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Natural antioxidants and susceptibility of low density lipoproteins to oxidation

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

at the University of Otago, Dunedin,
New Zealand.

1999
Abstract

In patients with diabetes mellitus, increased oxidative stress may contribute to low density lipoprotein (LDL) oxidation and elevated levels of circulating products of inflammation. A randomised, placebo-controlled study was conducted in patients with type 2 diabetes mellitus to compare the effect of four weeks of supplementation with tomato juice (500 ml/day), alpha-tocopherol (800 IU/day) or vitamin C (500 mg/day) on LDL oxidation, circulating levels of C-reactive protein (C-RP), soluble vascular cell adhesion molecule-1 (sVCAM-1) and intercellular adhesion molecule-1 (sICAM-1), and circulating products of lipid peroxidation. Plasma lycopene levels increased nearly three fold with the consumption of tomato juice and LDL resistance (lag time) to copper ion stimulated oxidation increased by 42% (P=0.001). The magnitude of the change with tomato juice was comparable to the corresponding increase during supplementation with a pharmacological dose of alpha-tocopherol (54%, P=0.001). Alpha-tocopherol also decreased plasma C-RP (-49%, P=0.004) and increased plasma cholesterol concentration (9%, P=0.022). Circulating levels of adhesion molecules, erythrocyte TBARS, plasma indices of lipid peroxidation and plasma glucose did not change significantly during the study. These findings indicate drinking tomato juice is an effective way to increase plasma lycopene levels and the intrinsic resistance of LDL to oxidation in diabetic patients. Alpha-tocopherol increases the resistance of LDL to oxidation and reduces systemic inflammatory activity but these potentially anti-atherogenic actions are opposed by a concomitant increase in plasma cholesterol levels.

The second study in this thesis focused on the development of a model which may reflect LDL oxidation in the arterial intima more closely than the frequently used copper ion induced oxidation of isolated LDL. Low density lipoproteins can bind to proteoglycans rich in heparin and chondroitin sulphate in the arterial intima and consequently may become a target for atherogenic modification by myeloperoxidase (MPO). Experiments were conducted to examine the susceptibility to peroxidase/hydrogen peroxide (H$_2$O$_2$) catalysed oxidation of resolubilised LDL, that has been precipitated from serum with heparin and from native LDL with heparin, chondroitin sulphate, dextran sulphate, and polyethyleneglycol. In addition, the effects of antioxidants and components of human serum on the oxidation of heparin-LDL in a peroxidase/ H$_2$O$_2$ system were investigated. The LDL from complexes with glycosaminoglycans and dextran sulphate were oxidised rapidly by horse radish peroxidase (HRP) and H$_2$O$_2$ (mean $t_{	ext{max}}$ for conjugated diene formation of 3-5 minutes) while there was little oxidation of native LDL and polyethylene glycol-LDL during the 30 minute incubation period. Aggregated LDL was not oxidised by HRP/H$_2$O$_2$. The formation of thiobarbituric acid reacting substances (TBARS) paralleled the change in conjugated dienes during oxidation of heparin-LDL. Heparin-LDL was also more rapidly oxidised than native LDL by MPO/H$_2$O$_2$. Oxidation of heparin-LDL by peroxidases did not require free tyrosine and was almost totally inhibited by butylated hydroxytoluene (BHT) and ascorbate, and was unaffected by vitamin E or urate. Increasing concentrations (0-14.9%) of
betalipoprotein deficient serum (BLPDS) significantly (P<0.0001) inhibited the formation of TBARS during heparin-LDL oxidation catalysed by HRP and MPO. The inhibitory activity was removed by dialysis and gel-filtration of BLPDS and was not restored by addition of physiological levels of ascorbate, tyrosine and reduced thiols (cysteine) to gel-filtered BLPDS. These results indicate that LDL can form complexes with glycosaminoglycans rendering them particularly susceptible to oxidation by peroxidases. Furthermore LDL oxidation may be inhibited by small, water soluble compounds in the human serum but not by vitamin E. These findings may be relevant to the formation of oxidatively modified LDL in the artery wall.
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### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycosylation end-products</td>
</tr>
<tr>
<td>BLPDS</td>
<td>Betalipoprotein deficient serum</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>C-RP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CsLDL</td>
<td>Chondroitin sulphate LDL</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DexLDL</td>
<td>Dextran sulphate LDL</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>FSB</td>
<td>Fluorescent schiff bases</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HepLDL</td>
<td>Heparin LDL</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MPA</td>
<td>Metaphosphoric acid</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear transcription factor-kappa B</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PegLDL</td>
<td>Polyethyleneglycol LDL</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>Soluble intercellular cell adhesion molecule-1</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>Soluble vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reacting substances</td>
</tr>
</tbody>
</table>
Chapter 1

1.1 Introduction

Oxidation of low density lipoprotein (LDL) that becomes trapped in the artery wall is regarded as an important step in the development of atherosclerosis.\(^1,2\) There is evidence that mildly oxidised LDL enhances the expression of inflammatory cytokines, chemokines and cellular adhesion molecules\(^1\) by endothelial cells and monocytes. These molecules promote the adhesion of monocytes to the vascular endothelium followed by transmigration of adhered cells into the intima where they are retained and transformed into macrophages.\(^1,2\) Cultured macrophages avidly internalise oxidised LDL via unregulated receptors resulting in lipid-filled cells.\(^1,2\) A similar process may occur in the artery wall to generate foam cells, the hallmark of the early atherosclerotic lesion. Oxidative activities may also contribute to changes in plaque morphology, rupture and thrombus formation that can lead to angina pectoris, myocardial infarction (MI) and ischaemic stroke.\(^3-5\)

Reliable methods to determine the susceptibility of LDL to oxidation in vivo are not available. Monitoring the formation of conjugated dienes as a measure of intrinsic resistance of isolated LDL to copper ion mediated oxidation has emerged as a popular ex vivo model.\(^6,7\) However models which more closely reflect LDL oxidation in vivo, by the inclusion of elements present in the artery wall and physiological pro-oxidants, are the current focus of investigation by a number of research groups.\(^8-16\)

Decreasing oxidative stress through the antioxidant protection of LDL is a strategy which may have significant consequences in reducing the development of atherosclerotic heart disease and its clinical sequelae.\(^17-22\) A number of studies indicate that dietary supplementation with high doses of vitamin E increases the resistance of isolated LDL to oxidation.\(^23-27\) Also monocytes isolated from healthy subjects after supplementation with vitamin E show reduced adhesion to cultured endothelial cells and depressed release of interleukin-1\(\beta\) (IL-1\(\beta\)).\(^28\) In people under increased oxidative stress, due to smoking, supplementation with high doses of vitamin C has been demonstrated to reduce the susceptibility of LDL to oxidation \(^29\) and inhibit monocyte adhesion.\(^30\) Lycopene, a major carotenoid in human plasma, inhibits the oxidative modification of isolated LDL.\(^31\) Tomato products in the diet are a rich source of lycopene and drinking tomato juice has been demonstrated to increase plasma lycopene levels in healthy subjects.\(^32-35\) Regular consumption of tomato juice appears to improve the resistance of isolated LDL to oxidation but this has not been tested in a randomised placebo-controlled trial.\(^33-36\) Also, the effect of tomato juice on markers of inflammatory activity has not been studied.
Patients with type 2 diabetes are at increased risk of developing coronary heart disease (CHD) compared with the general population. Increased oxidative stress and enhanced LDL oxidation are believed to contribute to this excess risk of arterial disease. In diabetic patients, circulating levels of inflammatory cytokines, C-RP, soluble vascular cell adhesion molecule-1 (sVCAM-1) and intercellular adhesion molecule-1 (sICAM-1) are elevated, suggesting pro-atherogenic inflammatory activity is increased. Furthermore, antioxidant protection may be inadequate and levels of some antioxidants including ascorbate and lycopene are low. Antioxidants may attenuate oxidative stress and inflammatory activity, however this has not been extensively tested in patients with type 2 diabetes.

Outline of thesis

A review of the current literature examining the interrelationship between natural antioxidants, LDL oxidation and diabetes is presented in Chapter 2. Some detail is also included on the methodology employed to investigate LDL oxidation and the relevance of these measures to atherosclerosis. Chapter 3 outlines an intervention trial which was conducted to examine the effect of natural antioxidants on LDL oxidation and inflammatory activity in patients with type 2 diabetes. A draft manuscript has been prepared from this chapter for submission to the journal “Diabetes Care.” Chapter 4 characterises a new model for assessing the susceptibility of LDL to oxidative modification ex vivo. A shorter version of this chapter was recently accepted for publication by in the journal “Atherosclerosis.” Conclusions are presented in Chapter 5.
Chapter 2

2.1 Atherosclerosis

2.1.1 Pathogenesis of atherosclerosis

Atherosclerosis is the underlying primary cause of most cardiovascular diseases (CVD).\(^{(50,51)}\) Atherosclerosis is a progressive multifactorial disease of large and medium sized arteries, whereby the arterial wall loses elasticity and the lumen is narrowed due to thickening of the intima.\(^{(50,52)}\) In the development of atherosclerosis the lipid-filled fatty streak is the earliest lesion and this may progress via an intermediate lesion to a fibrous plaque. This plaque encroaching on the lumen may eventually rupture, resulting in occlusive thrombosis and subsequent myocardial infarction (MI) or ischemic stroke.\(^{(1,4,5,50-54)}\)

2.1.2 Role of oxidised LDL in atherosclerosis

In the early stages of atherosclerosis, increased plasma LDL, endothelial dysfunction and permeability, and an imbalance between cholesterol influx and efflux result in the formation of foam cells in the artery wall.\(^{(55)}\) The majority of the foam cells are derived monocyte-macrophages, and many of the properties exhibited by LDL which has been oxidatively modified could explain why monocytes migrate from the circulation into the subendothelial space, differentiate to macrophages and accumulate intracellular lipid to be converted to foam cells.

Low density lipoprotein appears to be readily transported across the endothelium. Components of the extracellular matrix, namely proteoglycans and their long carbohydrate side-chains glycosaminoglycans, can interact with LDL, resulting in structural alterations and retention in the arterial wall.\(^{(56)}\) The distribution of proteoglycans in the arterial intima varies with anatomical location and is increased in areas of intimal thickening associated with lesion development.\(^{(8)}\) It is postulated that associations of LDL with proteoglycans and glycosaminoglycans increase both the susceptibility of LDL to oxidative modification and the opportunity for this modification to occur.\(^{(8)}\) Subendothelial retention of LDL is an essential process in the development of the early lesion.\(^{(53,57,58)}\)

Oxidative waste and enzymes secreted from the vascular cells probably mediate LDL oxidation in the subendothelial space.\(^{(2)}\) Initially LDL is minimally oxidised and the modified lipids formed are considered to have pro-inflammatory characteristics, upregulating inflammatory genes and the expression of cytokines and chemokines.\(^{(59,60)}\) Several of these molecules participate in to the adherence of circulating mononuclear cells in the endothelium, transmigration of the adhered cells
into the intima and transformation of monocytes into macrophages.\(^{(59)}\) The expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) result in the firm adhesion of monocytes. Monocyte chemotactic protein-1, together with cell adhesion molecules, attract and guide monocytes into the arterial intima. Macrophage colony-stimulating factor causes monocyte differentiation into macrophages. Differentiation of monocytes also produces monocyte chemotactic protein-1 that can stimulate further amplification of the recruitment of monocytes.\(^{(61)}\) Activated macrophages may continue to oxidise the lipids in LDL and modify apolipoprotein B, leading to a loss of recognition by the LDL receptor.\(^{(62-64)}\) Oxidatively modified lipoprotein is taken up by unregulated receptors on macrophages, resulting in massive accumulation of cholesterol. Such cholesterol-loaded cells are called foam cells and represent the first stage in fatty streak formation.

In addition, oxidised LDL may be pro-atherogenic in other ways. Oxidised LDL may form immune complexes\(^{(65)}\) and aggregates, and these are readily internalised by macrophages.\(^{(66,67)}\) The release of cytotoxic lipid peroxidation products from oxidised LDL in the intima can be considered an irritant for the endothelial cell layer,\(^{(55,68)}\) and may result in endothelial cell necrosis, platelet aggregation, release of growth factors that promote smooth muscle cell proliferation, accumulation of inflammatory cells and alterations to eicosanoid homeostasis. Furthermore, oxidatively modified LDL stimulate procoagulant activities by endothelial cells.\(^{(69,70)}\)

### 2.1.3 Evidence for oxidised LDL in vivo

Several lines of evidence provide support to the hypothesis that oxidatively modified LDL is an atherogenic agent.\(^{(1,2,50,55,71,72)}\) Oxidised LDL\(^{(73-75)}\) and the major cytotoxin in oxidised LDL, 7β-hydroperoxycholesterol-5-en-3β-ol,\(^{(76)}\) have been isolated from atherosclerotic lesions. Low density lipoprotein isolated from aortic plaques, obtained at autopsy, share a number of structural and functional similarities with LDL which has been oxidised by incubation with copper ions.\(^{(74)}\) Oxidised LDL forms auto-antibodies against oxidised-specific-lipid-protein epitopes\(^{(65,77)}\) which have been reported as predictive markers of the progression of carotid atherosclerosis and MI.\(^{(78)}\) Furthermore, a number of structurally unrelated antioxidants have been shown to retard lesion formation in hypercholesterolaemic animals.\(^{(79-81)}\)

### 2.1.4 Potential oxidants of LDL

Low density lipoproteins circulating in the plasma are well protected from oxidation by a variety of antioxidant defence systems.\(^{(82,83)}\) However in microdomains of the arterial wall, antioxidants may be depleted and the lipoproteins exposed to oxidative stress. The precise mechanism is uncertain although a number of candidates have been suggested as potential pro-oxidants of LDL.

Vascular cells in the artery wall release enzymes, such as lipoxygenase\(^{(84)}\) and myeloperoxidase (MPO),\(^{(85,86)}\) which may instigate the oxidation of LDL.\(^{(87-90)}\)
Myeloperoxidase and hydrogen peroxide (H$_2$O$_2$) secreted by phagocytes convert chloride to hypochlorous acid (HClO) and L-tyrosine to a tyrosyl radical, and these products are capable of oxidising LDL. Hypochlorous acid targets reactive amino groups on apolipoprotein B, and unsaturated fatty acids, and cholesterol, modifying LDL to a form which is readily internalised by cultured macrophages. There is strong evidence that myeloperoxidase is responsible for oxidation of LDL in vivo. Concentrations of o,o'-dityrosine, a marker of tyrosyl radical damage, and 3-chlorotyrosine, a marker of MPO modified tyrosine, are markedly higher in atherosclerotic lesions compared with normal tissue or the circulation.

During normal metabolism vascular cells also release a number of reactive oxygen species, including superoxide anion radicals and nitric oxide. Superoxide anion radicals may interact with nitric oxide to produce peroxynitrite, which is capable of promoting LDL peroxidation. Peroxynitrite reacts with tyrosine to form 3-nitrotyrosine. Levels of 3-nitrotyrosine are higher in atherosclerotic lesions than in the circulation indicating peroxynitrite may stimulate oxidative reactions in the LDL in vivo.

Incubating LDL with vascular cells, such as macrophages, endothelial cells, smooth muscle cells, or neutrophils, has been demonstrated to stimulate the oxidation of the LDL in vitro. However this process requires low concentrations of transition metal ions, such as copper or iron. Copper ions initiate LDL oxidation by first binding to the LDL and subsequently copper is reduced by the LDL. In the case of iron, LDL cannot reduce iron so an external reductant such as a superoxide anion radical, hydrogen peroxide or thiol is required to initiate oxidation. Vascular cells could provide these reductants in vivo.

Metal containing proteins, such as ceruloplasmin and hemin also stimulate LDL oxidation in vitro. The metal is released from the protein by reactive oxygen species, hydrogen peroxide or glycation, and oxidation proceeds in a process which is similar to transition metal ion stimulated oxidation. Glucose may undergo auto-oxidation and also stimulate LDL oxidation in the presence of transition metal ions.

The oxidation of LDL by transition metal ions has been extensively studied in vitro however in the artery wall this process appears unlikely to be the major initiator of LDL oxidation. Proteins in the extracellular fluid of the artery wall would probably bind any free transition metal ions and prevent them from catalysing the oxidation of LDL. Transferrin, the major carrier of iron in the plasma, also binds iron and copper with high affinity. Free metal ions are unlikely to exist in the plasma or normal arterial tissue except in some situations they may be released as a result of cellular dissolution in injured tissue or with glycation of the ceruloplasmin. The protein moiety on LDL forms o-tyrosine and m-tyrosine when oxidised by copper ions, thus levels of o-tyrosine and m-tyrosine in the atherosclerotic lesions provide a possible strategy to evaluate if transition metals ions are responsible for catalysing LDL oxidation in vivo. In fatty streaks or intermediate atherosclerotic lesions levels of o-tyrosine and m-tyrosine appear similar to the levels in the circulation.
with diseases where high levels of trace metals are deposited in the tissues, such as haemochromatosis and Wilson’s disease, do not appear to have an increased predisposition to premature atherosclerosis. (129-131) Studies examining iron stores in the body and risk of CHD have not resulted in clear conclusions. (132-134)

### 2.1.5 Oxidised LDL and inflammatory activity

The oxidised lipids in minimally modified LDL may induce an inflammatory response through the stimulation of the transcription factor nuclear factor-kappa, B (NF-κB). (135-139) The activation of NF-κB leads to a co-ordinated increase in the expression of many genes whose products mediate inflammatory and immune responses. (140, 141) including genes which encode pro-inflammatory cytokines, interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α), macrophage colony stimulating factor; chemokines, in particular monocyte chemotactic protein-1; inflammatory enzymes; cellular adhesion molecules, ICAM-1, VCAM-1, E-selectin; and immune receptors. Interleukin-1β and TNF-α may also cause the activation of NF-κB, thus amplifying and perpetuating local inflammatory responses.

#### 2.1.5.1 C-reactive protein

Levels of C-reactive protein (C-RP), the classical acute-phase plasma protein, can rise by more than 1000 fold following tissue damage, infection or inflammation. (142, 143) C-reactive protein is synthesised rapidly and exclusively by hepatocytes in response to increased levels of the pro-inflammatory cytokines, IL-1β and TNF-α (142) and synergistically enhanced with IL-6. (144) The oxidised lipids from LDL have been demonstrated to increase pro-inflammatory cytokines and stimulate an acute phase response. (142)

Inflammation may be elevated in response to the underlying atherosclerotic disease process and also as part of the disease. (145) Inflammatory activity in arterial lesions may be an important component of changes in plaque morphology, rupture and thrombus formation leading to MI. (3, 4) Deposits of C-RP have been isolated from lesions in human aortas. (146, 147) Plasma concentrations of C-RP are elevated in patients with acute ischaemia (148) or MI (149, 150) and have been found to predict the progression of ischaemia in patients with unstable angina. (151) C-reactive protein is associated with both the propensities for MI in patients with angina and mortality in people with multiple risk factors for atherosclerosis. (152-155) Ridker and co-workers demonstrated plasma concentrations of C-RP predict the risk of future MI and stroke in healthy men (156) and this prediction was independent of the other known CVD risk factors, including plasma lipid levels. (157) Furthermore, this risk was observed with apparently small elevations in plasma C-RP concentration (3 mg/l) which are in the normal reference range. (155, 156)
2.1.5.2 Cell adhesion molecules

The adhesion of leukocytes to endothelial cells and their subsequent transendothelial migration is mediated by adhesion molecules on both the endothelium and monocytes. Quiescent endothelial cells lack adhesion molecules whilst cells activated by cytokines, such as IL-1β and TNF-α, have increased expression of these molecules.

Strong associations between macrophage accumulation and adhesion molecules, in particular VCAM-1, expression have been reported. Soluble forms of VCAM-1 and ICAM-1 are present in the circulation, and appear to indicate cellular expression of these molecules. Recently sICAM-1 was reported to be an independent predictor of future MI in apparently healthy men. Also, Rohde and co-workers found levels of sVCAM-1 and sICAM-1 were correlated with early atherosclerosis, as assessed by intimal-medial thickness. These findings are in agreement with case-control studies showing elevated levels of sVCAM-1 and sICAM-1 in patients with evidence of atherosclerosis compared with asymptomatic control subjects. In contrast, Peter and co-workers reported levels of sVCAM-1 were raised while sICAM-1 was normal in patients with peripheral artery vascular disease. Differences in the sensitivity of analytical methods and the inclusion of subjects with subclinical disease may have contributed to the variance in the findings.

Dyslipidaemia is associated with raised levels of sVCAM-1 and sICAM-1. In particular, low HDL is associated with enhanced cytokine induced expression of endothelial cell adhesion molecules. Also oxidised lipid, lysophosphatidylcholine, and oxidatively modified LDL increase the expression of VCAM-1 and ICAM-1 on cultured endothelial cells. However, Khan and co-workers reported that oxidised LDL did not increase the expression of ICAM-1 directly but increased the expression of TNF-α resulting in increased ICAM-1. The variance in findings may depend on differing methods for preparing oxidised LDL and cell culture conditions. Intercellular adhesion molecule-1 and VCAM-1 expression on human endothelial cells has been demonstrated to be increased when LDL oxidation is catalysed in the presence of low levels of copper ions, suggesting it is a specific product of mildly oxidised LDL which may be responsible for this expression.

2.2 Lipid oxidation products

2.2.1 Formation of lipid oxidation products from polyunsaturated fatty acids

Reactive oxygen species are constantly produced by metabolic reactions in the human body. When an oxygen radical interacts with a polyunsaturated fatty acid, a hydrogen atom is abstracted from the fatty acid liberating a carbon radical which then undergoes molecular rearrangement to form a conjugated diene. These in
turn may react with oxygen, resulting in a peroxyl radical. The peroxyl radical may have several possible fates including the abstraction of a hydrogen atom from an adjacent polyunsaturated fatty acid side chain, thereby propagating the oxidative injury. Further decomposition of the peroxyl radical catalysed by copper, iron and metal complexes (e.g. haeme, ceruloplasmin) results in additional radicals, and finally the formation of aldehydes (e.g. malondialdehyde (MDA)) and hydrocarbon gases. Rearrangement of conjugated dienes, after the addition of oxygen, results in the formation of isoprostanes F₃α. Thus attack by one oxygen radical may result in the production of many radicals and the propagation of peroxidation.

2.2.2 Measurement of lipid oxidation products

The measurement of lipid oxidation products in blood is difficult as LDL oxidation is likely to occur outside the circulation in the micro-domains of the arterial wall. Even if some oxidised lipoproteins are in the blood their concentration may not directly reflect those at the site of their formation in the artery wall. Extensively oxidised LDL is rapidly removed from the circulation by scavenger receptors so residence time in the plasma will be exceedingly short and levels are likely to be very low. Alternatively, the levels of oxidation products from isolated LDL or erythrocytes, provide a site-specific index of oxidative damage. However the isolation procedures may alter the concentration of lipid oxidation products. Numerous methods are available for the measurement of lipid oxidation products, however none are entirely satisfactory.

Lipid peroxides are among the first peroxidation products to form from the oxidation of fatty acids in LDL. Levels can be quantified by iodimetry, whereby the peroxides oxidative capacity to convert iodide to iodine can be determined using a commercial reagent spectrophotometrically at λ 365 nm. This method is convenient to perform but lacks specificity. Nevertheless this assay may provide a useful index of early lipoprotein peroxidation. A colourimetric assay using N-benzoyl methylene blue also provides a measure of lipid hydroperoxides in isolated lipids. However, the extraction of lipids from the plasma may result in loss and an under estimation of peroxide levels. Plasma concentrations of lipid hydroperoxides can be determined colourimetrically by ferrous iron oxidation in xylene orange. Specificity in this assay is achieved by subtracting a background where lipid hydroperoxides are reduced by triphenyl-phosphine but the sensitivity of this measure is reduced due to the high background readings. A number of sophisticated procedures have also been developed to measure hydroperoxides and cholesterol hydroperoxides, including high performance liquid chromatography (HPLC) analysis with chemiluminescence, fluorimetric post column detection and electrochemical detection. These methods are extremely sensitive and specific. However when they are applied to plasma samples, the water soluble antioxidants must be removed or the lipid isolated prior to analysis, and this may result in the loss of peroxides. The specialist equipment required for these procedures may also limit their use in many laboratories.

Increased levels of lipoperoxides result in the rearrangement of the fatty acid double bonds forming conjugated dienes, and these may be measured with
absorbance at \( \lambda \) 234 nm. However due to their transitory nature they are not a good index of peroxidation when measured at a single time point.\(^{(186)}\)

Isoprostanes are a marker of arachidonic acid peroxidation\(^{(187,188)}\) which form from esterified phospholipids and are subsequently released, presumably in the free form, through the action of phospholipases in vivo.\(^{(189)}\) Isoprostanes are stable adducts which circulate in the plasma and are excreted in the urine. One of the most abundant \( \text{F}_2 \)-isoprostanes in the plasma is 8-epi PGF\(_{2\alpha}\)\(^{(190-192)}\) which is sensitive primarily to lipid oxidation and to a minor extent platelet cyclooxygenase.\(^{(193)}\) Recently IPF\(_{2\alpha}1\) was identified in urine and it appears to provide a sensitive marker of arachidonic acid oxidation which is independent of cyclooxygenase.\(^{(194)}\) Isoprostanes can be quantified in these fluids by gas chromatography/ mass spectrometry.\(^{(190,191,195)}\)

As lipid peroxidation continues, lipid hydroperoxides decompose to form secondary products such as alkanes and aldehydic compounds. The reaction between MDA and TBARS yields a pink MDA-TBA adduct.\(^{(196)}\) This coloured complex can be quantified spectrophotometrically from its visible absorbance (\( \lambda \) max 532 nm)\(^{(197)}\) or by spectrofluorometry (excitation 532 nm, emission 553 nm)\(^{(198,199)}\) and it is readily extractable into organic solvents such as butanol.\(^{(200)}\) However other aldehydes and substances, such as bilirubin, DNA, sugars, prostaglandins and thromboxanes, may interfere with determinations of MDA.\(^{(201-203)}\)

Quantification of the MDA-TBA adduct by HPLC almost entirely eliminates this interference providing a specific and sensitive measurement of MDA-TBA.\(^{(204,205)}\) However, this does not alleviate the problems associated with the conditions used to form the TBA-MDA adduct.\(^{(206)}\)

Many aldehydic products are highly reactive and can react with the \( \varepsilon \)-amino groups of lysine residues in apolipoprotein B, forming Schiff bases and oxycholesterol derivatives. Schiff bases (FSB) may be quantified with fluorescence.\(^{(207)}\) However advanced glycosylated end-products (AGE-products) also exhibit fluorescence over the same range\(^{(208)}\) and may interfere with this measurement. Volatile hydrocarbons and aldehydes, such as pentane, ethane, hexane and 4-hydroxynoneal, may be determined by head-space gas chromatography of exhaled air.\(^{(209,210)}\) These hydrocarbons have been linked with increased lipid peroxidation in vivo, however interference by other hydrocarbons and methodological difficulties may limit use.\(^{(211)}\)

Lysine residues are positively charged at physiological pH, hence the formation of Schiff's bases during the modification of LDL results in an increased net-negative charge of apolipoprotein B, which can be measured as increased anodic relative electrophoretic mobility of LDL upon agarose gel electrophoresis. Thus the proportion of LDL to total LDL has been used as a measure of minimally modified LDL in vivo.\(^{(212)}\)

Auto-antibodies to epitopes of oxidatively modified LDL, such as MDA-LDL provide a very sensitive and specific measure of LDL oxidation. The relevance of this measure may be limited by the lack of information concerning the chemical characterisation of modified epitopes of circulating oxidised LDL in vivo.\(^{(213)}\)
2.3 Measurement of LDL susceptibility to oxidation

Following the development of lipid oxidation products formed in isolated LDL when stimulated with a pro-oxidant, provides information on compositional changes which affect susceptibility to oxidation. A number of assays exist to evaluate this ex vivo oxidation, the most popular of these models uses copper ions to initiate the oxidation of isolated native LDL. Recently, other models which may better mimic the conditions believed to exist in the micro-domains of the arterial wall have also been studied.

2.3.1 Copper ion stimulated oxidation

2.3.1.1 Kinetics of LDL oxidation

The susceptibility of LDL to copper ion stimulated oxidation may be determined by following the temporal profiles of the formation of conjugated dienes, TBARS, fluorescence at λ 430 nm, lipid peroxides, or relative electrophoretic mobility. During the lag and propagation phases these measurements are very similar, and only after the peroxide maximum is reached do the different indices separate producing dissimilar kinetic traces. (6, 80, 214)

The duration of the lag time, as assessed by a change in absorbance at λ 234 nm, provides a reliable and reproducible index of susceptibility to oxidation of the polyunsaturated fatty acids in LDL. (7, 23, 214-216) The kinetics of this oxidation are highly dependent on the experimental conditions, in particular temperature, pH, copper concentration, LDL preparation and concentration. (217-220) Low density lipoproteins appear to have a discreet number of copper binding sites, mainly on apolipoprotein B, (112) which display either high or low affinity binding for copper ions depending on the concentration of copper ions. The characteristic lag phase is only present under experimental conditions in which the copper ions bind predominantly to the low affinity sites, when the copper concentration is high (≥1.6 µmol/l) and the sites are not saturated. In contrast, at lower copper concentrations the high affinity sites bind copper ions and no lag phase is observed. Low density lipoprotein cholesterol is isolated in the presence of EDTA to limit artefactual oxidation. (175) However, EDTA must be removed before copper stimulated oxidation of LDL and desalting columns are the preferred method of removing EDTA as prolonged dialysis has been associated with lipid oxidation and antioxidant depletion. (221) The temperature of the incubation medium must be stable throughout the assay to avoid variations in the rate of oxidation. Ideally the assay should be conducted at 37°C, although, lower temperatures may be used and the rate of oxidation will be reduced accordingly. Thus conducting copper ion induced oxidation of isolated LDL under standardised conditions is essential for comparisons of this data.
2.3.1.2 Intrinsic factors influencing susceptibility of LDL to oxidation

The duration of the lag time appears stable for an individual\(^{(222,223)}\) but there is considerable variation between individuals.\(^{(224,225)}\) A number of factors intrinsic to LDL are thought to be responsible for this variation, such as antioxidant concentrations, composition of fatty acids, distribution of LDL subfractions, intrinsic peroxides, protein to cholesterol ratio, and apolipoprotein B sulphhydryl groups.\(^{(7,226)}\)

Low density lipoprotein contains a number of antioxidants including tocopherols, carotenoids and ubiquinol-10.\(^{(227)}\) Alpha-tocopherol is the predominant antioxidant, with each LDL particle containing five to nine alpha-tocopherol molecules.\(^{(227,228)}\) It has been reported that alpha- and gamma-tocopherol explain at least 30\% of the variation in lag time with copper induced oxidation of LDL.\(^{(225,229)}\) Lycopene, beta-carotene, and ubiquinol-10 are present in similar amounts, albeit substantially reduced compared with alpha-tocopherol, in the LDL.\(^{(229-231)}\) Enriching LDL in vitro with antioxidants such as alpha-tocopherol,\(^{(80,224,227,230,232,233)}\) lycopene,\(^{(31)}\) beta-carotene,\(^{(31,232)}\) and ubiquinol-10\(^{(183)}\) facilitates significant reductions in the susceptibility of LDL to copper ion stimulated oxidation.

The fatty acid composition of LDL is primarily determined by diet.\(^{(234,235)}\) Diets high in linoleate increase the levels of total oxidation products formed with copper ion oxidation.\(^{(235,236)}\) However fatty composition does not appear to greatly influence the resistance of LDL to oxidative modification. Abbey and co-workers reported that lag time was identical when normolipidemic men consumed a linoleate-rich diet or an oleate-rich diet for several weeks.\(^{(237)}\) In contrast, Reaven and co-workers reported increased oxidative modification of LDL in mildly hyperlipidemic men on a diet highly enriched with polyunsaturated fats but examination of their data suggested the lag time was not reduced.\(^{(236)}\)

Levels of small dense LDL are associated with lipoprotein abnormalities, such as increased total cholesterol, VLDL and low HDL.\(^{(238,239)}\) Low density lipoproteins which are small and dense are more susceptible to oxidation.\(^{(240-244)}\) Also this LDL subfraction is depleted in antioxidants, in particular vitamin E and carotenoids.\(^{(240,245)}\)

2.3.1.3 Extrinsic factors influencing susceptibility of LDL to oxidation

The environment surrounding LDL in the micro-domains of the artery wall is believed to contain a number of compounds which also modulate LDL oxidation.\(^{(246,247)}\) The composition of this fluid is known to vary with location and permeability of the endothelium.\(^{(248)}\) These elements are usually removed during the isolation of LDL from plasma. Thus the complex composition of these components in the extracellular fluid and matrix are difficult to reproduce under conditions in vitro.
Recently, the effects of some of these elements on copper ion stimulated oxidation of LDL has been studied in vitro. High density lipoproteins protect LDL from copper ion stimulated oxidation in vitro\(^{(249)}\) and prevent the production of minimally modified LDL by vascular cells in a co-culture model\(^{(250)}\). High density lipoprotein contains enzymes, platelet activating factor acetylhydrolase and paraoxonase, which have been shown to protect against LDL oxidation in co-culture systems\(^{(251)}\). High concentrations of albumin and other plasma proteins, including transferrin, and \(\alpha\)-lactalbumin, appear to sequester free copper ions and prevent copper induced oxidation\(^{(125)}\). At physiological concentrations the water soluble antioxidant vitamin C has been demonstrated to scavenge free radicals\(^{(89,252-254)}\) (aqueous peroxyl, tyrosyl and superoxide anion radicals) and oxidants\(^{(183,255)}\) (hypochlorite, peroxynitrite) in the fluids similar to those surrounding LDL in the micro-domains of the artery wall. Vitamin C may oxidise the histidine residues of apolipoprotein B rendering them unable to bind copper ions and thus limit LDL oxidation\(^{(252,256,257)}\). Furthermore, ascorbic acid can destroy lipid hydroperoxides in the LDL\(^{(256)}\). Ascorbic acid and several other water soluble antioxidants, glutathione and ubiquinol-10, are able to regenerate LDL associated \(\alpha\)-tocopherol from the \(\alpha\)-tocopheroxyl radical at the water-lipid interface\(^{(258,259)}\).

Kontush and co-workers have established a model which allows total lipoprotein oxidisability to be evaluated with the plasma components present; diluted plasma is oxidised by copper ions and the increase in conjugated dienes is monitored\(^{(10)}\). In this system plasma albumin and thiol groups are strongly correlated with duration of lag phase and \(\alpha\)-tocopherol, carotenoids and vitamin C, influence the maximal rate of oxidation. These results suggest antioxidants present in the extracellular fluid may have an important role in reducing LDL oxidation in vivo. Furthermore, antioxidant enrichment of plasma in vitro\(^{(260)}\) or through supplementation of the diet of rabbits\(^{(10)}\) reduced the susceptibility of plasma to oxidation. However in this model of oxidation, the plasma was diluted extensively resulting in the components of the plasma in very low concentrations compared with the situation in vivo and a high ratio of copper ions to plasma lipoproteins.

The formation of complexes between LDL and arterial proteoglycans, such as heparin and chondroitin sulphate has been found to make LDL more susceptible to oxidation in vitro\(^{(14,122)}\). Low density lipoprotein that has been precipitated with proteoglycans or glycosaminoglycans in vitro and then resolubilised is thought to imitate LDL which has formed reversible associations with proteoglycans in the arterial intima\(^{(261,262)}\). Low density lipoproteins which have been isolated from the plasma in this way show increased susceptibility to copper ion oxidation\(^{(14)}\). This precipitated resolubilised LDL has an altered structure\(^{(14,263)}\) and is bound and internalised more efficiently than native LDL by the apolipoprotein B/E receptor of cultured macrophages\(^{(264)}\). It is possible this LDL is structurally altered by this association resulting in increased access of the copper ions to the inner core components which are highly susceptible to oxidation.
2.3.1.4 Association of lag time with CHD

A number of case-control studies have reported an association between increased susceptibility of LDL to copper ion oxidation, as assessed by lag time, and risk of CAD. Regnström and co-workers examined the susceptibility of LDL to oxidation in patients who had an acute MI and recorded that the duration of lag time was inversely related to the severity of coronary atherosclerosis.\(^{(216)}\) De Rijke and co-workers reported oxidative resistance was lower in by-pass patients who displayed progression of their atherosclerosis compared with controls who remained stable.\(^{(265)}\) Furthermore, CAD patients had significantly shorter lag times compared with control subjects\(^{(266)}\) and this relationship was independent of other risk factors for CAD. This finding is in agreement with a number of other studies in CAD patients\(^{(267-269)}\) although not all.\(^{(270\text{--}271)}\) Angiography is a sensitive technique for the diagnosis of CAD however studies which have used less sensitive methodology may result in the inclusion of asymptomatic patients with subclinical CAD in the control group, and this may obscure any differences between cases and controls. Several studies have reported LDL from patients with known risk factors for CVD, such as hypertension, hypercholesterolemia, display increased susceptibility to oxidation.\(^{(272-275)}\) This provides further support to the hypothesis that the susceptibility of LDL to oxidation may be related to the rate of atherosclerosis. However, case-control studies can not determine cause and effect. Prospective studies are required to determine whether increased susceptibility of LDL to oxidation precedes the formation of arterial lesions.

2.3.2 Other models of LDL oxidation

At present there is considerable interest in the development of models which may more closely reflect LDL oxidation in the micro-domains of the artery wall than copper ion stimulated oxidation of isolated LDL. These models have focused on oxidative modification of LDL by physiological pro-oxidants and in the presence of extracellular components. Hæmin is a iron-containing product of hæmoglobin degradation. It is able to enter hydrophobic domains and oxidise LDL, in vitro, particularly in the presence of hydrogen peroxide.\(^{(13)}\) When native LDL is oxidised by hæmin/H\(_2\)O\(_2\) there is a lag time in lipid peroxidation, measured by hæmin degradation, and this can be prolonged by high concentrations of ascorbate and vitamin E.\(^{(13)}\) The degradation of hæme in the presence of H\(_2\)O\(_2\) appears to be accelerated with low levels of betalipoprotein deficient serum (BLPDS) or human serum albumin.\(^{(12)}\) Furthermore, elevated levels of TBARS are also formed when glycosaminoglycan treated LDL is oxidised with hæmin/H\(_2\)O\(_2\) in the presence of low levels of BLPDS.\(^{(12)}\) Moreover the addition of physiological levels of ascorbate and vitamin E do not appear to influence the maximal rate of hæmin degradation under these conditions. The low levels of albumin which stimulated peroxidation of native and glycosaminoglycan treated LDL in these in vitro experiments are comparable with the concentration in regions of the artery wall.\(^{(276)}\) The action of BLPDS may be mediated through interactions between hæme and albumin, and it appears redox dependent with antioxidants reducing the maximal rate of oxidation.
Activated phagocytes may release MPO, a hæme-containing enzyme, and hydrogen peroxide (H₂O₂). Atherosclerotic lesions contain products of LDL which appear to have been oxidised by MPO/ H₂O₂, supporting the hypothesis that MPO is a potential pro-oxidant of LDL in vivo. The mechanism underlying peroxidase-catalysed oxidation of LDL differs from LDL oxidation by copper ions. Peroxidases do not directly oxidise lipids but radicals derived from protein, tyrosine or alpha-tocopherol appear to mediate oxidation. (11)

Horse radish peroxidase (HRP), an enzyme derived from plants, also oxidises isolated LDL via a protein radical. (11) Horse radish peroxidase has been suggested as a model for transition metal ion independent oxidation of LDL in vivo. The role of alpha-tocopherol and other radical forming antioxidants in reducing LDL susceptibility to oxidation in HRP/H₂O₂ system has been controversial. Santanam and co-workers found lag time was reduced with increasing levels of alpha-tocopherol and this was consistent with a pro-oxidant role for alpha-tocopherol. (277) In contrast, Kalyanaraman and co-workers found alpha-tocopherol reduced the initial rate of conjugated diene formation in a concentration dependent manner. (11) The prevailing pro or antioxidant behaviours of alpha-tocopherol may reflect the levels of free alpha-tocopherol. The antioxidant behaviour of alpha-tocopherol was predominant when LDL was enriched with alpha-tocopherol before isolation rather than when it was added directly to the LDL stock. In the HRP/H₂O₂ system, the radical forming capacity of the antioxidant may be pivotal in determining its behaviour. Probuco (277) and butylated hydroxytoluene (BHT) (11,277) inhibited oxidation at very low concentrations whereas diphenylphenylenediamine increased oxidation. (277) All are powerful antioxidants, but probucol and BHT are incapable of forming propagating radicals. (277) In the HRP/H₂O₂ system tyrosine also forms a radical (tyrosyl radical) and this may be capable of inducing oxidation. However tyrosine stimulated oxidation appears to be dependent on the levels of free tyrosine and is also influenced by the concentrations of alpha-tocopherol in LDL. (277) The effect of other elements present in the serum and extracellular matrix have not been studied in a peroxidase stimulated model of LDL oxidation.

### 2.4 Natural antioxidants

The Mediterranean diet, rich in fruits, vegetables, fish and olive oil, is correlated with a lower incidence of CHD. (279,280) These diets contain a variety of anti-atherogenic agents, including antioxidants such as carotenoids, tocopherols, vitamin C, flavonoids and other polyphenolic compounds. (281-283) Several of these antioxidants are the focus of this thesis, namely the carotenoid lycopene, vitamin E and vitamin C, and will be reviewed in more detail in the following section.
2.4.1 Lycopene

2.4.1.1 Antioxidant properties of lycopene

The antioxidant and lipophilic properties associated with lycopene, along with its characteristic deep red colour, are a function of the structure of this carotenoid. Lycopene has a polyene chain backbone, consisting of forty carbon atoms linked by a system of alternate double and single bonds, with flexible end groups. High performance liquid chromatography enables specific lycopene isomers to be separated and quantified. In nature, lycopene occurs in the all-trans-isomer but undergoes trans-to-cis isomerisation if exposed to light, heat or during some chemical reactions. Plasma mainly contains a mixture of all-trans and 5-cis-lycopene isomers, whereas in the tissues cis-isomers predominate. The biological significance of the various isomers of lycopene is uncertain.

Lycopene is one of the major carotenoids in LDL where it is incorporated within the inner core probably due to its hydrophobic properties. Lycopene is similar in structure to a polyunsaturated fatty acid, with an allylic methylene group at the C-4 position and a high number of double bonds. Thus, lycopene may be oxidised in preference to a polyunsaturated fatty acid. The rapid reaction of lycopene with reactive oxygen species may reduce the availability of free radicals to react with other molecules such as unsaturated lipids, and thereby reduce the level of damage caused to these molecules. Studies have shown that lycopene effectively reduces LDL oxidation in vitro, by quenching singlet oxygen and scavenging peroxyl radicals and this results in the formation of a lycopene carbon centred radical. This carbon centred radical is stable but the lycopene radical formed from it by interaction with oxygen is not, and is capable of inducing peroxidation. Therefore in the presence of oxygen, lycopene may behave as a pro-oxidant. However, in healthy tissue the partial pressure of oxygen is low and antioxidant rather than pro-oxidant reactions are favoured. Also other antioxidants in the immediate vicinity, such as vitamin E and vitamin C may limit the pro-oxidant behaviour of lycopene.

In plasma the major oxygen radical of lycopene, lycopene-5,6-expoxide, is quickly removed. Supplementing healthy people with lycopene has been demonstrated to reduce LDL oxidation. Agarwal and co-workers compared the effect of three high lycopene treatments (spaghetti with tomato sauce, tomato juice and Oleoresin®) with a placebo treatment on LDL oxidation in nineteen healthy subjects. After seven days all of the lycopene regimens increased plasma lycopene three fold, and oxidation was reduced by 25%, as determined by TBARS, in the LDL and serum. Lycopene may improve the intrinsic resistance of LDL to oxidation. A preliminary uncontrolled study reported plasma lycopene increased three fold following seven days of tomato juice consumption in healthy people and this corresponded to a 50% increase in lag time. A similar study by Steinberg and co-workers reported tomato juice supplementation over four weeks increased the resistance of LDL to oxidation by 25%. The lower magnitude of the change in lag time in this study
may be an artefact of cigarette smoking, as previously smokers have been found to have lower levels of serum lycopene and higher levels of oxidation products compared with non smokers.\textsuperscript{(35)} The potential reduction in LDL oxidation with increasing plasma lycopene is in agreement with a large study comparing risk factors for CHD in middle aged men. Intrinsic resistance of LDL to oxidation was 15\% shorter in the region with mean plasma lycopene levels which were two fold lower and CHD rates were higher.\textsuperscript{(19)}

\textbf{2.4.1.2 Dietary sources of lycopene}

Lycopene intake from the diet is estimated to be 1-4 mg a day and typically contributes 30\% of the total carotenoid intake.\textsuperscript{(305-307)} Dietary intake of lycopene reduces with age and cigarette smoking\textsuperscript{(308,309)} and in some populations seasonal variations have been observed.\textsuperscript{(306,310,311)}

Tomatoes are rich in lycopene, with tomato products frequently supplying over 85\% of the lycopene in the diet.\textsuperscript{(312-315)} The lycopene concentrations of tomato products depend on the variety of tomato\textsuperscript{(314)} and processing conditions.\textsuperscript{(316,317)} The bioavailability of lycopene from tomato products is improved with heat treatment,\textsuperscript{(316)} due to the release of lycopene from the plant cell wall components and improved lipid solubility.\textsuperscript{(288,317,318)} Lycopene is also available as a dietary supplement, Oleoresin\textsuperscript{®}, which contains 5\% all-trans-lycopene in the extracted lipid components of tomatoes.

Dietary intake of lycopene is the primary determinant of plasma levels in healthy people.\textsuperscript{(319,320)} Increasing the consumption of tomato products in the diet has been shown to substantially increase blood levels of lycopene.\textsuperscript{(32,34-36)} However, plasma lycopene levels often show poor correlations with total fruit and vegetable intake\textsuperscript{(321)} or dietary lycopene.\textsuperscript{(320)} Inadequacies with food composition databases and dietary assessment methodologies may be key factors in this apparent discrepancy. Many food composition databases do not have complete and accurate data for lycopene.\textsuperscript{(322)} Historically carotenoids were included in food composition databases due to their role as precursors of vitamin A consequently carotenoid data was often absent if the carotenoid lacked provitamin A activity, as is the case with lycopene.\textsuperscript{(323)} Furthermore, prior to the use of HPLC carotenoid analysis lacked specificity and sensitivity.\textsuperscript{(285)} It has been demonstrated that dietary estimates of lycopene are almost three fold higher when dietary data is re-analysed with specialist carotenoid food composition databases.\textsuperscript{(324,325)} However the New Zealand food composition database currently does not include lycopene data.\textsuperscript{(326)} Therefore it would be difficult to accurately estimate lycopene intake in the New Zealand population. Researchers\textsuperscript{(305,306)} have found good correlations between plasma lycopene and intake of dietary lycopene when estimated from a four day diet record and analysed by specialist carotenoid databases.\textsuperscript{(285,315)} In contrast, food frequency questionnaires are often used to successfully estimate the intake of other carotenoids but this form of dietary assessment is unsuitable for lycopene as it is concentrated in a small number of foods.\textsuperscript{(324,325)}
2.4.1.3 Bioavailability, metabolism and regulation of lycopene

In foods lycopene is tightly bound to macromolecules, including proteins and fibre. Mastication destabilises protein-lycopene complexes facilitating dispersion. Co-ingestion of lycopene with lipid may extract it from the food matrix into the lipid phase of the chyme, then as a result of the action of bile salts and pancreatic lipases, lycopene-containing lipid droplets enter the duodenum and form multilamellar lipid vesicles. Studies have shown lycopene is better absorbed from tomatoes when consumed with lipid (spaghetti sauce versus tomato juice). Conversely non-absorbable fat analogues, including sucrose polyesters and drugs which affect cholesterol absorption significantly reduce serum lycopene. The lycopene supplement, Oleoresin® appears to have lower bioavailability compared with tomato products especially those which are heat treated which may reflect differences in the bioavailability of isomers. Other carotenoids may interact with lycopene during absorption although the effect has been inconclusive. Lycopene concentration in the blood increased by 176% after healthy volunteers took 20 mg/day of beta-carotene for two years in a large placebo-controlled clinical trial. Conversely, short term beta-carotene supplementation trials did not appear to significantly alter plasma lycopene. It is unclear if other carotenoids facilitate or inhibit lycopene absorption.

The movement of lycopene from the mixed lipid micelle into the mucosal cells of the duodenum appears to take place via passive diffusion. Lycopene is taken up into the mucosal cell and packaged into chylomicrons with phospholipids and triglycerides from the meal, thus lipid increases bioavailability. Typically, only a small percentage of the lycopene from the meal will be taken up by the intestinal cell and if this is not incorporated into chylomicrons within a few days it is lost when the mucosal cells are sloughed off into the lumen of the gastrointestinal tract. Lycopene is incorporated into the chylomicrons and VLDL, and during the following 48 hours concentrations rise in the other lipoprotein fractions. Three quarters of the lycopene in the blood is in LDL while the remainder is in HDL. The transfer rate between beta-carotene and presumably lycopene, and native lipoproteins is very slow (more than 18 hours) compared to free cholesterol (20 minutes) and even alpha-tocopherol (70 minutes). Lycopene requires triglyceride lipolysis by lipase to transfer into biological lipid structures (mixed micelles, lipoproteins, membranes). The exact mechanism in vivo is unclear but it is independent of lecithin: cholesterol acyl transferase, cholesteryl ester transfer protein or a specific carotenoid binding protein.

Lycopene in the blood of healthy individuals varies between 0.22-1.06 µmol/l and contributes between 21-43% of the total carotenoids. There is wide variation in plasma lycopene between people and levels reduce with age probably reflecting different dietary patterns. Plasma levels are relatively stable in an individual and in contrast to other carotenoids, lycopene levels in the blood do not appear to be influenced by BMI, gender, age.
menopausal status,\(^{(344)}\) menstrual cycle,\(^{(345)}\) or moderate alcohol consumption.\(^{(308,346)}\)

Lycopene accumulates in a number of tissues in the body, including the liver, adrenal glands, testes, ovary, adipose and kidney.\(^{(291,347,348)}\) Levels of lycopene are higher in the tissues with more LDL receptors.\(^{(347)}\) Mononuclear cells also appear to contain significant quantities of lycopene.\(^{(349)}\) In feeding studies in healthy adults plasma lycopene levels reach a maximum in about three days\(^{(317)}\) and lycopene stores appear to take twelve to thirty-three days to be depleted in healthy adults with non linear reduction suggesting that there are at least two significant body stores,\(^{(350,351)}\) probably the liver and adipose tissue.\(^{(291)}\) The regulatory mechanism for lycopene accumulation in tissues is unknown.\(^{(352)}\)

### 2.4.1.4 Relationship between lycopene and CHD

Mortality from CHD has been inversely correlated with increased fruit and vegetable intake, and in particular tomato products demonstrate strong correlations.\(^{(353)}\) Tomatoes contain a number of anti-atherogenic compounds with the antioxidant lycopene is in the highest concentration.\(^{(354)}\) Palgi and co-workers reported total carotenoid intake is inversely correlated with CHD mortality.\(^{(355)}\) Lycopene did not show a significant relationship, however in this study poor food compositional data may have obscured associations.\(^{(322)}\) Recently, Kristenson and co-workers reported plasma lycopene was two fold higher in middle-aged men in Sweden compared with men in Lithuania where mortality from CHD is four times lower.\(^{(19)}\) Also, in the ten European countries involved in the EURAMIC study high adipose lycopene was found to be protective against MI.\(^{(18)}\)

The Austrian Stroke Prevention Study reported that low plasma lycopene was significantly associated with subclinical ischaemic brain damage.\(^{(356-358)}\) In contrast, Street and co-workers found patients who had an MI did not have lower plasma lycopene than control subjects,\(^{(359)}\) although lengthy storage of blood samples (15 years) in this study may have influenced the findings as stability data is only available for samples stored up to four years at -70°C.\(^{(360-362)}\) In summary epidemiological evidence appears to support a protective role for lycopene against CHD, which may be conferred from levels of lycopene obtained from the diet. Randomised controlled intervention trials are required to determine the effect of lycopene on risk of CHD.

### 2.4.2 Vitamin E

#### 2.4.2.1 Antioxidant properties of vitamin E

Vitamin E refers to eight compounds and their isomers, tocopherols and tocotrienols. These compounds share a similar 6-chromanol ring structure but differ in the methylene group positions and phytyl side-chain structure.\(^{(363)}\) Alpha-tocopherol and gamma-tocopherol are the predominant vitamin E homologues present in LDL and biological membranes. Alpha- and gamma-tocopherol differ
only in the methylene group substitutions on the chromanol ring and this is responsible for the differences in antioxidant potential observed between these homologues.\(^{(364,365)}\) High performance liquid chromatography allows specific, sensitive and rapid analysis of vitamin E homologues and isomers.\(^{(366)}\)

Vitamin E is located near the surface of the LDL or membrane\(^{(367)}\) with the chromanol head group orientated toward the outer surface while the phytol side chain is buried within the hydrophobic region.\(^{(368-372)}\) This orientation allows the chromanol ring to be in close proximity with the carbonyl groups of the fatty acids. As a result of its location and structure alpha-tocopherol may be oxidised in preference to the fatty acids. The reaction between alpha-tocopherol and a peroxyl radical results in the formation of a tocopheroxyl radical, and this radical may propagate further oxidation.\(^{(231,278,373,374)}\) However water soluble antioxidants, such as ascorbate and ubiquinol-10, are capable of reducing tocopheroxyl radicals to tocopherol.\(^{(231,278,374-376)}\) The orientation of the phenolic hydroxyl group is directed toward the surface at the water-lipid interface\(^{(367)}\) and favours regeneration of tocopherol.\(^{(258,377)}\) Tocopherol is in low concentrations compared with the fatty acids in LDL, thus regeneration of tocopherol may be critical.\(^{(373)}\) However, under conditions of higher oxidative stress or as the tocopheroxyl radical penetrates the LDL core, tocopherol recycling may be insufficient to prevent oxidation.\(^{(378,379)}\)

A number of randomised placebo controlled clinical studies have shown that enriching LDL with alpha-tocopherol through supplementation extends the lag time to copper ion stimulated oxidation of LDL. Supplemental doses of alpha-tocopherol appear to be required to modify LDL oxidation\(^{(23-27)}\) although dose response studies remain unclear. Several dose response studies (alpha-tocopherol 60-1200 IU/day) have reported levels of greater than 400 IU/day for more than two weeks are required to prolong lag time significantly.\(^{(27,28)}\) Conversely, Princen and co-workers found a significant increase in lag time with supplementation of 25 IU/day, although the magnitude of this change was small (5.3 %).\(^{(26)}\)

Recently, vitamin E has been found to modulate endothelial cell interactions with immune cells. This effect has been demonstrated to be attenuated through stimulation of the genes for the expression of adhesion molecules on the endothelial cells and the expression of integrins on the surface of monocytes. Enrichment of human aortic endothelial cells with vitamin E significantly reduces the adherence of these cells in a dose-dependent manner.\(^{(380)}\) Elevated levels of sICAM-1, due to increasing concentrations of native LDL, are also attenuated with vitamin E\(^{(380)}\). Furthermore, when human endothelial cells are activated, the IL-1\(\beta\) induced expression of cell adhesion molecules ICAM-1, VCAM-1, and E-selectin are increased significantly. This effect is attenuated in a dose-dependent manner if the cells are pre-enriched with vitamin E.\(^{(381,382)}\) In response to oxidative stress monocytes produce cytokines and adhesion molecules (integrins). Supplementing healthy volunteers\(^{(28)}\) and athletes\(^{(383)}\) with high doses of alpha-tocopherol (\(\geq\)1200 IU/day) for several weeks reduces the release of IL-1\(\beta\) by monocytes. In addition monocytes incubated with alpha-tocopherol displayed reduced expression of agonist stimulated integrins.\(^{(384)}\) In summary, increasing cell and LDL content of alpha-tocopherol decreases the expression of cell adhesion molecules in vitro and
dietary supplementation with alpha-tocopherol reduces the cellular release of inflammatory cytokines. However the effect of supplementation with alpha-tocopherol, and other antioxidants, on circulating levels of ICAM-1 and VCAM-1 is not yet well understood.

2.4.2.2 Dietary sources of vitamin E

Vegetable oils are the major dietary source of vitamin E(385) and this is primarily gamma-tocopherol. In oils, the contribution of alpha-tocopherol is usually only 7% to 15% with the exception of sunflower, canola, safflower and olive oils in which alpha-tocopherol predominates (e.g. sunflower oil, 48.7 mg/100g). Other important dietary sources of vitamin E are polyunsaturated margarines, some nuts and wheat germ.(326) A recommendation to increase intakes of vitamin E from the dietary sources would also result in a substantial increase in dietary fat due to the association of vitamin E with lipids in foods.

Most countries have set recommended daily intakes of vitamin E between 7-10 mg/day for adults(386) and this level is readily achieved from the New Zealand diet.(387) However, the dose of vitamin E which may be required to alter oxidative parameters may be significantly higher (40 fold) than usual dietary levels,(23-27,388) The safety of pharmacological doses of vitamin E has been extensively tested and a dose of up to 900 mg per day is considered to be safe and without side effects in healthy volunteers.(389-392)

2.4.2.3 Bioavailability, metabolism and regulation of vitamin E

Approximately 40% of alpha-tocopherol is absorbed at levels of vitamin E in the dietary range, but this percentage decreases with pharmacological doses.(393) Concurrent intake and digestion of dietary fat facilitates absorption of vitamin E through increasing pancreatic secretions and bile salts. Vitamin E is taken up directly by enterocytes in the intestine then secreted into the lymphatic system via chylomicra, before being taken up by the liver and released back into the circulation in association with VLDL.(394) Alpha-tocopherol which is secreted from the liver in VLDL may travel with the VLDL core during the conversion to LDL, or return to the liver in VLDL remnants.(395) In the plasma, almost all of the alpha-tocopherol circulates in association with lipoproteins (96%)(396) where it is able to transfer readily between LDL and HDL without the assistance of a transfer protein.(397-399)

The liver is the primary site of regulation of circulating levels of vitamin E,(395,400,401) and it exhibits a distinct preference for the alpha-tocopherol homologue,(395,402,403) In addition, the RRR-alpha-tocopherol is favoured over other stereoisomers and the synthetic form (racemic or all-rac-alpha-tocopherol).(403-405) This discrimination between the tocopherol isomers and homologues is related to a specific tocopherol binding protein which recognises the fully saturated methylated aromatic ring, the saturated phytol side chain and the stereochemical RRR-configuration of the methyl groups branching the side chain.(406-409) Tocopherol-binding protein appears to be critical for the regulation of plasma alpha-tocopherol within a narrow range of concentrations, with pharmacological doses of
alpha-tocopherol increasing levels by as little as two fold. Excess vitamin E, including non alpha-tocopherol homologues are probably lost through excretion in the bile.

The uptake of tocopherol from plasma by the tissues occurs by both a LDL receptor mechanism and also by receptor-independent pathways. Adipose tissue is the major peripheral tissue depot, containing up to 90% of the alpha-tocopherol. Tissue levels can be increased in response to high-doses of alpha-tocopherol (800 IU/day), although the observed increase is relatively modest (10% to 20%). Monocytes and erythrocytes have elevated levels of alpha-tocopherol with high dose supplementation, and it is presumed based on the available information that levels are concomitantly increased in endothelial cells.

### 2.4.2.4 Relationship between vitamin E and CHD

Several large observational studies have reported dietary intake of vitamin E is inversely related to mortality from CHD. Prospective studies also show higher vitamin E intakes are associated with reduced risk of CHD. The largest of these studies, the Nurse’s Health and Health Professional’s studies found a 34% to 44% reduction with the highest quintile of vitamin E intake and the greatest benefit in people who consumed 100-250 IU per day for more than two years over the four to eight year follow-up. In agreement with these findings the cross-cultural MONICA Study reported the incidence of CHD was inversely related to vitamin E concentration in the plasma. Furthermore, 60% of the difference between the high and low CHD regions in the MONICA study could be explained by plasma vitamin E.

Low plasma vitamin E has been reported in patients with angina and in elderly people with subclinical ischaemic brain damage. In contrast, several other case-control studies have reported less compelling findings between CVD and blood or adipose tissue levels of vitamin E. However, the inclusion of control subjects with asymptomatic subclinical disease may confound the relationship between vitamin E and CVD in these studies. Also epidemiological data suggest the beneficial effects of vitamin E may be confined to high doses and a number of case-control studies did not include people who took vitamin E supplements.

Case-control studies are unable to determine if an observed reduced risk is directly attributable to vitamin E or whether vitamin E is a biomarker for some other dietary or lifestyle factor that improves coronary risk. Randomised controlled intervention trials are required to determine the role of vitamin E in the development of coronary disease. The most significant trial showing a protective effect for vitamin E is the Cambridge Heart Antioxidant Study (CHAOS). Nonfatal MI was reduced by 77% in CAD patients taking 400 or 800 IU alpha-tocopherol daily for an average of sixteen months. Comparing these findings with cholesterol lowering trials, the benefits of vitamin E on non fatal MI are observed in a shorter time. However fatal MI actually tended to increase in the CHAOS study in those patients taking vitamin E. Although it can not be completely dismissed that high
dose vitamin E may be harmful, other factors require consideration when interpreting this finding. In particular, few MI events occurred in the entire study population, especially fatal MI and the difference was not significant between the vitamin E and placebo groups for fatal MI (36 verses 26). In addition, most of the fatal MI events occurred early in the study and survival curves show the onset of treatment benefit was seen after 200 days. With respect to mortality from MI, the CHAOS study may have been underpowered and not of sufficient duration to evaluate vitamin E treatment. In agreement with findings of the CHAOS study another trial in patients with CAD found supplementing with 100 IU of vitamin E daily for two years was independently associated with a reduction in progression of CAD, as determined by angiography.(20,21) In contrast to these findings, two large intervention trials have reported no benefit of vitamin E supplementation on CVD in healthy populations. The Alpha-tocopherol/Beta-Carotene Cancer (ATBC) Prevention Study reported CAD events in apparently healthy male smokers did not change over six years with supplementation of 50 mg daily of vitamin E.(427) Similarly, a large vitamin E supplementation trial in 29,000 people in Linxian, China, found no reduction in CVD after five years of supplementation with 30 IU/day of alpha-tocopherol (428). In the latter study the population had a poor nutritional status at baseline, including low plasma levels of vitamin E. In addition, the level of supplementation used in both studies may have been too low, as doses were lower than the level reported to offer protection in the health professional studies (418,421) Furthermore, the primary objective of both the ATBC study and Linxian trial was cancer prevention and this may have influenced the selection of subjects in these studies. Moreover, in the case of the Linxian trial, the prevalence of CVD was extremely low in this region of China. In summary, vitamin E may play a protective role in the development of CVD, but pharmacological doses of vitamin E may be required to achieve this protection.

2.4.3 Vitamin C

2.4.3.1 Antioxidant properties of vitamin C

A number of compounds exhibit the biological activity of ascorbic acid and these are collectively known as vitamin C.(429) The predominant form of vitamin C in the tissues and foods is ascorbic acid. Also the steroisomer of ascorbic acid, erythorbic acid, is often added during food processing as an antioxidant.(430) Dehydroascorbic acid is the most significant oxidised product of ascorbic acid often present in low concentrations in the tissues.

While there are numerous methods available for the analysis of vitamin C in the plasma, HPLC and fluorescent methods are the most sensitive and specific.(431) High performance liquid chromatography can readily identify ascorbic and dehydroascorbic acid.(432) Metaphosphoric acid is usually added to serum or plasma when the sample is collected to stop degradation of vitamin C. However, the addition of metaphosphoric acid to the sample coupled with the time delay associated with HPLC may increase levels of dehydroascorbic acid. Recently, a
number of automated fluorescent methods have been developed measuring the fluorescent product which forms when dehydroascorbic acid is coupled with o-phenylenediamine. Total vitamin C may be reliably quantified using fluorescence after the conversion of ascorbic acid to dehydroascorbic acid by the ascorbic acid oxidase. These methods more cost effective and convenient than HPLC while still retaining the specificity and sensitivity for total vitamin C.

Ascorbic acid is part of a complex system providing protection against free radical damage both in extracellular fluids and cells. Vitamin C has been shown to scavenge aqueous superoxide and hydroxyl radicals and act as a chain-breaking antioxidant in lipid peroxidation. Free radicals in the aqueous phase react with ascorbic acid to produce ascorbyl free radicals. The ascorbyl free radical is relatively stable and is an effective free radical scavenger, by reacting with a second ascorbyl radical forming one molecule of ascorbic acid and a second of dehydroascorbic acid which is subsequently reduced. Conversely, the addition of vitamin C to extensively oxidised LDL in the presence of metal ions may actually stimulate lipid peroxidation via the formation of the alkoxy radical.

Vitamin C may reduce the oxidation of LDL, especially when oxidative stress is high. Resisnce of LDL to copper ion stimulated oxidation was significantly increased after subjects who were habitual smokers when given pharmacological doses of vitamin C. In contrast, vitamin C did not alter LDL oxidation in a similar study in non smoking men. Smokers have lower natural antioxidant levels and increased free radical activity which may result in elevations of hydroperoxides in LDL. The effect of vitamin C on oxidative resistance of LDL is difficult to determine using oxidation procedures where LDL is isolated from plasma however added vitamin C completely inhibits LDL oxidation in vitro.

Vitamin C may also affect monocyte adhesion through specific actions on cell adhesion molecules. Cultured monocytes isolated from smokers had elevated expression of the integrin CD11b and supplementing these people with high doses (500 mg/day) of vitamin C reduced CD11b expression and reduced monocyte adhesion to the endothelial cells. The expression of other cell adhesion molecules, including ICAM-1 and VCAM-1, are decreased with vitamin C treatment in subjects under oxidative stress due to smoking. The effect of vitamin C on these adhesion molecules in other people under oxidative stress has not be studied.

2.4.3.2 Dietary sources of vitamin C

Most of the vitamin C in the New Zealand diet is obtained from foods of plant origin, primarily citrus fruits, green vegetables, peppers, tomatoes, berries and potatoes. The recommended daily intakes for vitamin C vary between 30-80 mg daily and most people in New Zealand would consume in excess of this amount.
2.4.3.3 Bioavailability, metabolism and regulation of vitamin C

Vitamin C is nearly completely absorbed at low doses (less than 30 mg/day) and this only reduces slightly with increasing intakes under 180 mg/day. Above this amount absorption decreases dramatically. Delaying gastric emptying or dividing the dose over several hours will increase the total level absorbed. Ascorbic acid is absorbed by active transport, driven by ATP hydrolysis, or indirectly via a sodium gradient. Also the absorption of ascorbic acid and its entry into cells may be facilitated by conversion into dehydroascorbic acid, which penetrates membranes better than ascorbic acid at physiologic pH. After entry into the cell, dehydroascorbic acid can be reduced to ascorbic acid. It is not known whether this regeneration is chemically or enzymatically driven under physiological conditions.

Absorbed ascorbic acid equilibrates rapidly with the extracellular pool. Ascorbic acid is present in all tissues in the body, and generally is in higher concentrations in tissues than in the plasma. Levels are particularly high in the glandular organs, including adrenals, pituitary and spleen, although muscle provides the largest reservoir due to its mass. Maximum body pool for vitamin C is a little over two grams and this is achieved with an intake of 120 mg/day in healthy adults. Vitamin C body pool size increases with intake up to this maximal size, and as the renal threshold is exceeded (approximately 45 µmol/l) an increased proportion of the intake is excreted.

2.4.3.4 Relationship between vitamin C and CHD

Several prospective studies have shown an inverse relationship between high vitamin C intakes and risk of mortality from CVD. Conversely, other prospective studies have found either no association between vitamin C intake and risk or a weak non significant trend. A contributing factor to these inconsistent findings may be the methods of dietary assessment. A plasma vitamin C concentration of less than 12 µmol/l has been significantly associated with increased CVD mortality and levels of greater than 26 µmol/l appear protective. These findings have also been supported by case-control studies examining vitamin C in the blood and CHD. Leukocyte vitamin C is thought to reflect vitamin C status better than plasma, and reduced leukocyte vitamin C was reported in CAD patients compared with healthy controls. In a preliminary intervention study, supplementation with 500 mg/day of ascorbate significantly reduced coronary angiographic restenosis.

2.5 Diabetes and atherosclerotic disease

Diabetes mellitus is a heterogeneous condition comprising of several distinct pathophysiologic disorders of carbohydrate metabolism, each of which ultimately manifests with hyperglycaemia. Type 1 diabetes mellitus is primarily due to autoimmune destruction of the pancreatic islet beta cells. The more prevalent form of diabetes mellitus, type 2, results from a combination of insulin resistance and
relative insulin deficiency. Type 2 diabetes mellitus in New Zealand has been estimated to affect 10.4 people per 100,000, which is similar to rates in the USA, Britain and Europe. Type 2 diabetes directly caused 373 deaths in NZ in 1987, although mortality statistics probably underestimate the importance of diabetes, as most deaths related to diabetes result from complications, particularly coronary heart disease (CHD). Seventy-five to 80% of adult diabetic patients die from CHD, cerebrovascular disease, or peripheral vascular disease.

2.5.1 Risk factors

Mortality from atherosclerotic vascular diseases is two to three fold higher in diabetic populations compared with non diabetic populations. Diabetic patients have numerous cardiovascular risk factors, such as hypertension, obesity and hyperlipidaemia however epidemiological data indicate that traditional risk factors account for less than half of the excess risk of CHD in this group. People with type 2 diabetes may have other risk factors which increase their risk of developing atherosclerosis.

Hyperglycaemia induces a number of adverse biochemical reactions which may be responsible for atherogenic complications. Recently, attention has focused on the role of increased oxidative stress generated by hyperglycaemia and protein glycation. Raised levels of glucose in the blood have been demonstrated to react non-enzymatically with amine groups on proteins forming a Schiff base followed by Amadori rearrangement to fructoselysine. This reversible glycosylation of the amino group underlies the formation of glycated haemoglobin, a marker of chronic glycaemic control. Lipoproteins, apolipoproteins and collagen in the arterial wall are also vulnerable to glycation as a result of hyperglycaemia, and glycation is elevated even when metabolic control is good in diabetic patients. Numerous metabolic abnormalities are associated with glycation of LDL, including diminished recognition of LDL by the regulated LDL receptor, retardation of the clearance of LDL from the plasma, enriched uptake of LDL by macrophages, increased platelet aggregation, increased binding of the LDL in the vessel walls and generation of oxygen free radicals. Glycation may also result in the oxidation of fructoselysine to form an irreversible AGE-product, such as Nε-(carboxymethyl)lysine, pentosidine, and pyrroline. In diabetic patients AGE-products have been significantly correlated with vascular complications.

Diabetic patients frequently have raised plasma triglycerides and this may result in changes to their lipoproteins and an increase in coagulation activities. The diabetic dyslipidaemia which is associated with hypertriglyceridaemia is characterised by chylomicronaemia, increased VLDL and remnants both with raised levels of apolipoprotein E and triglyceride enriched LDL and HDL.

Chylomicronaemia and increased VLDL and remnants are associated with increased cholesterol deposition in the artery wall. Triglyceride enriched LDL tends to be smaller and denser, and this phenotype has been correlated with increased CHD in
non diabetic populations (475). Hypertriglyceridaemia increases the coagulation activities (476) of thrombogenic factors and reduces the concentrations of tissue plasminogen activator inhibitor (477). Also, platelet aggregation and concentrations of lipoprotein (a) are increased in diabetic patients and these may further contribute to coagulation (40).

Insulin resistance and hyperinsulinaemia appear to play an important role in the pathogenesis of atherosclerosis in diabetes (37). Central adiposity is associated with insulin resistance and compensatory hyperinsulinaemia (40). Insulin, with other growth factors (478), can potentially promote cell cholesterol ester accumulation. Also, high levels of insulin may have a direct affect on arterial wall function (40). Patients with type 2 diabetes have multiple pro-atherogenic risk factors and a number of these may increase oxidative stress in these patients.

### 2.5.2 Oxidative stress

#### 2.5.2.1 Products of oxidation

Oxidative stress is increased in patients with type 2 diabetes, as evidenced by elevated levels of a number of products of lipid oxidation. Recently concentrations of the F₂isoisoprostane, 8-epi PGF₂α, were found to be three fold higher in the plasma of diabetic patients (187,479). In agreement with these findings, circulating levels of TBARS have been reported to be elevated in diabetic patients compared with healthy controls (43,49,480-484) and levels were further increased if metabolic control was poor or cholesterol levels were raised. Higher levels of lipid hydroperoxides (485) and cholesterol peroxides (486) have been measured in the plasma of diabetic patients compared with healthy control subjects. Lipid hydroperoxides are predominantly associated with lipoproteins in the plasma and mainly carried by HDL in healthy individuals (374). Lipid hydroperoxides are reported to be higher in the HDL fraction of diabetic patients (487). Increased levels of auto antibodies to MDA-LDL (39) and an increased proportion of negatively charged LDL (488) a small oxidised fraction of LDL, provide support for the presence of oxidatively modified LDL in diabetic patients. Erythrocytes in diabetic patients also have raised levels of MDA (482,489) and show increased susceptibility to oxidation (490).

#### 2.5.2.2 Antioxidants

Patients with type 2 diabetes appear to have inadequate antioxidant protection. Plasma lycopene levels were reported to be 50% lower in diabetic patients compared with age-matched control subjects (49). All of the lycopene in the plasma is associated with lipoproteins thus it is probable that LDL lycopene levels are also significantly reduced. Duration of diabetes (319) rather than dietary intake appears to predict the lower levels of lycopene observed in diabetic patients (491).

Vitamin E in the plasma is primarily associated with the lipoproteins (396). Thus, the lipoprotein abnormalities often observed in diabetic patients affect the
The distribution of vitamin E has been found to be depleted in diabetic patients. However, raised levels of LDL in the plasma of diabetic patients may restore vitamin E concentrations to the normal range. Total vitamin C in diabetic patients is often similar to healthy individuals but the proportion of ascorbic acid is reduced. Hyperglycaemia may reduce the renal reabsorption of ascorbic acid. Continuous infusion of glucose in people without diabetes has been demonstrated to increase urinary ascorbic acid and diabetic patients with microalbuminuria have elevated ascorbic acid excretion. Also intracellular ascorbate is reduced in rats with diabetes. Consistent with reduced antioxidant levels in the plasma, total antioxidant capacity was reported to be lower in diabetic patients compared with non-diabetic subjects. These alterations in the levels of important intrinsic antioxidants in the LDL, including vitamin E and lycopene, and altered levels of ascorbic acid in the extracellular fluid may critically undermine the antioxidant defence system in diabetic patients.

2.5.3 LDL susceptibility to oxidation

Diabetic patients often have higher levels of small dense LDL and the proportion may increase with poor blood glucose control. This LDL subfraction is more susceptible to oxidation and depleted in intrinsic antioxidant levels. Yoshida and co-workers and Lintott have reported the decreased resistance of isolated LDL to copper ion stimulated oxidation, indicated by a 20% shorter lag time in diabetic patients. In contrast Mol and co-workers using similar experimental procedures did not report a significant difference in lag time. However, the LDL isolated in the latter study contained very high levels of vitamin E and these levels were similar among the patients and the healthy control subjects. The ratio of vitamin E to peroxides in the LDL isolated from diabetic patients is a strong determinant of lag time. Several studies have also reported levels of TBARS from LDL oxidation are increased in diabetic patients compared with healthy controls.

The increased oxidative stress associated with diabetes may be attenuated by antioxidants. In a randomised placebo-controlled trial, conducted in twenty-one men with type 2 diabetes, supplementation with 1600 IU/day of alpha-tocopherol for ten weeks increased plasma and LDL alpha-tocopherol four fold and reduced the susceptibility of LDL to copper ion stimulated oxidation by 60%. Fuller and co-workers reported comparable findings in a similar study in patients with diabetes after taking 1200 IU/day alpha-tocopherol for eight weeks compared patients following the placebo treatment. Plasma and LDL alpha-tocopherol increased 2.4 fold and lag time increased by 41%. This study included twenty-eight patients with type 1 (46%) and type 2 (54%) diabetes mostly men and four women, who were slightly younger, slimmer but with higher glycated hemoglobin levels than the patients in the previous study. The doses of vitamin E taken in these studies were very high, over three fold higher than the pharmacological doses which reduced
LDL oxidation in non diabetic subjects.\(^{(23-27)}\) Mol and co-workers recently reported 600 IU/day of alpha-tocopherol supplemented for four weeks increased lag time by 52% in eleven patients with type 2 diabetes.\(^{(481)}\) However, this study was not placebo-controlled and the basal alpha-tocopherol levels were extremely high.

### 2.5.4 Inflammation markers and cell adhesion molecules

Hyperglycæmia may increase inflammatory proteins by altering signal transduction pathways which increase the expression of genes for these proteins through the activation of NF-κB.\(^{(463)}\) Studies in animals have established the signal transduction pathways, diacylglycerol (DAG) and protein kinase C (PKC) can be sustained chronically in a number of vascular cells in response to hyperglycæmia.\(^{(509,510)}\) Advanced glycosylation end-products may modulate cellular functions through a specific cell surface receptor (RAGE) or RAGE which has complexed with a lactoferrin-like polypeptide.\(^{(463)}\) The binding of AGE-products to these receptors results, probably through multiple pathways, in the induction of cellular oxidative stress.\(^{(463)}\) Receptors for AGE-products are present on endothelial cells, mononuclear phagocytes and smooth muscle cells.\(^{(511)}\)

Furthermore, the identification of RAGE as a member of the immunoglobulin superfamily suggests that in addition to interacting with AGE-products, it might serve other functions, such as mediation of cell-cell recognition growth factors, cytokine or other immune responses.\(^{(463,512)}\) Lipid peroxidation and AGE-product formation appear to be mutually reinforcing\(^{(38,513)}\) therefore the generation of a variety of epitopes of oxidised, Amadori, and AGE-product modified LDL may occur in diabetic patients.

Consistent with these activities, diabetic patients have elevated levels of some of the acute phase response proteins and cell adhesion molecules. Levels of plasma C-RP have been reported to be higher in patients with diabetes,\(^{(42)}\) especially those with syndrome X.\(^{(41)}\) The expression of VCAM-1 and ICAM-1 is raised on the vascular endothelial cells of diabetic rabbits.\(^{(514)}\) Diabetic patients have elevated levels of sVCAM-1 and sICAM-1.\(^{(43,515,516)}\) Furthermore, during an oral glucose tolerance test when blood glucose levels increase acutely, there is a corresponding increase in circulating sICAM-1 in diabetic and non diabetic subjects.\(^{(515)}\) Consistent with this finding, high glucose induces ICAM-1 over expression in cultured human endothelial cells and increased ICAM-1 mediated adhesion of monocyte binding to human aortic endothelial cells.\(^{(517)}\) Also cultured human endothelial cells exposed to AGE-products show increased expression of VCAM-1.\(^{(518)}\) It has been reported the accumulation of AGE-products on the vessel walls of diabetic patients may trigger the increase in VCAM-1 expression and the subsequent release of sVCAM-1.\(^{(519)}\)

### 2.5.5 Summary

Overall, it is evident that high dose alpha-tocopherol supplementation decreases the susceptibility of LDL to oxidation in patients with diabetes. However
there is little information on the comparative effects of increased intake of other
dietary antioxidants on LDL susceptibility to oxidation and circulating levels of cell
adhesion molecules and marker of systemic inflammation, C-RP.

2.6 Aims

The studies in this thesis were designed to:

(a) Compare the effect of tomato juice, or supplementation with high doses of
vitamin E or vitamin C on the susceptibility of LDL to oxidation and
circulating levels of a marker of systemic inflammation and cell adhesion
molecules in patients with type 2 diabetes mellitus.

(b) Establish a model of LDL oxidation which incorporates several factors that
may be involved in the oxidation of LDL in the artery wall and determine
characteristics of the model including response to antioxidants.
Chapter 3

Antioxidant supplementation, LDL oxidation and products of inflammatory activity in patients with type 2 diabetes

3.1 Introduction

Patients with type 2 diabetes are at greater risk of developing CHD(37) and commonly measured risk factors for CHD account for only part of this increased risk.(40) Recently, attention has focused on the role of increased oxidative stress generated by hyperglycaemia and protein glycation.(38,119,464) Diabetic patients show evidence of enhanced susceptibility of LDL to oxidation,(505,506,520) chronic systemic inflammation(41,42) and elevated levels of circulating cell adhesion molecules.(43,44,521)

There is epidemiologic and clinical evidence that high intake or levels of vitamin E, vitamin C and lycopene may be associated with a decreased risk of coronary heart disease.(17,18,423) Laboratory studies have also demonstrated potentially antiatherogenic activities of these compounds. Supplementing people with high doses of alpha-tocopherol reduces susceptibility of LDL to oxidation ex vivo (23-27,481,494,508) and decreases the release of pro-inflammatory cytokines from isolated monocytes.(28,383) Also, enriching cultured monocytes with vitamin E decreases the LDL-induced production of cell adhesion molecules, including ICAM-1 and VCAM-1, and their adhesion to human endothelial cells in vitro.(380-382) In individuals who are under increased oxidative stress, due to smoking, high doses of vitamin C have been demonstrated to lead to a small increase in the resistance of LDL to oxidation(29) and reduce monocyte adhesion to cultured endothelial cells.(30) The regular consumption of tomato juice markedly increases the resistance of LDL oxidation in healthy people.(34) Tomatoes and tomato products are the main source of lycopene in the diet(315) and this increased resistance of LDL to oxidation may be due to the nearly three fold increase in circulating levels of the antioxidant lycopene after consumption of tomato juice.(33,34,36) Lycopene is a lipophilic antioxidant present in plasma lipoproteins, including LDL,(245,338,339,341) and it has been shown to inhibit LDL oxidation when added to lipoproteins in vitro.(31) Plasma levels of lycopene appear to be low in patients with type 2 diabetes.(49) The effect of regular consumption of tomato
juice and supplementation with high levels of vitamin E or vitamin C on susceptibility of LDL to oxidation and plasma concentrations of cellular adhesion molecules in diabetic patients has not been extensively studied. The present study was designed to compare the effect of dietary supplementation with tomato juice, vitamin E or vitamin C on the susceptibility of LDL to oxidation and circulating levels of lycopene, sVCAM-1, sICAM-1 and C-RP in patients with type 2 diabetes mellitus.

3.2 Methodology

3.2.1 Participants

Patients with type 2 diabetes were recruited from the Diabetes Clinic, Dunedin Hospital, local general practitioners and by advertisement in the newspaper. Inclusion criteria were (a) age less than 75 years and (b) glycated haemoglobin < 10% and fasting plasma glucose < 11 mmol/l. Exclusion criteria included (a) presence of hepatic or renal disease and (b) cigarette smoking, (c) use of dietary supplements, including antioxidants or (d) treatment with insulin, lipid-lowering drugs or hormone-therapy currently or during the preceding 6 months. The protocol was approved by the Southern Regional Health Authority Ethics Committee and all participants gave informed consent.

At entry to the study patients provided a full clinical history of their hypertension and CVD. Clinical details pertaining to the duration of their diabetes and treatment regimen were obtained. Past smoking habits, medication use, blood pressure and BMI were also recorded.

3.2.2 Study design

The study was a randomised, placebo-controlled parallel trial. A total of 57 patients were randomised to receive either: 800 IU/day α-tocopherol (natural source d-α-tocopherol, Red Seal) n=12; 500 mg/day vitamin C (Redoxin, Roche) n=12; 250 ml twice daily commercial tomato juice (Campbells Foodstuffs, Australia) n=16; or a placebo supplement (gelatine capsule containing pharmaceutical starch) n=17. The tomato juice did not contain added sugar but erythorbic acid (stereoisomer of ascorbic acid) had been added as a preservative. Randomisation was carried out independently using a computer generated scheme with stratification by gender (Excel, Microsoft Office for Windows 95, Microsoft Office Inc.). All patients were instructed not to change their usual dietary habits for the duration of the study and four day checklists were used to estimate the frequency of consumption of tomato products during the study. Patients were asked to take the study supplements with meals. Supplement compliance was evaluated during the study by counting unused supplements.

During the initial four weeks all patients received the placebo supplement and then proceeded to their assigned treatment for the following four weeks. Blood samples, blood pressure and BMI measurements were taken on two occasions at baseline, end of placebo period and at the end of the study. The mean of these two
values was used as a more reliable measure of the variables during the study. Blood samples were collected for analysis: plasma concentrations of glucose, total cholesterol, HDL, HDL2, HDL3, triglycerides, apolipoprotein A1, apolipoprotein B, α-tocopherol, α-carotene, β-carotene, lycopene, vitamin C, FSB, TBARS, sVCAM-1, sICAM-1, C-RP; LDL levels of α-tocopherol, peroxides, protein, free cholesterol, cholesterol ester, phospholipid, triglyceride; erythrocyte TBARS; Conjugated diene and TBARS formation in response to copper stimulated oxidation of isolated native LDL.

3.2.3 Plasma, LDL and erythrocyte preparation

Patients reported to the study centre early in the morning, after an overnight fast. Venous blood was collected into tubes containing disodium EDTA, sodium fluoride or heparin. Blood was kept on ice, for a maximum of two hours, before plasma and erythrocytes could be isolated by slow speed centrifugation at 4°C. Several aliquots of sodium fluoride and EDTA plasma were stored at −80°C and samples were later analysed for concentrations of glucose and lipids, respectively. Butylated hydroxytoluene was added to aliquots of EDTA plasma and heparin plasma to be assayed for lipid soluble antioxidants and TBARS, respectively. Samples were stored at −80°C until analysis. The remaining EDTA plasma was flushed with argon and stored at 4°C (for a maximum of 24 hours) until it could be isolated rapidly at 291110 x g by ultracentrifugation on a single-step discontinuous gradient in a Beckman NVT 65 rotor. The LDL isolated by this procedure did not contain appreciable levels of albumin. Several aliquots of LDL were stored at −80°C and this was later analysed for the concentrations of alpha-tocopherol, cholesterol, triglyceride, cholesterol ester, phospholipid, and protein. Concentrations of LDL peroxides, the formation of conjugated dienes and levels of TBARS were determined on the remaining LDL, which had been stored for up to 72 hours at 4°C under a layer of argon. Native LDL, for conjugated diene or TBARS determinations, were desalted into phosphate buffered saline (PBS) by gel-filtration in Ecocnopac PD-10 columns (Bio Rad) immediately preceding the assay (within 60 minutes). The PBS had been Chelex treated to remove any metal ions. Packed erythrocytes were prepared, immediately after removal of plasma and buffy coat, by washing erythrocytes 3 times with PBS. All laboratory analyses were determined without knowledge of the antioxidant regimen of the patient.

3.2.4 Composition of plasma, LDL and erythrocytes

Commercial enzymatic kits and calibrators (Boehringer Mannheim) were used to determine concentrations of plasma glucose, cholesterol, triglycerides, and LDL cholesterol, free cholesterol, cholesterol esters, phospholipids and triglycerides. Plasma apolipoprotein A1 and apolipoprotein B were measured by immunoturbidimetry. High density lipoprotein cholesterol was measured in the supernatant after precipitation of apolipoprotein B-containing lipoproteins with dextran/magnesium chloride. Glycated hæmoglobin was determined in whole blood using a commercial kit (Piece, Glycotest 2). The protein content of LDL was measured by the Lowry method. Lipid peroxides in freshly isolated LDL were
measured using an iodometric method (177) with hydrogen peroxide standards and the addition of BHT and EDTA.

High performance liquid chromatography was used to determine concentrations of plasma \( \alpha \)-tocopherol, lycopene, \( \alpha \)-carotene, \( \beta \)-carotene (526) and LDL \( \alpha \)-tocopherol. (527) All sample preparation for lipid-soluble antioxidant analyses were performed under low level lighting. The six samples collected from each patient were stored at \(-80^\circ\)C for several months, to allow analysis in a single batch. Each sample was extracted in duplicate from the plasma sample.

When the plasma lipid-soluble antioxidants were measured, a thawed EDTA plasma sample (0.25 ml) containing BHT (0.1 mmol/l) was mixed with 0.25 ml of sodium dodecyl sulphate (10 mmol/l), and deproteinised with 0.5 ml of ethanol containing an internal standard (tocopherol acetate, 40 \( \mu \)mol/l). The lipid phase was extracted with 1 ml of hexane, and 0.7 ml of this upper phase was evaporated to dryness under oxygen-free nitrogen at 40°C. The evaporated organic layer was reconstituted with 0.25 ml of mobile phase (methanol/acetonitrile/chloroform 47:47:6 by volume). The chromatographic system consisted of a Waters 501 pump (Millipore Waters Chromatography Division), a 5 µm prepacked guard column (Spherisorb ODS-2, Alltech Associates Ltd.) with a 0.5 µm stainless steel frit, a 150 x 4.60 mm 5 µm (Phenomenex Prodigy ODS-3) analytical column at 30°C with an isocratic mobile phase with a flow rate of 2.0 ml/minute, UV/VIS detection (Jasco UV-975 Intelligent Detector) at \( \lambda \) 292 nm (\( \alpha \)-tocopherol), 474 nm (lycopene), 450 nm (\( \alpha \)-carotene and \( \beta \)-carotene) and quantification by integrated peak areas (Shimadzu C-R3A chromatopac). The integrator was calibrated by 15 replicate injections of \( \alpha \)-tocopherol, lycopene, \( \alpha \)-carotene, \( \beta \)-carotene and tocopherol acetate standards. The calibration standards (Roche) were quantified by spectrophotometry (Ultrospec 2000 UV Visible Spectrophotometer, Pharmacia Biotech) using the specifications presented in Table 3.1. The quantity of antioxidant detected at the following times, \( \alpha \)-tocopherol \( R_t \) = 4.7 minutes, lycopene \( R_t \) = 8.1 minutes, \( \alpha \)-carotene \( R_t \) = 13.1 minutes, \( \beta \)-carotene \( R_t \) = 14.0 minutes, was adjusted by recovery of the internal standard measured at \( R_t \) = 5.8 minutes. The intra-sample (n=25) and inter-sample (n=30) coefficients of variation were as follows; 3.1%, 5.5% \( \alpha \)-tocopherol; 5.6%, 7.1% lycopene; 7.3%, 8.2% \( \alpha \)-carotene; 5.7%, 6.8% \( \beta \)-carotene. Recovery experiments were performed on a pooled plasma sample, spiked with the various antioxidants across the expected concentration range, and the mean results (n=8); 99.1% \( \alpha \)-tocopherol, 98.4% lycopene, 96.9% \( \alpha \)-carotene and 95.9% \( \beta \) carotene.

When the LDL \( \alpha \)-tocopherol was measured, 0.1 ml of thawed LDL sample containing BHT (0.1 mmol/l) was combined with 0.1 ml of ethanol and 0.1 ml of ethanol containing 0.1 mg/ml of internal standard (tocopherol acetate dissolved in ethanol, 0.21 mmol/l). This mixture was vortexed and partitioned twice against 0.2 ml of hexane, the organic supernatants were pooled and evaporated to dryness under oxygen-free nitrogen at 40°C. The evaporated organic layer was reconstituted with 0.1 ml of ethanol. The chromatographic system consisted of a 10 x 4.60 mm guard column (Direct-Connect, Alltech Associates Ltd.) and a 250 x 4.60 mm 10 \( \mu \)m (Versapack) C\(_{18}\) analytical column and with a 97% methanol mobile phase at 2.5 ml/minute, UV detection (Shimadzu SPD-6AV) at \( \lambda \) 294 nm and quantification by integrated peak areas (Shimadzu C-R3A chromatopac). The integrator was
calibrated by 10 replicate injections of α-tocopherol and tocopherol acetate quantified by spectrophotometry (Ultrospec 2000 UV visible Spectrophotometer, Pharmacia Biotech) (Table 3.1). The quantity of vitamin E detected at $R_1 = 4.2$ minutes was corrected by recovery of the internal standard measured at $R_1 = 5.8$ minutes. The intra-sample ($n=20$) coefficient of variation was 1.9 %.
### TABLE 3.1  Tocopherol and carotenoid extinction coefficients and absorptivity

<table>
<thead>
<tr>
<th></th>
<th>E&lt;sub&gt;1%1cm&lt;/sub&gt;</th>
<th>Maximal wavelength nm</th>
<th>Absorptivity A.mol⁻¹.l⁻¹</th>
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</thead>
<tbody>
<tr>
<td>α-tocopherol</td>
<td>0.718</td>
<td>292&lt;sup&gt;E&lt;/sup&gt;</td>
<td>3.26</td>
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<tr>
<td>Lycopene</td>
<td>2.565</td>
<td>474&lt;sup&gt;C&lt;/sup&gt;</td>
<td>186.3</td>
</tr>
<tr>
<td>α-carotene</td>
<td>0.250</td>
<td>450&lt;sup&gt;H&lt;/sup&gt;</td>
<td>145.5</td>
</tr>
<tr>
<td>β-carotene</td>
<td>0.369</td>
<td>450&lt;sup&gt;H&lt;/sup&gt;</td>
<td>136.9</td>
</tr>
<tr>
<td>Tocopherol acetate</td>
<td>0.447</td>
<td>292&lt;sup&gt;E&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

-<sup>E</sup> Tocopherol and tocopherol acetate were measured in ethanol
-<sup>C</sup> Lycopene was measured in chloroform
-<sup>H</sup> α-carotene and β-carotene were measured in hexane
Plasma vitamin C was determined by fluorimetry on a Cobas Fara autoanalyser (Roche) as described by Vuilleumier and Peck. Measurements were performed on the clear supernatant obtained from thawed samples of heparin plasma which had been stored with 5% metaphosphoric acid (plasma/MPA 1:9 by volume) and centrifuged at 4000 x g for 4 minutes at 4°C. The ascorbate oxidase was purchased from Boehringer Mannheim Biochemica (10 mg lyophilisate, approximately 1700 U), the lyophilisate was dissolved in 5 ml of acetate buffer (2 mol/l, pH 6.2) and stored in aliquots of 0.25 ml at -80°C until required. Recovery experiments were performed on a pooled plasma sample which had been spiked with ascorbic acid across the expected concentration range, and the mean result was 92.4% (n=10). Samples for each patient were analysed in a single run and in triplicate. The intra-sample (n=25) coefficient of variation for the assay was 1.7%.

Circulating plasma fluorescent Schiff bases were determined essentially by the method developed by Tsuchida and co-workers. First the proteins were separated from the lipid components by adding 4 ml of ethanol/diethyl ether (3:1 by volume) to 0.1 ml of EDTA plasma. This mixture was shaken for 5 minutes, centrifuged at 1310 x g for 5 minutes (4°C) and the solvents were discarded, this procedure was repeated with the resulting sediment. The sediment was dissolved in 2 ml of distilled water and the fluorescence intensity of this solution was measured at an excitation $\lambda$ 350 nm and an emission of $\lambda$ 460 nm (Aminco-Bowman Spectrophotofluorometer), with the sample sensitivity set at 10 and both slit widths at 5 nm. The instrument was calibrated daily to read 100 relative fluorescence units against a quinine sulphate solution (0.1 µg/ml of 0.1 sulphuric acid).

Plasma concentrations of MDA were determined by HPLC. A 0.5 ml aliquot of thawed heparin plasma containing BHT (18.9 µmol/l) was combined with 6 ml of chloroform and 3 ml of methanolic BHT (0.25 g/l) and the mixture was shaken for 15 minutes at room temperature. Aqueous sodium chloride (2 ml of 0.9% weight by volume) was added and centrifuged at 1200 x g for 15 minutes at room temperature. A salt layer formed at the interface of the two phases, the upper phase was discarded and the chloroform layer was transferred to another tube, and the procedure was repeated on the chloroform layer, before the chloroform was removed under oxygen-free nitrogen at room temperature. The residue was taken up in 0.3 ml of dimethyl sulphoxide (DMSO) (containing 2.5 g/BHT) and then 0.5 ml of aqueous Triton X-100 (0.5% weight by volume, prepared daily) in 50% (by volume) aldehyde-free acetic acid and 0.2 ml hydrous ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) (1.5 g/l in 50% by volume aqueous aldehyde-free acetic acid) were added sequentially, with vortex mixing between each addition. The capped tube was heated in boiling water for 60 minutes, then cooled rapidly on crushed ice. A further 2 ml of 50% Triton X-100 and 0.5 ml sodium hydroxide (8 mol/l) were added before application on the C$_{18}$ mini-cleanup columns (500 mg Bond-Elut, Alltech Associates Ltd.) preconditioned with 10 ml propan-2-ol followed by 2 rinses of distilled water (10 ml). Non-binding substances were eluted with distilled water and bound adduct, malondialdehyde(thiobarbituric acid)$_2$ (MDA(TBA)$_2$) was eluted in 3.0 ml of 70% (by volume) aqueous methanol, 50 µl of this eluate was subjected to HPLC separation using the following chromatographic system: a 100 x 4.60 mm i.d. C$_{18}$ (Alltech Associates Ltd.) analytical column with 70% (by volume) methanol mobile phase at 3.0 ml/minute, and a fluorescence detector (Shimadzu RF-530, excitation 515 nm, emission 553 nm) and integrator (Shimadzu C-R3A chromatopac).
Integrated peak areas for samples were quantified by a MDA(TBA), standard which was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane.(528) Chromic acid washed glassware was used throughout this assay.

Thiobarbituric acid reacting substances in erythrocytes were determined in triplicate essentially by the method developed by Jain et al (1989).(489) Butylated hydroxytoluene and EDTA were added to the samples to be assayed for TBARS prior to heating with acid to protect the polyunsaturated fatty acids from oxidation.(200,529)

Plasma sVCAM-1 (sVCAM-1, R&D Systems), sICAM-1 (sICAM-1, R&D Systems) and C-RP (Hemagen CRP 150 Kit, Hemagen Diagnostics) were measured by an immunoassay. Due to economic constraints, only one sample at each phase of the trial was analysed for C-RP, sICAM-1 and sVCAM-1 for each patient. However all the samples from each patient were analysed in the same batch in duplicate. The intra-sample coefficients of variation for the assays were as follows: 4.9% sVCAM-1; 3.3% sICAM-1; 8% C-RP.

3.2.5 Oxidation of LDL

The oxidation of isolated LDL was monitored by the formation of conjugated dienes or TBARS. When conjugated dienes were measured, isolated LDL (100-300 µl, 0.39 µmol cholesterol) was added to Chelex treated PBS (final volume was 2 ml). An aliquot of copper sulphate (32 µl, final concentration 1.6 µmol/l) was added with rapid mixing and the absorbance at λ 234 nm (Ultrospec 2000 UV Visible Spectrophotometer, Pharmacia Biotech) was monitored every 60 seconds over 4 hours. The temperature of the sample during this incubation was 25 ± 1°C. The lag phase was defined, from the plot of absorbance values against time, as the intercept of the lag and propagation phase tangents. The inter-assay coefficient of variation for the lag time in LDL oxidation was 5% (n=12). Maximal and rate of propagation of conjugated diene formation were also determined.

When TBARS were measured, the LDL (100-200 µl, 0.2 µmol cholesterol), and copper sulphate (30 µl, final concentration 2.5 µmol/l) were added to Chelex treated PBS and the final volume was adjusted to 1.2 ml. The mixture was incubated at 37°C for 3 and 5 hours. At the end of this period, BHT (10 µl of 4 mmol/l in methanol) and EDTA (50 µl of 4 mmol/l) were added and TBARS measured essentially by the method of Beuge and Aust.(197) Thiobarbituric acid solution (2 ml), containing 0.375% thiobarbituric acid in 15% trichloracetic acid and 0.25 mol/l hydrochloric acid, was added to the incubation mixture and to the standards containing 1,1,3,3-tetraethoxypropane (1.84-4.60 nmol). Tubes were heated on a boiling water bath for 30 minutes and after cooling were centrifuged at 2800 x g for 30 minutes. The optical density of the clear supernatant was read at λ 532 nm (Ultrospec 2000 UV Visible Spectrophotometer, Pharmacia Biotech) against the blank and amount of TBARS formed was calculated using standards. Rates of LDL oxidation were expressed as mmol MDA/mol LDL cholesterol.
3.2.6  Statistical analysis

The study was able to detect a change in lag time in LDL oxidation of 10 minutes at a power of 90% and \( P=0.05 \). Variables required logarithmic transformation to give approximately symmetrical distributions for statistical analysis. Analysis of variances with repeated measures were used to investigate the association between parameters of oxidative stress and markers of inflammation with the antioxidant regimens. Baseline values were used as a covariate in these analyses. When a significant difference was present among the treatment groups, unadjusted paired t-tests were used to test for within-group changes during the study. Multiple linear regression, adjusting for baseline LDL lag time, was performed to evaluate the relative impact of the antioxidant treatments on final lag time. Pearson’s product-moment correlation analysis was used to test C-RP levels, at baseline and at the end of the placebo phase and sICAM-1 and sVCAM-1. Two-sided tests of significance were used and a \( P \) value of less than 0.05 was considered statistically significant. Baseline differences between the treatment groups were examined using analysis of variances for continuous variables and \( \chi^2 \) for categorical variables. The statistical analysis was carried out using SPSS release 6.1 (SPSS for Windows, SPSS Inc.). Unless otherwise stated all data are expressed as mean ± SD.
### TABLE 3.2 Characteristics of the patients at entry to the study (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato Juice n=15</th>
<th>Placebo n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>56 ± 14</td>
<td>56 ± 9</td>
<td>63 ± 8</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>6/6</td>
<td>6/6</td>
<td>10/5</td>
<td>10/3</td>
</tr>
<tr>
<td>Median duration of diabetes, years</td>
<td>3</td>
<td>1.5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>BMI, Kg/m²</td>
<td>31.5 ± 7.4</td>
<td>30.7 ± 6.3</td>
<td>30.9 ± 7.0</td>
<td>31.8 ± 4.1</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>84 ± 11</td>
<td>89 ± 12</td>
<td>89 ± 8</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>141 ± 10</td>
<td>140 ± 12</td>
<td>144 ± 13</td>
<td>142 ± 16</td>
</tr>
<tr>
<td>Glycated haemoglobin (HbA₁c), %</td>
<td>6.73 ± 0.93</td>
<td>6.68 ± 0.98</td>
<td>6.00 ± 0.69</td>
<td>6.62 ± 1.66</td>
</tr>
<tr>
<td>Fructosamine, µmol/l</td>
<td>270 ± 36</td>
<td>258 ± 21</td>
<td>269 ± 32</td>
<td>264 ± 20</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/l</td>
<td>8.3 ± 2.1</td>
<td>8.7 ± 1.5</td>
<td>8.2 ± 1.3</td>
<td>9.1 ± 2.4</td>
</tr>
</tbody>
</table>
TABLE 3.3  Medication, prevalence of IHD, obesity and raised blood pressure (numbers and percentages)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato Juice n=15</th>
<th>Placebo n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischaemic heart disease</td>
<td>8 (67)</td>
<td>5 (42)</td>
<td>10 (67)</td>
<td>8 (62)</td>
</tr>
<tr>
<td>Obesity (BMI &gt;30, Kg/m²)</td>
<td>6 (50)</td>
<td>5 (42)</td>
<td>6 (40)</td>
<td>9 (69)</td>
</tr>
<tr>
<td>Systolic blood pressure ≥ 90 mmHg</td>
<td>6 (50)</td>
<td>2 (17)</td>
<td>4 (27)</td>
<td>6 (46)</td>
</tr>
<tr>
<td>Control of hyperglycaemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet only</td>
<td>4 (33)</td>
<td>5 (42)</td>
<td>6 (40)</td>
<td>4 (31)</td>
</tr>
<tr>
<td>Oral hypoglycaemics</td>
<td>8 (67)</td>
<td>7 (58)</td>
<td>9 (60)</td>
<td>9 (69)</td>
</tr>
<tr>
<td>Biguanides</td>
<td>2 (17)</td>
<td>0</td>
<td>4 (27)</td>
<td>2 (15)</td>
</tr>
<tr>
<td>Sulphonylureas</td>
<td>8 (67)</td>
<td>7 (58)</td>
<td>7 (47)</td>
<td>7 (54)</td>
</tr>
</tbody>
</table>

χ² test comparing antioxidant groups with the placebo group, with level of significance P<0.05.
TABLE 3.4 Concentrations of plasma lipids and lipoproteins (mean ± SD)

|                  | α-Tocopherol (n=12) | Vitamin C (n=12) | Tomato Juice (n=15) | Placebo (n=13) | P  
|------------------|---------------------|------------------|---------------------|----------------|------
| **Cholesterol, mmol/l** |                     |                  |                     |                |      
| Baseline         | 5.65 ± 1.16         | 5.96 ± 0.97      | 5.87 ± 1.02         | 6.48 ± 1.14    |      
| End of placebo   | 5.57 ± 1.30         | 5.92 ± 0.94      | 5.82 ± 1.00         | 6.49 ± 1.19    |      
| End of study     | 6.07 ± 1.50†        | 5.97 ± 0.88      | 5.73 ± 0.97         | 6.52 ± 1.09    | 0.022
| **HDL, mmol/l**  |                     |                  |                     |                |      
| Baseline         | 1.16 ± 0.22         | 1.13 ± 0.23      | 1.04 ± 0.29         | 0.95 ± 0.16    |      
| End of placebo   | 1.16 ± 0.22         | 1.15 ± 0.25      | 1.02 ± 0.30         | 0.96 ± 0.16    |      
| End of study     | 1.20 ± 0.23         | 1.15 ± 0.22      | 1.07 ± 0.35         | 0.98 ± 0.20    | 0.693
| **Triglycerides, mmol/l** |         |                  |                     |                |      
| Baseline         | 2.09 ± 1.01         | 1.95 ± 0.93*     | 2.49 ± 0.74         | 3.03 ± 1.76    |      
| End of placebo   | 2.00 ± 1.02         | 1.97 ± 1.00      | 2.57 ± 0.63         | 2.99 ± 1.52    |      
| End of study     | 2.28 ± 1.20         | 2.14 ± 1.10      | 2.53 ± 1.04         | 2.83 ± 1.46    | 0.070
| **Apolipoprotein A1, g/l** |     |                  |                     |                |      
| Baseline         | 1.24 ± 0.21         | 1.19 ± 0.22      | 1.14 ± 0.28         | 1.12 ± 0.17    |      
| End of placebo   | 1.26 ± 0.14         | 1.24 ± 0.26      | 1.28 ± 0.40         | 1.11 ± 0.10    |      
| End of study     | 1.37 ± 0.21         | 1.27 ± 0.25      | 1.21 ± 0.29         | 1.14 ± 0.21    | 0.316
| **Apolipoprotein B, g/l** |           |                  |                     |                |      
| Baseline         | 0.95 ± 0.31         | 0.86 ± 0.17      | 0.95 ± 0.24         | 1.13 ± 0.31    |      
| End of placebo   | 0.94 ± 0.33         | 0.92 ± 0.23      | 1.00 ± 0.22         | 1.17 ± 0.19    |      
| End of study     | 1.04 ± 0.37         | 1.06 ± 0.18†     | 0.92 ± 0.19         | 1.20 ± 0.34    | 0.027

* ANOVA comparing vitamin C group with placebo group, with level of significance P<0.05.
† Unadjusted paired t-tests comparing the placebo phase with vitamin C supplementation, with level of significance P<0.05.
‡ Unadjusted paired t-tests comparing the placebo phase with vitamin C supplementation, with level of significance P<0.01.
TABLE 3.5  Concentrations of plasma antioxidants (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol</th>
<th>Vitamin C</th>
<th>Tomato Juice</th>
<th>Placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=12</td>
<td>n=12</td>
<td>n=15</td>
<td>n=13</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol, µmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>24.44 ± 8.57</td>
<td>22.69 ± 4.13</td>
<td>26.13 ± 4.88</td>
<td>23.89 ± 5.34</td>
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</tr>
<tr>
<td>End of placebo</td>
<td>24.34 ± 8.78</td>
<td>22.21 ± 5.01</td>
<td>25.49 ± 4.46</td>
<td>25.36 ± 5.75</td>
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</tr>
<tr>
<td>End of study</td>
<td>56.71 ± 23.66 †</td>
<td>22.55 ± 4.76</td>
<td>26.74 ± 5.97</td>
<td>24.36 ± 6.54</td>
<td>0.001</td>
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<tr>
<td>Lycopene, µmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>0.35 ± 0.24</td>
<td>0.41 ± 0.27</td>
<td>0.39 ± 0.23</td>
<td>0.31 ± 0.26</td>
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</tr>
<tr>
<td>End of placebo</td>
<td>0.31 ± 0.19</td>
<td>0.41 ± 0.27</td>
<td>0.39 ± 0.26</td>
<td>0.33 ± 0.28</td>
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<tr>
<td>End of study</td>
<td>0.41 ± 0.23</td>
<td>0.44 ± 0.32</td>
<td>1.08 ± 0.39 †</td>
<td>0.28 ± 0.21</td>
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<tr>
<td>Vitamin C, µmol/l</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>39.57 ± 19.51</td>
<td>40.74 ± 15.43</td>
<td>38.87 ± 25.50</td>
<td>24.96 ± 15.64</td>
<td></td>
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<tr>
<td>End of placebo</td>
<td>42.71 ± 18.03</td>
<td>37.70 ± 12.37</td>
<td>43.83 ± 28.56</td>
<td>27.34 ± 15.35</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>39.96 ± 13.71</td>
<td>64.74 ± 14.34 †</td>
<td>55.96 ± 23.40</td>
<td>29.68 ± 21.10</td>
<td>0.089</td>
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* Repeated measures ANOVA by treatment and group with baseline covariate correction.
† Unadjusted paired t-tests comparing the placebo phase with treatment phase, with level of significance P<0.001.
<table>
<thead>
<tr>
<th>Time</th>
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<td>Baseline</td>
<td>Baseline</td>
<td>Baseline</td>
<td>Baseline</td>
</tr>
<tr>
<td>0.00</td>
<td>1.5 ± 0.3</td>
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<td>0.05</td>
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<tr>
<td>0.10</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>0.15</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>0.20</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
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TABLE 3.6
Low density lipoprotein composition and intrinsic resistance to oxidation (mean ± SD)
<table>
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<tr>
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<th>α-Tocopherol n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato Juice n=15</th>
<th>Placebo n=13</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma FSB, U/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9.00 ± 2.05</td>
<td>9.24 ± 2.43</td>
<td>10.84 ± 1.76</td>
<td>11.13 ± 2.32</td>
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</tr>
<tr>
<td>End of placebo</td>
<td>9.83 ± 2.74</td>
<td>9.95 ± 2.35</td>
<td>11.72 ± 2.47</td>
<td>11.51 ± 2.72</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>9.41 ± 2.03</td>
<td>9.88 ± 2.37</td>
<td>11.38 ± 3.25</td>
<td>10.62 ± 2.29</td>
<td>0.614</td>
</tr>
<tr>
<td><strong>Plasma TBARS, ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>90.49 ± 27.03</td>
<td>96.45 ± 19.83</td>
<td>109.84 ± 23.32</td>
<td>111.22 ± 35.80</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>83.72 ± 21.60</td>
<td>94.03 ± 18.78</td>
<td>103.17 ± 22.83</td>
<td>109.21 ± 29.23</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>87.88 ± 25.45</td>
<td>88.93 ± 19.70</td>
<td>108.09 ± 23.52</td>
<td>110.69 ± 32.27</td>
<td>0.206</td>
</tr>
<tr>
<td><strong>RBC TBARS, nmol MDA/ml PC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.12 ± 0.25</td>
<td>1.20 ± 0.19</td>
<td>1.17 ± 0.25</td>
<td>1.12 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>1.17 ± 0.15</td>
<td>1.14 ± 0.22</td>
<td>1.18 ± 0.24</td>
<td>1.23 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>1.20 ± 0.19</td>
<td>1.23 ± 0.25</td>
<td>1.20 ± 0.27</td>
<td>1.29 ± 0.18</td>
<td>0.731</td>
</tr>
</tbody>
</table>

Abbreviations: MDA, malondialdehyde; PC, packed cells.

* Repeated measures ANOVA by treatment and group with baseline covariate correction.
TABLE 3.8  Concentrations of plasma C-RP and soluble cell adhesion molecules (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato Juice n=15</th>
<th>Placebo n=13</th>
<th>P *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma C-RP, mg/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.5 ± 3.8</td>
<td>2.9 ± 3.3</td>
<td>3.8 ± 2.2</td>
<td>3.1 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>5.6 ± 3.1</td>
<td>3.1 ± 3.3</td>
<td>3.5 ± 2.2</td>
<td>2.9 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>2.9 ± 3.6 †</td>
<td>3.1 ± 2.9</td>
<td>4.1 ± 2.1</td>
<td>3.1 ± 2.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Plasma sICAM-1, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>257 ± 85</td>
<td>256 ± 59</td>
<td>244 ± 70</td>
<td>297 ± 131</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>276 ± 66</td>
<td>244 ± 55</td>
<td>246 ± 60</td>
<td>289 ± 112</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>272 ± 55</td>
<td>243 ± 62</td>
<td>240 ± 49</td>
<td>287 ± 112</td>
<td>0.893</td>
</tr>
<tr>
<td>Plasma sVCAM-1, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>533 ± 148</td>
<td>582 ± 353</td>
<td>619 ± 179</td>
<td>626 ± 232</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>526 ± 139</td>
<td>584 ± 370</td>
<td>632 ± 189</td>
<td>635 ± 244</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>534 ± 153</td>
<td>585 ± 354</td>
<td>632 ± 160</td>
<td>640 ± 256</td>
<td>0.997</td>
</tr>
</tbody>
</table>

* Repeated measures ANOVA by treatment and group with baseline covariate correction.
† Unadjusted paired t-test comparing placebo with treatment phase, with level of significance P=0.009.
3.3 Results

Five people withdrew during the study, due to difficulties donating a blood sample (n=2), alterations in medication (n=2) and problems tolerating the tomato juice (n=1). The baseline characteristics of the participants are detailed in Tables 3.2 and 3.3. The mean age of the patients was 58 ± 9 years. Forty percent of the patients were female and four were less than 50 years of age. Patients had been diagnosed with diabetes mellitus for a median of 2.5 years and the majority (86%) of patients had been diagnosed with diabetes for less than six years. Patients had elevated fasting plasma glucose, blood glycated haemoglobin and BMI at baseline and these remained stable during the study. A third of the study group controlled their diabetes by diet alone and the remainder were treated with oral hypoglycaemic drugs. Nine patients (17%) controlled their hyperglycaemia with gliclazide. Many of the participants had multiple risk factors for atherosclerosis and more than half had clinical evidence of coronary heart disease (60%). Consequently a number of the patients were taking medications regularly, including ACE inhibitors (37%), calcium blocking agents (19%) and low-dose aspirin (21%). The proportion of patients regularly taking calcium blocking agents, aspirin or gliclazide was similar between the treatment groups, however ACE inhibitor medication use was significantly lower (P<0.05) in the patients randomised to receive alpha-tocopherol and vitamin C compared with those on placebo. Medication regimens were unchanged during the study.

Plasma lipids, lipoproteins and apolipoproteins concentrations were similar between the treatment groups at baseline (Table 3.4), with the exceptions of plasma triglyceride which were lower in the patients randomised to receive vitamin C (P<0.05) and plasma HDL which was higher in those in the α-tocopherol group (P<0.05). Concentrations of plasma total cholesterol (9% n=12; P<0.001) and apolipoprotein B (15% n=12; P<0.05) increased with α-tocopherol and vitamin C supplementation, respectively. The concentration of LDL in the plasma is not presented as it was not measured directly and an estimation using the Friedwald formula is not considered appropriate when plasma triglycerides are elevated. (530)

Plasma concentrations of antioxidants during the study are presented in Table 3.5. Plasma concentrations of α-tocopherol, vitamin C and lycopene were similar between the treatment groups at baseline and at the end of the placebo phase, but increased significantly after the supplement phase for the respective groups, suggesting both compliance with the treatment regimen and good bioavailability of the antioxidant supplements. Plasma concentrations of α-carotene and β-carotene were not changed by the tomato juice supplementation (data presented in appendix D). At baseline plasma lycopene, expressed per mol of plasma cholesterol, was significantly lower in the diabetic patients compared with younger non diabetic subjects measured in the same laboratory (patients 0.37 ± 0.24 mmol/mol n=52; control subjects 1.05 ± 0.41 n=18; P=0.001). This was also the case for α-tocopherol (patients 1.77 ± 0.35 mmol/mol n=52; control subjects 2.09 ± 0.50 mmol/mol n=18; P=0.001). The frequency checklist for tomato products showed consumption was
low amongst the patients at baseline and this remained similar during the study (data presented in appendix E).

Oxidation characteristics and the composition of the LDL are presented in Table 3.6. Lag time was significantly lengthened after supplementation with tomato juice (42% n=15; P<0.001) and α-tocopherol (54% n=12; P<0.001) but remained unchanged in those patients who received placebo and vitamin C supplements. Multiple linear regression analysis demonstrated in these patients, supplementing with tomato juice or α tocopherol increased the lag time by 31 (P=0.004) and 44 (P=0.001) minutes, respectively, after adjusting for initial lag time (b=0.4 P=0.026). In contrast, supplementation with vitamin C (b= -2 P=0.838) or the initial levels of plasma α-tocopherol (b= -2 P=0.329), lycopene (b= -7 P=0.802) or cholesterol (b=6 P=0.191) did not significantly influence the final lag time. Lag time remained stable during the placebo phase. The coefficient of variation between the two measures of lag time at each time point was 3.3% (n=57). At baseline the rate of conjugated diene formation during the propagation phase was 8.43 ± 2.3 nmol/µmol/minute and the maximal level was 1056 ± 134 nmol/µmol. The maximal and rate of conjugated diene formation were similar between the groups at baseline and levels were unaffected by the antioxidant treatments (data presented in appendix D). The levels of TBARS formed at 3 hours (17.92 ± 4.12 mmol MDA/mol cholesterol) and 5 hours (18.11 ± 4.01 mmol MDA/mol cholesterol) were unaffected by the antioxidant or placebo supplementation regimens (data presented in appendix D). The chemical composition of the LDL was similar between the groups at baseline, with the exception of patients randomised to receive vitamin C who had a significantly higher percentage of phospholipid (P<0.05) and less triglyceride (P<0.05). The chemical composition of LDL did not change during the study, with the exception of α-tocopherol levels which were increased after supplementation with this antioxidant (111% n=12 P<0.001). The peroxide content in the LDL remained unchanged with the antioxidant supplements.

Indices of lipid oxidation in the plasma and erythrocyte TBARS are presented in Table 3.7. Levels were similar between the treatment groups at baseline and did not change significantly with the antioxidant regimens. At baseline FSB levels were significantly higher than levels measured previously in healthy subjects in the laboratory (8.8 ± 1.6 U/ml n=10).

Plasma levels of C-RP and cell adhesion molecules are reported in Table 3.8. At baseline the concentrations of C-RP, sVCAM-1, and sICAM-1 were not different between the groups, however there was a non significant trend for the patients randomised to receive α-tocopherol to have higher C-RP. Supplementation with α-tocopherol reduced plasma C-RP by 51% (P=0.009) while no effect was observed with tomato juice, vitamin C or placebo supplementation. C-reactive protein was stable during the placebo phase, as demonstrated by the strong correlation between baseline and the end of the placebo measures (r=0.998 P<0.001). Also plasma C-RP concentrations did not change in patients with baseline levels above 3 mg/l who were not receiving α-tocopherol (baseline 6.3 ± 1.8 mg/l; end of placebo 5.0 ± 2.0 mg/l; end of supplementation 5.4 ± 1.9 mg/l; n=23; P=0.340). Patients with 4 or more risk factors for CHD associated with syndrome X, including raised blood pressure (systolic blood pressure ≥160 or diastolic blood pressure ≥95 or anti-hypertensive medication), obesity (BMI ≥30 Kg/m²), low HDL (≤1.1 mmol/l) and
high triglycerides (≥1.5 mmol/l) had significantly higher baseline levels of C-RP compared with those with one or zero risk factors (syndrome X 4.5 ± 2.3 mg/l n=16; without syndrome X 1.7 ± 2.6 mg/l n=12; P=0.007). Glucose, glycated haemoglobin, age and duration of diabetes were similar between patients with and without risk factors for syndrome X.

Levels of cell adhesion molecules were not altered when concentrations of plasma α-tocopherol and lycopene were increased as a result of the supplementation regimens. Plasma sICAM-1 and sVCAM-1 levels displayed a strong correlation (r=0.576 P=0.001).

### 3.4 Discussion

Regular consumption of tomato juice increased the intrinsic resistance of LDL to oxidation in diabetic patients by 42%. The magnitude of this increase was comparable with a preliminary study in healthy subjects which used similar methodology\(^\text{34}\) and several other non placebo-controlled studies which have reported tomato juice reduces LDL susceptibility to oxidation in people without diabetes.\(^\text{33,35,36}\) Tomato juice contains a number of antioxidants which could potentially influence the susceptibility of LDL to oxidation. In particular, it is a rich source of the lipophilic antioxidant lycopene.\(^\text{354}\) Enriching LDL in vitro with lycopene has been demonstrated to increase the intrinsic resistance of LDL to oxidation.\(^\text{31}\) In the lipoprotein, lycopene may attenuate oxidative damage by reducing the availability of free radicals.\(^\text{293}\) Lycopene may be oxidised in preference to the unsaturated fatty acids as a consequence of its similar structure, namely the allylic methylene group at the C-4 position\(^\text{284,293,294}\) and high number of double bonds.\(^\text{284,295}\) Furthermore, the resulting lycopene radical is unlikely to promote lipid peroxidation in vivo.\(^\text{304}\)

In the present study, plasma lycopene increased almost three fold following tomato juice consumption for several weeks and this increase was consistent with data in non diabetic subjects.\(^\text{32,34-36}\) The good bioavailability of lycopene from the tomato juice used in this study was probably due to extensive heat treatment of the juice\(^\text{316}\) which releases lycopene from the plant cell wall components.\(^\text{288}\) Drinking the juice with meals would have further improved absorption of lycopene by co-ingestion with fat.\(^\text{317}\) This increase in plasma lycopene would be expected to enrich LDL with lycopene, as in the circulation lycopene is associated primarily with lipoproteins.\(^\text{345}\)

Tomato juice contains a small amount of vitamin E\(^\text{354}\) however plasma alpha-tocopherol levels were not increased after the tomato juice regimen. This finding indicates that increased alpha-tocopherol intake was not responsible for the reduced susceptibility of LDL to oxidation observed with tomato juice in the present study. However in isolated LDL, the antioxidant action of lycopene may be attenuated by lower alpha-tocopherol levels. Fuhrman and co-workers have suggested that lycopene is only effective in reducing LDL oxidation when high concentrations of alpha-tocopherol are also present in the LDL (≥16 µmol/g LDL)
cholesterol). In the present study, the intrinsic resistance of LDL to oxidation was improved in spite of apparently low levels of LDL alpha-tocopherol (levels at baseline 7.26 ± 1.19 µmol/g cholesterol compared with at end of tomato juice supplementation 6.99 ± 1.19 µmol/g cholesterol). It is possible the antioxidant capacity of the lycopene may be dependent on its location within the LDL particle. When tomato products are fed to individuals, lycopene is incorporated into the chylomicron and the VLDL fractions, and during the following 48 hours concentrations rise in the other lipoprotein fractions. Lycopene is predominantly transported by the LDL fraction where it is associated with the hydrophobic inner core components and is therefore well-placed to protect the majority of the LDL lipids from oxidation. In contrast, enriching the LDL with lycopene, in vitro, may have incorporated it near the surface of the lipoprotein. The present study findings do not support the hypothesis that high levels of alpha-tocopherol in the LDL are required to elicit the antioxidant action of lycopene.

Tomatoes contain substantial levels of vitamin C and during processing tomato juice frequently has vitamin C added, further elevating these concentrations. There was a non significant trend for vitamin C levels to increase during tomato juice supplementation in the present study. However, vitamin C alone did not appear to be responsible for the increased resistance to oxidation during supplementation with tomato juice, as the resistance of LDL was unchanged in patients who were randomised to receive high doses of vitamin C. However the trend toward increased plasma vitamin C levels may have enhanced the antioxidant effects of tomato juice in the present study as vitamin C may regenerate or spare alpha-tocopherol and carotenoids.

Vitamin C supplementation of people who are under oxidative stress, due to cigarette smoking, has been reported to have a modest effect on LDL resistance to oxidation (14%). In this situation vitamin C may improve LDL resistance to copper induced oxidation by reducing the levels of lipid hydroperoxides in the LDL and disrupting the copper metal-binding sites on apolipoprotein B. The diabetic patients in the present study are also under oxidative stress but vitamin C failed to reduce lipid hydroperoxide levels or alter the susceptibility of the LDL to oxidation. It is possible that the lower dose of vitamin C (500 mg/day) and the corresponding lower plasma vitamin C levels achieved during the supplementation period were insufficient to decrease LDL peroxides or alter LDL conformation, and increase the intrinsic resistance of LDL to oxidation. Also vitamin C supplementation may not normalise plasma ascorbate in diabetic patients, as demonstrated by Sinclair and co-workers.

Enriching LDL with alpha-tocopherol has been shown previously to improve the resistance of the LDL to oxidation in non diabetic volunteers. However, pharmacological doses (~400 IU/day) of alpha-tocopherol for several weeks have been required to show significant improvements in lag time. The LDL isolated from diabetic patients has been reported to be depleted in alpha-tocopherol and a proportion is likely to be small dense LDL which appears more difficult to enrich with alpha-tocopherol. Thus it is unlikely non pharmaceutical doses of alphatocopherol would influence LDL oxidation. In the present study, supplementation with 800 IU/day of alpha-tocopherol significantly increased both plasma and LDL.
alpha-tocopherol levels and this resulted in a 54% extension of the LDL lag time. The magnitude of this improvement was only marginally shorter (15%) than a similar study in diabetic men supplemented for ten weeks with a higher dose of alpha-tocopherol (1600 IU/day). (494)

Alpha-tocopherol reduced plasma C-RP levels by 51% and this may indicate an improvement in systemic inflammatory status. At baseline, plasma levels C-RP were similar to the elevated levels previously reported in diabetic patients. (41) Inflammatory activity as a result of oxidative stress or atherosclerosis may contribute to high C-RP levels. (152, 155) Most of the patients in the current study had multiple risk factors for CHD and those with several risk factors had higher levels of C-RP which is in agreement with data reported in diabetic patients with syndrome X. (41) Few patients reported infections but it cannot be excluded that undiagnosed and asymptomatic infections also contributed to the elevated C-RP levels. (142, 143)

The mechanism underlying the reduction in C-RP with supplementation of alpha-tocopherol in diabetic patients is uncertain. However, it appears to be specific to alpha-tocopherol, in as much as increased levels of the lycopene or vitamin C did not alter C-RP levels. Devaraj and Jialal reported supplementation with high doses of alpha-tocopherol in healthy subjects inhibited the release of IL-1β from isolated monocytes. (28) The pro-inflammatory cytokines IL-1β and IL-6 increase the synthesis of C-RP by hepatic cells in vitro. (533)

Baseline levels of plasma MDA, FSB and erythrocyte TBARS appeared to be elevated in the diabetic patients and this is consistent with increased oxidative stress. Levels of plasma MDA were comparable with the high levels recorded in people who smoked in a study which had used the same method of MDA estimation. (534) Erythrocyte TBARS levels were similar to concentrations documented in diabetic patients (489) and plasma FSB levels were markedly higher than levels in healthy subjects determined in Dr Sutherland's laboratory. While tomato juice and alphatocopherol supplementation improved the resistance of isolated LDL to oxidation, these treatments did not change circulating levels of lipid oxidation products. The unchanged levels of plasma MDA during supplementation with high doses of alpha-tocopherol was in contrast to a study by Mol and co-workers which reported a decrease in plasma TBARS in diabetic patients after four weeks supplementation with 600 IU/day of alpha-tocopherol. (481) However variance in the levels of plasma glucose and alpha-tocopherol, and methods used to measure MDA may contribute to these dissimilar findings. In the present study, glycated haemoglobin and fasting plasma glucose were lower, and it is possible alpha-tocopherol may be less effective in decreasing circulating levels of lipid oxidation products under these conditions of lower gluco-oxidative stress. Determining the concentration of lipid oxidation products in the plasma is difficult and the possibility cannot be excluded that these determinations were not sufficiently specific or sensitive to detect a small decrease in lipid oxidation products during antioxidant supplementation. However in the present study, plasma MDA was determined using an HPLC method which had been designed to reduce the interference from molecules that are not products of lipid oxidation in the plasma (199) and this method has previously identified oxidative stress in smokers and patients with peripheral vascular disease. (534) Also, the present findings appear consistent with a recent placebo-controlled alpha-tocopherol intervention study in elderly non diabetic subjects where isoprostane F₂α levels, a
highly specific index of lipid oxidation, were used to determine lipid oxidation products. Erythrocytes are vulnerable to oxidative damage and provide an accessible cellular measure of peroxidation. The unchanged levels of erythrocyte TBARS in the diabetic patients in the present study suggest that although plasma antioxidant levels were increased following supplementation, membrane lipid oxidation and cellular oxidant stress were not inhibited. These results are in contrast to a similar study in non diabetic patients where erythrocyte TBARS were reduced during alpha-tocopherol supplementation. It is possible that cells from diabetic patients are less responsive than normal cells to the effects of increased levels of alpha-tocopherol.

At baseline, plasma levels of sICAM-1 were similar to the elevated levels previously reported in diabetic patients, whereas levels of sVCAM-1 were not raised compared to data published previously in diabetic and non diabetic subjects. Lysophosphatidycholine, a product of oxidised LDL, can stimulate the expression of adhesion molecules on cultured cells and studies have reported the expression of cell adhesion molecules is depressed when cultured cells are enriched with antioxidants, such as alpha-tocopherol or probucox. Circulating forms of ICAM-1 and VCAM-1 can be measured in the plasma and they appear to reflect the cellular expression of these molecules. Ceriello and co-workers have previously reported a decrease in plasma sICAM-1 levels in patients with type 2 diabetes following the intravenous administration of the antioxidant glutathione. However in the present study, the antioxidant regimens failed to reduce levels of sICAM-1 or sVCAM-1. It is possible the supplementation period may have been too short and the cellular concentration of antioxidants may not have been sufficiently elevated to reduce the cellular expression of adhesion molecules. Also plasma glucose levels remained elevated during the study which may have maintained elevated sICAM-1 levels, as glycaemic control has previously been demonstrated to have a major influence on sICAM-1 levels in diabetic patients.

Supplementing diabetic patients with high doses of alpha-tocopherol and vitamin C in the present study had deleterious effects on plasma cholesterol and apolipoprotein B, respectively. After supplementation with high doses of alpha-tocopherol the diabetic patients showed a significant increase in plasma cholesterol levels and a trend for apolipoprotein B to rise. This finding is not due to measurement variability. The measurement of plasma cholesterol conformed to strict quality control (CV 3%). Also, two measurements of plasma lipids and apolipoproteins were made at each time point. It is unlikely that there were changes in the usual diet and that these were responsible for the increase in plasma cholesterol levels. Patients were instructed to maintain their usual dietary habits during the study and plasma cholesterol levels did not change noticeably in the placebo group. Reaven and co-workers have previously reported a trend for increased plasma cholesterol levels, in patients with type 2 diabetes treated with vitamin E and the magnitude of this trend was comparable with the increase in plasma cholesterol after vitamin E supplements in the current study. Supplementation with vitamin C increased plasma apolipoprotein B levels but not plasma cholesterol in the diabetic patients in the present study. Few studies have examined the effect of supplementation with vitamin C on apolipoprotein B levels in...
diabetic patients. It has been clearly established that high plasma cholesterol concentrations are associated with an increased risk of CAD in diabetic patients and high plasma apolipoprotein B levels with normal cholesterol levels have also been associated with an increased risk of coronary atherosclerosis. Thus this increase in apolipoprotein B with high dose vitamin E and vitamin C supplementation regimens in diabetic patients requires further investigation in a larger number of subjects and over a longer period of supplementation.

The findings of the present study require some caution with interpretation as the number of patients in the treatment groups was relatively small and the treatment period was comparatively short. Nevertheless, the length of supplementation was sufficient to establish markedly increased levels of circulating antioxidants. The patients in this study were treated with a number of medications which have previously been shown to reduce the susceptibility of LDL to oxidation and plasma C-RP in non diabetic subjects. It is unlikely these regimens were responsible for the improvements in lag time and C-RP levels observed after alpha-tocopherol and tomato juice supplementation in the present study, as patient medication regimens remained stable throughout the study.

In summary pharmacological doses of alpha-tocopherol increased the intrinsic resistance of LDL to oxidation and reduced chronic systemic inflammation, as indicated by a decrease in plasma C-RP. These changes may be associated with a reduced risk of CAD, according to several case-control studies. Furthermore, similar doses of alpha-tocopherol have previously been associated with a reduction in non fatal MI in high risk patients in two randomised placebo-controlled intervention trials. However these benefits may be opposed by the potentially adverse effects the high doses of vitamin E appeared to have on plasma cholesterol levels in these diabetic patients. Further vitamin E intervention studies with a higher number of subjects for a longer duration are required to confirm these findings and the relationship between vitamin E and CVD in diabetic patients. The regular consumption of tomato juice by diabetic patients also improved the intrinsic resistance of LDL to oxidation and the magnitude of this change was almost equal to the value observed with high doses of vitamin E. High lycopene status appears to be associated with a low rate of CAD in case-control studies in non diabetic populations. Intervention trials are required to establish if the improved intrinsic resistance of LDL to oxidation confers an anti-atherogenic benefit in patients with type 2 diabetes.
Chapter 4

Oxidation of heparin-treated LDL by peroxidases. A new model of LDL oxidation in the artery wall

4.1 Introduction

Oxidative modification of LDL that has become trapped in the arterial intima by interaction with glycosaminoglycan groups on proteoglycans is believed to play an important role in the development of atherosclerosis. Proteoglycans and glycosaminoglycans can form reversible and irreversible complexes with LDL in vitro. There is evidence that these interactions also occur in the artery wall where they may lead to deposition of LDL in atherosclerotic lesions and proteoglycan-LDL complexes have been isolated from human and rabbit arterial lesions. Associations of LDL with proteoglycans and glycosaminoglycans appears to increase the susceptibility of LDL to oxidative modification and the opportunity for this modification to occur. Isolated LDL that has complexed with proteoglycans and glycosaminoglycans is abnormally susceptible to oxidation by copper ions and by cultured cells in a transition metal ion mediated process. These complexes promote lipid accumulation in cultured macrophages which mimics foam cell formation. Human LDL that has been precipitated with proteoglycans or glycosaminoglycans in vitro and then resolubilised is thought to imitate LDL that has formed reversible associations with proteoglycans in the arterial intima. This precipitated-resolubilised LDL has an altered structure and is bound and internalised more efficiently than native LDL by the apolipoprotein B/E receptor of cultured macrophages.

Myeloperoxidase (MPO), a heme-containing enzyme which is present in inflammatory cells and in arterial lesions, may oxidise LDL in the artery wall. High levels of 3-chlorotyrosine, a specific marker of MPO-catalysed oxidation, were measured in the LDL isolated from human atherosclerotic intima compared with levels in circulating LDL. Also hypochlorous acid produced during MPO activity chlorinates proteins and lipids and modifies LDL to a form which is readily internalised by cultured macrophages. The mechanism underlying peroxidase catalysed oxidation of LDL differs from LDL oxidation by transition metal ions. Peroxidases do not directly oxidise lipids but radicals...
derived from protein, alpha-tocopherol and tyrosine mediate oxidation.\(^{(11)}\) Hydrogen peroxide generated by activated phagocytes accelerate peroxidase-catalysed LDL oxidation. Horse radish peroxidase (HRP), an enzyme derived from plants, also oxidises isolated LDL via a protein radical\(^{(11)}\) and has been suggested as a model for transition metal ion independent oxidation of LDL.\(^{(88)}\)

The environment surrounding LDL in the micro-domains of the artery wall is believed to contain a variety of compounds which could modulate LDL oxidation.\(^{(246,247)}\) Plasma proteins, other lipoproteins and water soluble antioxidants are present in the extracellular fluid\(^{(248)}\) and have been demonstrated to inhibit transition metal ion oxidation of LDL in vitro.\(^{(125,249,252,256)}\) Serum which is betalipoprotein deficient (BLPDS) has been proposed as a surrogate for the fluid surrounding LDL in the artery wall.\(^{(12)}\) However, BLPDS appears to increase the oxidation of glycosaminoglycan LDL (HepLDL) when catalysed with the low molecular chelate of iron, haemin.\(^{(12)}\) The aim of the present study was to determine the characteristics of peroxidase-catalysed oxidation of HepLDL including the effect of antioxidants, tyrosine and BLPDS. This model may approximate the situation in the arterial intima where LDL oxidation is believed to occur in vivo.

4.2 Methodology

4.2.1 Materials

Chondroitin sulphate C, 1,1,3,3-tetraethoxypropane, vitamin E (d-\(\alpha\)-tocopherol), butylated hydroxytoluene (BHT), tyrosine, horse radish peroxidase (HRP) and myeloperoxidase (MPO) (from human leukocytes) were obtained from Sigma Chemical Company. Heparin (Multiparin, from porcine mucosa) was purchased from Fisons PTY Ltd. L-ascorbic acid was obtained from JT Baker, NJ and uric acid and polyethyleneglycol 20,000 from BDH. Ecocnopac PD-10 columns were purchased from Bio Rad.

4.2.2 Preparation of lipoprotein fractions

Lipoproteins were isolated from serum or EDTA plasma from normolipidaemic donors. Heparin LDL (HepLDL) was isolated by the following method.\(^{(12)}\) Sodium citrate buffer (64 mmol/l, pH 5.12) containing heparin (50,000 IU/l) was added to serum (0.5 ml) and incubated at 23°C for 10 minutes before centrifuging at 2800 x g for 15 minutes (23°C). The precipitate (HepLDL) was washed 3 times with hypotonic HEPES buffer (5 mmol/l with sodium chloride 20 mmol/l, calcium chloride 4 mmol/l and magnesium chloride 2 mmol/l, pH 7.2) then redissolved in 4% sodium chloride in PBS. The redissolved HepLDL was used immediately.

Native LDL (d 1.019-1.063 g/ml) was isolated by sequentially ultracentrifuging EDTA plasma adjusted to the appropriate densities for 20 hours and 24 hours respectively, at 40,000 rpm in a Beckman 50.3 Ti rotor at 10°C. The isolated LDL was dialysed twice against PBS (16 hours) in the dark at 4°C. The
dialysed LDL was stored in the dark at 4°C under argon and was used within 2 days. Native LDL was also isolated rapidly by ultracentrifugation of EDTA plasma on a single-step discontinuous gradient in a Beckman NVT 65 rotor. The LDL isolated by this procedure was desalted into PBS by gel-filtration on Ecocnopac PD-10 columns. The PBS was Chelex treated to remove any metal ions. Chondroitin sulphate treated LDL (CsLDL) was prepared by adding an aliquot (200 µl) of chondroitin sulphate C solution (13 mg/ml in PBS) to native LDL (2.6 mg protein in 3 ml PBS) and adjusting the pH to 4.6. The precipitate was washed with PBS and redissolved in 4% sodium chloride in PBS. An insoluble complex between LDL and dextran sulphate (DexLDL) was prepared by adding 0.12 ml of dextran sulphate/magnesium chloride reagent to 0.5 ml of native LDL solution (2-4 mmol/l cholesterol) followed by 3 ml of hypotonic HEPES buffer. The precipitate was sedimented, washed and redissolved in 4% sodium chloride in PBS as described for the preparation of HepLDL. Native LDL (1 volume) was also precipitated by adding 2 volumes of 9.5% polyethylene glycol (MW 20,000) solution in phosphate buffer pH 6.5, resulting in PegLDL. Aggregated native LDL was prepared essentially according to the method of Khoo and co-workers. Isolated LDL was passed through a Ecocnopac PD-10 gel-filtration column equilibrated with PBS containing EDTA (0.01%) and the resulting solution was vortexed for 1 minute.

4.2.3 Oxidation of LDL

Formation of conjugated dienes or TBARS were used to monitor oxidation of LDL which had been rapidly isolated and desalted within the preceding 60 minutes. When conjugated dienes were measured, isolated LDL (50-100 µl, 0.1 µmol cholesterol) was added to H₂O₂ (10 µl of 12.9 µmol/ml in PBS) and added to 1.8 ml PBS and distilled water (0.20-0.25 ml) to maintain approximate isotonicity in the incubation medium. An aliquot of HRP (10 µl of 119 U/ml in PBS) or MPO (10 µl of 250 U/ml in PBS) was added with rapid mixing and the absorbance at 234 nm was monitored during incubation of the mixture at room temperature (23°C). The final volume of the incubation mixture was 2.12 ml. Tonicity of the incubation medium and use of Chelex-treated PBS or untreated PBS did not appreciably influence oxidation rates. Maximal absorbance was determined by spectrophotometer (Ultrospec 2000 UV Visible Spectrophotometer, Pharmacia Biotech) at λ 234 nm and time taken to attain half-maximal absorbance (t₅₀₃ₐ₅₄) were determined from the plot of absorbance values against time.

When TBARS were measured, the LDL (100-150 µl, 0.2 µmol cholesterol), the serum fraction (0-200 µl), H₂O₂ (10 µl of 12.9 µmol/ml in PBS), and HRP (10 µl of 119 U/ml in PBS) or MPO (10 µl of 250 U/ml in PBS) were added to PBS (1 ml) and the final volume adjusted to 1.4 ml. The mixture was incubated at 23°C for 30
minutes. At the end of this period, BHT (10 µl of 4 mmol/l in methanol) and EDTA (50 µl of 4 mmol/l) were added and TBARS were measured essentially by the method of Beuge and Aust. Thiobarbituric acid solution (2 ml) containing 0.375% thiobarbituric acid in 15% trichloracetic acid and 0.25 mol/l hydrochloric acid was added to the incubation mixture and to the standards containing 1,1,3,3-tetraethoxypropane (1.84-4.60 nmol). Tubes were heated on a boiling water bath for 30 minutes and after cooling were centrifuged at 2800 x g for 30 minutes. The optical density of the clear supernatant was determined by a spectrophotometer (Ultrospec 2000 UV Visible Spectrophotometer, Pharmacia Biotech) at λ 532 nm against the blank and amount of TBARS formed was calculated using standards. Rates of LDL oxidation were expressed as nmol TBARS/ µmol LDL cholesterol/ 30 minutes.

4.2.4 Analytical methods

Commercial enzymatic kits and calibrators (Boehringer Mannheim) were used to determine concentrations cholesterol. Serum paraoxonase activity was measured by monitoring the rate of phenylacetate hydrolysis at 270 nm following the addition of diluted serum. A fluorimetric method was used to measure TBARS in unoxidised HepLDL.

4.2.5 Statistical analysis

The means of variables were compared with two-tailed t-tests, with P ≤ 0.05 considered statistically significant. Analysis of variances was used to compare the effects of several concentrations of hydrogen peroxide on tₜₘₐₓ and maximal absorbance at 234 nm, and varying levels of BLPDS on TBARS formation during HepLDL oxidation. The statistical analysis was carried out using SPSS release 6.1 (SPSS for Windows, SPSS Inc.). Unless otherwise stated all data are expressed as mean ± SD.
Figure 4.1 Oxidation of HepLDL and native LDL by HRP and \( H_2O_2 \)

A Change in optical density at 234 nm during the oxidation of HepLDL [open circle] or native LDL [open triangle] (0.47 µmol cholesterol/ml) by HRP (0.56 U/ml) and \( H_2O_2 \) (58 µmol/l) incubated in PBS at 23°C. HepLDL incubated in PBS without the oxidants [circle]. The figure represents a typical experiment from over 6 individual experiments with HepLDL and 5 experiments with native LDL. B Time-course of TBARS formation during the oxidation of HepLDL [open circle] (0.14 µmol/cholesterol/ml) by HRP (0.85 U/ml) and \( H_2O_2 \) (87 µmol/l) incubated in PBS at 23°C. The figure represents the average of two experiments and the bars represent the range of values at each time point.
Figure 4.2 Oxidation of HepLDL and native LDL by MPO and H\textsubscript{2}O\textsubscript{2}

Change in optical density at 234 nm during the oxidation of HepLDL [open circle] or native LDL [circle] (0.47 \(\mu\)mol cholesterol/ml) by MPO (0.09 U/ml) and H\textsubscript{2}O\textsubscript{2} (58 \(\mu\)mol/l) incubated in PBS at 23\(^\circ\)C. The figure represents a typical experiment from 2 separate experiments with HepLDL and native LDL.
Figure 4.3  Effect of ascorbate and vitamin E on the oxidation of HepLDL by HRP and H$_2$O$_2$

A  Change in optical density at 234 nm during oxidation of HepLDL (0.47 µmol cholesterol/ml) by HRP (0.56 U/ml) and H$_2$O$_2$ (58 µmol/l) incubated in PBS at 23°C with [circle] and without ascorbate [open circle] (79 µmol/l). Also, ascorbate (79 µmol/l) was incubated with the oxidants [open square]. The figure represents a typical experiment from 3 independent experiments.  

B  Vitamin E solution in ethanol (27.2 mmol/l) was added to serum (10 µl and 272 nmol vitamin E/ml serum) and the mixture was incubated at 37°C for 1.5 hours. A control [open circle] incubation of serum was added to ethanol (10 µl/ml serum). HepLDL was isolated from serum [circle] and 0.47 µmol HepLDL cholesterol/ml was oxidised with HRP (0.56 U/ml) and H$_2$O$_2$ (58 µmol/l). The mean vitamin E content of the control HepLDL was 1.3 ± 1.0 nmol/µmol cholesterol and of vitamin E enriched HepLDL was 107.7 nmol/µmol cholesterol. The figure represents a typical experiment from 3 independent experiments.
Figure 4.4 Effect of added tyrosine on the oxidation of HepLDL by HRP and H₂O₂

Change in optical density at 234 nm during the oxidation of HepLDL (0.47 µmol/l cholesterol/ml) by HRP (0.56 U/ml) and H₂O₂ (58 µmol/l) incubated in PBS at 23°C with tyrosine (48 µmol/l) [circle] or without tyrosine [open circle]. The stock solution (20.44 mmol/l) of tyrosine was prepared by dissolving tyrosine in dilute hydrochloric acid. The addition of 5 µl of tyrosine solution to the PBS did not appreciably alter the pH of the incubation medium (pH 7.39). The figure represents a typical experiment from 3 independent experiments.
Figure 4.5 Effect of BLPDS on oxidation of HepLDL by HRP or MPO and H$_2$O$_2$

A Formation of TBARS after 30 minutes oxidation of HepLDL (0.14 µmol cholesterol/ml) with BLPDS (1.9%, 7.5%, 11.2%, 14.9% by volume) by HRP (0.85 U/ml) and H$_2$O$_2$ (58 µmol/l) incubated in PBS at 23°C. Similar incubations but without HepLDL were also performed and TBARS were subtracted from the levels measured when HepLDL was also present. Values are averages of 3 separate experiments and the bars represent the range. B A similar experiment in which MPO (0.14 U/ml) replaced HRP is displayed. Values are averages of 2 separate experiments and the bars represent the range
### TABLE 4.1 Effect of serum components on TBARS formation in HepLDL oxidised by HRP/H$_2$O$_2$

<table>
<thead>
<tr>
<th>Additions</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLPDS (14.9% by volume)</td>
<td>10 ± 4*</td>
</tr>
<tr>
<td>Dialysed BLPDS (14.9 % by volume)</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>Gel-filtered BLPDS (14.9% by volume)</td>
<td>88 ± 15</td>
</tr>
<tr>
<td>+ Mg$^{2+}$ (54 mmol/l)</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>+ ascorbate (84 μmol/l)</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>+ tyrosine (48 μmol/l)</td>
<td>122 ± 23</td>
</tr>
<tr>
<td>+ cysteine hydrochloride (134 μmol/l)</td>
<td>98 ± 2</td>
</tr>
</tbody>
</table>

*Values are mean ± SD of 3 separate experiments.
*BLPDS compared with control, with level of significance P<0.001.
4.3 Results

Conjugated diene formation when native LDL and glycosaminoglycan LDL stimulated with HRP/H$_2$O$_2$ is presented in Figure 4.1A. Conjugated diene formation increased rapidly with no lag-time during HRP catalysed oxidation of HepLDL while there was little change with native LDL. The mean t$_{\text{max}}$ was 3 ± 1 minutes (n=5) for the oxidation of HepLDL and values for CsLDL were within the range of those observed in HepLDL. Similar results were obtained when native LDL that had been isolated by ultracentrifugation of plasma, precipitated with heparin or chondroitin sulphate and then resolubilised, was oxidised by HRP. There was no change in absorbance at $\lambda$ 234 nm when HepLDL was incubated alone or with H$_2$O$_2$ in the absence of HRP. The formation of TBARS paralleled that of conjugated dienes during HRP oxidation of HepLDL, this is displayed in Figure 4.1B. Thiobarbituric acid reacting substances were virtually undetectable (10 - 30 µmol/mol cholesterol) in freshly isolated HepLDL. Redissolving the HepLDL precipitate in 4% sodium chloride/PBS was not responsible for the increased susceptibility of HepLDL to oxidation. When an aliquot of a suspension of HepLDL precipitate in hypotonic HEPES buffer was added to PBS (in which it was dissolved) and oxidised with HRP/ H$_2$O$_2$, the rate of TBARS formation (26.5 ± 5.7 mmol/mol cholesterol/30 minutes, n=3 experiments) was not significantly different compared with the corresponding rate with HepLDL that had been redissolved in 4% sodium chloride/PBS. Addition of EDTA (0.14 mmol/l) did not reduce the formation of TBARS during oxidation of HepLDL by HRP/ H$_2$O$_2$.

When a suspension of precipitated native LDL in polyethyleneglycol solution was treated with HRP/ H$_2$O$_2$ (maximum final polyethyleneglycol concentration, 0.17 mmol/l) absorbance at 234 nm increased slowly to a mean value of 0.029 ± 0.013 mmol/mol cholesterol (n=3) at 30 minutes which was significantly (P=0.0004) lower than the corresponding value for the oxidation of HepLDL (0.236 ± 0.029 mmol/mol cholesterol/ 30 minutes with t$_{\text{max}}$, 5.9 ± 0.6 minutes, n=3) in the presence of polyethyleneglycol (0.17 mmol/l). The precipitated LDL suspended in polyethyleneglycol solution redissolved to give virtually zero absorbance at 680 nm (a measure of turbidity) when the suspension was added to PBS prior to addition of the oxidants. Aggregated LDL did not dissolve in 4% sodium chloride/PBS and a suspension of the lipoprotein in PBS was not oxidised to TBARS by HRP/H$_2$O$_2$.

The formation of conjugated dienes during the incubation of HepLDL with MPO/ H$_2$O$_2$ was rapid with no lag-phase compared with the corresponding oxidation of native LDL which had a lag-time of greater than 150 minutes, displayed in Figure 4.2. The t$_{\text{max}}$ for the formation of dienes from HepLDL was approximately 50 minutes.

Figure 4.3 shows the effect of antioxidants, ascorbic acid and vitamin E, on the oxidation of HepLDL by HRP/H$_2$O$_2$. In the presence of ascorbate, conjugated diene formation increased within the first two minutes and then declined toward zero during the 30 minute incubation period. Optical density at $\lambda$ 234 nm also increased rapidly when ascorbate was treated with the oxidants. Production of TBARS was
inhibited, by 98% in each of the two separate experiments, when HepLDL was oxidised with HRP/H₂O₂ in the presence of 79 µmol/l ascorbate. The rate of diene formation during oxidation of vitamin E enriched HepLDL (isolated from serum enriched with vitamin E and contained increased levels of vitamin E) was similar to the rate in control HepLDL (isolated from serum incubated with ethanol). In separate experiments (n=3) with materials from different subjects, additional of vitamin E in ethanol at concentrations of 1 µmol/l, 2 µmol/l and 3 µmol/l and a final ethanol concentration of 0.9% did not significantly affect tₙₜₘₐₓ (control: 2.7 ± 0.3 minutes; 1 µmol/l vitamin E: 3.0 ± 1.3; 2 µmol/l vitamin E: 3.3 ± 1.2 minutes; 3 µmol/l vitamin E: 3.0 ± 1.0 minutes, ANOVA P=0.88) and the ratio maximum absorbance in the presence of vitamin E/ control maximum absorbance in the absence of vitamin E and with added ethanol (ANOVA P=0.29). Added BHT (18.9 µmol/l) significantly (P<0.001) decreased the maximum amount of conjugated dienes formed (−75 ± 13%, n=3) and the production of TBARS to 2% of control values (n=2) during oxidation of HepLDL by HRP/H₂O₂. Tyrosine (48 µmol/l) inhibited the early rapid formation of conjugated dienes during oxidation of HepLDL, presented in Figure 4.4. After 30 minutes, amounts of dienes formed were similar in the presence or absence of tyrosine.

Figure 4.5 plots the effect of increasing concentrations of BLPDS on the formation of TBARS during oxidation of HepLDL with HRP and MPO. Oxidation was followed by measuring TBARS formation because conjugated dienes cannot be readily measured in the presence of BLPDS which absorbs strongly at λ 234 nm. Added BLPDS significantly inhibited TBARS formation (ANOVA P<0.0001) particularly at concentrations between 7.5 – 14.9%. The rate of TBARS formation increased (0.1 – 1.2 nmol/30 minutes) with increasing concentrations of BLPDS alone, and quantities of TBARS produced were markedly smaller than levels when HepLDL was also present. Levels of TBARS derived from BLPDS alone were subtracted from values obtained during oxidation of HepLDL in the presence of BLPDS. Oxidation of HepLDL by MPO/H₂O₂ was also inhibited by increasing concentrations of BLPDS. This inhibition was less marked compared with corresponding inhibition of HRP catalysed oxidation of HepLDL.

The effect of BLPDS (14.9% by volume) that had been dialysed or gel-filtered to remove small water soluble molecules and the addition of magnesium ions, ascorbate, tyrosine and cysteine hydrochloride to gel-filtered BLPDS on the formation of TBARS during oxidation of HepLDL by HRP/H₂O₂ is summarised in Table 4.1. Both dialysed and gel-filtered BLPDS failed to inhibit this oxidation significantly. Gel-filtered BLPDS with added magnesium ions (equivalent to the level in BLPDS) and physiological levels of ascorbate, tyrosine and cysteine hydrochloride did not inhibit TBARS formation. Added urate (19.2 – 76.9 µmol/l) also did not appreciably alter TBARS formation during oxidation of HepLDL catalysed by HRP/H₂O₂. The inhibitory effect was still present in BLPDS that was prepared from serum that had been heated for 1 hour at 58°C and had only 3% of its initial activity of paraoxonase, an enzyme which metabolises oxidised lipids (control: 23.5 ± 0.7 nmol/ µmol/ 30 minutes; heated BLPDS: 2.1 ± 1.9 nmol/ µmol/ 30 minutes; n=3 experiments, P<0.0001).
4.4 Discussion

Low density lipoproteins which have been resolubilised from complexes with glycosaminoglycans are more susceptible than native LDL to peroxidase-catalysed oxidation and were only partially inhibited by BLPDS when MPO was the catalyst. This enhanced susceptibility to oxidation may contribute to increased levels of myeloperoxidase-modified LDL and oxidised LDL that are present in atherosclerotic arteries.\(^{(74,102)}\)

In the presence of \(\text{H}_2\text{O}_2/\text{HRP}\) or MPO rapidly oxidised HepLDL compared with native LDL. Following HRP catalysed oxidation of HepLDL by monitoring the lipid oxidation products conjugated dienes and TBARS gave similar profiles. The model of HRP/\(\text{H}_2\text{O}_2\) catalysed HepLDL oxidation displayed appropriate characteristics of linearity with respect to HepLDL, HRP and \(\text{H}_2\text{O}_2\) levels. Furthermore, oxidation occurred at levels of \(\text{H}_2\text{O}_2\) which may occur in vivo.

The increased susceptibility of HepLDL (and other glycosaminoglycan treated LDL) to peroxidase oxidation appeared to be due to changes in LDL as a result of the interaction with glycosaminoglycan and not as a result of precipitation and resolubilisation of LDL. Precipitation with polyethylene glycol which sterically excludes LDL from solution\(^{(547)}\) and does not form a complex with the lipoprotein, did not increase the oxidisability of the resolubilised LDL by HRP. Levels of lipid oxidation products were low in freshly isolated HepLDL suggesting that the isolation procedure did not oxidatively stress HepLDL and thereby increase its susceptibility to peroxidase catalysed oxidation. When LDL is precipitated with heparin, apolipoprotein B-containing remnant lipoproteins such as intermediate density lipoproteins are also precipitated. However these remnants were not uniquely responsible for the rapid oxidation of HepLDL as native LDL which had been precipitated with heparin also displayed rapid oxidation.

Changes in lipoprotein structure caused by the interaction of LDL with glycosaminoglycans may underlie the rapid rate of peroxidase stimulated lipid peroxidation in HepLDL compared with native LDL. These structural changes may increase the efficiency of peroxidase induced formation of radicals from apolipoprotein B and alpha-tocopherol which may mediate oxidation of LDL lipids by MPO and HRP. Also, the altered molecular structure in HepLDL may increase the proximity of the radicals to lipids. Camejo and co-workers have reported that the interaction with arterial proteoglycans and glycosaminoglycans in vitro alters the structure of both lipid and protein components of LDL\(^{(16,263)}\) and this could be as a result of disruption of the molecular organisation of the cholesterol ester core\(^{(263)}\) and apolipoprotein B moiety.\(^{(14,261,548)}\) Thus, it is possible that altered protein conformation may increase the accessibility of sites on apolipoprotein B to peroxidase catalysed radical formation and may place apolipoprotein B radicals in a better position to attack the lipid substrate in HepLDL. The increased access of the peroxidase enzymes to apolipoprotein B in glycosaminoglycan treated LDL is consistent with previous reports that have shown that the protein in LDL which has interacted with proteoglycans has increased susceptibility to fragmentation.\(^{(16,548)}\) It has also been suggested that the physical state of lipids and their proximity to
radicals generated in the lipoprotein may be important in the oxidation by peroxidases.\textsuperscript{(277)}

Glycosaminoglycans induced changes in lipoprotein structure that may also alter the effect of alpha-tocopherol on LDL oxidation by HRP. Santanam and co-workers have reported that addition of small amounts of alpha-tocopherol accelerates peroxidase oxidation in native LDL apparently by increasing numbers of alpha-tocopherol radicals.\textsuperscript{(277)} At higher levels of alpha-tocopherol, the oxidation is inhibited suggesting that sufficient free alpha-tocopherol may remain to inhibit oxidation.\textsuperscript{(11,277)} In contrast, the present data indicate that HRP/H\textsubscript{2}O\textsubscript{2} oxidation of LDL that has interacted with heparin glycosaminoglycan is unaffected not only by additions of small quantities of alpha-tocopherol to the incubation but also by massive prior enrichment of HepLDL with alpha-tocopherol. It is possible that molecular disorganisation\textsuperscript{(263)} may allow HRP increased access to alpha-tocopherol in the cholesterol ester-rich hydrophobic core of HepLDL resulting in faster production of alpha-tocopherol radicals and decreased free alpha-tocopherol. The HRP catalysed lipid oxidation in HepLDL is probably near or at maximal rate and may therefore be unaffected by extra alpha-tocopherol radicals generated from added alpha-tocopherol. Lipid oxidation in HepLDL was markedly inhibited by BHT, a lipophilic antioxidant whose radicals are sterically hindered and may be inaccessible for propagation of the radical chain reaction.\textsuperscript{(277)} Thus, increased generation of alpha-tocopherol radicals that are not sterically hindered and can accelerate lipid oxidation\textsuperscript{(277)} may contribute to the inefficiency of alpha-tocopherol as an antioxidant in the HepLDL and HRP/H\textsubscript{2}O\textsubscript{2} system. It remains to be determined whether rapid oxidation reduces free alpha-tocopherol to levels that no longer inhibit lipid oxidation in alpha-tocopherol enriched HepLDL.

Ascorbate inhibited HepLDL oxidation by HRP, possibly by decreasing levels of apolipoprotein B and alpha-tocopherol radicals. The protein component of LDL is largely at the surface of the LDL and therefore readily accessible to a water soluble antioxidant. Horse-radish peroxidase may oxidise ascorbate to products which react with LDL protein and decrease the formation of apolipoprotein B radicals. This proposed mechanism is essentially analogous to that which governs the inhibition of copper ion oxidation of LDL by ascorbate. Retsky and co-workers have reported that during copper ion oxidation of LDL in the presence of ascorbate there is oxidation of ascorbate to dehydroascorbate (and other products) which may react with LDL protein and inhibit the subsequent copper ion catalysed lipid oxidation.\textsuperscript{(252)} In the present study, ascorbate appeared to have been oxidised before it appreciably inhibited the oxidation of HepLDL. There was a large and rapid increase in absorbance at \(\lambda\) 234 nm when ascorbate and oxidants were incubated in PBS suggesting that ascorbate had been modified. The momentary increase in absorbance at \(\lambda\) 234 nm when HRP and H\textsubscript{2}O\textsubscript{2} were added to a solution of HepLDL in the presence of ascorbate may be due mainly or solely to the formation of oxidation products of ascorbic acid that absorb at this wavelength and not to increased conjugated diene formation. The gradual decline of this early peak in absorbance at \(\lambda\) 234 nm may reflect a reaction between the ascorbate oxidation products and HepLDL. These findings suggests that there may be differences in the antioxidant mechanism of ascorbate in the presence of transition metal ions and HRP. The
reduced form of ascorbate can convert alpha-tocopherol radicals to free alpha-tocopherol in LDL.(278) However, it is uncertain whether this regenerating activity of ascorbate remains after it has been modified by HRP/H₂O₂.

Free tyrosine also inhibited the oxidation of HepLDL by HRP but only during the early stages of the incubation. Tyrosine may have initially competed with HepLDL for oxidation until eventually levels of the amino acid were decreased and no longer competed effectively for oxidation. Santanam and co-workers reported the oxidation of native LDL by myeloperoxidase was inhibited by high levels of added tyrosine.(277) The requirement of free tyrosine for the oxidation of native LDL by MPO (89) may only be essential when the alpha-tocopherol content of the lipoprotein is low.(277) In the present study, MPO readily oxidised HepLDL in the absence of free tyrosine. Freshly isolated HepLDL is not depleted of alpha-tocopherol,(12) which may mediate MPO catalysed lipid oxidation. Also, the altered lipoprotein structure may allow the oxidation of HepLDL by MPO in the absence of tyrosine.

Added BLPDS decreased the oxidation of HepLDL catalysed by HRP but even at the highest concentration only partially inhibited the corresponding oxidation catalysed by myeloperoxidase. The inhibitor(s) in BLPDS was a small, water soluble molecule which did not appear to be ascorbate, urate, tyrosine or reduced thiols. The mechanism underlying the greater sensitivity of HRP oxidation to inhibition by BLPDS is uncertain but may relate to the position of the catalytic hæme unit in HRP. In HRP the hæme unit is relatively exposed whereas in myeloperoxidase it is buried deep in a hydrophobic cleft(549) and may be less accessible to components of BLPDS. The finding that BLPDS only partially inhibited the oxidation of HepLDL by myeloperoxidase appears to be consistent with evidence that myeloperoxidase oxidises LDL in the artery wall(102) where components of extracellular fluid can modulate the process. Levels of BLPDS used in the present study were in the range which corresponds with the concentration of many of the compounds in extracellular fluid.(550)

In conclusion, the present data suggest that LDL which has formed complexes with glycosaminoglycans is abnormally susceptible to oxidation by peroxidases including a physiological oxidant MPO. These experiments support the hypothesis that LDL which becomes trapped in the artery wall by interaction with proteoglycans may be susceptible to oxidation by MPO. Also peroxidase oxidation of LDL may be favoured at low levels of water-soluble antioxidants. Proteoglycans and glycosaminoglycans are negatively charged and may attract MPO which has a basic pK and will be positively charged at physiological pH. An interaction of this type could bring the enzyme and LDL into close proximity and potentiate lipoprotein modification. Oxidation of LDL-proteoglycan complexes by MPO may increase levels of MPO-modified LDL and potentially accelerate lesion development.
Chapter 5

Conclusions

Over recent years there has been considerable interest in the hypothesis that supplementation with antioxidants may reduce the risk of CHD. This interest has been generated largely by evidence that the oxidation of LDL is an important step in atherogenesis. Also, recently published research has indicated that other steps in lesion formation, including the expression of pro-inflammatory cytokines and cell adhesion molecules, may be mediated by oxidant sensitive mechanisms. One of the studies in this thesis has compared the effect of supplementation with a tomato juice, which is rich in the carotenoid lycopene, and high doses of vitamin E or vitamin C on LDL oxidation and markers of inflammation in patient with type 2 diabetes. These patients have a high risk of CAD and may benefit from antioxidant therapy as they are often under increased oxidative stress. The supplementation regimens with tomato juice, alpha-tocopherol and vitamin C each resulted in plasma concentrations of lycopene, alpha-tocopherol and vitamin C increasing over two fold. Lycopene and alpha-tocopherol are primarily associated with lipoproteins in the plasma, thus this enrichment would significantly enhance the intrinsic antioxidant protection of LDL. The resistance of isolated LDL to copper ion stimulated oxidation was increased by 42% after the patients consumed tomato juice for several weeks, and this was comparable to the 54% found after the alpha-tocopherol regimen. An extension in resistance to oxidation may conceivably reflect a lower risk of atherosclerosis, however, the relationship between lag time of isolated LDL to copper ion stimulated oxidation and atherosclerosis has not yet been established with absolute certainty. Alpha-tocopherol supplementation also decreased plasma C-RP levels, suggesting a reduction in systemic inflammation. On the basis of recent epidemiological studies this change could be associated with a reduced risk of MI. Expression of cell adhesion molecules in diabetic patients does not appear to be affected by high intakes of dietary antioxidants with differing structures and modes of action. Supplementation with high doses of vitamin E and vitamin C increased plasma cholesterol and apolipoprotein B, respectively, and this change would be expected to increase the risk of CAD, thus the effects of high dose supplementation with vitamins E and C requires careful evaluation of the possible risks and benefits in diabetic patients. Overall, this research has identified changes with potential relevance to CHD in diabetic patients during supplementation with dietary antioxidants. In the future, studies with larger numbers of patients and of a longer duration are required to confirm the effects of vitamin E supplementation on plasma lipids and markers of systemic inflammation.

Copper ion oxidation of isolated LDL may not reflect the complexity of LDL oxidation in the artery wall. Interactions of LDL with proteoglycans and glycosaminoglycans, and the presence of extracellular fluid and peroxidases are
factors which may have an important influence on LDL oxidation in the arterial intima. A second study established a model of LDL oxidation which incorporated several of these elements. Low density lipoproteins which have complexed with glycosaminoglycans were rapidly oxidised by peroxidases, including MPO and HRP. This rapid oxidation was inhibited by vitamin C and small water soluble compounds in the human serum, but not by added vitamin E. This model predicts that LDL which becomes trapped in the artery wall by interactions with proteoglycans may be susceptible to oxidation by MPO particularly when levels of water soluble antioxidants are low. These findings may be relevant to oxidation of LDL in the micro-domains of the artery wall which become depleted of antioxidants. Finally, the model may provide new insights on the effect of antioxidant supplementation on LDL oxidation in diabetic patients.
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Appendices
Appendix A

Upritchard JE, Sutherland WHF, Mann JI. The effect of supplementation with tomato juice, vitamin E and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. *Submitted to “Diabetes Care” 1999.*
THE EFFECT OF SUPPLEMENTATION WITH TOMATO JUICE, VITAMIN E AND VITAMIN C ON LDL OXIDATION AND PRODUCTS OF INFLAMMATORY ACTIVITY IN TYPE 2 DIABETES

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Short title: Antioxidants, LDL oxidation and inflammatory markers in diabetes

Key words: Antioxidants, LDL, oxidation, inflammation, type 2 diabetes.

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ABSTRACT

OBJECTIVE – To compare the effects of dietary supplementation with tomato juice, vitamin E and vitamin C on susceptibility of low density lipoprotein (LDL) to oxidation and circulating levels of C-reactive protein (C-RP) and cell adhesion molecules in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS – Fifty-seven patients with well-controlled type 2 diabetes and aged < 75 years were treated with placebo for 4 weeks and then randomised to receive either tomato juice (500 ml/day), vitamin E (800 IU/day), vitamin C (500 mg/day) or continued placebo treatment for 4 weeks. Susceptibility of LDL to oxidation (lag time) and plasma concentrations of C-RP, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were measured at the beginning of the study, after the placebo phase and at the end of the study.

RESULTS – Plasma lycopene levels increased nearly 3-fold (P=0.001) and the lag-time in isolated LDL oxidation by copper ions increased by 42% (P=0.001) in patients during supplementation with tomato juice. The magnitude of this increase in lag time was comparable with the corresponding increase during supplementation with vitamin E (54%). Plasma C-RP levels decreased significantly (~49%, P=0.004) in patients who received vitamin E. Circulating levels of cell adhesion molecules and plasma glucose did not change significantly during the study.

CONCLUSIONS – This study indicates that drinking commercial tomato juice increases plasma lycopene levels and the intrinsic resistance of LDL to oxidation almost as effectively as supplementation with a high dose of vitamin E in patients with diabetes. Supplementation with vitamin E also decreases plasma C-RP levels which may indicate reduced systemic inflammatory activity. These findings may be relevant to strategies aimed at reducing risk of myocardial infarction in patients with diabetes.
Patients with type 2 diabetes are at increased risk of developing coronary heart disease (CHD) compared with the general population (1). Increased oxidative stress (2) and enhanced oxidation of low density lipoproteins (LDL) (3) are believed to contribute to this excess risk of arterial disease (1). In vitro, high glucose levels increase LDL oxidation (4) and glycated LDL is abnormally susceptible to oxidative modification (5). Also, levels of small dense LDL are increased in diabetic subjects (6) and these particles are more readily oxidised than larger, more buoyant LDL (7). Oxidation of low density lipoprotein (LDL) that becomes trapped in the artery wall is widely regarded as an important step in the development of atherosclerosis (8,9). There is evidence that mildly oxidised LDL enhances the expression of proinflammatory cytokines, chemoattractants and cellular adhesion molecules (9) by endothelial cells. These molecules promote adhesion of monocytes to the vascular endothelium followed by transmigration of adhered cells into the intima where they are retained and transformed into macrophages (8,9). Cultured macrophages avidly internalise oxidised LDL via scavenger receptors to form lipid-filled cells (8,9). A similar process may occur in the artery wall to generate foam cells that are seen early in the development of the atherosclerotic lesion. In diabetic patients, circulating levels of proinflammatory cytokines (10), C-reactive protein (C-RP) (10) and soluble vascular cell adhesion molecule-1 (VCAM-1) and soluble intercellular adhesion molecule-1 (ICAM-1) (11,12), are elevated suggesting stimulation of proatherogenic inflammatory activity. Plasma C-RP is a sensitive marker of systemic inflammation and chronically high levels predict increased risk of future coronary events (13-15).

There is epidemiologic and clinical evidence that high intake or levels of vitamin E, lycopene and vitamin C may be associated with decreased risk of CHD (17-18). Laboratory studies suggest that these compounds may potentially attenuate a number of the steps in the postulated pathway of atherosclerotic lesion formation. Supplementation with high doses of vitamin E markedly reduces susceptibility of isolated LDL to oxidation and inhibits secretion of proinflammatory cytokines (19). Enriching cultured endothelial cells (20, 21) or LDL (20) with vitamin E decreases expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) induced by native or oxidised LDL. Lycopene, a major carotenoid in human plasma, also inhibits the oxidative modification of isolated LDL (22). Tomato products in the diet are the main source of plasma lycopene and supplementation with tomato juice increases plasma lycopene levels in healthy subjects (23). Vitamin C in the aqueous milieu can protect plasma lipids and LDL from oxidative damage (24). Also, supplementation with vitamin C normalises low plasma vitamin C levels and enhanced monocyte adhesion in smokers (25).
In diabetic patients, antioxidant protection may be inadequate and levels of some antioxidants including vitamin C and lycopene (26) are frequently low. Supplementation with vitamin E increases isolated LDL resistance to oxidation in patients with type 2 diabetes (27). However, information regarding the effects of food products such as tomato juice rather than supplements on susceptibility of LDL to oxidation and circulating levels of antioxidants and inflammatory products in patients with type 2 diabetes is sparse. The present study was therefore designed to compare the effects of supplementation with tomato juice, vitamin E and vitamin C on these factors in patients with type 2 diabetes in a randomised, placebo-controlled trial.

RESEARCH DESIGN AND METHODS

Patients

Patients with type 2 diabetes under the age of 75 years and with HbA1c < 10% and fasting plasma glucose < 11 mmol/l were recruited from the Diabetes Clinic, Dunedin Hospital, from local general practitioners and by advertisement in the newspaper. Exclusion criteria included, presence of hepatic or renal disease, cigarette smoking, use of dietary antioxidant supplements and treatment with insulin, lipid-lowering drugs or hormone therapy during the preceding 6 months.

At recruitment, a medical history was obtained from the patients. Past smoking habits and medication use were recorded. Blood pressure and body mass index (BMI) were measured. Patients were instructed not to change their usual dietary habits for the duration of the study and to take the experimental dietary supplements with meals. A checklist was used to estimate the frequency of tomato product consumption during the study. Patients were also instructed to return any unused supplements and compliance with study protocol was assessed during the study by counting returned supplements.

Study design and protocol

The study was a randomised, placebo-controlled parallel trial. Randomisation was carried out independently using a computer generated scheme (Excel, Microsoft Office for Windows 95). A total of 57 patients were randomised to receive either: (a) 800 IU/day vitamin E (d-α-tocopherol from a natural source, Red Seal); 500 mg/day vitamin C (Redoxin, Roche); 250 ml tomato juice which did not contain added sugar (Campbells Foodstuffs, Australia) twice daily; or a placebo gelatine capsule containing pharmaceutical starch. During the initial 4 weeks of the study, all patients received the placebo capsule and then proceeded to their assigned supplement for the following 4 weeks. Blood samples, blood pressure and BMI were taken on two occasions and 3 days apart, at baseline, end of placebo and end of intervention. The mean of values measured at these time points was used as a more reliable measure of variables during...
the study. The study was approved by the Ethics Committee of the Southern Regional Health Authority.

Patients reported to the study centre in the early morning after an overnight fast. Venous blood was collected into tubes containing disodium EDTA, sodium fluoride or heparin. Blood was kept on ice for a maximum of 2 hours before plasma was separated by low-speed centrifugation at 4°C. Metaphosphoric acid (900µl, 5% solution) was added to an aliquot of plasma (100µl) to be assayed for vitamin C and these aliquots and others were stored at -80°C. A sample of EDTA plasma to be used for the isolation of LDL was flushed with argon and was stored at 4°C in the dark for a maximum of 24 hours.

**Separation and oxidation of LDL**

Native LDL was rapidly separated by ultracentrifuging EDTA plasma for 2 hours at 60,000 rpm on a single-step discontinuous gradient in a Beckman NVT 65 rotor (28). The LDL isolated by this procedure did not contain appreciable levels of albumin (29). The LDL was desalted into phosphate buffered saline (PBS) by gel-filtration in Econopac PD-10 columns (28). The PBS was Chelex treated to remove any transition metal ions.

Oxidation of LDL was performed essentially as described by Puhl and coworkers (30). LDL (0.39 µmol cholesterol) was added to 2ml PBS in a quartz cuvette at ambient temperature in an air conditioned room that was maintained at constant temperature. The oxidation was initiated by the addition of copper ions (1.6 µmol/l) and was followed by monitoring the formation of conjugated dienes at 234 nm. The temperature in the cuvette was constant within one degree of 27°C. The interassay coefficient of variation for the lag-time in LDL oxidation was 5%.

**Analytical methods**

Cholesterol and triglycerides in plasma and lipoprotein fractions were measured using commercial enzymatic kits and calibrator (Boehringer Mannheim). High density lipoprotein cholesterol was measured in the supernatant after precipitation of apolipoprotein B (apoB)-containing lipoproteins with dextran/magnesium chloride (31). Plasma apoA1 and B were measured by immunoturbidimetry (32). Plasma glucose was measured enzymatically using a commercial kit (Boehringer Mannheim). HbA1c was measured using a commercial kit (Pierce, Glycotest 2). Concentrations of VCAM-1 and ICAM-1 were measured in duplicate by ELISA (R&D Systems) in one of the two plasma samples obtained at each time-point in the study. The coefficient of variation for the assays were 3.3% (VCAM-1) and 4.9% (ICAM-1). Plasma C-RP concentration
was measured in duplicate by a commercial ELISA assay (Hemagen) with a coefficient of variation of 8% and sensitivity of 0.2 mg/l. In the plasma VCAM-1, ICAM-1 and C-RP assays, all samples from a patient were measured in the same run. High pressure liquid chromatography (HPLC) was used to measure plasma α-tocopherol and lycopene levels (33) and LDL α-tocopherol content (34). Plasma vitamin C was measured by fluorimetry (35).

**Statistical analysis**

The study was able to detect a change in lag-time in LDL oxidation of 10 minutes at a power of 90% and P=0.05. Variables were log-transformed before statistical analysis. The multivariate analysis of variances (MANOVA) procedure in SPSS with repeated measures and with covariate correction for baseline values was used to test for differences in the response of variables to the various dietary supplementation regimen. When a significant difference was detected among the treatment groups, unadjusted paired t-tests were used to test for within-group changes during the active treatment phase of the study. Mean (95% confidence interval) was also calculated for changes during active treatment. Two-sided tests of significance were used and a P value <0.05 was considered as statistically significant. Unless otherwise stated all data are expressed as mean±SD.

**RESULTS**

Five patients withdrew during the study due to difficulties in donating a blood sample (n=2), alterations in medications (n=2) or problems with consuming the tomato juice (n=1). The baseline characteristics of the patients are detailed in Table 1. The majority of participants (86%) had diagnosed diabetes for less than 6 years. A third of the study group controlled their diabetes by diet alone and the remainder were receiving treatment with oral antihyperglycemic drugs. A majority of patients had multiple risk factors for CHD and many had clinical evidence of cardiovascular disease. Several patients were receiving treatment with ACE inhibitor drugs which are known to reduce the susceptibility of isolated LDL to oxidation (36). However, treatment with ACE inhibitor drugs remained unchanged during the study. There were no significant changes in BMI during the study.

**Plasma antioxidants**

Plasma concentrations of antioxidants in the patients during the study are shown in Table 2. Levels of α-tocopherol, vitamin C and lycopene were similar between treatment groups at baseline and at the end of the placebo phase of the study and increased significantly in the appropriate treatment group indicating both compliance with the supplementation regimen and good bioavailability of antioxidants in the
supplements. The frequency checklist of tomato products in the diet showed that at baseline, 82% of patients consumed raw tomatoes, 37% baked beans, 8% pizza, 13% canned tomatoes, 5% tomato soup, 67% tomato sauce and 21% spaghetti/sauce at least once during a 4 day period and these proportions were similar at the end of the study (excluding tomato juice supplement).

**LDL oxidation**

Oxidation characteristics and α-tocopherol content of LDL from the participants is shown in Table 3. The lag-time in conjugated diene formation increased significantly in patients treated with tomato juice [30 (95%CI: 17, 43) min] and vitamin E [40 (95%CI: 24, 57) min] and remained unchanged in those who received placebo [-6 (95%CI: -24, 12) min] and vitamin C [4 (95%CI: -11, 18) min]. The coefficient of variation between the two measures of the lag-time at baseline was 3.3% (n=57). The LDL content of α-tocopherol increased significantly only in the group which was supplemented with this antioxidant. Rate of oxidation during the propagation phase and maximum concentration of conjugated dienes formed did not change significantly during the study. The chemical composition (protein and lipids) of LDL also did not change significantly in patients during the study (data not shown).

**C-RP and cell adhesion molecules**

Plasma concentrations of C-RP and adhesion molecules in the patients during the study are shown in Table 4. Plasma C-RP levels decreased significantly (P=0.001) in patients supplemented with vitamin E [-3.5 (95%CI: -1.3, -5.7) mg/l] and did not vary significantly in those treated with vitamin C [-0.1 (95%CI: -2.2, 2.0) mg/l], tomato juice [0.6 (95%CI: -0.6, 1.8) mg/l] and placebo [0.8 (95%CI: -0.2, 1.8) mg/l]. At baseline, plasma C-RP tended to be higher in patients who were treated with vitamin E. Two patients had infections (colds) during the study. Concentrations of circulating adhesion molecules were similar between the treatment groups at baseline and did not change significantly during the study (Table 4).

**Plasma lipids and glucose**

Plasma lipids, lipoproteins, apolipoproteins and fasting glucose concentrations were similar between the treatment groups at baseline, excepting plasma triglyceride levels were lower in patients who were randomised to receive the vitamin C supplement (Table 5). Plasma total cholesterol concentration increased significantly [0.50 mmol/l (95% CI: 0.19, 0.81) mmol/l] in patients treated with vitamin E and did not change significantly in those treated with tomato juice [-0.10 (95%CI: -0.45, 0.25) mmol/l], vitamin C [0.04 (95%CI: -0.17, 0.25) mmol/l] and placebo [0.03 (95%CI: -0.25, 0.30) mmol/l]. Plasma apoB concentration increased significantly in patients receiving
vitamin C [0.14 (95%CI: 0.004, 0.28) g/l] and did not change significantly in the placebo group [0.03 (95%CI: -0.10, 0.16) g/l]. Fasting glucose did not change significantly during the study.

CONCLUSIONS

These data indicate that regular supplementation with tomato juice increases plasma lycopene levels nearly 3-fold and the intrinsic resistance of LDL to oxidation by approximately 42% in diabetic patients. The magnitude of these changes were similar to those reported previously in healthy subjects during supplementation with tomato juice in a preliminary study (37). Furthermore, the magnitude of the present increase in LDL resistance to oxidation during tomato juice supplementation was comparable with the corresponding increase during supplementation with vitamin E. In addition, treatment with vitamin E markedly decreased plasma levels of C-RP that is a risk factor for myocardial infarction (13-15).

The increase in LDL resistance to oxidation during dietary supplementation with tomato juice is presumably due to increased LDL content of lycopene. Carotenoids are transported by lipoproteins, and substantially by LDL, in the blood (38). Thus, the 3-fold increase in plasma lycopene in the present study undoubtedly included an increase in LDL lycopene levels. Fuhrman and coworkers have reported that enrichment of LDL with lycopene in vitro increases its resistance to copper ion oxidation (22). Vitamin C is usually added to commercial tomato juice but this antioxidant alone is not responsible for the increased resistance to oxidation of LDL isolated during supplementation with tomato juice in our data. The susceptibility of LDL to oxidation was unchanged in patients who were randomised to receive a high dose of vitamin C. Also, LDL content of α-tocopherol, a major antioxidant in lipoproteins, did not change appreciably in patients randomised to receive tomato juice. It is unlikely that ACE inhibitor therapy was responsible for the increase in LDL resistance to oxidation in these patients. Numbers of patients receiving ACE inhibitor drugs (that are reported as inhibiting LDL oxidation (36)) were comparable in the groups of patients treated with tomato juice and placebo while LDL susceptibility to oxidation was clearly decreased in those receiving tomato juice but not in those receiving placebo. Furthermore, treatment with ACE inhibitor drugs remained unchanged during the study.

Plasma C-RP levels in the current patients with type 2 diabetes were comparable with elevated levels reported previously (39). The mechanism(s) responsible for these elevated C-RP levels is unclear. It is possible that obesity, oxidative stress, atherosclerotic disease and enhanced release of proinflammatory cytokines induced by advanced glycation end products may contribute to augmented inflammatory activity in
patients with type 2 diabetes (10, 40). In the present study, few of the patients reported infections but we cannot exclude the possibility that undiagnosed and asymptomatic infections also contributed to their elevated C-RP levels.

The decrease in plasma C-RP in patients with type 2 diabetes during supplementation with vitamin E in this study may indicate an improvement in systemic inflammatory status. It is possible that vitamin E decreases the secretion of proinflammatory cytokines which promote the synthesis of C-RP in the liver. Devaraj and Jialal (19) have reported that dietary supplementation of healthy subjects with a high dose of vitamin E inhibits the release of IL-1β from isolated monocytes. The proinflammatory cytokine IL-1β stimulates the expression of IL-6 which in turn increases the synthesis of C-RP (15, 41). The decrease in IL-1β secretion induced by vitamin E appears to be independent of its antioxidant properties and relies on a decrease in 5-lipoxygenase activity (42). Our data suggest that plasma C-RP levels may also be unaffected by increased antioxidant protection and greater intrinsic resistance of LDL to oxidation in patients with type 2 diabetes. In patients supplemented with vitamin C and tomato juice, levels of ascorbate and lycopene were increased respectively and in those treated with tomato juice LDL resistance to copper ion oxidation was also increased but plasma C-RP remained unchanged.

The expression of cell adhesion molecules is also stimulated by proinflammatory cytokines (15) and by oxidised LDL in vitro in a process which can be inhibited by addition of antioxidants (20, 21). However, our data suggest that supplementation with natural antioxidants and increased intrinsic resistance of LDL to oxidation may not inhibit enhanced expression of VCAM-1 and ICAM-1 in patients with type 2 diabetes. This finding appears to be in keeping with a recent study which has reported a neutral effect of treatment with smaller amounts of vitamin E (75 mg/day) and vitamin C (150 mg/day) on plasma levels of VCAM-1 in male smokers (43). Hyperglycemia (11) and increased levels of advanced glycation end-products (44) may have continued to enhance the expression of cell adhesion molecules in patients during the present study.

The increases in plasma total cholesterol and apoB levels in patients receiving vitamin E and vitamin C respectively must be interpreted with caution. These increases may not be clearly different from the corresponding changes in the placebo group as the 95% confidence intervals for the changes overlapped appreciably. Few if any, published placebo-controlled studies have reported an increase in plasma cholesterol in humans during vitamin E supplementation. However, a trend toward an increase in plasma cholesterol has been reported in patients with type 2 diabetes (27). Further studies of
the effect of supplementation with vitamins E on plasma cholesterol in a larger number of patients with type 2 diabetes are warranted. This study has limitations that must be considered. Numbers of patients in the treatment groups were relatively small. Thus, care should be taken in extrapolating the present findings to other populations. Also, the treatment period was comparatively short. On the other hand, the length of the supplementation period was sufficient to establish markedly increased levels of circulating antioxidants. Patients were taking a number of medications to control diabetes and hypertension or reduce risk of a cardiovascular event. However, these treatments are characteristic of patients with type 2 diabetes and remained unchanged during the study.

In conclusion, this study indicates that regular consumption of tomato juice markedly increases plasma lycopene levels and increases the resistance of isolated LDL to oxidation almost as effectively as a high dose of vitamin E in patients with type 2 diabetes. However, vitamin E supplementation also decreases plasma C-RP indicating lower systemic inflammatory activity. These changes are consistent with a lower risk of CHD according to epidemiological studies (13,17,45). Our findings may have application in the development of strategies aimed at reducing the risk of CHD in patients with type 2 diabetes.

ACKNOWLEDGEMENTS
The authors are grateful to the participants in the study and to Clare Robertson and G Peter Herbison for statistical advice. They also thank Sylvia de Jong for measurement of plasma lipids. The study was supported by a grant from the Laurenson Fund, Otago Medical Research Foundation. JEU received a postgraduate scholarship from the University of Otago.

REFERENCES


Table 1 – Baseline characteristics of patient groups.

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<th></th>
<th>Vitamin E n=12</th>
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<th>Tomato juice n=15</th>
<th>Placebo n=13</th>
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<td>56±9</td>
<td>63±8</td>
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<td>6/6</td>
<td>10/5</td>
<td>10/3</td>
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<td>Duration of diabetes (years)</td>
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<td>1.9±1.3</td>
<td>4.9±5.5</td>
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<td>BMI (kg/m²)</td>
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<td>31.8±4.1</td>
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<td>HbA₁c (%)</td>
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<td>6</td>
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<td>Biguanides (n)</td>
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<td>Aspirin (n)</td>
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Values are mean±SD and numbers of patients in categories.
Abbreviations: M, male; F, female; BMI, body mass index; HbA₁c, glycated hemoglobin; BP, blood pressure; IHD, clinical ischemic heart disease; OHAs, oral hypoglycemic agents.
Table 2 – Plasma antioxidants at baseline (B), end of placebo (PL) and end of supplementation (S).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin E</th>
<th>Vitamin C</th>
<th>Tomato juice</th>
<th>Placebo</th>
<th>P*</th>
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<tr>
<td>α-tocopherol (µmol/l)</td>
<td></td>
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<tr>
<td>B</td>
<td>24.4±8.6</td>
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<td>26.1±4.9</td>
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<td>24.3±8.8†</td>
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<tr>
<td>S</td>
<td>56.7±23.7†</td>
<td>22.6±4.8</td>
<td>26.7±6.0</td>
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<td>B</td>
<td>0.35±0.24</td>
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<td>0.41±0.27</td>
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<td>S</td>
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<td>0.44±0.32</td>
<td>1.08±0.39†</td>
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<td>B</td>
<td>39.6±19.5</td>
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<td>PL</td>
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<td>S</td>
<td>40.0±13.7</td>
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<td>56.0±23.4</td>
<td>29.7±21.1</td>
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Values are mean±SD.
* Repeated measures ANOVA with baseline values as a covariate.
† Significantly different at P<0.001 (paired t-test).
Table 3 – Low density lipoprotein lag time, rate and concentration of conjugated dienes formed during copper ion oxidation and content of α-tocopherol at baseline (B), end of placebo (PL) and end of supplementation (S).

<table>
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<th>Vitamin E n=12</th>
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<tr>
<td>B</td>
<td>74±16</td>
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<td>74±16†</td>
<td>63±15</td>
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<td>S</td>
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<td><strong>Rate (nmol/µmol LDL cholesterol/min)</strong></td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>1.10±0.34</td>
<td>1.09±0.38</td>
<td>1.13±0.32</td>
<td>1.15±0.39</td>
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<tr>
<td>PL</td>
<td>1.13±0.34</td>
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<td>1.12±0.32</td>
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<tr>
<td>S</td>
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<td>1.14±0.38</td>
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<tr>
<td><strong>Concentration (nmol/µmol LDL cholesterol)</strong></td>
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<tr>
<td>B</td>
<td>139±26</td>
<td>137±12</td>
<td>146±17</td>
<td>142±15</td>
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<tr>
<td>PL</td>
<td>127±16</td>
<td>132±29</td>
<td>144±17</td>
<td>147±15</td>
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<tr>
<td>S</td>
<td>138±22</td>
<td>137±22</td>
<td>149±16</td>
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<td>B</td>
<td>2.7±0.9</td>
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<tr>
<td>S</td>
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<td>2.4±0.8</td>
<td>2.7±0.5</td>
<td>2.4±0.4</td>
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</table>

Values are mean±SD.

* Repeated measures ANOVA with baseline values as a covariate.
† Significantly different at P<0.001 (paired t-test).
<table>
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<tr>
<th></th>
<th>Vitamin E n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato juice n=15</th>
<th>Placebo n=13</th>
<th>P*</th>
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<td>C-RP (mg/l)</td>
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<tr>
<td>B</td>
<td>4.5±3.8</td>
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<td>3.1±2.5</td>
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<td>ICAM-1 (ng/ml)</td>
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<tr>
<td>B</td>
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<td>244±70</td>
<td>297±131</td>
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<tr>
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<td>276±66</td>
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<td>289±112</td>
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<tr>
<td>S</td>
<td>272±55</td>
<td>243±62</td>
<td>240±49</td>
<td>287±112</td>
<td>0.893</td>
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<td>VCAM-1 (ng/ml)</td>
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<tr>
<td>B</td>
<td>533±148</td>
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<tr>
<td>PL</td>
<td>526±139</td>
<td>584±370</td>
<td>632±189</td>
<td>635±244</td>
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<tr>
<td>S</td>
<td>534±153</td>
<td>585±354</td>
<td>632±160</td>
<td>640±256</td>
<td>0.997</td>
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</table>

Values are mean ±SD.

Abbreviations: C-RP, C-reactive protein; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

* Repeated measures ANOVA with baseline values as a covariate.
† Significantly different at P<0.01 (paired t-test).
Table 5 - Plasma lipids, lipoproteins, apolipoproteins and glucose concentrations at baseline (B), end of placebo (PL), and end of supplementation (S).

<table>
<thead>
<tr>
<th></th>
<th>Vitamin E n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato juice n=15</th>
<th>Placebo n=13</th>
<th>P*</th>
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<tbody>
<tr>
<td>TC (mmol/l)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>B</td>
<td>5.65±1.16</td>
<td>5.96±0.97</td>
<td>5.87±1.02</td>
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<td>5.57±1.30†</td>
<td>5.92±0.94</td>
<td>5.82±0.93</td>
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<tr>
<td>S</td>
<td>6.07±1.50†</td>
<td>5.97±0.86</td>
<td>5.73±0.97</td>
<td>6.52±1.09</td>
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<tr>
<td>HDL-C (mmol/l)</td>
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<td></td>
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<tr>
<td>B</td>
<td>1.14±1.21</td>
<td>1.10±1.25</td>
<td>1.01±1.29</td>
<td>0.93±1.17</td>
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</tr>
<tr>
<td>PL</td>
<td>1.14±1.21</td>
<td>1.12±1.26</td>
<td>0.99±1.30</td>
<td>0.95±1.18</td>
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<tr>
<td>S</td>
<td>1.18±1.25</td>
<td>1.13±1.22</td>
<td>1.03±1.33</td>
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<tr>
<td>TG (mmol/l)</td>
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<tr>
<td>B</td>
<td>1.86±1.68</td>
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<td>2.50±1.28</td>
<td>2.70±1.58</td>
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<tr>
<td>S</td>
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<td>1.91±1.65</td>
<td>2.35±1.48</td>
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<td>ApoA-1 (g/l)</td>
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<tr>
<td>B</td>
<td>1.23±1.17</td>
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<td>1.16±1.25</td>
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<tr>
<td>PL</td>
<td>1.25±1.12</td>
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<td>1.23±1.30</td>
<td>1.10±1.10</td>
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<tr>
<td>S</td>
<td>1.35±1.17</td>
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<tr>
<td>ApoB (g/l)</td>
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<td>B</td>
<td>0.89±1.48</td>
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<td>0.92±1.29</td>
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<td>0.98±1.25</td>
<td>1.16±1.18</td>
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<tr>
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<td>0.97±1.50</td>
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<tr>
<td>Fasting glucose (mmol/l)</td>
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<tr>
<td>B</td>
<td>8.4±2.1</td>
<td>8.7±1.5</td>
<td>8.2±1.3</td>
<td>9.1±2.4</td>
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<tr>
<td>PL</td>
<td>8.9±1.6</td>
<td>8.4±2.3</td>
<td>8.1±1.2</td>
<td>9.3±2.8</td>
<td></td>
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<tr>
<td>S</td>
<td>9.1±1.7</td>
<td>8.8±2.3</td>
<td>8.3±1.3</td>
<td>9.3±2.8</td>
<td>0.699</td>
</tr>
</tbody>
</table>

Values are mean±SD
Abbreviations: TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; apoA-1 apolipoprotein A-1, apoB, apolipoprotein B.
* Repeated measures ANOVA with baseline values as a covariate.
† Significantly different at P<0.01 (paired t-test).
Appendix B

Upritchard JE, Sutherland WHF. Oxidation of heparin-treated low density lipoprotein by peroxidases.Accepted by "Atherosclerosis" 1999.
Oxidation of heparin-treated low density lipoprotein by peroxidases

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Abstract

Low density lipoproteins (LDL) can bind to glycosaminoglycans and proteoglycans rich in heparin and chondroitin sulphate in the arterial intima and may become a target for atherogenic modification by myeloperoxidase activity. We have examined the susceptibility of resolubilized LDL, that has been precipitated from serum with heparin (HepLDL), to peroxidase–H2O2-catalysed oxidation and the effects of antioxidants and components of human serum on the oxidation. HepLDL was oxidised rapidly by horse radish peroxidase (HRP) and H2O2 (mean t1/2 max for conjugated diene formation, 3 min) while there was little oxidation of native LDL or native LDL precipitated with polyethylene glycol and resolubilised during the 30 min incubation period. The formation of thiobarbituric acid reacting substances (TBARS) essentially paralleled that of conjugated dienes during oxidation of HepLDL. HepLDL was also more rapidly oxidised than native LDL by myeloperoxidase–H2O2. Oxidation of HepLDL by peroxidases did not require free tyrosine, was almost totally inhibited by butylated hydroxytoluene (BHT) and ascorbate, and was unaffected by vitamin E and urate. Increasing concentrations (0–14.9%) of β-lipoprotein deficient serum (BLPDS) significantly (P < 0.0001) inhibited the formation of TBARS during HepLDL oxidation catalysed by HRP and partially inhibited the corresponding myeloperoxidase-catalysed oxidation. This inhibitory activity was removed by dialysis and gel-filtration of BLPDS and was not restored by addition of magnesium ions used in the isolation of BLPDS, or physiological levels of ascorbate, tyrosine and reduced thiols (cysteine) to gel-filtered BLPDS. The results indicate that LDL from complexes with glycosaminoglycans are highly susceptible to oxidation by peroxidases, particularly at low levels of water soluble antioxidants, and that vulnerability of these LDL to myeloperoxidase oxidation remains in the presence of serum components that should exist in the arterial intima. These findings may be relevant to the oxidative modification of LDL that becomes trapped by binding to arterial proteoglycans and to the formation of myeloperoxidase-modified LDL in the artery wall. © 1999 Elsevier Science·Ireland Ltd. All rights reserved.

Keywords: Peroxidases; Low density lipoproteins; Heparin; Glycosaminoglycans; Antioxidants; Serum inhibitors

1. Introduction

Oxidative modification of low density lipoproteins (LDL) that have become trapped in the arterial intima by interaction with glycosaminoglycan groups on proteoglycans is believed to have an important role in the development of atherosclerosis [1–3]. Proteoglycans and glycosaminoglycans can form reversible and irreversible complexes with LDL in vitro [4,5]. There is evidence that these interactions also occur in the artery wall where they may lead to deposition of LDL in atherosclerotic lesions [6]. Proteoglycan–LDL complexes in different states of aggregation have been isolated from human and rabbit arterial lesions [3,7,8]. These complexes promote lipid accumulation in cultured macrophages [8] which mimics foam cell formation; an early stage of atherogenesis. Human LDL that has been precipitated with proteoglycans or glycosaminoglycans in vitro and then resolubilised is thought to imitate LDL that has formed reversible associations with proteoglycans in the arterial intima [3]. This precipitated-resolubilised LDL has an altered
structure [9–11] and is bound and internalised more efficiently than native LDL by the apo B, E receptor of cultured macrophages [12].

Oxidation of LDL produces a number of biologically active molecules and modifies apo B. As a result, the lipoprotein no longer binds to the apo B, E receptor and is avidly taken up by macrophages via the scavenger receptor resulting in foam cell formation [2]. Oxidatively modified LDL has been isolated from human atherosclerotic lesions [13]. It has been postulated that reversible associations of LDL with proteoglycans and glycosaminoglycans increase both the susceptibility of LDL to oxidative modification and the opportunity for this modification to occur [6]. Isolated LDL that has complexed with proteoglycans and glycosaminoglycans is abnormally susceptible to oxidation by copper ions and by cultured cells [5,14] in a transition metal ion mediated process. However, proteins in the extracellular fluid of the artery wall probably bind any free transition metal ions and prevent them from catalysing LDL oxidation [15,16]. In contrast, activity of oxidants which do not depend on free transition metal ions should be unaffected by protein binding of these ions.

An earlier study from our laboratory [17] indicated that hemin-catalysed oxidation of LDL which has been isolated by precipitation with heparin glycosaminoglycan (HepLDL) is stimulated by low concentrations of β-lipoprotein deficient serum (BLPDS) and human serum albumin comparable to concentrations in the human arterial intima-media. Serum BLPDS contains most of the compounds that are found in extracellular fluid. Other oxidants, which may have an important role in LDL oxidation in vivo [2], have not been tested in this model.

There is recent evidence [18] that myeloperoxidase, a heme-containing enzyme which is present in inflammatory cells [19] and in arterial lesions [20], oxidises LDL in the artery wall. High levels of 3-chlorotyrosine, which is a specific marker of myeloperoxidase-catalysed oxidation, were measured in LDL isolated from human atherosclerotic intima compared with levels in circulating LDL [18]. Hypochlorous acid produced during myeloperoxidase activity chlorinates proteins and lipids, and modifies LDL to a form which is readily internalised by cultured macrophages [21]. Hydrogen peroxide accelerates peroxidase-catalysed LDL oxidation and can be generated by activated phagocytes. The mechanism underlying peroxidase-catalysed oxidation of LDL differs notably from that which governs LDL oxidation by transition metal ions. In contrast to transition metal ions, peroxidases do not directly oxidise lipids in LDL [22]. Radicals derived from protein, vitamin E and tyrosine appear to mediate oxidation of LDL by peroxidases [23]. Horse radish peroxidase (HRP), an enzyme derived from plants, also oxidises isolated LDL via a protein radical [23] and has been suggested as a model for transition metal ion independent oxidation of LDL in vivo [24]. The aim of the present study was to determine the characteristics of peroxidase-catalysed oxidation of HepLDL including the effect of antioxidants, tyrosine and BLPDS. This model system may approximate the situation in the arterial intima.

2. Materials and methods

2.1. Materials

Chondroitin sulphate C, 1,1,3,3-tetraethoxypropane, vitamin E, butylated hydroxytoluene (BHT), tyrosine, HRP and myeloperoxidase from human leukocytes were obtained from Sigma (St. Louis, MO). Heparin (Multiparin, from porcine mucosa) was purchased from Fisons. L-Ascorbic acid was obtained from JT Baker (NJ) and uric acid and polyethylene glycol 20 000 from BDH (UK).

2.2. Preparation of lipoprotein fractions

Lipoproteins were isolated from serum or EDTA plasma from normolipidemic donors. HepLDL was isolated as described previously [17]. Briefly, sodium citrate buffer (64 mmol/l, pH 5.12 containing heparin (50 000 I.U./l) was added to serum (0.5 ml). The tubes were left at room temperature for 10 min then centrifuged at 2800 × g for 15 min at room temperature. The precipitate (HepLDL) was washed three times with hypotonic Hepes buffer (5 mmol/l with sodium chloride 20 mmol/l, calcium chloride 4 mmol/l and magnesium chloride 2 mmol/l, pH 7.2) then desalted in 4% sodium chloride in phosphate buffered saline (PBS). The redissolved HepLDL was washed immediately. Native LDL (d 1.019–1.063 g/ml) was isolated by sequential ultracentrifuging EDTA plasma at 10°C [25] or on a single-step discontinuous gradient in a Beckman NVT 65 rotor [26]. The LDL isolated was desalted either by dialysis (twice against PBS (2.1) in the dark at 4°C) or by gel-filtration on Ecocnopac PD-10 columns equilibrated with PBS. The PBS was Chelex treated to remove any metal ions. Chondroitin sulphate treated LDL (CsLDL) was prepared as described previously [17]. Native LDL (1 volume) was also precipitated by adding 2 volumes of 9.5% polyethylene glycol (mol. wt. 20 000) solution in phosphate buffer pH 6.5 [27]. Aggregated native LDL was prepared essentially according to the method of Khoo et al. [28]. The BLPDS fraction was prepared by the addition of dextran sulphate–magnesium chloride to serum [29]. The apo B-containing lipoproteins were pelleted by centrifugation in a Beckman microfuge for 5 min and BLPDS was isolated in the supernatant. Gel-filtered BLPDS
was prepared by loading 1 ml BLPDS onto a PD-10 gel-filtration column equilibrated with PBS. A volume of PBS (2 ml) was added to the column and the ensuing eluate was discarded. The gel-filtered BLPDS was eluted with 1 ml PBS.

2.3. Oxidation of LDL

Formation of conjugated dienes or thiobarbituric acid reacting substances (TBARS) was used to monitor oxidation of lipoproteins. When conjugated dienes were measured, the LDL species (50–100 µl, 0.1 µmol cholesterol) and H$_2$O$_2$ (10 µl of 12.9 µmol/ml in PBS) were added to 1.8 ml PBS and distilled water (0.20–0.25 ml) to maintain approximate isotonicity in the incubation medium. An aliquot of HRP (10 µl of 119 U/ml in PBS) or MPO (10 µl of 250 U/ml in PBS) was added with rapid mixing and the absorbance at 234 nm was monitored during incubation of the mixture at room temperature (23°C). The final volume of the incubation mixture was 2.12 ml. Tonicity of the incubation medium and use of Chelex-treated PBS or untreated PBS did not appreciably influence the oxidation rates. Maximal absorbance at 234 nm and time taken to attain half-maximal absorbance ($t_{1/2 \text{ max}}$) were determined from the plot of absorbance values against time.

When TBARS were measured, the LDL species (100–150 µl, 0.2 µmol cholesterol), the serum fraction (0–200 µl), H$_2$O$_2$ (10 µl of 12.9 µmol/ml in PBS), and HRP (10 µl of 119 U/ml in PBS) or myeloperoxidase (10 µl of 250 U/ml in PBS) were added to PBS (1 ml) and the final volume was adjusted to 1.4 ml. The mixture was incubated at room temperature (23°C) for 30 min. At the end of this period, BHT (10 µl of 4 mmol/l in methanol) and EDTA (50 µl of 4 mmol/l) were added and TBARS were measured essentially by the method of Beuge and Aust [30]. Rates of LDL oxidation were expressed as nmol TBARS/µmol LDL cholesterol/30 min.

2.4. Analytical methods

Cholesterol was measured using a commercial kit and standards from Boehringer Mannheim (Germany). Serum paraoxonase activity was measured by monitoring the rate of phenylacetate hydrolysis at 270 nm following the addition of diluted serum [31]. A fluorimetric method [32] was used to measure TBARS in unoxidized HepLDL.

2.5. Statistical analysis

Student's t-test was used to compare mean values. Analysis of variance (ANOVA) was used to compare the effects of several concentrations of H$_2$O$_2$ on $t_{1/2 \text{ max}}$ and maximal absorbance at 234 nm and varying levels of BLPDS on TBARS formation during HepLDL oxidation. A two-sided test of significance was used and $P < 0.05$ was considered statistically significant. Results are mean ± S.D. unless stated otherwise.

3. Results

Fig. 1A shows the oxidation of native LDL and LDL that had interacted with glycosaminoglycans. Conjugated diene formation increased rapidly with no lag-
time during HRP-catalysed oxidation of HepLDL, while there was little change with native LDL. The mean $t_{1/2\,\text{max}}$ was $3 \pm 1$ min ($n = 5$) for the oxidation of HepLDL and values for CsLDL were within the range of those observed for HepLDL. Similar results were obtained when native LDL that had been isolated by ultracentrifugation of plasma, precipitated with heparin or chondroitin sulphate and then resolubilised was oxidised by HRP. There was no change in absorbance at 234 nm when HepLDL was incubated alone or with H$_2$O$_2$ in the absence of HRP. The formation of TBARS paralleled that of conjugated dienes during HRP oxidation of HepLDL (Fig. 1B). TBARS were virtually undetectable (10–30 pmol/µmol cholesterol) in freshly isolated HepLDL. Redissolving the HepLDL precipitate in 4% sodium chloride–PBS was not responsible for the increased susceptibility of HepLDL to oxidation. When an aliquot of a suspension of HepLDL precipitate in hypotonic Hepes buffer was added to PBS (in which it dissolved) and oxidised with HRP–H$_2$O$_2$, the rate of TBARS formation (26.5 ± 5.7 nmol/µmol cholesterol/30 min, $n = 3$ experiments) was not significantly different compared with the corresponding rate with HepLDL that had been redissolved in 4% sodium chloride–PBS. Addition of EDTA (0.14 mmol/l) did not reduce the formation of TBARS during oxidation of HepLDL by HRP–H$_2$O$_2$.

When a suspension of precipitated native LDL in polyethylene glycol solution was treated with HRP–H$_2$O$_2$ (maximum final polyethylene glycol concentration, 0.17 mmol/l) absorbance at 234 nm increased slowly to a mean value of 0.029 ± 0.013 ($n = 3$) at 30 min which was significantly ($P = 0.0004$) lower than the corresponding value for the oxidation of HepLDL (0.236 ± 0.029 with $t_{1/2\,\text{max}}$ 5.9 ± 0.6 min, $n = 3$) in the presence of polyethylene glycol (0.17 mmol/l). The precipitated LDL suspended in polyethylene glycol solution redissolved to give virtually zero absorbance at 680 nm (a measure of turbidity) when the suspension was added to PBS prior to addition of the oxidants. Aggregated LDL did not dissolve in 4% sodium chloride–PBS and a suspension of the lipoprotein in PBS was not oxidised to TBARS by HRP–H$_2$O$_2$.

The formation of conjugated dienes during incubation of HepLDL with myeloperoxidase–H$_2$O$_2$ was rapid, with no lag-phase compared with the corresponding oxidation of native LDL, which had a lag-time of greater than 150 min (Fig. 2). The $t_{1/2\,\text{max}}$ for the formation of dienes from HepLDL was approximately 50 min.

The rate of HepLDL oxidation ($t_{1/2\,\text{max}}$, absorbance at 234 nm and maximal absorbance) did not change significantly (ANOVA $P > 0.60$) at H$_2$O$_2$ concentrations between 6 and 60 µmol/l (HRP concentration, 0.56 U/ml) or at HRP concentrations between 0.125 and 0.50 U/ml (H$_2$O$_2$ concentration, 30 µmol/l) in three separate experiments. In another experiment, maximum absorbance at 234 nm increased linearly ($r^2 = 0.994$) as increasing concentrations (0.017–0.070 µmol/ml) of HepLDL were oxidised with HRP–H$_2$O$_2$.

Fig. 2. Oxidation of HepLDL with myeloperoxidase. HepLDL or native LDL (0.047 µmol cholesterol/ml), myeloperoxidase (0.09 U/ml) and H$_2$O$_2$ (28 µmol/l) in PBS were incubated at room temperature (23°C). Absorbance at 234 nm was monitored. The figure shows results of a representative experiment from two separate experiments.

Fig. 3 shows the effect of the antioxidants ascorbic acid and vitamin E on oxidation of HepLDL by HRP–H$_2$O$_2$. In the presence of ascorbate, conjugated diene formation increased within the first 2 min and then declined toward zero during the 30 min incubation period. Optical density at 234 nm also increased rapidly when ascorbate was treated with the oxidants. Production of TBARS was inhibited by 98% in each of two separate experiments when HepLDL was oxidised with HRP–H$_2$O$_2$ in the presence of 79 µmol/l ascorbate. The rate of diene formation during oxidation of HepLDL that had been isolated from serum enriched in vitamin E and contained increased levels of vitamin E, was similar to the rate in control HepLDL isolated from serum incubated with ethanol. In separate experiments ($n = 3$) with materials from different subjects, addition of vitamin E in ethanol at concentrations of 1, 2 and 3 µmol/l and a final ethanol concentration of 0.9% did not significantly affect $t_{1/2\,\text{max}}$ (control: 2.7 ± 0.3 min; 1 µmol/l vitamin E: 3.0 ± 1.3; 2 µmol/l vitamin E: 3.3 ± 1.2 min; 3 µmol/l vitamin E: 3.0 ± 1.0 min, ANOVA $P = 0.88$) and the ratio maximum absorbance in the presence of vitamin E/control maximum absorbance in the absence of vitamin E and with added ethanol (ANOVA $P = 0.29$). Added BHT (18.9 µmol/l) significantly ($P < 0.001$) decreased the maximum amount of conjugated dienes formed (−75 ± 13%, $n = 3$) and the production of TBARS to 2% of control values ($n = 2$) during oxidation of HepLDL by HRP–H$_2$O$_2$. Tyrosine
(48 µmol/l) inhibited the early rapid formation of conjugated dienes during oxidation of HepLDL (Fig. 4). After 30 min, the amounts of dienes formed were similar in the presence or absence of tyrosine.

Fig. 5 plots the effect of increasing concentrations of BLPDS on the formation of TBARS during oxidation of HepLDL with HRP and myeloperoxidase. Oxidation was followed by measuring TBARS formation because conjugated dienes cannot be readily measured in the presence of BLPDS which absorbs strongly at 234 nm. Added BLPDS significantly inhibited TBARS formation (ANOVA $P < 0.0001$) particularly at concentrations between 7.5–14.9%. The rate of TBARS formation increased (0.1–1.2 nmol/30 min) with increasing concentrations of BLPDS alone and the quantities of TBARS produced were markedly smaller than the levels when HepLDL was also present. The levels of TBARS derived from BLPDS alone were subtracted from the values obtained during oxidation of HepLDL in the presence of BLPDS. Oxidation of HepLDL by myeloperoxidase–$\text{H}_2\text{O}_2$ was also inhibited by increasing concentrations of BLPDS. This inhibition was less marked compared with the corresponding inhibition of HRP-catalysed oxidation of HepLDL.

234 nm. Added BLPDS significantly inhibited TBARS formation (ANOVA $P < 0.0001$) particularly at concentrations between 7.5–14.9%. The rate of TBARS formation increased (0.1–1.2 nmol/30 min) with increasing concentrations of BLPDS alone and the quantities of TBARS produced were markedly smaller than the levels when HepLDL was also present. The levels of TBARS derived from BLPDS alone were subtracted from the values obtained during oxidation of HepLDL in the presence of BLPDS. Oxidation of HepLDL by myeloperoxidase–$\text{H}_2\text{O}_2$ was also inhibited by increasing concentrations of BLPDS. This inhibition was less marked compared with the corresponding inhibition of HRP-catalysed oxidation of HepLDL.

The effect of BLPDS (14.9%, v/v) that had been dialyzed or gel-filtered to remove small water soluble molecules and the addition of magnesium ions, ascorbate, tyrosine and cysteine hydrochloride to gel-filtered BLPDS on the formation of TBARS during oxidation of HepLDL by HRP–$\text{H}_2\text{O}_2$ is summarised in Table 1. Both dialyzed and gel-filtered BLPDS failed to inhibit this oxidation significantly. Gel-filtered BLPDS with added magnesium ions (equivalent to the level in BLPDS) and physiological levels of ascorbate, tyrosine and cysteine hydrochloride did not inhibit TBARS formation. Added urate (19.2–76.9 µmol/l) also did not appreciably alter TBARS forma-
tion during oxidation of HepLDL-catalysed by HRP. The inhibitory effect was still present in BLPDS that was prepared from serum that had been heated for 1 h at 58°C and had only 3% of its initial activity of paraoxonase, an enzyme which metabolises oxidised lipids (control: 23.5 ± 0.7 nmol/µmol/30 min; heated BLPDS: 2.1 ± 1.9 nmol/µmol/30 min; n = 3 experiments, P < 0.0001).

Table 1

<table>
<thead>
<tr>
<th>Additions</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLPDS (14.9%, v/v)</td>
<td>10 ± 4*</td>
</tr>
<tr>
<td>Dialysed BLPDS (14.9%, v/v)</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>Gel-filtered BLPDS (14.9%, v/v)</td>
<td>88 ± 15</td>
</tr>
<tr>
<td>+Mg²⁺ (54 mmol/l)</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>+Ascorbate (84 µmol/l)</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>+Tyrosine (48 µmol/l)</td>
<td>122 ± 23</td>
</tr>
<tr>
<td>+Cysteine hydrochloride (134 µmol/l)</td>
<td>98 ± 2</td>
</tr>
</tbody>
</table>

* Values are mean ± S.D. of three separate experiments.
* P<0.0001 compared with control.

4. Discussion

Our data indicate that LDL which has been resolubilised from complexes with glycosaminoglycans is more susceptible than native LDL to peroxidase-catalysed oxidation that is only partially inhibited by BLPDS when myeloperoxidase is the catalyst. This enhanced susceptibility to oxidation may contribute to increased levels of myeloperoxidase-modified LDL and oxidised LDL that are present in atherosclerotic arteries [13,18]. The increased susceptibility of HepLDL (and other glycosaminoglycan treated LDL) to peroxidase oxidation appears to be specifically due to changes in LDL as a result of the interaction with a glycosaminoglycan and not as a result of precipitation and resolubilisation of LDL. Precipitation with polyethyleneglycol which sterically excludes LDL from solution [33] and does not form a complex with the lipoprotein, did not increase the oxidisability of the resolubilised LDL by HRP. Levels of lipid oxidation products were low in freshly isolated HepLDL suggesting that the isolation procedure did not oxidatively stress HepLDL and thereby increase its susceptibility to peroxidase-catalysed oxidation.

Changes in lipoprotein structure caused by the interaction of LDL with glycosaminoglycans may underlie the rapid rate of lipid peroxidation in HepLDL compared with native LDL when they are incubated with peroxidases. These structural changes may increase the efficiency of peroxidase induced formation of radicals from apo B and vitamin E which mediate oxidation of LDL lipids by myeloperoxidase and HRP. Also, the altered molecular structure in HepLDL may increase the proximity of the radicals to oxidisable lipids. Camejo and coworkers [9-11] have reported that interaction with arterial proteoglycans and glycosaminoglycans in vitro alters the structure of both lipid and protein components of LDL. Low angle X-ray kinetic studies indicate disruption of the molecular organisa-
tion of the cholesteryl ester core [9] and apo B in the lipoprotein [3,6,34]. Thus, it is possible that altered protein conformation may increase the accessibility of sites on apo B to peroxidase-catalysed radical formation and may place apo B radicals in a better position to attack oxidisable lipid substrate in HepLDL. Increased access of the peroxidase enzymes to apo B in glycosaminoglycan treated LDL seems to be in keeping with previous reports that protein in LDL which has interacted with proteoglycans has an increased susceptibility to tryptic fragmentation [11,34]. It has also been suggested previously that the physical state of lipids and their proximity to radicals generated in the lipoprotein may be important in the oxidation by peroxidases [22].

Glycosaminoglycan induced changes in the lipoprotein structure may also alter the effect of vitamin E on LDL lipid oxidation by HRP. A previous study has reported that the addition of small amounts of vitamin E accelerates peroxidase oxidation of native LDL apparently by increasing the numbers of α-tocopherol radicals [22]. At higher levels of vitamin E, the oxidation is inhibited, suggesting that sufficient free vitamin E may remain to inhibit lipid oxidation [22,23]. In contrast, the present data indicate that HRP–H₂O₂ oxidation of LDL that has interacted with heparin glycosaminoglycan is unaffected not only by additions of small quantities of vitamin E to the incubation but also by massive prior enrichment of HepLDL with vitamin E. It is possible that molecular disorganisation [9] may allow HRP increased access to vitamin E in the cholesteryl ester-rich hydrophobic core of HepLDL, resulting in faster production of α-tocopherol radicals and decreased levels of free vitamin E. The HRP-catalysed lipid oxidation in HepLDL is probably near or at a maximal rate and may therefore be unaffected by any extra α-tocopherol radicals generated from added vitamin E. Lipid oxidation in HepLDL was markedly inhibited by BHT, a lipophilic antioxidant whose radicals are sterically hindered and may be inaccessible for propagation of the radical chain reaction [22]. Thus, increased generation of α-tocopherol radicals that are not sterically hindered and can accelerate lipid oxidation [22] may contribute to the inefficiency of vitamin E as an antioxidant in the HepLDL–HRP–H₂O₂ system. Whether rapid oxidation reduces free vitamin E to levels that no longer inhibit lipid oxidation in HepLDL that has been massively enriched with vitamin E remains to be determined.

Ascorbate markedly inhibited HepLDL oxidation by HRP possibly by decreasing levels of apo B and vitamin E radicals. The protein component of LDL is largely at the surface of LDL and therefore readily accessible to the water soluble antioxidant. HRP may oxidise ascorbate to products which react with LDL protein and decrease the formation of apo B radicals. This proposed mechanism is essentially analogous to that which governs the inhibition of copper ion-catalysed oxidation of LDL by ascorbate. Retsky et al. [35] have reported that during copper ion oxidation of LDL in the presence of ascorbate there is oxidation of ascorbate to dehydroascorbate (and other products) which reacts with LDL protein and inhibits subsequent copper ion-catalysed lipid oxidation. In the present study, ascorbate appeared to have been oxidised before it appreciably inhibited the oxidation of HepLDL. There was a large and rapid increase in absorbance at 234 nm when ascorbate and oxidants were incubated in PBS, suggesting that ascorbate had been modified. The momentary increase in absorbance at 234 nm, when HRP and H₂O₂ were added to a solution of HepLDL in the presence of ascorbate, may be due mainly or solely to the formation of oxidation product(s) of ascorbic acid that absorb at this wavelength and not to increased lipid conjugated diene production. The gradual decline of this early peak in absorbance at 234 nm may reflect reaction between the ascorbate oxidation product(s) and HepLDL which not only reduces the increased absorbance due to oxidation of ascorbate but also inhibits the oxidation of HepLDL lipids. Incubation of ascorbate with copper ions does not result in an increase in absorbance at 234 nm (data not shown). This finding suggests that there may be differences in the antioxidant mechanism of ascorbate in the presence of transition metal ions and HRP. The reduced form of ascorbate can convert α-tocopherol radicals to free α-tocopherol in LDL [36]. However, it is uncertain whether this regenerating activity of ascorbate remains after it has been modified by HRP–H₂O₂.

Free tyrosine also inhibited the oxidation of Hep-LDL by HRP but only during the early stages of the incubation. Tyrosine may have initially competed with HepLDL for oxidation until eventually levels of the amino acid were decreased and no longer competed effectively for oxidation. In a previous study, oxidation of native LDL by myeloperoxidase was inhibited by high levels of added tyrosine [22]. Some studies [37], but not others [22], have reported that oxidation of native LDL by myeloperoxidase requires the presence of free tyrosine. It has been suggested that added tyrosine may be required only when lipoprotein vitamin E content is low [22]. In the present study, myeloperoxidase readily oxidised HepLDL in the absence of free tyrosine. Freshly isolated HepLDL is not depleted of vitamin E [17] which may mediate myeloperoxidase-catalysed lipid oxidation. Also, altered lipoprotein structure may allow the oxidation of HepLDL by myeloperoxidase in the absence of tyrosine.

Added BLPDS markedly decreased oxidation of HepLDL-catalysed by HRP, but even at the highest concentration only partially inhibited the corresponding oxidation catalysed by myeloperoxidase. The in-
hition(s) in BLPDS was a small, water soluble molecule which did not appear to be ascorbate, urate, tyrosine or reduced thiols. The mechanism underlying the greater sensitivity of HRP oxidation to inhibition by BLPDS is uncertain but may relate to the position of the catalytic heme unit in HRP. In HRP the heme unit is relatively exposed whereas in myeloperoxidase, by contrast, it is buried deep in a hydrophobic cleft [38] and may be less accessible to components of BLPDS. Our finding that BLPDS only partially inhibited the oxidation of HepLDL by myeloperoxidase appears to be consistent with evidence that myeloperoxidase oxidises LDL in the artery wall [18] where components of extracellular fluid can modulate the process. The levels of BLPDS used in the present study were in a range which may correspond with the concentration of many compounds in extracellular fluid [39].

In conclusion, the present data suggest that LDL from complexes with glycosaminoglycans is abnormally susceptible to oxidation by peroxidases including myeloperoxidase which is a physiological oxidant. These experiments support the hypothesis that LDL which becomes trapped in the artery wall by interaction with proteoglycans may be susceptible to oxidation by myeloperoxidase. Our data also suggest that this peroxidase oxidation of LDL may be favoured at low levels of water-soluble antioxidants. Proteoglycans and glycosaminoglycans are negatively charged and may attract myeloperoxidase which has a basic pK and will be positively charged at physiological pH. An interaction of this type could bring the enzyme and LDL into close proximity and potentiate lipoprotein modification. Oxidation of LDL-proteoglycan complexes by myeloperoxidase may increase levels of myeloperoxidase-modified LDL and accelerate lesion development.

Acknowledgements

This study was supported by funds from the Nye Special Lipid Fund. JEU received a postgraduate scholarship from the University of Otago.

References


Appendix C

Chapter 3 Baseline data
Clinical characteristics of the patients at entry to the study

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>32/20</td>
</tr>
<tr>
<td>Duration of diabetes, years</td>
<td>4.0 ± 5.0</td>
</tr>
<tr>
<td>BMI, Kg/m²</td>
<td>31.2 ± 6.2</td>
</tr>
<tr>
<td>Glycated haemoglobin, %</td>
<td>6.48 ± 1.13</td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>8.6 ± 1.18</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>141 ± 25</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>86 ± 16</td>
</tr>
<tr>
<td>Systolic blood pressure ≥ 90 mmHg</td>
<td>18 (35)</td>
</tr>
<tr>
<td>Clinical heart disease, n (%)</td>
<td>31 (60)</td>
</tr>
<tr>
<td>Control of hyperglycaemia</td>
<td></td>
</tr>
<tr>
<td>Diet only, n (%)</td>
<td>19 (37)</td>
</tr>
<tr>
<td>Oral hypoglycaemics, n (%)</td>
<td>33 (63)</td>
</tr>
</tbody>
</table>

Values are mean ± SD; except in the case of high blood pressure, heart disease and control of hyperglycaemia where number of patients (%) are reported.

Medication taken by patients at baseline

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biguanides, n (%)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Sulphonylureas, n (%)</td>
<td>29 (56)</td>
</tr>
<tr>
<td>Gliclazide, n (%)</td>
<td>9 (17)</td>
</tr>
<tr>
<td>ACE Inhibitor, n (%)</td>
<td>19 (37)</td>
</tr>
<tr>
<td>β-andrenoceptor blocking agent, n (%)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Calcium blocking agent, n (%)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>11 (21)</td>
</tr>
</tbody>
</table>

Values are numbers and percentages
Plasma lipids, lipoproteins and antioxidants at baseline

<table>
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<tr>
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<th>Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=52</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>6.00 ± 1.09</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>1.06 ± 0.24</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;2&lt;/sub&gt;-cholesterol, mmol/l</td>
<td>0.26 ± 0.13</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;3&lt;/sub&gt;-cholesterol, mmol/l</td>
<td>0.81 ± 0.16</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>2.41 ± 1.21</td>
</tr>
<tr>
<td>Apolipoprotein A1, g/l</td>
<td>1.17 ± 0.22</td>
</tr>
<tr>
<td>Apolipoprotein B, g/l</td>
<td>0.97 ± 0.27</td>
</tr>
<tr>
<td>α-tocopherol, µmol/l</td>
<td>24.4 ± 5.9</td>
</tr>
<tr>
<td>Lycopene, µmol/l</td>
<td>0.37 ± 0.24</td>
</tr>
<tr>
<td>Vitamin C, µmol/l</td>
<td>36.0 ± 20.3</td>
</tr>
</tbody>
</table>

Values are mean ± SD

Products of inflammation and oxidation at baseline

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=52</td>
</tr>
<tr>
<td>Plasma sICAM-1, ng/ml</td>
<td>263 ± 91</td>
</tr>
<tr>
<td>Plasma sVCAM-1, ng/ml</td>
<td>592 ± 233</td>
</tr>
<tr>
<td>Plasma C-RP, mg/l</td>
<td>5.6 ± 5.3</td>
</tr>
<tr>
<td>Plasma FSB, U/ml</td>
<td>10.1 ± 2.3</td>
</tr>
<tr>
<td>Plasma TBARS, ng/ml</td>
<td>102.6 ± 27.8</td>
</tr>
<tr>
<td>RBC TBARS, nmol MDA/ml PC</td>
<td>1.15 ± 0.22</td>
</tr>
</tbody>
</table>

Abbreviations: MDA, malondialdehyde; PC, packed cells
Values are mean ± SD
### Composition of LDL isolated from patients at baseline

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<th>Diabetic Patients</th>
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</thead>
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<tr>
<td>n=52</td>
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</tr>
<tr>
<td>Free cholesterol, %</td>
<td>9.02 ± 1.46</td>
</tr>
<tr>
<td>Cholesterol ester, %</td>
<td>29.93 ± 2.51</td>
</tr>
<tr>
<td>Phospholipids, %</td>
<td>23.79 ± 4.09</td>
</tr>
<tr>
<td>Triglycerides, %</td>
<td>8.19 ± 1.83</td>
</tr>
<tr>
<td>α-tocopherol, mmol/mol cholesterol</td>
<td>2.64 ± 0.66</td>
</tr>
<tr>
<td>Peroxides, mmol/mol cholesterol</td>
<td>9.59 ± 2.73</td>
</tr>
</tbody>
</table>

Values are mean ± SD

### Intrinsic resistance of LDL isolated from patients at baseline

<table>
<thead>
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<th>Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=52</td>
<td></td>
</tr>
<tr>
<td>Lag time, minutes</td>
<td>70.2 ± 20.1</td>
</tr>
<tr>
<td>Rate conjugated diene formation, nmol/µmol/min</td>
<td>8.4 ± 2.3</td>
</tr>
<tr>
<td>Total conjugated diene formation, nmol/µmol</td>
<td>1055.6 ± 133.7</td>
</tr>
<tr>
<td>3 hour TBARS, nmol MDA/µmol cholesterol</td>
<td>17.92 ± 4.12</td>
</tr>
<tr>
<td>5 hour TBARS, nmol MDA/µmol cholesterol</td>
<td>18.11 ± 40.01</td>
</tr>
</tbody>
</table>

Values are mean ± SD
Appendix D

Chapter 3 Tomato product inventory
Tomato product consumption by the patients at baseline and at the end of the study

<table>
<thead>
<tr>
<th>Food</th>
<th>Baseline</th>
<th>End of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomatoes-raw</td>
<td>82%</td>
<td>77%</td>
</tr>
<tr>
<td>Tomatoes-canned</td>
<td>13%</td>
<td>18%</td>
</tr>
<tr>
<td>Tomatoes-cooked</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>0</td>
<td>1%</td>
</tr>
<tr>
<td>Tomato soup</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Tomato sauce</td>
<td>67%</td>
<td>64%</td>
</tr>
<tr>
<td>Baked beans/tomato sauce</td>
<td>37%</td>
<td>38%</td>
</tr>
<tr>
<td>Spaghetti/tomato sauce</td>
<td>21%</td>
<td>17%</td>
</tr>
<tr>
<td>Tomato paste (in cooked meal)</td>
<td>2%</td>
<td>3%</td>
</tr>
<tr>
<td>Pizza (with tomato)</td>
<td>8%</td>
<td>6%</td>
</tr>
</tbody>
</table>

Percentage of the group consuming item once over 4 day period.
Data collated from a qualitative tomato product checklist.
Appendix E

Chapter 3  Additional data
## Concentrations of plasma alpha and beta carotene (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol</th>
<th>Vitamin C</th>
<th>Tomato Juice</th>
<th>Placebo</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-carotene, µmol/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.05 ± 0.04</td>
<td>0.12 ± 0.11</td>
<td>0.07 ± 0.04</td>
<td>0.08 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>0.06 ± 0.05</td>
<td>0.12 ± 0.11</td>
<td>0.07 ± 0.05</td>
<td>0.09 ± 0.05</td>
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</tr>
<tr>
<td>End of study</td>
<td>0.04 ± 0.04</td>
<td>0.10 ± 0.09</td>
<td>0.06 ± 0.04</td>
<td>0.08 ± 0.04</td>
<td>0.069</td>
</tr>
<tr>
<td><strong>β-carotene, µmol/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.31 ± 0.16</td>
<td>0.51 ± 0.38</td>
<td>0.33 ± 0.19</td>
<td>0.31 ± 0.2</td>
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<tr>
<td>End of placebo</td>
<td>0.32 ± 0.17</td>
<td>0.49 ± 0.36</td>
<td>0.36 ± 0.26</td>
<td>0.30 ± 0.24</td>
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<td>End of study</td>
<td>0.26 ± 0.17</td>
<td>0.42 ± 0.29</td>
<td>0.47 ± 0.21†</td>
<td>0.28 ± 0.22</td>
<td>0.013</td>
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* Repeated measures ANOVA by treatment and group with baseline covariate correction.
† Unadjusted paired t-tests comparing the placebo phase with treatment phase, with level of significance P<0.001.
Formation of TBARS in LDL after copper stimulated oxidation (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocopherol</th>
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<th>Placebo</th>
<th>P*</th>
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<td>n=12</td>
<td>n=12</td>
<td>n=15</td>
<td>n=13</td>
<td></td>
</tr>
<tr>
<td>3 hour TBARS, mmol MDA/mol cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>15.27 ± 3.45*</td>
<td>17.40 ± 3.13</td>
<td>19.12 ± 4.59</td>
<td>19.48 ± 3.98</td>
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<tr>
<td>End of placebo</td>
<td>13.00 ± 3.61</td>
<td>14.23 ± 3.64</td>
<td>18.22 ± 3.85</td>
<td>20.25 ± 4.29</td>
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</tr>
<tr>
<td>End of study</td>
<td>13.62 ± 5.65</td>
<td>12.79 ± 4.05</td>
<td>18.35 ± 4.22</td>
<td>18.73 ± 4.14</td>
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</tr>
<tr>
<td>5 hour TBARS, mmol MDA/mol cholesterol</td>
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</tr>
<tr>
<td>Baseline</td>
<td>15.53 ± 3.48*</td>
<td>17.62 ± 2.75</td>
<td>19.18 ± 4.57</td>
<td>19.71 ± 3.87</td>
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</tr>
<tr>
<td>End of placebo</td>
<td>12.38 ± 4.38</td>
<td>14.74 ± 3.33</td>
<td>18.32 ± 5.89</td>
<td>20.42 ± 4.30</td>
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</tr>
<tr>
<td>End of study</td>
<td>12.95 ± 6.06</td>
<td>11.65 ± 3.84</td>
<td>18.51 ± 4.48</td>
<td>18.96 ± 4.62</td>
<td>0.134</td>
</tr>
</tbody>
</table>

* Repeated measures ANOVA by treatment and group with baseline covariate correction.

* ANOVA comparing antioxidant groups with placebo group at baseline, with level of significance P<0.05.
Formation of conjugated dienes in LDL after copper stimulated oxidation (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>α-Tocophorol n=12</th>
<th>Vitamin C n=12</th>
<th>Tomato Juice n=15</th>
<th>Placebo n=13</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal conjugated dienes, nmol/µmol LDL cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>139 ± 26</td>
<td>137 ± 12</td>
<td>146 ± 17</td>
<td>142 ± 15</td>
<td></td>
</tr>
<tr>
<td>End of placebo</td>
<td>127 ± 16</td>
<td>132 ± 29</td>
<td>144 ± 17</td>
<td>147 ± 15</td>
<td></td>
</tr>
<tr>
<td>End of study</td>
<td>138 ± 22</td>
<td>137 ± 22</td>
<td>149 ± 16</td>
<td>147 ± 19</td>
<td>0.501</td>
</tr>
<tr>
<td>Rate conjugated dienes, nmol/µmol LDL cholesterol/minute</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>1.10 ± 0.34</td>
<td>1.09 ± 0.38</td>
<td>1.13 ± 0.32</td>
<td>1.15 ± 0.39</td>
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<tr>
<td>End of placebo</td>
<td>1.13 ± 0.34</td>
<td>1.09 ± 0.39</td>
<td>1.12 ± 0.32</td>
<td>1.15 ± 0.40</td>
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</tr>
<tr>
<td>End of study</td>
<td>1.12 ± 0.34</td>
<td>1.10 ± 0.39</td>
<td>1.12 ± 0.32</td>
<td>1.14 ± 0.38</td>
<td>0.693</td>
</tr>
</tbody>
</table>

* Repeated measures ANOVA by treatment and group with baseline covariate correction.
* ANOVA comparing antioxidant groups with placebo group at baseline, with level of significance P<0.05.