibition of serum CETP[346]. As mice do not express CETP[171], the effects of alcohol on atherosclerosis observed in this model are not produced by alterations in the activity of this enzyme.

The ratio of serum HDL to total cholesterol was significantly decreased by dietary alcohol.

In human epidemiological studies and in previous experiments using the C57BL/6 mouse model, atherogenesis has been found to be inversely correlated with this ratio[12,13,172,173]. As illustrated in Figure 7.1, this relationship was not observed in the present study. While it appears reasonable to suggest that alcohol increased fatty streak formation due to, in part, reductions in the ratio of serum HDL to total cholesterol, the lack of significant correlation between this ratio and lesion size suggests that dietary alcohol may have also altered fatty streak formation independently of changes in serum lipoprotein concentration. Alcohol has previously been shown to influence enzymes important in lipid metabolism[351] and alter immune function[174], however, precisely how alcohol affects fatty streak formation in C57BL/6 mice is currently unknown.

The aim of this experiment was to model differences between human moderate drinkers and abstainers. To my knowledge, no published studies have revealed significant differences in the dietary habits of such groups of humans. Therefore, the control diet was not altered to compensate for the calories from alcohol received by mice in the alcohol-fed groups. These additional calories could have lowered the amount of atherogenic diet consumed by mice receiving alcohol, reducing fatty streak formation. The observation that alcohol increased fatty streak formation suggests that factors other than food intake were important in determining atherogenesis.

No significant differences in fatty streak formation were observed between mice receiving red wine and mice receiving white wine. This supports the findings of Klurfeld and Kritchevsky who also did not observe any significant differences in fatty streak lesion development in rabbits receiving either red or white wine[336]. However, the results of the present study are in contrast to those of a study by Hayek *et al* which demonstrated that both red wine and polyphenolic compounds isolated from red wine reduced atherogenesis compared to alcoholised water[335]. In the same study, components of red wine were found to decrease the susceptibility of LDL to *in vitro* oxidation, decrease LDL aggregation, and reduce foam cell formation[335]. Total serum antioxidant capacity was not significantly altered by red wine in the present experiment. This test has previously detected a significantly higher serum antioxidant capacity as a result of feeding diet containing 2% dietary vitamin E to C57BL/6 mice (Chapter 6[410]). It may not be sensitive enough, however, to measure

the more subtle changes which are expected to result from a moderate intake of red wine.

In conclusion, the results of this trial demonstrate that administering moderate amounts of alcohol to C57BL/6 mice decreases the ratio of serum HDL to total cholesterol and increases fatty streak formation. As well as producing a serum lipoprotein profile considered to be more atherogenic, dietary alcohol may also influence atherogenesis independently of serum lipoprotein concentration. The mechanisms by which alcohol alters the serum lipoprotein profile and directly affects atherogenesis in C57BL/6 mice require further investigation.

Chapter 8

The effect of dietary conjugated linoleic acids on fatty streak development in the C57BL/6 mouse model

Conjugated linoleic acids (CLAs) are a group of dietary fatty acids which have been suggested to reduce atherosclerosis[258]. Fat from grass-fed ruminants contains a higher concentration of CLAs than fat from grain-fed ruminants[259] and so identification of beneficial properties of CLAs could be used to promote New Zealand meat and dairy products.

Introduction

Conjugated linoleic acids (CLAs) are positional isomers of linoleic acid which have been shown to have anticarcinogenic properties[261] and have been suggested to reduce atherogenesis[258]. CLAs are produced by bacteria and are found at highest concentrations in food products derived from ruminants[258]. Meat from ruminants contains approximately 5.5mg CLAs per gram of fat while milk fat contains around 7mg CLAs per gram[259]. Fats from vegetables and non-ruminant animals contain approximately 0.7mg CLAs per gram[259].

Two animal experiments investigating the effect of dietary CLAs on atherosclerosis have been reported. In a study using hamsters, atherogenic diets containing CLAs produced significantly smaller aortic fatty streaks (thought to be an early lesion of atherosclerosis[2]) than diets containing no free fatty acids[193]. However, no significant differences were observed when fatty streak formation in CLA-fed hamsters was compared to that in hamsters fed a diet containing linoleic acid[193]. Supplementation of cholesterol-fed rabbits with 0.5g CLAs per day did not significantly reduce fatty streak lesion development[260]. Although it was initially thought that CLAs may be able to protect LDL particles from oxidation[193,260,264,266], a recent *in vitro* investigation[267] showed no antioxidant activity attributable to these fatty acids.

When fed an atherogenic diet, C57BL/6 mice develop fatty streaks in the intima of the aortic sinus within 15 weeks. This enables trials involving relatively large numbers of animals

without excessive cost, and, as these mice are inbred, they possess little genetic variation. Limitations of mice as a model of atherosclerosis include the absence of lipoprotein (a) and cholesteryl ester transfer protein, both of which are known to influence human atherogenesis[171].

The purpose of this study was to investigate the effect of dietary CLAs on atherogenesis using C57BL/6 mice.

Materials and methods

Experimental animals

Sixty, six-week-old female C57BL/6 mice were housed in conditions previously described in Chapter 2. The mice were acclimatised for two weeks, during which time they were fed standard mouse food. At the start of the trial their mean weight was 20.6g.

Experimental design

Twenty mice were randomly allocated to each of the three dietary groups. Two treatment groups received atherogenic diets containing either 0.5% CLAs or 0.25% CLAs. To ensure that all diets contained equal amounts of free fatty acids, the 0.25% CLAs group also contained 0.25% linoleic acid. Mice in the control group were fed a diet containing 0.5% linoleic acid. The mice were fed *ad libitum* for 15 weeks. They were then fasted for between 8 and 12 hr, and killed by carbon dioxide inhalation.

Experimental diets

The atherogenic diets used were similar to those reported by Nishina *et al*[378] and are illustrated in Table 8.1. All diets contained 14.5% triglyceride, 0.5% free fatty acids, 1% cholesterol and 0.5% cholic acid. Solutions containing either >95% CLAs or >99% linoleic acid were purchased from Nu-Chek-Prep Inc, MN, USA. The preparation and feeding of the diets has been described in Chapter 2.

Sample collection and processing

The methods used to measure serum lipoprotein and triglyceride concentrations have been

Ingredient	Dietary Group		
	Control	0.25% CLAs	0.5% CLAs
	grams/kilogram, as fed		
Sucrose	480.64	480.64	480.64
Casein	200	200	200
Corn oil	45	45	45
Olive oil	50	50	50
Anhydrous milk fat	50	50	50
Cellulose	50	50	50
Salt mix	50	50	50
Vitamin mix	50	50	50
dl-methionine	3	3	3
dl-α-tocopherol	1.36	1.36	1.36
CLAs	0	2.5	5
Linoleic acid	5	2.5	0
Cholesterol	10	10	10
Cholic acid	5	5	5

Table 8.1. Composition of diets used to investigate conjugated linoleic acids. CLAs is an abbreviation for conjugated linoleic acids.

described in Chapter 2.

The size of fatty streaks in the aortic sinus was quantified using methods described in Chapter 2.

The liver from one mouse in each cage was fixed in neutral buffered formalin, embedded in paraffin, sectioned, and then stained with haematoxylin and eosin for routine histological examination.

Statistical analysis

Differences among dietary groups were investigated using analysis of variance techniques. Relationships between serum lipids, body weight, and fatty streak formation were analysed using linear regression techniques.

Results

A summary of the measurements made at the completion of the trial is presented in Table 8.2. During the trial, four mice were removed from each dietary group. In all instances, removal was due to weight loss presumably resulting from the reluctance of mice to eat the experimental diet. Although the accurate measurement of food intake is not practicable using this model, no differences in food disappearance rates were found and no significant differences in body weight were observed among the treatment groups.

No significant differences in mean serum total cholesterol concentration or mean serum HDL cholesterol concentration were observed among the dietary groups. Compared to controls, feeding 0.5% CLAs significantly (p=0.008) increased the ratio of serum HDL cholesterol to total cholesterol. Although approaching significance (p=0.09), the ratio observed in mice fed 0.25% CLAs was not significantly different from that of controls. When data from both groups of CLA-fed mice were combined, dietary CLAs significantly increased the ratio of serum HDL to total cholesterol (p=0.01). Serum triglyceride concentrations were significantly higher in controls than in mice receiving 0.5% CLAs (p=0.01), but not 0.25% CLAs (p=0.16) When data from the two CLA-fed groups were combined, the inclusion of CLAs into the atherogenic diet significantly lowered serum triglyceride concentration (p=0.03).

	Dietary Group		
	Control n=16	0.25% CLAs n=16	0.5% CLAs n=16
Total aortic fatty streak area (mm^2)	0.13 (0.13) a	0.33 (0.27) b	0.25 (0.22) ab
Serum total cholesterol (mmol/l)	4.17 (0.74) a	3.57 (0.72) a	3.90 (1.16) a
Serum HDL cholesterol (mmol/l)	1.39 (0.21) a	1.40 (0.34) a	1.61 (0.31) a
HDL cholesterol: total cholesterol	0.34 (0.08) a	0.40 (0.09) ab	0.43 (0.10) b
Serum triglyceride (mmol/l)	0.56 (0.13) a	0.51 (0.08) ab	0.47 (0.10) b
Final weight (grams)	22.01 (1.18) a	21.08 (1.22) a	21.34 (1.51) a

Table 8.2. ***Results of trial investigating conjugated linoleic acids.*** All measurements were taken after C57BL/6 mice had been fed an atherogenic diet for 15 weeks. Mice in the 0.5% CLAs group received diets containing 0.5% conjugated linoleic acids. Mice in the 0.25% CLAs group received diets containing 0.25% conjugated linoleic acids and 0.25% linoleic acid. Mice in the control group received diets containing 0.5% linoleic acid. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different (p<0.05). HDL indicates high density lipoprotein. CLAs is an abbreviation for conjugated linoleic acids.

Fatty streak lesions similar to those described in Chapter 3 were present in the intima of the aortic sinus in all mice. Mice receiving diets containing 0.25% CLAs (p=0.01), but not 0.5% CLAs (p=0.12), developed a significantly greater area of fatty streaks than controls. When data from both groups of

mice receiving CLAs were combined, the addition of CLAs to the diet significantly increased aortic fatty streak development (p=0.02). In this study, fatty streak area was not correlated with the serum concentration of total cholesterol (p=0.5), HDL cholesterol (p=0.4), or triglyceride (p=0.3), or with either the ratio of HDL to total cholesterol (p=0.9), or body weight (p=0.8). The relationship between the ratio of HDL to total cholesterol and lesion development is illustrated in Figure 8.1.

All livers examined in this study were enlarged and pale tan in colour. Histological examination revealed diffuse hepatic lipidosis which did not differ in severity among groups on the basis of subjective assessment.

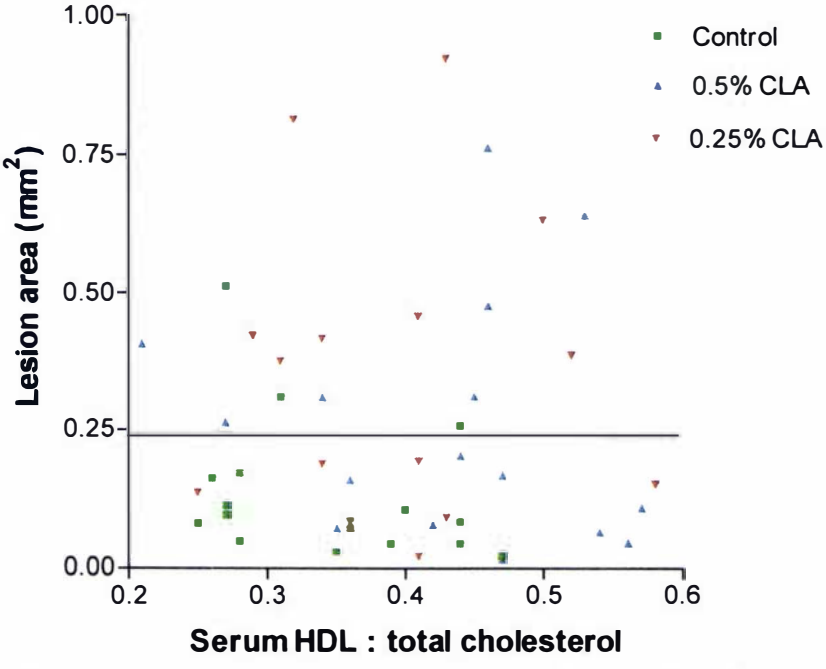

Figure 8.1. ***Relationship between fatty streak area and the ratio of serum HDL to total cholesterol in mice receiving dietary conjugated linoleic acid.*** Mice in the '0.5% CLA' group received atherogenic diets containing 0.5% CLAs. Diets fed to mice in the '0.25% CLA' group received diets containing 0.25% CLAs plus 0.25% linoleic acid. Mice in the 'Control' group received diets containing 0.5% linoleic acid. r^2=0.0001, p=0.9, n=48. HDL is an abbreviation for high density lipoprotein, CLA is an abbreviation for conjugated linoleic acid.

Discussion

In C57BL/6 mice, dietary CLAs significantly increased aortic fatty streak formation. This

is in contrast to a study by Lee *et al*[260] in which no significant differences in fatty streak development were observed between rabbits supplemented daily with either 0.5g CLAs or 0.5g coconut oil. The results of the present study are also in contrast to a study using hamsters in which an atherogenic diet containing CLAs resulted in significantly less atherogenesis than a control diet containing no free fatty acids. However, this result is difficult to interpret because control hamsters were also significantly heavier, raising the possibility that they may have consumed more of the atherogenic diet[193]. When CLA-fed hamsters were compared to hamsters fed a control diet containing free linoleic acid, no differences in body weight or fatty streak formation were observed[193].

In the present study, dietary CLAs increased the ratio of serum HDL cholesterol to total cholesterol. As was observed in the study by Lee *et al*[260], this increase appeared to be the result of non-significant changes in both serum total cholesterol and HDL cholesterol concentration. In hamsters, dietary CLAs significantly lowered serum total cholesterol concentrations, although, HDL cholesterol concentrations were unaltered by CLAs in this model[193]. Hepatic lipid metabolism has been shown to be affected by CLAs[430] which may explain how these conjugated fatty acids produce changes in the serum lipoprotein profile.

In human epidemiological studies and in previous experiments using the C57BL/6 mouse model, atherogenesis has been found to be inversely correlated with the ratio of serum HDL to total cholesterol[12,13,172,173]. In the present trial, as illustrated in Figure 8.1, lesion development was not correlated with this ratio or with any other parameter measured. This suggests that CLAs increase fatty streak formation by a mechanism independent of the serum lipoprotein profile, triglycerides or body weight.

It is possible that CLAs promote atherogenesis by stimulation of the immune system. Interleukin-2 production[431], lymphocyte proliferation[432], and macrophage phagocytic activity[433] have all been found to be increased in cells cultured from animals supplemented with CLAs. In C57BL/6 mice, foam cells are the predominant cell type present in fatty streaks[168]. Foam cells are produced when macrophages become engorged with lipid[120] suggesting that promotion of the immune system, especially macrophage phagocytosis, could be expected to increase fatty streak formation. CLAs may also promote atherogenesis

by their ability to, depending on the concentration, both decrease and increase lipoprotein lipase activity[434]. Lipoprotein lipase has been suggested to be an important factor in determining early atherogenesis[112] so that alteration of enzyme activity may influence fatty streak formation.

It is interesting to note that CLAs did not promote atherogenesis in either the cholesterol-fed rabbit or hamster atherogenesis models[193,260]. This suggests that the mechanism by which CLAs increase fatty streak development in C57BL/6 mice may be unique to this model. The most important question raised by this observation is whether or not CLAs will also promote atherogenesis in humans.

The addition of CLAs to the diet resulted in a significant decrease in serum triglyceride concentration. This is in contrast to previous animal studies where no reduction in triglyceride concentrations were observed in rabbits supplemented with CLAs[260] or when CLA-fed hamsters were compared to hamsters fed linoleic acid[193].

When the two groups of mice fed CLAs were considered separately, only the group receiving 0.5% CLAs developed a ratio of serum HDL to total cholesterol and a serum triglyceride concentration significantly different from that in controls. The addition of 0.25% CLAs to the atherogenic diet resulted in changes which approached, but did not attain, statistical significance, suggesting that dietary CLAs may have a dose-dependent effect on both these lipid parameters. However, fatty streak development was significantly greater than controls only in mice receiving diets containing 0.25% CLAs. It is possible, therefore, that dietary CLAs at a level of both 0.5% and 0.25% promoted atherogenesis, but lesion formation was ameliorated in mice receiving 0.5% by the development of a less atherogenic serum lipoprotein profile.

No significant differences in body weight were observed among animals in the three dietary groups. During preliminary studies, it was observed that diets containing either CLAs or linoleic acid appeared to be less palatable than diets containing triglyceride. Free fatty acids have a 'soapy' taste and differences in feed intake[434] and weight gain[193,430] have been observed when animals fed diets containing CLAs were compared to those fed diets containing

triglyceride. Therefore, when using animals to investigate the physiological effects of CLAs, it is essential that all experimental diets contain equal quantities of free fatty acid. If this is not done, it becomes difficult to differentiate between the effects of CLAs and the effects of differences in food intake.

In conclusion, the addition of CLAs to an atherogenic diet fed to C57BL/6 mice resulted in the development of a serum lipoprotein profile considered to be less atherogenic. If human supplementation with CLAs produces a less atherogenic lipoprotein profile without promoting atherogenesis then CLAs may be useful in preventing atherosclerotic disease. However, in the present study, dietary CLAs increased the development of aortic fatty streaks and the possibility that dietary CLAs also promote atherogenesis in humans requires further investigation. Considering the increased atherogenesis observed in this trial and the lack of significant atherosclerosis reduction seen in previous animal studies[193,260], CLAs cannot currently be regarded as antiatherogenic.

Chapter 9

In vitro investigations

Because of the limitations observed in the C57BL/6 mouse model, to further investigate factors which may influence the oxidation of LDL, two sets of *in vitro* experiments were performed. The first was a brief examination of the effect of dietary supplementation with garlic and garlic products on *in vitro* oxidation of LDL. Garlic was chosen because of recent reports which have described strong antioxidant properties associated with it[435-439]. The second study attempted to investigate the role of LDL oxidation in atherogenesis by examining genetic differences in the rate of macrophage-mediated LDL oxidation between strains of mice resistant and susceptible to atherosclerosis.

The effect of daily supplementation with aged garlic extract, raw garlic, and α-tocopherol on low density lipoprotein oxidation.

Introduction

The oxidation of LDL is believed to be a critical process in the development of atherosclerosis[117,139]. The presence of oxidised LDL in the intima of an artery leads to the production of macrophage-derived foam cells[120], the main cell type present in fatty streaks, believed to be the earliest lesion of atherosclerosis[2]. Once an atheroma has developed, oxidised lipids can produce surface fissures which result in larger and more occlusive atherosclerotic lesions[117,124,139]. Therefore, the use of antioxidants as dietary supplements to protect LDL particles against oxidation may reduce both the development and the progression of atherosclerosis[413].

Whereas the *in vitro* antioxidant properties of garlic (*Allium sativum)* are well recognised[435-439], studies investigating the effect of garlic supplementation on the oxidation resistance of LDL isolated from human subjects have been inconclusive. In one, daily supplementation with 600mg garlic powder for two weeks significantly protected isolated LDL from oxidation[440]. However, daily supplementation with 900mg garlic powder for 12 weeks in another study produced no significant changes[441]. It has also been reported that garlic supplementation produces changes in the serum lipoprotein profile which are believed to be

associated with lowering an individual's atherosclerosis risk[442] and in an experiment using cholesterol-fed rabbits, garlic was found to promote atheroma regression[443].

Aged garlic extract (AGE) is an aqueous ethanolic extract of garlic which has been shown to possess *in vitro* antioxidant properties[444-446]. AGE has also been found to reduce atherosclerotic lesion formation in a rabbit model[454]. Although less effective than raw garlic[442], significant reductions in serum LDL cholesterol concentration were observed after prolonged supplementation with AGE[447].

Alpha-tocopherol is transported around the body in LDL particles and is able to scavenge lipid peroxyl radicals, increasing the resistance of the particle to oxidation[133,272]. Both human and animal experiments have consistently shown that supplementation with α-tocopherol at doses as low as 200mg per day protects LDL from oxidation[284,293].

The aim of this study was to determine if supplementing the diet of human subjects with either raw garlic or AGE could alter the oxidation resistance of their LDL particles. So that these results could be compared with those of a known inhibitor of LDL oxidation, α-tocopherol was also included in the study.

Materials and methods

Study design

Nine human volunteers were supplemented daily with 6g of raw garlic, 2.4g of AGE, or 0.8g of dl-α-tocopherol acetate. Subjects in the study took each supplement for 7 days, interrupted by a 7-day washout period. To diminish the possibility that variation in assay technique could affect results, the study population was randomly divided into three groups of three subjects. During treatment weeks, all three groups received a different supplement, constituting a Latin square design. At the beginning and end of each 7-day supplementation period, a fasting blood sample was taken and the concentration of plasma lipoproteins and the oxidisability of LDL were measured. While it would have been desirable to incorporate a placebo group into this study, this was made impossible by the inclusion of raw garlic.

Raw garlic was dosed at a rate of 6g per day because in preliminary studies this was found to be the highest daily level that could be tolerated. As crushed garlic contains between 2.5 and 4.5mg allicin per gram[448], subjects in the trial received at least 15mg allicin per day. Garlic powder supplements were not used because some of the more unstable antioxidant substances contained in garlic have been shown to be lost during the manufacturing process[448]. Although some supplement manufacturers recommend α-tocopherol dose rates of 1g/day, 0.8g/day was used in this trial because this has been found to be safe and effective at reducing lipoprotein oxidation[449]. The dose of AGE given in this study is the highest daily dose recommended by the manufacturer and resulted in an intake of 1.2mg S-allylcysteine per day.

Materials

To provide the desired dose of garlic, whole garlic cloves weighing between 3 and 4g were purchased locally. At the beginning of the supplementation period, two garlic cloves with a combined weight of 7g were issued for each of the 7 days. Immediately prior to consumption, the outer sheaths and end pieces of the garlic cloves were removed, leaving approximately 6g. To ensure maximum allicin production, the garlic was crushed to release alliinase from cell vacuoles and left at room temperature for two minutes[448]. Because allicin is unstable[448], the entire dose of raw garlic was then eaten immediately. AGE was administered using two 'High Potency Kyolic' (Wagner ProBiotics, USA) capsules. Two 'Red Seal 400mg vitamin E' capsules (Red Seal Natural Health Ltd, New Zealand) provided the daily dose of α-tocopherol. To reduce the possibility of stomach irritation, all supplements were taken with the evening meal. The laboratory reagents used in this study were purchased from BDH Chemicals New Zealand Ltd, New Zealand.

Subjects

The subjects included in this study were all non-smoking volunteers. The study population consisted of 4 females and 5 males with a mean (±SD) age of 36.5 ± 12.1 years and a mean body mass index of 22.3 ± 3.4 kg/m^2. None had consumed significant quantities of garlic or taken any medication, including dietary supplements, during the month preceding the study. Although no dietary restrictions were enforced during the study period, subjects were requested not to alter their normal diets. Informed, written consent was obtained from

all subjects.

Lipoprotein analysis

A 12hr-fasting sample of venous blood was collected into a 10ml tube containing 17.5mg EDTA. After plasma had been separated by centrifugation, plasma cholesterol and triglyceride concentrations were measured using a Hitachi 704 autoanalyser (Boehringer Mannheim GmbH, Germany). Plasma HDL cholesterol concentrations were determined after selective precipitation of lipoproteins containing apoB using polyethylene glycol 6000[380].

LDL oxidation studies

LDL was isolated using sequential ultracentrifugation according to the method of Havel *et al*[450] (Appendix). Briefly, plasma density was adjusted to 1.019g/l and then centrifuged at 100 000 x g for 20hr. After the IDL and VLDL had been removed, the plasma was then adjusted to a density of 1.063g/l and centrifuged at 100 000 x g for a further 24hr so that the LDL, which floats on the surface, could be harvested. To prevent LDL oxidation during isolation, 10mM EDTA was present throughout the isolation steps and centrifugation was performed at 4°C. Once separated, the LDL was gassed with N_2 and stored at 4°C for a maximum of 48hr.

Resistance to oxidation of LDL was determined by continuously measuring the formation of conjugated dienes according to the method of Esterbauer *et al*[451]. EDTA was removed from the LDL solution using an Econopac PD-10 desalting column (Bio-Rad Laboratories, CA, USA) preconditioned with 20ml of phosphate buffered saline (PBS) that had been treated with Chelex 100 resin (Bio-Rad Laboratories, CA, USA). Protein concentration in each sample was determined using the method of Lowry *et al*[452] (Appendix) and the LDL samples were diluted to 75µg LDL protein/ml using PBS. After the addition of 5µM Cu^{2+}, the LDL solution was incubated at 30°C and absorbance at 234nm was measured every 90 seconds using an Ultrospec III spectrophotometer (Pharmacia LKB, Sweden). As illustrated in Figure 9.1, the lag time of LDL oxidation was estimated from the intercept of the tangents to the slow and fast increase of diene formation[451].

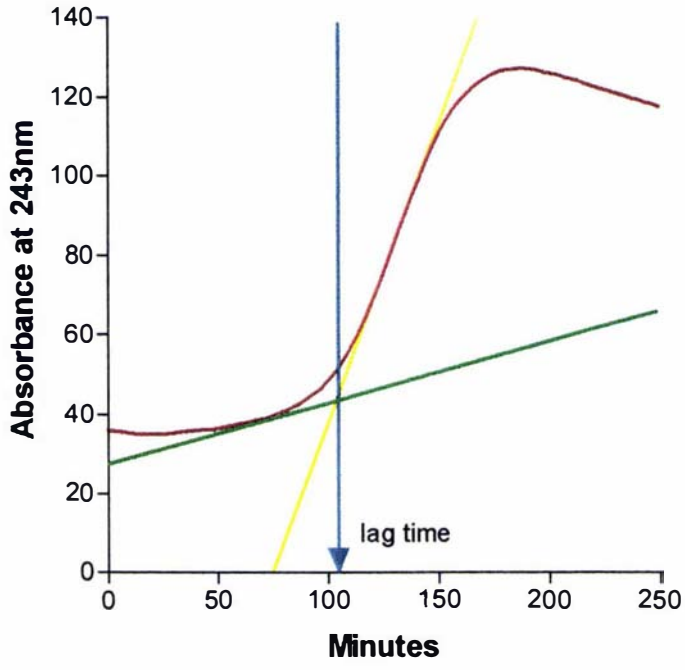

Figure 9.1. ***Sample graph showing kinetics of conjugated diene formation.*** The reaction was performed at 30°C with 75μg/l LDL protein and 5μM Cu^{2+}. Derivation of the lag time of LDL oxidation is shown. The green line indicates the slow increase in diene formation while the yellow line indicates the fast increase. LDL is an abbreviation for low density lipoprotein.

Statistical analysis

Comparisons between mean values of baseline and treatment groups were performed using analysis of variance techniques.

Results

All nine participants completed the six-week trial without significant changes in body weight, diet, or lifestyle. Daily supplementation with 6g raw garlic resulted in no serious side effects, although body odour was noticed by all participants. No adverse effects of either AGE or α-tocopherol supplementation were observed. Compliance during the trial, as assessed by questioning participants, was excellent.

The results of the study are summarised in Table 9.1, with the effect of supplementation on oxidation lag times illustrated in Figure 9.2. LDL isolated from subjects given α-tocopherol acetate was significantly ($p<0.005$) more resistant to Cu^{2+}-mediated oxidation than LDL

isolated from subjects at baseline or after receiving either AGE or raw garlic. Daily AGE supplementation produced LDL which was significantly ($p<0.01$) more resistant to oxidation than LDL isolated at baseline. The oxidation resistance of LDL from subjects receiving raw garlic was not significantly different to LDL from subjects at baseline or subjects receiving AGE. The increase in LDL oxidation resistance which resulted from α-tocopherol acetate supplementation appeared to be consistent. In contrast, supplementation with AGE or raw garlic resulted in considerable variation in oxidation resistance. No significant changes in serum lipoprotein or triglyceride concentration were observed during the trial period.

	Supplementation			
	BL	Vit E	AGE	RG
Serum total cholesterol (mmol/l)	5.11 (0.79) a	4.84 (0.94) a	4.90 (0.92) a	5.21 (0.81) a
Serum HDL cholesterol (mmol/l)	1.58 (0.51) a	1.55 (0.48) a	1.50 (0.39) a	1.66 (0.55) a
HDL cholesterol: total cholesterol	0.31 (0.10) a	0.33 (0.11) a	0.31 (0.08) a	0.33 (0.11) a
Serum triglyceride (mmol/l)	1.14 (0.40) a	1.19 (0.32) a	1.00 (0.32) a	1.06 (0.43) a
Lag time (minutes)	86.5 (10) a	167 (36) b	119 (49) c	109 (43) a c

Table 9.1. ***Results of human antioxidant supplementation study.*** Measurements were made after supplementation with one of, 0.8g α-tocopherol acetate (Vit E), 2.4g aged garlic extract (AGE), or 6g raw garlic (RG). Measurements made at the beginning of the study are indicated in the baseline column (BL). All figures are means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($p<0.01$). HDL indicates high density lipoprotein.

Discussion

LDL isolated from human subjects supplemented with 2.4g aged garlic extract per day was significantly more resistant to Cu^{2+}-mediated oxidation than LDL isolated from subjects prior to supplementation. Whereas AGE has previously been shown to inhibit *in vitro* LDL oxidation[445,446,453], these results suggest that the antioxidant compounds contained in AGE may also be able to prevent *in vivo* LDL oxidation. This supports the suggestion that an antioxidant effect may have contributed to the reduced atherogenesis observed in rabbits supplemented with AGE[454].

Daily supplementation with 6g raw garlic did not significantly increase

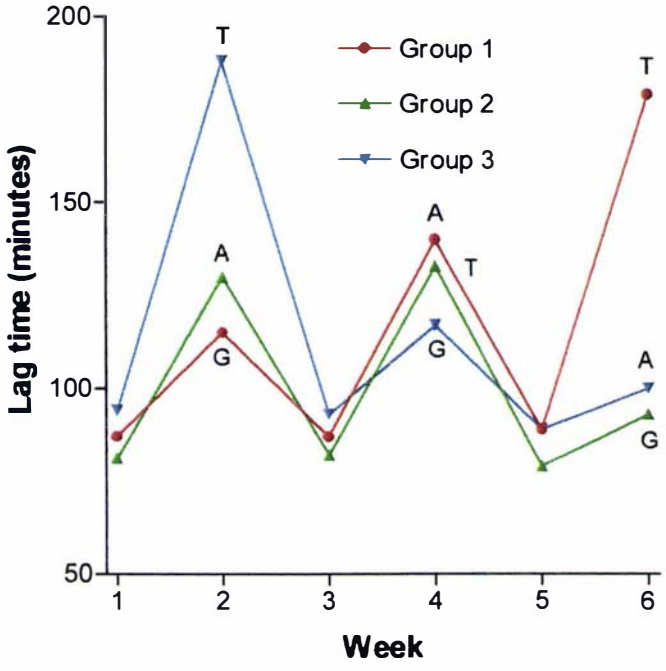

Figure 9.2. ***Effect of dietary supplements of LDL oxidation lag times.*** Subjects received one of, 6g raw garlic (G), 2.4g aged garlic extract (A), or 0.8g α-tocopherol acetate (T) for 7 days. The value shown is the mean lag time of LDL oxidation in each group of three subjects. No supplements were taken on weeks 1, 3, and 5. In week 2, subjects in group 1 received garlic, group 2 received AGE, and group 3 received α-tocopherol acetate. In week 4, group 1 received AGE, group 2 α-tocopherol acetate, and group 3 garlic. In week 6, group 1 received α-tocopherol acetate, group 2 garlic, and group 3 AGE. LDL is an abbreviation for low density lipoprotein.

the lag time of LDL oxidation. This supports the study by Simons *et al*[441], but is in contrast to the results of Phelps and Harris[440]. Both of these previous studies used garlic powder tablets to provide daily doses of 12 and 8mg allicin respectively[439,440]. In the present trial, at least 15mg allicin per day was consumed and, as raw garlic was used, there was no possibility that any antioxidant substances contained in garlic could have been lost, as may occur during the production of the dried garlic powder. Therefore, the results of this study, combined with the results of Simons *et al*, suggest that neither raw garlic nor standard garlic supplements are likely be effective in reducing *in vivo* LDL oxidation.

The *in vitro* antioxidant action of AGE is believed to be derived largely from its content of S-allylcysteine[445]. In the body, this is metabolised to N-acetyl-S-allylcysteine[455], which has been shown to be a less powerful antioxidant[445]. The *in vitro* antioxidant action of garlic is believed to be mainly attributable to allicin[437,439]. Allicin is rapidly metabolised in the body,

first to diallyl disulphide and then to allyl mercaptan, neither of which possess antioxidant activity[448,456]. Compared to α-tocopherol acetate, supplementation with either AGE or garlic provided a more variable degree of oxidation resistance to an individual's LDL particles. This variation may be due to inter-subject differences in the rate at which the active antioxidant compounds, S-allylcysteine and allicin, were metabolised into the less active N-acetyl-S-allylcysteine and allyl mercaptan. Subjects who responded well to AGE did not appear to be more or less likely to respond well to garlic, although this study lacked the statistical power to properly investigate this relationship.

Supplementation with 0.8g α-tocopherol acetate protected LDL particles from oxidation significantly better than supplementation with either AGE or raw garlic. Previous studies investigating α-tocopherol have demonstrated an increase in oxidation lag times of around 30%[282,283] and the degree of protection observed in the present study (an average increase in lag time of 90%) was greater than expected. It is unclear why α-tocopherol acetate protected LDL so effectively in this study.

As shown in Figure 9.2, the oxidation resistance of LDL particles isolated at baseline and at the completion of the 7-day washout periods remained similar throughout the study. This provides evidence that the washout period used in this study was sufficient to return the oxidation resistance of LDL particles to pre-supplementation levels. It also suggests that any changes in the diet of the study participants during the trial did not significantly alter the oxidation resistance of their LDL particles.

None of the dietary supplements examined in this trial significantly affected serum lipoprotein or triglyceride concentrations. This was expected because of the short supplementation periods used.

While it is accepted that dietary antioxidants can increase the resistance of LDL to oxidation, the relationship between LDL oxidisability and atherogenesis is less certain. Results of epidemiological studies examining the relationship between antioxidants and atherosclerosis are difficult to interpret due to inconsistency of results and the presence of confounding factors[457]. Animal experiments are also inconclusive, with many antioxidant

chemicals affecting blood lipids or weight gain[410]. Currently, there is insufficient evidence to determine whether or not antioxidants can reduce atherogenesis. However, if antioxidants are found to be antiatherogenic, the combined antioxidant and serum cholesterol-lowering[447] actions of AGE may make it useful in preventing atherogenesis.

Oxidation of LDL by peritoneal macrophage cell cultures

Introduction

A fatty streak, believed to be the earliest lesion of atherosclerosis, is an accumulation of macrophage-derived foam cells within the intima of an artery[2,142]. Foam cells develop when macrophages are exposed to oxidised LDL. This is because, unlike phagocytosis of native LDL, macrophage phagocytosis of oxidised LDL is rapid and unregulated[458,459]. The oxidation of LDL by cells in the artery wall, including macrophages[459], smooth muscle cells[460], and endothelial cells[460,461] is considered to be an important process of atherogenesis[117].

Inbred strains of mice exhibit marked variation in fatty streak development after receiving an atherogenic diet[383]. While this variation is, in part, explained by differences in the serum lipoprotein profile, it is likely that other mechanisms are also involved[426]. One such mechanism, which has been found to be variable among strains of mice, is macrophage function[426].

Since LDL oxidation by macrophages is important in atherogenesis[459] and differences in macrophage function occur between strains of mice[426], it is possible that one source of variation in atherogenesis between inbred strains may be related to differences in the oxidation rate of LDL by macrophages. To test this hypothesis, the oxidation rate of LDL incubated with macrophages from atherosclerosis-susceptible C57BL/6 mice could be compared with that of LDL incubated with macrophages from atherosclerosis-resistant C3H/HeJ mice.

To perform this experiment, an assay of LDL oxidation which was specific, sensitive, and suitable for large numbers of samples was required. Because the LDL oxidation was to be

mediated by cultured cells, the test also had to function in cell culture medium. The most common method for determination of cell-mediated LDL oxidation is measurement of the uptake of I^{125}-labelled LDL by macrophages[459]. This assay, however, is labour intensive, making it unsuited to large numbers of samples[459]. In comparison, colorimetric assays are cheaper, safer, and easier. Unfortunately, because of interference from the cell culture medium, neither the conjugated diene assay nor the thiobarbituric acid-reactive substances assay could be used[462,463]. The triiodide assay has been used to measure macrophage-mediated LDL oxidation in cell culture medium[331]. However, since this assay measures the concentration of all peroxides, not just lipid peroxides, it suffers from low specificity[462,464]. The ferrous oxidation-xylenol orange assay (FOX2 assay)[464] is more specific than the triiodide method[462], although this assay had not previously been validated for use in cell culture experiments.

The aim of the experiments reported in this section was to evaluate the ability of the FOX2 assay to detect lipid peroxides in cell culture medium. If this assay was found to be suitable, it could then be used to investigate possible differences in the rate of LDL oxidation when cultured with macrophages from atherosclerosis-susceptible and resistance strains of mice.

Materials and methods

Harvesting peritoneal macrophages

Resident peritoneal macrophages were obtained from Swiss white mice that were between 6 and 10 weeks old. After euthanasia, the mice were soaked in a solution containing 70% alcohol for 5 min. The skin of the ventral abdomen was removed and 10ml ice-cold PBS injected into the peritoneal cavity using a 23 gauge needle. The abdomen was then gently manipulated to suspend the cells and the PBS was aspirated. The macrophages were washed twice by centrifugation in PBS for 10 min at 500 x g. After the second wash, the cell pellet was resuspended in 1ml Ham's F-10 medium (ICN Biomedicals Inc, OH, USA) containing 5% foetal calf serum and 50μg/ml gentamycin sulphate.

A sample of cells was stained with a solution containing 0.5% trypan blue and counted using a Neubauer haemocytometer (American Optical Corp, NY, USA). Macrophages were

plated on a 48 well tissue culture plate (Becton Dickinson Labware, CA, USA) at a density of 1 x 10^6 macrophages per well. After a 6hr incubation period, the wells were washed twice with warm PBS to remove any non-adherent cells, and fresh medium was added. The cells were incubated at 37°C in air containing 5% CO_2 for a minimum of 2 days and a maximum of 1 week prior to use.

LDL isolation

LDL was isolated, desalted, and assayed for protein content as described earlier in this chapter.

Macrophage-mediated oxidation

Foetal calf serum inhibits LDL oxidation. All experiments were performed, therefore, in either serum-free Ham's F-10 or modified PBS (MPBS). MPBS is PBS which has Cu^{2+} (0.01μM) and Fe^{2+} (3μM) added to it. LDL was filter-sterilised using a Millex-GP 0.22μm filter unit (Millipore, MA, USA) before being added to the cell cultures at a concentration of 100μg LDL protein/ml. During oxidation studies, the cell cultures were incubated at 37°C in air containing 5% CO_2

To determine the concentration of lipid peroxides, the method of Jiang *et al* was employed[465]. Samples of the medium (20μl) were mixed with 180μl FOX2 reagent (Appendix) in a 96 well Falcon flexible assay plate (Becton Dickinson Labware, CA, USA). Lipid peroxides oxidise Fe^{2+} to Fe^{3+} which reacts with xylenol orange dye [o-cresolsulfonphthalein-3,3'-bis(methylaminodiacetic acid) sodium salt], to produce a blue/purple complex. To ensure maximum colour development, the reaction was incubated for 30 min at room temperature before the absorbance at 550nm was measured against hydrogen peroxide standards using a SLT 340 ELISA plate reader (SLT-Labinstruments GmbH, Austria).

Macrophage LDL uptake studies

LDL at a concentration of 100μg LDL protein/ml was incubated for 6hr with either peritoneal macrophages (1 x 10^6 cells/well) or cell-free controls. The cell medium containing LDL was then collected and centrifuged at 500 x g for 10 min to remove any

dead cells. A sample of the supernatant was assayed for protein concentration as previously described.

To observe the uptake of the pre-incubated LDL by macrophages, resident peritoneal macrophages were cultured in 8-well Lab-Tek Chamber Slides (Nalge Nunc, IL, USA). Cells were cultured in Ham's F-10 with 5% foetal calf serum and 50μg/ml gentamycin sulphate at a density of 1 x 10^6 macrophages per chamber. LDL which had been incubated with either macrophages or cell-free controls was added to each chamber to produce a final concentration of 50μg LDL protein/ml. The slides were incubated at 37°C for 20hr and then stained using Oil red O, Meyer's haematoxylin method, and light green counterstaining. The amount of lipid phagocytosis was estimated by measuring the average number of lipid vacuoles in 50 macrophages. Vacuole number in the two sets of macrophages was compared using a two-sample t-test.

Results and discussion

Oxidation of LDL by macrophages in Ham's F-10 medium

As can be seen from Figure 9.3, the FOX2 assay did not detect any significant lipid peroxide formation when LDL was incubated with macrophages. This is in contrast to a previous experiment in which the triiodide method detected significant quantities of LDL oxidation products after a 4hr incubation with macrophages[331]. The positive control for this experiment, LDL incubated with PBS containing 10μM Cu^{2+}, did produce detectable levels of peroxides, suggesting that either the macrophages were not oxidising LDL or that the cell culture medium was interfering with the ability of the FOX2 assay to detect this oxidation.

As illustrated by the photomicrographs in Figure 9.4, macrophages incubated with LDL that had been previously exposed to macrophages contained significantly more Oil red O-positive vacuoles (1.2 vs 0.1 vacuoles per macrophage, $p<0.01$) than macrophages incubated with cell-naive LDL. Because macrophages phagocytose oxidised LDL faster than native LDL, this suggests that the LDL was being oxidised by the macrophages but that the FOX2 assay was unable to detect this oxidation.

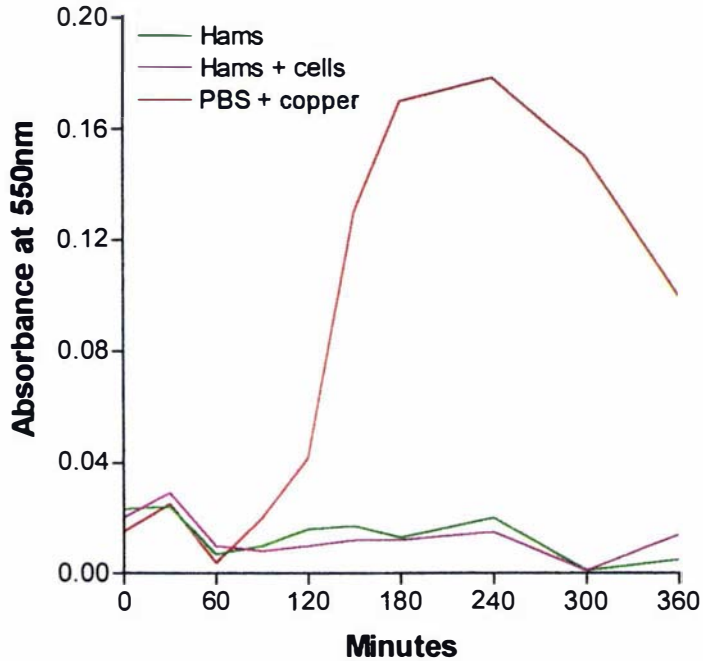

Figure 9.3. ***Kinetics of macrophage-mediated LDL oxidation.*** 'Hams + cells' indicates LDL was incubated with macrophages in serum-free Ham's F-10. 'Hams' indicates LDL was incubated in cell-free, serum-free Ham's F-10. 'PBS + copper' indicates LDL was incubated with phosphate buffered saline containing 10μM Cu^{2+}. LDL was added to all the plates at a protein concentration of 100μg/ml. All incubations were performed at 37°C in air with 5% CO_2. Samples (20μl) were mixed with 180μl FOX2 reagent and the absorbance measured. LDL is an abbreviation for low density lipoprotein.

To investigate whether or not the culture medium was interfering with the FOX2 assay, 10-250μM hydrogen peroxide solutions were formulated in Ham's F-10, distilled water, MPBS, or PBS. The peroxide concentrations were measured and, as illustrated in Fig 9.5, Ham's F-10 was found to interfere with the FOX2 assay, making it unable to accurately detect peroxides at concentrations below 100μM.

Oxidation of LDL by macrophages in MPBS

Ham's F-10 medium is used in cell culture oxidation studies because, unlike other culture mediums, it contains trace amounts of copper and iron which have been demonstrated to be necessary for LDL oxidation[458]. MPBS contains the same concentration of these metal ions as Ham's F-10, but, as demonstrated in Figure 9.5, peroxides are able to be detected accurately by the FOX2 assay when contained in this solution. Unfortunately, when macrophages were cultured in MPBS, they rapidly became rounded and lost adherence.

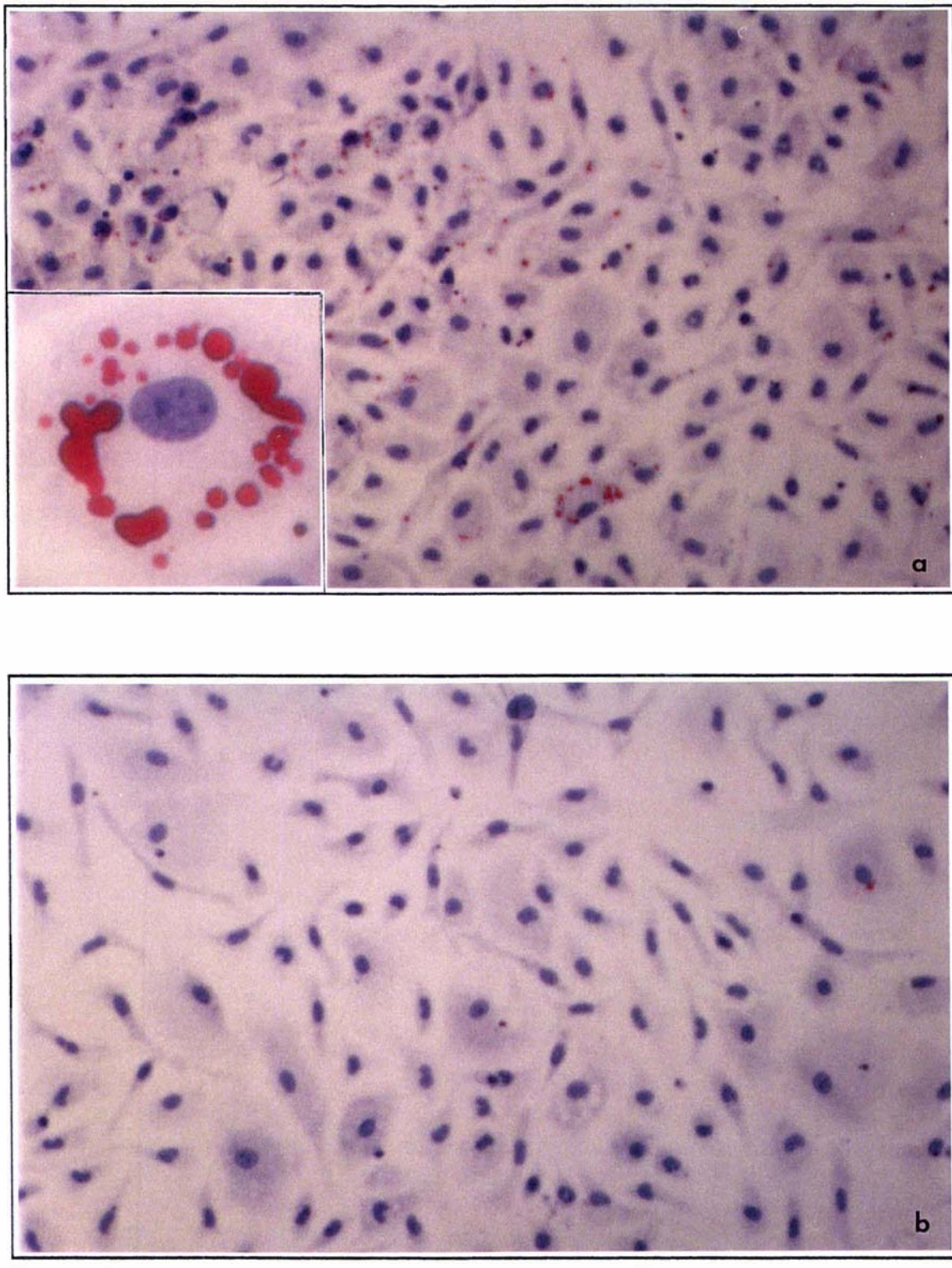

Figure 9.4. ***Photomicrographs of macrophage cell cultures.*** In (a) the macrophage cell culture was incubated for 20hr with LDL that had previously been exposed to macrophages. Some macrophages contain numerous red-staining lipid globules (inset). The number of lipid globules contained in (a) was significantly greater than that observed in (b) which is a macrophage culture incubated for 20hr with un-exposed LDL. This suggests that the LDL was being modified by the initial incubation period in a way which promoted its subsequent uptake by macrophages. Magnification: (a) x 210, inset x 860, (b) x 210.

This suggests that macrophages are unable to perform their normal metabolic functions in MPBS and, as can be seen from Figure 9.6, no significant quantity of lipoprotein oxidation products were detected when LDL was incubated with macrophages cultured in MPBS.

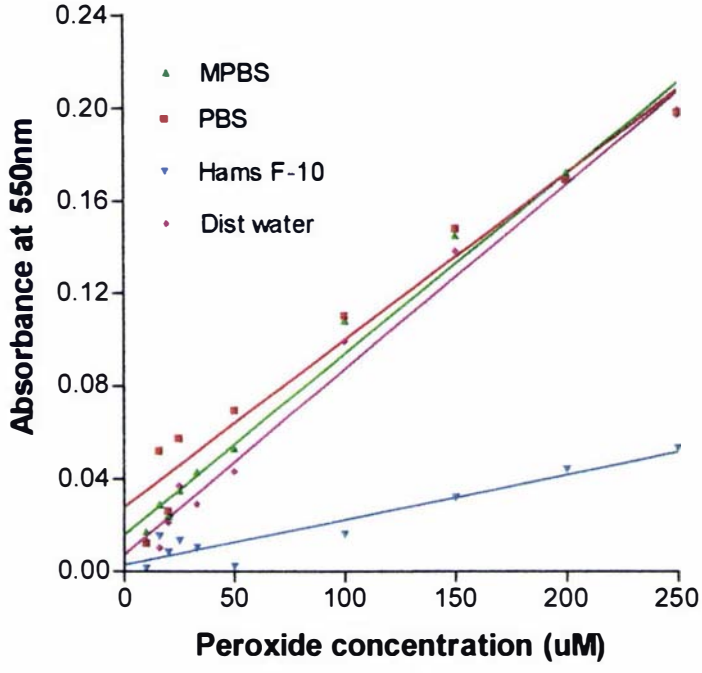

Figure 9.5. ***The absorbance at 550nm of solutions containing FOX2 reagent and peroxides in the range of 10 to 250μM.*** Hydrogen peroxide solution samples (20μl) were mixed with 180μl FOX2 solution and incubated for 30 min at room temperature prior to measurement. 'PBS' indicates peroxide solutions were formulated in phosphate buffered saline. 'MPBS' indicates solutions were formulated in PBS with 0.01μM Cu^{2+} and 3μM Fe^{2+} added. 'Hams F-10' indicates solutions were formulated in serum-free Ham's F-10 cell culture medium. 'Dist water' indicates solutions were formulated in distilled water.

Conclusion

It is believed that LDL becomes oxidised when it is incubated with macrophages contained in Ham's F-10 medium[331,458,459]. However, due to interference from the culture medium, this oxidation cannot be measured using the FOX2 reagent. Oxidised LDL can be accurately measured in MPBS, but macrophages appear unable to oxidise LDL when cultured in this solution. Therefore, the results of this preliminary study confirm that the most appropriate way to measure cell-mediated oxidation of LDL is by measuring macrophage uptake of radiolabelled LDL. Unfortunately, due to the expense and labour input, this method could

not be used in this research programme and the hypothesis that the rate of macrophage-mediated LDL oxidation influences an individual's risk for atherosclerosis remains untested.

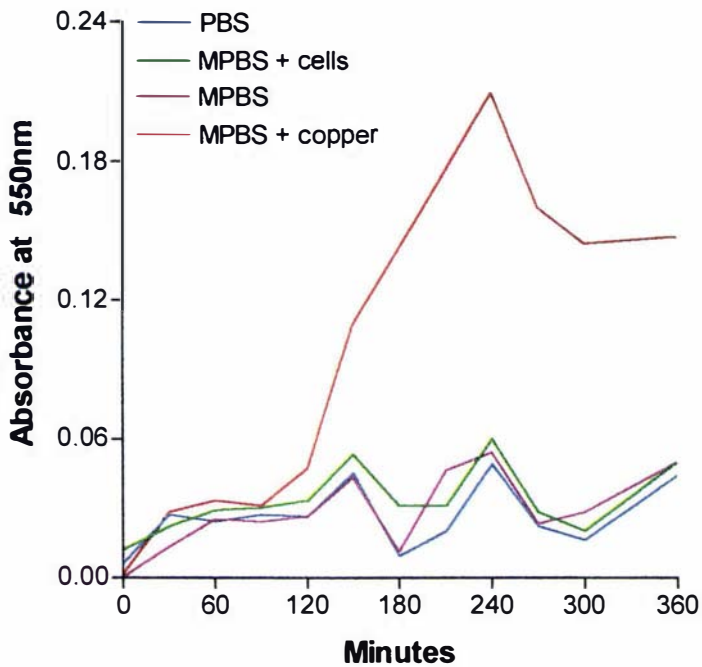

Figure 9.6. ***Kinetics of macrophage-mediated LDL oxidation.*** 'PBS' indicates that LDL was incubated in phosphate buffered saline (PBS). 'MPBS + cells' indicates LDL was incubated with macrophages cultured in PBS which contained 0.01μM Cu^{2+} and 3μM Fe^{2+}. 'MPBS' indicates LDL was incubated in PBS which contained 0.01μM Cu^{2+} and 3μM Fe^{2+}. 'MPBS + copper' indicates LDL was incubated with PBS containing 10.01μM Cu^{2+} and 3μM Fe^{2+}. All incubations were performed at 37°C in air containing 5% CO_2. Samples (20μl) were mixed with 180μl FOX2 reagent. LDL is an abbreviation for low density lipoprotein.

Chapter 10

General discussion

Atherosclerosis is a vascular disease characterised by chronic inflammatory changes in the walls of arteries associated with the accumulation of lipid[2]. Atherosclerosis causes disease when these lesions, or emboli originating from them, disrupt the flow of blood through the artery. Because atherosclerotic disease causes the death of 45% of people who live in the Western world[1,10] it has been the subject of intense research for the past 40 years. As reviewed in Chapter 1, Part 5, this research has identified risk factors which are considered to be important in mediating the development of atherosclerosis. While the influence of some of these factors is still debatable[194,197], there appears to be a consensus that increases in serum LDL[194] and decreases in serum HDL[51] cholesterol concentration increase an individual's risk for atherosclerotic disease.

Despite extensive research into the effects of diet on atherosclerosis, it is still unclear what dietary factors, if any, influence atheroma formation. Information regarding the effects of dietary factors on atherosclerosis is derived from four types of research. Firstly, epidemiological studies can be used to identify associations between dietary factors and disease rates within a population. Secondly, the effect of diet on proposed risk factors for atherosclerosis, such as serum LDL cholesterol concentration, can be examined experimentally. Unfortunately, because accurate measurement of atheroma formation in living human subjects is difficult, it remains controversial whether or not alterations in risk factors caused by diet significantly influence human atherosclerosis. Thirdly, *in vitro* studies can be used to investigate the effect of diet on isolated components of atherogenesis. However, such studies have severe limitations when extrapolating to the *in vivo* situation. Fourthly, dietary factors can be investigated using animal models of atherosclerosis.

When fed a special diet, C57BL/6 mice develop aortic fatty streaks, considered by some to resemble early human atherosclerotic lesions[168], and have been proposed as an animal model for human atherogenesis. Unfortunately, in our studies and those of others, the diet required to induce fatty streak formation in these mice has been found to be unpalatable, and, despite efforts to improve it, acceptance of the diet by mice remained poor throughout the research

programme. This resulted in 15% of mice having to be removed from the experiments because of chronic weight loss.

Human apoB-transgenic mice were also used during this research programme. When compared to non-transgenic mice, these mice develop a serum lipoprotein profile considered to be more atherogenic and a greater area of fatty streaks[393]. However, because transgenic mice offered no clear advantages, and a greater number of non-transgenic mice were available, the C57BL/6 mouse model was used for all studies investigating dietary factors.

C57BL/6 mice fed experimental diets containing hydrogenated coconut oil (mainly saturated fatty acids) developed a higher ratio of serum HDL to total cholesterol than mice fed diets containing olive oil (mainly unsaturated fatty acids). This is in contrast to the results of human studies which have shown that, compared to dietary unsaturated fats, saturated fats lower this ratio[235,236,243,246]. This discrepancy between mice and human subjects can be explained by considering the mechanism by which dietary fats affect serum lipoprotein concentrations. In humans, dietary fats alter the concentration of serum lipoproteins by changing the activity of hepatic LDL receptors[85]. In C57BL/6 mice, LDL receptor activity is inhibited by dietary cholic acid[169], making the subtle effects of dietary fats on receptor activity insignificant. This illustrates the problems inherent in using animal models to investigate the effect of dietary fats on atherosclerosis. Many commonly used animal models including LDL receptor-knockout mice, apoE-deficient mice, apoB-transgenic mice, cholesterol-fed rabbits, WHHL rabbits, and cholesterol-fed hamsters, develop high serum cholesterol concentrations because of alterations in LDL receptor activity[176,186,190]. Until the identification of an animal model in which atherosclerotic lesions can be induced without altering LDL receptor activity, it is unlikely that animal models will provide useful information on the atherogenicity of dietary fats.

It remains controversial whether or not changes in the serum lipoprotein profile associated with dietary fats significantly influences human atherogenesis[10,195,196,228]. Although some animal studies have observed increased fatty streak formation as a result of feeding diets high in saturated fats[173,229], these studies lack consistency and, given the problems associated

with using animal models, their reliability is questionable. In contrast, fatty streak formation in C57BL/6 mice was found to be correlated with the ratio of dietary saturated to unsaturated fat. This unexpected observation appeared to be due to alterations in the ratio of serum HDL to total cholesterol which were associated with the different types of dietary fatty acid. Therefore, while the results of this study should not be interpreted as evidence that saturated fats will reduce atherogenesis in humans, they do suggest that human atherosclerosis may be significantly altered by changes in the serum lipoprotein profile that are associated with dietary fats.

Another group of dietary fats examined in this research programme were the conjugated linoleic acids (CLAs). Only small concentrations of CLAs were included in the experimental diets, and, as these lipids were not expected to alter LDL receptor activity, it was considered appropriate to investigate them using the C57BL/6 mouse model. In C57BL/6 mice, dietary CLAs increased the ratio of serum HDL to total cholesterol. In two previous experiments examining CLAs, these fatty acids have also been found to produce changes in the serum lipoprotein profile which are expected to be less atherogenic[193,260]. In our studies using C57BL/6 mice, the effect of CLAs on serum lipoproteins appeared to be dose-dependent. Commercial preparations of CLAs have recently become available as dietary supplements[466] and it would be interesting to determine how these affect the serum lipoprotein profile of humans.

Surprisingly, despite producing a serum lipoprotein profile considered to be less atherogenic, CLAs promoted fatty streak formation in C57BL/6 mice. CLAs have been shown to stimulate the immune system[431-433] and alter lipoprotein lipase activity[434], both of which may influence atherogenesis[112,375]. CLAs are a class of lipid which appear likely to become a fashionable dietary supplement, however, how CLAs affect human atherogenesis is unknown. While it is possible that they may reduce atherosclerosis by changing the serum lipoprotein profile, they may also, as in the C57BL/6 model, increase atherosclerosis in humans independently of changes in the serum lipoprotein concentration.

Although some epidemiological studies have indicated an association between dietary

antioxidants and reduced risk of coronary heart disease[413,467], the evidence is difficult to interpret. For example, the results of the Health Professionals Follow-up Study indicate that people who took dietary vitamin E supplements suffered lower rates of major coronary artery disease[273]. However, 'people who take antioxidants develop less atherosclerosis' is not the same as 'antioxidants protect against atherosclerosis'. Epidemiological studies are unable to determine if people taking antioxidants developed less atherosclerosis because of the antioxidant supplement or because, for example, of the glass of water that they used to wash it down with. To determine causality, placebo-controlled prospective studies are required. However, the results of prospective studies examining the effect of antioxidants on atherosclerosis have been inconsistent. Of the six studies which have been completed, a beneficial effect of antioxidants was reported in one[281], increased cardiovascular mortality rates were reported in two[280,303], and no effect of antioxidants was observed in the remaining three[279,280,302].

There is little evidence from animal studies that antioxidants protect against atherosclerosis. To my knowledge, no published study examining antioxidants in an animal atherogenesis model has demonstrated a significant decrease in atherosclerotic lesion formation without concurrent significant changes in either serum cholesterol concentration or body weight[287,289-295]. During the experiments reported here, the effects of three different classes of dietary antioxidants on fatty streak formation were investigated using the C57BL/6 mouse model. The addition of vitamin E to the atherogenic diet significantly lowered serum total cholesterol concentration but did not reduce fatty streak formation. In contrast, dietary butylated hydroxytoluene lowered serum HDL concentration and increased fatty streak development. Unfortunately, because both antioxidants affected enzymes important in lipid metabolism, it was impossible to determine if the antioxidant properties of either chemical influenced fatty streak formation. High doses of the oxidisable phenolic compounds contained in red wine have also been found to alter lipid metabolism[468]. However, no differences in serum lipid concentration or fatty streak development were observed between mice consuming low doses of either red or white wine. It appears that most antioxidants affect either lipid metabolism or body weight in animal models. In order to provide unequivocal evidence for an effect of antioxidants in animal atherosclerosis models, it is

essential that a chemical which does not alter either of these parameters is identified.

Therefore, it is difficult to draw firm conclusions regarding the effect of antioxidants on human atherosclerosis. Evidence that they have a protective effect is derived mainly from epidemiological studies. These are difficult to interpret due to inconsistency in the results and the impossibility of excluding bias. In contrast, a large number of animal trials have investigated antioxidants without finding any beneficial effects. However, because it is unlikely that the process of human atherosclerosis is accurately reproduced in any animal model, it is difficult to use this data to exclude the possibility that antioxidants will alter atherogenesis in humans.

Because the dose rates of vitamin E taken by humans are much lower than those used in animal experiments, human supplements are not associated with any significant changes in the serum lipoprotein profile[423]. Dietary supplementation with vitamin E has, however, been shown to increase the oxidation resistance of LDL particles to *in vitro* oxidation. As reported in Chapter 9, daily supplementation of human subjects with aged garlic extract, but not raw garlic, also increases LDL oxidation resistance. Whether or not this *in vitro* effect influences *in vivo* human atherogenesis is unknown, but in animal models increased LDL oxidation resistance has not been associated with a reduction in atherosclerotic lesion formation[285,291-294].

It was hoped to continue investigating the role of LDL oxidation in atherogenesis by studying possible genetic differences in the rate of oxidation of LDL by macrophages. To do this, LDL could have been incubated with macrophages cultured either from atherosclerosis-susceptible or atherosclerosis-resistant mice. Unfortunately, no data could be produced from pilot experiments and the hypothesis that genetic differences in the rate of macrophage-mediated LDL oxidation influences an individual's atherosclerosis risk remains untested.

Epidemiological studies have revealed an association between a moderate intake of alcohol and lower rates of atherosclerotic disease[342,344,353]. While this does not indicate a causal

relationship, evidence from human studies has also shown that alcohol increases serum HDL cholesterol concentration, suggesting a possible protective mechanism[51,346].

Animal studies investigating the effect of alcohol on serum lipoproteins and atherogenesis, however, have been inconclusive[174,354,357]. In the experiment reported in Chapter 6, C57BL/6 mice received a level of alcohol comparable to that of a human moderate drinker, unlike previous animal experiments in which high levels of alcohol were administered[174,354]. Surprisingly, this level of alcohol lowered serum HDL cholesterol concentrations, a finding which contradicts the results of human studies[346]. Mice, unlike human beings, have no serum CETP activity and the results of this trial support the hypothesis that alcohol raises serum HDL cholesterol concentrations by altering the activity of this protein[346]. It may be more appropriate to investigate the effects of dietary alcohol using an animal which has serum CETP activity, for example, the CETP transgenic mouse. The mechanism by which alcohol decreased serum HDL cholesterol concentrations in C57BL/6 mice is unknown.

Mice receiving moderate amounts of alcohol developed a greater area of fatty streaks than controls. It is probable that this was associated, at least in part, with the reduction in serum HDL cholesterol concentration. However, no correlation between serum lipoprotein concentrations and fatty streak formation was observed, suggesting that alcohol may also influence atherogenesis independently of changes to serum lipoproteins. Because this trial used an animal model to investigate the effects of a unique human dietary factor on a unique human disease, it is difficult to predict how alcohol will affect human atherogenesis using data derived from this experiment.

The C57BL/6 mouse model was used in most of the experiments reported in this thesis because these mice develop lesions which resemble early human atherosclerosis[168] in a relatively short period of time. However, like most animals, C57BL/6 mice do not develop atherosclerosis naturally. Therefore, their lipid metabolism has to be drastically altered to increase serum cholesterol concentrations and produce fatty streaks. This then raises an important question regarding the validity of this, and all other, animal models of atherosclerosis. Are the processes by which aortic fatty streaks develop in C57BL/6 mice

comparable to the processes which lead to the development of atherosclerosis in humans? Because of the current uncertainty surrounding the processes involved in early human atherogenesis[108,112], it is impossible to definitively answer this question.

To conclude, the results of the experiments reported in this thesis have demonstrated the difficulties inherent in investigating a disease which only occurs naturally in humans. Like most animal models of atherosclerosis, the C57BL/6 mouse model that was used in the majority of the research reported in this thesis can only be expected to, at best, reflect some of the processes involved in human atherogenesis. The results obtained using animal models of atherogenesis must, therefore, like the results of *in vitro* studies, always be interpreted with caution. It may be best to think of results from experiments using animal and *in vitro* models of atherosclerosis as pieces of a large jigsaw puzzle. In many cases it is difficult to know the importance of each piece until the complete picture has been put together.

Appendix

Polyethylene glycol 6000 solution.

Into 90ml of distilled water was dissolved 1.5g glycine, 0.1g EDTA, and 20g PEG6000. The pH was adjusted to 10 using a 400g/l NaOH solution and the final volume was adjusted to 100ml using distilled water.

Staining protocol for Oil red O.

Stock Oil red O solution was mixed (6ml) with distilled water (4ml) and filtered before use. The slides were then immersed in this solution for 30 min at room temperature before being differentiated in 70% alcohol for 5 min. After a 5 min wash in distilled water, the slides were stained with Meyer's haematoxylin for 4 min, washed, then placed in Scott's tap water for 2 min. After the slides had been washed again for 2 min, they were counterstained with a 0.2% light green solution for 30s and coverslipped using aqueous mounting solution.

Determination of total oxidisable phenolic substances in wine.

A stock 5g/l gallic acid (3,4,5-trihydroxybenzoic acid) solution was made by dissolving 500mg gallic acid in 100ml water. Standards were prepared by diluting 1, 2, 3, 5, and 10ml of this stock solution to 100ml using distilled water. 1ml of each of these solutions and 1ml of the wine (or a 1 in 10 dilution for red wine) were then transferred into a 100ml flask. To each flask, 79ml water and 5ml Folin and Ciocalteu's phenol reagent were added. After 30s, 15ml of a 200g/l Na_2CO_3 solution was added. This solution was incubated at room temperature for 2hr before the absorbance at 765nm was measured.

Solutions used in LDL isolation.

1.072g/ml solution. 107g NaCl was dissolved in 960ml distilled water. To this, was added 40ml of a 2.8g/l EDTA solution.

1.019g/ml solution. 29.5g NaCl was dissolved in 1l distilled water.

1.225g/ml solution. 283g NaBr was dissolved in 960ml distilled water containing 40ml of a 2.8g/l EDTA solution.

1.063g/ml solution. 85g NaBr was dissolved in 1l distilled water.

Determination of LDL protein concentration.

Standards in the range of 250 to 1000 μg protein/ml were prepared using bovine serum albumin. To samples of these standards and to triplicate, diluted 0.1ml samples of the LDL solution, 0.5M NaOH was added to produce a final volume of 1ml. To this was added 5ml of a 100mg/l $CuSO_4$, 200mg/l $KNaC_4H_4O_6$, 20g/l Na_2CO_3 solution. This was then incubated at room temperature for 10 min before adding 0.5ml of a 50% Folin and Ciocalteu's phenol reagent solution diluted in distilled water. After being incubated for 30 min at room temperature, the absorbance at 720 nm was measured.

Synthesis of the FOX2 reagent.

Firstly, 880mg BHT was dissolved in 900ml of methanol. To this was added 50ml of a 100g/l sulphuric acid solution, 25ml of a 3.04g/l xylenol orange solution, and 25ml of a 3.92g/l ammonium ferrous sulphate hexahydrate solution.

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Dietary Antioxidants Do Not Reduce Fatty Streak Formation in the C57BL/6 Mouse Atherosclerosis Model

John S. Munday, Keith G. Thompson, Kerry A.C. James, B. William Manktelow

Abstract—Epidemiological studies and animal trials have suggested that dietary antioxidants protect against atherosclerosis. To test this hypothesis, C57BL/6 mice were fed atherogenic diets supplemented with either vitamin E or butylated hydroxytoluene (BHT). Three groups of 20 mice were fed for 15 weeks on diets containing 1% cholesterol and 0.5% cholic acid. The diet of two groups was supplemented with either 2% vitamin E or 1% BHT. The control group received no antioxidant supplements. The lowest mean serum cholesterol concentration was measured in mice supplemented with vitamin E. Mean serum HDL cholesterol concentrations were highest in the control group, which also had the highest ratio of HDL cholesterol to total cholesterol. Mice fed BHT developed a significantly greater area of aortic fatty streak lesions than the other two groups. However, despite having a more atherogenic lipoprotein profile, mice fed vitamin E developed a level of fatty streak formation similar to the control group. At the end of the trial, mice consuming the vitamin E- and BHT-supplemented diets had higher serum total antioxidant levels than the control mice. Because of changes to lipid metabolism caused by both vitamin E and BHT, the results of this study cannot be used to support the hypothesis that antioxidants confer protection against atherosclerosis. The results do, however, raise the possibility that other studies demonstrating an antiatherogenic action of vitamin E and BHT may have been influenced by their effects on lipid metabolism. (***Arterioscler Thromb Vasc Biol.* 1998;18:114-119.)**

Key Words: atherosclerosis ■ antioxidants ■ BHT ■ vitamin E ■ mice

The earliest lesion of atherosclerosis is believed to be the fatty streak,[1] which consists of a subendothelial collection of foam cells (macrophages containing lipid droplets), small amounts of extracellular lipid, and increased numbers of smooth muscle cells.[2] Oxidation of LDL is considered to be an important step in the development of fatty streaks.[3] Macrophages do not engulf native LDL quickly enough to form foam cells,[4] but oxidized LDL is recognized by macrophage scavenger receptors,[4,5] leading to fast, unregulated LDL uptake and foam cell formation.[1,3,6] Oxidation of LDL occurs after it has become trapped in the subendothelial matrix because at that site it loses the protection of plasma antioxidants.[6,7] Dietary antioxidants may, however, delay the oxidation of LDL,[8–11] enabling it to leave the subendothelial space before oxidation occurs.[8] This may reduce incorporation of LDL into foam cells and therefore reduce the atheroma development and progression.[8] This concept has led to the hypothesis that low plasma antioxidant status is a risk factor for atherosclerosis.[12]

Studies on the possible beneficial effects of vitamin E on heart disease began in 1949,[13] but the results of such studies have been inconclusive. Vitamin E was reported to decrease the severity of atherosclerosis in a Dutch-belted rabbit model[14,15] and in primates,[16] but had no effect in a WHHL rabbit model.[9,17,18] In a rabbit model in which atheroma formation was induced by damaging the endothelium, vitamin E increased the severity of atherosclerosis.[19] The results of epidemiological studies investigating the relationship between vitamin E and atherosclerosis have also been inconclusive.[20–28]

The antioxidant BHT has been shown to reduce atherosclerosis in a model using WHHL rabbits.[29] This chemical is widely used at low levels in foods and cosmetics,[30,31] although its chronic toxicity makes it unlikely to be used as an antioxidant supplement in human diets.[30] Another synthetic antioxidant, diphenyl-1,4-phenylenediamine, has also been shown to decrease atherosclerosis in rabbit[32] and mouse[33] atherosclerosis models.

C57BL/6 mice fed an atherogenic diet for 15 weeks develop fatty streaks in their aortic sinus.[34,35] This well-established mouse model was used in the present study to examine the effect on subendothelial fatty streak formation of dietary supplementation with the antioxidants vitamin E and BHT.

Materials and Methods

Experimental Animals

Sixty 6-week-old female C57BL/6 mice were obtained from the Animal Breeding Station, Department of Laboratory Animal Sciences, University of Otago, New Zealand. Five mice were housed in each solid-floored cage and kept in a room maintained at 22°±1°C with a

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Department of Veterinary Pathology and Public Health, Massey University (J.S.M., K.G.T., B.W.M.), and New Zealand Institute for Crop and Food Research Limited (K.A.C.J.), Palmerston North, New Zealand.

Address correspondence to John S. Munday, Department of Veterinary Pathology and Public Health, Massey University, Private Bag 11222, Palmerston North, New Zealand.

E-mail jsmunday@massey.ac.nz

Selected Abbreviations and Acronyms

ACAT = acyl-CoA/cholesterol acyltransferase
BHT = butylated hydroxytoluene
WHHL = Watanabe heritable hyperlipidemic rabbit model

humidity level of 60%±5% and air exchange 12 times per hour on a 12-hour light-dark cycle. The mice were acclimatized for 20 weeks, during which time they were fed a normal commercial mouse diet. At the start of the trial the mean mouse weight was 26.3±2.6 g. Throughout the experiment, the mice were given free access to food and water.

Experimental Design

Twenty mice were randomly allocated into one of the three dietary groups. Two treatment groups received diets supplemented with either 2% vitamin E acetate (10 IU/g diet) or 1% BHT by weight. The control group received the same diet but without supplementary antioxidant.

The mice were fed ad libitum for 15 weeks and were weighed weekly. At the end of the trial period, the mice were fasted for between 8 and 12 hours, then killed by carbon dioxide inhalation.

All procedures involving animals in this study were conducted under guidelines established by the Massey University Animal Ethics Committee and with their prior approval.

Experimental Diets

The composition of the experimental diets was based on that of Nishina et al.[34] All mice were fed diets containing 5% corn oil, 5% olive oil, 5% anhydrous milk fat, 1% cholesterol, and 0.5% cholic acid (Table 1). The vitamin and mineral mixes were formulated by staff at the Food Evaluation Unit, Crop and Food Research Ltd, Palmerston North according to guidelines established by the United States National Research Council.[36] Dry vitamin E 50%, type CWS/F containing 500 IU of vitamin E per gram, in the form of DL-α-tocopherol acetate was obtained from Roche Products (NZ) Ltd. All other chemicals were purchased from either Sigma Chemical Co or BDH Chemicals New Zealand Limited. To prepare the diets, the dry ingredients were mixed together and then water was added to form a dough. The dough was cut into strips, dried in an oven at 29°C for 3 days and then stored at −20°C until used. One week's supply of diet was removed from the freezer at a time and kept refrigerated at 4°C until used. Mice were provided daily with fresh diet from the refrigerator.

TABLE 1. Composition of Diets

	Dietary Treatment, g/k, as fed		
Ingredient	**BHT**	**Vitamin E**	**Control**
Sucrose	477	472	482
Casein	200	200	200
Corn oil	50	50	50
Olive oil	50	50	50
Anhydrous milk fat	50	50	50
Cellulose	50	50	50
Salt mix	50	50	50
Vitamin mix	50	50	50
DL-Methionine	3	3	3
BHT	5	0	0
Vitamin E acetate	0	10	0
Cholesterol	10	10	10
Cholic acid	5	5	5

Composition of the antioxidant-containing and the control diet fed to C57BL/6 mice for 15 weeks.

Sample Collection and Processing

Immediately after euthanasia, between 0.5 and 1.0 mL of blood was collected from each mouse by cardiac puncture. The blood was transferred to a test tube and the serum separated by centrifugation at 1500*g* for 15 minutes.

Serum cholesterol and triglyceride concentrations were determined using a Hitachi 704 autoanalyzer (Boehringer Mannheim GmbH, Mannheim, Germany). Because the densities of both mouse and human lipoproteins are the same,[37] it was possible to determine serum HDL cholesterol concentrations after selective precipitation of VLDL and LDL using polyethylene glycol 6000.[38–42] The LDL cholesterol concentration could not be calculated from triglyceride and HDL cholesterol concentrations because the Friedewald formula[43] has been found to be inaccurate in mice.[44]

Serum total antioxidant status was measured using a commercial kit (Randox Laboratories Ltd) run on a Hitachi 704 autoanalyzer. The kit contains a reagent that is oxidized at a known rate by a peroxidase. The ability of the test sample to inhibit this reaction is determined by measuring the formation of oxidation products of the reagent.

The liver from one mouse in each cage was fixed in neutral buffered formalin, embedded in paraffin, then sectioned and stained with hematoxylin and eosin for routine histological examination.

Morphological Evaluation of Fatty Streaks

The size of fatty streaks in the aortic sinus was quantified using a method similar to that previously described.[35] After euthanasia, the heart was placed in 0.9% saline for 1 hour, then in 0.9% saline containing 4% formalin for 1 to 7 days. The ventricles were removed by cutting across the heart in a plane that included the base of both auricles. The hearts were then passed through ascending concentrations of gelatin at 37°C for 18 hours, frozen, and sectioned at 40 μm with a cryostat. Sections were discarded until the aortic sinus was recognized by the appearance of aortic valves and the rounded appearance of the aortic wall. Twenty-four consecutive 10-μm sections were then cut, mounted onto gelatin-coated slides, and stained using oil red O and Meyer's hematoxylin method with light green counterstaining. The areas of intimal lipid deposition stained red with oil Red O were quantified using the SigmaScan Scientific Measurement System (Jandel Scientific, San Rafael, Calif). The cross-sectional area of lesions in every second section was measured so that a total of 12 sections from a 240-μm segment of aorta were examined per heart. All lesion assessment was performed blind by the same researcher.

Statistical Analysis

Differences among dietary groups were analyzed using analysis of variance techniques. Because the groups contained an uneven number of animals by the completion of the trial, differences between means were analyzed using an unbalanced design. Lesion size, total serum cholesterol, total antioxidant status, triglyceride, and lipoprotein cholesterol concentrations were modeled using linear regression. All statistics were calculated using the SAS statistics package (SAS Institute Inc).

Results

During the trial, three mice were removed from the control and vitamin E groups, and two from the BHT group after failing to adapt to the trial diet and losing weight. The mean weights of the remaining mice in each trial group are shown in Table 2. At the completion of the trial, the mice in the BHT group were significantly lighter than those in the control group.

Antioxidant Status

Total antioxidant status was significantly higher in serum from mice fed diets containing either BHT or vitamin E than from control mice (Table 2).

TABLE 2. Summary of Measurements Made After 15 Weeks on an Atherogenic Diet

	Dietary Group		
	1% BHT (n=18)	2% Vitamin E Acetate (n=17)	Control (n=17)
Total aortic fatty streak area (mm^2)	0.483 (0.265)[a]	0.312 (0.105)[b]	0.300 (0.156)[b]
Serum total cholesterol (mmol/L)	6.13 (0.81)[a*]	4.68 (0.85)[b*]	5.53 (1.54)[a]
Serum HDL cholesterol (mmol/L)	2.00 (0.42)[a]	1.96 (0.51)[a]	2.70 (0.59)[b]
HDL cholesterol: total cholesterol	0.33 (0.08)[a*]	0.42 (0.10)[b]	0.50 (0.08)[c*]
Serum triglyceride (mmol/L)	0.48 (0.24)[a]	0.58 (0.17)[ab]	0.82 (0.69)[b]
Serum total antioxidant status (mmol/L)	1.40 (0.22)[a]	1.43 (0.29)[a]	1.22 (0.26)[b]
Final weight (g)	24.21 (2.21)[a*]	25.62 (1.93)[ab]	26.35 (2.52)[b*]

The vitamin E group was fed a diet containing 2% vitamin E acetate, the BHT diet contained 1% butylated hydroxytoluene. The control group was fed a diet containing no antioxidants. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($P<.05$). *Indicates that the differences between the means are significant at $P<.01$.

Serum Lipids

The mean serum lipoprotein and triglyceride concentrations of each group of mice are shown in Table 2. The mean total cholesterol concentration of mice supplemented with vitamin E was significantly lower than that of the other two groups. The mean serum HDL cholesterol concentrations of mice in the control group was significantly higher than those in the BHT and vitamin E groups. The highest mean ratio of serum HDL cholesterol to total cholesterol was observed in the control group, whereas the BHT group had a significantly lower ratio than the other groups. The ratio of serum HDL cholesterol to total cholesterol was negatively correlated with the serum total antioxidant status ($r^2=.16$, $P=.005$; n=52) (Fig 1).

Mean serum triglyceride concentrations in mice from the BHT group were higher than in mice from the control group.

Pathology

After 15 weeks, all mice developed lipid-containing lesions in the intima of the aortic sinus, similar to those previously described.[45] The lesions were most common close to the origins of the coronary arteries and at the base of the aortic valves, and consisted of subendothelial collections of macrophages containing numerous oil red O–positive lipid globules. These globules were found extracellularly around areas of macrophage accumulation and were also present in the underlying media.

The mean area of the aortic lesions in each group of mice is shown in Table 2. The mean lesion area in mice receiving the diet containing BHT was significantly greater than in mice in the control and vitamin E groups. The ratio of HDL to total cholesterol was not significantly correlated with mean lesion area for individual mice ($r^2=.07$, $P=.07$; n=52) or when mean values of the dietary groups were considered ($r^2=.75$, $P=.33$; n=3) (Fig 2).

The livers of mice from all groups were enlarged and pale tan in color. Histological examination revealed diffuse hepatic lipidosis that did not differ in severity among groups on the basis of subjective assessment.

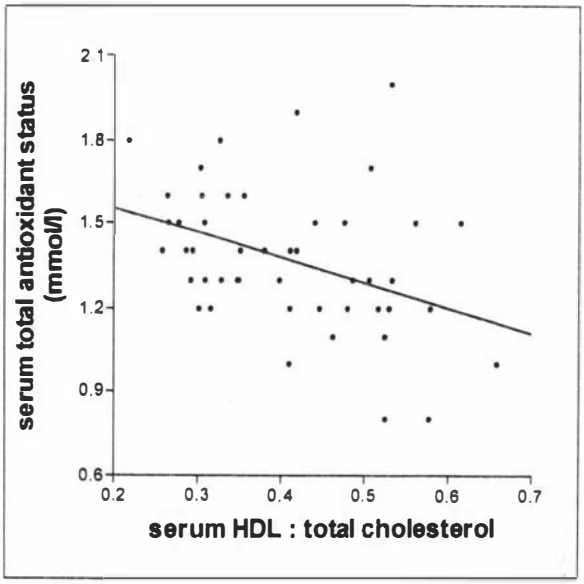

Figure 1. Relationship between serum total antioxidant status and the ratio of serum HDL to total cholesterol. $r^2=.16$, $P=.005$; n=52.

Discussion

The addition of either vitamin E or BHT to an atherogenic ration fed to C57BL/6 mice did not reduce the severity of fatty streak formation in the aortic sinus. Both antioxidants did, however, significantly change the serum lipoprotein profile.

The failure to observe an effect of vitamin E on aortic fatty streak reduction in this trial is in agreement with previous studies that used cholesterol-fed[46,47] and WHHL rabbits.[9,17,18] In contrast, two studies in which a low cholesterol, atherogenic diet was fed to rabbits for 10 months reported an atherosclerosis-reducing effect of vitamin E.[14,15] In these last two studies, the vitamin E-supplemented diet was associated with lower serum cholesterol concentrations, and the beneficial effect of vitamin E observed may have reflected exposure to this, rather than an antioxidant effect.[14,15] In a further study, in which vitamin E was added to an atherogenic diet fed to nonhuman primates, vitamin E resulted in significant reductions in arterial stenosis at some sample sites, although no significant differences were observed in the aorta.[16]

In our study, mice receiving the diet supplemented with vitamin E had a significantly lower mean total serum choles-

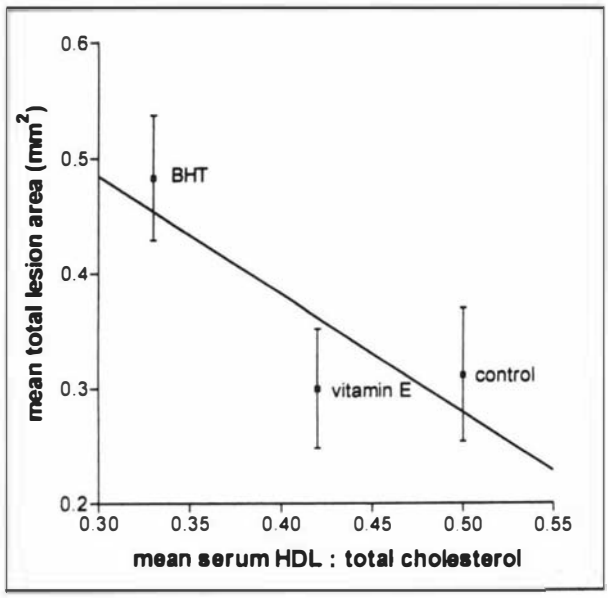

Figure 2. Relationship between mean total lesion area and mean ratio of serum HDL cholesterol to total cholesterol. Error bars indicate one standard error. BHT indicates mice were fed 1% butylated hydroxytoluene, mice in the vitamin E group were fed diets containing 2% vitamin E acetate. $r^2=.75$, $P=.33$; n=3.

terol concentration than controls. A hypocholesterolemic effect of vitamin E has been previously reported in rats[48] and rabbits.[10,14,15,17,49] Significant changes in serum cholesterol concentrations have not been reported in human subjects receiving vitamin E supplements[50,51]; however the doses of vitamin E used in human studies (600 to 800 mg/d per person) were far lower than those used in animal studies. Vitamin E probably lowers serum cholesterol by increasing the activity of cholesterol 7α-hydroxylase,[49,52] the enzyme responsible for controlling the rate at which hepatic cholesterol is converted to bile acids.[53]

Mean serum HDL cholesterol concentrations in the mice supplemented with either vitamin E or BHT were significantly lower than in controls. This is consistent with previous observations in which vitamin E has been shown to reduce serum HDL cholesterol concentrations in rabbits.[46] In another rabbit study, BHT also showed a definite, if not significant, trend toward lowered HDL.[29] In our study, the ratio of HDL to total cholesterol was negatively correlated to serum total antioxidant status, suggesting that the proportion of cholesterol carried in the HDL fraction may be reduced by dietary antioxidants.

The addition of 1% BHT to the atherogenic diets of C57BL/6 mice resulted in significantly more aortic fatty streak development than in controls. This is in contrast to previous trials using rabbits, which reported either a reduction in atherosclerosis[29] or no change[15] in animals given BHT. Furthermore, total serum cholesterol concentrations in the C57BL/6 mice fed BHT were not significantly different from controls. Again, this is in contrast to previous rabbit,[29] rat,[30] and mouse[54] BHT studies that reported an increase in serum cholesterol concentrations because of an inhibition of acyl-CoA/cholesterol acyltransferase (ACAT) activity.[54] Because of the presence of cholic acid in the atherogenic diets, ACAT has little influence in determining serum cholesterol concentrations in the C57BL/6 mouse model.[55] This may explain why BHT failed to increase serum cholesterol concentrations in this model.

The inhibition of ACAT activity by BHT may also reduce foam cell production. Foam cells develop after cholesterol contained in modified LDL is phagocytosed by a macrophage.[56] In the macrophage, ACAT is responsible for forming cytoplasmic cholesterol droplets by esterifying cholesterol, and evidence suggests that the excretion of cholesterol from macrophages is most rapid when ACAT activity is low.[57] It is possible, therefore, that BHT reduces foam cell formation by decreasing ACAT activity and so promoting the clearance of cholesterol from macrophages rather than as a result of any antioxidant action. Macrophages from C57BL/6 mice, when compared with macrophages from atherosclerosis-resistant mice, posses high ACAT activities when challenged with a high cholesterol diet.[57] BHT may not depress ACAT activity sufficiently in this model to cause the reduction in foam cell formation that has been observed in rabbits.

Serum triglyceride concentrations were significantly lower in mice consuming a diet containing BHT than in controls. This does not support findings of previous rabbit trials in which dietary BHT greatly increased serum triglyceride concentrations.[15,29] However, in our trial the mice that were fed diets supplemented with BHT for 15 weeks weighed less than those fed the control diet. Obesity is thought to be a cause of hypertriglyceridemia,[58] and this may explain the lower levels reported here. Decreased weight gain because of 1% BHT has also been observed in trials with rats.[30] In the rabbit studies in which increased triglyceride concentrations were reported, no differences in weight gain between the BHT and control groups were reported.[15,29]

In a previous trial using the C57BL/6 mouse atherosclerosis model to examine the effect on fatty streak formation of diets containing different proportions of saturated fat, the lesion area was correlated to the HDL to total cholesterol ratio ($r^2=.1$, $P=.01$; n=73)(unpublished data, 1996). This relationship was not observed in the present trial, ($r^2=.07$, $P=.07$; n=52), despite the fact that all the mice were fed essentially the same diet. These results could perhaps be interpreted as evidence of an antiatherosclerotic effect of antioxidants. As illustrated in Fig 2, despite having a significantly lower HDL to total cholesterol ratio, which would be expected to result in greater fatty streak formation, mice fed vitamin E had a similar mean lesion area to the controls. However, because both antioxidants studied in this trial alter lipid metabolism their effect on foam cell formation may be independent of their antioxidant action.

No published study examining the effect of dietary vitamin E supplementation on atherosclerosis has demonstrated a decrease in atherosclerosis without a concurrent decrease in serum cholesterol.[9,14–18,46,47] This is despite LDL particles from animals fed vitamin E becoming enriched with antioxidant and being more resistant to in vitro oxidation.[9,17,18,46,59]

Because of the apparent effects on lipid metabolism of the antioxidants investigated in this trial, our results cannot be used to support the hypothesis that antioxidants confer protection from atherosclerosis. As most antioxidants appear to have an

effect on blood lipoprotein profiles[15,29,32] or body weight,[33] it is difficult to evaluate the benefits of antioxidants from animal feeding trials. In vitro investigations into the oxidizability of LDL have been performed,[8,9,11,46,60–63] but convincing data on a causal relationship between LDL oxidizability and atherosclerosis development is lacking.[9,46] Results of epidemiological studies examining the relationship between antioxidants and atherosclerosis are also difficult to interpret because of inconsistency of results and the presence of confounding factors.[20–27] Therefore, until more conclusive proof of a protective action of antioxidants is produced, discretion is needed before dietary antioxidant supplementation can be recommended to reduce the risk of atheroma-associated coronary heart disease.

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