A Study of Natural Killer Cells in Renal Failure and in Patients at Cardiovascular Risk

by

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List of Abbreviations

AAA	Abdominal aortic aneurysm
ACAT	Acyl CoA:cholesterol acyltransferase
ACS	Acute coronary syndrome
α-GalCer	Alpha-galactosylceramide
AFCAPS/TexCAPS	Air Force/Texas Coronary Prevention Study
AGEs	Advanced glycation end-products
ALERT	Assessment of LEscol in Renal Transplantation
APC	Allophyocyanin
APCs	Antigen presenting cells
ATP III	Adult Treatment Panel III
AURORA	A Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Haemodialysis
AVERT	Atorvastatin versus Revascularisation Treatment
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CAC	Coronary artery calcification
CARE	Cholesterol and Recurrent Events
Ca x P	Calcium x phosphate product
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease

CHS	Cardiovascular Health Study
CKD	Chronic kidney disease
cLDL-C	Calculated LDL cholesterol
CKD	Chronic kidney disease
CKD-MBD	Chonic kidney disease-Mineral bone disorder
CMV	Cytomegalovirus
CRP	C-reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
DC	Dendritic cells
DMAPP	Dimethylallyl pyrophosphate
ECL	Enhanced chemiluminescence
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
FACS	Fluorescence-activated cell-sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FPP	Farnesyl pyrophosphate
FTI	Farnesy transferase
GFR	Glomerular filtration rate
GGPP	Geranylgeranyl pyrophosphate
GGTI	Geranylgeranyl transferase

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GPP	Geranyl pyrophosphate
GTP	Guanosine triphosphate
HD	Haemodialysis
HDL-C	High-density lipoprotein cholesterol
HL	Hepatic lipase
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPS	Heart Protection Study
hsCRP	High sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cells
ICAM	Intracellular adhesion molecule
IDL	Intermediate density lipoprotein
IFN-γ	Interferon-gamma
IL-6	Interleukin-6
IPP	Isopentenyl pyrophosphate
IQR	Inter-quartile range
ITAM	Immunoreceptor tyrosine-based activation motif
JUPITER	Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
KIR	Killer cell Ig-like receptor
LAT	Linker for activation of T-cells

LCL-C	Low-density lipoprotein cholesterol
LCAT	Lecithin cholesterol acyltransferase
LIPID	Long-term Intervention with Pravastatin in Ischemic Disease
LOX-1	Lectin-like oxidized LDL receptor-1
LPL	Lipoprotein lipase
MBCD	Methyl-beta-cyclodextran
M-CSF	Monocyte-colony stimulating
MHCs	Major histocompatibility complexes
MI	Myocardial infarction
MIRACL	Myocardial Ischemia Reduction With Aggressive Cholesterol Lowering
MMPs	Matrix metalloproteinases
MONICA	MONItoring of trends and determinants in CArdiovascular disease
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NCEP	National cholesterol education panel
NIDDM	Non-insulin dependent diabetes mellitus
NK cell	Natural killer cell
NKT	Natural killer T-cell
NKRs	Natural killer receptors
NO	Nitric oxide
Non-HDL-C	Non-high density lipoprotein cholesterol
oxLDL	Oxidised LDL cholesterol
PBMCs	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PE	Phycoerythrin
PEM	Protein energy malnutrition
PEW	Protein energy wasting
PRRs	Pattern-recognition receptors
PTH	Parathyroid hormone
PTX-3	Pentraxin-3
PVD	Peripheral vascular disease
RBD	Ras binding domain
RENAAL	Reduction of Endpoints in NIDDM with Angiotensin II Antagonist Losartan
RF	Risk factors
RR	Relative risk
RRT	Renal replacement therapy
SAA	Serum amyloid A
SAP	Serum amyloid P
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error
SHARP	Study of Heart and Renal Protection
sST2	Soluble ST2
TC	Total cholesterol
TCR	T cell receptor

Th	T helper
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TRPC	Transient receptor potential channel
VCAM-1	Vascular cell adhesion molecule-1
VSMCs	Vascular smooth muscle cells
VLDL	Very low-density lipoprotein
WCC	White cell count
WHS	Women's Health Study
WOSCPOS	West of Scotland Coronary Prevention Study
4D	Die Deutsche Diabetes Dialyse Studie
4S	Scandinavian Simvastatin Survival Study

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Declaration

The experimental design of the work presented in this thesis was that of the author and supervisor, Professor Alan Jardine. The work presented in this thesis was performed by the author, except where the assistance of others is acknowledged.

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not previously been submitted for a higher degree.

Ray Kay Wan

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Summary

Cardiovascular disease (CVD) is the leading cause of death in the UK, accounting for more than a third of all deaths, with atherosclerotic coronary artery disease (CAD) being the commonest type of CVD. Recently, it has become recognised that in addition to traditional cardiovascular (CV) risk factors such as dyslipidaemia, hypertension, smoking, and diabetes, inflammation plays an important role in the development and progression of atherosclerosis. A concept has emerged that atherosclerosis to some extent can be viewed as a chronic inflammatory autoimmune disease in which the adaptive immune system is targeted against vascular self-antigens modified by hypercholesterolaemia, involving both the innate and adaptive immune response. Much work has been done in determining how the immune system is involved, however relatively little is known about natural killer (NK) cells – an important component of the innate immune system which acts against virally infected cells and neoplastic transformation. In addition NK cells possess cytolytic ability and provide an early source of immunoregulatory cytokines. Recently, there has been increasing evidence to support a role for NK cells in the development of atherosclerosis.

The work in this thesis examines NK cell function with the aim of determining whether any changes in the function of this immune cell could have a role in the development of CVD. In order to do this, we chose two patient populations at high CV risk and compared NK cell subsets and function to healthy controls. Firstly, 66 patients with dyslipidaemia on a variety of lipid lowering treatments attending a lipid clinic, and secondly 143 patients with chronic kidney disease (CKD) including 11 with end-stage

renal disease (ESRD) on hospital haemodialysis (HD). It is known that CVD is the leading cause of death in patients with CKD, and in ESRD patients have a 20-100 fold risk of premature CV death compared to age matched controls from the general population. The increased CV risk results from additional risk factors that are unique to this patient population, but in particular, these patients have an immune dysfunction that is not completely understood and a resultant inflammatory state.

We determined T-cell, NKT-cell, and NK cell subsets from peripheral blood mononuclear cells (PBMCs) by flow cytometry. We then isolated NK cells from PBMCs and assessed NK cell function using a 51-Chromium release assay. These results were then correlated with clinical and laboratory results. In the patients with dyslipidaemia, we did not find any correlations between lipid levels and NK cell numbers, subsets, or cytoxicity. The presence of statin therapy or any other lipid lowering treatment did not result in a reduction in NK cell cytotoxicity. In the CKD patient group, we found a correlation between NK cell cytotoxicity and creatinine, although this did not retain significance after multivariate analysis. Interestingly, we also found a correlation between NK cell cytotoxicity and serum phosphate level, which did remain significant after multivariate regression. We are the first to report a relationship between phosphate and NK cytotoxicity. This is an interesting finding as there is increasing evidence supporting a role for hyperphosphataemia in CVD and increased mortality in both the general population and particularly in patients with ESRD. Phosphate has been shown in some studies to be an independent predictor of inflammation, and may provide the link between the high risk of CVD and CKD.

The next part of this thesis was an *in vitro* study of membrane cholesterol in NK cells. The cell membrane supports cholesterol-rich microdomains termed “lipid rafts”, which concentrate receptors and signal transduction molecules to facilitate high efficiency signal transduction. Statins have a number of pleiotropic effects which have been explained by reduced production of isoprenoid intermediates, and depletion of cell membrane rafts. This study aimed to investigate the effects of membrane cholesterol manipulation on NK cell function, and specifically, whether the actions of statins on NK cells are due to depletion of membrane cholesterol or inhibition of isoprenylation. The NK92MI cell line was used. Cells were either cholesterol loaded, or cholesterol depleted using statins, and NK cell function assessed using a 51-Chromium release assay. Cholesterol was successfully incorporated into the membrane and rafts and was concentration dependent. The addition of cholesterol to statin treated cells restored the cholesterol content in the cell membrane and in rafts. NK cell cytotoxicity decreased with statin treatment in correspondence with raft levels, however in contrast with the increased raft levels of cells which were cholesterol loaded, NK cytotoxicity was also decreased. Measurement of active Ras (a small G-protein that is localised by isoprenylation in membrane rafts when activated), showed that statin treatment reduced Ras within the raft which was not rescued by the addition of cholesterol, suggesting that statins deplete membrane cholesterol and rafts as well as inhibiting isoprenylation. Replenishment of membrane cholesterol restores non-isoprenylated, raft-associated proteins, but does not correct the functional effects of statins.

The final part of this thesis aimed to evaluate the relationship between NK cells and potential CV risk biomarkers in these two patient populations at high risk of CVD. High sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6), pentraxin-3 (PTX-3), adiponectin, and soluble ST2 (sST2) levels were determined by ELISA, and correlated with NK cell numbers, phenotype, and function, as well as other routine biochemical and haematological parameters. We were not able to determine any definite relationships between the biomarkers studied and NK cell function, although there was an association between PTX-3 and NK cytotoxicity that was only found in inflamed (hsCRP>2mg/L) patients.

In conclusion, these studies have provided further insight into the role of NK cells in a group of patients that have not previously been studied: patients with a range of CKD, in addition to patients with dyslipidaemia. This study is the first to associate NK cell cytotoxicity with serum phosphate levels which may have clinical implications. Further studies are needed to clarify whether other immune abnormalities occur in the context of hyperphosphataemia, and the causes and consequences of this. We have also demonstrated that statins deplete membrane lipid rafts as well as inhibit isoprenylation, suggesting a novel dual mechanism of action which merits further investigation.

CHAPTER 1

Introduction

1.1 Background

Cardiovascular disease (CVD) is the leading cause of death in the UK, accounting for almost 198,000 deaths per year. More than a third of all deaths (35%) are from CVD. Coronary heart disease (CHD) is the commonest type of cardiovascular disease causing death in the UK, resulting in around 94,000 deaths in the UK each year [1]. Ischaemic stroke makes up the other main form of CVD. Atherosclerosis is the main pathophysiological process causing CVD. Atherosclerosis is a multi-factorial, complex process that is now widely accepted as an inflammatory disease, and consists of focal lesions (plaques) in the arterial tree. Atherosclerotic lesions are composed of cell debris, lipid, inflammatory cells, including lipid-laden macrophage foam cells, and a covering cap of smooth muscle cells and collagen. It is fissuring and rupture of these plaques that leads to thrombus formation, occlusion of vessels, and acute ischaemia. Recently, it has emerged that both the innate and acquired immune system are involved in atherosclerosis, in keeping with the inflammatory nature of this disease.

1.2 Dyslipidaemia

Dyslipidaemia is a heterogeneous disorder with multiple aetiologies, although in western societies, the majority of dyslipidaemia is secondary to lifestyle and dietary habits. Amongst the numerous risk factors for CVD, the relationship between elevated serum cholesterol and CVD has long been recognised [2]. Over the last two decades, multiple

clinical and epidemiological studies have further established this relationship beyond reasonable doubt, with studies showing that lowering serum cholesterol leads to decreased CVD mortality. Indeed it has been shown that even more intensive lowering of low-density lipoprotein cholesterol (LDL-C) by statins is associated with greater clinical benefits [3]. The guidelines of the National Cholesterol Education Program's Adult Treatment Panel III (ATP III) [4] are summarised in table 1. Low-density lipoprotein cholesterol is the primary target of cholesterol lowering therapy.

Table 1.1 ATP III Classification of LDL, Total, and HDL Cholesterol (mg/dL)*

LDL cholesterol	
<100	Optimal
100-129	Near or above optimal
130-159	Borderline high
160-189	High
≥190	Very high
Total cholesterol	
<200	Desirable
200-239	Borderline high
≥240	High
HDL cholesterol	
<40	Low
≥60	High

*ATP indicated Adult Treatment Panel; LDL, low-density lipoprotein; and HDL, high-density lipoprotein

Table 1.2 Classification of serum triglycerides

Triglyceride Category	ATP II Levels	ATP III Levels
Normal triglycerides	<200 mg/dL	<150 mg/dL
Borderline-high triglycerides	200-399 mg/dL	150-199 mg/dL
High triglycerides	400-1000 mg/dL	200-499 mg/dL
Very high triglycerides	<1000 mg/dL	≥500mg/dL

Table 1.3 ATP III classification of HDL cholesterol

Serum HDL cholesterol (mg/dL)	
<40 mg/dL	Low HDL cholesterol
≥60 mg/dL	High HDL cholesterol

Table 1.4 Definitions of dyslipidaemia

Category	Value (mg/dl)		
	LDL	HDL	Triglycerides
CAD,PVD,CVD	>100	<40	>150
2 NCEP RF's	>130	<40	>150
<2 NCEP RF's	>160	<40	>150
Diabetes Mellitus	>100	<40	>150

Risk Factors (RF) are defined as hypertension, tobacco use, diabetes mellitus, family history of CAD, men > 55 years, women > 60 years

1.3 Cholesterol

Cholesterol is a fat-like substance (lipid) that is present in cell membranes and is a precursor of bile acids and steroid hormones. Cholesterol travels in the blood in distinct particles which contain both lipid and proteins (lipoproteins). There are three major classes of lipoproteins: low density lipoproteins (LDL), high density lipoproteins (HDL), and very low density lipoproteins (VLDL). There is another lipoprotein class, intermediate density lipoprotein (IDL) that lies between VLDL and LDL. In clinical practice, IDL is included in the LDL measurement.

1.3.1 Atherogenic dyslipidaemia

Atherogenic dyslipidaemia is characterised by high triglycerides, elevated small dense LDL-C and low HDL-C. This pattern of dyslipidaemia confers a high risk of CHD. LDL cholesterol comprises 60-70% of total serum cholesterol. LDL is the major atherogenic lipoprotein and is the primary target of cholesterol-lowering therapy as outlined by NCEP [4]. HDL cholesterol makes up 20-30% of total serum cholesterol. HDL cholesterol levels are inversely associated with CHD risk. In an observational study, it was found to have a 2-3% decrease in the risk of CHD for every 1 mg/dL increase in HDL [5]. The mechanism is not clear, however it has been suggested that the anti-atherogenic effect of HDL-C may be a result of reverse cholesterol transport, and anti-oxidant and anti-inflammatory properties [6]. Triglycerides have been reported in prospective epidemiological studies to have a positive relationship with incidence of CHD [7, 8]. Non-lipid risk factors of CHD such as hypertension, obesity, diabetes and smoking are

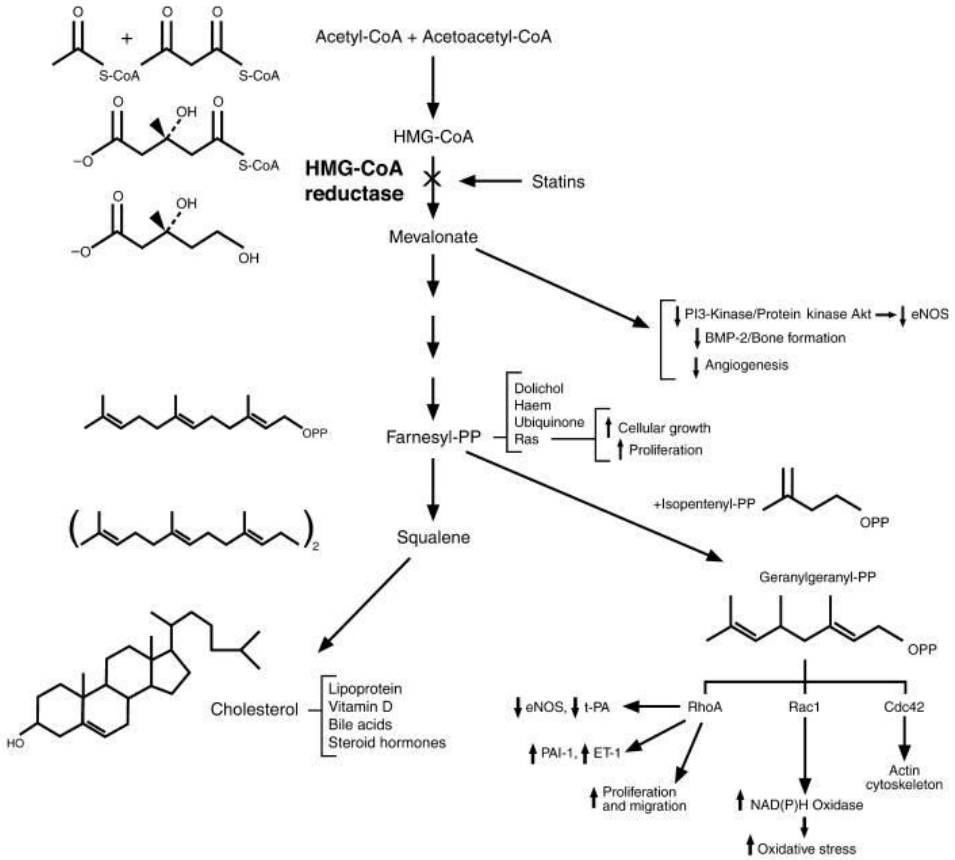
interrelated with triglycerides, along with emerging risk factors (insulin resistance, glucose intolerance, prothrombotic state) [9].

1.3.2 Biosynthetic Pathway

Cholesterol is a four-ring, 27-carbon compound, synthesised from acetyl CoA. The first step is when acetyl CoA is derived from an oxidation reaction in the mitochondria and is transported to the cytoplasm. Two moles of acetyl CoA are condensed, forming acetoacetyl-CoA. Acetoacetyl-CoA and a third mole of acetyl CoA are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the action of HMG-CoA synthase. HMG-CoA is converted to mevalonate, in a rate limiting step catalysed by the enzyme HMG-CoA reductase. Mevalonate is then activated by three successive phosphorylations, yielding 5-pyrophosphomevalonate. After phosphorylation, an ATP-dependent decarboxylation yields isopentenyl pyrophosphate (IPP), an activated isoprenoid molecule. Isopentenyl pyrophosphate is in equilibrium with its isomer, dimethylallyl pyrophosphate (DMAPP). One molecule of IPP condenses with one molecule of DMAPP to generate geranyl pyrophosphate (GPP), catalysed by GPP synthase. GPP further condenses with another IPP molecule to produce farnesyl pyrophosphate (FPP). This step is catalysed by FPP synthase. FPP condenses with another IPP molecule to yield geranylgeranyl pyrophosphate (GGPP), catalysed by GGPP synthase. The head-to-tail condensation of two molecules of FPP is catalysed by squalene synthase and yields squalene. Squalene undergoes a two-step cyclization to yield lanosterol, which is converted to cholesterol through a series of nineteen additional

reactions (Figure 1.1). There is a regulatory system co-ordinating the biosynthesis of cholesterol with the availability of dietary cholesterol.

Figure 1.1 The cholesterol biosynthesis pathway (adapted with permission: Liao et al [10])



1.3.3 Isoprenylated Proteins

Recently, numerous investigators have highlighted the role played by intermediate metabolites of the cholesterol biosynthetic pathway in the pathogenesis of atherosclerosis [10, 11]. As a result of statin inhibition (as discussed in detail below), the downstream pathways affected are those that depend on the supply of intermediate products of the cholesterol biosynthetic pathway. In particular, FPP and GGPP, have attracted interest because they act as adjuncts in post-translational prenylation of various cell-signalling proteins [12].

Protein isoprenylation allows the covalent attachment, subcellular localisation, and intracellular trafficking of water soluble membrane-associated proteins. Members of the Ras and Rho GTPase family are major substrates for post-translational modification by isoprenylation. As will be discussed further below, by inhibiting L-mevalonic acid synthesis, statins also prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as FPP and GGPP. These intermediates serve as important lipid attachments for the posttranslational modification of a variety of cell signalling proteins, particularly small GTPase, and by inhibiting its synthesis, statins deplete cells of these lipids and thus elicit the retention of small GTPase in the cytosol, where they cannot exert their biological actions.

1.4 Atherosclerosis

1.4.1 Atherosclerosis and Dyslipidaemia

The endothelium is a monocellular layer lining the inside of vessels that normally provides a non-adhesive, non-thrombogenic surface for blood constituents, acting as a dynamic interface regulating blood vessel functions. In addition to playing a crucial role in regulating vascular tone, the endothelium controls other processes such as inflammation, coagulation and thrombosis [13]. Normal endothelial function can be altered by traditional risk factor abnormalities. This altered endothelial function leads to a cascade of events that cause plaque formation with subsequent inflammation, altered thrombosis, altered vessel tone, and biochemical interactions. Plaque enlargement then results in vascular shear stress, plaque fissure or rupture, platelet adhesion, and finally, vessel thrombosis.

In human and experimental atherosclerosis, hypercholesterolaemia is the major precipitating factor initiating inflammatory activation of the vascular endothelium. Certainly the importance of hypercholesterolemia has been well documented in animal models. Mice that are homozygous for a targeted disruption of the low density lipoprotein (LDL) receptor gene (*LDLD*$^{-/-}$ mice) fed a high cholesterol diet showed a marked rise in total plasma cholesterol associated with the development of massive xanthomatous infiltration of skin and subcutaneous tissues, and gross atheroma of the aorta and coronary ostia [14]. Similarly, mice genetically modified to lack apolipoprotein

E (apoE$^{-/-}$), a ligand for receptors that clear remnants of chylomicrons and very low density lipoproteins (VLDLs), have grossly elevated cholesterol and foam cell-rich deposits in their proximal aortas by 3 months [15]. The importance of hypercholesterolaemia in humans as a risk factor for coronary heart disease was recognised in the 1950s [16]. Subsequently, the indisputable clinical effects of lowering LDL-C levels have been demonstrated in large clinical trials using statins, showing reductions in deaths from coronary artery disease and in the incidence of acute myocardial infarction in patients with established coronary heart disease and elevated [17] or average [18] cholesterol levels, as well as in subjects with hypercholesterolaemia without overt coronary heart disease [19].

The earliest detectable event in atherogenesis is the accumulation of plasma lipoproteins in the subendothelium, or intima, of focal areas of the arterial tree [20]. Retention here sequesters lipoproteins within the intima, isolating them from plasma antioxidants, which together with increased plasma levels of LDL-C may result in enhanced oxidation or other modifications of LDL within the vascular wall. Endothelial cells normally resist leukocyte adhesion, however pro-inflammatory stimuli, such as hypercholesterolaemia (the retained lipoproteins), hyperglycaemia, hypertension, and smoking, activate the overlying endothelium to secrete chemokines, and trigger the endothelial expression of adhesion molecules for monocytes and T-cells, such as P-selectin and vascular cell adhesion molecule-1 (VCAM-1) [21]. These immune cells then migrate across the endothelial barrier and into the arterial intima. A similar activation can be caused by disturbed flow haemodynamics, common at branching points of the arterial tree –

common sites for atherosclerotic lesions [22]. Once embedded in the intima, the monocytes differentiate into macrophages, stimulated by the monocyte-colony stimulating factor (M-CSF). These macrophages encounter native and modified lipoproteins, most of which are bound to the matrix, and ingest the lipoprotein particles [23, 24], which are taken up by a class of cellular pattern-recognition receptors (PRRs) called scavenger receptors, which are expressed on macrophages [25]. Cholesterol molecules contained in these LDL particles accumulate in the cytoplasm of the macrophage, where it is esterified, generating cholesterol ester droplets, transforming the macrophage into a foam cell, the prototypic cell of the atherosclerotic lesion. Oxidised phospholipids moieties of oxidised lipoproteins signal to many of the cells in the evolving plaque, especially to the endothelium overlying the accumulating oxidised LDL and foam cells. This increases the expression of adhesion molecules as described above, that attract monocytes and lymphocytes to this localised activated endothelium. Not all macrophages however, are transformed into foam cells. Some act to promote inflammation in the artery.

1.4.2 Atherosclerosis and Inflammation

In addition to traditional risk factors, inflammatory factors also activate the endothelium resulting in endothelial dysfunction. Activation by cytokines or other inflammatory mediators leads to increased expression of a variety of cell surface adhesion molecules as described above. Endothelial dysfunction has been shown to correlate with inflammatory markers and other accepted markers or predictors of cardiovascular disease, such as increased high-sensitivity C-reactive protein (hs-CRP) levels in patients with coronary

artery disease [26]. C-reactive protein has been shown to stimulate the expression of VCAM-1 *in vitro* [27], in keeping with the idea of endothelial dysfunction and inflammation.

1.4.2.1 Clinical trials

Nested case-control studies within the Multiple Risk Factor Intervention Trial [28], the Cardiovascular Health Study [29], the Physicians' Health Study [30], the British Regional Heart Study [31], the Women's Health Study [32, 33], and the Helsinki Heart Study [34] showed that measurements of baseline levels of CRP predicted the risk of future cardiovascular death, myocardial infarction, or stroke. In the MONICA (MONItoring of trends and determinants in Cardiovascular disease) Augsburg cohort, a study of over 900 men between the ages of 45 to 64 years, CRP was significantly associated with the risk of coronary events during a follow up period of eight years, even after adjusting for potential confounders [35]. Elevation of the inflammation markers CRP and serum amyloid A (SAA) have been shown in a subgroup analysis of the Cholesterol and Recurrent Events (CARE) study to be associated with the highest risk of development of recurrent non-fatal myocardial infarction or a fatal coronary event, although this risk was attenuated becoming no longer significant among those patients randomised to statin treatment [36], which will be discussed further in section 1.6.2.

1.4.2.2 C-Reactive Protein

It is now widely recognised that atherosclerosis is a specific example of a chronic inflammatory response, mainly to dyslipidaemia and other risk factors as described

above. The chronic inflammatory influence of hypercholesterolaemia is thought to be mediated by the induction of cytokines and chemokines [37], up-regulation of endothelial adhesion molecules, and immune reactions against oxidised moieties on lipoproteins [38]. In keeping with this, atherosclerotic lesions contain abundant immune cells, particularly macrophages and T-cells, smooth muscle cells, and matrix components. Among the numerous inflammatory biomarkers, the largest amount of published data supports a role for C-reactive protein (CRP) not only as a robust and independent risk marker in the prediction of primary and secondary adverse cardiovascular events, with prognostic value exceeding that of LDL cholesterol [39], but also as an active participant in atherogenesis.

CRP is a primitive acute phase inflammatory protein that is produced in the liver and released in response to acute injury, infection or inflammation, and in particular the inflammatory cytokine interleukin-6 (IL-6). Discovered in 1930 by Tillet and Frances, measurement of CRP has become part of routine biochemical testing. Its' name relates to the ability of this protein to precipitate pneumococcal C-polysaccharide in the presence of calcium, and it is a member of a family of proteins called the pentraxins. Several epidemiological studies have shown that CRP is an important risk factor for atherosclerosis and coronary heart disease, and indeed higher levels of CRP have been shown to be related to increased risk of coronary events in patients with both stable [40] and unstable angina [41]. The most important studies to show that the addition of CRP to global risk assessment by the Framingham Risk Score (which is based on age, gender, hypercholesterolaemia and smoking status) leads to an improved prediction of

cardiovascular disease are MONICA [42], Cardiovascular Health Study (CHS) [43], and Women's Health Study (WHS) [39]. However, the mechanisms underlying this association are not clear, more specifically, whether CRP is merely a marker of the inflammatory response, or whether it plays a direct role in the pathogenesis of atherosclerosis is uncertain. CRP has been demonstrated within the arterial plaque [44] and has also been shown to bind to LDL [45], thus potentially being entrapped in the intima by deposited lipids. It has also been shown to bind to complement factor C1q and factor H and activate the classical complement system [46]. Deposition of CRP in the arterial intima at sites of atherogenesis has been demonstrated to precede the appearance of monocytes, and has also been shown to be chemotactic for freshly isolated human blood monocytes which express a CRP receptor [44] supporting the notion that CRP itself may directly contribute to early atherogenesis.

CRP has also been found to cause adverse changes to cultured endothelial cells, resulting in up-regulation of the cellular adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 [27]. CRP also directly affects endothelial function by altering nitric oxide (NO) bioavailability. Nitric oxide is synthesised by the endothelial nitric oxide synthase enzyme (eNOS), and acts locally as a potent vasodilator. CRP down regulates eNOS transcript and destabilizes eNOS mRNA, with resultant decreases in both basal and stimulated NO release [47]. It has also been shown to facilitate endothelial cell apoptosis and inhibit angiogenesis [47] and in a rat balloon injury model, local administration of CRP increased neointimal formation, which was attenuated by angiotensin receptor blockade [48]. More recently, human aortic endothelial cells

cultured with CRP showed up regulation of Lectin-like oxidized LDL receptor-1 (LOX-1), which binds oxidized LDL, generating superoxide anions, decreasing NO production, and activating the transcription factor nuclear factor κB (NF-κB) [49]. In addition this receptor LOX-1 increases monocyte binding to endothelial cells.

The increased expression of adhesion molecules in the vascular wall is an important factor in the development of atherosclerosis and may enhance the local inflammatory response within atherosclerotic plaques by recruiting monocytes and lymphocytes. In addition, the up regulation of the LOX-1 receptor and the resulting effects leads to an endothelial cell phenotype that is pro-inflammatory, pro-oxidant and atherogenic. Therefore, lowering CRP levels may have beneficial effects on the evolution of atherosclerosis and may reduce the risk of coronary events. This will be discussed in detail in section 1.6.2.

1.4.3 Atherosclerosis and the immune system

Recently, the current concept of atherosclerosis as an inflammatory disease has allowed the description of atherosclerosis as a chronic inflammatory autoimmune-like disease that occurs in the context of enhanced plasma lipid levels [50-52]. The inflammatory component of atherosclerosis can be thought of as an innate immune response involving monocytes and macrophages which are responding to the excessive uptake of lipoproteins, and secondly an adaptive immune response involving antigen-specific T cells.

The innate immune system is critical to the initial inflammatory response, and primarily involves the monocyte-macrophage as described above. There is increasing evidence that other immune cells may also have a role, including natural killer (NK) cells, dendritic cells, mast cells, and B cells [53]. NK cells will be discussed in detail below. Dendritic cells play an important role in antigen presentation, expressing high levels of scavenger receptors and class II major histocompatibility complexes (MHCs), which present antigens to cells of the adaptive immune system. Additionally, mast cells when activated release histamine, leukotrienes, platelet-activating factor, proteases, and cytokines.

The adaptive immune system responds to endogenous neoantigens, such as apoptotic cells or oxidised LDL [38], or exogenous antigens, resulting in the activation of T-cells and B-cells. A T cell response is generated when the naïve T cell encounters an antigen-presenting cell (APC) that presents an antigenic peptide for which the T cell is specific. $CD8^+$ T cells recognise peptides presented on MHC class I, whereas $CD4^+$ T cells recognise MHC class II [54]. The primary activation takes place in secondary lymphoid organs such as the peripheral lymph nodes to which dendritic cells that have ingested antigens migrate. The T cell then leaves and migrates to non-lymphoid tissue for a second activation by APCs that present the same antigen. Therefore naïve T cells are rarely found in non-lymphoid tissues. This also applies to atherosclerotic plaques, where most T cells that are found in human lesions are effector or memory T cells [55, 56]. It has also been shown that the proportion of activated T cells increase with severity of coronary disease [57]. The mechanism for T-cell and macrophage entry into the arterial wall are similar, however specific antigens, such as those mentioned, and also microbial

antigens, are needed to activate the T-cell. Because the most prevalent type of T-cell in the lesion is the $CD4^+$ Th1 cell, effector responses to antigenic stimulation include secretion of interferon-γ (IFN-γ), which results in the activation of macrophages and endothelial cells.

1.4.3.1 T-cells

Atherosclerotic lesions are characterised by an infiltration of leukocytes at all stages of disease progression. The presence of T cells in human atherosclerotic plaques was first described in 1985 [58], and in human and animal models of atherosclerosis the most prominent cells infiltrating evolving lesions are macrophages and T-cells [59, 60]. $CD4^+$ T cells are the predominant T cell subset in atherosclerotic lesions in $apoE^{-/-}$ and $LDLR^{-/-}$ mice [61]. Mice lacking adaptive immunity ($apoE^{-/-}$/*scid/scid* mice) have reduced atherosclerosis [62], and reconstitution of the immunodeficient *scid/scid* mice with transfer of $CD4^+$ T cells from atherosclerotic donors accelerates disease [62], suggesting that $CD4^+$ T cells play a proatherogenic role. Additionally, depletion of $CD4^+$ T cells by depleting anti-CD4 antibodies reduced fatty streak development in C57BL/6 mice fed an atherogenic diet [63]. There is less evidence to define the exact role of $CD8^+$ T cells in atherogenesis. One study showed that $ApoE^{-/-}$ $CD8^{-/-}$ mice showed no change in lesion formation compared with $ApoE^{-/-}$ mice [64], although $CD8^+$ T cells have been shown to promote atherogenesis when triggered by antigenic stimulation [65].

T cell receptor (TCR) $\alpha\beta^+$ T cells are present in much larger numbers than TCR $\gamma\delta^+$ cells in atherosclerotic lesions and therefore would seem to have a more important role in

atherogenesis. Indeed TCR $\gamma\delta^+$ cells represent less than 5% of the T cell population in the peripheral human blood [66], although they are enriched in specific tissues such as skin, spleen, gastrointestinal mucosa, and also at sites of chronic inflammation such as the joint synovium in inflammatory arthritis [67]. TCR$\alpha\beta$-deficient apoE$^{-/-}$ mice have been shown to develop less atherosclerosis, whereas mice that were deficient in TCR $\gamma\delta^+$ cells were only marginally affected [64].

Whilst there is abundant evidence to support the participatory role of T-cells in atherosclerosis, the importance of other cells of the innate immune system remains undefined. More recently, two less prominent immune components – natural killer (NK) cells and natural killer T (NKT) cells have also been shown to contribute.

1.4.3.2 NKT cells

NKT cells are a subset of lymphocytes characterised by that fact that they have surface markers and functions of both T cells and NK cells. Makino *et al* coined the term NKT cells in 1995 [68] to describe a heterogeneous subset of mouse T lymphocytes that share characteristics with NK cells and appear to function by linking the innate and adaptive immune systems. A number of criteria have developed to define this class of lymphocyte as different from NK cells; (i) the ability of NKT cells to show autoreactivity to the non-classical MHC molecule CD1d; (ii) the expression of a specific TCR reservoir of the NKT cell; (iii) the presence of NK cell receptors; (iv) the responsiveness of the cell to the synthetic CD1d ligand, α-galactosylceramide (α-GalCer), which is derived from marine sponges [69]. Once activated, all NKT cell populations have the capacity to exert

immunoregulatory functions by releasing large quantities of T helper (Th) 1 or Th 2 cytokines.

Given that NKT cells link the two arms of the immune system it would seem logical that they participate in the pathogenesis of atherosclerosis. In human atherosclerotic lesions, macrophage foam cells have been shown to strongly express all four human CD1 proteins (CD1a, -b, -c, and –d) [70]. The TCR Vα14Jα18 mRNA of NKT cells has been detected within atherosclerotic lesions in mice [71]. Also activation of NKT cells by feeding mice an atherogenic diet can decrease NKT cell number in the liver and spleen [71]. Interestingly, NKT cells were not found in the vascular wall in control mice, which may suggest that the development of the atherosclerotic lesions involves recruitment of NKT cells from peripheral blood into atherosclerotic plaques. This group has also shown that NKT cells are decreased in the peripheral blood of patients with both stable and unstable angina [72]. One possible explanation for this observation is that activated NKT cells secrete tumour necrosis factor-α, IFN-γ, and other cytokines, and subsequently undergo activation-induced apoptosis [66]. Alternatively, it has also been reported that activation of NKT cells may instead lead to a down regulation of the TCR and NK1.1/cd161 to prevent over stimulation [73]. Other studies supporting the proatherogenic role of NKT cells have shown that in apoE$^{-/-}$ mice, exogenous administration of α-GalCer increased atherosclerosis, whereas CD1d-deficient mice showed reduced atherosclerotic lesion development [71, 74]. The α-GalCer-driven increase in atherosclerosis was accompanied by a dramatic increase of IFN-γ, α-GalCer and IL-4 by NKT cells [75]. Further evidence comes from in vitro studies, where macrophages incubated with oxidised LDL display

increased expression of CD1d, which in turn can induce NKT cells to produce IFN-γ [71]. Whilst the exact role of NKT cells has not yet been defined, it does appear that CD1d-mediated activation of NKT cell leads to expression of inflammatory cytokines that drive the progression of atherosclerosis.

1.4.3.3 NK cells

NK cells are a crucial component of the innate immune system against virally infected cells and neoplastic transformation. They have cytolytic ability and also provide an early source of immunoregulatory cytokines [76]. NK cells participate in innate immunity through the production of cytokines such as IFN-γ, TNF-α, IL-10, and GM-CSF, as well as various chemokines that generate an immediate immune response. They have spontaneous cytotoxic activity against some virally-infected, leukaemic, and other cancerous cells, and also mediate antibody-dependent cellular cytotoxicity through FcγRIII (CD16), a receptor molecule on the surface of NK cells that specifically binds the Fc part of an antibody [77], or antibody-coated (opsonized) target and signals through associated subunits containing an immunoreceptor tyrosine-based activation motif (ITAM) to direct antibody-dependent cellular cytotoxicity [78]. Additionally, IFN-γ can elicit innate and adaptive immune responses by increasing the expression of major histocompatibility complex (MHC) class I and II molecules and by activating macrophages. The cytotoxic function of NK cells is the result of a balance between activating and inhibitory signals delivered by specific membrane receptors [77, 79]. Thus, NK cells express receptors (NKRs) that can be either activatory or inhibitory of NK cytotoxicity. Three major superfamilies of NKRs have been described in humans –

the killer cell Ig-like receptor (KIR) superfamily, which primarily recognises HLA-A, -B, and –C; the C-type lectin superfamily, which includes CD94 and NKG2 receptors, recognising HLA-E; and a class of natural cytotoxicity receptors (NCRs) with unknown ligands [79]. The inhibitory receptors specifically interact with MHC class I molecules, inhibiting killing of target cells bearing the appropriate class I allotypes, in other words, these receptors are crucial for distinguishing normal cells from foreign or transformed cells.

NK cells comprise approximately 10-15% of all circulating lymphocytes [79] and are defined phenotypically by their expression of CD56 and lack of expression of CD3 [80]. Additionally, NK cells are not a homogeneous population. It has been recognised that two distinct populations of human NK cells can be identified, based upon their cell surface density of the CD56 antigen. Resting $CD56^{dim}$ cells comprise 90% of total NK cells and are the more cytotoxic subset, and also the mature NK cell subset [79, 81]. The $CD56^{bright}$ NK-cell subset (comprising the remaining 10%) mediates low or no cytotoxicity but has the capacity to produce large amounts of various immunoregulating cytokines [80], and are considered immature NK cells [82]. In contrast to NKT-cells, NK cells do not express TCR molecules. The NK cell subsets also show differences in their NK receptor repertoires, for example, most $CD56^{bright}$ NK cells lack expression of CD16, and as would be expected, greater than 95% of $CD56^{dim}$ NK cells are $CD16^{bright}$ [79].

The roles of NK-cell subsets *in vivo* is not entirely clear. For example, normal ratios of NK-cell subsets are altered during aging – numbers of $CD56^{dim}$ NK cells are expanded in

elderly populations, whereas there is no significant change in the numbers of $CD56^{bright}$ NK cells [81, 83, 84]. Functionally, NK cells in the elderly are less responsive to IL-2-induced proliferation [85], perhaps as a consequence of and increased percentage of $CD56^{dim}$ NK cells. Additionally, in patients with chronic heart failure, NK cells have been shown to be present in lower circulating numbers, and to exhibit impaired cytolytic function [86].

The potential role of NK cells in atherosclerosis has been receiving more attention recently. NK cells have been detected in mouse aortic atherosclerotic-like lesions [87], and more recently in human atherosclerotic lesions [88, 89], providing convincing support of the participation of NK cells in the atherogenic process. More in depth study has been hampered by the lack of an animal model that is selectively deficient in NK cells. Initial studies used the *beige* mutation in mice and found conflicting results [90, 91]. However, the mutation in the *beige* mouse model, which involves the *Lyst* gene, results in a complicated phenotype that is more complex than decreased NK cell activity [92, 93]. Recently, transgenic mice have been developed with defective natural cytotoxicity and a selective deficiency in functional $NK1.1^{+}$ $CD3^{-}$ cells, while maintaining functionally normal T and B lymphocytes [94]. Whitman *et al* have shown that *ldl-r-/-* mice deficient in functional NK cells, fed an atherogenic diet (cholesterol and saturated fat-enriched) had a statistically significant reduction in atherosclerotic lesions [87]. This was achieved by transplanting the bone marrow of lethally irradiated *ldl-r-/-* mice with bone marrow cells obtained from Ly49A transgenic mice or gender-matched nontransgenic littermates.

There have been few comparable studies in human atherosclerotic disease. NK cell activity in humans has been studied in patients with vascular disease with conflicting results. A significant reduction in circulating NK cells with a concomitant decrease in NK cell function has been found in patients with coronary artery disease when compared with healthy controls, although the actual cytotoxicity per NK cell did not differ between groups, suggesting that the NK cell defect in these patients is a quantitative one [95]. The authors observed that this may in part be due to long term treatment of patients with coronary artery disease with statins and beta-blockers. However, they suggested that conversely a loss of NK cell activity might lead to increased susceptibility to atherosclerotic-related pathogens with resulting consequences such as increased plaque burden and enhanced formation of vulnerable plaques [96]. This group went on to find increased percentages of apoptotic NK cells in peripheral blood of coronary artery disease patients when compared with healthy subjects, possibly related to increased oxidative stress [97]. The mechanism of NK cell deficiency influencing atherosclerosis is unclear, although the influence that NK cells have on multiple cytokines, in particular IFN-γ undoubtedly plays an important role. Interferon gamma has been implicated in the atherosclerotic process directly and indirectly and will be discussed further below.

In contrast, it has also been shown that patients with abdominal aortic aneurysms (AAA) have significantly higher percentages of peripheral blood NK cells than patients with peripheral vascular disease and control subjects [98]. This is of particular relevance as AAA can be thought of as a chronic inflammatory disorder of uncertain aetiology. Not

only were there higher numbers of NK cells, but the NK cells from patients with an AAA had increased cytotoxicity, and CRP values. CRP will be discussed in further detail below.

1.5 Cell Membrane Lipids

The Singer-Nicholson fluid mosaic concept remains the classical model of how the cell membrane is organised [99], proposing that the lipid bilayer functions as a neutral two-dimensional solvent. However, it has been found that lipids exist in several phases in model lipid bilayers, including gel, liquid-ordered, and liquid disordered, in order of increasing fluidity. The lipid composition of the cell membrane is highly complex, consisting of up to 500 different lipid species, cholesterol being a major lipid component. Eukaryotic cells, viewed in transverse section, are organised in heterogeneous multi-layers. This begins with the extracellular matrix, followed by a semipermeable lipid bilayer, the plasma membrane consisting of lipids and embedded proteins, and ending in a cytoskeletal meshwork loosely attached to the plasma membrane via anchoring proteins. More recently, the lateral organisation of the cell surface has received the most interest.

1.5.1 Lipid Rafts

Since the fluid mosaic model of the plasma membrane, numerous studies revisiting the architecture of the plasma membrane have provided a much more complex picture of the cell surface organisation. Of particular interest is the relatively recent proposal of

membrane lipid rafts. Lipid rafts are dynamic assemblies of proteins and lipids – microdomains - that float freely within the liquid-disordered bilayer of cellular membranes, but have the ability to cluster, forming larger ordered platforms [100]. A comprehensive definition was adopted at the Keystone Symposium on Lipid Rafts and Cell Function, 2006; "Membrane rafts are small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalise cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions". These microdomains are in liquid ordered phase and are resistant to solubilisation with non-ionic detergents. Lipid rafts are enriched in glycosphingolipids, sphingomyelin, and cholesterol, creating an environment which attracts and segregates specific proteins, while excluding others [100]. The attachment of lipid anchors, particularly those containing saturated fatty acyl chains, facilitates interaction with the liquid-ordered phase, leading to localisation of the modified protein to the lipid raft. Proteins with raft affinity include glycosylphosphatidylinositol (GPI)-anchored proteins [101, 102], proteins modified by dual acylation, typically myristoylation and/or palmitoylation such as Src-family kinases, or the α-subunits of heterotrimeric G proteins [103]. Rafts have been associated with a variety of signalling and sorting properties of many membrane components, and conversely, cholesterol depletion has been shown to disturb sorting and signalling properties of many membrane proteins. The proposed platforms are composed of cholesterol and sphingolipids in the outer exoplasmic leaflet, where the preponderance of saturated hydrocarbon chains in cell sphingolipids allows for cholesterol to be tightly intercalated, connected to phospholipids and cholesterol in the inner cytoplasmic leaflet

of the lipid bilayer [104]. Cholesterol is thought to act as a spacer between the hydrocarbon chains of the sphingolipids and to function as a dynamic glue that holds the raft assembly together.

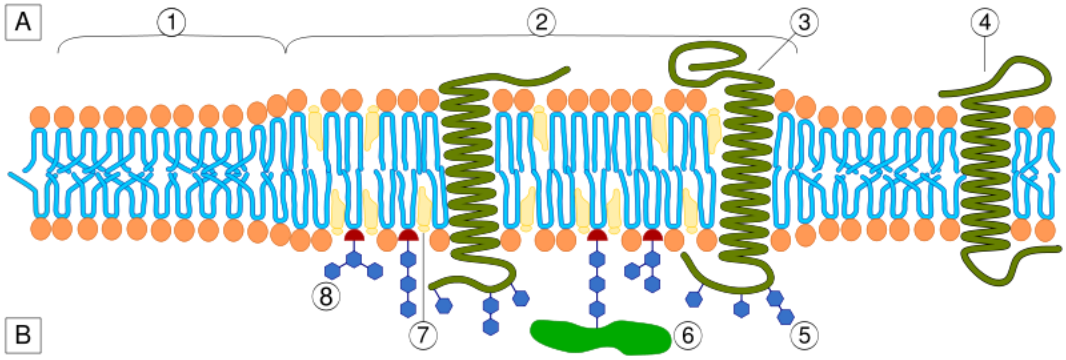


Figure 1.2 Organisation of the plasma membrane and microdomains. (A) Intracellular space or cytosol; (B) Extracellular space or vesicle/Golgi apparatus lumen; (1) Non-raft membrane; (2) Lipid raft; (3) Lipid raft associated transmembrane protein; (4) Non-raft membrane protein; (5) Glycosylation modifications (on glycoproteins and glycolipids); (6) GPI-anchored protein; (7) Cholesterol; (8) Glycolipid.

Biological functions attributed to lipid rafts include endocytosis, pinocytosis, and sorting and transport of proteins, however the most important role of rafts at the cell surface may be their function in signal transduction. It has been suggested that rafts form concentrating platforms for individual receptors, activated by ligand binding [105]. Raft binding recruits proteins to a new microdomain, where the phosphorylation state can be modified by local kinases and phosphatases, resulting in downstream signalling. Upon receptor stimulation, small lipid rafts may coalesce into bigger structures or become more rigid, possibly by associating to the cytoskeleton. Specialised microdomains called 'caveolae' constitute a distinct subset of lipid rafts. They are cell surface flask-shaped invaginations that contain caveolin as a major structural protein [106]. Lipid rafts have a relatively short lifespan, however caveolae appear to be more stable structures with a lower turnover rate. Caveolae and lipid rafts not only differ in stability, shape, lifespan and protein content, but also in proposed roles.

1.6 Statins

The introduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, in the late 1980s represented a breakthrough in the treatment of hyperlipidaemia and CVD in both primary and secondary prevention of CVD. In recent years, statins have emerged as the most important class of lipid lowering agents. They are potent inhibitors of cholesterol biosynthesis through inhibition of HMG-CoA reductase, the rate-limiting step of cholesterol synthesis, resulting in a dramatic reduction

in circulating LDL-cholesterol. In addition, the reduction of LDL-cholesterol leads to up-regulation of LDL receptors on the cell membrane and increased LDL clearance.

1.6.1 Role in cardiovascular disease

Several landmark clinical trials, such as the Scandinavian Simvastatin Survival Study (4S) (the first randomised controlled trial to show significant risk reduction in cardiovascular mortality in patients with coronary artery disease) [17], Cholesterol and Recurrent Events (CARE) [18], West of Scotland Coronary Prevention Study (WOSCOPS) [19], Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) [107], Air Force/Texas Coronary Prevention Study (AFCAPS/TexCAPS) [108], and the Heart Protection Study (HPS) [109], have demonstrated beyond doubt the beneficial effects of statin therapy for both primary and secondary prevention of cardiovascular disease. Lowering of serum cholesterol has been thought to be the primary mechanism underlying the therapeutic benefits of statin therapy in CVD. In the Atorvastatin versus Revascularisation Treatment (AVERT) study [110], aggressive cholesterol reduction with high dose atorvastatin was compared to coronary angioplasty in patients with stable angina. Those in the atorvastatin group had a significantly longer time to first ischaemic event, as well as a lower incidence of ischaemic events (although this was not statistically significant). In keeping with the findings of this study, other trials of angioplasty versus medical therapy in stable angina have not shown any benefit in terms of reduction of incidence of myocardial infarction or coronary mortality [111, 112]. Given the current understanding of atherosclerosis as an inflammatory disease, some of the benefits of

statin therapy are likely to be due to anti-inflammatory effects which will be discussed further below.

1.6.2 Clinical evidence of anti-inflammatory and pleiotropic effects

The strong association between serum cholesterol levels and coronary artery disease has led to the general assumption that the mechanism underlying the beneficial effects in CVD of statins is cholesterol reduction. However, subgroup analyses of large clinical trials have highlighted the possibility that the beneficial effects of statins extend beyond cholesterol reduction. Subgroup analysis of the WOSCOPS and CARE studies found that despite comparable serum cholesterol levels among the statin-treated and placebo groups, the statin-treated patients had a significantly lower risk of coronary heart disease than age-matched placebo-controlled individuals [113]. Statins have also been found to reduce cardiovascular events in normo-cholesterolaemic patients with coronary heart disease or cardiovascular risk [108]. Further strength is added to the possibility of cholesterol-independent benefits in that meta-analyses of lipid-lowering trials suggest that the risk of myocardial infarction in statin treated patients is significantly lower than in patients treated with other lipid-lowering agents, despite comparable reduction in serum cholesterol levels [114, 115]. In line with this, the level of efficacy and early treatment benefits as a result of statin therapy have so far been greater than the beneficial effects of non-statin lipid-lowering therapies. Angiographic studies in patients on statins have found that the reduction in lesion progression achieved was too mild to fully explain the reduced mortality and reduction in the incidence of coronary events [116], attributing the beneficial effects to plaque stabilization and remodelling. In the Myocardial Ischemia

Reduction With Aggressive Cholesterol Lowering (MIRACL) trial [117], a benefit in reduction of recurrent ischaemic events was seen as early as 16 weeks after acute coronary ischaemia, a period which may be too short for appreciable changes in vascular remodelling to have occurred. These observations have led to the consensus that statins indeed exert cholesterol independent, or "pleiotropic" effects. Various inflammatory markers have been studied to analyse the effect of statins on the inflammatory state. As discussed in section 1.4.2.2 high sensitivity CRP (hsCRP) has been widely studied. Human studies have shown that along with a reduction in cholesterol, statin treatment leads to a reduction in CRP [118-120]. In a recent trial, long-term treatment with statins was associated with a reduction in serum CRP levels and a better clinical outcome after acute myocardial infarction [119].

It also seems that the higher the dose of statin, the greater the beneficial effects [3, 117, 121]. However, it is difficult to ascertain whether the benefits are due to lower cholesterol levels or to greater pleiotropic effects. Statins also appear to have therapeutic benefits in diseases that are unrelated to elevated serum cholesterol levels, for example rheumatological diseases [122], stroke [123], Alzheimer disease [124], and multiple sclerosis [125]. Additionally, there is interest in the potential immunomodulatory effect of statins, in particular with regards to solid organ transplantation [126, 127].

1.6.3 Mechanism of action

1.6.3.1 Statins and cholesterol lowering

The majority of serum cholesterol is derived from hepatic synthesis, and the predominant pharmacological site of action in terms of lipid lowering is the liver. HMG-CoA reductase is the crucial rate-limiting enzyme in the cholesterol biosynthetic pathway, inhibition of this enzyme by statins results in a dramatic reduction in circulating LDL-cholesterol. Additionally, statins result in up regulation of the LDL receptor and therefore increased LDL clearance. Until recently, lowering of serum cholesterol has been thought to be the primary mechanism underlying the therapeutic benefits of statin treatment in cardiovascular disease.

1.6.3.2 Statins and anti-inflammatory effects

In addition to the clinical evidence outlined above, in vitro studies uniformly support the anti-inflammatory role of statins. As already discussed in section 1.4.1 one of the earliest stages in atherogenesis is the adhesion of monocytes to the endothelium as a result of various adhesion molecules. Statins have been found to interfere with this proinflammatory pathway of adhesion and migration at the levels of protein expression and function. Lovastatin and cerivastatin have been found to reduce the expression of CD11b-dependent adhesion of monocytes and inhibit the adhesion of leukocytes to endothelial cells [128, 129]. Statins selectively inhibit leukocyte adhesion by direct interaction with the leukocyte-function antigen-1 (LFA-1) [130]. Atorvastatin, lovastatin,

pravastatin, fluvastatin, and simvastatin reduce expression of the chemokine monocyte chemoattractant protein-1 (MCP-1) [131, 132]. Migration of monocytes/macrophages as well as vascular wall cells such as endothelial and smooth muscle cells depends on adhesion molecules and chemokines, but also on the activity of matrix metalloproteinases (MMPs), matrix-degrading enzymes [133] which may participate in weakening of the fibrous cap, making atherosclerotic lesions more likely to rupture. Statins lower the expression and function of a broad range of MMPs in many cells involved in atherogenesis [134, 135]. Additionally statins may have a protective role once the atherosclerotic plaque has ruptured by diminishing expression of procoagulant tissue factor in macrophages [135] and endothelial cells [136], promoting fibrinolytic activity [137], and modulating platelet function [138].

1.6.3.3 Statins and immunomodulation

The triggers of the inflammatory response observed in atherosclerosis have not yet been completely defined. It is suggested that autoantigens expressed in the atherosclerotic plaque may induce an immune response, and possible antigens include heat-shock protein 70 [139], LDL and non-LDL oxidative epitopes [140], and infectious agents [141]. Constitutive expression of MHC-II molecules is restricted to professional antigen presenting cells (APC), although several cell types can express MHC-II molecules upon cytokine stimulation, in particular by IFN-γ. Statins are able to inhibit this MHC-II expression on a variety of cell types, particularly IFN-γ induced expression of MHC-II molecules on endothelial cells, macrophages and microglia [142-144]. Statins are also able to suppress constitutive and IFN-γ induced expression of the co-stimulatory

molecules CD40, CD80 and CD86 on lymphocytes, macrophages, microglia and endothelial cells, and inhibit the up regulation of these molecules during maturation of dendritic cells (DC) [144-147]. Statin treatment has consequently been shown to result in suppression of DC maturation and therefore their ability to properly stimulate T cell proliferation [146]. In addition to MHC-TCR and co-stimulatory molecule interactions, cytokine signalling by APC is also an important factor in T cell activation. The effect of statins on cytokines has been widely studied both *in vitro* and *in vivo*. They have been found to inhibit the production of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-6 [148, 149], and additionally reduce the secretion of other inflammatory mediators as described above.

Different effects of statins on lymphoid cell function have been shown. T cell proliferation *in vitro* is suppressed and cytokine production inhibited [144, 150, 151]. Natural killer cell function has been shown to be reduced following treatment with simvastatin in healthy volunteers both *ex vivo* and *in vitro*. In patients with cardiovascular disease, both T cell proliferation and NK cell cytotoxicity have been shown to be reduced following statin treatment [150, 152, 153].

1.6.3.4 Statins and isoprenoid biosynthesis

The cholesterol biosynthetic pathway has been described in section 1.3.1. The inhibition of mevalonate biosynthesis by statins does not solely lead to lowered cholesterol levels, but also to reduced production of intermediate products of the mevalonate pathway (Figure 1) such as FPP and GGPP. This leads to inhibition of various cellular processes

that depend on the synthesis of these molecules. The role of these isoprenoid intermediates is as lipid attachments for the post-translational modification (isoprenylation) of a variety of proteins important in intracellular signalling, including the γ subunit of heterotrimeric G proteins and the small GTP-binding protein Ras, and Ras-like proteins such as Rho and Rac. Isoprenylation is a mechanism of post-translational lipid modifications leading to targeting of proteins to the cell membrane of other subcellular compartments. Isoprenylation is important for proper membrane localisation and function of these small GTP-binding proteins [154] and members of the Ras and Rho GTPase family are the major substrates for isoprenylation [155]. The Rho signal transduction pathway is involved in the activation of inflammatory cytokines and chemokines. Rho also plays an important role in the formation and maintenance of the actin cytoskeleton and thereby affects intracellular transport, mRNA stability, and gene transcription. The Ras proteins are responsible for cell proliferation and hypertrophy, whereas the Rac proteins are involved in the production of reactive oxygen species through activation of NADPH oxidase. Inhibition of HMG-CoA reductase by statins results in a decrease in farnesylated and geranylgeranylated proteins, and a subsequent dose-dependent reduction in downstream signalling pathways mediated by Rho, Ras, and Rac. Statins have been shown to induce changes in the actin cytoskeleton and assembly of focal adhesion complexes by inhibiting RhoA and Rac1 isoprenylation [10]. Inhibition of RhoA by statins also increases eNOS expression and decreases severity of cerebral ischaemia in a mouse model of ischaemic stroke [156]. Statins also increase the expression of tissue-type plasminogen activator and inhibit the expression of plasminogen activator inhibitor-1 [137] and endothelin-1 by mechanisms involving

inhibition of geranylgeranylation [157]. As Ras and Rho also regulate the cell cycle, the antiproliferative effects of statins may act via this mechanism, indeed statins inhibit vascular smooth muscle cell proliferations in transplant-associated arteriosclerosis [152] and may have clinical benefits in inhibiting certain breast cancers [158]. Additionally, statin inhibition of Rac1 geranylgeranylation and Rac1-mediated NAD(P)H oxidase activity attenuates angiotensin II induced reactive oxygen species production in vascular smooth muscle cells and cardiac myocytes [159, 160]. Finally, statins have been shown to affect T cell proliferation by reducing the functional activity of Ras-dependent ERK pathways and Rho-dependent p38 activation [151].

1.6.3.5 Statins and lipid rafts

As discussed in section 1.5, cholesterol plays an important role in the intracellular transport of proteins to the cell membrane. Within the cell membrane, lipid rafts play an important role in the recruitment and concentration of cell signalling molecules to the plasma membrane, and have important roles in both T and B cell signalling. As cholesterol is one of the major components of lipid rafts, statin treatment leads to disruption of these rafts [105, 145, 153]. Further evidence for this theory that statins have some of their actions by causing the disruption of lipid rafts, comes from studies showing that statin treatment of various cell types does not affect membrane expression of molecules known not to be associated with lipid rafts such as CD4 or the transferrin receptor (CD71) [145]. It has also been shown that *in vitro* statin treatment results in disruption of lipid rafts, resulting in impaired Fcγ-receptor signalling at the level of tyrosine kinase activation [161] and more recently, statin treatment of NK cells *in vitro*

caused reduction of NK cytotoxicity and reduction of lipid rafts [153], suggesting lipid raft disruption as a potential mechanism for some of the pleiotropic effects of statins. Further support for this has been shown where simvastatin treatment of healthy volunteers lead to a reduction in lymphocyte lipid raft levels [150]. With cell signalling molecules concentrated within lipid rafts, their disruption results in impairment of immune receptor signalling, reduced activation of downstream signal transduction cascades important for the induction of inflammatory mediators, thereby affecting immune activation.

1.7 Chronic Kidney Disease

Chronic kidney disease (CKD) has been defined as persistent kidney damage (confirmed by renal biopsy or markers of kidney damage) and/or glomerular filtration rate (GFR) <60 mL/min/1.73m^2 for longer than three months [162]. CKD is a large and increasing problem worldwide, in fact using the above definition, there are an estimated 11 million individuals and rising with CKD [162]. With our aging population, the rates of treated end-stage renal disease (ESRD) in the elderly has increased by more than 50% over the last decade [163]. Whilst only a minority progress to ESRD, the significance of CKD is gaining increasing importance following the recognition that even mild renal impairment is associated with higher mortality from any cause [164-167], but in particular, from cardiovascular causes [165, 166, 168, 169]. Indeed, patients with CKD have a higher probability of death than of receiving renal replacement therapy.

1.7.1 CKD and cardiovascular disease

We now know that cardiovascular disease is the leading cause of death in patients with CKD [170]. The cardiovascular mortality rate in dialysis patients is approximately 30 times the risk in the general population and remains 10 to 20 times higher even after stratification for age, gender, and presence of diabetes. Indeed cardiovascular disease accounts for more than half the deaths in patients with ESRD. The high incidence of cardiovascular disease is due in part to a high incidence of traditional cardiovascular risk factors such as diabetes, hypertension, LVH, anaemia, advanced age, and hypercholesterolaemia. Additionally, there are factors that are unique to this patient population, such as vascular calcification, uraemic toxins, hyperphosphataemia, hyperhomocysteinaemia, chronic inflammation, and oxidative stress (Table 5). Additionally, decreased glomerular filtration rate has also been shown to be a risk factor for cardiovascular disease [168, 171] and it seems that the risk of death is inversely proportional to the GFR [172].

In recent years, attention has focused on identifying those risk factors specific to the state of uraemia to attempt to explain the massively increased rates of mortality in this patient population. In the early stages of CKD, the more traditional risk factors are more important, as generally, many patients who develop CKD have a history of hypertension, diabetes, hyperlipidaemia etc. As renal function worsens, the non-traditional risk factors become more important, for example, the degree of anaemia and secondary hyperparathyroidism will worsen with decreasing GFR. Among the so-called non-traditional risk factors, inflammation has been shown to be an epidemiologically

important risk factor for cardiovascular disease in the general population [32]. In the ESRD population, inflammation strongly predicts all-cause [173] and cardiovascular mortality rates [173, 174].

Traditional Risk Factors	Non-traditional Risk Factors
Age	Albuminuria/Proteinuria
Male gender	Hyperhomocysteinaemia
Hypertension	Anaemia
Diabetes Mellitus	Abnormal calcium/phosphorous metabolism
Smoking	Increased oxidative stress
Dyslipidaemia	High lipoprotein (a)
Left ventricular hypertrophy	Malnutrition/inflammation
Family history	Haemodynamic overload
Sedentary lifestyle	Elevated asymmetrical dimethylarginine levels
Menopause	

Table 1.5 Cardiovascular risk factors in CKD patients

1.7.2 CKD and dyslipidaemia

The prevalence of dyslipidaemias in CKD patients is very high. In one study of patients on both haemodialysis and peritoneal dialysis patients, the prevalence of dyslipidaemia was approximately 67% [175], where dyslipidaemia had been defined as at least one abnormal lipid parameter. In fact, peritoneal dialysis seems to be associated with a relatively more atherogenic lipid profile than haemodialysis [176], which may be related to the continuous glucose load. Typically in ESRD patients have either normal or slightly increased LDL, increased VLDL and IDL, leading to elevated triglyceride levels, and decreased levels of HDL. The hypertriglyceridaemia results from increased synthesis and decreased clearance, the later as a result of low expression and activity of lipoprotein lipase (LPL) and hepatic lipase (HL). The characteristic low levels of HDL arises from low activity of lecithin cholesterol acyltransferase (LCAT), which esterifies cholesterol taken up by HDL, therefore enabling HDL to acquire subsequent cholesterol particles [177]. The low levels of HDL cholesterol are also a consequence of increased cholesterol ester transfer protein (CETP) activity and of acyl CoA:cholesterol acyltransferase (ACAT), as well as decreased activity of LPL and HL [178].

Patients with ESRD additionally undergo qualitative changes in dyslipidaemia with a shift to a more atherogenic profile, with a shift of LDL particle size toward a small, dense apo-B-rich LDL predominance. Lipoprotein (a) is an LDL-like particle whose protein moiety contains apolipoprotein (a) which has been shown to be an independent risk factor for CVD in both the general and the CKD population [179]. Levels of lipoprotein (a) have been shown to be increased in CKD, in particular in patients with nephrotic

syndrome and in peritoneal dialysis due to significant protein losses and subsequent apolipoprotein overproduction [180, 181]. Therefore, even when the concentration of LDL cholesterol is normal, LDL particles are more atherogenic in ESRD. Of particular note, in nephrotic syndrome, hypercholesterolaemia is a typical feature, triggered by increased apolipoprotein production. This is followed by an increase in cholesterol biosynthesis resulting in profound hypercholesterolaemia. There is also impaired lipoprotein clearance. Animal studies show that cholesterol synthesis is increased in CKD [182]. Gene expression and activity of HMG-CoA reductase is significantly augmented in experimental CKD [183].

As described above, in the general population, the relationship between hyperlipidaemia and CVD (predominantly coronary artery disease) is well established, as are the proven benefits of lipid-lowering with statins. There is only limited epidemiological and even more limited interventional data on the relationship between dyslipidaemia and CVD in ESRD and the clinical implications, creating uncertainties with regards to the impact of dyslipidaemia on CVD in ESRD, and also with regards to treatment. In patients receiving maintenance haemodialysis, reports suggest either no relationship or paradoxical correlations, the so-called "reverse epidemiology" paradox, where a lower total cholesterol level has been associated with a higher risk of death [184-186], or conversely, a higher serum cholesterol has been found in long-term dialysis survivors [187]. These studies have often used registry data, and either all cause mortality or unspecified CV mortality. Similar "J" or "U"-shaped relationships between lipid levels and all cause mortality have been reported in other populations and are thought to reflect

a high prevalence of co-morbid disease (specifically malignancy and associated malnutrition) in patients with low cholesterol levels. It is likely that similar relationships are present in patients with advanced renal failure, further compounded by the atypical mix of CV outcomes in this population. The notion that hyperlipidaemia is not harmful – and even protective – in ESRF may also be flawed by the tendency to look at hypercholesterolaemia rather than dyslipidaemia. "Uraemic dyslipidaemia" involves quantitative and qualitative changes in a variety of lipid subfractions that may contribute to premature CVD, even though total and LDL cholesterol levels are low. Additionally, the so-called reverse epidemiology phenomenon may simply be an observation confounded by the effects of protein energy malnutrition (PEM) and inflammation [188] and will be discussed in further detail below.

The clinical implications of dyslipidaemia in CKD patients are not solely limited to the development of atherosclerotic CVD. There is growing evidence that hyperlipidaemia contributes also to renal disease progression. Animal models have shown that a high-fat diet (hypercholesterolaemia) causes macrophage infiltration and foam cell formation in rats, leading to glomerulosclerosis [189]. Human studies have supported this; a relationship between serum cholesterol levels and GFR decline has been shown in several studies [190-192]. This has also been shown in patients with type 2 diabetes and overt nephropathy; a *post hoc* analysis of the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study [193] showed that both total cholesterol and LDL cholesterol measured at baseline were independent risk factors for ESRD. In line with these findings, statins have shown beneficial effects in different

models of progressive renal failure [194], although there is not yet a large interventional study on the effect of statin therapy in the progression of renal damage, there is evidence from *post hoc* analyses to suggests that statins are likely to be effective in the treatment of renal disease. The use of statins in CKD will be discussed in further detail below.

1.7.3 CKD and inflammation

The inflammatory state of renal failure, particularly in its advanced stages is widely recognised. As mentioned above, a factor that contributes to the cholesterol paradox is the presence of malnutrition and inflammation. Protein-energy malnutrition is present in a large proportion of patients with CKD and is a strong risk factor for CV mortality in dialysis patients [195]. Low serum albumin, an index of malnutrition, is also highly associated with increased mortality in patients with ESRD [184], and correlated with high concentrations of acute phase proteins, such as CRP indicative of systemic inflammation [196, 197]. Available evidence suggests that CRP is a precise objective index of the inflammatory activity and that it accurately reflects generation of pro-inflammatory cytokines, such as IL-6 and TNF-α. Accordingly, elevated serum levels of pro-inflammatory cytokines have been demonstrated to be associated with increased mortality in dialysis patients [198, 199]. Inflammation is now an established risk factor for coronary artery disease, and has an inconsistent relationship with serum lipids in the general population. In ESRD intercurrent illness, inflammation and malnutrition in ESRD may confound the relationships between dyslipidaemia and CVD. This hypothesis has been explored in a study that examined the relationship between markers of inflammation and CVD in a cohort of 1243 haemodialysis patients followed,

prospectively for 10 years [200]. In patients with low serum cholesterol levels the mortality rate was significantly higher even after adjusting for possible confounders affecting baseline levels of serum cholesterol, such as age, body mass index (BMI) and serum albumin. However, in the subgroup of patients with normal serum albumin, hypercholesterolaemia was a significant risk factor for death, and low total cholesterol was in fact associated with a lower risk of death. In addition, death from CV events was more prevalent in patients with high levels of serum cholesterol than in those with low levels.

Thus, there is a U-shaped, or J-shaped relationship between cholesterol levels and mortality. Total cholesterol levels appear to influence survival in two ways; first as a marker of malnutrition and poor health, which identifies patients at high risk of death, and second as a more traditional CV risk factor (and a causative factor in atherosclerosis). More recently, the effect of inflammation and malnutrition has been investigated in more detail. Liu and colleagues [201] followed up 823 chronic haemodialysis patients for a median of 2.4 years, and categorised the study population into two subgroups based on the presence or absence of inflammation or malnutrition (defined as achievement of specified cut-offs for any of serum albumin, CRP and IL-6 derived from studies of the general population). Overall, 77% of the patients had evidence of inflammation/malnutrition, which was associated with older age, increased co-morbidity, prevalent CVD, diabetes, and lower total cholesterol levels. A U-shaped association between cholesterol levels and mortality was seen in the overall cohort and in the subgroup with inflammation/malnutrition. However, in the absence of

inflammation/malnutrition, total cholesterol was positively associated with all-cause mortality and even more strongly associated with CV mortality. This was also true when the analysis was repeated with non-high-density lipoprotein cholesterol (non-HDL-C) as the independent variable. This is further supported by the finding that CRP is an independent predictor of the number of atherosclerotic plaques in the carotid arteries of dialysis patients [202], and a strong relationship between elevated CRP levels and atherosclerosis has also been documented in ESRD patients [203].

Whether there is a mechanistic relationship between hypocholesterolaemia and hypoalbuminaemia, both are negative "acute-phase" responses to inflammatory stimuli. Elevated levels of pro-inflammatory cytokines are one of the reported causes of hypocholesterolaemia [198]. Hypocholesterolaemia and hypoalbuminaemia are also markers of malnutrition and an indicator of energy intake. PEM is a recognised complication of ESRD to which anorexia, low nutrient intake, nutrient losses during dialysis, intercurrent illness, oxidative and carbonyl stress and chronic inflammation contribute [204]. Both PEM and inflammation may reduce serum concentrations of acute phase proteins, such as albumin, transferrin, pre-albumin, and cholesterol-carrying lipoproteins, thus acting as markers of concomitant illness that may themselves predispose to CVD.

Causes of inflammation in ESRD are multi-factorial and include patient-specific processes, such as infections and clotted access grafts, however increased production and decreased renal clearance of pro-inflammatory cytokines, co-morbid disease processes,

accumulation of advanced glycation end-products (AGEs) and multiple factors associated with dialysis itself, may also contribute to inflammation in ESRD.

End-stage renal disease	Additional causes in dialysis	Additional causes in peritoneal dialysis
Reduced renal clearance of cytokines	Graft and fistula infections	Peritonitis
Accumulation of AGEs	Bioincompatibility of dialysis membrane	Bioincompatibility of peritoneal dialysis solution
Chronic heart failure	Exposure to endotoxins and other cytokine-inducing substances from contaminated dialysate	Exposure to endotoxins and other cytokine-inducing substances from contaminated dialysate
Atherosclerosis		
Unrecognised persistent infections		

Table 1.6 Causes of inflammation in end-stage renal disease

1.7.4 CKD and immune dysfunction

Although the medical determinants of mortality in patients with ESRD are well recognised, the contribution of immunologic parameters to survival is unclear. Patients with ESRD undergoing haemodialysis display a state of immunodeficiency, and dysregulation of cytokine metabolism has long been appreciated. It has been shown that higher levels of circulating proinflammatory cytokines are associated with mortality [198], while immune parameters reflecting improved T-cell function are associated with survival in ESRD patients on haemodialysis , independent of other medical risk factors. Immune dysfunction in uraemia is associated with alterations in both the innate and adaptive branches of the immune system.

All classes of the innate immune system pattern recognition receptors (secreted, endocytic, signalling) are affected in ESRD [205], resulting in impaired function of the effector cells. Monocytes from patients with ESRD have been shown to have impaired function [206] and when normal monocytes are cultured in uraemic serum their function also becomes impaired [207]. Bactericidal activity of neutrophils have been found to be reduced in haemodialysis patients [208]. Natural killer cell activity in renal failure will be discussed in further detail below. ESRD results in various cytokine disturbances involving both anti-inflammatory and pro-inflammatory cytokines, supporting the idea of uraemia and dialysis as a chronic inflammatory state. The increased levels of cytokines in ESRD is thought to be as a result of reduced rate of removal and increased generation [209], and the development of PEM and atherosclerosis has been discussed above.

The increased rate of infections, together with an impaired response to vaccination and a common failure of tuberculin skin test to diagnose latent tuberculosis indicates that adaptive immunity is impaired in patients with ESRD [210]. Non response to vaccination is closely associated with an impaired proliferation of T-cells *in vitro* and a reduced production of the autocrine T-cell growth promoting cytokine IL-2 [211]. Lymphocyte numbers as well as the CD4/CD8 relation are diminished in dialysis patients [212], and reduced lymphocyte counts have been identified as predictors of mortality in haemodialysis patients [213]. In addition to this however, there is also reduced co-stimulation by antigen presenting cells. In other words, not only is the quantity of T-cell activation affected, but the quality is also reduced. The altered T-cell function has been attributed to impaired function of APCs [210], possibly secondary to a disorder in toll-like receptor expression or activity. A reduction in B-cells has also been shown and suggested that this is due to increased apoptosis [214].

The causes of the immune dysfunction seen in ESRD are multiple and are related both to the state of uraemia and to the dialysis that corrects uraemia. The accumulation of uraemic toxins, interactions between blood and dialyser, endotoxins in water, access-related infections, peritoneal dialysis solutions with high glucose concentration, low pH and the presence of glucose degradation products all present chronic stimuli to the inflammatory response. It is interesting to speculate that with the well established associated between inflammation and cardiovascular disease there may also be evidence for a causal relationship between infections and cardiovascular disease [215], and

accordingly a link between the immune dysfunction in ESRD and cardiovascular disease through these infections and inflammation.

1.7.4.1 CKD and natural killer cell function

Studies in the immune dysfunction of renal failure have focused mainly on T- and B-cell adaptive immunity. However, there is increasing interest in the NK cell compartment with some conflicting reports, some suggesting that a NK deficit occurs in haemodialysis patients [216], and more recent studies suggesting that NK cell counts are enhanced [212]. It is possible that this change may be related to improvements in modern dialysis technique and efficiency, water purity, and membranes. A decrease in the cytotoxic activity of the NK cells compared with controls has been found in patients on haemodialysis [217], in particular those dialysed with cuprophan membranes. Natural killer cell activity has also been shown to be related to the total time on haemodialysis, with a shorter time on dialysis being related to lower NK cell activity [217]. More recently, it has been demonstrated that patients on chronic haemodialysis have a normal number of lymphocytes and NK cells, however there is decreased NK cell activity as indicated by decreased expression of the ζ-chain on NK cells, an early marker of NK cell activation [218]. The high incidence of cardiovascular disease in this patient population together with the participation of NK cells in atherosclerosis make this an important area to investigate further.

1.7.5 CKD and Phosphate

Hyperphosphataemia is invariably present among patients with advanced CKD, resulting from decreased excretion and increased parathyroid hormone (PTH) through its action to release calcium and phosphate from bone. Several studies have found an association between higher phosphate levels and cardiovascular events and mortality in patients with both CKD and normal renal function [219-223]. Notably, in one study of 3490 older, predominantly male veterans with CKD stages 3 to 4, each 1mg/dl higher serum phosphate concentration was associated with a statistically significant 23% greater adjusted risk for all-cause mortality [220]. This is in keeping with data from two large, national, randomised, cross-sectional samples of 6407 haemodialysis patients who had all dialysed for at least one year, which showed that patients with a serum phosphate level of > 6.5mg/dL had a 27% higher mortality risk than patients with serum phosphate levels of 2.4 to 6.5 mg/dL [223]. A high calcium-phosphate product (Ca x P) in excess of 72 mg^2/dL^2 was also associated with a relative risk (RR) of mortality of 1.34 ($P < 0.01$) compared with patients with a Ca x P between 42 and 52 mg^2/dL^2. The increased mortality risk was not associated with serum calcium concentrations suggesting that the increased risk results from the phosphate levels. The exact mechanism responsible for the increased mortality in patients with hyperphosphataemia is not entirely clear, however is thought to be primarily cardiovascular in nature. Several observational studies in populations with CKD have suggested a role for phosphate in vascular calcification of smaller arterial beds, in particular the coronary arteries. Recent studies have shown that patients with ESRD on dialysis have a higher prevalence and severity of coronary artery calcification (CAC) than healthy subjects of the same age and sex [224, 225].

Cardiovascular calcification has been linked to an increased risk of cardiovascular events, including myocardial infarction, fatal arrhythmia, congestive cardiac failure, and valvular heart disease [226, 227]. In addition to the traditional cardiovascular disease risk factors, studies in dialysis patients have shown a correlation between CAC and a number of dialysis-related factors, such as dialysis vintage, hyperphosphataemia, high Ca x P product, and vitamin D therapy [225, 227]. The exact mechanisms resulting in vascular calcification have not been completely elucidated. In vitro, phosphate acts directly on cultured vascular smooth muscle cells to initiate phenotype transformation, characterised by loss of contractility, expression of bone-specific markers, and calcification of matrix proteins [228, 229]. Thus high phosphorous levels seem to stimulate osteoblastic differentiation of vascular smooth muscle cells and directly enhance extracellular calcification. It would therefore appear that the high serum phosphate levels play an important role in contributing to the markedly increased prevalence of cardiovascular calcification and therefore cardiovascular mortality in patients with CKD.

1.8 Other Biomarkers

The role of inflammation in atherosclerosis is undisputed, and much interest has developed surrounding the association of inflammatory biomarkers with cardiovascular disease, and in particular the possibility of a pathogenic role of these biomarkers in the atherosclerotic process. Not only has an association been established, but inflammatory markers have been shown to predict and correlate independently with major cardiovascular end points such as myocardial infarction and death [30, 39]. The potential

of identifying a biomarker that will be able to facilitate the diagnosis of cardiovascular disease is hugely attractive, potentially resulting in more accurate risk stratification and treatment selection, particularly when in a reasonable proportion of patients, the traditional cardiovascular risk factors do not provide information on the presence of atherosclerosis, and other specific factors such as inflammation need to be considered.

1.8.1 High Sensitivity CRP

High sensitivity CRP is the term applied to a test that detects serum CRP concentration at lower levels than previous generations of laboratory tests. High sensitivity CRP detects the same CRP molecule but its lower limit of detection is substantially lower, it can therefore detect much lower levels of inflammation, so when testing CRP for vascular risk assessment, hsCRP is used as the levels of CRP are low in comparison to other inflammatory states such as sepsis. As discussed previously, elevated serum hsCRP has been shown to be a stronger predictor of incident cardiovascular events in healthy men than LDL cholesterol and to be additive to the Framingham risk score [39]. In healthy men and women, a significant relationship has been found between high blood pressure, hsCRP and IL-6 [230]

1.8.2 Interleukin-6

The plasma cytokine IL-6 plays an important role in mediating inflammation, and is a central stimulus for the acute-phase response, in particular it induces the hepatic synthesis of CRP [231], and thus circulating levels of IL-6 and CRP are physiologically linked. Elevations of IL-6 however have been shown to have independent associations with

cardiovascular events, even after controlling for CRP. In the Physician's Health Study, elevated levels of IL-6 in apparently healthy males predicted up to a 2.3-fold increase in myocardial infarction over 6 years [232]. Among these men, the number of risk factors at baseline was correlated with the plasma concentration of IL-6. Baseline levels of IL-6 have also been found to predict cardiovascular events in the Women's Health Study [33]. In addition, IL-6 levels have also been shown to be a better predictor of mortality than CRP [233, 234]. IL-6 and CRP have also been shown to be associated with traditional risk factors for cardiovascular disease independent of lipid levels, supporting a separate or concurrent pathway to atherosclerosis [235]. More recent studies have suggested that CRP and IL-6 may not always track each other, and in actual fact divergent levels may be seen under certain conditions, suggesting that IL-6-independent pathways may exist in the regulation of circulating levels of CRP [235].

1.7.5 Pentraxin-3

The pentraxins are a family of proteins which includes CRP, considered to be markers of the acute phase of inflammation, structurally distinguished by a characteristic pentameric structure [236]. Pentraxin 3 (PTX3) is a long pentraxin that is structurally related to, although distinct from, prototypic classic short pentraxins such as CRP and serum amyloid P (SAP). PTX3 was the first long pentraxin to be discovered, and its expression is induced in response to inflammatory stimuli, for example infections, IL-1, and TNF-α, but not IL-6 [237-240]. PTX3 is produced by a number of different cell types including in particular two of the major cellular components of atherosclerotic lesions – macrophages and endothelial cells – as well as monocytes and fibroblasts, however

unlike CRP it is not produced in the liver. It is found in advanced atherosclerotic lesions [241], and as it is produced from vascular endothelial cells and not hepatocytes, it is interesting to speculate that PTX3 levels may more directly reflect the inflammatory status of the vasculature, and therefore have a greater sensitivity and specificity for cardiovascular inflammation. In a mouse model, systemic administration of microbial products and inflammatory cytokines, or ligation of the left coronary artery results in expression of PTX3 at high levels [239]. It is induced by oxidized LDL in smooth muscle cells [242] and interestingly PTX3 has been found to be suppressed by statin treatment in human umbilical vein endothelial cells (HUVEC) by cDNA microarray analysis [243]. PTX3 levels have been found to be increased in patients with unstable angina [244] and myocardial infarction [245], and to be predictive of outcome.

1.7.6 Adiponectin

Adipose tissue is now considered an endocrine organ as it is able to release many biologically active substances – adipocytokines, or adipokines – in response to specific extracellular stimuli or to variations in metabolic conditions. These adipokines may directly contribute to obesity-linked metabolic and vascular diseases [246]. Adiponectin is an adipocyte-specific plasma protein [247] that circulates in high concentrations (approximately 0.01% of total plasma protein) [248]. The relationship of this protein to fat and body mass is the converse of leptin, another adipokine. Adiponectin levels are significantly reduced among obese subjects when compared to lean, healthy controls [248], and in fact weight loss results in a marked increase in plasma adiponectin levels among both normal controls and type 2 diabetics [249]. Plasma adiponectin

concentrations are higher in woman than men, low in diabetics, and inversely related to plasma glucose, insulin, and triglyceride levels [249]. Hypoadiponectinemia has also been observed in patients with coronary artery disease, and is highly associated with coronary artery disease prevalence even after adjustment for established coronary artery disease risk factors such as diabetes mellitus, dyslipidaemia, hypertension, smoking, and BMI [250] in men. In patients with ESRD, adiponectin has been shown to be a strong and independent (inverse) predictor of cardiovascular outcomes [251].

Adiponectin appears to play a protective role in experimental models of vascular injury with anti-inflammatory and anti-atherogenic properties. Plasma adiponectin rapidly accumulates in the subendothelial space of the injured human artery. It has been shown to inhibit monocyte adhesion to endothelial cells and macrophage-to-foam cell transformation, as well as TNF-α secretion from macrophages and vascular smooth muscle cell proliferation in vitro [252-255]. Other effects include increased insulin sensitivity and free fatty acid oxidation, and decreased hepatic glucose production and intracellular triglycerides [256]. It would therefore seem that adiponectin acts as a modulator of the inflammatory response in the vascular wall, and hypoadiponectinemia may cause an excessive inflammatory response in coronary arteries. Indeed in humans, low levels of adiponectin appear to be closely correlated with severity of endothelial dysfunction [257]. In keeping with this, an inverse correlation has been demonstrated between the levels of adiponectin and CRP [258] and it has recently been reported that adiponectin significantly decreases CRP mRNA and protein levels [246].

1.8 Statin trials in chronic kidney disease

1.8.1 4-D Study

Despite the enormously increased incidence of cardiovascular disease in patients with ESRD, observational studies have so far not demonstrated a positive relationship between total cholesterol and mortality in haemodialysis patients. As discussed above, this is complicated by the phenomenon of "reverse epidemiology" and confounded by invariable underlying comorbidity. A reverse relationship between lipid levels and mortality exists where low cholesterol is in fact associated with higher mortality rates [201], however the question of whether lipid lowering is beneficial in this patient population remains a contentious issue. Aside from the high cardiovascular risk, this patient group may actually benefit more from statin therapy given the prominent oxidative stress and inflammation that is know to occur, as well as the highly atherogenic lipid profile that occurs, that cannot be detected by measuring and correlating serum cholesterol on its own.

The 4D study (Die Deutsche Diabetes Dialyse Studie) [259] was a study of atorvastatin versus placebo in 1255 type 2 diabetics on regular haemodialysis, those patients who represent the highest risk for cardiovascular disease. Atorvastatin lowered mean LDL-cholesterol levels by 42% to 72mg/dl without a significant reduction in the primary end-point (a composite of cardiac death, non-fatal MI, and fatal or non-fatal stroke). The investigators speculated that the results reflected a different pathogenesis of vascular

events in diabetic patients with ESRD, or that the intervention was too late in the natural history of such complex disease, although atorvastatin therapy did reduce the incidence of MI. Therefore, an alternative explanation is that in the complex mixture of CVD that affects patients with ESRD, the relationship between cholesterol and MI is present (and lipid-lowering reduces this end-point) but that MI is not the dominant CV end-point.

1.8.2 AURORA

A more recent trial, the AURORA study (A Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Haemodialysis: An Assessment of Survival and Cardiovascular Events) was designed to investigate the effects of statin therapy in a more wide-ranging population of patients with ESRD on regular haemodialysis [260]. 2776 patients were randomly assigned to double-blind treatment with rosuvastatin or placebo. Approximately half of the patients had diabetes, and the inclusion criteria specified patients aged 50-80 years, who had been on haemodialysis for at least three months and not expected to receive a renal transplant within a year. This study population were therefore at higher cardiovascular (and all cause) risk than the general maintenance haemodialysis population. Rosuvastatin treatment was associated with modification in serum lipids comparable to the effects of statin therapy in other populations and an 11% reduction in CRP, however despite these favourable effects, those patients randomised to rosuvastatin showed no reduction in the composite cardiovascular end-point (non-fatal MI, cardiovascular death or stroke), any individual end-points, or all-cause mortality. Although these findings support the 4D study, there were limitations in the AURORA study. In particular, the exclusion of patients already on statin treatment which

presumably includes those patients at highest risk of coronary disease, and also the age restrictions.

1.8.3 ALERT

The relationship between lipid concentrations and cardiovascular events is less clear in renal transplant recipients than in other populations. The ALERT trial (Assessment of LEscol in Renal Transplantation) is the only major cardiovascular outcome trial in this patient population [261]. This was a multicentre, randomised, double-blind, placebo-controlled trial in 2102 cyclosporin treated adult renal transplant recipients with a total cholesterol between 4.0-9.0 mmol/L, randomly assigned to fluvastatin (Lescol) or placebo, and followed up for 5-6 years. For the primary end-point, the occurrence of a major adverse cardiac event (a composite of cardiovascular death, non-fatal MI and coronary intervention), there was a 13% reduction that did not reach statistical significance. Failure to achieve statistical significance resulted from the fact that the study was underpowered to detect a significant reduction in the chosen primary endpoint as the recruited population had an event rate lower than registry data suggested. However, fluvastatin therapy was associated with a reduction in risk of MI, of cardiac death, and of the secondary end-point of cardiac death and non-fatal MI, consistent with the beneficial effects of statins in other populations.

The ALERT extension trial was a 2 year open-label extension of the core trial during which all patients were offered fluvastatin treatment [262]. The patients randomised to fluvastatin had a significantly reduced risk of the primary end-point (major adverse

cardiac events) by 21%, and a 29% reduction in cardiac death or non-fatal MI. This is in keeping with reductions of between 9% and 37% seen in other primary prevention trials of statin therapy, suggesting that fluvastatin achieves a similar cardioprotective effect in renal transplant recipients.

1.8.4 JUPITER

Although this trial was not carried out in the CKD population, it is mentioned here due to the support it lends to the inflammatory nature of cardiovascular disease. The JUPITER study (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) was a randomised, double-blind, placebo-controlled, multicentre trial of 17,802 patients, investigating rosuvastatin versus placebo in apparently healthy persons with LDL-C levels below current treatment threshold (<130 mg/dL), but with elevated levels of hsCRP (>2.0mg/dL) [263]. By 12 months, in the rosuvastatin group, the median LDL-C had fallen by 50%, and the median hsCRP level had fallen by 37%. The rosuvastatin group had a 44% lower incidence of the primary end-point ($P < 0.00001$) (defined as the composite of cardiovascular death, myocardial infarction, stroke, hospitalisation for unstable angina, or arterial revascularisation), as well as a 20% reduction in all-cause mortality. The study was terminated early after an average follow-up period of 1.9 years due to statistically significant reductions in the incidence of acute MI, stroke, revascularisation, unstable angina, and cardiovascular death in the rosuvastatin group. This trial lends support to the idea that where LDL-C is concerned "lower is better". The JUPITER study does not offer any further clarification as to whether hsCRP is a mechanistic participant in atherosclerosis, or simply a biomarker, but

certainly adds weight to the use of hsCRP as a non-lipid marker to identify those who may benefit from statin therapy.

1.9 Background Work to Aims and Hypothesis

The background to this thesis was previous work that had been carried out in our research group on statins and lymphocyte function, as briefly described in section 1.6.3.5. This work was carried out to investigate the hypothesis that the pleiotropic effects of statins may result from lipid raft disruption, rather than by the reduction of isoprenoid intermediates, and that the disruption of membrane rafts, and therefore cell signalling apparatus, may be one mechanism by which statins exert their immunomodulatory and anti-inflammatory effects.

Both T lymphocytes and NK cells have been implicated in experimental and human atherosclerosis, although NK cells are less well studied. It has been shown already that NK cell function is sensitive to both in vitro (Figure 1.3) and in vivo statin treatment, with reductions in NK cell cytotoxicity that recover following withdrawal of treatment, and that the changes in NK cell cytotoxicity appear to parallel changes in LDL-C in statin treated patients [150, 153].

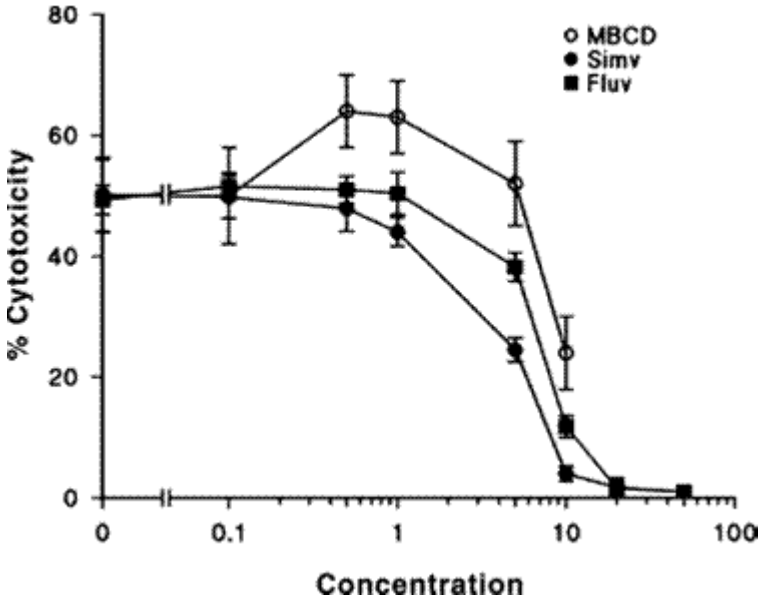


Figure 1.3 Pre-incubation of NK92MI cells with either fluvastatin (Fluv) or simvastatin (Simv) reduced NK killing of K562 cells (% killing of K562 cells) in a dose-dependent manner, with simvastatin being more potent than fluvastatin. Incubation of cells with methyl-beta-cyclodextran (MBCD), a cholesterol depleting agent, also results in reduced NK cell cytotoxicity. (Reproduced with permission) [153]

As many isoprenylated proteins are localised in membrane rafts, membrane cholesterol depletion and inhibition of isoprenylation may have similar physiological effects. This was investigated by using specific inhibitors of farnesyl and geranylgeranyl transferase. Proteins that are incorporated into the cell membrane by unrelated processes were studied. Inhibition of farnesyl and geranygeranyl transferase have been shown to have little effect on NK cell cytotoxicity, suggesting that NK cell cytotoxicity is less susceptible to inhibition of isoprenylation than proliferation and function of other cells types (Figure 1.4).

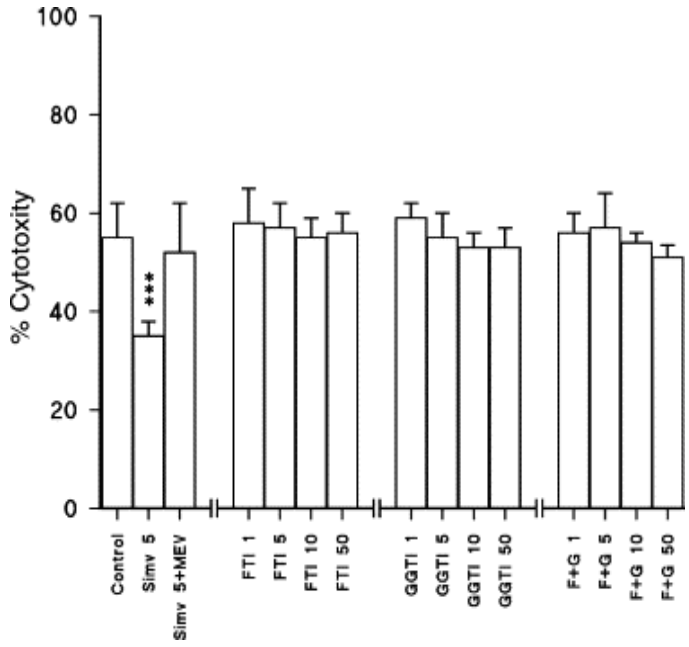


Figure 1.4 NK92MI cell cytotoxicity (% killing of K562 cells) after treatment for 48 hours with varying concentrations of farnesyl (FTI) and geranylgeranyl transferase (GGTI) inhibitors was not affected. This also shows that one millimolar of mevalonate (rescued) the inhibition of 5μM simvastatin. $p < 0.001$ t-test vs. control. (Reproduced with permission) [153]

It has been shown that fluvastatin disrupted lipid rafts in a U937 monocyte line [161], and that statin therapy lead to a reduction in raft-associated proteins in healthy volunteers treated for 4 weeks with simvastatin 40mg daily [150]. These findings were also confirmed using an immortalised NK cell line (NK92MI) with fluvastatin and simvastatin [153]. Levels of raft associated proteins were restored with mevalonate rescue, however incubation of cells with FTI and GGTI had no measurable effect on raft associated proteins supporting the notion that inhibition of cholesterol synthesis by fluvastatin and not isoprenoid synthesis is responsible for membrane raft disruption.

1.11 Aims and Hypothesis

The general hypothesis was that membrane and plasma cholesterol (and therefore the density of membrane rafts) may change in parallel. If increased plasma lipids are associated with an increase in membrane rafts (and raft associated proteins), this may provide a generalisable link to CVD. Therefore a treatment that lowers circulating cholesterol is likely to reduce membrane cholesterol, and correspondingly, cell functions that are dependent on membrane cholesterol (and cholesterol rafts). Our original intention had been to confirm whether changes in lipid rafts did parallel changes in serum cholesterol, and whether this was an effect unique to HMG-CoA reductase inhibitors (providing an alternative explanation for the pleiotropic effects of statins), or whether all forms of lipid lowering therapies resulted in a reduction of membrane lipid rafts, with a

corresponding reduction in cell function. NK cells were chosen as we had experience with this cell line, and pilot experiments had been carried out as described above. Also, the previous work from our group described above interestingly showed a correlation between the activity of NK cells and circulating lipid levels, consistent with a wider link between NK cells and CV risk. Thus the core of this thesis was the role of lipid rafts and isoprenoids in NK cell cytotoxicity.

Preliminary experiments found that using the centrifugation apparatus available to our laboratory (SW60-ti rotor) to isolate lipid rafts, the amount of venous blood required to yield a sufficient number of PBMCs was too much to be feasible. The focus of this study therefore moved to examining NK cell function in patients at particular cardiovascular risk, namely patients with CKD, to determine whether any changes in the function of this important immune cell could have any role in the hugely increased cardiovascular risk which develops in these patients.

1.11.1 Aims

The aims of this project were:

- To evaluate the relationship between lipid profiles and NK cell function and phenotype
- To determine if NK cell function and phenotype alters with differing stages of CKD
- To examine the relationship between NK cell cytotoxic activity and traditional and novel predictors of CV risk and renal function
- To examine the effects of manipulation of membrane cholesterol (depleting and replenishing) on NK cell function
- To establish the relationship between NK cell function, renal function, and biomarkers of inflammation and CVD

1.11.2 Hypothesis

The studies performed are to examine the hypothesis that changes in NK cell function may relate to serum cholesterol, reflecting changes in cell membrane and specifically lipid raft function, and that NK cell function is altered in renal failure, supporting a role for NK cells in atherosclerosis and CVD.

1.12 Outline of the studies contained within this thesis

Four principle studies were performed and are detailed in Chapters 3-6:

Chapter 3: A study of natural killer cells in patients with dyslipidaemia

Chapter 4: A study of natural killer cells in chronic kidney disease

Chapter 5: An *in vitro* study of membrane cholesterol in natural killer cells

Chapter 6: A study of biomarkers in patients at high risk of cardiovascular disease

CHAPTER 2

Materials and Methods

2.1 Subjects

Subjects were recruited from out-patients attending the general nephrology and low-clearance renal clinics, the lipid clinic, and the regular dialysis unit. None of the patients had infections or malignancy, and none were immunosuppressed. We had initially planned to exclude diabetic subjects (as a potential confounder), but to increase numbers, diabetics were later included. Fifty millilitres of blood was taken into BD vacutainer collection tubes from a peripheral vein at routine visits to their clinic. In the patients on haemodialysis, blood samples were taken within 15 minutes of the start of the dialysis session in all the patients on dialysis. Biochemical, haematological, and clinical parameters (observations and blood samples taken at the same time the study samples were taken) were recorded. All patients and controls gave written consent, the forms being approved by the North Glasgow Hospitals University NHS Trust Ethics Committee.

2.2 Blood sampling

50ml of venous blood was drawn into BD vacutainer tubes. Routine biochemistry and haematology were analysed in the hospital laboratories for haemoglobin, electrolytes, urea, creatinine, glucose, and lipid profile (total cholesterol, low-density lipoproteins, high-density lipoproteins, and triglycerides). Approximately 20ml was used immediately for peripheral mononuclear cell extraction, and then NK cell isolation as described below.

Approximately 15ml was centrifuged and frozen within 30 minutes of collection to -70^{o}C for later use.

2.3 Materials

2.3.1 Biochemicals

Unless otherwise stated, all chemicals were purchased from Sigma (Poole, UK). Tissue culture products including foetal calf serum (FCS), L-glutamine, RPMI 1640 penicillin and streptomycin were purchased from Gibson (Paisley, UK).

2.3.2 Immunochemicals

The antibodies used in this study are shown in table 2.1.

Specificity (clone)[1]	Species	Usage[2]	Source[3]
Anti-human CD16/FITC (DJ130c)	Mouse	FACS	DakoCytomation
Anti-human CD56/RPE (C5.9)	Mouse	FACS	DakoCytomation
Anti-human CD3/APC	Mouse	FACS	DakoCytomation
Negative control, Mouse IgG1	Mouse	FACS	DakoCytomation
LAT	Rabbit	Western Blot	Upstate
Lyn	Rabbit	Western Blot	Upstate
HMG-CoA reductase	Rabbit	Western Blot	Upstate
ERK	Rabbit	Western Blot	NEB
pERK p44/42 MAP kinase (Thr202/Tyr204)	Rabbit	Western Blot	NEB
Anti-rabbit:HRP	Donkey	W 2y antibody	Amersham

Table 2.1 Antibodies. [1]Clone number and/or species in which antibody was raised. [2]FACS: fluorescent activated cell scanning; W 2y antibody: western blot secondary antibody. [3]Manufacturer; DakoCytomation, Cambridgeshire, UK; Upstate, Upstate Biotechnology, Lake Placid, USA; Amersham, Amersham, UK; New England Biolabs, Ipswich, UK

2.4 Cell biology techniques

2.4.1 Cell Culture

2.4.1.1 NK-92MI Cell Line

NK-92MI is an interleukin-2 (IL-2) independent natural killer cell line derived from the NK-92 cell line by stable transfection with human IL-2 cDNA in a retroviral vector by particle-mediated gene transfer. The parental NK-92 cell line was an IL-2 dependent cell line derived from peripheral blood mononuclear cells from a 50 year old Caucasian male with rapidly progressive non-Hodgkin's lymphoma [264]. NK-92 cells were routinely cultured in Alpha minimum essential medium without ribonucleosides and deoxyribonucleosides and containing 2mM L glutamine, 1.5g/L sodium bicarbonate, 0.2mM inositol, 0.1mM 2-mercaptoethanol, 0.02mM folic acid, 12.5% horse serum, 12.5% foetal bovine serum, penicillin (10U/ml) and streptomycin (10µg/ml) (Gibco, Paisley, UK). They were maintained at 37^{o}C in a water-saturated atmosphere containing 5% carbon dioxide.

2.4.1.2 K562 Cell Line

The human erythroleukaemic cell line K562 was established from the pleural fluid of a 53 year old female with chronic myelogenous leukaemia in terminal blast crisis [265]. The cell population has been characterized as highly undifferentiated and of the granulocytic series [266]. The K562 cells were routinely cultured in RPMI 1640 medium supplemented with FCS (10% v/v), glutamine (2mM), penicillin (10U/ml) and

streptomycin (10µg.ml). They were maintained at 37 ^{o}C in a water saturated atmosphere containing 5% carbon dioxide, in 25cm^{2} tissue culture flasks in a volume of 10mls.

2.4.1.3 Human peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 mls of human peripheral venous blood freshly drawn into lithium heparin tubes. The blood was centrifuged on a Ficoll-Hypaque (1.077g/ml) gradient (Histopaque-1077; Sigma, Poole, UK). The cells at the interface were collected and washed with phosphate buffered saline (PBS). The cellular pellet was resuspended in K562-media (RPMI 1640 medium with 10% foetal calf serum, 100 U/ml penicillin and 100µg/ml streptomycin) and incubated in 6cm Petri plates at 37^{o}C for 45 minutes, to allow adherent monocytes to attach. Non-adherent cells were collected and counted on a haemocytometer.

2.4.2 NK cell isolation

NK cells were isolated from the PBMCs collected using the MACS NK cell human isolation kit (Miltenyi Biotec). An indirect magnetic labeling system for the isolation of untouched NK cells from human PBMCs. Isolation of highly pure NK cells is achieved by depletion of magnetically labeled cells. PBMCs were resuspended in buffer (PBS with 0.5% bovine serum albumin (BSA) and 2mM EDTA), and were labeled with 10µl of NK Cell Biotin-Antibody Cocktail per 10^{7} total cells. After incubation for 10 minutes at 4-8^{o}C, the cells were washed with 30µL of buffer per 10^{7} total cells, and then 20µL of NK Cell MicroBead Cocktail per 10^{7} total cells was added. The cells were incubated at 4-8^{o}C for 15 minutes and then washed with buffer, adding 10 – 20 X the labeling volume,

and then centrifuged for 10 minutes at 300 x g for 10 minutes. The supernatant was removed, and up to 10^8 cells were resuspended in 500µL of buffer. MS columns (Miltenyi Biotec) were used. The columns were placed in the magnetic field of a MACS Separator and prepared by rinsing with 500µL of buffer which was then discarded. The cell suspension was applied onto the column and the eluate, representing the unlabeled enriched NK cell fraction was collected. The column was washed with 500µL of buffer x 3, and the eluate collected. Cell viability was assessed by trypan blue exclusion, the percentage of viable cells being calculated as follows: (Number of unstained cells/Total number of cells) X 100 = Percent viable cells. The flow-through cells were retained and fluorescence-activated cell-sorting (FACS) was used to ensure pure isolation.

2.4.3 Natural Killer Cell Cytotoxicity Assay

The human chronic myelogenous leukaemia cell line – K562, was used as a target for NK cells. The K562 cells, at a concentration of 2 x 10^6 cells/ml were incubated with 5MBq of ^{51}Cr at 37^{o}C for 2 hours, and washed thoroughly with PBS. The effector cell concentration was established and equalised in all patient samples (the isolated NK cells described in section 2.4.2), and the target cell concentration was then adjusted to 5:1 E/T ratio. One hundred microlitres of effector cells and 100µl of target cells were mixed in V-bottomed plates; six replicates were carried out. Controls included 100µl of target cells plus 100µl of media as a negative control, and 50µl of target cells (in suspension) as a positive control. After incubation for 3 hours at 37^{o}C, plates were centrifuged at 400 x g (1000rpm) for 5 minutes, 100µl of supernatant was collected and released radioactivity was measured by gamma counter. Cytotoxicity was determined as the release of

radiolabel from “killed” K562 cells [percentage killing = (sample – negative control)/(positive control – negative control) x 100] [267].

2.4.4 Flow Cytometry

Three colour flow cytometry (BD Biosciences FASCalibur) of PBMCs prior to NK cell isolation was carried out to determine NK cell phenotype and distribution of NKT- and T-cells. Phycoerythrin (PE)-anti-CD56, fluorescein isothiocyanate (FITC)-anti-CD16, and allophyocyanin (APC)-anti-CD3 (DakoCytomation Denmark A/S) were used. CD56+CD16+CD3- were defined as NK cells, CD56+CD16+CD3+ were defined as NKT cells, and CD56-CD16-CD3+ were defined as T cells. Analysis was carried out using CELL Quest Prosoftware. Ten microlitres of PBMCs was transferred to 2 FACS tubes, and made up to 100μl with K562-media. Into one tube was added 5 μl of isotype controls (FITC, PE, APC), and into the other tube, 5 μl of the above antibodies. The cells were incubated in the dark for 30 minutes, washed in media, and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 500 μl of formyl saline. The lymphocyte subsets were expressed as a percentage of gated events, with 10,000 gated events recorded.

Peripheral blood mononuclear cell isolation, NK cell purification, NK cell cytotoxicity assays and FACS were carried out by myself with the assistance of Dr Dianne Hillyard and Ms Helen Miller (Renal Research Group, BHF Glasgow Cardiovascular Research Centre).

2.4.5 Cholesterol Loading

Soluble cholesterol (Sigma, Poole, UK) was dissolved in NK92MI media to a stock concentration of 1mg/ml and added to cells at various concentrations. The NK92MI cell line was used. NK92MI cell were treated with increasing concentrations of cholesterol (as a cholesterol-methyl-beta-cyclodextran complex). To load cells, varying concentrations ranging from 5 - 100μg/ml of the cholesterol-MBCD complex were added to 1 x 10^6 cells/ml to a volume of 2 ml RPMI media. Cells were then incubated at 37^{o}C for increasing lengths of time; from 30 minutes to overnight incubation. They were then washed with PBS.

2.5 Biochemical Assays

2.5.1 Cell Membrane Preparation

The NK-92MI cell line was used. Cells were washed twice in cold nuclear preparation buffer (10mM Tris-HCl, pH 7.4, 2mM Magnesium Chloride, 140mM Sodium Chloride). Cells were then lysed by three dry ice freeze-thaw cycles in nuclear preparation buffer containing 2% (v/v) Tween 40, protease inhibitors (1mM pheylmethylsulfonyl fluoride and 1μg/ml each of chymostatin, leupeptin, antipain and pepstatin) and phosphatase inhibitors (1mM vanadate and 1mM NaF). Cell debris and nuclei were removed by centrifugation at 15,000 x g (13,000 rpm) for 5 minutes and membranes were precipitated by ultracentrifugation (Beckman Instruments, High Wycombe, UK) of the supernatant at 100,000 x g (55,000 rpm), 4^{o}C for 1 hour. Following ultracentrifugation, the supernatant

represents the cytosolic fraction. Membrane pellets were resuspended in cold membrane preparation buffer.

2.5.2 Lipid Raft Isolation

The cells being used were lysed by mixing with 2.5ml of MNE (25mM, pH 6.5, 150mM NaCl, 2mM EDTA) containing 1% Triton X-100 (v/v). The samples were then mixed (1 in 2) with 80% sucrose MNE (2.5ml), 30% sucrose (7ml) was then carefully layered on top, and finally 5% sucrose MNE (1.3ml) was layered on top. Samples were kept on ice throughout and the rotor and rotor buckets were chilled to fridge temperature. The samples were then subjected to sucrose gradient centrifugation at 32,000 rpm for 16 hours at 4^{o}C in a SW60-ti rotor and a Beckman Coulter Optima L-80 XP Ultracentrifuge (Beckman Instruments, High Wycombe, UK).

2.5.2.1 Lipid raft fractions

Lipid rafts were collected from the 5% and 30% sucrose interface, diluted 1:1 with MNE and micro-centrifuged. The pellet was resuspended in RIPA lysis buffer (1% (v/v) Triton X-100, 50mM Tris-HCl, pH 7.5, 0.25% (w/v) sodium-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM vanadate and 1mM NaF) for subsequent analysis by SDS-PAGE and Western Blot. LAT or Lyn antibodies were used as markers for rafts.

2.5.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the NuPAGE Pre-Cast Gel System (Invitrogen, Paisley, UK). The NuPAGE system works with bis-tris buffered (pH 6.4)

polyacrylamide gels, available at 3 different acrylamide concentrations (10% (w/v), 12% and 4-12% gradient gels) allowing different separation ranges to be selected. By varying the running buffer used, the separation range can be altered further. Samples were denatured in NuPAGE LDS (Lithium Dodecyl Sulphate) sample buffer by heating to 70^{o}C for 10 minutes.

2.5.4 Western Blot Analysis

Proteins were transferred to nitrocellulose (Schleicher and Schuel GmbH) using the Novex XCell II blotting apparatus. Transfer efficiency was ascertained by staining with Ponceau S solution for five minutes followed by washing with dH_2O. Ponceau S was removed by washing with TBS (20mM Tris.HCl, pH 7.6, 136mM NaCl) containing 0.1% (v/v) Tween 20 (TBST). Nitrocellulose membranes were blocked in TBST with 4% (w/v) non-fat powdered milk (e.g. Marvel) for at least 1 hour at room temperature. Typically, blots were incubated with primary antibodies at a final concentration of 0.1 - 1μg/ml in 4% milk-TBST overnight at 4^{o}C. Blots were then washed vigorously three times for five minutes with TBST and incubated with a HRP conjugated secondary antibody diluted 1 in 2000 for two hours at room temperature. Blots were washed extensively with large volumes of TBST (typically 5 x 5 minute washes) followed by a final wash in H_2O prior to developing. Blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham, UK).

2.6 Manufactured kits

2.6.1 Amplex Red Cholesterol Assay (Manufactured Kit)

Cholesterol content in cell membrane preparations and cytosol was quantified using the Molecular Probes' Amplex Red Cholesterol Assay Kit (Invitrogen) as per manufacturer's instructions. This kit provides a fluorometric method for quantification of cholesterol, measuring H_2O_2 detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a highly sensitive and stable probe for H_2O_2, producing highly fluorescent resorufin. NK-92 cells at a density of 1 x 10^6 cells/ml were loaded with cholesterol as described in section 2.4.5, and cell membrane preparations made as described in section 2.5.1. The membrane pellet was suspended in 200µL of Amplex Red reaction buffer (0.1M potassium phosphate, 0.05 M NaCl, 5mM cholic acid, and 0.1% TritonX-100), 50µL aliquots of the lysates were pipetted in triplicate into a 96-well plate. Fifty-microlitre aliquots of Amplex Red working solution was added to each well. (300 µM Amplex Red reagent, 2U/ml horseradish peroxidase, 2U/mL cholesterol oxidase and 0.2U/mL cholesterol esterase). Plates were incubated for 1 hour at 37^{o}C protected from light. Fluorescence was measured on a Wallac – Victor2 (Perkin Elmer, Beaconsfield, UK) microplate reader, using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. A cholesterol standard curve was also determined for each assay using a provided cholesterol reference standard.

2.6.2 Ras Assay

The Ras small GTPase was assayed using an Upstate biotech Ras activation assay kit (TCS Biologicals, Claydon, Buckingham, UK). The kit comprised of glutathione agarose bound GST fusion proteins of the Ras binding domain (RBD) of Raf-1, which binds Ras:GTP. Briefly, cells were stimulated and then lysed with Mg^{2+} lysis buffer (MLB – 25mM HEPES, pH 7.5, 150mM NaCl 1% Igepal CA-630, 10mM $MgCl_2$, 1mM EDTA, and 2% glycerol). Typically, 500µg cell lysate at a concentration of 1mg/ml was used for each assay sample. Active Ras was precipitated with 5µl Raf-1 RBS agarose for 30 minutes. Precipitates were washed three times with 500µl MLB followed by SDS-PAGE (section 2.5.3) and Western blotting (section 2.5.4). Ras was detected with specific monoclonal antibody included in the kit. The Ras assay was carried out by Dr Dianne Hillyard (Renal Research Group, BHF Glasgow Cardiovascular Research Centre).

2.7 Measurement of biomarkers

Blood for measurement of biomarkers was collected in plain tubes and allowed to clot at room temperature. Samples were then centrifuged at 3000rpm for 15 minutes at ambient temperature, the supernatant serum was then collected and immediately frozen and stored at -70^{o}C until later analysis.

2.7.1 High Sensitivity CRP measurement

High sensitivity CRP measurements were performed by the Dr Lynne Cherry (Senior Research Technician, Department of Vascular Biochemistry, Glasgow Royal Infirmary)

and staff of the routine lipids section of the Biochemistry Department at Glasgow Royal Infirmary using a Hitachi Modular P analyzer. The assay was an immunoturbidimetric assay, and kits were supplied by Roche Diagnostics GMBH, D-68298 Mannheim.

2.7.2 Interleukin-6 measurement

Interleukin-6 was quantified using a DuoSet ELISA kit (R&D Systems, Abingdon, UK).

2.7.3 Pentraxin-3 measurement

Pentraxin-3 was quantified using DuoSet ELISA kit (R&D Systems, Abingdon, UK).

2.7.4 Adiponectin measurement

Adioponectin was quantified using DuoSet ELISA kit (R&D Systems, Abingdon, UK).

The ELISAs for IL-6, Pentraxin-3, and Adiponectin were carried out by myself with the assistance of Dr Dianne Hillyard and Ms Helen Miller (Renal Research Group, BHF Glasgow Cardiovascular Research Centre).

2.7.5 Serum Soluble ST2 measurement

Serum sST2 was quantified using human ST2/IL-1 R4 ELISA kit (R&D Systems, Abingdon, UK). The ELISA was performed by Dr Grace Murphy, Division of Immunology, Infection and Inflammation, Glasgow Biomedical Research Centre.

2.8 RNA Extraction

HMG-CoA reductase: We measured gene expression for HMG-CoA reductase by real time PCR and quantified enzyme protein by Western blotting. Quantitative real time PCR was used to quantify of mRNA transcripts following reverse transcription (Invitrogen, Paisley, UK) of RNA extracted from NK cells using the RNeasy mini-kit (Qiagen, Crawley, UK). Primers were designed using Primer-3 software to generate a DNA product of around 100 base pairs to maximise reaction efficiency (HMG-CoA Reductase - For: 5'GACCATCTGCATGATGTCCA 3', Rev: 5'GGCCAGCAATACCCAAAATA 3'. 18S (rRNA small subunit) – For: 5'AAACGGCTACCACATCCAAG 3', Rev: 5'CGCTCCCAAGATCCAACTAC 3' (VHBio, Gateshead, UK)). Reactions were performed in triplicate 20µl volumes in 96-well optical reaction plates, using SYBR Green PCR Master Mix (Finnzymes, Espoo, Finland) containing modified *Thermus brockianus* DNA Polymerase, SYBR Green I dye, optimized PCR buffer, 5 mM $MgCl_2$, and dNTP mix. Each reaction was performed with either 0.1µl diluted cDNA or reverse transcription control. Forward and reverse primers were each present at a concentration of 300nM (150nM for 18S). The thermal cycling profile started with a 15 minute hot-start at 95°C followed by 35 cycles - 30 seconds at 95°C, 30 seconds at 56°C and 60 seconds at 72°C. Results were analysed using Bio-Rad Icycler software (Bio-Rad, Hemel Hempstead, UK). The RNA extraction and real time PCR was carried out by Dr Dianne Hillyard (Renal Research Group, BHF Glasgow Cardiovascular Research Centre).

CHAPTER 3

A study of natural killer cells in patients with dyslipidaemia

3.1 INTRODUCTION

There is an established relationship between lipids and atherosclerotic cardiovascular disease. The growing interest in atherosclerosis as an inflammatory disorder has now identified inflammation as a key regulatory process that links multiple risk factors for atherosclerosis and its complications. These findings are providing new insight into the pathophysiology of atherosclerosis and are opening new avenues for treatment of this important and prevalent disease. No cholesterol lowering treatment has been as effective as HMG-CoA reductase inhibitors. As discussed previously, not only do these agents successfully lower cholesterol levels, they appear to have several cholesterol-independent, or "pleiotropic" effects [154, 268]. For example, there is substantial evidence that statins modulate immune responses. In experimental models, statin treated rats showed reduced chronic rejection in addition to decreased graft atherosclerosis, following heart and liver transplants [269, 270]. Similarly in humans, increased 1-year survival and reduced rates of acute rejection in heart transplant recipients receiving pravastatin, compared with controls receiving standard immunosuppression have been reported [152]. There have been inconsistent findings in studies of renal transplant recipients with regards to rejection rates [127, 271]. In other studies, reduced levels of CRP, a marker of inflammation, have been observed [119]. Numerous studies have shown that statins have inhibitory effects on immune cells [130, 150, 161, 272] which appears to be a cholesterol-independent effect, although it is accepted that hypercholesterolemia promotes the development of a pro-inflammatory phenotype in large vessels with disease. Whilst the role of plasma lipoproteins in atherogenesis is

accepted, the physiological relevance of their immunoregulatory properties is still not clear. It is known that in experimental animal models and human studies, diet and nutritional status affect immune cell function [273]. It has been shown that a T helper-1-skewed cytokine profile of NKT cells which was induced by feeding mice an atherogenic diet was responsible for exacerbation of atherosclerosis by these cells [71]. These findings suggest that dietary factors, in particular lipids, may influence adaptive immunity via NKT cells. It is also known that obesity influences host immunity [274]. In vitro, NK cells express different lipoprotein receptors, and in experimental studies, responses of NK cell activity to lipoproteins have been shown to vary according to the concentrations of lipoproteins [275], and in addition NK cells stimulated with different lipoproteins secrete a variety of cytokines, such as IL-2, which may in turn affect NK cell activity [276].

The purpose of this study was to attempt to evaluate the physiological relevance of the immunoregulatory properties of plasma lipoproteins by evaluating the relationship of serum lipid profile with NK cell phenotype and function in a group of patients with dyslipidaemia, on various lipid lowering therapies.

3.2 METHODS

3.2.1 Patients

Blood samples were taken from 36 healthy volunteers and 66 patients attending a specialist lipid disorder clinic. All patients were fasting. 50 millilitres of blood was taken into BD vacutainer collection tubes. Biochemical, haematological, and clinical

parameters including age, gender, BMI, presence of vascular disease – ischaemic heart disease, history of previous cerebrovascular accident or transient ischaemic attack, or peripheral vascular disease – was recorded, along with smoking status and drug therapy. None of the patients had infection or malignancy, and none were immunosuppressed. In all 99 patients, NK cells were isolated and NK cell phenotype and distribution of NKT- and T-cells was determined. Routine biochemical parameters including urea and electrolytes, albumin, phosphate, calcium, glucose, lipid profile, and creatinine were obtained from biochemistry laboratory reports, and full blood counts were obtained from haematology laboratory reports.

3.2.2 Human peripheral blood mononuclear cell isolation

Approximately twenty millilitres of blood was used. Peripheral blood mononuclear cells were isolated from peripheral venous blood as described in section 2.4.1.3 using Ficoll-Hypaque centrifugation (Histopaque-1077; Sigma, Poole, UK).

3.2.3 NK cell isolation

From the PBMCs isolated, NK cells were then isolated using the MACS NK cell human isolation kit (Miltenyi Biotec) as described in section 2.4.2.

3.2.4 51-Chromium Release Assay

Natural killer cell cytotoxicity was measured using a standard 51-Chromium Release Assay, using the human chronic myelogenous leukaemia cell line – K562 as target cells,

as described in section 2.4.3. Percentage killing was calculated: (sample – negative control)/(positive control – negative control) x 100.

3.2.5 Flow Cytometry

Three colour flow cytometry of PBMCs was carried out (BD Biosciences FASCalibur) before isolation of NK cells to determine NK cell phenotype and distribution of NKT- and T-cells as described in section 2.4.4. The NK-, NKT-, and T-cells were expressed as a percentage of total lymphocyte count, and the $CD56^{bright}$ and $CD56^{dim}$ subsets were expressed as a percentage of NK cells.

3.2.6 Statistical Analysis

Statistical analysis was carried out using SPSS statistical package (version 15.0). Comparisons between groups were made using the independent samples T-test. The association between continuous variables was analysed using Pearson correlation test. Non parametric data were logarithmic transformed and Pearson correlation was used. Multiple linear regression was used to assess the contribution of different factors to changes in lymphocyte subsets and NK cell cytotoxicity. A probability value of $p<0.05$ was considered significant.

3.3 RESULTS

3.3.1 Patient Characteristics and Laboratory Data

The clinical and laboratory data are shown in Table 3.1. The pattern of drug treatment is shown in Table 3.2 and Figures 3.1 and 3.2. No-one in the control group was on lipid lowering therapy. There were significant differences between the control and lipid clinic group with regards to age (41.6±12.8 vs. 57.9±10.1, $p<0.001$), BMI (24.7±4.8 vs. 30.3±6.0, $p<0.05$), creatinine (82.1±10.8 vs. 98.5±25.4, $p<0.001$), glucose (5.0±0.9 vs. 6.4±2.2, $p<0.005$), and haemoglobin (13.4±1.3 vs 14.5±1.4, $p<0.005$). However there was no significant difference between groups between serum phosphate or albumin. With regards to lipids, there were significant differences between HDL-C (1.4±0.3 vs. 1.19±0.3, $p<0.005$) and triglycerides (1.2±0.7 vs. 2.9±1.6, $p<0.001$) between groups, however there were no significant differences between total cholesterol and LDL-C.

In the lipid patient group, there was no difference in creatinine between those on ace inhibitors and those not. In terms of lipid profile, there was a significant difference in total cholesterol (6.04±1.65 vs 4.99±0.95, $p < 0.05$) and LDL-C (3.61±1.43 vs 2.6±0.75, $p < 0.05$) between those patients not taking a statin and those who were. There was no difference in HDL-C or triglycerides however. There was also no difference in creatinine, phosphate, adjusted calcium or albumin in those taking a statin compared with those who were not. LDL-C was lower in the lipid patients not taking ezetimibe (2.64±1.05 vs 3.36±1.04, $p < 0.05$), although of thirteen patients taking ezetimibe only four of these were not also on dual therapy with a statin. There were no differences in lipid fractions in those patients in the lipid group whether they were taking omacor, or a fibrate, or not.

In the control group, there was a significant correlation between creatinine and age ($r = 0.506$, $p < 0.01$). Creatinine did not correlate with any other routine biochemistry or haematological parameters, and specifically there were no correlations between creatinine and serum lipids. Age correlated with serum glucose ($r = 0.486$, $p < 0.05$). Haemoglobin correlated with serum albumin ($r = 0.434$, $p < 0.05$). Total cholesterol correlated significantly with haemoglobin ($r = 0.491$, $p < 0.01$) and also with albumin ($r = 0.472$, $p < 0.01$). LDL-C correlated with age ($r = 0.466$, $p < 0.05$). HDL-C correlated with phosphate ($r = 0.385$, $p < 0.05$), albumin ($r = 0.369$ $p < 0.05$), and glucose ($r = -0.414$, $p < 0.05$).

In the lipid patient group, creatinine also correlated with age ($r = 0.252$, $p < 0.05$), but also negatively with haemoglobin ($r = -0.290$, $p < 0.05$). Haemoglobin also correlated with albumin ($r = 0.428$, $p < 0.01$). White cell count correlated with adjusted calcium ($r = 0.384$, $p < 0.01$) and albumin ($r = -0.316$, $p < 0.05$), whilst platelets correlated with haemoglobin ($r = -0.304$, $p < 0.05$), WCC ($r = 0.269$, $p < 0.05$) and albumin ($r = -0.253$, $p < 0.05$). Total cholesterol correlated with creatinine ($r = -0.264$, $p < 0.05$) although neither LDL-C or HDL-C had any correlations with routine biochemistry or haematology.

Characteristic	Controls (mean ± SD)	Lipid (mean ± SD)	*P*
Age (years)	41.6 ± 12.8	57.9 ± 10.1	<0.001
Sex (M/F)	10/26	46/17	ns
Weight (kg)	68.6 ± 14.9	88.9 ± 18.0	ns
Height (cm)	164.9 ± 13.4	171.5 ± 8.3	ns
BMI (kg/m^2)	24.7 ± 4.8	30.3 ± 6.0	<0.05
Creatinine (μmol/L)	82.1 ± 10.8	98.5 ± 25.4	<0.001
Phosphate (mmol/L)	1.1 ± 0.1	1.0 ± 0.2	ns
Albumin (g/L)	42.0 ± 2.7	40.7 ± 3.5	ns
Glucose (mmol/L)	5.0 ± 0.9	6.4 ± 2.2	<0.005
Haemoglobin (g/L)	13.4 ± 1.3	14.5 ± 1.4	<0.005
Total cholesterol (mg/dl)	4.9 ± 1.1	5.2 ± 1.2	ns
LDL-C (mg/dl)	2.9 ± 0.8	2.9 ± 1.0	ns
HDL-C (mg/dl)	1.4 ± 0.3	1.2 ± 0.3	<0.005
Triglycerides (mg/dl)	1.2 ± 0.7	2.9 ± 1.6	<0.001

Table 3.1 Clinical characteristics of the controls and the lipid patients.

Drug	Number (%)
Statin	50.8
Ezetimibe	20.6
Fibrate	14.3
Omacor	22.2

Table 3.2 Shows the percentage of patients on various lipid lowering drugs

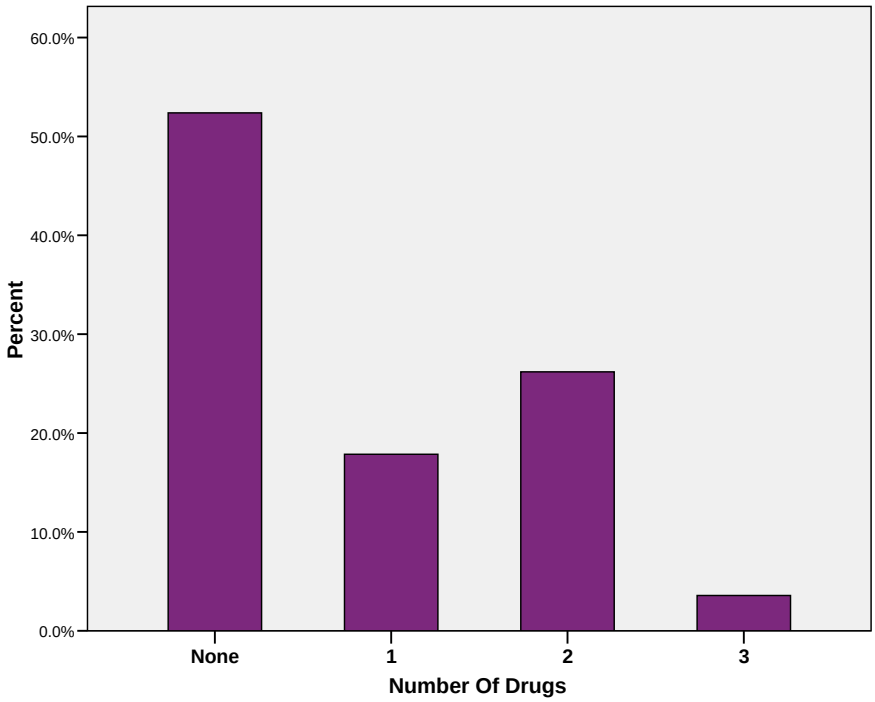


Figure 3.1 Shows the number of lipid lowering drugs being taken by the subjects sampled (number of subjects is shown as a percentage)

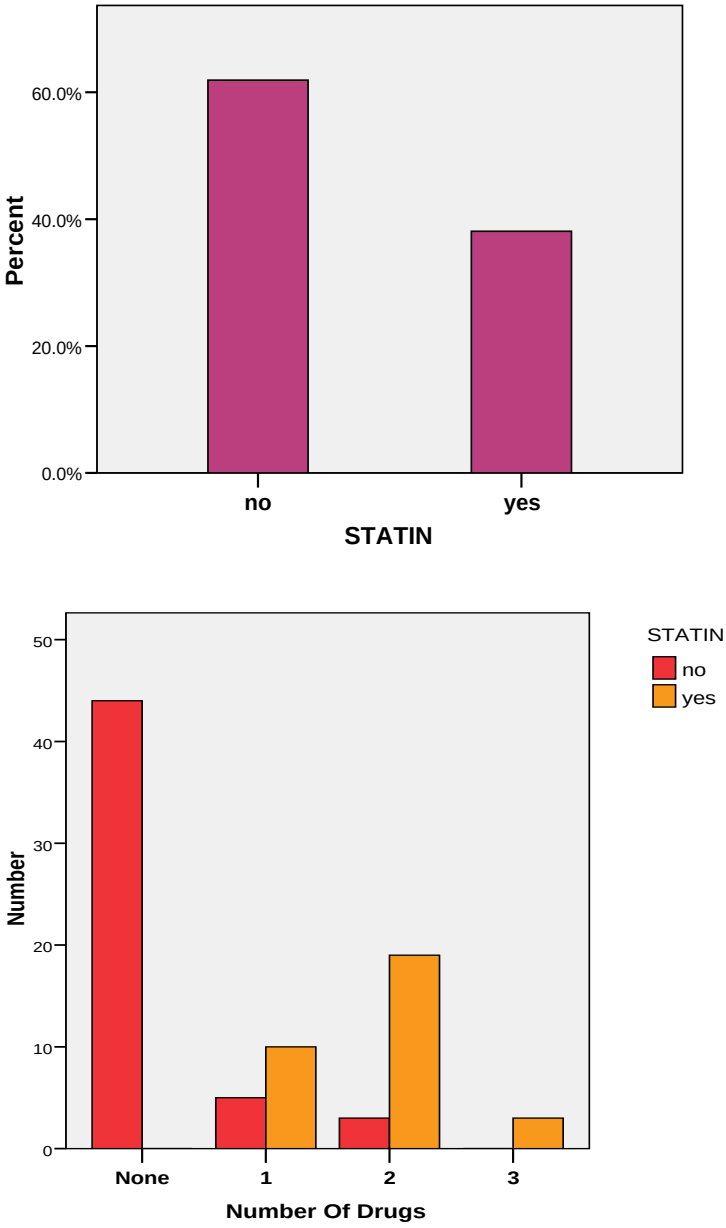


Figure 3.2 (a) Shows the percentage of subjects on or not on statin treatment (b) shows the how many lipid lowering drugs subjects are taking, further divided into whether or not this includes statin therapy.

3.3.2 Distribution of peripheral blood lymphocytes

There was no difference in total lymphocyte count between groups. There was also no difference in number of NK cells or T cells between groups. There was however a significant difference between NKT cells ($p<0.01$), and $CD56^{bright}$ ($p<0.001$) and $CD56^{dim}$ cells ($p<0.001$) (Figure 3.3) between groups (Table 3.3). This was true when comparing the control group with patients in the lipid group whether or not they were on a statin.

In the control group, total cholesterol, LDL-C and triglycerides had no correlations with lymphocyte subsets. HDL-C however correlated with T cells ($r = 0.382$, $p < 0.05$), $CD56^{bright}$ cells ($r = 0.429$, $p < 0.01$) and $CD56^{dim}$ cells ($r = -0.432$, $p < 0.01$) (Figure 3.4). NK cells correlated with creatinine (0.392, $p < 0.05$) (Figure 3.5), haemoglobin ($r = 0.405$, $p < 0.05$), WCC ($r = -0.393$, $p < 0.05$), and phosphate ($r = -0.464$, $p < 0.01$). In addition to correlating with HDL-C, T cells also correlated with glucose ($r = -0.403$, $p < 0.05$). Interestingly, there was a significant correlation between $CD56^{dim}$ cells and age ($r = 0.350$, $p < 0.05$) as has been previously shown.

In the lipid group, neither total cholesterol, LDL-C nor HDL-C had any correlations with lymphocyte subsets. Triglycerides however correlated with T cells ($r = 0.314$, $p < 0.05$). There were no correlations between NKT cells or $CD56^{dim}$ cells, but NK cells correlated with haemoglobin ($r = 0.262$, $p < 0.05$) and adjusted calcium ($r = -0.371$, $p < 0.01$), and $CD56^{bright}$ cells correlated with haemoglobin ($r = -0.254$, $p < 0.05$). The presence of statin therapy in the lipid group did not result in any difference in NK-, NKT-, or T-cell numbers, or in the distribution of NK cell phenotype, between those taking a statin and

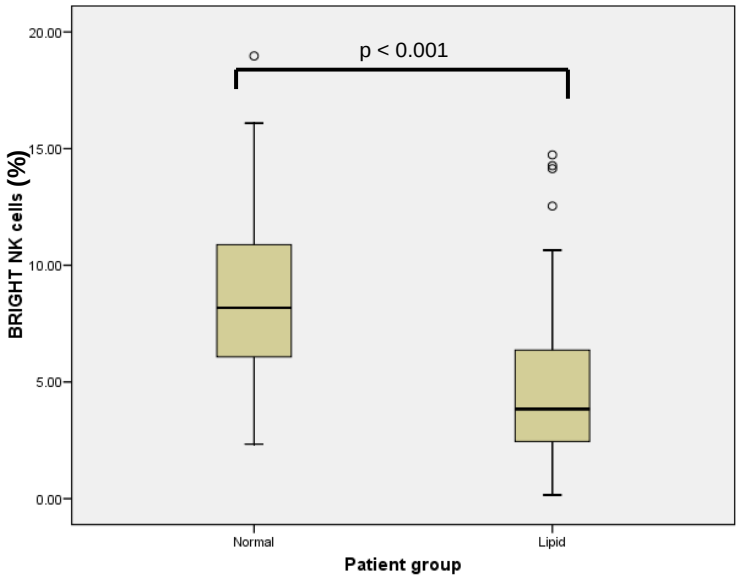
those who were not. Notably there was no significant age difference in those prescribed a statin versus those who were not. There was no difference in lymphocyte distribution between those lipid patients taking a fibrate or omacor, and those who were not. There were no correlations between BMI and lymphocyte subsets in the lipid patient group.

Excluding those patients who by ATP III classification of lipid levels [4] have "optimal" LDL-C levels (those with LDL-C < 100 mg/dL) (23 of 36 controls; 33 of 63 lipid clinic patients), therefore including only those patients who have a raised LDL-C, does not unmask any novel inter-relationships, however it does strengthen the correlation between triglycerides and T cells ($r = 0.430$, $p < 0.05$) in the lipid group. Similarly, in the control group, it strengthens the correlation between creatinine and NK cells ($r = 0.535$, $p < 0.01$), and between HDL-C and T cells ($r = 0.551$, $p < 0.01$).

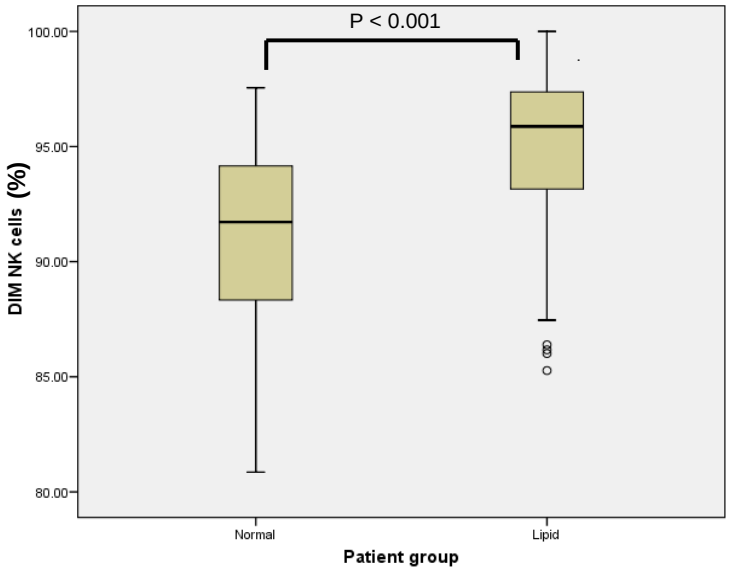
Examining only those patients who have an eGFR > 60 ml/min/1.73m^2 (33 of 36 controls, mean eGFR 80.73±11.36 ml/min/1.73m^2; 50 of 63 lipid clinic patients, mean eGFR 78.24±11.5 ml/min/1.73m^2), in the control group, the correlation between creatinine and NK cells is strengthened ($r = 0.452$, $p<0.01$), and the correlation of HDL-C with T-cells still exists. In the lipid group, the correlation between creatinine and age is lost, but creatinine does still correlate significantly with $CD56^{bright}$ cells ($r = -0.292$, $p<0.05$), but not $CD56^{dim}$ cells.

Blood cells	Controls	Lipid patients	*P*
Lymphocytes (x10^9/L)	2.05±0.50	2.14±0.73	ns
T cells (%)[a]	65.66±12.26	65.50±8.61	ns
NKT cells (%)[a]	4.23±3.35	6.79±8.61	< 0.05
NK cells (%)[a]	11.71±5.61	13.16±5.95	ns
$CD56^{bright}$ cells (%)[b]	8.72±3.93	4.81±3.36	< 0.001
$CD56^{dim}$ cells (%)[b]	91.01±4.14	94.81±3.67	< 0.001

Table 3.3. Lymphocyte subset counts in control patients and lipid clinic patients. [a]Percentage of lymphocytes; [b]percentage of NK cells.



(a)



(b)

Figure 3.3. Boxplot graphs showing the distribution of (a) $CD56^{bright}$ and (b) $CD56^{dim}$ cells in the normal and lipid patient groups.

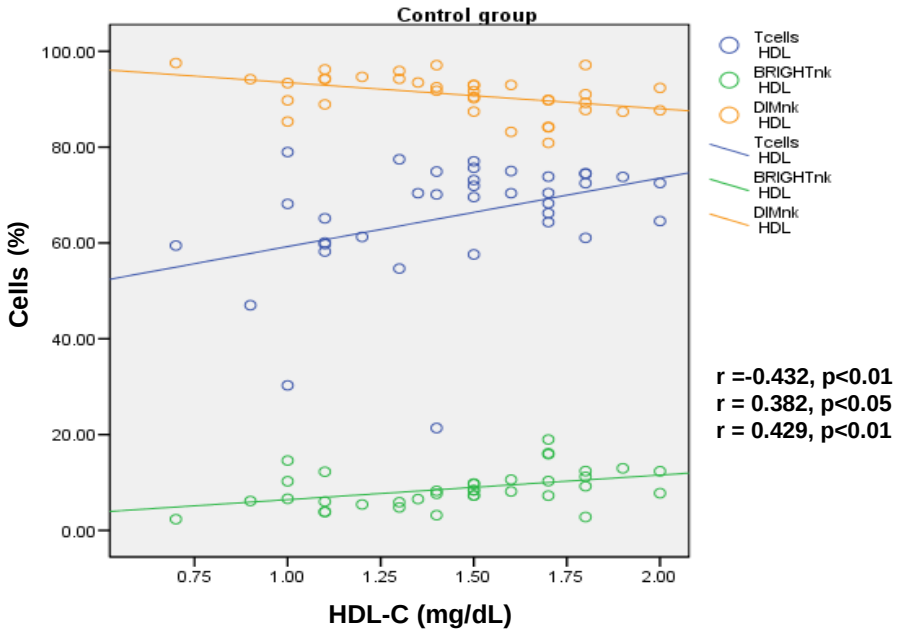


Figure 3.4. A scatterplot showing the relationship between HDL-C and T cells and NK cell subsets in the control group. T-cells are expressed as a percentage of lymphocytes, and $CD56^{bright/dim}$ cells are expressed as a percentage of NK cells, as per table 3.3.

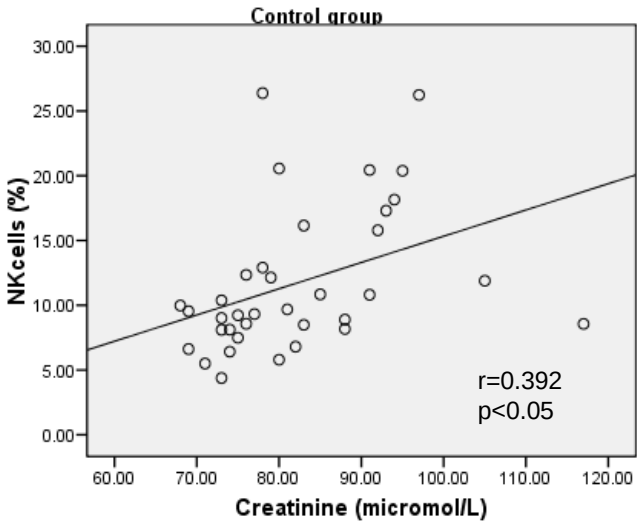


Figure 3.5. A scatterplot showing the relationship between NK cells (expressed as percentage of total lymphocytes) and creatinine in the control group.

3.3.3 Natural killer cell cytotoxicity

There was no significant difference in mean NK cell cytotoxicity between the control and the lipid patient groups. When the controls were compared with only those on statin therapy in the lipid patient group there was also no significant difference in NK cell cytotoxicity. There was a significant negative correlation between NK cell cytotoxicity and LDL-C in both the control ($r = -0.485$, $p < 0.05$) and lipid patient groups ($r = -0.282$, $p < 0.05$) (Figure 3.6). In the lipid patient group NK cell cytotoxicity also correlated with albumin ($r = 0.259$, $p < 0.05$). In both groups, NK cell cytotoxicity did not correlate with creatinine or phosphate, or any routine haematological parameters, or age. In the lipid patient group there was no correlation between NK cell cytotoxicity and BMI or blood pressure. Blood pressures were not available for the control group.

Looking at the lipid patient group, there was no difference in NK cell cytotoxicity between those taking a statin and those who were not. NK cell cytotoxicity was significantly lower in those patients who were taking ezetimibe (37.07 ± 11.37 vs 46.83 ± 12.08, $p < 0.05$). Again, it should be noted again that of the thirteen patients taking ezetimibe, only 4 of these were not also on a statin. None of the patients taking ezetimibe were on a fibrate or omacor. There was also no difference in NK cell cytotoxicity between those patients taking a fibrate or not, and those taking omacor or not. Finally, there was no difference in NK cell cytotoxicity in those patients taking an ACE inhibitor.

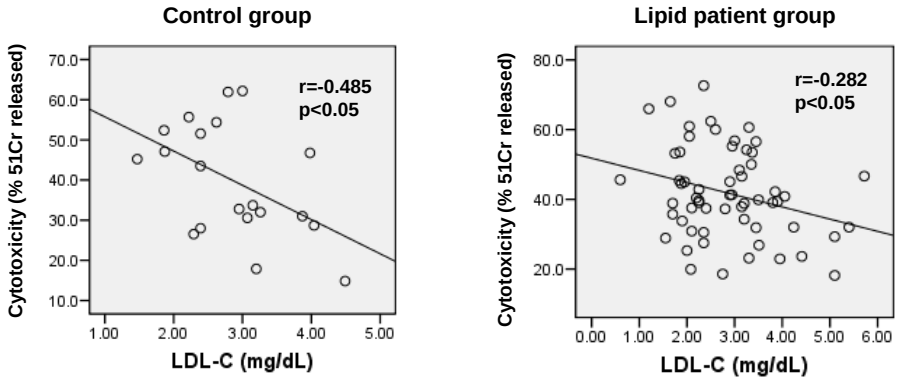


Figure 3.6. Scatterplots showing the relationship between NK cell cytotoxicity and LDL-C in both the control and the lipid patient groups

3.4 DISCUSSION

The present study considers potential relationships between serum lipids and the immune system. It is known of course that hyperlipidaemia, specifically increased total cholesterol and LDL-C, is associated with increased cardiovascular mortality which results from increased atherosclerosis. That the process of atherosclerosis is an inflammatory condition involving the immune system has been discussed at length previously. The relationship between hyperlipidaemia and mortality is less clear cut in the elderly. Some studies have shown that lower HDL-C levels are a stronger predictor of mortality than total cholesterol in the elderly [277, 278]. However, there appears to be a J-shaped relationship between total cholesterol and mortality, even with adjustment for health status and indicators of frailty [279, 280]. Many studies over the past few decades have suggested that having a low serum cholesterol is associated with increased age-adjusted mortality from non-atherosclerotic diseases, including chronic diseases and malignancy, which is not simply attributable to smoking or alcohol consumption [281]. A potential explanation for observed relationships between cholesterol and non-atherosclerotic disease is a link between serum lipids and immune function. The immune system provides resistance to infections and tumours, and plays a role in most, if not all, chronic diseases.

We have found that there are significantly higher numbers of NKT cells in the lipid group patients, and although NK cell numbers were not different between groups, there were proportionately more $CD56^{dim}$ cells in the lipid group patients, and significantly fewer

$CD56^{bright}$ cells. This applies whether on statin therapy or not. This would suggest that NK cell cytotoxicity would be higher in the lipid patient group however this was not the case. Additionally, there was no significant difference in NK cell numbers between groups that may have accounted for the lack of difference in cytotoxicity, although NK cell numbers were equalised for the cytotoxicity assay. It is possible that lipid lowering alters NK cell phenotype, but perhaps more likely is that NK cell phenotype is altered with a disease state, in other words by dyslipidaemia. It should be noted that there was no difference in NK cell subsets or activity whether or not patients had ischaemic heart disease or diabetes, although the number of diabetics included was very small (7 out of 66) as previously stated, diabetics were included later on in the study. The patients in the lipid patient group were significantly older, which may also explain the alterations in NK cell subsets, as $CD56^{dim}$ cells are know to be expanded in elderly populations [84]. Reassuringly, this was confirmed in the current study where a significant correlation was shown between $CD56^{dim}$ cells and age in the control group.

In the control group, there was a positive correlation between HDL-C and T cells and HDL-C and $CD56^{bright}$ cells (with a negative correlation with $CD56^{dim}$ cells) which is in keeping with the higher HDL-C values in the control group. Interestingly, NK cell numbers correlated with creatinine in the control group. It may be that this is simply a reflection of muscle mass. There were no differences in NK-, NKT-, or T-cell numbers between patients on different lipid lowering therapies, and notably there was no difference whether or not on statin treatment. This is in keeping with findings from a previous double-blind, randomised, placebo-controlled study [282] of 24 adult renal

transplant recipients, receiving cyclosporin for immunosuppression. They randomised patients who had high or borderline high cholesterol levels to receive 10mg of simvastatin or placebo, and quantified NK cells by FACS at randomisation and at day 90, to investigate whether a reduction in NK cell numbers with statin therapy could serve as an explanation for reduced rates of graft rejection that have been observed in statin treated recipients of organ transplantation [152, 271]. Whilst cholesterol levels were significantly reduced, they found no changes in numbers of NK cells.

We did not find a difference in NK cell cytotoxicity between control and lipid patient groups whether or not they were on statin therapy. These results are consistent with other studies where no changes in NK cell cytotoxicity in patients with high or low cholesterol levels [283], or with statin treatment [283]. One particular study found no change in NK cell numbers and no reduction in NK cell activity in 25 stable renal transplant recipients on either cyclosporin or azathioprine, all treated with 20mg of pravastatin [284]. Patients were assessed at 6 and 12 weeks, and in fact an increase in NK cell activity at 12 weeks in the cyclosporin treated group was observed.

Interestingly we found a negative correlation between NK cytotoxicity and LDL-C in both groups. This is not consistent with findings from studies previously carried out by our group which showed significant and reversible reductions in NK cell cytotoxicity *ex vivo*, in subjects treated with simvastatin, and also a correlation between NK cell cytotoxicity and total and LDL-C [150]. The current study involves larger numbers, and more importantly, NK cells have been purified in this study, whereas the previous studies

were carried out using a crude lymphocyte preparation. Another previous study from this group in 146 diverse patients or various treatments including statins and immunosuppressants, did not find lower NK cell cytotoxicity with statin treated patients, but found total cholesterol and weight to be independently associated with NK cell cytotoxicity [153]. It is interesting to consider that rather than directly as a result of statin treatment itself, changes in immune cell function may be a consequence of changes in circulating cholesterol levels, and thus changes in cell membrane cholesterol. The negative correlation between NK cell cytotoxicity and LDL-C does not appear to be simply a lipid lowering effect as the relationship is also shown in the control group.

A study by Jonasson *et al.* [95] investigated NK cell subset distribution and cytotoxicity in 95 patients with coronary artery disease (CAD). These patients had significantly higher total cholesterol, LDL-C and triglycerides, and lower HDL-C. They found lower numbers of NK cells and $CD56^{dim}$ cells in the patients with CAD, and reduced NK cytotoxicity, although this was thought to be due to reduced NK cell numbers in the CAD group. Conversely, higher NK cell cytotoxicity has been found to be related to HDL-C in a study of 47 elderly males [285]. The relationship between NK activity and LDL-C may relate to oxidative stress. NK cells are particularly susceptible to oxidative stress and in several studies, oxidised LDL cholesterol (oxLDL), a key component of human atherosclerotic tissue particularly abundant in unstable lesions [286], has been shown to inhibit NK cell cytotoxicity [97, 287]. Another interesting theory is that loss of NK cell function increases susceptibility to atherosclerosis-related infections, for example Chlamydia pneumoniae, and in the context of an increased LDL-C may result in increased plaque burden.

There were limitations to this study; the control group was not aged-matched and thus the changes in NK cell distribution could be potentially attributable to the older age of the lipid group patients. The small number of diabetics included and the incomplete collection of data regarding drug history were limitations. It should be noted also that the resuspension medium contained 12.5% foetal bovine serum which is rich in lipoproteins and could confound results. However all assays of NK cell cytotoxicity were carried out uniformly.

CHAPTER 4

A Study of Natural Killer Cells in Chronic Kidney Disease

4.1 INTRODUCTION

Chronic renal failure is an immunodeficient state involving defects in both cellular and humoral immunity [288]. These patients are at higher risk of infectious complications, and among dialysis patients particularly, this leads to a high morbidity and mortality, second only to cardiovascular disease as a cause of death [289]. Paradoxically however, this often coexists with signs of activation of the immune system. Infectious complications and cardiovascular disease are closely linked to immune function, with immune suppression resulting in the increased incidence of infections, and immune activation leading to inflammation that may contribute to CVD.

The innate immune system provides a first line of defence against infection, in addition to playing a role in the initiation and subsequent direction of adaptive immune responses, and contributing to the removal of pathogens that have been targeted by the adaptive immune response. The high frequency of bacterial infections in dialysis patients suggests that polymorphonuclear leukocyte dysfunction may be involved in the immune deficiency in this population. It has been shown that higher levels of circulating pro-inflammatory cytokines are associated with mortality, while immune parameters reflecting improved T-cell function are associated with survival in ESRD patients on haemodialysis, independent of other medical risk factors [199, 213]. It has also been previously shown that haemodialysis induces a decrease in the absolute numbers of peripheral blood lymphocytes, with higher numbers of NK- and NKT cells [212], and a decrease in the cytotoxic activity of the NK cells compared with controls [290]. A

similar relationship has been shown in patients with cardiovascular disease where reduced lymphocyte counts have been identified as predictors of mortality [291, 292]. Whilst the association between inflammation and cardiovascular disease is well documented [293, 294], the immune dysfunction in renal failure is not completely understood and has been attributed to uraemic toxins, malnutrition, oxidative stress, altered renal metabolism of immunologically active proteins, for example pro-inflammatory cytokines, and dialysis therapy [209]. Previous studies have focused mainly on T- and B-cells. The role of NK cells in chronic renal failure is less well studied, with conflicting results. Both reduced NK cells numbers and activity [216, 290, 295, 296], and also enhancement [212, 217] have been reported, focusing mainly on dialysis patients. Our interest in this study was related to the emerging role of NK cells in atherosclerosis, and the high incidence of cardiovascular disease in CKD. The aim of this study was to examine the function and phenotype of NK cells in patients with a range of chronic renal failure, including dialysis patients, and to correlate this with clinical and laboratory parameters.

4.2 METHODS

4.2.1 Patients

Blood samples were taken from 143 patients across the range of renal function. Fifty millilitres of blood was taken into BD vacutainer collection tubes. There were 36 healthy volunteers, 14 patients with CKD stages 1 and 2, 34 patients with CKD stages 3 and 4, 48 patients with CKD stage 5, and 11 patients on haemodialysis (HD). Patients were

divided into 3 groups – controls, CKD 1-4, and CKD 5 which included the dialysis patients. In the patients on HD, blood samples were taken within 15 minutes of the start of the dialysis session in all the patients on dialysis. Biochemical, haematological, and clinical parameters including age, gender, BMI, and renal diagnosis were recorded. None of the patients had infection or malignancy, and none were immunosuppressed. In all 143 patients, NK cells were isolated and NK cell phenotype and distribution of NKT- and T-cells was determined. Routine biochemical parameters including urea and electrolytes, albumin, phosphate, calcium, glucose, lipid profile, and creatinine were obtained from biochemistry laboratory reports, and full blood counts were obtained from haematology laboratory reports. LDL-C was not available for all patients as some blood samples were obtained from afternoon clinics and afternoon or evening dialysis shifts, and therefore patients could not be asked to fast. LDL-C was calculated (cLDL-C) using the Freidwald formula [297].

4.2.2 Human peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells were isolated from peripheral venous blood as described in section 2.4.1.3 using Ficoll-Hypaque centrifugation (Histopaque-1077; Sigma, Poole, UK).

4.2.3 NK cell isolation

Natural killer cells were isolated using the MACS NK cell human isolation kit (Miltenyi Biotec) as described in section 2.4.2.

4.2.4 51-Chromium Release Assay

Natural killer cell cytotoxicity was measured using a standard 51-Chromium Release Assay, using the human chronic myelogenous leukaemia cell line – K562 as target cells, as described in section 2.4.3.

4.2.5 Flow Cytometry

Three colour flow cytometry of PBMCs was carried out (BD Biosciences FASCalibur) before isolation of NK cells to determine NK cell phenotype and distribution of NKT- and T-cells as described in section 2.4.4. The NK-, NKT-, and T-cells were expressed as a percentage of total lymphocyte count, and the $CD56^{bright}$ and $CD56^{dim}$ subsets were expressed as a percentage of NK cells.

4.2.6 Statistical Analysis

Statistical analysis was carried out using SPSS statistical package (version 15.0). The association between continuous variables was analysed using Pearson correlation test. Non parametric data were logarithmic transformed and Pearson correlation was used. Analysis of variance (ANOVA) was used to test differences between groups with post hoc Scheffe's test. Multiple linear regression analysis was used to assess the contribution of different factors to changes in lymphocyte subsets and NK cell cytotoxicity. Significant was taken to mean $p<0.05$.

4.3 RESULTS

4.3.1 Patient Characteristics and Laboratory Data

The clinical and laboratory data are shown in Table 4.1 and the primary causes of renal disease are shown in Table 4.2. The renal diagnosis was unavailable for 22 patients. There was a statistically significant difference in age between the controls and the CKD1-4 and CKD5 groups. There was also a statistically significant difference between creatinine and eGFR between all groups as would be expected. Haemoglobin was lower in the CKD5 group. There was no difference in total white cell count between groups, but there was a statistically significant difference in platelet count between all 3 groups, being lowest in the CKD5 group. There was a significant difference in serum phosphate between the CKD5 group and all other groups ($p<0.001$), shown in Figure 4.1, even when the HD patients were excluded from analysis. There was no difference between adjusted calcium between groups. Serum albumin was significantly lower in the CKD1-4 and CKD5 groups (Figure 4.2), and serum glucose was significantly lower in the CKD1-4 group.

There was no significant difference in total cholesterol between groups, but HDL-C was significantly lower in the CKD1-4 ($p<0.05$) and CKD5 ($p<0.001$) groups compared with controls. Triglycerides were significantly higher in the CKD1-4 groups compared with controls ($p<0.05$), but there was no difference in cLDL-C between groups, or in non-HDL-C.

Creatinine correlated significantly with haemoglobin ($r = -0.367$, $p<0.01$), platelet count ($r = -0.355$, $p<0.01$), albumin ($r = -0.196$, $p<0.05$), and phosphate ($r = 0.513$, $p<0.01$) (Figure 4.3). Haemoglobin had a significant negative correlation with phosphate ($r = -0.438$, $p<0.01$) and positive correlation with albumin ($r = 0.470$, $p<0.01$). Phosphate and albumin inversely correlated ($r = -0.275$, $p<0.01$). There was a negative correlation between total cholesterol and creatinine ($r = -0.260$, $p<0.01$) (see Figure 4.4), and HDL-C and creatinine ($r = -0.371$, $p<0.01$). Phosphate had a significant correlation with triglycerides ($r = -0.234$, $p<0.01$), but not other lipid fractions. Calculated LDL-C correlated weakly but significantly with adjusted calcium ($r = 0.186$, $p<0.05$), and with platelet count ($r = 0.199$, $p<0.05$). There was also a significant correlation between creatinine and age ($r = -0.21$, $p<0.05$), and creatinine and SBP ($r = 0.27$, $p<0.05$) and DBP ($r = 0.322$, $p<0.001$) (BP was not available for control patients).

Clinical Parameters	Controls	CKD1-4	CKD5&HD
Patient number	36	48	59
Age (years)	41.6 ± 12.8	60.2 ± 16.3	61.9 ± 14.5
Gender (M/F)	10/26	21/27	40/19
BMI (kg/m^2)	24.8 ± 5.0	28.2 ± 8.2	27.2 ± 8.9
Creatinine (μmol/L)	82.0 ± 10.9	148.7 ± 55.1	528.6 ± 178.2
eGFR (ml/min)	79.5 ± 12.2	45.9 ± 23.4	10.0 ± 3.6
Cholesterol (mmol/L)	4.9 ± 1.1	4.8 ± 1.2	4.5 ± 1.2
Haemoglobin (g/dL)	13.4 ± 1.3	12.8 ± 1.7	11.8 ± 1.3
WBC count (x 10^9/L)	7.1 ± 2.4	7.6 ± 2.2	6.9 ± 1.7
Platelets (x 10^9/L)	290.4 ± 50.2	249.8 ± 65.8	218.0 ± 71.4
Phosphate (mmol/L)	1.1 ± 0.1	1.1 ± 0.2	1.5 ± 0.5
Adjusted calcium (mmol/L)	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.2
Serum albumin (g/L)	41.7 ± 2.5	38.4 ± 4.1	37.1 ± 4.8
Serum glucose (mmol/L)	5.0 ± 0.9	7.3 ± 4.7	5.3 ± 1.7

Table 4.1. Clinical characteristics of patients. Values are expressed as mean ± SD.

Renal Diagnosis	Frequency (%)
Unknown aetiology	14.0
Renovascular	12.6
Glomerulonephritis	12.6
Chronic pyelonephritis/interstitial nephritis	9.1
Diabetes	4.2
Polycystic	7.7
Other	9.1

Table 4.2. Renal diagnosis

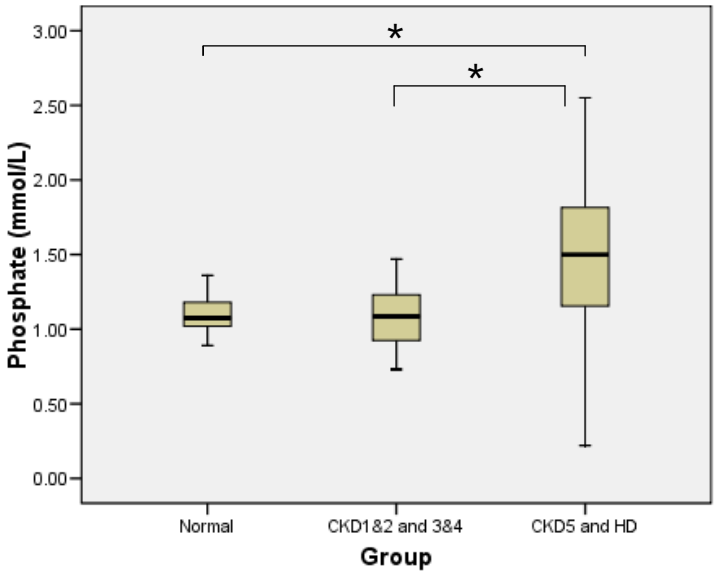


Figure 4.1. A boxplot showing mean phosphate level. (* p<0.001).

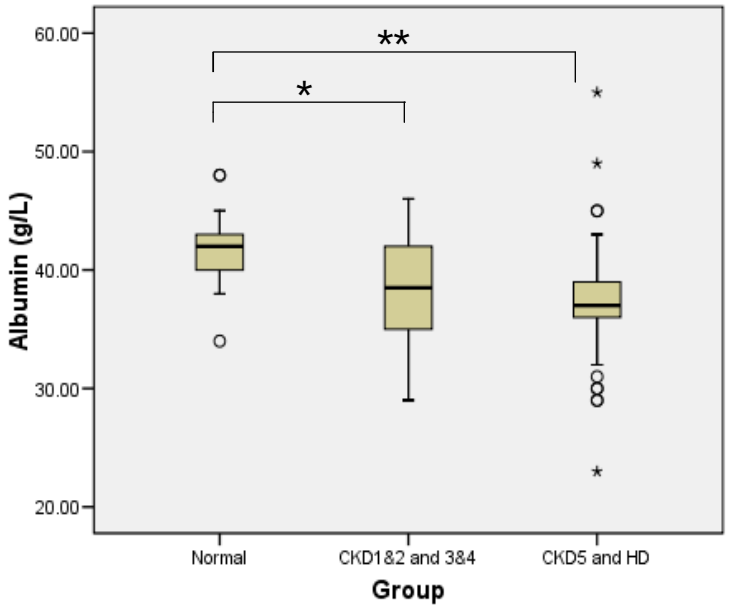


Figure 4.2. Mean serum albumin level. (* p<0.05, ** p<0.001).

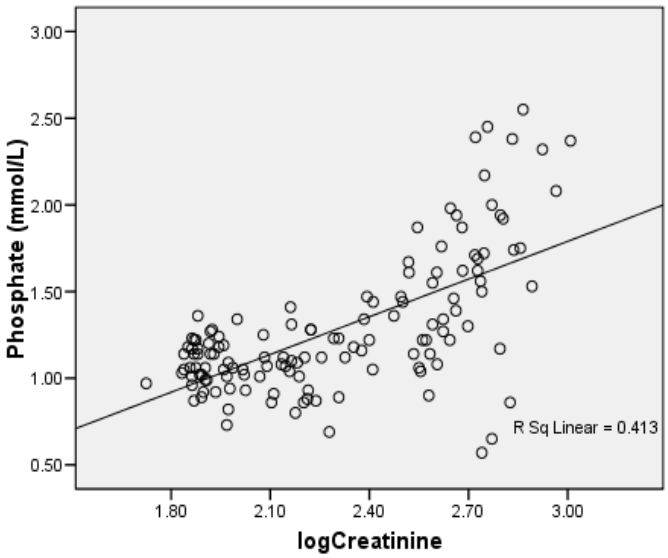


Figure 4.3. A scatterplot showing the relationship between creatinine (logarithmic transformed) and phosphate.

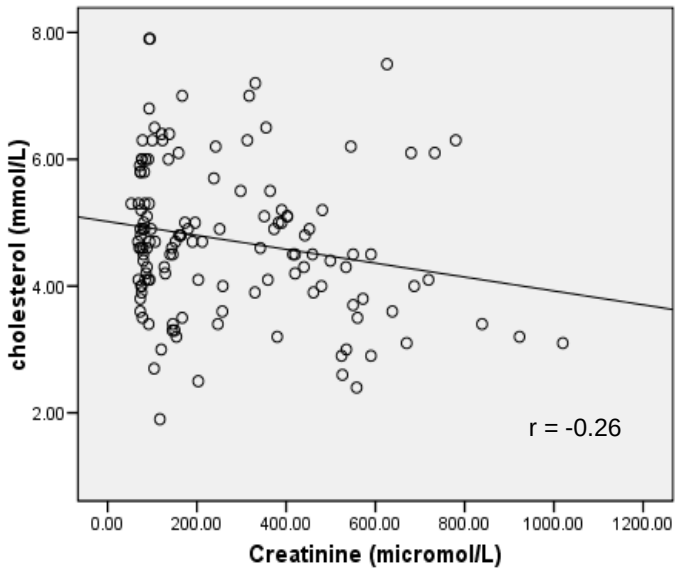


Figure 4.4. A scatterplot showing the relationship between total cholesterol and creatinine.

4.3.2 Distribution of Peripheral Blood Lymphocytes

The distribution of peripheral blood lymphocyte subsets is shown in Table 4.3. White blood cell counts were similar in all groups but there was a significant reduction in the total lymphocyte count in the CKD5 groups compared with controls and the CKD1-4 group ($p<0.05$) as shown in Figure 4.5. There was no significant difference in NK-, NKT- or T-cell numbers between groups, although when patients were re-analysed according to CKD stage (controls, CKD1&2, CKD3&4, CKD5, and HD patients), there was a significant reduction in T cells in the haemodialysis group ($p<0.05$) compared with all other groups except the CKD3&4 group (Figure 4.6). There were no differences in $CD56^{bright}$ or $CD56^{dim}$ cells between groups, although on analysing according to CKD stage there was a significant reduction in $CD56^{dim}$ NK cells in the HD groups vs the CKD1&2 groups ($p<0.05$).

(a)

Blood cells	Controls	CKD1&2	CKD3&4	CKD5	HD
White blood cells (x10^9)	7.1±2.4	7.9±2.2	7.4±2.3	7.1±1.9	6.3±1.6
Lymphocytes (x10^9)	2.1±0.5	2.2±0.5	1.8±0.8	1.7±0.6	1.2±0.5*
CD56-CD16-CD3+ [a]	65.7±12.3	66.0±4.7	59.2±11.5	62.1±13.1	45.7±20.3†
CD56+CD16+CD3+ [a]	4.2±3.4	5.1±4.3	4.1±4.0	6.1±6.1	4.4±3.0
CD56+CD16+CD3- [a]	11.7±5.6	11.8±3.5	13.3±8.0	13.6±8.2	10.3±6.0
• CD56 dim [b]	91.0±4.1	95.3±2.8	93.2±5.2	92.6±5.0	88.2±8.7‡
• CD56 bright [b]	8.7±4.0	5.2±2.6	6.5±4.8	6.8±5.2	11.1±8.7

(b)

Blood cells	Controls	CKD1-4	CKD5
White blood cells (x10^9)	7.12±2.35	7.60±2.23	6.98±1.87
Lymphocytes (x10^9)	2.05±0.50	1.89±0.71	1.58±0.62**
CD56-CD16-CD3+ [a]	65.7±12.3	61.2±10.4	59.0±15.9
CD56+CD16+CD3+ [a]	4.2±3.4	4.4±4.1	5.7±5.6
CD56+CD16+CD3- [a]	11.7±5.6	12.9±7.0	12.9±7.9
• CD56 dim [b]	91.0±4.1	93.8±4.7	91.8±6.0
• CD56 bright [b]	8.7±4.0	6.1±4.3	7.6±6.2

* $P < 0.05$ v all groups
† $P < 0.05$ v controls, CKD1&2, and CKD5
‡ $P < 0.05$ v CKD1&2
** $P < 0.05$ v controls and CKD1-4

Table 4.3. WCC, lymphocyte and lymphocyte subset counts (expressed as a percentage of total lymphocytes). (a) shows patients divided into groups according to CKD stage (b) shows patients divided into 3 groups. (CD56+CD16+CD3- represents NK cells, CD56+CD16+CD3+ represents NKT cells, CD56-CD16-CD3+ represents T cells). **[a]**Percentage of lymphocytes; **[b]**percentage of NK cells.

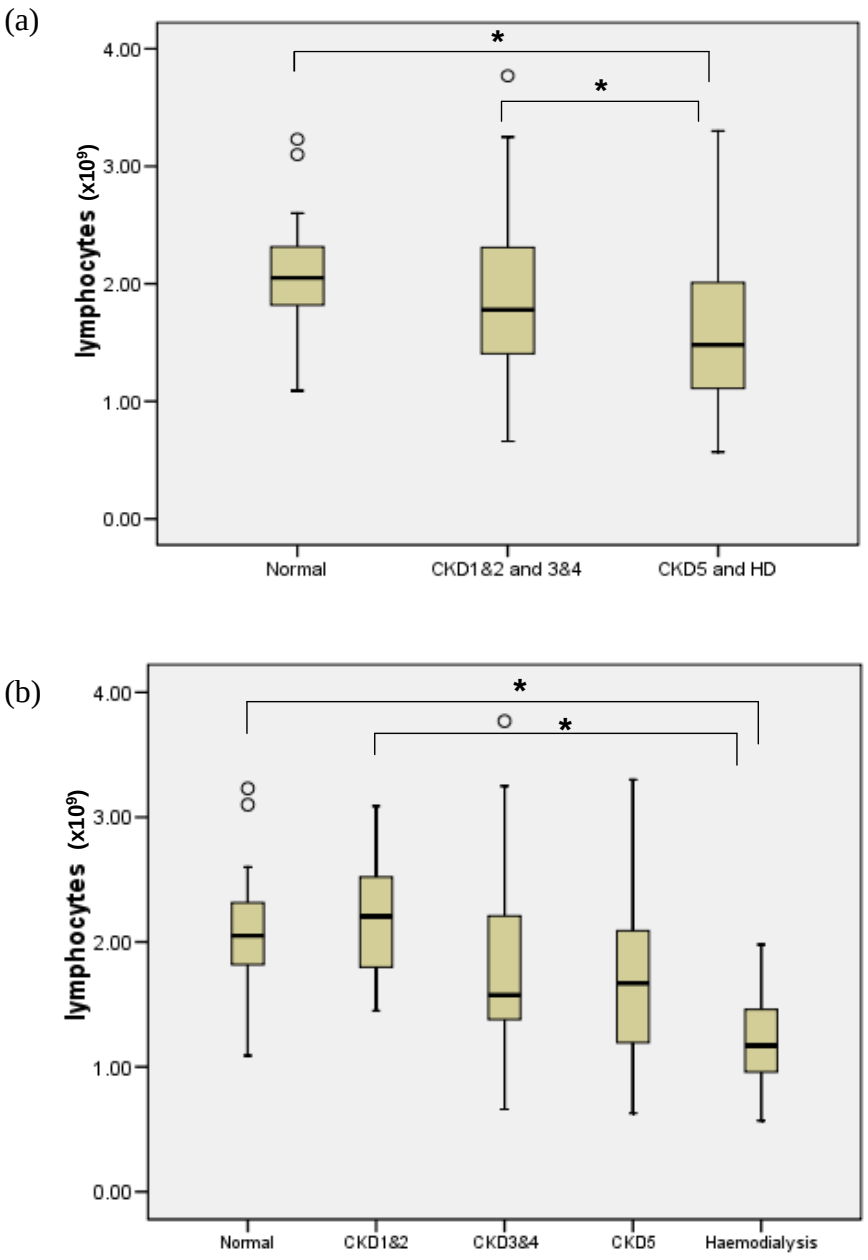


Figure 4.5. Distribution of peripheral blood lymphocytes between groups. (a) shows the 3 groups described in section 4.2.1. (b) shows the patients divided according to CKD stage, including HD patients to show the significant difference in lymphocytes in the HD group. (*p<0.05).

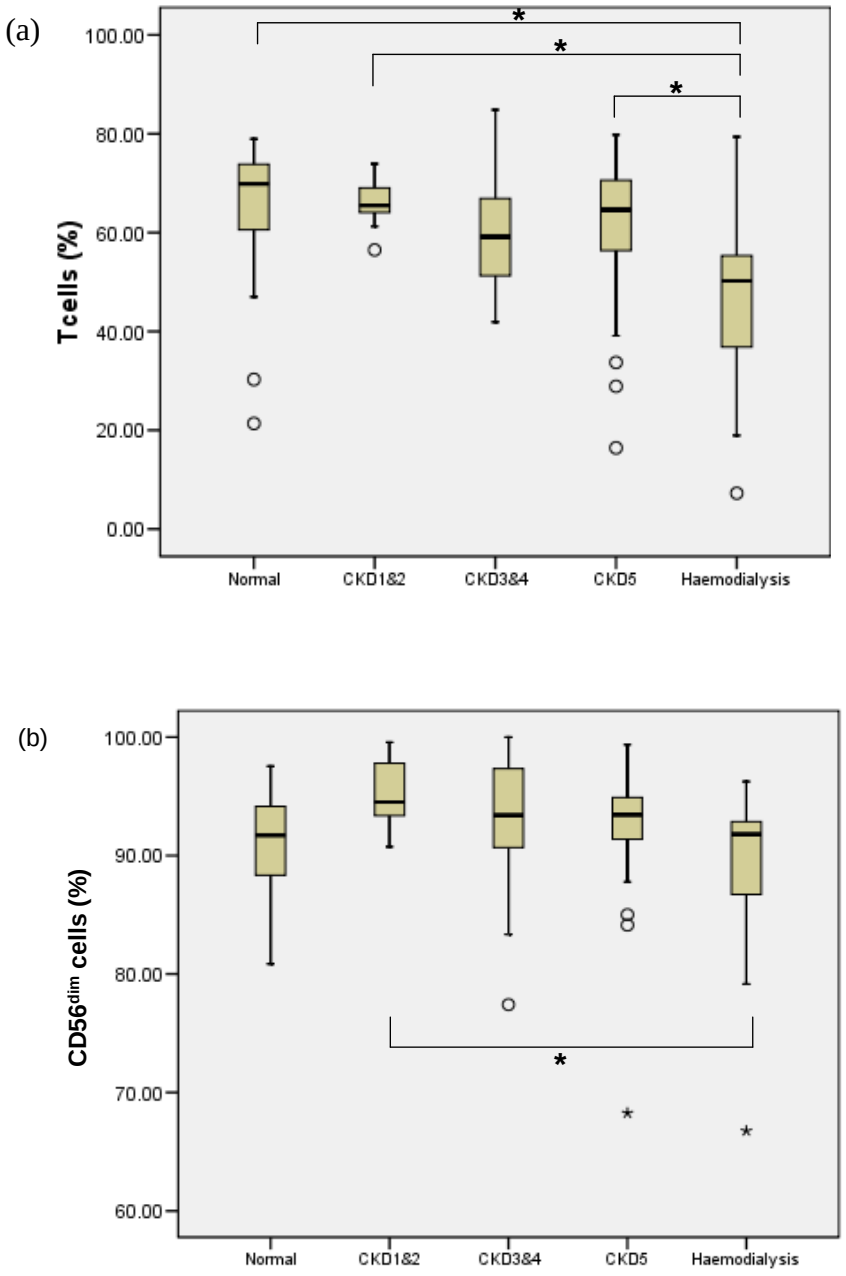


Figure 4.6. (a) Distribution of T cells between groups (expressed as a % of lymphocytes) (b) Distribution of NK dim cells (expressed as a % of NK cells) between groups (*$p<0.05$).

There were no significant correlations between lipid fractions and NK or NKT cell number or NK cell phenotype. There was a weak but significant correlation between T-cells and total cholesterol ($r = 0.185$, $p < 0.05$) and HDL-C ($r = 0.176$, $p < 0.05$) (Figure 4.7), and also between T-cells and cLDL-C ($r = 0.195$, $p < 0.05$). There was a significant negative correlation between creatinine and T-cells ($r = -0.222$, $p < 0.001$), and a positive correlation between albumin and T-cells ($r = 0.178$, $p < 0.05$). There was a weak negative correlation between glucose and $CD56^{bright}$ NK cells ($r = -0.191$, $p < 0.05$). As has been shown in previous studies, there was an inverse relationship between age and T cells ($r = -0.410$, $p < 0.001$) which remained statistically significant adjusting for sex, creatinine, eGFR, haemoglobin, white cell count, platelet count, statin treatment, total cholesterol, HDL-C, triglycerides, BMI, phosphate, albumin, adjusted calcium, and glucose (β coefficient -0.515, SD 0.132, $p<0.001$). There were also significant correlations between age and NK cells ($r = 0.20$, $p<0.05$), NKT cells ($r = 0.25$, $p < 0.01$), $CD56^{bright}$ ($r = -0.26$, $p<0.01$) and $CD56^{dim}$ cells ($r=0.26$, $p<0.01$). The relationship between age and NK cells, and age and $CD56^{dim}$ cells also remained significant when adjusted for the above variables (β coefficient 0.178, SD 0.79, $p<0.05$; β coefficient -0.21, SD 0.9, $p<0.05$ respectively). Albumin correlated positively with T cells ($r = 0.18$, $p<0.05$) but this did not retain significance in multiple regression analysis. There was no significant correlation with NK cell phenotype and creatinine. There were no correlations between T cells, NK and NKT cells, or NK cell phenotypes and BMI or blood pressure. There was a very strong negative correlation between NK bright and NK dim cells ($r = -0.94$, $p < 0.001$) as would be expected.

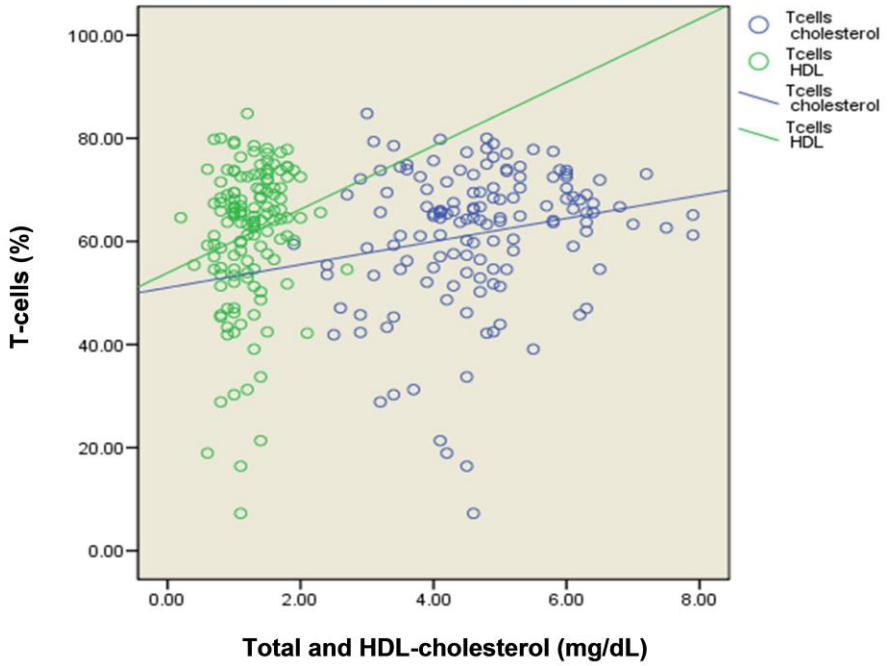


Figure 4.7. Scatterplot showing the relationship between T cells and total cholesterol (in blue) and HDL-C (in green). T-cells are expressed as a percentage of lymphocytes.

4.3.3 Natural Killer Cell Cytotoxicity

There was no difference in NK cell cytotoxicity between groups, even when the HD patients were excluded from the CKD5 group. There was a significant correlation between NK cell cytotoxicity and creatinine ($r = 0.208$, $p<0.05$) [Figure 4.8(a)], which remained when the HD patients were excluded from analysis ($r = 0.203$, $p<0.05$). There was also a significant correlation between NK cell cytotoxicity and serum phosphate when analysed with the HD patients ($r = 0.301$, $p<0.01$) and without ($r = 0.201$, $p<0.05$) [Figure 4.8(b)]. The correlation between phosphate and NK cytotoxicity including the

haemodialysis group remained significant after multivariate analysis (β coefficient 14.6, $p = 0.006$), adjusting for age, total cholesterol, cLDL-C, HDL-C, triglycerides, log creatinine, haemoglobin, white cell count, platelet count, adjusted calcium, albumin, glucose, weight, and BMI (Table 4.4). There was no significant correlation between NK cell cytotoxicity and albumin despite the relationship between albumin, creatinine and phosphate.

There was no relationship between NK cytotoxicity and any of the lipid fractions. Despite the strong negative correlation between NK bright and NK dim cells, and significant correlations between total NK cells and NK bright ($r = -0.39$, $p < 0.001$) and NK dim ($r = 0.352$, $p < 0.001$) cell phenotypes, there was no significant correlation between NK cell phenotype and NK cell cytotoxicity. Additionally, there was no correlation with age or blood pressure.

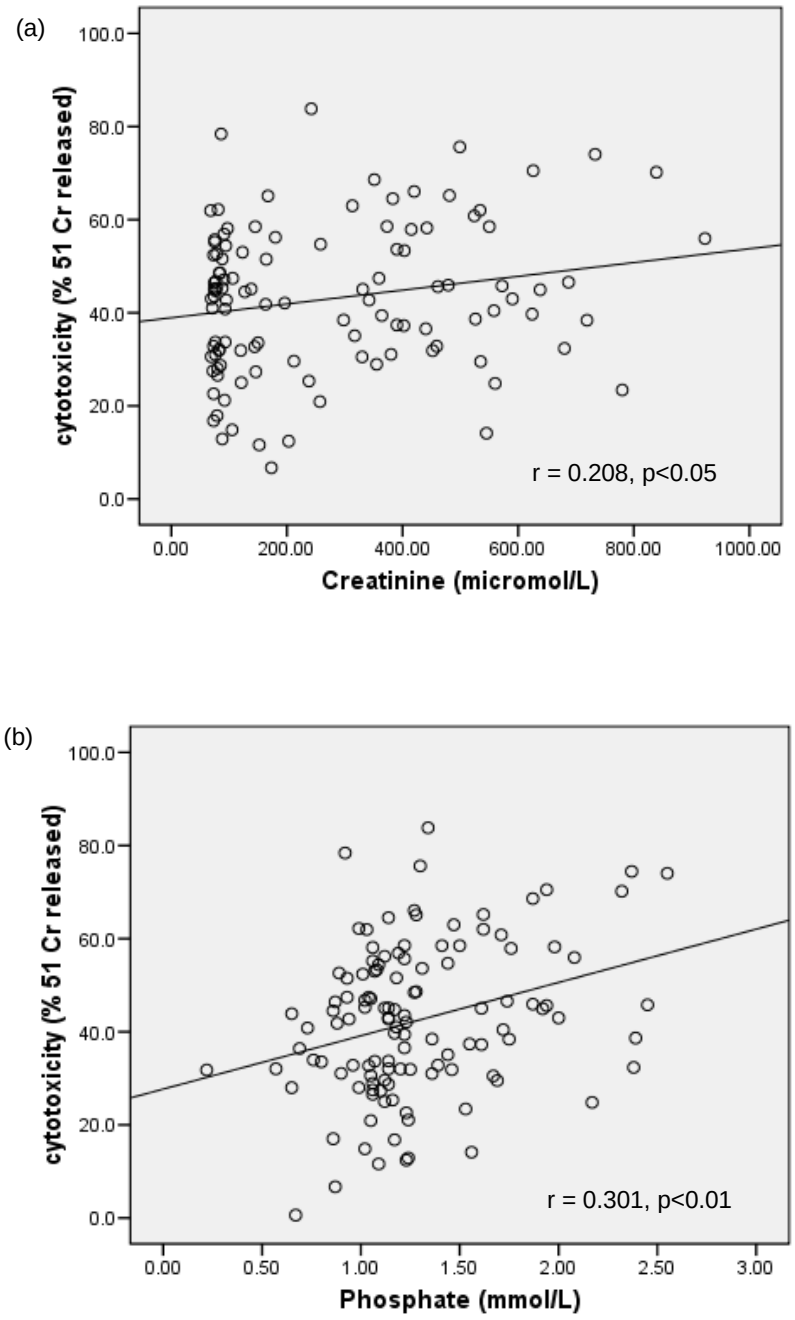


Figure 4.8. Scatterplots showing the relationship between NK cell cytotoxicity and (a) serum creatinine, (b) serum phosphate. NK cell cytotoxicity is expressed as percentage of ^{51}Cr released.

Variable	Multivariable beta coefficient	*P*
Phosphate (mmol/L)	14.627	0.006
Log creatinine (μmol/L)	-0.087	0.636
Age (years)	-0.101	0.415
Total cholesterol (mmol/L)	0.130	0.297
cLDL-C (mmol/L)	0.106	0.388
HDL-C (mmol/L)	-0.004	0.973
Triglycerides (mmol/L)	0.043	0.733
Haemoglobin (g/dL)	0.114	0.410
WCC (x 10^9/L)	-0.083	0.505
Platelets (x 10^9/L)	0.100	0.441
Adjusted calcium (mmol/L)	-0.144	0.246
Albumin (g/L)	0.018	0.883
Glucose (mmol/L)	-0.141	0.259
Weight (kg)	-0.158	0.194
BMI (kg/m^2)	-0.211	0.087

Table 4.4 Multivariate predictors of NK cell cytotoxicity

4.4 DISCUSSION

It has recently been recognised that atherosclerosis is a multi-factorial, highly complex disease that is now widely accepted as an inflammatory disease, and the participation of NK and NKT cells have been demonstrated [69, 87, 89, 298]. In addition, NK cells have been identified in atherosclerotic lesions [89], and atherogenic diet fed *ldl-r-/-* mice deficient in functional NK cells had a statistically significant reduction in atherosclerotic lesions [87]. There have been few comparable studies in human atherosclerotic disease. Human NK cells comprise 15% of all lymphocytes, and are defined phenotypically by their expression of CD56 and lack of expression of CD3. Additionally, NK cells are not a homogeneous population. As mentioned previously it has been recognized that two distinct populations of human NK cells can be identified, based upon their cell surface density of CD56 [79]. Resting $CD56^{dim}$ cells are the more cytotoxic subset, whereas the $CD56^{bright}$ NK-cell subset mediates low or no cytotoxicity but has the capacity to produce large amounts of various immunoregulating cytokines such as IFN-γ in response to interleukin (IL)-12 and IL-18. All three of these cytokines have been shown to have proatherogenic effects [299].

Natural killer cell function in dialysis patients has been analysed previously [212, 216, 217, 290, 295, 296], but these studies have compared dialysis patients with healthy controls, or included patients with CKD but in much smaller numbers, with conflicting results. Our study aimed to assess NK cell function in patients throughout the range of renal failure, to look for alterations in NK cell phenotype related to renal function, and to correlate this with clinical and laboratory parameters. Conflicting results from previous

studies looking at NK cells in renal failure and dialysis patients may be explained by small patient numbers, and changes in practice in terms of use of statins, phosphate binders, and dialysis membranes.

In the present study, we have confirmed the expected correlations between albumin, haemoglobin, phosphate, and creatinine in this group of patients. We found a significant reduction in T cells in the RRT group that has been well described [300] and an inverse relationship between T cells and age, and although we did not find any alterations in NK cell counts with increasing renal failure, we did find a correlation between age and total NK cell numbers and $CD56^{dim}$ NK cells as has been previously described [301], and interestingly an inverse relationship between age and $CD56^{bright}$ NK cells. The most interesting finding was the correlation between NK cell cytotoxicity and phosphate, and although there was a correlation between NK cytotoxicity and creatinine, this correlation did not retain significance after multivariate linear regression analysis. However, by the strong association creatinine has with phosphate it is intriguing to speculate on an association. Clearly the relationship between increasing creatinine and rising cardiovascular risk is indisputable. There is an abundance of emerging evidence of the contribution that phosphate has in cardiovascular disease [223], particularly in the dialysis population in the context of cardiovascular calcification [302], and an association between phosphate and increased mortality has been described [223]. Phosphate has also been found to have a role in proliferation of vascular smooth muscle cells – a contributor to the development of atherosclerotic lesions – in vitro [228], and phosphate binders have been shown to have anti-inflammatory effects, and favourable effects on lipid profiles

[303]. The phosphate binder sevelamer has also been shown to prevent uraemia-enhanced atherosclerosis progression in apoE-/- mice [304]. Our findings lend support to the role of phosphate and NK cells in cardiovascular disease, particularly in patients with CKD.

In patients with chronic heart failure however, NK cells have been shown to be present in lower circulating numbers, and to exhibit impaired cytolytic function [86]. Reductions in NK cell number with a loss of NK cell function thought to be a quantitative rather than qualitative defect has also been described in patients with coronary artery disease [95] who were found to have evidence of an enhanced inflammatory response by higher levels of C-reactive protein and IL-6, although the authors were unable to exclude statin treatment as a cause for the reduced NK cell activity. An interesting speculation by the authors was that a loss of NK cell activity might increase susceptibility to atherosclerosis-related pathogens. They went on to find increased numbers of apoptotic NK cells in peripheral blood of coronary artery disease patients compared with healthy controls, and that the NK cells were more sensitive to spontaneous apoptosis ex vivo [97]. A recent study in patients with renal failure found that although NK cell numbers were lower in haemodialysis patients compared to healthy controls and undialysed patients with CKD, NK cytotoxicity was positively correlated to dialysis duration [295], a finding that is in keeping with the inflammatory state of renal failure and dialysis.

The role of NK cell subsets *in vivo* is not entirely clear. For example, normal ratios of NK cell subsets have been found to be altered during ageing – numbers of $CD56^{dim}$ NK

cells are expanded in elderly populations, with no significant change in the numbers of $CD56^{bright}$ NK cells [301]. Functionally, NK cells in the elderly are less responsive to IL-2-induced proliferation, perhaps as a consequence of an increased percentage of $CD56^{dim}$ NK cells and reduced $CD56^{bright}$ NK cells. Our findings are in keeping with this although we found a reduction in the $CD56^{bright}$ subset.

In summary, our results suggest a link between NK cell activity and renal function, and in particular serum phosphate, which has been shown to have a role in cardiovascular disease. The finding of increased NK cell numbers and increases in the $CD56^{dim}$ subset with age may have importance given our ageing population and increasing numbers of older patients being accepted for RRT. The state of chronic inflammation, altered mineral metabolism, uraemic toxins, dialysis, and other factors such as cytomegalovirus (CMV) infection, shown to be significantly associated with atherosclerotic disease in patients with end stage renal failure [305], that are specific to renal failure make understanding of the immune dysfunction in this group of patients important. The high incidence of cardiovascular disease in this patient population with more evidence of the contributing role of the immune system, and in particular NK cells, to atherosclerosis stresses the need for further studies to evaluate the role they have to play in CKD patients and their link with vascular calcification.

There were limitations to this study; again, the control group was not aged-matched and thus any changes in NK cell distribution or indeed cytotoxicity could be potentially attributable to the older age of the CKD patients. The small number of diabetics included

and the small number of HD patients could potentially affect results, although no major differences were found when data were analysed excluding the HD patients. The blood samples being taken at the beginning of the dialysis session (within the first 15 minutes) in the HD patients may not give a true reflection of lymphocyte numbers and function as there is evidence that certainly T-cell function can change acutely during HD [306]. It would have been preferable to take samples immediately prior to connection to the dialysis machine. The fact that the samples were non-fasting, hence a laboratory LDL-C value was not available was a major limitation, however due to the organisation of the renal clinics and dialysis shifts, with the majority of the patients being seen in afternoon clinics, it was not felt possible to ask patients to fast for such a prolonged period. Once again, it should be noted also that the resuspension medium contained 12.5% foetal bovine serum which is rich in lipoproteins and could confound results. However all assays of NK cell cytotoxicity were carried out uniformly.

CHAPTER 5

An *in vitro* study of membrane cholesterol in natural killer cells

5.1 INTRODUCTION

Conventional pharmacology is focused on membrane receptor and sub-cellular secondary messenger proteins. However, the cell membrane that supports these proteins and their interactions is neither inert nor homogeneous. Within the plasma membrane, functional cholesterol-rich membrane microdomains – lipid rafts – have high concentrations of receptors and signal transduction molecules [100, 105]. This arrangement facilitates high efficiency signal transduction.

Drugs that inhibit cellular cholesterol synthesis by HMG-CoA reductase – statins – have proven benefits in the prevention of cardiovascular events in patients with dyslipidaemia and increased cardiovascular risk. Numerous reports however, suggest that statins have effects that extend beyond the lowering of lipid levels, the so-called pleiotropic effects [154, 268] that include inhibition of cell proliferation and in particular, immunosuppressive [126, 127] actions. Amongst these pleiotropic effects, statins have been demonstrated to impair NK cell cytotoxicity *in vivo* [307] and *in vitro* [150]. Two mechanisms have been proposed to explain this observation: reduced production of isoprenoid intermediates in the cholesterol biosynthetic pathway which participate in cell signalling, and alternatively by depletion of cell membrane lipid rafts. Natural killer cells appear particularly sensitive to the effects of statins, an observation that may reflect the critical dependence of NK cell activation and cytotoxicity on membrane rafts.

The aim of this study was to investigate the effects of membrane cholesterol on NK cell function and specifically, whether the actions of statins on NK cells are due to depletion of membrane cholesterol or inhibition of isoprenylation.

5.2 METHODS

These were *in vitro* experiments and the NK92MI cell line was used.

5.2.1 Cell Culture

NK92MI cells were maintained at 37^{o}C, with 5% CO_2 in a water saturated atmosphere in alpha minimum essential medium without ribonucleosides and deoxyribonucleosides and supplemented with 2mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 0.2nM inositol, 0.1mM 2-mercaptoethanol, 0.02mM folic acid, 12.5% horse serum and 12.5% fetal bovine serum (all from Gibco, Paisley, UK). K562 cells were maintained in RPMI supplemented with 2mM L-glutamine, 10 U/ml penicillin and 10g/ml streptomycin.

5.2.2 Stock Solutions of cholesterol and statin

Soluble cholesterol (Sigma, Poole, UK) was dissolved in NK92MI media to a stock concentration of 1mg/ml and added to cells at the concentrations indicated. Simvastatin was dissolved in 50μl DMSO, then diluted to a stock concentration of 200ug/ml in NK92MI media and added to cells at the concentrations indicated.

5.2.3 Cholesterol Loading

NK92MI cells were treated with increasing concentrations of cholesterol (as a cholesterol-methyl-beta-cyclodextran complex [MBCD]) as described in section 2.4.5. To load cells, varying concentrations ranging from 5 - 100μg/ml of the cholesterol-MBCD complex was added to 1 x 10^6 cells/ml to a volume of 2 ml RPMI media. Cells were then incubated at 37^{o}C for increasing lengths of time; from 30 minutes to overnight incubation. They were then washed with PBS.

To cause raft disruption, NK92MI cells were spun down, resuspended in 1ml of media and seeded at a density of 1 x 10^6 cells/ml in 6-well plates, in 2 ml of media in combination with either 1mM methyl-beta-cyclodextran or 2μmol of simvastatin.

5.2.4 51-Chromium release assay

NK92MI cells were seeded at a density of 1 x 10^6 cells/ml in 2.0 ml of media and treated with various concentrations of soluble cholesterol (Sigma, Poole, UK). K562 cells were used as target cells for NK cells, and a 51-Chromium release assay was carried out to assess NK cell function, as described in section 2.4.3.

5.2.5 Cell Membrane Preparations

Cell membranes were prepared as described in section 2.5.1. Membrane pellets were resuspended in cold membrane preparation buffer for analysis by SDS-PAGE and Western blot.

5.2.6 Cholesterol measurement

Cholesterol quantification was performed with the Amplex Red Cholesterol Assay Kit (Molecular Probes, Invitrogen, Paisley, UK) as described in the manufacturer's protocol, and in section 2.6.1.

5.2.7 Isolation of Lipid Rafts

The treated NK92MI cells were lysed with MNE and subjected to sucrose gradient centrifugation and sucrose layering as described in section 2.5.3. Volumes were adjusted to contain equal protein levels (as determined by Bradford's assay). A fraction of the sample was retained to compare expression in whole cell lysates and the rest subjected to sucrose gradient centrifugation at 45,000 rpm (287.500 x g) for 18 hours in a SW60-ti rotor (Beckman Instruments). The samples for centrifugation were mixed (1 in 2) with 80% sucrose MNE, with 30% then 5% sucrose MNE layered on top. Rafts were collected from the 5% and 30% sucrose interface as described in section 2.5.3.1.

5.2.8 Western Blotting

Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (NuPage pre-cast gel SDS-PAGEsystem, Invitrogen, Paisley, UK). Nitrocellulose membranes were incubated in blocking buffer (tris-buffered saline containing 0.1% Tween 20 and 4% non-fat milk) and incubated overnight at $4^{o}C$ with the specified primary antibody diluted 1:1000 [Anti-LAT and anti-HMG-CoA Reductase (Upstate, Lake Placid, USA); Lyn (Santa Cruz, USA); ERK (New England Biolabs, Ipswich, UK)]. Membranes were incubated with appropriate horse-radish peroxidase conjugated

secondary antibodies for 1 hour, and developed using the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

5.2.9 Ras activation

Ras activity was measured using a Ras Activation Assay Kit which is western blot based, as described in section 2.6.2. Ras activation assays were carried out by Dr Dianne Hillyard (Renal Research Group, BHF Glasgow Cardiovascular Research Centre).

5.2.10 HMG-CoA Reductase

We measured gene expression for HMG-CoA reductase in NK92MI cells that had been treated with either soluble cholesterol or 5μmol simvastatin, by real time PCR and quantified enzyme protein by Western blotting as described in section 2.7. Real time PCR was carried out by Dr Dianne Hillyard (Renal Research Group, BHF Glasgow Cardiovascular Research Centre).

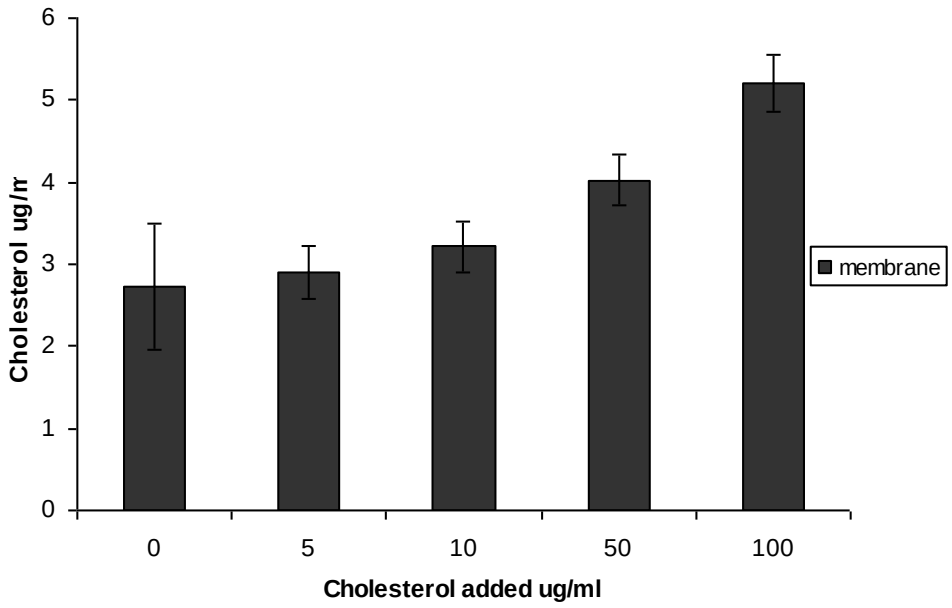
5.3 RESULTS

5.3.1 Membrane and raft cholesterol measurements

NK92MI cells were treated with increasing concentrations of cholesterol (as cholesterol-MBCD complex), and incubated at 37^{o}C for 1 hour. Complete membranes were then isolated from the NK92MI cells. The hour long incubation resulted in increasing levels of membrane cholesterol with increasing concentrations of cholesterol. There was no significant change in cytosolic cholesterol (Figure 5.1).

Next, NK92MI cells were treated uniformly with 50μg/ml of cholesterol, for increasing lengths of time. MBCD (not complexed to cholesterol) was used as a control to cause membrane cholesterol depletion. Complete membranes were then isolated to measure membrane and then raft cholesterol. Short incubations with MBCD-cholesterol doubled the incorporation of cholesterol into NK cell membranes. Longer periods of exposure returned membrane cholesterol towards baseline levels (Figure 5.2).

(a)



(b)

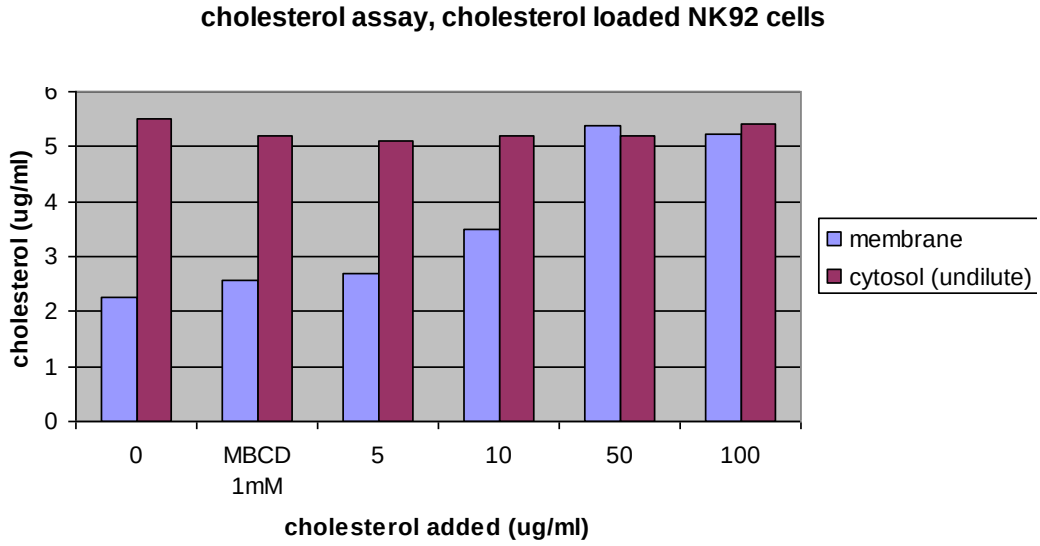


Figure 5.1 (a) A bar chart showing membrane cholesterol content increases with increasing concentrations of cholesterol added. (Representative of 2 experiments, expressed as the mean ± SD) (b) Membrane cholesterol increases with increasing concentrations of added cholesterol but cytosol cholesterol remains unaltered.

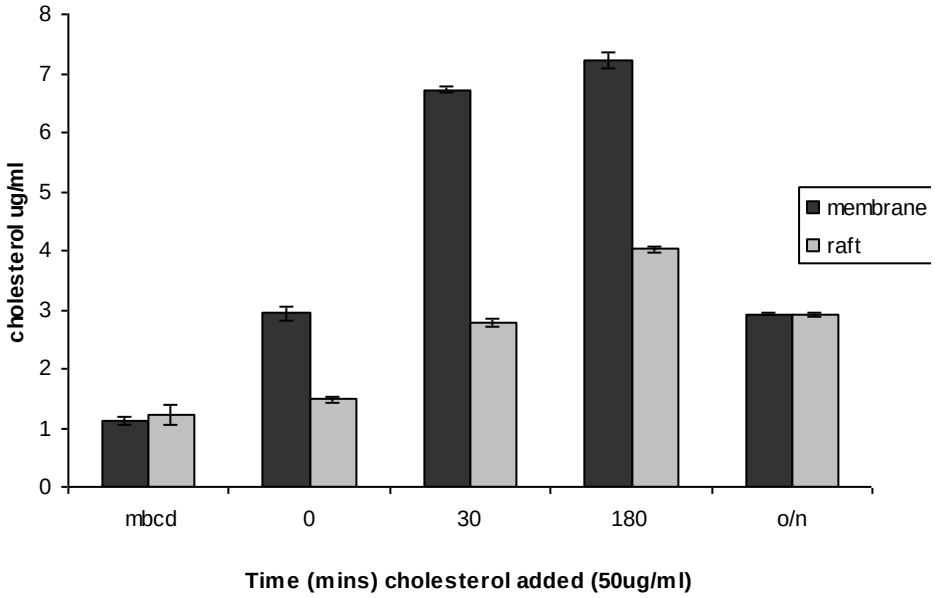
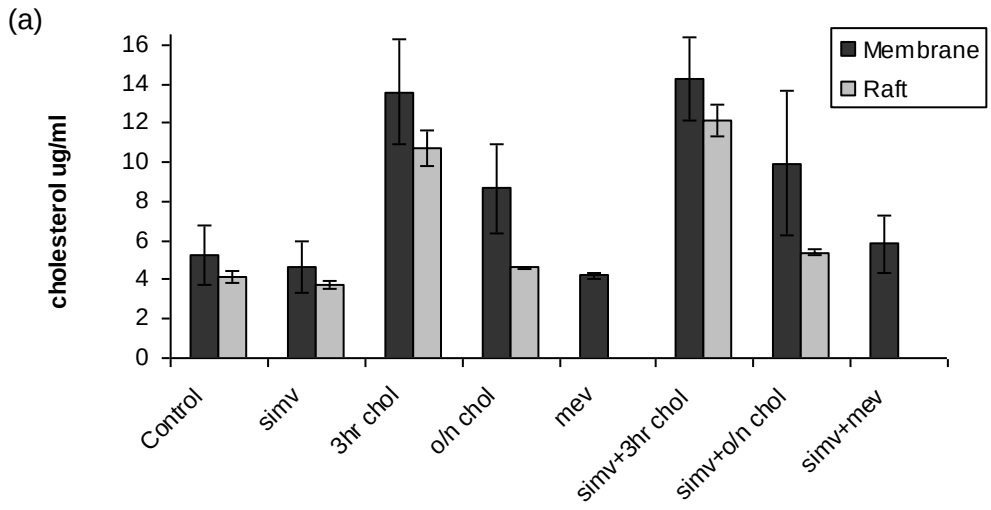


Figure 5.2 Increasing duration of incubation time with cholesterol-MBCD results in increasing levels of membrane cholesterol incorporation in both the whole cell membrane and raft fraction. However, prolonged (overnight) incubation resulted in levels returning to baseline. MBCD was used as a control to cause membrane cholesterol depletion. (Data are expressed as the mean ± SD, and are representative of 2 experiments).

Cholesterol-rich membrane microdomains were then investigated. Rafts were isolated from NK92MI cells which were loaded with cholesterol-MBCD as described above, by sucrose density centrifugation. The cholesterol content of the raft fraction increased with prolonged exposure to exogenous cholesterol, and then tended to fall, approaching baseline levels after overnight exposure (Figure 5.2).

It has been hypothesised that statins disrupt signalling pathways by inhibiting prenylation of small G protein signalling molecules [308]. Following statin treatment, mevalonate has been shown to restore both isoprenoid and cholesterol synthesis, in contrast to the restoration of membrane cholesterol alone by soluble cholesterol. Thus, we investigated the effect of excess cholesterol on simvastatin treated cells. NK92MI cells were treated with 5μmol of simvastatin for 48 hours. This has previously been shown to be the length of time required for simvastatin to alter cell function [150] without causing significant statin-induced apoptosis. This concentration of simvastatin has also been shown to be clinically relevant. Following the incubation with simvastatin, cells were then incubated with soluble cholesterol-MBCD, to restore membrane raft cholesterol. Mevalonate rescue was also studied. Cell membranes and rafts were then isolated. Simvastatin reduced cholesterol content in membranes, however no change in raft-cholesterol could be detected using this assay. This is likely to be a reflection of the sensitivity of the assay in view of previous observations [153]. Addition of soluble cholesterol reversed the effects of simvastatin on membrane cholesterol content as, although to a lesser extent, did mevalonate. Raft cholesterol content was similarly increased by short-term, and also to a lesser extent by longer-term, exposure to soluble cholesterol-MBCD (Figure 5.3).

Figure 5.3


(a)
cholesterol ug/ml
16
14
12
10
8
6
4
2
0
Membrane
Raft
Control
simv
3hr chol
o/n chol
mev
simv+3hr chol
simv+o/n chol
simv+mev

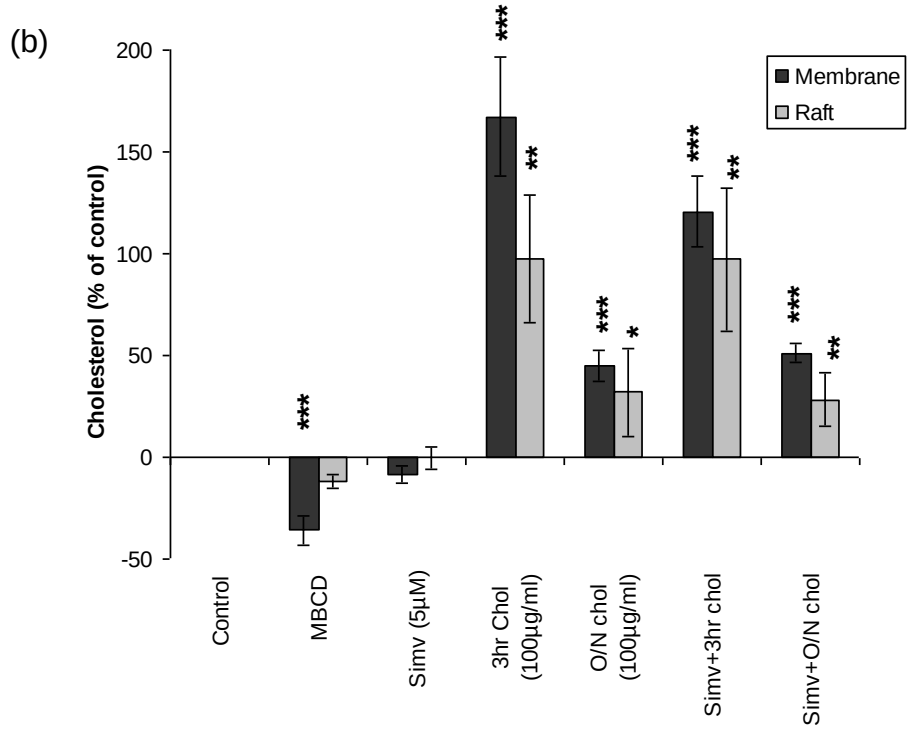


Figure 5.3 Cholesterol and mevalonate rescue statin inhibition of membrane and raft cholesterol. NK92MI cells were treated with 5μM of simvastatin for 48 hours and the resulting cholesterol inhibition was rescued by incubation with cholesterol for either 3 hours or overnight, or with 1mM mevalonate incubation overnight. (a) 3 hour cholesterol and overnight cholesterol successfully rescued the inhibition of membrane cholesterol by simvastatin. Mevalonate also rescued the inhibition but to a lesser extent. (b) 3 hour cholesterol and overnight cholesterol also successfully rescued the inhibition of raft cholesterol content by simvastatin. (This was the average of 5 experiments. The data are expressed as mean±SD. Students t-test was used to compare with control. *p<0.05, **p<0.005, ***p<0.0005).

In order to measure raft-associated signal proteins, specifically LAT and Lyn, isolated raft fractions were also subjected to SDS PAGE and Western Blotting (Figure 5.4). LAT is palmitoylated and Lyn is merystolated, and are therefore unlikely to be affected by reduced levels of isoprenoids [309]. LAT and Lyn levels were both shown to be reduced by simvastatin treatment. Short term exposure to soluble cholesterol increased both LAT and Lyn levels in the raft fractions whereas longer exposure reduced levels towards baseline, corresponding with changes in membrane cholesterol levels seen in Figure 5.1. Short term soluble cholesterol also rescued statin inhibition, with longer term exposure only rescuing LAT (but not Lyn), again corresponding to cholesterol levels in Figure 5.2.

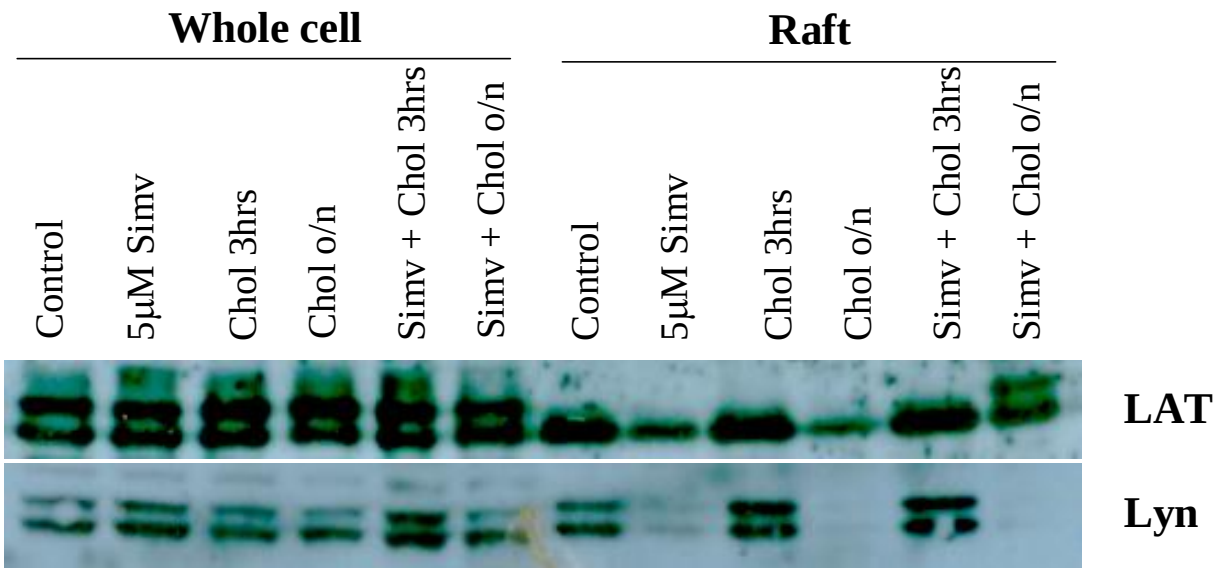


Figure 5.4. Simvastatin reduces raft markers (and therefore rafts). Rafts were isolated from NK92MI cells treated with simvastatin and cholesterol and western blot was carried out for the raft markers LAT and Lyn. Simvastatin reduced both LAT and Lyn, and therefore rafts. Short term incubation (3 hours) increased LAT and Lyn (and rafts), whereas longer term incubation with cholesterol (overnight) reduced levels to baseline. Cholesterol loading for 3 hours rescued statin inhibition, whereas overnight cholesterol only rescued LAT (and not Lyn).

5.3.2 Natural killer cell cytotoxicity

The functional effect of raft disruption on NK cells was investigated by cytotoxicity assay. The NK92MI cells were incubated with 100μg/ml of soluble cholesterol for increasing duration, and then incubated with Chromium-51 laden target K562 cells in a Chromium release cytotoxicity assay. NK cell cytotoxicity decreased with time exposed to soluble cholesterol (Figure 5.5), in contrast with the increased raft levels in Figures 5.3 and 5.4. Additionally, as would be expected NK cell cytotoxicity decreased with simvastatin and MBCD treatment, corresponding with decreased raft levels in Figure 5.3.

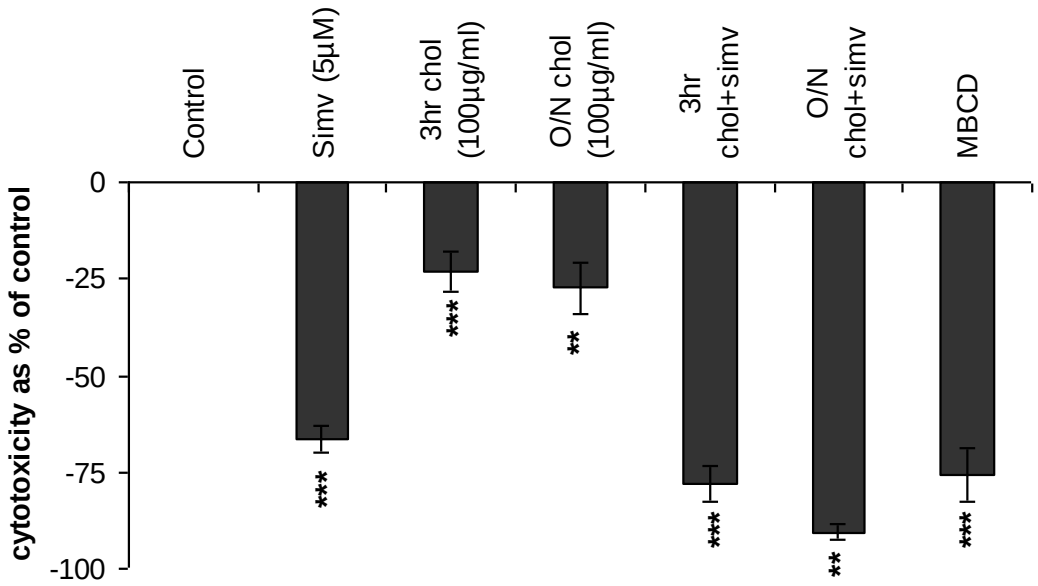


Figure 5.5. Shows that NK cytotoxicity decreases with time incubated with cholesterol. Data are expressed as a percent change from time 0. The data are expressed as the mean ± SD. Student's t-test was used to compare with controls. $^{**}p<0.005$, $^{***}p<0.0005$. (This was the average of 6 experiments).

5.3.3 Isoprenylated Proteins

The discrepancy found between changes in membrane cholesterol, raft associated proteins and cytotoxicity described above, did not fit with the original hypothesis that changes in membrane cholesterol (membrane rafts) was the predominant mechanism through which statins exert their effect on NK cells (outlined in the introduction chapter). We therefore measured active Ras by Western Blotting in the cell membranes. Ras is a small G-protein that is localised in membrane rafts when activated and, unlike LAT or Lyn, the localisation is dependant on isoprenylation [12, 310]. Treatment of NK cells with simvastatin reduces Ras isoprenylation within the raft fraction of the cell membrane, and this reduction is not rescued by the addition of soluble cholesterol (Figure 5.6). Surprisingly, soluble cholesterol alone also reduced active Ras concentration.

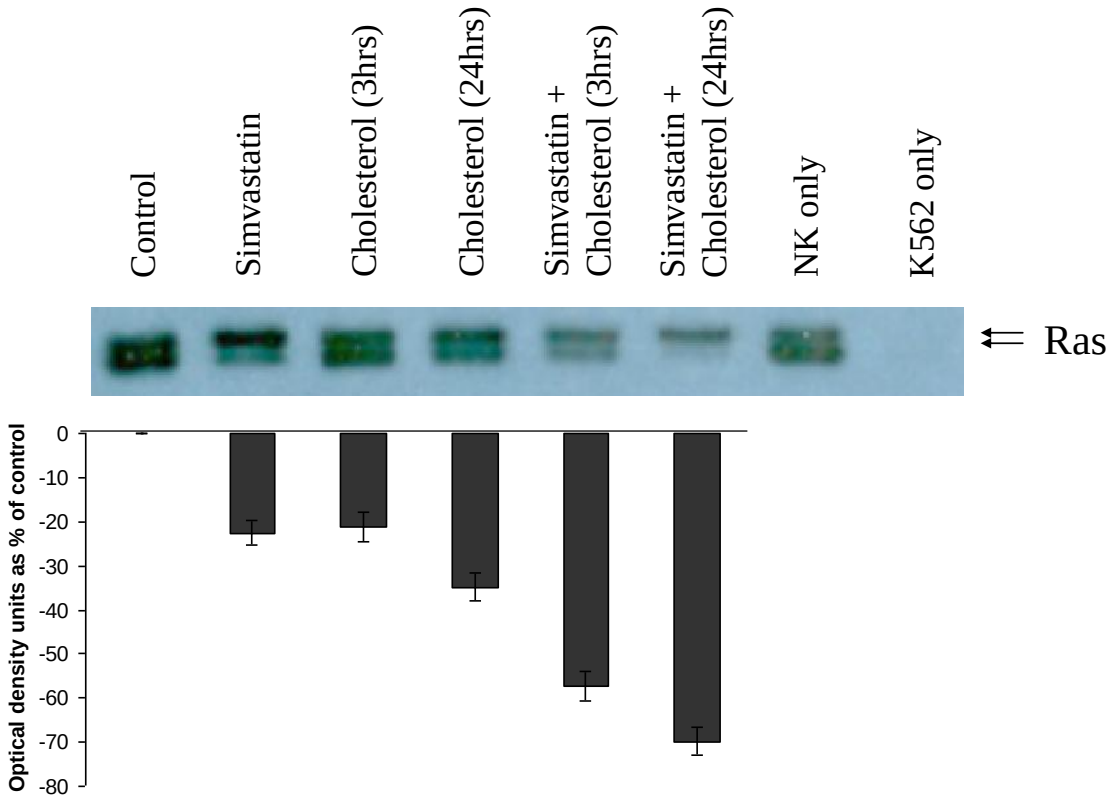


Figure 5.6 Soluble cholesterol dose not affect prenylation of Ras and does not rescue inhibition of isoprenylation by simvastatin. Fixed target K562 cells were used to activate Ras in NK92MI cells. Active Ras was measured by a Ras activity kit and intensity of bands measured by densitometry. The lower band represents isoprenylated Ras. Simvastatin inhibits isoprenylation of Ras and therefore its activity. Soluble cholesterol had no effect on prenylation of Ras but did reduce Ras activity and did not rescue the inhibition of isoprenylation caused by simvastatin. Fixed K562 cells did not contribute any Ras activity. The data are expressed as the mean ± SEM.

These findings suggest that statins deplete membrane cholesterol and raft associated proteins in addition to inhibiting isoprenylation. Replenishment of membrane cholesterol restores non-isoprenylated, raft-associated proteins such as LAT and lyn, but does not correct the functional effects of statins on NK cells. One unexpected observation was the contrasting effects of long versus short exposure to soluble cholesterol. One hypothesis is that this may be a consequence of negative feedback of supplemented exogenous cholesterol on HMG-CoA reductase, resulting in a similar effect to the addition of statins. To investigate this, NK92MI cells were treated with 100μg/ml soluble cholesterol, and/or 5 μmol of simvastatin for 3 hours or overnight, and then HMG-CoA reductase gene expression was quantified using real time PCR. Results are shown in Figure 5.7. Simvastatin alone increased HMG-CoA reductase greater than 2-fold, whereas soluble cholesterol reduced its expression. The addition of soluble cholesterol to simvastatin treated cells reduced the expression, perhaps due to negative feedback of the exogenous cholesterol on the HMG-CoA reductase. This was also confirmed by protein expression, shown by Western blot. ERK was used as a protein loading control as it has been previously shown to be unaffected by statins [161].

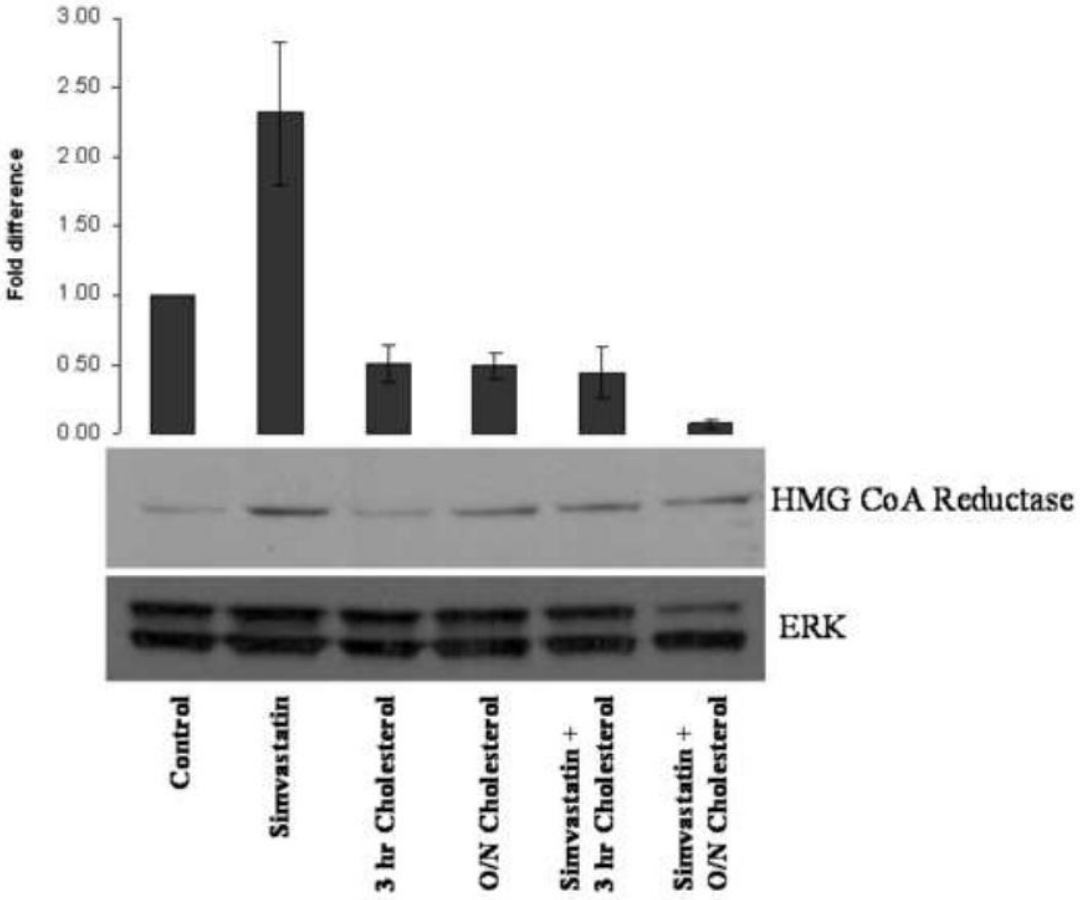


Figure 5.7. Real time PCR of HMG-CoA reductase; shows that prolonged exposure to soluble cholesterol does down regulate HMG-CoA reductase gene. This was also confirmed by protein expression, shown by western blot. ERK was used as a protein loading control (as it is unaffected by statin treatment). (Average of 6 experiments).

5.4 DISCUSSION

Cholesterol is a major and essential component of the plasma membrane as well as of lipid rafts, contributing to the normal functioning of the cells. It is now established that atherosclerosis is an inflammatory disorder with cholesterol acting as the major precipitating factor for the inflammatory process, however the mechanism linking the progression of cholesterol-related disease and inflammation remains unclear.

It has previously been shown that statin therapy inhibits lymphocyte function [150] and NK cell cytotoxicity. These actions are part of the spectrum of pleiotropic effects of statins that are thought to be independent of circulating cholesterol and are widely believed to be mediated by a reduction in intracellular isoprenoids [311]. However, more recent studies have suggested that statins in fact cause depletion of membrane cholesterol and raft-associated proteins that are not isoprenylated, and that inhibition of membrane cholesterol synthesis, and specifically disruption of membrane lipid rafts, is responsible for the pleiotropic effects [153]. What may actually be more physiologically relevant is the effect of increasing membrane cholesterol levels. In these studies I have explored this hypothesis by modification of membrane cholesterol to see whether cholesterol enrichment of rafts might alter functionally important raft structural properties. The results suggest that the pleiotropic effects of statins on NK cell function probably involve both inhibition of isoprenylation and cholesterol depletion, and that isoprenylated proteins including Ras play a central role in NK cell mediated cytotoxicity.

With previous work from this group showing that the depletion of cell membrane lipid rafts using MBCD reduced raft associated proteins and accordingly NK cell cytotoxicity [153], I attempted to augment membrane rafts by the use of a cholesterol-MBCD complex to cholesterol load the cells. The MBCD acts as a vector through which cholesterol is incorporated directly into the cell membrane. Consistent with this mechanism of action, the amount of cholesterol within the membrane and the raft fractions was able to be increased, using short term incubations. This did not however result in an increase in NK cell cytotoxicity as originally hypothesised. Following depletion of membrane rafts and raft-associated proteins with simvastatin, membrane cholesterol was restored with the use of cholesterol-MBCD. This reversed the effects of statins on membrane cholesterol, and also on some raft associated proteins, specifically Lyn and LAT. These proteins are myristoylated and palmitoylated respectively. Despite the apparent restoration of rafts and raft-associated proteins however, NK cell cytotoxicity remained impaired, suggesting that raft function had not returned to normal as lipid rafts have been shown to be required for NK cell activation [312]. Further investigation determined the absence of active Ras – an isoprenylated protein – within these reconstituted rafts. The findings of these studies thus suggests that both isoprenylation and intact membrane lipid rafts are essential for NK cell cytotoxicity at least *in vitro.* It is therefore possible to impair NK cytotoxicity through depletion of rafts, for example with MBCD or statins, or effective isoprenylation inhibitors. The variable pleiotropic effects of statins on different cell types is likely to reflect the relative dependence of cell functions on isoprenylation or rafts. Where both are essential – as in NK cell cytotoxicity – then statins are likely to be particularly effective. Although Ras is

an example of an isoprenylated protein, other specific isoprenylated proteins required for NK cell cytotoxicity are unclear.

The finding of altered cell function in the face of increased membrane cholesterol is interesting given the current understanding of the inflammatory nature of atherosclerosis which occurs in the context of hyperlipidaemia. In line with this, increases in T cell membrane cholesterol associated with aging have been shown to inhibit T cell signalling through lipid rafts by impairing the recruitment of signalling proteins [313]. Elevated membrane cholesterol has also been found to alter macrophage signalling and function [314], more specifically, cholesterol loading inactivates RhoA in macrophages, inhibiting cell migration [315] – the Rho family of small GTPases are involved in migration through regulation of F-actin organization and actin-myosin contraction [316]. This has been offered as an explanation as to why macrophages are retained and accumulate within atherosclerotic lesions that are rich in lipids. Variation in the cholesterol content of cells is likely to be a physiological regulator of membrane receptor function that affects all cells, indeed we have shown here that NK cell function is also related to circulating cholesterol levels at least *in vitro*. In vivo, cells are continuously exposed to cholesterol in an equilibrium where cholesterol desorbs from albumin to cell membranes. The effects of diet, specifically malnutrition states, and also catabolic disease states can lead to marked variations in serum albumin concentrations which may be reflected in the cholesterol membrane content of cells. Similarly, high serum or tissue-free cholesterol availability may lead to increases in the cholesterol content of cell membranes which

could translate to alterations in the activation of immune cells, which in the case of high cholesterol levels could contribute to the progression of atherosclerotic lesions.

CHAPTER 6

A study of biomarkers in patients at high risk of cardiovascular disease

6.1 INTRODUCTION

Inflammation is now widely regarded as a contributor to morbidity in cardiovascular disease, and inflammatory markers are a subject of much interest, as potential non-traditional cardiovascular risk factors. Inflammatory proteins such as CRP have been shown to predict and correlate independently with, major cardiovascular end-points such as myocardial infarction and death [39, 40, 317]. The traditional risk factors for cardiovascular disease, such as family history, hypertension, smoking and dyslipidaemia, explain the majority of morbidity and mortality, however, these traditional risk factors do not fully account for the cardiovascular burden in certain populations, such as diabetics, and in particular, those with CKD. Attention has therefore been recently focusing on whether the non-traditional risk factors are in fact independently associated with cardiovascular risk.

C-reactive protein, the archetypal biomarker has been independently demonstrated to have associations with cardiovascular events and mortality among apparently healthy men and women [30, 33] as mentioned above. It has also been shown to be associated with comorbidity and clinical events in haemodialysis patients [318] and also patients with CKD stages 3 to 5, along with other markers of inflammation such as hypoalbuminaemia [319]. An elevated serum hsCRP level has been shown to be a stronger predictor of incident cardiovascular events in healthy men than LDL-C and to be additive to the Framingham risk score [39]. Evidence supporting a role for CRP in atherogenesis is mounting. It has been demonstrated in atherosclerotic plaques [44] and

causes adverse changes to cultured endothelial cells [27]. It has been shown to be chemoattractant for monocytes [44], to directly affect endothelial function by altering NO bioavailability [27], and to up regulate Lectin-like oxidized LDL receptor-1 (LOX-1), which in turn promotes endothelial dysfunction [49]. Additionally, local administration of CRP in a rat balloon injury model increased neointimal formation [48].

Interleukin-6 plays a pivotal role in inflammation as it is an inducer of the hepatic acute phase response, with CRP the prototype of this response. It is therefore unsurprisingly up-regulated in renal failure leading to elevated levels of IL-6 in ESRD patients [199]. Along with CRP, IL-6 has been shown to be a strong predictor of mortality in both haemodialysis [173, 198] and peritoneal dialysis [320] patients, and in patients with normal renal function [232]. More specifically, IL-6 has been shown to mediate malnutrition in dialysis patients, itself a strong predictor of outcome [321] and stimulates breakdown of muscle proteins [322]. Like CRP, evidence is emerging of a direct role of IL-6 in atherogenesis, for example, it has been demonstrated that injection of recombinant IL-6 exacerbates early atherosclerosis in apoE-deficient mice [323]. A clinical study showed that elevated circulating IL-6 levels were independently associated with progressive carotid atherosclerosis during the first 12 months of dialysis treatment [324].

Adiponectin is produced by adipocytes and found in the circulation. It appears to play a protective role in experimental models of vascular injury and has unusual properties that inhibit inflammation and enhance insulin sensitivity, glucose transport, and fatty acid

oxidation. It has also been shown to suppress the attachment of monocytes to endothelial cells [252]. Low circulating adiponectin is generally found in populations at enhanced risk of cardiovascular disease [325], and low adiponectin levels have been shown to predispose healthy individuals to the later development of insulin resistance [326]. Adiponectin is also known to be inversely related to BMI. An inverse relationship between adiponectin and CRP has been shown which has prompted the suggestion that hypoadiponectinaemia can result in a low-grade systemic chronic inflammatory state [258]. Plasma adiponectin levels are generally elevated in patients with CKD [251], with previous studies showing conflicting results with elevated levels associated both with a positive and an inverse relationship with mortality [251, 327, 328].

The long pentraxin 3 (PTX-3) is a member of the pentraxin family of acute-phase proteins, and is involved in innate immunity. The physiological function of PTX-3 is similar to CRP but unlike CRP it is produced by many different cell types, including endothelial cells, smooth muscle cells, fibroblasts and monocytes [329]. Levels are known to be associated with CVD and levels are known to be higher in renal failure. It has been only recently studied in the setting of CKD, and of much interest is the hypothesis that PTX-3 levels may represent a more accurate picture of local inflammation as it is produced at sites of disease activity. The relationship between PTX-3 and NK cell function has not been previously examined.

Serum soluble ST2 receptor (sST2) is an IL-1 receptor that has recently been described as a biomarker of biomechanical stress of the myocardium that has been shown to be

physiologically linked to cardiac hypertrophy, fibrosis, and ventricular dysfunction. It is associated with increased mortality following MI and in also in both acute and chronic cardiac failure, and higher levels have been found in various autoimmune diseases and also in severe sepsis [330]. The role of sST2 in has not been studied in CKD patients.

Patients with dyslipidaemia and patients with CKD are amongst the highest risk of CVD, most likely attributable to the increased risk of inflammation. Inflammation is strongly associated with adverse outcomes such as loss of physical function, dyslipidaemia, and anaemia. It is also associated with CV events, increased hospitalisation, and death. The causes of inflammation are multifactorial, but some sources are recognisable and treatable, and therefore recognition is important. Additionally, it is known that proinflammatory cytokines play a significant role in the pathogenesis of atherosclerosis. As such there is much interest in the role of biomarkers and in particular in the prognostic information on outcomes. The aim of this study was to evaluate the relationship between NK cells and potential CV risk biomarkers in a population at high risk of CVD, specifically in patients with CKD, and additionally, to investigate the association of biomarker levels with renal function and other routine laboratory parameters. If there was a relationship with these CV risk markers, it would lend further support to the role that NK cells have to play in atherosclerosis.

6.2 METHODS

6.2.1 Patients

Blood samples were taken from 207 patients from six groups of patients. There were 36 healthy volunteers who acted as the normal controls, 63 patients with treated dyslipidaemia recruited from the lipid clinic on a variety of lipid lowering therapies as described in chapter 3, 14 patients with CKD stages 1 and 2, 34 patients with CKD stages 3 and 4, 49 patients with CKD stage 5, and 11 haemodialysis patients. Patients were recruited from the lipid clinic, nephrology clinics and the regular dialysis unit, following consent.

6.2.2 Blood Sampling

Fifty millilitres of blood was taken into BD vacutainer tubes, as described in section 2.2. In the patients on haemodialysis, blood samples were taken within 15 minutes of the start of the dialysis session. Biochemical, haematological, and clinical parameters including age, gender, BMI and renal diagnoses (in the CKD patients) were recorded. None of the patients were known to have active infection, none had malignancy, and none were immunosuppressed. Routine biochemical parameters including urea and electrolytes, albumin, phosphate, calcium, glucose, lipid profile, and creatinine were obtained from biochemistry laboratory reports, and full blood counts were obtained from haematology laboratory reports. In all patients, NK cells were isolated and NK cell phenotype and distribution of NKT- and T-cells was determined.

6.2.3 Human peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells were isolated from peripheral venous blood as described in section 2.4.1.3 using Ficoll-Hypaque centrifugation (Histopaque-1077; Sigma, Poole, UK).

6.2.4 NK cell isolation

Natural killer cells were isolated using the MACS NK cell human isolation kit (Miltenyi Biotec) as described in section 2.4.2.

6.2.5 51-Chromium Release Assay

Natural killer cell cytotoxicity was measured using a standard 51-Chromium Release Assay, using the human chronic myelogenous leukaemia cell line – K562 as target cells, as described in section 2.4.3.

6.2.6 Flow Cytometry

Three colour flow cytometry of PBMCs was carried out (BD Biosciences FASCalibur) before isolation of NK cells to determine NK cell phenotype and distribution of NKT- and T-cells as described in section 2.4.4. The NK-, NKT-, and T-cells were expressed as a percentage of total lymphocyte count, and the $CD56^{bright}$ and $CD56^{dim}$ subsets were expressed as a percentage of NK cells.

6.2.7 Measurement of biomarkers

Blood for measurement of biomarkers was collected in plain tubes and allowed to clot at room temperature. Samples were then centrifuged at 3000rpm for 15 minutes at ambient temperature, the supernatant serum was then collected and immediately frozen and stored at -70^{o}C until later analysis.

High sensitivity CRP, IL-6, pentraxin-3, adiponectin and serum soluble ST2 measurements were performed as described in section 2.6.

6.2.8 Statistical Analysis

Statistical analysis was carried out using SPSS statistical package (version 15.0). Non parametric data were expressed as medians. The Wilcoxon rank sum test was used to compare groups, and the Kruskal-Wallis test was used to compare more than 2 groups. The association between continuous variable was analysed using the Pearson correlation test. The data that were non-parametric were logarithmic transformed and the Pearson correlation test was used to examine the association between variables, or alternatively non-parametric correlations were carried out with the Spearman rank correlation. Analysis of variance (ANOVA) was used to test differences between groups with post hoc Scheffe's test. Multiple linear regression analysis was used to assess the contribution of different factors to changes in lymphocyte subsets and NK cell cytotoxicity.

6.3 RESULTS

6.3.1 Patient characteristics and laboratory data

Three patients were excluded from analysis due to abnormally high biomarker results, suggestive of an occult infection. One was from the lipid group, one from the CKD 3&4 group, and one from the CKD 5 group, leaving a total of 204 patients. The baseline characteristics and biochemical and haematological parameters have already been described in Chapters 3 and 4, and are summarised in Table 6.1. The creatinine and eGFR results in the HD patient group are included for data but I acknowledge that eGFR in particular cannot be used in a comparable way with the other groups.

	Controls (n=36)	Lipid (n=62)	CKD1&2 (n=14)	CKD3&4 (n=33)	CKD5 (n=48)	HD (n=11)
Sex (m/f)	10/26	45/17	7/7	13/20	32/16	8/3
Age (years)[a]	41.7*	58.0	47.4	65.2	62.6	59.0
BMI (kg/m^2) [a]	24.8 5.02	30.4±5.94	26.8±5.01	29.4±9.11	27.8±9.49	24.3±4.37
Creatinine (µmol/l) [a]	82.1±10.8	98.3±25.5	85.9±14.3	172.1±41.4**	495.7±146.9**	604.0±296.6**
eGFR (ml/min/1.73m^2) [a]	79.5±12.2	71.5±17.7	78.1±12.2	33.0±10.1**	10.2±2.9**	16.1±12.8**
Albumin (g/l) [a]	41.8±2.7	40.7±3.5	39.1±4.1	38.2±4.2†	36.2±3.9†	41.2±6.1
Total cholesterol [a]	4.9±1.1	5.3±1.2	5.2±1.3	4.7±1.2	4.7±1.2	3.8±1.0††
hsCRP (mg/l)[b]	1.84 (0.59-2.63)	2.02 (0.82-6.32)	2.52 (1.21-5.03)	2.09 (1.12-6.22)	5.97 (2.35-13.12)‡	4.14 (2.99-6.14)
Interleukin-6 (pg/ml) [a]	13.7 ± 27.3	14.5 ± 33.7	40.6 ± 79.0	5.8 ± 21.1	30.1 ± 61.0	28.6 ± 69.6
Pentraxin-3 (pg/ml) [b]	1063.0 (506.0-1407.0)	909.0 (515.0-1293.5)	636.5 (350.0-1197.3)	608.0(327.5-832.0)	1746.0 (1125.0-2616.0)[#]	1901.0 (1542.0-2061.0)[#]
Adiponectin (ug/ml) [b]	3.8 (2.7-5.4)	2.6 (1.6-3.8)[##]	6.6 (5.6-7.6)	5.8 (4.0-7.6)	5.3 (4.2-5.8)	45.8 (32.7-55.2)§
Soluble ST-2 (pg/ml) [b]	20.9 (0.4-79.4)	37.4 (1.4-106.2)	72.6 (7.2-237.6)	27.4 (0.4-76.3)	42.3 (6.4-149.0)	43.7 (0.4-76.8)

[a]Mean ± SD, [b]Median (IQR)

* p<0.05 v lipid, CKD3&4, CKD5 and HD groups
** p<0.05 v control, lipid and CKD1&2 groups
† p<0.05 v control, lipid, CKD1&2 and HD groups
†† p<0.05 v lipid group
‡ p<0.05 v control and lipid groups
[#] p<0.05 v control, lipid, CKD1&2 and CKD3&4 groups
[##] p<0.05v control, CKD1&2, CKD3&4 and CKD5groups
§ p<0.01 v control, lipid, CKD1&2, CKD3&4 and CKD5 groups

Table 6.1. Patient characteristics and laboratory data.

6.3.2 High Sensitivity CRP

There were significant differences in hsCRP between the CKD5 group and the control and lipid groups ($p<0.05$). Median values of hsCRP for each group and interquartile range are detailed in Table 6.1. High sensitivity CRP correlated significantly with creatinine ($r = 0.362$, $p<0.01$) [Figure 6.1(a)] which remained significant when the HD patients were excluded from analysis ($r = 0.363$, $p<0.01$). This correlation also remained significant this time excluding both the HD patients, and also the CKD5 patients, as this group had significantly higher levels of hsCRP ($r = 0.206$, $p<0.05$). In keeping with the correlation with creatinine, there was a correlation between hsCRP and phosphate ($r = 0.255$, $p<0.01$), which again remained significant when the HD patients were excluded ($r = 0.265$, $p<0.01$), but not when the CKD5 patients were excluded also. It should be noted that unsurprisingly, serum phosphate was significantly higher in the CKD5 group compared with all others ($p<0.05$). There were also correlations between hsCRP with age ($r = 0.196$, $p<0.01$), inversely with HDL-C ($r = -0.211$, $p<0.01$), haemoglobin ($r = -0.249$, $p<0.01$), white cell count ($r = 0.276$, $p<0.01$), and strongly with albumin ($r = -0.436$, $p<0.01$) [Figure 6.1(b)]. There were no correlations with BMI or weight.

There was no association between hsCRP and NK cell cytotoxicity, NK- and NKT-cell number. There was also no correlation between hsCRP and NK cell phenotype. There was however a significant correlation between hsCRP and T cells ($r = -0.177$, $p<0.05$).

Including all patient groups, but selecting only those with a hsCRP of less than 2 mg/dL (Figure 6.2), using the cut-off used in the JUPITER trial [263], thereby excluding

"inflamed" patients, the correlation between hsCRP and creatinine remained significant ($r = 0.268$, $p<0.05$), as did the correlation between hsCRP and phosphate ($r = 0.217$, $p<0.05$). The correlations with age, HDL, haemoglobin were lost, but correlations between albumin ($r = -0.281$, $p<0.01$) and white cell count ($r = 0.296$, $p<0.01$) remained. There were no correlations with any of the lipid fractions. No correlations were unmasked between hsCRP and NK cell cytotoxicity, number, or phenotype in those "non-inflamed patients", but HDL-C did now correlate with $CD56^{bright}$ cells ($r = 0.380$, $p<0.01$) and $CD56^{dim}$ cells ($r = -0.371$, $p<0.01$). Interestingly, in patients with hsCRP < 2mg/dL, the correlation between NK cell cytotoxicity and creatinine and phosphate described in chapter 4 is lost. However, when "inflamed" patients with hsCRP > 2 are analysed, there is a significant correlation between NK cell cytotoxicity and creatinine ($r = 0.212$, $p<0.01$) and phosphate ($r = 0.338$, $p<0.01$). In keeping with this, the correlations between hsCRP and creatinine and phosphate remain significant ($r = 0.229$, $p<0.05$; $r = 0.202$, $p<0.05$). There was no significant difference in creatinine within groups when comparing those with hsCRP > or < 2 to account for this. To compare creatinines within groups, once patients had been coded into those with hsCRP > or < 2mg/dL, data were split and organised according to group code, a Mann-Whitney U test was then carried out using a grouping variable of hsCRP cut off of > or < 2mg/dL.

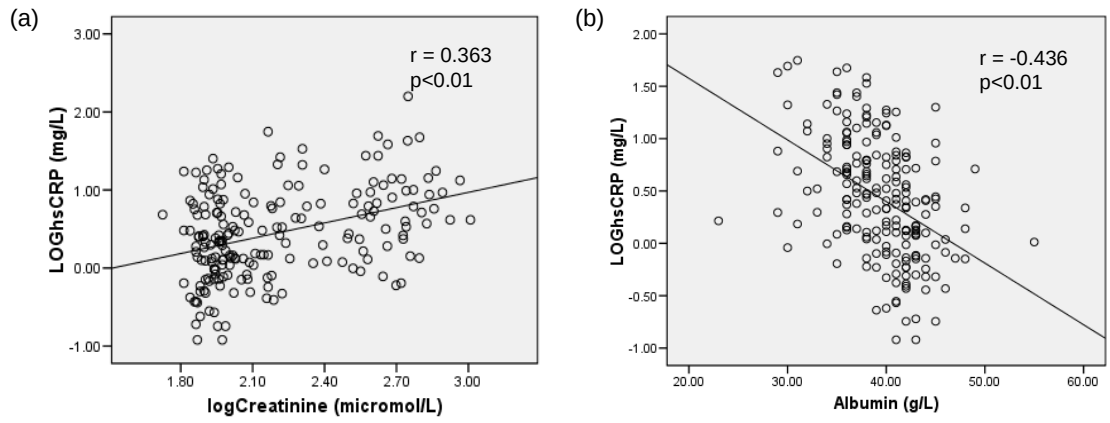


Figure 6.1. Scatterplots showing the relationship between (a) hsCRP and creatinine and (b) hsCRP and albumin.

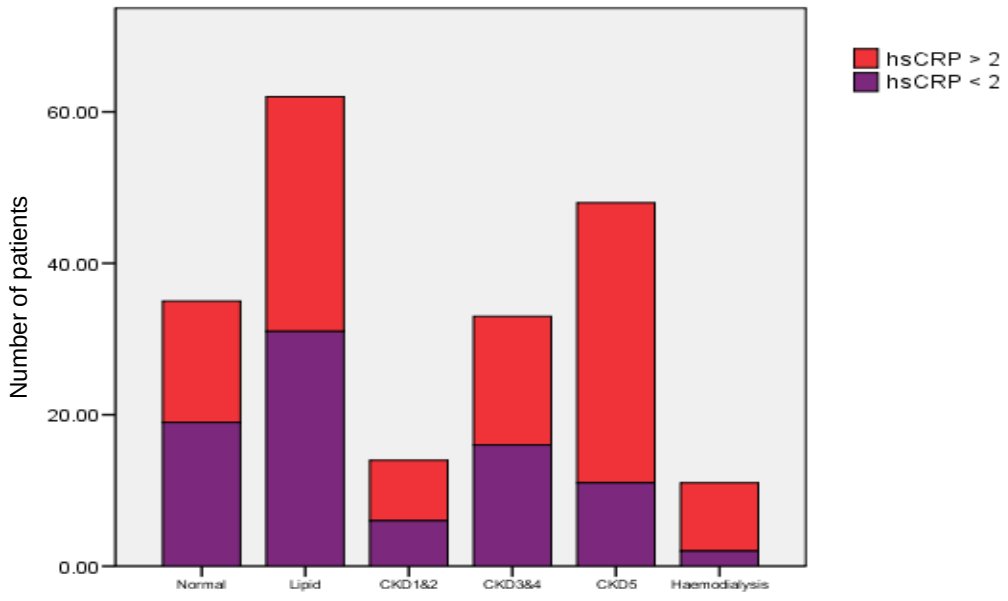


Figure 6.2. Bar chart showing the number of patient with hsCRP > or < 2mg/dl in the different groups

6.3.3 Interleukin-6

Unfortunately, the ELISA kit used was not sensitive enough to detect very low levels, and the lower limit of detection of IL-6 was 1.0 pg/ml. There were no significant differences in IL-6 levels between groups. Mean values are summarised in Table 6.1. There was no correlation with age or sex. There were no significant correlations with creatinine, phosphate, adjusted calcium, albumin, glucose, haemoglobin and white cell count. There was a correlation between IL-6 and platelets ($r = -0.295$, $p<0.01$). There were also significant correlations between IL-6 and cLDL-C ($r = 0.237$, $p<0.05$), but not with other lipid fractions. This correlation remained when only those with a hsCRP < 2mg/L were analysed ($r = 0.388$, $p<0.05$) but lost when hsCRP > 2mg/dL.

Interleukin-6 did not correlate with NK cell cytotoxicity, NK-, NKT-, or T-cells. No correlations were unmasked by separating analysis into "inflamed" or "not-inflamed" patients, by using a cut-off level of hsCRP greater than or less than 2mg/dL.

6.3.4 Pentraxin-3

There were significant differences in PTX-3 between groups as shown in Figure 6.3 and Table 6.1. Pentraxin-3 correlated significantly with creatinine ($r = 0.327$, $p<0.01$) (Figure 6.4). There were also significant correlations between PTX-3 and phosphate ($r = 0.158$, $p<0.05$), glucose ($r = -0.203$, $p<0.01$), and platelets ($r = -0.229$, $p<0.01$). The correlation between PTX-3 and creatinine is lost when analysis includes only those patients with hsCRP < 2mg/dL, however this relationship is strengthened when only those with hsCRP > 2mg/dL are included ($r = 0.429$, $p<0.01$). When all patients were included in analysis,

there were no correlations between PTX-3 and any lipid fractions. When those patients with hsCRP < 2mg/dL were analysed, PTX-3 correlated with total cholesterol ($r = 0.367$, $p<0.05$). Looking at those inflamed patients with hsCRP > 2mg/dL, PTX-3 correlated inversely with total cholesterol ($r = -0.216$, $p<0.05$), triglycerides ($r = -0.208$, $p<0.05$), and non-HDL-C ($r = -0.215$, $p<0.05$).

Overall, there were no correlations between PTX-3 and NK cytotoxicity, NK-, NKT-, or T-cells. However there was a correlation with NK cell phenotype; PTX-3 correlated with $CD56^{bright}$ cells ($r = 0.173$, $p<0.05$), and inversely with $CD56^{dim}$ cells ($r = -0.206$, $p<0.01$). Excluding patients with hsCRP > 2mg/dL, PTX-3 correlates with NK cytotoxicity ($r = -0.234$, $p<0.05$), and also with NK cell phenotype, with $CD56^{bright}$ cells ($r = 0.242$, $p<0.05$) and $CD56^{dim}$ cells ($r = -0.271$, $p<0.05$). There were no significant correlations with age, sex, BMI, or blood pressure. In only those patients with a hsCRP > 2mg/dl, there was a significant correlation between PTX-3 and BMI ($r = -0.233$, $p<0.05$). There was no correlation between PTX-3 and presence of known IHD.

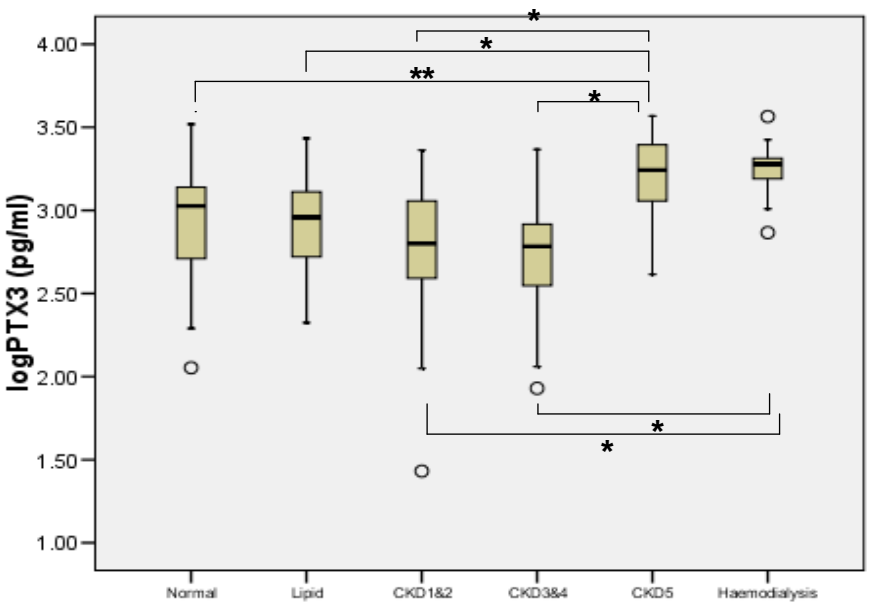


Figure 6.3. A boxplot diagram showing the differences in PTX-3 between groups and median values shown by the black line. Pentraxin-3 values have been logarithm transformed. * $p < 0.01$, ** $p < 0.05$

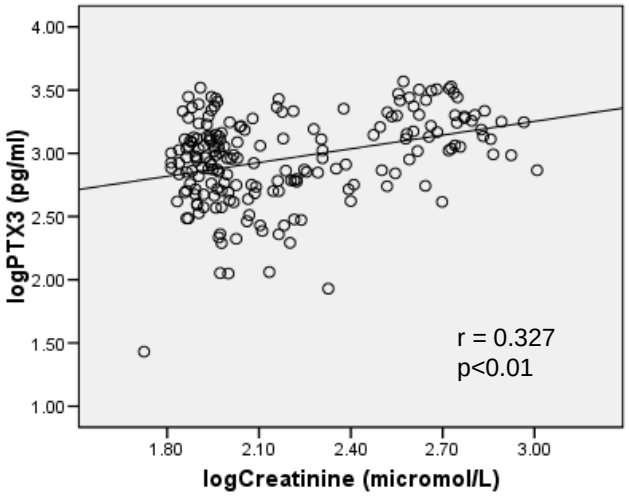


Figure 6.4. A scatterplot showing the relationship between creatinine and pentraxin-3. Both have been logarithmic transformed.

6.3.5 Adiponectin

There were significant differences between groups as shown in Figure 6.5. Median values and interquartile range for each group are detailed in Table 6.1. Adiponectin was significantly higher in the HD group and lower in the lipid patient group. There was no correlation between adiponectin and age. Adiponectin levels were higher in women, with a mean of 8.01±12.03ug/ml, and 6.55±10.94ug/ml for men (Figure 6.6). There were no correlations with lipid fractions. There was a significant correlation with creatinine ($r = 0.317$, $p<0.01$) and eGFR ($r = -0.366$, $p<0.01$), but not with phosphate, adjusted calcium, serum albumin or glucose. The correlation with creatinine remained significant when the haemodialysis patients were removed from analysis ($r = 0.262$, $p<0.01$). This correlation remained significant after adjusting for age, sex, BMI, total cholesterol, LDL-C, phosphate, albumin, glucose, and BP.

Adiponectin correlated with haemoglobin ($r = -0.315$, $p<0.01$), platelets ($r = -0.190$, $p<0.01$), and total lymphocytes ($r = -0.281$, $p<0.01$). There was also an inverse correlation with T-cells ($r = -0.398$, $p<0.01$) (Figure 6.7), and also with $CD56^{bright}$ cells ($r = 0.218$, $p<0.01$) and $CD56^{dim}$ cells ($r = -0.240$, $p<0.01$). However, there were no correlations with NK-, or NKT-cells, or with NK cell cytotoxicity. Adiponectin correlated negatively with BMI ($r = -0.211$, $p<0.05$) but did not correlate with blood pressure.

Analysing the patients with a hsCRP < 2mg/dl, the correlation between adiponectin and creatinine is lost, but there is a correlation between adiponectin and non-HDL-C (r = -

0.232, $p<0.05$). The correlation between adiponectin and BMI is strengthened ($r = -0.459$, $p<0.01$). In those patients with a hsCRP > 2mg/dl, adiponectin now correlates with total cholesterol ($r = -0.277$, $p<0.01$), triglycerides ($r = -0.277$, $p<0.01$), and non-HDL-C ($r = -0.310$, $p<0.01$). The correlation with creatinine is still significant ($r = 0.342$, $p<0.01$), as is the correlation between haemoglobin ($r = -0.221$, $p<0.05$), and platelets ($r = -0.210$, $p<0.05$). In these presumably "inflamed" patients, adiponectin now correlated more strongly with T-cells ($r = -0.490$, $p<0.01$), and the correlation with $CD56^{bright}$ cells and $CD56^{dim}$ cells still exists ($r = 0.275$, $p<0.01$; $r = -0.290$, $p<0.01$). Interestingly, the relationship with BMI is lost.

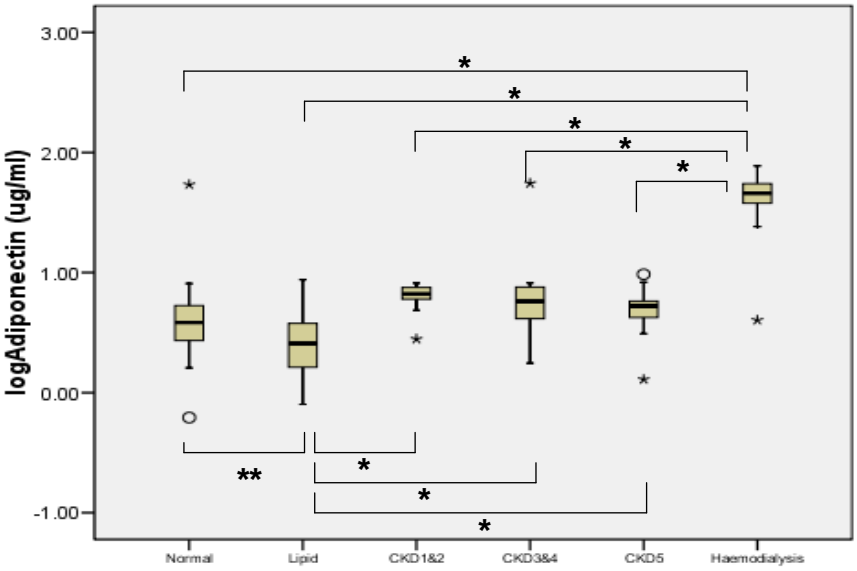


Figure 6.5. A boxplot diagram showing the differences in adiponectin levels between groups. The black line represents the median. Adiponectin values have been logarithmic transformed. * $p < 0.01$, ** $p < 0.05$

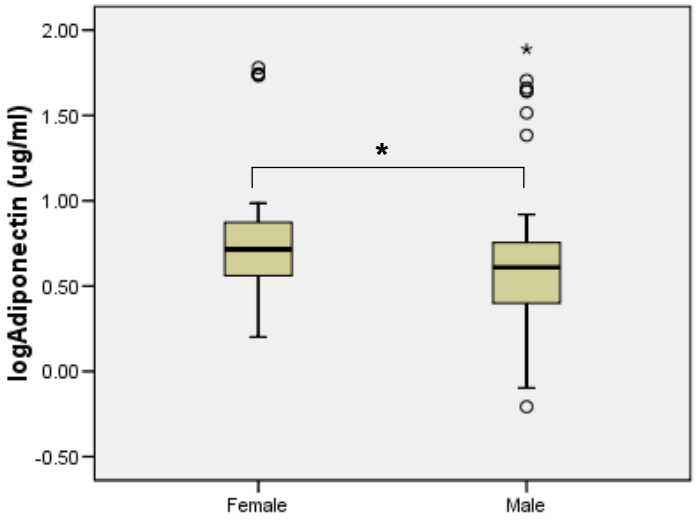


Figure 6.6. A boxplot diagram showing the difference in adiponectin levels between males and females. * $p < 0.05$

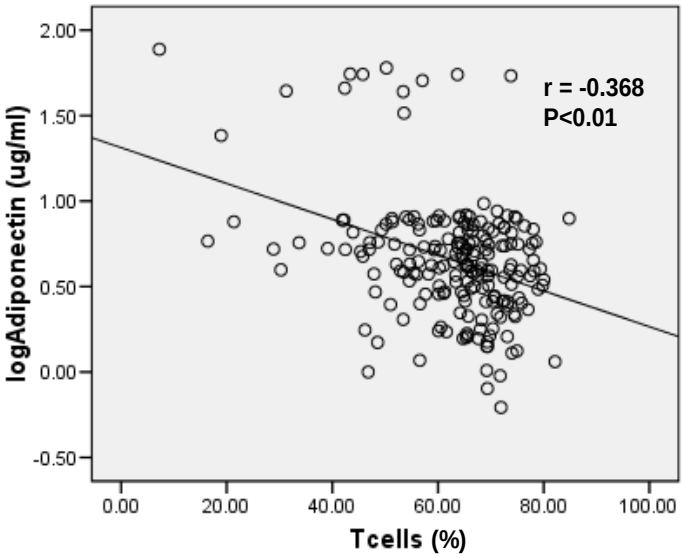


Figure 6.7. A scatterplot showing the relationship between adiponectin and T-cells. Adiponectin was logarithm transformed. T-cells are expressed as a percentage of total lymphocytes.

6.3.6 Serum soluble ST2

There were no significant differences between serum sST2 levels between groups, and the median and interquartile range of each group is shown in Table 6.1. There were no correlations between sST2 and age, sex, BMI, or BP. There were no correlations between creatinine, phosphate, albumin, glucose, or any lipid fractions. There were no correlations between routine haematological parameters. There were also no correlations between NK cytotoxicity, NK-, NKT-, or T-cells, or NK cell phenotype. No correlations were unmasked by analysing only those patients with a hsCRP > 2mg/dl. In those patients with hsCRP < 2mg/dl, sST2 correlated negatively with phosphate ($r = -0.338$, $p<0.01$).

6.3.7 Correlations between biomarkers

High sensitivity CRP correlated with PTX-3 ($r = 0.171$, $p<0.05$), but not with any of the other biomarkers studied. Interleukin-6 correlated with PTX-3 ($r = 0.426$, $p<0.01$). Pentraxin-3 also correlated with adiponectin ($r = 0.239$, $p<0.01$). Serum sST2 correlated with IL-6 ($r = 0.508$, $p<0.01$), and PTX-3 ($r = 0.311$, $p<0.01$) (Figure 6.8). It is worth remembering that hsCRP correlates with creatinine ($r = 0.362$, $p<0.01$). Although a correlation between creatinine and cytotoxicity was found in patient with CKD (Chapter 4), when all patients in this study were analysed there was no correlation between creatinine and NK cell cytotoxicity, and there is no correlation between any of the biomarkers studied and NK cytotoxicity.

	All patients (r)				
	hsCRP	IL-6	PTX-3	Adiponectin	sST2
hsCRP	-	0.070	0.171*	0.082	0.038
IL-6	0.070	-	0.426**	0.214**	0.508**
PTX-3	0.171*	0.426**	-	0.239**	0.311**
Adiponectin	0.082	0.214**	0.239**	-	-0.019
sST2	0.038	0.508**	0.311**	-0.019	-

Table 6.2 Correlations among inflammation markers in all patients (r, Spearman rank correlation). *$p<0.05$, **$p<0.001$.

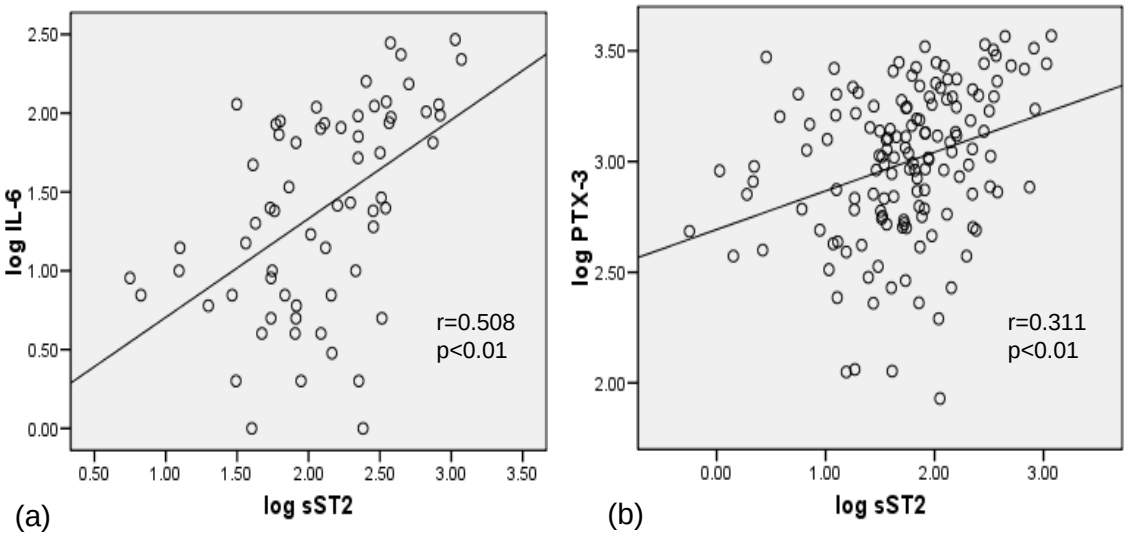


Figure 6.8. Scatterplots showing the relationship between (a) sST2 and IL-6, and (b) sST2 and PTX-3.

If the patients are again separated by hsCRP > or < 2mg/dl, in the patients who have hsCRP > 2mg/dl, creatinine now correlates with NK cytotoxicity ($r = 0.194$, $p<0.05$), however there is still no correlation between NK cytotoxicity and any of the biomarkers studied. The correlations described in the first part of this section remain. In those patients with hsCRP < 2mg/dl, creatinine still correlates with hsCRP, but now does not correlate with NK cytotoxicity, however NK cytotoxicity now correlates with PTX-3 ($r = -0.234$, $p<0.05$) (Figure 6.9). Additionally, hsCRP now correlates with sST2 ($r = -0.252$, $p<0.05$). Pentraxin-3 no longer correlates with hsCPR, IL-6, or adiponectin. Serum sST2 still correlates with IL-6 and PTX-3, but now also with hsCRP ($r = -0.252$, $p<0.05$).

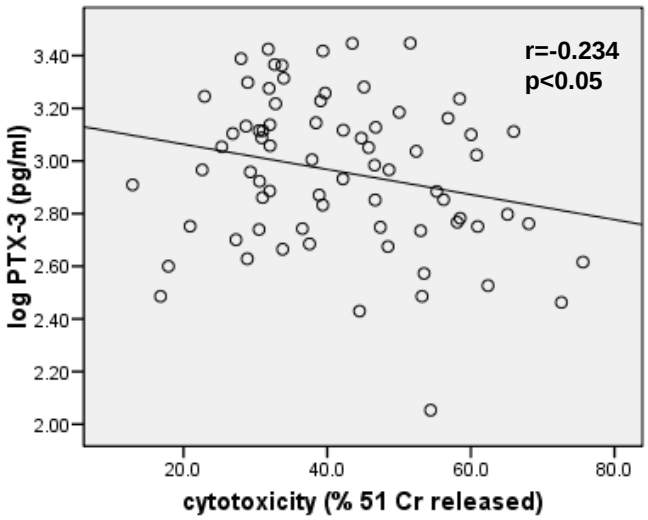


Figure 6.9 A scatterplot showing the relationship between PTX-3 and NK cell cytotoxicity in patients with hsCRP < 2mg/dl

6.4 DISCUSSION

Inflammatory and immunopathological processes play a crucial role in the pathogenesis of atherosclerosis. Moreover, patients with CKD have inflammatory and immunological alterations that differ from the non-CKD population, which are not completely understood, but are almost certainly responsible for the increase in mortality seen in these patients, in particular in ESRD patients, from both CV (not explained by traditional CV risk factors) and infective causes. The role of NK cells in both atherosclerosis and their contribution in ESRD has not been widely studied, but there is mounting evidence of their involvement. The aim of this study was to evaluate the relationship between NK cells and potential CV risk biomarkers in a population at high risk of CVD, specifically in patients with CKD and dyslipidaemia, and examine the relationships with biochemical and haematological parameters.

To date, CRP is the most common biomarker to assess the inflammatory status, although it remains unclear whether CRP is a marker or a mediator of inflammation and vascular disease. This study demonstrates expected correlations between hsCRP and creatinine and phosphate. It has shown a relationship between PTX-3 and creatinine and phosphate, and also total cholesterol in non-inflamed patients, with an inverse relationship with total cholesterol and BMI in inflamed patients. PTX-3 correlated positively with $CD56^{bright}$ cells and inversely with $CD56^{dim}$ cells, and in inflamed patients there was a relationship between PTX-3 and NK cytotoxicity. Adiponectin has also been shown to correlate with creatinine and was significantly higher in HD patients. It correlated positively with $CD56^{bright}$ and negatively with $CD56^{dim}$ cells, and inversely with T-cells. The well

described inverse correlation between adiponectin and BMI was confirmed. Splitting analysis into inflamed and non-inflamed patients, additional correlations become apparent. There were no significant correlations between serum sST2 and routine biochemical parameters or NK cells. PTX-3 correlated with hsCRP, IL-6 and adiponectin, and sST2. Serum sST2 also correlated with IL-6.

This study has confirmed the well recognised process of an inflammatory state in CKD with correlations between hsCRP and creatinine. It is known that a relationship exists between CRP and CV events in healthy men and women, and patients with CKD and ESRD [318, 319]. The relationship between hsCRP and phosphate is interesting. Numerous studies have shown that both the inflammatory state of CKD and abnormal mineral metabolism results in increased cardiovascular mortality, however the relationship between these two factors has not been examined in great depth. Two studies have shown an association with a high calcium x phosphorus product and a high CRP [331, 332], and in a more recent study in patients with CKD stages 3 to 4, serum phosphate was significantly associated with CRP and IL-6, and was an independent risk factor for the presence of an inflammatory state [333], suggesting that hyperphosphataemia itself may promote the development of inflammation in these patients. The anti-inflammatory effect of phosphate binders also supports this [334]. The relationship found between phosphate and PTX-3 in this study is interesting, and gives more weight to the correlation between phosphate and NK cell cytotoxicity described in Chapter 4. The relationships may of course simply be a function of renal function, but evidence is increasing to support hyperphosphataemia as an independent risk factor for

inflammation and CVD. There were no actual correlations between hsCRP and NK cell cytotoxicity, however, in inflamed patients a correlation developed between NK cell cytotoxicity and creatinine. This association would appear to fit with the general inflammatory state of patients with advancing renal failure, where NK cells are more active and may contribute to the increasing rate of CV disease. The inverse correlation of hsCRP with albumin is consistent with inflammation.

IL-6 did correlate with cLDL-C, but this relationship was lost in inflamed patients. One hypothesis would be that the normal relationships between CV risk and lipids are lost in inflamed patients, and this may be a function of more advanced renal dysfunction, where there is known to be a "J-shaped" relationship between lipid levels and all cause mortality. IL-6 did not however correlate with hsCRP, and had no relationship with NK cells. It must be acknowledged that the ELISA kit used had a lower limit of detection of 1.0pg/ml and therefore may simply have been too insensitive to detect any meaningful relationships.

Whereas CRP is produced primarily in the liver, PTX-3 is an acute phase protein produced by many cell types, including dendritic cells, endothelial cells, VSMCs, fibroblasts and monocytes. Because of this, it has been postulated that PTX-3 levels are a more accurate indicator of disease activity produced at actual sites of inflammation. Its production is induced by inflammatory mediators such as lipopolysaccharide, IL-1β and TNF-α, but not IL-6. It has a similar physiological function to CRP and is involved in innate immunity [238] with associations with CAD and PVD [335, 336]. It is also

known to increase following MI, and to be a predictor of outcome [337]. Levels of PTX-3 are known to be significantly increased in HD patients [335] and also in patients with CKD, correlating negatively with GFR, with higher levels when inflammation, PEW and CVD exist together, where it has been found to be associated with all-cause mortality in incident dialysis patients [336]. One study looking at PTX-3 levels in both HD and CKD patients (mean creatinine clearance 56.54ml/min) divided patients into those with high (>2ng/ml) and low (<2ng/ml) PTX-3 levels, and found significant differences in HDL-C and BMI, and a negative correlation between PTX-3 and HDL-C and BMI in those patients with high levels of PTX-3, supporting the idea of malnutrition and inflammation [338]. Reassuringly, the levels of PTX-3 for controls in this study are comparable to previous studies [245, 335]. The relationship between PTX-3 and TC in non-inflamed patients fits with an association between PTX-3 and CAD and PVD. The inverse relationships with TC and BMI in inflamed patients support the idea of PEM, inflammation and the concept of "reverse epidemiology" know to occur in inflamed patients, such as those with ESRD. In concordance with the development of an association between NK cell cytotoxicity and creatinine in inflamed patients, the relationship between PTX-3 and NK cytotoxicity in inflamed patients follows, supporting the notion that in inflamed patients, NK cells may become more active, and possibly therefore contribute to increasing the incidence of CVD seen in inflamed patients.

Adiponectin is an adipocytokine produced by adipose tissue and is considered to be anti-inflammatory and anti-atherogenic, and therefore protective against CVD [339]. Circulating levels of adiponectin are known to be paradoxically lower in the obese, and

also in type 2 diabetics, to be higher in women than men, and adiponectin is know to correlate negatively with plasma glucose, insulin, triglycerides and insulin-resistance [340]. In other words, hypoadiponectinaemia is found in populations at enhanced risk of CVD. In patients with CKD and ESRD however, levels are markedly increased despite the high CV risk known to exist. Whether the increased levels can be completely explained by elimination of adiponectin by the kidneys has not yet been completely elucidated, although levels are known to fall after successful renal transplantation, and interestingly, the reverse epidemiology relationships that exist in ESRD become normalised following renal transplantation. It also remains unclear as to whether the relationships with CV risk in the general population also apply, as adiponectin has been found to be both an independent inverse predictor of CV outcomes in CKD populations [251, 327] even when adjusted for stage of CKD [325], and also to be an independent predictor of total mortality in both CKD [328] and HD patients [327]. The levels of adiponectin found in this study are comparable to previous studies [325]. A correlation with creatinine was confirmed, as well as the markedly increased levels in HD patients. The positive correlation with $CD56^{bright}$ and negative correlation with $CD56^{dim}$ cells is interesting, suggesting that there might be a decrease in NK cell cytotoxicity. It has been postulated that the high levels of adiponectin in CKD patients are a compensatory response to inflammation and the malnutrition that occurs. Indeed in one study which found adiponectin to be associated with increased mortality, when malnourished patients (those with an albumin ≤ 3.5g/dL) were excluded, adiponectin levels were not a significant predictor of mortality [327]. Additionally, adiponectin has been demonstrated to be a negative regulator of IL-2-induced NK cell activation [341], providing further

evidence that it may act as an in vivo regulator of anti-inflammatory functions. The inverse correlations found with TC, triglycerides and non-HDL-C in inflamed patients would seem consistent with this theory that adiponectin secretion is increased to try to counteract the inflammation in CKD as cholesterol levels are known to fall in malnourished and inflamed patients, another example of reverse epidemiology. The correlation found between adiponectin and IL-6 supports this. The relationship between adiponectin and BMI is difficult to interpret in patients with CKD and in particular ESRD, where paradoxically a higher BMI confers a survival advantage, although lower adiponectin levels are seen with higher BMIs. The inflammation/PEM seen in these patients will confound the effects of adiponectin on clinical variables.

Serum soluble ST2 is a member of the IL-1 receptor family and is a biomarker of mechanical stress which is up regulated in isolated cardiomyocytes exposed to mechanical strain. ST2 is expressed in a transmembrane form (ST2 ligand [ST2L]) and a soluble circulating form (sST2). IL-33 has recently been identified as the ligand for ST2L. ST2L serves a negative regulatory function for toll-like receptor (TLR) signalling which when uncontrolled can result in excessive inflammation. It has been suggested that sST2 acts as a decoy receptor by binding IL-33, thereby inhibiting signalling by ST2L [342]. Too much sST2 in the context of potential stretch-induced injury to the heart may therefore result in inadequate cardioprotection from IL-33, with an increased risk for cardiac remodelling, ventricular dysfunction, or death. Both forms are induced in cardiomyocytes and fibroblasts exposed to biomechanical stress. After pressure overload by transverse aortic constriction, $ST2^{-/-}$ mice had more left ventricular hypertrophy,

myocardial fibrosis, and chamber dilation, and decreased fractional shortening and survival compared with wild-type littermates [342]. Recombinant IL-33 treatment was found to reduce hypertrophy and fibrosis leading to improved survival after transverse aortic constriction in wild-type mice but not $ST2^{-/-}$ littermates. In patients with acute MI, elevated sST2 levels are associated with an increased risk of mortality or heart failure [343]. Increased levels have also been found in patients with acute dyspnoea, whether or not this is due to decompensated cardiac failure, with baseline values predictive of 1 year mortality [344]. High levels of ST2 have also been found in patients with severe chronic heart failure [345], in various immune disorders such as asthma, pulmonary fibrosis and various autoimmune diseases, and more recently in patients with severe sepsis, irrespective of the source of sepsis, which correlated with disease severity and mortality [330].

In this study, no significant correlations between sST2 levels and renal function were found. There were no correlations with any biochemical parameters, and there were no correlations with any lipid fractions. No correlations were found with either NK cell function or phenotype. In the non-inflamed patients, there was a correlation with phosphate however this correlation is difficult to explain in the non-inflamed patients. Interestingly, sST2 did correlate with IL-6 and PTX-3 in all patients, in keeping with previous findings of higher levels in inflammatory states such as severe chronic heart failure and sepsis. A correlation exists between hsCRP and sST2 in non-inflamed patients, suggesting that perhaps PTX-3 is a better inflammatory biomarker in this group of patients than CRP. It is perhaps not surprising that no correlations were found given

that any patients with infection or a known inflammatory process were excluded. This was a group of stable out-patients, therefore none were in overt heart failure. The inflammation that exists in CKD is due to the uraemic milieu and likely to represent a different inflammatory process than that occurring in sepsis, autoimmune disease and cardiac failure. Although many similarities exist with chronic heart failure patients, for example PEW and malnutrition, this is probably more relevant to patients with ESRD on RRT, of which there were only 11 in this study. Patients with ESRD are continuously subjected to cardiac stressors, in particular chronic volume overload which results in left ventricular hypertrophy, and undoubtedly biomechanical strain on cardiac myocytes. This is a patient group that should be investigated in more detail, as to date there are no studies looking at ST2 levels in patients with ESRD. If a biomarker existed that could indicate whether there was significant subclinical "cardiac stress" which was predictive of outcome, strategies to improve volume status more rigidly, for example nocturnal or daily dialysis could be considered.

There were several limitations to this study. Firstly, the small number of diabetic patients and the small number of HD patients recruited may be too small to provide statistically significant information. The fact that samples from some of the CKD and all of the HD patients were non-fasting may affect the accuracy of the lipid profile. As pointed out already, the IL-6 assay was unfortunately not sensitive enough to detect very low levels, and therefore results may be skewed as the lower limit of detection was 1.0pg/ml and may not have been sensitive enough to detect any meaningful relationships. Additionally, a single plasma sample at a certain time point may fail to reflect the natural

course of the process studied, or provide as accurate a measurement as an average. The presence of a range of comorbid conditions in a population on multiple drugs represents an additional limitation on the interpretation of the data.

CHAPTER 7

General discussion and conclusions

7.0 GENERAL DISCUSSION

7.1 Natural killer cells and dyslipidaemia

The aim of this thesis was to assess the role of NK cells in CVD, and to address the hypothesis that changes in NK cell function may result in their participation in the development of atherosclerosis, particularly in high risk patient groups. The groups of patients studied were those with dyslipidaemia attending a lipid out-patient clinic, and patients with CKD, who will be discussed in the next section. The limitations of the studies have been discussed at the end of each chapter.

The role of plasma lipoproteins in atherogenesis is well recognised, but the physiological relevance of their immunoregulatory properties is not fully established. Previous work by this research group assessed T-cell proliferation and NK cell cytotoxicity in a group of thirteen healthy volunteers before and after treatment with 40mg simvastatin for four weeks [150]. This study showed that simvastatin treatment reduced NK cytotoxicity ex vivo by 30±34%, with levels returning to normal following cessation of therapy. Overall, there was a close relationship between the reduction in LDL-C and inhibition of NK cell killing. A further study assessed NK cytotoxicity in a wide ranging and varied patient population including immunosuppressed renal transplant recipients, patients with CKD and ESRD on HD, and patients with CAD, and found a trend towards higher levels of cytotoxicity at higher cholesterol levels [153], identifying total cholesterol and weight as the main factors associated with cytotoxicity.

In the study detailed in Chapter 3, we specifically sampled patients attending a lipid clinic to ensure that they had dyslipidaemia, and were therefore at higher risk of CVD. These patients were on a variety of lipid lowering treatments and it was notable that TC and LDL-C were higher in those patients not on statin treatment. We did not find any correlations between lipid levels and NK cell numbers, phenotype, or cytotoxicity, although triglycerides did correlate with T cell numbers. The presence of statin therapy did not affect NK-, NKT-, or T-cell numbers. We did not confirm a reduction in NK cytotoxicity in patients on statin treatment or indeed on any other lipid lowering treatment, and unexpectedly, there seemed to be an inverse correlation between NK cytotoxicity and LDL-C which did not appear to be a lipid lowering treatment effect as this relationship existed in the control group also. We did confirm a positive correlation between $CD56^{dim}$ cells and age. The differences in the findings of this study compared to the two previous studies described above may be partially explained by the fact that the cytotoxicity assays in this study used isolated NK cells, whereas previously a crude lymphocyte preparation was used. Another possibility could be a recent finding that lipophilic, but not hydrophilic statins inhibit NK cytotoxicity [346]. A significant proportion of the patients sampled were on rosuvastatin, a hydrophilic statin, the previous studies used the lipophilic statins simvastatin and fluvastatin.

The original hypothesis that membrane and plasma cholesterol change in parallel, and that changes in immune cell function may occur as a consequence of changes in circulating cholesterol levels (and thus changes in cell membrane cholesterol) has not been answered in this study. The idea that treatments that lower circulating cholesterol

are therefore likely to reduce membrane cholesterol, resulting in impaired or altered cell functions that are dependent on membrane cholesterol has also not been confirmed. Our findings are consistent with other previous studies as detailed in Chapter 3 however there are numerous conflicting studies. There is an abundance of experimental, clinical and epidemiologic data which has established hypercholesterolaemia as a major causative factor in atherogenesis. Additionally, numerous prospective epidemiologic studies have indicated that low serum cholesterol is associated with increased age-adjusted mortality from non-atherosclerotic diseases, not just in ESRD populations [281], and the link would appear to be between serum lipids and immune function. Lymphocytes receive cholesterol, essential for maintaining cell membrane structure, from circulating lipoproteins as well as synthesising cholesterol *de novo*. A concept that has emerged is that atherosclerosis to some extent can be viewed as an autoimmune disease in which the adaptive immune system is targeted against vascular self-antigens modified by hypercholesterolaemia [347]. Hypocholesterolaemic healthy volunteers have been shown to have lower numbers of circulating lymphocytes and T-cells, but no changes were noted in NK cell numbers between hypo- and hypercholesterolaemic subjects [283]. One group studied distribution of NK cell subsets and NK cytotoxic activity in patients with stable CAD and acute coronary syndrome (ACS) [95]. They found significantly lower numbers of NK cells and $CD56^{dim}$ cells in the ACS and stable CAD groups compared with controls, and additionally lower NK cell cytotoxicity in the ACS and CAD groups. There was however no difference in the calculated cytolytic activity per NK cell between groups, so they have described the impaired NK cell function in the CAD patients as a mainly quantitative defect. They also found no correlations between NK cell parameters

and lipid levels. Another group also looked at lymphocyte subtypes in patients with stable CAD and ACS. Conversely, they found that the relative number of NK cells was significantly raised in patients with ACS in comparison to controls, and in fact followed the pattern of ACS > stable CAD > controls [348]. The elevation of NK cell ratio was accompanied by a decrease in the total T-cell count in both the ACS and stable CAD groups, which was hypothesised to be due to migration of T-cells from the peripheral circulation to the site of atherosclerotic lesions. The possibility remains that the higher numbers of $CD56^{dim}$ cells seen in the lipid patient group in the present study may be a characteristic of the disease itself, although this was not possible to determine given the varied drug treatment. For example, increased NK cell apoptosis has been found in CAD patients compared with controls [97], and furthermore the NK cells were more susceptible to oxidized lipids *ex vivo*. Increased spontaneous apoptosis of $CD56^{dim}$ cells has also been demonstrated in patients with malignancy [349]. Statins are known to protect against oxidative stress, and statin treatment may explain why higher numbers of $CD56^{dim}$ cells were seen in the lipid patient group.

7.2 Natural killer cells and chronic kidney disease

In Chapter 4, we investigated whether any changes in the distribution of NK cell subsets or NK cell-mediated cytotoxicity could be observed in patients with CKD, the aim of the study being to provide a quantitative, functional and phenotypic multivariate analysis of NK parameters in relation to clinical and laboratory parameters of patients with CKD. Chronic renal failure is an immunodeficient state which is not completely understood, and results in a high morbidity and mortality from cardiovascular disease and infectious

complications. It is also associated with a 30% increase in malignant tumours [350], diminished serological responses to vaccination, and reduced fertility. It is known that patients with CKD and those with ESRD undergoing HD have decreased lymphocyte counts [212, 213] with altered function, but there is comparatively little information regarding the role of NK cells in CKD, and the studies in the current literature report conflicting results. Of the previous studies examining NK cell function and renal failure, some report increases in NK cell numbers [212, 217, 351] or decreases in numbers and activity [216, 217, 352] or no change [353]. All of these studies were small and involved only patients on RRT. None were carried out on undialysed patients with CKD in relation to renal function and these studies did not examine NK cell subsets. One more recent study [295] did involve both patients on HD and "uraemic undialysed" patients, although it is unclear what the degree of renal dysfunction was in these undialysed patients. They found that NK cell counts were lower in HD patients compared with controls, and although not statistically significant NK cell counts in the undialysed patients were lower and at an intermediate level between the HD patients and controls. Multivariate analysis of the undialysed group showed that creatinine clearance (Cockcroft and Gault) was positively correlated to NK cell counts. They found no alterations in NK cytotoxicity in HD patients compared to controls, but did find that cytotoxicity was positively correlated with time on dialysis, with patients on HD for over 5 years exhibiting higher cytotoxic potential. They did not however examine NK cell cytotoxicity in the undialysed CKD group in whom they had noted a positive relationship between NK cell counts and creatinine clearance.

To the best of my knowledge, we are the first to have examined NK cell numbers, subtypes, and function in a group of patients across the range of CKD. We did find a correlation between NK cell cytoxicity and creatinine, although this did not retain significance after multivariate analysis. There was also a correlation between NK cytotoxicity and phosphate which remained significant after stepwise multivariate regression. We are the first to report a relationship between phosphate and NK cytotoxicity. This is an interesting finding in light of the increasing evidence supporting a role for phosphate in cardiovascular disease and increased mortality, not only in the ESRD population [223] but also in populations without CKD [219], and the emerging evidence for the role of NK cells in atherosclerosis. In cross-sectional studies, each 1-mg/dl rise in serum phosphorous was associated with a 23% increased risk of death [220]. Hyperphosphatemia is also correlated with an increased risk of soft tissue and vascular calcification, which may provide the link between CKD and CVD. There has been much interest recently in the use of calcium containing versus non-calcium containing phosphate binders, as there is evidence that use of the non-calcium containing phosphate binder sevelamer leads to better survival [354]. In the future, in treatment of hyperphosphatemia in CKD patients, vascular risk reduction and mortality need to be considered in addition to effects of mineral homeostasis and reduction of serum phosphate level.

7.3 Membrane cholesterol and natural killer cells

It has long been accepted that hypercholesterolaemia correlates with the development of atherosclerosis, and more recently that progression of cholesterol-related disease requires

inflammation, but the mechanisms linking those events are as yet unknown. Reductions in membrane cholesterol have been shown to affect cell function, presumably by altering membrane lipid organisation. High serum cholesterol availability therefore might also activate immune cells that contribute to the progression of atherosclerosis. Activation of immune cells by cholesterol may explain the association that has been found between atherosclerosis and CRP [33]. We have postulated that some of the reported pleiotropic effects of statins on cell signalling and immunity may reflect altered cell membrane, and in particular lipid raft structure and function due to reduced cholesterol availability. As a consequence, hypocholesterolaemia as is seen in malnourished, acutely or chronically unwell patients such as those with malignancy, cardiac failure or ESRD, could lead to reduced inflammatory responses, which may contribute to the increased susceptibility these patients have to infections. One study found that cholesterol loading macrophages with MBCD affected F-actin organisation, increased membrane pinocytic activity, and decreased cell migration [314], changes that were similar to those observed when macrophages were treated with modified lipoproteins, suggesting that the changes induced by modified lipoproteins are partly attributable to changes in cellular cholesterol levels. These findings may explain how macrophages within atherosclerotic lesions continue to take up retained lipoproteins, becoming foam cells that are then unable to migrate from the atherosclerotic lesions. Another study investigated the effects of cholesterol depletion (using MBCD) on polymorphonuclear neutrophil rafts, to assess the effects of raft disruption on G protein-coupled Ca^{2+} mobilisation [355]. Regulated Ca^{2+} entry is a primary mechanism of immune cell activation and therefore inflammation, and receptors that activate phospholipases can initiate Ca^{2+} entry through transient receptor

potential calcium (TRPC) channels. They found that cholesterol bioavailability regulated membrane raft composition and the TRPC1 redistributed to raft fractions in response to cholesterol. If Ca^{2+} entry can be regulated in this way by cholesterol availability, this may provide the link to the regulation of immune cell activity. The association between hypercholesterolaemia and inflammation may therefore in part reflect cholesterol-dependent changes in lipid raft structure that regulate immune cell calcium entry [355].

Although we have concentrated on the actions of statins in this study, variation in the cholesterol content of cells is likely to be a physiological regulator of membrane receptor function that affects all cells. The cholesterol within the cell membrane comes from recycled circulating lipids and that produced by HMG-CoA reductase within individual cells. HMG-CoA reductase is regulated by exogenous lipid, as we have shown here, and NK cell function is also related to circulating cholesterol levels. The effect of diet, specifically starvation, on NK cell function is well known and may contribute to susceptibility to infection in malnourished individuals. Cholesterol excess or deficiency may alter cell signalling in ways that are therefore modifiable by dietary or pharmacologic cholesterol control. However, the relationship between circulating and membrane cholesterol, and cell function, and the concept of statins as membrane modifying agents merits further study.

7.4 Biomarkers in chronic kidney disease

Cardiovascular disease is the leading cause of morbidity and mortality in patients with ESRD, and even the presence of mild CKD has been shown to be associated with a

higher prevalence of CVD [2] therefore early identification of patients who are at heightened CV risk may facilitate more aggressive and focused treatment. To do this, there is an increasing need to identify an ideal biomarker that will be able to facilitate the clinical diagnosis of CVD. In fact CKD itself may be considered a biomarker of CVD. The CV risk is increased very early on in the evolution of CKD, at a GFR of about 75ml/min, and increases continuously with decreasing renal function [356]. In this group of patients in particular, prediction models using traditional risk factors underestimate CVD risk. Additionally, the pattern of CVD in ESRD differs from the general population and although there is an increase in atheromatous CAD and its sequelae, there is a broader range of CVD with CV calcification, LVH, and sudden cardiac death, possibly related to "uraemic cardiomyopathy" and associated heart failure or arrhythmia, which may not be as dependent on cholesterol levels. To further complicate matters, the conventional relationships between existing biomarkers and CV risk may be reversed in the ESRD population, with many confounders, particularly inflammation, making interpretation more complex. Decreased renal clearance of inflammatory markers and the high incidence of co-morbid conditions may also contribute to a confusing picture.

In Chapter 6, we have not been able to determine any definite relationships between the biomarkers studied and NK cell function. There was an association between PTX-3 and NK cytotoxicity that was found only in inflamed (hsCRP>2mg/L) patients. There was also a positive correlation between adiponectin and $CD56^{bright}$ cells which are the cytokine producing NK cell. This would be consistent with the idea of increased adiponectin (and perhaps other cytokine) production in increasingly inflamed patients to

try to counteract the inflammation that occurs in CKD. In mice, atherosclerotic lesions are generally driven by the increased expression of proinflammatory cytokines, produced by NK cells. The activation of NK cells eventually leads to enhanced stimulation of macrophages, B and T cells which further perpetuates this process [69]. We were able to confirm established relationships between hsCRP, PTX-3, adiponectin and renal function, as well as lesser know relationships – hsCRP and phosphate, and PTX-3 and phosphate. Taken together with the significant correlation between phosphate and NK cytoxicity found in Chapter 4, this provides more support for serum phosphate itself being associated with inflammation, and as a result perhaps leading to formation of atherosclerotic lesions.

7.5 Clinical implications and further work

There are a number of clinical implications from the findings and observations in this thesis. The relationships between NK cell cytotoxicity and creatinine, and NK cell cytotoxicity and phosphate are important given the increasing evidence implicating hyperphosphataemia in increased CV risk in both the general population [219] and in the CKD population [223]. In considering treatment of hyperphosphataemia in patients with CKD; at present, the current KDIGO guidelines for chronic kidney disease-mineral bone disorder (CKD-MBD) [357] suggest that in CKD stages 3-5 serum phosphorous is maintained in the normal range, and in CKD stage 5D, elevated phosphorous levels should be lowered towards the normal range, using phosphate-binding agents. A recommendation is made to restrict the dose of calcium-based phosphate binder (and vitamin D supplements) in the presence of persistent hypercalcaemia and arterial calcification. Whilst hypercalcaemia can be easily monitored, the presence of arterial

calcification may be asymptomatic. The presence of vascular calcification is associated with all-cause and CV mortality in ESRD [358], and few studies have addressed interventions to decrease vascular calcification. It would therefore seem sensible to adopt an approach of prevention, therefore in considering treatment, not only the reduction of phosphate, but an assessment of vascular risk reduction and mortality need to be considered. No prospective studies have been carried out to examine the benefits of targeting different phosphate levels to determine the effect on patient-level end points. Further studies need to be carried out to determine whether we should be lowering phosphate more aggressively and in a more targeted fashion as part of specific CV risk reduction. Further studies could be carried out in a larger patient population with CKD, examining NK cell function in those treated with calcium and non-calcium based phosphate binders. In vitro studies examining the effect on NK cell function of uraemic serum from hyperphosphataemic patients and patients with well controlled phosphate levels and Ca x P product would be an interesting next step. Additionally, studies to determine whether other immune abnormalities occur in the context of hyperphosphataemia would be of interest.

Whilst in our patient cohort we did not confirm the previously observed relationships between NK cell function and lipid levels *in vivo*, we have shown that NK cell function is related to circulating cholesterol levels at least *in vitro*. Variation in the cholesterol content of cells is likely to be a physiological regulator of membrane receptor function that affects all cells. These findings suggest that cholesterol excess or deficiency may alter cell signalling in ways that are modifiable by dietary or pharmacologic cholesterol

control. In other words, immune functions are affected by nutritional status which is particularly important in CKD/ESRD patients. Further work needs to be done to tease out the reverse relationships that exist in this population, when inflammation has been excluded. The evidence for lipid lowering in ESRD remains unclear, with the 4D study [259] showing no benefit in treating diabetic dialysis patients with atorvastatin vs placebo, and the AURORA study [260] showing no benefit in treating a more wide-ranging population of dialysis patients with rosuvastatin vs placebo. The SHARP study (Study of Heart and Renal Protection) [359] to assess the effects of cholesterol lowering with a combination of simvastatin and ezetimibe, aiming to recruit around 9000 patients with CKD is ongoing, and may shed more light on this important area.

The relationship between $CD56^{dim}$ cells and ageing that is already established and confirmed in this thesis may have significance in the future in view of our ageing general, and therefore CKD population who will accrue increasing CV risk. Establishing the role that NK cells play in atherosclerosis is therefore important. Whether increasing NK cytotoxicity results in an increased inflammatory response (as indicated by the relationships with the biomarkers in Chapter 6), or whether conversely given their capacity to control infections, a loss of NK cell activity might lead to increased susceptibility to atherosclerosis-related pathogens (with consequences, such as increased plaque burden and enhanced formation of vulnerable plaques) remains unclear. The pleiotropic effects of statins on NK cell function probably involve both inhibition of isoprenylation and cholesterol depletion. It may be that in the future, the clinical use of

statins will not only be simply for cholesterol reduction, but for immunomodulation in patients at high risk of CVD, for example as part of treatment of hyperphosphataemia.

There is little doubt now that immune cells and inflammatory mediators which are present either in the circulation or at the site of inflammation can result in atherosclerotic lesions. It may be that in the future, along with conventional CV risk reduction therapies such as anti-platelet and lipid lowering agents, immune-based therapies for prevention and treatment of CV disease may become a reality.

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