THE ROLE OF ANTI-INFLAMMATORY PROPERTIES OF HIGH DENSITY LIPOPROTEINS IN ATHEROPROTECTION.

A thesis submitted by

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DECLARATION

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

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"When you come to a fork in the road, take it"

Yogi Berra

ABSTRACT

It is well established that high density lipoproteins (HDL) protect against the development of atherosclerotic cardiovascular disease. However, the mechanisms that confer this benefit remain unclear. In addition, to its well recognised ability to promote reverse cholesterol transport, in vitro studies and hypercholesterolaemic animal models have demonstrated that HDL possess anti-inflammatory, antioxidant and antithrombotic properties. Furthermore, in vitro studies have found that the anti-inflammatory properties of HDL are influenced by the phospholipid composition, suggesting a possible link to dietary fatty acid intake. The studies that contribute to this thesis explore the anti-inflammatory properties of HDL during an acute model of vascular injury in normocholesterolaemic rabbits, their ability to stabilise atherosclerotic plaque and their activity during the postprandial state.

The vascular protective properties of reconstituted HDL (rHDL) were investigated in a normocholesterolaemic rabbit model of acute vascular inflammation, the periarterial collar. Infusions of rHDL profoundly inhibited the recruitment of neutrophils into the arterial wall in response and generation of reactive oxygen species in response to application of the periarterial collar. In addition, the early expression of proinflammatory adhesion molecules and chemokines by the endothelium was inhibited. These profound effects of discoidal HDL were seen in the absence of elevating plasma HDL and appeared to be independent of their ability to promote cholesterol efflux.

It is recognised that one origin of discoidal HDL is from the metabolism of chylomicrons. The effect of infusing phospholipid specific chylomicron-like emulsions on the anti-inflammatory properties of HDL was investigated. Following infusion of these emulsions, isolated HDL demonstrated a greater ability to inhibit the in vitro expression of the adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), by endothelial cells in response to cytokine stimulation. This suggests that the functional properties of HDL may change during the postprandial state.

These properties of HDL were then investigated in a hypercholesterolaemic, aortic balloon denudation rabbit model of established atherosclerosis. Infusions of HDL were comparable to atorvastatin in their ability to rapidly promote the formation of a more stable plaque phenotype, characterised by an increase in smooth muscle cells, reduction in matrix metalloproteinases and increase in the anticoagulant thrombomodulin. The effect of altering the composition of rHDL on these properties was then investigated. The ability of rHDL to promote plaque stabilisation was diminished when rHDL contained the protein apolipoprotein A-II.

The effect of the postprandial state on the anti-inflammatory properties of HDL was investigated in humans. Following the consumption of a fat enriched meal, the ability of isolated HDL to inhibit in vitro expression of VCAM-1 by activated endothelial cells was studied. When the meal comprised a polyunsaturated fat, the anti-inflammatory property of HDL improved. In contrast, when a saturated fat was consumed, the anti-inflammatory property of HDL diminished. These results suggest that dietary fatty acid composition can have a profound impact on atheroproperties of HDL.

This thesis expands our knowledge of the contribution of the in vivo anti-inflammatory properties of HDL. The results suggest that small amounts of HDL can have a profound beneficial influence in the setting of acute vascular inflammation and established atherosclerotic plaque. This highlights the important role that HDL plays in both the early and advanced stages of atherogenesis. In addition, the consumption of dietary fat has a striking impact on this activity, providing another mechanism by which the consumption of dietary fat modifies the risk of atherosclerotic cardiovascular disease.

DEDICATION

This thesis is dedicated to my wife, *Katherine*. The studies that contribute to this thesis were performed in three different states and therefore required substantial travel and time away from home. This was received with an incredible degree of tolerance. She shared my enthusiasm during the good times and frustrations during the bad. In addition, she acted as independent counsel and helped put things in perspective. Without her unconditional love and support it would not have been possible to complete the presented studies. I will love her always.

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PUBLICATIONS AND PRIZES

REVIEWED PAPERS

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2. **Nicholls S**, Drummond G, Rye K, Dusting G and Barter P. Reconstituted High Density Lipoproteins Inhibit The Pro-oxidant And Proinflammatory Vascular Changes Induced By A Periarterial Collar In Normocholesterolemic Rabbits. Circulation. (submitted)
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ABSTRACTS

1. **Nicholls SJ** and Barter PJ. The role of lipids in atherogenesis. ANZ Journal of Surgery. 2002; 72(suppl):A118-9.
2. **Nicholls S**, Drummond G, Rye KA, Dusting G and Barter P. Reconstituted high density lipoprotein attenuates superoxide expression and the increase in proinflammatory adhesion molecules and chemokines induced by a periarterial collar. Heart, Lung and Circulation. (In press)
3. **Nicholls S**, Rye KA and Barter P. Infusion of chylomicron-like emulsions enhance the ability of rabbit high density lipoprotein to inhibit the cytokine induced expression of vascular cell adhesion molecule-1. Heart, Lung and Circulation. (In press)
4. **Nicholls S**, Rye KA and Barter P. Enhanced anti-inflammatory property of high density lipoprotein following infusion of chylomicron-like emulsions. Atherosclerosis. 2003;4(2):142.

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PRIZES

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4. **Nicholls SJ**. New insights into atherogenesis. Combined meeting of the Australian and New Zealand Society of Vascular Surgery, Michael E DeBakey International Surgical Society and the ANZ Chapter of the International Union of Angiology. Gold Coast. September 2002.
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12. **Nicholls S**, Drummond G, Cutri B, Hime N, Rye KA, Dusting G and Barter P. Prothrombotic features of a periarterial collar are inhibited by infusions of reconstituted high density lipoprotein. Presented at the International Atherosclerosis Society satellite symposium 'Update on HDL lipidology: new lessons from basic and clinical medicine'. Okinawa. October 2003.
13. **Nicholls SJ**. New developments in lipid therapy. Cardiology In The Vines. Hunter Valley. October 2003.
14. **Nicholls, SJ**. Preventive treatment for vascular disease. Cardiology Direction 2003. Canberra. October 2003

ABBREVIATIONS

ABCA1	ATP binding cassette A-1
AI-DPPC	rHDL containing apoA-I and DPPC
AI-PLPC	rHDL containing apoA-I and PLPC
AII-PLPC	rHDL containing apoA-II and PLPC
AP-1	Activator protein-1
Apo	Apolipoprotein
ApoA-I	Apolipoprotein A-I
ApoA-II	Apolipoprotein A-II
ApoA-I(M)	Apolipoprotein A-I_{Milano}
ApoE	Apolipoprotein E
BHT	Butylated hydroxy toluene
BSA	Bovine serum albumin
CAD	Coronary artery disease
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CNP	C-naturetic peptide
COX	Cyclo-oxygenase
d	Density
DAB	Diaminobenzamine
DETCA	Diethyldithiocarbamate
DPPC	1,2-dipalmitoyl phosphatidylcholine
DPPC-rHDL	rHDL containing DPPC

DPPC-TG	Chylomicron-like emulsions containing DPPC
DMPC	1,1-dimyristoyl-sn-glycero-3-phosphocholine
ECL	Enhanced chemiluminescence
EDTA-Na_2	Ethylenediaminetetra-acetic acid, disodium salt
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
FMD	Flow mediated dilatation
FPLC	Fast performance liquid chromatography
HBSS	Hank's balanced salt solution
HDL	High density lipoproteins
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
hs-CRP	High sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IDL	Intermediate density lipoprotein
IFN	Interferon
IL	Interleukin
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LFAI	Lipid-free apoA-I
LPS	Lipopolysaccharide
LSF	Lysosulfatide

MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MMP-9	Matrix metalloproteinase-9
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NEFA	non-esterified fatty acid
NF-κβ	Nuclear factor-κβ
NHS	Native horse serum
NO	Nitric oxide
NOS	Nitric oxide synthase
PAF-AH	Platelet activating factor-acetyl hydrolase
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PECAM	Platelet endothelial cellular adhesion molecule
PKC	Protein kinase C
PLPC	1-palmitoyl-2-linoleoyl phosphatidylcholine
PLPC-rHDL	rHDL containing PLPC
PLPC-TG	Chylomicron-like emulsions containing PLPC
PLTP	Phospholipid transfer protein
PON	Paraoxonase
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
POPC-TG	Chylomicron-like emulsions containing POPC
rHDL	Reconstituted HDL

ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid A
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cell
SPC	Sphingosylphosphorylcholine
SR-B1	Scavenger receptor-B1
TBS	Tris-buffered saline
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour necrosis factor-α
TRL	Triglyceride rich lipoproteins
VCAM-1	Vascular cell adhesion molecule-1
VHDL	Very high density lipoprotein
VLA-4	Very late antigen-4
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell

CHAPTER ONE

INTRODUCTION

The role of inflammation in the formation of atherosclerotic plaque has gained increasing attention. Inflammatory factors promote not only the formation of atheroma within the arterial wall, but are also a key factor in determining its likelihood to cause clinical events. As a result, features of this inflammatory cascade are seen as potential targets for both the development of markers of cardiovascular risk and prophylactic strategies.

High density lipoproteins (HDL) are the atheroprotective lipoprotein fraction in circulating plasma. In addition to their well characterised role in the promotion of reverse cholesterol transport, it has been demonstrated that HDL are anti-inflammatory. Previous studies have demonstrated that HDL regulate the expression of proinflammatory adhesion molecules and chemokines by the endothelium, a key early event in atherogenesis.

The studies outlined in this thesis focus on the *in vivo* anti-inflammatory properties of HDL. The aims of the studies were to determine whether HDL modifies *in vivo* models of acute and chronic arterial inflammation and to investigate the influence of dietary fat consumption on this activity. The studies highlight the role of HDL in preventing both the early stages of atheroma formation and the pathological phenomena that lead to the development of clinical events.

1.1 ATHEROSCLEROSIS

1.1.1 Impact of atherosclerosis

Atherosclerotic cardiovascular disease is a global burden. It is the leading cause of morbidity and mortality in the western world (1). As the prevalence of the metabolic syndrome increases in developing nations, cardiovascular disease is expected to reach pandemic proportions. It is

estimated that by 2020 cardiovascular disease will become the greatest public health problem globally (2). Coronary heart disease is the leading cause of death in Australia (3). It is estimated that in the United States, a person suffers a myocardial infarct every 29 seconds. Of these, one quarter experience sudden death (4). The costs to the community in terms of hospitalisation, investigation and treatment are substantial. Cardiovascular disease is the largest financial burden on the health budget (5). Therefore interventions that prevent the development of atherosclerotic plaques and their subsequent clinical complications are of immense interest.

1.1.2 Established risk factors of atherosclerosis

Population studies have established the presence of several patient characteristics that are independently associated with an increased incidence of cardiovascular events (6). These characteristics, or 'risk factors', can be divided into those that can be modified and those that can not. Modifiable risk factors include smoking, hypertension, low plasma HDL, hypercholesterolaemia and obesity. Factors that can not be modified include a family history of cardiovascular disease, male gender and age greater than 55 years. Population studies have established that an individual's risk of developing clinical cardiovascular events is proportional to their number of risk factors (7). However, not all patients with cardiovascular disease can be predicted on the basis of the presence of these established risk factors. Indeed, only 50% of patients with incident cardiovascular disease are reported to have identifiable risk factors (8). Therefore, the search has continued to identify novel markers that can lead to more effective prediction of the likelihood of developing cardiovascular events. These markers focus on inflammatory, infective and prothrombotic processes, all of which contribute to clinical events

(9). The ability to optimise risk prediction in individuals will identify those most likely to benefit from prophylactic measures.

1.1.3 Evolving paradigms of atherogenesis

Atherosclerosis has traditionally been thought to represent a simple 'plumbing' problem, resulting in the deposition of cholesterol in the walls of arteries (10). As the deposition accumulates, the plaque encroaches on the lumen leading to complete occlusion. The likelihood of developing clinical events increases in parallel with the degree of luminal stenosis. However, emerging insights into atherogenesis have revealed that it is a systemic process, as opposed to the focal deposition of cholesterol. Atherogenesis results from a complex cascade of events including endothelial dysfunction, oxidative stress, inflammation, apoptosis and thrombogenicity (11). These processes result in the formation of atheromatous deposits at different points of the vascular tree. Whilst the development of critical arterial stenoses can result in clinical symptoms, particularly on exercise, it has been established that many clinical events result from complications in mildly stenosed plaques. In particular, the majority of myocardial infarctions occur in patients with stenoses less than 50% in the culprit vessel (12).

Atherosclerosis is not a process that begins in middle age. Autopsy studies of young soldiers killed during the Korean war revealed a marked prevalence of macroscopic aortic atheroma (13). In addition, the Pathological Determinants of Atherosclerosis in Youth (PADY) study revealed that most teenagers have evidence of fatty streaks in some part of the arterial tree (14). Furthermore, an intravascular ultrasound of donated hearts shortly after transplantation revealed that about one-fifth of apparently healthy teenagers had macroscopic atheroma in their coronary

arteries (15). Therefore there is strong evidence to suggest that this chronic process begins early in life.

The earliest stage of atherogenesis involves dysfunction of the endothelial layer (11). Endothelial dysfunction precedes the formation of atheromatous deposits within the arterial wall. In the setting of established risk factors, the nonadherent endothelial layer expresses various adhesion factors and chemokines that promote the adhesion of circulating inflammatory cells and subsequent transmigration into the arterial wall (11). Following migration to the intimal space, monocytes undergo morphologic changes to become macrophages (16). Macrophages engulf oxidised low density lipoprotein (LDL) to become foam cells, the sentinel cells of atheromatous plaques (17). The accumulation of foam cells in the intimal space comprise the fatty streak, the earliest macroscopic change in the arterial wall (11).

Foam cells secrete a series of chemokines and growth factors that contribute to ongoing development of plaque (17). They promote expression of adhesion molecules and chemokines leading to a continuing infiltrate of inflammatory cells into the arterial wall. In addition, foam cells promote the migration and proliferation of vascular smooth muscle cells. Vascular smooth muscle cells produce the collagen that forms the fibrous cap (18). The fibrous cap, overlying the deposition of inflammatory cells, lipid and necrotic material represents the mature atheromatous plaque.

Atherosclerotic plaques may undergo one of several pathological pathways. They remain quiescent or continue to accumulate in the arterial wall, with increasing encroachment of the

lumen. Alternatively, the fibrous cap can undergo erosion or frank rupture, exposing circulating blood to the plaque contents (19). The plaque core is highly thrombogenic and this contact stimulates thrombus formation in the arterial lumen, leading to a compromise of blood flow and clinical ischaemic events (20).

This carefully orchestrated process results from the interaction of a complex series of pathological events, promoted by a vast range of risk factors, both established and those yet to be identified. As such, these pathological phenomena provide a unique opportunity to identify new strategies to prevent the formation of atherosclerotic plaque and its clinical complications.

1.1.4 Role of lipids and lipoproteins

Dyslipidaemic states are major determinants of atherosclerotic risk. Lipids such as cholesterol and triglyceride circulate in the plasma, incorporated in lipoprotein particles. Lipids are required in the physiologic state for the maintenance of cell membrane homeostasis and cellular metabolic processes. The typical lipoprotein particle comprises a hydrophobic core of cholesterol ester and triglyceride, surrounded by a monolayer of phospholipid, unesterified cholesterol and various apolipoproteins. Lipoproteins are classified according to their density when isolated by ultracentrifugation (21).

The triglyceride rich lipoproteins include chylomicrons and very low density lipoproteins (VLDL), which are derived from the intestine and liver respectively (22). In plasma, chylomicrons undergo rapid lipolysis, by lipoprotein lipase (LPL), to become remnant particles. VLDL are rapidly remodelled to become cholesterol-rich LDL, following the action of lipolytic

enzymes and lipid transfer factors including cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). LDL provides a cholesterol source for cellular homeostasis and synthesis of steroid hormones. Alternatively it may be taken up by the liver, following binding of the LDL receptor (23). High density lipoproteins (HDL), the smallest and most dense lipoproteins, promote the transport of cholesterol from peripheral tissue to the liver (24).

Population studies have established that the plasma concentration of LDL (25), its variant lipoprotein (a) (26) and triglyceride (27) are each independent predictiors of cardiovascular events. In addition, an increased concentration of remnant particles (28) and an excessive plasma triglyceride response to a meal, impaired postprandial lipaemia (29), are markers of increased risk. Pharmacologic strategies that reduce LDL (30) and triglyceride (31,32) and elevate HDL (31,33) are associated with a profound reduction in clinical events.

It is well established that LDL diffuses into the subendothelial space, as promoted by a concentration gradient (34). This process does not involve receptor mediated endocytosis. LDL becomes trapped in this space by proteoglycans in the extracellular matrix (35). The presence of LDL has no deleterious effects, until it is modified, predominately by oxidation. Oxidised LDL is a potent inducer of adhesion molecules and chemokines, promoting the rapid accumulation of inflammatory cells (36). Monocytes undergo transformation to become macrophages (16). Macrophages engulf oxidised LDL, via the scavenger receptor CD36, to become foam cells (37). This process is not subject to a concentration gradient and can continue unabated. Foam cells are the sentinel cell of the developing atheromatous plaque. They play a pivotal role, through the

elaboration of chemokines and growth factors, orchestrating the complex cascade of events that promote atherogenesis.

1.2 HIGH DENSITY LIPOPROTEINS

HDL are the smallest and most dense lipoproteins circulating in plasma. The basic structure of HDL consists of a hydrophobic core containing triglyceride and esterified cholesterol, which is surrounded by a surface monolayer comprising of phospholipid, unesterified cholesterol and apolipoproteins (38). Greater than 90% of the apolipoprotein composition of HDL consists of apoA-I and apoA-II (39). Present in small amounts are apoA-IV, apoA-V, apoC-I, apoC-II, apoC-III, apoD, apoE, apoJ and apoL (39,40). The majority of apolipoprotein synthesis takes place in the liver, whilst a small amount of apoA-I is formed in the intestine (41,42). HDL are formed by the lipidation of these synthesised apolipoproteins. In addition, a small proportion of discoidal HDL is derived from chylomicron particles following the hydrolytic activity of the enzyme LPL (43). The HDL plasma fraction displays marked heterogeneity in terms of size, shape, density, composition and surface charge (38). This heterogeneity results from the constant remodelling of HDL by various plasma factors, including lecithin:cholesterol acyltransferase (LCAT), CETP and PLTP, all of which are transported in the plasma on HDL (44-46).

HDL are either discoidal or spherical in shape. Discoidal HDL, which comprise of low amounts of cholesterol, are present in low levels in the plasma. With the accumulation of increasing amounts of cholesterol ester, a process mediated by LCAT, HDL assumes its more common spherical appearance (47). HDL can be classified into three subfractions, on the basis of density adjusted ultracentrifugation (38). These include HDL_2 ($1.063<d<1.125$ g/ml), HDL_3,

(1.125<d<1.21 g/ml) and VHDL (1.21<d<1.25 g/ml). In addition five different subpopulations have been identified on the basis of size (7.0-12 nm diameter) using gradient gel electrophoresis (48). HDL varies with regard to its apolipoprotein composition. As a result, two major subpopulations have been identified in plasma. HDL can contain apoA-I alone or apoA-I in combination with apoA-II (49). The majority of apoA-II containing HDL particles are found within the smaller HDL_3 subpopulations (50). HDL also differ in their surface charge, as determined by the degree of migration on agarose gel electrophoresis. They can be classified, relative to plasma proteins, as possessing alpha, pre-alpha, pre-beta or gamma mobility (51-53). The majority of HDL, circulating in the spherical form, possess alpha mobility. Lipid depleted apoA-I and discoidal HDL both possess pre-beta mobility. HDL with gamma mobility have been found to contain apoE as the sole apolipoprotein. It is unclear whether this heterogeneity of HDL contributes to any functional differences between the various subpopulations *in vivo*.

1.2.1 Epidemiology of HDL and cardiovascular disease

Population studies have established that HDL has an atheroprotective role. Both the Framingham (54) and PROCAM (55) cohorts established an inverse correlation between the plasma concentration of HDL and long term incidence of cardiovascular events. Every 0.026 mmol/L (1 mg/dL) increase in plasma HDL is associated with a 1% reduction in coronary events (56). Multivariate analysis of the Framingham data established that after age, plasma HDL was the strongest independent predictor of clinical events (57). Furthermore, in angiographic studies, low plasma HDL was found to be the strongest independent biochemical risk factor (58). This powerful relationship was highlighted in the lipid intervention trials. Studies establishing the efficacy of both statins (30,59) and fibrates (31,32) demonstrated an inverse relationship between

baseline plasma HDL and the placebo event rate. In addition, the baseline plasma HDL appeared to predict the degree of benefit conferred by these agents (30,60,61).

However, it is unclear if very high levels of plasma HDL confer increasing protection from clinical events. In the PROCAM (62) and ECAT (58) cohorts, subjects with plasma HDL above the 80^{th} percentile were afforded no additional protection from clinical events than those in the $60\text{-}80^{th}$ quintile. In addition, an elevated plasma HDL has been demonstrated to increase risk in some metabolic conditions, such as hypertriglyceridaemia (63,64). What has evolved is that a low plasma HDL is a clear predictor of clinical events, and that the threshold used for this definition depends on the presence of additional risk factors. The PROCAM cohort demonstrated that the plasma HDL threshold that indicates an increased risk of events is elevated in the presence of concomitant diabetes mellitus, elevated LDL cholesterol or increased global cardiovascular risk (65).

In addition, studies of cohorts with reduced plasma HDL in the setting of genetic anomalies have provided variable results. Several groups have demonstrated that patients with homozygous deficiencies in apoA-I, LCAT or ATP binding cassette (ABCA1), resulting in low plasma HDL, are associated with premature coronary artery disease (66). In addition, heterozygous deficiency of ABCA1 is associated with greater carotid intimal medial thickness (67). However, in those with heterozygous apoA-I deficiency (66) or carriers of apoA-I mutants (68), a plasma HDL below the 10^{th} percentile is not always associated with increased cardiovascular risk. Furthermore, it is well characterised that those who carry the apoA-I_{Milano} mutant have low

plasma HDL, due to increased catabolism, are protected against developing cardiovascular events (68).

It has been suggested that HDL subpopulations or apolipoprotein levels may have a greater predictive ability than plasma HDL (69). However, the data is inconsitent and the majority of reports are from small, case-controlled studies. Two large prospective population studies, the Physicians Health Study (70) and the Atherosclerosis Risk in the Community (ARIC) study (71) both found no superiority of plasma levels of HDL_2, HDL_3 or apoA-I over plasma HDL alone. However, the PRIME cohort did demonstrate that plasma apoA-I was a stronger predictor of risk than plasma HDL (72). As a result of the inability of any of these factors to consistently prove to be superior predictors of risk, they have not replaced plasma HDL in guidelines for risk prediction and initiation of lipid lowering therapy.

1.2.2 Human intervention studies

Established lipid modifying agents are associated with variable effects on the plasma concentration of HDL. Accordingly, whilst the landmark lipid intervention trials have highlighted the ability of plasma HDL to predict cardiovascular risk (30-32,59), it is uncertain what degree of the benefit was due to raising HDL. Statins raise HDL by 5-10% (73). The statin trials established that as the baseline plasma HDL increased, the placebo event rate and subsequent proportional reduction in events seen with active therapy both decreased (30,59). As the clinical benefit in these studies correlates with the degree of LDL lowering (74), it is unclear what degree of benefit is derived from HDL elevation. However, posthoc analysis of the 4S trial demonstrated that raising HDL was more efficient than LDL lowering in terms of clinical event reduction (30).

Fibric acid derivatives (fibrates) raise HDL by 10-15% (60,61,75). Intervention trials have demonstrated a marked benefit of these agents, particularly in the setting of the metabolic syndrome (60,61,75). In a similar fashion, baseline plasma HDL was a strong predictor of both the placebo event rate and proportional reduction in events seen with active therapy (60,61). In addition, the elevation of plasma HDL with fibrates reduces events. It was determined in the Helsinki Heart Study that every 1% elevation in plasma HDL correlated with a 3% reduction in events (60). This benefit compares with a 1% event reduction for every 1% reduction in plasma LDL in the statin trials (30). Post hoc analysis of the secondary prevention trial, VA-HIT, found similarly that the overall 22% reduction in clinical events seen in subjects randomised to gemfibrozil, was due to the 6% elevation in plasma HDL. Whilst there was no relationship between triglyceride lowering and clinical benefit, posthoc analysis of VA-HIT determined that 20% of the clinical benefit of gemfibrozil was derived from its HDL raising properties (61).

Nicotinic acid is the most potent HDL raising agent currently available, elevating plasma concentrations by up to 30% (76,77). The HATS study was an angiographic study that demonstrated that the combination of simvastatin and nicotinic acid resulted in angiographic regression over a 3 year followup (33). In addition, there was a 90% reduction in clinical events compared with the placebo group, although the trial was not powered to formally study this end point. This result was placed in the context of the inability of statin therapy alone to induce atherosclerotic regression and highlighted the importance of raising HDL in addition to statin therapy in patients with established atherosclerosis.

Several reports of infusing reconstituted HDL (rHDL) in humans have now emerged in the context of endothelial function and atherosclerotic burden. In subjects with hypercholesterolaemia, a single infusion of rHDL increased flow mediated dilatation at four hours (78). In addition, forearm blood flow measured by venous plethysmography, that was impaired in ABCA1 heterozygotes with low plasma HDL, was restored to that of normal controls four hours after an infusion of rHDL (79). These preliminary studies highlight the ability of a single infusion of rHDL to raise plasma HDL and improve vascular reactivity. Furthermore, weekly infusions of rHDL containing high dose apoA-I_{Milano} apoA-I(M) to patients following an acute coronary syndrome promotes atherosclerotic regression in a six week period (80).

1.2.3 Effect of HDL on experimental atherosclerosis

The use of animal models of experimental atherosclerosis and vascular injury has provided further evidence for the beneficial properties of manipulating plasma HDL at the level of the arterial wall. Various approaches have been employed including the administration of native or rHDL, lipid free apoA-I, transgenic models, apoA-I variants and mimetics and exogenous phospholipid.

1.2.3.1 Infusion of HDL and apoA-I

Badimon *et al* were the first to demonstrate the potential of HDL to inhibit atherogenesis in an animal model. Weekly infusions of HDL-VHDL fractions isolated from pooled rabbit plasma, containing 50 mg of protein, significantly reduced the development of aortic fatty streaks in cholesterol fed New Zealand White rabbits. (81) In a followup study, the effect of HDL-VHDL

on a model of established atherosclerosis was assessed. When administered weekly for 30 days, HDL-VHDL promoted a reduction in fatty streak surface area compared with animals sacrificed at the start of the treatment phase. This result suggested that in addition to inhibiting atherogenesis, HDL may actually promote plaque regression. (82)

Beneficial properties have been demonstrated using rHDL. rHDL containing 40 mg/kg apoA-I was administered on alternate days for up to three weeks to apoE knockout mice that underwent application of periarterial collars around the carotid arteries. With no change in plasma HDL cholesterol, infusions of rHDL resulted in early reductions in VCAM-1 expression, macrophage infiltration and modification of LDL. This resulted in an inhibition of neointimal formation at three weeks. Infusion of phospholipid alone had no effect, supporting the prevailing view that the atheroprotection of HDL is due to apo A-I. It is unclear which phospholipid type was used.(83)

Miyazaki *et al* demonstrated that the administration of apoA-I alone was beneficial. The weekly administration of purified rabbit apoA-I to cholesterol fed rabbits inhibited progression of atherosclerotic burden. This effect was similar in groups stratified to receive either 1mg or 40mg of apo A-I in each infusion. The ability to promote plaque regression was not demonstrated. The authors proposed that it was the apoA-I that was responsible for the atheroprotection of HDL.(84)

1.2.3.2 Transgenic models

1.2.3.2.1 Transgenic expression of apoA-I

The transgenic expression of human apoA-I has been consistently demonstrated to prevent the development of experimental atherosclerosis. The first studies investigated C57BL/6 mice, a

strain associated with low plasma HDL and an increased propensity to develop atherosclerosis when fed an atherogenic diet. The transgenic expression of human apoA-I elevated plasma HDL and apoA-I and inhibited fatty streak formation.(85) Attention then focussed on hyperlipidaemic models. Two groups almost simultaneously reported the atheroprotective properties of human apoA-I transgenic expression in chow-fed apoE knockout mice. Paszty *et al* found that human apoA-I expression resulted in a polydispersity of HDL particle size, elevation of plasma HDL and a six-fold reduction in aortic lesion area compared with apoE knockout controls.(86) Using a similar model, Plump *et al* found a wide variety in the degree of transgenic expression. Animals with low levels of human apoA-I displayed similar atherosclerotic burden to controls. In contrast, transgenic animals with a high level of human apoA-I demonstrated a significant reduction in aortic lesion area. In addition, the lesions that developed were predominantly fibrous and depleted of lipid. Linear analysis found that 78% of the atheroprotective benefit in this study was related to the increase in plasma HDL.(87) Further studies demonstrated that the expression of human apoA-I alone or in combination with the expression of apo[a], in mice subjected to an atherogenic diet, was atheroprotective. This highlighted the potential benefit of apoA-I expression in models of increased risk of atherosclerosis, independent of the lipoprotein profile (88).

This work was extended to determine if the expression of human apoA-I influenced the progression of established atherosclerosis. Rong *et al* transplanted atherosclerotic aortas from cholesterol fed apoE knockout mice into either apoE knockouts or apoE knockouts that expressed human apoA-I. After 5 months the aortic atherosclerosis in apoE knockout recipients predictably progressed. In contrast, expression of human apoA-I retarded atherosclerotic progression. This

benefit was associated with substantial phenotypic changes of plaque, including an increase in smooth muscle cells and reduction of macrophages. This provided strong evidence that raising HDL stabilised plaque in addition to retarding atherosclerotic progression.(89)

These results demonstrate a clear benefit derived from the transgenic expression of apoA-I in murine models of atherosclerosis. Transgenic expression of human apoA-I was subsequently studied in rabbits. When fed cholesterol enriched chow for 14 weeks, transgenic animals demonstrated a 50% reduction in lesion area and aortic lipid composition compared with controls. In vitro studies demonstrated a superior ability of serum from transgenic animals to promote cholesterol efflux, supporting the contribution of reverse cholesterol transport to the atheroprotection of HDL.(90)

Several groups have extended these findings to investigate the role of genetic transfer of apoA-I into animal atherosclerotic models. Major *et al* demonstrated that the transplantation of bone marrow from macrophage apoA-I expressing animals to apoA-I/apoE double knockout mice, fed an atherogenic diet, resulted in a reduction in lesion area to levels comparable with animals expressing apoE(91). Benoit *et al* studied the role of somatic gene transfer of human apoA-I to apoE knockout mice that also expressed human apoA-I. The sustained, incremental elevation of plasma HDL reduced lesion area.(92) Furthermore, Tangirala *et al* performed a single hepatic genetic transfer of human apoA-I to LDL receptor deficient mice, fed an atherogenic diet. The resulting increase in hepatic apoA-I synthesis and plasma HDL promoted atherosclerotic regression.(93) The development of helper-dependent adenoviral vectors has generated long term

hepatic expression of human apoA-I, with atheroprotective properties in both apoE (94) and LDL receptor (95) knockout models.

1.2.3.2.2 Transgenic expression of apoA-II

Whilst the atheroprotection of apoA-I has been clearly established, the role of apoA-II in models of atherosclerosis is less clear (96). Whilst population studies appear to demonstrate a similar inverse relationship with clinical events as with apoA-I, this correlation with apoA-II remains uncertain (97). ApoA-II deficiency is rare in humans and does not appear to be associated with either a dramatic change in the lipid profile or an increase in cardiovascular risk (98). It also remains uncertain whether the presence of apoA-II can compensate for states of apoA-I deficiency. In cohorts with documented apoA-I deficiency, but normal levels of apoA-II, there appears to be a greater risk of events. This would suggest that if apoA-II was anti-atherogenic, this property is not adequate by itself to compensate for the loss of the atheroprotection of apoA-I.(99)

In a similar fashion, the results of transgenic studies have been variable and largely depend on the species origin of apoA-II. In studies of the transgenic expression of murine apoA-II it would appear that the evidence supports a pro-atherogenic role (100,101). Expression of apoA-II in these animals results in an increase in plasma HDL concentration and size. The large HDL particles comprise esterified cholesterol, apoA-II and apoE. In addition, these animals demonstrate greater atherosclerotic lesion area, in both animals receiving normal chow and an atherogenic diet.(100) Further studies have demonstrated that HDL isolated from these animals are less efficient at promoting cholesterol efflux from macrophages, are unable to protect against

LDL oxidation, stimulate the formation of lipid hydroperoxides in the arterial wall and are pro-inflammatory, promoting monocyte transmigration. These features are associated with a marked decrease in the paraoxonase content of circulating HDL.(101) Furthermore, on the background of the apoE knockout mouse, deletion of apoA-II results in reductions in both HDL concentration and particle size, in addition to conferring a less atherogenic lipid profile, associated with enhanced lipoprotein remnant clearance (102).

It would appear that the transgenic expression of human apoA-II is, on balance, anti-atherogenic (103,104). Tailleux *et al* studied the role of transgenic expression of human apoA-II in mice. This resulted in a bimodal distribution of HDL particles, containing human apoA-II by itself or in association with murine apoA-I and apoA-II. In addition, murine apoA-I and apoA-II were decreased in the plasma as a result of reductions in both production and catabolism. A reduction in non-HDL cholesterol was seen in transgenic animals. Despite, a reduction in the ability to promote cholesterol efflux *in vitro* using serum from transgenic animals, a 55% reduction in aortic lesion area resulted. Lesion area was found to correlate with plasma VLDL and IDL, but was independent of plasma HDL. The reduction in non-HDL cholesterol, in addition to increases in activity of LCAT and hepatic lipase (HL), were thought to contribute to the atheroprotection seen in the human apoA-II transgenic animals.(103) In contrast, however, when human apoA-II was co-expressed with human apoA-I, the anti-atherogenic effect of apoA-I was reduced (104). These results highlight the complex role that apoA-II plays in atherogenesis.

1.2.3.2.3 Transgenic expression of apoA-IV

ApoA-IV has been demonstrated *in vitro* to participate in reverse cholesterol transport through its promotion of cholesterol efflux, activation of LCAT and binding to hepatocytes (105). Duverger *et al* transgenically expressed human apoA-IV in the livers of C57BL/6 and apoE knockout mice, fed an atherogenic diet. This had a differential effect on plasma HDL with a 35% increase in C57BL/6 mice, but no effect in apoE knockout mice. Regardless, the transgenic expression resulted in a 70-90% reduction in aortic lesion area in both models (105).

1.2.3.2.4 Genetic deletion of apoA-I

Much attention has been given to genetic deletion of apoA-I to investigate the effects of loss of function. However, while the transgenic expression of apoA-I in animal models has consistently demonstrated an atheroprotective benefit, knockout models of apoA-I have provided variable results. In contrast to the anticipated detrimental effect of deleting apoA-I, many groups have found that this alteration alone does not result in any significant atherosclerotic burden (106). However, it appears that when combined with an atherogenic lipid profile, the deletion of apoA-I and consequent reduction in plasma HDL cholesterol does result in an increase in lesion area.

Two research groups have studied the effect of apoA-I deletion on the background of human apoB expression. The resulting reduction in plasma HDL had no effect on atherosclerotic burden in chow fed animals with normal plasma LDL (107). However, when subjected to an atherogenic diet and the appearance of elevated triglyceride and LDL, a significant increase in atherosclerotic lesion area was seen in animals with low plasma HDL.(108) Similarly, apoA-I deletion promotes atherosclerosis in the apoE knockout mouse. In comparison with the apoE knockout mice, the

apoE/apoA-I double knockouts demonstrated an increase in atherosclerotic lesion area, despite lower circulating plasma cholesterol (109). Moore *et al* studied the role of apoA-I deletion in the presence of a more modest hyperlipidaemia using chow fed LDL receptor knockout mice. In this setting, the deletion of apoA-I caused increases in both atherosclerotic area and oxidative stress, despite having a similar plasma HDL concentration as wild type animals. This supports an anti-atherogenic effect of apoA-I, but suggests that this benefit results from more than just increasing plasma HDL. Moreover, this study highlights the importance of the quality of circulating HDL cholesterol in atheroprotection (110). In addition, mice with deletion of the nuclear receptor RORα, when subjected to an atherogenic diet, demonstrated a reduction in intestinal expression of apoA-I mRNA, low plasma HDL and severe atherosclerosis (111).

1.2.3.3 Infusion of apoA-I variants and mimetics

Several rare familial disorders of HDL deficiency result from the expression of apoA-I variants (66). One such mutant, apoA-I(M), involves a cysteine for arginine substitution at amino acid 173. This results in the formation of a disulfide bridge and dimerism of the apoA-I(M) molecules. The presence of the disulfide bridge limits the degree of conformational flexibility of the protein and thus reduces the size and heterogeneity of the associated HDL particles (112).

The North Italian hamlet of Limone sul Garda is home to a well studied population of heterozygous apoA-I(M) carriers. This cohort is characterised by severely low plasma HDL and moderate hypertriglyceridaemia, who are relatively protected from coronary heart disease.(68) The low plasma HDL results from increased catabolism (113). It has been proposed that apoA-I(M) possesses enhanced atheroprotective properties. These include enhanced promotion of

cholesterol efflux (114), antioxidant activity (115) and inhibition of platelet activity (116). However, it has been reported that the cellular expression of apoA-I(M) does not result in any greater cholesterol efflux than in cells that express wild type apoA-I (117). ApoA-I(M) can be expressed in a recombinant form and has therefore been studied either individually or complexed with phospholipid in animal studies.

Soma *et al* demonstrated that infusing rHDL containing 40 mg of recombinant apoA-I(M) and egg phospholipid reduced neointimal formation induced by the application of carotid periarterial collars in cholesterol fed rabbits. This was associated with a reduction in cellular proliferation in the intima and media of vessels. There was no benefit observed whether infusions were commenced on the day of injury or whether egg phospholipid was administered alone (118). Ameli *et al* infused rHDL containing 40 mg of recombinant apoA-I(M) and phosphatidylcholine from five days before to five days following balloon denudation of the iliac artery in the cholesterol fed rabbit. Three weeks after balloon injury recombinant apoA-I_{Milano} reduced macrophage infiltration and neointimal formation by greater than 50%, despite no change in arterial cholesterol content. This strongly suggested that the benefit of apoA-I(M) was independent of the promotion of the reverse cholesterol transport pathway (119).

rHDL containing apoA-I(M) and phosphatidylcholine have also been studied with regard to their ability to modify established atherosclerotic plaque. Shah *et al* administered rHDL containing apoA-I(M) to 20 week old apoE knockout mice on alternate days for five weeks. Infusion of rHDL halted atherosclerotic progression, in addition to promoting a reduction in both the lipid and macrophage content of the experimental plaque (120). Further research subsequently

demonstrated that a single infusion of rHDL containing apoA-I(M) in apoE knockout mice significantly depleted plaque of lipids and macrophages after 48 hours (121). In addition, Chiesa *et al* infused rHDL containing apoA-I(M) directly into the carotid artery of cholesterol fed rabbits, that had undergone perivascular electric injury. After 90 minutes the local infusion of rHDL resulted in a 30% regression in plaque volume in association with lipid depletion, as determined by intravascular ultrasound (122). These studies strongly suggest that the administration of rHDL containing apoA-I(M) can have profound and rapid effects on established atherosclerotic plaque.

The benefit of rHDL containing apoA-I(M) has also been extended to non-atherosclerotic processes involving the arterial wall. The local administration of rHDL containing recombinant apoA-I(M) and the phospholipid 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) was also studied in a porcine model of in-stent restenosis. rHDL was infiltrated intramurally prior to stent deployment. At 28 days, rHDL infiltration resulted in a 50% reduction in lumen loss compared with vehicle, as a result of reduced neointimal hyperplasia.(123)

Together, these studies demonstrate that the administration of rHDL, either locally or systemically, results in profound beneficial properties. It remains unclear whether these benefits are due to the apoA-I(M) alone or due to its combination with phospholipid. To date, no animal studies have directly compared the effect of apoA-I(M) with wild-type apoA-I. Regardless, these studies add further strong support to the beneficial properties of HDL on the arterial wall.

Peptide analogues of human apoA-I have been shown *in vitro* to form complexes with phospholipid, promote cholesterol efflux, activate LCAT and protect LDL from oxidative modification through the removal of hydroperoxide metabolites of arachidonic and linoleic acid (124). Garber *et al* administered an analogue *via* intraperitoneal injection daily for sixteen weeks to C57BL/6J mice fed an atherogenic diet. HDL isolated from animals that received the analogue demonstrated an enhanced ability to inhibit the *in vitro* development of lipid hydroperoxides and induction of monocyte chemotaxis. Administration of the analogue resulted in a 40% reduction in atherosclerotic burden.(124) Further studies have focussed on the oral administration of these analogues. Proteins synthesized from D-amino acids appear to be resistant to proteolysis by human enzymes. Therefore, it is an attractive strategy to develop proteins that can be administered orally. The oral administration of an analogue, synthesised from D-amino acids, in LDL receptor deficient mice fed an atherogenic diet and chow fed apoE knockout mice significantly reduced atherosclerotic lesion area (125). This striking benefit was demonstrated in the absence of any change in plasma HDL.

1.2.3.4 Administration of exogenous phospholipid

Phospholipid circulates in the plasma incorporated into lipoproteins. Phosphatidylcholine stimulates the *in vitro* synthesis of apoA-I by neonatal swine epithelial cells (126). Navab *et al* proposed that the administration of oral phospholipid might stimulate the *in vivo* synthesis of apoA-I and increase atheroprotection. Female apoE knockout mice receiving drinking water which contained 1,2–dimyrystoyl-sn-glycero-3-phosphocholine (DMPC) demonstrated an increase in jejunal apoA-I synthesis, plasma apoA-I and HDL, in association with a decrease in atherosclerotic lesion area. Furthremore, HDL isolated from these animals demonstrated an

enhanced ability to inhibit *in vitro* monocyte chemotaxis. When the study was repeated using ten month old mice, the administration of DMPC promoted atherosclerotic regression.(127)

1.3 ROLE OF INFLAMMATION

The concept that atherosclerosis is an inflammatory condition is well established. Virchow proposed more than a century ago that the accumulation of atheromatous deposits in the arterial wall represented a process of chronic inflammation (128). Whilst the focal theory of cholesterol deposition subsequently became popular, an increasing amount of evidence supports the central role of inflammation in atherogenesis (11). It has become apparent that atherogenesis involves both innate and acquired arms of the immune mediated response and that the complex interaction between these processes promotes the accumulation of inflammatory cells in the arterial wall (129). Pathological studies demonstrate the presence of monocytes, macrophages, lymphocytes and to a lesser extent, neutrophils, in atheromatous plaques (130). As a result, the atherosclerotic plaque incorporates features of both acute and chronic inflammatory reactions. The accumulation of these cells within the arterial wall has a profound impact on the propagation of atherogenic events. Plaques, rich in macrophages, are more likely to have features of fibrous cap rupture (131). In addition, circulating inflammatory markers in the plasma correlate with the extent of atherosclerotic cardiovascular disease (132) and clinical events (133). Furthermore, anti-inflammatory strategies have been associated with atheroprotective properties in both animal models of atherosclerosis (134) and human intervention studies (135).

1.3.1 Role of adhesion molecules

Adhesion molecules are a heterogeneous group of cell surface proteins that mediate interactions between the cell and other cells and the extracellular matrix (136). They are essentially expressed by all cells and play a physiological role through their ability to focus an inflammatory response to a tissue bed. However, they can also play a pathological role promoting chronic inflammatory processes, such as atherosclerosis. Pathological studies have consistently demonstrated the focal presence of adhesion molecules in atherosclerotic plaque (137,138). In addition, the plasma concentration of soluble forms of adhesion molecules correlate with cardiovascular risk (139). Adhesion molecules can be classified into three families: (i) selectins, (ii) immunoglobulin superfamily and (iii) integrins (136). Each family differs in the pattern of cellular expression and the specific role they play in the inflammatory process.

Selectins share a similar structure consisting of extracellular lectin and epidermal growth factor like domains, linked to a small cytoplasmic domain by a short transmembrane domain (136). Their ligands consist of fucosylated and sialylated carbohydrates and mucin-like glycoproteins. The three selectins currently identified are named according to its predominant cell of expression. L-selectin is expressed constituitively on leukocytes (136). E-selectin is expressed on endothelial cells, activated by proinflammatory cytokines via the nuclear transcription factor, nuclear factor kappa beta (NF-κβ) (140). P-selectin is expressed mainly on activated platelets and to a lesser degree endothelial cells. P-selectin is stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells and are rapidly recruited to the cell surface following activation (141). These proteins mediate the rolling and loose tethering of leukocytes on the luminal surface of the

endothelial cell. In addition, they promote interactions between leukocytes and activated platelets (142).

The immunoglobulin superfamily is a large family of proteins containing variable numbers of extracellular immunoglobulin domains. The five members of the intercellular adhesion molecule (ICAM) family interact with leukocyte specific β2 integrins. ICAM1-3 are constitutively expressed on endothelial cells and leukocytes. In addition, ICAM-2 is also expressed on platelets. Interestingly, ICAM-1 is upregulated in response to inflammatory cytokines, whereas ICAM-2 expression is decreased. Vascular cell adhesion molecule-1 (VCAM-1) is expressed on endothelial cells in response to inflammatory cytokines and interacts with the integrin α4β1, known as the very late antigen (VLA-4). These molecules promote firm adhesion and arrest of leukocytes on the endothelial surface. Platelet endothelial cellular adhesion molecule-1 (PECAM-1) is expressed on endothelial cells, leukocytes and platelets, although their main role is to mediate binding between adjacent endothelial cells. In addition, they promote transmigration of leukocytes across the endothelial cell layer. (143)

The integrins consist of a non-covalent association between an α chain and a β chain spanning across cell membranes. Integrins are normally expressed on a wide variety of cells, with low affinity for ligands. Following cell activation, the conformation changes promoting adhesion. These proteins are expressed on leukocytes and platelets, facilitating interaction with the endothelial surface. (144)

It is well established in pathological studies, that VCAM-1 and ICAM-1 are expressed in atherosclerotic plaque (145). In addition, their expression is implicated in transplant vasculopathy (146). The expression of VCAM-1 and ICAM-1 by endothelial cells and smooth muscle cells is increased *in vitro* following stimulation by proinflammatory cytokines and lyso-phosphatidylcholine (147,148). In addition, animal studies have demonstrated their increased expression in response to cholesterol feeding (149), altered shear stress (150) and balloon injury (151). Their expression following balloon injury parallels the development of abnormal acetylcholine induced vasodilatation (151). Animal studies have demonstrated that the expression of proteins involved in the activation of NF-κβ is enhanced in regions of altered shear stress, leading to an increased probability of developing fatty streaks. This regional priming of the endothelium is increased with cholesterol feeding (152).

Genetic studies support a major role for adhesion molecules in the promotion of atherogenesis. In particular, results suggest that VCAM-1 is a critical component in the early stages. Homozygous VCAM-1 deletion is associated with embryonic lethality. However, VCAM-1 domain 4 deficient mice have been developed that have been cross bred with apoE knockout mice. These mice demonstrate a reduction in VCAM-1 expression, monocyte adherence and fatty streak formation. This suggests a dose dependence of VCAM-1 in the promotion of atherogenesis (153). The administration of a monoclonal antibody against VCAM-1 reduces neointimal formation following carotid injury in the mouse (154). In addition, antibody blockade of the VCAM-1 ligand, VLA-4, reduces neointimal formation following carotid injury in primates (155). Furthermore, the atheroprotective benefit of antibodies directed against the CD40 ligand are proposed to be mediated via their ability to inhibit VCAM-1 expression (134). The development

of ICAM-1 knockout mice has produced variable results. Whilst it has been demonstrated to be atheroprotective in cholesterol fed mice (156), other reports have suggested that it has no influence on the background of LDL receptor deletion (157). Furthermore, genetic deletion of P-selectin and E-selectin are each associated with atheroprotection in cholesterol fed mice (158,159).

Various selectins and members of the immunoglobulin superfamily also exist in a soluble plasma form (136). The precise mechanism leading to their generation remains unclear, although it is likely to involve proteolytic cleavage. In addition, it is uncertain whether they possess any functional properties in circulating plasma. There have been variable reports describing the significance of these types of soluble proteins as markers of cardiovascular risk. Soluble levels of ICAM-1 and VCAM-1 appear to correlate with the presence of cardiovascular risk factors (136). In addition, there is some evidence that their levels decline in response to treatment with statins (160) and hormone replacement therapy (161). In population studies of subjects with no evidence of atherosclerotic disease, the level of soluble ICAM-1 appears to independently predict cardiovascular events (162). Similarly, in subjects with established coronary artery disease various studies have demonstrated the ability of soluble ICAM-1 (163) and VCAM-1 (164) to predict events. As VCAM-1 is not expressed constitutively on normal endothelium, it seems logical that its soluble form would be a stronger predictor in the presence of established atherosclerosis.

1.3.2 Role of proinflammatory chemokines

Chemokines are a heterogeneous family of low molecular weight proteins (8 to 12 kDa) that play important roles in the trafficking of leukocytes and promotion of cell proliferation (165). Comprising more than 50 members, chemokines are classified into four families on the basis of the pattern of cysteine residues in their amino acid backbone (166). These families include CC, CXC, C and CX_3C classes. The prototype chemokine in the CC class is monocyte chemoattractant protein-1 (MCP-1). The CXC family is subdivided on the basis of the presence (ELR+), represented by interleukin-8 (IL-8), or absence (ELR-), represented by interferon-inducible protein-10 (IP-10), of an ELR (Glu-Leu-Arg) amino acid motif in the amino terminal domain. There is currently only one identified member which belongs to the C and CX_3C classes, these being lymphotactin and fractalkine respectively. Fractalkine is unique in that it has a long mucin stalk, with chemokine and adhesion molecule domains (167). These chemokines interact with a family of G protein-coupled receptors with variable degrees of specificity (168). This results in the ability of individual chemokines to promote trafficking of specific leukocyte classes. Binding results in the activation of numerous secondary messengers including protein kinase C and elevation of intracellular calcium (169).

The predominant role of chemokines is to promote the trafficking of leukocytes. This is a critical component of the inflammatory response. All cells in the arterial wall are capable of producing chemokines in response to various vasoactive and physical factors (170). Vasoactive substances include oxidised LDL, angiotensin II, platelet derived growth factor and proinflammatory cytokines such as TNF-α, IL-1β and IFN-γ. Established cardiovascular risk factors, abnormal shear stress and vascular injury such as balloon angioplasty are all potent inducers of chemokine

production by endothelial cells. The common pathway for each of these factors appears to act through activation of the transcription factor NF-κβ. This occurs in the earliest stages of atherogenesis and, in concert with the expression of adhesion molecules, promotes the tethering of circulating leukocytes to the endothelial cell surface and subsequent migration into the arterial wall. Contact between monocytes and endothelial cells, both on the surface and in the subendothelial space, amplifies the signals leading to further elaboration of chemokines (170). Chemokine generation within the arterial wall produces a chemokine gradient which in turn promotes migration of leukocytes, adhering to the endothelial cell surface, in to the arterial wall. In addition, it appears that chemokines stimulate proliferation and migration of endothelial cells and smooth muscle cells, and as a result may promote angiogenesis (171). It has also been demonstrated that chemokines promote the synthesis of the procoagulant tissue factor by macrophages (172).

Numerous chemokines have been identified in atherosclerotic plaque, predominately located in the vicinity of the necrotic lipid core and macrophage rich areas (171). Circulating levels of MCP-1 (173) and IL-8 (174) in plasma are elevated in patients with acute coronary syndromes. In addition, their levels are increased in patients with heart failure and left ventricular dysfunction (175). This may reflect the elevated cytokine levels in these disease states. Furthermore, animal studies support the central role of chemokines in atherogenesis. Using well established genetic models of atherosclerosis, deletion of MCP-1 (176) or receptors for CC (177), CXC (178) and CX_3C (179) classes, results in reduced lesion area. As a result, inhibiting chemokine synthesis or receptor binding presents an attractive target for atheroprotection. For example, administration of

atorvastatin has been found to reduce plaque composition of MCP-1 in a rabbit model of atherosclerosis (172).

Macrophage-colony stimulating factor (M-CSF) promotes the role of monocytes and macrophages in atherogenesis (180). M-CSF regulates the growth, survival and function of monocytes and macrophages, acts as a chemotactic factor and stimulates production of cytokines and growth factors. M-CSF expression is increased in atherosclerotic plaque (181), and oxidised LDL is a potent inducer of its *in vitro* expression (16). Furthermore, in animal models of atherosclerosis, genetic deletion of M-CSF reduces atherosclerotic burden and its macrophage composition (182).

1.4 ANTI-INFLAMMATORY EFFECTS OF HDL

Given the pivotal role for inflammation in the pathogenesis of atherosclerosis, it has been tempting to speculate that part of the atheroprotective properties of HDL result from their ability to inhibit the inflammatory cascade. Population studies have supported an inverse correlation between the plasma concentration of HDL and inflammatory markers such as hs-CRP (183). In addition, patients with chronic inflammatory conditions such as rheumatoid arthritis demonstrate not only an inverse relationship between plasma CRP and HDL, but also an increase in plasma HDL in response to disease modifying immunosuppressive agents (184). Furthermore, manipulation of plasma HDL in animal models of atherosclerosis depletes plaque of inflammatory cells (89,121). However, as cholesterol is a potent inflammatory stimulus, it remains unclear whether these *in vivo* effects of HDL occur independent of their ability to promote cholesterol efflux. It has been proposed that HDL possess anti-inflammatory properties

that interact with all stages of atherogenesis from the early migration of inflammatory cells into the arterial wall to the inflammatory composition of established atheroma.

1.4.1 Effect of HDL on proinflammatory cytokines

HDL inhibit the generation of proinflammatory cytokines. HDL binds directly to lipopolysaccharide (LPS) and reduces the expression of its principal receptor, CD14, on the surface of monocytes (185). The infusion of rHDL has been demonstrated to inhibit the systemic release of proinflammatory cytokines that results from the infusion of either LPS in humans (185) or E. coli in rabbits (186).

1.4.2 Effect of HDL on cell adhesion molecules

Several groups have demonstrated that HDL inhibit the expression of cell surface adhesion molecules by activated endothelial cells *in vitro* (187,188). Native human HDL (187,189) and rHDL (187,188,190) inhibit the cytokine induced expression of VCAM-1, ICAM-1 and E-selectin by human umbilical vein endothelial cells in a concentration dependent manner. This was accompanied by a reduction in mRNA levels. This activity is greatest when cells are preincubated for up to 16 hours with HDL prior to cytokine stimulation (187). In addition, adhesion molecule expression does not change if HDL are removed from the incubation prior to cytokine activation (187). This suggests that the reduction in adhesion molecule expression is a function of a change to the endothelial cell, rather than competitive binding with the cytokine. However, not all studies have demonstrated that HDL inhibits adhesion molecule expression. Two studies have found that neither native nor rHDL inhibit cytokine stimulated adhesion molecule expression by either

arterial or venous endothelial cells (191,192). The reason for these discordant results remains unclear.

Further studies demonstrated that HDL, isolated from different human subjects, varied substantially in their ability to inhibit adhesion molecule expression (193). Using rHDL, it has been possible to investigate the role of different HDL components on this activity. The anti-inflammatory properties of HDL were not altered by changing the apolipoprotein composition, size, shape or lipid content of the particle. However, this property is influenced by the phospholipid composition (194). rHDL containing the polyunsaturated fatty acid, linoleic acid in the *sn~2* position inhibit adhesion molecule expression to the greatest degree. When this *sn~2* position is occupied with fatty acids of increasing degrees of saturation, the ability of HDL to inhibit adhesion molecule expression decreases. This hierarchy matches that seen when activated endothelial cells are incubated with the free fatty acids alone (195). However, the degree of preincubation required for free fatty acids to demonstrate this property is much greater than that required by HDL. In addition, the degree of inhibition seen with phospholipid vesicles alone is less than that seen when the fatty acids are incorporated into HDL particles (194). This suggests that through incorporation of fatty acids into their phospholipid bilayer, HDL increases the efficiency of fatty acids to modulate adhesion molecule expression by endothelial cells.

The mechanism by which HDL inhibit cell adhesion molecule expression remains unclear. A recent report found that native and rHDL also inhibit the endothelial cell expression of VCAM-1, ICAM-1 and E-selectin, in response to stimulation with C-reactive protein (196). In this study, oxidised phospholipid was found to be an important factor. In addition, this anti-inflammatory

property of HDL differed from the scenario of cytokine stimulation, in terms of its need for preincubation with endothelial cells. These differences suggest that at least two mechanisms exist leading to the inhibition of adhesion molecule expression by HDL.

As adhesion molecule expression is mediated via activation of NF-κβ it would seem logical that HDL exerts its beneficial anti-inflammatory effects, by the inhibition of this nuclear factor. However, conflicting results have been reported. Cockerill *et al* reported that neither native or rHDL inhibits nuclear translocation or DNA binding of NF-κβ. This report also found that HDL promotes cellular expression of the inducible form of cyclo-oxygenase (COX2) leading to an increased generation of prostacyclin (197). Prostacyclin has been demonstrated to potent anti-inflammatory and antithrombotic properties (198). In addition, HDL acts as an exogenous source of substrate for prostacyclin synthesis (199,200). In contrast, Park *et al* found that HDL did inhibit activation of the transcription factors NF-κβ and activator protein-1 (AP-1) and the subsequent nuclear translocation of NF-κβ (201). The reason for this discrepancy is unclear. In addition, it has been demonstrated that oxidised forms of HDL activate NF-κβ and its nuclear translocation, associated with an increase in the generation of intracellular ROS (202). A beneficial effect on NF-κβ may result from a reduction in oxidative stress. It is well established that NF-κβ is activated by ROS and maintained in an inactive state by low levels of nitric oxide (203). Thus, the ability of HDL to inhibit ROS generation and promote the synthesis of NO may contribute to the inhibition of adhesion molecule expression, *via* their effects on NF-κβ.

HDL may inhibit adhesion molecule expression through alterations in bioactive sphingolipids. Xia *et al* demonstrated that HDL inhibit the activity of sphingosine kinase, resulting in a reduced

generation of sphingosine-1-phosphate (S1P) (204). S1P has been demonstrated to activate the Erk and NF-κβ pathways (205,206). Nofer *et al* recently proposed that the ability of HDL to inhibit the cytokine induced expression of E-selectin was mediated via the lysosphingolipids, sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF). It was demonstrated that these phospholipid species inhibit E-selectin expression in a concentration dependent manner, although not to the same degree as HDL particles. In addition, it was demonstrated that this activity was mediated through Akt activation and interaction with the endothelial differentiation gene family of G proteins (207).

The ability of HDL to modify adhesion molecule expression has also been demonstrated *in vivo*. Alternate daily infusions of rHDL to apoE knockout mice with carotid periarterial collars demonstrated a 40% reduction in VCAM-1 expression and monocyte infiltration as early as one week, associated with a dramatic reduction in the development of neointimal hyperplasia at three weeks (83). In addition, a single infusion of rHDL has been demonstrated to inhibit E-selectin expression in intradermal vessels following subcutaneous administration of interleukin-1 in a normocholesterolaemic porcine model (208). In contrast, the transgenic expression of human apoA-I on a background of apoE knockout mice, was demonstrated to have no effect on endothelial VCAM-1 expression, monocyte adherence or lipid infiltration, when studied at an early age (209). The authors of this study proposed that the inhibition of foam cell formation and atheroma development documented with apoA-I transgenic expression in apoE knockout mice resulted from beneficial properties following the early stages of VCAM-1 expression and monocyte adhesion to the endothelium and proposed that the benefit was likely to result from inhibition of chemokine induced monocyte trafficking and foam cell formation, in addition to

promoting cholesterol efflux from foam cells. Interestingly, elevation of plasma HDL in this study was not associated with an increase in serum paraoxonase activity. This lack of increase in antioxidant activity may have contributed to the lack of anti-inflammatory properties in this study.

1.4.3 Effect of HDL on proinflammatory chemokines

HDL has been demonstrated to have a beneficial effect on the expression of proinflammatory chemokines, their receptors and functional sequelae. HDL inhibits the expression of MCP-1 in response to oxidised LDL. This property is associated with the antioxidant components of HDL and results in reduced *in vitro* monocyte transmigration (210). In addition, transplantation of atherosclerotic aorta into apoE knockout mice expressing human apoA-I resulted in a reduced plaque expression of MCP-1 (89). HDL inhibits the expression of the chemokine receptor CCR2 on monocytes (211). CCR2 expression on monocytes, isolated from postmenopausal subjects with elevated LDL, was found to correlate inversely with plasma HDL (211). Following a two month period of oestrogen supplementation, monocyte CCR2 expression decreased two-fold in subjects with high LDL and low HDL. In addition, incubation of cultured monocytes with HDL resulted in a reduction in monocyte cholesterol, CCR2 expression and chemotaxis in response to MCP-1. This property was thought to be a consequence of cholesterol efflux.

1.4.4 Effect of HDL on monocytes and macrophages

Transmigration of monocytes into the arterial wall is an early event in atherogenesis. *In vitro* studies have demonstrated that HDL inhibits monocyte transmigration in response to oxidised

LDL (210). This property appears to be related to paraoxonase (PON) and platelet activating factor-acetylhydrolase (PAF-AH) on HDL (212,213). It has been subsequently been demonstrated that this property of HDL can be influenced in acute inflammatory states. In these states, HDL accumulates serum amyloid A (SAA) and loses its anti-inflammatory activity (212). Furthermore, HDL isolated from apoA-II transgenic mice induce monocyte transmigration, in association with a reduction in PON content (101). In subsequent studies, it was determined that following inoculation with the Influenza virus, HDL demonstrated a progressive and transient loss of its anti-inflammatory property (214). The relevance of this functional property of HDL was then studied in patients with documented coronary heart disease (CHD) without low plasma HDL concentrations. In this study it was found that HDL isolated from these subjects did not inhibit monocyte chemotaxis as efficiently as HDL isolated from controls without CHD. In fact, HDL from subjects with high plasma HDL actually promoted monocyte chemotaxis. It was subsequently determined that the anti-inflammatory property of HDL improved in these subjects following administration of simvastatin (215).

HDL_3 and apoA-I inhibit M-CSF stimulated monocyte spreading, chemotaxis and the surface expression of β2-integrins, β1-integrins and the scavenger receptors CD163 and CD36. These features were reproduced by cyclodextrin promoted cholesterol efflux from monocytes, but not seen when monocytes were isolated from Tangier patients. This suggested that the potent *in vitro* anti-inflammatory properties of HDL and apoA-I are likely to be due to regulation of cellular cholesterol pools leading to reduced expression of CDC42, a member of the family of GTP-binding proteins (216).

HDL also has beneficial effects on the presence of macrophages within the arterial wall. HDL inhibits the formation of foam cells. Macrophages within the arterial wall are derived from monocytes, whose transmigration is inhibited by HDL (210). Foam cells form following the uptake of oxidised LDL by macrophages. HDL inhibits the oxidation of LDL (217). In addition, HDL promotes cholesterol efflux from macrophages (218). Elevation of HDL, via transgenic expression of human apoA-I (89) or infusion of rHDL containing apoA-I(M) (121) deplete the macrophage composition of established atherosclerotic plaque. It remains uncertain whether this property is dependent on HDL mediated lipid efflux. Furthermore, *in vitro* studies have demonstrated that HDL inhibits the expression of proinflammatory factors by stimulated macrophages (211). This suggests that HDL can inhibit the accumulation of macrophages, their transition to foam cells and their ability to promote ongoing inflammatory activity in the arterial wall.

1.4.5 Effect of HDL on lymphocytes

Despite the evidence establishing a role of lymphocytes in atherogenesis (219), little is known about the relationship of lymphocytes with HDL. Lymphocytes undergo adhesion and chemotaxis in response to the same adhesion molecules and chemokines as monocytes. The ability of HDL to inhibit the expression of these proinflammatory factors should inhibit the accumulation of lymphocytes in the arterial wall. This has not been addressed in studies of experimental atherosclerosis. *In vitro* studies have demonstrated a complex relationship between HDL and lymphocytes. ApoA-I inhibits contact between monocytes and T lymphocytes which normally results in the production of chemokines IL-1β and TNF-α (220). In addition, incubation with HDL results in reduced activation and function of natural killer cells (221). Furthermore,

HDL inhibits the activation of perforin, a major mediator of cell lysis (222). However, variable effects have been reported with regards to the effect of HDL on lymphocyte proliferation. Studies both *in vitro* (223) and in animals (224) have found that HDL inhibit proliferation. However, it has been demonstrated that lymphocytes possess specific receptors that promote binding of HDL, and through this binding, it has been proposed that HDL acts as source of fatty acids, required for cell proliferation (225-227).

1.4.6 Effect of HDL on neutrophils

Neutrophils have been implicated in the generation of reactive oxygen species (ROS) (228)and early stages of atherogenesis (229). Studies suggest a complex interaction between neutrophils and HDL. Incubation with HDL prevents the expression of CD11b/CD18 on the surface of neutrophils, adhesion of neutrophils to endothelial cells and their subsequent chemotaxis (230). In addition, HDL inhibit neutrophil degranulation, phagocytic activity and generation of ROS (231). They prevent the inhibition of neutrophil NOS activity by oxidised LDL and the release of proinflammatory interleukins from stimulated neutrophils (232,233). HDL from septic patients, however, actually enhance the functional properties of neutrophils (234). This effect may be related to an increased proportion of serum amyloid A in HDL. *In vivo* models have demonstrated that administration of HDL reduces CD11b/CD18 expression on neutrophils and their accumulation in renal tissue in a model of ischaemia-reperfusion injury (235). However, there is some suggestion that *in vitro* exposure to neutrophils promotes the degradation of HDL apolipoproteins leading to an impairment of their ability to promote cholesterol efflux (236).

1.5 OTHER FACTORS IN ATHEROGENESIS

1.5.1 Role of endothelial dysfunction

The endothelium has endocrine, paracrine and autocrine properties that influence vascular tone, the mobilisation of inflammatory cells to tissue and thrombogenicity (237). Normal endothelium provides a surface that inhibits the adherence of circulating inflammatory cells, permeability of the arterial wall and tendency of circulating blood to thrombose. The functional properties of the endothelium promote the redistribution of blood flow in shock, direct appropriate inflammatory responses to foreign agents and provide adequate haemostasis in the setting of haemorrhage. However, in the setting of established cardiovascular risk factors, the inappropriate alteration in the elaboration of substances can have a profound impact on the arterial wall.

The principal product of the endothelium is nitric oxide (NO), formed from the amino acid L-arginine by the endothelial isoform of NO synthase (eNOS). In addition to producing NO in a constitutive fashion, eNOS activity can be stimulated to increase NO production in response to a variety of physiological agonists, shear stress and pharmacological agents. NO possesses numerous beneficial properties including promoting vasodilatation, in addition to the inhibition of proinflammatory chemokine and adhesion molecule expression, vascular smooth muscle cell proliferation and platelet adhesion and aggregation. Furthermore, NO regulates apoptosis and angiogenesis. (238)

However, the endothelium produces a variety of chemical messengers in addition to NO. Some factors promote vasodilation, angiogenesis and inhibition of thrombus formation, cellular proliferation and inflammation. These factors include endothelium-derived hyperpolarising

factor, prostacyclin, tissue plasminogen activator and naturetic peptides. Other factors promote vasoconstriction, thrombosis and cellular proliferation and include endothelin, angiotensin-II, plasminogen activator inhibitor-1 and thromboxane A2. The balance between these factors determines the role played by the endothelium. (239)

Dysfunction of the endothelium is the earliest change in the arterial wall during atherogenesis (11). The abnormalities that result have a direct impact on the development of both microscopic and macroscopic changes in the arterial wall. The functional changes result in an endothelium that favours vasoconstriction and the adherence of inflammatory and thrombogenic components of the circulating blood. A dysfunctional endothelium is characterised by the expression of (i) cell surface adhesion molecules, (ii) proinflammatory chemokines, (iii) prothrombotic substances and (iv) the development of abnormal vascular reactivity. Numerous techniques have been developed to assess the ability of the endothelium to regulate vascular reactivity both *in vitro* and *in vivo*. The intracoronary administration of substances which induce NO release result in dilatation of the coronary artery. In patients with established coronary atherosclerosis on angiography, these stimuli result in vasoconstriction, indicative of endothelial dysfunction (240).

It has subsequently been demonstrated that endothelial dysfunction is present prior to the appearance of atherosclerosis. Each established risk factor, including elevated LDL-cholesterol, hypertrigylceridaemia, low plasma HDL-cholesterol, hypertension, smoking, diabetes, aging, menopause and hyperhomocysteinaemia, is associated with endothelial dysfunction (237,241). In addition, children with a family history of premature coronary artery disease (CAD) have been demonstrated to have abnormal vascular reactivity (242). The degree of endothelial dysfunction

has been found to predict both the presence and extent of atherosclerosis (243). In addition, in patients with established clinical atherosclerosis, the presence of endothelial dysfunction predicts clinical events and poor prognoses (244).

1.5.2 Role of oxidative stress

Increasing evidence has accumulated to implicate a central role of oxidative stress in atherogenesis (245). In addition to the pivotal pro-atherogenic properties of oxidised LDL and products of lipid peroxidation, it is now recognised that a range of ROS play an important role in the development of arterial pathology including atherosclerosis, hypertension and restenosis (246). All cells in the arterial wall generate ROS by the activation of the mitochondrial electron transport chain (247), numerous cellular enzymes (245) and the uncoupling of the enzyme eNOS (248). Each of these processes respond to factors including vasoactive stimuli, such as angiotensin II, tumour necrosis factor-α, interleukin-1 and platelet derived growth factor, and physical forces, such as abnormal shear (249).

ROS have variable cellular effects at different concentrations. When present in low concentrations ROS act as intracellular second messengers promoting a variety of pro-atherogenic events (245). At higher concentrations, they promote irreversible cell damage and apoptosis (250,251). ROS promote many pro-atherogenic events including lipid peroxidation, expression of proinflammatory adhesion molecules and chemokines, proliferation and apoptosis of vascular smooth muscle cells, matrix metalloproteinase activation and expression of tissue factor (247).

Genetic models of atherosclerosis, such as apoE and LDL receptor knockout mice, demonstrate increased products of oxidative stress (252,253). Animal models that target deletion of important elements in the generation of oxidative stress, including receptors for oxidised LDL (CD36) (254), 12/15-lipoxygenase (255) and subunits of NAD(P)H oxidase (256), are atheroprotective. In addition, administration of vitamin E, in doses that inhibit lipid peroxidation, reduce atherosclerotic burden in cholesterol fed apoE knockout mice (252). Furthermore, cholesterol fed rabbits develop increased aortic superoxide, in association with activation of NAD(P)H activity and abnormal acetylcholine induced vasodilatation (257). These features are reversed following the administration of superoxide dismutase (258).

Increased products of oxidative stress have been demonstrated in subjects with cardiovascular risk factors and established atherosclerosis (246). The degree of oxidative products found in carotid plaque correlates with clinical events (259). However, clinical trials of antioxidants have been disappointing. Administration of multivitamin cocktails has been found to have no effect in large, randomised prevention trials (260,261). In addition, multivitamins reduce the benefit seen with the combination of nicotinic acid and simvastatin (33). However, it is unclear if these interventions resulted in a reduction in oxidative stress in these patients. As a result, the search continues to identify effective *in vivo* antioxidant strategies.

1.5.3 Role of smooth muscle cells

Smooth muscle cells (SMC) play an important role in atherosclerosis and restenosis. They accumulate in the intima in response to growth factors including platelet derived growth factor, fibroblast growth factor and transforming growth factor-β (TGF-β). In addition, heparin and

plasminogen activator inhibitor-1 (PAI-1) possess strong mitogenic properties (262). SMC orginiate from a diverse range of sources, including medial SMC (263), adventitial fibroblasts (264), endothelial cells (263) and circulating bone marrow derived cells (265). In addition, macrophages within the atherosclerotic plaque may undergo phenotypic change to become smooth muscle cells, in response to cholesterol efflux (266). Upon proliferation in the intimal space, SMC change from a contractile to synthetic phenotype, leading to the elaboration of extracellular matrix components that strengthens the fibrous cap overlying atherosclerotic plaque (18). Therefore, SMC proliferation plays a dichotomous role in atherogenesis. It promotes expansion of atherosclerotic burden, whilst at the same time reduces its propensity to rupture.

1.5.4 Role of matrix metalloproteinases

Matrix metalloproteinases (MMP) play a major role in atherogenesis. Their degradation of connective tissue promotes vascular remodelling and increases the propensity of the fibrous cap to rupture (267). Pathological studies have identified increased MMP expression and activity, colocalised with macrophages, in atherosclerotic plaque (268). In addition, the amount of MMP-9 in carotid plaque *ex vivo* correlates with clinical events and the degree of histological inflammation (269). Lipid lowering interventions reduce MMP-1 expression and activity in experimental atheroma (270). In addition, the transgenic expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in the apoE knockout mouse reduces lesion size and macrophage infiltration (271). Circulating plasma levels of MMP-9 are increased in the settings of acute ischaemic syndromes (272). Furthermore, genetic polymorphisms in the promoter sequence of MMP-3 are associated with a greater prevalence of acute myocardial infarction (273).

Reducing the ratio of MMP to TIMP activity is a potential therapeutic target in the prevention of ischaemic events. Potential strategies focus on both the inhibition of MMP production and activity and promotion of TIMP expression. Statins reduce MMP expression *in vitro* (274) and an animal model of experimental atherosclerosis (275). Furthermore, the administration of pravastatin prior to carotid endarterectomy is associated with a reduction in MMP expression and activity on *ex vivo* plaque analysis (276).

1.5.5 Role of apoptosis

Pathological studies have demonstrated the presence of apoptotic cells, colocalised with the expression of apoptotic effectors, in human plaque (277). Inefficient removal of the apoptotic cells leads to their accumulation and subsequent secondary necrosis in the plaque's necrotic core (278). Apoptotic cells are proatherogenic through the activation of the coagulation cascade and promotion of synthesis of proinflammatory cytokines (279). In addition, loss of normal function of these cells contributes to the atherogenic process (280). Endothelial cell apoptosis contributes to exposure of plaque contents to circulating blood. Apoptosis of SMC contributes to regression of atherosclerotic burden. However, it also promotes weakening of the fibrous cap, as less collagen is produced and therefore increases the propensity to plaque rupture.

1.5.6 Role of thrombogenicity

The majority of ischaemic events result from the formation of thrombus overlying a breakdown in the barrier which separates plaque contents from circulating blood. Thrombus formation compromises the arterial lumen, resulting in ischaemia. Pathological studies have demonstrated

the presence of fresh arterial thrombus in the setting of acute coronary syndromes (281). Thrombus formation represents the result of an orchestrated cascade of events influencing (i) platelet activation and aggregation, (ii) activation of the coagulation cascade, (iii) the role of endogenous anticoagulants and (iv) the inhibitory activity of endogenous fibrinolytic substances.

Platelets play a critical role in the development of intracoronary thrombosis. Following rupture of the fibrous cap, platelets rapidly adhere to components of the plaque's core and provide the nidus for thrombus formation. The elaboration of nitric oxide and prostacyclin, in response to normal shear stress, maintains circulating platelets in a quiescent state (282). However, these endogenous anti-platelet factors are reduced in the presence of cardiovascular risk factors, promoting their activation and aggregation (283). In addition, platelets promote the inflammatory cascade via the elaboration of proinflammatory chemokines, induced by the release of microparticles from the surface of activated platelets and the binding of activated platelets to monocytes (284). Platelet factors stimulate SMC proliferation (285). Furthermore, platelets are a source of ROS (286). These properties highlight that platelets play a central role in atherogenesis, one that begins at the very early stages of endothelial dysfunction.

The major promoters of the coagulation cascade are tissue factor and activated platelets. Tissue factor is expressed by activated endothelial cells, circulating leukocytes, SMC in the subendothelial space and macrophages in the plaque core (20). These factors promote activation of the coagulation cascade early, in the setting of endothelial dysfunction, and following rupture of the fibrous cap. The activity of tissue factor is regulated, in physiological conditions, by the presence of tissue factor pathway inhibitor, which circulates bound to lipoproteins (287). A

relative imbalance between tissue factor and its inhibitor is characteristic of established atherosclerosis. Lipid lowering interventions reduce tissue factor expression in an experimental model of atherosclerosis (288).

Endogenous factors that inactivate coagulation factors limit thrombus formation. These include antithrombin, heparin cofactor II and thrombomodulin which bind directly to activated clotting factors. In addition, thrombomodulin, when complexed to thrombin, activates protein C and protein S, which both inactivate clotting factors (289). Antithrombin and heparin cofactor II are produced by the liver. Thrombomodulin, in contrast, is produced constitutively by endothelial cells. This synthesis is reduced in endothelial dysfunction, contributing to the prothrombotic state. Uncontrolled thrombus formation is further controlled by the presence of endogenous fibrinolytic factors. Plasminogen activators convert plasminogen to plasmin, a fibrin-degrading protease. This process is regulated by the action of plasminogen activator inhibitors (PAI). The balance between plasminogen activators and their inhibitors has a major influence on thrombus stability (290).

It has become increasingly recognised that increased thrombogenicity is present in the early stages of atherogenesis (284). The endothelium normally functions to provide a surface in contact with circulating blood that inhibits thrombus formation. This is achieved through the elaboration of anti-thrombotic substances, including NO, prostacyclin, thrombomodulin and tissue plasminogen activator. However, the synthesis and release of these substances is reduced in endothelial dysfunction. Furthermore, dysfunctional endothelial cells produce PAI-1. As a result,

clinical studies have found a correlation between abnormal vascular reactivity and the presence of thrombogenic plasma markers (291).

A complex relationship exists between inflammation and thrombogenicity (284). This is largely mediated *via* the elaboration of chemokines that activate both inflammatory cells and platelets. In addition, activation of circulating monocytes and platelets occurs, following their contact (292). Futhermore, release of microparticles from the surface of activated platelets act as potent proinflammatory chemokines (293). The role of CD40 and its ligand CD40L has received considerable attention. Soluble levels of CD40L in the plasma correlate with the risk of clinical events (294). In addition, manipulation in animal models, using either CD40 knockout mice (295) or the administration of antibodies directed against CD40L (296) results in atheroprotection.

1.6 FUNCTIONS OF HDL

1.6.1 Reverse cholesterol transport

HDL plays a pivotal role in reverse cholesterol transport, the process involving the transportation of cholesterol from peripheral tissues to the liver (24). Lipid deplete apoA-I is synthesised in the liver and intestine and acts as an efficient acceptor of free cholesterol, effluxed from cells (41,42). HDL promotes cholesterol efflux by three mechanisms including aqueous diffusion, scavenger receptor-BI (SR-BI) mediated flux and ABCA1 mediated flux (297). The aqueous diffusion of free cholesterol between acceptor and donor particles is a bidirectional and inefficient process. Net flux of cholesterol by aqueous diffusion is determined by the degree of contact between donor and acceptor, the ability of cholesterol to desorb from the donor plasma membrane and the concentration gradient promoting net flux of free cholesterol to the

acceptor.(298) The scavenger receptor SR-BI is a member of the CD36 family which acts as a multi-ligand receptor for HDL, LDL, oxidised LDL, acetylated LDL and unilamellar vesicles. The most prominent role of SR-BI is to facilitate selective hepatic uptake of cholesterol from HDL. However, it is apparent that SR-BI can promote bidirectional exchange of cholesterol between cells and extracellular acceptors. Cholesterol efflux, mediated by SR-BI, requires the presence of a phospholipid containing acceptor.(299)

ABCA1-mediated efflux of free cholesterol, in contrast, is unidirectional and results in transfer of cholesterol and phospholipid preferably to lipid-free apolipoproteins (218). Early studies of cholesterol enriched fibroblasts and macrophages from patients with Tangier disease found that these cells lacked the ability to release phospholipid and cholesterol to lipid-free apolipoproteins, but maintained normal efflux to mature forms of HDL (300,301). Subsequent work found that the genetic basis for this disease involved mutations in ABCA1, a member of the ATP-binding cassette transporter family (302,303). ABCA1 is expressed in the plasma membrane and late endosomal components (304). It is proposed that the lipid-poor apolipoprotein interacts with ABCA1 either directly or *via* a lipid domain in the plasma membrane (297). In addition, the mechanism of ABCA1 mediated efflux remains uncertain, although two models have been proposed. The molecular efflux model proposes that there is sequential efflux of phospholipid generating pre-β nascent HDL, which then acts as the acceptor for effluxed cholesterol (305). Alternatively, the membrane solubilisation model proposes that cholesterol and phospholipid are effluxed simultaneously to the protein acceptor (306).

Free cholesterol on the surface of the HDL particle is rapidly esterified by the enzyme LCAT (307). The resulting esterified cholesterol is stored in the core of the particle, thus maintaining the concentration gradient promoting movement of free cholesterol from the cell to HDL surface. HDL particles accumulating a core of esterified cholesterol increase in size and become mature, α-migrating HDL. These particles dispose of the cholesterol in two ways. Firstly, they interact with SR-BI located on the surface of hepatocytes and cells involved in steroidogenesis (308). Cholesterol is transferred to the cell *via* a process involving selective endocytosis. The HDL particle itself is not taken up by the cell and therefore remains free to return to the circulation. The other pathway for HDL cholesterol disposal involves transfer of esterified cholesterol to triglyceride rich intermediate density lipoproteins (IDL) *via* CETP (309). CETP coordinates the exchange of esterified cholesterol and triglyceride between lipoproteins. This pathway is responsible for disposal of the majority of esterified cholesterol in HDL. LDL resulting from the hydrolysis of triglycerides in IDL then deliver esterified cholesterol to the peripheral tissue where it is used for cellular homeostasis or to the liver where it is taken up and either excreted in the bile or recycled for endogenous cholesterol synthesis.

1.6.2 Antioxidant effects of HDL

HDL has been demonstrated to possess various antioxidant properties. These include inhibition of both LDL oxidation by transition metal ions (310) and the formation of lipid hydroperoxides mediated by the enzyme 12/15-lipooxygenase (217). In addition, HDL protects the vascular wall from the deleterious effects of bioactive oxidised products (210,311,312) and restores the balance of NO to ROS (313). Furthermore, it has been demonstrated that erythrocyte markers of oxidative stress are inversely correlated with plasma HDL (314).

HDL circulates in the plasma associated with numerous antioxidant substances including apoA-I, LCAT, PON, PAF-AH and glutathione peroxidase (69). There are numerous mechanisms by which these substances inhibit oxidative stress. HDL can scavenge oxygen derived free radicals (315). HDL acts as a reservoir for the transfer of bioactive lipid peroxides from LDL (310). HDL is the principal carrier of lipid hydroperoxides in plasma (316). These products can then be transferred to the liver, where they are taken up and metabolised. It is theoretically possible that these lipid peroxides may be transferred back to LDL *via* the action of CETP. ApoA-I has been demonstrated to possess antioxidant properties. It promotes the depletion of lipid peroxides in LDL associated with sulfoxidation of methionine residues, generating oxidised apoA-I (317). In addition, HDL isolated from apoA-I overexpression in transgenic mice has been demonstrated to have a greater *in vitro* ability to prevent the oxidation of LDL (318).

Alternatively, HDL can directly hydrolyse phospholipid and cholesterol ester hydroperoxides on LDL, converting them to nonatherogenic compounds (315). This is mediated by PON, PAF-AH, LCAT and glutathione peroxidase. PON hydrolyses phospholipid hydroperoxides on both LDL and HDL (315). As HDL loses its ability to promote cholesterol efflux (319) and prevent platelet aggregation when oxidised (320), this adds further support to a protective role for PON, which has been demonstrated in transgenic animal models. PON knockout mice develop more atherosclerosis in response to an atherogenic diet (321). In addition, HDL isolated from these animals does not prevent oxidation of LDL *in vitro*. Furthermore, overexpression of PON in cholesterol fed apoE knockout mice reduces lesion area (322).

HDL also protects against the pro-atherogenic effects of oxidised LDL. Oxidised LDL promotes *in vitro* monocyte transmigration, endothelial cell apoptosis, calcification of vascular cells and the development of abnormal vascular reactivity in organ baths. Coincubation with HDL prevents these deleterious changes (210,312,323,324). In addition, HDL restores the balance of NO to superoxide. LCAT deficient mice, with low plasma HDL, have an increased generation of vascular superoxide (325). Furthermore, coincubation of endothelial cells with apoA-I mimetics prevents uncoupling of eNOS, in response to LDL, favouring generation of NO over superoxide (313).

1.6.3 Effect of HDL on matrix metalloproteinases

The anti-inflammatory properties of HDL should theoretically inhibit MMP activity. HDL prevents the *in vitro* release of MMPs by cytokine and oxidised LDL stimulated monocytes (326). In contrast, native and oxidised forms of HDL stimulate and inhibit the expression of variable metalloproteinases by endothelial cells (327). In addition, there is some suggestion that metalloproteinases may have a detrimental effect on HDL. Their coincubation results in proteolytic degradation of apoA-I and impaired ability of HDL to promote cholesterol efflux from macrophage foam cells (328).

1.6.4 Effects of HDL on vascular reactivity

HDL promotes the maintenance of normal, endotheliuim-dependent vasoreactivity. Plasma HDL is correlated with the degree of NO dependent coronary vasodilatation (329,330). In addition, plasma HDL is a strong independent predictor of endothelium-dependent vasodilatation in

peripheral arteries of healthy subjects (331), subjects with cardiovascular risk factors (332,333) and subjects with established CHD (334). Furthermore, plasma HDL correlates with the overall coronary flow reserve (335).

HDL promote normal vascular reactivity in *ex vivo* organ baths and *in vivo*. Native HDL and rHDL prevent the inhibition of acetylcholine induced vasodilatation induced by incubation of vascular rings with oxidised LDL or lyso-phosphatidylcholine (312,336). Transgenic expression of human apoA-I normalised the impaired sensitivity of aortic segments to relaxation by acetylcholine, induced by high fat feeding in the apoE knockout mouse (337). Recent studies have demonstrated the ability of a single infusion of rHDL to rapidly restore vascular reactivity in subjects with either hypercholesterolaemia (78) or low plasma HDL in the setting of heterozygous ABCA1 deficiency (79).

This benefit is likely to result from the ability of HDL to promote the synthesis of vasoprotective factors by the endothelium. HDL increases the expression of eNOS in endothelial cells (338). Activation of eNOS is mediated by the binding of lipidated apoA-I to the scavenger receptor, SR-BI (339). The precise mechanism by which this interaction results in enhanced eNOS activity remains unclear. Several mechanisms have been proposed including modification of cholesterol distribution within the plasma membrane, which alters local morphology and eNOS activity (340), and activation of protein kinase pathways leading to the generation of intracellular ceramide, which can phosphorylate and activate eNOS (341). Alternatively, it is possible that HDL acts as a vehicle to transport oestradiol to the plasma membrane where this interaction results in an increase in eNOS activity. The increase in NO is also influenced by the activity of

HDL sphingolipids, which act through intracellular mobilisation of calcium and Akt-mediated phosphorylation of eNOS (342). In addition, NO is depleted in the presence of ROS (245). The inhibition of the *in vitro* generation of ROS by HDL provides another mechanism by which NO bioavailability is promoted.

HDL influences the elaboration of other vasoactive substances. HDL promotes the synthesis and prolongs the half life of prostacyclin (PGI_2) (343,344). PGI_2 acts synergistically with NO to promote vasodilatation, and has anti-thrombotic and anti-proliferative properties. (198) Plasma HDL has been found to correlate with the concentration of PGI_2 metabolites (345). The incubation of HDL with endothelial cells promotes the synthesis of PGI_2 (346). HDL may be a source for the arachidonic acid substrate, required for PGI_2 formation, either from its own phospholipid reserve or *via* activation of membrane bound phospholipase (199,200). In addition, HDL also increases expression of the cyclooxygenase (COX-2) which mediates PGI_2 synthesis (197). HDL also increases the expression of the vasodilator C naturetic peptide (CNP) (347). However, it is unclear whether HDL has any influence on endothelin production. It has been demonstrated that HDL both stimulates (348) and inhibits (349) generation of endothelin by endothelial cells. However, the beneficial finding was demonstrated in a more physiological model of endothelin synthesis.

1.6.5 Antithrombotic effects of HDL

HDL have been demonstrated in both *in vitro* and *in vivo* studies to possess powerful antithrombotic properties. This benefit results *via* reduction of blood viscosity, inhibition of platelet aggregation and the coagulation cascade and promotion of both endogenous

anticoagulants and fibrinolysis. Plasma HDL is inversely correlated with blood viscosity (350). This relationship is mediated through several actions. HDL competes with LDL for binding sites on the membranes of erythrocytes (351). This inhibits the ability of LDL to induce erythrocyte aggregation. ApoA-I stabilises the membrane of erythrocytes (352). HDL inhibits binding of fibrinogen to erythrocytes (353). These actions contribute to a reduced propensity of erythrocytes to aggregate and contribute to blood viscosity.

HDL inhibits *in vitro* activation and aggregation of platelets (354). Platelets from subjects with a low plasma HDL have an increased tendency to aggregation and are more likely to form acute platelet-dependent thrombus (355). In addition, infusion of rHDL inhibits *ex vivo* platelet activation (356). This relationship is related to several properties of HDL. Numerous binding sites for HDL have been identified on platelet surfaces (357,358). HDL inhibits the secretion of α-granules and dense granules from activated platelets (359). In addition, apoE containing HDL induces the activity of the enzyme NOS in platelets (360,361). The NO that is generated inhibits platelet aggregation (362). HDL activates protein kinase C (PKC) which stimulates the Na^+/H^+ transporter resulting in the development of an alkaline cytoplasm and inhibition of intracellular calcium release (363). In addition, PKC inhibits the activity of phosphatidylinositol specific phospholipase C, a signal mediator of platelet agonists thrombin and collagen (359). HDL binds to glycoprotein IIa on the platelet surface, which inhibits binding with the fibrinogen receptor (359). In addition, apoA-I stabilises prostacyclin which inhibits platelet aggregation (364).

HDL inhibit activation of the coagulation cascade. HDL inhibits tissue factor synthesis by endothelial cells (353). It is uncertain whether HDL contains tissue factor pathway inhibitor. In

addition, transgenic expression of human apoA-I results in a depletion of tissue factor in an animal model of atherosclerosis (89). HDL inhibits activation of the coagulation factor X (365). In addition, HDL prevents the calcium ionophore-induced formation of the prothrombinase complex, comprising factors Va, Xa, II, phospholipids and calcium, on the platelet surface (352). This property results from the ability of HDL to inhibit the translocation of anionic phospholipids to the outer leaflet of the platelet plasma membrane (352). Furthermore, HDL promotes endogenous anticoagulation and fibrinolysis. HDL augments the ability of activated protein C to inactivate factors Va and VIIIa (366). In addition, HDL increases the ability of protein S to activate protein C. Cardiolipin and phosphatidylethanolamine found on the surface of HDL may promote these beneficial properties.(366) In addition, plasma PAI-1 is inversely correlated with plasma HDL (367). Furthermore, HDL inhibits the *in vitro* expression of PAI-1 by activated endothelial cells (368).

1.6.6 Effect of HDL on cell death

HDL prevents endothelial cell apoptosis, in response to a number of stimuli including oxidised LDL (369), TNF-α (370), the remnants of triglyceride rich lipoproteins (371) and the deprivation of growth factors (372). This protection is mediated *via* several pathways. The apolipoprotein component of HDL prevents elevation in cytosolic calcium and proteases in response to oxidised LDL and TNF-α (369,370). In addition, bioactive lysosphingolipids on HDL inhibit the mitochondrial generation of ROS and subsequent apoptotic effectors (373). However, this anti-apoptotic activity is impaired when HDL are glycated (374), are enriched with either apoA-II (369) or apoC-I (375), or are isolated from sera of individuals with the apoE 4/4 genotype (376).

In addition, HDL protects cells from irreversible damage and death due to non-apoptotic mechanisms. HDL protect endothelial cells from damage induced by complement activation. HDL binds to the complement factor C9, preventing its incorporation into the effector C_{5a-9} complex (377). HDL also carries the protein protectin (CD59) that inhibits complement mediated cell lysis (378).

1.6.7 Effects of HDL on cellular proliferation

The relationship between HDL and cellular proliferation is complex. HDL has been demonstrated to promote the proliferation of numerous cell lines *in vitro* (379). In contrast, other groups have found that HDL have little or no effect on the regulation of cell growth (380). The promotion of endothelial cell proliferation by HDL appears to be mediated *via* two distinct pathways. The apolipoprotein component of HDL promotes cellular proliferation *via* a protein kinase C pathway (381), whilst the lipid component activates phospholipase C with an elevation of intracellular pH and calcium (382).

Much of the controversy with regards to the influence of HDL on cellular proliferation comes from studies involving vascular smooth muscle cells (VSMC). There are variable reports of the effect of HDL on VSMC proliferation. Studies have demonstrated that when incubated with HDL, a greater proportion of these cells are found to be in the replicative phase of the cell cycle (383). HDL induces the expression of cyclins which promote the transition of a cell to the synthetic phase of the cell cycle (384). In addition, HDL promotes various changes in VSMC required for normal cell proliferation. These include the phosphorylation of G-proteins, induction of transcription of key growth factors and activation of pathways including protein kinase C,

mitogen-activated protein kinase and phosphatidylinositol and phosphatidylcholine specific phospholipases. The promotion of these factors by HDL appears to be mediated *via* the action of bioactive lysosphingolipids (384). However, when studied *in vivo* HDL inhibit VSMC proliferation. In particular, infusing rHDL was found to reduce proliferation in association with inhibiting the development of neointimal hyperplasia induced by a periarterial collar (118).

It remains uncertain whether the promotion of VSMC proliferation by HDL would be beneficial. Potentially such proliferation increases overall plaque burden. However, these *in vitro* properties have been demonstrated using greater concentrations of HDL than seen in physiological tissue. It has been proposed that VSMC would only be exposed to such concentrations when endothelial integrity was impaired. In this situation, the proliferation of VSMC may be favourable as it promotes the production of collagen and strengthening of the fibrous cap (69).

1.6.8 Effects of HDL on angiogenesis

HDL induces tube formation in a coronary artery endothelial model on a matrix gel (385). This potential role in promoting angiogenesis may result from their beneficial impact on proliferation, migration and apoptosis of endothelial cells. As angiogenesis may have both a beneficial and deleterious impact on atherogenesis, it is unclear whether this property of HDL is desirable.

1.6.9 Effects of HDL on vascular calcification

HDL exert a beneficial effect on vascular calcification. HDL prevent *in vitro* osteogenic differentiation and calcification of calcifying vascular cells in response to stimulation by

proinflammatory chemokines and oxidised LDL. This benefit is mediated *via* the lipid component of HDL. Further studies have demonstrated that oxidative modification of HDL promotes osteogenic differentiation (311).

1.6.10 Effects of HDL on ischaemia-reperfusion injury

Restoration of blood flow to a vascular bed remains the primary aim in episodes of acute arterial occlusion. In addition to the ischaemic insult, cells are exposed to a range of oxidative and inflammatory mediators during reperfusion. The injury that results from such ischaemia-reperfusion can compromise end organ function even further (386). It would seem logical that the antioxidant and anti-inflammatory properties of HDL would result in a favourable impact on the outcome of ischaemia-reperfusion. The infusion of native and rHDL has been demonstrated to have beneficial properties, characterised by reduced inflammatory and oxidative changes, in models of coronary (387) and renal artery (235) ischaemia. In addition, their benefit has been extended to systemic organ dysfunction in the setting of endotoxic (388) and haemorrhagic (389) shock.

1.7 ANIMAL MODELS OF ATHEROSCLEROSIS

The development of well validated animal models of atherosclerosis have increased our understanding of the biological processes that take part in atherogenesis. They provide an ideal tool to assess the ability of interventions to modify plaque burden and composition. The development of genetically modified mouse models, involving deletion of the genes involved in the expression of apolipoprotein E (390) and the LDL receptor (391), in combination with

'western' type diets promote the development of atherosclerotic changes in the aortic root. This, however, is not a typical site of atherosclerosis in humans. The rabbit has proven to be an ideal animal model for the investigation of atherosclerosis. When subjected to cholesterol enriched feeding, the rabbit develops an increase in apolipoprotein B containing particles in the plasma and fatty streaks in the aortic wall (392). Unlike the mouse, the rabbit does not require genetic modification to demonstrate these changes.

Various techniques have been developed to simulate processes that contribute to atherogenesis. Manipulation of the artery, using application of periarterial collars (393) and electric current (394), balloon injury (395) and arterial ligation (396) promotes pro-oxidant and proinflammatory changes that lead to the formation of neointimal hyperplasia. In addition, the combination of aortic balloon denudation with cholesterol feeding results in the development of accelerated and localised atherosclerotic plaque which bears a similar pathologic resemblance to that seen in humans (270).

One major limitation of established animal models, is their low propensity to undergo spontaneous rupture. Various approaches have been utilised to provoke rupture of experimental plaque, including the administration of sympathomimetic agents (397) and the use of balloon inflation within the plaque itself (398). However, this does not allow for suitable investigation of the ability of an intervention to prevent spontaneous rupture of pathologically vulnerable plaque. As this is the predominant mechanism that leads to clinical ischaemic events, there has been an active search to identify suitable models of spontaneous plaque rupture. Combining genetic deletion of both the scavenger receptor SR-BI and apoE results in mice with a high incidence of

spontaneous plaque rupture, myocardial infarction and premature death (399). The mechanism by which the SR-BI deletion promotes plaque rupture remains uncertain. In addition, selective in breeding of a population of Watanabe hyperlipidaemic rabbits, known to have a low incidence of spontaneous rupture, results in a cohort with almost universal rupture (400). These models provide an exciting background to investigate factors that reduce plaque vulnerability.

1.8 USE OF RECONSTITUTED HDL IN RESEARCH

Given the marked heterogeneity of HDL in terms of size, electrophoretic mobility and composition, it is difficult to assess the effects of these variables on the function of native HDL. The use of rHDL appears to overcome this problem. These particles, formed by the cholate dialysis method, incorporate phospholipid and apolipoprotein, with or without cholesterol (401). These particles are prepared in discoidal form, and can then be converted to a spherical particles following the incubation with cholesterol and LCAT (307). For example, it has been demonstrated that discoidal particles are rapidly converted to spheres following intravenous administration to rabbits (402). This reconstitution method allows for the selective preparation of particles which vary in size and composition. These particles have been extensively applied to assess the role of HDL composition on both HDL remodelling by plasma factors (403) and its potential antiatherogenic function both *in vitro* (190) and *in vivo* (83).

1.9 ROLE OF DIET IN ATHEROGENESIS

It has become increasingly apparent that dietary factors have a profound impact on atherosclerotic risk. Epidemiologic studies have established the relationship between dietary

intake of cholesterol and fatty acids with atherosclerotic burden and clinical cardiovascular events (25,404). Numerous studies have defined the role that these dietary factors play in modifying the mechanisms that contribute to plaque formation. It is recognised that diet can have an influence on plasma lipoproteins and directly on the arterial wall. As a result, dietary strategies have assumed a central role in atheroprotective guidelines.

1.9.1 Postprandial lipoproteins

As the typical western diet comprises the consumption of numerous, large meals, humans spend a majority of the twenty four hour cycle in the postprandial state. Therefore factors generated during this period are exposed to vascular endothelium for prolonged periods. As a result, it has been proposed that atherogenesis is a 'postprandial phenomenon'. This extends an early proposition by Heberden, in the eighteenth century, that the circulatory system is altered during the postprandial period (405).

This state is characterized by the appearance of numerous metabolic changes, including elevations in plasma glucose and TRLs. The increase in TRLs results from the intestinal assembly of chylomicrons and hepatic synthesis of VLDL. Circulating TRLs are hydrolysed by an increase in activity of LPL and HL, mediated *via* increased insulin secretion. This results in the formation of remnant particles, non-esterified fatty acid release, small and dense forms of LDL and reduced levels of HDL. This postprandial lipid profile is pro-atherogenic (406).

Epidemiologic studies have demonstrated that the presence of these postprandial lipoproteins are associated with increased atherogenesis. The plasma concentration of triglyceride (27) and

remnant particles (407) predict cardiovascular events. In addition, an exaggerated triglyceride response to a meal is associated with an increased prevalence of CAD (408). Furthermore, interventions that reduce the level of TRLs slow atherosclerotic progression (409) and reduce clinical events (32).

In vitro studies support the role of postprandial factors in atherogenesis. Non-esterified fatty acids (NEFA) promote the expression of proinflammatory adhesion molecules and chemokines by endothelial cells (410). Remnant particles reduce acetylchloline-induced vasodilatation (411). In addition, they promote adhesion and activation of monocytes and platelets (410). These particles also activate clotting factors VII and XII (412). These properties appear to be mediated *via* an increase in oxidative stress. Furthermore, it has been demonstrated that VLDL activates the promoter gene for PAI-1 promoting fibrinolytic resistance (413).

It has been demonstrated that the consumption of a fatty meal is associated with an impairment of endothelial function, as assessed by flow mediated dilatation (FMD) (414). However, some groups have not demonstrated such an effect (415). In addition, the infusion of a fat rich emulsion promotes a transient reduction in FMD (416). These responses are associated with markers of oxidative stress and an increase in circulating pro-inflammatory cytokines. This impairment of vascular reactivity can be prevented by the consumption of vitamins or antioxidant rich foods (417). Furthermore, the consumption of a fat rich meal is associated with increased platelets and PAI-1 activity (418,419). These factors contribute to the documented increase in angina that occurs during the postprandial period (420).

1.9.2 Dietary fatty acids

Population studies have established that the degree of saturated fat consumption correlates with plasma cholesterol and the incidence of clinical events (404). In contrast, consumption of mono- and polyunsaturated fat reduces clinical events (404,421). Furthermore, secondary prevention studies have found that consumption of a Mediterranean diet, rich in monounsaturated fat, reduces clinical events in subjects with established CHD (422).

Dietary fatty acid consumption influences the levels of circulating lipoproteins (423). Saturated fat intake raises plasma triglyceride and LDL cholesterol. In contrast, mono- and polyunsaturated fat intake lowers plasma triglyceride and LDL cholesterol. Consumption of any of these fatty diets promotes an elevation of HDL cholesterol. However, it is uncertain whether this HDL cholesterol is dysfunctional.

In addition, the consumption of dietary fatty acids is a major determinant of the phospholipid composition of circulating lipoproteins. Animal studies have demonstrated that radiolabelled phospholipid, incorporated into chylomicron-like emulsions, are rapidly transferred to other lipoprotein fractions (424). In addition, chronic fatty acid consumption is reflected in tissue composition of adipose tissue biopsies (425). This transfer of fatty acids can have a profound impact on lipoprotein and cellular function.

Fatty acids differ in their susceptibility to oxidative modification (426). As a result, consumption of a diet enriched with polyunsaturated fatty acids renders their LDL particles more susceptible to oxidation (427). Given the major pro-atherogenic role of oxidised LDL, this impact of dietary

fatty acid consumption can have a profound impact on atherogenicity. In addition, dietary fatty acids have a major influence on inflammation and thrombogenicity. Consumption of monounsaturated fats are associated with a reduced surface expression of ICAM-1 and macrophage-associated adhesion molecule-1, Mac-1 (CD11b), on circulating monocytes (428). In addition, plasma PAI-1 activity is reduced (429). Moreover, consumption of polyunsaturated fats confers strong anti-thrombotic properties (430).

These functional properties have been confirmed in animal models that demonstrated induction of atherosclerosis following the consumption of saturated fat (431). The use of unsaturated fats have produced variable results, but it would appear that use of mono- and polyunsaturated fats are atheroprotective (432,433). In contrast, when polyunsaturated fats are oxidised, atherosclerotic burden increases (434). These studies highlight a complex and important link between dietary fatty acid consumption and atherosclerotic risk and support the need to promote the central role that modifying dietary fatty acid intake can play in atheroprotection.

1.10 SCOPE OF THIS THESIS

The work presented in this thesis extrapolates the *in vitro* findings that HDL possess certain properties, that contribute to their marked atheroprotection, in addition to the promotion of reverse cholesterol transport. In particular we aim to demonstrate that HDL have significant *in vivo* properties in the models of acute vascular inflammation and plaque stabilisation. In addition, we have tested the hypothesis that HDL phospholipid composition contributes to these in vivo properties.

In the studies discussed in Chapter three we have investigated the role of infusing rHDL particles containing different phospholipids into rabbits that have received periarterial collars around the carotid arteries as a model of atherosclerosis. We have assessed the effects of rHDL on the early proinflammatory, pro-oxidant and prothrombotic factors.

In the studies discussed in Chapter four we have aimed to extrapolate the *in vitro* findings that HDL phospholipid composition affects their ability to inhibit VCAM-1 expression by activated cells. In particular, we have infused phospholipid specific chylomicron-like emulsions into rabbits and assessed whether this affects the composition of HDL and their ability to inhibit *in vitro* VCAM-1 expression.

In the studies reported in Chapter five we have investigated the effect of infusing HDL into rabbits with established atherosclerotic plaque in order to determine their ability to change plaque composition consistent with plaque stabilisation. We have compared administration of HDL with that of atorvastatin.

In the studies reported in Chapter six we have investigated the effect of infusing rHDL into rabbits with established atherosclerotic plaque to determine their ability to induce plaque stabilisation. We have altered the phospholipid and apolipoprotein composition of the rHDL particle to assess their effects on this property of rHDL.

In the studies described in Chapter seven we have performed a human intervention study and investigated the effect of a single fatty meal on endothelial function, arterial compliance and the ability of HDL to inhibit the *in vitro* expression of VCAM-1 by activated endothelial cells.

The studies presented in this thesis aim to demonstrate that HDL have profound anti-inflammatory, antithrombotic and antioxidant effects *in vivo* that occur independent of their ability to promote cholesterol efflux. In addition, the studies aim to assess whether the influence of HDL phospholipid composition on their *in vitro* properties can be extrapolated to the *in vivo* setting and to relate this to dietary fatty acid consumption. The studies endeavour to highlight the role of modification of HDL in the prevention of stages of atherogenesis.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 ISOLATION OF APOA-I AND APOA-II

ApoA-I and apoA-II were prepared from pooled expired, autologously donated human plasma which was donated by Gribbles Pathology, Adelaide, South Australia. ApoA-I was also prepared from pooled rabbit plasma, treated with streptomycin and penicillin (Quality Farms Australia, Lara, Victoria). HDL ($1.06<d<1.21$ g/mL) were isolated from the plasma by sequential ultracentrifugation in a Beckman L8-70M ultracentrifuge using a 55.2 Ti rotor (Beckman Instruments, Fullarton, CA, USA). The 1.06 g/mL spin was carried out at 55,000 rpm for 16 h. Two 26 h spins at 1.21 g/mL were carried out at 55,000 rpm. All spins were conducted at 4°C. The isolated HDL were extensively dialysed against 5 mM ammonium bicarbonate (3x 5L) and then delipidated (435). The resulting apoHDL was dissolved in 20 mM Tris, pH 8.2, lyophilised, and stored at -20°C before further separation of apolipoproteins.

ApoA-I and apoA-II were isolated from apoHDL by anion-exchange chromatography on a 2.6 x 24.0 cm column of Q Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) attached to a Fast Performance Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech). The column was pre-equilibrated with 20 mM Tris, 6 M urea (pH 8.5). The apoA-I and apoA-II were resolved by a modification of the method of Weisweiler (436). The apoA-I and apoA-II were eluted from the column at a flow rate of 3 mL/min with a linear gradient of 80-140 mM NaCl. The eluent was collected in 9 mL fractions. The purified apoA-I and apoA-II appeared as single bands following electrophoresis on a homogeneous 20% SDS-polyacrylamide PhastGel (Amersham Pharmacia Biotech) and Coomassie staining. The isolated apolipoproteins were dialysed against 20 mM ammonium bicarbonate (3 x 5L), lyophilised, and stored at -20°C. Prior to use, the apolipoproteins were reconstituted in 10 mM Tris-HCl, 3.0 M guanidine-HCl, 0.01%

(w/v) EDTA-Na_2 (pH 8.2) and exhaustively dialysed against Tris-buffered saline (TBS), pH 7.4 (5 x 1L), containing 10 mM Tris-HCl, 150 mM NaCl, 0.006% (w/v) NaN_3, and 0.005% (w/v) EDTA-Na_2.

2.2 ISOLATION OF NATIVE HDL FROM POOLED PLASMA

Total HDL was isolated from pooled rabbit plasma (Qualtiy Farms of Australia, Lara VIC), treated with streptomycin and penicillin. HDL (1.06<d<1.21 g/mL) were isolated from the plasma by sequential ultracentrifugation in a Beckman L8-70M ultracentrifuge using a 55.2 Ti rotor (Beckman Instruments, Fullarton, CA, USA). The 1.06 g/mL spin was carried out at 55,000 rpm for 16 h. Two 26 h spins at 1.21 g/mL were carried out at 55,000 rpm. All spins were conducted at 4°C. The isolated HDL were extensively dialysed against 5 mM ammonium bicarbonate (3x 5L) and endotoxin free phosphate buffered saline (PBS, pH 7.4). The apoA-I concentration of each isolated sample of HDL was determined by an immunoturbidometric assay (437) and divided into 25mg aliquots which were then stored at -80°C.

2.3 ISOLATION OF NATIVE HDL FROM INDIVIDUAL PLASMA SAMPLES

The total HDL fraction (1.063<d<1.21 g/mL) was isolated from plasma samples of individual rabbit and human subjects by sequential ultracentrifugation in a Beckman TL-100 ultracentrifuge using a T100.4 rotor (Beckman Instruments, Fullarton, CA, USA). The 1.06 g/mL spin was carried out at 100,000 rpm for 16 h. Two 26 h spins at 1.21 g/mL were carried out at 100,000 rpm at 4°C. The isolated HDL were extensively dialysed against 5 mM ammonium bicarbonate (3x

5L) and endotoxin free PBS (pH 7.4). The apoA-I concentration of each isolated sample of HDL was determined by an immunoturbidometric assay.

2.4 PREPARATION OF RECONSTITUTED HDL

Discoidal rHDL containing phosphatidylcholine (PC) and apoA-I were prepared by the cholate dialysis method (401). Solutions of 1-palmitoyl-2-linoleoyl PC (PLPC) and 1,2-dipalmitoyl PC (DPPC) (100 mg/mL in chloroform:methanol, 2:1 v/v) were prepared. Either PLPC or DPPC were placed in glass tubes and dried as a thin film on the walls of the glass tubes under nitrogen. The tubes were lyophilised at room temperature overnight and then placed on ice. A solution of sodium cholate (30 mg/mL in TBS, pH 7.4) was added to the glass tubes to give a molar ratio of PC:cholate of 200:200. TBS, pH 7.4 was added to each tube to bring the volume to 0.5 ml. The tubes were vortexed every 15-20 min until the contents were optically clear. A 6-8 mg/mL solution of apoA-I was added to each tube (2 mg apoA-I/tube) in a molar ratio of PC:apoA-I of 200:1. The tubes remained on ice for 2 h, after which the contents of all tubes were pooled and dialysed extensively against TBS, pH 7.4 (5 x 1 L) over five days to remove the cholate.

2.5 ELECTROPHORESIS

2.5.1 Non-denaturing gradient gel electrophoresis

Particle sizes of native HDL, reconstituted HDL and chylomicron-like emulsions were determined by electrophoresis on non-denaturing polyacrylamide gradient gels (3-40% prepared according to the method of Rainwater *et al* (438). The gels were run in a Gel Electrophoresis Apparatus GE-2/4 LS (Pharmacia Fine Chemicals, Stockholm, Sweden) attached to an

Electrophoresis Power Supply EPS 400/400 (Amersham Pharmacia Biotech). Samples were pre-mixed with 40% (w/v) sucrose, 0.01% (w/v) bromophenol blue made up in the electrophoresis buffer. The electrophoresis was carried out at 150-180 volts for a total of 3000 volt-hours in electrophoresis buffer, 0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA-Na_2, pH 8.4. A calibration standard (High Molecular Weight Calibration Kit for Native Electrophoresis, Amersham Pharmacia Biotech) containing thyroglobulin (Mol. Wt. 669,000 Da; Stokes' diameter 17.0 nm), ferritin (440,000 Da; 12.1 nm), catalase (232,000 Da), lactase dehydrogenase (140,000 Da; 8.1 nm) and BSA (67,000 Da; 7.0 nm), was subjected to electrophoresis simultaneously with samples for calculation of particle size.

Following electrophoresis, the gels were fixed with 10% (w/v) sulphosalicylic acid for 1 h and then stained for 3 h with 0.04% (w/v) Coomassie G-250, 5% (v/v) perchloric acid. Finally, the gels were de-stained with 5% (v/v) acetic acid for 20-28 h. Gels were scanned using a Sharp JX 610 High Resolution Scanner and densitometric analysis of the gel scans was performed using ImageMaster™ Software (Amersham Pharmacia Biotech). Particle sizes were determined by comparing the migration distance of the particles with that of the known protein standards.

2.5.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using homogeneous 20% polyacrylamide PhastGels and SDS buffer strips in a PhastSystem (Amersham Pharmacia Biotech). Molecular weight references came from a Low Molecular Weight Calibration Kit for SDS Electrophoresis (Amersham Pharmacia Biotech) which contained phosphorylase b (Mol Wt. 97,000 Da), albumin (66,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100

Da) and α-lactalbumin (14,400 Da). Electrophoresis was carried out for 95 volt-hours. For coomassie staining, the gels were preserved with glycerol:acetic acid:water (10:10:80, v/v/v), stained with 0.1% (w/v) PhastGel Blue R (Amersham Pharmacia Biotech) and, destained with methanol:acetic acid:water (30:10:60, v/v/v).

2.5.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate isolated HDL samples on the basis of surface charge (439). A Bio-Rad Mini Sub gel electrophoresis system (Hercules, CA, USA) was used for this procedure. The running buffer contained 10 mM barbitone/50 mM sodium barbitone, pH 8.7. The 0.6% agarose gel was prepared by dissolving 0.18 g agarose in 30 ml running buffer. The mixture was poured into a gel tray and left at room temperature for about 25 minutes or until the gel set. The samples containing about 20 μg protein were pre-mixed with a small amount of tracking dye containing 40% (w/v) sucrose and 0.01% (w/v) bromophenol blue before being applied to the gel. The gel was electrophoresed at 100 volts at room temperature for 1 h. Gels were then fixed with ethanol:water:acetic acid 6:3:1 (v/v/v) for 10 min, stained with Coomassie Brilliant Blue G for 30 minutes, destained with 45% ethanol overnight and scanned as for non-denaturing polyacrylamide gradient gels.

2.5.4 Two dimensional gel electrophoresis

Two dimensional gel electrophoresis was used to separate HDL subpopulations on the basis of their surface charge and size (51). This involved the sequential application of agarose gel

electrophoresis, non-denaturing polyacrylamide gradient gel electrophoresis and immunoblotting for the presence of apoA-I.

Samples were subjected to agarose gel electrophoresis as described. Prior to the fixation stage each individual lane was isolated, rotated 90 degrees and placed on top of a 3-40% non-denaturing gradient gel. This gel was then re-subjected to electrophoresis. Prior to the fixation stage the samples were transferred electrophoretically from the gel to a nitrocellulose membrane (MFS Membrane filters mixed cellulose ester, Advantec MFS, Inc., CA, USA) using a Bio-Rad Trans-blot electrophoresis unit (Bio-Rad Laboratories). The buffer used for this procedure was 0.025 M Tris/0.2 M glycine/20% (v/v) methanol, pH 8.3. The transfer was carried out at 200 mA, at 4°C for 24 h. The membrane was then immunoblotted with polyclonal sheep anti-human apoA-I antiserum 1:5,000 (Boehringer Mannheim GmbH, Germany) in Blotto which contained 50 mg/mL of skim milk powder and small amounts of thimerosal and anti-foam. This procedure was carried out at room temperature for 1 h. The membrane was washed with 0.05% Tween 20 in TBS (3 x 5 min) then Blotto (3 x 5 min) and incubated at room temperature for 1 h with 1:10,000 anti-sheep/goat antiserum conjugated to horseradish peroxidase (Silenus Laboratories Pty. Ltd., Hawthorn, Australia). The membrane was then washed with 0.05% Tween 20 in TBS (3 x 5 min) and TBS (3 x 5 min). The transferred bands were detected by ECL (Amersham Pharmacia Biotech). (Liang 1994)

2.6 IMMUNOHISTOCHEMICAL STAINING

2.6.1 Staining of frozen tissues

5 μm thick sections of frozen tissue were cut using a cryotome and placed on coated slides. Sections were fixed by immersion in a mixture of acetone and methanol (150:150 (v/v)) at room temperature for 5 min and then allowed to air dry. The primary antibody was applied to cover the tissue section and allowed to incubate at room temperature overnight. Slides were washed twice with PBS (pH 7.4) and incubated with a secondary antibody against either anti-mouse immunoglobulin (4 μL/mL) or anti-goat immunoglobulin (2 μL/mL) for 30 min. The slides were then re-washed twice with PBS (pH 7.4). Tertiary antibody incubations were then performed for 60 min using a streptavidin biotin complex. Slides were then washed with PBS (pH 7.4) three times. A diaminobenzamine (DAB) solution (pH 7.65-7.7) containing 0.2 M HCl (13.6 mL), 0.2 M Tris (12.4 mL), water (24 mL) and H_2O_2 (50 μL) was applied to the slides for 7 min, and washed three times with running water. Background counter staining of sections was performed by sequential immersion in haematoxylin, acid alcohol, lithium carbonate, graded alcohol solutions and histolene. Slides were then air dried and coverslips were applied.

2.6.2 Staining of paraffin embedded tissues

5 μm thick sections were cut, mounted on silane-coated slides and dried at 60°C for 20 min. Sections were dewaxed by immersion with three exchanges in xylene (2 min) followed by three exchanges in absolute alcohol (2 min). Endogenous peroxidase activity was blocked by incubation with 0.5% H_2O_2 (8.3 mL) in methanol (500 mL) at room temperature for 30 min. Slides were rinsed twice in PBS (pH 7.4). Sections were then subjected to microwave antigen retrieval with 10 mM sodium citrate (pH 6) containing 5M NaOH (25 mL), citric acid (10.5 g)

and water (5 L). Sections were placed into plastic racks and loaded into 250 mL of 10 mM sodium citrate (pH 6) in microwave pots. Sections were run in a microwave on HIGH, maintaining a temperature 95-99°C for 10 min after the solution begins to boil. The pots were removed and allowed to cool to 50°C. Slides were rinsed twice with PBS (pH 7.4). Sections were incubated in 3% horse serum (NHS) for 30 minutes. The primary antibody, diluted in 3% NHS, was applied to the slides and incubated at room temperature overnight. Slides were rinsed twice with PBS (pH 7.4). A vector biotinylated secondary antibody (1/250 diluted in 3% NHS) was applied and incubated for 30 min. Slides were rinsed twice with PBS (pH 7.4). A streptavidin peroxidase tertiary antibody (1/1000 diluted in 3% NHS) was applied for 60 min. Slides were rinsed twice with PBS (pH 7.4). A DAB solution was applied as a peroxidase substrate solution for 7 min. Slides were rinsed twice with PBS (pH 7.4). Sections were lightly counter stained with Mayer's haematoxylin, dehydrated as described above and mounted with coverslips. Plaque composition was determined by computer immunohistochemical analysis (288). Digital micrographs were obtained of the entire circumference of arterial sections using an Olympus BX40 microscope at 20X magnification. All images were analysed using ImagePro Plus (Cybernetics). A threshold was determined to represent positive immunohistochemical staining, by an independent pathologist, blinded to the treatment status of the animals. The threshold was determined for each individual antibody studied. The perimeter of atherosclerotic plaque was traced on each micrograph obtained. The percentage of plaque area that contained staining, above the pre-determined threshold, was calculated by the computer. The results from each of the micrographs of an artery were then averaged to provide a total percentage of plaque area that stained positive for the antibody applied for the whole arterial section studied. An example of this analysis is provided in figure 2.1.

2.7 ISOLATION AND CULTURE OF HUVECS

Umbilical veins were donated from the delivery suites of the Women's and Children's, Burnside War Memorial and Royal Prince Alfred Hopsitals. Human umbilical vein endothelial cells (HUVECs) were isolated as described by Wall *et al* (440). Cells were cultured on gelatin coated culture flasks in medium M199 with Earles Salts (Trace Biosciences, Australia) supplemented with 20% foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Victoria), 20 mmol/L HEPES, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, non essential amino acids, penicillin, streptomycin, 20 mg/mL endothelial growth supplement (Collaborative Research, Australia) and 20 mg/mL heparin (Sigma). The HUVECs were passaged every two or three days. Cell viability was tested with the standard dye exclusion method and by assessing plating efficiency of the cultures. In addition, the cells were continually observed with light microscopy to ensure that they maintained confluent cultures with a healthy 'cobblestone' morphology. HDLs were not toxic to the cells as proven by the fact that in initial studies PECAM-1 was not inhibited in the presence of HDLs (187). Moreover, cell viability (assessed as discussed above) was not altered in the presence of HDLs.

2.8 CHEMICAL ANALYSES

All chemical assays were performed on a Hitachi 902 Autoanalyser (Roche Diagnostics, Mannheim, Germany).

Phospholipid concentrations were determined using a modification of the method described by Takayama *et al* (441). A buffer solution (500 mmol/L TrisHCl, 21.25 mmol/L Phenol, 5.44 mmol/L $CaCl_2.2H_2O$, pH 7.8) was made up with water to a final volume of 50 mL. The reagent consisted of the buffer (800 μL), phospholipase D (800 IU), choline oxidase (11.2 IU), peroxidase (17.75 IU), 4-aminoanti-pyrene (80 μL of 12 mg/mL), Triton X-100 (3.2 mL of 5 g/mL) and water (3 mL). The final volume of the reagent was 8 mL. Choline chloride (15 mmol/L) in 5 g/mL Triton X-100 was used as the standard.

Protein concentrations were measured using the bichichioninic acid assay (442). Reagent A was the bichinchoninic acid solution (50 mL) mixed with Triton X-100 (500 μL). Reagent B was a copper (II) sulphate pentahydrate solution (4% (w/v)). Reagent A and reagent B were mixed together (800:16 (v/v)). A fatty-acid free BSA solution (1.0 mg/mL) was used as the standard.

Concentrations of apoA-I and apoA-II were measured immunoturbidometrically using sheep anti-human apoA-I and anti-human apoA-II anti-serum (Boehringer Mannheim, Germany). The reagent for the apolipoprotein assays was a solution of saline (0.9% (w/v) NaCl) containing 40% (v/v) polyethylene glycol 600 (Boehringer Mannheim), 0.1% Tween-20 (BDH Chemicals) and 0.025% (v/v) buffer concentrate (Behring).

Triglyceride concentrations were assayed as describe by Wahlefeld (443). A buffer concentrate (pH 7.6) containing Tris (1500 mmol/L), $MgSO_4.7H_2O$ (180 mmol/L), EDTA-Na_2 (100 mmol/L), 4-chlorophenol (30 mmol/L), potassium ferrocyanide (1.3 mg), sodium cholate (30 mmol/L), Triton X-100 (3 mL) and water (final volume 50 mL) was prepared. Reagent 1 consisted of the

buffer concentrate (2 mL), peroxidase (3 IU), L-glycerin-3-phosphate-oxidase (100 IU), adenosine-5'-triphosphate (11 mg) and water (final volume 20 mL). Reagent 2 contained the buffer concentrate (1 mL), lipase (final concentration 30 IU), ampyrone (1.42 mg) and water (final volume 10 mL). Prior to use, glycerokinase (1 mg/mL) was added to reagent 1 (10 μL/1 mL reagent 1). The standards for the triglyceride assay were obtained from Roche Diagnostics.

Total cholesterol concentration was determined as described (439) using a commercially available kit (Roche Diagnostics). Unesterified cholesterol was measured as as described by Stahler *et al* (444). Buffer 1 (pH 7.7) consisted of Na_2HPO4 (41.9 mmol/L), phenol (20 mol/L), methanol (3.75 mL) and water. Buffer 2 (pH 7.7) was Na_2H_2PO4 (360 mmol/L), $NaH_2PO4.2H_2O$ (41.9 mmol/L), 4-aminoant-pyrene (0.41 mg/mL), polyoxyethylene-9-lauryl ether (200 μL), methanol (3.75 mL) and water. The final volumes of buffer 1 and 2 were both 50 mL. The enzyme reagent contained cholesterol oxidase (12 IU/mL), peroxidase (8 IU/mL) and Tris-HCl (10 mmol/L, pH 7.4). The reagent for the assay contained buffer 1, buffer 2 and the enzyme reagent in the ratio of 1:1:0.01 (v/v/v). The standards were the same as for the total cholesterol assay. The concentration of esterified cholesterol was determined as the difference between total cholesterol and unesterified cholesterol concentrations.

The concentration of non-esterified fatty acids were determined using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan).

2.9 USE OF THE RABBIT AS AN EXPERIMENTAL MODEL

All animal experiments that contribute towards this thesis were performed using the New Zealand White rabbit. This rabbit model is well validated in the study of both lipoprotein metabolism and atherosclerosis (445). Murine animal models have been used extensively in the study of the vascular biological events that contribute to atherogenesis. In particular, the apoE (390) and LDL receptor (391) knockout models result in marked hyperlipidaemia and development of fibrofatty lesions in the arterial wall. They have subsequently been used to assess the impact of numerous interventions, thought to be of benefit in atheroprotection.

However, murine models have several major limitations. Unlike humans, the majority of cholesterol circulates in plasma in HDL particles. This is, in part, a reflection that murine models lack CETP and therefore do not exhibit the marked transfer of esterified cholesterol to apoB containing particles, as seen in humans. In contrast, rabbits express CETP, carry the majority of circulating cholesterol in LDL and, as a result, represent a more suitable model to assess HDL metabolism (309).

The murine model, also differs from humans in the distribution and characterisation of atheroma that develops. In addition, the lesions that form in these models do so predominately in the aortic root. This anatomic distribution contrasts with that seen in humans. Furthermore, murine models of atherosclerosis do not exhibit evidence of spontaneous plaque rupture (446). The rabbit model, in contrast, develops atheroma in an anatomic distribution that is more similar to humans. In addition, hyperlipidaemic rabbit models are associated with a small incidence of spontaneous

plaque rupture (400). The rabbit model is also well validated with regards to the role that inflammation plays in all stages of atherosclerosis (447).

2.10 STATISTICAL ANALYSES

All results are expressed as mean ± SEM unless otherwise specified. Statistical comparisons were made using Students t-tests and analysis of variance using the statistical program in GraphPad Prism 4.0 (GraphPad, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

2.11 CONTRIBUTION BY THE CANDIDATE TO THE THESIS

I was personally responsible for all the techniques involved in this thesis with the exception of harvesting and the initial culturing of the HUVECs, and the vascular studies in Chapter 7. All methods used were established techniques.

2.12 OTHER METHODS

Methods specific to the work in individual studies are presented in the relevant chapters.

2.13 CHEMICALS AND REAGENTS

Acetic acid glacial	BDH Chemicals 100015N
Acetone	BDH Chemicals 10003.4Q
Acrylamide	Bio-Rad 161-0107

Adenosine-5'-triphosphate	Sigma Chemicals A-5394
Agarose	Sigma Chemicals A-6013
4-Aminoantipyrine	Sigma Chemicals A-7699
Ammonium bicarbonate	BDH Chemicals 103025E
Ammonium persulfate	Bio-Rad 161-0700
Anti-foam	Sigma Chemicals A-5758
Anti-human apolipoprotein A-II (from sheep)	Calbiochem 178464
Anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep)	Amersham Biosciences NA931V
Anti-sheep/goat antiserum conjugated to horseradish peroxidase	Southern Biotechnology 6150-05
Barbitone	BDH Chemicals 10415.3P
Barbitone, sodium	BDH Chemicals 103654E
Bichinchoninic acid	Sigma Chemicals B-9643
Boric acid	BDH Chemicals 10054.3R
Bovine serum albumin, fatty acid-free (BSA)	Sigma Chemicals A-6003
Bromophenol blue	BDH Chemicals 44305
Butylated hydroxy toluene (BHT)	Sigma Chemicals B-1378
Calcium chloride	BDH Chemicals 27588
Chelex 100 resin	Bio-Rad 142-2832
Chloroform	BDH Chemicals 10077.6B
Cholesterol, unesterified	Sigma Chemicals C-8667
Cholesterol oxidase	Sigma Chemicals C-5421

Cholic acid, sodium salt	Sigma Chemicals C-1254
Choline oxidase	Sigma Chemicals C-5896
4-Chlorophenol	Sigma Chemicals C-4914
Citric acid	BDH Chemicals 277814N
Coomassie Brilliant Blue G-250	Bio-Rad 161-0406
Coomassie Brilliant Blue R-350 (PhastGel)	Amersham Pharmacia 17-051801
Copper (II) sulphate pentahydrate	Sigma Chemicals C-2284
Diethyldithiocarbamate	Sigma Chemicals D-9428
Diethylenetriamine pentaacetic acid (DETAPAC)	Sigma Chemicals D-1133
Diethyl ether	UNILAB Analytical Reagents 465
3-dimethylaminopropionitrile	Sigma Chemicals D9413
β,γ-dipalmitoyl-L-α- phosphatidylcholine (DPPC)	Auspep 850355
ECL	Amersham Biosciences RPN2106
Ethanol	BDH Chemicals 10107.2500P
Ethylenediaminetetraacetic acid, disodium salt (EDTA-Na_2)	BDH Chemicals 10093.5V
Glycerokinase	Roche Diagnostics 127-159
L-glycerin-3-phosphate oxidase	Roche Diagnostics 775-797
Glycerol	BDH Chemicals 10118.6M
Glycine	BDH Chemicals 10119.CU
Guanidine (aminomethanamidine) hydrochloride	Sigma Chemicals G-4505

Halothane	Astrazenica
Hank's balanced salts, modified	Sigma Chemicals H-4891
Heparin, sodium salt	Sigma Chemicals H-3393
HEPES	Gibco BRL 11344-041
High molecular weight standard electrophoresis calibration kit	Amersham Pharmacia 17-0445-01
20% Homogeneous polyacrylamide gel	Amersham Pharmacia 17-0624-01
Hydrocholoric acid	BDH Chemicals 103078R
Ketamine	Parnell
β-Linoleoyl-γ-palmitoyl-L-α-phosphatidylcholine (PLPC)	Avanti Polar Lipids 850458
Lipase	Sigma Chemicals L-9518
Low molecular weight standard electrophoresis calibration kit	Amersham Pharmacia 17-0446-01
Lucigenin	Sigma Chemicals M-8010
Magnesium sulphate	BDH Chemicals 10151
Methanol	BDH Chemicals 10158.BG
Mouse IgG1, negative control	DakoCytomation X0931
N,N'-Methylene-bis-acrylamide	Bio-Rad 161-0201
NEFA kit	Wako Pure Chemicals 279-75401
β-Nicotinamide adenine dinucleotide, reduced form	Sigma Chemicals N-6005

β-Oleoyl-γ-palmitoyl-L-α- phosphatidylcholine (POPC)	Avanti Polar Lipids 850457
Palmitic acid	Sigma Chemicals P-9767
Peroxidase	Roche Diagnostics 413-470
Phenol	Sigma Chemicals P-5566
Phosphate buffered saline	Sigma Chemicals D-5652
Phospholipase D	Sigma Chemicals P-8023
Polyclonal goat anti-human apoA-I antiserum	Calbiochem 178463
Polyethylene glycol 8000	Sigma Chemicals P-2139
Polyoxyethylene-9-lauryl ether	Sigma Chemicals P-9641
Potassium bromide	BDH Chemicals 101954F
Potassium chloride	BDH Chemicals 10198
Potassium ferrocyanide	Sigma Chemicals P-9387
Purified anti-human CD54	BD Pharmingen 555510
Purified anti-human CD106	BD Pharmingen 555645
SDS buffer strips	Amersham Pharmacia 17-0516-01
Silver nitrate	Ajax Chemicals 449
Sodium acetate	Sigma Chemicals S-8625
Sodium azide	Sigma Chemicals S-2002
Sodium bromide	BDH Chemicals 301164S
Sodium carbonate	BDH Chemicals 10240.4H
Sodium chloride	BDH Chemicals 10241.3000
Sodium dodecyl sulphate (SDS)	BDH Chemicals 442442F

Sodium HEPES	Amresco 0485-500G
Sodium hydroxide	BDH Chemicals 10242.4X
Sodium pentobarbitone	Virbac
Streptomycin/Penicillin	Sigma Chemicals P-4333
Sucrose	BDH Chemicals 10274.4B
Thimerosal	Sigma Chemicals T-5125
Tissue-Tek Optimal Cutting Temperature (OCT) fixative	Sakura 4385
Total cholesterol reagent kit	Roche Diagnostics 2016630
Triolein	Sigma Chemicals T-7140
Tris (hydroxymethyl) aminomethane	Sigma Chemicals T-1378
Triton X-100	Merck Chemicals 30632
Tween 20	Bio-Rad 170-653
Urea	BDH Chemicals 10290.BG
Xylazine	Troy Laboratories

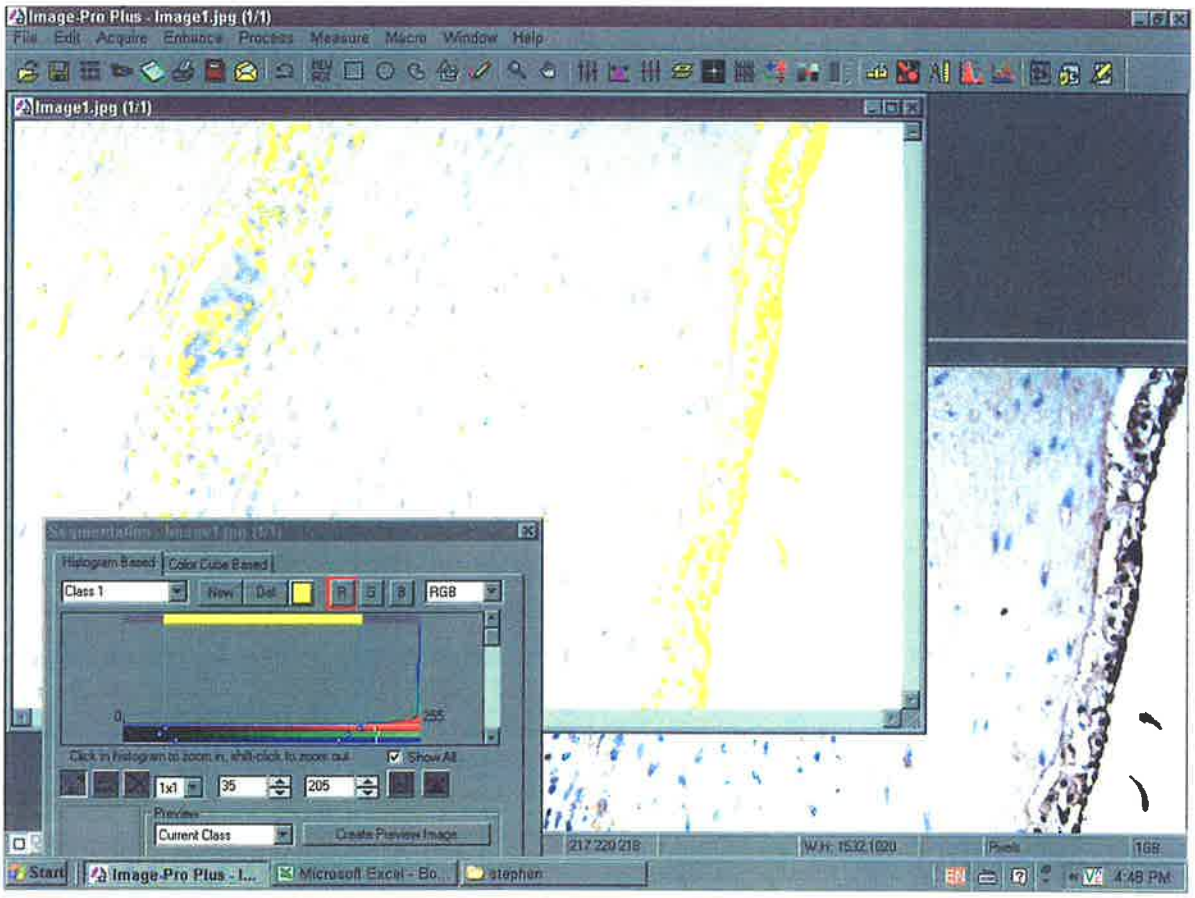

Figure 2.1. Representative image of computer analysis of immunohistochemical staining of plaque composition. The digital micrograph is on the right and the degree of staining, determined by the computer to be above the threshold, is on the left.

CHAPTER THREE

ROLE OF HDL IN A MODEL OF ACUTE VASCULAR INFLAMMATION

3.1 INTRODUCTION

The anti-atherogenic properties of HDL are well established (54). Despite this, the mechanism of the protective effect is still not clear. The best documented of the potentially anti-atherogenic functions of HDL relates to their role in promoting reverse cholesterol transport (448). However, HDL have additional non-lipid transporting properties that may contribute to the inhibition of atherogenesis.

In studies conducted *in vitro*, HDL inhibit the adhesion of monocytes to endothelial cells following stimulation with oxidised LDL (210). Native HDL (187) and rHDL (188,190) inhibit the expression of the adhesion molecules, VCAM-1 and ICAM-1, in activated HUVECs. These anti-inflammatory properties of HDL have also been demonstrated *in vivo*. For example, infusion of rHDL reduces endothelial adhesion molecule expression in cholesterol-fed apoE knockout mice following insertion of carotid periarterial cuffs (83). In addition, infusion of rHDL inhibits the development of a local inflammatory infiltrate following the subcutaneous administration of interleukin in a porcine model (208). Furthermore, the transgenic expression of human apoA-I in mouse models of established atherosclerosis results in a reduction in the macrophage composition of plaque (89).

HDL also inhibit the generation of ROS and oxidative damage. *In vitro* studies have demonstrated that HDL inhibit both the oxidation of LDL (310) and the endothelial dysfunction that results from coincubation of arterial strips with oxidised LDL (449). These properties have been attributed to the enzymes PON (450) and PAF-AH (451) which circulate with HDL. PON1 knockout mice demonstrate increased oxidative stress in macrophages (452). In addition, apoA-I

analogues reduce the *in vitro* generation of superoxide by endothelial cells in the presence of LDL (313). Antioxidant properties of HDL have also been demonstrated *in vivo*, with the transgenic expression of human apoA-I reducing oxidative stress in apoE knockout mice (453). The *in vivo* antioxidant role of HDL in normocholesterolemic animal models of inflammation has not been addressed.

In the studies reported in this chapter we have infused rHDL in to chow-fed rabbits that have undergone application of carotid periarterial collars. The periarterial collar model induces acute inflammatory and pro-oxidant changes (454,455). The aims of these studies were to determine if rHDL possessed anti-inflammatory and antioxidant properties in this normocholesterolaemic model and whether this effect was influenced by the phospholipid composition of rHDL.

3.2 METHODS

3.2.1 Animals

Male New Zealand White rabbits (Nanowie Small Animal Production Unit, Modewarre, Australia) weighing approximately 3kg were maintained on a normal laboratory chow diet throughout the study. All procedures were approved by the Howard Florey Institute Animal Ethics Committee (protocol 02-009).

3.2.2 Preparation of lipid free apoA-I

HDL was isolated from pooled samples of rabbit plasma (Quality Farms of Australia, Lara, Australia) by sequential ultracentrifugation in the 1.06-1.21 g/mL density range. The HDL were

lyophilised and delipidated (435). ApoA-I was isolated from the apoHDL by chromatography on a Q-sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden) attached to an FPLC system (Amersham Biosciences) (436). The purified apoA-I was lyophilised and stored at –20°C until used. Lyophilised apoA-I was reconstituted in 3M guanidine hydrochloride and dialysed against endotoxin free phosphate-buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO) containing 0.2 g/L KH_2PO_4, 0.2 g/L KCl, 8 g/L NaCl and 1.15 g/L Na_2HPO_4 before being infused or used to prepare rHDL. The concentration of apoA-I was determined using an immunoturbidometric assay (437).

3.2.3 Preparation of reconstituted HDL

Discoidal rHDL containing apoA-I complexed to either 1-palmitoyl-2-linoleoyl phosphatidylcholine (PC) (PLPC, Sigma) or 1,2-dipalmitoyl PC (DPPC, Sigma) in a molar ratio of PC to apoA-I of 200:1, were prepared using the cholate dialysis method (401). The resulting rHDL were dialysed extensively against endotoxin free PBS before use. Protein (442) and phospholipid (441) concentrations were determined by enzymatic assay. The rHDL were subjected to agarose gel electrophoresis and 3-40% non-denaturing gradient gel electrophoresis (438) to determine their surface charge and Stokes' diameter.

3.2.4 Administration of reconstituted HDL

19 rabbits were randomly allocated to receive treatment with saline (n=6), PLPC-rHDL (n=4), DPPC-rHDL (n=4) or lipid free apoA-I (LFAI, n=5). Each treatment of rHDL and LFAI contained 25 mg of apoA-I (8 mg/kg). The treatment was administered via a marginal ear vein on

each of three occasions: 24 hours prior to collar implantation, directly before the surgical procedure and 24 hours after the collar implantation. The animals were sacrificed 48 hours after the collar implantation.

3.2.5 Collar implantation

The rabbits were anaesthetised using intravenous propofol (5 mg/kg) followed by intramuscular ketamine/xylazine (50/10 mg/kg). The carotid arteries were exposed surgically and cleared of connective tissue along a 30mm length. Hollow, non-occlusive silastic collars (length 20mm; internal diameter along bore 4mm; internal diameter at ends 1mm) were then placed around each artery and held in place with a nylon sleeve (454). The space inside the collar was filled with sterile saline (0.9%). Muscle, fat and skin layers were sutured, the wound dressed with antibiotic, and animals allowed to recover for 48 hours before being sacrificed.

3.2.6 Tissue harvesting

Forty-eight hours after insertion of the collar, blood was sampled from a marginal ear vein. Animals were then heparinised (1000U, i.v.) prior to euthanasia with an overdose of sodium pentobarbitone (90 mg/kg, i.v.). The collared segment and approximately 10mm of noncollared artery proximal to the collar were excised and placed in ice-cold Krebs-HEPES buffer (composition in mmol/L: NaCl 99.0, KCl 4.7, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, Na-HEPES 20.0 and glucose 11.0, pH 7.4). Collars were removed and arteries cleaned of fat and connective tissue. Three ring sections (~3mm) were cut from both the area enclosed by the collar and from the proximal noncollared segment of artery. One section from each segment

was snap frozen in Tissue-Tek OCT (Sakura, Tokyo, Japan) fixative for immunohistochemical analysis. The other sections were used to determine generation of ROS.

3.2.7 Detection of ROS by lucigenin-enhanced chemiluminescence

Levels of ROS in the carotid arteries were measured by 5μmol/L lucigenin-enhanced chemiluminescence (456). Ring segments were incubated for 45 min at 37°C in Krebs-HEPES buffer containing diethyldithiocarbamate (DETCA, 3mmol/L) to irreversibly inactivate endogenous Cu^{2+}/ Zn^{2+} superoxide dismutase. Some rings were further treated with NADPH (10μmol/L), the preferred substrate of NADPH oxidase which is the predominant source of superoxide in rabbit carotid arteries (454). Each segment was then transferred to a separate well of a white, opaque 96-well plate containing 300μL of 5μmol/L lucigenin in Kreb-HEPES buffer as well as the appropriate drug treatment. DETCA was excluded from the lucigenin assay solution. The 96-well plate was loaded into a TopCount Single Photon Counter (Packard Bioscience, Australia) and photon emission per second was measured (6 second count time per cycle, 12 cycles, one minute delay between cycles). Ring segments were dried for 2-3 days in a 65°C oven and presence of ROS was normalised to dry tissue weight (counts/s/mg).

3.2.8 Immunohistochemistry

Frozen tissues were sectioned in 5 μm slices. Sections were fixed with methanol/acetone (1:1) at room temperature for 5 minutes. Arterial wall infiltration by inflammatory cells was determined using mouse monoclonal antibodies against macrophages (RAM11, DakoCytomation, Glostrup, Denmark), neutrophils (CD18, Serotag, Oxford, UK) and lymphocytes (CD43, Serotag). In

addition, the endothelial expression of pro-inflammatory and prothrombotic factors was determined using mouse monoclonal antibodies against rabbit VCAM-1 and ICAM-1 (gifts from Dr. M. Cybulsky), and MCP-1 (a gift from Dr. A. Matsukowa), a goat polyclonal antibody against human E-selectin (R&D, Minneapolis, MN), mouse monoclonal antibodies against human PAI-1 (American Diagnostica) and CD40 (Serotec) and a goat polyclonal antibody against rabbit thrombomodulin (American Diagnostica) were applied and incubated overnight at room temperature. Endothelial integrity was determined using a mouse monoclonal antibody against human CD31 (DakoCytomation). Sections were incubated with biotinylated anti-mouse or anti-goat immunoglobulins for 30 minutes and then incubated with alkaline-phosphatase labelled streptavidin solution for 60 minutes. Slides were rinsed in PBS (pH 7.4) after each incubation. Peroxidase activity was revealed by diaminobenzamine. Slides were counterstained with haematoxylin and mounted. These slides were subsequently graded (457,458) independently by four pathologists who were blinded to the treatment status of the animal. The degree of endothelial staining was graded using a scale that incorporated both the strength of staining and the amount of endothelial surface involved (0=no staining, 1=weak staining of less than 50% of endothelium, 2=strong staining of less than 50% or weak staining of greater than 50% of endothelium, 3=strong staining between 50 and 99% of endothelium and 4=strong staining of 100% of endothelium). The collar-induced change in expression was calculated as the difference in mean score between noncollared and collared segments of each individual artery. Infiltration of the arterial wall by neutrophils, demonstrated by CD18 staining, was determined by quantitative immunohistochemistry. Digital micrographs were acquired with an Olympus BX40 microscope and the percentage of total vessel wall area occupied by positive staining was determined using ImagePro Plus (Cybernetics).

3.2.9 Plasma analyses

Plasma collected at the commencement of the study and prior to sacrifice of the animal was stored at -80°C in EDTA until required for analysis. All chemical analyses were carried out on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Triglyceride (443) and free cholesterol (444) concentrations were determined enzymatically. Total cholesterol was determined using a Roche Diagnostics kit. HDL cholesterol was determined by enzymatic assay following precipitation of apolipoprotein B containing lipoproteins with polyethylene glycol (459). ApoA-I concentrations were determined by an immunoturbidometric assay using a sheep anti-rabbit apoA-I immunoglobulin (437). Soluble VCAM-1 and ICAM-1 concentrations were determined using commercially available ELISA kits (R&D).

3.2.10Data analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made by Student's t-tests and one way ANOVA using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered significant.

3.3 RESULTS

3.3.1 Generation of reactive oxygen species

Application of the periarterial collar increased the presence of ROS in the vessel wall both in the unstimulated state without NADPH (126.9±48.1 and 6.6±2.9 counts/s/mg in the collared and non-collared segments respectively, $p<0.005$) and after stimulating by incubation with NADPH (540.3±169.1 and 130.3±42.1 counts/s/mg in the collared and non-collared segments respectively, $p<0.01$). (Fig 3.1) The collar-induced increase in vascular ROS that was observed in both the unstimulated (Fig 3.1A) and the stimulated (Fig 3.1B) states was significantly reduced in the vessels isolated from animals infused with rHDL. Infusion of PLPC-rHDL inhibited the collar-induced increase in vascular ROS in the unstimulated state by 92% (10±4.1 and 126.9±48.1 counts/s/mg in the PLPC-rHDL and saline infused animals respectively, $p<0.03$) and in the stimulated state by 85% (82.3±34.4 and 540.3±169.1 counts/s/mg in the PLPC-rHDL and the saline-infused animals respectively, $p<.001$). The collar-induced increase in ROS in the stimulated state was also inhibited 90% by infusion of DPPC-rHDL (56.7±29.8 and 540.3±169.1 counts/s/mg in the DPPC-rHDL and saline-treated animals respectively, $p<0.001$) (Fig 3.1B).

3.3.2 Effect of rHDL on neutrophil infiltration

It was subsequently determined whether the in vivo anti-oxidant properties of infusing rHDL was associated with a reduction in neutrophil infiltration of the arterial wall in response to application of the periarterial collar. Representative staining for CD18 of collared and non-collared arterial segments is shown in figure 3.2. Application of the periarterial collar promoted a dense infiltration of neutrophils in the vessel wall (30±9% of total vessel wall area in the collared versus 0.7±.2% in the non-collared segments, $p<0.005$). The collar-induced neutrophil

recruitment was inhibited by 73% by infusion of PLPC-rHDL (from 30±9% of the total vessel wall area in the saline infused rabbits to 8.2±3.7% in the animals infused with rHDL, $p< 0.03$). Collar induced neutrophil recruitment into the artery wall was inhibited 94% by infusion of DPPC-rHDL (from 30±9% of the total vessel wall area in the saline infused rabbits to 1.8±1.4% in the animals infused with rHDL, $p< 0.02$). The difference in degree of neutrophil infiltration in collared segments of animals infused with PLPC-rHDL and DPPC-rHDL was not statistically significant. (Fig 3.3)

3.3.3 Effect of lipid free apoA-I on neutrophil infiltration

Given the profound ability of rHDL to inhibit the infiltration of neutrophils into the vessel wall following application of a periarterial collar, it was subsequently determined what was the effect of infusing lipid-free apoA-I. (Fig 3.2) The collar-induced neutrophil recruitment was inhibited by 73% by infusion of lipid-free apoA-I (30±9% of the total vessel wall area in the saline infused rabbits to 8±2.9% in the animals infused with lipid-free apoA-I, $p<0.02$). This property did not differ in comparison with animals infused with rHDL. (Fig 3.3)

3.3.4 Endothelial adhesion molecule and chemokine expression

It was subsequently determined whether the ability of rHDL and lipid-free apoA-I to inhibit neutrophil recruitment into the arterial wall was associated with an influence on the endothelial expression of pro-inflammatory adhesion molecules and chemokines following application of a periarterial collar. Representative sections of collared and noncollared segments, stained for endothelial adhesion molecule and chemokine expression, are shown in figures 3.4-3.7. Staining

with an antibody directed against CD31 demonstrated that the endothelium was intact (not shown). The collar induced change in expression of endothelial VCAM-1, ICAM-1, E-selectin and MCP-1 are shown in figure 3.8. Forty-eight hours after implantation of a periarterial collar there was an increase in the expression of endothelial VCAM-1, (3.43 vs. 0.91 in the collared and non-collared segments, respectively, $p<0.05$), ICAM-1 (3.34 vs. 2.27, $p<0.05$) and MCP-1 (1.63 vs. 1.06, $p<0.05$). Constitutive endothelial expression of E-selectin in non-collared segments did not increase following application of the collar at 48 hours (2 vs. 1.83, NS). Compared with saline, infusion of rHDL inhibited the collar-induced increase in endothelial expression of VCAM-1 (by 53.9% and 54.4% with PLPC-rHDL and DPPC-rHDL respectively, $p<0.005$), ICAM-1 (by 50% and 74.6% with PLPC-rHDL, $p<0.01$ and DPPC-rHDL, $p<0.005$ respectively) and MCP-1 (by 76.5% with PLPC-rHDL, $p<0.01$). Although DPPC-rHDL inhibited the collar-induced change in MCP-1 by 50% compared with saline, this did not reach statistical significance ($p=0.09$). The collar-induced change in MCP-1 did not significantly differ between PLPC-rHDL and DPPC-rHDL groups. When the PLPC and DPPC groups were combined, the infusion of rHDL significantly inhibited the collar-induced change in MCP-1 staining (by 63%, $p<.0.05$).

In addition, similar benefits were seen in animals that were infused with lipid-free apoA-I. (Fig 3.4-3.7) Compared with saline, infusion of lipid-free apoA-I inhibited the collar-induced increase expression of VCAM-1 by 40.5% ($p<0.01$), ICAM-1 by 94.1% ($p<0.0005$) and MCP-1 by 76.5% ($p<0.05$). (Fig 3.8) At 48 hours, the collar-induced increase in adhesion molecule and chemokine expression was not associated with an infiltration of macrophages or lymphocytes in the arterial wall, as determined by staining for RAM11 and CD43 respectively. Furthermore, medial staining

for MCP-1 and E-selectin was detected. This did not differ between non-collared and collared segments.

3.3.5 Endothelial expression of thrombogenic factors

The effect of infusing lipid-free apoA-I and rHDL on the endothelial expression of thrombogenic factors induced by application of a periarterial collar was determined. The collar induced change in expression of endothelial PAI-1, CD40 and thrombomodulin are shown in figure 3.9. Forty-eight hours after implantation of a periarterial collar there was an increase in the expression of endothelial PAI-1, (2 vs. 1.2 in the collared and non-collared segments, respectively, $p<0.05$), CD40 (1.73 vs. 1.18, $p<0.05$) and a reduction in thrombomodulin (3.23 vs. 3.82, $p<0.05$). Compared with saline, infusion of rHDL inhibited the collar-induced increase in endothelial expression of PAI-1 (by 100% with PLPC-rHDL, $p<0.05$) and CD40 (by 87% with PLPC-rHDL, $p<0.05$). In contrast, infusion of lipid-free apoA-I had no impact on the collar-induced increase in PAI-1 and CD40. The collar-induced decrease in thrombomodulin expression was not altered inhibited by infusing rHDL or lipid-free apoA-I.

3.3.6 Plasma analyses

Plasma concentrations of triglyceride, total and free cholesterol, HDL cholesterol and apoA-I at the commencement of the study and at the time of sacrifice of the animals are presented in table 3.1. No significant differences were found between groups. Application of the periarterial collar had no apparent effect on the plasma concentrations of soluble VCAM-1 in saline infused animals (360.5±47.1 vs. 305.3±16 ng/ml at the commencement of the study and prior to sacrifice

respectively) or ICAM-1 (84.6±3.7 vs. 87.3±7.3 ng/ml at the commencement of the study and prior to sacrifice respectively). Infusions of rHDL and lipid-free apoA-I did not significantly modify the plasma concentration of VCAM-1 (287±9.9, 349.8±9.6, 284.3±6.4 and 305.3±16 ng/ml in the PLPC-rHDL, DPPC-rHDL, lipid-free apoA-I and saline infused animals respectively) or ICAM-1 (83.5±2.8, 81.8±1.8, 80.3±0.3 and 87.3±7.3 ng/ml in the PLPC-rHDL, DPPC-rHDL, lipid-free apoA-I and saline infused animals respectively). (Table 3.1)

3.4 DISCUSSION

The studies outlined in this chapter demonstrate that HDL possess profound *in vivo* anti-inflammatory and anti-oxidant properties in an acute, non-atherosclerotic inflammatory model in normolipidemic rabbits. Our studies further establish that the ability of HDL to inhibit the expression of pro-inflammatory adhesion molecules and chemokines that have previously been observed *in vitro* and hypercholesterolaemic animal models, is apparently independent of cholesterol efflux. In addition, HDL have a favourable influence on the development of a prothrombotic endothelium induced by a periarterial collar.

The two most dramatic effects of infusing rHDL in these studies were the marked reduction in neutrophil recruitment and a virtual abolition of the collar-induced generation of ROS. It has been reported elsewhere that insertion of a carotid periarterial collar results in a rapid influx of neutrophils into the arterial wall (455). The finding in the present study that infusion of rHDL almost completely inhibited this accumulation further highlights the anti-inflammatory potential of HDL. The mechanism by which HDL inhibit neutrophil recruitment is uncertain but may relate to the observed HDL-mediated inhibition of ICAM-1, a factor known to promote

neutrophil infiltration into the artery wall (460). Neutrophils are an important component of many acute inflammatory conditions, including myocardial ischaemia-reperfusion injury (461) and stroke (462). The results of the present study suggest that a protective role of HDL may therefore extend beyond the ability of these lipoproteins to promote cholesterol efflux.

Anti-inflammatory properties of HDL have been demonstrated *in vitro* (187,188,190) and *in vivo* (83,89,208) in many, but not in all (191,209), previous studies. In contrast to previous *in vitro* studies, the present *in vivo* study did not reveal any difference between the effects of rHDL containing PLPC and DPPC (194). The anti-inflammatory effects of HDL *in vitro* have been observed in cells that were not loaded with cholesterol and may therefore have been unrelated to the cholesterol efflux. *In vivo*, however, most studies have been conducted in models of hypercholesterolemia and atherosclerosis. As an example, infusion of rHDL into hypercholesterolemic, apoE knockout mice has been shown to inhibit the endothelial expression of VCAM-1, the macrophage accumulation and the neointimal formation induced by a carotid periarterial collar (83). There has been one report of an effect of rHDL *in vivo* in the absence of atherosclerosis in a porcine model of acute inflammation induced by the intradermal injection of interleukin-1 (208). In this model, a single infusion of rHDL inhibited the expression of endothelial E-selectin in the intradermal vessels, although effects on neutrophil recruitment and generation of ROS have not been reported.

The anti-inflammatory effects of rHDL in the present study were demonstrated with relatively low doses of rHDL. In contrast to previous studies in which 40-50 mg/kg of apoA-I was infused, the rabbits in the present study received rHDL containing only 8 mg/kg of apoA-I per infusion.

The fact that the concentration of apoA-I and HDL cholesterol were not increased at the time of sacrifice (24 hours after the last dose of rHDL) presumably reflects the relatively small amount that was administered. Despite this, the anti-inflammatory effects that were observed in these rabbits were profound.

Application of periarterial collars has been reported previously to increase vascular ROS in association with abnormal vascular reactivity and neointima formation (454,463). The increased chemiluminescent signal, probably reflecting superoxide, is mediated by increased activity of NADPH oxidase (454). To minimise the possibility that the lucigenin used in the chemiluminescent assay may have contributed to the production of superoxide, we used a concentration of lucigenin, that was not sufficient to participate in superoxide production by redox cycling (464). We also considered the possibility that HDL may have interfered with the ability of DETCA to inactivate superoxide dismutase, but dismissed this on the grounds that HDL was not present in the wells during the incubation.

The inhibition of arterial ROS by rHDL may have been the consequence of direct antioxidant properties of these lipoproteins. It may also have been secondary to the inhibition of neutrophil recruitment into the arterial wall, since neutrophils are known to generate superoxide. Regardless of the mechanism, a comparable inhibition of arterial ROS in vivo may explain the favourable effects of HDL infusion on endothelial function in humans (78,79). The production of ROS by the artery wall is increased in conditions associated with endothelial dysfunction (465). It is also known that the generation of ROS such as superoxide and hydrogen peroxide can be detrimental to the vessel wall, either directly through the promotion of the inflammatory cascade or

secondary to their ability to reduce the bioactivity of nitric oxide (247). Nitric oxide has well documented anti-inflammatory and antithrombotic properties that are potentially atheroprotective (466).

An additional benefit of infusing rHDL was its promotion of an anti-thrombotic endothelium. Application of the periarterial collar induces rapid thrombogenic changes at the level of the endothelium. These include an increase in expression of the antifibrinolytic PAI-1 and CD40, a pivotal mediator of the interaction between inflammation and platelet activation. In addition, constitutive expression of the anticoagulant thrombomodulin is reduced. Infusion of rHDL inhibited the increase in PAI-1 and CD40 and reduced the decrease in thrombomodulin, although this failed to meet statistical significance. Whilst *in vitro* data suggests that HDL possess antithrombotic properties (352-354,365,366,368), little is known about their *in vivo* properties. These findings provide further support to the favourable influence exerted by HDL on the arterial wall. The mechanism that confers these benefits is uncertain. The profound anti-inflammatory and antioxidant properties of HDL result in a reduction in prothrombotic stimuli. In addition, promotion of nitric oxide has substantial antithrombotic effects.

It was also demonstrated that infusing lipid-free apoA-I had a beneficial impact on the arterial wall. Lipid-free apoA-I possessed similar anti-inflammatory properties to that seen when rHDL was administered. This provides further evidence that the administration of apoA-I, by either transgenic expression (87) or direct infusion (84), has a protective influence on the arterial wall. It is unclear if apoA-I *per se* has a direct impact on the arterial wall, or whether the benefit results from rapid lipidation of the circulating apoA-I to form new HDL particles. However, the anti-

thrombotic properties of rHDL were not seen when lipid-free apoA-I was administered. The reason for this discrepancy is uncertain. It does suggest that the protein and phospholipid components may differ with regard to the influence they exert on different functions of HDL.

In conclusion, these studies provide compelling evidence that the anti-inflammatory and anti-oxidant properties of HDL that have been well documented *in vitro*, also operate *in vivo via* mechanisms additional to their role in promoting reverse cholesterol transport. The findings have potential implications in the management of acute inflammatory states as prevail in acute coronary syndromes.

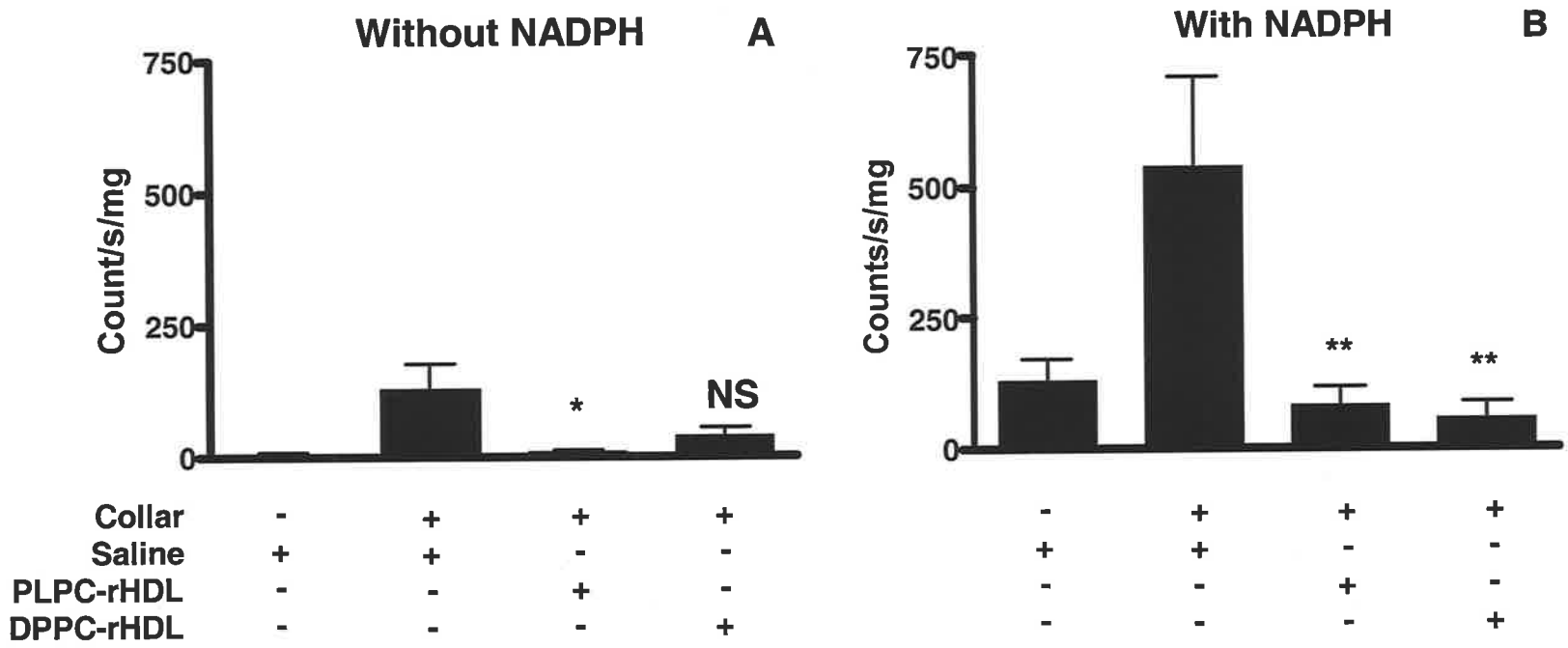

Figure 3.1. Levels of ROS, as determined by lucigenin-enhanced chemiluminescence, performed on collared and non-collared vascular segments from animals infused with saline, PLPC-rHDL or DPPC-rHDL were incubated in the absence (Fig 3.1A) or presence (Fig 3.1B) of NADPH (10 μmol/L). The detected chemiluminescent signal was expressed per dry weight of the segment. Results are expressed as mean ± S.E.M (*P<0.03, **P<0.001 for comparison with saline infused animals).

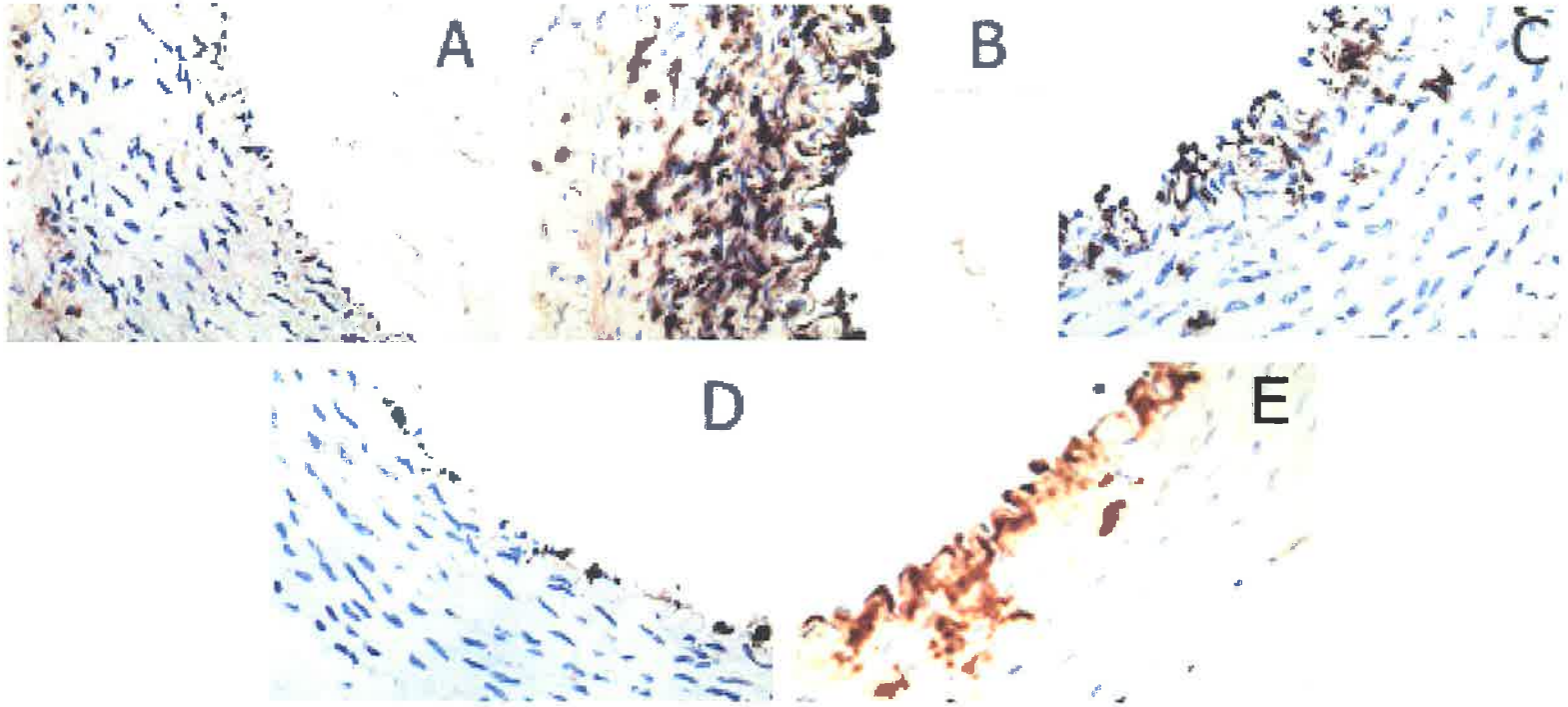

Figure 3.2. Representative immunohistochemical staining (x40 magnification) for the presence of neutrophils (CD18) in non-collared segments from saline infused animals (panel A), collared segments from saline infused animals (panel B), collared segments from PLPC-rHDL infused animals (panel C), collared segments from DPPC-rHDL infused animals (panel D) and collared segments from lipid-free apoA-I infused animals (panel E).

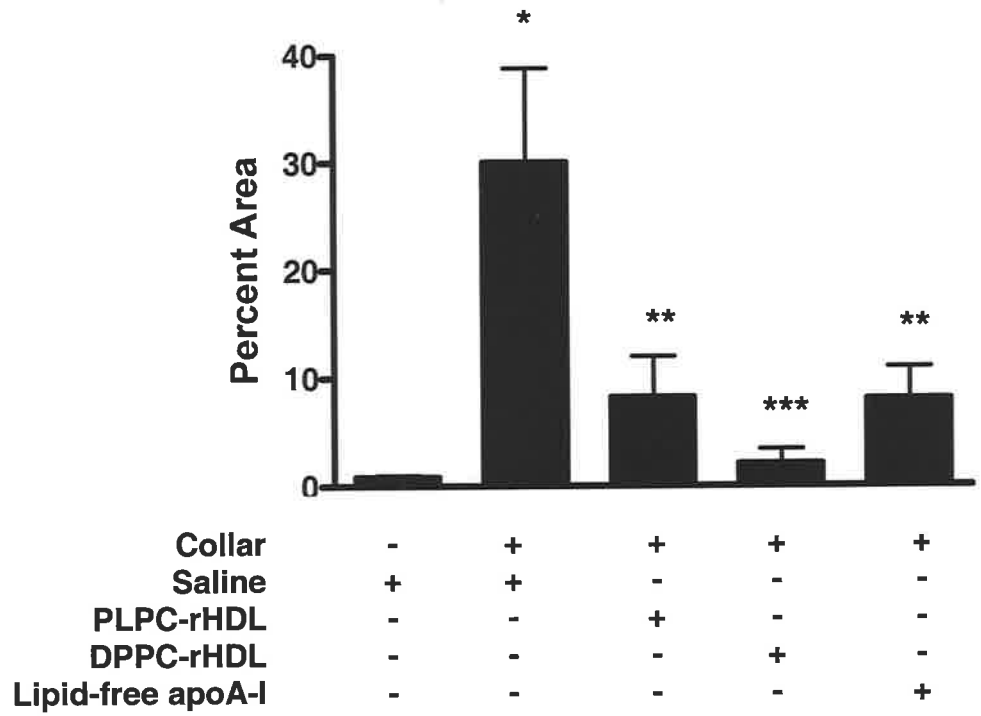

Figure 3.3. Percentage of total arterial wall area infiltrated by neutrophils, as determined by CD18 immunohistochemical staining. Results are expressed as mean ± S.E.M. (*P<0.005 for comparison with non-collared segments; **P<0.03 and ***P<0.02 for comparisons with saline infused animals).

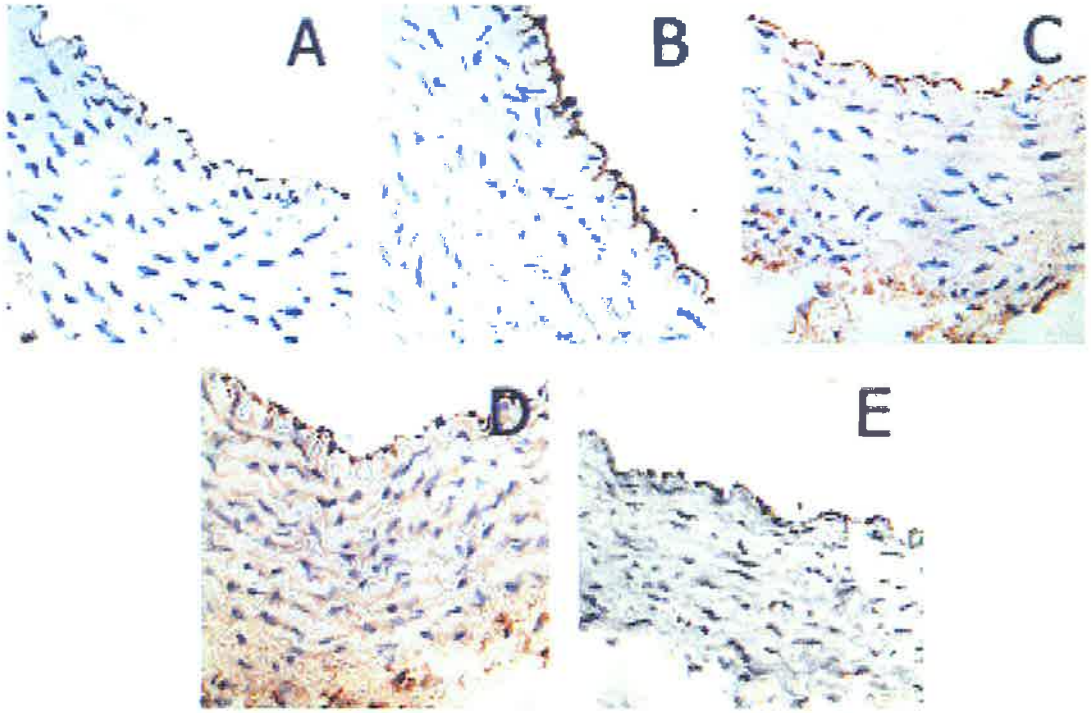

Figure 3.4. Representative immunohistochemical staining (x40 magnification) for the presence of endothelial VCAM-1 (panels A-E) demonstrated in non-collared segments from saline infused animals (panel A), collared segments from saline infused animals (panel B), collared segments from PLPC-rHDL infused animals (panel C), collared segments segments from DPPC-rHDL infused animals (panel D) and collared segments from lipid-free apoA-I infused animals (panel E).

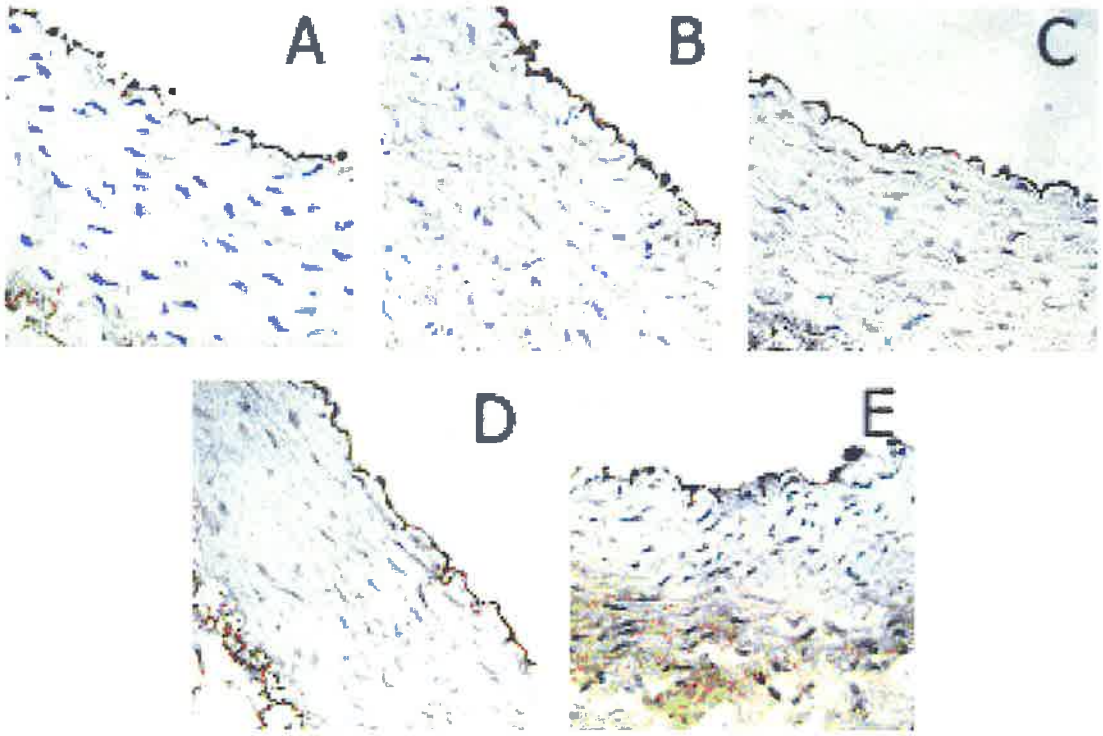

Figure 3.5. Representative immunohistochemical staining (x40 magnification) for the presence of endothelial ICAM-1 (panels A-E) demonstrated in non-collared segments from saline infused animals (panel A), collared segments from saline infused animals (panel B), collared segments from PLPC-rHDL infused animals (panel C), collared segments segments from DPPC-rHDL infused animals (panel D) and collared segments from lipid-free apoA-I infused animals (panel E).

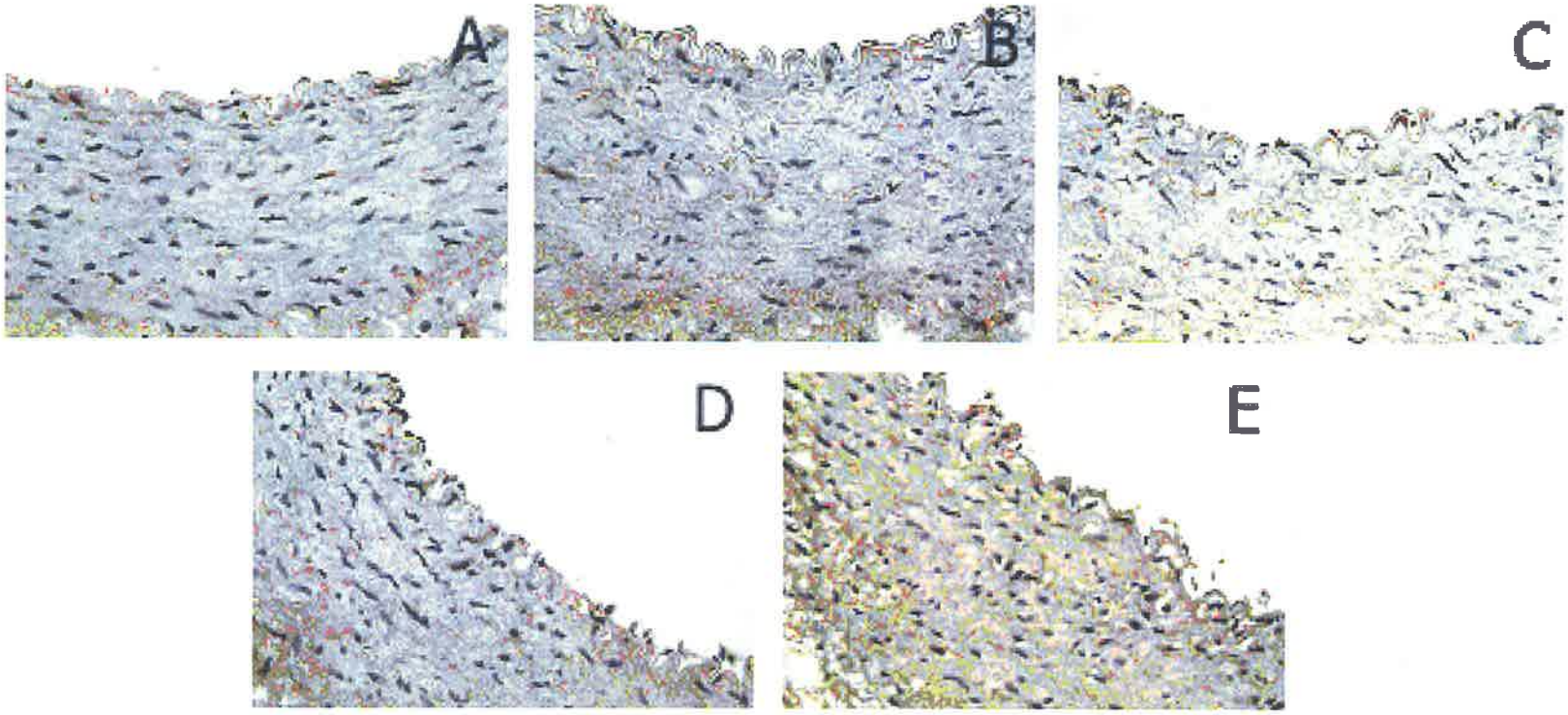

Figure 3.6. Representative immunohistochemical staining (x40 magnification) for the presence of endothelial MCP-1 (panels A-E) demonstrated in non-collared segments from saline infused animals (panel A), collared segments from saline infused animals (panel B), collared segments from PLPC-rHDL infused animals (panel C), collared segments segments from DPPC-rHDL infused animals (panel D) and collared segments from lipid-free apoA-I infused animals (panel E).

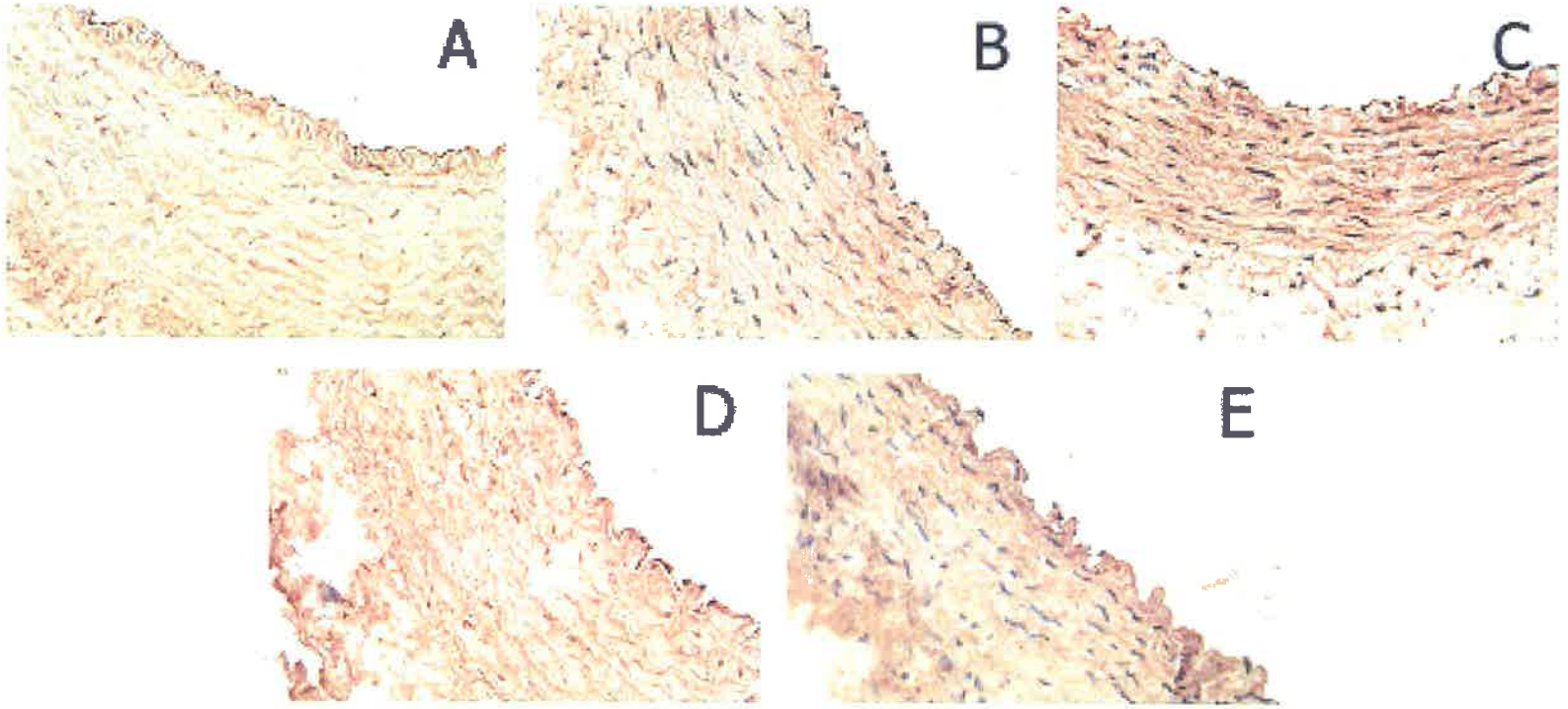

Figure 3.7. Representative immunohistochemical staining (x40 magnification) for the presence of endothelial E-selectin (panels A-E) demonstrated in non-collared segments from saline infused animals (panel A), collared segments from saline infused animals (panel B), collared segments from PLPC-rHDL infused animals (panel C), collared segments segments from DPPC-rHDL infused animals (panel D) and collared segments from lipid-free apoA-I infused animals (panel E).

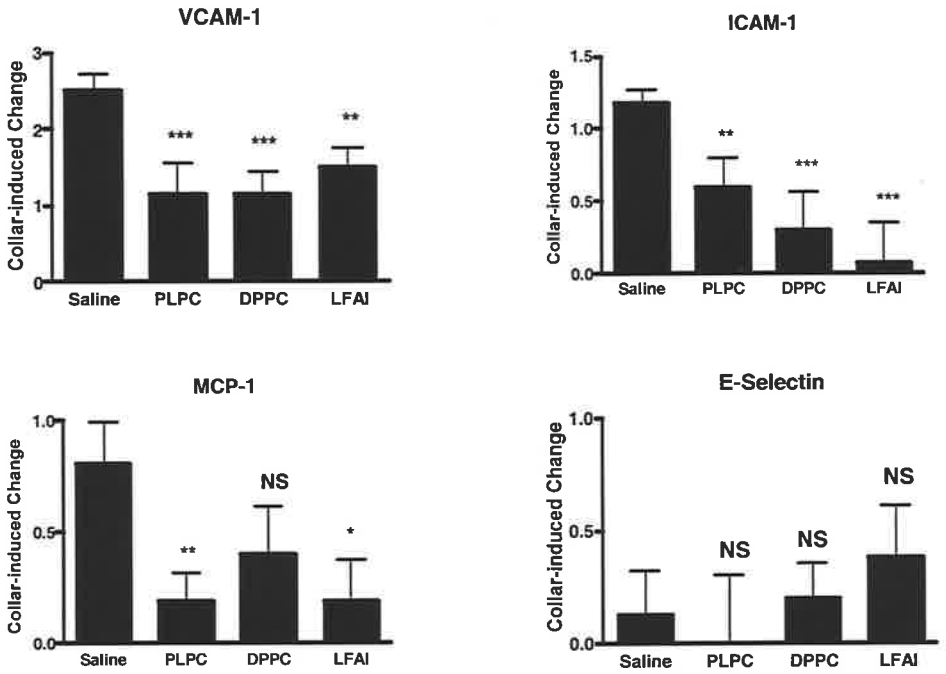

Figure 3.8. Collar-induced change in the endothelial expression of VCAM-1, ICAM-1, MCP-1 and E-Selectin as determined by the difference in score between the collared and non-collared segment of artery in animals infused with saline, PLPC-rHDL (PLPC), DPPC-rHDL (DPPC) or lipid-free apoA-I (LFAI). Results are expressed as mean ± S.E.M. (*P<0.05, **P<0.01 and ***P<0.005 and NS non significant for comparisons with saline infused animals).

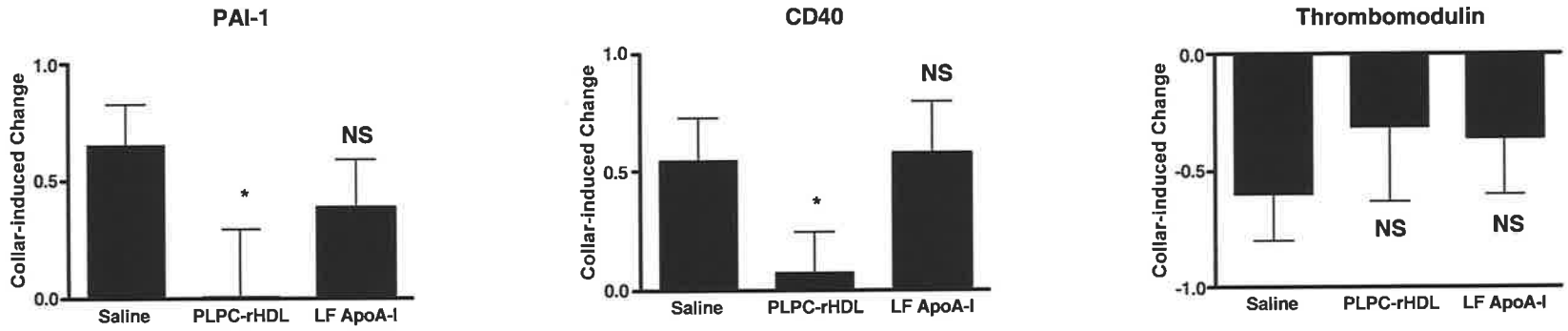

Figure 3.9. Collar-induced change in the endothelial expression of PAI-1, CD40 and Thrombomodulin as determined by the difference in score between the collared and non-collared segment of artery in animals infused with saline, PLPC-rHDL or lipid-free apoA-I. Results are expressed as mean ± S.E.M. (*P<0.05 and NS non significant for comparisons with saline infused animals).

	Baseline				Time of Sacrifice			
	Control	LFAI	PLPC-rHDL	DPPC-rHDL	Control	LFAI	PLPC-rHDL	DPPC-rHDL
Total Cholesterol (mmol/L)	1.18 ± 0.46	1.05±0.23	1.58 ± 0.24	1.61 ± 0.19	1.03 ± 0.29	0.85±0.23	1.41 ± 0.31	1.61 ± 0.21
Free Cholesterol (mmol/L)	0.31 ± 0.09	0.27±0.03	0.39 ± 0.06	0.37 ± 0.05	0.37 ± 0.1	0.33±0.04	0.45 ± 0.03	0.44 ± 0.07
Triglyceride (mmol/L)	0.58 ± 0.1	0.61±0.21	0.73 ± 0.31	0.62 ± 0.01	0.81 ± 0.16	0.52±0.09	0.71 ± 0.27	0.46 ± 0.11
HDL Cholesterol (mmol/L)	0.6 ± 0.2	0.31±0.05	0.38 ± 0.09	0.38 ± 0.09	0.38 ± 0.19	0.28±0.09	0.42 ± 0.15	0.64 ± 0.13
LDL Cholesterol (mmol/L)	0.41 ± 0.3	0.81±0.33	0.72 ± 0.36	0.95 ± 0.18	0.34 ± 0.24	0.71±0.39	0.67 ± 0.36	0.65 ± 0.23
ApoA-I (mg/ml)	0.69 ± 0.38	0.72±0.13	0.81 ± 0.29	0.71 ± 0.03	0.6 ± 0.27	0.61±0.08	0.57 ± 0.11	0.48 ± 0.03
Soluble VCAM-1 (ng/mL)	360.5 ± 47.1	315.4±17.3	416.4 ± 22.4	388.6 ± 9.1	305.3 ± 16	284.3±6.4	287 ± 9.9 *	349.8 ± 9.6
Soluble ICAM-1 (ng/mL)	84.6 ± 3.7	82.2±0.62	99.1 ± 9.7	84.5 ± 2.2	87.3 ± 7.3	80.3±0.3	83.5 ± 2.8	81.8 ± 1.8

Table 3.1. Plasma lipid profiles and soluble adhesion molecule concentrations from animals infused with saline, PLPC-rHDL and DPPC-rHDL at baseline and immediately prior to sacrifice. Results are expressed as mean ± S.E.M. (*P<0.001 for comparison with value at baseline).

CHAPTER FOUR

ROLE OF INFUSING CHYLOMICRON-LIKE EMULSIONS ON THE ANTI-INFLAMMATORY PROPERTIES OF HDL

4.1 INTRODUCTION

In the preceding chapter it was demonstrated that the infusion of discoidal rHDL containing apoA-I and phospholipid was effective in blunting the acute inflammatory response in rabbit carotid arteries to application of a periarterial collar. A potential physiological origin of such discoidal HDL is as a catabolic product of the hydrolysis of chylomicrons (467). To investigate this possibility, rabbits were infused intravenously with artificial chylomicron-like emulsions consisting of triolein and phosphatidylcholine.

It has been reported that these artificial chylomicron-like emulsions behave metabolically like native chylomicrons (468). They also donate phospholipid to HDL in a process that may generate discoidal HDL (469). We hypothesised that the generation of discoidal HDL from chylomicron-like emulsions *in vivo* may result in an enhanced anti-inflammatory potential of the HDL fraction, isolated after the infusion of such an emulsion.

4.2 METHODS

4.2.1 Animals

Male New Zealand White rabbits weighing approximately 3kg were maintained on a normal laboratory chow diet throughout the study. All procedures were approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science (17/01).

4.2.2 Preparation of chylomicron-like emulsions

Phospholipid specific chylomicron-like emulsions were prepared as described by Martins (469). Phospholipid (37.5 mg) was mixed with triolein (100 mg) and cholesterol (1.93 mg), in

a molar ratio of 5:25:0.5 and dissolved in chloroform:methanol (2:1 (v/v)). Butylated hydroxy toluene (BHT, final concentration 0.12 mmol/L) was added to the mixtures to inhibit phospholipid oxidation. The mixtures were evaporated to dryness under a stream of N_2 for 2 hours and maintained under a vacuum overnight. The lipids were resuspended in 12 mL of Tris-buffered saline (TBS, pH 7.4) containing 150 mM NaCl, 0.03% (w/v) EDTA-Na_2, 0.006% (w/v) NaN_3, 0.2% (w/v) Chelex (Bio-Rad Laboratories, CA, USA), 50 µmol/L diethylenetriamine-pentaacetic acid (DETAPAC) and 10 µmol/L BHT and sonicated for 30 minutes at 55-56°C. The density of the solutions was adjusted to 1.21 g/mL and they were placed in SW41 tubes. The emulsions were overlayered with density solutions of 1.065, 1.02 and 1.006 g/mL. The microemulsions were then isolated by ultracentrifugation as the fraction of d<1.21 g/mL. The individual phosphatidylcholine (PC) species used included 1-palmitoyl-2-linoleoyl PC (PLPC), 1-palmitoyl-2-oleoyl PC (POPC) and 1,2-dipalmitoyl PC (DPPC). Triglyceride, phospholipid and cholesterol composition was determined by enzymatic assay.

4.2.3 Experimental protocol

23 rabbits were fasted overnight and received a single bolus of (i) saline (n=5) or phospholipid specific chylomicron-like emulsions containing (ii) PLPC (PLPC-TG, n=6), (iii) POPC (POPC-TG, n=6) or (iv) DPPC (DPPC-TG, n=6) as the sole phospholipid. When chylomicron-like emulsions were infused, they contained 20 mg triolein. Animals were euthanased 20 minutes after the infusion, with an overdose of sodium pentobarbitone (90 mg/kg, i.v.). Blood was collected by cardiac puncture immediately prior to sacrifice of the animals. HDL was isolated from fresh plasma. The remaining plasma was stored in EDTA containers at -80°C until analysed for lipid profiles.

4.2.4 Isolation of rabbit HDL

HDL was isolated from samples of rabbit plasma by sequential ultracentrifugation in the 1.063-1.21 g/mL density range. Isolated HDL was dialysed extensively against endotoxin free phosphate buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO) containing 0.2 g/L KH_2PO_4, 0.2 g/L KCl, 8 g/L NaCl and 1.15 g/L Na_2PO_4 prior to incubation with HUVECs. Cholesterol (444), triglyceride (443), protein (442) and phospholipid (441) composition of HDL were determined by enzymatic assays. The apoA-I concentration of HDL was determined by an immunoturbidometric assay using a sheep anti-rabbit apoA-I immunoglobulin (437).

4.2.5 Characterisation of isolated HDL

The molar composition of triglyceride, phospholipid, cholesterol and apoA-I were determined. Two-dimensional gel electrophoresis (agarose gel electrophoresis followed by non-denaturating polyacrylamide gradient gel electrophoresis) was carried out as previously described to determine the distribution of HDL subpopulations (51). For immunoblotting, the HDL were transferred electrophoretically from the gradient gels to nitrocellulose membranes. ApoA-I was detected by enhanced chemiluminescence (Amersham Life Sciences, Inc.).

HDL PC composition was determined by mass spectrometry. HDL samples were extracted with chloroform/methanol (2:1). 0.1mL of the organic phase was diluted 1:5000 in acetonitrile/0.05% formic acid and 0.02mL of the sample was subjected to ion-spray mass spectrometry by infusion at 0.01mL/min. Mass spectra were acquired using an API-100 ion-spray mass spectrometer (PE/Sciex) using an ion-source voltage equal to 5,000V and an orifice voltage equal to 70V. Data were collected at 0.1 amu resolution over a mass/charge (m/z) range of 100-1,000. Data were imported into Microsoft Excel and the relative PC

amounts calculated, taking into account isotopic distribution and consequent spectral overlap where necessary. It was assumed that each PC species ionized with equal efficiency. Individual PC species were expressed as the proportion of total HDL PC.

4.2.6 HUVEC isolation, culture and incubation conditions

HUVECs were isolated and cultured as described previously (187). Cells were cultured on gelatin-coated culture flasks in medium M199 with Earles Salts (Trace Biosciences, Australia) supplemented with 20% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), 20 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, nonessential amino acids, 12 μg/ml penicillin, 16 μg/ml gentamicin, 20 mg/ml endothelial growth supplement (Collaborative Research, Australia) and 20 mg/ml heparin (Sigma). Confluent preparations of HUVECs (passage 2-4) were washed with 10 mM EDTA-Na_2 (5 ml) in PBS, trypsinised and replated onto 24-well gelatin-coated plates at a density of $3x10^5$ cells/ml (500 μl/well). After a 5 hour reattachment period a portion of the medium (100 μl) was removed and replaced with PBS alone (100 μl) or with HDL in PBS (100 μl). HDL was added to achieve a final concentration of 1, 2, 4 and 8 uM apoA-I. The cells were then preincubated for 16 hours before being activated with TNF-α (100 U/ml). Five hours after addition of cytokine the cell surface expression of VCAM-1 was measured by flow cytometry.

4.2.7 Flow cytometry analysis

Cells were washed with FACS wash (RPMI 1640, containing 10 mM HEPES, 3.1 mM NaN_3 and 2.5% fetal calf serum). Cells were incubated with mouse monoclonal antibody to VCAM-1 (51-10C9) for 30 minutes at 4°C, washed again with FACS wash, and incubated for a

further 30 minutes at 4°C with FITC-conjugated secondary antibody (Immunotech FITC conjugated $F(ab)_2$ fragment goat (mouse IgG)). Cells were washed twice with PBS and harvested by incubation for 30 seconds at room temperature with trypsin. After 30 seconds FACS wash was added to neutralize the trypsin. The cells were pelleted by centrifugation and the pellet was resuspended in FACS fixative (PBS containing 111 mM glucose, 3.1 mM NaN_3 and 350 mM formaldehyde). The expression of VCAM-1 was then measured as fluorescence intensity using an Epics XL-MCL flow cytometer (Coulter, Hialeah, FL) which counted 10,000 cells in each sample. Each sample counted 10,000 cells. Controls included replacement of the primary antibody with an isotype-matched non-relevant antibody and exclusion of TNF-α. Expression of VCAM-1 by cells was reported relative to expression by cells stimulated by TNF-α in the absence of HDL.

4.2.8 Plasma analysis

Plasma collected at the commencement of the study and prior to sacrifice of the animal was stored at -80°C in EDTA until required for analysis. All chemical analyses were carried out on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Triglyceride, protein, phospholipid and free cholesterol concentrations were determined enzymatically. Total cholesterol was determined using a Roche Diagnostics kit. HDL cholesterol was determined following the precipitation of apolipoprotein B containing lipoproteins by polyethylene glycol (459). Non-HDL cholesterol was calculated as the difference between total and HDL cholesterol. ApoA-I was determined by an immunoturbidometric assay using a sheep anti-rabbit apoA-I immunoglobulin.

4.2.9 Data analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made by one way ANOVA with Bonferroni correction where appropriate using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered significant.

4.3 RESULTS

4.3.1 Effect of infusing emulsions on plasma lipids and lipoproteins

As expected from the relatively small amounts given to each rabbit (20 mg triolein per rabbit), infusion of the chylomicron-like emulsions had no significant effect on the concentrations of plasma lipids (Table 1). Nor were there significant differences in the overall chemical composition of HDL (Table 2). However, mass spectrometry revealed predictable changes in HDL PC composition following infusion of the different emulsions (Table 3). Compared with saline infused animals, the proportion of HDL-PLPC (40.9±1.2% vs. 31.1±.6%, $p<0.05$), HDL-POPC (20.8±1.2% vs. 12.4±.3%, $p<0.05$) and HDL-DPPC (7.95±3.2% vs. 1.0±0.1%, $p<0.05$) was increased with infusions of PLPC-TG, POPC-TG and DPPC-TG respectively.

4.3.2 Effect of infusing emulsions on the anti-inflammatory activity of HDL

Infusion of the chylomicron-like emulsions had a significant effect on the ability of the subsequently isolated HDL to inhibit the expression of VCAM-1, by activated endothelial cells (Fig 1). This effect was seen, regardless of the phospholipid composition of the emulsion. At an apoA-I concentration of 4uM, the HDL, isolated from animals infused with saline, PLPC-TG, POPC-TG and DPPC-TG inhibited VCAM-1 expression by 21±4.9%,

55.9±13%, 52.6±11.9% and 89.5±3% ($p<0.05$ for each comparison with saline) respectively (Fig 1). At an apoA-I concentration of 8uM, the isolated HDL from animals infused with saline, PLPC-TG, POPC-TG and DPPC-TG inhibited VCAM-1 expression by 45.8±7%, 78.7±8.7%, 79±8.6% and 91±1.9% ($p<0.05$ for each comparison with saline) respectively (Fig 1). HDL isolated from animals infused with the emulsions containing the saturated phospholipid, DPPC, demonstrated a greater anti-inflammatory activity than emulsions containing either PLPC or POPC at apoA-I concentrations of 2μM ($p<0.001$ for comparison with PLPC-TG and POPC-TG) and 4μM ($p<0.05$ for comparison with PLPC-TG and POPC-TG).

4.3.3 Characterisation of HDL

In an attempt to define how the infusion of the chylomicron-like emulsions may have changed the HDL so as to enhance the anti-inflammatory properties, HDL isolated from animals infused either with saline or with the emulsions were further analysed. Compared with saline infused animals, the HDL isolated from the different groups did not differ with regard to overall composition (Table 2), electrophoretic mobility on agarose gels or particle size, as assessed by non-denaturing gradient gel electrophoresis. In all cases the HDL comprised alpha-migrating particles with diameters of 11.6 nm and 9.5 nm for HDL_2 and HDL_3 respectively. Two-dimensional gel electrophoresis was also performed. This did not reveal an increase in pre-β forms of HDL.

4.4 DISCUSSION

This study represents the first demonstration that the ability of HDL to inhibit the expression of VCAM-1 by activated endothelial cells increases following the infusion of emulsions

containing triolein and phospholipid. This enhanced anti-inflammatory property of HDL is apparent rapidly following the infusion of relatively small amounts of the emulsions.

Experimental studies have demonstrated that HDL possess anti-inflammatory properties. *In vitro*, HDL inhibits the adhesion of monocytes to endothelial cells following stimulation by oxidised LDL (210). HDL also inhibit the expression of VCAM-1, ICAM-1 and E-selectin by cytokine stimulated endothelial cells (187,188,190). In addition, the infusion of HDL inhibits endothelial adhesion molecule expression *in vivo*. The periarterial collar induced expression of endothelial VCAM-1 and macrophage accumulation in the apoE knockout mouse is inhibited by infusions of rHDL (83).

This current study shows that the ability of HDL to inhibit expression of VCAM-1 by activated endothelial cells is considerably enhanced following the infusion of relatively small amounts of emulsions. The mechanism for the enhancement is uncertain. It was previously demonstrated *in vitro* that the anti-inflammatory potential of rHDL is influenced by its phospholipid composition, with particles containing PLPC being superior to those containing either POPC or DPPC (194). The HDL PC composition was altered *in vivo* following the infusion of emulsions in this study. However, this did not result in a similar anti-inflammatory hierarchy as seen in the previous *in vitro* studies using rHDL. It should be noted that the degree of phospholipid enrichment did differ with infusion of different emulsions. Altering the HDL content of POPC and PLPC by 30% and 60% respectively resulted in a comparable increase in anti-inflammatory activity. However, a nearly 8 fold increase in the HDL content of DPPC, normally expressed in minimal amounts, resulted in a much greater increase in anti-inflammatory activity. It is tempting to speculate that even small changes in HDL phospholipid may confer these beneficial properties. It has been demonstrated that the ability

of HDL to promote vasodilatation and smooth muscle cell proliferation, in addition to inhibiting apoptosis, is due to the presence of bioactive phospholipids on its surface (342,373,384). The relationship between HDL phospholipids and their anti-inflammatory properties remains to be determined. However, the results of this study do not support a predictable relationship between enrichment of HDL with individual PC species and subsequent alteration in their anti-inflammatory activity. This suggests that some other factor conferred the beneficial properties seen following the infusion of emulsions containing triolein and phospholipid.

We considered the possibility that the enhanced inhibitory activity of HDL may have related to changes in the particle size, the surface charge or the subpopulation distribution of HDL. These possibilities were excluded by finding that infusion of the emulsions had no effect on HDL size, electrophoretic mobility or subpopulation distribution. It is possible that the enhanced inhibitory activity was the result of formation of a minor subpopulation of highly inhibitory particles, the presence of which was below the level of detection by the techniques used. One such subpopulation may be discoidal HDL, formed as a by-product of lipolysis of the emulsions (467). In chapter three, it is demonstrated that the infusion of small amounts of discoidal rHDL exert profound anti-inflammatory effects *in vivo*. It is uncertain how such particles could be identified within the bulk HDL.

It was of interest to note that the HDL isolated from animals infused with emulsions containing a saturated fatty acid appeared to demonstrate the greatest anti-inflammatory activity. The explanation for this is not known, although such an effect would make teleological sense in view of the greater pro-inflammatory effects of saturated fat in the diet

(410) and would be consistent with a proposition that the anti-inflammatory properties of HDL following a fat meal may increase in proportion to the pro-inflammatory stimulus.

The postprandial state is characterised by the appearance in the plasma of chylomicrons and remnant particles. These lipoproteins are potentially atherogenic (28,406,410). They have been demonstrated to promote oxidative stress, inflammation, reduced vascular reactivity and thrombogenicity. Population studies have established that postprandial lipemia correlates with cardiovascular risk (408). Vascular reactivity declines following both the infusion of triglyceride rich emulsions (416) and the consumption of a fatty meal (414) in normal subjects. In addition, the plasma concentration of proinflammatory cytokines and soluble adhesion molecules rises within the first few hours following the consumption of a fatty meal in healthy, normolipidaemic subjects (470). Furthermore, postprandial lipoproteins have been shown in vitro to promote monocyte chemotaxis, activation, adhesion to endothelial cells and aggregation with platelets (410). As the typical western diet includes the consumption of several meals during the course of a typical day, the presence of these postprandial factors may have a substantial impact in promoting atherogenesis and its complications. The studies reported in this chapter demonstrate that the anti-inflammatory properties of HDL are enhanced following the infusion of very small amounts of emulsions containing triolein and phospholipid. It is uncertain what would be the effect of elevating plasma triglyceride to postprandial levels, *via* the infusion of greater amounts of emulsions.

In conclusion, this study demonstrates that following the infusion of emulsions containing triolein and phospholipid, HDL undergo a rapid change that results in enhanced anti-inflammatory properties. Such a finding raises the possibility that the anti-inflammatory properties of HDL may be regulated in response to the degree of pro-inflammatory

lipoproteins circulating in the plasma during the postprandial state. As humans in the western world spend the majority of their lives postprandial, the significance of such regulation may be of major importance in protecting against the pro-atherogenic effects of typical western diets.

	Saline		PLPC-TG		POPC-TG		DPPC-TG	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Total Cholesterol (mmol/L)	1.11±0.21	1.27±0.18	0.92±0.13	1.11±0.27	0.91±0.05	0.96±0.09	0.88±0.08	0.93±0.11
Triglyceride (mmol/L)	0.53±0.07	0.41±0.05	0.47±0.09	0.92±0.12	0.35±0.1	0.51±0.09	0.46±0.01	0.5±0.08
HDL Cholesterol (mmol/L)	0.31±0.03	0.3±0.06	0.35±0.05	0.29±0.03	0.33±0.12	0.28±0.02	0.32±0.03	0.25±0.05
Non-HDL Cholesterol (mmol/L)	0.8±0.2	0.97±0.19	0.57±0.11	0.83±0.26	0.58±0.05	0.68±0.1	0.55±0.08	0.68±0.08
ApoA-I (mg/ml)	0.53±0.14	0.6±0.05	0.59±0.2	0.69±0.07	0.47±0.03	0.66±0.09	0.56±0.03	0.37±0.03
Phospholipid (mmol/L)	1.33±0.08	0.8±0.11	1.2±0.16	0.92±0.19	0.99±0.17	0.71±0.15	1.16±0.01	0.43±0.06*

Table 4.1. Plasma lipid profiles at baseline and 20 minutes following infusion of either (i) PLPC-TG, (ii) POPC-TG, (iii) DPPC-TG or (iv) saline. Data are expressed as mean±SEM. * $P<0.001$ compared with saline infused animals.

	Phospholipid	ApoA-I	Triglyceride	Unesterified Cholesterol	Esterified Cholesterol
PLPC-TG	28.3±1.3	39.3±1.1	11.5±3.4	4.4±0.3	16.5±1.5
POPC-TG	24.2±1.7**	37.2±3.1	22.5±5*	3.9±0.3	12.2±1
DPPC-TG	31.1±1.1	43.7±2.4	7.4±1	4.2±0.2	13.5±0.6
Saline	27.3±0.6	34±1*	17.1±3.4	4.4±0.1	17.2±2

Table 4.2. Chemical composition of HDL isolated from animals infused with either (i) PLPC-TG, (ii) POPC-TG, (iii) DPPC-TG or (iv) saline. Data are expressed as percentage of total molar mass (mean±SEM). * $P<0.05$ and ** $P<0.01$ compared with DPPC-TG infused animals.

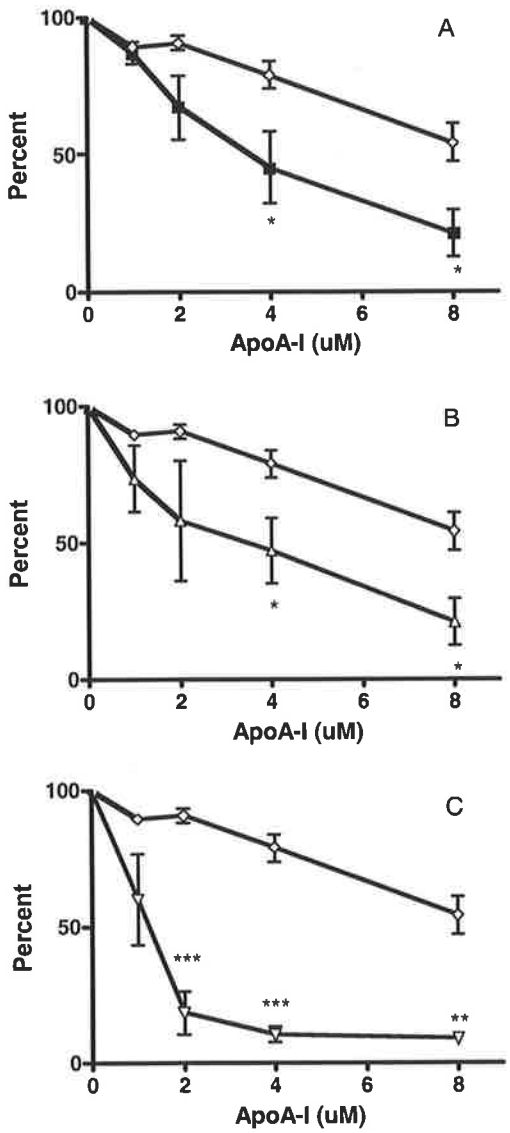

Figure 4.1. Inhibition of cytokine-induced expression of endothelial VCAM-1 by HDL isolated from animals infused with chylomicron-like emulsions containing either PLPC (panel A, ■), POPC (panel B, ▲) or DPPC (panel C, ▼) compared with saline (◆). Data are expressed as percentage of VCAM-1 expression by activated HUVECs in the absence of HDL (mean±SEM) * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with saline infused animals.

	PLPC-TG	POPC-TG	DPPC-TG	Saline
DPPC	1.92±0.43	0.82±0.23	7.95±3.14*	0.97±0.05
PLPC	40.92±1.24*	29.95±0.81	33.17±1.71	31.1±0.55
POPC	10.18±0.5	20.78±1.22*	10.27±0.5	12.37±0.32
PAPC	10.28±0.71	10.51±0.49	11.82±1.13	11.35±0.16
SLPC	36.75±1.73	37.77±0.97	36.82±1.19	44.2±0.48

Table 4.3. Phospholipid composition of HDL isolated from animals infused with either (i) PLPC-TG, (ii) POPC-TG, (iii) DPPC-TG or (iv) saline. Data are expressed percentage of total phosphatidylcholine (mean±SEM). *P<0.05 compared with saline infused animals.

CHAPTER FIVE

ROLE OF HDL IN PLAQUE STABILISATION

5.1 INTRODUCTION

In chapter three it was demonstrated that HDL possess profound beneficial properties in a model of acute vascular inflammation. It remains to be determined what is the significance of these properties in an *in vivo* model of chronic arterial inflammation, atherosclerotic plaque.

The majority of atherosclerotic plaques remain clinically quiescent throughout life. However, it has been demonstrated that a number of plaques undergo erosion or frank rupture of the overlying fibrous cap (19). This exposes circulating blood to highly thrombogenic plaque contents. The formation of thrombus over a variably stenotic plaque compromises the arterial lumen and precipitates ischaemia. The severity of angiographic stenosis does not predict the propensity of a plaque to rupture (12). In contrast, pathological studies have established that plaques, rich in inflammatory tissue, are vulnerable and thus more likely to be complicated by rupture of the fibrous cap (131).

In patients with established atherosclerotic disease it is important to identify those at a greater risk to develop ruptured plaques and tailor interventions that are likely to stabilise their plaques and thus prevent clinical events. In experimental models, interventions that target hyperlipidaemia result in a more stable pathologic plaque. In a well validated model of atherosclerotic plaque, aortic balloon denudation in the cholesterol fed rabbit, Aikawa *et al* have demonstrated that resumption of a normal chow diet results in both a normalisation of the plasma lipid profile and a reduction in the inflammatory content of plaque (270). In addition, it has been suggested that the 3-hydroxy 3-methyl-glutaryl coenzyme A reductase inhibitors (statins) may promote plaque stabilisation in addition to their ability to lower LDL cholesterol (471). The

benefit of these agents appears to exceed their ability to promote plaque regression or halt progression of overall burden judged by angiography. In addition, evidence from observational studies (472) and randomised clinical trials (473) suggests that early administration of statin therapy following an acute ischaemic event results in a significant reduction in early recurrent events. It has been demonstrated both in vitro (471) and in animal models of atherosclerosis (474) that statins possess properties, in addition to LDL lowering. Such pleiotropic properties may contribute to the ability of these agents to passivate atherosclerotic plaque.

In vitro and animal studies have demonstrated that HDL possess powerful anti-inflammatory, antithrombotic and antiapoptotic properties (475). It is therefore possible that HDL may stabilise plaque. Transgenic expression of human apoA-I (89) and the infusion of rHDL containing recombinant apoA-I_{Milano} (121) both reduce the inflammatory composition of established atherosclerotic plaque in animal models. In addition, the studies outlined in chapter four demonstrate that the infusion of small amounts of rHDL have profound anti-inflammatory properties, that appear to be independent to their ability to promote cholesterol efflux.

In the studies outlined in this chapter, the ability of HDL to confer a stable plaque phenotype has been investigated. Using the model of balloon aortic denudation in the cholesterol fed rabbit, atherosclerotic plaque was established in the abdominal aorta. The ability of infusing HDL was compared with oral atorvastatin, with regard to their ability to influence the inflammatory and thrombogenic components of plaque histology.

5.2 METHODS

5.2.1 Animals

Male New Zealand White rabbits (Institute of Medical and Veterinary Science, Gilles Plains, Australia) aged 12 weeks were maintained on a diet comprising 0.2% cholesterol enriched chow (GlenForest Stock Feed, Western Australia) throughout the study. All procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee (protocol 43/02).

5.2.2 Preparation of native rabbit HDL

HDL was isolated from pooled samples of rabbit plasma (Quality Farms of Australia, Lara, VIC, Australia) by sequential ultracentrifugation in the 1.06-1.21 g/mL density range. HDL was dialysed against endotoxin free phosphate-buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO) containing 0.2 g/L KH_2PO_4, 0.2 g/L KCl, 8 g/L NaCl and 1.15 g/L Na_2PO_4 before use. ApoA-I concentrations were determined by an immunoturbidometric assay (437).

5.2.3 Establishment of experimental atherosclerosis

Atherosclerotic plaque was induced in 30 rabbits by a combination of a high cholesterol diet, containing 0.2% cholesterol enriched chow, and balloon aortic denudation of the abdominal aorta (447). The diet, comprising 150 g/day of the cholesterol enriched chow and water provided ad libitum, was commenced one week prior to aortic denudation. Balloon denudation of the abdominal aorta was then performed under general anaesthesia. Animals were anaesthetised using intramuscular ketamine/xylazine (20/3 mg/kg) and maintained using inhaled halothane (1-3 mL/min). The right carotid artery was exposed surgically and cleared of connective tissue along a

30mm length. A bolus of heparin (100 U/kg) was administered via a cannula placed in the marginal ear vein. An arteriotomy incison was made, through which a 4F Fogarty embolectomy balloon catheter (Edwards BioLifesciences) was inserted into the artery. The catheter was advanced retrogradely, using fluoroscopic guidance, into the descending aorta and placed distal to the aortic bifurcation in the right iliac artery. The balloon was inflated using moderate resistance and pulled back to the level of the diaphragm. The balloon denudation of the abdominal aorta was repeated a further three times. The catheter was then removed from the carotid artery. The proximal carotid artery was ligated. Skin layers were sutured, the wound dressed with antibiotic, and animals allowed to recover. All animals continued the high cholesterol diet for a further 16 weeks to establish atherosclerotic plaque.

5.2.4 Experimental protocol

Animals then entered a five day treatment phase where they received either (i) rabbit HDL containing 25mg apoA-I infused intravenously on days 1 and 3 (n=8), (ii) atorvastatin (Pfizer, Groton, CT, USA) 5 mg/kg/day administered mixed in the cholesterol enriched chow with 3% peanut oil (n=7) or (iii) no treatment (n=15). The high cholesterol diet was continued throughout the treatment phase. On the fifth day of the treatment phase, blood was sampled from a marginal ear vein. Animals received heparin (100 U/kg, i.v.) five minutes prior to euthanasia to prevent postmortem thrombosis. Animals were euthanased with an overdose of sodium pentobarbitone (90 mg/kg, i.v.). The aortic root was cannulated and the aorta was flushed with 500 mL phosphate-buffered saline (PBS, pH 7.4), followed by perfusion fixation with 500 mL of 4% paraformaldehyde in PBS at 100 mmHg. Following perfusion fixation, the aorta was removed and immersed in fresh fixative.

5.2.5 Immunohistochemistry

Specimens were paraffin-embedded and serial 5 μm slices were cut. One section was stained with haematoxylin and eosin. The other sections were used for immunohistochemical analysis which were stained as outlined in chapter 2. Antibodies applied included mouse monoclonal anti-rabbit RAM11 (DAKO, 1/200), mouse monoclonal anti-rabbit smooth muscle actin (Sigma, 1/60000), mouse monoclonal anti-rabbit tissue factor (American Diagnostica, 1/500), sheep polyclonal anti-human von Willebrand factor (Binding Site, 1/1500), mouse monoclonal anti-human PAI-1 (American Diagnostica, 1/1000), goat polyclonal anti-rabbit thrombomodulin (American Diagnostica, 1/2000), goat polyclonal anti-rabbit MCP-1 (kindly donated by Dr A Matsukawa, 1/50), goat polyclonal anti-human fractalkine (Santa Cruz, 1/500) and mouse monoclonal anti-human MMP-9 (Oncogene, 1/200). Digital micrographs of sections were acquired using an Olympus BX40 microscope. The percentage of plaque area containing positive staining was determined using ImagePro Plus (Cybernetics).

5.2.6 Plasma analyses

Plasma collected at both the commencement of the study and treatment phase and prior to sacrifice of the animal was stored at -80°C in EDTA until required for analysis. All chemical analyses were carried out on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Triglyceride (443) and free cholesterol (444) concentrations were determined enzymatically. Total cholesterol was determined using a Roche Diagnostics kit. HDL cholesterol was determined by enzymatic assay following precipitation of apolipoprotein B

containing lipoproteins with polyethylene glycol (459). ApoA-I concentrations were determined by an immunoturbidometric assay using a sheep anti-rabbit apoA-I immunoglobulin (437).

5.2.7 Data analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made by Student's t-tests and one way ANOVA using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered significant.

5.3 RESULTS

5.3.1 Plasma lipid profile

Plasma total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol are presented in table 5.1. Administration of the cholesterol enriched diet predictably increased plasma total cholesterol (1.51±0.16 and 12.86±2.2 mmol/L in control treated animals at baseline and at the time of sacrifice respectively). Infusion of HDL did not alter the plasma concentration of HDL cholesterol (0.39±0.02 and 0.38±0.04 mmol/L in HDL infused animals when sampled prior to treatment and at the time of sacrifice respectively).

5.3.2 Plaque cellular composition

Representative sections of atherosclerotic plaque staining for the presence of macrophages and smooth muscle cells are shown in figures 5.1-2. The plaque ratio of smooth muscle cells to

macrophages was 2.6 fold higher in HDL treated animals (12.3±5.9 vs. 4.7±0.9 in HDL and untreated animals respectively, P<0.05) and 4 fold higher in atorvastatin treated animals (18.4±7.7 vs. 4.7±0.9 in atorvastatin and untreated animals respectively, P<0.01). (Fig 5.3) This effect resulted largely from an increase in plaque smooth muscle cell composition by 39% in HDL treated animals (50.5±3.6 vs. 36.3±3.8 percent plaque area in HDL and untreated animals respectively, P<0.03) and 31% in atorvastatin treated animals (47.4±4.5 vs. 36.3±3.8 percent plaque area in atorvastatin and untreated animals respectively, P<0.05). (Fig 5.3) In addition, there was a trend towards a reduction in macrophage composition by 43% in HDL treated animals (7±3.5 vs. 12.4±2.3 percent plaque area in HDL and untreated animals respectively, NS) and by 54% in atorvastatin treated animals (5.7±3.3 vs. 12.4±2.3 percent plaque area in atorvastatin and untreated animals respectively, NS), although this failed to meet statistical significance. (Fig 5.3) There was no difference in the degree of plaque smooth muscle cell and macrophage infiltration of atherosclerotic plaque between HDL and atorvastatin treated animals.

5.3.3 Thrombogenic features of atherosclerotic plaque

Representative staining of thrombogenic features of atherosclerotic plaque are shown in figures 5.4-8. Plaque composition of thrombomodulin increased by 112% in HDL treated animals (10.4±3 vs. 4.9±0.7 percent plaque area in HDL and untreated animals respectively, P<0.03) and by 45% in atorvastatin treated animals (7.2±1.3 vs. 4.9±0.7 percent plaque area in atorvastatin and untreated animals respectively, NS), although this failed to meet statistical significance. (Fig 5.6) In addition, a trend toward a reduction in plaque composition of PAI-1 by 22% in HDL treated animals (23.6±6 vs. 30.3±2.8 percent plaque area in HDL and untreated animals respectively, NS) although this failed to reach statistical significance. (Fig 5.6) Neither treatment

modified the plaque composition of tissue factor (15.1±3.6, 30.8±9.9 and 22±1.9 percent plaque area in HDL, atorvastatin and untreated animals respectively, NS) or von Willebrand factor (39.1±12.8, 50.6±11.2 and 49±4.9 percent plaque area in HDL, atorvastatin and untreated animals respectively, NS) compared with untreated animals. (Fig 5.9)

5.3.4 Atherosclerotic plaque composition of matrix metalloproteinases

Representative staining of the matrix metalloproteinase MMP-9 in atherosclerotic plaque are shown in figure 5.10. Plaque composition of MMP-9 decreased by 42% in HDL treated animals (21.6±7.4 vs. 37.3±2.6 percent plaque area in HDL and untreated animals respectively, $P<0.05$) and by 45% in atorvastatin treated animals (20.4±6.2 vs. 37.3±2.6 percent plaque area in atorvastatin and untreated animals respectively, $P<0.05$). (Fig 5.11)

5.3.5 Atherosclerotic plaque composition of proinflammatory chemokines

Representative staining of the proinflammatory chemokines MCP-1 and fractalkine in atherosclerotic plaque are shown in figures 5.12-13. HDL and atorvastatin did not significantly alter the plaque composition of MCP-1 (40.6±7.2, 46.6±6.9 and 42.2±4 percent plaque area in HDL, atorvastatin and untreated animals respectively, NS) and fractalkine (26.6±4.8, 19.5±5.2 and 25.1±4.2 percent plaque area in HDL, atorvastatin and untreated animals respectively, NS). (Fig 5.14)

5.4 DISCUSSION

The studies described in this chapter demonstrate that the infusion of HDL rapidly promotes the development of a stable phenotype in a model of established experimental atherosclerosis. This is characterised by an increase in the proportion of smooth muscle cells to macrophages and reductions of both plaque thrombogenicity and matrix metalloproteinase composition.

It has become apparent that the overall burden of atherosclerosis does not strongly predict the likelihood of developing clinical events. Studies have demonstrated that the majority of acute coronary syndromes result from complications of atheromatous plaque deemed to be mild or moderately stenotic on conventional angiography (12). In contrast, the composition of atherosclerotic plaque appears to be a major determinant of its propensity to rupture and promote occlusion of the arterial lumen. Pathologic studies have demonstrated that atherosclerotic plaque containing lipid and inflammatory material is more likely to rupture (131). Rupture of the fibrous cap overlying atherosclerotic plaque exposes circulating blood to the thrombogenic plaque core leading to thrombus formation (20). The evolving thrombus occludes luminal blood flow and precipitates ischaemia in the vascular bed. Strategies that reduce the inflammatory and thrombogenic components of atherosclerotic plaque are therefore likely to reduce the propensity of a plaque to rupture and incite thrombus formation.

Studies have consistently demonstrated that the administration of statins is associated with a marked reduction in clinical events, in both primary (59,476) and secondary (30,477) prevention. This benefit appears to correlate with the degree of LDL lowering that results from HMG CoA reductase inhibition. However, recent studies have demonstrated that early administration of

statins in the setting of acute coronary syndromes is associated with an early clinical benefit, prior to effective LDL lowering (473). It has been proposed that this benefit may reflect the finding that statins possess other, pleiotropic, properties (471). *In vitro* studies have found that statins are anti-inflammatory (478) and anti-thrombotic (474,479). In addition, they reduce the expression and activity of metalloproteinases (474). These actions are thought to are related to the ability of statins to decrease synthesis of isoprenoids, mediators involved in the activation of Rho/Rho kinase signalling pathways (480). The study outlined in this chapter confirms previous findings of the benefits of statins in animal models of established atherosclerosis. As a result, the development of strategies to passivate plaque should be compared to the effect of statins.

It has been demonstrated that HDL possess non-lipid transporting properties (475). These include anti-inflammatory and anti-thrombotic roles. These properties influence not only the very early stages of atherogenesis but the setting of mature atheroma. Elevation of plasma HDL, either by the transgenic expression of human apoA-I (89) or infusion of rHDL containing large amounts of recombinant apoA-I_{Milano} (121) promotes the development of a more stable plaque phenotype, characterised by a reduction in macrophage and increase in smooth muscle cells. The current study extends these findings by demonstrating that the infusion of relatively low amounts of HDL influence plaque phenotype in a manner comparable to that of statins. This benefit represents predominantly an increase in the proportion of smooth muscle cells, accompanied by a reduction in macrophage content.

In addition, this is the first *in vivo* evidence that the infusion of HDL has a favourable influence on the plaque composition of MMP-9, thrombomodulin and PAI-1. These results suggest that in

addition to promoting changes in the cellular composition of plaque, HDL also influences the expression of factors that participate in the development of acute ischaemic syndromes. Matrix metalloproteinases degrade components of the fibrous cap (267). As weakening of the fibrous cap increases its propensity to rupture, the findings of the current study provide an important mechanism by which HDL reduces the incidence of clinical events. This extends *in vitro* findings that HDL inhibits the release of metalloproteinases by monocytes following stimulation with oxidised LDL (326). It is uncertain what degree of improvement, in terms of loss of MMP-9 activity, accompanies the demonstrated 40-50% reduction in plaque expression.

Furthermore, it is the formation of thrombus within the arterial lumen that compromises blood flow and therefore the ability of HDL to reduce the thrombogenic potential of experimental plaque is important. This extends *in vitro* evidence that HDL have favourable effects on the thrombotic process (351-354,365,368). The experimental model that was used is characterised by a dense accumulation of highly thrombogenic tissue factor and von Willebrand factor. The previous demonstration that the transgenic expression of human apoA-I depletes established atheroma of tissue factor was found after a five month exposure to elevated plasma HDL (89). As the current study involves the infusion of low amounts of HDL in a short period time, it is not surprising that the atheroma was not depleted of either of these components. However, HDL did rapidly promote the expression of factors that contribute to the limitation of thrombus formation. Thrombomodulin and PAI-1 regulate the endogenous anticoagulation and fibrinolytic systems respectively (289). Their constitutive expression contributes to the inhibition of thrombosis on or within the arterial wall. However, in the settings of endothelial dysfunction and atherosclerosis, their expression is reduced. The results of chapter four demonstrate that the infusion of rHDL

inhibits the endothelial expression of PAI-1 induced by application of a periarterial collar. The current study extends this benefit of HDL to established atheroma.

The mechanism by which HDL confers this stable plaque phenotype remains uncertain. Several groups have demonstrated that depletion of plaque lipid is associated with a reduction in its inflammatory and thrombogenic components (121,447). The promotion of cholesterol efflux from the plaque reduces the stimuli for macrophage accumulation. In addition, HDL has been demonstrated *in vitro* to inhibit the synthesis of proinflammatory chemokines that may promote the accumulation of macrophages (210). This was extended to the *in vivo* setting in chapter four in a model of acute vascular inflammation. In the current study, we found that the plaque composition of MCP-1 and fractalkine, chemokines implicated in the early stages of atherogenesis, were not altered in this model of mature atheroma.

The major cellular change in this study was an increase in the proportion of smooth muscle cells. HDL have beneficial effects on the viability of smooth muscle cells (383). These include mitogenic and anti-apoptotic properties. It is possible that these *in vitro* benefits also occur *in vivo*. It has also been demonstrated that cholesterol loaded macrophages become morphologically like smooth muscle cells following cholesterol efflux (266). It is therefore possible that such efflux, promoted by HDL, contributes to the increase in smooth muscle cells.

In addition, products of oxidative stress, including oxidised LDL and ROS, are potent promoters of the accumulation of inflammatory cells within the arterial wall (245). HDL possess anti-oxidant properties (315). HDL inhibits the oxidative modification of LDL and generation of

vascular ROS, as demonstrated in chapter four. Furthermore, nitric oxide has anti-inflammatory and anti-thrombotic properties, in addition to its ability to maintain vascular tone (238). HDL promotes the generation of nitric oxide, via the activation of eNOS (338). This mechanism is likely to contribute to the restoration of endothelial function seen in human subjects following the infusion of rHDL (78,79). It may also contribute to the ability of HDL to rapidly stabilise atherosclerotic plaque.

In contrast to previous studies, the benefit of HDL on established atheroma, in addition to that seen with rHDL in chapter four, was seen in the absence of an elevation in plasma HDL. This further supports the powerful impact that even small amounts of HDL can have on the arterial wall. In addition, this benefit is seen within a few days, which would suggest that therapeutic strategies that manipulate HDL may result in a similar early benefit as that seen with statin agents. It is possible that the combination of a statin and strategies that elevate plasma HDL may result in an incremental benefit. However, these properties of HDL have been demonstrated in an animal model with a low incidence of spontaneous plaque rupture. Whilst the infusion of rHDL has recently been demonstrated to have a profound and rapid impact on atherosclerotic burden in humans (80), it is unclear what the effect will be on plaque composition.

In summary, the findings of this chapter demonstrate that the infusion of HDL results in a rapid promotion of a stable plaque phenotype, comparable to that seen with administration of a statin. These results highlight the potential benefit that can be gained through strategies that target plasma HDL in the setting of established atherosclerotic cardiovascular disease.

	Baseline				Sacrifice			
	TC	TG	HDL-C	LDL-C	TC	TG	HDL-C	LDL-C
Control	1.51±0.16	0.83±0.1	0.31±0.03	0.82±0.12	12.86±2.2	0.74±0.22	0.4±0.09	12.12±2.12
HDL	1.44±0.12	0.65±0.12	0.39±0.02	0.76±0.09	12.4±4.22	1.1±0.56	0.38±0.04	11.51±4.14
Atorvastatin	1.81±0.19	0.68±0.08	0.32±0.02	1.18±0.18	11.1±3.84	0.88±0.4	0.3±0.05	10.37±3.67

Table 5.1. Plasma lipid profile of animals at baseline, prior to treatment and at the time of sacrifice. Results expressed as mmol/L (mean±SEM). TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol.

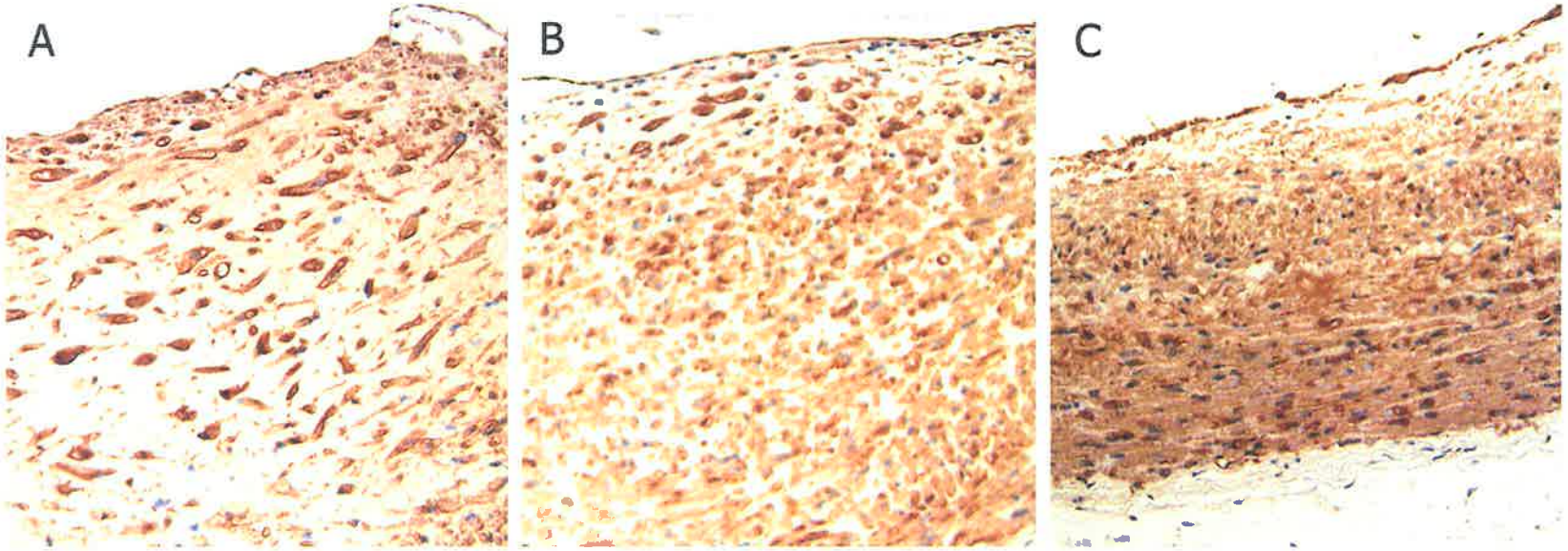

Figure 5.1. Representative staining of plaque smooth muscle actin in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

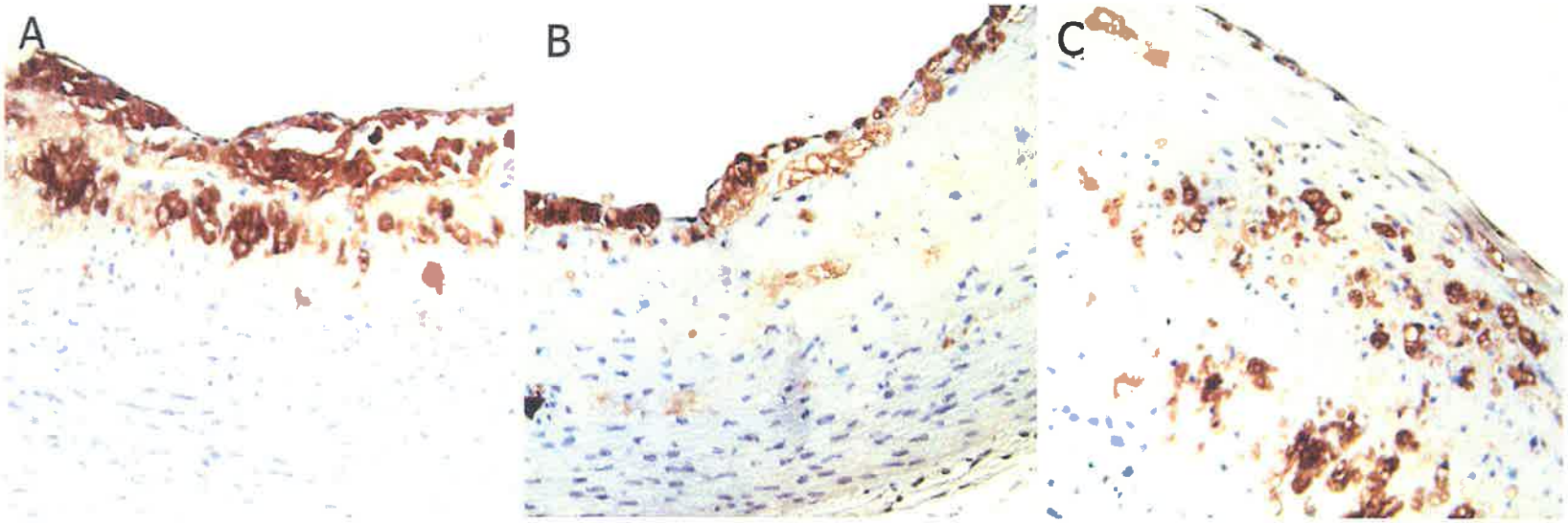

Figure 5.2. Representative staining of plaque macrophage composition in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

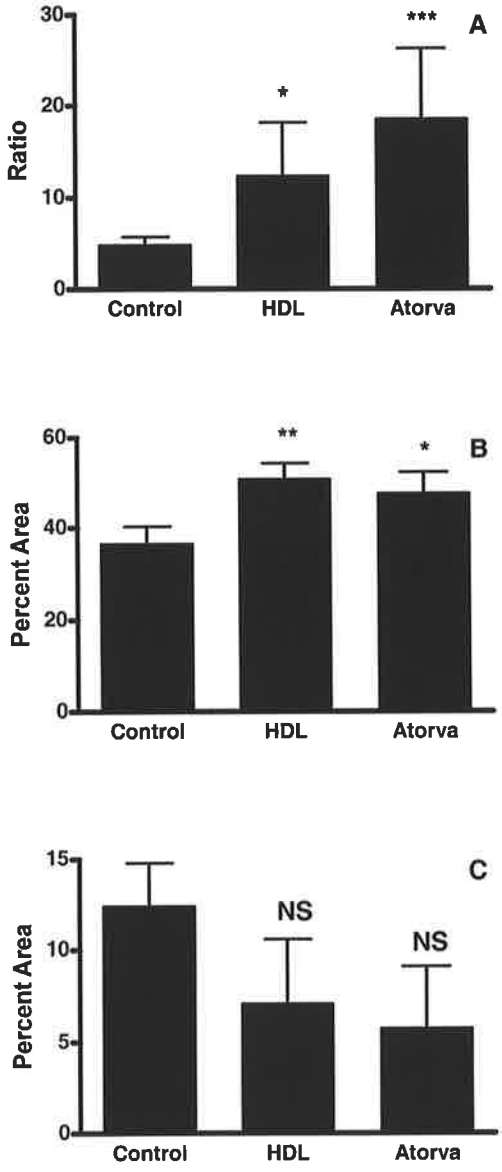

Figure 5.3. Effect of administration of HDL and atorvastatin on the ratio of smooth muscle cells to macrophages in atherosclerotic plaque (panel A), plaque smooth muscle cell composition (panel B) and plaque macrophage composition (panel C). Results expressed as mean±SEM. NS nonsignificant, * $P<0.05$, ** $P<0.03$ and *** $P<0.01$ compared with untreated animals.

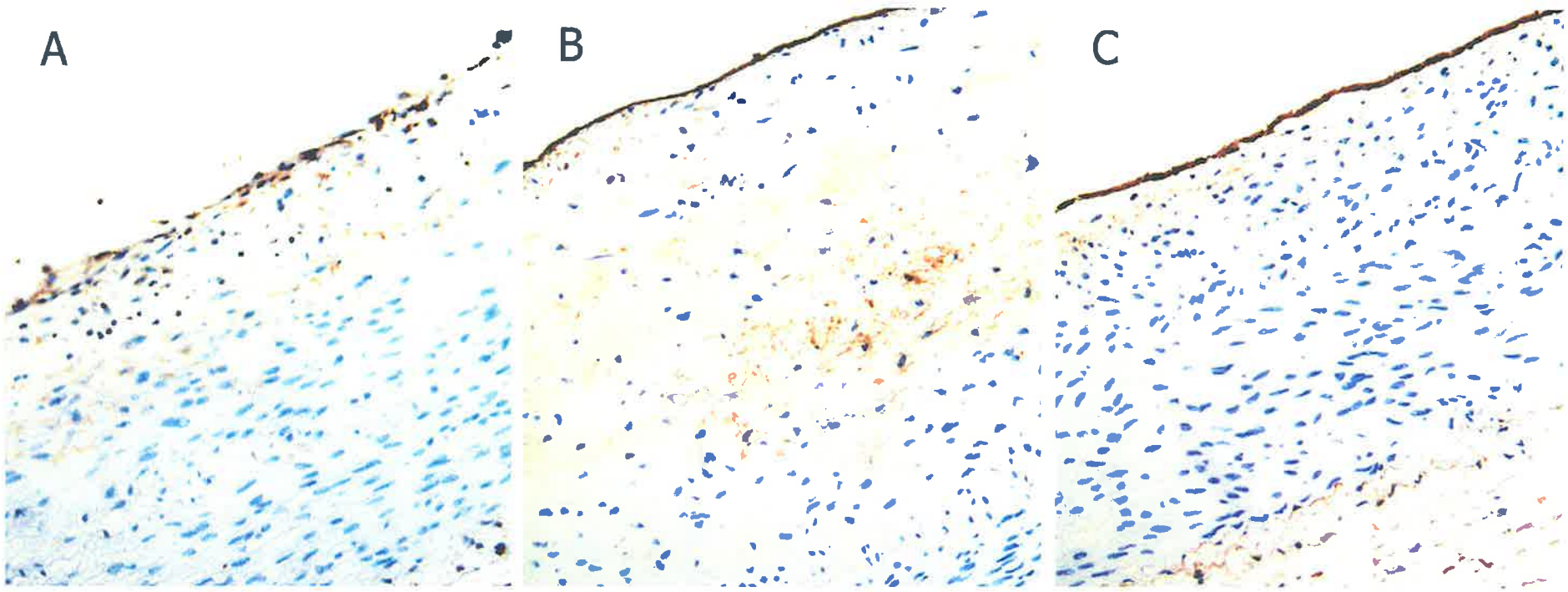

Figure 5.4. Representative staining of plaque thrombomodulin in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

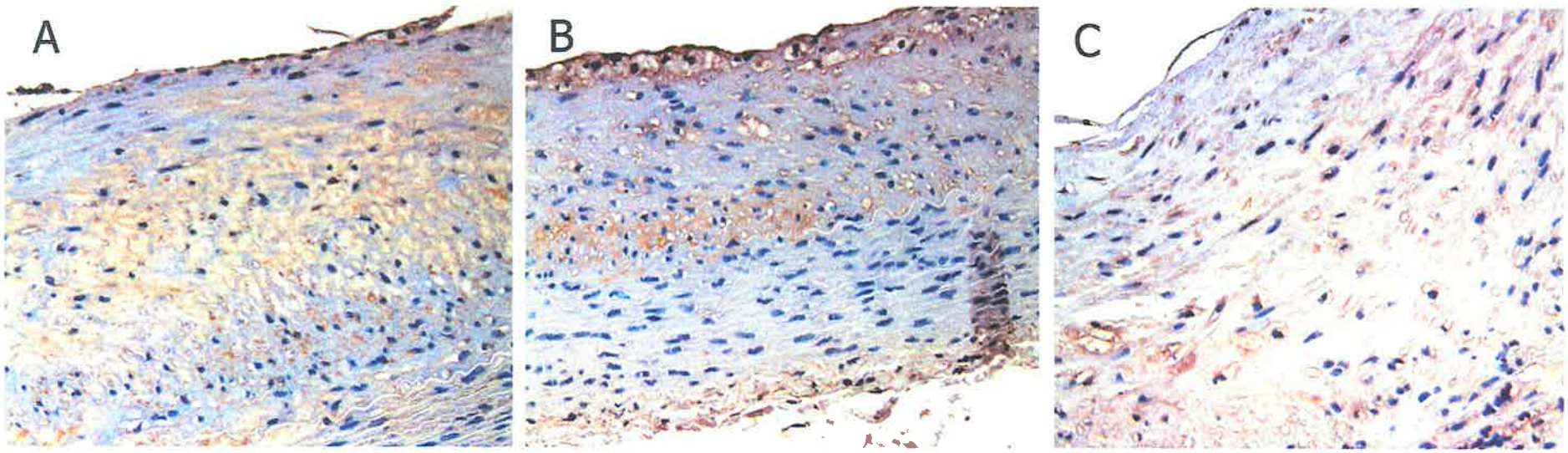

Figure 5.5. Representative staining of plaque PAI-1 in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

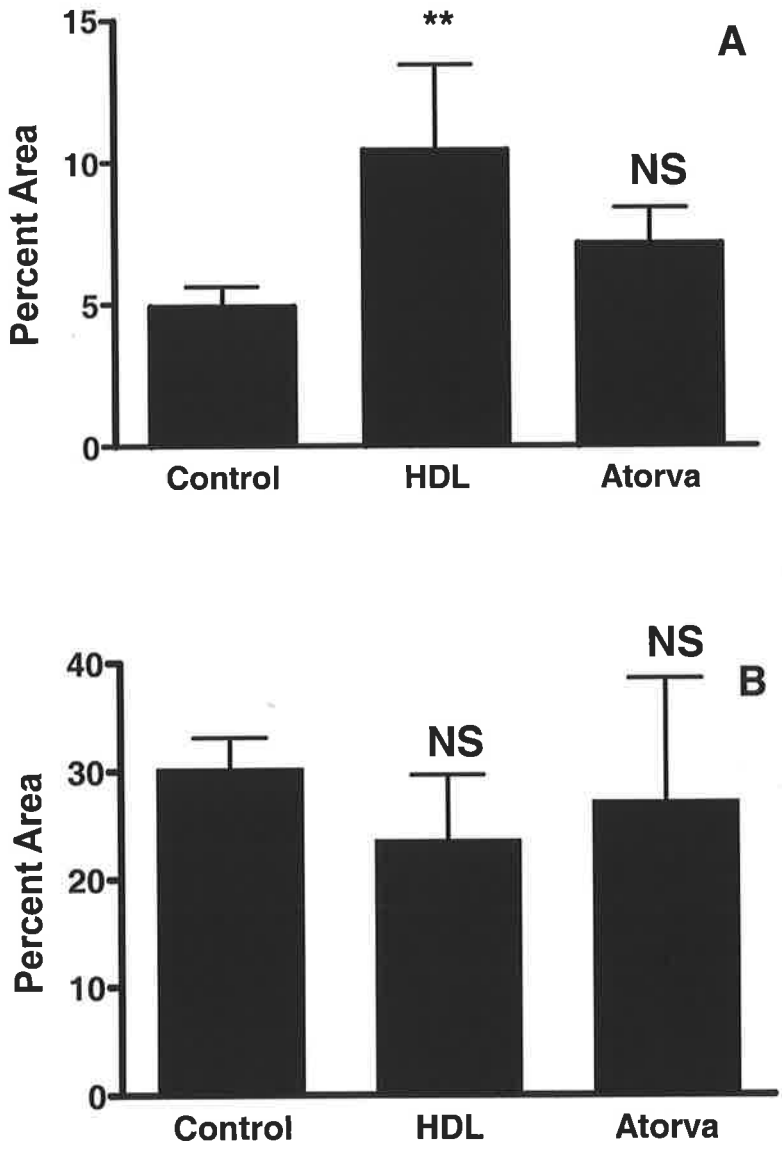

Figure 5.6. Effect of administration of HDL and atorvastatin on the plaque composition of thrombomodulin (panel A) and PAI-1 (panel B). Results expressed as percentage of plaque area that revealed positive staining (mean±SEM). NS nonsignificant and ** $P<0.03$ compared with untreated animals.

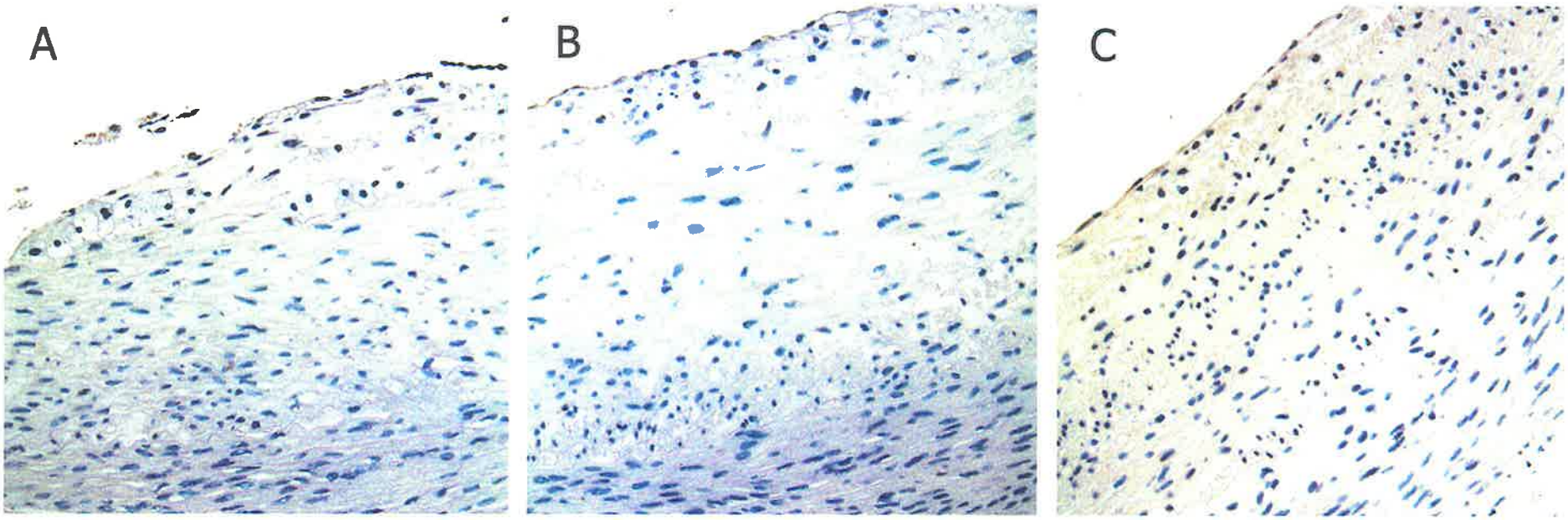

Figure 5.7. Representative staining of plaque tissue factor in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

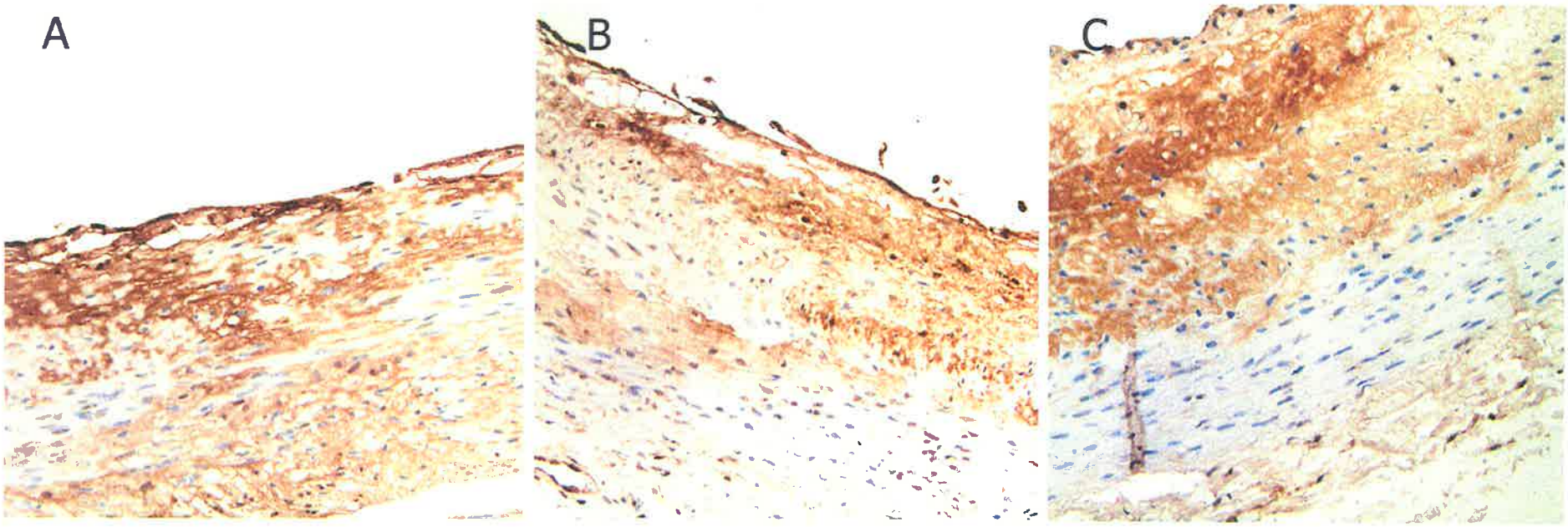

Figure 5.8. Representative staining of plaque von Willebrand factor in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

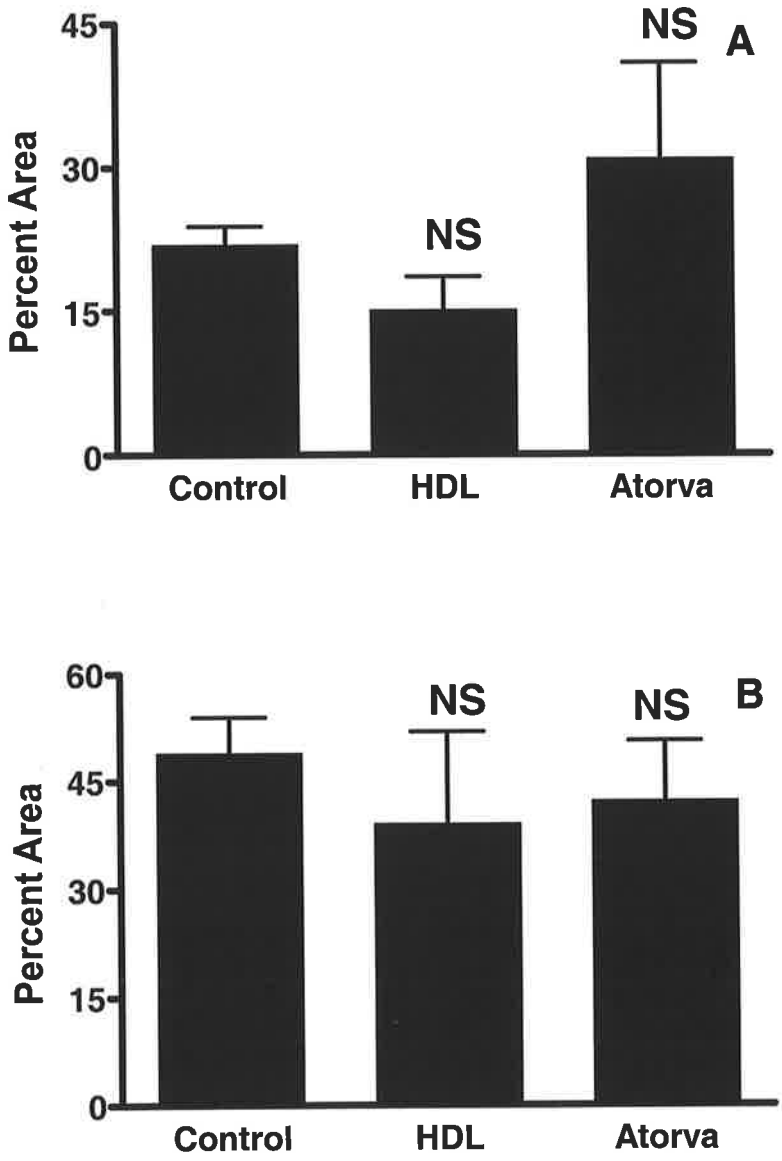

Figure 5.9. Effect of administration of HDL and atorvastatin on the plaque composition of tissue factor (panel A) and von Willebrand factor (panel B). Results expressed as percentage of plaque area that revealed positive staining (mean±SEM). NS nonsignificant compared with untreated animals.

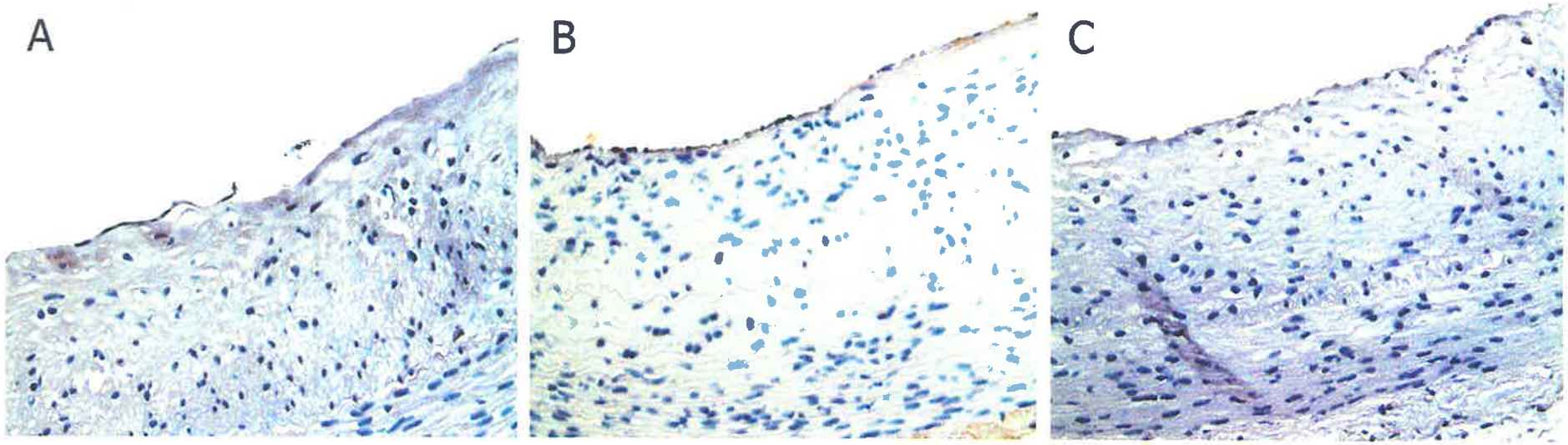

Figure 5.10. Representative staining of plaque MMP-9 in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

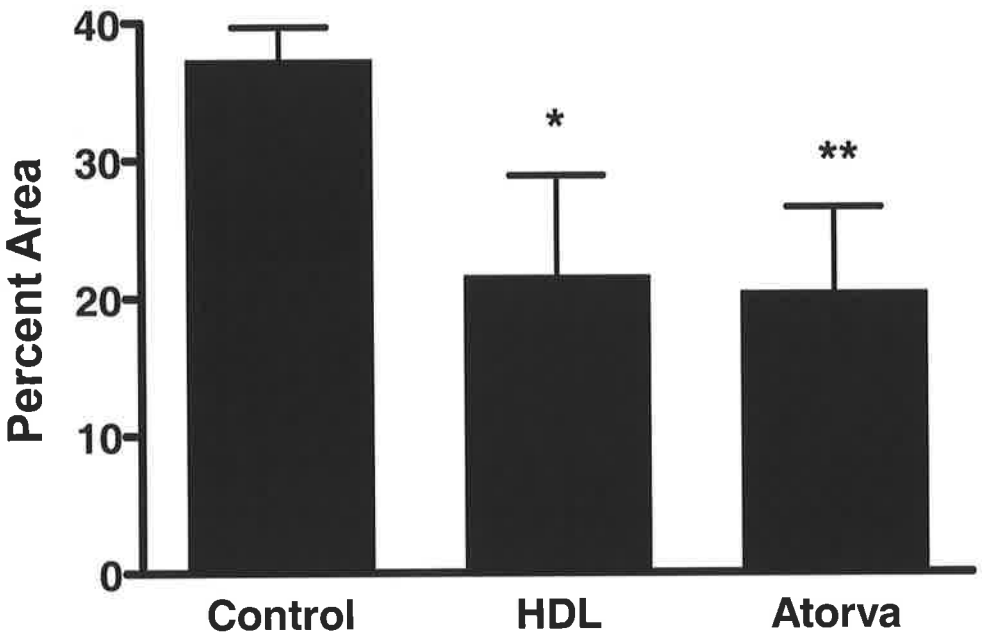

Figure 5.11. Effect of administration of HDL and atorvastatin on the plaque composition of MMP-9. Results expressed as percentage of plaque area that revealed positive staining (mean±SEM). * $P<0.05$ and ** $P<0.03$ compared with untreated animals.

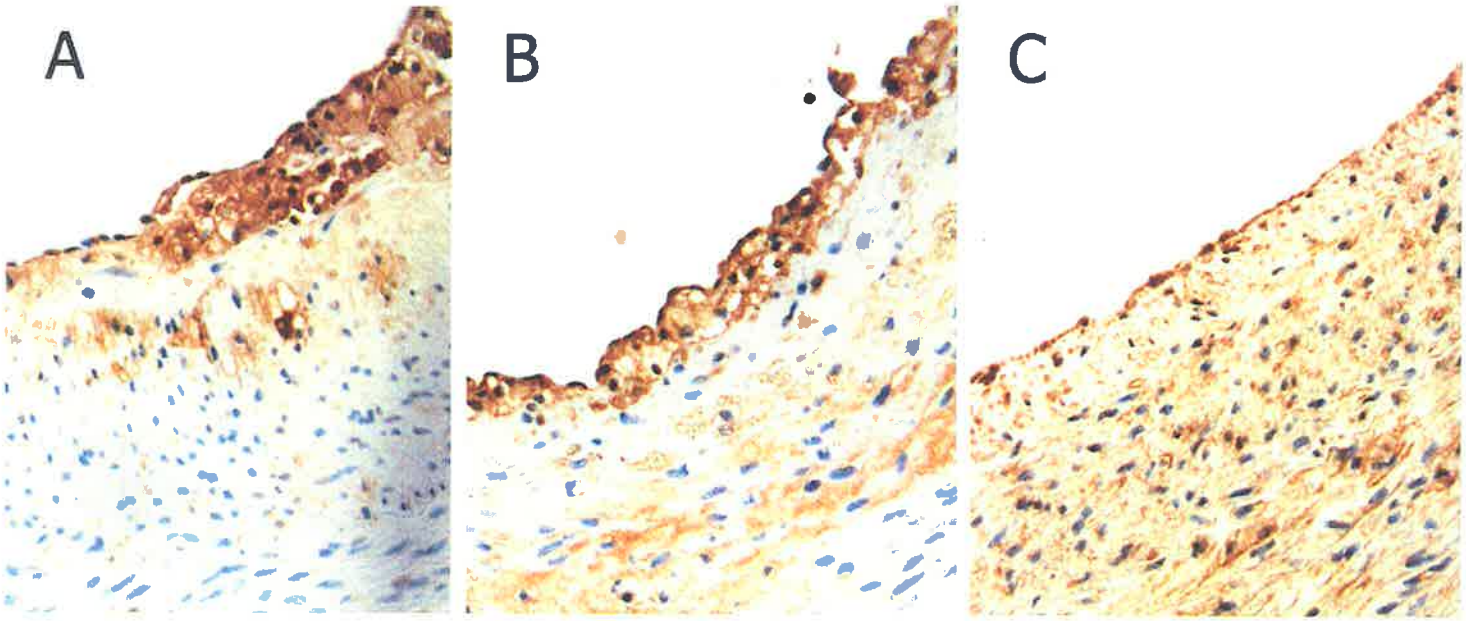

Figure 5.12. Representative staining of plaque MCP-1 in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

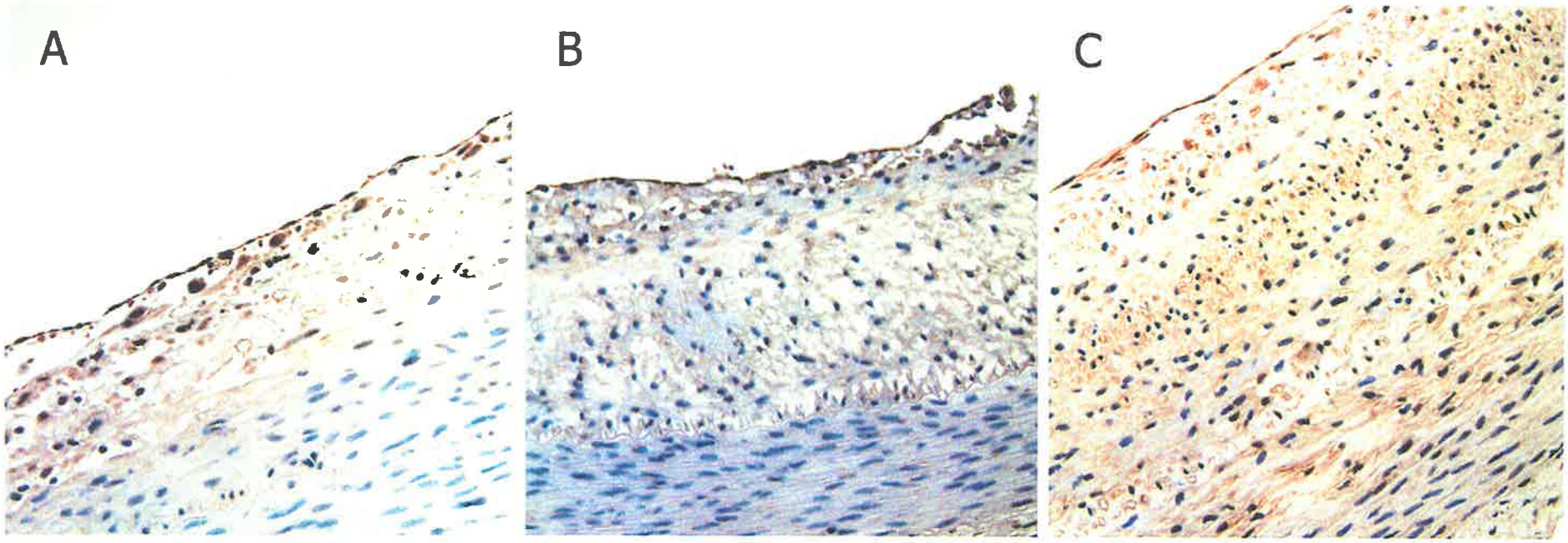

Figure 5.13. Representative staining of plaque fractalkine in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

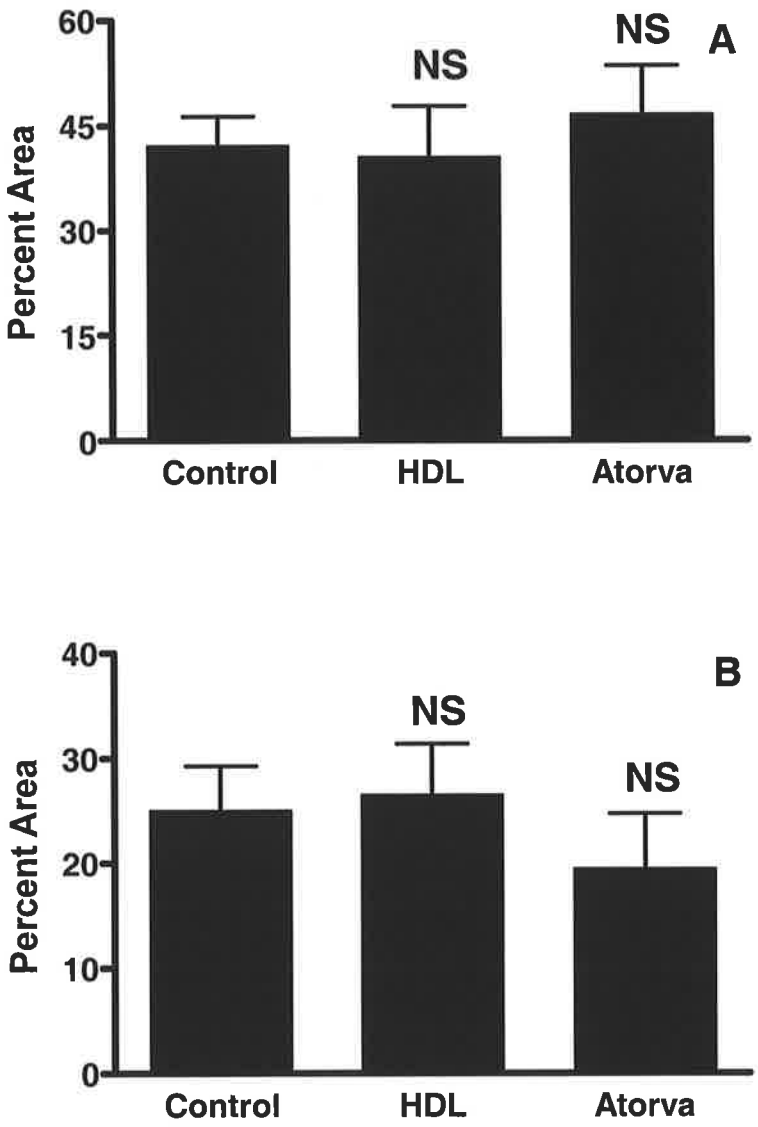

Figure 5.14. Effect of administration of HDL and atorvastatin on the plaque composition of MCP-1 (panel A) and fractalkine (panel B). Results expressed as percentage of plaque area that revealed positive staining (mean±SEM). NS nonsignificant compared with untreated animals.

CHAPTER SIX

ROLE OF HDL COMPOSITION IN PLAQUE STABILISATION

6.1 INTRODUCTION

In the previous chapter it was demonstrated that the infusion of HDL promotes the development of a stable plaque phenotype in a rabbit model of experimental atherosclerosis. This was characterised by an increase in smooth muscle cells and reduction in macrophage content. In addition, HDL induced favourable changes on thrombogenic factors and matrix metalloproteinases. These benefits were seen rapidly and were comparable to the benefit of oral atorvastatin. These results provided further support for the promotion of HDL as an effective atheroprotective strategy.

The use of rHDL allow for the investigation of how the components of HDL affect their metabolism and function. The aim of the studies outlined in this chapter are to determine if rHDL passivate established atherosclerotic plaque and whether this property is influenced by the (i) phospholipid and (ii) apolipoprotein composition of rHDL.

6.2 METHODS

6.2.1 Animals

Male New Zealand White rabbits (Institute of Medical and Veterinary Science, Gilles Plains, Australia) aged 12 weeks were maintained on a diet comprising 0.2% cholesterol enriched chow (GlenForest Stock Feed, Western Australia) throughout the study. All procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee (protocol 43/02).

6.2.2 Preparation of lipid free apoA-I and apoA-II

HDL was isolated from pooled samples of rabbit plasma (Quality Farms of Australia, Lara, Australia) by sequential ultracentrifugation in the 1.063-1.21 g/ml density range. The HDL were lyophilised and delipidated (435). ApoA-I and apoA-II were isolated from the apoHDL by chromatography on a Q-sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden) attached to an FPLC system (Amersham Biosciences) (436). The purified apoA-I and apoA-II were lyophilised and stored at –20°C until used. Lyophilised apoA-I and apoA-II were reconstituted in 3M guanidine hydrochloride and dialysed against endotoxin free phosphate-buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO) containing 0.2 g/L KH_2PO_4, 0.2 g/L KCl, 8 g/L NaCl and 1.15 g/L Na_2PO_4 before being used to prepare reconstituted HDL.

6.2.3 Preparation of rHDL

Discoidal rHDL containing apoA-I complexed to either 1-palmitoyl-2-linoleoyl phosphatidylcholine (PC) (PLPC, Sigma) or 1,2-dipalmitoyl PC (DPPC, Sigma) or apoA-II complexed to PLPC in a molar ratio of PC to apoA-I of 200:1, were prepared using the cholate dialysis method (401). The resulting rHDL were dialysed extensively against endotoxin free PBS before use. Protein (442) and phospholipid (441) concentrations were determined by enzymatic assay. The rHDL were subjected to agarose gel electrophoresis and 3-40% non-denaturing gradient gel electrophoresis (438) to determine their surface charge and Stokes' diameter.

6.2.4 Establishment of experimental atherosclerosis

Atherosclerotic plaque was induced in 31 rabbits by a combination of a high cholesterol diet, containing 0.2% cholesterol enriched chow, and balloon aortic denudation of the abdominal aorta (447). The diet, comprising 150 g/day of the cholesterol enriched chow and water provided ad libitum, was commenced one week prior to aortic denudation. Balloon denudation of the abdominal aorta was then performed under general anaesthesia. Animals were anaesthetised using intramuscular ketamine/xylazine (20/3 mg/kg) and maintained using inhaled halothane (1-3 mL/min). The right carotid artery was exposed surgically and cleared of connective tissue along a 30mm length. A bolus of heparin (100 U/kg) was administered via a cannula placed in the marginal ear vein. An arteriotomy incison was made, through which a 4F Fogarty embolectomy balloon catheter (Edwards BioLifesciences) was inserted into the artery. The catheter was advanced retrogradely, using fluoroscopic guidance, into the descending aorta and placed distal to the aortic bifurcation in the right iliac artery. The balloon was inflated using moderate resistance and pulled back to the level of the diaphragm. The balloon denudation of the abdominal aorta was repeated a further three times. The catheter was then removed from the carotid artery. The proximal carotid artery was ligated. Skin layers were sutured, the wound dressed with antibiotic, and animals allowed to recover. All animals continued the high cholesterol diet for a further 16 weeks to establish atherosclerotic plaque.

6.2.5 Experimental protocol

Animals then entered a five day treatment phase where they received either (i) rHDL containing 25 mg apoA-I (n=6), (ii) rHDL containing 31 mg apoA-II (n=5), (iii) rHDL containing 25 mg apoA-I and PLPC (n=5) or (iv) no treatment (n=15). Infusions of rHDL were administered

intravenously via the marginal ear vein on days 1 and 3 of the treatment phase. The high cholesterol diet was continued throughout the treatment phase. On the fifth day of the treatment phase, blood was sampled from a marginal ear vein. Animals received heparin (100 U/kg, i.v.) five minutes prior to euthanasia to prevent postmortem thrombosis. Animals were euthanased with an overdose of sodium pentobarbitone (90 mg/kg, i.v.). The aortic root was cannulated and the aorta was flushed with 500 ml phosphate-buffered saline (PBS, pH 7.4), followed by perfusion fixation with 500 ml of 4% paraformaldehyde in PBS at 100 mmHg. Following perfusion fixation, the aorta was removed and immersed in fresh fixative.

6.2.6 Immunohistochemistry

Specimens were paraffin-embedded and serial 5 μm slices were cut. One section was stained with haematoxylin and eosin. The other sections were used for immunohistochemical analysis which were stained as outlined in chapter 2. Antibodies applied included mouse monoclonal anti-rabbit RAM11 (DAKO, 1/200), mouse monoclonal anti-rabbit smooth muscle actin (Sigma, 1/60000), mouse monoclonal anti-rabbit tissue factor (American Diagnostica, 1/500), mouse monoclonal anti-human PAI-1 (American Diagnostica, 1/1000), goat polyclonal anti-rabbit thrombomodulin (American Diagnostica, 1/2000) and mouse monoclonal anti-human MMP-9 (Oncogene, 1/200). Digital micrographs of sections were acquired using an Olympus BX40 microscope. The percentage of plaque area containing positive staining was determined using ImagePro Plus (Cybernetics).

6.2.7 Plasma analyses

Plasma collected at both the commencement of the study and treatment phase and prior to sacrifice of the animal was stored at -80°C in EDTA until required for analysis. All chemical analyses were carried out on a Roche Diagnostics/Hitachi 902 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Triglyceride (443) and free cholesterol (444) concentrations were determined enzymatically. Total cholesterol was determined using a Roche Diagnostics kit. HDL cholesterol was determined by enzymatic assay following precipitation of apolipoprotein B containing lipoproteins with polyethylene glycol (459). ApoA-I concentrations were determined by an immunoturbidometric assay using a sheep anti-rabbit apoA-I immunoglobulin (437).

6.2.8 Data analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made by Student's t-tests and one way ANOVA using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered significant.

6.3 RESULTS

6.3.1 Plasma lipid profile

Plasma total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol are presented in table 6.1. Administration of the cholesterol enriched diet resulted in a predictable increase in total cholesterol (1.51±0.16 and 12.86±2.2 mmol/L in the control treatment group at baseline and sacrifice respectively). Infusion of rHDL did not result in a significant change in plasma HDL

(0.31±0.03, 0.27±0.05 and 0.29±0.08 mmol/L in AI-PLPC, AI-DPPC and AII-PLPC infused animals prior to treatment and 0.34±0.04, 0.3±0.06 and 0.29±0.08 mmol/L in AI-PLPC, AI-DPPC and AII-PLPC infused animals at the time of sacrifice).

6.3.2 Plaque cellular composition

6.3.2.1 Role of HDL phospholipid composition

Representative sections of atherosclerotic plaque staining for the presence of macrophages and smooth muscle cells are shown in figures 6.1 and 6.2. The plaque ratio of smooth muscle cells to macrophages was 4.2 fold higher in PLPC treated animals (19.7±7.9 vs. 4.7±0.9 in PLPC and untreated animals respectively, $P<0.01$) and 4.1 fold higher in DPPC treated animals (19.5±10.5 vs. 4.7±0.9 in DPPC and untreated animals respectively, $P<0.02$). (Fig 6.3) This effect resulted largely from an increase in plaque smooth muscle cell composition by 91% in PLPC treated animals (69.4±3.1 vs. 36.3±3.8 percent plaque area in PLPC and untreated animals respectively, $P<0.01$) and 90% in DPPC treated animals (69.1±4.5 vs. 36.3±3.8 percent plaque area in DPPC and untreated animals respectively, $P<0.01$). (Fig 6.4) In contrast, there was no significant difference in macrophage composition in PLPC, DPPC and untreated animals (9.1±4, 8±2.9 and 12.4±2.3 percent plaque area respectively). (Fig 6.5) Plaques from animals treated with PLPC and DPPC did not significantly differ with regards to the plaque composition of smooth muscle cells or macrophages.

6.3.2.2 Role of HDL apolipoprotein composition

Representative sections of atherosclerotic plaque staining for the presence of macrophages and smooth muscle cells are shown in figures 6.1 and 6.2. The plaque ratio of smooth muscle cells to macrophages was 4.2 fold higher in apoA-I treated animals (19.7±7.9 vs. 4.7±0.9 in HDL and untreated animals respectively, P<0.01) and 1.7 fold higher in apoA-II treated animals (7.9±4.9 vs. 4.7±0.9 in apoA-II and untreated animals respectively, NS). (Fig 6.3) This effect resulted largely from an increase in plaque smooth muscle cell composition by 91% in apoA-I treated animals (69.4±3.1 vs. 36.3±3.8 percent plaque area in apoA-I and untreated animals respectively, P<0.01) and 29% in apoA-II treated animals (46.9±7.8 vs. 36.3±3.8 percent plaque area in apoA-II and untreated animals respectively, NS). (Fig 6.4) In contrast, there was no significant difference in macrophage composition in apoA-I, apoA-II and untreated animals (9.1±4, 11.6±4.2 and 12.4±2.3 percent plaque area respectively). (Fig 6.5) Plaques from animals treated with apoA-I had 48% greater smooth muscle cells and 22% less macrophages than apoA-II resulting in a 2.5 fold higher ratio of smooth muscle cells to macrophages.

6.3.3 Thrombogenic features of atherosclerotic plaque

6.3.3.1 Role of HDL phospholipid composition

Representative staining of thrombogenic features of atherosclerotic plaque are shown in figures 6.6, 6.8 and 6.10. Plaque composition of thrombomodulin increased by 4.1 fold in PLPC treated animals (19.9±6.8 vs. 4.9±0.7 percent plaque area in PLPC and untreated animals respectively, P<0.01) and by 2.8 fold in DPPC treated animals (13.9±2.1 vs. 4.9±0.7 percent plaque area in DPPC and untreated animals respectively, P<0.01). (Fig 6.7) In addition, plaque composition of PAI-1 decreased by 24% in PLPC treated animals (23±4 vs. 30.3±2.8 percent plaque area in

PLPC and untreated animals respectively, NS) and 19% in DPPC treated animals (24.5±8.7 vs. 30.3±2.8 percent plaque area in DPPC and untreated animals respectively, NS). (Fig 6.9) Neither treatment modified the plaque composition of tissue factor (22±6.1, 28±9.9 and 22±1.9 percent plaque area in PLPC, DPPC and untreated animals respectively, NS) compared with untreated animals. (Fig 6.11)

6.3.3.2 Role of HDL apolipoprotein composition

Representative staining of thrombogenic features of atherosclerotic plaque are shown in figures 6.6, 6.8 and 6.10. Plaque composition of thrombomodulin increased by 4.1 fold in apoA-I treated animals (19.9±6.8 vs. 4.9±0.7 percent plaque area in apoA-I and untreated animals respectively, $P<0.01$) and by 3.3 fold in apoA-II treated animals (16.1±4.6 vs. 4.9±0.7 percent plaque area in apoA-II and untreated animals respectively, $P<0.01$). (Fig 6.7) In addition, plaque composition of PAI-1 decreased by 24% in apoA-I treated animals (23±4 vs. 30.3±2.8 percent plaque area in apoA-I and untreated animals respectively, NS) and 49% in apoA-II treated animals (15.5±4.7 vs. 30.3±2.8 percent plaque area in apoA-II and untreated animals respectively, $P<0.02$). (Fig 6.9) Neither treatment modified the plaque composition of tissue factor (22±6.1, 17.1±5.5 and 22±1.9 percent plaque area in apoA-I, apoA-II and untreated animals respectively, NS) compared with untreated animals. (Fig 6.11)

6.3.4 Atherosclerotic plaque composition of matrix metalloproteinases

6.3.4.1 Role of HDL phospholipid composition

Representative staining of the matrix metalloproteinase MMP-9 in atherosclerotic plaque are shown in figure 6.12. Plaque composition of MMP-9 decreased by 35% in PLPC treated animals (24.2±4.8 vs. 37.3±2.6 percent plaque area in apoA-I and untreated animals respectively, $P<0.05$). However, there was no effect on plaque MMP-9 content in DPPC treated animals (37.8±5.5 vs. 37.3±2.6 percent plaque area in DPPC and untreated animals respectively, NS). (Fig 6.13) The difference in plaque MMP-9 content between PLPC and DPPC treated animals was statistically significant ($P<0.05$).

6.3.4.2 Role of HDL apolipoprotein composition

Representative staining of the matrix metalloproteinase MMP-9 in atherosclerotic plaque are shown in figure 6.12. Plaque composition of MMP-9 decreased by 35% in apoA-I treated animals (24.2±4.8 vs. 37.3±2.6 percent plaque area in apoA-I and untreated animals respectively, $P<0.05$) and by 60% in apoA-II treated animals (15.1±4.2 vs. 37.3±2.6 percent plaque area in apoA-II and untreated animals respectively, $P<0.01$). (Fig 6.13)

6.4 DISCUSSION

The studies described in this chapter extend the results presented in chapter five to demonstrate that the infusion of rHDL also rapidly promotes the development of a stable phenotype in a model of established experimental atherosclerosis. The results also demonstrate that this property of rHDL is influenced by its phospholipid and apolipoprotein composition.

The current findings extend previous reports that rHDL have a beneficial impact on the arterial wall *in vivo*. rHDL inhibit the in vitro expression of adhesion molecules by activated endothelial cells (188,190). Infusion of rHDL has also been demonstrated to have favourable anti-inflammatory properties in animal models. The expression of adhesion molecules and macrophage accumulation within the arterial wall, induced by application of carotid periarterial collars, was inhibited by infusion of rHDL in cholesterol fed apoE knockout mice (83). In addition, a single infusion of rHDL inhibited the expression of E-selectin by subcutaneous vessels induced by the intradermal infiltration of interleukin-1 (208). Futhermore, the studies presented in chapter four demonstrate that the infusion of rHDL inhibits the expression of adhesion molecules and proinflammatory chemokines, infiltration of neutrophils and expression of vascular ROS induced by the application of carotid periarterial collars in normocholesterolaemic rabbits. These benefits have now been extended to human subjects. A single infusion of rHDL restores endothelial function in subjects with hypercholesterolaemia (78) and low plasma HDL (79), whilst weekly infusions of rHDL rapidly promote atherosclerotic regression when assessed by intravascular ultrasound (80). The mechanism that confers this benefit of rHDL is uncertain. It has been demonstrated that discoidal forms of HDL are efficient promoters of cholesterol efflux (481). It is possible that these forms of HDL are also more efficient with regards to the other established functional properties of HDL.

The use of rHDL in experimental models allows for the assessment of the effect of its components on their metabolism and function. The predominant HDL apolipoproteins are apoA-I and apoA-II (39). It is uncertain whether these proteins confer differential functional properties on HDL. The current study demonstrates that when rHDL contain apoA-II they lose their ability

to promote favourable cellular changes in established atherosclerotic plaque. In particular rHDL no longer promotes an increase in smooth muscle cells and reduction in macrophages, as seen when rHDL contain apoA-I. These results provide further support for the proposition that apoA-II is potentially pro-atherogenic (96). In contrast, the effect of rHDL apolipoprotein composition on thrombogenic factors and MMP-9 are less clear. Whilst there did not appear to be any effect of HDL apolipoprotein composition on thrombomodulin expression, rHDL containing apoA-II appeared to have a favourable effect on the expression of PAI-1 and MMP-9. These variable results highlight the complexity of the relationship between apoA-II and atherosclerotic risk.

In contrast to the well established protective role of apoA-I, the influence of apoA-II on atherogenic risk remains uncertain. Population studies have demonstrated that plasma apoA-II is inversely related to clinical events (97). In addition, apoA-II deficiency is rare in humans and is not associated with an increase in cardiovascular risk (98). However, a normal level of apoA-II is not able to compensate for the increased risk associated with apoA-I deficient states (99). Animal models have produced variable results. The effect on atherogenesis appears to be influenced by both the animal model studied and the genetic origin of the apoA-II (100,101,103,104). The current study similarly reveals variable effects on plaque composition. The development of animal models with a high incidence of spontaneous plaque rupture would provide an ideal environment to assess the functional significance of these findings.

In addition, the phospholipid composition of rHDL has a variable effect on the composition of established atherosclerotic plaque. Phospholipid composition had no impact on the atheroma's cell phenotype. In contrast, rHDL containing the polyunsaturated phospholipid PLPC appeared to

have a beneficial effect on the expression of thrombogenic factors and MMP-9. rHDL containing PLPC promoted a greater increase in plaque thrombomodulin than rHDL containing DPPC. In addition, the plaque composition of tissue factor was greater in animals treated with rHDL containing DPPC. Furthermore, whilst rHDL containing PLPC reduced the plaque expression of MMP-9, particles containing DPPC had no effect. These variable results extend the findings of previous studies that have addressed the effect of rHDL phospholipid composition on its function. *In vitro* studies have demonstrated that while rHDL containing polyunsaturated phospholipids inhibit expression of VCAM-1 by activated endothelial cells, when the phospholipid contains saturated fatty acids this anti-inflammatory activity is lost (194). These results may reflect the differences in the anti-inflammatory properties between different human subjects (193). In addition, they suggest that HDL may act as a vehicle to carry dietary fatty acids to cells in the arterial wall where they have a differential effect on their response to proinflammatory stimuli. However, the studies outlined in chapters three and four found no marked effect of altering phospholipid composition of either chylomicron-like emulsions or rHDL on the anti-inflammatory properties of HDL.

In summary, the findings of this chapter demonstrate that the phospholipid and protein composition of rHDL have variable but important impacts on its ability to passivate experimental atherosclerotic plaque. These results highlight how HDL heterogeneity may have a profound influence on their atheroprotective role.

	Baseline				Sacrifice			
	TC	TG	HDL-C	LDL-C	TC	TG	HDL-C	LDL-C
Control	1.51±0.16	0.83±0.1	0.31±0.03	0.82±0.12	12.86±2.2	0.74±0.22	0.4±0.09	12.12±2.12
AI-PLPC	1.69±0.18	0.66±0.12	0.46±0.05	0.93±0.13	9.73±2.75	0.47±0.19	0.34±0.04	9.22±2.7
AI-DPPC	1.65±0.16	1.04±0.21	0.37±0.02	0.89±0.08	15.25±2.99	0.96±0.55	0.3±0.06	14.54±2.91
AII-PLPC	1.54±0.35	0.64±0.13	0.41±0.07	0.84±0.3	12.94±3.37	0.4±0.14	0.29±0.08	12.47±3.35

Table 6.1. Plasma lipid profile of animals at baseline, prior to treatment and at the time of sacrifice. Results expressed as mmol/L (mean±SEM). TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol.

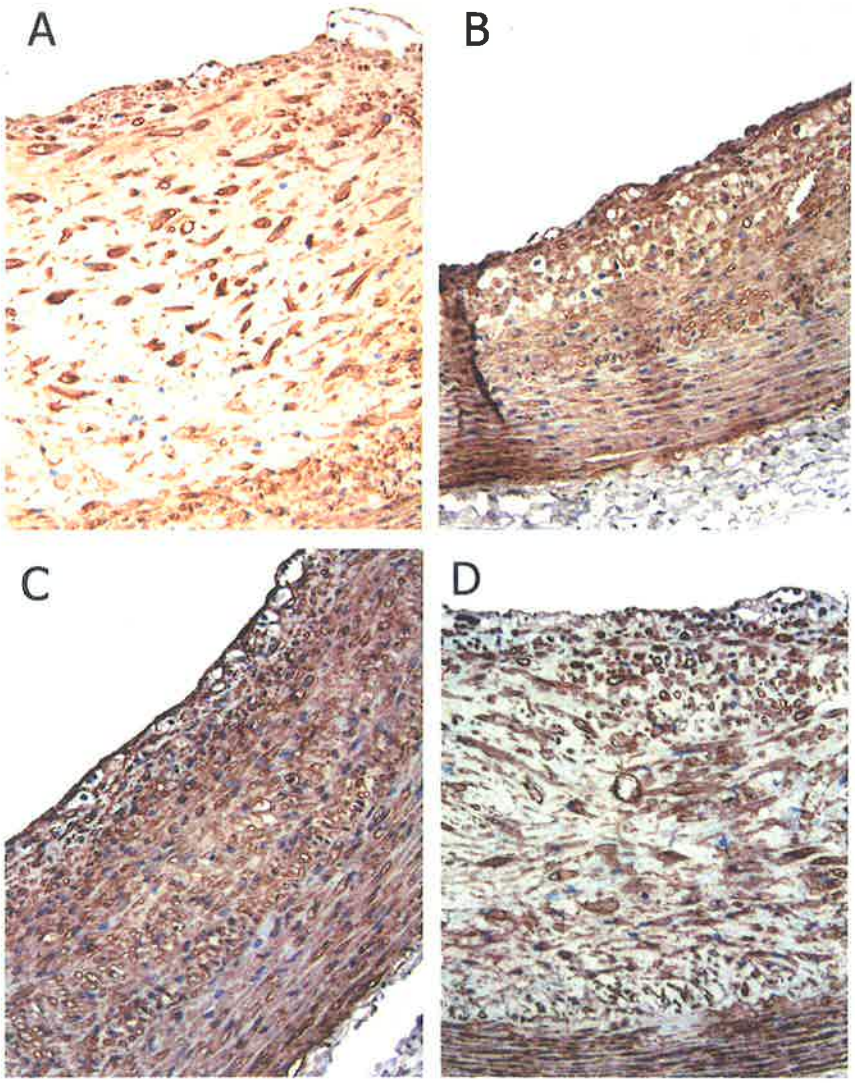

Figure 6.1. Representative staining of plaque smooth muscle actin in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

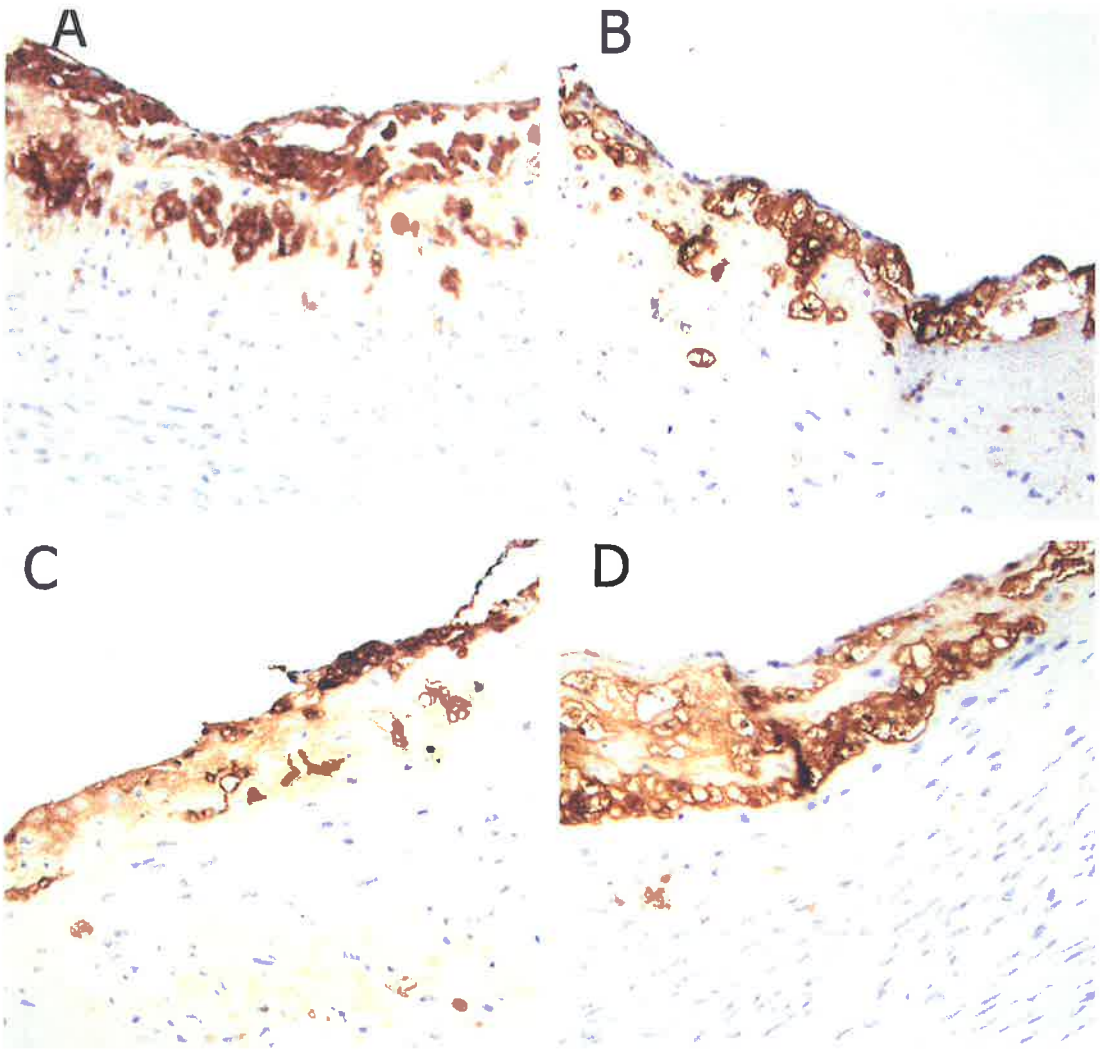

Figure 6.2. Representative staining of plaque macrophages in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

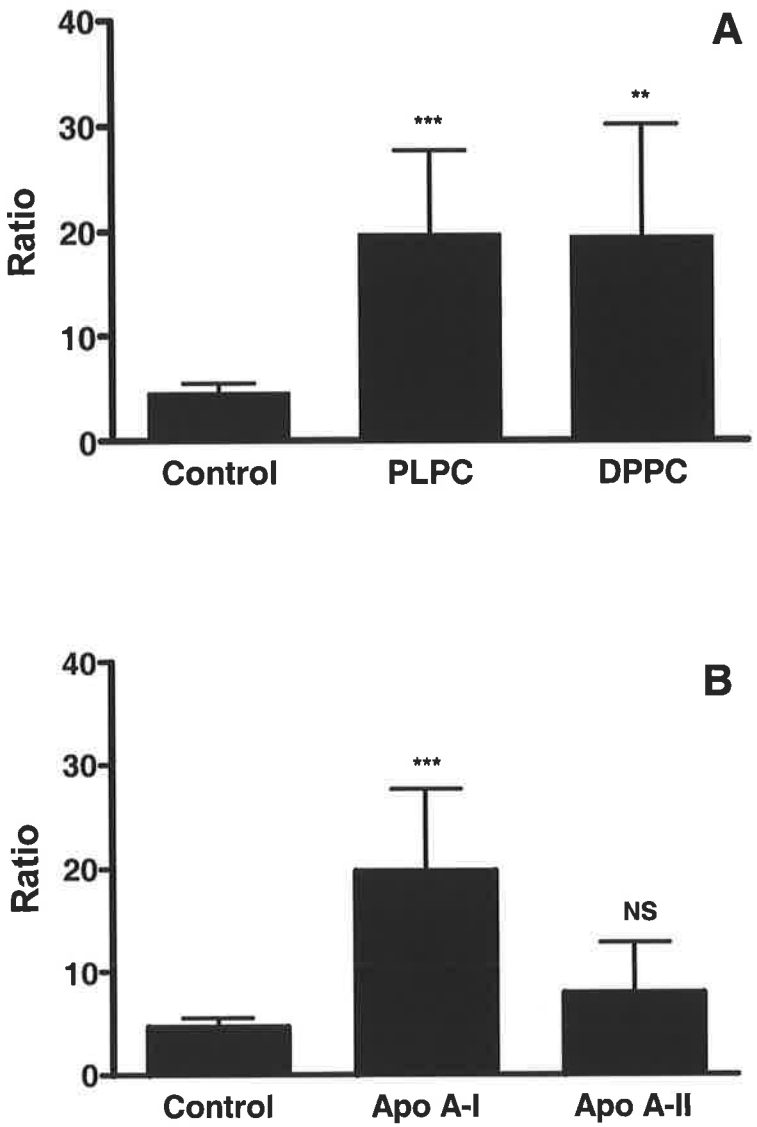

Figure 6.3. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the ratio of smooth muscle cells to macrophages in established atherosclerotic plaque. Results expressed as mean ± SEM. NS non significant, ** $P<0.02$ and *** $P<0.01$ for comparisons with untreated animals.

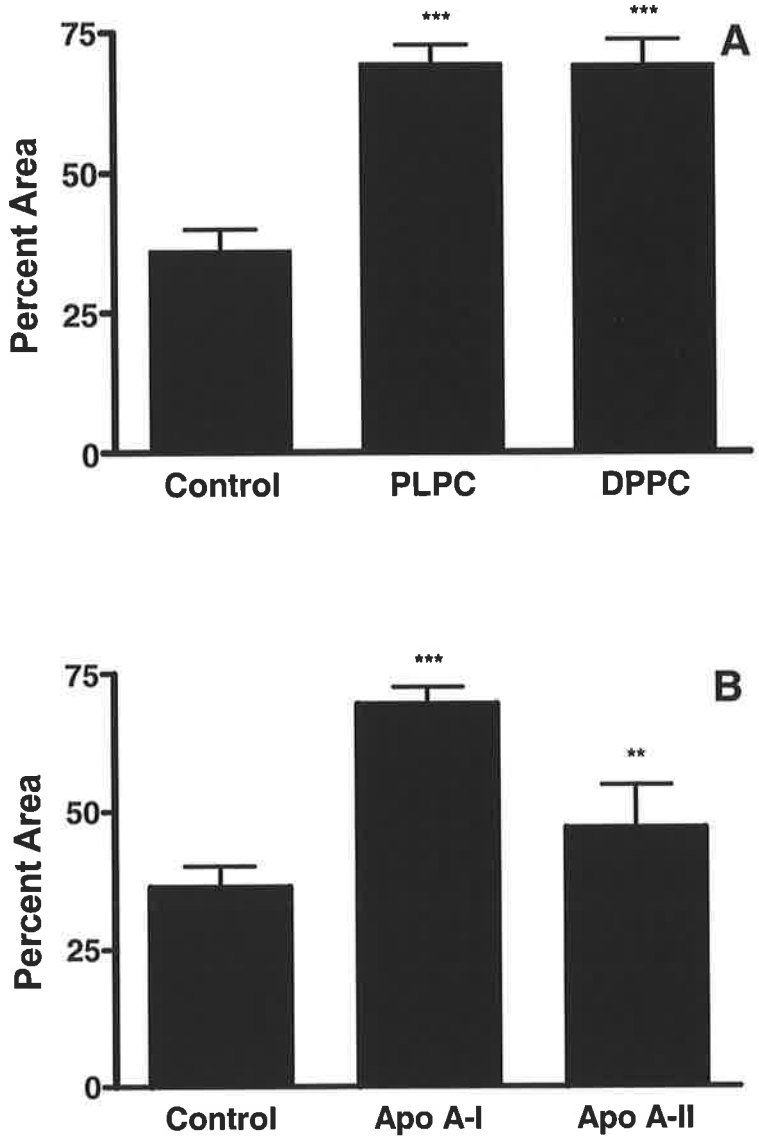

Figure 6.4. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the expression of smooth muscle cells in established atherosclerotic plaque. Results expressed as percentage of plaque area (mean ± SEM). NS non significant and *** $P<0.01$ for comparisons with untreated animals.

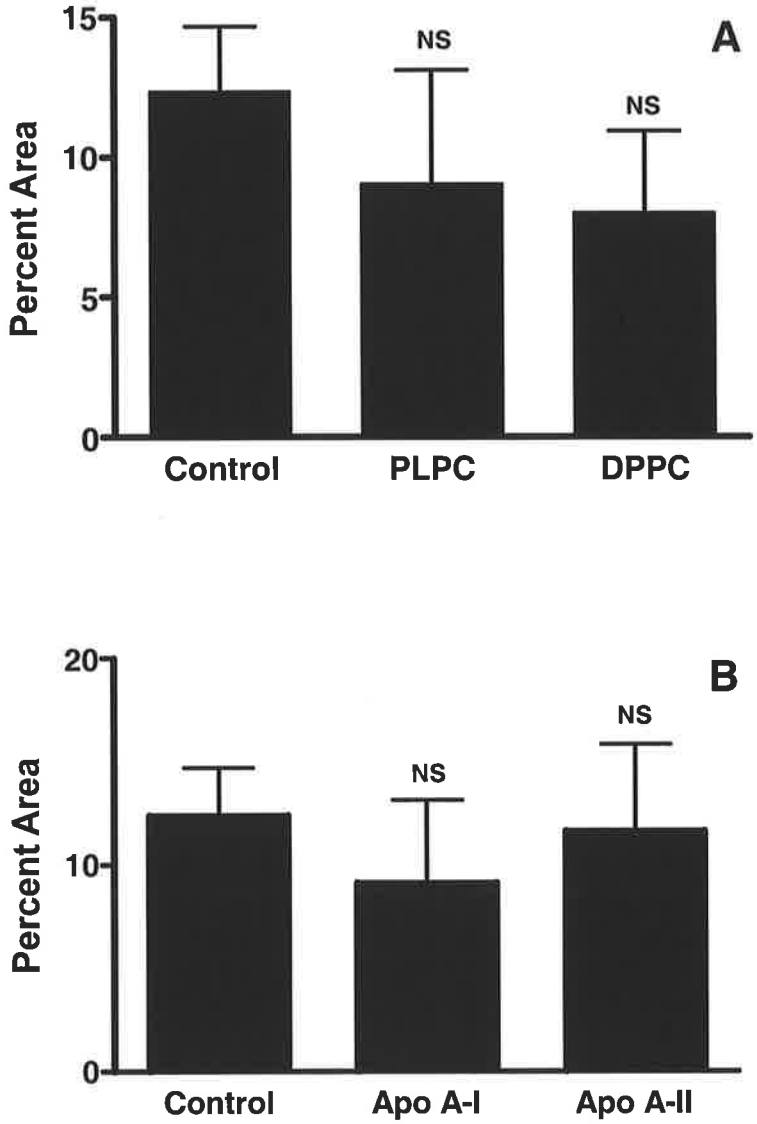

Figure 6.5. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the expression of macrophages in established atherosclerotic plaque. Results expressed as percentage of plaque area (mean ± SEM). NS non significant for comparison with untreated animals.

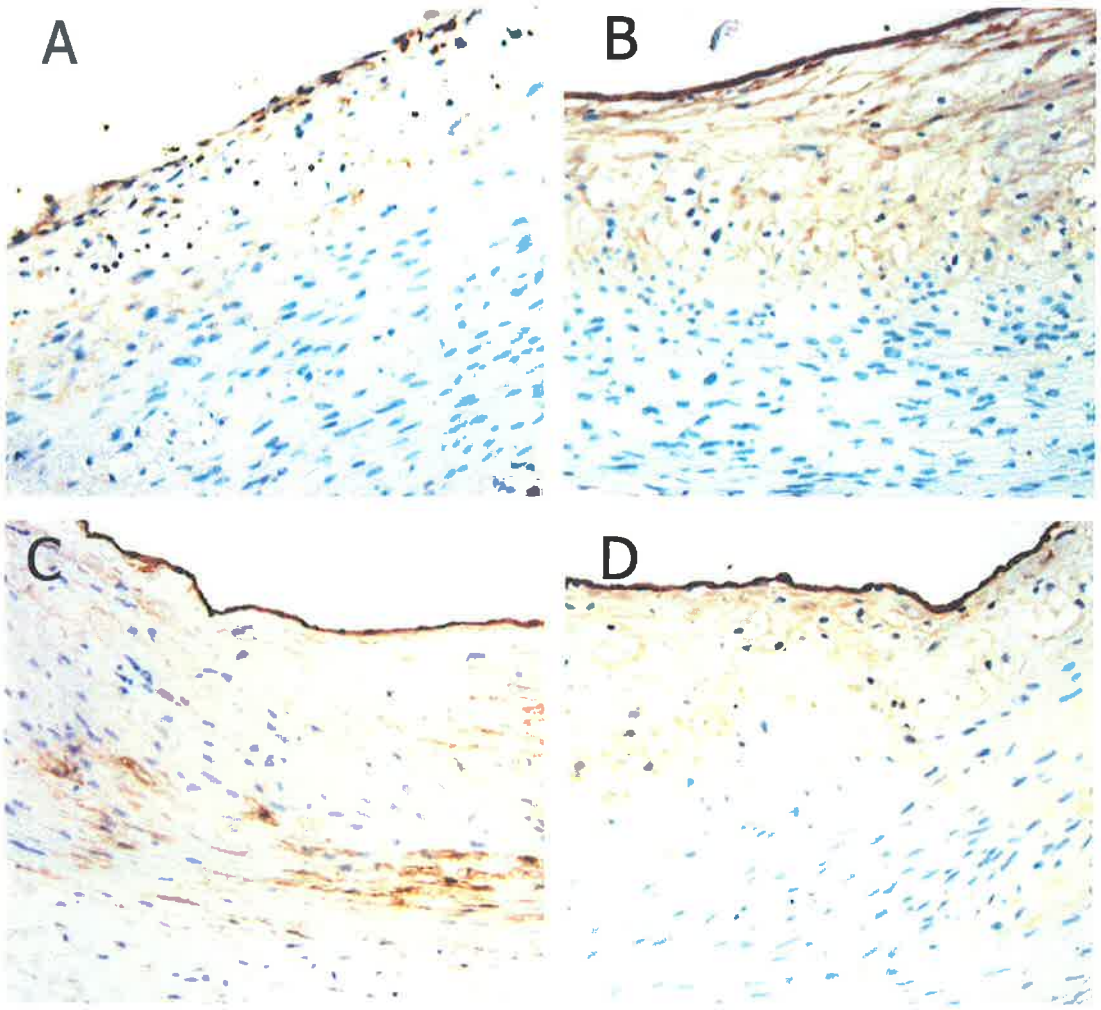

Figure 6.6. Representative staining of plaque thrombomodulin in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

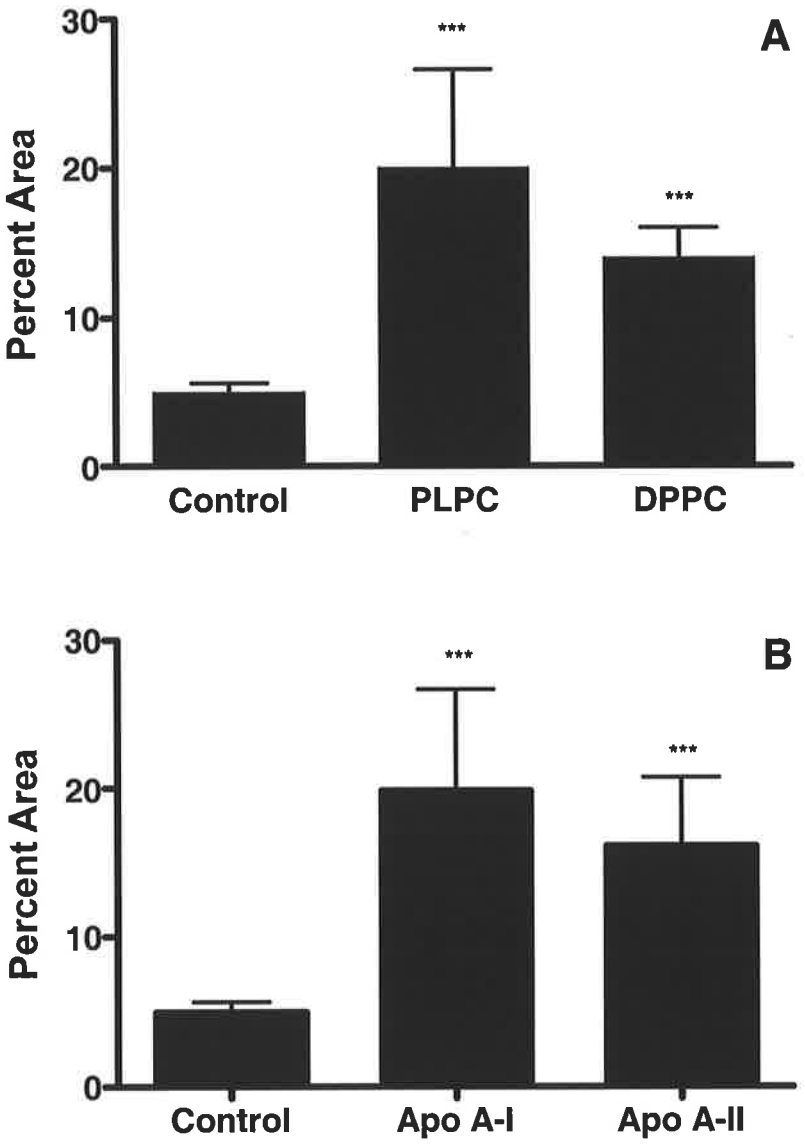

Figure 6.7. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the expression of thrombomodulin in established atherosclerotic plaque. Results expressed as percentage of plaque area (mean ± SEM). *** $P<0.01$ for comparison with untreated animals.

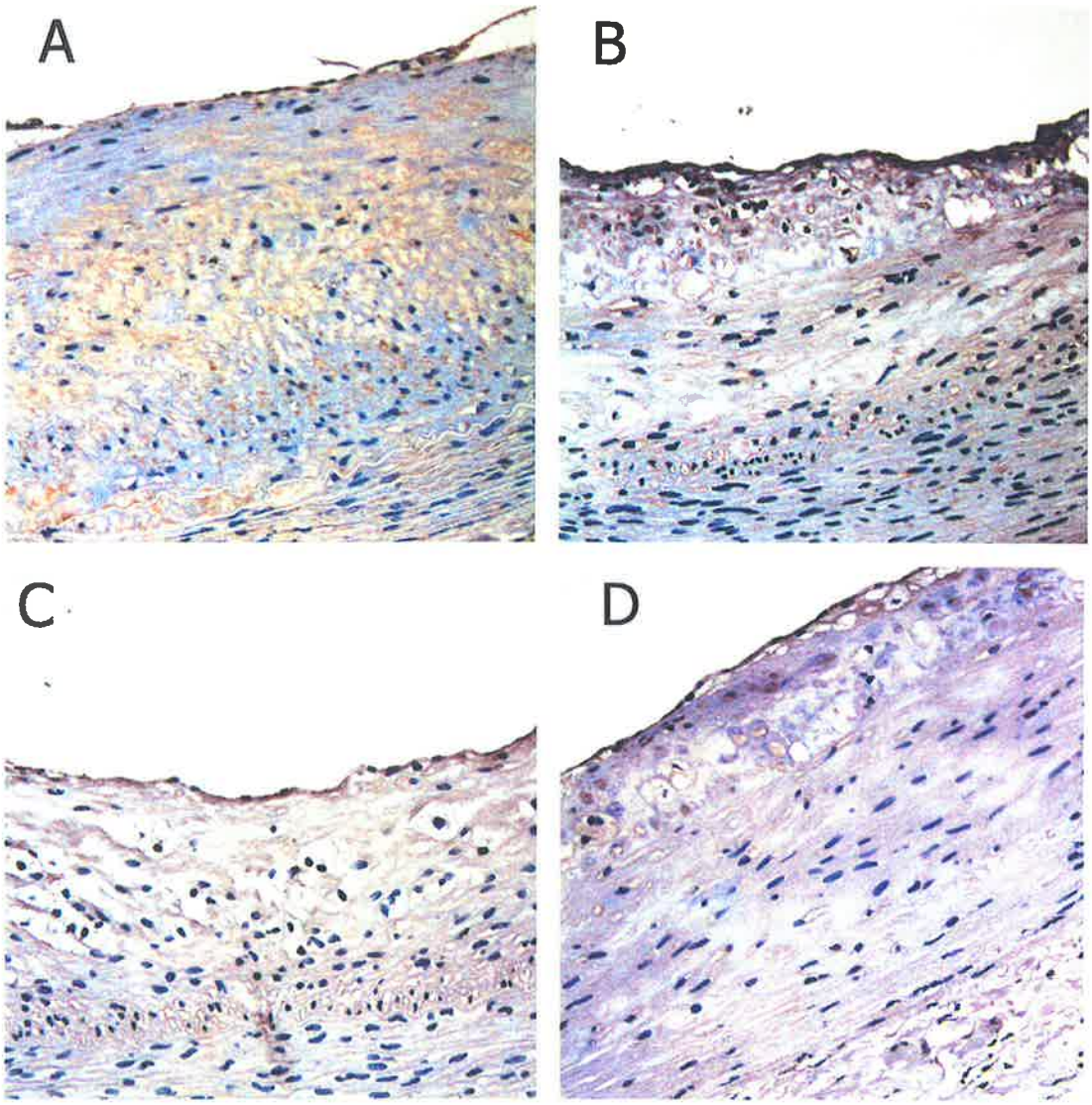

Figure 6.8. Representative staining of plaque PAI-1 in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

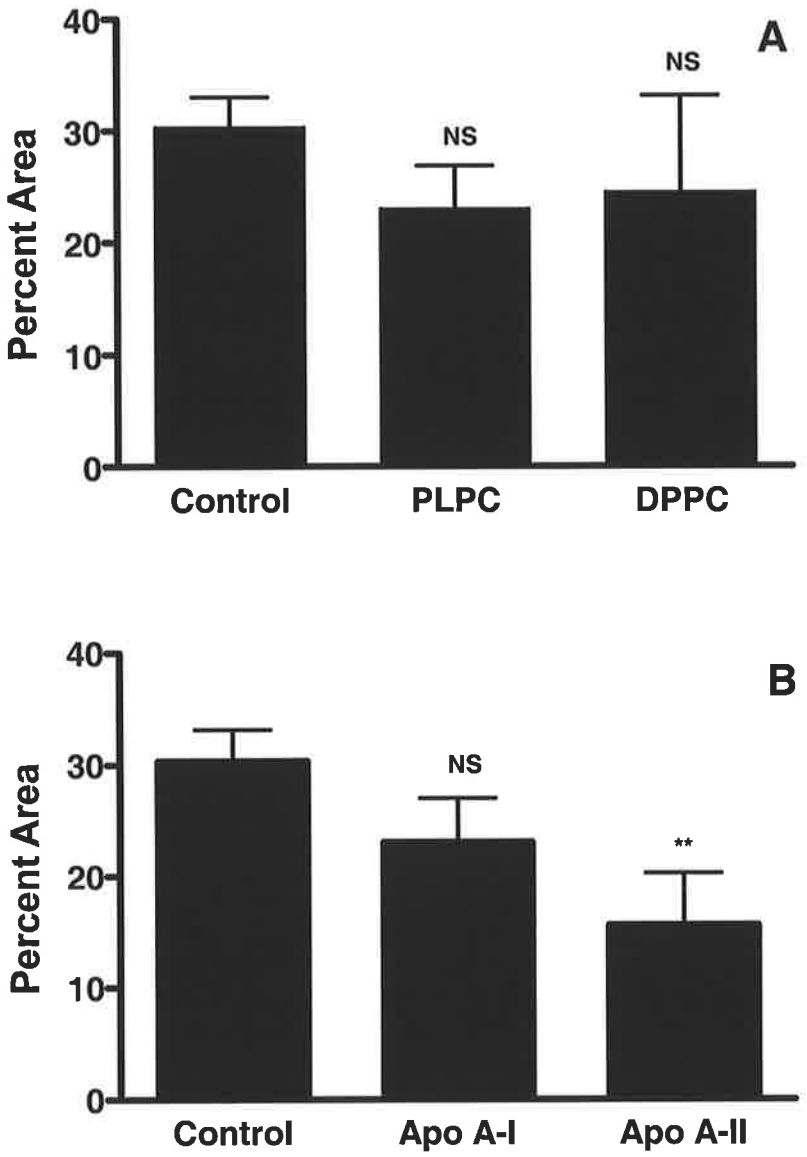

Figure 6.9. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the expression of PAI-1 in established atherosclerotic plaque. Results expressed as percentage of plaque area (mean ± SEM). NS non significant and ** $P<0.02$ for comparison with untreated animals.

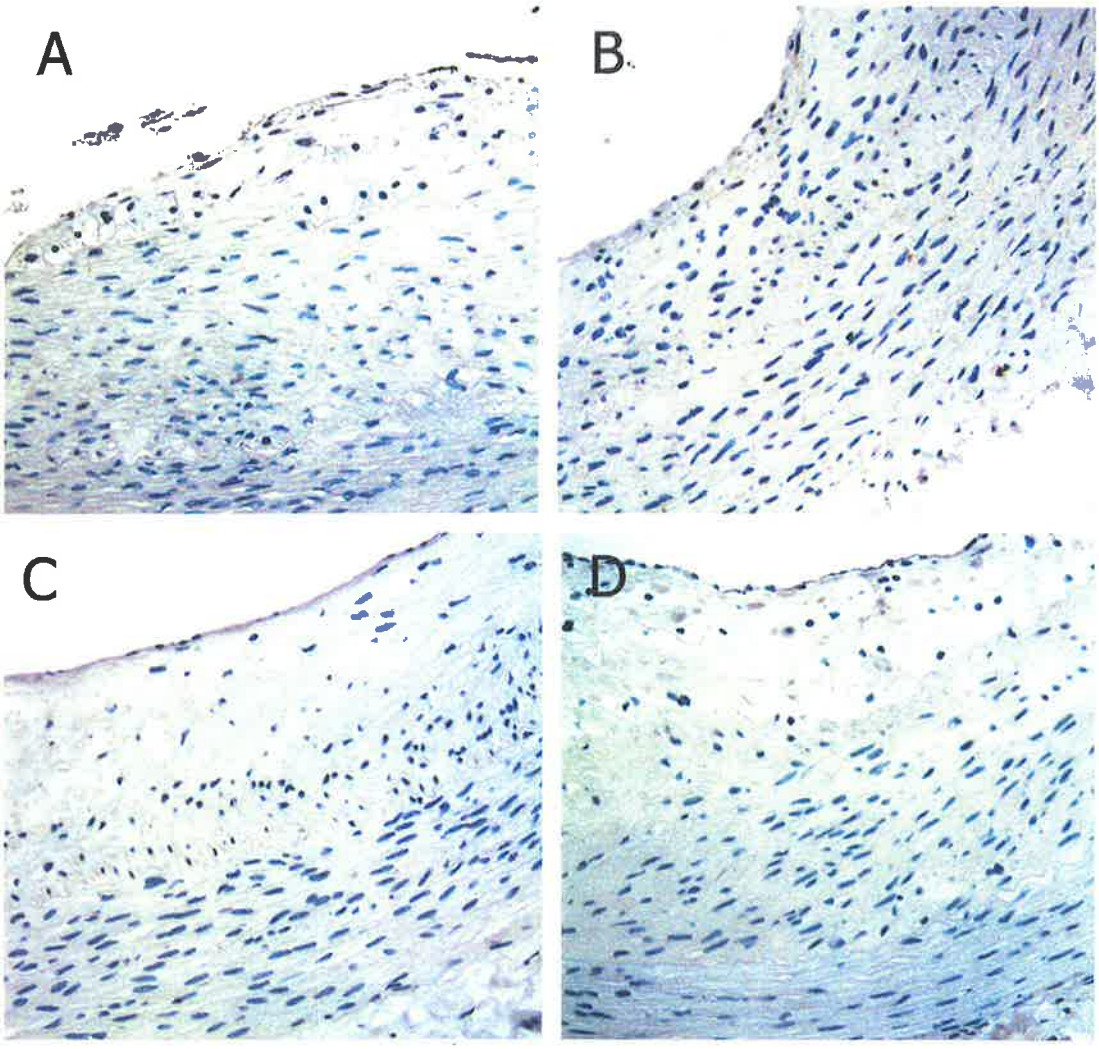

Figure 6.10. Representative staining of plaque tissue factor in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

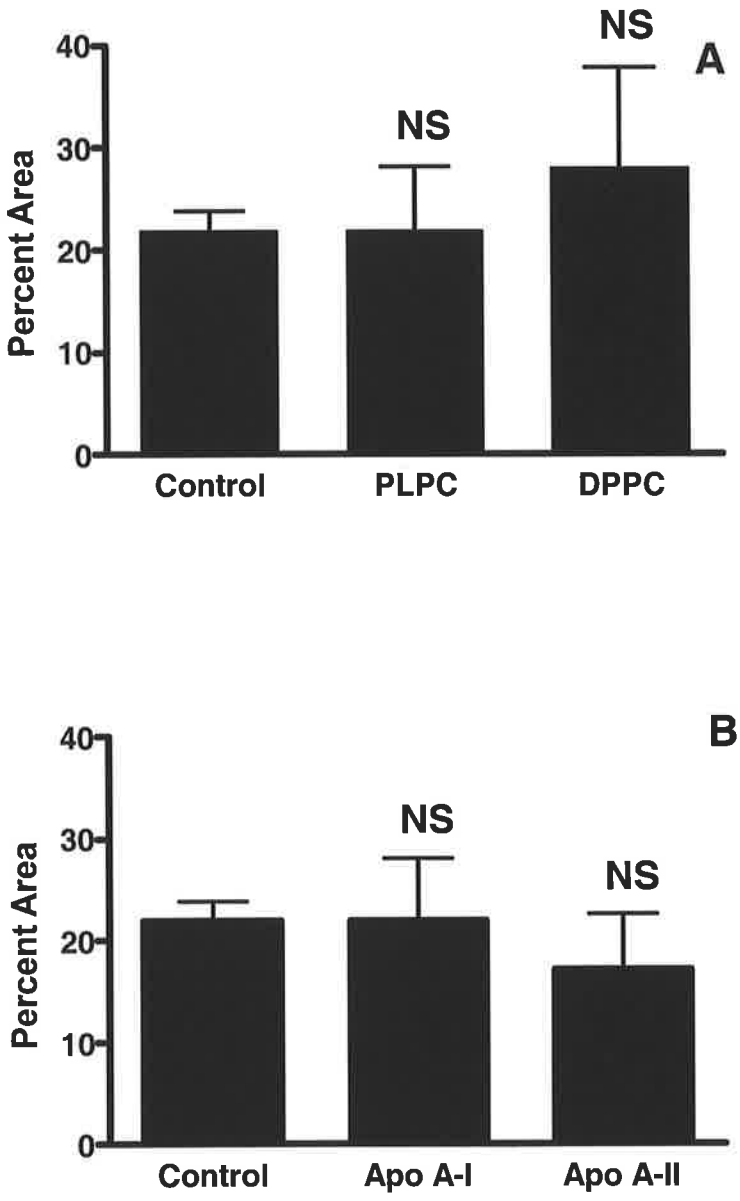

Figure 6.11. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the expression of tissue factor in established atherosclerotic plaque. Results expressed as percentage of plaque area (mean ± SEM). NS non significant for comparison with untreated animals.

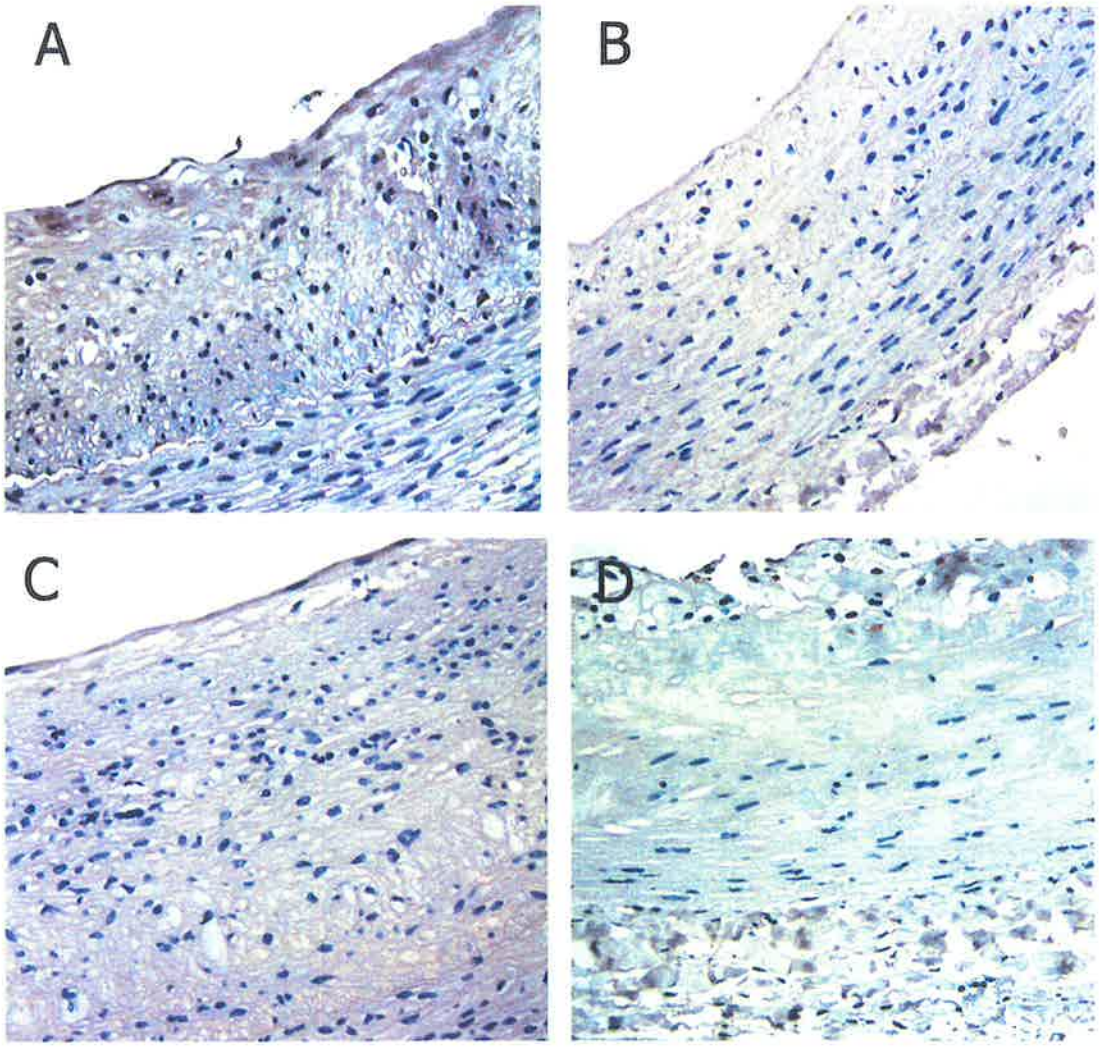

Figure 6.12. Representative staining of plaque MMP-9 in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

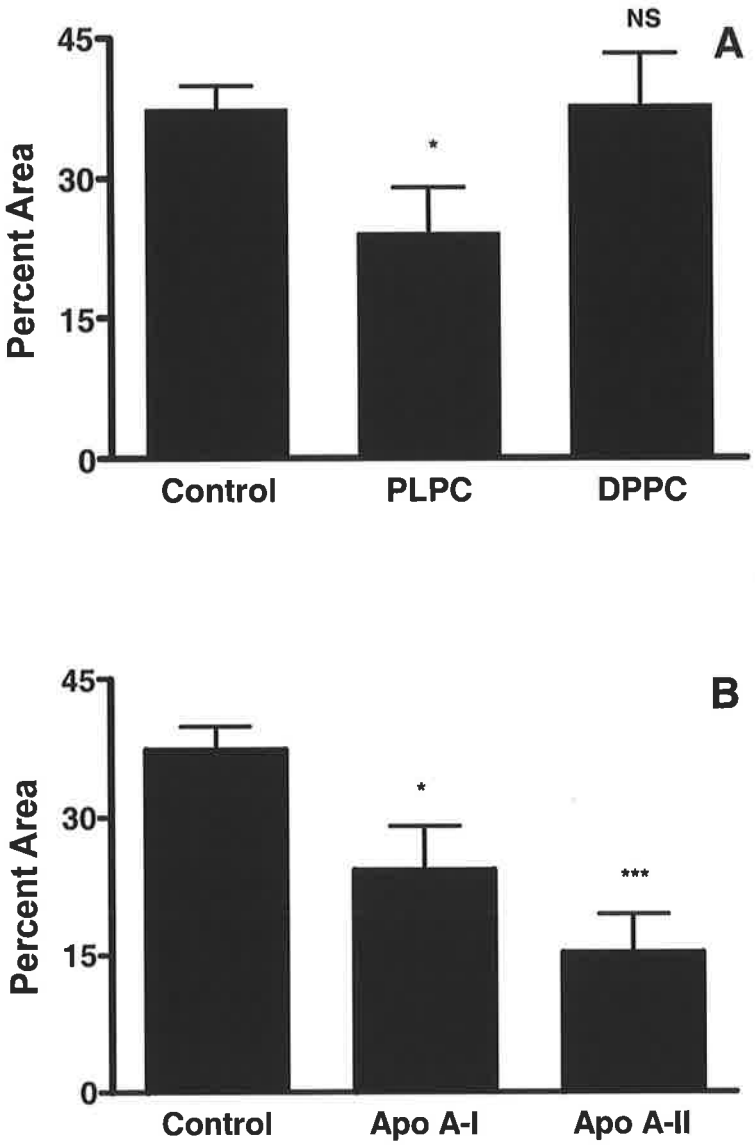

Figure 6.13. Effect of phospholipid (A) and apolipoprotein (B) composition of rHDL on the expression of MMP-9 in established atherosclerotic plaque. Results expressed as percentage of plaque area (mean ± SEM). NS non significant, * $P<0.05$ and *** $P<0.01$ for comparison with untreated animals.

CHAPTER SEVEN

EFFECT OF DIETARY FATTY ACIDS ON HDL AND ENDOTHELIAL FUNCTION

7.1 INTRODUCTION

There is a complex relationship between the consumption of dietary fat and atherosclerotic cardiovascular disease. Population studies have established that the intake of dietary fat correlates with clinical events (404). In addition, dietary fatty acid composition has a major influence on atherogenesis. Animal studies have demonstrated that the administration of dietary saturated fat increases atherosclerotic burden (431). Furthermore, human studies have demonstrated that dietary interventions including an increased consumption of monounsaturated fat result in fewer clinical events in subjects with established atherosclerotic disease (422).

The precise mechanisms that link the consumption of dietary fatty acids and atherosclerotic risk remains uncertain. It has been demonstrated that the consumption of different dietary fatty acids vary in their ability to promote the development of an atherogenic lipid profile, in addition to circulating pro-inflammatory and thrombogenic factors (423,428-430). In addition, considerable attention has focussed on the response of endothelium-dependent vasodilatation following a fatty meal. Some (414), but not all (415), groups have demonstrated that flow mediated dilatation, a measure of endothelial function in conduit vessels, is impaired following the consumption of a high fat meal. It is unclear if this response is influenced by the fatty acid composition of the meal.

The anti-inflammatory properties of HDL are well established. *In vitro* studies have demonstrated that HDL isolated from different human subjects differ in their ability to inhibit the expression of VCAM-1 by activated endothelial cells (193). Subsequent studies demonstrated that the phosphatidylcholine composition of rHDL influences its ability to inhibit VCAM-1 expression (194). As the *sn-2* fatty acid of the phospholipid became more saturated, the ability of rHDL to

inhibit VCAM-1 expression decreased. The studies outlined in chapter three demonstrated that the anti-inflammatory properties of HDL were enhanced following the infusion of chylomicron-like emulsions. These results suggest that there may be a link between dietary fat consumption and the anti-inflammatory properties of HDL.

The aims of the studies outlined in this chapter were to characterise the vascular biological response to the consumption of a fatty meal and to determine if the fatty acid consumption of the meal affects (i) the ability of HDL to inhibit *in vitro* expression of adhesion molecules by activated endothelial cells, (ii) the ability of serum to induce adhesion molecule expression by activated endothelial cells and (iii) vascular reactivity, as determined by flow mediated dilatation and venous strain gauge plethysmography.

7.2 METHODS

7.2.1 Experimental protocol

The study was approved by the research ethics committee of the Central Sydney Area Health Service (RPAH Division). 10 healthy volunteers, aged 18-40, without cardiovascular risk factors or established atherosclerotic disease, provided informed consent to participate in the study. Each subject attended on two separate occasions. Female subjects attended within seven days from the commencement of menstruation to control for the effect of hormonal variation during the menstrual cycle on vascular function. Following an overnight fast, subjects underwent (i) assessment of flow mediated dilatation of the right brachial artery, (ii) venous strain gauge plethysmography of the left forearm and (iii) collection of venous blood. Subjects then consumed one of two meals comprising a slice of carrot cake and a milkshake containing 100 g/kg of fat.

The two test meals differed in fatty acid composition. The first meal contained safflower oil (fatty acid composition: 75% polyunsaturated, 13.6% monounsaturated and 8.8% saturated fat). The second meal contained coconut oil (fatty acid composition: 89.6% saturated fat, 5.8% monounsaturated and 1.9% polyunsaturated fat). 68.9% of the total fat content of each meal was provided in the form of the milkshake. The remainder was provided in the cake. 90% of the fat in the cake was derived from the oil. Measurements were made prior to and at three and six hours following consumption of the meal. The order of meals ingested was determined by random allocation and status was blinded to the investigators at the time that the arterial and cellular measurements were made.

7.2.2 Plasma analyses

Plasma was stored at -80°C until analysed. Plasma concentrations of cholesterol (444), triglyceride (443), HDL-cholesterol and non-esterified fatty acids (NEFAs) (482) were determined by enzymatic assay. HDL cholesterol was determined following precipitation of apoB containing lipoproteins by centrifugation with polyethylene glycol (459). LDL cholesterol was calculated using the Friedewald equation (483). Plasma insulin was determined by a microparticle enzyme immunometric assay (MEIMA, Abbott, Japan) (484).

7.2.3 Characterisation of HDL

Total HDL (1.063>d>1.21 g/mL) was isolated from plasma by sequential ultracentrifugation, as described in chapter 2, and were dialysed extensively against endotoxin free PBS prior to use. Cholesterol, triglyceride, phospholipid (441) and protein (442) composition of HDL was

determined by enzymatic assay. ApoA-I composition was determined immunoturbidometrically using a sheep anti-human apoA-I antibody (437). HDL size was determined by non-denaturating polyacrylamide gel electrophoresis (438), which was subsequently analysed using ImageMaster. Surface charge of HDL was determined by agarose gel electrophoresis.

7.2.4 Determination of endothelial adhesion molecule expression

The cell surface expression of adhesion molecules be HUVECs in response to HDL and serum isolated from subjects was assessed with an ELISA technique (485). HUVECs were isolated and cultured as described in chapter 2. Confluent cell monolayers were established in 96-well plates. For the HDL study, samples were added to media containing 10% heat-inactivated serum at a concentration of 0, 2, 4 or 8 μM apoA-I and incubated with cells for 16 hours. For the serum study, the media contained 10% heat-inactivated serum, isolated from subjects, without HDL. Cells were incubated for a further 5 hours in the basal or stimulated state following the addition of TNF-α (0.2 ng/ml). The media was then removed. Wells were rinsed twice with Hank's Balanced Salt Solution (HBSS) without phenol red (200 μL/well). Monoclonal antibodies against ICAM-1 (1 mg/mL), VCAM-1 (1 mg/mL) or isotype mouse immunoglobulin G (100 mg/mL) (0.1 mg in 100 ml HBSS with 10% heat-inactivated human serum) were added to wells and incubated on ice for 30 minutes. Wells were rinsed three times with HBSS without phenol red plus 0.05% Tween-20 (250 μL Tween-20 in 500 mL HBSS, 200 μL/well). A sheep anti-mouse antibody/horseradish peroxidase conjugate diluted 1/500 in 100 mL HBSS with 10% heat-inactivated human serum and 0.05% Tween-20 (100 μL/well) was added to the wells and incubated on ice for 30 minutes. Wells were rinsed four times with HBSS and 0.05% Tween-20 (200 μL/well). The ABTS substrate (150 μL/well, Kirkegaard and Perry Laboratories,

Gaithersburg, MD, USA) was added and incubated at room temperature for 15 minutes. Colour development was stopped by the addition of 10% acetic acid solution (50 μL/well). Results were expressed as units of optical density, measured at 414 nm with an ELISA plate reader (Titretek Multiscan, Flow Tubes, Rickmansworth, UK).

7.2.5 Brachial artery reactivity

All studies were performed using an ATL HDL 5000 machine (Phillips, Bothell, Washington) with a wide-band 12- to 5-MHz linear-array transducer. Brachial artery diameter was measured from B-mode ultrasound images. Scans were obtained at rest, during reactive hyperaemia and again at rest. The artery was scanned longitudinally 2-15 cm above the elbow. A resting scan was performed and arterial flow velocity was measured using a Doppler signal. Increased flow was induced by inflation of a pneumatic tourniquet around the forearm to a pressure of 250 mmHg for 4.5 minutes followed by its release. The second scan was continuously recorded from 30 seconds prior to until 90 seconds following deflation of the cuff. In addition, a flow velocity recording was obtained during the first 15 seconds following deflation. The vessel was then allowed to recover for 10 minutes, after which a repeat resting scan was obtained. Nitrate-induced dilatation was determined as the last measurement of each day. A metered dose of sublingual nitrate (glyceral trinitrate spray 400 μm) was administered and scans were obtained 3 minutes later. All ultrasound studies were recorded on super-VHS tape and later analysed vessel diameter was measured by two independent observers, who were blinded to the experimental details. Measurements were made from the anterior to posterior 'm' lines at the end of diastole, indicated by the start of an R wave on a continuously recorded electrocardiograph. For the reactive hyperaemia measurements, diameters were recorded 45-60 seconds following deflation of the

cuff. Vessel diameter following reactive hyperaemia and administration of sublingual nitrate was expressed as the percentage relative to the average resting diameter. This method has been shown to be accurate and reproducible, with low interobserver error, for the measurement of both flow-mediated dilatation (FMD) and nitrate induced dilatation (486).

7.2.6 Venous occlusion strain gauge plethysmography

Forearm blood flow in the left forearm was determined by venous occlusion strain gauge plethysmography using calibrated mercury-in-silastic strain gauges (Hokanson, Bellevue, WA, USA) (415). The gauges were applied to the widest muscular segment of the forearm. Venous occlusion pressure (60 mmHg) was applied with a cuff placed around the upper arm. Circulation to the hand was prevented by the inflation of a paediatric cuff around the wrist to suprasystolic pressure (220-250 mmHg). Following the measurement of forearm blood flow at rest the upper arm cuff was inflated to 250 mmHg for 5 minutes to induce ischaemia. After the cuff was released to venous occlusion pressure postischaemic hyperaemia, a measure of vasodilator capacity, was determined. Peak reactive hyperaemia was determined by measuring forearm blood flow immediately following release of the upper arm cuff to venous occlusion. Using an automated cuff controller, forearm blood flow was measured every 10 seconds for the first 80 seconds, every 15 seconds for the next 120 seconds and every 30 seconds for the next 150 seconds. Postischaemic volume was determined as the area under the flow versus time curve for the first 115 seconds following release of suprasystolic pressure. Flow curves were recorded using a computer based chart recorder (MacLab/8e System, ADInstruments, Castle Hill, Australia). Arterial inflow was measured by the application of a straight regression line from the

initial part of the upward flow curve. The slope reflects the change in forearm volume per unit time.

7.2.7 Data analysis

All results are expressed as mean±SEM. Statistical comparisons were made using paired Students t tests and one-way ANOVA where appropriate. $P<0.05$ was determined to be statistically significant.

7.3 RESULTS

7.3.1 Baseline characteristics

10 healthy subjects (5 male, 5 female) participated in the study. Mean age was 30.5±2 years and mean body mass index was 24±1.1 kg/m^2. All subjects were free of established atherosclerotic risk factors and clinical cardiovascular disease. No subjects consumed lipid modifying therapies. All studies were uncomplicated and all subjects attended for both visits.

7.3.2 Plasma analyses

Plasma lipid profiles are presented in figure 7.1. The postprandial state was characterised by an increase in plasma triglyceride at 3 hours. There was no significant change in total cholesterol, HDL and LDL concentrations. Plasma insulin was significantly elevated 3 hours following the consumption of the saturated fat. The elevation of plasma insulin following the polyunsaturated fat meal just failed to reach statistical significance (P 0.057 compared with baseline). Plasma

insulin returned to baseline levels at 6 hours. (Fig 7.2) Plasma NEFA concentrations increased 6 hours following the consumption of both fatty meals, although was this effect was greater following the saturated fat (627±24.6 vs. 473.6±70.6 μmol/L, P<0.03). (Fig 7.3)

7.3.3 Characterisation of HDL

Isolated HDL did not differ in overall chemical composition following consumption of either polyunsaturated or saturated fat (Table 7.1). Isolated HDL were predominately α-migrating on agarose gel electrophoresis. There was a non-significant increase in HDL particle size following consumption of both meals. (Table 7.2)

7.3.4 Influence of HDL on adhesion molecule expression

HDL isolated from plasma following the consumption of the polyunsaturated and saturated fat enriched meals had a striking and differential effect on ICAM-1 expression by activated endothelial cells. (Fig 7.4) At an apoA-I concentration of 2 μM and 8 μM, HDL isolated following a polyunsaturated meal decreased ICAM-1 expression by activated cells by 11.8±4% and 12.3±5.2% at 3 hours (P<0.05 for each comparison with baseline) and 11.7±3.1% and 13.1±4.2% at 6 hours (P<0.05 for each comparison with baseline) respectively. In contrast, at an apoA-I concentration of 2 μM, 4 μM and 8 μM HDL isolated 6 hours following the consumption of a saturated meal increased ICAM-1 expression by activated endothelial cells by 15.2±5.4%, 26.4±11% and 11.6±4.4% respectively (P<0.05 for each comparison with baseline). HDL isolated 3 hours following the consumption of the saturated meal had no effect on ICAM-1 expression, compared with baseline. The influence of the fatty acid composition of the meal on

the effect of HDL on ICAM-1 expression was statistically significant at 6 hours, at all concentrations of HDL studied.

A similar effect was seen when the expression of VCAM-1 by activated endothelial cells was studied. (Fig 7.5) At an apoA-I concentration of 2 μM and 4 μM, HDL isolated following a polyunsaturated meal decreased VCAM-1 expression by activated cells by 15.8±5.6% and 14.3±6.7% at 3 hours ($P<0.05$ for each comparison with baseline) and 11.7±6.7% and 13.1±5.5% at 6 hours ($P<0.05$ for each comparison with baseline) respectively. In contrast, at an apoA-I concentration of 2 μM, 4 μM and 8 μM HDL isolated 6 hours following the consumption of a saturated meal increased VCAM-1 expression by activated endothelial cells by 14.7±4.4%, 14.8±4.4% and 37.8±16.1% respectively ($P<0.05$ for each comparison with baseline). HDL isolated 3 hours following the consumption of the saturated meal had no effect on VCAM-1 expression compared with baseline. The influence of the fatty acid composition of the meal on the effect of HDL on VCAM-1 expression was statistically significant at 6 hours at all concentrations of HDL studied.

7.3.5 Influence of serum on expression of adhesion molecules

Serum isolated from subjects following the consumption of the fatty meal exerted a differential effect on the expression of adhesion molecules by activated endothelial cells. (Fig 7.6) Isolated serum following the consumption of either meal had no effect on the expression of ICAM-1. In contrast, a non-significant 16.3±9.1% reduction in the expression of VCAM-1 was seen when serum isolated 3 hours after the consumption of a polyunsaturated fat.

7.3.6 Influence of dietary fatty acid composition on vascular function

The fatty acid composition of the meal did not affect the resting brachial artery diameter and FMD (Table 7.3). There was a nonsignificant reduction in FMD at 3 hours following the consumption of both meals, which returned to baseline levels at 6 hours. (Fig 7.7) GTN-mediated dilatation, a marker of endothelium-independent vasodilatation, did not differ between meals (14.3±2.3% vs. 14.9±3.2% following consumption of the polyunsaturated and saturated meals respectively). In addition, the different meals had no influence on resting forearm blood flow, peak flow and total hyperaemia, as measured by venous occlusion strain gauge plethysmography (Table 7.3). Compared with baseline levels, consumption of a meal enriched with a polyunsaturated fat increased total hyperaemia by 33% at 3 hours (12.2±2 vs. 9.6±1.3 mL/100 mL at 3 hours and baseline respectively) which was not statistically significant (P 0.068). In contrast, total hyperaemia did not change at all following the consumption of the saturated fat (11.1±1.2 vs. 11.4±2.2 mL/100 mL at 3 hours and baseline respectively). (Fig 7.8)

7.4 DISCUSSION

This study demonstrates that the fatty acid composition of a fatty meal has a profound influence on the expression of pro-inflammatory adhesion molecules by activated endothelial cells. In association with elevations in plasma triglyceride, insulin and NEFA concentrations, these findings suggest that postprandial serum, following the consumption of a polyunsaturated fat, may be potentially beneficial, by reducing the cellular expression of the pro-inflammatory factors ICAM-1 and VCAM-1. In contrast, the consumption of a saturated fat results in HDL that display a reduced ability to inhibit the expression of adhesion molecules.

The expression of adhesion molecules on the surface of endothelial cells is a key early event in atherogenesis (136). Adhesion molecules facilitate the rolling and tethering of circulating inflammatory cells on the endothelial surface, where they subsequently migrate into the arterial wall under the influence of various chemotactic factors. Pathological studies have demonstrated the presence of adhesion molecules overlying atherosclerotic plaque (145). In addition, their presence on the endothelial surface precedes the development of macroscopic changes in the arterial wall (11). Furthermore, animal studies have demonstrated that reducing the functional activity of adhesion molecules *via* the use of monoclonal antibodies (154) and genetic deletion (153) is atheroprotective. Interventions that reduce endothelial adhesion molecule expression could therefore be beneficial.

It is well established that HDL possess anti-inflammatory properties, both *in vitro* and *in vivo*. The results of chapter four suggest that this beneficial activity occurs, independently to the ability of HDL to promote cholesterol efflux. Both native (187) and reconstituted (188,190) forms of HDL inhibit the *in vitro* expression of adhesion molecules by cytokine-stimulated endothelial cells. Further studies demonstrated that HDL, isolated from different human subjects, display a marked variety in their ability to inhibit adhesion molecule expression (193). It was subsequently determined that altering the phosphatidylcholine composition of rHDL had a profound influence on this activity (194). An increase in the degree of saturation of the fatty acid in the *sn-2* position of the phospholipid resulted in a reduction in the ability of rHDL to inhibit VCAM-1 expression by activated endothelial cells. These results were further extended by the studies outlined in chapter three that demonstrate that, regardless of their phospholipid composition, the infusion of

chylomicron-like emulsions results in an enhanced ability of HDL to inhibit VCAM-1 expression.

The results outlined in this chapter extend these findings further to demonstrate that the consumption of a fatty meal can have a significant influence on the anti-inflammatory properties of HDL. These studies highlight that dietary fatty acids can have variable effects on the functional properties of HDL rapidly following the consumption of a meal. The consumption of a polyunsaturated fat resulted in the generation of HDL that reduce the expression of adhesion molecules. In contrast, the incorporation of a saturated fat in the meal results in HDL that may be less protective, *via* a reduced ability to inhibit adhesion molecule expression.

These results have important implications for the role of dietary fat intake in the prevention of atherosclerotic cardiovascular disease. It is well established that dietary fatty acid composition has a major influence on the development of clinical events in population studies (404,421). The results of these studies suggest that a diet enriched in polyunsaturated fat is likely to result in the generation of circulating serum that inhibits the expression of pro-inflammatory factors on the endothelial cell surface. In addition, fatty acid consumption appears to have an influence on the ability of HDL to perform its protective roles. It is well established that dietary fatty acids are rapidly transferred from chylomicrons to other circulating lipoproteins, including HDL (469). This transfer has the potential to result in structural and functional changes of HDL. In this study, the detrimental effect that a saturated fat, in comparison to a polyunsaturated fat, has on the properties of HDL, adds further evidence to support the need to reduce saturated fat consumption.

In this study we also investigated the effect of dietary fatty acid composition on vascular function. It is well recognised that the consumption of a high fat meal is characterised by an increase in vascular flow which correlates with the rise in plasma insulin that occurs during the postprandial period (415). However, altering the fatty acid composition of the meal appeared to have an influence on the change in total hyperaemic response to ischaemic stimuli, although this just failed to meet statistical significance. Whilst total hyperaemia appeared to increase following the consumption of a polyunsatured fat, there was no such response to a meal enriched with a saturated fat. These results, in combination with the effects of dietary fatty acid composition on the expression of cellular adhesion molecules, in the presence and absence of HDL, provide further impetus for the proposition that dietary polyunsaturated and saturated fats might have differential effects on the arterial wall that may contribute to their differential effects on atherogenesis. In contrast, there was no difference between the meals with regard to their effect on endothelium-dependent vasodilatation in conduit vessels.

There was a nonsignificant reduction in brachial artery FMD at 3 hours following the consumption of both meals. It has been reported that the consumption of a fatty meal has variable effects on endothelium-dependent vasodilatation, a well validated measure of endothelial function. Whilst some groups have reported an impairment of FMD following the consumption of a high fat meal (414), others have reported no change (415). In addition, several groups have measured endothelium-dependent vasodilatation in the microvasculature, using venous strain gauge plethysomography and found that the consumption of a high fat meal has no influence (415). The reason for these discrepant results is unclear, but may relate to a lack of standardisation of experimental meals and protocols.

In summary, varying the fatty acid composition of a meal has profound differential effects on the expression of pro-inflammatory adhesion molecules by activated endothelial cells and their regulation by HDL. These results have important implications to support the need to reduce the dietary intake of saturated fat, in the prevention of atherosclerotic cardiovascular disease.

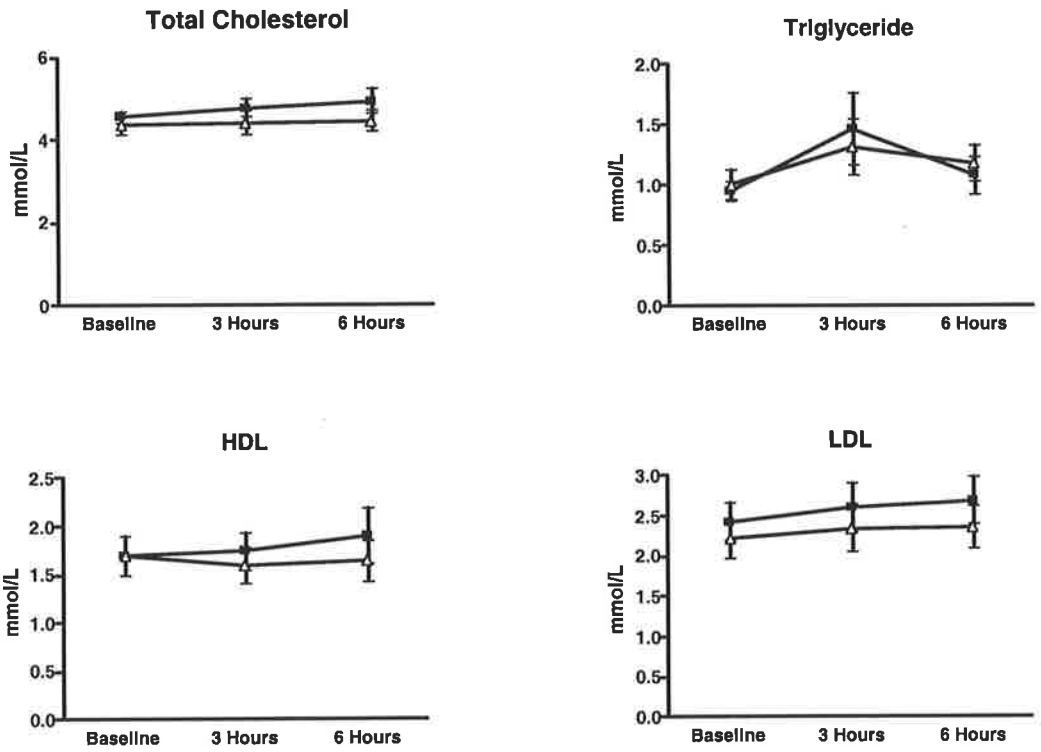

Figure 7.1. Change in the plasma concentration of lipid parameters following the consumption of a meal enriched with a polyunsaturated fat (■) or a saturated fat (▲). Results are expressed as mean ± SEM.

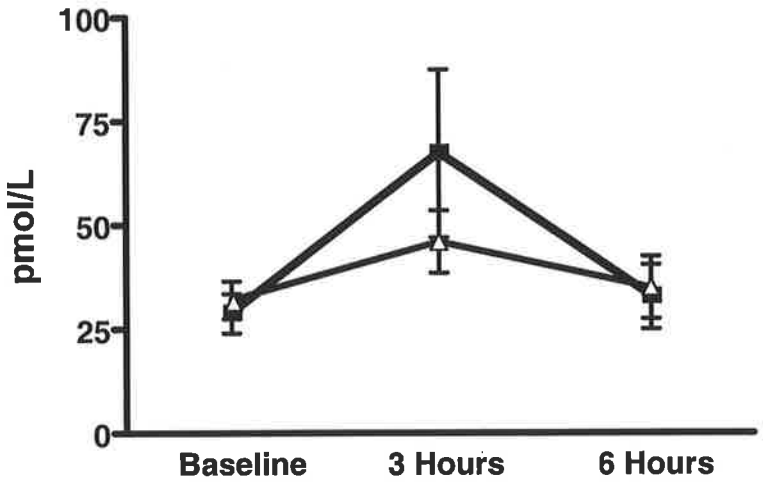

Figure 7.2. Change in the plasma concentration of insulin following the consumption of a meal enriched with a polyunsaturated fat (■) or a saturated fat (▲). Results are expressed as mean ± SEM.

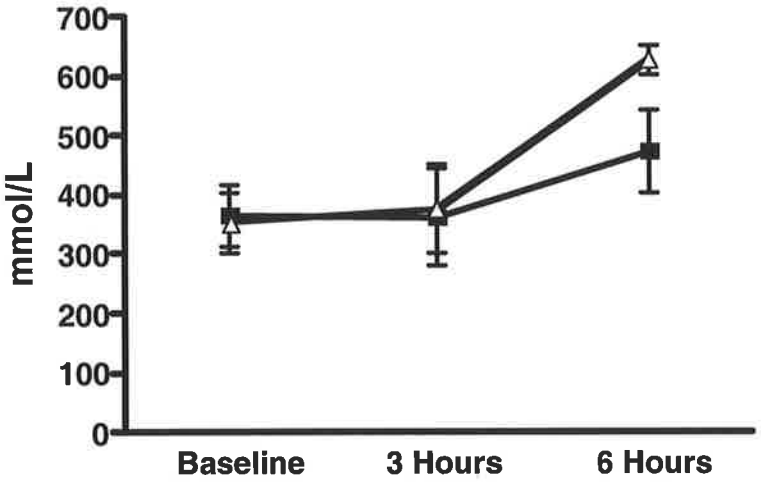

Figure 7.3. Change in the plasma concentration of non-esterified fatty acids (NEFA) following the consumption of a meal enriched with a polyunsaturated fat (■) or a saturated fat (▲). Results are expressed as mean ± SEM.

	Protein	Phospholipid	Triglyceride	Cholesterol Ester	Unesterified Cholesterol
Polyunsaturated					
Baseline	50.5±1.9	27.9±1.6	2.2±0.3	16.2±1	3.3±0.3
3 Hours	50.2±1.7	29.2±1	2.4±0.4	15.1±0.8	3.2±0.2
6 Hours	51.8±1.3	27.1±1.1	2.5±0.3	15.5±0.7	3.2±0.2
Saturated					
Baseline	53.1±1.4	26.6±1.6	2.6±0.3	14.5±0.8	3.3±0.3
3 Hours	51.3±1.6	27.7±1.4	2.1±0.3	16.2±1.2	2.7±0.2
6 Hours	49.4±1.1	29.9±1	2.2±0.3	15.5±1	3.1±0.2

Table 7.1. Stoichiometric composition of HDL isolated from subjects following consumption of a meal enriched with either a polyunsaturated or saturated fat. Results expressed as percentage of total mass of HDL (mean±SEM).

	HDL_2 (nm)	HDL_3 (nm)
Polyunsaturated		
Baseline	10.81±0.07	8.92±0.11
3 Hours	10.93±0.09	9.07±0.12
6 Hours	10.96±0.06	9.11±0.07
Saturated		
Baseline	10.92±0.1	9.04±0.11
3 Hours	10.93±0.09	9.05±0.12
6 Hours	11.02±0.08	9.11±0.1

Table 7.2. Stokes' diameter of HDL_2 and HDL_3 isolated from subjects who consumed a meal enriched with a polyunsaturated or saturated fat. Results expressed as mean±SEM.

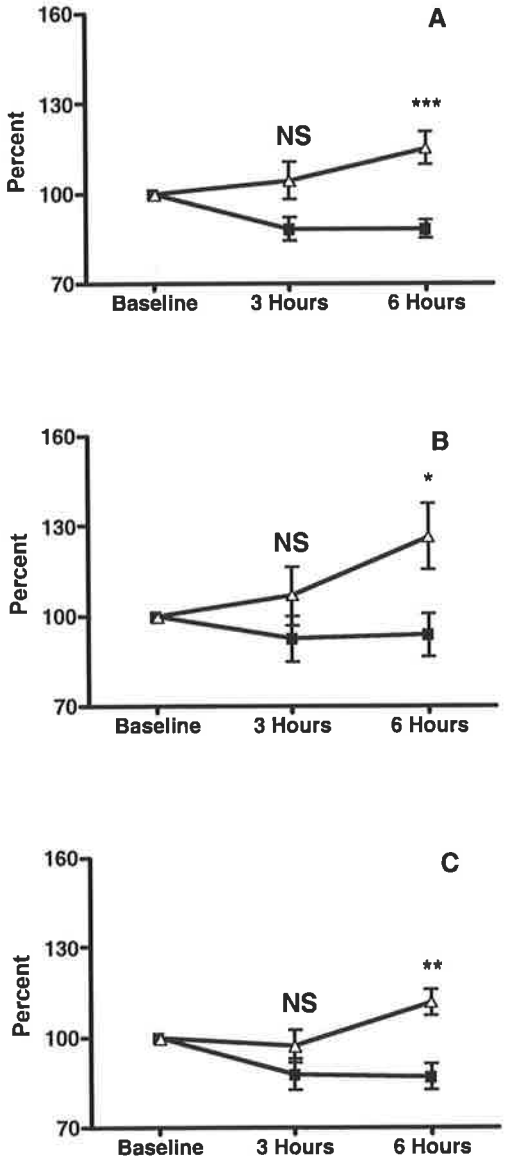

Figure 7.4. Effect of HDL, at a concentration of 2, 4 and 8 μM apoA-I (panels A, B and C), isolated from subjects who consumed a meal enriched with a polyunsaturated fat (■) or saturated fat (▲) on expression of ICAM-1 by HUVECs stimulated with TNF-α (0.2 ng/mL). Results expressed as percentage change from baseline, mean ± S.E.M. NS non significant, * $P<0.05$, ** $P<0.002$, *** $P<0.0001$ for comparison between meals.

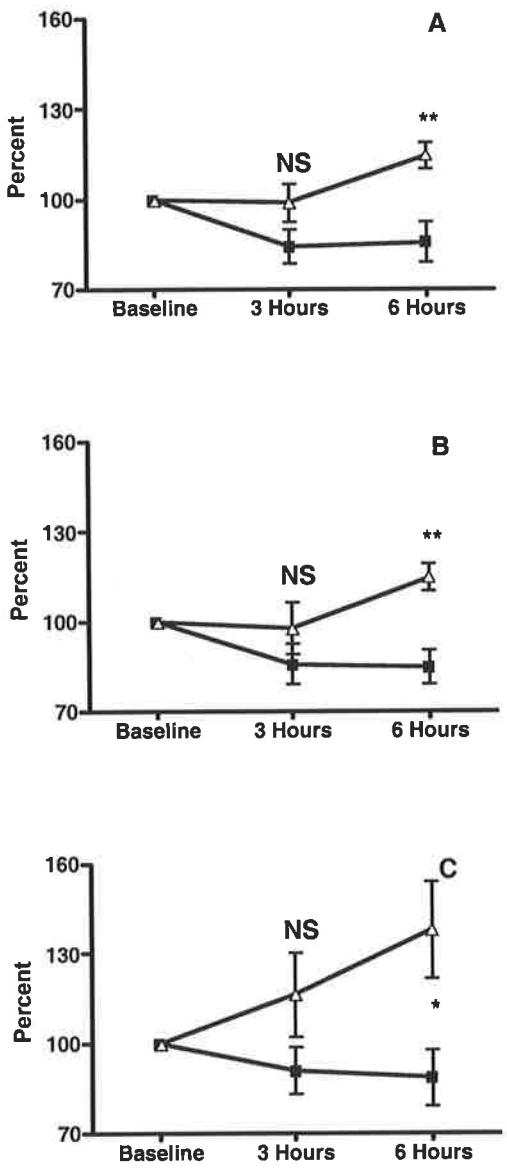

Figure 7.5. Effect of HDL, at a concentration of 2, 4 and 8 μM apoA-I (panels A, B and C), isolated from subjects who consumed a meal enriched with a polyunsaturated fat (■) or saturated fat (▲) on expression of VCAM-1 by HUVECs stimulated with TNF-α (0.2 ng/mL). Results expressed as percentage change from baseline, mean ± S.E.M. NS non significant, * $P<0.05$, ** $P<0.002$ for comparison between meals.

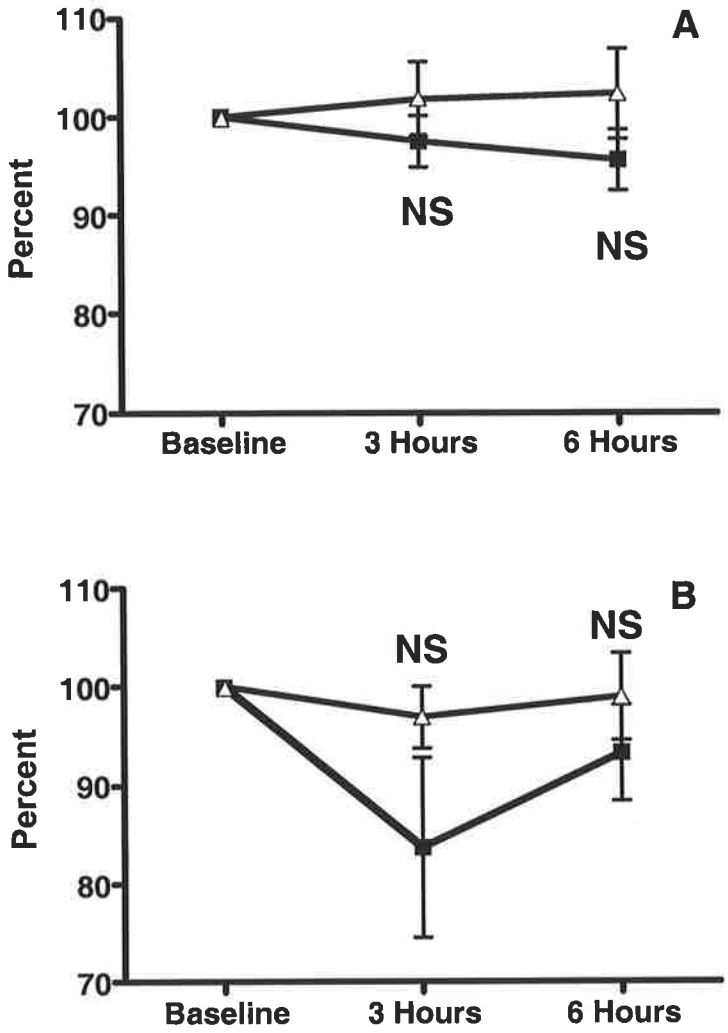

Figure 7.6. Effect of serum isolated from subjects who consumed a meal enriched with a polyunsaturated fat (■) or saturated fat (▲) on expression of ICAM-1 (panel A) and VCAM-1 (panel B) by a HUVEC monolayer as measured by ELISA. Results expressed as the fold change between cells in the basal state and cells stimulated with TNF-α (0.2 ng/ml), mean ± S.E.M. NS nonsignificant for comparison between meals.

	Polyunsaturated			Saturated		
	Baseline	3 Hours	6 Hours	Baseline	3 Hours	6 Hours
Baseline diameter (mm)	3.7±0.25	3.69±0.25	3.67±0.26	3.7±0.27	3.72±0.25	3.74±0.25
Flow-mediated dilatation (%)	5.6±1.56	4.37±1.08	5.08±1.64	5.7±1.13	4.6±1.07	5.45±1.73
FBF_{rest} (ml/min/100 ml)	1.38±0.23	1.86±0.22	1.68±0.28	1.48±0.19	1.53±0.18	1.36±0.13
Peak flow (ml/min/100 ml)	19.57±1.05	18.28±1.48	17.95±1.27	19.85±1.02	17.45±1.02	18.71±1.15
Total hyperaemia (ml/100 ml)	9.63±1.29	12.19±1.97	10.82±1.59	11.41±2.19	11.1±1.18	10.38±1.91

Table 7.3. Effect of dietary fatty acid composition on vascular measures of endothelial function, measured by brachial artery flow-mediated dilatation and venous occlusion strain gauge plethysmography. Results expressed as mean±SEM.

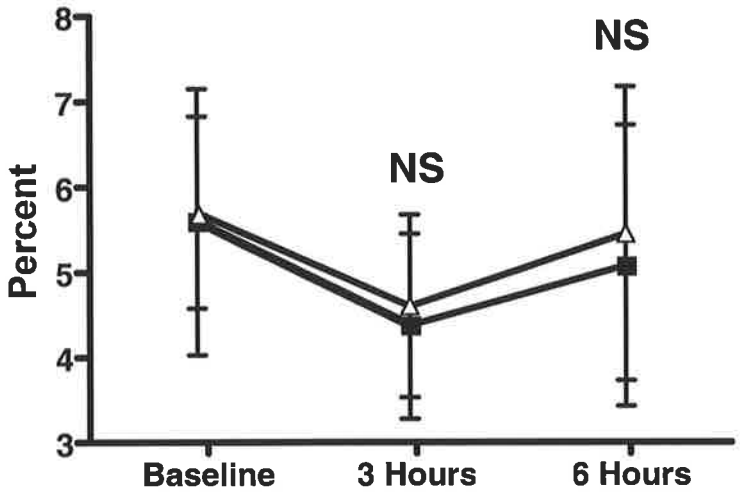

Figure 7.7. Flow-mediated dilatation of brachial artery following consumption of a fatty meal enriched with either a polyunsaturated (■) or saturated (▲) fat. Results expressed as percentage change in arterial diameter with ischaemic stimulus (mean±SEM). NS nonsignificant comparison between meals.

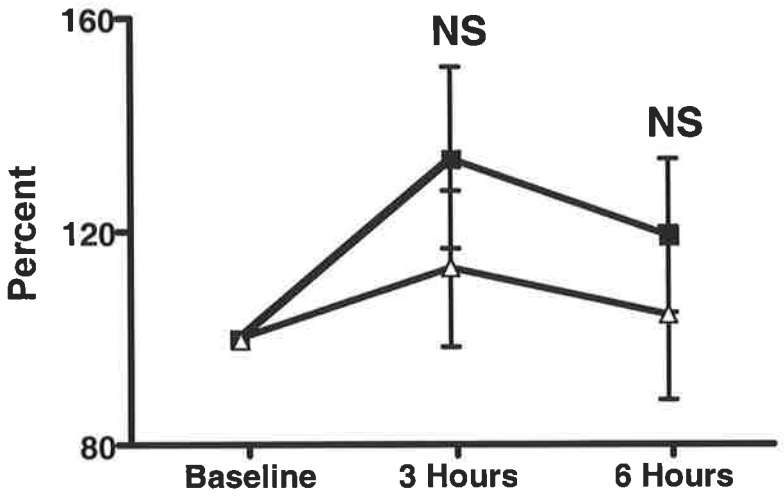

Figure 7.8. Change in total hyperaemia, measured by venous occlusion strain gauge plethysomography, following consumption of a fatty meal enriched with either a polyunsaturated (■) or saturated (▲) fat. Results expressed as percentage change compared with baseline level (mean±SEM). NS nonsignificant comparison between meals.

CHAPTER EIGHT

GENERAL DISCUSSION

The concept that high density lipoproteins possess beneficial properties continues to gain momentum. There has been an evolution from the initial discovery that patients with coronary heart disease tended to have a low plasma concentration of HDL to the prospect that the therapeutic manipulation of HDL is an attractive target in atheroprotection. Population studies have clearly established that a low plasma HDL is a strong independent predictor of clinical events. In addition, elevation of plasma HDL in animal models is atheroprotective. Futhermore, elevation of HDL in clinical trials is associated with marked benefit. Subsequent studies have demonstrated that the infusion of rHDL in humans has a profound impact in promoting the regression of atherosclerotic plaque.

The studies outlined in this thesis extend our understanding of functional properties of HDL that may contribute to their atheroprotective benefit. In particular, the major findings provide further evidence to support the concept that HDL are anti-inflammatory. This property was studied in models of acute and chronic vascular inflammation. In addition, this activity is modified in the postprandial period. HDL were found to have a beneficial impact on the pro-inflammatory and pro-oxidant changes induced by the application of a periarterial collar, a model of acute vascular injury. This benefit was demonstrated in normocholesterolaemic animals and suggests that these properties may be independent to the ability of HDL to promote cholesterol efflux. The anti-inflammatory properties were then extended to a model of chronic vascular inflammation. Without an elevation of plasma HDL, the infusion of small amounts of both native and reconstituted HDL promoted the stabilisation of plaque phenotype in a model of established atherosclerosis. Furthermore, it was demonstrated that following the consumption of dietary fat, the anti-inflammatory properties of HDL are modified. These studies provide further evidence that HDL can have a favourable impact on the arterial wall at all stages of the atherogenic process.

In chapter three it was demonstrated that the infusion of rHDL has beneficial properties in the setting of acute vascular inflammation and oxidative stress. These benefits extend previous findings to suggest that HDL possess functional properties in addition to promoting cholesterol efflux. In addition, the benefit of rHDL exceeded that seen with infusing lipid free apoA-I alone. Furthermore, this study provides the first *in vivo* evidence that HDL inhibit the infiltration of neutrophils into the arterial wall. Given the role of neutrophils in acute infection, autoimmune disease, ischaemia-reperfusion injury and atherogenesis, these results have important implications that HDL can have a favourable impact on numerous disease processes.

In chapter four it was demonstrated that the infusion of native HDL had a favourable impact on established atherosclerotic plaque. This resulted in the rapid promotion of a stable plaque phenotype that was comparable to that of administering a statin. As it has been proposed that plaque passivation is one mechanism by which statins exert an early clinical benefit, the current findings suggest that a similar advantage may be obtained from the rapid manipulation of plasma HDL. This property was further studied with the use of rHDL in chapter five. This result suggested that reconstituted forms of HDL possess a greater potential to reduce plaque vulnerability. In addition, it provides insight into how the components of HDL influence its functional properties. These studies add further evidence to the proposition that apoA-I and apoA-II differ in their effect on atherogenic risk.

It has also been demonstrated that following the ability of HDL to inhibit the *in vitro* expression of adhesion molecules, by activated endothelial cells, is modified following the infusion of chylomicron-like emulsions and the consumption of a fatty meal. These results suggest that the *in vivo* anti-inflammatory properties of HDL may be altered during the postprandial period. As the western diet involves the consumption of frequent meals during

the typical day, we spend most of our time in the postprandial state. Therefore, such modifications of the functional properties of HDL can have a profound impact on the atherogenic process. In addition, the results of chapter seven suggest that the fatty acid composition of a meal has a profound influence on the anti-inflammatory properties of HDL. These results provide another mechanistic link between dietary fat consumption and vascular risk.

A consistent finding in the studies presented in this thesis was that the beneficial properties of infusing either native or reconstituted HDL was seen rapidly and with the administration of relatively small amounts. Previous reports of the atheroprotective benefits of HDL in animal models have been associated with marked elevations of the plasma concentration of HDL. In contrast, the beneficial impact of HDL in models of acute and chronic vascular inflammation, described in this thesis, were seen without an elevation in plasma HDL. The dramatic effects of infusing very small amounts of discoidal forms of HDL raises the possibility that a lot of the benefit seen following the infusion of chylomicron-like emulsions may reflect the generation of very small amounts of lipid deplete pre-β forms of HDL. These features highlight the importance of qualitative changes in circulating HDL.

In addition, the studies described found that altering the phospholipid composition of HDL had no effect on their beneficial properties. In the experimental settings of acute and chronic vascular inflammation, altering the degree of fatty acid saturation of phosphatidylcholine did not modify the benefit of infusing rHDL. Furthermore, altering the phospholipid composition of chylomicron-like emulsions did not have an effect on the ability of HDL to inhibit the *in vitro* expression of VCAM-1 by activated endothelial cells. This finding occurred despite a predictable change in phospholipid composition of isolated HDL, consistent with a degree of transfer from infused emulsions. Each of these results are in contrast to the previous *in vitro*

finding that the PC composition of rHDL has a profound influence on their *in vitro* anti-inflammatory properties.

The reasons for the discrepancy between this finding and those presented in this thesis remains unclear. It is possible that there are fundamental differences between endothelial cells of human origin, as used in the previous *in vitro* study, and arterial endothelial cells of rabbits, as studied in the *in vivo* studies presented in this thesis. As a result, whilst rHDL PC composition had an important influence on the ability of one type of endothelial cell to express adhesion molecules in response to cytokine stimulation, it may have little impact on other types of endothelial cells. In addition, the discrepancy may also represent differences in the functional properties of rHDL between the *in vitro* and *in vivo* settings. The effect of infusing phospholipid alone, in the form of unilamellar vesicles, warrants further investigation *in vivo*.

Various factors influencing the anti-inflammatory properties of HDL remain uncertain. The time course of the benefit of infusing rHDL in the setting of acute vascular injury, induced by the application of a periarterial collar, is unclear. It has been demonstrated that the neutrophil influx induced by a periarterial collar commences within six hours of its application. The study presented in chapter three only looked at a single time point, 48 hours, and therefore it remains to be determined whether rHDL has an even earlier benefit in this arterial model. In addition, it should be determined if a similar benefit can be demonstrated with only a single infusion of rHDL. Furthermore, it should be determined whether the benefit is transient or sustained, influencing the ability of the collar to promote neointimal hyperplasia. The use of rHDL allows for further determination of the effect of HDL composition. It is uncertain whether apoA-II shares this beneficial property. In addition, it is unclear whether modification of rHDL, through oxidation or glycation, affects this functional benefit. This has important

implications as HDL are modified in states such as diabetes mellitus and the metabolic syndrome.

The mechanisms that promote the ability of HDL to passivate atherosclerotic plaque remain uncertain. It is unclear whether the increase in plaque smooth muscle cells results predominantly from either the promotion of their migration and proliferation or by inhibiting SMC apoptosis. It would appear that this effect on both SMC and macrophages result from a combination of promoting cholesterol efflux, in addition to direct anti-inflammatory and antioxidant properties. In addition, it remains to be determined whether the changes in protein expression within the plaque confer functional differences such as the inhibition of thrombus formation and collagen degradation. The true test would involve the assessment of infusing HDL in an experimental animal model of atherosclerosis that is associated with a high incidence of spontaneous plaque rupture, arterial thrombosis and myocardial infarction. The ongoing development of animal models that possess this activity should make it possible to test this hypothesis. Further studies should also address whether the combination of these strategies results in an incremental benefit.

The effect of apolipoprotein composition remains unclear. The role of apoA-II in either the promotion or inhibition of atherogenesis remains confusing. Further studies should address whether the benefit of apoA-I is reduced when rHDL also contains apoA-II. In addition, many groups have demonstrated the benefit of the variant protein apoA-I_{Milano}. It remains to be determined whether this variant possess mythical properties in excess of wild type apoA-I. A direct comparison has yet to be performed. In addition, the influence of apoE and its various genotypes on these functional properties of rHDL remain to be determined.

The postprandial period is characterised by a complex cascade of events that have the potential to have an important impact on atherogenesis. The results of the studies presented in chapters six and seven suggest that the anti-inflammatory properties of HDL are also potentially modified during this period. The mechanism that confers the enhanced anti-inflammatory property of HDL following the infusion of chylomicron-like emulsions remains to be determined. The effect of infusing chylomicron-like emulsions on the intracellular signalling processes that regulate the expression of cellular adhesion molecules is unknown. A greater understanding of this process may highlight the factors of HDL that play an important role. In addition, the studies represent a snap shot taken 20 minutes following infusion of chylomicron-like emulsions. Future studies should address the time course of this phenomenon. It is uncertain how rapidly this effect is seen and how long it persists. Furthermore, the effects were demonstrated following the infusion of relatively small amounts of triglyceride, which predictably did not alter the plasma triglyceride concentration. It would be interesting to determine what would be the effect on HDL following the infusion of a much greater amount of triglyceride. Finally, does this relationship occur in healthy human subjects and if so, how is it affected in pathologic states such as the metabolic syndrome.

In a similar fashion, the mechanisms that confer a reduced anti-inflammatory role of HDL following the consumption of a meal enriched with a saturated fat remain to be established. In the studies outlined in chapter seven, this modification of HDL activity resulted, without an alteration of HDL size, surface charge or chemical composition. It is also unclear whether the ability of postprandial serum following different meals to have variable effects on VCAM-1 expression by activated endothelial cells operates *via* a similar mechanism to that seen with HDL. It should also be noted that the lack of a significant change in vascular function following the consumption of either fatty meal reflects the wide variation in physiological response of normal vasculature to the postprandial state.

The consumption of dietary fat has a complex relationship with vascular risk. Population studies have established a clear correlation between the amount of dietary fat consumed, its composition and the incidence of cardiovascular disease. In addition, dietary intervention studies suggest that altering the fatty acid composition can have a profound impact on the incidence of clinical events in subjects with established CHD. It has been demonstrated that the postprandial period is characterised by the appearance of circulating lipoproteins that have a detrimental effect on the arterial wall. The findings presented in this thesis add to the complex body of evidence that suggests that the consumption of dietary fat can have variable effects. Further large standardised studies are required to further clarify the true relationship between dietary fat consumption and atherogenesis. These results will provide further impetus to the implantation of dietary guidelines as a central step in atheroprotection.

In addition to ongoing investigation of the mechanisms which confer these beneficial properties on HDL, and the role of HDL composition, it should be remembered that HDL circulates in a state where it is constantly remodelled by a series of factors. Therefore, the influence that these lipolytic enzymes and lipid transfer factors have on the functional properties of HDL should be further explored. Effective chemical inhibitors of CETP have been developed and their ability to promote endogenous HDL has been demonstrated. It is uncertain whether this will equate with an increased atheroprotective benefit. In addition, it remains unclear whether remodelling of HDL by an increasing family of lipases alters its functional activity at the level of the arterial wall. Whilst the effect of hepatic lipase on atherogenesis is complex, the role of endothelial lipase has not been investigated. The benefits that result from the direct administration of HDL provide further support for the proof of concept that HDL is atheroprotective. It may result, however, that the pharmacologic manipulation of these factors that remodel HDL become the focus for prophylactic strategies.

Therefore, it is imperative that we have a greater understanding of how these factors modify the beneficial activity of HDL.

In summary, the studies outlined in this thesis provide further support that HDL possess non-lipid transporting properties that influence the atherogenic process. These benefits appear rapidly and with the administration of relatively small amounts. The importance of these properties has important implications at all stages of atherogenesis. They have a dramatic influence on both acute and chronic arterial inflammatory models. In addition, they change during the complex period that characterises the consumption of a meal. These results highlight the importance of HDL and the need to identify effective strategies to promote its benefit at the level of the arterial wall.

CHAPTER 9

AMENDMENTS

Chapter One

1. Addition of a figure depicting the heterogeneous structure, composition and size of HDL.

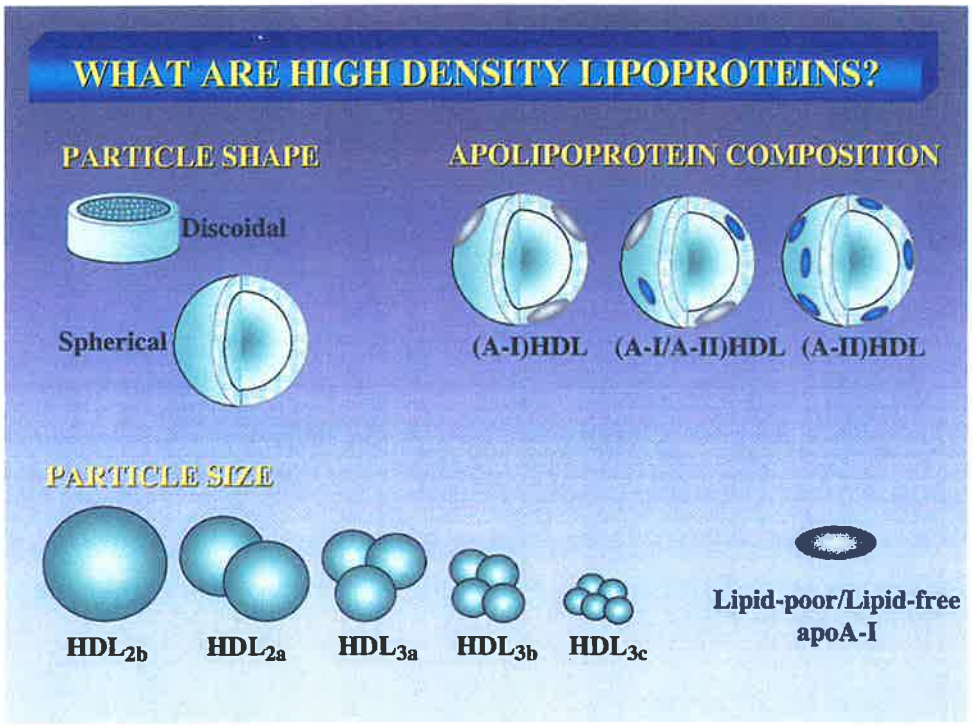

Figure 1.1. Circulating HDL represents a heterogeneous group of particles that differ in shape, size and composition as result of constant remodelling by plasma factors.

2. Addition of a figure summarising the role of HDL in cholesterol homeostasis

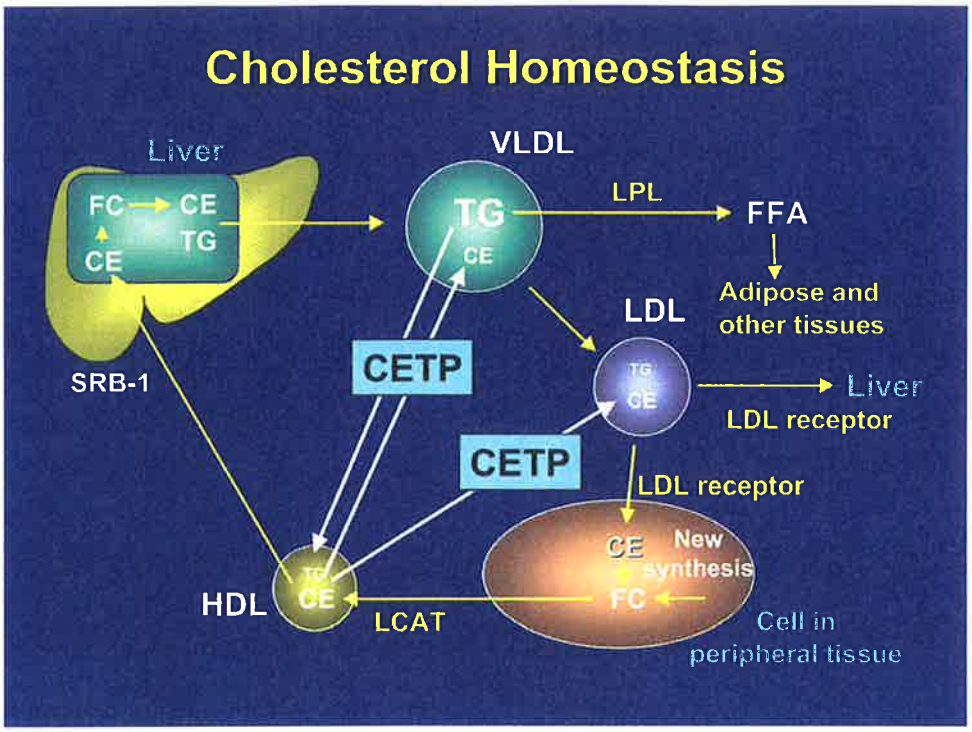

Figure 1.2. Summary of cholesterol homeostasis. CE cholesterol ester, CETP cholesteryl ester transfer protein, FC free cholesterol, HDL high density cholesterol, LCAT lecithin:cholesterol acyltransferase, LDL low density lipoprotein, LPL lipoprotein lipase, SRB-1 scavenger receptor B type 1, TG triglyceride, VLDL very low density lipoprotein.

3. Addition of table summarising the evidence that promoting plasma HDL beneficial effects on the overall burden and composition of atherosclerotic plaque in animal models.

Beneficial Effects on Atherosclerotic Burden and Composition
Infusion of native HDL
Infusion of reconstituted HDL
Infusion of lipid-free apoA-I
Transgenic expression of apoA-I
Transgenic expression of apoA-II
Transgenic expression of apoA-IV
Administration of apoA-I variants and mimetics
Administration of exogenous phospholipid
Detrimental Effects on Atherosclerotic Burden and Composition
Transgenic expression of apoA-II
Genetic deletion of apoA-I

Table 1.1. Summary of the effect on atherosclerotic burden and composition of experimental interventions that modify plasma HDL in animal models.

4. Addition of a table summarising the potential antiatherogenic properties of HDL.

Promote reverse cholesterol transport
Anti-inflammatory properties
Reduce expression of pro-inflammatory cytokines
Reduce expression of adhesion molecules
Reduce expression of pro-inflammatory chemokines
Inhibit monocyte chemotaxis
Inhibit proliferation and activation of lymphocytes
Inhibit neutrophil chemotaxis and degranulation
Anti-oxidant properties
Inhibit oxidative modification of LDL
Inhibit lipid hydroperoxide formation
Restore balance of nitric oxide to superoxide
Prevent metalloproteinase release from stimulated monocytes
Promote vascular reactivity
Anti-thrombotic properties
Reduce blood viscosity
Inhibit platelet activation and aggregation
Inhibit tissue factor synthesis
Inhibit activation of coagulation factors
Promotes activity of endogenous anticoagulant proteins C and S
Inhibits expression of PAI-1
Prevent cellular death
Inhibits endothelial cell apoptosis
Inhibits complement mediated cell lysis
Beneficial effects on cellular proliferation
Promote endothelial cell proliferation
Variable effects on smooth muscle cell proliferation
Promote angiogenesis
Inhibit vascular calcification
Prevent ischaemia-reperfusion injury

Table 1.2. Potential antiatherogenic properties of HDL.

5. Page 7, paragraph 2, sentence 2 has been revised to state 'In addition, an increased concentration of remnant particles (28) and an excessive plasma triglyceride response to a meal are markers of increased risk.'
6. Page 7, paragraph 3, sentence 3 has been revised to state 'Modification of LDL, predominantly by oxidation, generates particles that are major factors in stimulating atherogenesis.'
7. Page 8, paragraph 3, sentence 3 has been revised to state 'HDL can be classified into three subfractions, on the basis of their density in plasma.'
8. Page 10, paragraph 1, sentence 2 has been deleted.
9. Page 25, paragraph 2, sentence 8 has been revised to state 'E-selectin mediates the rolling and loose tethering of leukocytes on the luminal surface of the endothelial cell.'
10. Page 31, paragraph 3, sentence 3 has been revised to state 'In addition, patients with chronic inflammatory conditions such as rheumatoid arthritis demonstrate and inverse relationship between plasma CRP and HDL. Moreover, an increase in plasma HDL occurs in response to disease modifying immunosuppressive agents.'
11. Page 60, paragraph 2, sentence 3 has been revised to state 'In addition, the combination of aortic balloon denudation with cholesterol feeding results in the development of accelerated and localised atherosclerotic plaque which bears a similar pathologic resemblance, although predominantly containing foam cells, to that seen in humans.'
12. Page 61, paragraph 2 has been amended by the addition of the following sentences at the end stating 'For example, experimental models has demonstrated that have established that hepatic and endothelial lipase mediated hydrolysis of rHDL are influenced by both the apolipoprotein and phospholipid composition of the rHDL particle. In addition, the ability of rHDL to inhibit cytokine induced expression of

VCAM-1 by endothelial cells, *in vitro*, is influenced by the rHDL phospholipid composition.'

13. Page 64, paragraph 2, sentence 4 has been revised to state 'However, it is uncertain whether these HDL particles are dysfunctional.'

23.1 Chapter Two

1. Page 70, paragraph 1 has been amended to include a final sentence stating 'Purity of isolated apolipoproteins was confirmed by SDS-polyacrylamide gel electrophoresis.'
2. Page 70, paragraph 2 has been amended to include a sentence stating 'Purity of isolated native HDL was confirmed by subjecting samples to non-denaturing gradient and agarose gel electrophoresis.'
3. Page 80, paragraph 3, sentence 5 has been revised to state 'The rabbit model, in contrast, develops atheroma, containing predominantly foam cells, in an anatomic distribution that is more similar to humans.'
4. Page 71, paragraph 2 has been amended to include two final sentences stating 'Samples were divided for this dialysis step to ensure complete removal of cholate. The resultant particles possessed chemical and electrophoretic properties as previously described (401).'
5. Page 78, paragraph 1, sentence 1 has been revised to state 'Phosphatidylcholine concentrations were determined using a modification of the method described by Takayama *et al* (441).'

23.2 Chapter Three

1. An additional figure has been included to depict the application of the periarterial collar and its demonstrated effects.

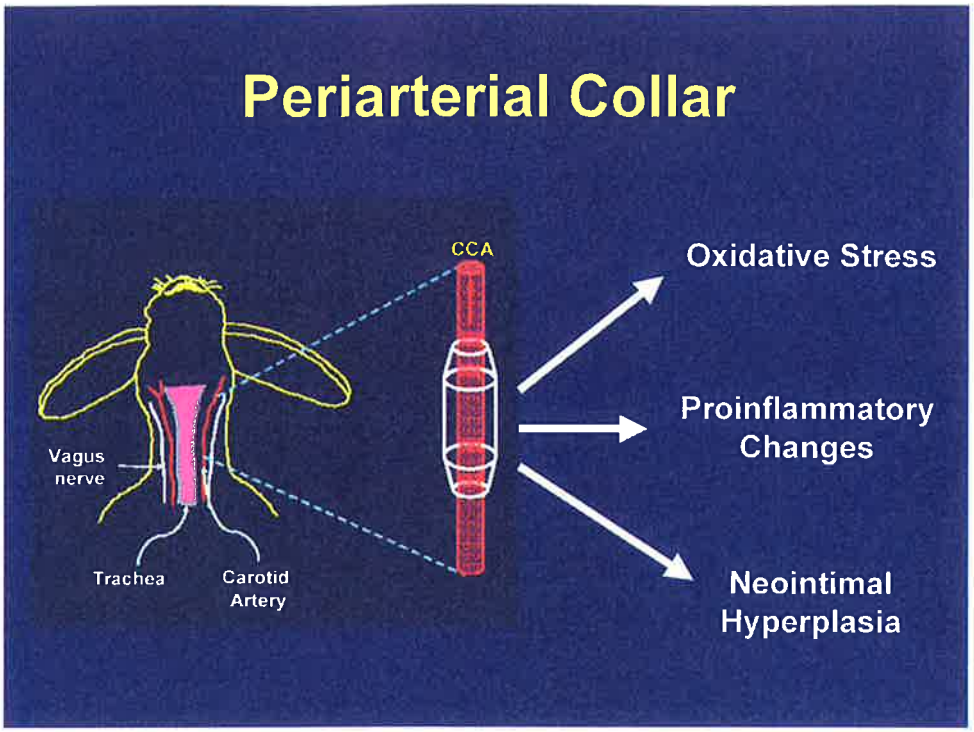

Figure 3.10. Schematic demonstration of application of a silastic, non-occlusive collar surrounding the carotid artery and its demonstrated effects on the arterial wall.

2. Page 94 has been amended by the addition of two final sentences stating 'As outlined in chapter two, a threshold of staining was determined by an independent pathologist, blinded to the treatment status of the animals. Staining above this threshold was deemed to be positive.'
3. Page 100, paragraph 2, sentence 2 has been revised to state 'Our studies further establish that the ability of HDL to inhibit the expression of pro-inflammatory adhesion molecules and chemokines that have previously been observed *in vitro* and hypercholesterolaemic animal models, may be independent of cholesterol efflux.'

23.3 Chapter Four

1. Page 117, paragraph 1, sentence 7 has been revised to state 'The microemulsions floated up with ultracentrifugation and were isolated.'

2. Page 119, paragraph 2 has been amended by the addition of an opening sentence that states 'Cellular expression of VCAM-1 was subsequently performed by a well validated, previously described, flow cytometric analysis protocol (187).'
3. Page 121, paragraph 2 has been amended to include an additional sentence at the beginning stating 'Infused emulsions had a composition of triglyceride 10.3 mmol/L, cholesterol 0.3 mmol/L and phosphatidylcholine 4.7 mmol/L.'
4. Page 124, paragraph 2 has been amended to include an additional final sentence stating 'It is also possible that an alteration in anti-inflammatory activity may result from changes in the antioxidant properties of isolated HDL. Further studies should investigate whether infusing chylomicron-like emulsions has any impact on the activity of the established antioxidant factors of circulating HDL, such as PON and PAF-AH.'
5. Page 133, paragraph 2, sentence 4 has been revised to state 'In addition, the studies outlined in chapter three demonstrate that the infusion of small amounts of rHDL have profound anti-inflammatory properties, that appear to be independent to their ability to promote cholesterol efflux.'

23.4 Chapter Five

1. Figure 5.1 has been replaced with the following

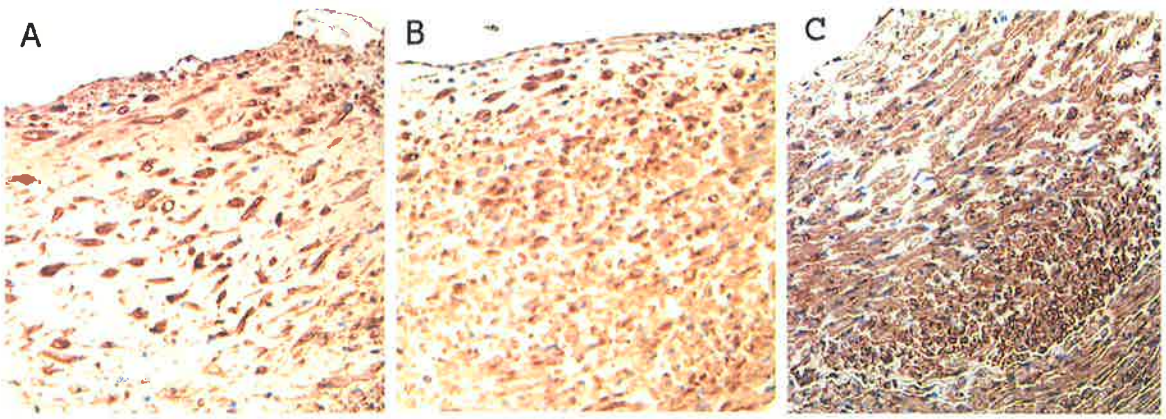

Figure 5.1. Representative staining of plaque smooth muscle actin in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

2. Figure 5.10 has been replaced with the following

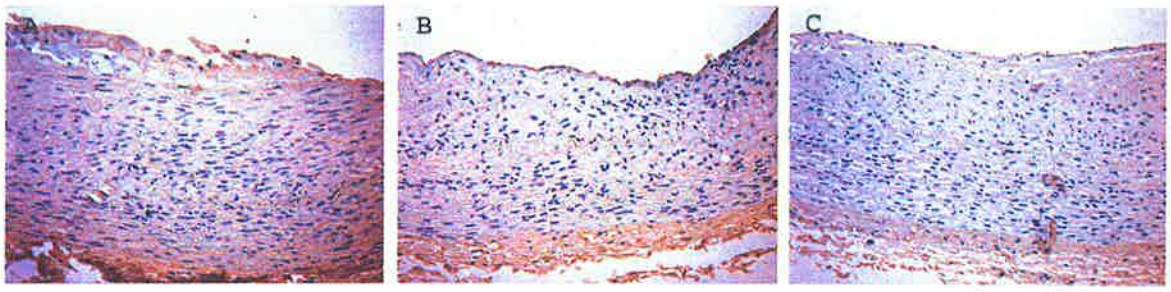

Figure 5.10. Representative staining of plaque MMP-9 in untreated animals (panel A) and animals treated with HDL (panel B) and atorvastatin (panel C).

3. Page 134, paragraph 2, sentence 1 has been revised to state 'HDL was isolated from pooled samples of chow fed rabbit plasma (Quality Farms of Australia, Lara, VIC, Australia) by sequential ultracentrifugation in the 1.06-1.21 g/mL density range.'

4. Page 136, paragraph 1 has been amended by the addition of two final sentences stating 'As outlined in chapter two, a threshold of staining was determined by an independent pathologist, blinded to the treatment status of the animals. Staining above this threshold was deemed to be positive.'

5. Page 140, paragraph 3, sentence 3 has been revised to state 'However, recent studies have demonstrated that early administration of statins in the setting of acute coronary syndromes is associated with an early clinical benefit (473).'

6. Page 141, paragraph 3, sentence 1 has been revised to state 'In addition, this is the first in vivo evidence that the infusion of HDL has a favourable influence on the plaque composition of MMP-9 and thrombomodulin.'

7. Page 144, paragraph 2, sentence 1 has been revised to state 'In contrast to previous studies, the benefit of HDL on established atheroma, in addition to that seen with rHDL in chapter three, was seen in the absence of an elevation in plasma HDL.'

8. Page 144, paragraph 2, sentence 5 has been revised to state 'However, these properties of HDL have been demonstrated in animal model, characterised by predominantly

foamy lesions and a low incidence of spontaneous plaque rupture. The development of experimental models with a higher incidence of spontaneous plaque rupture should help to clarify the significance of these findings.'

23.5 Chapter Six

1. Figure 6.12 has been replaced with the following

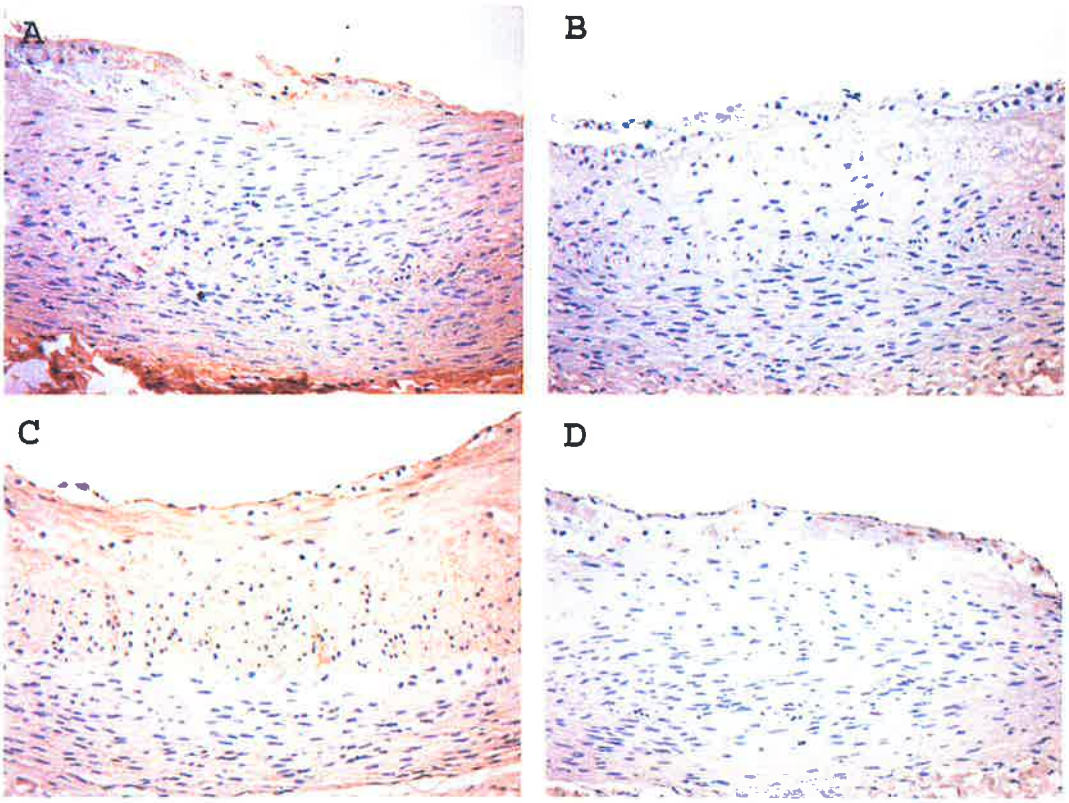

Figure 6.12. Representative staining of plaque MMP-9 in untreated animals (panel A) and animals that were infused with apoAI-PLPC (panel B), apoAI-DPPC (panel C) and apoAII-PLPC (panel D).

2. Throughout this chapter, in sections focussing on the effect of rHDL apolipoprotein composition on atherosclerotic plaque, apoA-I and apoA-II treated animals are denoted by apoA-I-PLPC and apoA-II-PLPC treated animals respectively.
3. Throughout this chapter, in sections focussing on the effect of rHDL phospholipid composition on atherosclerotic plaque, PLPC and DPPC treated animals are denoted by apoA-I-PLPC and apoA-I-DPPC treated animals respectively.

4. Page 172, paragraph 2 has been amended by the addition of an extra sentence at the end stating 'The development of experimental models of atherosclerosis with a higher incidence of spontaneous plaque rupture than that seen in the current model will help to clarify the functional significance of these histological changes.'

23.6 Chapter Seven

1. Page 192, paragraph 1 has been amended to include a final sentence that states 'The degree of adhesion molecule expression in the presence of each concentration of postprandial HDL was expressed as the proportion of expression demonstrated following the incubation with the same concentration of fasting HDL.'
2. Page 197, paragraph 2 has been revised to state 'This study demonstrates that the fatty acid composition of a fatty meal has an potential influence on the expression of pro-inflammatory adhesion molecules by activated endothelial cells. In association with elevations in plasma triglyceride, insulin and NEFA concentrations, these findings suggest that HDL, following the consumption of a polyunsaturated fat, may be potentially beneficial, by reducing the cellular expression of the pro-inflammatory factors ICAM-1 and VCAM-1. In contrast, the consumption of a saturated fat results in HDL that display a reduced ability to inhibit the expression of adhesion molecules.'

23.7 Chapter Eight

1. Page 215, paragraph 2, sentence 1 has been revised to state 'In chapter five it was demonstrated that the infusion of native HDL had a favourable impact on established atherosclerotic plaque.'
2. Page 217, paragraph 3 has been amended with the addition of a sentence following sentence 5 stating 'Moreover, only a single dose of apoA-I was used in each infusion. Future studies should explore whether even lower amounts of rHDL are effective.'

3. Page 219, paragraph 1, sentence 2 has been revised to state 'The results of the studies presented in chapters four and seven suggest that the anti-inflammatory properties of HDL are also potentially modified during this period.'

CHAPTER 10

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