INFLAMMATORY MARKERS AS NOVEL PREDICTORS OF CARDIOVASCULAR DISEASE

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"The folly of mistaking a paradox for a discovery, a metaphor for a proof, a torrent of verbiage for a spring of capital truths, and oneself for an oracle, is inborn in us."
Introduction to the Method of Leonardo da Vinci (1895)

"On a toujours cherché des explications quand c'était des représentations qu'on pouvait seulement essayé d'inventer."
Unsourced

Paul Valéry (1871-1945)

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ABBREVIATIONS

Ab	Antibody (prefix m; monoclonal)
ABI	Ankle brachial index
ACS	Actue coronary syndrome
Ag	Antigen
APC	Activated protein C
apo	Apolipoprotein
APR	Acute phase response
BMI	Body mass Index
bp	Binding protein
BP	Blood pressure
CD	Cluster of differentiation
CHD	Coronary heart disease
CHF	Chronic/congestive heart failure
CI	Confidence intervals
COX	Cyclooxygenase
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DC	Dendritic cell
ECG	Electrocardiogram
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
F1+2	Prothrombin fragment 1+2
FEV_1	Forced expiratory volume in one second
GM-CSF	Granulocyte monocyte colony stimulating factor
HDL	High density lipoprotein
HRT	Hormone replacement therapy
HSP	Heat shock protein

ICAM	Intercellular adhesion molecule
ICE	Interleukin-1 converting enzyme
IFN-	Interferon-
IL-	Interleukin-
IMT	Intima media thickness
IS	Ischaemic stroke
KO	Knock-out
LACI	Lacunar infarct
LDL	Low density lipoprotein
LV	Left ventricle
LVEF	Left ventricle ejection fraction
LPS	Lipopolysaccharide
MABP	Mean arterial blood pressure
MCP	Macrophage chemoattractant protein
MCPT	Maximum carotid plaque thickness
MHC	Major histocompatability complex
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MMSE	Mini mental state exam
MS	Multiple sclerosis
MBP	Myelin basic protein
NEFA	Non-esterified fatty acids
NO	Nitric oxide
NOS	Nitric oxide synthase (prefixes; e, endothelial, i, inducible)
NHS	National health service
NYHA	New York Heart Association
OCSP	Oxfordshire communities stroke project score
OD	Optical density
OR	Odds ratio
oxLDL	Oxidised low density lipoprotein
PACI	Partial anterior circulation infarct
PAMP	Pathogen associated molecular pattern

PCI	Percutaneous coronary intervention
POCI	Posterior circulation infarct
R	Receptor (suffix)
RA	Rheumatoid arthritis
RBP-4	Retinol binding protein-4
ROS	Reactive oxygen species
s	Soluble (prefix)
SAP	Serum amyloid protein
SCID	Severe combined immunodeficient
SLE	Systemic lupus erythrematosus
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SR-A	Scavenger receptor A (CD204)
SR-S	Self-reported stoke
SSS	Scandinavian stroke score
STROOP	Speed of information processing test
TACE	Tumour necrosis factor alpha converting enzyme
TACI	Total anterior circulation infarct
TAT	Thrombin-antithrombin complex
TCR	T-cell receptor
TGF	Transforming growth factor
TIA	Transient ischaemic attack
TIDAL	Instrumental activities of daily living score
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
t-PA	Tissue plasminogen activator
Trig	Triglycerides
VLDL	Very low density lipoprotein
vWF	Von Willebrand factor
WCC	White cell count
WHO	World health organisation

DECLARATION

I declare that I am the author of this thesis and that no part of the work has previously been reported in another thesis, with the exception of small sub-sections of Chapter 3, where results from a previous thesis are included for reasons of comparison with my own novel findings. The relevant author is clearly and fully credited where appropriate, and inclusion of their results in no way detracts from the novelty of my own findings.

The experimental work on the four novel inflammatory biomarkers was carried out by myself in the Division of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow Royal Infirmary. Other inflammatory markers, not central to this thesis, were measured by myself (~50% of the work load) or by a technical colleague in consultation with myself.

I performed all aspects of the literature review and the data compilation, discussing and drawing conclusions where appropriate.

I performed basic statistical analysis for each of the studies. Advanced statistical analysis in case-control and cohort studies were performed by the collaborating statistician and study database holder, secondary to the nature of the blinded testing study designs, as described in Chapter 2.

Paul Welsh

SUMMARY

Inflammation is widely considered to be an important contributing factor in atherogenesis and the risk of atherothrombotic complications. Baseline measurements of some inflammatory markers are known to be predictive risk factors for future cardiovascular disease (CVD) events in prospective epidemiological studies. Inflammatory markers dominant in the literature are acute phase response (APR)-associated and include fibrinogen, C-reactive protein (CRP) and, more recently, interleukin- (IL-) 6. This thesis reviews the literature and suggests the need for further research into novel inflammatory markers of CVD risk. The broad aim was to expand on limited existing data and ascertain if circulating levels of four novel inflammatory markers (tumour necrosis factor α [TNFα], IL-18, soluble CD40 ligand [sCD40L], and matrix metalloproteinase-9 [MMP-9]) are associated with classical cardiovascular risk factors, and with future CVD events in several epidemiological studies.

In studies of pre-analytical variables, all four markers had commercially available assays acceptable for epidemiological use, but only IL-18 and TNFα displayed assay stability and the ability to be measured in plasma or serum.

Due to limited serum samples, MMP-9 and sCD40L were less extensively measured. Results suggest a moderate positive association of MMP-9 with coronary heart disease (CHD) risk (although confounded by smoking and markers of general inflammation), while serum sCD40L may be moderately *inversely* related to CHD risk. More data is required for these markers.

IL-18 and TNFα displayed similar degrees of short-term biological variability and regression dilution as CRP. Population distributions of both cytokines were consistent with limited previous reports. Both displayed associations with conventional vascular risk factors (such as age, gender, HDL cholesterol, and smoking), although interestingly, associations with epidemiological measures of obesity were poor. Both cytokines demonstrated moderate associations with vascular disease in a retrospective CHD study. In 3 prospective CHD or CVD studies, IL-18 demonstrated consistent but moderate

associations with risk of vascular events in age- and sex-adjusted models (Odds ratio [OR]~1.6 in the top versus bottom third of the population). The association became borderline significant after adjustment for conventional risk markers. Associations of TNFα with risk of CHD in these studies were inconsistent, and more data are needed.

In 3 prospective stroke studies, TNFα demonstrated some moderate associations with acute stroke outcome and recurrent stroke risk, but not with incident stroke in the elderly with vascular disease. IL-18 demonstrated no association with risk or outcome in any stroke study.

Meta-analysis in 4 suitable prospective studies showed (in full adjustment models) that IL-18 (OR 1.18 [95% CI 0.95-1.48] comparing extreme thirds) and TNFα (OR 1.05 [0.67-1.64]) have at best weak independent associations with CVD risk. Therefore these markers are unlikely to add significantly to clinical vascular risk prediction models, although these cytokines may still be of biological significance and potential therapeutic targets. More data is required for these markers.

In conclusion IL-18, TNFα, MMP-9 and sCD40L may show weak associations with CVD. However, despite animal and tissue models indicating that they may play pivotal roles in atherogenesis, circulating concentrations of these inflammatory markers have limited independent vascular risk associations. Elevated circulating levels of APR-associated markers may sensitively reflect exposure to a wide range of adverse pro-inflammatory stimuli including lifestyle exposures, whereas some other inflammatory markers may not.

CHAPTER 1

LITERATURE REVIEW OF CARDIOVASCULAR DISEASE, INFLAMMATION AND EPIDEMIOLOGY

1.1 CARDIOVASCULAR DISEASE INTRODUCTION

1.1.1 The Global Cardiovascular Disease Epidemic

Cardiovascular disease (CVD) is the primary cause of mortality and morbidity worldwide, and is projected to remain so indefinitely ([1]Murray and Lopez, 1997). In terms of global burden of disease, in 1999 the World Health Organization (WHO) placed Coronary heart disease (CHD) in sixth place and stroke in seventh place, but by 2020 they will have moved to first and fourth place, respectively (www.who.int/cardiovascular diseases/). CHD is the end result of accumulation of atheromatous plaques within the walls of the arteries that supply the myocardium. Recent estimates suggest that half of currently healthy 40-year-old males will develop CHD in the future, and one in three healthy 40-year-old women (AHA, 2007).

The WHO estimates that cardiovascular disease killed 14.7 million people in 1990, rising to 17 million in 1999 and with a projected further increase to 24.8 million in 2020 ([1]Murray and Lopez, 1997; Poulter et al, 2003; www.who.int/cardiovascular_diseases/). CVD is acknowledged to be causally linked to the lifestyle of the individual (including behaviours such as smoking, a sedentary lifestyle, a poor diet) a fact popularised by the media. In the developed world increasing urbanization of inner cities has caused a major change in behaviour patterns at all levels of the social spectrum. Widely available motor transportation and mechanisation of traditionally manual labour jobs has meant an increase in the proportion of people leading sedentary lifestyles. In addition, urbanisation promotes tobacco-smoking, and an unhealthy diet (high saturated fat and salt, low fruit and vegetables). Physical (in)activity and poor diet are likely to be the two most significant factors in the pandemic of obesity in the developed world. Indeed the WHO estimates that 60% of the world's population is insufficiently physically active, and in

1999 61% of US adults were considered overweight or obese (body mass index [BMI] 25+ kg/m^2) (Bonow et al, 2002), rising to 64.5% in 2004 (Health, United States, 2003. Atlanta, Georgia Centres for Disease Control and Prevention, National Centre on Vital Statistics), a trend increasingly prevalent in the Westernised world.

Cardiovascular disease is also a major problem in the developing world ([2]Murray and Lopez, 1997). This may largely be a consequence of improving medical treatment and increasing affluence (with the exception of sub-Saharan Africa), meaning the population is subjected less to malnutrition and communicable disease and is hence more likely to die of chronic degenerative diseases associated with older age (Yusuf et al, 2001). China is a particularly apt model of this "Epidemiological shift" (Yusuf et al, 2001) in developing countries, where increasingly Westernised lifestyles, jobs, smoking and unhealthy diets has brought about the emergence of cardiovascular disease as the major cause of morbidity and mortality after a lag period of one or two decades (Tsung 1998, Novak, 1998).

It is clear then that cardiovascular disease prevention is an important social and clinical issue, but it is also an economic one. It is thought that CVD costs the UK economy £26bn per year, (£474 per capita, and 57% of which are in direct healthcare costs) (British Heart Foundation Database. *Coronary heart disease statistics*, 2007 Edition). For this reason much recent research has focused on identifying individuals most at risk of a primary cardiovascular event, and administering effective prophylaxis, such as aspirin, beta-blockers and statins, as well as advice on lifestyle choices. Although this approach has had some success, more research is required to understand the molecular mechanisms through which cardiovascular disease progresses, to aid informed development of novel preventative approaches.

1.1.2 The Development of Atherosclerosis in Cardiovascular Disease

The presence of atherosclerotic lesions within the vasculature is virtually a pre-requisite for acute ischaemic events. Advanced lesions within the coronary arteries can cause luminal occlusion leading to stable and unstable angina pectoris. Erosion or rupture of

vulnerable lesions can lead to the atherothrombotic complications of myocardial infarction (MI), stroke, renal failure, heart failure, sudden death and peripheral vascular disease (Cohn, 2004; Hansson et al, 2005) (Figures 1.1-1.5). Indeed, although vascular pathology is usually discussed and managed on an organ by organ basis, atherosclerosis and atherothrombosis are generalised processes. Patients may only clinically present with arterial pathology in a specific area, but the disease is almost invariably ubiquitous, and is the root cause of the associated morbidity and mortality. For example, one study of 1500 patients with leg artery atherosclerosis (intermittent claudication) found that in a 4-10 year follow-up, 60% of deaths were cardiac, 17% were cerebrovascular, and 8% were due to other vascular causes such as ruptured aortic aneurysm (Laing et al, 1991). In addition a recent study has demonstrated that the ankle brachial blood pressure index (ABI) (a marker of peripheral atherosclerosis and subclinical stenosis) is associated with the site and number of arterial beds affected by atherosclerosis in vascular patients and to a lesser extent with the patient risk factor profile (Fowkes et al, 2006). These studies show that the atherosclerosis underlying the claudication pathology is also by far the main cause of mortality in this patient group. Considering the ubiquitous nature of atherosclerosis in the Westernised population, and its diffuse nature in the vasculature, it is therefore desirable to fully understand the cellular and molecular interplay involved in the development of atherosclerosis so that individuals at risk of progressing to an event may be identified, and possible pathways of therapeutic intervention may be examined.

Until the mid-1970s atherosclerosis was considered a bland lipid storage disease where lesion swelling and stenosis were thought to be the primary cause of luminal occlusion leading to ischaemic heart disease and stroke (Ross and Harker, 1976). Indeed, much historical and current evidence supports the role of plasma lipids (cholesterol and triglycerides) and lipoproteins (low-density lipoprotein [LDL] and very low density lipoprotein [VLDL]) in atherogenesis (Kannel et al, 1979; Martin et al, 1986; Lloyd-Jones et al, 2001). To suggest that lipid storage alone is the cause of atherogenesis is however a vast over-simplification. If atherosclerosis was purely a lipid storage disease it would be a reasonable assumption that the main driving force would be hyperlipidaemia, with the extent of atherosclerosis being proportional to sustained blood lipid concentration in most cases. Although hyperlipidaemia is a major risk factor for future

cardiovascular death, almost half of all coronary events occur in individuals without clinical hyperlipidaemia (Braunwald, 1997), and other risk factors such as smoking and blood pressure are of similar importance. Furthermore, hyperlipidaemia is only weakly related to ischaemic stroke (Shahar et al, 2003).

The development of atherosclerosis *in vivo* is currently thought to be a more complex process, requiring persistent lipid presence, endothelial dysfunction and inflammation (Libby, 2002, Willerson and Ridker, 2004, Szmitko, 2003, Hansson, 2005) (Figures 1.1-1.5). This model gives inflammatory cells and cytokines a pivotal role in the development of the lesion.

Figure 1.1 Low density-lipoprotein (LDL) infiltrates the arterial wall and is modified by oxidation processes. It is then phagocytosed by macrophages which become active inflammatory lipid-laden foam cells characteristic of early atherosclerotic fatty streaks. Figures 1.1-1.5 adapted from Hansson, 2005.

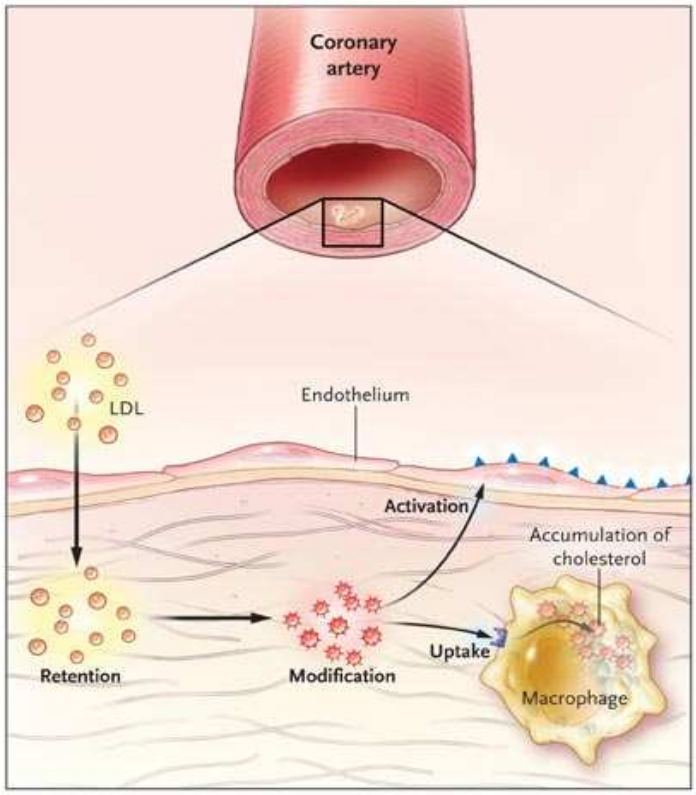


Figure 1.2 Activated local endothelium starts to produce P- and E- selectins and integrins such as vascular cell adhesion molecule-1 (VCAM-1). Monocytes and activated lymphocytes from the periphery roll, adhere and extravasate into the tissue. Macrophages activated via toll like receptors (TLRs) produce inflammatory cytokines, chemokines, proteases, and free radicals which perpetuate inflammation and damage tissue.

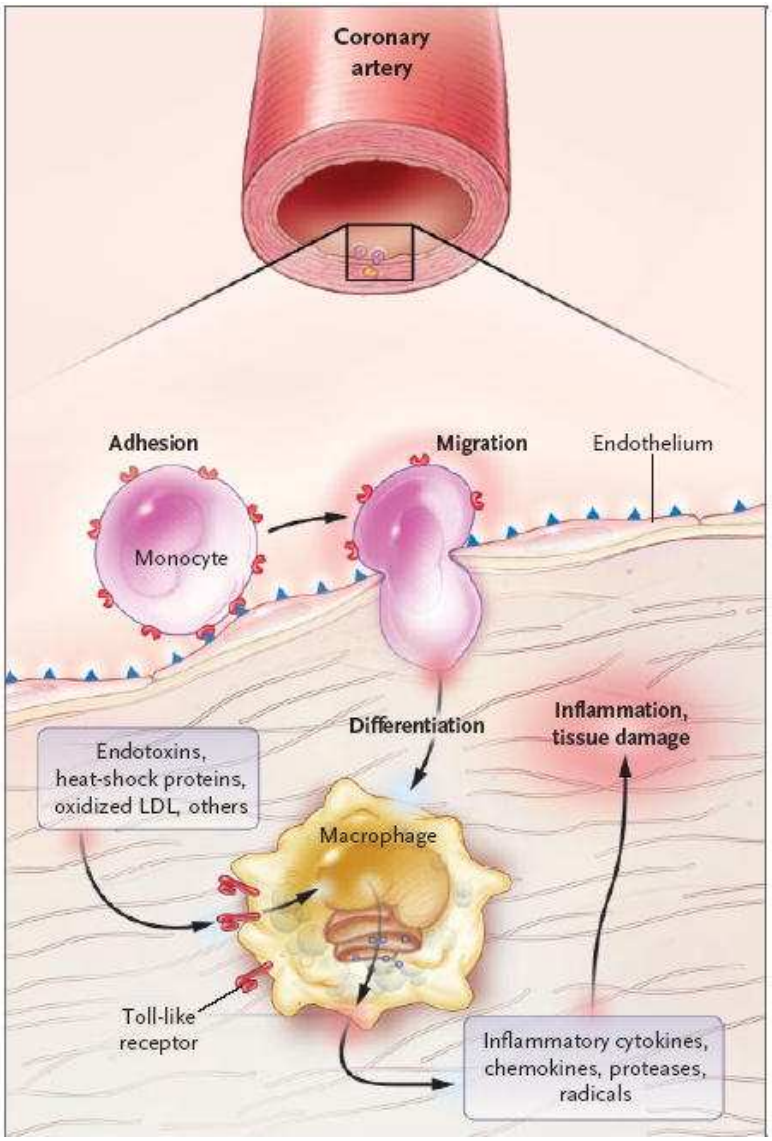


Figure 1.3 A Th1 type adaptive response (see text 1.2.5) becomes established and interferon- (IFN-) γ is produced. This stimulates further activation of macrophages and proliferation of smooth muscle cells (SMCs). A stable fatty lesion develops with the collagenous cap manufactured by the SMCs.

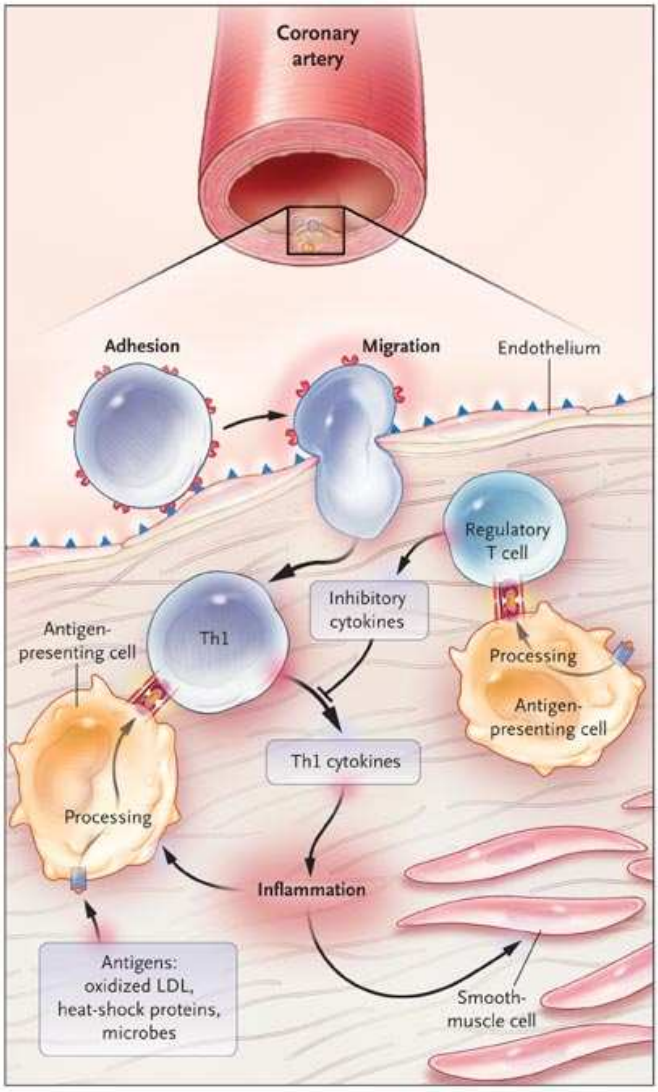


Figure 1.4 Production of IL-6 and other cytokines (interleukin-1, TNFα) leads to stimulation of the hepatic acute phase response (APR) releasing acute phase reactants such as fibrinogen, serum amyloid A, and C-reactive protein (CRP).

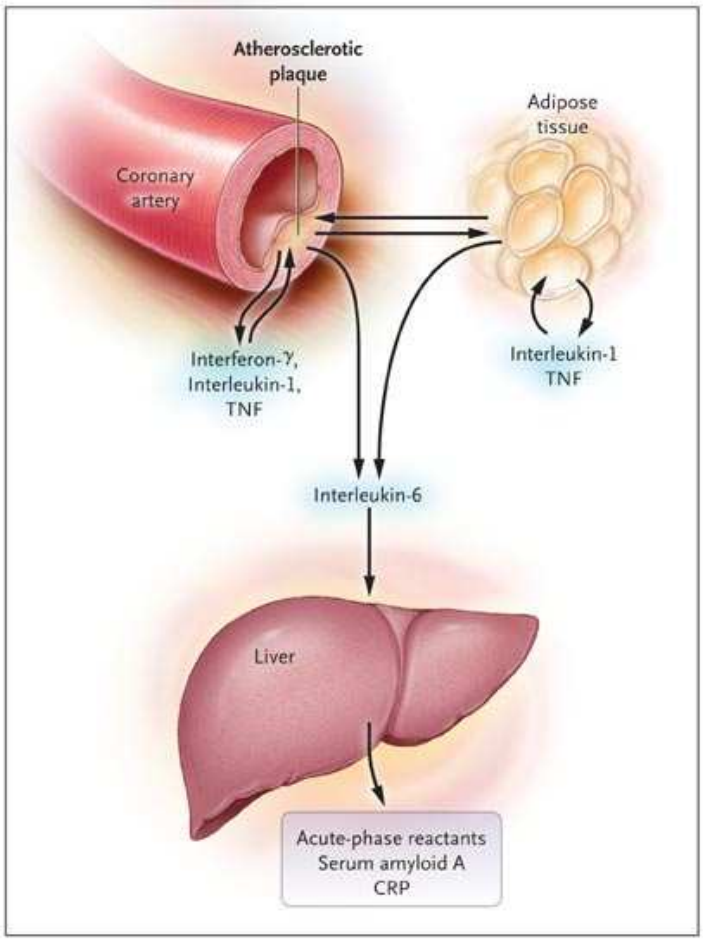
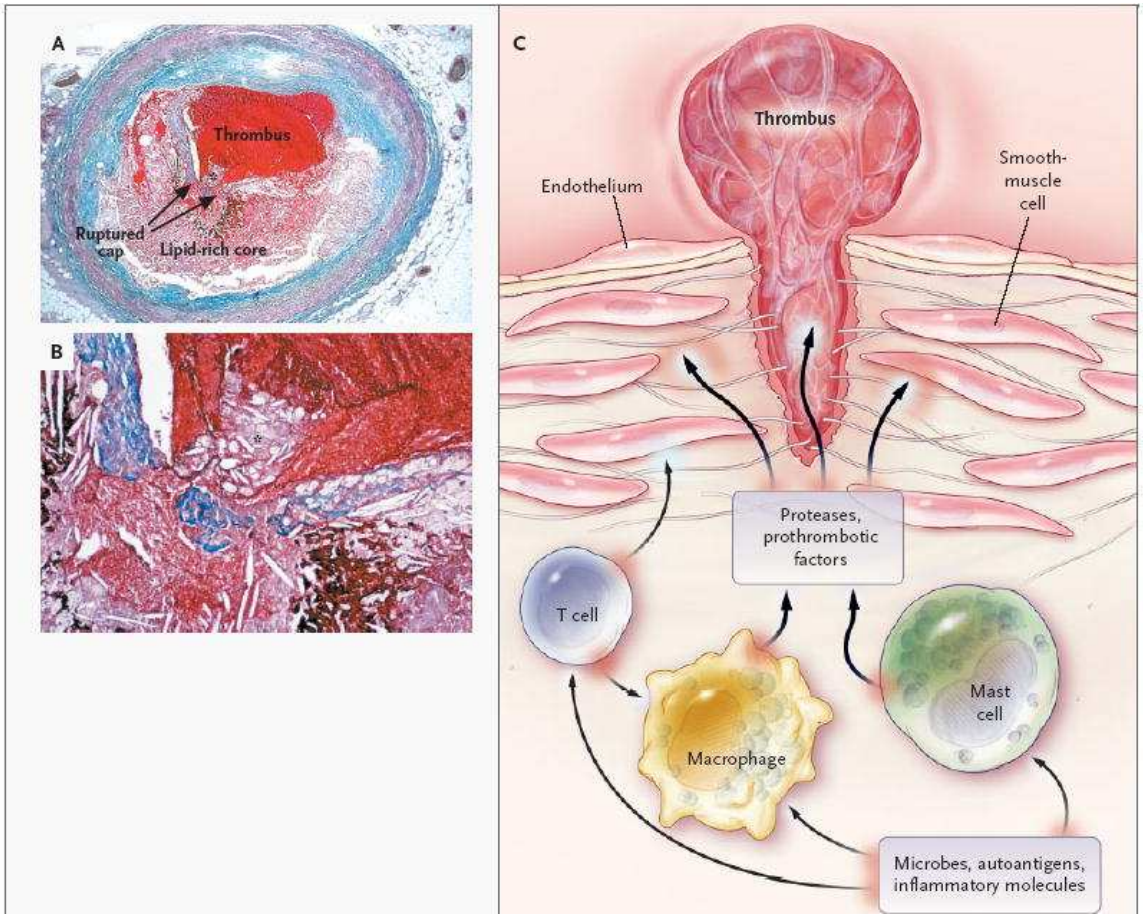


Figure 1.5 (A) A cross-section of a coronary artery depicting a massive thrombus occluding the coronary artery after rupture(stained with Trichrome, rendering luminal thrombus and haemorrhage red and collagen blue). The area in between arrows shows the ruptured fibrous cap. (B) Following rupture, lipids can be seen seeping into the lumen (high powered micrograph). (C) Cellular involvement in atherothrombosis. Immunological cells as well as endothelial and smooth muscle cells within the plaque may play an important role in promotion and stabilisation of the thrombus.



1.1.3 Endothelial Dysfunction

Endothelial dysfunction is usually defined as a diminished ability of the endothelium to produce the vasorelaxing compound nitric oxide (NO), or a profound imbalance in the relative amounts of other vasoactive compounds such as angiotensin II and oxidants (Verma and Anderson, 2002). Using this definition, endothelial dysfunction is confined to describing inappropriate vasomotor constriction of the vasculature, hence aggravating luminal occlusion caused by established atherosclerotic lesions. In the current model of atherosclerotic development however, diminished NO production by the endothelium is an intricate player in the development of pathophysiological inflammatory and molecular processes in the vasculature. (Szmitko et al, 2003). In addition to vasodilatory effects, NO also generates an anti-thrombotic environment by limiting platelet aggregation at the endothelium (de Graaf et al, 1992), prevents leukocyte adhesion to the endothelium by suppressing expression of adhesion molecules (Gauthier et al, 1995), and it maintains vascular smooth muscle in a non-proliferative state (Cornwell et al, 1994). This is a delicate balance, and local changes in vascular environments (such as LDLcholesterol, free radicals, infectious microorganisms, low shear stress, and angiotensin II-induced hypertension) can cause endothelial activation, in part by reducing the intracellular concentration of NO ([1]Ross, 1999).

1.1.4 Initiation of the Atherosclerotic Lesion; Where Inflammation and Endothelial Dysfunction Meet

Atherosclerosis is initiated and exacerbated in the presence of systemic risk factors such as elevated LDL, hypertension, high blood sugar, and by-products of tobacco smoking. Although the entire vascular endothelium is exposed to these stimuli, atherosclerotic lesions develop preferentially at bifurcations, branch points, and inner curvatures of arteries, suggesting that local factors contribute to disease susceptibility. It is widely accepted that the complex patterns of blood flow in these regions expose the endothelium to "disturbed" haemodynamic forces (low shear stress; Lowe, 2003), which may in turn cause physical disruption of the endothelium and concomitant inflammation (Gimbrone et al, 1997; Garcia-Cardena et al, 2001).

In chronically hyperlipaemic plasma the vasculature is exposed to LDL cholesterol, which penetrates the endothelial cell layer around areas of low shear stress and moves into the intima, and then under oxidising conditions forms oxidised LDL (oxLDL) (Alderman et al, 2002) (Figure 1.1). This modified lipoprotein can bind to the endothelium and causes an increase in superoxide production, at the expense of lowering vascular concentration of NO, resulting in endothelial activation (Cominaciniet al, 2001). Activation of the endothelium begins the process of localised inflammation, allowing leukocyte extravasation through selectin and integrin production. Rapid externalisation of Weibel-Palade bodies results in P-selectin and E-selectin expression on the endothelial surface (Dong et al, 2000). The selectins weakly bind Lewis X-antigens on the surface of leukocytes, allowing loose "rolling" along the surface of the endothelial barrier. Endothelial expression of integrins such as VCAM-1 (vascular cellular adhesion molecule) allows arresting adhesion of Mac-1 (CD18/CD11b) expressing monocytes, which can then extravasate into the tunica intima underlying the endothelium (Piqueras et al, 2000) (Figure 1.2). Interestingly, VCAM-1 is expressed in large quantities by endothelial cells in animal models of atherosclerosis as part of the initial vascular response to cholesterol accumulation (Cybulsky and Gimbrone, 1991). This expression is not uniform and is patchy in appearance, and subsequent fatty streaks develop at the site of VCAM-1 expression only, and so too have a patchy appearance (Nakashima et al, 1998). This probably reflects areas of low shear stress and flow turbulence, hence disturbing the integrity of the endothelium sufficiently for lipids to migrate through to the intima. Accordingly, the same haemodynamic stress may ultimately cause the rupture of more complex plaques.

Maturing monocytes underlying the tunica intima express cytokines such as TNFα which help initiate and perpetuate the immune response and endothelial activation. Chemokines such as interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 allow recruitment of monocytes to areas of concentrated inflammation (Wang et al, 1996.)

1.1.5 Early Development and Progression of the Lesion

Maturing monocytes become tissue macrophages and express scavenger receptors, such as SR-A, CD36 and importantly oxLDL receptor-1 (LOX-1) (Bobryshev, 2006). LOX-1 is particularly important as it allows phagocytosis of modified LDL, and providing that blood lipid levels remain high, the macrophages gorge themselves and become lipid laden "foam cells." (Jovinge et al, 1996; Sano et al, 2004). The fatty streaks develop as more monocytes arrive and mature in the pro-inflammatory cytokine network (Figure 1.3).

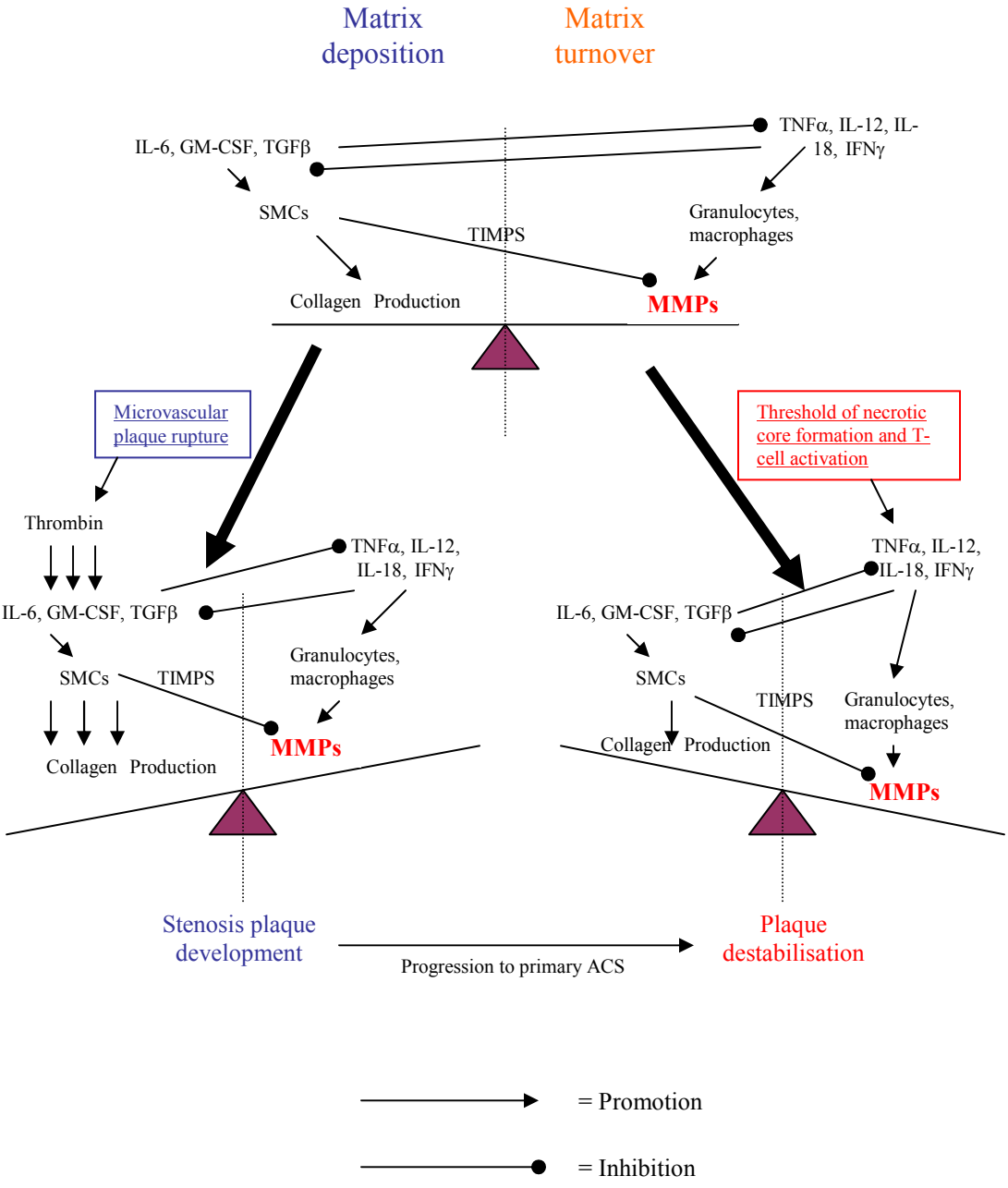
Angiotensin II, produced by the activated endothelium, is a vasoconstrictor that opposes NO action. NO normally regulates angiotensin II production, although this feedback is disturbed during endothelial dysfunction. This molecule may be important in the initial development of a pro-inflammatory local cytokine network through its action on smooth muscle cells (SMCs) which produce interleukin-6 (IL-6) and hence help initiate the systemic acute phase response (APR) through hepatocyte activation (Funakoshi et al, 1999).

IL-6 also acts in an autocrine manner to further activate the local SMCs (Klouche M et al, 1999) which then proliferate (Schonbeck and Libby, 2001; Porreca et al, 1997). SMCs control the maintenance of the extracellular matrix (ECM) through collagen production (Ford et al 1999) which is normally offset by matrix turnover and is mediated by matrix metalloproteinases (MMPs) (Galis et al, 1999). The MMPs are a family of zinc atom-containing endopeptidases, consisting of collagenases, gelatinases and stromyelysins. Degradative turnover of the ECM is controlled both by collagen production and tissue inhibtors of MMPs (TIMPs) both of which are produced by SMCs (Squire et al, 2004). Disturbance of matrix homeostasis is a major cause of vascular pathology.

In this way the plaque becomes more lipid-laden and cellular, but in the early stages of formation matrix deposition by proliferating SMCs provides mechanical support for the increasingly complex structure of the lesion (also contributing to luminal occlusion). Plaque development has long been believed to be an absolute and irresistible progressive

state, although some evidence suggests that this may not be the case. Coronary angiographic studies in a cohort of patients at different time points, suggests that plaque development may progress in a punctuated non-continuous fashion (Yokoya et al, 1999). The reasons for this are not clear, but several mechanisms have been proposed. One hypothesis is that neoangiogenesis in the plaque can result in fragile new microvasculature, which is prone to rupturing. The resulting thrombosis leads to thrombin generation, causing SMC proliferation and migration through a platelet derived transforming growth factor (TGF)-β dependant mechanism (Libby, 2002). This increase in SMC population would tip the balance in favour of collagen deposition, and progression of the lesion to a more bulky fibrous stenosis (Figure 1.6).

Figure 1.6 A representation of inflammatory factors contributing to progression of plaque development and atherothrombosis through collagen disruption and de-stabilisation of the lesion. The role of MMPs is pivotal in this process. Adapted from Van den Steen et al, 2001.



1.1.6 Activation of the Adaptive Response in the Developing Lesion

The presence of T-cells in plaque was first noted in 1985 by Hansson's group (Jonasson et al, 1985). Most T-cells found in lesions are CD4+, although some are CD8+, effector or memory cells (i.e. not immunologically naive) (Stemme et al, 1992) and the proportion of activated cells increases with disease severity (Hosono et al, 2003).

How and why these T-cells become activated is a more problematic issue. Recent findings suggest that, as in most infections, dendritic cells (DCs) activated in the lesion migrate to draining lymph nodes, and there activate naive T-cells (Galkina et al, 2006). DCs resembling Langerhans cells of the skin are found within the lesion, and are likely to capture antigen, such as modified LDL, through continuous macropinocytosis (Bobrysev, 2005; Alderman et al, 2002). "Danger signals" such as cytokines, stimulation of Toll-like receptors (TLRs) (perhaps by modified LDLs; [1]Miller et al, 2005), and apoptotic bodies cause the DCs to become activated, and they move to the draining lymph node. Here they theoretically have access to a naive T-cell repertoire that may be largely immunologically ignorant of "altered-self" antigens such as oxidised and modified LDL antigens. The T-cell repertoire are probably only ever exposed to these antigens except under pro-inflammatory stimulus from DCs matured in the lesion (Alderman et al, 2002), and in theory this may cause cognate T-cells to clonally expand and initiate the adaptive response.

Despite the logical role of oxLDL antigens in stimulating T-cell responses, molecular genetic studies have shown that T-cells found in lesions are highly heterogeneous in terms of T-cell receptor (TCR) specificity (Stemme et al, 1991) and only 10% of clones derived from T-cell plaques respond to oxidised LDL in an MHC class II-dependant manner (Stemme et al, 1995). In theory these T-cells could be activated by some superantigen or inflammatory stimulus more proximal to the lesion. Alternatively, there may be a great number of "altered-self" antigens within the developing lesion. Indeed heat shock proteins and pathogen-derived antigens among others have been suggested as other sources of foreign or "altered-self" antigen (Robertson and Hansson 2006).

Naïve CD4+ T-cells have the potential to become activated in one of two categorised functional manners; T helper (Th)-1 type or Th2 type. Briefly, Th1 cells are primarily noted for their interferon (IFN)γ producing capability and consequential stimulation of a largely macrophage mediated cellular response. Th2 cells are noted for the production of IL-4 and IL-13, which are required to drive B-cell clonal expansion and an antibody mediated response. In the prevalent response T-cell activation tends to skew towards a general "consensus" i.e. generally Th1 and Th2 have an inverse relationship. This happens by (poorly understood) multi-factorial mechanisms, perhaps including DC maturation signals, molecular interactions in the immunological synapse, epigenetic limitations, and through a positive feedback response in the cytokine milieu (e.g. IL-12 from macrophages promotes differentiation of Th1 cells, which in turn produce IFNγ) (Figure 1.3). Th1 versus Th2 skewing is excellently reviewed elsewhere (Crane and Forrester, 2005). In atherogenesis, IFNγ is produced by the vast majority of T-cells in the lesion (Hanson et al, 1989; Stemme et al, 1995) and the majority of evidence suggests that inflammatory components of atherogenesis are Th1 driven.

In spite of this B-cells are also activated in the atherogenic immune response and several types of potentially self-reactive "altered-self" specific antibodies have been described, particularly antibodies to various forms of altered LDL. However, studies of mice aberrant in B-cell responses suggest that B-cells are atheroprotective, in contrast to macrophages and T-cells (Stoll et al, 2006). This may reflect a pro-atherothrombotic role for athero-antibodies, but an overall protective role for Th2 responses, probably primarily through the consequent limitation of classical Th1 cytokines.

1.1.7 The Vulnerable Lesion and Atherothrombosis:

Regardless of the mechanisms of adaptive response activation, activated T-cells are present in developing lesions and IFNγ is produced locally by almost all of them (Stemme et al, 1995). This Th1 cytokine is known to possess several proatherogenic and prothrombotic effects, including increasing matrix turnover both through direct action on SMCs and activation of MMP producing macrophages (Mallat et al, 1995; Libby, 2002;

Robertson and Hansson, 2006.) Certainly IFNγ absence or impairment in various mouse models results in reduced vascular pathology (Robertson and Hansson, 2006).

Concomitantly, oxLDL accumulates in the lesion and causes apoptosis of SMCs (and macrophages i.e. necrotic core formation) (Kataoka et al, 2001). SMCs are a major source of TIMPs, and in their relative absence MMPs become more physiologically active. Despite the presence of large populations of scavenger cells, overwhelming debris means many of the apoptotic cells will progress to secondary necrosis, leading to further inflammation. This all leads to a microenvironment of matrix degradation prevalence, a change from the early balance where collagen deposition allowed development of a stable bulky stenosis (Figure 1.6).

Over time the plaque becomes destabilised as the fibrous cap thins, and the mechanical structure holding the lesion containing the necrotic core and inflammatory cells is compromised. One study has suggested that coronary artery lesions with high densities of foam cells and thin fibrous plaques account for only 10-20% of the overall plaque population, and yet cause 80-90% of clinical events due to propensity to rupture (Brown et al, 1993). It is now recognised that plaque stability, rather than absolute size has the determining role in the potential to rupture (Mallat et al, 1999). Since the plaques accumulate in regions of non-laminar flow, haemodynamic factors put mechanical stress on the plaque, hence providing a mechanism for erosion of the endothelial layer and propensity to rupture, exposing procoagulant stimuli underneath the endothelial layer (such as tissue factor) to the blood stream. Therapeutic prevention of (or limitation of the likelihood to experience) plaque rupture therefore is the primary goal of vascular medicine. Elucidation of modifiable causal risk factors in atherogenesis and subsequent atherothrombotic events is the first step towards this goal.

1.2 EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE

1.2.1 The Concept of "Risk Factors"

The concept of risk factors is derived from epidemiological studies trying to ascertain the factor(s) responsible for chronic diseases historically associated with infection and nutritional deficiency. As public health has improved and chronic diseases associated with aging (such as cardiovascular disease and cancer) have become more common, the focus of this type of research has subsequently adapted. Hence epidemiological methods have been long been used to assess which factors are statistically linked to a variety of disease states. For CVD, the concept of "risk factors" was popularised initially in the Framingham Heart Study (Kannel, 1961). Under the initial definition the risk *factor* (from the latin verb "facere" meaning "to make") must be causal in the development of disease progression. Technically, where a factor has been associated with risk of a disease but has not yet been proven causal, the term "risk marker" should be employed, although the names are often used interchangeably.

In some diseases there is a clear-cut cause, e.g. infections where a single common pathogen gives rise to the clinically observed symptoms and disease state (e.g. HIV virus infection giving rise to the acquired immunodeficiency syndrome, AIDS.) CVD however, is not so clear-cut. The complex molecular and cellular interplay involved in the development of atherosclerosis, and the further complications determining likelihood, position and size of a damaging thrombus mean that there are many factors that contribute before clinical presentation of an end-point event. Determining which of these factors has most influence over progression of disease is a key issue, either for use as a predictive marker or therapeutic target.

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1.2.2 Current Cardiovascular Risk Factors

Current major cardiovascular risk factors are summarised in Table 1.1. Seminal epidemiological work, such as the Framingham Study (Kannel, 1961) helped identify the classical or conventional risk factors for CVD that all health care professionals, and most

lay people are aware of. In general these are: male sex, increasing age, family history, smoking habit, presence of diabetes, obesity (especially high levels of visceral adiposity), hypertension, hyperlipidaemia and a sedentary lifestyle. Most of these risk factors inter-relate in some way either directly or indirectly. Age, sex and family history are obviously non-modifiable, so the five main risk factors usually considered clinically are smoking, diabetes, hyperlipidaemia, adiposity, and blood pressure. These major risk factors are currently combined in scores or charts which predict risk of CVD, e.g. the Joint British Societies chart (Figure 1.7). This current chart utilises the total:HDL cholesterol ratio as a measure of atherogenic blood lipids and hence takes into account the fact that not all cholesterol is equal in terms of CHD risk. VLDL and LDL cholesterol play direct roles in atherogenesis, but HDL cholesterol, in contrast, plays a role in reverse lipid transport (and other anti-atherogenic processes) and hence reduces risk of coronary heart disease (Barter, 2004). So although in general hyperlipidaemeia is a risk factor for CHD, this can be tempered if a high part of the total circulating cholesterol is HDL.

Importantly there is scientific evidence that each of the risk factors shown in Figure 1.7 is known to be causally involved in increasing the risk of CHD, either through increasing lipids available to generate plaques, or altering vascular biology to make the vasculature more susceptible to plaques, or increasing the likelihood of rupture and thrombosis (or some combination). Use of specific therapeutic intervention has shown that lowering each of these factors (e.g. by stopping smoking, lowering blood pressure, lowering of blood cholesterol by statins [hydroxymethylglutaryl-CoA reductase inhibitors] or control of obesity and diabetes) results in diminished risk in the general healthy population, as well as those with established vascular disease (Gotto et al, 2005; Lawes et al, 2002; Rodgers et al, 2006). These established risk factors are believed to account for somewhere in the region of 70-90% of incident CHD cases (Beaglehole and Magnus, 2002; Emberson et al 2003 & 2004).

Table 1.1 Major conventional risk factors for CVD

Classification of Factor	Examples of Risk
Non-modifiable	Sex (♂>♀), age, diabetes, social class (historical)
Modifiable	Smoking, blood pressure, sedentary lifestyle, adiposity (BMI or waist circumference), cholesterol (total or total:HDL)

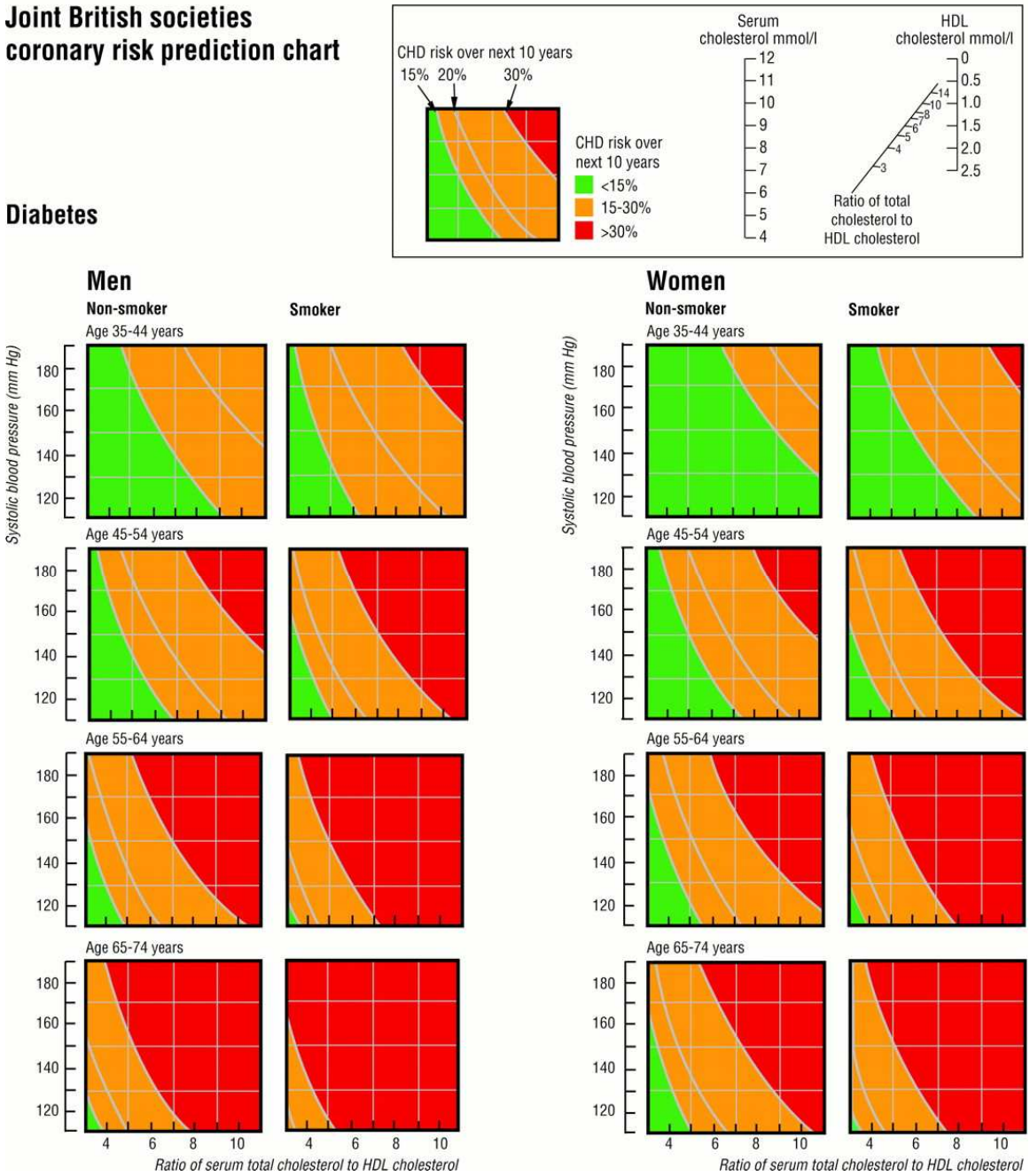
Table 1.2 Emerging potential risk markers for CVD

Classification of Factor	Examples of Risk
Metabolic/ Dietary markers	Triglycerides, impaired glucose tolerance, metabolic syndrome, leptin, adiponectin
Novel lipids	LP(a), apoA, apoB
Inflammatory markers	Fibrinogen, PV, WCC, hsCRP, IL-6, TNFα, IL-18, sCD40L, MMPs, αoxLDL Abs
Thrombotic markers	t-PA, PAI-1, D-dimer, vWF, homocysteine
Markers of endothelial dysfunction/ oxidative stress	oxLDL, sICAM-1, NO, brachial artery reactivity, glutathione
Non-invasive imaging biomarkers	Ankle brachial pressure index (ABI), MRI angiography, US carotid IMT, CT coronary calcification
Invasive imaging biomarkers	Intravascular US, coronary angiography

BMI, body mass index; MRI, magnetic resonance imaging; US, ultra sound; IMT, intima-media thickness; CT, computerised tomography; s, soluble; LP(a), lipoprotein(a); apoA/B, apolipoprotein A/B; hsCRP, high sensitivity C-reactive protein; IL, interleukin; αoxLDL Abs, anti oxLDL antibodies; PV, plasma viscosity; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; vWF, von Willebrand factor.

Figure 1.7 Clinical Joint British Societies risk assessment chart for CHD (example shown is in diabetics).

(From: Joint British recommendations on prevention of coronary heart disease in clinical practice: summary. *BMJ* .2000; 320: 705-708)



1.2.3 Emerging CVD Risk Markers

Emerging CVD risk factors are summarized in Table 1.2 and fall into numerous overlapping categories. Considerable interest has been shown in novel metabolic and dietary markers in recent times. The metabolic syndrome (The WHO criteria, 1999; defined as presence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance, plus any two of hypertension, dylipidaemia, obesity, and microalbuminuria – although there are several proposed clinical definitions) has attracted considerable attention as a predictor of type 2 diabetes as well as cardiovascular disease. Recent meta-analysis shows it is a predictor of CVD (Odds ratio [OR] 1.74 95% confidence intervals [CI] 1.29 – 2.35) (Galassi et al, 2006) although the clinical utility in diagnosing the syndrome as a CHD risk marker over and above the Framingham risk assessment is extremely dubious despite enthusiasm for its use particularly in the USA ([1]Wannamethee et al, 2005; [1]Sattar, 2006). In other examples of individual dietary hormones and lipids, triglycerides have recently been confirmed to be moderately associated with incident CHD during meta-analysis (Sarwar et al, 2006) as have lipoprotein(a) (LP(a)) ([1]Danesh et al, 2000) and apolipoprotein B:A ratios (Thompson et al, 2006). In contrast the hormone adiponectin is often reported to be inversely associated with CHD, but any association was recently shown to be weak on meta-analysis ([2]Sattar et al, 2006).

Some haemostatic markers have also shown association with incident CVD, perhaps due to their roles in thrombus formation, longevity and degradation. These include fibrin D-dimer fragment ([1]Danesh et al, 2001), von Willebrand factor (vWF; Whincup et al, 2002), and tissue plasminogen activator (t-PA; [1]Lowe et al, 2004).

In addition to these, measurements of subclinical plaque progression and stenosis have also been shown to have associations with incident CVD. The simplest measure of peripheral arterial resistance is the ABI which shows a low sensitivity, but high specificity association with CVD and CHD risk (Doobay et al, 2005; Fowkes et al, 2006). More recent computer imaging shows both non-invasive, and (the less clinically

attractive) invasive procedures can predict CHD risk (Rigatelli and Rigatelli, 2005; Simon et al, 2006).

Typically all of these markers have risk associations with CVD and CHD that can be described as moderate, but are clearly not as useful as the Framingham-based tests in clinical practice.

1.2.4 Inflammatory Risk Markers

As the aforementioned role of inflammation in atherogenesis would suggest, a great deal of work has been done looking for inflammatory markers that are risk factors for CHD (Libby et al, 2002). The most extensively examined markers are fibrinogen, C-reactive protein (CRP), white cell count (WCC), and IL-6. All of these markers are acute phase response associated, and hence circulating levels reflect the current inflammatory state of the individual, WCC and CRP being the routine clinical measures of inflammation. They have been used in epidemiological studies to examine the hypothesis "elevated baseline inflammation predicts risk of CVD."

Meta-analysis of prospective studies in generally healthy populations has shown that WCC (Wheeler et al, 2004), CRP (Danesh et al, 2004), and fibrinogen (Fibrinogen Studies Collaboration, 2005) are risk predictors for CHD. Based on 6944 first non-fatal MI events, a 1g/L increase in circulating fibrinogen is associated with an OR of 2.42 (95% CI 2.24 – 2.60), which once adjusted for most conventional risk factors is attenuated to OR 1.80 per 1g/L increase (Fibrinogen Studies Collaboration, 2005). CRP has been promoted as a risk marker in cardiovascular disease, and much publication space has been devoted to this, particularly from the USA ([1]Verma et al, 2005). Recent meta-analysis (Danesh et al, 2004) has shown however that after adjustment for the baseline values of established risk factors, the OR for CHD was 1.45 (95% CI 1.25 - 1.68) in a comparison of participants in the top third of the group with respect to baseline CRP values with those in the bottom third. There is however evidence of publication bias in early smaller studies that overestimated relative risk (Danesh et al, 2004). Regression to the mean over subsequent studies has seen the OR approach a lower value. Publication

bias is an important confounding factor in the epidemiological study of newer risk markers.

CRP level measurements have been suggested to add to established risk factors for individual risk prediction for cardiovascular prevention in the USA (Pearson et al, 2003) but not in Europe. The conference that debated this decision in the USA assessed several potential inflammatory markers to be added to Framingham risk assessments. The criteria for marker consideration included: (1) the ability to standardize the assay and to control the variability of the measurement; (2) independence from established risk factors; (3) association with CVD clinical end points in observational studies and clinical trials; (4) the presence of population norms to guide interpretation of results; (5) ability to improve the overall prediction beyond that of traditional risk factors; (6) generalization of results to various population groups; and (7) acceptable cost of the assays. (Pearson et al, 2003). Several other markers were considered possible candidates as inflammatory risk markers in this debate (including WCC, fibrinogen and IL-6), but it was decided that CRP best-fitted the above criteria, and was generally the most comprehensively studied of the markers. (Pearson et al, 2003).

1.2.5 Are CRP and Fibrinogen Useful as Clinical Prognostic Markers?

Some authors question the validity of adding inflammatory markers to clinical risk assessment protocols (Lowe, 2005; Lowe and Pepys, 2006; Lloyd-Jones et al, 2006). Generally in epidemiological studies of these markers they have univariable (unadjusted) ORs for CHD of 1.5 - 3.0 in the top third of the population versus bottom third of the population distribution, and then the risk association attenuates to around 1.0-1.5 upon adjustment for classical risk factors. Although a 50% increase in risk above conventional risk factors sounds impressive, a recent study (Pepe et al, 2004) suggests that when a marker is considered concomitantly with classical risk factors, it needs a multivariable OR of 3.0 in order to improve the C-statistic (area under the receiver-operator curve) by an additional 5% or more, which appreciably increases the risk assessment capability. Indeed, as considered by Lloyd-Jones et al (Lloyd-Jones et al, 2006) assuming that the correlation between CRP levels and the existing Framingham risk score is $r = 0.3$ (Albert

et al, 2003) a multivariable OR of 3.4 in top versus bottom quartiles of CRP distribution would be required to improve the C-statistic from 0.80 to 0.85. Neither CRP, fibrinogen, nor any other novel risk factor has been reported to attain that independent level of risk association.

In fact, when considering recent publications, CRP seems to add very little to Framingham risk assessments. The Atherosclerosis Risk in Communities study (ARIC) examined incident CHD in 15792 people followed for 20 years and examined 19 novel as well as all conventional risk factors (Folsom et al, 2005). A basic risk factor model (age, race, sex, total and HDL cholesterol, systolic BP, antihypertensive medication, smoking and diabetes) gave a C-statistic of 0.8 (that is 80% of CHD events were detectable). Including CRP only added 0.003 to this figure, and other novel markers did not add greater than 0.011 (Folsom et al, 2005). This makes sense considering the close association of CRP with conventional risk markers. One study has shown in 15341 people that high levels of CRP (>3mg/L) were attributable to conventional CHD risk factors in 78% of men and 67% of women ([2]Miller et al, 2005). These results were confirmed in the Framingham study, showing that traditional risk factors had a C-statistic of 0.78 for CVD events, a value unchanged by the addition of CRP ([1]Wilson et al, 2005). Subsequently Ridker's group published data on the Women's Heath Study (WHS) (Cook et al, 2006) arguing that CRP adds accuracy to classifying women by stratification of risk (i.e. 5%-10%, or 10-20% risk over 10 years) by reassigning ~20% of women to more accurate categories. However, the model used only incorporated age, BP and smoking as conventional risk factors, and examining the women in "categories" of risk loses statistical information, even if the model reflects clinical assessment.

There is further debate as to whether CRP or fibrinogen could be used as "tie-breakers" in order to categorise patients at the threshold of being classified as at high or moderate risk, a theory currently being tested by the Jupiter (The Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin) Trial ([1]Ridker, 2003; Gotto, 2007). In the WHS Cook et al (2006) point out that when using the improvement in C-statistic as the criteria for use as a clinical prognostic marker, cholesterol measurements (total, LDL or HDL) actually add similarly little value to the

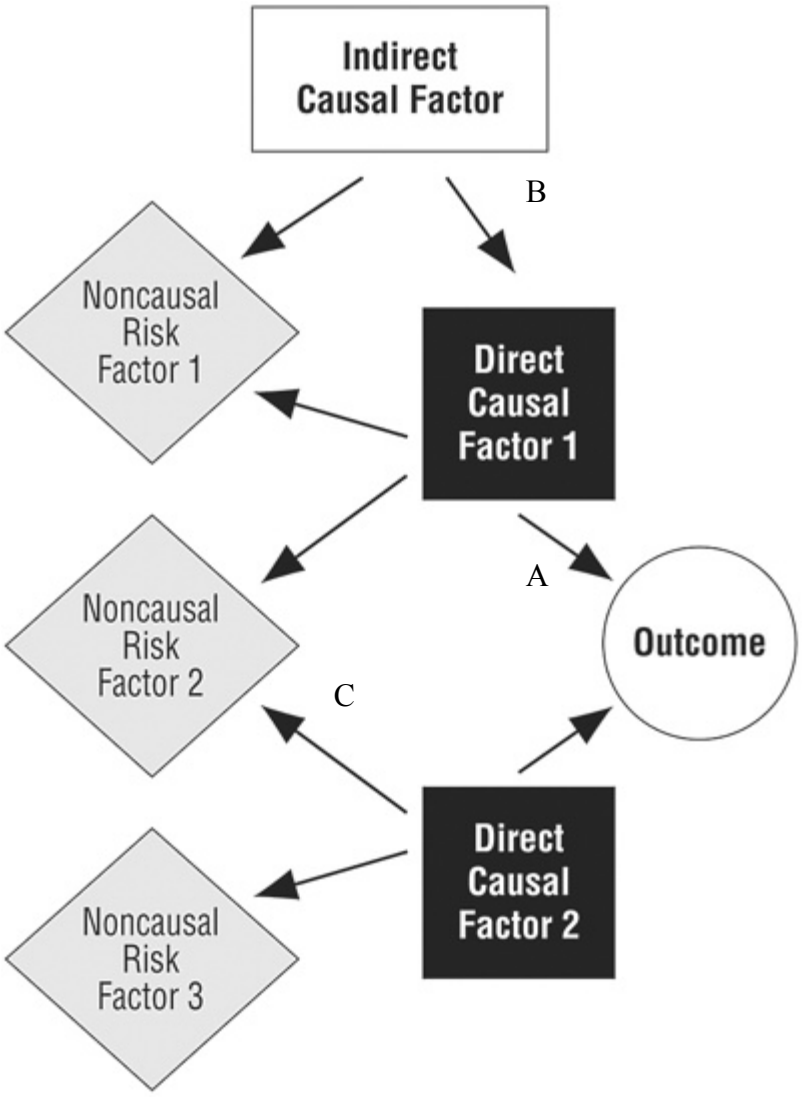
Framingham assessment as CRP (although it is worth noting only in their age/BP/smoking/unisex model). So then why include measurements of cholesterol and not CRP? The logical answer is that cholesterol is known to be directly causal in risk of CHD (through atherogenesis) whereas evidence of inflammatory markers playing causal roles in atherogenesis/thrombosis is less compelling. Using statins to lower cholesterol level will reduce the risk of CHD even if other risk factors are absent. Hence cholesterol is not only causal, but modifiable (either through lifestyle or therapeutic interventions), and so inclusion in risk assessment is rational.

1.2.6 Problems in Implicating Causality for Risk Factors

The argument for measurement of inflammatory markers such as fibrinogen and CRP in CVD risk assessment now begs the question "are they causally involved in chronic atherogenesis and/or acute atherothrombotic events, and hence the risk of CHD?"

One of the problems associated with epidemiological studies is that observations of association in themselves cannot be used to infer causality (Lowe et al, 2005). Hypothetical illustrations of problems encountered in epidemiologically implicating a factor as causal are shown in Figure 1.8.

Figure 1.8 An illustration of the distinctions between 3 types of risk factors. Arrows denote causation. Bidirectional arrows, which may exist in real life, are not included, and this example does not include all possible relationships. (From Brotman et al, 2005).



From Figure 1.8, it is evident that directly causal factors are invariably associated with a biological outcome (A), presuming they can be accurately measured. However, in complex biological systems these causal factors can often have their own causal factors in turn, which may also be associated with the biological outcome, so they can be said to be indirectly causal (B). In addition both direct and indirect causal factors may promote distal noncausal factors, which may then also be associated with outcome, but only by co-incidence (C). Adding further complexity (and not illustrated in the figure) there are often interactions between these types of factors and also reverse interactions, which result in confounding. In many studies of populations with prevalent disease the biological outcome itself may result in altered levels of risk factors (reverse causation bias), and since in most studies of CVD there is at least subclinical atherosclerosis even in healthy populations, reverse causation bias cannot be ruled out. Complex biological systems mean that empirical observations in epidemiology cannot, in isolation, infer causality, and there is therefore a need for evidence-based approaches as well.

There are 3 main ways to study causality of a hypothetical risk factor

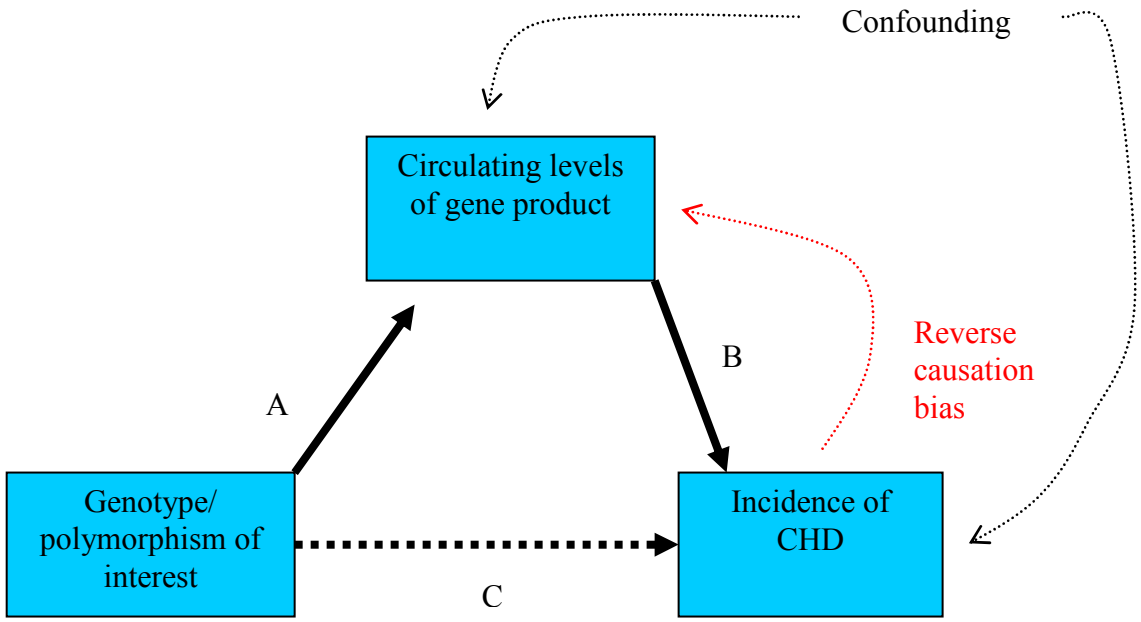
1. "Experimental" animal and pathology studies of molecular and cellular interactions.
2. Genetic epidemiological studies coupled with molecular epidemiology observations - "Mendelian randomisation studies."
3. Randomised, double-blinded, placebo-controlled trials using specific therapeutic inhibitors.

Experimental animal studies are useful in that they are scientifically cleaner because of the potential to remove confounding factors in elegant experiments. However, whilst they attempt to model disease, they are unlikely to replicate it. Tissue studies are usually done *in vitro* or *in situ* and hence are limited, and since animals are physiologically and biologically distinct from humans they often experience disease, especially disease brought about through genetic aberrations or experimental stimulation in a different way to humans.

Mendelian randomisation studies are an epidemiologically based method of inferring causality, and take advantage of the randomized inheritance of genes, which are not confounded by external biological or sociological factors ([1]Davey Smith and Ebrahim , 2005). In these studies inheritance of functional polymorphisms of genes of interest (such as promoter polymorphisms of CRP or fibrinogen) are studied in relation to the risk of CVD or CHD. As such these polymorphisms provide a background for a "natural" randomized, controlled trial of exposure, assuming the polymorphism has a known and quantifiable biological effect on the action or circulating level of the gene product, and that circulating levels of that gene product are associated with CHD outcome. If then the genotype is shown to have influence on CHD incidence in prospective studies, then there is triangulation, and strong implication of causality (see Figure 1.9) (Davey Smith and Ebrahim, 2004, [1]Davey Smith and Ebrahim 2005).

The gold standard to infer a factor as being causal is a randomised double-blinded placebo controlled trial of a drug which *specifically* targets the marker in question e.g. studies suggest stains lower cholesterol levels and risk of CHD in any population. This level of causal proof has not yet been achieved with any inflammatory marker in CHD risk

Figure 1.9 Random inheritance of alleles is independent from reverse causation bias, or external confounders seen in other areas of epidemiology. Complete triangulation in molecular (B) and genetic (A) risk association observations provides strong evidence of causality, whilst no association between genotype and CHD incidence (C) would implicate confounders and reverse causation underlying observation B. Adapted from [1]Davey Smith et al, 2005.



1.2.7 Are CRP and Fibrinogen Causal in CHD?

CRP is highly evolutionarily conserved with homologous proteins in all vertebrates, indicating an important physiological role. Human CRP binds with high affinity to phosphorylcholine residues and other constituents of micro-organisms, but intrinsically it also binds to modified lipoproteins (oxLDL) (Pepys et al, 1985; Chang et al, 2002), and various particles related to necrotic and apoptotic cells (Pepys et al, 2003, and 2005; Chang et al, 2002). It is thought to opsonise all these particles through activation of the classical complement pathway (Mold et al, 1999) and hence mediate innate defense and help prevent autoimmunity. It therefore may play a role in promoting the development of foam cells through the opsonization of modified LDLs in the endothelial wall. CRP is also reported to be a procoagulant (Lagrand et al, 1997; Singh et al, 2005), to mediate endothelial dysfunction (Yaron et al, 2006), and to behave in a cytokine-like manner. This seems inherently unlikely, as no other cytokine is thought to be so variable in expression (up 10,000-fold circulating increases) and indeed would be dangerous in the uncontrolled inflammation they would elict if they were (Pepys et al, 2003).

Animal models of CRP action have thus far proven problematic. Serum amyloid P component (SAP) is the major acute phase reactant in mice, and in rats native CRP does not activate complement, and although transgenic or extrinsic human CRP can be used to activate complement in rats, studies using these animals are heavily artificial (de Beer et al, 1982; Pepys et al, 2003). Earlier studies suggested that addition of extrinsic CRP to rodent models exacerbated atherosclerosis, but more recent models have shown that moderately elevated CRP levels in ApoE*3-Leiden transgenic mice do not have any effect on early atherogenesis (Trion et al, 2005) and furthermore, injection of even enormous doses of purified human CRP into mice and rats neither elicits inflammation nor produces any clinical ill effects (Clapp et al, 2005). Indeed, it is now believed that earlier inferences that CRP *in vitro* directly induced endothelial activation and other pro-inflammatory properties were due to azide and LPS contamination in commercial products (Taylor et al, 2005; Pepys et al, 2005). Nevertheless, Pepys et al (2006) have shown that a small human CRP antagonist reduced the area of myocardial infarcts in an animal model of MI. The relevance of this to rodent and human atherosclerosis remains

to be determined further studies, although the potential epidemiological implications of being able to use this CRP antagonist in human double blinded placebo-controlled trials are obvious (Pepys et al, 2006).

Evidence of a causal link for fibrinogen in atherosclerosis is mixed. Intuitively, fibrinogen may have a role in the development of atherothrombosis in established atherosclerosis since it is a haemostatic factor as well as an inflammatory marker as an APR protein. Fibrinogen may directly increase CHD risk because of its role in platelet aggregation, increasing plasma viscosity, and fibrin formation (Kannel, 2005) and hence may partially mediate the thrombogenic effect of other environmental risk factors. For example, epidemiological studies show that fibrinogen levels correlate with the number of cigarettes smoked and fall after smoking cessation ([2]Wannamethee et al, 2005), and fibrinogen decline may be a mechanism for CHD risk reduction following smoking cessation (McCallum, 2005).

In molecular and animal studies, fibrinogen and its degradation products may promote expression of proinflammatory cytokines such as IL-6 and IL-1β (Lee et al, 1999; Perez et al, 1999). Despite this, mouse models show that transgenic hyperfibrinogenaemia has no effect on diet induced atherogenesis in C57/B6 or ApoE KO mice (Gulledge et al, 2003; Rezaee et al, 2002). Further analysis of these mice show that they do have augmented fibrin formation and degradation, altered coagulation cascade function, and altered vascular response to injury (Kerlin et al, 2004) hence perhaps alluding to a role for fibrinogen in thrombosis rather than atherogenesis.

Meta-analyses of Mendelian randomized trials have so far failed to implicate either CRP ([2]Davey Smith et al, 2005; Timpson et al, 2005; Casas et al, 2006) or fibrinogen (Keavney et al, 2006) as being causal agents in increasing the risk of CHD. This must be considered in light of known limitations of these new studies, which include that the study must be sufficiently powered to pick up small hypothetical increases in OR often associated with small increases in levels of markers from polymorphisms in promoters, and that the genetic biology of the markers (including potential linkage disequilibrium confounders) should be well recognised (Davey Smith and Ebrahim, 2004; Davey Smith

et al, 2003). Canalisation of gene carriers is also a potential confounder. Although sound in principle, these studies have yet to prove consistently useful tools in epidemiology.

Much has recently been published about the pleiotropic effects of statins, particularly their seeming ability to dampen inflammation (Schonbeck et al, 2004). Some authors have ascribed this alternative function to being a "CRP lowering" function, and speculate that this CRP lowering provides some evidence of a causal role of CRP in CHD risk ([2]Ridker, 2003). While it is true that statins lower CRP levels concomitantly with cholesterol (Jain and Ridker, 2005), this is largely due to inhibition of activation of nuclear transcription factor kappa b (NFκB), and activator protein-1 (AP-1) which are responsible for aiding transcription of many pro-inflammatory cytokines including CRP (Blanco-Colio et al, 2003; Jain and Ridker, 2005). Since this protective effect is both immunosuppressive and immunomodulatory in a multi-factorial manner however, lower incidences of acute CHD cannot be attributed to CRP level reduction. Hence attempts to disentangle influences of statins on CRP and CHD outcomes in the PROVE-IT (PRavastatin Or atorVastatin Evaluation and Infection Therapy) trial (Ridker et al, 2005) are essentially impossible.

Incidental evidence of a non-causal role for fibrinogen and CRP in CHD also exist; there is a lack of specificity linking them with human CHD in observational studies. As markers they also predict risk of all-cause and non-vascular mortality, and with an OR almost identical to observed vascular risks (Fibrinogen Studies Collaboration, 2005; Lowe, 2005; Mendall et al, 2000; [1]Lee et al, 2006). Inflammatory markers such as CRP and IL-6 have also recently been shown to correlate with a decline in cognitive function in the elderly in the Edinburgh Artery Study, although this could arguably be mechanistically linked to vascular disease through stenosis and silent ischaemic events (Rafnsson et al, 2007). Lack of CVD risk specificity argues against a causal role, since elevated circulating levels of markers may be attributable to lifetime exposure to adverse risk factors for diseases that may or may not be cardiovascular related (Lowe, 2005)

1.2.8 IL-6 and CHD Risk Prediction

The literature therefore appears to confirm that both CRP and fibrinogen are markers of CHD risk, but has not as yet convincingly causally implicated either marker. Since both of these inflammatory markers are proteins of the acute phase response, it stands to empirical reason that stimulators of the acute phase response may also be risk markers (at the very least due to indirect associations with acute phase reactants). In this regard the pro-inflammatory cytokine IL-6 has recently been extensively examined in the literature. In the interest of putting other inflammatory markers measured in this thesis into context, a brief overview of IL-6 and its associations with CVD are included here.

IL-6 is a 26 kDa pleiotropic cytokine mainly produced by endothelial cells, macrophages, adipocytes and lymphocytes (Le et al, 1989; Kerr et al, 2001). As mentioned previously, IL-6 is implicated as a pivotal APR-inducing cytokine in IL-6 KO mice, although there is evidence it is not exclusively responsible for this (Fattori et al, 1994; Kopf et al, 1994). In addition to its role in the APR it is thought to stimulate haemostasis and influence T- and B-cell differentiation (Kerr et al, 2001) as well as stimulating endothelial cells to produce chemokines (in complex with soluble IL-6 receptor [sIL-6R]) (Romano et al, 1997) and plays a role in the transition between acute and chronic inflammatory responses (Gabay, 2006).

Mouse models indicate that exogenous addition of IL-6 exacerbates atherogenesis, indicating a role for IL-6 (Huber et al, 1999) through the mechanisms described above. Despite this IL-6 KO models indicate that basal physiological levels of IL-6 may be required to maintain plaque integrity (Schieffer et al, 2004; Song et al, 2004). This observation may be consistent with some of the condition-specific pro- versus anti-inflammatory actions of IL-6 (Kerr et al, 2001; Gabay, 2006).

In epidemiology, major correlates of IL-6 are age, smoking and acute phase response markers (Woodward et al, 1999). A recent meta-analysis of 10 available prospective studies (Harris et al, 1999; [1and2]Ridker et al, 2000; Volpato et al, 2001; Jenny et al, 2002; Pradhan et al, 2002; Luc et al, 2003; [2]Lowe et al, 2004; Pai et al, 2004), including 2089

cases of non-fatal MI or CHD death, demonstrated that men in the top third of the population for IL-6 expression had an OR 1.67 (95% confidence intervals [CI] 1.35 – 2.05) independent of conventional risk markers (Mann et al, submitted). This does not include more recent data, such as that from the Edinburgh Artery Study (Tzoulaki et al, 2007), although the data is again in broad agreement with the meta-analysis; OR 1.85 (1.33 to 2.58) in the top versus bottom tertile in a fully adjusted model.

In the same study (Mann et al, submitted), a meta-analysis of the well studied IL-6 promoter polymorphism -174 G/C was performed, since this allele is generally thought to be associated with elevated circulating concentrations of IL-6. In this analysis, and in contrast with some published literature, no association between the SNP and circulating levels of IL-6 was observed (in 9 studies with 5871 participants) and unsurprisingly nor was there any association with CHD risk. Hence IL-6, like fibrinogen and CRP, is associated with atherogenesis in some molecular and animal models, and is associated with CHD risk in epidemiological studies to a similar extent.

1.2.9 Rationale for the Study of Novel Risk Markers:

Having examined the evidence currently available for the role of inflammation in vascular disease and event risk prediction, several points can be made. Low-grade inflammation is thought to have a role in the development of atherosclerosis, and is likely to be causal on some level in atherogenesis and subsequent propensity to plaque rupture. Further weight is given to this argument by the observation that people with high-grade systemic Th1 autoimmune responses (such as systemic lupus erythematosus [SLE], insulin dependant diabetes mellitus, and rheumatoid arthritis [RA]) experience accelerated atherosclerosis and propensity to cardiovascular events compared with healthy controls with similar risk factors (Sattar and McInnes, 2005; Sherer et al, 2006). Indeed, the risk of myocardial infarction in women with RA was twice that of women without RA in the Nurses' Health Study (Solomon et al, 2003). Circulating markers of the acute phase response predict risk of vascular disease and consequent vascular events, but have not been shown to be mechanistically causal in a convincing way. In the absence of pharmaceutical therapies to specifically lower individual acute phase reactants, this

debate cannot currently be resolved by double-blinded placebo controlled trials. In addition these markers may lack the predictive capabilities required to add information by simultaneous determination with the Framingham assessment (conventional risk markers).

There is a rationale then to examine novel markers of inflammation as risk factors for cardiovascular events, provided there is existing pathological/animal data indicating they have roles early on in the development of an inflammatory response and are thought to have a strong causal mechanism in the development of atherosclerosis and/or propensity of established plaques to rupture. Ideally, these markers should not be involved in the APR as a "primary" function; all inflammatory markers are of course in some way linked to the APR, but information is required regarding markers further upstream of the APR. I hypothesized that, in particular, inflammatory markers implicated in the classical Th1-type response characteristic of an atherogenic environment, and highly expressed in diseases such as RA, would be associated with risk of incident CVD. Since the thesis aims to add information to the debate on causality of inflammation in CVD any markers it considers would, ideally, be potential future therapeutic targets.

From these criteria I constructed a short-list of possible markers for examination:

- Interferon γ (IFNγ)
- Tumour necrosis factor α
- Interleukin (IL)-1β
- IL-12
- IL-18
- IL-8
- Matrix metalloproteinase (MMP)-9
- Soluble CD40 ligand (sCD40L / sCD154)

IFNγ is a cytokine, produced by activated Th1 cells, which is a powerful stimulus for macrophage activation, resulting in IL-12 production to form a positive feedback loop,. Both these processes are important in atherosclerotic development (Gupta et al, 1997;

Lee et al, 1999). Neither cytokine, however, is easily detectable in the circulation of normal healthy people, and hence are unsuitable for population-based stuides. Similarly, IL-1β is a potent pro-inflammatory cytokine expressed early in an inflammatory response, and IL-8 is an important macrophage chemokine, but neither of which are detectable in the majority of healthy people.

Of the other four markers, all have a generally strong putative role in risk of CVD events, but the literature is inconclusive. IL-18 shares structural homology with IL-1β and functional homology with IL-12 and plays an important role in RA, and so was considered an interesting possible marker. TNFα is an early pro-inflammatory cytokine and like IL-1β, is also important in RA and perhaps the metabolic syndrome, and recent development of a high sensitivity assay allows detection of circulating concentrations in normal people (<1pg/ml). MMP-9 may play a role in plaque rupture, and sCD40L has an enigmatic role in the circulation, but appears to be associated with atherogenesis.

In light of this, and the data reviewed below, I set out to examine TNFα, IL-18, MMP-9, and sCD40L as markers of CVD risk. Brief literature reviews of each marker are included in which a general overview of the expression, regulation and biological function of the marker is given before considering the potential causal roles of the markers in atherogenesis, and existing epidemiological data on each. The aims of the thesis are discussed at the end of this chapter.

1.3 TNFα LITERATURE REVIEW

1.3.1 Introduction:

TNFα has a long scientific history associated with CVD. For more than 2.5 millennia it has been recognised that patients with end-stage chronic heart failure (CHF) share clinical features with individuals with neoplasia or inflammatory disorders (Katz, 1962). Clinical wasting of these patients was a puzzling phenomenon, which was thought to be symptomatic of the body consuming itself, worsening the condition. In the mid-1800s

Bruns reported regression of tumours in humans following some bacterial infections (Bruns et al, 1868). It was demonstrated that these effects were caused by an inducible serum factor mediator, concomitantly named tumour-necrotizing factor (O'Malley et al, 1962) which was later re-named tumour necrosis factor (TNF) (Carswell et al, 1975). Following this discovery, the issue of cardiac cachexia syndrome was eventually resolved (Levine et al, 1990) when it was shown that TNFα was responsible for mediating the symptoms in the different conditions.

TNFα is now recognised as being a pluripotent cytokine, and the mechanisms of many of its biological activities are still not clearly understood. It seems to be capable of inducing diverse, and at times, contradictory physiological effects depending on the experimental setting. It is known that TNFα can cause apoptosis, septic shock, inflammation and cachexia systemically (Beutler et al, 1988). Yet, in contrast, it can induce growth through mitosis, it immunologically protects the host, and may also help induce obesity by metabolic mechanisms (Ferrari et al, 1999).

1.3.2 TNFα Expression, Synthesis, and Regulation

The TNFα and TNFβ genes are single copy genes located on the short arm of human chromosome 6 near the MHC region. TNFα is produced by neutrophils, activated T and B cells, NK cells, macrophages, astrocytes, SMCs, endothelial cells, adipose tissue, and some transformed cells whereas TNFβ is restricted to production by lymphocytes (Ruddle, 1992)

Human TNFα is a polypeptide of 157 amino acid (Ruddle, 1992) and the biologically active form of TNFα has a homotrimeric structure that is the hallmark of the TNF superfamily (Jones, 1989). TNFα, unlike TNFβ, has no signal peptide sequence but is initially manufactured as a larger peptide with an N-terminal sequence containing hydrophilic and hydrophobic domains. It is hence expressed in a membrane bound form initially (Luettig et al, 1989) and is biologically active in this form, physiologically acting in a lytic manner (Perez et al, 1990). It is currently thought that membrane bound and the

soluble proteolytically cleaved (possibly via TNFα converting enzyme protease– TACE) TNFα have distinct in vivo roles (Idress et al, 2000).

Most inflammatory settings result in an early local upregulation of TNFα production, which helps establish an innate immune response. In the acute stages of inflammation TNFα is known to largely regulate itself in an autocrine fashion (Hensel et al, 1987 and Xaus et al, 2000) although it also induces specific negative regulatory elements to maintain tight control of its own production (Carballo et al, 1998). TNFα also initiates the production of many other macrophage and DC derived cytokines, particularly IL-1β and IL-8 ([1]Papadakis et al, 2000).

In most classical hypothetical immune responses it is the presence of the highly conserved pathogen associated molecular patterns (PAMPs) that initiates the immune response through stimulation of TLRs, and results in the production of early TNFα and IL-1β (Akira et al, 2003). This begs the question of what the early stimulus is for the production of cytokines at a developing fatty streak in the arteries. Jovinge (Jovinge et al, 1996) originally demonstrated that human monocytes and macrophages become activated and release TNFα in response to oxLDL, probably through LOX-1 and SR-A mediated uptake. An interesting twist in these results came from results showing that early modified LDLs (not fully oxidized mmLDLs) are recognised by TLR4 and induce signalling and responses superficially similar to LPS, including TNFα production ([1]Miller et al, 2005). Theoretically, the TLR4 signalling would provide a potent stimulus for the initial and sustained TNFα expression whilst local levels of mmLDLs and oxLDLs are elevated in the vessel tunica intima underlying the disrupted endothelium.

1.3.3 TNFα Receptors and Signalling

TNFα has two functional receptors, the p55 TNFR1 and the p75 TNFR2. These receptors display limited extracellular homology and almost no intracellular homology, alluding to distinct signalling mechanisms and consequences (Idriss et al, 2000). TNFR1 is almost ubiquitously expressed, whereas TNFR2 has more restricted expression on cells with

haematopoetic origin (MacEwan et al, 2002). TNFα homotrimer binding to p55 or p75 results in clustering and cross-linking of the TNFRs on the cell surface (Idriss et al, 2000). TNFα signalling is excellently reviewed elsewhere (MacEwan et al, 2002 and Aggarwal, 2003) and is too complex and controversial an issue to describe in depth here.

Membrane bound TNFα can also act as a receptor as well as a ligand for soluble p55 (and perhaps p75) and this "reverse-signalling" has physiological consequences (Eissner et al, 2000 and Waetzig et al, 2005). The level of complexity implicated by two functional TNFRs (soluble and membrane bound), two functional ligands (also, soluble and membrane bound), a range of *in vitro* versus *in vivo* phenomena, as well as a staggering array of complex and partially understood signalling and reverse signaling has meant slow development in this field, and generally it is probably appropriate to focus on the larger physiological picture of TNFα than the molecular detail.

1.3.4 General Biological Effects of TNFα

The many and varied biological effects of TNFα are likely to be caused by a variety of restrictive signalling factors such as TNFα concentration, whether the TNFα ligand is soluble or membrane bound, other interacting cytokines in the microenvironment milieu, target cell expression of TNF receptor(s), target tissue specific expression of adaptor molecules and kinases, and target tissue specific epigenetic configuration of promoter regions (Goeddel, 1999).

Systemically, TNFα effects can be categorized in two manners that reflect the general inflammatory response; the acute and chronic phases. A high grade acute response with excessive TNFα levels can lead to systemic vasodilation and a consequential drop in blood pressure leading to clinical shock and multiple organ failure as well as (at slightly lower concentrations) fever, disseminated intravascular coagulation, gastrointestinal and acute renal tubular necrosis, vascular leakage syndrome, adrenal haemorrhage and catabolic hormone release (Tracey et al, 1994). Low-grade chronic exposure can lead to weight loss (change in metabolic rates), protein catabolism, hepatosplenomegaly, subendocardial inflammation, insulin resistance, enhanced tumour metastasis, endothelial

activation and acute-phase protein production by hepatocytes (Tracey et al, 1994). As one would expect from the hypothesis of plasma TNFα as an underlying causal agent in atherogenesis, some of these symptoms may be consistent with the systemic development of atherosclerosis. In support of this acute versus chronic TNFα categorization, an interesting study has suggested that mouse cardiac tissue can produce different isoforms of the NF−κB transcription factor with divergent effects dependant on the exposure time to TNFα (Haudek et al, 2001).

TNFα is regarded as a pivotal pro-inflammatory cytokine in "normal" physiological function. In mouse knock-out models it has been shown that p55-null mice are moderately resistant to LPS induced shock but succumb quickly to *L.Monocytogenes* infection (Pfeffer et al, 1993) whilst p75-null are also resistant to TNFα induced shock (Erickson et al, 1994). These examples demonstrate the potential importance of the cytokine in fighting infection, but also demonstrate the other side of the coin where inappropriate and excessive expression of the cytokine can be directly linked pathophysiology in the host. In line with the latter, TNFα has been implicated as pivotal in many human Th1 type autoimmune responses such as rheumatoid arthritis (Maini and Taylor, 2000) diabetes (Rabanovitch, 1998) and IBD (Crohns disease) ([2]Papadakis et al, 2000). It is interesting that these autoimmune conditions (notably RA) have overlapping risk factor clustering with cardiovascular disease. TNFα has been cited as a potential common causative immunopathogenic factor across these disease states.

1.3.5 Pathological and Animal Studies of TNFα in CVD

There is a wealth of literature on the role of TNFα in CHF. The myocardium itself has the potential, under mechanical stress and volume overload, to produce large quantities of TNFα and TNFRs p55 and p75, whilst there is no biosynthesis in the non-failing heart (Torre-amione et al, 1999). Mice with targeted over-expression of TNFα develop left ventricular (LV) dilation (Kubota et al, 1997), characterized by disproportionate thinning of the left ventricle due to myocardial necrosis, adversely affecting cardiac diastolic function in the long-term. This phenomenon of LV dilation that is thought to involve

time-dependant changes in the MMP/TIMP regulation of extra cellular matrix ([1]Li et al, 2000). In addition to this, TNFα induces negative inotropic effects on cardiac cells. Cardiocyte shortening is substantially depressed when exposed to TNFα at graded Ca2+ concentrations through a mechanism that is thought to involve elevated intracellular Ca2+ concentrations, hence impairing flux (Yokoyama et al, 1993). This impaired contractility would be devastating in an already failing heart. Furthermore, TNFα has been implicated in the production of pathological concentrations of NO, as well as directly causing apoptosis in myocytes and endothelial cells (Panas et al, 1998; Finkel et al, 1992), both contributing to impaired cardiac and vascular function. Clearly, these conditions may overlap with atherogenesis in terms of prevalent pathologies in many patients with chronic vascular disease.

There is strong molecular evidence for a role of TNFα in the development of atherothrombosis. Circulating TNFα is present in the vasculature at baseline in all individuals, although it does increase with age (Paolisso et al, 1998) and blood vessels themselves from older mice produce increased levels of TNFα in response to stimulation (Belmin et al, 1995). TNFα can also be detected in many human atheromas (Barath et al, 1990). It is produced in murine and in human atherosclerotic lesions, primarily by macrophages/foam cells, activated T-cells, smooth muscle cells, and endothelial cells (Barath et al, 1990; Kaartinen et al, 1996; Reckless et al, 1999). On a local level within the lesion per se, TNFα has the potential to promote cellular infiltration to the plaque via endothelial activation (Berk et al, 2001) and may induce endothelial dysfunction (Picchi et al, 2006). It also promotes the production of other cytokines as well as chemokine expression (Zhao et al, 2003), the expression of matrix metalloproteinase-9 (Saren et al, 1996) (hence increasing plaque instability) and it can promote angiogenesis (Leibovich et al, 1987). The main potential proatherogenic effects are summarized in Table 1.3.

Despite this strong putative molecular role for TNFα in atherogenesis and CVD, *in vivo* mouse models report evidence both for (Elhage et al, 1998; Niemann-Jonsson et al, 2000; [1]Li et al, 2000; Branen et al, 2004; Canault et al, 2004) and against (Schreyer et al, 1996; Reckelss et al, 1999; Hansen et al, 2001; Schreyer et al, 2002) a causal role. Schreyer et al (1996) originally reported that C57Bl/6J mice lacking the p55 TNFR had exacerbated

lesions in the aortic sinus, 2.3 times larger than wild types. This counter-intuitive result was accompanied by excessive SR-A expression by macrophages, thought to be symptomatic of the fact that TNFα modulates scavenger expression. The same group later published results suggesting that lymphotoxin, but not TNFα, was required for 'normal' wild-type lesion development in a KO model (Schreyer et al, 2002). These results have been refuted in similar models by different groups. Indeed, the model used was recently re-assessed (Canault et al, 2004) and it was found that soluble TNFα was required for normal lesion development in the aortic sinus. In agreement with a vital role for TNFα in atherosclerosis, Branen et al (2004) demonstrated in ApoE KO mice that whether they restricted TNFα action by TNFR1 pellet administration, by conditional TNFα KO, or by total TNFα KO, mice exhibited diminished lesion development relative to a WT ApoE mouse (50-83% reduction in lesion size, range dependant on model). These discrepancies illustrate the caution that must be employed when considering knock-out models. Differences are probably partly to do with varying models, and partly due to the complex nature of TNFα biology. It has recently been confirmed in the ApoE KO model (more widely studied than the Schreyer et al model), that it is p75 that is required for normal atherogenesis (Chandrasekharan et al, 2007). Overall, the animal and tissue evidence seems to point towards an important role for TNFα in atherogenesis.

Table 1.3 Illustrative table of potential direct and indirect proatherogenic stimulators and processes induced by TNFα expression in experimental settings

Inflammatory stimuli produced	Molecule	Major biological property and cellular source	Reference
Cytokine	IFNγ	Inhibition of SMC production of collagen/TIMPS. Produced by Th1 cells/macrophage	Mytar et al, 1995
	IL-1β	Activation of all major inflammatory cells/endothelium. Pyrogen. Produced by innate cells	Marucha et al, 1991
	IL-6	Induction of APR. T-cell differentiation. Produced by macrophage and T-cells	Ng et al, 1994
Chemokine	RANTES	T-cell/monocyte recruitment via CCR1/3/5. Produced by endothelial cells and monocytes	Hiranoet al, 2003
	IL-8	Neutrophil/T-cell recruitment via CXCR1/2. Produced by activated macrophages	[1]Papadakis et al, 2000
	MCP-1	Recruitment of monocytes/ T-cells via CCR2. Produced by endothelial cells	Murao et al, 2000
Selectins/ integrins	ICAM-1	Endothelial arresting adhesion of LFA-1/Mac-1 expressing PBMCs. Expressed on activated endothelium	Raab et al, 2003
	E-Selectin	Endothelial rolling adhesion of Lewis^x Ag expressing PBMCs. Expressed on activated endothelium	Raab et al, 2003
Metabolism	Triglycerides	Increased circulating triglyceride-rich lipoproteins in blood. Produced by hepatocytes.	Feingold et al, 1989
	Decreased Lipoprotein lipase	Increased free circulating fatty acids and hence increased triglycerides produced by liver	Kawakami and Cerami, 1981
Coagulation	PAI-1	Decrease turnover of spontaneous clots by plasminogen. Produced by endothelium	Schleef, 1988
	Tissue factor	Increased thrombotic potential after erosion or rupture in plaque. Produced by endothelium.	Esmon, 2004

Table 1.3 Continued

	Decreased Thrombomodulin	Limits protein C activation and promotes thrombus. Acts on endothelium	Conway and Rosenberg, 1988
Other	MMPs	Collagen turnover and destabilisation of venerable plaques. Produced by foam cells	Rajavashisth et al, 1999
	Heparin –binding Epidermal growth factor-like growth factor	Increased mitogenic potential of SMCs in vasculature produced by macrophages	Yoshizumi et al, 1992

1.3.6 TNFα and CVD in Genetic Epidemiology Studies

In terms of genetic epidemiology, TNFα is a very well studied marker. There are eight known TNFα promoter functional SNPs, and these have been investigated in a variety of diseases, including RA, ankylosing spondylitis, multiple sclerosis, asthma, and CVD (Bayley et al, 2004). Associations with disease and risk factors have so far been variable. For instance, the fairly common -308G/A polymorphism may be associated with increased transcription of the TNFα gene *in vitro* (Wilson et al, 1997). The polymorphism has been suggested to associate with metabolic markers, such as body fat and the hormone leptin levels (Fernandez-Real et al, 1997; Herrmann et al, 1998).

The less frequent polymorphism -238G/A is in complete linkage disequilibrium with -308, and was shown to be associated with insulin resistance (Day et al, 1998), and associated with the risk of MI in a retrospective study of 1213 cases (Bennet et al, 2006) (although the-308 polymorphism was not itself significantly associated with any factor in either of these studies). Another study failed to find either polymorphism associated with metabolic factors (Koch et al, 2000). In a further study of 641 Caucasians there was no association of the -308 SNP with the number of significantly diseased vessels (>50% occlusion), or angiographically demonstrable coronary disease (Wang and Oosterhof, 2000). A very recent meta-analysis has examined the association of the -308 polymorphism with ischaemic heart disease and ischaemic stroke, using >17,000 subjects from 23 studies (Pereira et al, 2007). Overall, no association was found; Odds ratio [OR], 1.07; 95% confidencde intervals [CI] 0.94-1.21 and OR, 0.99; 95% CI, 0.70-1.41 for IHD and stroke respectively.

Although this fails to implicate TNFα as a causal marker, it is important to re-emphasise the limitations of Mendelian randomized studies. Even if a hypothetical polymorphism results in a hypothetically un-confounded increase in the circulating levels of the gene transcript by as much as 10% (potentially a very large influence for an individual SNP) in the general population, this increase probably does not account for much increase in risk. The statistical power required to pick up the increase in risk with small confidence intervals is staggering. For instance, as previously described a 1g/L increase in circulating

fibrinogen results in an independent increase in OR of CHD of 1.8. In the general population however, a 10% increase in fibrinogen is only ~0.25g/L, which translates to an OR of just 1.2, and the confidence intervals of the above study of >17000 people (Pereira et al, 2007) would only just be sufficient to detect this in theory. This conservative example illustrates the problems associated with genetic epidemiological studies.

1.3.7 TNFα and CVD in Molecular Epidemiology Studies

There has been a strong suggestion in the literature that TNFα is associated with metabolic disorders. Adipose tissue is often considered a major producer of TNFα (Kern et al, 2001; Hotamisligil et al, 1993 and 1995). Indeed it appears that weight reduction in obese subjects without (Ziccardi et al, 2002) and with other CVD risk factors (Samuelsson et al, 2003) results in lowered circulating TNFα levels. Furthermore, systemic TNFα is a candidate mediator of insulin resistance (Borst et al, 2004) as well as having potential roles in influencing dylipidaemia and glucose metabolism (Feingold et al, 1989 and 1992; Jovinge et al, 1998). TNFα may hence be involved in both the local processes of atherogenesis and atherothrombosis as well as the more global metabolic syndrome, which as been linked with CHD risk.

In two population studies TNFα levels correlated well with lipid parameters in the expected directions (Jovinge et al, 1998 and Skoog et al, 2002) and non-fasting glucose and pro-insulin correlate positively with TNFα (Jovinge et al, 1998). These results generally reaffirm the idea that TNFα is a candidate mediator of obesity-related insulin resistance and metabolic syndrome (Borst et al, 2004). A recent study suggests TNFα may play at least some role related to cholesterol levels and metabolism, from observations that a high fat meal results in depressed endothelial function and elevated circulating TNFα, a difference more exaggerated in those with pre-existing metabolic syndrome (Esposito et al, 2007). Whether TNFα is causally involved in these processes is currently unclear.

Another study contradicts all this, finding that in young healthy families (170 families), that TNFα levels are only associated with total cholesterol (in females) and, interestingly, had strong associations with IL-6 (another adipokine), but not CRP or soluble adhesion molecules (Haddy et al, 2003).

Aside from metabolic markers of disease, TNFα does not correlate (either at all or only very weakly) with ultrasonic measurements of carotid intima media thickness (IMT) or maximal carotid plaque thickness (MCPT) in healthy people, nor with degree of angiographically determined coronary artery disease (CAD) and nor does it correlate with ABI (Skoog et al, 2002; Elkind et al, 2002 and 2005; Sukhija et al, 2007). Strong associations between ABI and extent of atherosclerosis have been reported (Fowkes et al, 2006) so these results are perhaps not surprising. In addition, one recent study showed that TNFα did not associate with 24 hour ambulatory blood pressure in healthy normotensive people (Abramson et al, 2006). These results showing a lack of correlation of TNFα with prevalent "vascular condition" do not support, but neither do they preclude, a role for TNFα in CVD.

There are a large number of studies which have suggested that circulating levels of TNFα are elevated in patients with prevalent CVD such as angina pectoris (Blabay et al, 2001; Mizia-Stec K et al, 2002; Waehre et al, 2002; [1]Cesari et al, 2003; Mizia-Stec K et al, 2003; Wang et al, 2004; Pasqui et al, 2006). In addition, a retrospective study (Jovinge et al, 1998) noted a very significant difference in TNFα levels (4.1 pg/ml ± 1.6 vs 2.5 pg/ml ± 0.4, $p<0.0001$) between young male 4-6 month post-infarct patients (n= 92 and 60) and matched healthy controls (n=63). In these retrospective studies, although acute inflammation may have reduced, the elevated inflammation is likely to be partially a consequence of reverse causation and partially due to post-infarct pathologies (such as CHF).

In terms of TNFα being a prospective risk marker for CVD events, there are only very limited data in the literature and the available data appears quite disparate. This data is summarised in Table 1.4. Studies published after commencement of this thesis (October

2004) are not discussed in this section, since these were not available in considering assessing TNFα as a risk marker.

The two earliest studies in Table 1.4 (Koukkunen et al, 2001 and the CARE trial) examine patients with prevalent CHD, and who have (or are likely to have; Koukkunen et al study) experienced ischaemic events. Inflammation caused by reverse causation in these patients may confound associations of baseline inflammation with risk, although inflammatory markers may still be useful prognostic tools in these patients. This is a general limitation to consider in nested case-control prospective studies comprising participants with prevalent CHD. Interestingly, the CARE trial, which studies recurrent MI, found TNFα to be a poor prognostic indicator (the paper is essentially negative since the 95th percentile represents only 27 people; Table 4.1). Koukkunen et al however found TNFα to be a reasonable prognostic marker in a similar patient group to the CARE study. Unfortunately it is difficult to assess the study fully because of the limited details divulged in the publication abstract (full paper not available).

The ABC study (Table 1.4) was the only prospective study of TNFα and CVD in healthy people at commencement of this thesis ([2]Cesari et al, 2003). In this study the 180 CHD cases had elevated levels of TNFα, and the top tertile of TNFα expression were at significantly increased risk even after adjustment for classical risk factors: OR 1.6 (95% CI 1.23-2.26). In contrast, TNFα had no association with the risk of stroke, although both prospective observations were hampered by a very small number of cases. This finding therefore requires verification in larger studies.

Table 1.4 Publications of TNFα as a prospective risk marker of CVD; methods for systematic review of the literature detailed in chapter 2.

Publication	Study	Population	Follow-up	End-point	n cases	Proportion of population at increased risk*	OR at sig † (95% CI)	Linear increase in OR?
[3]Ridker et al, 2000	CARE	Previous MI	8.9 months	CHD death or MI	272	> 95th percentile	2.5 (1.3–5.1)	No (yes after 90%)
Koukkunen et al, 2001	-	Unstable angina	17 months	CHD mortality	? Events from 263 entry patients	3rd tertile	3.5 (??-??)	Yes
[2]Cesari et al, 2003	ABC	Healthy 70-79 years old	7 years	CHD category	180	3rd tertile	1.6 (1.23-2.26)	Yes
				Stroke category	60	ns	-	-
Tuomisto et al, 2006	FINRISK	General population	9 years	CHD event	151	2nd quartile	2.11 (1.18-4.14)‡	No
				CVD event	205	2nd quartile	2.05 (1.15-3.36)	No
				All cause death	183	4th quartile	2.15 (1.23-3.76)	No
Sukhija et al, 2007	-	Chest pain and coronary angiography	6 months	MI, coronary re-vasularisation, CHD death	55	ns (unadjusted)	1.14 (0.61-2.14)	-

* Point in population distribution at which publication reports a statistically determined increased risk (either $p<0.05$ or OR lower 95% CI > 1.00)

† Odds ratio of event at the point of significance stated in the table after adjustment for conventional risk factors (variables are paper specific) unless otherwise indicated

‡ OR of combined top 3 quartiles

ns: Not significant

1.3.8 Clinical Trials of Anti-TNFα Therapies and use in CVD

Specific intervention of TNFα in human trials is possible thanks to the development of anti-TNFα therapies, such as etanercept, infliximab and adalimumab. TNFα is the only marker examined in this thesis with such existing antagonists that are approved for use in humans. The drugs were originally developed for use in RA, but interest is growing in using them in a variety of disease states, including CVD.

Infliximab is a human-murine (25% murine) chimeric monoclonal antibody with high affinity and specificity for TNFα (Knight et al, 1993). Etanercept is a dimeric fusion protein, and consists of the extracellular portion of TNFR p55 linked to Fc IgG1, and forms less strong bonds with monomeric and membrane bound TNFα than the infliximab antibody (Scallon et al, 2002). Adalimumab is a recombinant human monoclonal IgG1 antibody that is more humanized than the others (Keystone, 2004).

Most clinical trials of anti-TNFα in CVD were performed on patients with CHF, due to the well documented association of the condition with elevated TNFα (1.3.5). On the basis of pre-clinical studies showing that etanercept was sufficient to reverse deleterious negative inotropic effects of TNFα *in vitro* (Kapadia et al, 1995) phase I clinical trials began. Small short-term studies were promising (Deswal et al, 1999). After a longer term world-wide study however, the RENEWAL trial (Randomized Etanercept Worldwide evaluation; n=1500) suggested that there was no benefit to patients, on the basis of all-cause mortality and hospitalization for heart-failure (Mann et al, 2004). Infliximab was evaluated in a phase II randomised double-blind placebo-controlled pilot study where there was a dose dependant increase in death and CHF hospitalizations with infliximab compared to placebo at 14 (21%) and 28 (26%) weeks (Packer et al, 2002). It is interesting to speculate that in CHF patients treated with infliximab, that the ability of the drug to bind membrane bound TNFα in stressed cardiac tissue may mediate apoptosis, and worsen the ability of the heart to cope (Scallon et al, 1995 and Lugering et al, 2001)

In light of these disappointing results, prescription of anti-TNFα therapies to patients with moderate to advanced heart failure (measured on the New York Heart Association

[NYHA] chronic heart failure scale as Grades III and IV) has been restricted. This coupled with some of the more ubiquitous side-effects of anti-TNFα therapy, such as hypersensitivity reactions, autoimmune reactions, acytopoenia, and some neurologic effects (Suryaprasad et al, 2003) as well as immunosupression, with a very high occurrence of respiratory infection and tuberculosis reactivation among patients (Gardam et al, 2003) is clearly a problem.

In more recent times, a cautious optimism has crept back in to the study of the potential uses of these drugs in CVD. In a small cohort (n=46, controls =13) of RA patients given adalimumab there was a significant rise in HDL cholesterol level, and a fall in CRP and IL-6 levels (Popa et al, 2005). Also very recently, the new onercept therapy in 127 psoriatic arthritis patients induced significant reductions in the levels of CRP, Lp(a), and homocysteine, although levels of Apo B and triglycerides were also increased (Sattar et al, 2007). Indeed another recent study has found that TNFα blockade may reduce carotid IMT in patients with RA (Porto et al, 2007). That anti-TNFα therapies can bring about a positive change in known cardiovascular risk markers is encouraging. However, this study was done on high risk, high inflammatory RA patients, and this may have some bearing on mechanisms by which these results are achieved, since it is not clear how high-grade inflammation compares to low-grade inflammation in terms of CVD progression (Sattar and McInnes, 2005).

Prescription of TNFα blockers to patients with high-grade inflammatory disease and low quality of life is ethically acceptable (most are non-responders to conventional RA therapy); however prescription to patients that are overtly healthy individuals, or individuals with subclinical disease who will probably not ever experience a CVD event is another proposition entirely. These ethical issues mean that use of anti-TNFα therapy in CVD is a field that may have to wait for a new generation of drugs.

1.3.9 Conclusions:

Although there is strong molecular evidence for the involvement of TNFα in the development of the metabolic syndrome, atherosclerosis, and progression to incident

CVD, there is little in the way of molecular epidemiological studies to confirm these observations, especially in healthy populations. The existing epidemiological data regarding TNFα is generally hampered by small numbers, and genetic epidemiological studies are so disparate that no real conclusions can be drawn from the literature. Important roles for TNFα in RA have further led to speculation that it may be a possible further major CVD risk factor in patients with active high-grade inflammatory disease, and there is some evidence to corroborate this in studies of TNFα blockers in these patients. Further studies are required to elucidate the true association between TNFα and other CVD risk factors in the general population, as well as its association with incident cardiovascular events.

1.4 IL-18 LITERATURE REVIEW

1.4.1 Introduction

Interleukin-18 (IL-18) is now recognised to be a key cytokine in the regulation of innate and acquired immune responses. It is expressed in tissue microenvironments central to pathologies in infectious disease, autoimmune disorders, at least some cancers, as well as at sites of chronic inflammation including atherosclerosis (Gracie et al, 2003). Being a relatively recently discovered cytokine there are still many enigmas regarding its *in vivo* function, although these are rapidly being elucidated and expanded on.

IL-18 is a member of the IL-1β superfamily of cytokines, with a similar β-pleated sheet secondary structure (Bezan et al, 1996) to its homologue, although it is functionally related to IL-12, being synergistic in the production of IFNγ from Th1 cells (Okamura et al 1998). Indeed, IL-18 was originally named and identified as an IFNγ-inducing factor before the systematic name of IL-18 was given. The role of IL-18 in the production of IFNγ is particularly pertinent to atherosclerosis, since IFNγ has been shown to be important in progression of stable plaques to lesions with an unstable phenotype (Gupta et al, 1997).

1.4.2 IL-18 Expression, Synthesis, and Regulation

IL-18 mRNA is constitutively expressed in a wide variety of cells including macrophages (foam cells), Kuppfer cells, T and B cells, osteoblasts, keratinocytes, dendritic cells, microglia and astrocytes (Akira, 2000). IL-18 has also, more recently, been observed to be produced by adipocytes in relatively low levels *in vitro* (Skurk et al, 2005). Constitutive expression of mRNA has speculatively been attributed to absence of AUUUA destabilization sequences (Puren et al, 1999) although little is known of regulation of transcription or translation of IL-18, or for the biological stimuli for IL-18 expression (Gracie et al, 2003).

The 24kDa precursor protein pro-IL-18 requires processing to become the biologically active mature 18kDa protein. Intracellular interleukin-1β converting enzyme (ICE or caspase-1) may mediate this cleavage (Gu et al, 1997) and capase-1 deficient mice are resistant to ischaemic acute renal failure (Melnikov et al, 2001). Secreted pro-IL-18 may be cleaved to maturity extracellularly by proteinase-3 (Sugawara et al, 2001) although the full relevance of this pathway is not clear *in vivo*. Mature IL-18 may be degraded in the circulation by active caspase-3, and this represents a major regulatory pathway of IL-18 activity (Akita et al, 1997).

Mature IL-18 is also regulated through the production of an IL-18 binding protein (IL-18bp). When given to mice IL-18bp abrogates induction of IFNγ by IL-18 or LPS administration (Novick et al, 1999). IL-18bp belongs to the immunoglobulin superfamily and has limited homology to the type II IL-1 receptor (and hence binds IL-18 in a 1:1 ratio), but lacks a transmembrane signalling domain (Akira, 2000). The discovery of IL-1H, which has homology to IL-1 receptor antagonist, and binds the IL-18 receptor (via IL-1Rrp) but not the IL-1 receptor, has sparked speculation of another level of regulation (Pan et al, 2001). Heavy and multi-factorial regulation of IL-18 alludes to a potent physiological function for the cytokine. Circumstantial evidence for the immunological potency of IL-18 comes from the IL-18bp-like p13 protein encoded by the *ectromelia poxvirus* which inhibits IL-18 activity in vitro (Xiang et al, 1999), viral inhibition of Th1 responses being a major virulence factor.

1.4.3 IL-18 Receptor and Signalling:

The IL-1 and IL-18 receptors are very similar. Like IL-1β, the IL-18 functional receptor has a low-affinity α chain (IL-1Rrp) and a signal transducing β chain (AcPL) (Torigoe et al, 1997 and Born et al, 1998). Consistent with this receptor similarity, they share remarkable signalling similarity with each other, and the evolutionarily more ancient Toll-like receptors (TLRs) (Takeda et al, 2003). Upon ligand binding in these receptors, exposure of intracellular TIR domains (Toll/IL-1 receptor) results in recruitment of the adaptor proteins. The subsequent signalling process is reviewed elsewhere (Gracie et al, 2003). In light of the fact that TIR-mediated IL-18R signalling is evolutionarily conserved, spatially and temporally restricted expression of the IL-18R is clearly important to avoid dangerously exacerbated immune responses. Among the cells shown to express the IL-18R are NK cells, SMCs, macrophages, vascular endothelial cells, and T-cells (Leung et al, 2001, Gerdes et al, 2002)

1.4.4 General Biological Function of IL-18

As discussed previously, the Th1/Th2 paradigm may be important in atherosclerosis. Although Th1 cells are thought to be the major *in vivo* source of IFNγ, IL-18, and IL-12 act synergistically to increase IFNγ production from murine macrophages (Munder et al 1998), a potential pathway for autocrine macrophage regulation in chronic responses which has recently been confirmed elsewhere (Bastos et al, 2007). This mirrors several studies in T-cells where the combination of both cytokines was far more potent than either alone (Barbulescu et al, 1998).

The importance of IL-18 in the production of IFNγ is evident in IL-18 deficient mice. These mice have circulating levels of IL-12 similar to WT controls. However, preconditioning of the mice with *P Acnes* bacterium for 7 days followed by LPS injection shows IL-18-deficient mice attain circulating IFNγ levels one fifth that of WT mice (Takeda et al, 1998). These are similar to findings in mice treated with anti-IL-18 antibodies (Okamura et al, 1995) or caspase-1 null mice (Gahyur et al, 1997).

IL-18 is clearly important in the production of IFNγ. It is unsurprising then that IL-18 has a central role in the Th1/Th2 paradigm in the literature. IL-12 induces the expression of IL-18 receptors on the surface of naïve T-cells (Yoshimoto et al, 1998). Once established in this cytokine milieu Th2 associated IL-4 receptors are downregulated on the T-cells, and hence the IL-18R α chain is often used as a functional marker of Th1 cells in murine systems (Xu et al, 1998). In contrast, TCR ligation in the presence of IL-4 results in downregulation of the IL-18R α chain (Smeltz et al, 2001). It was originally thought that IL-18 could not contribute to Th2 responses. Gel-shift assays suggested that IL-18 did not induce translocation of NF-κB in Th2 cells but did in Th1 (Robinson et al, 1997). Whether this is an experimental artefact or if IL-18 induces other signalling pathways in Th2 cells is unclear, but evidence suggests that IL-13 (a Th2 cytokine) can be induced by IL-18 and IL-2 in synergy in the absence of IFNγ (Hosino et al, 1999). It hence seems possible that IL-18 can help drive a Th2 response in the absence of a Th1 cytokine milieu.

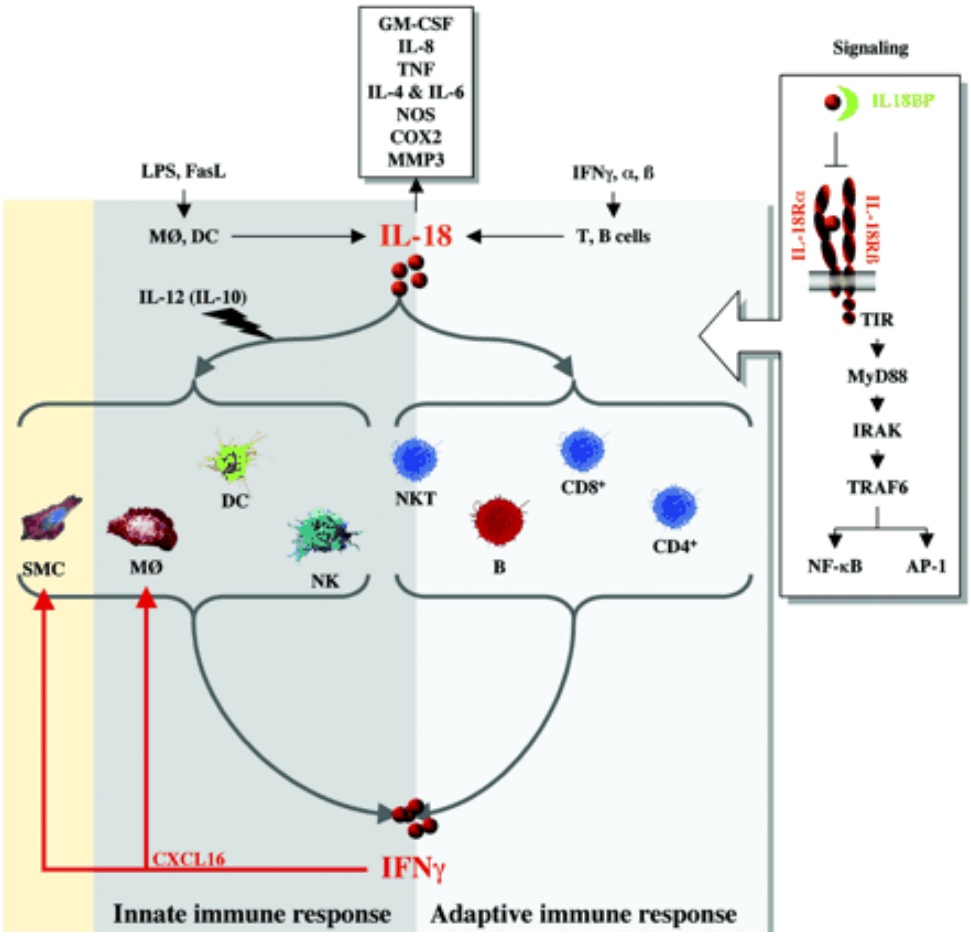
Interestingly, it has been shown that IFNγ can be produced in atherosclerosis models in the absence of T-cells (SCID mice) by macrophages and smooth muscle cells although IL-18 and IL-12 are still required to induce production (Gerdes et al, 2002, Tenger et al, 2005). It has also been shown that T-cells without cross-linked antigen receptors can be stimulated by IL-18 and IL-12 to become potent IFNγ producing cells without becoming antigen stimulated memory cells (Yoshimoto et al, 1998). These novel pathways in the production of IFNγ may have important ramifications for the process of atherogenesis and the development of unstable lesions.

1.4.5 Pathological and Animal Studies of IL-18 in CVD:

Mallat et al were the first group to show that IL-18 is expressed in human carotid plaques ([1]Mallat et al, 2001). They went on to demonstrate that transfection of ApoE -/- mice with intramuscular IL-18bp cDNA inhibited *de novo* lesion formations and slowed progression of more developed plaques ([2]Mallat et al, 2001). This work was of seminal importance in identifying IL-18 as a central player in atherogenesis, and was followed up

by Whitman (et al, 2002). They showed in the ApoE -/- model that exogenous addition of IL-18 increased lesion size two fold in the aortic arch and ascending aorta, but that this effect was almost totally abrogated in ApoE IFNγ double -/-. This directly implicates IFNγ as the major proatherogenic factor induced by IL-18. Yet this group also reported that IFNγ–deficiency had no effect on lesion size in female mice and the effect was restricted to male mice in a surprising gender-specific mechanism. Interestingly no studies have been published on IL-18 deficient female mice regarding atherosclerotic lesion development. Indeed, Elhage (et al, 2003) went on to show that ApoE IL-18 KO had reduced atherosclerosis compared to the ApoE counterparts, although the study, again, only used male mice. Whether this gender-specific IFNγ (and possibly IL-18) role is exclusive to the ApoE mouse strain, or if it is species specific, or indeed a trans-species phenomenon remains to be seen. The latter possibility would obviously have implications in the use of anti-IL-18 therapies and use of IL-18 as a marker for end-point cardiovascular events. However, despite this speculation a recent study has shown that exogenous IL-18 can mediate proatherogenic effects independently of T-cells (in SCID mice) (Tenger et al, 2005). This importantly suggests that IL-18 has some control of both innate and adaptive responses in the atherogenic setting, and hence represents a good potential therapeutic target. Mechanisms of potential ways IL-18 orchestrates proatherogeneic responses are illustrated in Figure 1.10 and in Table 1.5

Figure 1.10 Schematic diagram of the potential cells through which IL-18 can mediate proatherogenic effects (from Caligiuri et al, 2005)



MØ, macro-phages; DC, dendritic cells; NOS, NO synthase; COX2, cyclooxygenase 2; MMP3, stromelysin; TIR, Toll/IL-1R domain; IL-18BP, IL-18 binding protein; IRAK, IL-1 receptor–associated kinase; TRAF6, TNF receptor–associated factor 6; IL-18Rα, ß, $\alpha$and ß chains of IL-18 receptor.

Table 1.5 Illustrative table of potential direct and indirect proatherogenic stimulators and processes induced by IL-18 expression in experimental settings

Molecule type	Molecule	Major biological property and cellular source	Reference
Cytokine	IFNγ	Inhibition of SMC production of collagen/TIMPS. Produced by Th1 cells/macrophage	Whitman et al, 2002
	IL-1β	Activation of all major inflammatory cells/endothelium. Pyrogen. Produced by macrophages	Kirii et al, 2003
	TNFα	Local inflammation and endothelial activation. Produced by macrophage and T-cells	Bruunsgaard, 2000
	IL-6	Induction of APR. T-cell differentiation. Produced by macrophage and T-cells	Song et al, 2004
Chemokine	MCP-1	T-cell/monocyte recruitment via CCR2 Produced by endothelial cells	Harrington, 2000
	IL-8	Neutrophil/T-cell recruitment via CXCR1 Produced by activated macrophages	Boisvert et al, 2000
	MIP 1α	Recruitment of all major inflammatory cells via CCR1/5 Produced by PBMCs	Sheikine, 2004
Other	ICAM-1	Endothelial adhesion of LFA-1/Mac-1 expressing PBMCs Expressed on activated endothelium	Kondo, 2005
	GM-CSF	Recruitment and growth of endothelial cells and SMCs and DCs Produced by macrophages and T-	Werner, 2002

1.4.6 IL-18 in Genetic Epidemiology Studies:

There has only been one report of IL-18 polymorphisms and their association with CVD risk. The Atherogene investigators report (Tiret et al, 2005) demonstrated in 1288 patients that IL-18 haplotypes account for a small amount of heterogeneity in inter-individual IL-18 expression (around 2%) and that these haplotypes were also associated with cardiovascular disease (CVD) mortality (P=0.006). Haplotypes GCAGT and GCCAT were associated with opposite effects on risk (OR=0.57, 95% CI 0.36 to 0.92, versus OR=3.01, 95% CI 1.27 to 7.13, respectively). That IL-18 gene polymorphisms are associated with both circulating IL-18 levels and incident CVD (the haplotype association with incident CVD was abrogated when corrected for IL-18 expression) adds further evidence of a possible causal role to existing animal and tissue studies. These findings require corroboration, especially in light of sizeable confidence intervals, despite the reasonable study size.

Very recently several IL-18 polymorphisms have been investigated in relation to obesity and the need for coronary bypass artery grafting, and the study found some promoter polymorphisms to be associated with these clinical conditions (Thompson et al, 2007). The associations were not particularly strong, and more studies are required.

1.4.7 IL-18 in Molecular Epidemiology Studies:

Like TNFα, IL-18 may be associated with the metabolic syndrome and adipose tissue mass. In a study of 120 pre-menopausal women, 60 women who improved their diet over 2 years consequently lowered their BMI and a concomitant drop in circulating levels of cytokines, including IL-18 was observed (Esposito et al, 2003). In another study, serum IL-18 concentration measured in 955 subjects correlated with metabolic syndrome traits including body mass index (BMI), waist circumference, triglyceride, high-density

lipoprotein (inversely), and fasting glucose and insulin levels (Hung et al, 2005). In this study, after adjusting for age, gender, BMI, and insulin levels, increasing IL-18 levels in the top tertile were associated with an OR for metabolic syndrome of 2.28, strongly suggesting that elevated circulating IL-18 is either a cause or consequence of metabolic syndrome (Hung et al, 2005). These findings are contradicted in a later study of 261 healthy people, where IL-18 did not correlate with BMI, and only weakly with waist to hip ratio, although it did correlate with hypertension (Vilarrasa et al, 2006), and are also contradicted in the MONICA/KORA study, where IL-18 levels correlated with insulin sensitivity, predicted risk of type II diabetes, but did not associate with BMI or fat-free mass (Thorland et al, 2005; Herder et al, 2006). IL-18 is only produced in low levels by adipocytes *in vitro* (Skurk et al, 2005), and while this may be sufficient for adipocyte/leucocytes cross-talk, it is not yet consistently clear if this has any bearing on circulating levels of IL-18.

Several studies have reported that in patients with prevalent CVD disease, IL-18 levels are correlated with the extent of CAD, as measured by degree of stenosis, number of affected vessels, or to a lesser extent coronary plaque area (Yamashita et al, 2003; Yamaoko et al, 2003; Suchanek et al, 2005) as well as diabetes and smoking (Suchanek et al et al, 2005). A study of 366 patients who had carotid IMT measured at 12 sites showed that serum IL-18 was associated with mean IMT ($r = 0.36$; $p<0.001$) (Yamagami et al, 2005) even after adjustment for traditional risk factors and CRP ($r = 0.20$; $p<0.001$). However, a larger study (n=1111) recently suggested that only the univariate association was significant (Chapman et al, 2006). In addition, a recent study (Espinola-Klein et al, 2007) found in 720 patients that IL-18 levels were not different in patients with no significant CAD, those with CAD, or those with clinically relevant multi-vessel disease. Although these studies are not entirely consistent, overall there is evidence to suggest that in human populations that circulating IL-18 may be associated with some markers of the metabolic syndrome and degree of atherosclerosis.

Several small studies (Seta et al, 2000; Mallat et al, 2002; Rosso et al, 2005) have shown that circulating levels of IL-18 are elevated in patients with prevalent CHD, and that IL-18 levels correlate with the extent of myocardial necrosis (Seta et al, 2000) and with left

ventricle ejection fraction (LVEF) (Mallat et al, 2002). Recently, univariate comparisons between retrospective MI cases and controls have shown IL-18 levels to be elevated among cases (309.6 pg/ml ± 138.6 vs 285.4 pg/ml ± 115.7 $p<0.01$) (Hulthe et al, 2006). Another small study (Nairns et al, 2004) has shown that in patients requiring percutaneous coronary intervention, a cohort that recently experienced MI had elevated plasma levels of IL-18 compared to those who had not. The significance of this result improved when instead of using circulating IL-18, IL-18/IL-18bp ratio was used ($p<0.05$ vs $p<0.02$). This suggests that IL-18 may be active in post-MI dysfunction as opposed to acting as a passive marker of subsequent systemic inflammation.

There are limited prospective data available linking IL-18 to CVD risk. These are summarised in Table 1.6. Studies published after commencement of this thesis (October 2004) are not discussed in this section, since these were not available in considering assessing IL-18 as a risk marker. As can be seen in Table 1.6 before this thesis was undertaken, the published literature prospectively examining IL-18 was dominated by one group, who showed that IL-18 was predictive of risk of incident CVD in both healthy middle-aged men, and in a population with existing CAD (Blankenberg et al, 2002 [Atherogene]; Blankenberg et al, 2003 [PRIME]). Strikingly, IL-18 had fairly similar predictive properties in both cohorts (although the Atherogene cohort was divided in quartiles and not thirds). In the PRIME cohort IL-18 was suggested to be a better predictor of risk than total cholesterol/HDL cholesterol ratio or CRP when simultaneously introduced in a model additionally adjusted for BMI, smoking status, diabetes, and hypertension. The adjusted ORs (per tertile) were 1.65 (95% CI 1.19 to 2.29) for the lipid ratio, 1.63 (95% CI 1.17 to 2.27) for CRP, and 1.82 (95% CI 1.30 to 2.55) for IL-18, respectively (top versus bottom tertile). These reported associations are quite strong. In the Atherogene study, after adjustment for most potential confounders including ejection fraction and inflammatory variables, patients within the highest quartile of IL-18 had a 3.3-fold (95% CI, 1.3 to 8.4; P=0.01) increase in risk. Similarly in PRIME there was an OR~2 in the top tertile of IL-18 for combined endpoints in fully adjusted models. In both cases IL-18 was a better predictor than CRP. These interesting results clearly required further investigation.

Table 1.6 Publications of IL-18 as a prospective risk marker of CVD; methods for systematic review of the literature detailed in Chapter 2.

Publication	Study	Population	Follow -up	End-point	n cases	Proportion of population at increased risk*	OR at sig (95% CI)†	Linear increase in OR?
Blankenberg et al, 2002§	Atherogene	Stable/ unstable angina	3.9 years	CVD death	95	2nd quartile	1.44 (1.17-1.78)‡	Yes
[1]Blankenberg et al, 2003	PRIME	Healthy men 50-59 yrs	5 years	CHD death or MI or angina onset	335	2nd tertile	1.42 (1.13-1.79)‡	Yes
Kip et al, 2005	WISE	Women with suspected MI	4.7 years	MI, stroke, CHF, CHD death	136	?	?	?
Tiret et al, 2005§	Atherogene	CAD	5.9 years	Death from CVD	142	4th quartile	2.31 (1.42-3.73)	Yes
Blankenberg et al, 2006	HOPE	CAD, stroke, PVD, diabetes	4.5 years	MI, stroke, CHD death	501	3rd tertile	1.29 (1.03-1.62)	Yes
Koenig et al, 2006	MONICA Augsberg	Healthy men and women	11 years	Incident CHD	382	ns	1.21 (0.8-1.69)	n/a
Espinola-Klein et al, 2007	Atherogene	Coronary angiography	6.5 years	Cardiovascular Death	75	Above median	2.8 (1.6–4.9)	n/a

* Point in population distribution at which publication reports a statistically determined increased risk (either $p<0.05$ or OR lower 95% CI > 1.00)
† Odds ratio of event at the point of significance stated in the table after adjustment for conventional risk factors (variables are paper specific) unless otherwise indicated
‡ Increase in OR per quartile/tertile
§ Same study, different follow-up
ns: Not significant

1.4.8 Clinical trials of Anti-IL-18 Therapies and use in CVD:

Original interest in the inhibition of IL-18 came from rheumatic disease research, much as did anti-TNFα therapies. There are currently no IL-18 inhibitors approved for use in humans, although there are several drugs in phase one and two clinical trials. These drugs act at several levels of inhibition, including anti-IL-18 mAbs, recombinant / homologous IL-18bp, recombinant / homologous IL-1H, and ICE inhibitors. ICE inhibitors obviously have a direct effect on IL-1β production as well as IL-18. There have been several attempts at clinical trials of these drugs (such as Pralnacasan) although these have so far been unsuccessful due to excessive toxicity (Randle et al, 2001).

1.4.9 Conclusions:

Similarly to TNFα, there is pathological and limited genetic and epidemiological evidence that IL-18 may be important in atherogenesis, the metabolic syndrome, and in association with risk of subsequent cardiovascular events. As with TNFα, IL-18 plays a major role in the Th1 RA-associated chronic inflammatory response, and is a possible molecular link between RA and elevated CVD risk. At the outset of this thesis there was currently only one study that prospectively examines the association of IL-18 with risk in generally healthy populations. There is a requirement for more prospective data of IL-18 as a marker, produced by other laboratories, in order to confirm the current literature and to expand on it.

1.5 MMP-9 LITERATURE REVIEW

1.5.1 Introduction:

The matrix metalloproteinases (MMPs) are a family of zinc-containing endoproteinases that have similar structural domains, but differ in terms of substrate specificity, cellular source, expression, and regulation. Recent advances have seen the list of known MMPs

extend to in excess of twenty members identified in mammals (Woessner, 2002). These enzymes can be classified into a family based on several observations pertinent to the function of them all (Klein et al, 2004):

• Their enzymatic function centrally involves degradation of extracellular matrix components (ECM), although the family as a whole has a spectrum of specificity.
• They are zymogens and require to be enzymatically activated to become proteolytic.
• The active domain has an unusual, but functionally important Zn^{2+} atom.
• They are inhibited by a family of specific tissue inhibitors (TIMPS).
• Ca^{2+} is required for stability
• They function at neutral pH

Based on major substrate specificity, the MMPs were historically categorised into four subgroups (Creemers et al, 2001):
• Collagenases such as MMP-1 (interstitial collagenase). These can cleave fibrillar collagens (types I, II and III) which are dense and highly cross-linked.
• Stromelysins such as MMP-3. These are broad specificity proteinases for proteoglycans, lamanins, fibronectin, vitronectin and some collagens.
• Gelatinases, including MMP-9 and MMP-2 (gelatinase B and A respectively). These are well known for the ability to degrade gelatins (i.e. denatured collagen fragments).
• The last group are membrane-type MMPs (MT-MMPs) which degrade some ECM components and are able to activate soluble MMPs.

1.5.2 MMP-9 Expression, Synthesis, and Regulation:

MMP-9 (also known as gelatinase B and 92kDa type IV collagenase) was originally discovered as a neutrophil product (Sopata et al, 1974) where it is expressed in large quantities in tertiary granules (Cowland et al, 2000). It is also found in monocyte supernatents (Mainardi et al, 1984). Neutrophils and macrophages are still regarded as the major *in vivo* cellular sources of MMP-9, although lymphocytes, dendritic cells, connective tissue cells and epithelial cells can also be induced to express under inflammatory conditions ([1]Opdenakker et al, 2001). It noteworthy that whilst most

immunological cells express gelatinase A constitutively, gelatinase B requires adequate inflammatory triggering, and neutrophils produce the only biologically pre-stored MMP-9 in tertiary granules ([1]Opdenakker et al, 1991).

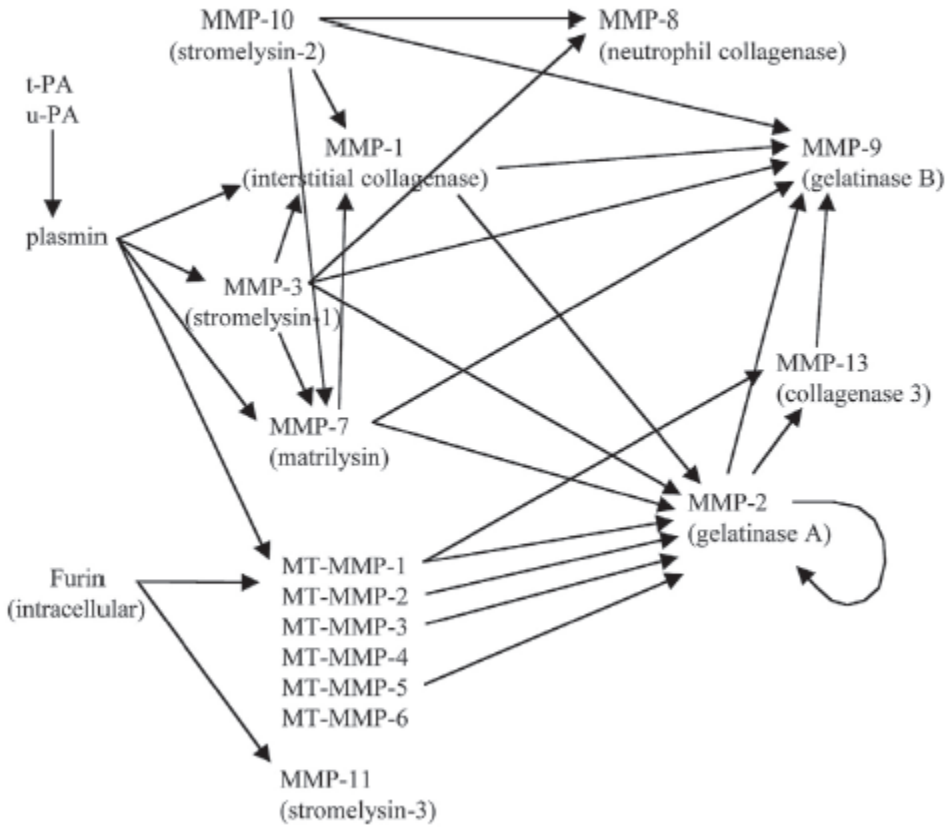
MMP-9 is regulated at the transcriptional level, with several cis elements in the promoter region playing a major role (Huhtala et al, 1991 and Gum et al, 1996). Chief stimulators of MMP-9 include IL-1β , platelet-derived growth factor, TNFα and EGF (Huhtala et al, 1991; Fabunmi et al, 1996; Kondapaka et al, 1997). In addition, a cell surface protein that induces general MMP expression has been identified in both normal and diseased human tissue and has been termed extracellular MMP inducer (EMMPRIN) (Spinale et al, 2000).

The MMP-9 molecule is initially expressed in a latent state and requires proteolytic cleavage to become activated. Extracellular cleavage can be mediated by a number of proteinases, although plasmin is thought to be the most potent activator of most MMPs *in vivo* (Murphy et al, 1994), via cleavage of a cysteine sulfhydryl switch. It is thought that plasmin cleaves the latent MMP, which induces a conformational change and renders the activation site ready to be cleaved by a second protease, usually another MMP (see Figure 1.11), although the first cleavage is probably the rate-limiting step for activation (Nagase H, 1997). In addition to this, in neutrophils, intra-granular hypochlorous acid can directly activate pro-MMP-9 (Peppin et al, 1986).

Once secreted, MMP-9 can still be regulated by degredation or by inhibition. α_2-macroglobulin is a universal protease inhibitor present in human serum and inhibits MMP-9 directly (Birkedal-Hansen et al, 1993). More specifically, there are a group of tissue inhibitors of matrix metalloproteinases (TIMPs) comprising TIMP-1,-2,-3 and -4 which bind to MMPs non-covalently in a 1:1 ratio (Gomez et al 1997). These TIMPs demonstrate limited specificity for MMPs, with TIMP-1 being the major inhibitor of MMP-9, but TIMP-2 and -3 also are (Gomez et al, 1997). TIMP-1 is similar to MMP-9 in that it requires to be induced, whilst TIMP-2, which has stronger specificity for gelatinase A, is produced constitutively (Gomez et al, 1997). Interestingly, TIMP-1 and -3 can bind pro-MMP-9 (Olson et al, 1997).

MMP-9 produced by macrophages is directly released in a pro-MMP-9/TIMP-1 complex ([2]Opdenakker et al, 1991) whilst neutrophils do not make TIMP-1 at all (Masure et al, 1991). It is interesting to speculate that this reflects different roles of each cell respectively in terms of inflammation and ECM turnover. Neutrophils represent a more acute response requiring ECM turnover as a pre-requisite for repair and angiogenesis, whilst monocytes require an inflammatory microenvironment to bring about ECM degredation, representing a more chronic response type.

Figure 1.11 The known activation network of MMPs. Arrows indicate the active form of one enzyme may participate in the activation of the other enzyme from its latent state. Note that MMP-9 is an end-product of the pathway, which may signify biochemical importance. From Van Den Steen et al, 2002



1.5.3 General biological function of MMP-9:

Gelatinases are identified on the basis of their ability to cleave the ¾ and ¼ collagen fragments yielded from the actions of interstitial collagenases on collagen. The substrate based division of MMPs was useful in the past, but increasingly it has become clear that functional profiles of these enzymes are gradually graded rather than absolute (and are not restricted to proteolysis of ECM components). MMP-9 is a good example of this since it can also degrade interstitial collagens (Okada et al, 1995) and type IV collagen in the basement membrane (Pauly et al, 1994) as well as proteoglycan core protein and elastin (Birkedal-Hansen, et al 1993), which are resistant to degradation by some other MMPs. Indeed, although MMP-9 is primarily regarded to functionally be a gelatinase, this function remains to be directly proven *in vivo.* However, broad specificity clearly may make MMP-9 a key player in ECM turnover.

The normal turnover of ECM is an important physiological process. The ubiquitous ECM provides biomechanical support to structures on all scales from individual cells to complex organs, and ECM turnover permits growth, repair and replacement of cellular tissue (Birkedal-Hansen, et al 1993) whilst controlling transformed cell growth though steric limitations. Interestingly, MMP-9 has been implicated in tumour cell invasion and metastasis largely due to its ability to degrade ECM, and in particular type IV collagen in the basement membrane (Himelstein et al, 1994).

Unsurprisingly for a molecule with such stark association with immune cells, the MMP-9 molecule also seems to possess some pro-inflammatory properties. It is postulated that MMP-9 plays a major role in leukocytosis and inflammation for several reasons. Firstly, it may be functionally important in allowing peripheral stem-cell mobilization (Pruijt et al, 1999) and extravasation of circulating leukocytes (Opdenakker et al, 1998) through targeted degradation of the basement membrane. This allows a localized cellular immune response to take place. Secondly, there is a positive feedback loop seen between IL-8 and

MMP-9. Active MMP-9 potentiates IL-8 tenfold by aminoterminal processing through cleavage to IL-8(7-77), and this more potent IL-8 is a stronger chemoattractant for more neutrophils, which degranulate locally and release MMP-9 (Van den Steen et al, 2000) (see Figure 1.12). The same study showed that some other chemokines such as CTAP-III, PF-4 and GRO-α were degraded by MMP-9, alluding to the fact that MMP-9 does not only amplify a cellular response through IL-8, but fine-tunes it (Van den Steen et al, 2000). Thirdly, further upstream in the immune response, MMP-9 can take the place of interleukin-1β converting enzyme (ICE) and cleave the pro-form of the cytokine, not to potentiate, but to activate (Schonbeck et al, 1998) although the physiological relevance of this in "normal" *in vivo* processes is not clear.

On a larger scale MMP-9 has a role in many important physiological processes and disease states (see Table 1.7). Many of the disease states MMP-9 has been implicated in are classical Th1 type inflammatory driven diseases, and it has been observed that Th1 cytokines enhance MMP-9 expression, whilst Th2 cytokines (such as IL-4, IL-10 and TGF-β) suppress expression of MMP-9 and/or enhance expression of TIMP-1 (Van den Steen et al, 2001).

Interestingly MMP-9 null mice themselves show very little if any signs of having immunological deficiencies (Van Den Steen et al, 2002) in pathogen free, or conventional animal house conditions (Dubois et al, 1999) a fact that has stepped up efforts in research for pharmacological inhibition of MMP-9.

Figure 1.12 Simplified diagram of the positive feedback inter-relationship between IL-8 and MMP-9. Adapted from [2]Opdenakker et al, 2001

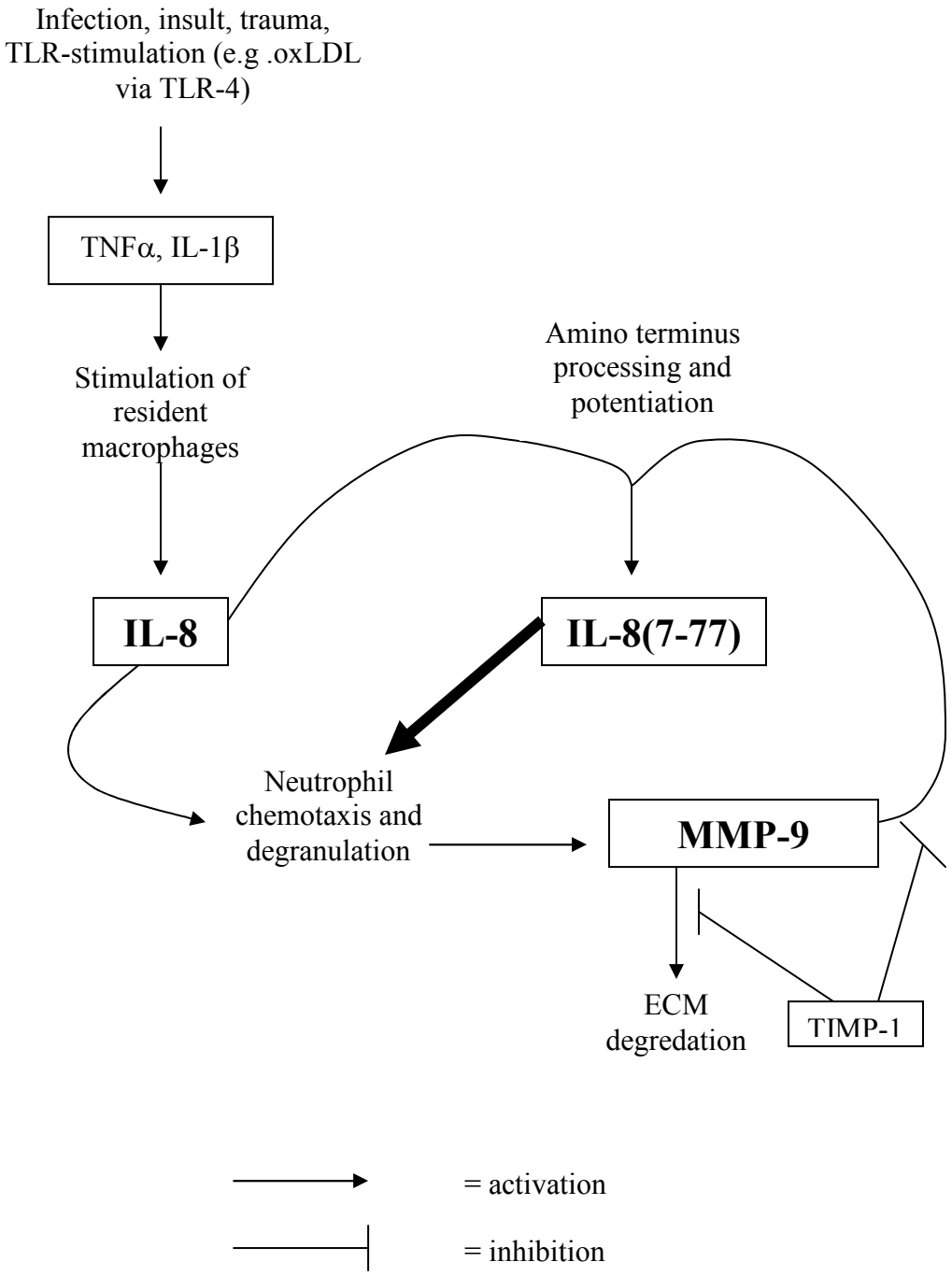


Table 1.7 Illustrative table of some of the physiological and pathological non-CVD functions of MMP-9. Adapted from Van den Steen et al, 2002

Physiological Function	References	KO mouse phenotype	References
Reproduction – Female menstrual cycle Blastocyte implanation	Van Den Steen et al, 2002; Librach et al, 1991	Impaired reproductive abilities	Dubois et al, 2000
Growth and development	Everts et al, 1992	Delayed ossification of long bones, reduced angiogenesis	Vu et al, 1998 Bergers et al, 2000
Inflammation and leukocyte mobilization	Kobyashi et al, 1999; Van Den Steen et al 2002; Opendakker et al, 2001	Impaired stem/progenitor cell recruitment, impaired neutrophil/ langerhans cell chemotaxis, prolonged delayed hypersensitivity	Hessig et al, 2002; D'Hase et al, 2000; Wang et al, 1999
Pathological Function	**References**	**KO mouse phenotype**	**References**
Tumour growth and progression	Himelstein et al, 1994	Impaired pro-angiogeneic function and metastasis of transformed cells	Bergers et al, 2000; Itoh et al, 1999
Autoimmune diseases e.g Rheumatoid arthritis and SLE (inititation by immunodominant antigen processing?)	Van Den Steen et al, 2002; Koolwijk et al, 1995; Ram et al, 2006	Resistant to experimental autoimmune encephalomyelitis	Dubois et al, 1999
Chronic wounds	Wysoki et al, 1993	Resistance to necrotizing tail lesions	Dubois et al, 1999
Bacterial sepsis	Paemen et al, 1997	Resistance to LPS shock	Dubois et al, 2002
Altzheimer's disease	Backstrom et al, 1996		

1.5.4 Pathological and animal studies of MMP-9 in CVD:

In chronic inflammation associated with build up of atherosclerotic plaques, neutrophils are usually present only in very low numbers in the vasculature (Hiuchi et al, 2002), hence any MMP-9 present is likely to be (largely) produced by macrophages and foam cells. Indeed macrophages have been shown to produce MMP-9 in both experimental (Galis et al, 1995) and human (Galis et al, 1994; Brown et al, 1995) atherosclerotic plaques.

A wealth of literature associates increased expression of MMP-9 with compensatory vascular remodelling during atherosclerotic lesion growth. It is currently believed that vascular expansion compensates for luminal occlusion caused by lesion swelling, but that this process may be (partially) causal in rendering the plaque prone to rupture in the longer term (Nishioka et al, 1997). Expansion of the vasculature requires the action of MMPs, as suggested by Pasterkamp et al (Pasterkamp et al, 2000) who reported that although constrictive and expanded coronary arteries contain similar macrophage loads in the plaque, both MMP-9 and MMP-2 are expressed in greater quantities in the expanding arteries. This role for MMP-9 is supported by experiments in MMP-9/ApoE double knock-out mice. It was shown that ApoE KO mice had increased artery size by 3.1 fold 14 days following induction of flow cessation (area measured encompassed external elastic lamina) whilst there was only a 1.6 fold increase in the double KOs (Lessner et al, 2004). The presence of MMP-9 in pathological vascular expansion (aneurisms) (Freestone et al, 1995) also supports MMP-9 playing a role in vascular remodelling. Another study has shown that MMP-9 KO mice on the 129/SvEv background have impaired SMC migration in an intimal lesion model (Galis et al, 2002). Although this observation may have limited relevance to humans (since the human intima already has SMCs), SMCs from the KO mice had impaired capability to contract collagen, suggesting a role not only in degradation, but also in reorganisation of collagenous matrix. This was supported by the observation that these KO mice had decreased intimal thickening as well as impaired geometric remodelling.

In the face of this evidence then, it is perhaps not surprising that MMP-9 has been associated with de-stabilisation of atherosclerotic lesions. Although the mechanisms of this process are not fully elucidated, especially in humans, it may be implied that as the plaque grows in an inflammatory environment MMP-9 compensates by opening up the lamina through geometrical remodelling. Although this temporarily relives potential occlusion, the plaque and the necrotic lipid core becomes bulkier, and further matrix turnover in the face of decreased collagen deposition due to SMC necrosis compromises the supportive collagenous matrix, rendering the lesion prone to stress-induced or spontaneous rupture. These potential mechanistic roles of MMPs in the development of ACS have been summarized previously in Figure 1.6.

This model is supported by several studies. Libby's group (Galis et al 1994) originally demonstrated that atherosclerotic plaques displayed locally increased concentrations of MMP-9 in the shoulders and regions of high-density foam cell accumulation. Furthermore, they showed via *in situ* zymography that this MMP-9 was active as opposed to the latent form found in normal arteries. In addition, the production of MMP-9 has been shown to be higher in unstable carotid plaques (Loftus et al, 2000). It was also shown *in vitro* that human monocyte-derived macrophages (HMDMs) can directly degrade fibrous caps from aortic of carotid plaques, and that this process could be limited by the addition of TIMP-1 (Prediman et al, 1995).

As well as playing a role in the build up to MI (and perhaps other ACS) MMP-9 had been implicated in playing a pathophysiological role in post-MI changes in myocardial tissue. The processes involved in these changes are not fully understood, but include left ventricular dilation, potentially leading to CHF or, in extreme cases, LV rupture (Thompson et al, 2002). Following the acute insult of ischaemia/reperfusion injury to the myocardial tissue, neutrophils rush to the area of insult and degranulate (Lindsey et al, 2001), releasing MMP-9 and resulting in further inflammation and tissue injury (Van den Steen et al, 2000; Frangogiannis et al, 2002). Romanic et al (Romanic et al, 2001) demonstrated that MMP-9 was upregulated within 24hrs of MI and that this was simultaneous with a decrease in TIMP-1, and MMP-9 knock-outs have smaller infarcts than controls (Romanic et al, 2002). Similarly, MMP-9 is also thought to play a role in

disruption of the blood brain barrier following focal cerebral ischemia (Fujimura et al, 1999) and so is important in pathologies following ischaemic events in both stroke and ACS.

Similarly to the acute inflammatory model of ischaemia/reperfusion, MMP-9 is also suggested to be involved in restenosis following percutaneous coronary intervention injury. MMPs in general are considered important contributers to intimal hyperplasia that follows either stent implantation or balloon angioplasty by allowing cellular infiltrates to extravasate following injury (Bendeck et al, 1996) and MMP-9 may be particularly important in these processes (Feldman et al, 2001).

1.5.5 MMP-9 and Plasma or Serum Measurement:

Manufacturer's instructions for the performance of MMP-9 ELISA (R&D Systems) indicate that citrated and EDTA plasmas are unsuitable for use in determination of circulating MMP-9 (see Chapter 2). Measurement of MMP-9 in serum has recently been called into question in the literature by one report, which suggests that serum samples are not suitable for use in measurement of MMP-9 due to "artificially high levels" in serum compared with plasma and EDTA (n=8 for each) (Gerlach et al, 2007). Although there is probably some basis for this argument, the authors make several assumptions:

1. That zymography and ELISA measurements are equivalent in measuring circulating MMP-9 (since they used zymography, but not ELISA techniques; something unlikely to be done in mass screening of a biomarker).
2. That because serum levels of MMP-9 are high and citrated or heparin samples are low, that these low levels reflect "true" circulating biological levels. This is not necessarily true, since it may reflect assay interference e.g. through chelation.
3. Tying in with the previous point, levels of plasma MMP-9 are very often below the level of detection in healthy populations (e.g. Sundstrom et al, 2004; ~20% of a healthy population had detectable circulating MMP-9) and therefore measurements are statistically weak for use in multivariate models of disease risk.

More research is required in this area, although this may have to wait for newer ELISA or other related detection technologies for MMP-9.

1.5.6 MMP-9 in Genetic Epidemiology Studies:

As mentioned previously in this report, MMP-9 expression is primarily regulated at the transcriptional level, and accordingly there has been some interesting epidemiological work done on a functional MMP-9 promoter polymorphism.

Zhang (Zhang et al, 1999) reported a novel –1562 C to T polymorphism in the MMP-9 promoter, and that this polymorphism showed increased expression functionality in transfection models. Studying this promoter, a sample of 584 male patients with MI and 645 controls from the ECTIM study (Etude Cas-Temoins de I'Infarctus du Myocarde) were genotyped, and although allele frequencies between cases and controls did not vary, 26% of those carrying 1 or 2 copies of the T allele had >50% stenosis in 3 coronary arteries, as opposed to only 15% of C/C homozygotes ($p<0.02$ after adjustment for age and referral centers) (Zhang et al, 1999). This suggests that although the polymorphism may not be associated with actual MI incidence, it is associated with angiographically determined symptoms of advanced atherogenesis, although in epidemiological terms the statistical association is fairly weak. A later study confirmed this finding in an autopsy study of 276 men (Pollanen et al, 2001) and showed MMP-9 genotype was associated with complex lesion area after adjustment for age, BMI, hypertension, diabetes and smoking ($p=0.012$). Another more recent study in 215 untreated hypertensive men showed that the T allele was associated with higher serum MMP-9, higher BP ($r=0.25$) and pulse wave velocity ($r=0.48$) (Zhou et al, 2007). Other studies have found the 1562C-T polymorphism not be associated with aneurysmal CAD (Lamblin et al, 2002), risk of MI ([1]Blankenberg et al, 2003), spontaneous cervical artery dissection (Wagner et al, 2004,) haemorrhagic transformation or parenchymal haematoma following recombinant tissue plasminogen activator (rt-PA) administration in stroke treatment (Montaner et al, 2003), nor differential levels of detectable MMP-9 activity in the plasma of healthy subjects at all (Demacq et al, 2006).

A different promoter polymorphism in the MMP-9 promoter, encompassing a $(CA)_n$ repeat element, has been implicated in intra-cranial aneurysm through action in the circle of Willis (Peters et al, 1999), although small study numbers (n=76) makes this study less robust. Another study suggests the number of repeats may be linked to plaques with thin fibrous cap and echolucent core (OR, 13.1; 95% CI, 1.6 to 100), although again this was only in 110 patients, and the confidence intervals are very wide (Fiotti et al, 2006).

1.5.7 MMP-9 in Molecular Epidemiological Studies:

Data regarding correlations of MMP-9 with other risk markers, particularly those associated with atherosclerosis, are sparse in the literature. One report in the Anglo-Scandinavian Cardiac Outcome Trial (ASCOT) showed that in 96 hypertensive patients, a clinical "package of care" treatment to reduce BP resulted in lowered MMP-9 levels (110 vs 80 ng/ml median; p= 0.035) as well as BP over 3 years ([1]Tayebjee et al, 2004). In line with some of the genetic studies above, molecular studies also suggest an association of MMP-9 with hypertension and arterial elasticity ([2]Tayebjee et al, 2004; Yasmin et al, 2005). A cross-section of the Framingham cohort was used to examine circulating MMP-9 plasma levels and correlations with CVD-specific echocardiographic left ventricular measurements (Sundstrom et al, 2004). Unfortunately, since the study used plasma (presumably citrated) only 20% (detectable n=138) of the cohort had detectable MMP-9. They did show that detectable plasma MMP-9 was significantly correlated with heart rate and antihypertensive medication. It also showed associations with increased LV internal dimensions, LV mass, and LV wall thickness in men but not in women, although these observations are hampered by small numbers of observations.

Circulating MMP-9 has been shown to be elevated in the blood of patients with acute coronary syndromes, including unstable (but not stable) angina (Kai et al, 1998; Inukubo et al, 2001; Fukuda et al, 2006). Interestingly, this could mean that MMP-9 is elevated in people who experience ischaemic events (silent or clinical) but not in those with only diffuse atherosclerosis, which may preclude use of MMP-9 as a marker of long-term risk. These are only small studies however, and more data is required. The FINRISK retrospective study has reported (cases n=120) that MMP-9 levels are significantly raised

in men with a history of MI compared with healthy controls (Renko et al, 2004). This is consistent with the hypothesis for a role for MMP-9 in post-ischaemic pathologies. Interestingly, there is a peak in MMP-9 expression immediately following MI in both coronary arteries and venous blood (Funayama et al, 2004; Squire et al, 2004), and MMP-9 levels correlate with echocardiographic and neurohormonal (N-terminal B-type natriuretic peptide) measures of LV dysfunction, and may be prognostic of post-MI CHF (Squire et al, 2004).

There are limited prospective data available for MMP-9, especially in healthy populations. These are summarized in Table 1.8. Studies published after commencement of this thesis (Oct 2004) are not discussed in this section, since these were not available in considering assessing MMP-9 as a risk marker. As can be seen, there was only one prospective study of MMP-9 at the outset of this thesis. The Atherogene study ([2]Blankenberg et al, 2003) examined 97 incidents of MI or fatal CVD in CAD patients, and found that MMP-9 was a moderate predictor of CHD; OR 1.4 (95% CI 1.2 to 1.8; $p<0.0001$), which attenuated to 1.3 after adjustment for confounders (95% CI 1.1 to 1.6; $p=0.005$). MMP-9 was not significantly associated with non-fatal MI, perhaps due to small numbers (n=41), although the unadjusted combined (fatal and non-fatal) OR was 1.3 (95% CI 1.11 to 1.52; $p<0.002$). The authors also show that (in this study) MMP-9 does not correlate strongly with acute-phase reactants, or associated, markers, and adds complementary information by simultaneous determination with IL-18. This isolated study suggests that MMP-9, generally similarly to other inflammatory markers, is a moderate marker of risk of MI in a population with prevalent CVD. More data is required to confirm and expand on this.

Table 1.8 Publications of MMP-9 as a prospective risk marker of CVD; methods for systematic review of the literature detailed in chapter 2.

Publication	Study	Population	Follow-up	End-point	n cases	Proportion of population at increased risk*	OR at sig (95% CI)†	Linear increase in OR?
[2]Blankenberg et al, 2003	Atherogene	CAD	4.1 years	CVD death or non-fatal MI	137	2nd quartile	1.30 (1.11–1.52)‡	Yes
Wu et al, 2005	-	Stable angina	17.7 Months	CVD event	48	ns	-	-
Cavusoglu et al, 2006	-	Patients undergoing angiography	2 years	All cause mortality	51	ns	0.78 (0.58-1.04)§	-
Eldrup et al, 2006	-	CAD patients	4.4 years	Stroke or CVD death	53	Above median	1.9 (1.1-3.5)	n/a

* Point in population distribution at which publication reports a statistically determined increased risk (either $p<0.05$ or OR lower 95% CI > 1.00)
† Odds ratio of event at the point of significance stated in the table after adjustment for conventional risk factors (variables are paper specific) unless otherwise indicated
‡ OR of increasing quartiles (univariable)
§ Univariate OR
ns: Not significant

1.5.8 Clinical Trials of Anti-MMP-9 Therapies and use in CVD:

Due to the wide range of diseases that MMPs in general play a role in there has been much interest in pharmacological inhibition, although this has not as yet proven successful in terms of acceptable toxicity of inhibitors.

Use of TIMPs as therapeutic inhibitors of MMPs is attractive, although non specific actions would precludes them from use in controlled trials to study specific MMP-9 inhibition. In any case, short TIMP half-life currently precludes therapeutic use (Curran et al, 2000).

Other ways inhibition may be achieved include at the transcriptional level, or directly on the enzyme itself. Known low molecular weight transcriptional inhibitors include xanthine derivatives, non-steroidal anti-inflammatories, and statins ([2]Wilson et al, 2005; Bellosta et al, 1998; McMillan et al 1996; Van den Steen et al, 2001). Hydroxamates, D-penicillamine, and tetracyclines have been described to inhibit MMP-9 activity directly (Cuzner et al, 1999). Although interesting these drugs have limited use in experimental models or in the clinic in terms of specific inhibition of MMP-9 or indeed MMPs in general. Many of these synthetic inhibitors were designed to be zinc-chelators, and reacted with other MMPs and proteases (Pavlaki et al, 2003). Lack of specificity may bring about additional unwanted side-effects in clinical trials of these drugs, e.g. in cancer therapies (Pavlaki et al, 2001; Hidalgo et al, 2001), whilst it renders them unsuitable in experimental models due to confounding effects. The most promising current compound is the covalent-binding mechanism based SB-3CT, which has specificity for gelatinases (although, again, not specifically MMP-9) and has shown some promise in the area of cancer research (Kruger et al, 2005). This research has also been extended to a potential use in acute stroke, since mice with focal cerebral ischaemia can be rescued from MMP-9-mediated laminin fragment induced neuronal apoptosis (Gu et al, 2005).

Interestingly, a novel approach to MMP inhibition in coronary intervention has seen an attempt to incorporate an MMP inhibitor (GM6001) into the fabric of the stent used, and

represents a novel pathway for localised anti-inflammatory treatment (Caldwell et al, 2003).

1.5.9 Conclusions:

Although MMP-9 is a molecule that has rightly received a lot of attentive research in the last decade, and its biology is becoming clearer, much of the research has been focused on cancer metastasis, and there are still glaring gaps in data, particularly in the cardiovascular area. Despite this there is a putative role for MMP-9 in bringing about mechanical instability in established plaques, and it may also play an auxiliary role in the inflammatory process. Consistent with this, the only study at the outset of this thesis which has prospectively examined MMP-9 as a marker of risk in CVD found it to be a moderate risk marker in a population with documented CAD. More epidemiological data in larger studies, and in healthy populations, are required to confirm expand on these findings.

1.6 sCD40L: BIOLOGY, AND EXPERIMENTAL AND EPIDEMIOLOGICAL ASSOCIATIONS WITH CVD

1.6.1 Introduction:

CD40 ligand (CD40L, CD154, gp39, TRAP, TBAM) is a member of the TNF superfamily, and interacts with CD40 (a TNF receptor superfamily member) in the trimeric manner typical of the family (Peitsch et al, 1993; Matsuura et al, 2001). Discovered in 1992 and originally thought to be largely restricted to expression on T-cells (Armitage et al, 1992; Graf et al, 1992), research into the functionality of CD40L has been dominated by adaptive immunology.

A growing appreciation of a wider cellular expression of the CD40/CD40L dyad, and the important role the dyad plays in inflammation and pathophysiological processes in general, mean the molecules are increasingly seen as central inflammatory players, rather

than an exclusive facet of lymphocyte biology. This review will focus on the role of membrane-bound CD40L (mCD40L) and its soluble form (sCD40L) in inflammation, how it interacts with its receptors, and in the role of the molecule more specifically in cardiovascular disease.

1.6.2 CD40L Expression, Synthesis, and Regulation:

Mapped to chromosome X q26.3 – q27.1 (Graf et al, 1992), the CD40L gene yields a 261 amino acid polypeptide with a small transmembrane domain. Much like its family relative TNFα, the molecule is externalised at the cell surface bound as a pre-formed trimer (Chan et al, 2000). Although perhaps not restricted to expression on T-cells (described below), most of our knowledge of the expression of CD40L is derived from studies employing activated mature CD4+ T-cells, which express high quantities of the protein.

Memory T-cells express CD40L on the cells surface as soon as 5 mins after activation, indicating expression of pre-formed CD40L (Casamayor-Palleja et al, 1995). De novo production of CD40L in naive T-cells is a more controversial issue, with T-cell receptor (TCR) stimulation and T-cell:antigen presenting cell co-stimulation playing an important role, but exactly which co-stimulation depends on the experimental setting (Jaiswal et al, 1996; Nusslein et al, 1996).

Other cells that have been shown to express CD40L include monocytes/macrophages, platelets, B-cells, basophils, eosinophils, mast cells, dendritic cells, endothelial cells, SMCs and epithelial cells (Gauchat et al, 1993; Gauchat et al, 1995; Grammer et al, 1995; Mach et al, 1997; Pinchuk et al, 1996; Henn et al, 1998; Gaweco et al, 1999). Induction of expression of CD40L in these cells is a poorly defined process and probably varies for different cell types; soluble factors such as IL-1β, TNFα, and IL-4 are thought to be important (Mach et al, 1997; Gauchat et al, 1994). Some of the cell types mentioned above are more classically known for expressing CD40 (typically being antigen presenting cells in an immunological context), but in some cases cross-linking of basal levels of CD40 on the cell surface with either CD40L or a CD40L-IgFc fusion protein

may result in expression of CD40L on the surface (Pinchuk et al, 1996). It should be noted that a more recent study specific to atheromas has re-examined the evidence that these "novel cell types" express CD40L using new labelling antibodies, and could not corroborate this claim phenotypically or in terms of mRNA production (Buchner et al, 2003). They found only CD4+ T-cells and platelets to express CD40L in the atheromatous lesions.

Soluble forms of CD40L have been described, and are currently very topical in the literature. The existence of these soluble forms was first noted, again in T-cells, in 1995 (Graf et al, 1995). The lighter soluble molecules have several isotypes although the molecular and functional differences between these are not clear (Graf et al, 1995; Ludewig et al, 1996; Wykes et al, 1998)

In terms of inhibition of CD40L, no cell-bound or soluble factor has been described as directly binding and inhibiting or cleaving either cell-bound or sCD40L. Indeed the only described physiological inhibition of expression of CD40L is at the transcriptional level, where transforming growth factor-β and IFNγ have been reported to inhibit mRNA expression in T-cells (Gauchat et al, 1994; Roy et al, 1993)

1.6.3 mCD40L, sCD40L, Platelets, and General Biological Functions:

Activated Th1 cells express CD40L and interact with CD40 to bring about cell mediated immunity (Grewal and Flavell, 1998). It has also been proposed that CD40 ligation on dendritic cells stimulates IL-12 production and helps skew a Th1-type response typical of an atherosclerotic environment (Van Kooten and Banchereau, 2000). Also well studied is the interaction between CD40L on Th2 cells and CD40 on B-cells to bring about humoral immunity. Ligation of CD40 on B-cells via recombinant CD40L or anti-CD40 Abs results in activation of B-cells as evidenced by surface marker expression (such as CD23, CD80, CD86) and soluble cytokines (such as IL-10, IL-6, TGF-β) (Schonbeck and Libby, 2001).

These specific functions of CD40L were for many years were the focal point of research, and it was widely considered that expression of CD40/CD40L must be largely restricted to immunological cells in order to prevent disseminated adaptive immunological activation. To some extent this may be true; controlled expression of the dyad is important as evidenced by the regression of many experimental autoimmune diseases in models where a disruptive antibody to the CD40/CD40L dyad is added (Howard and Miller 2004). As discussed above however, there may be scope for controlled expression of the dyad in other cell types.

Platelets warrant specific attention when talking about CD40L. The role platelets play in the development of atherosclerosis is now widely acknowledged, if poorly understood, and the concept that they play a role in development of plaques as well as thrombotic complications stretches back to Ross's first proposal of platelet interaction with injured epithelium in 1976 (Ross and Glomset, 1976). Although this hypothesis went out of favour, it was recently shown that introduction of activated platelets to ApoE -/- mice exacerbates atherosclerosis (Huo et al, 2003), suggesting some role in disease progression.

Henn et al first demonstrated that platelets store pre-formed CD40L (Henn et al, 1998). It is now believed that platelets contain around 95% of the circulating pre-formed CD40L (although it should be noted this view is not universal) and this is quickly expressed on the platelet surface following stimulation with various agonists such as thrombin (Henn et al, 1998) although it is distinct in kinetics from other markers of activation such as P-selectin (Hermann et al, 1998). Emphasising this, one recent model shows patients undergoing allogenic stem cell transplants and suffering subsequent graft versus host disease had elevated sCD40L, and this correlated very poorly with circulating sIL-2 receptor (a T-cell activation marker) levels, but correlates well with platelet number (Nagasawa et al, 2005). This may suggest that platelets are a major source of sCD40L.

mCD40L on platelets allows interaction with other platelets via CD40 (Henn et al, 2001) as well as the platelet integrin GPIIb/IIIa, and this interaction can stabilise arterial

thrombi via high-shear platelet aggregation, and hence conferring thrombogenic proclivity (Andre et al, 2002). CD40L may also bind to the promiscuous integrin Mac-1, mediating endothelial cell activation and facilitating extravasation of inflammatory cells (Zirlik et al, 2007).

It appears that platelet-platelet interaction is vital in the unknown hydrolysis process that sequesters mCD40L to sCD40L. It has been demonstrated that interrupting either CD40-CD40L or CD40L-GPIIb/IIIa interactions via blocking monoclonal antibodies prevents production of sCD40L, suggesting the need for a surface-bound proteinase (Henn et al, 2001; Furman et al, 2004). An MMP (Furman et al, 2004) or TACE (Schonbeck and Libby, 2001) have been suggested as a possible candidates for this proteolytic process.

Interestingly, the biological properties of sCD40L are largely unknown. While it is undisputed that mCD40L is proinflammatory, and acts as such in the atherosclerotic environment (discussed below), if studying CD40L as a biomarker, then it is the soluble form that is measured in plasma or serum, and yet its function remains enigmatic. Many investigators site evidence that recombinant sCD40L upregulates endothelial expression of adhesive proteins (Hollenbaugh et al, 1995), promotes chemokine production from PBMCs (Kiener et al, 1995), and induces pulmonary inflammation (Wiley et al, 1997). Yet Henn et al, who crucially discovered platelet production and release of sCD40L, also demonstrated in their work that under their experimental setting, natural sCD40L failed to induce an inflammatory response either in isolation or in combination with sub-optimal TNFα in human endothelial cells (Henn et al, 2001). Hence, the sequestering event may bring about some change in the function of the CD40L molecule. Indeed, even a study showing that sCD40L inhibits re-endothelialization of injured blood vessels used an *in* vitro technique and recombinant sCD40L (Urbich et al, 2002). T-cells appear to have different signalling mechanisms in the regulations of mCD40L and sCD40L (Matthies et al, 2006). Some work suggests that membrane polarised CD40 receptor/ligand clustering is required to bring about a highly concentrated, localised cellular activation site, and hence full signalling (Grassme et al, 2002). Sequestered CD40L (in a natural state) may be unable to accomplish this polarised clustering. It is also interesting that sCD40 has been implicated in *disrupting* signalling in T-cell interactions by acting as an antagonist

(Nemoto et al, 2002) although no experimental evidence yet describes such a role for sCD40L. Taken together this evidence clearly suggests that mCD40L and sCD40L may be physiologically different.

1.6.4 Pathological and Animal Studies of CD40L in CVD:

Three frequently discussed pro-atherothrombotic functions of sCD40L are illustrated in Figure 1.13. The most relevant of these with reference to the marker's potential to promote sub-clinical atherosclerosis and act as an active biomarker is its supposed pro-inflammatory activity.

Most of the reported studies in CVD to date have used models pertinent to mCD40L and not specifically sCD40L (reported in section 1.6.3). mCD40L and sCD40L are both found in human atheromas (Mach et al, 1997; Buchner et al, 2003) irrespective of which type of cell may be expressing them. This CD40L has the hypothetical *potential* to interact with CD40 (or other receptor) expressing cells in the vicinity and bring about a range of pro-atherogenic functions (listed in Table 1.9).

In animal models the evidence that the CD40 dyad is implicated in the development of atherosclerotic lesions is compelling. It was initially shown by Libby's group that LDL receptor-deficient mice fed a high cholesterol diet and given anti-CD40L antibodies had limited formation of initial lesions as compared with controls (Mach et al, 1998). Lutgens et al confirmed this finding, showing that ApoE-/- mice crossed onto a CD40L -/- background had drastically smaller plaque area as well as altered plaque composition (in this case, 77% fewer T cells in early plaques) (Lutgens et al, 1999). It was Libby's group again however which reported the most compelling finding in a later study (Schonbeck et al, 2000). They fed 8-10 week old LDL receptor-deficient mice a high-cholesterol diet for 13 weeks, before a group were sacrificed as a baseline control (n=8), and 22 continued on the diet in conjunction with either saline (n=8), rat IgG (n=8) or anti-CD40L treatment (n=6). This allowed them to study if the interruption of the CD40 dyad could limit evolution or cause regression of established lesions. They showed that although the CD40L antibody could not cause regression of lesions, it did moderately limit evolution.

More impressive however, was the fact that the mice given the CD40L antibody had the cellular composition of the lesions altered, and contained more SMCs and fewer macrophages as well as less lipid; a far more stable phenotype in terms of rupture. Interestingly, a very recent study has shown in CD40 LDLR double KO mice, that inhibition of atherosclerosis progression is not mediated by CD40 (atherogenesis being essentially normal in these mice) but perhaps by CD40L interactions with other cognate receptors, potentially including the Mac-1 integrin (Zirlik et al, 2007).

In contradiction to these reported animal models, one study shows that CD40L KO mice that receive cardiac allografts can become allospecific immunotolerant, but develop significant graft arteriosclerosis 8-12 weeks post-transplant (Shimizu et al, 2000). Although an exceptional experimental circumstance, this demonstrates that under extreme conditions CD40L may not necessarily be absolutely required for atherogenesis, although notably the authors here do not report phenotypic observations as to plaque morphology. It seems possible that the atherosclerosis progression in this report may be "trauma-propagated."

Generally these findings may have important implications for potential therapies of atherosclerosis and atherothrombotic complications, since they generally implicate CD40L as pivotal to the prevalent general inflammatory environment. It is currently impossible to untangle the influences of mCD40L and sCD40L or even relative importance of cognate receptors in these models. Such a model would require a selective antibody, or animals with a genetic alteration to the CD40L gene rendering the molecule non-sequestering. Since little is known of the mechanism of the sequestering process, this has not yet been achieved. Currently more experimental work in animal and tissue models work is required using human natural sCD40L.

Figure 1.13 Three putative functions of sCD40L released from platelets during thrombosis. sCD40L is released from platelet-rich thrombi and contributes to various steps in atherosclerotic lesion progression: (1) Inflammation; sCD40L induces the production and release of proinflammatory cytokines from vascular cells and matrix metalloproteinases from resident cells in the atheroma. (2) Thrombosis; sCD40L stabilizes platelet-rich thrombi. (3) Restenosis; sCD40L inhibits the reendothelialization of the injured vessel. Adapted from Andre et al, 2002

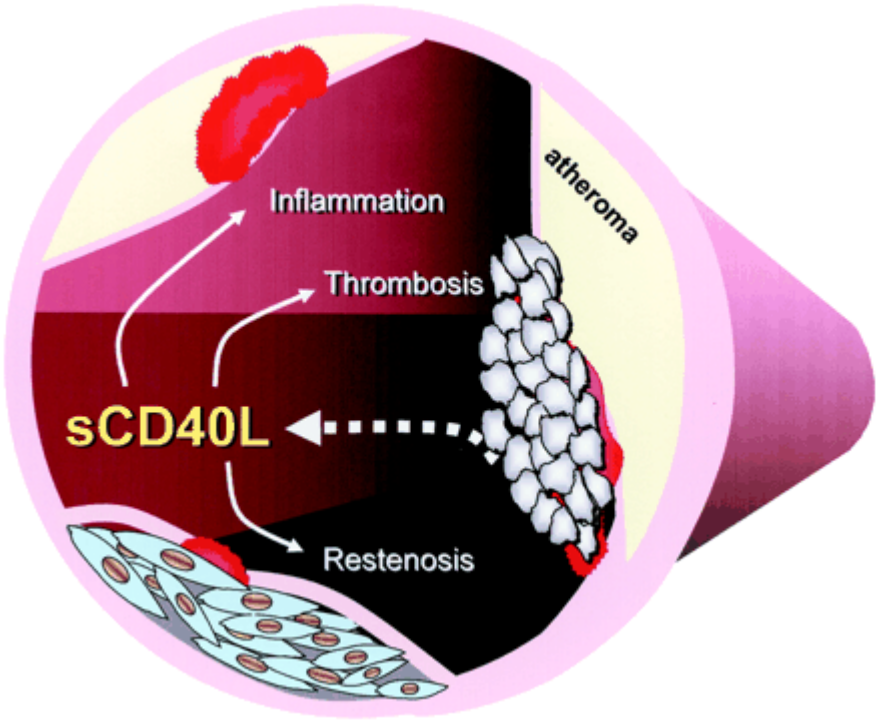


Table 1.9 Inflammatory processes initiated by the ligation of CD40 on various cell types. Note that hypothetically almost all aspects of atherothrombosis are potentially affected by CD40-CD40L interactions, i.e. initiation, perpetuation, necrotic core formation, destabilisation, and thrombus promotion

Mechanism of proatherogenic/ atherothrombotic function	**Primary responsible cell type(s) expressing cognate receptor**	**Resultant processes/ factors produced through CD40 ligation**	**References**
Increased extravasation of lymphocytes (vascular permeability)	Endothelial cells	Endothelial activation Production of adhesion molecules (VCAM-1, ICAM-1, E-selectin)	Karmann et al, 1995 Kotowicz et al, 2000. Zirlik et al, 2007
Inflammatory cell chemotaxis to site of plaque	Macrophage	Chemokine production (RANTES, IL-8, MIP-1α)	Kiener et al, 1995; Kornbluth et al, 1995
Localised inflammatory cytokine production	Endothelial cells, smooth muscle cells, macrophages, T-cells, NK-cells etc	TNFα, IL-1, IL-6, (IL-12, IFNγ – Th1)	Mach et al, 1997; Kiener et al, 1995; [1]Schonbeck et al, 2001
Destabilisation of established plaque	Macrophages	MMP expression (including MMP-9)	Schonbeck et al, 1999, [2]Schonbeck et al 2001; Mach et al, 1999
Thrombogenic proclivity	Endothelial cells, platelets,	Expression of tissue factor and promotion of platelet aggregation.	Miller et al, 1998 Zhou et al, 1998 Andre et al, 2002

1.6.5 Plasma or Serum in the Measurement of sCD40L:

The use of serum or plasma samples in the measurement of sCD40L is currently a controversial issue, and it has been addressed in a number recent studies. Ahn (et al, 2004) examined serum clotted on ice (serum-I) or at room temperature (serum-RT) as well as platelet poor plasma (PPP) in patients with thrombocytopenia (n=10), patients with thrombocytosis (n=12), and healthy controls (n=8). They found that serum-I appears to reduce much "*ex vivo"* release of sCD40L compared with serum-RT. In fact they report that serum-I levels are closer in magnitude to PPP, except in thrombocytosis patients, and the authors note this may be an artefact of more platelets releasing more sCD40L in the short time before cooling and separation compared to the other groups. Of note, although not reported, the data appears to show little difference in plasma levels between the different study groups (a median < 0.5ng/ml for all). This may allude to measurements being at the limit of sensitivity for the assay.

Interestingly, the authors (Ahn et al, 2004) go on to use a 0.1μM filter to demonstrate that a large proportion of "sCD40L" found in all sample types is in fact microparticle-bound. Therefore sCD40L is actually the sum of "true" sCD40L and microparticle-bound CD40L (mpCD40L). The proportion of mpCD40L is higher in serum-RT although the reason for this is not clear. The biological function of mpCD40L and its biochemical differences with sCD40L are also currently unknown.

In another recent report (Mason et al, 2005) PPP levels, and platelet surface expression of CD40L in patients with stable (n=40) and unstable (n=40) angina was examined. They found that PPP sCD40L did not differ between the groups, however, serum levels were lower (1.4 ± 1.3 vs 5.2 ± 3.7ng/ml, $p< 0.001$) and platelet membrane expression was higher (1.4 ± 0.7% vs 0.9 ± 0.6%, $p<0.03$) in *unstable* compare with stable angina. Speculatively, this may be a reflection of *in vivo* platelet activation. In patients with elevated baseline inflammation, platelets are activated and express surface mCD40L and sCD40L, and consequently there are fewer platelet granules to release upon serum activation. Hence, platelets in patients with prevalent CHD, platelets may appear exhausted *ex vivo*. This hypothesis has been partially corroborated in other work using

serum, where those with prevalent CVD have lower serum levels of sCD40L (Aukrust et al, 1999; Tanne et al, 2006) and at least non-significant trends have been observed in other work (Weber et al, 2007; Kiani et al, 2007). This is in contrast to many of the other biomarkers of CVD risk where higher circulating levels predict greater risk (the notable exception being high-density lipoprotein HDL; although this does not mean that sCD40L is acting in an atheroprotective protective manner.)

Other studies (Varo et al, 2006; Weber et al, 2006) broadly agree with these data, finding not only serum and anticoagulants to be important factors, but also centrifugation e.g. processing time, and sample condition (haemolysis, bilirubin etc) to be important factors in measuring sCD40L. Mason et al, (Mason et al, 2005) meanwhile reported that they used two different commercially available sCD40L ELISA kits to measure the same samples, and got very different results. Halldorsdottir (et al, 2005) report that a new Bender Medsystems high-sensitivity ELISA may preferable for the measurement of plasma sCD40L, and a new developmental electrochemiluminescence immunoassay (Elecsys®; Roche Diagnostics) also shows some promise (Weber et al, 2006). This means that while multi-centre trials are ongoing, the researchers must make every effort possible to keep conditions constant within a study, and results should be interpreted with caution until the full extent of these pre-analytical variables are determined. Review of the data is not helped by reports that do not fully explain methodologies used, or indeed contradict themselves in terms of anticoagulants used in the study (Yan et al, 2004).

Platelet degranulation is undoubtedly a problematic issue in serum measurements, however, EDTA anticoagulant also has undesirable and to some extent "non-specific" effects on the morphology and biochemistry of platelet function (Golanski et al, 1996), and all of the above study authors noted a trend towards higher sCD40L levels in EDTA anticoagulated compared with citrated plasma. This is almost certainly an *in vitro* artifact. While I would concede that circulating sCD40L levels are perhaps more accurately represented in citrated plasma, sodium citrate also has some platelet activating functions (Golanski et al, 1996). Indeed, very recent findings suggest that plasma levels of sCD40L are very much an *in vitro* artifact and are unrelated to prevalent disease in the individual (Ivandic et al, 2007). In addition, circulating levels of sCD40L in normal healthy patients

is often at, or below, the limit of detection in citrated samples (e.g. Malarstig et al, 2006; and chapter 3) in ELISAs that are not high-sensitivity (~100pg/ml). While it may be true that rigorous standardized conditions are required throughout studies for the measurement of sCD40L, this is certainly just as true for plasma samples as it is for serum.

In light of evidence that much sCD40L in circulation may be microparticle-bound in a poorly defined manner, that the effect of chronic inflammation on sCD40L expression is poorly understood, that the anticoagulant effect on sCD40L released is poorly (objectively) described, and that ELISAs by different manufacturers give very disparate results, it seems we are only beginning to learn of the complex underlying biology of the CD40L molecule. .

1.6.6 sCD40L in Genetic Epidemiology Studies:

Only one study has examined haplotypes of CD40L and risk of CVD events to date (Malarstig et al, 2006). In this study 2359 patients with non-ST elevation ACS had citrated blood taken although the number of MI cases obtained in follow-up is not stated by the authors and 7% of patients had undetectable sCD40L. The authors report the -3459 A-G polymorphism was associated with sCD40L levels. Patients hemi or homozygous for the G allele (23.6%) had ~10% increased levels of sCD40L. In the placebo group, patients above the median of sCD40L were at increased risk of MI; OR 2.5 (95% CI 1.6 – 3.9). Despite this, the G allele was not associated with risk of MI, although as previously discussed it is quite possible that the study was insufficiently powered to detect the corresponding increased risk due to a 10% increase in sCD40L levels.

1.6.7 sCD40L in Molecular Epidemiological Studies:

Due to the aforementioned issue regarding sCD40L levels in different sample types there is limited comparability between existing reports and limited conclusions can be drawn.

Data in larger population have so far demonstrated few associations of sCD40L with other inflammatory and classical risk markers. The largest such study to date was the

Dallas Heart Study performed by Libby's group (de Lemos et al, 2005), although the study used EDTA anticoagulated samples. sCD40L levels were measured in 2811 patients. Quartiles of sCD40L in the population showed no association with age, sex, race, BMI, diabetes, smoking, creatinine clearance, LDL cholesterol, HDL cholesterol, or CRP, nor was it associated with coronary artery calcium or aortic plaque mass. There was a weak association with total cholesterol and triglycerides. Lack of association with plaque mass is perhaps not surprising when it is considered (as discussed previously) sCD40L had a greater effect on plaque composition than on plaque mass in animal studies (Schonbeck et al, 2000). This report is supported by other MRI work in citrated plasma showing sCD40L levels correlate with degree of carotid artery lipid pool, but not actual stenosis size (Blake et al, 2003) and not with plaque size nor coronary calcification in a group of SLE patients (Kiana et al, 2007).

Lack of sCD40L correlation with conventional risk factors was seen in a study of 400 men with no history of CVD who had sCD40L levels measured in plasma (anticoagulant not stated) ([2]Verma et al, 2005). The study ascertained that circulating levels again did not correlate, or correlated very weakly with other risk markers, and also did not correlate with the subject's perceived Framingham Risk Score in univariate analysis ($r=0.074$, $p=0.14$).

In contrast to these findings, one report found that 36 patients with and 32 without overt CVD benefited from a multi-factorial cardiovascular risk clinical intervention "package of care" (Lim et al, 2004). Both groups had a drop in plasma sCD40L after 1 year (median of 47% and 60% of pre-intervention levels respectively) whereas IL-6 levels were not significantly changed in either group. The study is however small, and as with all such "package of care" studies it is impossible to directly implicate the cause of individually lowered biomarkers.

In serum samples, weak correlations with aspects of the metabolic syndrome have been demonstrated in 313 patients with CAD ([2]Lee et al, 2006). In agreement with this observation serum sCD40L levels may be elevated in children with hypercholesterolaemia (although numbers were very small; 16 children total) (Martino et

al, 2007). Significantly, the only very strong correlation reported with sCD40L levels is that of numbers of circulating platelet/monocyte aggregates and the levels of the platelet activation marker p-selectin in serum (Heeschen et al, 2003; Yan et al, 2004). The tendency of platelets to form aggregates can be seen as the "thrombotic proclivity" of the circulating blood, and hence may be a risk factor for ischaemic events. As discussed previously mCD40L has clear potential to play a role in this aggregation, but the physiological relationship to sCD40L is unclear.

Increased levels of sCD40L have been found in a range of cardiovascular diseases, such as diabetes, peripheral arterial disease (PAD), pulmonary hypertension, acute/chronic heart failure, and stable/unstable angina (UA) (Aukrust et al, 1999; Marx et al, 2003; Blann et al, 2005; [1]Varo et al, 2003; Damas et al, 2004; Ueland et al, 2005; Novo et al, 2005). The majority of these were performed on plasma samples, although the first such report was performed in serum and demonstrated elevated levels of circulating sCD40L in patients with stable and unstable angina, although it was quite a small study (total n=74) (Aukrust et al, 1999). The study also reported that platelets taken from patients suffering angina had decreased intra-granule stores of sCD40L, and that stimulation of these platelets from patients with a thrombin receptor agonist resulted in reduced release of sCD40L *ex vivo*. This is consistent with the preceeding "platelet exhaustion" hypothesis in patients with prevalent CVD. Despite this, another very small study has found that serum levels of sCD40L are raised in patients with prevalent CHD (Tsuzuki et al, 2005).

There were limited prospective data available for sCD40L, especially in healthy populations before commencement of this thesis. These are summarized in Table 1.10. Studies published after commencement of this thesis (October 2004) are not discussed in this section, since these were not available in considering assessing sCD40L as a risk marker. Libby's group were the first to show that sCD40L was predictive of CHD in patients after a primary acute event ([2]Varo et al, 2003). The study was a nested case (n=195) – control (n=195) study using citrated plasma and with end points of death, MI or CHF. After adjustment for conventional risk markers, CRP and cardiac troponin (cTnI), sCD40L was a risk marker for MI, and death/MI composite in the upper half of

the population (OR 1.9, $p<0.001$ for both). sCD40L was also an independent predictor of death (RR 1.9 $p<0.05$). In contrast, the MIRACL (Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering) study found sCD40L to only be a univariate predictor of incident CHD in a cohort with prevalent CHD, and then only in the 90th percentile (~30 cases) (Kinlay et al, 2004). Another paper has suggested that sCD40L levels in patients with previous ACS are associated with CHD events (Yan et al, 2004), however the study is not included in the table due to it being inadequate in meeting systematic review criteria (poorly reported results and contradictory methodology).

There has been only one prospective study that has examined sCD40L as a risk marker in healthy people. Libby and Ridker's groups ([3]Schonbeck et al, 2001) used a nested case-control study of healthy women (Women's Health Study using EDTA plasma samples) where 130 women subsequently developed MI, stroke or CVD death in a four year follow up. These cases were age and smoking status-matched with 130 women who remained disease free. Cases had higher baseline sCD40L levels than did controls (2.86 ± 0.35ng/ml vs 2.09 ± 0.19ng/ml, $p<0.02$). However, the OR of experiencing an event did not become significantly higher until sCD40L levels were in the 95th percentile (univariate RR 3.29, 95% CI 1.26 – 6.59, $p<0.02$). With only 11 cases above the 95th percentile of distribution to define the statistically significant RR, the result is unreliable, and the authors concede it is "limited data."

Table 1.10 Publications of sCD40L as a prospective risk marker of CVD; methods for systematic review of the literature detailed in chapter 2.

Publication	Study	Population	Follow-up	End-point	n cases	Sample type	Proportion of population at increased risk*	OR at sig (95% CI)†	Linear OR increase?
[3]Schonbeck et al, 2001	Womens Health Study	Healthy middle aged women	4 year	MI, Stroke, Fatal CVD event	130	EDTA	95th percentile ‡	3.29 (1.26–6.59)	Yes
[2]Varo et al, 2003	OPUS-TIMI16	Recent CVD event	10 months	New MI, CHF or death	195	Citrate	Above median ‡	1.8 (1.1 - 2.5) §	n/a
Kinlay et al, 2004	MIRACL	Patients with symptomatic CHD	4 months	MI, cardiac arrest, worsening angina, death	256	EDTA	90th percentile ‡	1.86 (1.25 – 2.77)	Yes
Heeschen et al, 2005	CAPTURE	Patients with acute chest pain and CAD	6 months	Incident CHD or mortality	193	Plasma (? Anti-coag)	>5.0 ug/L	2.45 (1.39- 4.32)	?
Malarstig et al, 2006	FRISC-II (Patients with ACS symptoms)	Placebo group	6 months	MI	? 1233 patients	Citrate	Above median	2.5 (1.6 - 3.9)	?
		Low mol weight heparin group	6 months	MI	? 1225 patients	Citrate	ns	1.1 (0.7 - 1.6)	n/a
Tanne et al, 2006	BIP	Chronic CHD	7.9 years	Incident CVD or sudden death	233	Serum	ns	0.70 (0.34–1.41) at 90th percentile	Linear inverse

Key as Table 1.4, 1.6 and 1.8

1.6.8 Clinical trials of Anti-sCD40L Therapies and use in CVD:

It is already acknowledged in the literature that safe, direct, non-toxic inhibition of the CD40-CD40L dyad may be prove to be impossible in the long-term without serious complications resultant from immunocompromisation of the patient. It may therefore be more desirable to direct therapies towards downstream signalling events caused by CD40L in the atheroma setting (Lutgens et al, 2007).

The non-specific effect of cardiovascular drugs on sCD40L levels and consequent risk of events has been investigated by some authors. In the CAPTURE trial (c7E3 Fab Anti-platelet Therapy in Unstable Refractory Angina) (Table 1.10) 1088 patients with ACS had been randomized into groups comparing the glycoprotein IIb/IIIa inhibitor abciximab with placebo controls (Heeschen et al, 2005). These patients had sCD40L measured at baseline (plasma; anticoagulant not stated). The authors found that patients with elevated sCD40L (>5.0ug/ml) were at increased risk of death or MI (OR 2.71, 95% CI 1.51-5.35, p=0.001). In the study patients with sCD40L levels in the top two quintiles benefited from abciximab treatment, with a reduction in risk from an OR of 1.12 in the third quintile to an OR of 0.35 in the fourth. Among troponin T-negative patients, high levels of sCD40L identified a subgroup whose risk was reduced by treatment with the drug (5.5% vs 13.6% for those receiving placebo; p=0.03). This extremely interesting study is hampered by the fact that, because the gp IIb/IIIa receptor can bind other ligands such as von Willebrand factor (vWF) and fibrinogen, effects cannot be only attributed to sCD40L based on these observation. The only way to eliminate this possibility is in a placebo controlled drugs trial involving a specific mAb against sCD40L, and this is not yet available.

In the MIRACL cohort (Kinlay et al, 2004) (Table 1.10) EDTA plasma levels of sCD40L were measured in 2908 subjects on admission within 24 and 96 hours of onset of unstable angina, or MI, with a further measurement at 16 weeks following high-dose atorvastatin (80mg/day) or placebo treatment. The study did not convincingly find that the drug alters circulating sCD40L after 16 weeks (all patients p=0.08). This may suggest that the protective effects of the drug on patients with high sCD40L levels are completely independent of any effects on the marker. This study may be consistent with previous findings in patients with stable coronary syndromes or familial history of CVD in which

the authors found that serum levels of sCD40L took 2 years to be lowered by statin treatment (Semb et al, 2003).

1.6.9 Conclusions:

Published literature in animal models clearly suggests that CD40L interactions with CD40 (and perhaps other novel receptors) play a role in the development of atherothrombosis. The potentially separate roles of sCD40L and mCD40L in this process have yet to be elucidated. Current epidemiological studies have failed to consistently show any association of plasma or serum sCD40L levels with conventional vascular risk factors, prevalent vascular disease, or risk of CHD events. This may be partially attributed to small studies and inadequate observations of standardized protocols for collection of blood samples in some studies. More data in larger and high quality studies is therefore required.

1.7 INFLAMMATORY MARKER ASSOCIATIONS WITH STROKE

"STROKE is one of the oldest recognized diseases, but remains one of the least understood. A description of one of the most classic stroke syndromes, the left middle cerebral artery embolism, may have been described in the Bible: 'If I forget you, O Jerusalem, let my right hand wither! Let my tongue cleave to the roof of my mouth, if I do not remember you, if I do not set Jerusalem above my highest joy!' (Psalm 137)." (from Elkind, 2003)

1.7.1 Introduction

Having examined the associations of CRP, fibrinogen and the four novel potential risk markers and their associations with atherosclerosis, vascular risk markers and (primarily) CHD, it is important to consider the associations of these markers also with stroke in a separate section. The aetiological differences between CHD and stroke have long been considered a puzzling phenomena, and inflammation may be at least a partial key in explaining these observations. This section of the literature review will briefly discuss the current data.

1.7.2 Atherosclerosis: Differences in Relationship with Stroke and CHD

The early introduction of this thesis discussed the diffuse nature of atherosclerosis and the consequential plethora of CVD-related morbidities this may cause as the atherosclerosis progresses and become clinically apparent. This is clearly because atherosclerosis plays causal roles in these pathologies, and the incidence of stroke (cerebrovascular accident) may be included in these pathologies. Despite this relationship it is important to differentiate stroke in its broadest definition from other CVDs, including CHD, due to the range of underlying aetiologies.

Stroke may either be haemorrahagic or ischaemic in origin. Haemorrhagic stroke more often has associations with micro-aneuyrsms than with atherosclerosis and as such can be considered as a separate vascular disease. However, even within ischaemic stroke there are several broad categories. While nearly all CHD is linked to coronary atheroma, only half of stroke is due to large vessel atheroma (Di Napoli et al, 2002). Other causes can be heart valve diseases, or cardiac arrhythmias (e.g. atrial fibrillation) leading to emboli in larger vessels, or lacunar stroke where occlusion occurs within the small vessels of the brain. A system for categorization of subtypes of ischemic stroke has been developed in the Trial of Org 10172 in Acute Stroke Treatment (TOAST) (Adams et al, 1993). It can generally be said however that the most severe ischaemic stroke cases usually occur as a result of thrombosis originating from larger vessels such as the common carotid artery or aortic arch.

1.7.3 Stroke, Cholesterol, and Statins

A major reason necessitating the differentiation of stroke and CHD is the different roles conventional risk factors seemingly play in the respective diseases. For instance, blood pressure is the single most important modifiable risk factor for primary and secondary stroke prevention (Cosentino et al, 2005). However, cholesterol measurements only weakly predict ischaemic stroke (Shahar et al, 2003). It could be argued this observed effect is due to composite pooling of strokes of different origin in investigating studies, although cholesterol is still only weakly related to isolated thrombotic stroke in slightly less powerful analyses (Oliver et al, 2000).

In accordance with these observations, fibrate drugs to lower circulating serum cholesterol failed to show efficacy in lowering risk of stroke on meta-analysis (Herbert et al, 1995). Also one study demonstrated that partial ileal bypass surgery reduced circulating LDL-cholesterol in patients with hypercholesterolaemia and previous MI, and it lowered risk of MI by 35%, but did not lower risk of stroke (Buchwald et al, 1990). In contrast with this lack of association with cholesterol, most major trials have reported that taking statins reduces the risk of incident stroke irrespective of the study population. In the 4S trial (Scandinavian Simavastatin Survival Study) of secondary prevention, the active therapy group had a 51% decreased risk of ischaemic stroke and 35% decreased risk of TIA (SSSS Study group, 1994). Similar results were found in the CARE trial (Plehn et al, 1999). In primary prevention trials the reduction in risk seems smaller if at all beneficial, as seen in the WOSCOPS study, although this observation was limited in strength by small numbers of incident stroke (46 vs 51 p=0.61) (WOSCOPS Study group, 1996) and the length of follow-up (since more recent data suggest that reduction of stroke risk in primary studies may take 3 years therapy to observe; Byington et al, 2001; Pedersen et al, 1998). Observations that taking statins reduce stroke risk are confirmed on meta-analysis ([2]Ross et al, 1999; Di Napoli et al, 2002). The reduction in risk of stroke from taking statins is also relatively independent of reduction in cholesterol level when comparisons are made with CHD. (Warshafsky et al, 1999) (Di Napoli et al, 2005). Indeed, in the Womens Health Study (n=15 632) a prospective cohort of initially healthy women, direct comparisons were made between CHD and stroke, and lipid levels were significant risk determinants for ischemic stroke but with a magnitude of effect much smaller than that observed for CHD (Everett et al, 2006). Recently, the meat-analysis of all statin tirals showed that statins reduce stroke risk (Cholesterol Trialist's Collaboration, 2005).

Clearly inflammation has a role in the subsequent degenerative neuronal damage in the acute phase following ischaemic stroke, and as such may be an attractive concomitant therapeutic target for use with recombinant tissue plasminogen activator (rt-PA) thrombolytic therapies (Lapchak and Araujo, 2007). However, progression from sub-clinical to the acute phase is a confounder of incident associations (del Zoppo and Hallenbeck, 2000). Prospective studies are required to establish the relationship between inflammatory markers and incident stroke.

1.7.4 CRP and Fibrinogen and the Prediction of Stroke

In a pattern of argument already discussed, many of the general points proponents of measuring CRP as a risk marker for CHD have used have also been applied to the clinical setting of stroke risk prediction (Pearson et al, 2003).

CRP has recently been shown to correlate with IMT of the carotid artery in 2 separate studies, and to differentially predict increased thickening of IMT over a number of years in 700 and 392 adults respectively (Schmidt et al, 2006, Lee et al, 2007) although the association with IMT was modest in both studies, and IL-6 appeared a superior marker compared to CRP in one study after adjustment for traditional confounders ($p<0.001$ vs $p<0.05$; Lee et al, 2007). Interestingly upon MRI scanning, the associations between severity and progression of small vessel disease-related brain abnormalities and CRP were nonsignificant (Schmidt et al, 2006) suggesting non-uniform associations of CRP with carotid atherosclerosis and lacunar pathology. Indeed, a separate study found that cholesterol and LDL were better markers of small vessel disease, whilst inflammatory markers were associated with extracranial large vessel disease (Suwanwela et al, 2006), underscoring potentially different aetiological roles of inflammation within subsets of ischaemic stroke.

A qualitative meta-analysis of the link between elevated circulating CRP and primary stroke is shown in Figure 1.14 (Di Napoli et al, 2005). As can be seen, those in the top population quartile of CRP expression have an elevated risk of stroke (~OR 1.5) comparable to that seen on meta-analysis for risk of CHD (Danesh et al, 2004), although no statistical data is presented in this report. More data has added to this debate recently. The Rotterdam Study showed prospectively in 498 primary strokes that CRP was very modestly associated with risk of all strokes (age- and sex-adjusted OR per SD, 1.14; 95% CI 1.04 - 1.24) and risk of ischemic stroke (age- and sex-adjusted OR per SD, 1.17; 95% CI 1.04 - 1.32) (Bos et al, 2006). The primary conclusion of this paper was that taking CRP levels into account did not improve the individual stroke risk prediction compared with Framingham assessment. Very recently these observations were confirmed in the prospective PROSPER study of elderly patients, where combined hard end points of stroke/TIA (n=120) or CHD events (n=638) were associated with elevated CRP, but CRP measurement themselves did not contribute extra information to Framingham (63.0% to

63.7% for all CVD), and nor were CRP levels associated with benefit in potential risk reduction from pravastatin administration ([1]Sattar et al, 2007). CRP was independently associated with stroke/TIA in PROSPER; OR 1.32 (1.03 - 1.79).

The Women's Health Study authors (Everett et al, 2006) assert that CRP is associated more closely with ischaemic stroke than with CHD (stroke upper tertile OR 2.76 (95% CI, 1.51 - 5.05) and 1.66 (95% CI 1.17 - 2.34). Despite this conclusion, the confidence intervals are large and overlap, so overall there is little strong epidemiological evidence that currently suggests that CRP is more strongly related to stroke than to CHD. Reviewing the above evidence, the associations of CRP to stroke and CHD seem remarkably similar.

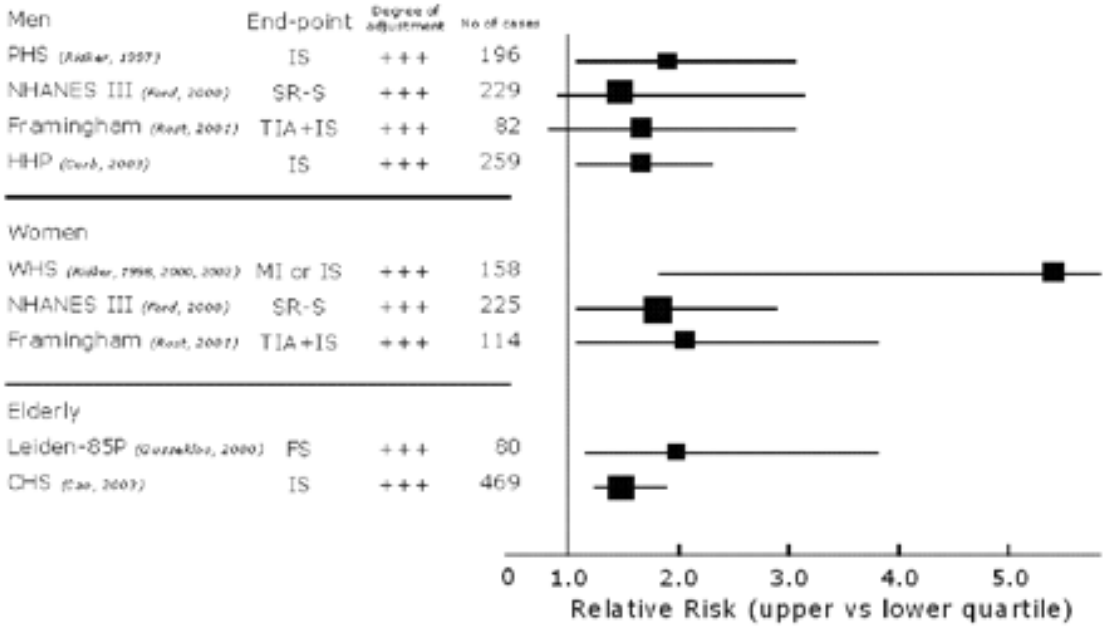
These broad data patterns in similarities in inflammatory risk markers for CHD and stroke are also seen for studies for fibrinogen. The Fibrinogen Studies Collaboration (2005) have published data on the association between fibrinogen and incident stroke, and again found broadly similar associations to those seen in CHD: for all types of stroke (n=2775) the age and sex-adjusted OR was 2.06 (95% CI, 1.83-2.33), ischemic stroke 2.08 (95% CI, 1.74-2.48), hemorrhagic stroke 1.44 (95% CI, 1.05-1.96), and stroke attributed to unclassified causes 2.36 (95% CI, 1.94-2.86) per 1g/L increase in fibrinogen. These results are comparable to data for combined OR for all vascular deaths and events; 2.35 (95% CI, 2.21-2.49) and was also similar to that for all nonvascular mortality 2.03 (95% CI, 1.90 – 2.08). Hence fibrinogen's association with stroke risk is similar to that seen in CHD risk and risk of non-vascular disease mortality, and although this does not preclude a causal role for fibrinogen it means there are potentially residual confounding biases in the data.

Pro-inflammatory cytokines in general are increased in production, both within the brain and in the periphery, following ischaemic stroke (Ferrarese et al, 1999; Vila et al, 2000). Following acute stroke, a range of studies have studies have examined the association between CRP levels and stroke outcome, and found some association. The largest of these studies involved 716 patients with cerebrovascular accidents, drew blood within 24hrs of the incident, and followed up for 12 months (Christensen and Boysen, 2004). This study found that CRP levels are higher in the first 24 hrs in severe (compared with moderate) stroke and correlates with disability (measured with the Modified Rankin

Score) and mortality for all causes 3 and 12 months after stroke. A smaller study suggests that CRP is not predictive of recurrent stroke after an index event, but it is predictive of death (adjusted OR 2.11; 95% CI 1.18-3.75) (Elkind et al, 2006), although this study failed to separate major stroke into subtypes due to its smaller size. These recent studies are consistent with general trends for relationships between CRP levels and acute stroke outcome in older smaller smaller studies (reviewed; Di Napoli et al, 2005). Similar associations with acute stroke outcome are seen for fibrinogen. One large recent study showing that among 900 unselected stroke patients that increased fibrinogen levels (>3.5g/L) are associated with one year mortality OR: 1.69 (95% CI 1.12-2.55) (Turaj et al, 2006).

These results, collectively, suggest that although inflammation may be important as a causal factor for stroke, as evidenced by the non-cholesterol benefits of statins, acute phase reactants such as CRP and fibrinogen may not fully explain the risk association of stroke with inflammation. Novel inflammatory markers in stroke risk therefore warrant further study.

Figure 1.14: Prospective studies relating baseline plasma C-reactive protein (CRP) levels to the risk of first cerebrovascular event (up to 2003). Relative risk compares top and bottom quartile of baseline measurements. Black squares indicate the relative risk in each study, with the size of square proportional to number of cases. All studies were adjusted for standard vascular risk factors. Adapted from Di Napoli et al, 2005.



IS, ischemic stroke; SR-S, self-reported stroke; TIA, transient ischemic attack; FS fatal stroke; PHS, Physicians' Health Study; NHANES III, 3rd National Health and Nutrition Examination Survey; Framingham, Framingham Heart Study; HHS, Honolulu Heart Study; WHS, Women's Health Study; Leiden-85P, Leiden Heart Study; and CHS, Cardiovascular Health Study.

1.7.5 IL-18 and TNFα and their Associations with Stroke:

Due to lack of availability of suitable samples in studies, sCD40L and MMP-9 are not examined in relation to stroke in this thesis and hence are not reviewed here.

IL-18 is expressed within human carotid plaques ([1]Mallat et al, 2001). A study of 366 patients who had carotid IMT measured at 12 sites showed that serum IL-18 was associated with mean IMT ($r = 0.36$; $p<0.001$) (Yamagami et al, 2005) even after adjustment for traditional risk factors and CRP ($r = 0.20$; $p<0.001$). However, a larger study (n=1111) recently suggested that only the univariate association of IL-18 with IMT was significant (Chapman et al, 2006). Clearly, if there is an independent association of IL-18 with carotid IMT, it is unlikely to be statistically strong. Likewise the direct association between circulating TNFα and measures of carotid thickening and atherosclerosis are consistently fairly weak (adjusted $r < 0.25$; Skoog et al, 2002; Elkind et al, 2005). These weak associations do not rule out a causal role for these markers in atherogenesis *per se.*

There is little published data on associations of TNFα or IL-18 with incident or prevalent stroke. For both IL-18 and TNFα there are data that report associations with composite "MI plus stroke risk" or "all CVD risk," (e.g. [2]Cesari et al, 2003, Wise et al, 2005, Blankenberg et al, 2006; see Tables 1.4 and 1.6) and although this is a perfectly reasonable methodology in terms of clinical risk assessment, it is not helpful in delineating associations with stroke risk specifically. This is especially true since CHD is far more common than stroke in prospective studies, particularly in non-elderly populations, hence resultant risk associations are naturally skewed to more strongly reflect associations with the more common end-point in these composite studies.

One small study of 23 patients with ischaemic stoke (Zaremba and Losy, 2003) showed that, compared with controls, patients had elevated circulating IL-18 levels within 24 hrs of stroke and that these levels correlated with early hypodense CT areas ($r=0.82$ $P<0.0001$). IL-18 levels also strongly correlated inversely with Scandinavian Stroke Scores (SSS) within 24 hrs ($r=-0.76$; $p<0.0001$) and with SSS scores at 2 weeks ($r=-0.77$; $p<0.0001$). Despite this a study of mice has shown that focal reversible occlusion of the

cerebral artery does not lead to IL-18 expression, and deletion of the IL-18 gene does not affect infarct volume (Wheeler et al, 2003). This finding may be an artifact of experiment however, since it is essentially a model of lacunar stroke in the absence of atheroscleosis or thrombosis, and was transient in occlusion time (15 to 30 mins). There are no published studies examining IL-18 and risk of incident stroke.

Similarly to IL-18, TNFα has been found to show an early and prolonged increase after stroke in 40 patients, but elevation was unrelated to lesion size, neurological impairment, or vascular risk factors, and the authors conclude it is elevated as part of the general acute phase response (Intiso et al, 2004). Clearly these types of small study tell us little on the association between these IL-18 and TNFα and stroke outcome, and the area requires clarification. The only published data examining stroke risk specifically and TNFα is from the ABC study ([2]Cesari et al, 2003) and contained only 60 incident strokes (see Table 1.4). In this study, TNFa was not associated with stroke risk, although there is clearly a need for larger studies.

1.7.6 Stroke Summary:

Ischaemic stroke has common risk factors with CHD, and this is as one would expect considering that they are general consequences of vascular disease brought about by atherosclerosis. While CHD events are almost always a consequence of rupture of plaques in the coronary arteries, lacunar and non-lacunar stroke may have distinct aetiologies, with reciprocal relative associations with inflammation and cholesterol. This may be why, while overall ischaemic stroke has poor associations with circulating cholesterol, risk of stroke is significantly reduced by statins; statins lower both generalised inflammation and cholesterol, and hence treatment is of benefit to both non-lacunar and lacunar stroke aetiologies.

It appears from general review of previous studies that "generalised inflammation" (as measured by CRP or fibrinogen) is not more strongly associated with stroke than CHD. If Th1 type inflammation plays a pro-atherosclerotic role and this is a greater aetiological risk factor for stroke than CHD, it may be that Th1 cytokines, such as IL-18 and TNFα,

are more important in stroke than CHD; this conjecture requires epidemiological investigation due to a current lack of data.

1.8 AIMS OF THESIS

1.8.1 General Aims

This thesis shall broadly aim to study the epidemiology of TNFα and IL-18, and to a lesser extent MMP-9 and sCD40L (due to a lack or serum), and their relationships with cardiovascular disease i.e. their technical and biochemical properties as markers (pre-analytical variables), their population distributions, their associations with other markers of CVD risk, and associations with risk of prevalent and incident CHD and stroke.

To achieve these goals, the following aims were drawn up:

1. To study assay-specific characteristics and the effects of pre-analytical variables on the markers:
- Anticoagulant suitability
- Intra/inter assay variation
- Long-term storage of samples
- Freeze-thaw cycles
- Short-term biological variation (20 weeks)*
- Long-term biological variation (4 years) i.e. regression dilution coefficient (British Regional Heart Study samples 16 vs 20 yrs)

2. To establish the population distribution of markers, as well as associations with conventional CVD risk markers in a cross-sectional study:
- MONItoring trends and determinants of CArdiovascular disease (4th Glasgow MONICA survey)*

3. To establish marker associations with prevalent CHD and stroke:
- GLAsgow Myocardial Infarction Study (GLAMIS)*

- Stroke In Progress (SIP) (Acute stroke)*

4. To establish marker associations with incident vascular events:

CHD

- British Regional Heart Study baseline samples (middle-aged men)
- Fletcher Challenge Study (New Zealand men and women)*

CVD

MONICA-4 (cross-sectional study)

Stroke

- PROSPER study (incident stroke in the elderly)*
- PROGRESS study (recurrent stroke)*

* Indicates that only plasma samples are available for this study, and so only IL-18 and TNFα can be measured.

1.8.2 General Hypothesis:

H_0: Null hypothesis

Circulating levels of the inflammatory markers TNFα, IL-18, sCD40L and MMP-9 are not related to risk of CVD independently of conventional risk markers.

H_1: Working Hypothesis

Circulating levels of the inflammatory markers TNFα, IL-18, sCD40L and MMP-9 (at least some of) are associated with CVD risk, the association is strong and reproducable, and relatively free from confounding by conventional risk factors. These result(s) would reflect the putative roles of TNFα and IL-18 in the Th1 response (and inflammatory atherogenesis), MMP-9's role in plaque rupture, and sCD40L's role in thrombosis and general inflammation. Differential abilities between these markers and markers of the acute phase response to predict risk of CVD independently of conventional risk factors might reflect underlying roles of these inflammatory markers in atherogenesis and atherothrombosis. There would hence be rationale for further experiments, and considering these and other inflammatory markers as candidates for clinical risk prediction and as specific therapeutic targets in secondary treatment and perhaps the prophylaxis of CVD.

CHAPTER 2

MATERIALS AND METHODS

2.1 REVIEW OF THE LITERATURE

2.1.1 Search Strategies

It is essential in modern literature reviews that systematic and thorough search strategies are employed in order that all studies pertinent to the area of interest are found. In order that a high level of coverage was obtained the following search strategies were used to obtain publications of interest. Searches were performed in Medline combining text words and MESH headings (using IL-18 as an example):

Text Words

(((Coronary Heart Disease) OR (Ischaemic heart disease) OR (Ischemic heart disease) OR (Cardiovascular Disease) OR (Cardiovascular mortality) OR (Myocardial infarction)).tw) AND ((Interleukin-18) or (IL-18).tw)

MESH Headings

(((Coronary Disease/) OR (Cardiovascular Diseases/) OR (Myocardial Infarction/) OR (Myocardial Ischemia/)) AND (Interleukin-18/))

Hits were restricted to English language and when searching for only epidemiological data, were restricted to human subjects as well. When searching for literature on TNFα or MMP-9 or sCD40L the words

((Tumour necrosis factor-alpha) OR (tumour necrosis factor α) OR (TNFα))

Or

((Matrix metalloproteinase-9) OR (MMP-9) OR (gelatinase-b))

Or

((Soluble CD40 ligand) OR (soluble CD40L) OR (sCD40L) OR (soluble CD154) OR (sCD154))

were substituted in place of interleukin-18. Searches were carried out at regular intervals to keep up to date with the literature.

2.2 SUBJECTS AND SAMPLES

2.2.1 Subject Recruitment

Since each study in this thesis is comprised of a different cohort of patients the study recruitment procedure, venupuncture procedure, sample handling, and cohort follow-up are described in detail in each relevant chapter.

2.2.2 Sample Handling and Storage

The careful collection, sample handling, and storage of blood samples is an essential component of any epidemiological study in order that potential confounding inter-sample differences are minimised. The accuracy of results attained from empirical population-based observations is ultimately a reflection of care taken with these procedures. For this reason this thesis will only consider studies where there is high quality and known history of the blood samples in question. In effect, whether samples were initially handled within our own laboratory (Chapter 3), locally (Chapters 4 and 5), or in multi-centre collaborations (other chapters) bloods were drawn by trained nurses or other health care professionals, and samples were handled in a standardised manner appropriate for epidemiological study. Individual study details are described in the relevant chapters.

2.3 AUTOMATED LABORATORY METHODS

2.3.1 Clottable Fibrinogen Assay (Clauss)

When discussing observations for the markers of inflammation in this thesis, fibrinogen results may also be included for reasons of comparison. For all markers measurements were made in our laboratory either by myself, or a colleague with appropriate technical experience.

Fibrinogen was measured by the Clauss method (von Clauss, 1957) using an automated coagulometer (MDA 180, Biomerieux, Basingstoke, UK) (Figure 2.1). When thrombin is added to diluted plasma, fibrinogen is enzymatically converted to fibrin, which in turn undergoes polymerisation to form a fibrin mesh. Factor XIII is activated by thrombin, and catalyses cross-linking to form a stable clot. The clot is detected by its interception of light. The elapsed time from addition of thrombin to clot formation is inversely proportional to the fibrinogen concentration in the sample. Fibrinogen concentration is inferred from a standard curve. Fibrinogen reagents were provided by the manufacturer, and the calibrant used was the 9th British Standard for blood coagulation factors from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, Herts) which is calibrated against the WHO international standard. In our laboratory the intra- and inter-assay coefficient of variation (CV) of fibrinogen measurements were 3.0% and 3.7% respectively (Poorhang, MSc thesis, 2005).

2.3.2 High Sensitivity-CRP Assay

The hs-CRP assay was performed by the immunonephelometric method using a BN ProSpec nephelometer (Dade Behring, Milton Keynes, UK) (Figure 2.2). Latex particles coated with monoclonal antibodies to human CRP are mixed with plasma samples. This results in an aggregate of latex and protein complexes in solution. The degree of aggregation determines solution turbidity, and is proportional to the concentration of CRP present. Plane-polarised light passed through the solution is scattered in a manner proportional to the concentration of CRP. CRP concentration is inferred from a standard curve. All reagents and calibrators were obtained directly from the manufacturer. In our laboratory the intra- and inter-assay CV of CRP measurements were 5.2% and 8.3% (Poorhang, MSc thesis, 2005).

Figure 2.1 The MDA 180 Coagulometer

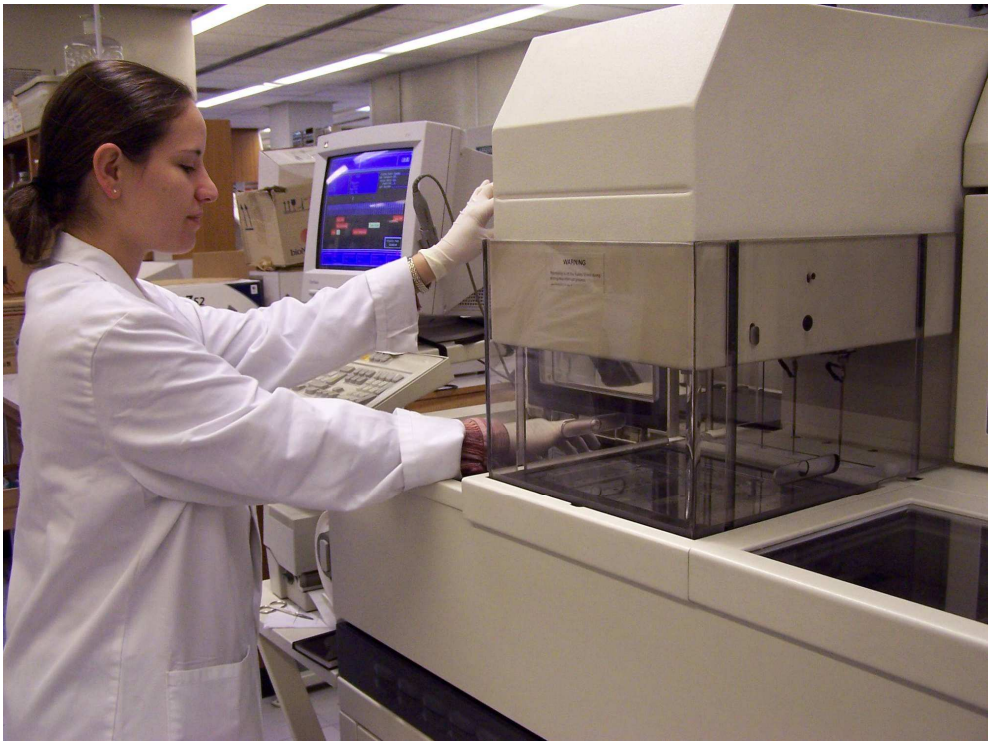


Figure 2.2 The BN ProSpec Nephelometer

2.4 ENZYME LINKED IMMUNOSORBENT ASSAY METHODS

2.4.1 Enzyme Linked Immunosorbent Assay Principle

Enzyme linked immunosorbent assays (ELISAs) employ the sandwich antibody principle. Plasma (or other appropriate medium) samples containing the antigen of interest and standards of known concentration are added to a plate of microtest wells which are coated with a primary antibody to the antigen. After incubation sufficient to allow >95% of the antigen to bind the primary "capture" antibody the wells are washed to remove any unbound material. Excess enzyme-linked secondary antibody, specific for a different epitope of the antigen is added to each well. The genetically engineered secondary antibody can be linked to a one of a range of enzymes (e.g. alkaline phosphatase in the IL-6 assay). Suitable incubation time allows end-point binding of the secondary antibody to the captured antigen, and the plate is washed to remove excess unbound conjugate antibody. A substrate for the specific enzyme on the secondary antibody is then added (e.g. Reduced Nicotinamide adenine dinucleotide phosphate [NADPH] for IL-6) bringing about a colourimetric reaction. The rate at which the coloured product develops is directly proportional to the rate limiting step in the ELISA: the amount of antigen present. An acid solution is added to stop the reaction at a point where the standard curve added is detectable and linear. The colour change in each well is measured photometrically using an appropriate wavelength on a plate reader (Multi Scan EX, Thermo Electron Corporation, UK). The plate reader is programmed to draw a standard curve and infer the concentrations of the plasma samples from this curve using each relevant optical density (OD). Examples of ELISA plates are shown in Figure 2.3 and 2.4. An example of a standard curve obtained for an ELISA is shown in Figure 2.5.

Figure 2.3 Part of an ELISA Procedure

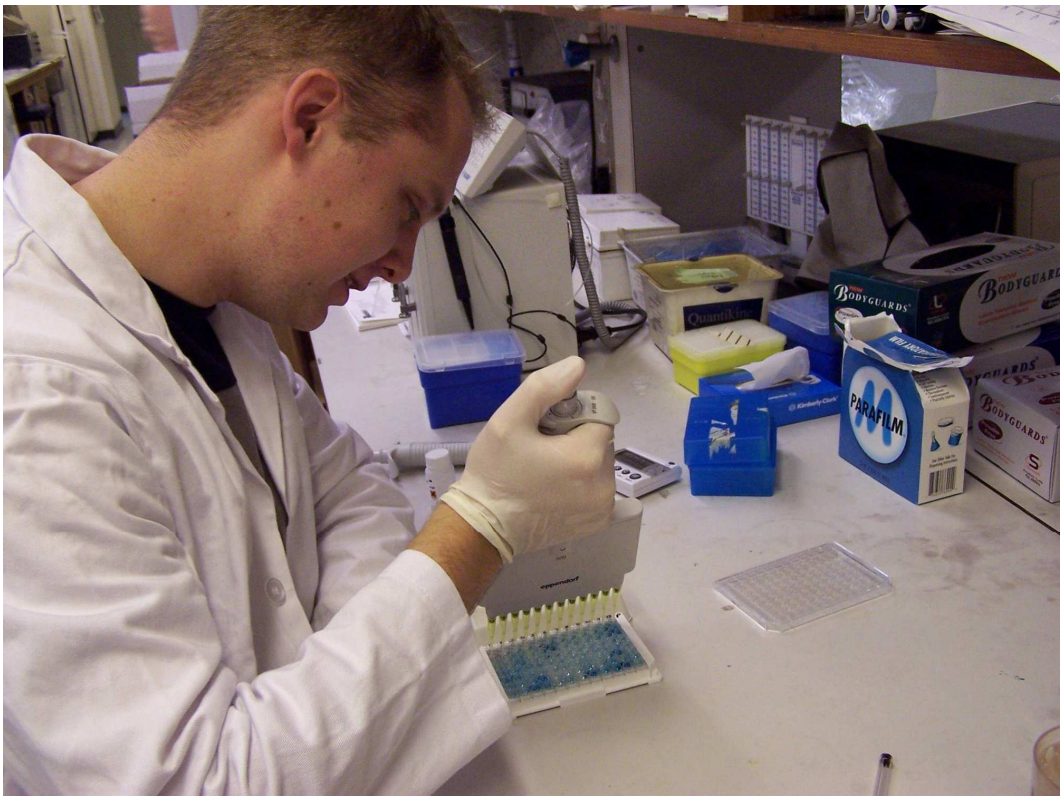


Figure 2.4: A Completed ELISA Plate

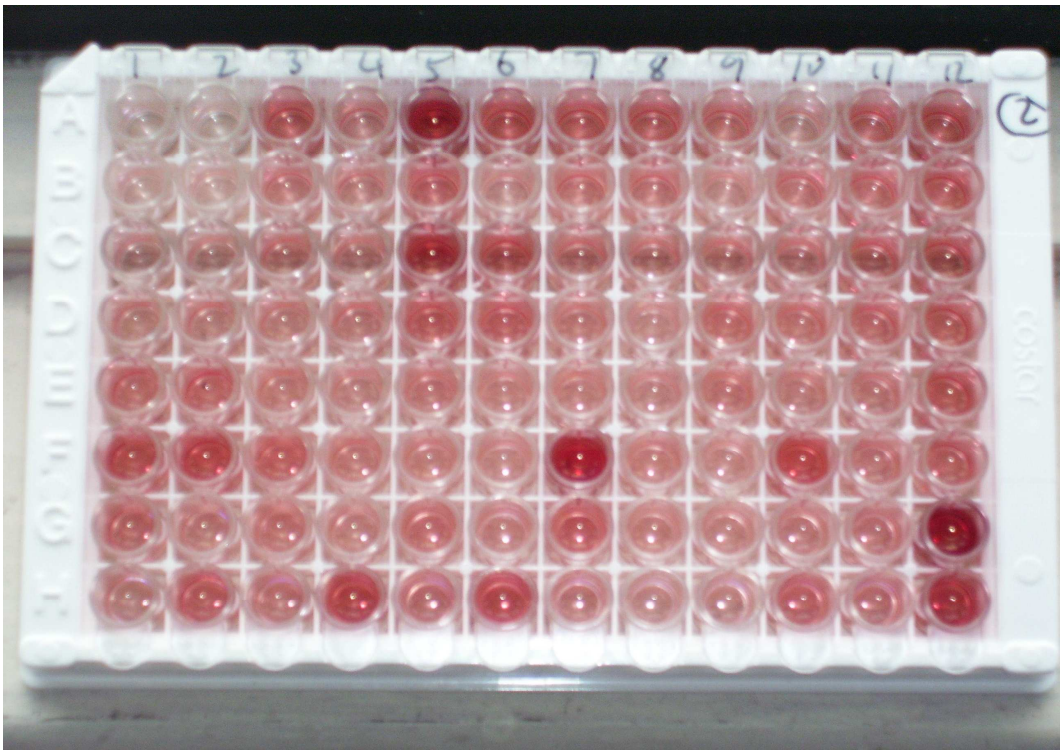
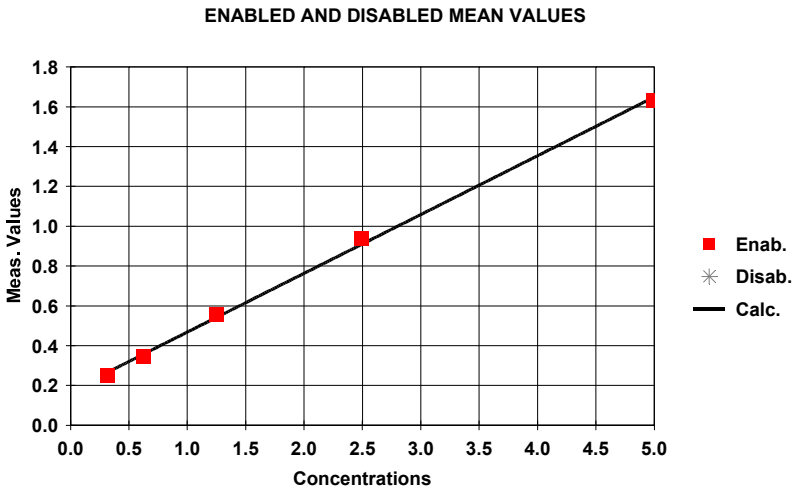


Figure 2.5 An abbreviated example of a standard curve readout for a IL-6 HS assay

Name	OD	Conc.
Cal_1	0.247	
	0.252	
	0.25	0.312
Cal_2	0.353	
	0.341	
	0.347	0.624
Cal_3	0.544	
	0.563	
	0.554	1.248
Cal_4	0.943	
	0.937	
	0.94	2.496
Cal_5	1.641	
	1.621	
	1.631	4.992

	Conc.	Meas.	CalcConc.	Residual
Cal_1	0.312	0.25	0.258	0.054
Cal_2	0.624	0.347	0.589	0.035
Cal_3	1.248	0.554	1.288	-0.04
Cal_4	2.496	0.94	2.598	-0.102
Cal_5	4.992	1.631	4.939	0.053



Fit type:	Linear regression (SVD): y = a+b*x
Meas. transformation:	Linear
Conc. transformation:	Linear
Parameters:	a b
	0.173 0.295
Corr. coeff. R2:	**0.997**

2.4.2 High Sensitivity IL-6:

High sensitivity IL-6 (IL-6 HS) was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim the assay is sensitive to < 0.1pg/ml and the reported CV is 7.2% (sample level dependant). The only deviation from manufacturer's instructions was to make the curve more sensitive at the bottom end of the range (since this is where circulating levels in healthy people generally are) and hence use a standard curve of 5.00, 2.50, 1.25, 0.625, 0.31, and 0 pg/ml. All R&D systems standard operating procedures can be procured from links on the website: http://www.rndsystems.com/product_detail_objectname_elisa_assay_product.aspx

2.4.3 High Sensitivity TNFα:

TNFα HS was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim the assay is sensitive to ~0.1pg/ml and the reported (sample level dependant) inter-assay CV is ~ 12.6%. The only deviation from manufacturer's instructions was to make the curve more sensitive at the bottom end of the range (since this is where circulating levels in healthy people generally are) and hence use a standard curve of 4.00, 2.00, 1.00, 0.50, and 0 pg/ml.

2.4.4 IL-18:

IL-18 was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim the assay is sensitive to 12.5 pg/ml and the reported (sample value dependant) inter-assay CV is <10%. The only deviation from manufacturer's instructions was to make the curve more sensitive at the end of the range for using 1:5 diluted serum or plasma samples (manufacturers suggested dilution) and hence use a standard curve of 200, 100, 50, 25, and 0 pg/ml.

2.4.5 MMP-9:

MMP-9 was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim the assay is sensitive to < 0.15 pg/ml and the

reported (sample value dependant) inter-assay CV is ~ 7.8%. The only deviation from the manufacturers instructions was to make the curve more sensitive at the end of the range for using 1:100 diluted serum samples (manufacturers suggested dilution) and hence use a standard curve of 20.0, 10.0, 5.0, 2.5, and 0 ng/ml.

2.4.6 sCD40L:

sCD40L was measured with a commercially available ELISA kit (R&D systems, Abingdon, UK). The manufacturers claim the assay is sensitive to 4.2 pg/ml and the reported (sample value dependant) inter-assay CV is ~ 6.0%. The only deviation from the manufacturers instructions was to make the curve more sensitive at the end of the range for using 1:5 diluted serum samples (manufacturers suggested dilution) and hence use a standard curve of 1000, 500, 250, 125, and 0 pg/ml.

2.5 QUALITY CONTROL

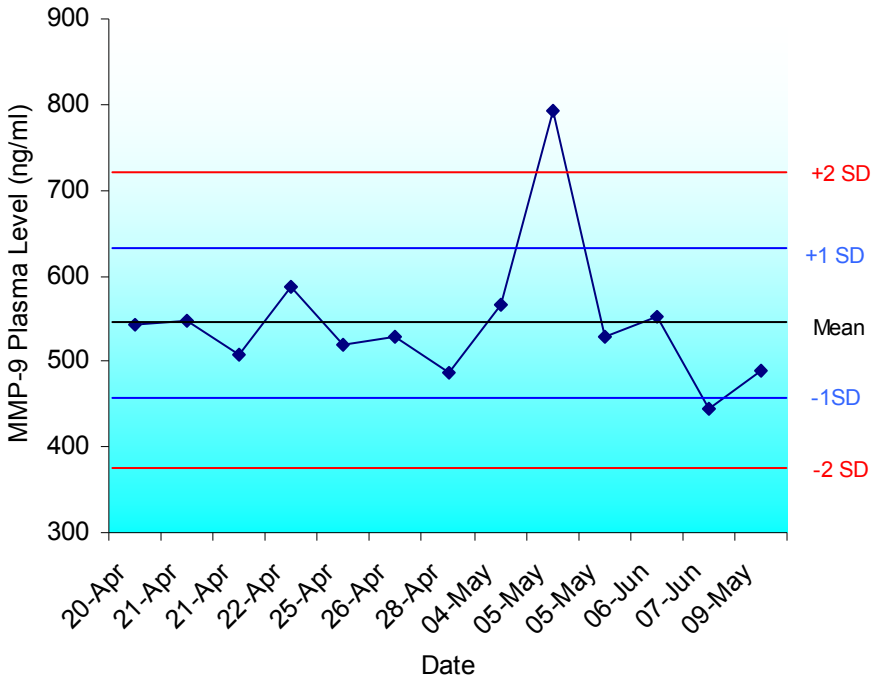
2.5.1 Internal QC:

An in-house pooled plasma (or serum for MMP-9 and sCD40L) was used to monitor inter-assay QC in all assays. 20 healthy volunteers over a range (25 – 60 years) of ages donated blood which was drawn into either citrated (4.5ml blood into 0.5ml 0.109M trisodium citrate) or prepared for serum (5ml Z serum sep. clot activator; left to clot for 30 mins at room temperature) vacutainers (Becton, Dickinson and Company, Oxford). Immediately after drawing (and clotting for serum) blood was centrifuged at 1500g for 10 mins. The plasma or serum supernatant was then removed and pooled into a common container for all samples from the 20 volunteers, and then mixed for a maximum of 15 minutes at room temperature. Samples were then aliquoted, and snap-frozen at -80^{0}C until required for assay. On automated analysers, a QC assay was performed at for least every 80 samples run through the machine. For ELISAs 2 separate QC samples were applied at the beginning and the end of each microplate (in order to monitor cross-plate stability).

The mean concentration, standard deviation (SD), and 95% confidence intervals (2SDs) of this QC were determined over 20 separate runs for each assay. A Levey-Jennings plot

(Levey and Jennings, 1950) was generated for each assay, and results for every assay were plotted over time. The principle of the plot is that the values of the QC should fluctuate randomly on either side of the assigned mean value over time, such that 95% of results obtained should sit within 2 standard deviations from the mean (Levey and Jennings, 1950). Assays that did not comply with these stipulations, or were outside 3SDs from the mean (i.e. 99% confidence intervals) were re-performed. An example of a Levey-Jennings plot for MMP-9 is shown in Figure 2.7.

Figure 2.6 A Levey-Jennings plot of MMP-9 internal QC results. Note that, in general, values tend to stay within two standard deviations, and fluctuate either side of the mean determined concentration. In this example there was one assay which was an outlier, and was subsequently repeated.



2.6 STATISTICAL ANALYSIS

2.6.1 General Statistical Overview

Detailed statistical methods are discussed in each relevant chapter for the individual study. For the smaller hospital based studies in Chapter 3 (pre-analytical variables) I performed all statistical analysis including analysis of the short-term biological variation (Fleiss et al, 1986; See chapter 3) with the exception of the statistical "Random Effects Model" (3.6.2) and the work was checked for accuracy by co-supervisor statistician Prof Mark Woodward. I performed basic statistics on the regression dilution studies, whilst more complex statistical analysis (for comparison with Emberson et al, 2004) was performed by a statistical team at the BRH Study main centre, supervised by our collaborators (Prof Peter Whincup and Dr Goya Wannamethee), in consultation with myself.

In larger more complex studies requiring large central databases, complex statistical models, and multivariate regression analysis (Chapters 4-9), statistical work was performed in collaberation of the co-ordinating group statistician, Prof Gordon Lowe, and I. Collaborating statisticians were either my co-supervisor (Prof Mark Woodward), our collaborators in the BRH Study (Prof Peter Whincup and Dr Goya Wannamethee), our collaborators in the Prosper study (Prof David Stott), or our collaborators in the SIP study (Prof Stott and Dr Mark Barber). In practice, all statistical analysis was performed using SAS, SPSS, Minitab, or Excel software packages.

2.6.2 Data Presentation, Skewedness, Medians and Means

Most data of central importance to this thesis, the measurement of cytokines in general populations or in selected cohorts, is on an interval scale and is non-normally distributed. This can present some problems in accurately representing the point of central tendancy in the data, and a number of techniques were employed to do this.

Skewedness and Kurtosis statistics were used to assess the data for non-parametric distribution. Limits of normal distributions were defined as at 2 standard errors of Skewdness and Kurtosis, where standard errors of Skewedness were approximated as

$\sqrt{\frac{6}{N}}$, and Kurtosis as $\sqrt{\frac{24}{N}}$ (Tabachnick and Fidell, 1996). Where an indication of the spread of data was required, skewed data was generally presented as untransformed medians with interquartile ranges (IQRs). Alternatively, data was log- or power-transformed to approximate normality, and medians and standard deviations were calculated on the transformed data, and were ultimately expressed as backtransformed data. The exception to this was the British Regional Heart Study (Chapter 6), where in order to compare with previously published data, data central tendancies were expressed as geometric means, defined as *n* results being multiplied (as opposed to being added in calculation of the arithmetic mean) and then the *n*th root of the resulting product being taken (as opposed to being dividing in the arithmetic mean).

2.6.3 Testing for Statistical Differences in Groups

Almost all tests in this thesis were comparisons of two unpaired samples with large amounts of data interval in each group. The two-tailed t-test is theortetically the best way to compare these groups in order to test the null hypothesis. The unpaired t-test requires that data is parametric for its use. Effort was made to normalise non-parametric data either by log- or power-transforming data as required to perform a t-test. Where this technique failed to induce normality, a Mann Whitney U test was employed to compare data sets (data not transformed). In univariate comparison of categorical variables, the chi-squared test was used to test the null hypothesis. Where there were comparisions of binary variables, and the numbers present were small (e.g. presence of baseline CVD in generally health populations), comparisons of groups were made by Fisher's exact test.

When testing two methods of comparing the same subjects (e.g. in Chapter 3), a general mistake is often made in the literature; comparisons are drawn using a test of correlation (a linear association) and perhaps a paired t-test (or Mann Whitney U). The problem with these is that it assumes that the effect of the different methods has uniform effects on the dependant variable, regardless of the value of the variable (i.e. the relationship is linear). This assumption may not be accurate (Woodward, 2005). In Chapter 3, the Bland-Altman test is employed to test the assumption of uniform effects of methodology on transformed data of paired samples (Bland and Altman, 1986; Woodward, 2005). Assuming the effect

is uniform, the correlation test and paired t-test become a valid methodology of comparion and test for differences on the data groups.

2.6.4 Correlation and Regression

The correlation coefficient indicates the strength and direction of a linear relationship between two variables. Where data was normally distributed, or transformed to normal distributions, a Pearson product-moment correlation coefficient (r) was used to determine the relationship between variables. This statistic can be found by dividing the normalised covariance between the two variables by the product of their standard deviations. The product r^2 is the proportion of change in variable X that can be attributed to change in variable Y (not necessarily causal). Where data was not parametric even after transformation, or the relationship of the variables was not linear, tests for correlation were performed uing Spearman's correlation coefficient. This ranking test of correlation does not require the assumption that the relationship between the variables is linear, nor does it require normally distributed data, although the test is less sensitive. Again, a correlation coefficient (r) was obtained. As previously, data from the British Regional Heart study was modelled in a different way to other studies in order to compare with previous data, and trends across thirds of the population distribution (by cytokine thirds) were fitted using a sum of least squares method.

In univariable analysis of the odds ratio of a CVD event in a population for a given cytokine, simple logistic regression analysis was used, coding for presence of a CVD event (1) or no CVD event (0) as a binary variable. The fitted line can be expressed as y= a + bX + error. Based on this relationship, the odds ratio of an event (p/q, where p is the probability of an event, and q is the probability of no event) can be implied from logit models; logit (p) = log (p/q) = a + bx $\Longrightarrow$ $\mathbf{p/q=e^{a+bX}}$ (i.e the odds ratio can be computed by raising e to the power of the logistic coefficient, and X could be defined as being by thirds or standard deviations of the variable in the population as required).

In a similar manner, multivariable logistic regression was used to assess the odds ratio of a CVD event for a cytokine in a population (by thirds or standard deviations) while mathematically holding constant a number of potential confounders such as classical CVD risk factors. This method adjusts for the effects of these potential confounders so

that, in theory, only the relationship of the cytokine with CVD event odds is investigated. In reality there are probably residual confounding factors.

Finally, in some models, forward setepwise regression analysis was used (Chapter 9). This analysis is performed in order to model the explanatory variables most strongly related to adverse outcome (death or dependency). In this case all conventional clinical markers of poor stroke outcome were included in a multivariable model from the outset. Those not significantly independently associated with outcome ($p>0.10$) were removed from the model stepwise until all clinical markers were significantly associated with outcome. Novel markers of risk of poor outcome were included in the model stepwise, and retained if significant ($p<0.05$), while removing markers which became non-significantly associated with outcome in the process ($p>0.10$).

2.6.2 Power Calculations

The studies used within this thesis were not specifically designed for the analysis of the novel inflammatory risk markers central to this thesis. The size of the studies, their offshoots, and the principal results are discussed in each individual chapter. Any power calculations performed on these studies would be retrospective.

Power calculations are a useful tool in the design of a proposed epidemiological study. However, it is my (and others; Hoenig and Heisey, 2001; Lenth et al, 2007) opinion that retrospective power calculations are limited in utility. If a study finds no association between a variable and an outcome the study was underpowered, or there was no relationship at all. The power calculation adds nothing to the odds ratio and 95% confidence intervals found in the results. The researcher is required to then ascertain if another prospective study is required, and hence could use a prospective power calculation to design another study, based on variences observed in the present study. If there was a significant association, ofcourse the study was adequately powered to detect it. In order to comply with University recommendations, power calculations for each study are included in the discussion (Chapter 12). Power calculations were performed by…….

CHAPTER 3

THE EFFECTS OF PRE-ANALYTICAL VARIABLES ON MEASUREMENTS OF IL-18, TNFα, MMP-9 AND sCD40L

3.1 INTRODUCTION

3.1.1 The Measurement of Pre-Analytical Variables:

When blood tests are carried out, either in the clinic or for epidemiological studies, there are several variables which will affect the results obtained before the sample is analysed. These include season, time of day, diet, stress, and physical activity of the patient, as well as technical aspects such as venipuncture technique, anticoagulation of the blood, processing speed and technique, freezing, storage, and assay-specific variables (such as assay variability) (Thomson, 1992).

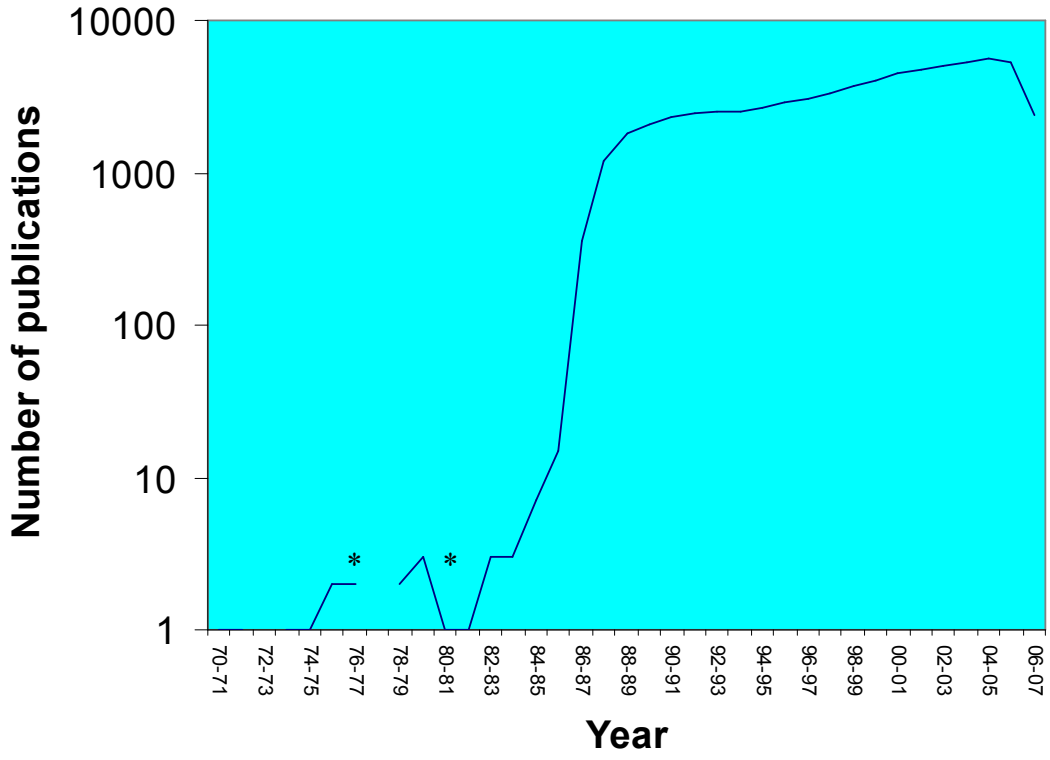
The number of published studies examining biological variables that are potential risk markers for cardiovascular disease has increased at a logarithmic rate in the last 15 years (See Figure 3.1; publications in Medline citing ((coronary heart disease(s) OR cardiovascular disease(s)) AND (risk factor(s) OR risk marker(s))) as keywords). Of the cumulative total of 33, 979 publications over the last 15 years illustrated in Figure 3.1, only 193 of them (~0.5%) also include ((pre-analytical variables) OR (anticoagulants) OR (blood specimen collection) OR (specimen handling)) as a key word. Despite this illustration of seeming relative disinterest in this subject area, the examination of pre-analytical variables is important prior to any epidemiological study, particularly with reference to the study of novel risk markers. The maintenance of high sample quality and observation of necessary specimen handling is ultimately reflected in the results obtained. Therefore, prior knowledge of the steps that must be taken to ensure minimisation of experimental error is essential. In addition to laboratory error, it is essential that biological variation is also accounted for when examining a risk marker.

It is not possible when performing the studies described in this chapter to comprehensively examine all potential confounding pre-analytical variables for the four novel inflammatory markers. However, the results presented in this chapter were

generally sufficient to validate the use of the samples from epidemiological studies included in other chapters.

The overall aim in this section was to validate IL-18, TNFα, MMP-9 and sCD40L for use in epidemiological studies (and to elucidate the requirements of these studies for accurate measurements of risk associations), and to assess potential use of these markers in meta-analysis.

Figure 3.1 Illustrative graph of the logarithmic increase in the number of published studies examining risk markers in cardiovascular disease in each year since 1970. The search was performed in Medline searching for publications citing ((coronary heart disease(s) OR cardiovascular disease(s)) AND (risk factor(s) OR risk marker(s))) as keywords. * Due to the semi-log scale, breaks in the graph indicate a y-value of 0



3.2 PLASMA, ANTICOAGULANTS AND SERUM SAMPLES

3.2.1 Introduction

In most epidemiological studies, either serum (generally used in the measurement of lipid variables) or plasma (used to measure other biochemical or haemostatic variables) samples may be available. Due to ubiquity in the measurement of lipid variables these aliquots tend to be exhausted first, and there tends to be an excess of plasma samples available for analytical purposes in most studies. It was necessary to ascertain which of the inflammatory markers assessed in this thesis could be assayed in serum or plasma.

In the literature, use of serum or plasma for the measurement of sCD40L and MMP-9 is controversial, and has been reviewed in Chapter 1 (1.5.5 & 1.6.5). As stated previously for MMP-9, "EDTA and citrate are not recommended anticoagulants for use in this assay due to their chelating properties." (R&D Systems kit insert Ref DMP900). I, therefore, used serum with the R&D Systems ELISA in this thesis because of lack of availability of heparinised plasma.

At the outset of this thesis there was no high-sensitivity sCD40L ELISA assay available for plasma sCD40L analysis, although one was recently released by Bender MedSystems (Vienna, Austria). This may prove more sensitive than the R&D ELISA for the measurement of sCD40L in plasma (Halldorsdottir et al, 2005) although this has not yet been investigated in peer-reviewed studies. I, therefore, used the R&D Systems ELISA in serum samples throughout this thesis.

The manufacturers state that the R&D Systems hs-TNFα assay can be performed from samples "using serum…[or]…..plasma using EDTA, heparin or citrate as an anticoagulant" (R&D Systems kit insert ref HSTA00D) and this has been confirmed elsewhere (Flower et al, 2000). There have been no such studies for IL-18, although the manufacturers (MBL) state that "serum and EDTA plasma can be used" (R&D Systems [suppliers] kit ref 7620).

I, therefore, devised a small study, aiming to ascertain which (if any) anti-coagulated plasmas were acceptable to use in epidemiological studies of IL-18, TNFα and sCD40L, basing our decision both on manufacturer recommendations, as well as our own results.

3.2.2 Subjects and Methods

20 healthy laboratory volunteers (age range 23-57 years) donated venous blood which was collected by venipuncture in citrated vacutainers (0.11M; 9:1 v:v) and standard K3 EDTA vacutainers (BD Vacutainers, Oxford) (1 citrate and 1 EDTA sample per subject). Blood samples were left on ice for a maximum of 30 minutes before centrifugation at 2000g for 10 mins, and then were separated into aliquots, which were snap-frozen at -80^{o}C. Samples were assayed on the same ELISA assay (for each marker) within 1 week of blood letting.

The hs-TNFα assay has been reported to be compatible with citrate, EDTA, heparin and serum (R&D Systems), and therefore only residual EDTA samples were assayed in this study to compare to manufacturer's reported results and to establish reference levels in healthy young people. IL-18 has been validated for EDTA and serum by the manufacturers, and therefore experimental comparisons were made between EDTA and citrate in order to validate citrated plasma. sCD40L was initially assayed in both EDTA and citrated samples (not recommended for use by the manufacturer), and compared to manufacturer's findings. Due to the reported chelation problems involving MMP-9 and plasma samples, MMP-9 was only run on serum samples throughout this study, and hence no results are reported here.

For IL-18 and sCD40L, EDTA and citrate data was analysed by comparing equality of means using a Bland-Altman plot (Bland and Altman, 1986) on log-transformed data (Woodward, 2005). Comparisons of the anti-coagulants on the log-transformed (normalised) data were made using paired t-tests and Spearman correlation coefficients. For TNFα, due to there only being summary statistics available from the manufacturers, EDTA plasma levels were compared only qualitatively with findings from our own samples. The objective was to ascertain whether or not it was acceptable to run large epidemiological studies using anti-coagulated plasma samples, and to ascertain if the

study findings of circulating levels of antigens were similar to the manufacturer's findings.

3.2.3 Results

Comparisons of EDTA and citrate measurements of IL-18 are shown in Table 3.1 and in Figure 3.2. Table 3.1 suggests that median levels of IL-18 in EDTA plasma are 14.4% higher than in citrate. This is confirmed in Figure 3.2, where the Bland-Altman plot shows that IL-18 levels are more often lower in citrated plasma (paired t-test $p<0.0004$. A random scatter of points suggests that the effects of taking EDTA or citrate has a uniform effect on levels, independent of the mean level of detectable of IL-18. Commensurate with this, citrate and EDTA values were highly correlated for IL-18 ($r = 0.88$). Both anti-coagulants were comparable to the manufacturers results in serum (means 126 pg/ml vs 186 and 215 pg/ml for citrate and EDTA respectively), which seemed reasonable given the different study populations (manufacturers age range not stated).

Results for TNFα in EDTA samples are shown in Table 3.2. This was performed only to ensure our results were comparable to the manufacturers, and hence results are qualitatively compared (given the different populations used and insufficient data from manufacturers for statistical tests). The results obtained by the manufacturers study and the current study were different (means 1.98 vs 0.41 pg/ml). This difference is clearly considerable (although only summary statistics were available for the manufacturer's findings so no statistical test was possible). It is worth noting that the assay is reportedly sensitive to ~0.2 pg/ml, which when compared with this small cohort of people, would appear to be a low value even within a young and healthy population.

sCD40L was shown to have far lower measurements in plasma when compared with the manufacturer's estimates for levels present in normal serum (Table 3.3) (~50-fold lower in citrate). Generally, measurements in citrate in particular were "flat" with little inter-individual variation, and although levels in EDTA were higher (60% comparing medians of citrate and EDTA), the mean detectable value of ~200 pg/ml in EDTA corresponds to an optical density (OD) readout of <0.15 on the standard curve of the assay (standard curve OD ranges from ~0.07 [Blank value] to ~1.60 [10,000 pg/ml]). The average

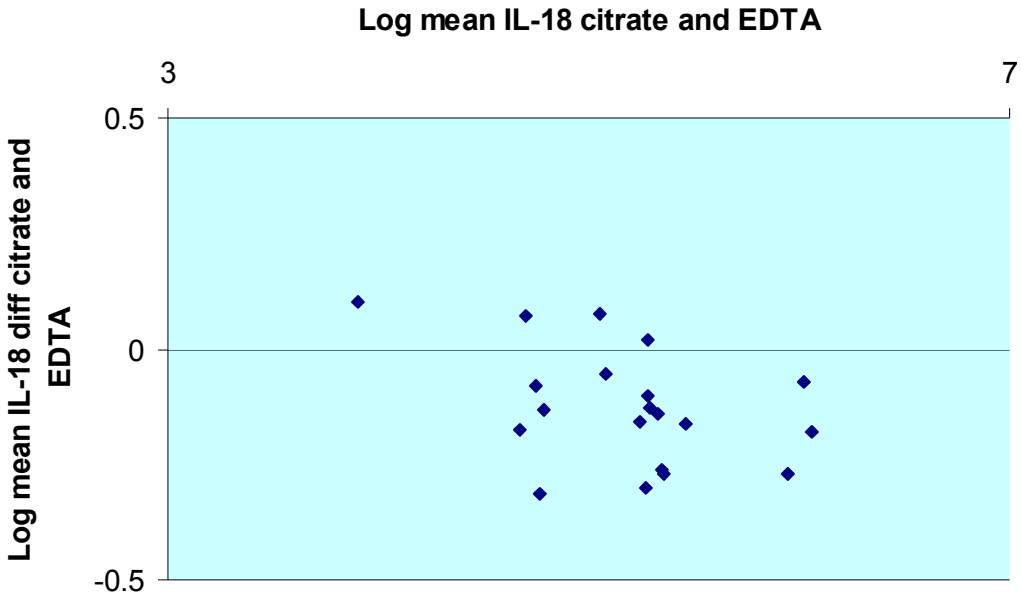
sCD40L value of plasma in healthy people is hence at the very limit of detection on the assay. As seen on the Bland-Altman plot, comparisons show that the noted trend for higher levels of sCD40L in EDTA than in citrate were significant ($p<0.0003$), and (unlike IL-18) Spearman correlations were relatively poor ($r = 0.50$), although the effects of the different anti-coagulants were uniform across sCD40L levels (random data scatter). This suggests that the differences caused by taking citrate or EDTA are unpredictable.

Table 3.1 Range, means and SDs for EDTA in trial study of IL-18.

	Manufacturer's Study	Plasma Validation Study	
	Serum n=46	Citrate n=20	EDTA n=20
Minimum (pg/ml)	36	52	47
Maximum (pg/ml)	258	399	428
Mean (pg/ml)	126	186	215
Median (pg/ml)	N/A	180	206
St Deviation	44	93	109

ND – Not detectible. N/A- Not determined

Figure 3.2 Bland-Altman analysis plotted on a log scale comparing IL-18 levels in citrate and EDTA plasma



Mean difference (transformed) = -0.126
Median EDTA levels 14.4% higher than citrate (untransformed)
t-test p<0.0004
Spearman correlation r = 0.88

Table 3.2 Range, means and SDs for anti-coagulants in trial study of TNFα.

	Manufacturer's Study	Plasma Validation Study
	EDTA n=35	EDTA n=20
Minimum (pg/ml)	ND	0.11
Maximum (pg/ml)	4.22	1.34
Mean (pg/ml)	1.98	0.41
Median (pg/ml)	N/A	0.31
St Deviation	N/A	0.29

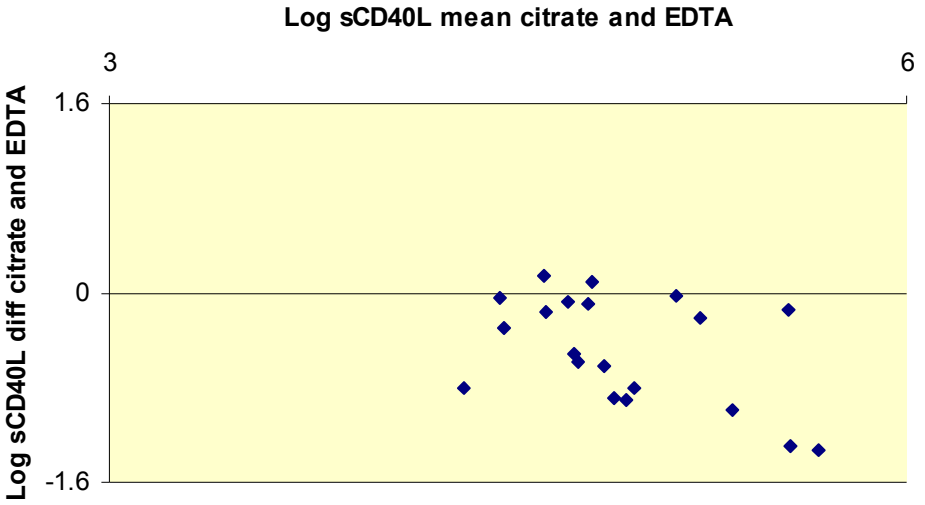
ND – Not detectible. N/A- Not determined

Table 3.3 Range, means and SDs for anti-coagulants in trial study of sCD40L.

	Manufacturer's Study	Plasma Validation Study	
	Serum n=58	Citrate n=20	EDTA n=20
Minimum (pg/ml)	ND	51	89
Maximum (pg/ml)	11451	241	565
Mean (pg/ml)	5461	115	203
Median (pg/ml)	N/A	103	165
St Deviation	N/A	42	130

ND – Not detectible. N/A- Not determined

Figure 3.3 Bland-Altman analysis plotted on a log scale comparing sCD40L levels in citrate and EDTA



Mean difference (transformed) = -0.478
Median EDTA levels 60% higher than citrate (untransformed)
t-test $p<0.0003$
Spearman correlation $r = 0.50$

3.2.4 Discussion

IL-18 was successfully validated for determination in both EDTA and citrated plasma. The manufacturers tested serum samples from 46 healthy volunteers, and these are comparable with EDTA and citrate plasma results reported here. The mean and standard deviation (SD) were slightly higher in plasma compared to the manufacturer's separate study in serum. In the current study, despite differences in absolute levels of IL-18 detected in citrate and EDTA, the correlation between anticoagulants was $r = 0.88$, suggesting results between the two are highly associated. On the basis of these findings the conclusion was that IL-18 could be measured in EDTA or citrated plasma as well as serum.

In the trial of TNFα with EDTA samples, the values obtained in this study were rather lower than the manufacturers range. It is possible that this was due to differences in the study populations used, although the observed differences were large. Since both our and the manufacturer's trials were used on relatively fresh EDTA anti-coagulated plasma the reason for this difference could not be verified. Despite this, the kit was sensitive enough to measure the lower levels we detected in our study, and a decision was made to press ahead with further small studies to further verify the suitability of the assay for epidemiological use. The results of the manufacturer suggest that, like IL-18, TNFα can be measured in plasma or serum, and this is confirmed elsewhere (Flower et al, 2000).

As stated previously, MMP-9 was not validated in this study due to chelation reactions in plasma, and use must be restricted to serum samples.

sCD40L was shown to have far lower measurements in plasma when compared with the manufacturer's estimates for levels present in normal serum. Measurements of citrate in particular looked "flat", and results were clustered below the bottom standard and at the limit of detection for the assay. This suggests that this particular assay, although widely used in the literature up until 2005, was not developed to be sensitive enough to accurately detect sCD40L levels this low, as shown by the much higher (~50 fold) serum levels. EDTA had significantly higher sCD40L levels than citrate, perhaps due to the ability of the anti-coagulant to preserve as well as activate platelets (Golanski et al,

1996), but correlation with citrated samples was relatively weak (r = 0.50). Hence despite previous suggestions that sCD40L is not suitable for measurement in serum (Ahn et al, 2004, Mason et al, 2005; Varo et al, 2006; Weber et al, 2006) there are severe contraindictions to the use of plasma in the ELISA technique. Indeed, even in platelet poor citrated plasma, carryover of very small platelet numbers may be sufficient to induce some *ex-vivo* sCD40L release, which may be sufficient to skew results (already at the limit of detection) to a meaningless artefact. Based on other results presented here (section 3.5), there is also the possibility that storage and freeze/thaw cycles may further reduce detectable levels of sCD40L, making older plasma samples hard to use.

In light of these results, I concluded that while IL-18 and TNFα may be used in studies with citrated or EDTA plasma as well as serum samples in epidemiological studies, sCD40L and MMP-9 can only be used in serum samples. While serum may not be ideal for measurement of sCD40L, the alternative of using plasma in technologies available at the outset of this thesis seemed even less useful.

3.3 INTRA- AND INTER-ASSAY VARIATION

3.3.1 Introduction

Laboratory error in the measurement of markers will be a contributing factor towards any underestimation of the association between the marker and its sequelae, such as cardiovascular disease. In addition, it is important to ascertain how sensitive an assay is. Since the inflammatory markers assessed in this thesis were measured by one operator using ELISA techniques, it may be assumed that operator error will be similar for each assay. Laboratory error is reported as the intra-assay (within assay) and inter-assay (between assay) variation. While the manufacturers report intra- and inter-assay variation values in the kit instructions, these require verification for the current operator. In this study I aimed to assess the level of intra- and inter-assay variation for each assay.

3.3.2 Methods

Inter-assay variation was determined by statistical analysis of the first 20 values obtained on different days (i.e. different assays over a period of up to 5 months) for the in-house

QC plasma (lot 777712; described in chapter 2) for each of the 4 cytokines. Intra-assay variation was determined by assaying a pooled QC plasma (not necessarily the in-house QC, but a pooled sample obtained by similar means; see 2.5.1) a number of times (number of times assay dependant; see results) on the same assay for each of the 4 cytokines. Where possible, the intra-assay CV was calculated using two different standards of different mean values in order to ascertain the effect of different antigen concentrations on the observed CV. Mean value, standard deviation (SD) and coefficient of variation (CV) were then calculated.

3.3.3 Results

Intra-assay variation is summarized in Table 3.4, and inter-assay variation in Table 3.5. IL-18 and TNFα, MMP-9 and sCD40L assays generally show levels of intra-assay variation comparable to other inflammatory markers measured by ELISA, such as IL-6 (CV 7.5%; data from Poorhang, MSc thesis, 2005). This is as expected, since the assays operate on the same immunodetection principle. Levels of variation rise sharply as the assays reach their limits of detection; this is especially applicable to TNFα, which at a level of 0.33 pg/ml has a CV of 36%, although it should be noted this level is particularly low. The second sCD40L sample used (n=5) did not have a substantially different level of sCD40L. In general, all markers had decreasing CV with increasing levels of antigen in the sample.

As expected, inter-assay variation (Table 3.5) is higher than intra-assay variation since it includes additional sources of variation. Direct comparisons of intra- and inter-assay results are not possible here due to the differences between the samples used. Despite this all markers showed an inter-assay variation of <15%. As seen in Table 3.5, all intra- and inter-assay variations were in reasonable agreement with the manufacturer's reported results, with the exception of sCD40L in the inter assay results (14.9% versus ~7%). This discrepancy may be attributable to two outliers in the in-house sCD40L inter-assay results (CV>20%) or it may reflect a higher concentration of standard used by the manufacturers to report inter-assay CV (levels not reported).

Table 3.4 Intra-assay variation in the four inflammatory markers

	n	Mean	SD	CV (%)	Manufacturer's reported CV (%)
IL-18 (pg/ml)	10	356	19.9	5.6	~10
	4	1716	74	4.5	~5
TNFα (pg/ml)	10	1.96	0.164	8.4	~8.5
	20	0.33	0.117	36	NR
MMP-9 (pg/ml)	10	551	24	4.4	~2
	5	878	32	3.6	NR
sCD40L (pg/ml)	10	2228	87	2.9	~5.5
	5	2068	78	3.8	~5.5

SD; standard deviation, CV; coefficient of variation (%), n; number; NR not reported

Table 3.5 Inter-assay variation in the four inflammatory markers

	n	Mean	SD	CV (%)	Manufacturer reported CV (%)
IL-18 (pg/ml)	17	196	20.3	10.4	~7
TNFα (pg/ml)	17	1.41	0.177	12.5	~11
MMP-9 (pg/ml)	17	543	56.5	10.4	~8
sCD40L (pg/ml)	17	2033	303.8	14.9	~7

SD; standard deviation, CV; coefficient of variation (%), n; number

3.3.3 Discussion

Intra- and inter-assay variation ranges for each marker were comparable to most other inflammatory markers within the normal healthy population range, and hence the assays were deemed appropriate for epidemiological use in terms of sensitivity.

3.4 SAMPLE PROCESSING

Sample processing is an important step in blood collection, since the length of time plasma or serum is left in contact with blood cells may alter subsequent levels of variables due to cell or platelet lysis and/or activation and may also influence levels of less biochemically stable markers left at room temperature. The epidemiological studies used in this thesis were all carried out employing reasonably prompt handling procedures, as detailed in each individual chapter. Since my laboratory had little input into the design of most of these studies prior to blood letting, sample processing is not investigated in this thesis. Previous studies have shown that TNFα is unaffected if left un-processed in whole blood for up to 4 hours (Flower et al, 2000). MMP-9 and sCD40L have been shown to be relatively stable for a number of hours in serum after centrifugation and sample separation (Weber et al, 2006; Makowski et al, 2003). There are no known studies of this type for IL-18.

3.5 SAMPLE STORAGE AND FREEZE THAW

3.5.1 Sample Storage Time Introduction

Degradation of stored blood samples is an important issue to assess, and can be caused by either time in storage itself, or increasing numbers of freeze-thaw cycles.

The time samples are kept frozen in storage is primarily a concern for large epidemiological studies rather than clinical processing and the testing of blood samples for individual patients, which are usually performed on fresh samples in clinical biochemistry departments. Blood samples from large cohort studies may be stored frozen for decades (such as the British Regional Heart Study samples; Walker et al, 2004) and

this may have a bearing on different markers of variable stability. Due to time constraints of this thesis, it is obviously not possible to study the effects of storage over such long periods.

In the literature, storage of frozen serum for 1 month has been reported to have no effect on MMP-9 (Souza-Tarla et al, 2005). Interestingly, however, in plasma MMP-9 may be unstable even when frozen at -80^{O}C, perhaps partially accounting for the relatively low circulating levels reported in most studies of plasma. One study found that, after 2 years, the detectable total MMP-9 dropped by 65% in plasma (Rouy et al, 2005). A literature search revealed no such studies for IL-18, TNFα or sCD40L, and none are reported by the manufacturers (R&D Systems).

In designing this study, it was important to define what information it was important to glean from the data. Plans for future studies involved comparing markers by thirds of the population in prospective studies, and this required us only to show that rank correlations of marker data sets were maintained across storage time (i.e. the actual information within the data sets are maintained as opposed to absolute values). For performing possible meta-analysis the marker requires to demonstrated more resistance to decay with time in storage in terms of absolute values (so studies may be combined without "experimental" heterogeneity in the pooled results).

In this experiment I aimed to study the effects of storage at -50^{o}C over 12-15 months on all four inflammatory markers in the plasma or serum of ten individuals. Storage at -50^{o}C will generally be the highest temperature epidemiological samples are stored at in modern laboratories. Data sets were analysed as outline in the relevant section.

3.5.2 Freeze-Thaw Cycles Introduction

Freeze/thaw cycles, like frozen storage, is primarily a concern for large epidemiological studies, rather than clinical processing and the testing of blood samples for individual patients. For the inflammatory markers in this thesis the manufacturer's instructions only state a non-committal recommendation of "avoid repeat freeze-thaw cycles" for each marker.

Some peer-reviewed studies suggest inflammatory markers are surprisingly robust, and show that 3 freeze thaw cycles had no effect on TNFα (or other cytokines such as IL-1β, IL-6 and IFNγ) or plasma sCD40L (Thavasu et al, 1992; Varo et al, 2006), and up to five freeze-thaw cycles for MMP-9 (Souza-Tarla et al, 2005) also had no effect. Again, no specific studies of IL-18 were found in the literature although the manufacturer of an ELISA kit for swine IL-18 has reported that 5 freeze-thaw cycles had no effect on detectable levels: (www.biosource.com/content/catalogcontent/tds3/moreinfo/KSC0181%20pr272%20revA2%20nov706%20(Sw%20IL-18)(BMS672).pdf).
The surprising resistance of these inflammatory markers to freeze/thaw cycles may reflect the measurement by ELISA techniques in these studies; ELISA antibody detection only requires the antigenic site to be intact, rather than the marker to be biologically active.

As with the section on storage stability, I required to ascertain whether markers maintained rank correlation with freeze-thaw biochemical stress, and whether absolute values were stable. In this study the aim was to examine the effect of 4 freeze-thaw cycles on each of the four inflammatory markers in the plasma or serum of ten individuals.

3.5.3 Subjects and Methods

The same subjects in the same blood let were used to study both storage and freeze-thaw variables.10 healthy laboratory volunteers (5 males: 5 females, age range 21-59 years) donated venous blood which was collected in a citrated vacutainer (BD, Oxford) (0.11M; 9:1 v:v) (IL-18 and TNFα assay) and in a serum clot accelerator vacutainer (MMP-9 and sCD40L) in the same venipuncture. Blood samples were left on the bench for 30 minutes (precisely) before centrifugation at 2000g for 10 mins, and then were separated into plasma or serum aliquots, which were snap-frozen at -80^{o}C. Appropriate aliquots were thawed as required.

In the storage study, baseline was defined as 3 weeks after freezing to allow for initial freezing effects on the samples. Due to limitations in the ELISA kits available in the laboratory at any given time point after setting up this study, IL-18 and TNFα were assayed at different time points from MMP-9 and sCD40L. IL-18 and TNFα were assayed at baseline, 6 and 15 months, and MMP-9 and sCD40L at baseline, 9 and 12 months. Due to unforeseen changes in the manufacture of the hs-TNFα assay by R&D Systems early in 2007, the ability to complete this experiment for TNFα has been restricted. The newly manufactured kit claims to have superior sensitivity and to resolve some "minor technical issues" with the previous lots. The newly manufactured kit has a linear r =0.82 correlation when results are compared directly with the older kit lot, but the conversion is non-linear at more extreme high and low observations. It is therefore hard to justify adjustment for these changes in a study of this small scale. Therefore the last study point at 15 months cannot be used in the storage study of TNFα. The storage study is the only study in this thesis affected by this kit change.

In freeze-thaw, after taking appropriate aliquots and initially snap-freezing at -80^{o}C, each aliquot from an individual was repeatedly thawed at 37^{o}C, assayed, and re-frozen at -80^{o}C (n= 4 cycles for all markers) leaving a minimum of 2 days in between freeze-thaw cycles.

Since the results of these experiments are very sensitive to inter-assay variations, each assay in both experiments was adjusted on the in-house QC result to give the average QC reading. This was a simple process of adjusting the data by the proportion:
(obtained QC value - mean QC value)/mean QC value

From this adjustment, the average magnitude of correction was ± 7.8% for all markers across 12 assays in total in the storage study (which is in broad agreement with the expected correction for the inter-assay CV range: table 3.5), and the median correction was 0.15%, suggesting random fluctuation in inter-assay variation. In the freeze-thaw study, the average magnitude of correction was ± 6.9% for all markers across a total of 16 assays and the median correction was ~0.1%, also suggesting random fluctuation in inter-assay variation.

Pairs of results from different storage points and freeze-thaw cycles were compared by Bland-Altman plot analysis (Bland & Altman, 1986) on a log or power-transformed scale (Woodward, 2005). The plots test the consistency of the effects of storage and freeze-thaws across different baseline marker levels by illustrating any trends in the data (a random scatter suggesting consistent effects independent of marker levels). Differences between transformed data sets were also tested by paired t-test and Spearman correlations.

3.5.4 Storage Results

Data on the effects of storage at -50^{o}C on the four markers are summarised in Table 3.6. Baseline measurements were not made until samples had been stored for three weeks.

The Bland-Altman analysis in Figure 3.4 shows that, after data transformation, the effects of storage on IL-18 levels are independent of mean IL-18 levels. There is an outlying point of relatively high IL-18 levels for which there appears to be more significant storage affects, however, it is a single point and possibly a chance finding. In agreement with this, the effect of 9 versus 15 month storage does not appear to have any additional degradative effect on the point. There is no significant change in mean IL-18 levels across storage points, although the rank correlation is not as high as for other markers ($r = 0.71$), but this does not worsen with further time in storage (9 versus 15 months). TNFα levels (Figure 3.5) were also stable across the single extra time-point measured at 6 months ($p=0.86$), and the correlation between points was high ($r = 0.90$). In contrast to IL-18 and TNFα, mean levels of serum MMP-9 (Figure 3.6) decreased substantially at each time point compared to baseline (22.9% and 28.5% at 9 and 12 months respectively). Despite this however, the rank order of the data is well maintained ($r \geq 0.84$ for both time-points), suggesting a uniform decay with time in storage, and this is confirmed by a random scatter of the Bland-Altman plot, showing a constant decay independent of MMP-9 levels.. For sCD40L (Figure 3.7), the 9 month time-point shows no decay compared to baseline, although the difference is significant by 12 months (5.4%, $p=0.03$). Again, the data suggests the effect of decay on detectable levels of sCD40L was uniform ($r =0.96$), and the data scatter on the plot was random, suggesting any decay is constant over all sCD40L levels.

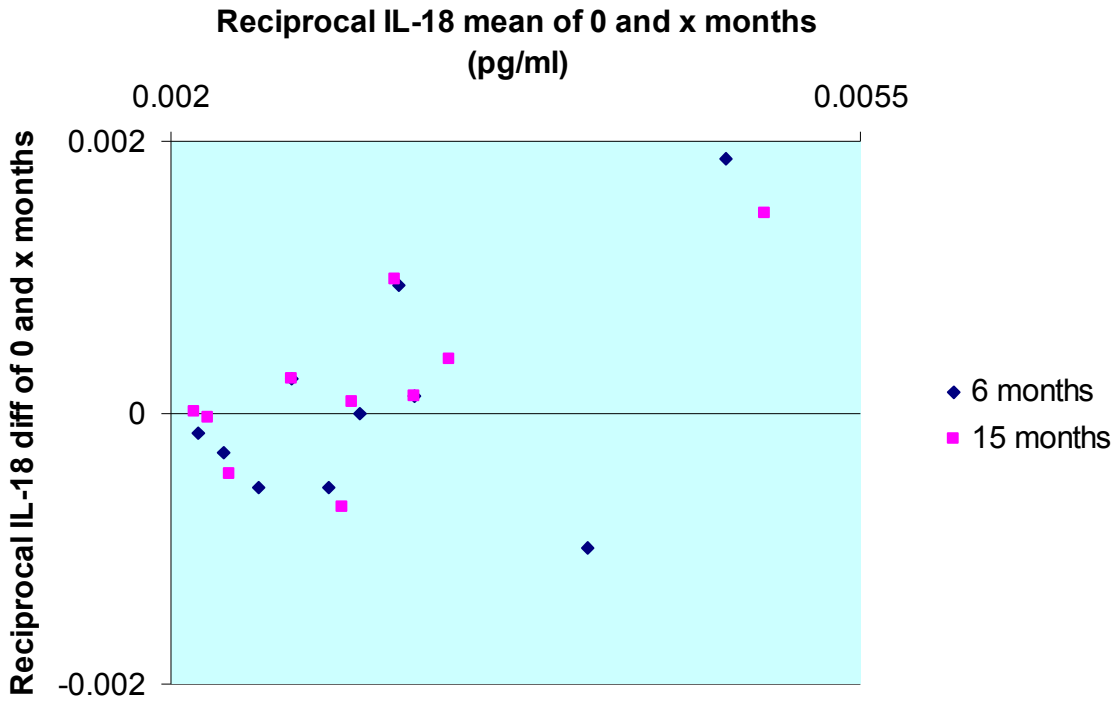
Table 3.6 Summary of data for the four inflammatory markers after storage at -50^{o}C at three time points; Baseline, time point 2 (12 months for IL-18 and TNFα, 9 months for MMP-9 and sCD40L), and time point 3 (15 months for IL-18, 12 months for MMP-9 and sCD40L).

	Median baseline	Median time point 2	Median time point 3
IL-18 (pg/ml)	351	352	363
TNFα (pg/ml)	1.86	1.96	n/a
MMP-9 (pg/ml)	1608	1239	1150
sCD40L (pg/ml)	6416	6469	6091

n/a – Not applicable

Figures 3.4-3.7 Bland-Altman plots showing the effect of minus 50°C storage at various time points (compared to baseline) for each of the four inflammatory markers.

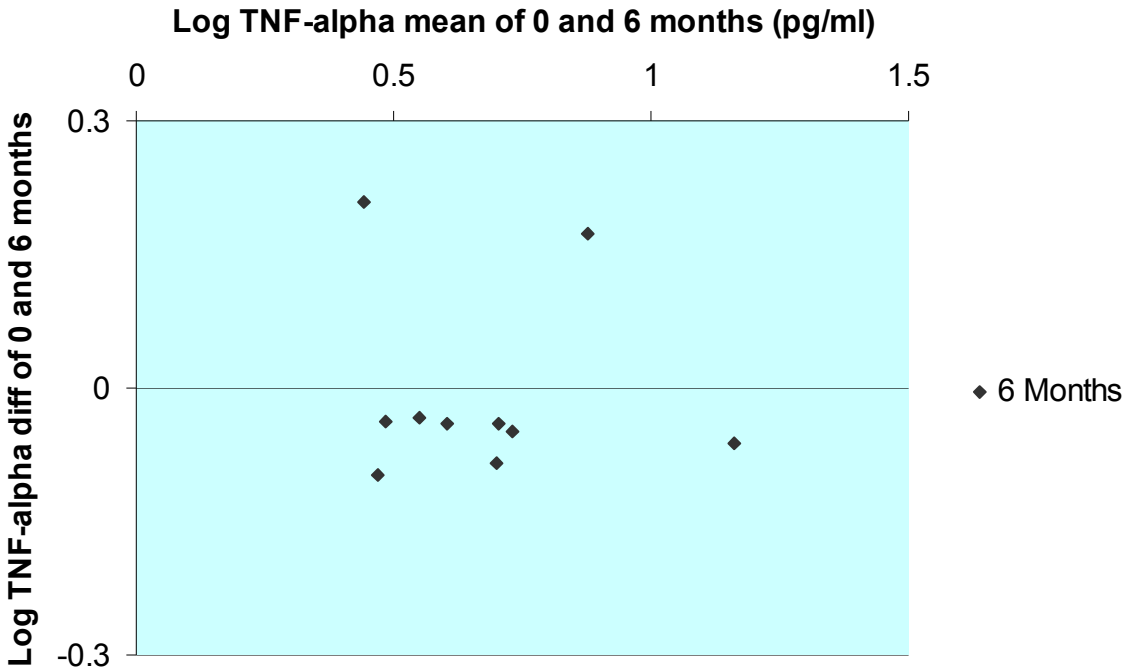
Figure 3.4 IL-18



6 Months
Mean difference (transformed) = $6.35E^{-05}$
Median difference (untransformed) = <1%
t-test p=0.81
Spearman correlation r = 0.71

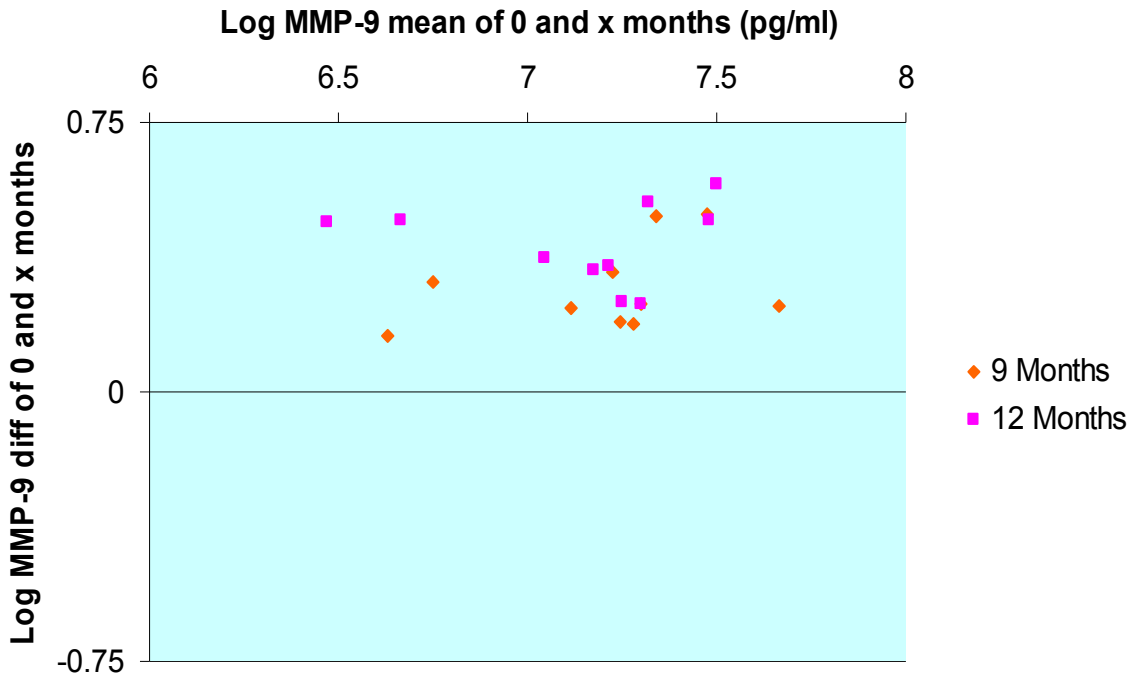
15 Months
Mean difference (transformed) = 0.000211
Median difference (untransformed) = 3.4%
t-test p=0.32
Spearman corr r = 0.69

Figure 3.5 TNFα



6 Months
Mean difference (transformed) = 0.006
Median difference (untransformed) = 5.4%
t-test p=0.86
Spearman correlation r = 0.90

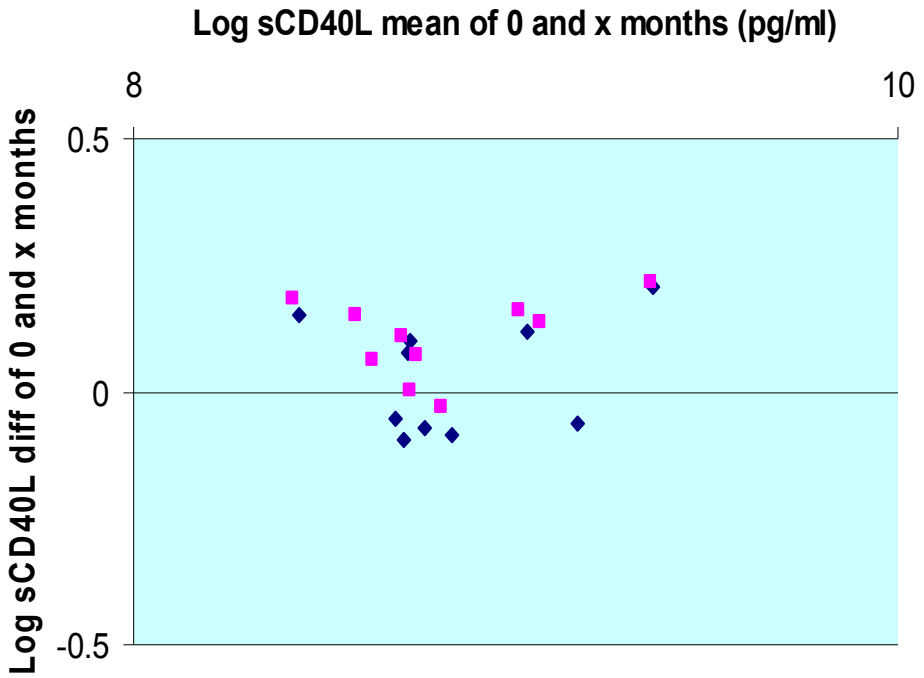
Figure 3.6 MMP-9



9 Months
Mean difference (transformed) = 0.288
Median difference (untransformed) = -22.9%
t-test p=0.0003
Spearman correlation r = 0.84

12 Months
Mean difference (transformed) = 0.409
Median difference (untransformed) = 28.5%
t-test p=0.0001
Spearman correlation r = 0.93

Figure 3.7 sCD40L



9 Months
Mean difference (transformed) = 0.029
Median difference (untransformed) = <1%
t-test p=0.44
Spearman correlation r = 0.80

12 Months
Mean difference (transformed) = 0.106
Median difference (untransformed) = -5.1%
t-test p=0.033
Spearman correlation r = 0.96

3.5.5 Freeze-Thaw Results

The effects of freezing and thawing cycles on the four markers are summarised in Table 3.7. The first thaw was at taken as a baseline value, since fresh plasma or serum was not assayed in this study.

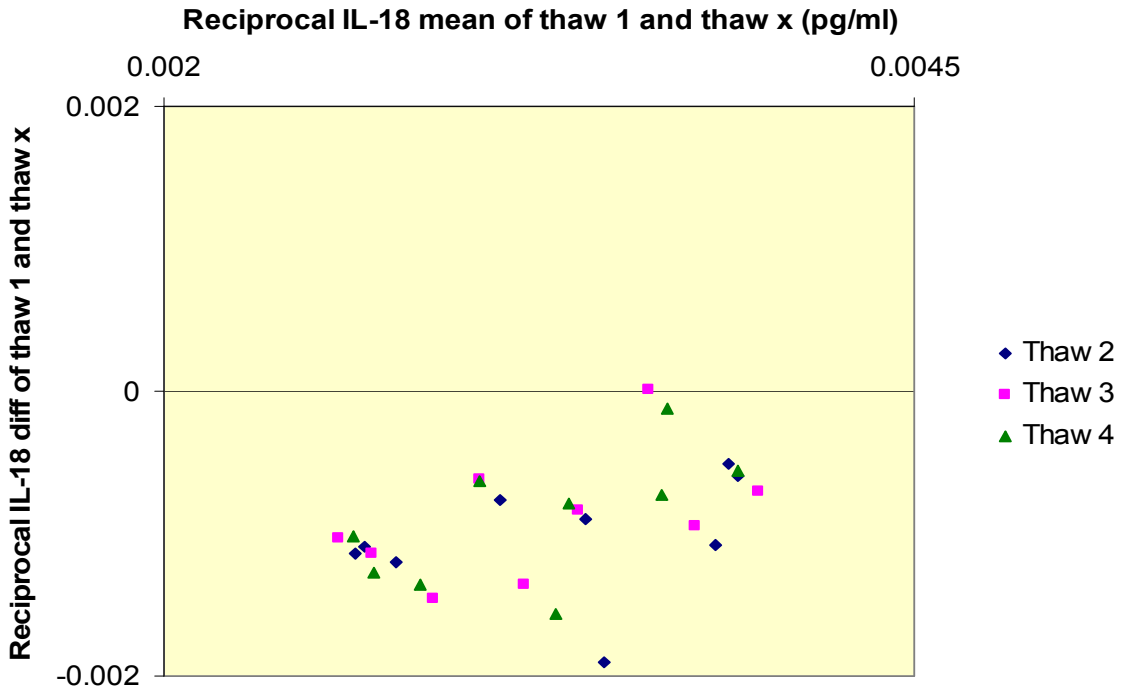
As seen in the Bland-Altman plot in Figure 3.8, IL-18 levels dropped 28.8% on the second compared to first thaw. Interestingly, this observation of decreasing detectable levels was not exacerbated on subsequent thaws. Spearman rank correlations of IL-18 data sets across thaws were maintained throughout ($r \geq 0.75$). Random data scatter shows that the effects of freeze-thaw were proportionately constant across IL-18 levels. TNFα levels (Figure 3.9) did not show any significant decreasing trend across the four thaws. The second thaw was significantly lower than the first (14.5%), but the equality of subsequent thaws to baseline suggest that this was a chance finding. Again, all data sets were correlated ($r \geq 0.78$). MMP-9 levels (Figure 3.10) decreased on the third and fourth thaws (27.1% and 27.6%, $p \leq 0.002$), and although the effect of thawing was constant across thaws, the data correlation was poor on the third thaw (r 0.59). This may be an outlier, since the correlation with baseline was better on the fourth thaw ($r = 0.77$). sCD40L levels did not decrease on the second thaw, but levels dropped significantly on the third thaw (8.2%) and dramatically on the fourth thaw (41.7%). The effect of these thaws was constant, and even on the fourth thaw there was very strong correlation with baseline values ($r = 0.94$).

Table 3.7 Summary of data for the four inflammatory markers after 4 freeze-thaw cycles.

	Median 1st thaw	Median 2nd thaw	Median 3rd thaw	Median 4th thaw
IL-18 (pg/ml)	351	250	269	267
TNFα (pg/ml)	1.86	1.59	2.07	2.19
MMP-9 (pg/ml)	1608	1634	1172	1164
sCD40L (pg/ml)	6416	6994	5889	3743

Figs 3.8-3.11 Bland-Altman plots showing the effect of four freeze-thaw cycles (compared to baseline [thaw 1]) on each of the four inflammatory markers

Figure 3.8 IL-18

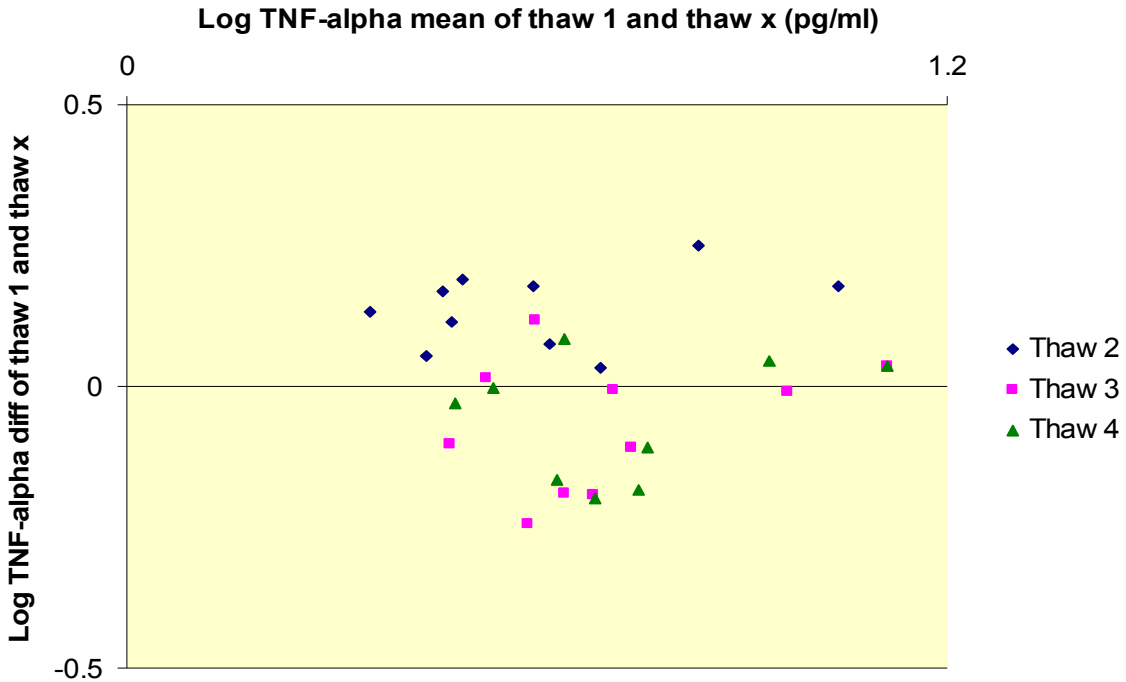


Thaw 2
Mean difference (transformed) = -0.001
Median difference (untransformed) = -28.8%
t-test p=0.0002
Spearman correlation r = 0.77

Thaw 3
Mean difference (transformed) = -0.0009
Median difference (untransformed) = -23.4%
t-test p=0.0004
Spearman correlation r = 0.79

Thaw 4
Mean difference (transformed) = -0.0009
Median difference (untransformed) = -23.9%
t-test p=0.001 (one point missing)
Spearman correlation r = 0.75

Figure 3.9 TNFα

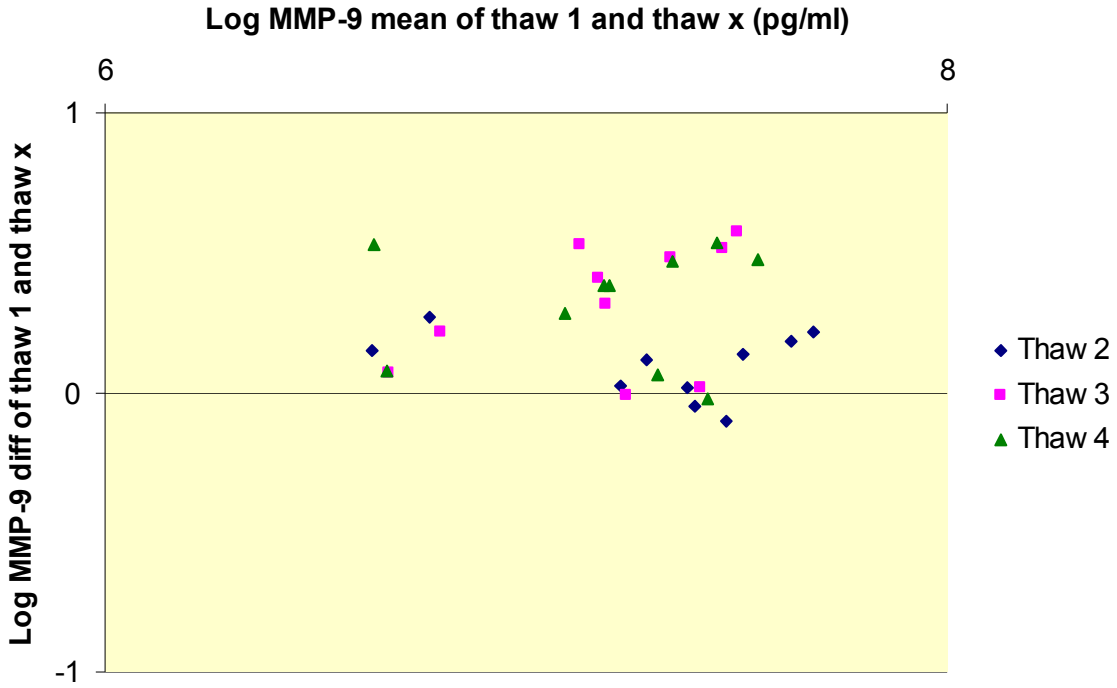


Thaw 2
Mean difference (transformed) = 0.137
Median difference (untransformed) = -14.5%
t-test p=0.0006
Spearman correlation r = 0.89

Thaw 3
Mean difference (transformed) = -0.069
Median difference (untransformed) = 11.3%
t-test p=0.1
Spearman correlation r = 0.78

Thaw 4
Mean difference (transformed) = -0.058
Median difference (untransformed) = 17.7%
t-test p=0.15 (one point missing)
Spearman correlation r = 0.87

Figure 3.10 MMP-9

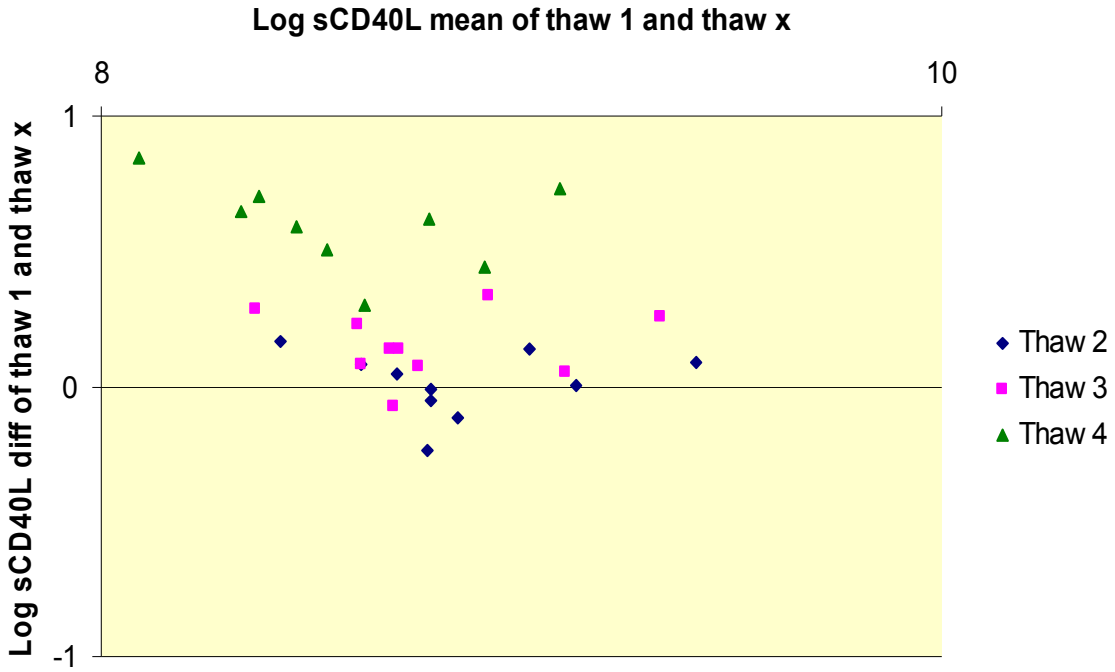


Thaw 2
Mean difference (transformed) = 0.095
Median difference (untransformed) = <1%
t-test p=0.04
Spearman correlation r = 0.98

Thaw 3
Mean difference (transformed) = 0.311
Median difference (untransformed) = -27.1%
t-test p=0.003
Spearman correlation r = 0.59

Thaw 4
Mean difference (transformed) = 0.318
Median difference (untransformed) = -27.6%
t-test p=0.002
Spearman correlation r = 0.77

Figure 3.11 sCD40L



Thaw 2
Mean difference (transformed) = 0.011
Median difference (untransformed) = 9.0%
t-test p=0.77
Spearman correlation r = 0.89

Thaw 3
Mean difference (transformed) = 0.152
Median difference (untransformed) = -8.2%
t-test p=0.006
Spearman correlation r = 0.83

Thaw 4
Mean difference (transformed) = 0.674
Median difference (untransformed) = -41.7%
t-test p=0.0003
Spearman correlation r = 0.94

3.5.6 Storage and Freeze-Thaw Discussion

In this experiment it is important to consider that the samples were taken from healthy participants with only small inter-individual differences in circulating levels of each marker. This may have important bearings on results, particularly the rank correlation analysis. For instance, for IL-18 and TNFα the baseline CV between all participants is <25%. Even though attempts were made to adjust for inter-assay variation effects, it is perfectly conceivable that residual intra-assay variation has a CV>5%, and this may have significant effects on the rank correlation between assays. Selection of only young and healthy individuals, therefore, may give an extreme view of the effects of storage and freeze-thaw conditions on these markers, and consequently results may be cautiously viewed as a possible exaggeration compared to the effects one would expect to observe in usual epidemiological studies of a general population including older subjects where the data set would have a higher CV.

Bearing in mind this limitation, the storage analysis study shows generally that keeping the samples frozen at -50^{o}C for over a year has little material impact, in terms of the rank order of the data sets, on the measurement of any of the four inflammatory markers. A previous study of MMP-9 has shown that storage for 2 years leads to a 65% fall in detectable levels (Rouy et al, 2005) and these results are consistent, demonstrating a fall of 28.5% of the median levels over 12 months. Despite this, the current study broadly found that the rank order of the data set is maintained since the effects appear to be uniform across all samples.

MMP-9 and sCD40L are most heavily affected in terms of absolute loss in detectable levels with each incremental freeze-thaw cycle, particularly sCD40L, which dropped by 41.7% of median baseline values after 4 cycles. A previous study of MMP-9 zymographic activity in EDTA plasma has shown no effect of several freeze-thaws, suggesting chelation of the molecule may confer some biochemical stability and allow it to maintain enzymatic potency (Souza-Tarla et al, 2005). This was clearly not the case here in serum measured by ELISA, since MMP-9 levels decreased on the third and fourth thaws.

Interestingly, sCD40L levels have recently been shown to be resistant to freeze-thaw cycles in platelet poor plasma, but levels may increase slightly in platelet rich plasma after freeze thaws (Varo et al, 2006). The ELISA used in that study (Varo et al, 2006) had a reported sensitivity of 10pg/ml, but the study reports values of up to ~16 ng/ml in serum; it is inherently unlikely the assay is sensitive over a range of ~2000-fold increases, and the sensitivity claim has not been peer-reviewed to my knowledge. Taken together the evidence suggests that in plasma samples any residual platelets (in platelet rich or poor plasma) are able to maintain or indeed elevate sCD40L levels due to the generally low levels already present being around the limit of detection for the technique. This is therefore experimental artefact, and further suggests that plasma samples are problematic in the measurement of sCD40L. The evidence presented here suggests that stored serum samples, or samples subjected to up to 4 freeze-thaw cycles, do not affect sCD40L or MMP-9 to the detriment of the integrity of the data set so long as all samples are prepared and treated in the same manner. This finding is important for epidemiological studies. Cross-study comparison of levels of the markers may prove more problematic.

In contrast, despite storage of samples or freeze-thaw cycles having little material effect on median IL-18 levels overall (which is in agreement with reported results from Biosource, the manufacturer of a swine IL-18 ELISA [see introduction]), I observed that IL-18 rank correlation within the data set was more substantially affected by storage for 15 months ($r = 0.70$ versus baseline) and 4 freeze thaw cycles ($r = 0.75$ versus baseline) than the other markers. Bearing in mind the limitations of this study, and the fact that the correlations were still significant, the conclusion is that IL-18 is suitable for use in epidemiological studies with the proviso that it be measured at the first thaw (within studies for this thesis) as the priority marker in order to minimise any possible effect of these pre-analytical variables.

This study has also shown that 4 freeze thaw cycles (and probably storage for up to a year; although this evidence is hampered by technical problems) has minimal impact on measurements of TNFα in terms of overall change in levels, which is in agreement with previous findings (Thavasu et al, 1992). It seems probable that TNFα is the most robust

of these inflammatory markers in terms of resistance to storage decay and freeze-thaw cycles, despite the very low circulating levels in healthy individuals.

In terms of epidemiological use, I conclude that all four markers are suitable to be examined as risk markers in prospective epidemiological studies, but based on the evidence here, it is possible only IL-18 and TNFα are suitable for meta-analysis of prospective studies, due to the increased labile nature of MMP-9 and sCD40L. Pooling of data sets for these markers may not be a suitable methodology. More data is required for all of the markers using a larger, wider population in order to confirm and expand on these findings.

3.6 SHORT-TERM BIOLOGICAL VARIATION

3.6.1 Short-Term Biological Variation

Normal fluctuations in the expression of circulating inflammatory and haemostatic markers occur within the time frame of weeks, days, and hours. For instance, there are diurnal and seasonal variations in circulating haemostatic factors (activated factor VII, tissue plasminogen activator, prothrombin fragment F_{1+2}, plasmin–plasmin inhibitor complex, fibrin D-Dimer, t-PA, vWF) and inflammatory markers (fibrinogen and CRP) (Kapiotis et al, 1997; Rudnicka et al, 2007). To some extent variation in circulating variables is caused by predictable external factors (e.g. physiological response to seasonal weather, activity, or eating patterns over 24 hours), and hence consequent variation is predictable at a population level. However, there are also less predictable fluctuations in short-term variation that are more prominent on the scale of individual patients (e.g. response to (sub)clinical infection or physical exertion). This source of biological variation could have implications for a marker's clinical utility, as well as perceived risk associations in epidemiological studies. In most clinical situations or epidemiological studies, only one blood sample from each subject is taken, but ideally multiple sampling would be required for more reliable estimation of the individual's habitual plasma level.

According to the literature, biological variation in inflammatory markers can be surprisingly large. For instance, CRP has been estimated to have an intra-person CV of 34 – 49.8% (Macy et al, 1997; Wilkins et al, 1998; Sakkinen et al, 1999; Ockene et al, 2001; at least 5 repeat measurements at a maximum of 1 month intervals). One of these studies has suggested that CRP has similar CV to that seen in total cholesterol (Ockene et al, 2001) although this claim has been refuted (Campbell et al, 2002 & 2003). The biological variability study from the British Regional Heart Study cohort (1 week repeat measurement in 297 men) reports a regression dilution ratio of 0.87 for total cholesterol, 0.74 for CRP, 0.84 for fibrinogen and 0.69 for systolic blood pressure (Emberson et al, 2004).

For more precise measurement of biological variability, markers should be repeatedly measured in a small number of individuals with the aim of ascertaining the number of repeat measurements required to estimate reliable usual level (or to minimise risk of mis-classification in individuals). The aim of this study was to examine the effect of short-term biological variability on IL-18 and TNFα in comparison to other new CVD risk predicotors (CRP, IL-6, fibrinogen). In doing this the aim was to estimate the number of blood samples which would be required for 75% and 90% reliability of such estimates in individuals (Fleiss et al, 1987), as well as estimating the effects of biological variation on observed risk associations in cohort studies. Due to a lack of serum samples, sCD40L and MMP-9 were not analysed. The results of the haemostatic markers, fibrinogen, CRP and IL-6 in this study have previously been reported elsewhere (Poorhang, MSc thesis, 2005), and are also reported here in a revised statistical analysis to compare with IL-18 and TNFα.

3.6.2 Subjects and Methods

14 healthy non-fasted volunteers, 7 men and 7 women aged between 37 and 56, all gave informed consent to repeated venipuncture for up to 21 weeks, and the study was approved by the Local Research Ethics Committee. 20 ml blood was drawn weekly into citrated (0.109M trisodium citrate) vacutainers. All samples were taken in the morning before 12MD, which minimises the effect of diurnal variation (Rudnicka et al, 2007). Due to subject withdrawal at various stages, the average number of visits was 17 (range 4-21).

Blood samples were centrifuged at 2000g, the plasma separated, and aliquots frozen at -80°C. Each sample was snap-thawed at 37^{o}C as required, and each aliquot was thawed for assay on a maximum of two occasions. All the time points for a given individual were assayed on the same ELISA (for each marker) to minimise inter-assay variation in the study, and each set of samples for an individual had experienced the same storage/freeze-thaw history. All available data was analysed.

Total within person variation (CV) in this study can be seen as the composite effect of biological CV and methodological CV (which is a constant; intra-assay CV). Therefore biological variation is given by the equation:

$$\text{Biological CV} = \frac{\text{Total CV} - \text{Methodological CV}}{1 - \text{Total CV}}$$

(Ernst et al, 1985)

More complex statistical analysis was performed in Excel in accordance with Fleiss (1986) after log-transformation to normalise the data. However, the Fleiss method is prone to statistical biases with decreasing number of values due to estimations of variance in components of random error and intra-subject error (Fleiss, 1986). Although this study is sizeable, it was deemed prudent to cross-examine the data with another statistical method. The "Random effects model" (a mixed model) removes these inherent biases, although the statistical analysis entailed is more complex. Therefore this analysis of the data was performed by using a random effects model (Hankinson et al, 1995). This involved regressing the correlated sets of values, all observations on all individuals taken together, against a random intercept. An unstructured correlation set was assumed, and log transformed used to induce approximate normality.

On comparison of both the Fleiss and the random effects models, the data were shown to be highly comparable (suggesting sufficient N to estimate variances in the Fleiss model). Due to high comparability, only the data from the random effects model is included here.

Key output data is presented with the following index:

R Reliability (the intra-class correlation coefficient); the proportion of variance due to within subject error. Calculated by the ratio of the within-subject mean square (also the within subject variance) to between subject mean square (from one-way ANOVAs).

nrep Number of replicates required to achieve desired reliability.

The reliability coefficient (R) obtained was used to determine the expected effect of short-term variation on marker associations with outcome in epidemiological studies. This means a hypothetical "true" association between a marker and a study outcome can be used to infer what the expected observed association will be when considering data "lost" to short-term variation (Hankinson et al, 1995).

Data examining all other inflammatory markers (than IL-18, TNFα, MMP-9 and sCD40L) in this section are from Poorhang, MSc thesis, 2005. The results presented here for those markers are analysed in a novel form (not used by Poorhang) are included for comparison.

3.6.3 Results

In total there were 243 time-points where blood samples were taken from 14 individuals. For technical reasons IL-18 and TNFα were assayed in fewer samples than the other markers (99% and 91% respectively), but all available data was analysed.

Table 3.8 shows the total variability in the 14 individuals over the period of the study. Total CV is broken down into methodological and biological CVs. As expected, by far the greatest proportion of total CV was biological in origin. Intra-person biological CV was lowest for fibrinogen (6.2%), greater for IL-18 (25.0%) and TNFα (24.8%), and highest for the acute phase response associated cytokines IL-6 (50.1%) and CRP (55.7%).

Despite the interesting observation that IL-6 and CRP as well as IL-18 and TNFα had highly comparable overall biological CVs, this belied considerable inter-individual

variation in observed CVs. Illustrating this, IL-6, CRP, IL-18 and TNFα CVs in individuals are shown as a function of plasma antigen level in Figure 3.12. As seen in the figure, although the average overall CV for IL-6 was ~54% the range spread from ~25% to ~150%. There were no significant interactions of CVs with circulating plasma levels of the marker. This means the CV is independent of plasma level of the marker in each case.

Expanding this theme, it would be ideal to compare biological CV between individual people; i.e. do some people display tendencies towards variability/stability across all inflammatory markers than others? Rank orders of CVs for all people in each marker are displayed in Table 3.9. As can be seen in this table, person 4 displayed consistently high biological variability (relative to the group), whereas there were others who were relatively stable across markers. There were too few people and too many variables to apply statistical models to this question reliably here, although the general trends are interesting to note. Thus, overall, some individuals appear to display greater propensity to a high degree of variation. It is hard to attribute this to a specific biological cause. The individual may have only had a cold(s) over a significant period of the study. Also interesting in Table 3.9, but as expected, IL-6 and CRP/fibrinogen demonstrated high correlations with each other throughout the data in terms of *intra*-individual CV. Other markers were relatively less well correlated.

Further illustrating differences in variation between individuals, Figure 3.13 shows two individuals at opposite ends of biological variability spectrum for CRP and fibrinogen. Note that even after 5 months it is hard to discern a "habitual" level of either marker for the variable individual. In Figure 3.14 the biological CV of fibrinogen (low overall CV) and IL-6 (high CV, although the correlation in CV between persons for the two markers is high) for two individuals is shown. Interestingly, erratic circulating IL-6 levels can be associated with concomitant erratic fibrinogen levels (person 1). In contrast, as seen in person 2, sharp peaks in IL-6 expression may also have no observed effect on fibrinogen (or any other inflammatory marker; data omitted for clarity). This is despite roles in the acute-phase response common to IL-6 (promoter) and fibrinogen (reactant), and a relatively strong correlation ($r = 0.49$). IL-18 and TNFα demonstrated no particularly strong association in variation with each other or any other inflammatory marker.

Using the random effects model, Table 3.10 was generated, and shows estimates of the coefficient (R) for each marker. According to study estimates, in order to achieve a reliability of 75% for an individual's true mean value, 1 measurement of fibrinogen (rounded whole number 95% confidence intervals; 1-2) is sufficient, while 2 measurements are required for IL-18 (1-3), CRP (1-4), and TNFα (1–4), although 8 measurements are required for IL-6 (1-14). To achieve a reliability of 90% it is estimated that 3 measurements are required for fibrinogen (1-6), 4 measurements for IL-18 (1-8), 6 measurements for CRP (1-12) and TNFα (1-12), and 22 measurements for IL-6 (2-43).

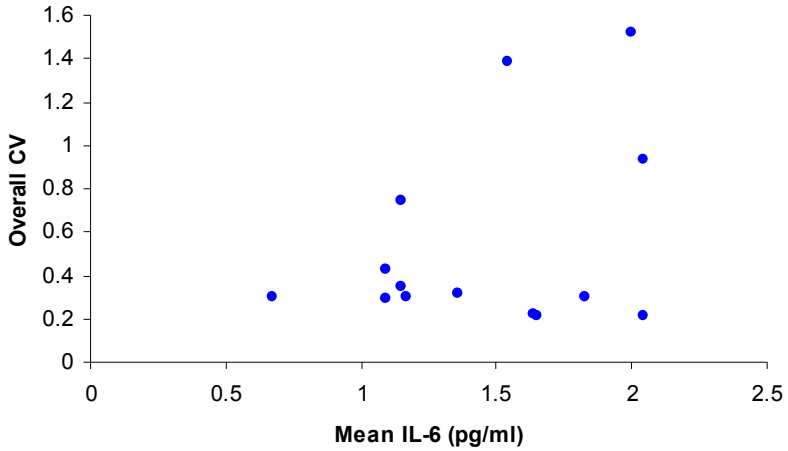
In addition to this analysis, the probable observed OR in prospective studies for each marker, based on a hypothetical "true" OR of 1.50 for each marker was estimated. These "expected/observed" ORs are also summarised in Table 3.10. Due to each marker being assigned the same hypothetical OR, the rank order of relative underestimations of the markers due to biological variation is the same as the rank order for the coefficient R. As can be seen from the table, estimates suggest the marker with the lowest biological CV, fibrinogen, would have an OR of 1.36 (a 28% underestimation of risk). In contrast the more variable markers would lose much of their associations with events in prospective studies: CRP OR 1.27 (46% underestimation) and IL-6 OR 1.12 (76% underestimation).

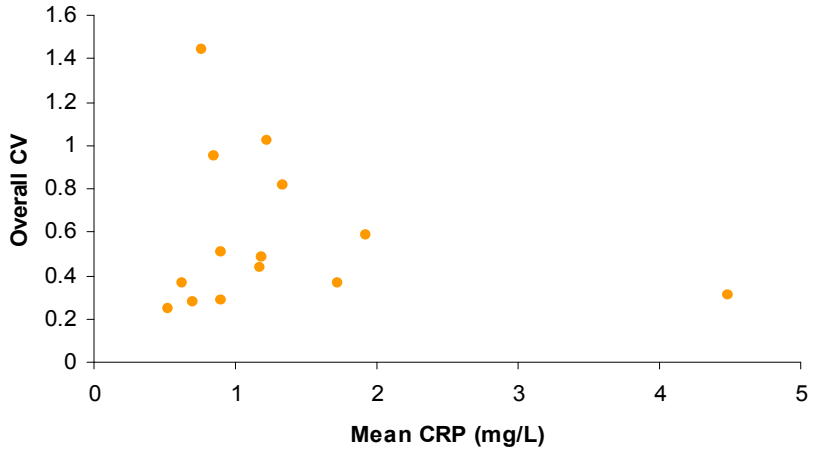
Table 3.8 Total short-term CVs for each marker and proportions thereof that are methodological (intra-assay) or biological in origin. CVs are obtained from 243 observations from 14 people (except IL-18 and TNFα; 221 observations from 13 people, 240 observations from 14 people respectively).

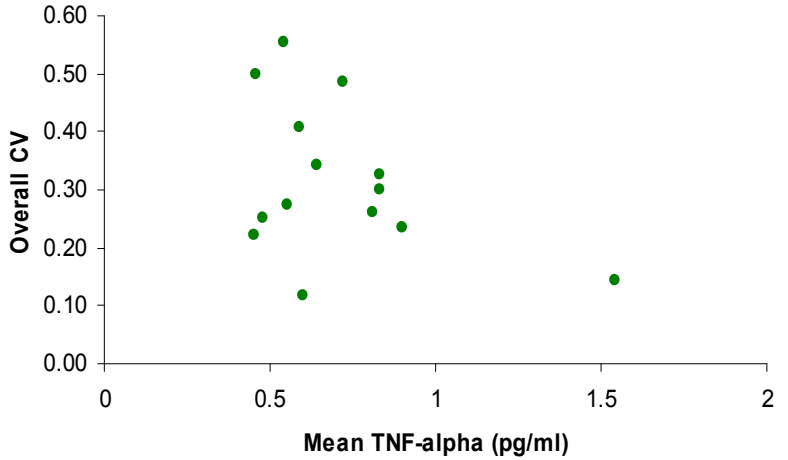
Variable	Mean of intra-person means	Mean of intra-person SDs	Mean of intra-person CVs (%)	Methodological CV %	Biological CV %
Fibrinogen (g/L)	3.05	0.25	8.31	2.6	6.2
CRP (mg/L)	1.31	0.7	57.8	4.7	55.7
IL-6 (pg/ml)	1.46	0.84	53.8	7.5	50.1
IL-18 (pg/ml)	299	91	29.2	5.6	25.0
TNFα (pg/ml)	0.71	0.21	31.1	8.4	24.8

SD: standard deviation, CV: coefficient of variation

Figure 3.12 Range of inter-individual CVs of inflammatory markers (CRP, IL-6, IL-18 and TNFα) as a function of circulating plasma level.







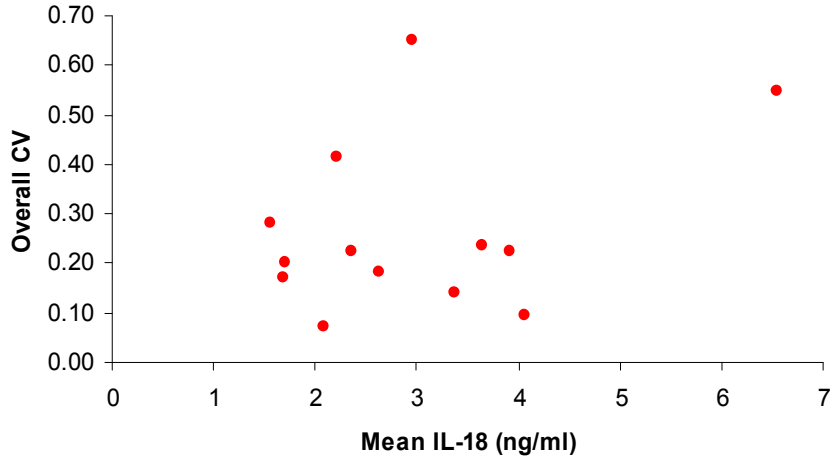


Table 3.9 Rank order of magnitude of CV for each person in each marker (1=highest CV for given marker). Table illustrates inter-individual differences in global inflammatory trends for variability. Correlation values displayed below are rank correlations comparing data sets for given markers in selected pairs.

	IL-18	TNFα	IL-6	CRP	Fib	Average
Person 1	2	13	11	14	9	10
Person 2	4	15	13	10	8	10
Person 3	13	2	14	13	14	11
Person 4	3	1	2	1	1	2
Person 5	11	12	6	5	5	8
Person 6	6	5	4	3	4	4
Person 7	1	11	1	8	12	7
Person 8	9	7	7	12	13	10
Person 9	8	4	5	6	3	5
Person 10	5	8	3	2	2	4
Person 11	12	10	8	4	10	9
Person 12	7	6	12	9	7	8
Person 13	n/a	3	10	11	11	9
Person 14	10	9	9	7	6	8

n/a, data missing.

CRP vs Fibrinogen	r = 0.78
IL-6 vs CRP	r = 0.72
IL-6 vs Fibrinogen	r = 0.49
IL-18 vs TNFα	r = 0.23
TNFα vs CRP	r = 0.17
IL-18 vs CRP	r = 0.04

Fig 3.13 Graphical comparison of CRP and fibrinogen in a person with high CV (person 4 in Table 3.9) and a person with low CV (person 8)

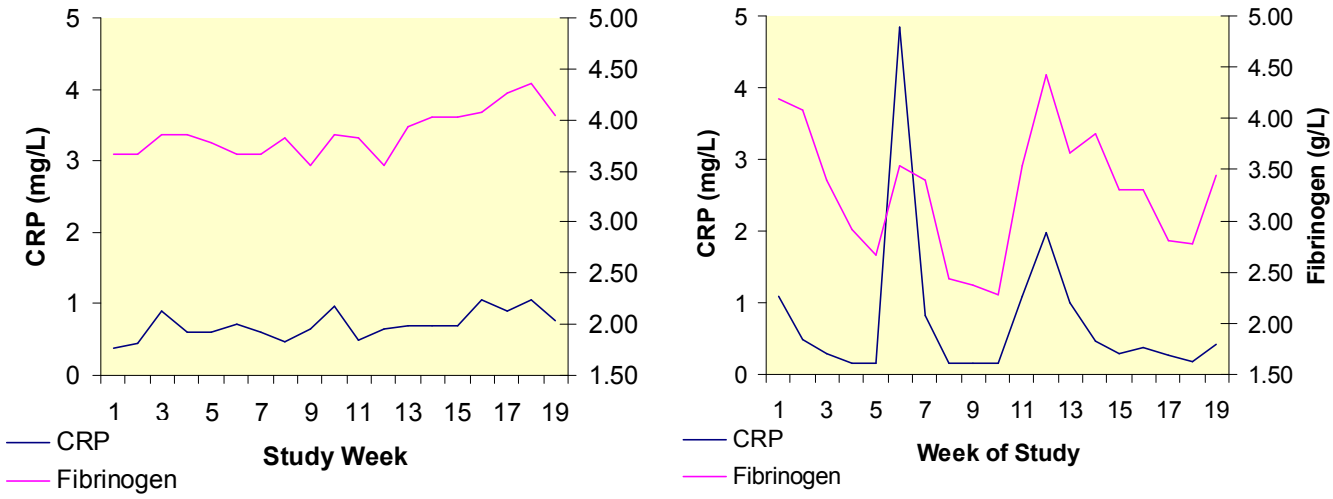


Figure 3.14 Graphical comparison of biological CV in fibrinogen (lowest biological CV) and IL-6 (highest biological CV) for two people over the course of the study.

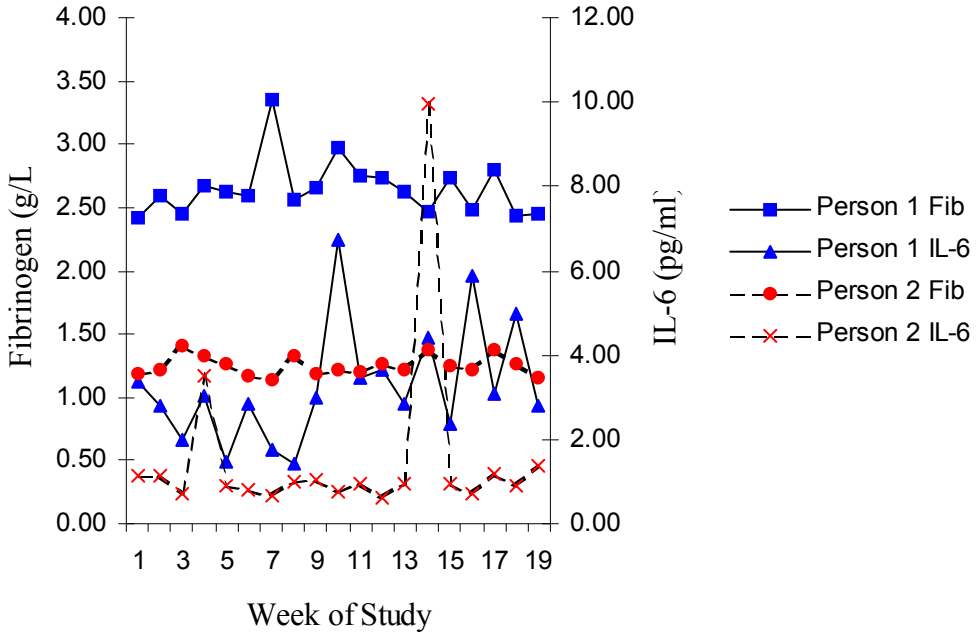


Table 3.10 Random Effects model statistics for reliability measurements in short-term variation.

	Intra-class correlation coefficient (R) R (95% CI)	n required measurements for 75% reliability nrep (95% CI)	n required measurements for 90% reliability Nrep (95% CI)	Hypothetical OR	Observed OR*
Fibrinogen	0.73 (0.60-0.94)	1 (1-2)	3 (1-6)	1.50	1.36
CRP	0.58 (0.43-0.89)	2 (1-4)	6 (1-12)	1.50	1.27
IL-6	0.28 (0.17 – 0.83)	8 (1-14)	23 (2-43)	1.50	1.12
IL-18	0.66 (0.52-0.93)	2 (1-3)	4 (1-8)	1.50	1.31
TNFα	0.58 (0.43-0.89)	2 (1-4)	6 (1-12)	1.50	1.27

CI: confidence intervals, n rep: number of required repeat measurements, OR: Odds ratio.

* Observed OR estimated to be found in prospective studies (assuming correct value for R coefficient).

3.6.4 Discussion

Overall, this study demonstrates the importance of considering the normal background biological variation in prospective studies of novel risk markers, and indeed in candidate markers for clinical risk assessment. From a clinical perspective, the risk of mis-classifying an individual into hypothetical "high, intermediate, or low" risk categories increases with decreasing reproducibility of a marker, and hence detrimentally affects the clinical utility of the marker as a tool. Biological variation also has impact on prospective epidemiological studies, which often only use one baseline measurement of an individual, and can lead to gross under-estimation of "true" risk association. These statements are of course not new, but the finding of such dramatic differences in biological variation between similar "general inflammatory" markers within the same study is striking.

Previous studies have shown similar biological variability in more conventional CVD risk markers. A meta-analysis of 30 studies published from 1970 to 1992 yielded estimates of biological CV of 6.1% for total cholesterol, 7.4% for HDL cholesterol, 9.5% for LDL cholesterol and 22.6% for triglycerides (Smith et al, 1993). Results presented here suggest that inflammatory markers are generally much more variable than serum lipids, although the CV of IL-18 and TNFα are comparable to the (by far) most variable lipid reported, triglycerides (Smith et al, 1993). This study further extends the exploration of biological CVs by examining implications of variability both at the epidemiological and clinical level.

Fibrinogen, CRP and IL-6

The finding that fibrinogen has a relatively low within-person CV over short time frames despite it being an acute phase reactant has been reported elsewhere (Sweetnam et al, 1998; Rosenson et al, 1994 & 2001; de Maat et al, 1996). Rosenson et al (1994 & 2001) recommended that 4 measurements of fibrinogen be performed to characterise a healthy person's "usual" level although due to a lack of comparison with other markers, this was concluded to be a high level of variability. According to study estimates, 4 replicates of fibrinogen would give a reliability ~90% (95% CI; 1-6 measurements). Fibrinogen was relatively less affected by short-term biological variability, and therefore appears to be a relatively reproducible marker in terms of biological variation.

In contrast, CRP shows considerable biological variability in healthy persons and hence appears to have more classical APR behaviour. In the present short-term biological variability study, results show a mean within-person CV of 55.7% for CRP, which is comparable to those obtained previously over similar time-frames (47.4% and 49.8%) (Macy et al, 1997; Sakkinen et al, 1999). These findings are in general agreement with previous studies, which recommended *at least* 3 sequential measurements of CRP (de Maat et al, 1996; Koenig et al, 2003) since our results suggest ~2 (1-4) measurements were required to attain only 75% reliability. According to these estimates, the 2 measurements recommended by the American Heart Association guidance (Pearson et al, 2003) would be insufficient to entirely counteract the inherent unreliability of CRP.

Perhaps consistent with its role as a chief regulator of the APR, estimates showed a mean within-person CV of 50.1% for biological variability in IL-6 levels, which is comparable with that of CRP in our study. However, our statistical analysis shows IL-6 to be much more variable than CRP, with 8 measurements required to obtain a reliability coefficient of 0.75 and 23 (2-43) measurements for a reliability coefficient of 0.90. This finding calls into question recent conclusions in a study of brain naturetic peptide (BNP; a putative CVD risk marker) (Clerico et al, 2006). The authors of this study show that BNP has a CV of 30-50%, suggest that, due to high CV, only those with very high BNP levels within the population could have BNP considered as a risk marker due to inherent uncertainty. In contrast, these findings show that actually little weight can be given to more extreme observations within a population as they likely have little physiological significance in terms of "habitual" levels (e.g. person 8 in Figure 3.13).

Assuming accuracy, our data suggest that prospective studies may be actually under-estimating risk associations of IL-6 by around 76% due purely to biological variation. On the other hand, data presented here (for IL-6 in particular) requires further study in order to narrow the confidence intervals; the finding that IL-6 is particularly unreliable could be a chance finding, and confidence intervals for IL-6 do overlap with other markers.

In some individuals, peaks of IL-6 and fibrinogen/CRP expression somewhat coincided, while in others they were dissociated (Figure 3.14). One interpretation of these observations may be that CRP and fibrinogen expression may be regulated by inflammatory factors other than IL-6 (e.g. leptin, adiponectin, oncostatin M; Hansson et

al, 2005) and conversely that aberrant expression of IL-6 need not result in simultaneous variability in expression of all acute phase response proteins. This may suggest that the APR is not an "absolute" event as seen in textbooks, although it is important to bear in mind data is lost even when individuals are examined at 1 week intervals.

IL-18 and TNFα

Consistent with pro-inflammatory roles somewhat removed from the acute-phase response, the cytokines IL-18 and TNFα had within-person CVs around half of that seen for CRP or IL-6 (25.0% and 24.8% respectively). The fact that they are highly comparable to each other is interesting considering the "upstream" pro-inflammatory Th1 type role they both play in initiating and maintaining immune responses. Lower biological CVs than CRP and IL-6 is perhaps suggestive of tighter transcriptional regulation in normal healthy people consistent with important immunoregulatory roles.

Analysis shows that IL-18 requires 1 measurement for 75% reliability and TNFα 2 measurements, while the requirement increases to 4 measurements for IL-18 (1-8) and 6 for TNFα (1-12) at 90%. According to our estimates, TNFα therefore is comparable to CRP in terms of reproducibility (despite having a CV around half that of CRP), while circulating IL-18 may be slightly better than either of these, and is the most reproducible of the pro-inflammatory cytokines in this study. Lower reproducibility of TNFα is probably at least partially due to the higher intra-assay CV in the normal range, and is hence partly a methodological issue. Both IL-18 and TNFα are estimated to have underestimated associations with risk in prospective studies by around 40-45%, which is similar to CRP.

In conclusion, there is a range to the extent of biological variation in different inflammatory markers. Such biological variation in inflammatory markers demonstrate limited intra-person cross-marker associations, and intra-marker cross-person associations. Despite this there may be clear trends in variation at the population level. Generally, normal biological variation of inflammatory markers (and indeed classical risk markers; Emberson et al 2003 & 2004) in healthy people will lead to varying degrees of under-estimation of risk association in prospective studies, and this has important limitations for clinical utility of potential risk markers. On the other hand prospective

studies which utilise only one baseline sample may drastically under-estimate a marker's risk associations. It is important therefore to consider these issues in such studies, particularly when considering novel risk markers.

3.7 LONG-TERM REGRESSION DILUTION

In addition to short-term biological variation, it has been shown for many classical risk markers that estimation of a baseline value based on one sample results in a long-term underestimate of the association with risk (Emberson et al, 2003 & 2004; Frost and White, 2005). For example, in middle-aged men taking a single measurement leads to an underestimate of all lipid measurements (25-35% on a log scale) and blood pressure (40-50%) within 4 years (Emberson et al, 2004). This leads to an underestimate of risk association, and is a potential explanation of the "50% myth" of conventional risk factors. The British Regional Heart Study estimates that once "regression dilution" is accounted for, classical risk factors may account for 70-90% of incident CHD, not just 50% (Emberson et al, 2003 & 2004).

The aim of this study was to assess the 4-year intra-individual variability and regression dilution in the inflammatory markers in a sizeable (>100 people) study, using the British Regional Heart Study stored plasma and serum samples. Use of the BRHS samples allows direct comparisons to be made with regression dilution results for other markers assayed in these samples, described in previous publications ([1]Emberson et al, 2004).

Methods, results and discussion of this study are included in Chapter 6.

3.8 GENERAL CONCLUSIONS

IL-18, TNFα, were found to be suitable for use in citrate or EDTA-anticoagulated plasma as well as serum, whereas acceptably high detectable levels levels of MMP-9 and sCD40L (above background assay noise) were only seen in serum samples in normal people. Therefore only serum samples will be used with the latter two markers in this thesis. All 4 inflammatory markers displayed acceptable intra- and inter-assay variations for use in epidemiological studies. Although generally higher in terms of CV than

automated methods such as CRP nephelometry and von Clauss clotted plasma fibrinogen detection, the ELISA assays employed are sensitive enough to measure circulating concentrations reproducibly in the normal range.

In further studies, TNFα was the most robust cytokine of the 4 markers, showing little variation over the six month period that the study was able to be used, or over 4 freeze-thaws. IL-18 was also relatively robust, although the data sets tended to show lower correlations after storage or freeze-thaws (more observations are required). MMP-9 and sCD40L were less robust and decayed faster in storage and with increasing freeze-thaw cycles. Larger studies in more varied populations are required to confirm and expand on these findings, although the possibility of accurately performing storage studies over decades is almost impossible in technical terms. In the meantime, I recommend that only IL-18 and TNFα can be used in meta-analysis, until such a time that there is sufficient evidence that statistical adjustment of pooled MMP-9 and sCD40L datasets will yield results suitable for meta-analysis..

Biological variability of risk markers is an area of current interest in the literature. The study presented here suggests that although many inflammatory markers display similar degrees of biological variability in the short-term (in terms of the reliability coefficient), such variability is clearly important to consider in epidemiological and clinical terms. In epidemiological studies these estimates predict that IL-18 risk associations will be underestimated by 38%, and TNFα by 46%. In clinical terms the estimates suggest that IL-18 and TNFα require at least 2 repeat measurements to achieve a reliability of 75%, and 4 and 6 measurements respectively to achieve reliability of 90%. The effect of 4-year regression dilution will be examined in Chapter 6.

CHAPTER 4

DISTRIBUTIONS OF IL-18, TNFα, AND IL-6 AND ASSOCIATIONS WITH CVD RISK FACTORS IN A RANDOM POPULATION SAMPLE: THE GLASGOW MONICA STUDY

4.1 INTRODUCTION

4.1.1 Population Distributions and Associations of IL-6, IL-18 and TNFα

Random cross-sectional studies of general populations provide the least bias-prone method of examining distributions of risk markers and their associations with other CVD risk factors, and data from large studies of this kind can potentially be used as reference ranges. I am not aware of any such studies examining IL-18 or TNFα published before commencing this thesis.

Despite lack of cross-sectional data from general populations before commencing this thesis, some studies had demonstrated trends suggesting inflammatory risk markers have associations with some classical CVD risk factors. TNFα is associated with age (Belmin et al, 1995; Paolisso et al, 1998) and is thought to correlate with BMI, and symptoms of the metabolic syndrome (Ziccardi et al, 2002; Samuelsson et al, 2003) as is IL-18 (Esposito et al, 2003). Previous prospective publications have reported that IL-18 shows relatively weak associations with HDL cholesterol (inversely) and triglycerides (but not total cholesterol), as well as weak but statistically significant associations with CRP and IL-6, but not with age, BMI, or fibrinogen levels (Blankenberg et al, 2002, 2003). Likewise in similar publications TNFα shows weak but statistically significant associations with triglycerides, HDL cholesterol (inversely), and other inflammatory markers (IL-6, IL-18 and CRP [moderate], although not fibrinogen) (Ridker et al, 2000; [2]Cesari et al, 2003). The ABC study found no gender-specific interactions with TNFα (or IL-6) concentrations ([2]Cesari et al, 2003), although there are reported mechanistic differences in TNFα expression between the sexes in this cytokine's association with BMI (Hube et al, 1999; Orel et al, 2004)

IL-6 has been investigated in a small cross-section (196 men and 221 women) of the Third Glasgow MONICA survey (Woodward et al, 1999) and was found to correlate significantly with age, fibrinogen, and current smoking, but not with other major risk factors or with prevalent cardiovascular disease. Clearly more cross-sectional data is needed to confirm and expand on these findings, preferably in a cohesive study of a range of inflammatory markers.

Some studies have reported that inflammatory markers in general (e.g. CRP, IL-6 and fibrinogen: Woodward et al, 2003; Mennen et al, 1999; Mukalmal et al, 2004; Volpato et al, 2004) but not TNFα (Volpato et al, 2004) have a relationship with alcohol consumption, perhaps reflecting alcohol consumption's J-shaped relationship with CVD risk (Emberson & Bennet, 2006). Although there are credible biological pathways to substantiate evidence that low alcohol consumption is associated with lower CVD risk (Emberson & Bennet, 2006), if inflammation is potentially a partial explanatory factor for the J-shaped relationship of alcohol consumption with CVD, this would be of obvious interest. In addition to these observations, inflammatory markers in general are known to be related to smoking habits (Woodward et al, 1997 & 2003, [2]Wannamethee et al, 2005). A potential relationship of IL-18 and TNFα with smoking requires clarification, since it would be a potential confounder of relationships with CVD.

Cross sectional data on inflammatory marker associations with socioeconomic status (SES) were sparse at the outset of this thesis, with one small (n=230) study showing that IL-6 and TNFα were weakly associated with SES in women (Steptoe et al, 2002), although there was a general association between IL-6 and CRP and SES in the Whitehall II study (Hemingway et al, 2003). In contrast, fibrinogen has long been known to be associated with social position (Markowe et al, 1985; Brunner et al, 1996, [2]Woodward et al, 2003). There were no published data linking IL-18 with SES at the outset of this thesis. It is possible that inflammation *per se* may be one plausible mechanism for the association between conventional risk markers, and social deprivation, as risk factors for CHD. This is of particular interest in Scotland. The ASSIGN score was recently introduced in Scotland to include social deprivation as a marker in risk assessment of CHD, since the association between social deprivation and CHD risk is not

explained fully by confounding from conventional risk factors (Brunner et al, 1996; [2]Woodward et al, 2003; Tunstall-Pedoe et al, 2006; [1]Ramsay et al, 2007; Woodward et al, 2007).

4.1.2 Aims of the Study

In this study, the aim was to use stored plasma samples from the fourth Glasgow MONICA survey as a cross-sectional study to study IL-18 and TNFα (and also CRP, fibrinogen and IL-6 for comparison), specifically

1. Reporting reference ranges of these cytokines.
2. Reporting their associations with each other.
3. Reporting their associations with some classical CVD risk markers (age, sex, BP, cholesterol, BMI, hormone use).
4. Investigating inflammatory marker associations with smoking, alcohol consumption, social deprivation scores - areas of current interest in the literature – and also analysing for sex-specific interactions with these factors.

4.2 METHODS

4.2.1 The World Health Organisation MONICA Study

It was realised, in the 1970s and 1980s, that more data were needed to explain rapidly changing incidence rates of cardiovascular disease across the globe, above and beyond data on death certificates (Havlik & Feinleib 1979). The Framingham study had already deduced the major conventional risk factors for coronary and stroke events, but the ways in which these and other risk factors contributed to global changes in incidence rates were unclear. Particularly still to be resolved were the reasons for the decline in mortality from both coronary heart disease and stroke in many countries, despite the continuing increase in prevalence in the countries of eastern and central Europe and the USA (Uemura et al. 1988).

The MONICA project was conceived in the early 1980s to MONItor the trends and determinants of CArdiovascular disease (Tunstall-Pedoe, 1989; WHO, 1989). In contrast to traditional cross-sectional studies of populations, the MONICA study aimed to longitudinally investigate the dynamic causes of change in incidence and mortality of cardiovascular disease and used a multitude of national research centres. It aimed to examine changes in six possible associations between four factorial categories:

1. Risk factors versus incidence
2. Medical care versus case fatality
3. Incidence versus case fatality
4. Medical care versus incidence
5. Risk factors versus case fatality
6. Medical care versus risk factors.

The study quickly became the largest study of cardiovascular disease in history, with 32 collaborating centres in 21 countries monitoring around 10 million people worldwide.

Details of the MONICA study population, methods, and publications are available on the MONICA manual website: http://www.ktl.fi/monica

In Scotland four MONICA surveys were performed in North Glasgow (1987, 1989, 1992 and 1995) obtaining age (10 year) and sex-stratified samples from GP registers. Previously, the first MONICA survey in Glasgow conducted a preliminary study of whole-blood viscosity, haematocrit, plasma viscosity and fibrinogen (Lowe *et al*, 1988) and the second survey conducted a preliminary study of haematocrit, red cell aggregation, white cell count and fibrinogen (Lowe *et al*, 1992). These studies suggested these rheological variables had associations with conventional cardiovascular risk factors, and with prevalent CVD, but lacked statistical power, partly due to insufficient prevalent CVD in the 25–64-year age range studied. The third Glasgow MONICA survey therefore extended the age range up to age 74 years (1992) and was used to examine the reference ranges and the demographic distributions of haemostatic and inflammatory (IL-6, CRP and fibrinogen) risk markers and their associations with each other and with conventional risk factors (Lowe et al, 1997; Woodward et al 1997, 1999, 2003). In the fourth Glasgow

MONICA survey, conducted in 1995, fibrinogen, plasma viscosity, and CRP were measured (Rumley et al, 2003; Woodward et al, 2003).

Stored plasma samples were available in the laboratory for measurement of newer inflammatory markers, hence it was a good opportunity to measure TNFα and IL-18 and establish their population distributions and associations in a cross-sectional study. Consent to assay new potential cardiovascular risk markers had been obtained at the time of the survey, which was approved by the local research ethics committee. Because stored serum was not available, sCD40L and MMP-9 could not be measured in this study.

4.2.2 Subjects and Methods:

The fourth MONICA Survey in North Glasgow was carried out in 1995 as one of a series of cross-sectional random samples of this community conducted as part of the international MONICA project (World Health Organisation Principal Investigators, 1988). In this survey, 1836 men and women aged 25 – 64 years old were randomly sampled from general practice registers and asked to attend a health screening clinic in a non-fasting state between 09.00 and 16.00 hours, where a blood sample was taken. Information on oral contraceptive use, menopause and hormone-replacement status as well as social class and alcohol use was obtained through the MONICA questionnaire and by interview. Smoking exposure was measured objectively by cotinine levels (a nicotine metabolite) (Woodward et al, 1991).

Subjects were rested for at least 20 minutes before venepuncture by trained nurses. One blood sample was anticoagulated with 0.11M trisodium citrate (9:1 v:v) and one with EDTA (K_2 EDTA 7.2mg) and samples were immediately centrifuged at 2000g for 5 min at room temperature. The middle layer of plasma was removed, aliquoted, and snap-frozen at -70^{o}C until assay. Citrated plasma was used for all analysis in this study.

IL-18, TNFα, IL-6, CRP, and fibrinogen were all assayed as described in Chapter 2. Aliquots used in this study were previously unthawed.

4.2.2 Statistical analysis

All analyses were, where appropriate, sex-specific. The distribution of each inflammatory variable was summarised through five percentiles: the point of central tendency (50^{th} percentile), the 25^{th} and 75^{th} percentiles, which indicate the spread, and the 5% and 95% percentiles, which show the range within which 90% of values lie, not influenced by outliers.

Each of the inflammatory variables included in this study had skewed distributions, and in each case a log transformation was found to induce approximate normality. Consequently, all tests on these variables were carried out after log transforms. These tests arose from comparisons of demographic groups, involving t-tests, tests of Spearman correlation coefficients and tests of trend from age-adjusted general linear models. The latter were used to test associations of inflammatory variables with alcohol drinking, cotinine levels and social deprivation, each defined as a four-grade ordinal variable, coded 1-4. Drinking was the number of units of alcohol drunk in the past week, divided into groups by UK national guidelines and, in subsequent analyses, into four equal parts by the sample distribution; cotinine was also divided into its observed quarters; and the level of social deprivation was defined from the Carstairs and Morris deprivation scores for the address at which the subject lived. Once again, deprivation was coded into its quarters, this time defined as the quarters for the north Glasgow population (Morrison et al, 1997). Further tests on the associations between inflammatory variables and social deprivation adjusted for classical CVD risk factors, and cross-adjusted all those inflammatory variables which had residual independent associations for each other.

4.3 RESULTS

4.3.1 Sample Availability

1836 men and women aged 25 – 64 years old were randomly sampled from general practice registers in North Glasgow in 1995. Due to refusal to consent and failed venepunctures, 1698 (92%) of the 1836 participants in this MONICA-4 survey had available plasma samples. This 92% differed significantly from the remaining 8% in that there were fewer females (52% v 67%; $p=0.0007$). There were no differences ($p>0.05$) in

age, systolic blood pressure, serum total cholesterol, body mass index, cotinine, alcohol drinking or social deprivation between those who gave plasma and those who did not. Due to these samples being residual, there was only a small amount of plasma available in some aliquots, and consequently the number of tests performed for each marker varied from 1136 to 1666 (Table 4.1).

4.3.2 Population Correlations

Table 4.1 shows the inter-correlations between inflammatory variables (adjusted for age) and their associations with age in both men and women. As expected, in general all inflammatory variables correlated strongly with each other. Particularly strong correlations were noted in both sexes for CRP and fibrinogen (male/female r = 0.55/0.47), CRP and IL-6 (M/F r = 0.53/0.47), and fibrinogen and IL-6 (M/F r = 0.35/0.31). These observations are consistent with these markers' respective roles in the acute phase response. Notable exceptions to strong inflammatory marker inter-associations were seen for TNFα and fibrinogen; (M/F r = 0.12/0.10). IL-18 was also more weakly correlated with fibrinogen than other inflammatory markers in both sexes (M/F r = 0.18/0.17). Inflammatory marker associations with age were all strong ($r \geq 0.20$), with the exception of IL-18 (M/F r = 0.16/0.14). There were, hence, no particularly notable sex-specific interactions of inflammatory markers with each other.

Table 4.2 shows the correlations of inflammatory markers with conventional risk markers measured in this study (with the exception of smoking and alcohol consumption, which are subsequently analysed in greater detail). Although none of the inflammatory markers correlated particularly strongly with total cholesterol ($r<0.11$), all showed strong inverse correlations with HDL cholesterol, IL-18 and TNFα being particularly strong in this respect (r= -0.19 and -0.21 respectively). Only IL-6 and CRP demonstrated strong associations with systolic BP (r =0.15). Inflammatory associations with BMI were strong, particularly for CRP and IL-6 (r = 0.31 and 0.21 respectively), and although IL-18 and TNFα correlated significantly, the association with BMI was considerably weaker than for the other inflammatory markers (r =0.10 and 0.13 respectively). Considering age- and sex- adjusted waist size measurement or waist-hip ratio (WHR) as measures of visceral fatness improved associations of IL-18 with "fat mass" compared with BMI (r =0.20 for

WHR) and also marginally improved similar associations of TNFα (r =0.16). In contrast, considering waist size and WHR had little impact on the association of IL-6, CRP or fibrinogen with "fatness" compared with BMI.

4.3.3 Distributions by Age and Sex of Inflammatory Markers

Table 4.3 shows the medians, three quartiles, and 5-95% ranges for inflammatory variables in men and women. Median levels of fibrinogen, CRP, IL-6, IL-18, and TNFα all showed increasing trends with age in both men and women (see Table 4.1 for correlations) across groups as expected. Comparing men and women in age adjusted analysis, IL-18 and TNFα medians were significantly higher in men than in women ($p<0.0001$ for both), but CRP ($p=0.55$), IL-6 ($p=0.33$), and PV ($p=0.85$) are not, while fibrinogen levels were lower in men ($p<0.0001$).

4.3.4 Effects Oral Contraceptive Use, Menopausal Age, and HRT in Women

Table 4.4 shows the effect of current oral contraceptive use on inflammatory markers in young (25-34 years) non-pregnant women still having periods, and Table 4.5 shows the effect of menopause and hormone replacement therapy (HRT) status among women aged 45-54 years. Current oral contraceptive use was associated with a very significant rise in circulating CRP levels, although this was highly specific to CRP and did not extend to any of the other inflammatory markers, including fibrinogen.

Post-menopausal women without HRT had significantly higher levels of IL-18 compared with the pre-menopausal group. Furthermore, women taking HRT had this menopause-associated effect of increased IL-18 levels reversed, so that circulating levels of IL-18 became comparable to the levels seen in the pre-menopause group ($p=0.84$). A similar pattern was seen for TNFα, for which there was a non-significant trend to higher median levels in post-menopausal women, an effect reversed by HRT ($p<0.07$). Menopausal status and HRT use had no effect on IL-6, CRP or fibrinogen.

4.3.5 Associations of Inflammatory Markers with Alcohol

Tables 4.6-4.8 show the effect of exposure to the categorical variables (alcohol, smoking, and social deprivation respectively) on the inflammatory risk markers. In this population 17.3% were teetotallers, 35.8% had never smoked any form of tobacco, and 45.6% were in non-manual employment.

As seen in Table 4.6, significant inflammatory marker associations with alcohol consumption (by groups according to UK guideline limits) were observed after adjustment for age, although many of these interactions were sex-specific (IL-6 p=0.0002; IL-18 p=0.08; TNFα p=0.02; CRP p=0.07; fibrinogen p=0.43). Results from analysis showed increased IL-6 expression with increased alcohol consumption only in men (median IL-6 1.56 versus 2.15 pg/ml in group 1 versus group 4; p for trend <0.0001), while there was a non-significant decreasing trend of IL-6 expression in women. This observation of increasing levels of inflammatory markers in men and decreasing levels in women with greater alcohol consumption was also mirrored for IL-18, TNFα, and CRP, although none of these trends reached significance (trends for IL-18 in men, TNFα in women and CRP in women were all borderline significant). Decreasing fibrinogen levels with increasing alcohol consumption was also seen in women (median fibrinogen 3.10 versus 2.96 g/l in group 1 versus group 4; p for trend 0.008) although the effect was nonlinear, and no significant trends were seen in men.

4.3.6 Associations of Inflammatory Markers with Smoking

Associations of smoking exposure, as measured objectively by the biochemical marker cotinine, with inflammatory markers are illustrated in Table 4.7. In this analysis there were fewer sex-specific differences (IL-6 p=0.05; CRP p=0.03; fibrinogen p=0.07) in terms of inflammatory marker association with cotinine levels, and IL-6, IL-18, and fibrinogen all had highly significant age-independent associations with cotinine levels both in men and women (p for trend <0.0002 for all). CRP had highly significant associations with cotinine levels in men (p for trend <0.0001), although the trend was slightly less strong, but still statistically significant in women (p for trend 0.04). There

were no strong associations between TNFα levels and cotinine in either sex after age adjustment, although there was a non-significant increasing trend in men.

4.3.7 Associations of Inflammatory Markers with Social Deprivation

As seen in Table 4.8, increasing social deprivation was significantly associated with increases in most of the inflammatory risk makers measured, and only CRP demonstrated a borderline sex-specific interaction after age adjustment (p=0.09). IL-6, IL-18 and TNFα all increased significantly in both men and in women with increasing deprivation ($p \leq 0.05$), while both sexes showed generally weaker increasing trends for fibrinogen. For CRP there was a linear increasing trend only in women (p=0.0004), while in men there was a non-linear non-significant increasing trend.

The association between inflammatory variables and social deprivation may be confounded by the relationship of inflammatory markers with sex, age, smoking, blood pressure, BMI, and cholesterol measurements since these variables (among others) are related to social deprivation. We therefore corrected the associations between the inflammatory variables and social deprivation for these potential confounders. This represents correction for all the ASSIGN cardiovascular risk markers (with the exception of social deprivation itself). In this analysis, the only inflammatory variables to show trends of association across quarters of social deprivation were 3 of the cytokines *in men only*; IL-6 (p for trend =0.004), IL-18 (p for trend =0.003), and TNFα (p for trend =0.008). After additional adjustment to correct for each other, these three cytokines remained independently associated with social deprivation in men (p for trend = 0.05, 0.01, 0.04 respectively). Only IL-18 had a borderline association with social deprivation in women in the fully adjusted model (p for trend across quarters= 0.07).

Table 4.1 Spearman correlations of inflammatory factors with age and correlations between inflammatory factors adjusted for age (**bold** p<0.0001; *italic* p>0.05; else 0.0001<p<0.05).

		IL-18	TNFα	CRP	Fibrinogen	Age
IL-6	M	**0.23**	**0.33**	**0.53**	**0.35**	**0.43**
	F	**0.19**	**0.24**	**0.47**	**0.31**	**0.35**
IL-18	M		**0.19**	**0.23**	**0.18**	**0.16**
	F		**0.22**	**0.20**	**0.17**	**0.14**
TNFα	M			**0.27**	0.12	**0.21**
	F			**0.24**	0.10	**0.28**
CRP	M				**0.55**	**0.27**
	F				**0.47**	**0.18**
Fibrinogen	M					**0.30**
	F					**0.22**

M: males; F: females

Table 4.2 Spearman correlations of inflammatory factors other with conventional risk markers (age- and sex-adjusted) (**bold** p<0.0001; *italic* p>0.05; else 0.0001<p<0.05).

	Total cholesterol	HDL Cholesterol	Systolic BP	BMI	Waist Size	WHR
IL-6	*0.03*	**-0.18**	**0.15**	**0.21**	**0.23**	**0.23**
IL-18	0.06	**-0.19**	0.07	**0.10**	**0.15**	**0.20**
TNFα	*0.05*	**-0.21**	*0.03*	**0.13**	**0.13**	**0.16**
CRP	0.11	**-0.18**	**0.15**	**0.31**	**0.32**	**0.30**
Fibrinogen	**0.10**	**-0.15**	*0.03*	**0.15**	**0.17**	**0.16**

WHR, Waist-hip ratio; BMI, body mass index

Table 4.3 Age-specific reference ranges for inflammatory factors. Values shown are median (first quartile, third quartile) and 5%-95% range.

		Age (years)			
	n	25-34	35-44	45-54	55-64
IL-6 (pg/ml)					
M	698	1.28 (0.95, 1.97)	1.52 (1.12, 2.27)	1.98 (1.40, 2.76)	2.63 (1.92, 3.81)
		0.64-3.60	0.71-4.78	0.79-6.47	1.18-9.39
F	739	1.32 (0.89, 2.11)	1.41 (1.03, 2.03)	1.87 (1.37, 2.89)	2.43 (1.49, 4.26)
		0.57-4.52	0.66-3.82	0.82-7.89	1.00-9.85
IL-18 (pg/ml)					
M	715	205 (163, 246)	254 (189, 323)	251 (191, 332)	251 (191, 340)
		122-407	128-460	132-443	132-469
F	769	187 (144, 247)	197 (141, 262)	213 (172, 283)	228 (186, 303)
		95-435	94-429	116-461	112-418
TNFα (pg/ml)					
M	715	1.49 (1.30, 1.86)	1.60 (1.31, 2.00)	1.65 (1.35, 2.11)	1.90 (1.50, 2.35)
		1.05-2.64	1.00-3.00	1.03-2.91	1.10-4.26
F	769	1.38 (1.12, 1.69)	1.39 (1.12, 1.75)	1.63 (1.19, 2.05)	1.83 (1.51, 2.17)
		0.79-2.77	0.80-2.54	0.87-2.05	1.06-3.40
CRP (mg/L)					
M	538	0.88 (0.40, 1.89)	1.05 (0.59, 2.78)	1.32 (0.77, 2.87)	1.96 (0.99, 4.47)
		0.25-5.82	0.21-7.90	0.30-6.90	0.40-10.59
F	598	1.29 (0.55, 2.94)	0.94 (0.49, 2.79)	1.58 (0.67, 3.32)	2.35 (0.98, 5.02)
		0.17-7.75	0.22-7.47	0.22-8.83	0.36-14.30
Fibrinogen (g/L)					
M	799	2.54 (2.29, 2.96)	2.67 (2.28, 3.11)	2.90 (2.40, 3.57)	3.11 (2.57, 3.92)
		1.83-3.71	1.96-4.30	2.00-4.77	2.10-5.54
F	862	2.87 (2.47, 3.49)	2.96 (2.50, 3.62)	3.02 (2.59, 3.67)	3.43 (2.84, 4.25)
		1.93-4.48	2.08-4.48	2.13-4.92	2.24-6.05

M: males; F: females

Table 4.4 Inflammatory factors in non-pregnant women aged 25-34 years who are still having periods, by current use or non-use of oral contraceptives. Values shown are median (first quartile, third quartile) and 5%-95% range. p-values are after log transformation.

	Current OC use		
	Yes (n=85)	No (n=124)	p-value
IL-6 (pg/ml)	1.13 (0.78, 2.00) 0.48-7.43	1.33 (0.91, 2.07) 0.61-3.43	0.71
IL-18 (pg/ml)	180 (145, 247) 106-566	190 (143, 246) 95-369	0.57
TNFα (pg/ml)	1.39 (1.01, 1.74) 0.81-2.78	1.37 (1.15, 1.66) 0.78-2.71	0.66
CRP (mg/L)	1.78 (0.80, 4.62) 0.20-10.0	0.84 (0.40, 1.71) 0.17-4.50	0.0001
Fibrinogen (g/L)	2.99 (2.52, 3.54) 1.88-4.36	2.75 (2.43, 3.32) 1.84-4.43	0.27

OC, oral contraceptives

Table 4.5 Inflammatory factors in non-pregnant women aged 45-54 years, by menopausal status and use of HRT. Values shown are median (first quartile, third quartile) and 5%-95% range. p values are after log transformation.

	Pre-menopausal (1) (periods, no HRT) (n=86)	p value (1 v 2)	Post-menopausal (2) (no periods, no HRT) (n=81)	p value (2 v 3)	All HRT (3) (n=42)
IL-6 (pg/ml)	1.70 (1.25, 2.76) 0.68-7.77	0.13	2.10 (1.49, 3.34) 1.01-7.88	0.11	1.70 (1.30, 2.40) 0.79-7.89
IL-18 (pg/ml)	188 (164, 238) 116-480	0.0002	268 (213, 361) 140-461	0.006	195 (144, 267) 105-533
TNFα (pg/ml)	1.59 (1.18, 2.00) 0.86-3.40	0.51	1.76 (1.36, 2.19) 0.71-3.91	0.07	1.35 (1.10, 1.86) 0.87-2.70
CRP (mg/L)	1.10 (0.46, 3.08) 0.21-8.85	0.27	1.27 (0.71, 3.24) 0.28-7.68	0.11	2.09 (1.23, 5.45) 0.43-8.83
Fibrinogen (g/L)	3.05 (2.49, 3.75) 1.92-4.92	0.30	2.93 (2.60, 3.86) 2.34-5.84	0.41	3.12 (2.81, 3.49) 2.14-4.23

HRT, hormone replacement therapy

Table 4.6 Medians of inflammatory markers in groups according to alcohol consumption.

		Group 1 (n=)	Group 2 (n=)	Group 3 (n=)	Group 4 (n=)	p-value for trend	p-value group 1 vs group 4
IL-6 (pg/ml)	M	1.56	1.70	1.69	2.15	**<0.0001**	**0.0002**
	F	1.78	1.63	1.51	1.51	0.12	0.12
IL-18 (pg/ml)	M	230	230	239	250	0.07	0.06
	F	217	196	205	207	0.46	0.21
TNFα (pg/ml)	M	1.58	1.56	1.65	1.74	0.17	0.37
	F	1.59	1.58	1.5	1.36	0.08	0.10
CRP (mg/L)	M	1.16	1.03	1.28	1.21	0.41	0.56
	F	1.43	1.71	1.16	1.19	0.09	0.18
Fibrinogen (g/L)	M	2.76	2.85	2.76	2.78	0.12	0.48
	F	3.10	3.34	2.9	2.96	**0.008**	0.13

Groups are defined by alcohol consumption by quarters of the population (Quarters are, M: 1 = 0-7; 2 = 8-17; 3 = 18-32; 4 = 33+, F: 1 = 0; 2 = 1-4; 3 = 5-11; 4 = 12+ units per week.)
p-values after log-transformations. Male and female p-trends after age-adjustment.

Table 4.7 Medians of inflammatory markers in groups according to smoking exposure (cotinine levels).

		Group 1 (n=504)	Group 2 (n=533)	Group 3 (n=412)	Group 4 (n=386)	% increase in median; group1 vs group 4	p-value for trend
IL-6 (pg/ml)	M	1.47	1.88	2.09	2.01	36.7	**<0.0001**
	F	1.52	1.55	1.58	1.86	22.4	**0.0002**
IL-18 (pg/ml)	M	207	242	228	265	28.0	**<0.0001**
	F	191	194	207	246	28.8	**<0.0001**
TNFα (pg/ml)	M	1.53	1.71	1.69	1.62	5.9	0.06
	F	1.49	1.52	1.53	1.62	8.7	0.17
CRP (mg/L)	M	1.02	1.00	1.39	1.88	84.3	**<0.0001**
	F	1.21	1.17	1.53	1.84	52.1	**0.04**
Fibrinogen (g/L)	M	2.67	2.64	2.84	3.11	16.5	**<0.0001**
	F	3.02	2.90	3.07	3.30	9.3	**<0.0001**

Groups are defined by levels of cotinine in quarters according to the population distribution of cotinine. p-values after log-transformations. Male and female p-trends after age-adjustment

Table 4.8 Medians of inflammatory markers in groups according to social deprivation.

		Group 1 (n=504)	Group 2 (n=533)	Group 3 (n=412)	Group 4 (n=386)	% increase in median; group1 vs group 4	p-value for trend	p-trend after full adjustment*
IL-6 (pg/ml)	M	1.61	1.79	2.05	1.88	16.8	**<0.0001**	**0.004**
	F	1.47	1.60	1.81	1.72	17.0	**0.05**	0.74
IL-18 (pg/ml)	M	219	223	245	262	19.6	**<0.0001**	**0.003**
	F	191	215	212	222	16.2	**0.0001**	0.06
TNFα (pg/ml)	M	1.52	1.65	1.68	1.74	14.5	**0.002**	**0.008**
	F	1.41	1.56	1.55	1.63	15.6	**0.01**	0.09
CRP (mg/L)	M	1.12	1.27	1.54	1.31	17.0	**0.19**	0.85
	F	1.11	1.35	1.70	1.85	67.0	**0.0004**	0.15
Fibrinogen (g/L)	M	2.66	2.84	2.89	2.87	7.9	0.06	0.95
	F	2.94	3.15	3.05	3.18	8.2	**0.02**	0.81

Groups are defined by Carstairs social deprivation scale in quarters; increasing group number implies increased deprivation.

p-values after log-transformations. Male and female p-trends after age-adjustment.

* Smoking, blood pressure, BMI, and HDL cholesterol, and total cholesterol

4.1 DISCUSSION

4.4.1 Population Distribution

No previous data in the literature has thus far established similar distribution ranges to those illustrated here for IL-18 or TNFα. In the absence of other published studies for comparison these data may be potentially useful as reference ranges. In this regard there are several positive and negative aspects of this study to take into account. The study was performed in a large random sample of the local population thought to be representative of the general population in the West of Scotland, it included a wide age range (25-64 years) of roughly equal numbers of men and women, and there was little in the way of selection criteria (subjects had to be registered with a GP). In addition to this, all markers measured were assayed with reagents calibrated against international standards (by the manufacturers) with the exception of IL-18, for which there is no recognised standard. The West of Scotland region is an area of relatively high cardiovascular risk (Scottish Health Survey, 2003 [Cardiovascular disease report]).

There were discrepancies with previous MONICA survey results, for fibrinogen, CRP and IL-6. CRP and fibrinogen levels in MONICA-4 were much higher in each age group (~10%) than in the MONICA-3 survey (Lowe et al, 1997, Woodward et al, 1997 & 2003). IL-6 levels reported here are also much higher than those previously reported in similar age groups (~2-fold) (Woodward et al, 1999) although the ~2-fold increase in circulating levels between age extreme groups in both sexes in this study is a consistent observation (Woodward et al, 1999). This change is probably due to change in the available IL-6 kit technologies (the new high-sensitivity kit) and in the international calibrants for these assays, rather than a genuine increase in circulating levels of these markers in the population over the last few years (3 years since MONICA-3), but these findings require further investigation.

4.4.2 Age Associations

In general, all markers of inflammation had positive associations with age, in agreement with previous MONICA-3 findings for IL-6, CRP and fibrinogen (Lowe et al, 1997,

Woodward et al, 1999, 2003) and findings elsewhere (Gimeno et al 2007; Miles et al, 2006; Fibrinogen Studies Collaboration, 2005). Recent cross sectional data from 1111 men and women (aged 27-77) in the Perth Carotid Ultrasound Disease Study (CUDAS) has shown that, similar to the present study, the Spearman association between IL-18 and age was weaker (r^2=0.10) than IL-6 or CRP (0.20 and 0.22 respectively) (Hung et al, 2005), a result also seen in the MONICA/KORA study (Thorland et al, 2005). It appears, therefore, that IL-18 levels in the general population may not be confounded by age to the same extent as other inflammatory markers. TNFα has long been known to increase with age in both mice and man (Belmin et al, 1995; Paolisso et al, 1998) and results presented here reflect this, TNFα being generally associated with age to a similar extent as CRP and fibrinogen.

4.4.3 Sex

Of the markers studied, only IL-18 and TNFα were significantly higher in men than in women. This difference in IL-18 between the sexes has been commented on in the CUDAS study (Chapmen et al, 2006), and the result is also confirmed in smaller studies (Yamagami et al, 2005). For TNFα there is the possibility that metabolic differences in male and female fat storage may contribute to sex-specific differences in circulating TNFα levels in the general population (see 4.4.4). The MONICA/KORA study group (Thorland et al, 2006) observed in a study of similar size to the present one that while CRP levels showed no sex-specific interactions, fibrinogen was lower and IL-6 was higher in men. Indeed, the present study showed non-significant trends towards confirming the association of IL-6 with male gender, and also found fibrinogen to be higher in women, which is confirmed in meta-analysis (Fibrinogen Studies Collaboration, 2005).

4.4.4 Metabolic Syndrome Components

IL-18 and TNFα have been suggested to be strongly associated with aspects of the metabolic syndrome in population studies (Hotamisligil et al, 1995; Kern et al, 1995; Jovinge et al, 1998; Ziccardi et al, 2002; Hotamisligil et al 2006). IL-18 was found to correlate with BMI, waist circumference, triglycerides, HDL cholesterol (inversely), and

fasting glucose and insulin levels in the CUDAS study (Hung et al, 2005), Some recent studies (Villarrasa et al, 2006; Herder et al, 2006) have suggested that IL-18 may not be associated with fat mass, however it does seem to be associated with type II diabetes and the metabolic syndrome (Thorland et al, 2005 & 2006; Hung et al, 2005) as does TNFα (Hotamisligil et al, 1995 & 2006). It is interesting to speculate that higher levels of these cytokines in men may reflect the generally higher prevalence of metabolic syndrome in UK men than in women (Tillin et al, 2005), although epidemiological studies cannot infer causality on the basis of an association.

The present study of a general population shows that IL-18 and TNFα are strongly associated inversely with HDL cholesterol levels, but are less strongly associated with BMI than other inflammatory markers. The latter observation is interesting, since both cytokines are thought to be produced in visceral adipose tissue (perhaps by infiltrating macrophages) and are often described as adipokines (Hotamisligil, 2006). Indeed, TNFα was described *in vitro* as the archetypal adipokine (Hotamisligil et al, 1993). The present discrepant observation may be explained by the often debated potentially "weak" association between BMI and visceral fat mass ([3]Wannamethee et al, 2005), but strong associations of IL-6 and CRP with BMI may conflict with this argument. However, when the study considered WHR instead of BMI as a marker of central adiposity, the associations of both IL-18, and to a lesser extent TNFα (although not IL-6 or CRP), with fatness improved. This is in line with previous observations (Hung et al, 2005; Thorland et al, 2005). It is likely, according to previous studies, that TNFα is associated relatively more strongly with subcutaneous fat in women than in men (and hence overall BMI) whereas in men TNFα is produced from visceral and subcutaneous fat in equal amounts (Hube et al, 1999; Orel et al, 2004).

Combined, these results may suggest that although inflammation in general is associated with relative body mass (i.e. muscle plus fat mass; BMI), some adipokines (e.g. IL-18) are more strongly associated with visceral fat (but perhaps only in a sex-dependant manner). TNFα is the prototypic pleiotropic cytokine, and is produced almost ubiquitously, even by muscle cells (Saghizadeh et al, 1996) which are often considered to be the metabolic antithesis of fat mass. It is hence possible that the association of TNFα with adipose tissue has been somewhat overestimated in some studies in terms of impact

on systemic concentrations of the cytokine. This speculation does not preclude a role for either marker in metabolic syndrome since they may be associated with other crucial factors such as insulin resistance. Whether low localised expression levels of TNFα and perhaps other cytokines are sufficient for biologically important cross talk between adipocytes and leukocytes without significant effect on circulating cytokine concentrations (Skurk et al, 2005), requires verification.

4.4.5 Female Hormones

Consistent with previous findings both in MONICA-3 and elsewhere (Woodward et al, 2003; Frohlich et al, 1999), we observed that modern oral contraceptives have a very specific effect of increasing circulating CRP levels ~2 fold in users aged 25-34 years, whilst not having any effect on fibrinogen, IL-6 or other inflammatory markers. The physiological mechanism for this is not clear. Modern oral contraceptives carry much lower hormone doses than previously, however it is still recommended that older pre-menopausal women with other cardiovascular risk factors avoid oral contraceptives due to slight excess risk of CVD (Vandenbroucke et al, 1998). The excess risk is found with haemostatic, as well as inflammatory, markers (Lowe et al, 1997, 2004) and hence cannot be solely attributed to inflammatory effects.

Among women aged 45-54 years, being post-menopausal had the specific effect of increasing IL-18 levels, while taking HRT abrogated this effect. As far as we are aware this is a novel epidemiological finding, although one recent study reports oestrogen steroids downregulate endometrial expression of IL-18 (Ledee et al, 2006). These observations may suggest that natural levels of oestrogen and progesterone during child-bearing pre-menopausal years attenuate the inflammatory environment of the endometrium, and possibly at other biological sites, and that these effects may be achieved exogenous dosages of oestrogen post-menopause. There is debate as to the requirements of the relative Th1/Th2 balance in the endometrium for successful reproduction (Chaouat et al, 2004), but IL-18 clearly has the potential to influence this balance via IFNγ, and the evidence in this report and in animals suggests oestro-progestative hormones may directly influence IL-18 expression. In addition to this effect on IL-18, there may be a borderline inhibitory effect of HRT on TNFα expression, but

valididity of the observation is hampered by small numbers of post-menopausal women in this cross-sectional study. Supporting this observation however, it has previously been noted *in vivo* that TNFα levels in the human endometrium fall in the late secretory phase of the menstrual cycle, and withdrawal of oestrogen stimulation leads to a rise in TNFα expression (Tabibzadeh et al, 1999). There is, hence, a plausible biological pathway by which hormone replacement in post-menopausal women may dampen down expression of both IL-18 and TNFα. It is hence clear that both the reproductive cycle and the age of women may have implications for circulating levels of both IL-18 and TNFα in epidemiological terms, although to an extent age and sex adjustment in multiple regression analysis will correct for a proportion of this error during epidemiological studies. If there were ever to be a case for IL-18 or TNFα being used clinically as any sort of biomarker, this subject would require more detailed analyses.

4.4.6 Alcohol

It is thought that suppression of inflammation may partly explain the cardiovascular benefits of moderate alcohol consumption. Associations between alcohol consumption and inflammatory markers have been previously reported (Lee et al, 1990; Krobot et al, 1992; Woodward et al, 1997; Imhov et al, 2001; Woodward et al, 2003; Mennan et al, 1999; Mukamal et al, 2004; Volpato et al, 2004; Levitan et al, 2005; Pai et al, 2006). The effect of alcohol consumption on inflammatory markers is open to a number of subjective biases in terms of how data is analysed, including how the population is divided based on drinking habits, and how those drinking habits are reported (although self-reporting may be the most effective method; Miadnik, 1988). Many studies examining both men and women report J-shaped dose response curve (Krobot et al, 1992; Woodward et al, 1997; Imhov et al, 2001; Volpato et al, 2004; Pai et al, 2006), however, by dividing the population into quarters there is a gain in statistical power over the range of alcohol intakes (as opposed to small numbers of observations in very low and very high alcohol intake groups), and by examining men and women separately there is evidence of sex-specific interactions in this study.

The finding of an age-independent reduction of circulating fibrinogen levels with increasing alcohol consumption in women only is consistent with previous findings (Lee

et al, 1990; Mennan et al, 1999) as are observations of inverse associations of CRP with alcohol consumption in women (Levitan et al, 2005). It may still be true that those with the highest alcohol consumption have increased fibrinogen levels, but such observations depend on cut-off points used for observation at the extremes of drinking habits (which may require a large study to gain adequate power), and adjustment for potential confounders in the population (Mennen et al, 1999; Volpato et al, 2004; Pai et al, 2006).

This cross-sectional study found that most inflammatory markers were differently associated with alcohol consumption in men and women when the data was viewed not adjusting for smoking or other potential confounders (apart from age). All cytokines followed the broad pattern of increasing trends among men, and decreasing trends among women (comparing extreme groups), although few of these patterns attained significance. One diet study has reported that moderate alcohol consumption reduces CRP and fibrinogen levels in men and in women (Sierksma et al, 2002), although all the participants were healthy non-smokers and numbers were small.

The results reported here confirm a previous study (Volpato et al, 2004) showing that alcohol intake has superficially minimal effect on circulating TNFα levels in either sex. Biological mechanisms supporting these observations have not been reported. Indeed, p55 TNFR is required for alcohol induced liver injury (Yin et al, 1999), as is biological TNFα action (Ponnappa et al, 2005), and one would hypothesise therefore that TNFα may be an important biological determinant of downstream physiological consequences of alcohol intake. Although TNFα may be required for such pathophysiological processes, it may still be the case that circulating levels of the cytokine are relatively unaffected by alcohol intake, and that hepatocytes (and other cellular systems) are primed to react pathophysiologically to it by separate signalling processes downstream of alcohol consumption.

4.4.7 Smoking

Inflammatory markers are known to be associated with lifetime smoking status (Woodward et al, 1997 & 2003; [2]Wannamethee et al, 2005), and results here reflect this, showing that in particular CRP levels are strongly positively associated with current

levels of cotinine in individuals, despite previous results suggesting increases in CRP levels are independent of the amount actually smoked (Ohsawa et al, 2005). This may be a facet of using cotinine as an objective biomarker. In the case of TNFα, a borderline positive association was also observed, but this was somewhat weaker than CRP, IL-6 or IL-18 due to plateau of the curve between group 2 and group 4 in both sexes, which is consistent with *in vitro* observations that high levels of nicotine may inhibit mononuclear cell TNFα production (Madrestma et al, 1996). IL-18 showed increases in expression comparable to IL-6 across quarters with a highly significant trend ($p<0.0001$). As far as I am aware this is the first time a relationship between IL-18 and cotinine (or similar smoking exposure measurements) has been observed in a large cross-sectional study.

4.4.8 Social Deprivation

Univariate analysis demonstrated that levels of all inflammatory markers were very strongly positively associated with social deprivation. These findings are in broad agreement with the published literature. In the Whitehall study of British civil servants, fibrinogen levels were higher among women and men with indicators of deprived childhood socioeconomic circumstances (fathers in manual jobs or short physical stature), participated in less leisure-time activity, had lower job grades as adults and lower levels of job control; all demographic indicators of lifetime social deprivation (Brunner et al, 1996). In the Scottish Heart Health Study, fibrinogen was the strongest "non-classical" risk predictor in explaining social influence (as indicated by housing tenure) on CHD in men (although the relationship was weaker in women) ([2]Woodward et al, 2003). Likewise for other inflammatory markers, recent studies have shown that IL-6 and TNFα are also biomarkers of lifetime socioeconomic status (education, income, and ownership of financial assets; Koster et al, 2006; Loukes et al, 2006).

A recent study has shown that fibrinogen and CRP are associated with lifetime socioeconomic status in 3745 women. When using socioeconomic status, as well as other risk markers, to adjust associations of fibrinogen with incident CHD events, this attenuates the CHD risk associated with a 1g/L increase in fibrinogen from 1.29 (1.12, 1.49) to 1.09 (0.93, 1.28) (Lawlor et al, 2005). In contrast, smoking was a risk factor for CHD (OR for ever smoking 2.27 [1.43, 3.58]), but adjusting associations with risk for

lifetime socioeconomic status and other conventional risk factors did not abrogate the risk association with CHD (1.63 [1.12, 2.36]). This suggests that smoking is far more likely to be a causal factor in CHD than fibrinogen and CRP, which may have confounding associations with socioeconomic status (Lawlor et al, 2005).

Although socioeconomic markers may confound the relationship of inflammatory markers with CHD this does not mean the association with inflammatory markers is unimportant; it may still represent a plausible biological pathway by which deprivation causes CHD. Contrary to this argument, recent data from older men (n=2682) in the British Regional Heart Study has shown that CRP, fibrinogen and IL-6 are associated with social class, but that these relationships are attenuated to null after adjusting for smoking, alcohol consumption, physical activity and BMI ([2]Ramsey et al, 2007). This would suggest that behavioural CVD risk factors are responsible for a confounded association between deprivation and inflammation, and argues against social deprivation *per se* resulting in elevated inflammatory markers.

The relationship between inflammatory markers and socioeconomic status/social deprivation is hence an important issue to explore, particularly in Scotland, where the new ASSIGN score (Woodeard et al, 2007) incorporates social deprivation (by postcode) as a marker of CVD risk due to the fact that conventional risk markers do not fully explain the greater cardiovascular risk experienced among the more socially deprived (Brunner et al, 1996; Tunstall Pedoe et al, 2006; [1]Ramsay et al, 2007). In the present study, after adjustment for conventional risk markers, which may confound associations with social deprivation (age, gender, smoking, blood pressure, BMI, total cholesterol, HDL-cholesterol – all ASSIGN risk markers), we found that IL-6, IL-18 and TNFα retained significant associations with social deprivation only in men across quarters of the population by Carstairs scores (p for trend <0.008 for all). IL-18 and TNFα had only borderline association with deprivation in women (p<0.09). Further cross adjustment of each of the three markers for each other left each independently associated with deprivation across quarters in men, with IL-18 most strongly associated (p for trend <0.01). The results suggest that fibrinogen and CRP are weakly (if at all) associated with Carstairs score in this population after only age and sex adjustment, and not at all after adjustment for other risk factors. This may reflect that the Carstairs score is a more

"immediate" reflection of social deprivation rather than lifetime socioeconomic status measured elsewhere (e.g. British Women's Heart and Health Study; Lawlor et al, 2005). Although lifetime socioeconomic status is probably, by definition, a better marker of long-term exposure to social CHD risk factors than Carstairs, the Carstairs score is a more accurate reflection of the postcode measurement used in the ASSIGN score (i.e. "current social class"), and is hence better placed to assess associations with relevance to the Scottish system. It is hence possible that lifetime socioeconomic status and immediate social deprivation scores are associated with different risk factors to different extents. Despite this, our observations are consistent with other observations that IL-6, IL-18, and TNFα are associated with lifetime education, income, and ownership of financial assets (Koster et al, 2006; Loukes et al, 2006) independently of other risk markers. This may suggest that IL-18, TNFα, and IL-6 are more strongly related to "current" deprivation than fibrinogen and CRP.

In this study, the discrepancy between socioeconomic status in men and women may reflect the long standing epidemiological problem of accurately measuring a women's social class (without using a lifetime questionnaire), since female socioeconomic position may (traditionally) vary over the course of her life to a greater extent than male; the social class a women marries into is often her reported social class in these studies and does not necessarily reflect lifetime social class. This is particularly true of the Carstairs score, which includes measurements of local male unemployment, households with no car, overcrowding (over 1 person per room), and head of household's social class categories IV and V. It is perhaps the case, then, that a more accurate picture of inflammatory associations with deprivation would be gleaned in females with a lifetime socioeconomic questionnaire. Indeed, this may be relevant to in the borderline associations of IL-18 and TNFα in the fully adjusted models in women, suggesting the difference between men and women in this respect is due to inability to measure deprivation as accurately in women as in men.

4.4.9 Conclusions

Inflammatory markers, in general, showed associations with CHD risk factors (age, sex, smoking, alcohol, social deprivation, metabolic syndrome, use of hormones). Although

IL-18 and TNFα do not show associations with BMI that would generally be expected of adipokines, the association with "fatness" was marginally improved by considering WHR instead of BMI. Inflammatory markers in general, including IL-18 and TNFα, demonstrated strong positive associations with smoking in this study. Increasing alcohol consumption was associated with divergent effects in the sexes, generally resulting in increased levels in men and decreased levels in women after age-adjustment although the trends were only strongly significant for IL-6 in men and for fibrinogen in women. All inflammatory markers had highly significant, but confounded, associations with social deprivation, which remained significant for IL-18 and TNFα and IL-6 (in men only) after full adjustment for the ASSIGN risk markers. This study suggests that IL-18 and TNFα are likely to have interesting and potentially informative associations with CHD risk in the general population, at the very least by proximal relationships with other cardiovascular risk markers, and these associations require further study.

The next 4 chapters report associations of IL-18, TNFα, sCD40L and MMP-9 with CHD risk.

CHAPTER 5

ASSOCIATIONS OF IL-18 AND TNFα WITH PREVALENT MYOCARDIAL INFARCTION: GLASGOW MYOCARDIAL INFARCTION STUDY (GLAMIS)

5.1 INTRODUCTION

5.1.1 Prevalent Case-Control Studies

When investigating the associations of new potential risk markers with CVD, case-control studies of prevalent CHD are a quick and relatively inexpensive useful first step, prior to assessment of the markers in prospective studies. Prevalent case-control studies are generally seen as far weaker in terms of proof of a statistical association of a biomarker with an event than prospective studies are. This is due to the various sources of bias additional confounding effects (such as reverse causality) that they entail. (Woodward, 2005).

The first use of the Glasgow ECTIM (Etude Cas-Témoin de l'Infarctus du Myocarde) study was to examine socioeconomic associations with prevalent MI in Glasgow, socioeconomic position being relatively unaffected by reverse causality bias. The study found, importantly, that social deprivation not only vastly increased likelihood of experiencing an event, but also lessened chance of survival at, and beyond, admission (Morrison et al, 1997). Using the Carstairs deprivation score, MI rate increased 1.7-fold in men and 2.4-fold in women from the least (Q1) to the most (Q4) deprived socioeconomic quarter (Morrison et al, 1997).

GLAMIS was used to assess the relative associations of the fibrinogen assays, CRP and IL-6 with CHD (Lowe et al, 2003). In a separate study, GLAMIS also demonstrated associations of homocysteine with prevalent MI, independent of conventional and inflammatory risk markers (Woodward et al, 2006).

The availability of stored plasma in this study provided the opportunity to investigate the associations of TNFα and IL-18 with other risk markers and with prevalent CHD in the

population. As there were no serum samples available in the Glasgow MONICA study, MMP-9 and sCD40L were not measured here.

5.1.2 Previously Published data on associations of IL-18 and TNFα with Prevalent Cardiovascular Disease

TNFα correlates well with the ankle-brachial index (ABI) (Bruunsgaard et al, 2000 & Skoog et al, 2002), an established marker of underlying atherosclerosis (Fowkes et al, 2006) and a CVD risk marker (Heald et al, 2006). Associations of TNFα with the degree of atherosclerosis in imaging studies have been weak. Several studies have suggested that TNFα is not associated with carotid IMT or maximal plaque thickness (MCPT) in a cross section of stroke-free people (Elkind et al, 2002 & 2005) or angiographic CAD degree of stenosis in people with prevalent CHD (Sukhija et al, 2007). Strong correlations between ABI and IMT have been reported (McDermott et al, 2005). The reasons for these discrepant results in studies of TNFα are not clear. Several studies have reported that in patients with prevalent CVD, IL-18 levels are correlated with the extent of CAD, as measured by degree of stenosis, number of affected vessels, and to some extent the coronary plaque area (Yamashita et al, 2003; Yamaoko et al, 2003; Suchanek et al, 2005). However, a larger study (n=1111) recently suggested that only the univariate association was significant (Chapman et al, 2006). In addition, a recent study (Espinola-Klein et al, 2007) found in 720 patients that IL-18 levels were not different in patients with no significant CAD, those with CAD, or those with clinically relevant multi-vessel disease. Although the latter study, in categorizing patients will lose a lot of power by categorizing (instead of measuring CAD as a continuous variable), any possible association of IL-18 with CAD (Yamashita et al, 2003; Yamaoko et al, 2003; Suchanek et al, 2005) would seem likely to be small overall.

Given these results, one would, therefore, expect significantly higher levels of both markers in patients with recent previous MI (since they would have greater prevalence of diffuse atherosclerosis and CAD). In line with this hypothesis, previous studies have suggested that circulating TNFα and IL-18 are increased in patient populations with previous MI (Jovinge et al, 1998; Bennett et al, 2006; Hulthe et al, 2006).

The first study to show this for TNFα examined young (<45 years) male postinfarct patients, but only described univariate differences between cases (n=153) and age-matched controls (n=63); 4.1pg/ml (±1.6) vs 2.5pg/ml (±0.4) $p < 0.0001$ (Jovinge et al, 1998). Another study (n cases = 1213) recently reported associations, this time in terms of odds ratios of MI risk in the top versus bottom quartile, but did not adjust for confounders; OR [95% CI]: 1.7 [1.1; 2.6] n cases=1213 (Bennet et al, 2006).

Univariate comparisons between retrospective cases (n=387) and controls (n=387) have also been made for IL-18; 309.6pg/ml (±138.6) vs 285.4pg/ml (±115.7) $p<0.01$ (Hulthe et al, 2006). Due to a lack of studies examining both inflammatory markers as well as classical and other inflammatory risk factors in the same cohort, data directly comparing the associations with prevalent CHD and the extent of confounding by other markers comparing IL-18 and TNFα are not known.

5.1.3 Aims

Hypothesising that IL-18 and TNFα levels would be higher in the survivors of MI than age-matched controls, the aim was to directly compare IL-18 and TNFα and their relationships with retrospective risk of MI in a case-control study, as well as with classical and inflammatory risk factors.

5.2 METHODS

5.2.1 Patient Recruitment

It was noted during the MONICA study that the incidence of CHD in middle-aged men is more than three times higher in Northern Ireland than in Northern France. The ECTIM (Etude Cas-Témoin de l'Infarctus du Myocarde) study was set up to investigate the demographic, clinical, genetic and biomarker differences between these populations in an attempt to explain this striking discrepancy (Moreel et al, 1992; Parra et al, 1992). Participants in the MONICA trials in Belfast, Toulouse, Lille, or Strasbourg who, according to MONICA databases, survived MI after 1992 were contacted and asked to

participate in the retrospective ECTIM case-control study (Parra et al, 1992). A high prevalence of CVD events were also (as expected) found in Glasgow in the MONICA study, and the Glasgow ECTIM study was subsequently set up as an extension of ECTIM. The Glasgow MONICA coronary event register contains all cases of MI and coronary death occurring in men and women aged 25-64 years resident in North Glasgow from the 1980s. Individuals surviving an MI event after July 1994 were asked to participate, and consequently a prevalent case-control study was set up. Although originally set up to overlap the ECTIM study of genetic markers, the Glasgow Myocardial Infarction Study GLAMIS was subsequently also established as a stand-alone case-control study of plasma markers which were not studies in ECTIM (Lowe et al, 2003). Some controls rejected by the ECTIM study due to genetic exclusion criteria were accepted in GLAMIS.

The aim of the Glasgow Myocardial Infarction Study (GLAMIS) was to recruit all men and women with MI in the North Glasgow MONICA study (Morrison et al, 1997), diagnosed by MONICA criteria (Tunstall-Pedoe et al, 1988) from July 1994-5, between 3 and 9 months after the event when acute-phase protein reactions due to myocardial trauma had settled. Cases were patients with MI in this population survey who were still alive, contactable and gave consent (75% response rate, n=490). Controls were selected from a random sample of the same North Glasgow population, obtained from general practice registers, who had no history or electrocardiograph evidence of MI, and who were frequency-matched for sex and age (within 1 year). Written informed consent was obtained from all participants, and the study was approved by the local research ethics committee.

Participants completed a detailed general health questionnaire including the Rose chest pain questionnaire, as well as questions on smoking (current/ex-smoker/never smoked cigarettes) and diabetes (yes/no) history. Weight and height were measured for calculation of BMI and an ECG and blood pressure were also recorded by a trained nurse. A forearm venous blood sample was taken after a full overnight fast; one serum and one citrated sample (0.11 mol/L, 9:1 v/v). Blood samples were centrifuged at 2000 × *g* for 10 min at room temperature within two hours of sampling, and aliquots stored at -70 °C until assay.

IL-18, TNFα, fibrinogen, CRP and IL-6 were assayed as described in Chapter 2. Aliquots used in this study were previously unthawed.

5.2.2 Statistical Analysis

Each of the inflammatory variables (IL-18, TNFα, CRP, IL-6 and fibrinogen) and continuous conventional cardiovascular risk factors considered (age, systolic blood pressure, serum total cholesterol and carbon monoxide in expired air) was compared between MI cases and controls using general linear models that adjusted for age and sex (Woodward et al, 2006). Skewed continuous variables (including all inflammatory markers) were log-transformed to approximate normality, or if normality was not achieved by these methods, were power-transformed as stated in the text. Spearman rank correlations were used to explore monotonic relationships, again adjusting for age and sex, between IL-18 and TNFα and all the continuous variables. Multivariable logistic regression models were used to obtain odds ratios for MI by the thirds of both IL-18 and TNFα in total population, adjusting both for age and sex, and additionally adjusting for all inflammatory variables and CVD risk factors considered in subsequent models.

5.3 RESULTS

5.3.1 Sample Availability

From the 75% response rate to the GLAMIS recruitment procedure, there were a total of 490 cases and 505 controls entered into the study. Of these, 446 cases (445 for TNFα) and 477 controls had citrated blood samples available for measurement of novel inflammatory variables due to previous sample attrition (93% of the total available). The baseline characteristics of subjects for whom samples were and were not available for the present study were not appreciably demographically different and were hence missing at random ($p>0.1$ for all).

5.3.2 Population Characteristics

Table 5.1 shows baseline demographic data for cases versus controls. As anticipated by design, there were no significant differences in age between cases and controls. Cases and controls were matched for age and sex (cases 74.4% male vs controls 72.6% male $p>0.5$). Cases and controls had similar percentages of current smokers (cases 46.9% vs controls 45.6% p = ns) but a higher percentage people who had ever smoked in the case population (cases 89.4% vs controls 74.4% $p <0.0001$). This is due to the fact that of 161 cases who were ex-smokers, 46% had given up in the last year compared with 6% in the controls. Cases were more likely to be diabetic (12% vs 2%; $p<0.0001$) and more likely to be taking prescribed medications for high blood pressure (39% vs 17%; $p<0.0001$) or high cholesterol (28% vs 1%; $p<0.0001$).

Cases had lower levels of blood pressure compared to controls, an effect probably attributable to treatment with medication following index MI. Cases and controls were significantly different for HDL cholesterol and triglyceride lipid measurements ($p<0.0001$) (although not total cholesterol) and body mass index (BMI) ($p=0.0007$) in the expected directions. Cases had very significantly higher levels of the APR associated inflammatory markers IL-6, fibrinogen, and CRP ($p<0.0001$ for all). In univariate comparisons of cases and controls both TNFα and IL-18 were also significantly higher in cases than in controls (29%; $p<0.0001$ and 6%; $p<0.01$ respectively).

5.3.3 Correlations of IL-18 and TNFα with Other Cardiovascular Risk Markers

TNFα levels in those who had ever smoked compared to those who had never smoked were not significantly higher in the case or in the control population (non-significant trend in control populations: median 0.69 vs 0.79pg/ml p= 0.38). For continuous variables, Tables 5.2 and 5.3 respectively show age- and sex-adjusted correlations of TNFα and IL-18 with classical and inflammatory risk markers. TNFα showed few strong correlations with classical risk factors, even in the control population (Table 5.2). TNFα had a moderate associations with age ($r^2 = 0.14$) triglycerides ($r^2 = 0.10$) and HDL cholesterol ($r^2 = -0.12$) in the control population, although these were absent in the cases (for lipid measurements this is probably attributable to fibrate prescription). In the group of haemostatic markers measured there were moderate associations of TNFα with t-PA

and D-dimer in case and control populations (r range =0.11-0.18), although not with vWF or PAI antigens. TNFα had expected strong associations with the other cytokines (IL-6 and IL-18) and with PV in both case and control populations, but surprisingly associations were not observed with the acute phase reactants (fibrinogen and CRP).

Similarly to TNFα, IL-18 levels showed no significant relationship with smoking, although there was a non-significant trend for controls who had ever smoked to have higher IL-18 levels (median 249 vs 277 pg/ml p=0.19). IL-18 showed strong associations with HDL cholesterol (r = -0.22) and associations with triglycerides (r = 0.16), although, similarly to TNFα, relationships with lipids were absent in the cases (Table 5.3). IL-18 also had modest associations with BMI (r = 0.14) and carbon monoxide expiration (r = 0.11), although these were also absent in case populations (probably due to weigh loss and altered smoking patterns following MI). IL-18 had significant associations with all haemostatic markers measured in the controls, and in particular associations with D-dimer and t-PA were strong (r = 0.25 for both). IL-18 also had expected strong associations with other cytokines in both case and control populations (IL-6 and TNFα), and a strong association with markers of the acute phase response (CRP and fibrinogen) as well as plasma viscosity, although again these associations were weakened or absent in the case population.

5.3.4 TNFα and IL-18 Associations with History of MI

Table 5.4 shows the risk of MI in the population by thirds of TNFα and IL-18 expression, and both cytokines are analysed by adjusting in three different models. After adjustment for matched variables (age and sex), those in the top third for TNFα expression had an elevated risk of MI (Odds ratio [OR] 2.19: 95% confidence intervals [CI] 1.58 – 3.03). After adjustment for classical risk factors the association attenuated to 1.66, and after further adjustment for acute phase reactants the relationship marginally attenuated to borderline significant 1.47 (95% CI 0.91 – 2.37).

In contrast, IL-18 showed a weak association with risk after adjustment for only matched variables (OR 1.38; 95% CI: 1.00 – 1.90). This relationship was attenuated to a null association after adjustment for classical risk factors (1.07), and classical risk factors and acute phase reactants (0.87). This loss of statistical association with outcome is broadly in agreement with the greater level of correlations IL-18 displayed with conventional and inflammatory risk markers than did TNFα.

Table 5.1 Baseline age- and sex-adjusted summary statistics for TNFα, IL-18 and continuous conventional risk factors in case and control populations.

	Cases		Controls		
	n	Medians (IQR)	n	Medians (IQR)	p-value
Age (years) *	490	57 (50 -61)	505	57 (51 – 61)	0.57
Systolic blood pressure (mmHg)	489	120 (105 – 137)	505	130 (116 – 143)	<0.0001
Diastolic blood pressure (mmHg)	489	77 (69 - 86)	505	83 (75 – 90)	<0.0001
Total cholesterol (mmol/L)	477	5.70 (4.95 – 6.48)	493	5.70 (5.01 – 6.42)	0.59
HDL cholesterol (mmol/L) †	477	1.08 (0.90 – 1.29)	492	1.29 (1.06 – 1.55)	<0.0001
Triglycerides (mmol/L) †	477	1.88 (1.35 – 2.63)	493	1.44 (1.05 – 2.11)	<0.0001
Body mass index (Kg/m^2) †	487	27.3 (24.7 – 30.4)	505	26.3 (23.8 – 29.7)	0.0007
Carbon monoxide (ppm) †	486	6 (3 – 15)	504	4 (3 – 15)	0.03
Fibrinogen (g/L)†	387	4.46 (3.80 – 5.26)	378	4.03 (3.44 – 4.69)	<0.0001
CRP (mg/L)†	391	2.92 (1.31- 6.32)	370	1.79 (0.80 – 4.23)	<0.0001
IL-6 (pg/ml) §	379	2.08 (1.42 – 3.17)	360	1.42 (1.07 – 2.16)	<0.0001
IL-18 (pg/ml) †	446	287 (212 – 404)	477	271 (200 – 373)	0.01
TNFα (pg/ml) ‡	445	0.99 (0.65 – 1.64)	477	0.77 (0.52 – 1.22)	<0.0001

* Adjusted for sex only
For transformed values summery statistics are shown after back transformation:
† Values computed on log scale
‡ Computed after power -0.1 transformation
§ Computed after power -0.5 transformation

Table 5.2 Age and sex-adjusted Spearman correlations of continuous variables with TNFα for MI cases and controls.

	Cases		Controls	
	r	p-value	r	p-value
Age	0.05	0.29	0.14	0.002
Systolic blood pressure	-0.008	0.86	-0.03	0.49
Diastolic blood pressure	0.00	0.94	-0.01	0.85
Total cholesterol	-0.03	0.51	-0.05	0.29
HDL cholesterol	0.06	0.17	-0.12	0.01
Triglycerides	0.06	0.21	0.10	0.03
Body mass index	0.08	0.08	0.06	0.18
Carbon monoxide	-0.09	0.05	0.04	0.37
Haematocrit	-0.02	0.62	0.03	0.51
PAI	0.03	0.51	0.05	0.30
t-PA	0.11	0.06	0.12	0.06
D-dimer	0.14	0.01	0.18	0.003
vWF	0.06	0.18	0.06	0.20
PV	0.11	0.02	0.11	0.01
Fibrinogen	0.07	0.19	0.09	0.08
CRP	0.05	0.30	0.07	0.17
IL-6	0.24	<0.0001	0.21	<0.0001
IL-18	0.24	<0.0001	0.23	<0.0001

Table 5.3 Age and sex-adjusted Spearman correlations of continuous variables with IL-18 for MI cases and controls.

	Cases		Controls	
	r	p-value	r	p-value
Age	0.01	0.77	0.00	0.97
Systolic blood pressure	0.00	0.96	0.04	0.41
Diastolic blood pressure	0.04	0.39	0.04	0.41
Total cholesterol	0.00	0.97	0.02	0.61
HDL cholesterol	0.09	0.06	-0.22	<0.0001
Triglycerides	0.07	0.12	0.16	0.0005
Body mass index	0.09	0.06	0.14	0.003
Carbon monoxide	0.04	0.46	0.11	0.01
Haematocrit	0.01	0.77	0.14	0.003
PAI	0.10	0.04	0.14	0.004
tPA	0.10	0.08	0.25	<0.0001
D-dimer	0.16	0.004	0.25	<0.0001
vWF	0.13	0.01	0.18	0.0004
PV	0.02	0.63	0.15	0.001
Fibrinogen	0.07	0.18	0.25	<0.0001
CRP	0.15	0.005	0.21	<0.0001
IL-6	0.22	<0.0001	0.25	<0.0001
TNFα	0.24	<0.0001	0.23	<0.0001

Table 5.4 Odds ratios (OR) of myocardial infarction for TNFα and IL-18, adjusted for three sets of risk factors. Analyses are by thirds (bottom third OR=1) based on distribution in the GLAMIS population.

	Odds ratio (95% CI) adjusted for:		
	Matched variables only	Classical risk factors*	Classical risk factors, fibrinogen, and CRP
TNFα			
Bottom third	1	1	1
Middle third	1.46 (1.06, 2.01)	1.06 (0.70, 1.59)	1.06 (0.66, 1.70)
Top third	2.19 (1.58, 3.03)	1.66 (1.10, 2.50)	1.47 (0.91, 2.37)
IL-18			
Bottom third	1	1	1
Middle third	1.28 (0.93, 1.76)	0.95 (0.62, 1.43)	0.75 (0.46, 1.21)
Top third	1.38 (1.00, 1.90)	1.07 (0.70, 1.62)	0.87 (0.53, 1.42)

* Age, sex, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, diabetes, smoking (current/ex/never), body mass index, use of medications for high blood pressure and total cholesterol.

5.4 DISCUSSION

5.4.1 Cytokine Expression Levels in the MONICA Study

Median IL-18 levels among controls were 271pg/ml (inter-quartile range 200-373) and for TNFα the median was 0.77pg/ml (0.52-1.22). Comparing the present study to the MONICA-4 study, median IL-18 levels are very similar (271 pg/ml among ~57 year old controls versus 251pg/ml in the 45-64 year old males), which would suggest a high comparability between the studies in terms of sample condition and the populations (which is as expected since they are both North Glasgow studies). Despite this, the same cannot be said for TNFα, for which levels appears to be around half of those seen in MONICA-4 (0.77 pg/ml among controls versus 1.65-1.90pg/ml range in the 45-64 year old males). The inter-quartile ranges between the studies are also similarly discrepant (~2-fold comparing extreme values in the IQR) and therefore the difference is a population-wide "shift" of all TNFα values. The reasons for this discrepancy are not clear. It is possible that the difference is partially due to differences in kit lots and drift of the calibrant between them. This would be a worrying conclusion, yet it may be the simplest one. As described in Chapter 3, if anything TNFα is more robust than IL-18, and it is unlikely therefore that sample quality plays a role in the discrepancy. Likewise, EDTA levels of TNFα are similar to those in citrate (MONICA-4) and may if anything be slightly higher (Chapter 3). These important findings require further monitoring and explanation.

5.4.1 Association of TNFα and IL-18 with Previous MI

The present study investigated the associations of TNFα and IL-18 with classical risk factors, acute phase response markers, and risk of MI in a case – control study. Results reported here show that that both TNFα and IL-18 are significantly elevated in persons with previous MI, independently of sex and age. This study also finds that these associations with MI are dependant on confounding associations with classical risk

factors and are also confounded by markers of the acute phase response, and after adjustment for both of these TNFα, but not IL-18, remains significantly associated with MI.

These patterns of association with MI for the two cytokines are in agreement with previous publications that studied the cytokines separately in case-control studies (Jovinge et al, 1998; Bennett et al, 2006, Hulthe et al, 2006) where it was observed in unassociated studies that IL-18 had a relatively weaker univariate association with MI than did TNFα. This study confirms these findings, and adds further data showing that after full adjustment for classical risk factors in multivariate analysis, TNFα remains associated with previous MI, whereas IL-18 does not. This result is slightly surprising, since in prospective studies in generally healthy populations ([2]Cesari et al, 2003; Blankenberg et al, 2003) as well as populations with prevalent CVD ([3]Ridker et al, 2000: Koukkunen et al, 2001; Blankenberg et al, 2002; Blankenberg et al, 2006; Table 1.4&1.6), trends suggest that if anything IL-18 may be a stronger prospective risk marker of MI than TNFα.

In the months immediately following index MI, and after acute-phase protein reactions have settled, physiological changes (such as cardiac cachexia and CHF), lifestyle changes (such as intentional weight loss and smoking cessation) and/or treatment with prescribed medication result may alter the relative circulating levels of IL-18 and TNFα, and hence there may be an alteration of observed associations with historical MI. For instance, although little is known of the role of IL-18 in cachexia, TNFα levels are known to be elevated in those with cardiac cachexia, and blockade of the cytokine may help normalize the anabolic response to overeating and allow fat-free mass to be gained (Marcora et al, 2006). In addition smoking cessation will likely lower circulating concentrations of most cytokines (see Chapter 4; [2]Wannamethee et al, 2005). Hence it is possible there are sub-groups of MI survivors that will display longer-term physiological responses to MI, and these responses will impact on pro-inflammatory cytokines, which will have bearings on the observed magnitude of associations with outcome.

Despite a lack of observed independent associations with previous MI and IL-18 in this study, IL-18 may still be a prospective risk marker within populations with prevalent CHD independently of conventional risk markers (Blankenberg et al, 2002, 2006). Speculatively, this may suggest that although there are changes in the medications, physiology, and lifestyles of populations with history of MI that abrogate independent associations of IL-18 with historical MI (and essentially make these populations appear closer to "normal" in terms of IL-18 expression) IL-18 may still ultimately predict risk of recurrent CHD events in these populations. In contrast with IL-18, the association between TNFα and retrospective MI is attenuated after adjustment for classical risk factors and markers of the acute phase response, but remains borderline significant in populations with historical MI. This is consistent with the central role TNFα may play in post-MI left ventricle dilatation and subsequent congestive heart failure (Torre-Amione et al, 1999; Sarzi-Puttini et al, 2005), as well as its role in cardiac cachexia (Marcora et al, 2006).

5.4.2 Associations of IL-18 and TNFα with Conventional Risk Markers

In agreement with previous publications (Jovinge et al, 1999; Bennett et al, 2006) and with the MONICA-4 study (Chapter 4), TNFα showed weak to moderate associations with some lipid parameters in the control population (HDL cholesterol and triglycerides) but not with total cholesterol. There was also evidence of correlations with D-dimer and a borderline association with t-Pa which suggests that TNFα participates in the fibrinolysis cascade, consistent with observations in patients with lung cell carcinoma and TNFα infusion experiments (Guadagni et al, 2004; van Hinsbergh et al, 1990). However, also consistent with MONICA-4 (chapter 4), TNFα did not demonstrate firm associations with other classical risk markers, such as blood pressure and BMI, although there was a correlation with age in the controls. Lack of association between BMI and TNFα contradicts proposed associations between obesity and TNFα (Ziccardi et al, 2002; Samuelsson et al, 2003), although the present study may biased by the poor general health of the north Glasgow control group, reflected by the high prevalence of smoking,

and the suboptimal average values of risk factors such as BMI and blood pressure. Interestingly, although TNFα showed expected associations with IL-18 and IL-6, it had no statistical association with the downstream markers of the APR; CRP and fibrinogen.

IL-18 had moderate associations with markers of the metabolic syndrome in the control population (HDL cholesterol, triglycerides, and BMI) and this is consistent with previous studies, which suggest it may also be associated with insulin levels (Esposito et al, 2003; Hung et al, 2005; Hulthe et al, 2006), although studies exist which also contradict this apparent association (1.4.7). Less controversially, associations with haemostatic markers in the control population (D-dimer, vWF, PAI and t-PA) as well as other inflammatory markers and markers of the acute phase response were observed for IL-18. These associations were generally absent or heavily attenuated in the case population and this is probably due to previously described changes in lifestyle, secondary consequences of MI such as cachexia, and prescribed medication.

Lack of an association of either cytokine with smoking is contradictory to results in the MONICA-4 study. This is likely due to the previously described recent cessation of smoking (and perhaps denial of previous/current smoking habits) in a population with a recent CVD event history.

5.4.3 Study Strengths and Limitations

This study comprised a relatively large number of patients (n=490) procured from a focused geographic area within a relatively short time-frame. This makes the study a statistically powerful resource. Despite this limitations of the study require consideration. As previously discussed, the North Glasgow population may be considered to be of generally sub-optimal cardiovascular health as reflected by the high-normal blood pressure, BMI and cholesterol levels of the control population in this study. Also, the present case-control study has the disadvantage of using a study design which, as previously discussed, is relatively weak for estimating causality (Woodward et al, 2005). In particular survivor bias may have had some effect on the results (cases had to be alive

several months after their MI), for instance lack of association between IL-18 and historical MI may imply that those with high IL-18 levels who experienced MI were less likely to survive. There is currently no causal evidence in favour of this argument, but it is important to highlight the potential confounding implicated by survivor bias in the interpretation of results.

The necessary lag between the event and recruitment into the study allowed time for changes to lifestyle and treatment that will usually occur after surviving a significant medical event. This is manifested here by the lower blood pressures in the case group, the lack of difference between the cases and controls in average cholesterol levels and the same percentage (45%) of current smokers amongst cases and controls at the time of surveying. Despite this a higher BMI was observed among cases, but IL-18 and TNFα were not associated with this variable among cases. These results reflect that that a lag time of a few months is not entirely sufficient for some risk factors to become attenuated, resulting in superficially disparate observations. Statistical correction measures included are that correlations and binary relationships with TNFα and IL-18 are presented separately for cases and controls, medication use is included as an adjustment variable in the analyses of association with MI, and smoking status is dichotomised as ever or never smoked cigarettes.

5.4.4 Conclusions

Despite these limitations, this study has shown that IL-18 is associated with classical risk markers for CHD that are also associated with the metabolic syndrome (HDL cholesterol, triglycerides and BMI), haemostatic markers, and other inflammatory markers, including markers of the APR in a control population. Heavy confounding may have implications limiting IL-18 utility as a risk marker (potentially even in prospective studies), but does not preclude (and may even allude to) a role for it in the development of incident CHD, particularly with reference to its associations with markers of the metabolic syndrome. This study also suggests that lifestyle changes and medical treatment following MI may

have profound effects on IL-18 associations with other risk markers, and indeed perhaps its association with risk.

In contrast, TNFα has few firm associations with classical risk markers (borderline associations with HDL cholesterol and triglycerides in controls) and although it associates with other cytokines (IL-6 and IL-18) it shows no association with markers of the APR, and hence multivariate adjustment leads to only moderate attenuation of the association with risk. This suggests that not all inflammatory markers associated with risk of CHD are acting as distal markers of the APR, although TNFα is undoubtedly at least partially confounded by it. Hence there is scope for inflammatory markers to play differential roles in both the conventional risk factor dependant and independent of the process of atherogenesis.

Further studies are needed to confirm these findings and to expand on them, particularly to elucidate reasons for cross-study discrepancies in IL-18 associations with conventional risk markers and TNFα's associations with degree of atherosclerosis (IMT or ankle brachial index). Prospective studies are required which can directly compare IL-18 and TNFα as prospective risk markers of CHD. Such studies are reported in Chapters 6 and 7.

CHAPTER 6

ASSOCIATIONS OF IL-18, TNFα, MMP-9 AND sCD40L WITH RISK OF CHD IN A NESTED CASE CONTROL STUDY OF MIDDLE-AGED MEN AND THE EXTENT OF 4-YEAR REGRESSION DILUTION IN THOSE MEN: THE BRITISH REGIONAL HEART STUDY

6.1 INTRODUCTION

6.1.1 Prospective Study of Risk Associations of Novel Inflammatory Risk Markers in Middle-Aged Men

Having examined associations of IL-18 and TNFα with retrospective risk of CHD, the logical next step was to further examine such associations in prospective studies, which are subject to less reverse-causality bias (GLAMIS: Chapter 5).

We had stored baseline serum samples in our laboratory from the British Regional Heart (BRH) Study, which were originally drawn in 1978-1980. The BRH study was set up to examine the factors responsible for the regional variation in coronary heart disease, hypertension, and stroke in Great Britain, and to determine the causes of these conditions (at both individual and regional levels) in order to provide a rational basis for recommendations towards their prevention. The focus of the study was on the prevalence and incidence of cardiovascular disease and their relations to established behavioural and biological risk factors (Walker et al, 2004).

Although these samples had been stored for a long period (~25 years), the cohort is large, well established, has had many conventional and novel risk factors measured, and follow-up of participans has been excellent (see methods). This well defined cohort therefore represents an ideal resource for the measurement of novel potential risk markers of CHD. It is a random sample of the 1980 UK general population of middle-aged men, with relatively few selection criteria, and over the 16 year follow-up the cohort yielded 643 incident coronary heart disease cases making it a powerful resource. The availability of

serum samples also allowed for the measurement of sCD40L and MMP-9 as novel risk markers, as well as IL-18 and TNFα. For these reasons the BRH study was considered one of the key studies of this thesis.

6.1.2 Previously Published Prospective Data Linking the Inflammatory Markers with Risk of CHD

Summary tables of all published prospective data examining IL-18, TNFα, MMP-9, sCD40L, and CHD risk are shown in Tables 1.4, 1.6, 1.8 and 1.10. At the outset of this thesis there was only very limited data examining associations of IL-18, TNFα, sCD40L and MMP-9 markers with incident CHD. Several studies had shown IL-18 (Blankenberg et al, 2002), TNFα (Ridker et al, 2001; Koukkunen et al, 2001), MMP-9 ([2]Blankenberg et al, 2003), and sCD40L ([2]Varo et al, 2003, Kinlay et al, 2004) were associated with risk of CHD in people with varying severities and degrees of prevalent vascular disease. However, these populations do not include "healthy" people and may be subject to reverse causality bias or selection bias compared to generally healthy populations.

The ABC nested case-control study of TNFα as a risk marker in healthy elderly people ([2]Cesari et al, 2003) indicated that TNFα was a moderate risk predictor of CHD (OR 1.6 [95% CI 1.23-2.26]) (Table 1.4). The PRIME Study of IL-18 in middle-aged men found a relatively strong association of IL-18 with risk of CHD among men in the top tertile (~80%) (Blankenberg et al, 2003) and the risk was independent of potential confounders. At the outset of this thesis there were no general or healthy populations studies for MMP-9 (Table 1.8), and the small Women's Health Study (n cases=130) was the only available data for sCD40L, which suggested significant associations with risk only at the 95th percentile (although the study was performed on plasma: see Chapter 3) ([3]Schonbeck et al, 2001).

Thus, data in general examining these inflammatory markers are suggestive of moderately strong associations with risk of CHD, although the data are limited. An

additional concern is that recent history suggests that the literature on novel biomarkers may be subject to publication bias in the early stages of investigation (e.g. CRP; Danesh et al, 2004). These markers therefore required further investigation on their association with risk of CHD.

6.1.3 Aims

The aim of this study was to establish the associations of serum IL-18, TNFα, MMP-9 and sCD40L with conventional risk markers and with risk of incident CHD in a long-term nested case-control study in a general population of middle-aged men. In addition, I aimed to use the cohort to estimate the 4-year regression dilution ratios of all four inflammatory markers (Chapter 3), to compare these with previously published findings for other risk markers in this cohort (Emberson et al, 2004) and to use them to assess the potential strength of associations once the bias is taken into account.

6.2 METHODS

6.2.1 Establishment of the British Regional Heart (BRH) Study

During the 1970s the epidemic of CHD was well-established in Britain. Pronounced regional variations in CVD prevalence were noted in epidemiological studies. Indeed, it is well known that the geographical phenomenon of a general South-North and East-West gradient in CHD is still evident in the UK today (see fig 6.1). For a time there was interest in the possibility that 'soft' drinking water was a risk factor for CHD and that variations in water hardness/softness, or the weather gradient, across Britain might account for these geographical variations. The study demonstrated that although water softness was not associated with incident CHD after adjustment for confounders (Pocock et al, 1980), classical risk factors accounted for 77% of the geographical variance in incident CHD levels (Morris et al, 2001). The BRH study has also contributed much valuable data on the associations of classical risk factors with incident CHD (Shaper et

al, 1985, 1986, 1991, 1994, 1997, 1998; Walker et al, 1987, 1995; Wannamethee et al, 1998[1&2], 2002), data on British CHD risk assessment (Brindle et al, 2003) and novel risk markers (Danesh et al, 2000[2], 2001; Whincup et al, 2000; Homocysteine Studies Collaboration, 2002) as well as the regression dilution effect for conventional and novel cardiovascular risk markers (Emberson et al, 2003 & 2004). An exhaustive list of publications up until 2004 has been published and discussed elsewhere (Walker et al, 2004).

The study included 24 towns and boroughs with populations >50 000 in 1978-80 (see fig 6.2) and various selection criteria including ranges of CVD prevalence (Walker et al, 2004). Initially almost 10 000 middle-aged men aged 40–59 were drawn at random age bands of each practice register and invited to take part. The cohort is socio-economically representative of British middle-aged men in 1980 (Table 6.1) and of senior citizens in the 2000s. Since then a wide range of personal, behavioural, risk factor exposure, and clinical data has been collected on follow up as the cohort moved from middle to old age (blood samples at baseline, 4, 16 and 20 years; Walker et al, 2004, Emberson et al, 2004). This makes the cohort a powerful epidemiological resource and longitudinal study.

Fig 6.1 Age-standardised death rates per 100,000 of the population from CHD in men aged under 65 years by local authority 2002/2004 illustrating a general South East-North West gradient in incidence. From The British Heart Foundation: www.heartstats.org

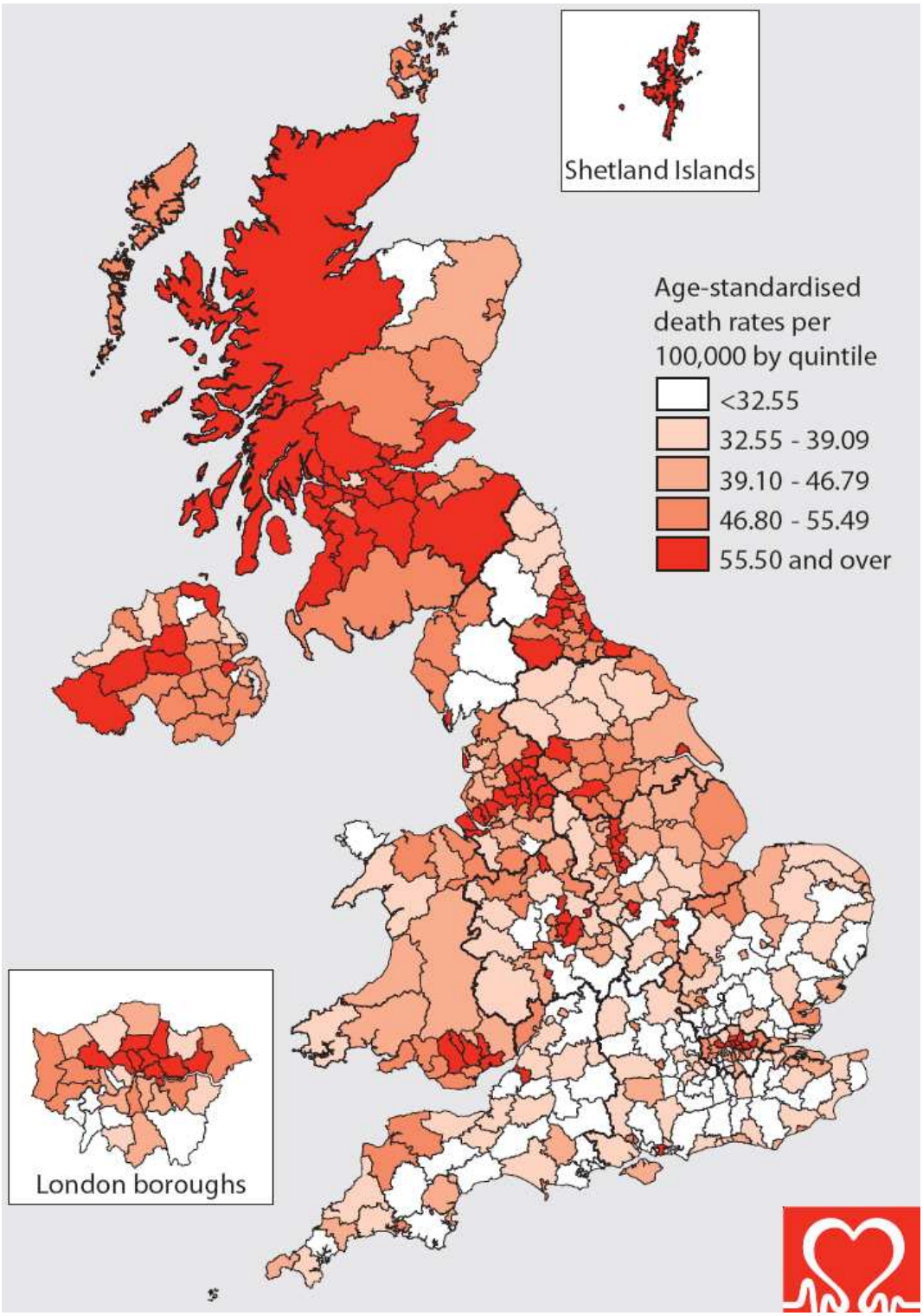


Fig 6.2 Illustration of towns involved in the BRHS. From Walker et al, 2004

Table 6.1 Social class distribution of participants in the British Regional Heart Study (BRHS) in relation to Census data. From Walker et al, 1987.

Social class	National Census 1981		24 towns Census 1981		BRHS participan ts 1978–80	
I	5.2		5.1		7.8	
II	23.8	39.5	23.2	41.5	22.5	39.6
IIINM	10.5		13.2		9.3	
IIIM	33.9		33.7		43.0	
IV	17.8	58.1	16.6	56.1	10.3	57.4
V	6.4		5.8		4.1	
Armed forces	2.4		2.4		3.0	

6.2.2 Subject recruitment and establishment of a case/control study

In 1978-1980, 7735 males aged 40-59 were randomly selected from general practice registers in each of 24 British towns and invited to take part in the British Regional Heart Study (response rate 78% from ~10,000 invitations). Nurses administered questionnaires, made physical measurements including blood pressure and body mass index (BMI), recorded an ECG and collected non-fasting venous blood samples. In 5661 men in 18 of the study towns, serum was initially stored at -20^{o}C for subsequent analysis (Shaper et al, 1981), and at -50^{o}C and –80^{o}C once the facility was available. In the cohort assessments, pre-existing CHD was defined on the basis of a history of a doctor diagnosis of CHD, a positive history of angina or possible MI on the Rose (WHO) chest pain questionnaire or an ECG consistent with definite or possible CHD.

All men were followed-up for all-cause mortality and for cardiovascular morbidity, with a follow up loss of <1% (Walker et al, 2004). Fatal CHD cases were ascertained through National Health Service Central Registers on the basis of a death certificate with ICD-9 codes 410-414. The diagnosis of non-fatal myocardial infarction was based on reports from general practitioners, supplemented by regular reviews of general practice records, and diagnosed in accordance with World Health Organisation criteria (Walker et al, 2004). After an average of 16 years follow-up, a nested case-control study was established, based on all 643 coronary heart disease cases (279 coronary heart disease deaths and 364 cases of non-fatal myocardial infarction) occurring between 1978 and 1996 and a total of 1278 controls (2 per case) were "frequency matched" to cases on town of residence and age in 5-year bands. These controls were randomly selected from among men who had survived to the end of the study period free from incident coronary heart disease, but could include a random sample of those with baseline CHD presuming no further incidents at follow-up. Emerging risk markers previously studies include CRP ([2]Danesh et al, 2000), soluble adhesion molecules (Malik et al, 2001), vWF (Whincup et al, 2002), t-PA (Lowe et al, 2004), D-Dimer (Danesh et al, 2001), and IL-6 (Mann et al, submitted).

IL-18, TNFα, MMP-9 and sCD40L were all measured as described in Chapter 2. Samples used in this thesis had been stored for ~25 years and had been thawed on ~4-5 previous occasions.

6.2.3 Statistical analysis

Highly skewed variables were log transformed where necessary, and this included all the four novel markers. Analysis of case-control samples was pre-specified by thirds of IL-18, TNFα, MMP-9 or sCD40L values in controls. Unmatched stratified logistic regression was fitted using unconditional maximum likelihood methods (SAS version 8.1). For associations between inflammatory markers and a variety of known and suspected CHD risk factors, trends across thirds of the population distribution for each inflammatory marker were analysed, and emphasis was mainly given to differences more extreme than 2.6 SD ($2P>0.01$) to make allowance for multiple comparisons.

6.2.4 Regression Dilution Ratio Study

The samples used to examine this pre-analytical variable were follow-up samples from the same cohort at 16 and 20 years (in towns Dewsbury and Maidstone; as per Emberson et al, 2004), hence this is a 4 year study of regression dilution in older (60 – 79 years at 20 year clinic attendance) males. These samples were therefore less old than the baseline samples, and had been subjected to fewer freeze-thaws (which is important with regard to pre-analytical variables; Chapter 3). The re-attendance and follow-up of the cohort is described in detail elsewhere (Walker et al, 2004). sCD40L and MMP-9 measurements were made on 139 pairs of serum samples, whilst IL-18 and TNFα were measured on 159 pairs of citrated plasma samples, each set of pairs representing a random selection of available samples. This was fewer samples than previously reported (Emberson et al, 2004) due to sample attrition (samples at both 16 and 20 years had to be adequate to assay).

The regression dilution ratio (RDR) is an estimate of the ratio of the uncorrected association (on a log scale) to the corrected association. Various methods have been described that measure this index (Frost et al, 2000). In this study the ratio was analysed using the Rosner method (Rosner et al, 1990) as in the original BRH study of regression dilution (Emberson et al, 2004), and the method estimates the RDR by regressing the follow-up measurement on the baseline, and obtaining the regression slope (regression dilution coefficient). As with the relative risk statistical analysis, this statistical work was performed by Professor P Whincup and colleagues.

6.3 RESULTS

6.3.1 Sample Availability

Due to the attrition of stored samples as a result of use in a number of previous studies (Danesh et al, 2000[2], 2001; Malik et al, 2001; Whincup et al, 2002; Lowe et al, 2004) a reduced number of residual serum samples were available for cytokine assays in 2004-5; IL-18 (595 cases, 1238 controls), TNFα (535 cases, 1155 controls), MMP-9 (465 cases, 1067 controls), and sCD40L (464 cases, 1063 controls). IL-18 and TNFα were assayed as the priority, and hence have a greater amount of data. The baseline characteristics of subjects for whom serum samples were and were not available for the present study were not appreciably different from the original case-control study ($p>0.10$ for case-control proportion, age distribution, residential town proportions, social class proportions).

6.3.2 IL-18 - Associations with Conventional Risk Markers, and with CHD Risk

The set of samples on which IL-18 levels were measured (the largest) were used to compare established vascular risk factors between cases and controls. The established factors showed highly significant differences between cases and controls in the expected directions (Table 6.2), although diabetes was not associated with CHD events. This could be due to very small numbers of diabetics (total <2% of the population) and undiagnosed latent cases within the population. This configuration of conventional risk factor

associations with outcome was also evident in the sets of case and control samples used to measure the other inflammatory markers (data not shown).

Table 6.2, shows that geometric mean IL-18 levels were significantly higher among cases than controls (proportional difference 9%, 95% CI 4 to 14% after age adjustment; $p<0.001$).

Examining possible associations of IL-18 with conventional risk markers (which may confound the association of IL-18 with risk), Table 6.3 illustrates correlations of IL-18 with other risk markers. Unsurprisingly, baseline IL-18 levels in the control population were strongly related to other inflammatory markers (CRP, IL-6 [both associations particularly strong], serum amyloid A, globulin, white cell count) and homocysteine as well as soluble cellular adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin) and haemostatic markers (vWF, fibrin D-dimer and t-PA), but not haematocrit (Table 6.3). IL-18 levels were also related to components of the metabolic syndrome such as dyslipidaemia (triglycerides and HDL cholesterol), as well as demonstrating weak associations with BMI and insulin levels. Among other classical risk markers IL-18 was not related to age but was strongly related to smoking, and weakly with diastolic and systolic BP as well as socioeconomic markers (non-manual occupation) (Table 6.3). IL-18 was not associated with evidence of CHD at entry to the study.

In comparisons of men in the top third compared with those in the bottom third of baseline IL-18 levels, the age-adjusted odds ratio for coronary heart disease was 1.55 (95% CI 1.21, 1.98) (Table 6.7). Adjustment for cigarette smoking slightly attenuated this odds ratio, while additional adjustment for other vascular risk factors reduced the odds ratio to borderline significance: 1.30 (95% CI 0.99, 1.69). Additional adjustment for other inflammatory markers (CRP and IL-6) attenuated the odds ratio to 1.12 (95% CI 0.84, 1.49) and hence nearly abrogated the association. The trend across thirds of IL-18 distributions was highly significant in the unadjusted model ($p=0.003$), but was attenuated with each extra step in the analysis models due to decreasing association of IL-18 with CHD, until the trend became non-significant in the final fully-adjusted model

(p=0.19). Repeating the analysis excluding men with established CHD had little material impact on the results, which is consistent with the absence of association with evidence of CHD at study entry (Table 6.3).

6.3.3 TNFα - Associations with Conventional Risk Markers, and with CHD Risk

Geometric mean TNFα levels were significantly *lower* among cases than controls (proportional difference -9%, 95% CI -2 to -16% after age adjustment; p=0.001) (Table 6.2).

As illustrated in Table 6.4, TNFα values showed positive associations with other inflammatory markers (CRP, IL-6, serum amyloid A, globulin, white cell count) homocysteine, and adhesion molecules (P-selectin, E-selectin, VCAM-1 and ICAM-1). Positive associations with haemostatic markers were limited to D-dimer and a borderline association with t-PA. There was a borderline inverse association with total cholesterol, although no association with other lipid markers was observed, or with other markers of the metabolic syndrome. TNFα had no associations with conventional risk markers: age, blood pressure, smoking, or evidence of baseline CHD.

In comparisons of men in the top third versus the bottom third of the TNFα control distribution, the age-adjusted odds ratio for CHD was *inverse;* 0.74 (95% CI 0.57 – 0.95) (Table 6.8). Adjustment for smoking status and other conventional risk factors did not significantly attenuate the inverse relationship: 0.74 (95% CI 0.57 – 0.98), an observation consistent with an absence of TNFα associations with such markers (Table 6.4). Further adjustment for IL-6 and CRP levels strengthened the inverse relationship with CHD, due to positive associations of TNFα with these inflammatory risk markers; 0.66 (0.49 – 0.88). None of the analysis models yielded a significant trend across thirds of the TNFα distribution (data not shown), with the top two thirds giving very similar odds ratios. Exclusion of men with evidence of baseline CHD had minimal effect on the odds ratio, but the inverse association became only borderline significant, due to widening of the confidence intervals with loss of statistical power.

6.3.4 MMP-9 - Associations with Conventional Risk Markers, and with CHD Risk

Geometric mean MMP-9 levels were higher among cases than controls (proportional difference 7%, 95% CI 1 to 13% after age adjustment; $p<0.03$) (Table 6.2).

Baseline MMP-9 levels in the control population were significantly positively associated with markers of systemic inflammation (CRP, IL-6, serum amyloid A, white cell count, and borderline with albumin) although not with homocysteine, most vascular adhesion molecules (E- and P-selectin, and ICAM-1, although not VCAM-1), and some haematological variables (haematocrit and fibrin D-dimer, but not t-PA or vWF) (Table 6.5). MMP-9 levels were inversely related to FEV_1 and strongly related to smoking as well as showing increasing trends with alcohol intake. Although it was weakly related to non-fasting glucose, MMP-9 showed no relationship to any other markers of the metabolic syndrome, and was not associated with other established risk factors such as age, evidence of CHD at entry, body mass index, blood pressure, physical activity, or any lipid measurements (Table 6.5).

In comparisons of men in the top third versus the bottom third of MMP-9 control distribution, the age-adjusted odds ratio for coronary heart disease was 1.37 (95% CI 1.04 - 1.82) (Table 6.9). Adjustment for smoking status attenuated the result to borderline significance: 1.28 (95% CI 0.95 - 1.74), although further adjustment for other vascular risk factors did not attenuate the result further. After further adjusting for CRP and IL-6 levels in the final model the association was attenuated to null: 1.13 (95% CI 0.82 – 1.56). None of the analysis models yielded a significant trend across thirds of the MMP-9 distribution, including in the unadjusted model (data not shown). Exclusion of men with evidence of baseline CHD had little material effect on the odds ratios observed, although the confidence interval was wider with the lower numbers of subjects.

6.3.5 sCD40L - Associations with Conventional Risk Markers, and with CHD Risk

Geometric mean sCD40L levels were borderline significantly *lower* among cases than in controls (proportional difference -7%, 95% CI -14% to +1% after age adjustment; p=0.068).

As illustrated in Table 6.6, baseline sCD40L levels in the control population were significantly positively associated with white blood cell count (although not CRP, IL-6, serum amyloid A, albumin, or homocysteine) and were strongly associated with the haemostatic markers t-PA and D-dimer (Table 6.6). There was also a strong positive association with p-selectin levels (but not other adhesion molecules). Serum sCD40L had inverse associations with age, vWF, fasting glucose, and triglycerides, as well as a positive association with HDL cholesterol. Serum levels of sCD40L showed no associations with other established risk factors such as age, evidence of CHD at entry, BMI, blood pressure, physical activity, or smoking, although there was a slight positive relationship with alcohol consumption (Table 6.6).

In comparisons of men in the top third versus the bottom third of sCD40L control distribution, the age-adjusted odds ratio for coronary heart disease was *inverse;* 0.75 (95% CI 0.57 – 0.99) (Table 6.10). Adjustment for smoking status and other conventional risk factors attenuated this relationship to borderline significance: 0.77 (95% CI 0.57 - 1.04). Further adjustment for CRP and IL-6 levels did not attenuate the odds ratio further: 0.77 (95% CI 0.57 – 1.05). None of the analysis models yielded a significant trend across thirds of the sCD40L distribution (data not shown). Exclusion of men with evidence of baseline CHD had little material effect on the odds ratios observed, although the confidence interval was wider with the lower numbers of subjects: 0.83 (95% CI 0.54 – 1.27).

6.3.6 4-Year Regression Dilutions Coefficients and Ratios of all Four Inflammatory Markers in Comparison to Previously Published Data

As seen in Table 6.11, regression dilution coefficients ranged from 0.25 for TNFα (corresponding to 4-fold underestimate of risk associations on the log scale), while IL-18 was more stable over the four years with a regression dilution coefficient of 0.71 (corresponding to a 1.41-fold underestimate). Coefficients for MMP-9 and sCD40L were intermediate and very similar (0.46 and 0.47 respectively). Overall therefore, IL-18 was the least prone to drift in expression levels over the 4 years compared to any other inflammatory risk marker, including CRP or fibrinogen (Table 6.1), and demonstrated a regression dilution ratio comparable to that of the total:HDL cholesterol ratio (Emberson et al, 2004). Regression dilution coefficients for MMP-9 and sCD40L were comparable to CRP. TNFα was the least stable marker measured, with a coefficient similar to that of clinical measurements of non-fasting insulin (Emberson et al, 2004). Despite this, TNFα had an overall correlation at 4 years compared to baseline that was similar to both MMP-9 and sCD40L.

As a very crude illustration of the possible implications of the impact of regression dilution on risk associations, I include adjusted odds ratios (based on this BRH cohort) for regression dilution. It should be noted that because the study is prospectively followed up for 16 years (not 4 years), these estimates are still likely to be underestimates, and no 95% confidence intervals are included for this reason; the figures are intended only as illustration as to the possible impact of regression dilution.

Age- and sex- adjusted odds ratios in the top tertiles become:

IL-18 OR 1.55 (1.21, 1.98) ⟶ OR 1.86 (1.31-2.62)

TNFα OR 0.74 (0.57, 0.95) ⟶ OR 0.30 (0.30-0.89)

MMP-9 OR 1.37 (1.04, 1.82) ⟶ OR 1.98 (1.09-3.67)

sCD40L OR 0.75 (0.57, 0.99) ⟶ OR 0.54 (0.30-0.98)

Hence, correction for regression dilution suggests that IL-18 and MMP-9 may be positively associated with CHD risk, and TNFα and sCD40L negatively associated. Ideally, the effect of regression dilution would be assessed in fully adjusted models, although for these inferences to be correct, adjustments would be made for conventional risk factors also adjusted for regression dilution over the same period. This has not been possible here.

Table 6.2 Baseline characteristics of the case and control populations. Values are means (standard deviation) unless otherwise specified. Case control numbers for TNFα, MMP-9 and sCD40L are stated in the text

	Cases (n=595)	Controls (n=1238)	p-value
Age (years)	52.6 (5.2)	52.5 (5.3)	matched
Current smoker (n,%)	304 (51.2)	521 (42.2)	0.0003
Evidence of coronary disease (n,%)	247 (41.5)	327 (26.4)	<0.0001
History of diabetes (n,%)	16 (2.7)	18 (1.5)	0.09
>2 drinks alcohol/day (n,%)	120 (20.2)	278 (22.5)	0.26
Non-manual occupation (n,%)	184 (32.3)	465 (38.7)	0.009
Physical activity (inactive/occasional) (n,%)	275(46.7)	493(40.4)	0.011
Physical measurements			
Body mass index (kg/m^2)	25.9 (3.4)	25.4 (3.3)	0.0008
Systolic blood pressure (mmHg)	151.8 (21.8)	146.7 (20.9)	<0.0001
Diastolic blood pressure (mmHg)	85.7 (13.8)	82.8 (13.2)	<0.0001
FEV_1 (L/min) (adjusted for age & size)	3.10 (0.65)	3.21 (0.71)	0.002
Blood sample			
Total cholesterol (mmol/L)	6.64 (1.06)	6.20 (0.99)	<0.0001
HDL cholesterol (mmol/L)	1.09 (0.27)	1.15 (0.27)	<0.0001
Triglyceride (mmol/L)*	1.95(0.69, 5.51)	1.70(0.57, 5.02)	<0.0001
C-reactive protein (mg/L)*	2.51(0.30, 20.95)	1.45(0.14, 15.11)	<0.0001
Interleukin-6 (pg/ml)*	2.75(0.81, 9.32)	2.29(0.65, 8.11)	<0.0001
Interleukin-18 (pg/ml)*	302 (117, 779)	275 (107, 711)	0.0007
TNFα (pg/ml)*	0.93(0.22, 3.96)	1.02(0.25, 4.15)	0.01
MMP-9 (pg/ml)*	741 (275, 2001)	692 (245, 1953)	0.026
sCD40L (pg/ml)*	257 (63, 1041)	275 (65, 1167)	<0.0001

* Indicates geometric mean after log transformation and back-transforming (95% CI)

Table 6.3 Relation of IL-18 (thirds) with other cardiovascular risk factors in controls

	Tertiles of IL-18						
Tertile Range (pg/ml)	Low (29-233) n=411		Middle (234-345) n=413		High (346-2068) n=414		
	Mean	se	Mean	se	Mean	se	p-trend
Age	52.5	0.3	52.1	0.3	52.8	0.3	0.61
current smoker (n)	133	32.4	188	45.7	200	48.3	<0.0001
>2 drinks alcohol/day (n)	98	23.8	86	20.9	94	22.7	0.56
Evidence of CHD at entry (n)	106	25.8	113	27.4	108	26.1	0.77
History of diabetes at baseline (n)	7	1.7	6	1.5	6	1.5	0.77
Non-manual occupations (n)	181	45.1	145	36.4	139	34.3	0.02
Physical Activity (inactive/occasional) (n)	152	37.7	169	41.6	172	41.8	0.66
Body mass index (kg/m^2)	25.1	0.2	25.5	0.2	25.6	0.2	0.05
Systolic blood pressure (mmHg)	144.6	1.0	146.9	1.0	148.4	1.0	0.03
Diastolic blood pressure (mmHg)	81.0	0.6	83.8	0.6	83.4	0.6	0.02
FEV_1 (L/min) (adjusted for age & size)	3.26	0.04	3.20	0.04	3.16	0.04	0.62
Total cholesterol (mmol/L)	6.14	0.05	6.23	0.05	6.21	0.05	0.39
HDL cholesterol (mmol/L)	1.20	0.01	1.15	0.01	1.11	0.01	<0.0001
Triglyceride (mmol/L)*	0.19	0.01	0.22	0.01	0.26	0.01	0.0001
Insulin (mU/L)	1.07	0.02	1.05	0.02	1.13	0.02	0.03
Glucose (mmol/L)	0.74	0.004	0.74	0.004	0.74	0.004	0.69
von Willebrand Factor (IU/dL)	2.00	0.01	2.01	0.01	2.05	0.01	<0.0001
Fibrin D-dimer (ng/ml)	1.86	0.02	1.88	0.02	1.93	0.02	0.01
t-PA (ng/ml)	0.97	0.01	1.01	0.01	1.06	0.01	<0.0001
Haematocrit (%)	41.91	0.45	42.79	0.45	42.45	0.45	0.7
Albumin (g/L)	44.7	0.1	44.6	0.1	44.3	0.1	0.003
Globulin (g/L)	28.9	0.2	28.9	0.2	29.3	0.2	0.1
White cell count (10^9/L)	0.81	0.02	0.83	0.02	0.88	0.02	0.003
Serum amyloid A protein (mg/L)	0.83	0.01	0.85	0.01	0.85	0.01	0.01
Homocysteine (mmol/L)	1.10	0.01	1.12	0.01	1.16	0.01	0.01
E-selectin (ng/ml)	1.74	0.01	1.77	0.01	1.82	0.01	<0.0001
P-selectin (ng/ml)	2.00	0.01	2.01	0.01	2.07	0.01	0.001
ICAM-1 (ng/ml)	2.43	0.01	2.47	0.01	2.52	0.01	<0.0001
VCAM-1 (ng/ml)	2.62	0.01	2.64	0.01	2.66	0.01	0.01
C-reactive protein (mg/L)	0.05	0.03	0.15	0.03	0.30	0.03	<0.0001
IL-6 (pg/ml)	0.31	0.01	0.36	0.01	0.41	0.01	<0.0001

Table 6.4 Relation of TNFα (thirds) with other cardiovascular risk factors in controls

	Tertiles of TNFα						
Tertile Range (pg/ml)	Low (0.05-0.76) n=389		Middle (0.77-1.16) n=384		High (1.17-30.0) n=382		
	Mean	se	Mean	se	Mean	se	p-trend
Age	52.4	0.3	52.6	0.3	52.5	0.3	0.92
current smoker (n)	159	41.0	160	41.7	164	43.0	0.53
>2 drinks alcohol/day (n)	83	21.3	90	23.5	88	23.0	0.99
Evidence of CHD at entry (n)	87	22.4	107	27.9	107	28.0	0.16
History of diabetes at baseline (n)	5	1.3	7	1.8	5.0	1.3	n/a
Non-manual occupations (n)	158	41.9	138	36.7	138.0	37.3	0.32
Physical Activity (inactive/occasional) (n)	143	37.3	154	41.1	160.0	42.1	0.25
Body mass index (kg/m^2)	25.2	0.2	25.5	0.2	25.4	0.2	0.45
Systolic blood pressure (mmHg)	145.4	1.1	146.9	1.1	147.3	1.1	0.26
Diastolic blood pressure (mmHg)	82.1	0.7	83.0	0.7	83.3	0.7	0.24
FEV_1 (L/min) (adjusted for age & size)	3.25	0.0.4	3.16	0.04	3.23	0.04	0.96
Total cholesterol (mmol/L)	6.26	0.05	6.18	0.05	6.14	0.05	0.07
HDL cholesterol (mmol/L)	1.16	0.01	1.16	0.01	1.15	0.01	0.33
Triglyceride (mmol/L)*	0.21	0.01	0.23	0.01	0.23	0.01	0.40
Insulin (mU/L)	1.06	0.02	1.08	0.02	1.09	0.02	0.38
Glucose (mmol/L)	0.743	0.004	0.740	0.004	0.737	0.004	0.23
von Willebrand Factor (IU/dL)	2.01	0.01	2.03	0.02	2.00	0.01	0.26
Fibrin D-dimer (ng/ml)	1.85	0.02	1.90	0.02	1.92	0.02	0.01
t-PA (ng/ml)	1.00	0.01	1.02	0.01	1.03	0.01	0.09
Hematocrit (%)	42.50	0.50	42.90	0.50	41.8	0.50	0.22
Albumin (g/L)	44.6	0.1	44.5	0.1	44.3	0.1	0.14
Globulin (g/L)	28.5	0.2	29.1	0.2	29.5	0.2	<0.0001
White cell count (10^9/L)	0.83	0.01	0.84	0.01	0.86	0.01	0.0002
Serum amyloid A protein (mg/L)	0.81	0.02	0.86	0.02	0.87	0.02	0.03
Homocysteine (mmol/L)	1.09	0.01	1.23	0.01	1.16	0.02	0.01
E-selectin (ng/ml)	1.75	0.01	1.76	0.01	1.81	0.01	<0.0001
P-selectin (ng/ml)	1.98	0.01	2.02	0.01	2.08	0.01	<0.0001
ICAM-1 (ng/ml)	2.45	0.01	2.48	0.01	2.49	0.01	0.001
VCAM-1 (ng/ml)	2.62	0.01	2.64	0.01	2.64	0.01	0.01
C-reactive protein (mg/L)	0.06	0.03	0.180	0.03	0.22	0.03	0.001
IL-6 (pg/ml)	0.30	0.01	0.36	0.01	0.42	0.01	<0.0001

Table 6.5 Relation of MMP-9 (thirds) with other cardiovascular risk factors in controls

	Tertiles of MMP-9						
Tertile	Low		Middle		High		
Range (ng/ml)	(70-555) n=358		(556-872) n=359		(873-3500) n=359		
	Mean	se	Mean	se	Mean	se	p-trend
Age (years)	52.1	0.3	52.6	0.3	52.5	0.3	0.27
Current smoker (n)	123	34.4	134	37.3	194	54.2	<0.0001
>2 drinks alcohol/day (n)	74	20.7	71	19.8	94	26.3	0.04
Evidence of CHD at entry (n)	91	25.4	81	22.6	104	29.0	0.40
History of diabetes at baseline (n)	2	0.6	7	2.0	7	2.0	n/a
Non-manual occupation (n)	137	39.3	133	37.7	130	37.7	0.44
Physical activity (inactive/occasional) (n)	138	38.9	143	40.4	149	42.5	0.51
Body Mass index (kg/m^2)	25.5	0.2	25.5	0.2	25.1	0.2	0.11
Systolic blood pressure (mmHg)	146.9	1.1	147.6	1.1	145.0	1.1	0.41
Diastolic blood pressure (mmHg)	83.5	0.7	83.2	0.7	81.9	0.7	0.23
FEV_1 (L/min) (adjusted for age & size)	3.30	0.04	3.25	0.04	3.09	0.04	0.002
Total cholesterol (mmol/L)	6.24	0.05	6.18	0.05	6.14	0.05	0.18
HDL cholesterol (mmol/L)	1.15	0.01	1.15	0.01	1.16	0.01	0.63
Triglyceride (mmol/L)	0.21	0.01	0.22	0.01	0.24	0.01	0.12
Insulin (mU/L)	1.08	0.02	1.10	0.02	1.05	0.02	0.21
Glucose (mmol/L)	0.745	0.004	0.743	0.004	0.73	0.004	0.032
Von Willebrand factor (IU/dL)	2.01	0.01	2.02	0.01	2.02	0.01	0.49
Fibrin D-dimer (ng/ml)	1.83	0.02	1.86	0.02	1.96	0.02	<0.0001
t-PA (ng/ml)	1.00	0.01	1.00	0.01	1.03	0.01	0.61
Haematocrit (%)	43.10	0.17	44.20	0.17	44.70	0.17	<0.0001
Albumin (g/L)	44.7	0.1	44.5	0.1	44.3	0.1	0.07
Globulin (g/L)	29.1	0.2	29.0	0.2	29.0	0.2	0.48
White cell count ($'10^9/L$)	0.79	0.01	0.84	0.01	0.90	0.01	<0.0001
Serum amyloid A protein (mg/L)	0.80	0.02	0.85	0.02	0.90	0.02	<0.0001
Homocysteine (mmol/L)	1.12	0.01	1.13	0.02	1.12	0.02	0.61
E-selectin (ng/ml)	1.76	0.01	1.77	0.01	1.80	0.01	0.002
P-selectin (ng/ml)	1.94	0.01	2.04	0.01	2.09	0.01	<0.0001
ICAM-1 (ng/ml)	2.45	0.01	2.46	0.01	2.50	0.01	<0.0001
VCAM-1 (ng/ml)	2.64	0.01	2.63	0.01	2.62	0.01	0.14
CRP (mg/L)	0.09	0.03	0.13	0.03	0.28	0.03	<0.0001
IL-6 (ng/ml)	0.27	0.01	0.36	0.01	0.44	0.01	<0.0001

Table 6.6 Relation of sCD40L (thirds) with other cardiovascular risk factors in controls

	Tertiles of sCD40L						
Tertile Range (ng/ml)	Low (9-206) n=359		Middle (207-386) n=356		High (387-2500) n=358		
	Mean	se	Mean	se	Mean	se	p-trend
Age (years)	52.8	0.3	52.4	0.3	52.1	0.3	0.06
Current smoker (n)	144	40.1	147	41.4	158	55.9	0.39
>2 drinks alcohol/day (n)	64	17.9	78	21.9	95	26.5	0.02
Evidence of CHD at entry (n)	87	24.2	94	26.4	94	26.3	0.68
History of diabetes at baseline (n)	5	1.4	6	1.7	5	1.4	n/a
Non-manual occupation (n)	133	37.9	140	40.8	126	36.0	0.79
Physical activity (inactive/occasional) (n)	134	37.6	153	43.7	142	40.3	0.62
Body Mass index (kg/m^2)	25.4	0.2	25.1	0.2	25.6	0.2	0.78
Systolic blood pressure (mmHg)	146.4	1.1	147.1	1.1	146.0	1.1	0.69
Diastolic blood pressure (mmHg)	83.0	0.7	82.9	0.7	82.6	0.7	0.77
FEV_1 (L/min)(adjusted for age & size)	3.23	0.04	3.27	0.04	3.15	0.04	0.27
Total cholesterol (mmol/L)	6.19	0.05	6.21	0.05	6.16	0.05	0.62
HDL cholesterol (mmol/L)	1.13	0.01	1.15	0.01	1.18	0.01	0.01
Triglyceride (mmol/L)	0.24	0.01	0.22	0.01	0.21	0.01	0.01
Insulin (mU/L)	1.10	0.02	1.04	0.02	1.09	0.02	0.27
Glucose (mmol/L)	0.752	0.004	0.737	0.004	0.731	0.004	0.0002
von Willebrand Factor (IU/dL)	2.04	0.01	2.01	0.01	2.01	0.01	0.02
Fibrin D-dimer (ng/ml)	1.82	0.02	1.85	0.02	1.97	0.02	<0.0001
t-PA (ng/ml)	0.98	0.01	1.00	0.01	1.05	0.01	<0.0001
Hematocrit (%)	43.80	0.17	43.90	0.17	44.30	0.17	0.21
Albumin (g/L)	44.4	0.1	44.4	0.1	44.6	0.1	0.18
Globulin (g/L)	29.0	0.2	29.1	0.2	29.1	0.2	0.50
White cell count (10^9/L)	0.83	0.01	0.84	0.01	0.86	0.01	<0.0001
Serum amyloid A protein (mg/L)	0.85	0.02	0.83	0.02	0.86	0.02	0.92
Homocysteine (mmol/L)	1.14	0.01	1.09	0.01	1.15	0.02	0.50
E-selectin (ng/ml)	1.77	0.01	1.76	0.01	1.80	0.01	0.20
P-selectin (ng/ml)	1.92	0.01	2.04	0.01	2.11	0.01	<0.0001
ICAM-1 (ng/ml)	2.46	0.01	2.47	0.01	2.48	0.01	0.21
VCAM-1 (ng/ml)	2.64	0.01	2.63	0.01	2.63	0.01	0.51
C-reactive protein (mg/L)	0.16	0.03	0.11	0.03	0.22	0.03	0.17
IL-6 (pg/ml)	0.37	0.01	0.37	0.01	0.36	0.01	0.88

Table 6.7 Odds ratio (95% CI) of coronary heart disease in men who had values of IL-18 in the top third of the distribution of controls relative to those who had values in the bottom third.

All 595 cases and 1238 controls:

IL-18 values (pg/ml)	Cases	Controls	Odds ratio (95% CI) adjusted for:			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
346-2068	242	414	1.55 (1.21, 1.98)	1.48(1.15, 1.90)	1.30 (0.99, 1.69)	1.12 (0.84, 1.49)
234-345	193	413	1.24 (0.96, 1.59)	1.19 (0.92, 1.54)	1.06 (0.81, 1.39)	1.04 (0.78, 1.38)
29-233	160	411	1	1	1	1
Total	595	1238				
p-trend‡			0.003	0.008	0.03	0.19

267 cases and 798 controls without baseline evidence of coronary heart disease

IL-18 values (ng/ml)	Cases	Controls	Odds ratio (95% CI) adjusted for:			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
346-2068	150	306	1.63 (1.19 2.22)	1.53 (1.12, 2.10)	1.29 (0.92, 1.80)	1.11 (0.77, 1.50)
234-345	105	300	1.16 (0.84 1.60)	1.09 (0.79, 1.52)	1.03 (0.73, 1.46)	1.05 (0.73, 1.51)
47-233	93	305	1	1	1	1
Total	348	911				
p-trend‡			0.03	0.06	0.34	0.76

*blood pressure, total cholesterol, HDL cholesterol, body mass index, history of diabetes, baseline CHD, social class, physical activity

† IL-6 and CRP fitted as logged variables

‡ Only included where significant in unadjusted model in Tables 6.6-6.9

Trends fitted using IL-18 as a continuous variable

Table 6.8 Odds ratio (95% CI) of coronary heart disease in men who had values of TNFα in the top third of the distribution of controls relative to those who had values in the bottom third.

All 535 cases and 1155 controls:

TNFα values (ng/ml)	**Cases**	**Controls**	**Odds ratio (95% CI) adjusted for:**			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation †
1.17-30.0	156	382	0.74(0.57, 0.95)	0.74 (0.57, 0.96)	0.74 (0.57, 0.98)	0.66 (0.49, 0.88)
0.77-1.16	164	384	0.75 (0.59, 0.97)	0.75 (0.59, 0.97)	0.75 (0.57, 0.98)	0.72 (0.54, 0.95)
0.05-0.76	215	389	1	1	1	1
Total	535	1155				

310 cases and 854 controls without baseline evidence of coronary heart disease

TNFα values (ng/ml)	**Cases**	**Controls**	**Odds ratio (95% CI) adjusted for:**			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
387-2500	93	275	0.81 (0.59, 1.12)	0.82 (0.60, 1.13)	0.87 (0.62, 1.22)	0.77 (0.54, 1.10)
207-386	91	277	0.79 (0.57, 1.09)	0.78 (0.57, 1.08)	0.80 (0.57, 1.12)	0.77 (0.55, 1.10)
9-206	126	302	1	1	1	1
Total	310	854				

*blood pressure, total cholesterol, HDL cholesterol, body mass index, history of diabetes, baseline CHD, social class, physical activity

† IL-6 and CRP fitted as logged variables

Table 6.9 Odds ratio (95% CI) of coronary heart disease in men who had values of MMP-9 in the top thirds of the distribution of controls relative to those who had values in the bottom third.

All 465 cases and 1076 controls:

MMP-9 values (ng/ml)	Cases	Controls	Odds ratio (95% CI) adjusted for:			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
873-3500	184	359	1.37 (1.04, 1.82)	1.27 (0.96, 1.69)	1.28 (0.95, 1.74)	1.13 (0.82, 1.56)
556-872	146	359	1.03 (0.77, 1.38)	1.01 (0.75, 1.35)	1.00 (0.73, 1.36)	1.01 (0.74, 1.14)
70-555	135	358	1	1	1	1
Total	465	1076				

268 cases and 800 controls without baseline evidence of coronary heart disease

MMP-9 values (ng/ml)	Cases	Controls	Odds ratio (95% CI) adjusted for:			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
873-3500	104	255	1.29 (0.94, 1.84)	1.17 (0.81, 1.67)	1.24 (0.85, 1.82)	1.07 (0.72, 1.61)
556-872	78	278	0.80 (0.55, 1.16)	0.79 (0.54, 1.14)	0.81 (0.54, 1.19)	0.82 (0.55, 1.23)
70-555	86	267	1	1	1	1
Total	268	800				

*blood pressure, total cholesterol, HDL, body mass index, history of diabetes, CHD, social class, physical activity

† IL-6 and CRP fitted as logged variables

Table 6.10 Odds ratio (95% CI) of coronary heart disease in men who had values of sCD40L in the top third of the distribution of controls relative to those who had values in the bottom third.

All 464 cases and 1073 controls:

sCD40L values (ng/ml)	Cases	Controls	Odds ratio (95% CI) adjusted for:			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
387-2500	135	358	0.75(0.57, 0.99)	0.74 (0.56, 0.97)	0.77(0.57, 1.04)	0.77 (0.57, 1.05)
207-386	150	356	0.87(0.66, 1.13)	0.87(0.66, 1.14)	0.86(0.65, 1.15)	0.88 (0.66, 1.18)
9-206	179	359	1	1	1	1
Total	464	1073				

267 cases and 798 controls without baseline evidence of coronary heart disease

sCD40L values (ng/ml)	Cases	Controls	Odds ratio (95% CI) adjusted for:			
Tertiles	N	N	Age and town only	Age, town and smoking status	Age, town, smoking & risk factors*	Age, town smoking, risk factors* and inflammation†
387-2500	78	264	0.77 (0.57, 1.10)	0.74 (0.54, 1.10)	0.80 (0.55, 1.16)	0.79 (0.54, 1.17)
207-386	89	262	0.93 (0.66, 1.30)	0.95 (0.68, 1.34)	0.88 (0.61, 1.26)	0.90 (0.62, 1.30)
9-206	100	272	1	1	1	1
Total	267	798				

*blood pressure, total cholesterol, HDL cholesterol, body mass index, history of diabetes, baseline CHD, social class, physical activity

† IL-6 and CRP fitted as logged variables

Table 6.11 Comparison of 4-year regression dilution ratios of MMP-9, sCD40L, IL-18 and TNFα with previously published results for other inflammatory and conventional risk markers (Emberson et al, 2004).

	No of pairs observations	Regression dilution coefficient	Regression dilution ratio (1/RD coeff)	Correlation (Spearman) r
IL-18	158	0.71	1.41	0.74
TNFα	158	0.25	4.00	0.36
MMP-9	138	0.46	2.17	0.49
sCD40L	138	0.47	2.13	0.32
CRP	297	0.52	1.92	n/a
Fibrinogen	297	0.61	1.64	n/a
Total:HDL cholesterol ratio	297	0.77	1.30	n/a
Systolic BP	297	0.61	1.64	n/a

n/a – data not available

6.4 DISCUSSION

6.4.1 Population Distributions of IL-18 and TNFα and Viability of Samples

The IL-18 levels among the control population (median 283 pg/ml [IQR 204-381]) were comparable to the median levels in the appropriate age groups of the MONICA-4 study (45-64 years ~251 pg/ml in men). This observation suggests that the BRH study samples are suitable for the measurement of IL-18 despite sample age and previous thaws. The comparison of this population with MONICA-4 also supports observations that the median IL-18 level among CHD cases in this study is higher than expected in middle-aged men (308 pg/ml [224-406]).

In contrast to IL-18, TNFα levels in this cohort (both cases and controls) were very much lower (around half) compared to those observed in the eldest age group of the MONICA-4 study (median 0.92 pg/ml [IQR 0.69-1.36] among BRH study controls compared to 1.90 pg/ml [1.50, 2.35] in MONICA-4). Despite this, the range of the 95% confidence intervals between in controls in the BRH study (0.25 – 4.15 pg/ml) is actually reasonably similar to the eldest age group in MONICA-4 (1.10 - 4.26 pg/ml). This suggests the data is skewed by an increased number of low observations in the current study. The reasons for this are not clear, but the possibility of sample degradation over time is hard to exclude. These observations are further discussed in Chapter 12.

6.4.2 General Observations

Before making general conclusions it is important to establish the relative strengths and limitations of the current study. Firstly, it is a large and powerful case - control study within a well-defined and established cohort of relatively unselected (male gender of age 40-59 selected from GP practices in one of 24 selected towns, but no medical exclusion criteria), demographically representative middle-aged men. For this reason it was an ideal, powerful resource to assess inflammatory markers as potential novel risk markers. Indeed, at the outset of this thesis (and even to date), the number of cases in this study made it the most powerful case - control study performed on any of these markers, even including studies examining total CVD events as end-points.

The potential limitations of the study lie in the quality of the serum samples used. While there is no question that the samples were obtained within rigorous guidelines, they are however old (>25 years) and have been thawed and re-frozen on a number of occasions

(~4-5 occasions), and the concession must be made that this may have unknown (and practically un-testable) effects on results obtained for any given marker dependant on its relative chemical stability. The thesis demonstrated in a preliminary study in Chapter 3 that the effects of storage (and indeed freeze-thaw cycles), although not negligible, appeared uniform on all samples tested in 10 normal people. However, extrapolating these preliminary, limited results to the BRH study is quite a proposition. Supporting the validity of the study, it has been shown that the IL-18 levels within controls in this population is comparable to those in the appropriate age groups for men in the MONICA-4 cohort, in which the plasma samples are much newer, and have been subjected to fewer freeze-thaws. The direct comparison of TNFα levels between the studies however shows different median values. There is no such opportunity for comparison for MMP-9 and sCD40L, although the levels obtained here are much lower (particularly for sCD40L) than those seen in the ten normal people in Chapter 3.

6.4.3 IL-18, Risk Factor Correlations, and Risk of CHD in Middle-Aged Men

In the present study, circulating IL-18 was strongly related to cigarette smoking and with level of triglyceride and HDL-cholesterol (inversely) in the control population. It was also associated with the inflammatory markers IL-6, C-reactive protein and other acute phase markers (including haemostatic factors) and adhesion molecules. In contrast to MONICA-4 there was no correlation with age, which is probably due to the narrow age range of BRH study subjects. There were also only weak associations with BMI and non-fasting insulin levels. IL-18 is generally thought to correlate with markers of the metabolic syndrome, although the present results and some more recent observations may question the strength of the association between IL-18 and BMI (see 4.4.2) and these men, were studied in 1978-80, when obesity was less prevalent in Great Britain compared to other recent studies. This weak association does not preclude a role for IL-18 in the metabolic syndrome, IL-18 may predict the risk of incident type II diabetes after adjustment for conventional CVD risk markers (Thorland et al, 2005). The association of IL-18 with metabolic syndrome markers will be considered in more detail in future chapters.

The results of the present study suggest that any independent association between IL-18 and CHD risk in middle-aged men is modest. Baseline circulating IL-18 concentrations were positively associated with CHD risk on univariable analysis. The strength of independent association between IL-18 and CHD is (OR 1.3) is more marginal than the

increased risk of ~1.8 reported from a smaller previous nested case control study of middle-aged men (Blankenberg et al, 2003). In our study (unlike that of Blankenberg) the association between IL-18 and CHD risk, although borderline after adjusting for conventional risk markers, was effectively abolished after adjustment for CRP and IL-6. IL-18 is, as previously discussed, pivotal in innate and adaptive Th1 inflammatory responses, and is present in human atherosclerotic lesions, and at higher concentrations in unstable plaques (Mallat et al, 2001). In animal models, IL-18 administration leads to increases in atherosclerotic lesion size and promotes increased numbers of T-lymphocytes in the lesion (Whitman et al, 2002). Despite this, absence of independent association with risk of CHD here does not add strong evidence to a role for IL-18 in atherogenesis. Our results are in broad agreement with recent results in the MONICA/KORA study of men and women (see table 1.6), where IL-18 was associated with ~1.4 increased risk (top versus bottom tertile) of CHD in a healthy population, but the risk was abrogated on adjustment for confounders (Koenig et al, 2006). However, more data is needed in other population cohorts to explore these results further.

6.4.3 TNFα, Risk Factor Correlations, and Risk of CHD in Middle-Aged Men

Baseline circulating levels of serum TNFα in the control population were positively associated with markers of inflammation as expected. A borderline inverse association with total cholesterol was noted, although no association with other lipid markers, or indeed any other conventional risk factors was observed. This profile of associations with other markers is very similar to that noted by another recent study, including no association with conventional risk markers, and a weak inverse association with total cholesterol (Tuomisto et al, 2006), although they also reported an inverse association with HDL cholesterol (non significant trend noted in BRH Study). In general, absence of strong associations of TNFα with conventional risk factors have been reported elsewhere (Ridker et al, 2000, [2]Cesari et al, 2003, Tuomisto et al, 2006).

Considering the weight of evidence that TNFα is associated with adipose tissue and the metabolic syndrome (Kern et al, 1995; Hotamisligil et al, 1995; Jovinge et al, 1998; Borst et al, 2004) it may be somewhat surprising that there was no observed association with BMI, insulin resistance or fasting glucose levels. This lack of association with metabolic syndrome markers was also noted for IL-18. Similarly there was no association of TNFα

with age. As with IL-18, this may be due to the narrow age range of the subjects. The MONICA-4 study (Chapter 4) found significant but weak associations of TNFα with both BMI and WHR, and results in other prospective studies (Tuomisto et al, 2006) are generally in agreement with this. As discussed for IL-18, the BRH study men were studied in 1978-1980 when obesity was less prevalent in Great Britain compared to other recent studies, so within the "normal" weight range circulating TNFα levels are poorly related to fat mass (measured by BMI or WHR), despite it being the prototypic adipokine (Hotamisligil et al, 1993).

Interestingly, this study has demonstrated an *inverse* association between thirds of TNFα levels and risk of incident CHD among middle aged men over a 20 year follow-up. The observation was only borderline significant on univariable analysis (0.74), but became stronger (0.66) after correcting for TNFα's positive associations with inflammatory risk markers and limited associations with conventional risk markers. It is hard to reconcile this observation with previous data (Table 1.4). A previous study found that only those with very high TNFα expression (population top 10%) were at risk for recurrent MI (Ridker et al, 2000), and non-linear associations of TNFα with risk of CHD have been found recently elsewhere (Tuomisto et al, 2006). Other published data, both in people with prevalent CVD and in normal populations is conflicting, probably partially due to low statistical power generally. It remains possible that this is a chance finding, although the confidence intervals in the fully adjusted model are reasonably convincing to the contrary (0.49-0.88). These findings require further investigation, which will be performed in future chapters.

6.4.4 MMP-9, Risk Factor Correlations, and Risk of CHD in Middle-Aged Men

Baseline circulating levels of MMP-9 were positively associated with circulating markers of inflammation, inversely related to FEV_1, and strongly associated with smoking. MMP-9 did not associate with most major CVD risk factors (BMI, blood pressure and cholesterol). These results are in broad agreement with the other reasonably powered prospective published study to date ([2]Blankenberg et al, 2003).

The elevated levels of MMP-9 observed in current smokers (who are at increased CHD risk) are of particular interest, because they may reflect greater expression of MMP-9 in tissues including arterial plaques, thus increasing the risk of plaque rupture. A previous study of MMP-9 risk with CHD has also shown an association with smoking

(Blankenberg et al, 2003), and mouse models have shown MMP-9 to be increased with smoke exposure in a TNFα-dependant manner ([2]Wright et al, 2007).

The univariable analysis showed that MMP-9 was associated with risk of incident CHD (OR 1.37, 95% CI 1.04-1.82), but this was heavily attenuated after adjusted for smoking (1.28, 95% CI, 0.95-1.74), although not by other conventional risk markers. The residual borderline association was nearly abrogated after adjustment for IL-6 and CRP. These findings suggest that MMP-9 has a moderate association with incident CHD in the general population, which is largely dependent on smoking and is also confounded by markers of the acute phase response.

The present study suggests a much more modest association of MMP-9 with incident CHD than that previously reported in a cohort of patients with unstable angina ([2]Blankenberg et al, 2003), possibly because such patients have a higher prevalence of unstable coronary artery plaques than the general population. The major hypothesized role of MMP-9 in CHD is in promoting plaque rupture, rather than directly in atherogenesis (see 1.5.4). It is interesting to speculate that MMP-9 may be a weaker marker of CHD risk in a generally healthy cohort than in those with established arterial disease who are often smokers. This is suggested in Table 1.8, where it is seen that one sizeable and one small study found significant independent associations in populations with prevalent disease ([2]Blankenberg et al, 2003; Eldrup et al, 2006) (although two other very small studies found no association), whereas the present study only finds a modest association dependant on smoking and classical risk markers a general population. These observations are consistent with a hypothesized role of MMP-9 to de-stabilise established plaques rather than to directly stimulate atherogenesis. They are also in agreement with other data showing that production of MMP-9 is higher in phenotypically unstable than stable plaques (Galis et al, 1994; Loftus et al, 2000; Sluijter et al, 2006). The BRH study shows some decrease in MMP-9 univariable risk associated with CHD when excluding those with baseline CHD (OR 1.29 vs 1.37), which would be consistent with this hypothesis, but the study is somewhat underpowered to make this observation.

Alternatively, discrepancies in results in the current study (compared to [2]Blankenberg et al, 2003) may reflect its greater size and hence more power and "regression to the mean", or may reflect the use of serum here, which may, according to some sources, be a artefactual confounder of the association with CHD (Gerlach et al, 2007). As discussed

previously (see 1.5.5) Gerlach et al suggest that MMP-9 levels should not be measured in serum samples due to such measurements "not being representative of circulating plasma levels of MMP-9." However, in the present study serum levels of MMP-9 still show an association with CHD and correlate with conventional risk markers, in a manner broadly similar to Blankenberg et al, 2003, suggesting serum levels are not merely an *in vitro* artifact.

6.4.5 sCD40L, Risk Factor Correlations, and Risk of CHD in Middle-Aged Men

Baseline circulating serum levels of sCD40L showed expected positive correlations with some other risk markers, particularly white blood cell count, and were also associated with circulating levels of P-selectin (Heeschen et al, 2003; Yan et al, 2004), which is a marker of platelet activation, as well as with D-dimer and t-PA. However, inverse associations were observed with some other CHD risk markers: vWF, fasting glucose, and triglycerides, as well as a positive association with HDL cholesterol. These associations are generally the reverse of that expected from a pro-inflammatory marker, and although the associations are not particularly strong, they are statistically significant. There were no associations with the more classical risk markers such as blood pressure, BMI, CHD at entry, physical activity, smoking or with age.

sCD40L was inversely associated with incident CHD on univariable analysis, to a similar degree seen in TNFα, and the relationship was not attenuated by age, town and smoking adjustment (~26%). The relationship became borderline significant after adjustment for classical risk markers, but was not attenuated by further adjustment for inflammatory risk markers. These results showing a borderline inverse association between serum sCD40L and CHD are consistent with a recently published study of the serum of people with prevalent vascular disease (Tanne et al, 2006), which also found a non-significant inverse association. These are the only two prospective studies reported in serum; all other studies in plasma have shown a positive relationship with CHD risk (see Table 1.10). It is difficult to compare these results directly to publications of plasma samples, due to the generally smaller size and wider confidence intervals in previous studies. One hypothesis to explain the inverse association of serum sCD40L with incident CHD may be that circulating platelets, which are thought to be the major sCD40L reservoir (Henn et al, 1998; Buchner et al, 2003), become "exhausted" during chronically elevated baseline inflammation. Hence, since extracting serum causes platelet degranulation, the measure

of total platelet pre-formed sCD40L is lower in those with low-grade chronic inflammation and hence propensity to experience CHD events. This would mean that although the measurement of risk is an *in vitro* artifact, it is a proximal marker of an *in vivo* phenomenon. This hypothesis is in line with a recent report that serum levels of sCD40L are lower and platelet mCD40L expression is higher in patients with unstable vs stable angina patients, whilst there was no difference between the groups in platelet poor plasma (Mason et al, 2005). This hypothesis has also been partially corroborated in other work using serum, where those with prevalent disease have lower serum levels of sCD40L (Aukrust et al, 1999; Tanne et al, 2006) and non-significant trends have been observed in other work with serum (Weber et al, 2007; Kiani et al, 2007).

As discussed previously, platelet degranulation is undoubtedly a problematic issue, however, EDTA anti-coagulant also has undesirable and to some extent "unspecific" effects on platelet function (Golanski et al, 1996), and all pre-analytical study authors noted a trend towards higher sCD40L levels in EDTA anti-coagulated compared with citrated plasma, which is almost certainly an *in vitro* artifact (Ahn et al, 2004; Weber et al, 2006; Varo et al, 2006). Despite this, circulating levels of sCD40L in healthy subjects is often at, or below, the limit of detection in citrated samples (Malarstig et al, 2006) in ELISAs that are not high-sensitivity (~90pg/ml), and that which is detectable may well be due to *in vitro* artifact, just as is the case in serum (Ivandic et al, 2007). In light of this perhaps sCD40L is better measured in serum after end-point degranulation, as observed in this study.

As with MMP-9, there is no reason to suspect that freeze-thaws or storage has adversely affected these results in terms of risk associations (although measured levels are certainly much lower than in Tables 3.3, 3.4, & 3.6 which are fresh samples from healthy people), but this is a possibility it is difficult to entirely exclude.

Further studies are required to address these analytical issues.

6.4.6 The Impact of 4-Year Regression Dilution on the 4 Inflammatory Markers

This is the only reported study to my knowledge of regression dilution ratios in these four inflammatory markers. They illustrate the inherent systematic underestimation bias present for any prospective marker in conventional prospective studies. With the

exception of TNFα; these cytokines appear as stable over time as some conventional risk markers, despite the assertion that biological instability is higher for "other" inflammatory markers compared to CRP (Pearson et al, 2003). The present study suggests that IL-18, MMP-9, and sCD40L may be as temporally biologically stable as CRP.

It is interesting to directly compare the data in this study of 4-year biological variation to that of the short-term biological variability in Chapter 3. Limited comparisons of these data are discussed in Chapter 12.

In BRH study, further examination of the effects of regression dilution on these markers could be performed by expanding the study to examine 1 week, 16 year, and 20 year (as well as 4 year) regression slopes for all the markers (Emberson et al, 2004)

6.4.7 Conclusions

This case control study from a cross section of middle-aged British men has found that IL-18 and MMP-9 both have positive associations with risk of CHD on univariable analysis, although the risk association comparing extreme thirds is relatively weak (increased risk ≤55%). After adjustment for conventional risk markers and inflammatory markers (CRP and IL-6) these associations were significantly attenuated, and both only have a non-significant (~10%) association with risk. TNFα and sCD40L demonstrated inverse associations with CHD risk in this study on univariable analysis, and the association was not attenuated in multivariable analysis for sCD40L (23%), and became stronger for TNFα (34%). All these risk associations are relatively modest, and the previously hypothesised association between IL-18 and TNFα and markers of the metabolic syndrome was also relatively weak. As expected, adjustment for regression dilution improves the univariable risk associations of all four inflammatory markers, particularly for TNFα, for which the RDR was 0.25. It may also improve multivariable associations, although adjustment for regression dilution in conventional risk markers would also be required to assess this. These results require further analysis for confirmation (particularly TNFα, since the results appear inconsistent with previous literature and may be a chance finding) in wider cohorts and in newer samples to eliminate the possibility of bias and confounding from this study.

I therefore further examined the associations of IL-18 and TNFα in a nested case-control study of CHD from another general population cohort – the Fletcher Challenge Study.

CHAPTER 7

ASSOCIATIONS OF IL-18, TNFα, AND IL-6 WITH RISK OF CHD IN A GENERAL POPULATION SAMPLE FROM NEW ZEALAND: THE FLETCHER CHALLENGE STUDY

7.1 INTRODUCTION

7.1.1 General Population Study of IL-18 and TNFα Risk Associations

Having examined the 4 novel inflammatory markers in the BRH Study, further work was required to corroborate and to expand on these findings. As discussed in Chapter 6, there are many strengths to the BRH Study, but also several possible critisisms. These require to be balanced by observations in other studies with differing strengths and limitations.

Stored plasma samples from the New Zealand Fletcher Challenge (FC) Study were available in our laboratory. New Zealand provides an interesting population for studying aetiology of cardiovascular disease. The population, like Scotland, has a high prevalence of CVD due to a generally high saturated fat diet, high smoking prevalence, and pronounced gradients in socioeconomic class across the population (with the native Maoris being generally more socially deprived) (Bell et al 1996; Bullen et al, 1996; Whitlock et al, 1997; Swinburn et al, 1998).

Using the Fletcher Challenge Cohort to examine risk associations of TNFα and IL-18 with CHD in a prospective case control study was an attractive possibility. Possible criticisms of the BRH study sample quality due to age and freeze-thaw cycles from the baseline samples, could be redressed by Fletcher Challenge plasma samples, which were more recently drawn, had always been stored at -80^{o}C, and the aliquots used for this study had not been subjected to previous freeze-thaw cycles. The study was also a cohort of a general population, unselected initially for age or sex. It hence removes some potential confounders of the larger BRH study. The cohort also has epidemiological strengths similar to the BRH; i.e. it is a good demographic representation of the New Zealand population (MacMahon et al, 1995), which has a high prevalence of CVD, and the cohort is well defined in terms of measurement and publication of conventional risk marker data. The difference in length of follow-up also removes potential confounders of

the BRH study, which was subject to greater regression dilution bias (16 versus 5 years follow-up) (Emberson et al, 2004). The disadvantages include a smaller number of cases (although still reasonably powerful) because it is a general population cohort followed-up for only 5 years, as well as a high proportion of "missing" samples, the samples used here being the final unthawed aliquots from the study.

The availability of stored plasma in this study provided the opportunity to investigate the associations of TNFα IL-18, as well as IL-6 for comparison in the population. As there were no serum samples available in the FC study, MMP-9 and sCD40L were not measured.

7.1.2 Previously Published Data on Associations of IL-18 and TNFα, and IL-6 with Risk of CHD in Generally Healthy Populations

As discussed in Chapter 1 & 6, at the outset of this thesis no study has examined risk associations for IL-18 and TNFα in healthy general populations. In contrast with these cytokines, IL-6 has been measured in a range of prospective studies, resulting in a meta-analysis (Mann et al, submitted) which shows that IL-6 predicts risk of CHD: OR 1.67 (1.35 – 2.05) in the top versus bottom tertile in fully adjusted models (see 1.2.8)

7.1.3 Aims

In using the Fletcher Challenge - University of Auckland Heart and Health Study we aimed to examine the associations of TNFα and IL-18, as well as IL-6, with conventional risk markers and with risk of incident CHD in a prospective nested case-control study of a general population. Since IL-6 is an established inflammatory CHD biomarker it forms a useful comparison to IL-18 and TNFα.

7.2 SUBJECTS AND METHODS

7.2.1 Establishment of Fletcher Challenge

As in other developed countries, CHD and stroke are leading causes of morbidity and mortality in New Zealand despite declining incidence since the early 1970s, and most of the conventional and genetic causes of CVD are postulated to play the same role in New

Zealand as elsewhere. However relatively little accurate information was available in the 1980s and early 1990s regarding the aetiology of heart disease and stroke in New Zealand populations, and the national Ministry of Health as well as the academic community in the country understandably required research into this, as well as data on the way in which risk factor demographics of the population were changing. At conception in 1991 the aim of the Fletcher Challenge-University of Auckland Heart and Health Study (FC) (comprising more than 10,000 participants) was to investigate the causes of common chronic diseases (e.g. CHD and stroke) as well as acute injuries (e.g. motor vehicle accidents) in New Zealand (MacMahon et al, 1995).

The FC Study has been used to determine the burden of many of these key risk factors within the population (Bell et al 1996; Bullen et al, 1996; Whitlock et al, 1997; Swinburn et al, 1998) and generally confirms similar findings to the CVD burden seen in Scotland and the UK. Further publications, data descriptions, and links can be found on the University of Auckland Heart and Health Study website: http://www.ctru.auckland.ac.nz/research/fletcher/index.html

The principal results of the Fletcher Challenge Study hence provided necessary data for estimating CVD prevalence and absolute CVD risk distributions in the New Zealand population using the Framingham risk prediction equations. This data has been used in a wide variety of studies for estimating CVD risk burden in the New Zealand population (Wells et al, 2006). The data is now fairly outdated for this purpose (Wells et al, 2006) and more recent data has been published on monitoring trends in cardiovascular risk factors in the New Zealand populations in the last twenty years up until 2003 (Metcalf et al, 2006). However, the demographics of the population has little bearing on the present study.

7.2.2 Subjects and Methods

The participants of the Fletcher Challenge study derive from two baseline samples obtained using the same protocol: one from an occupational workforce in New Zealand (the Fletcher Challenge Company) and the other a random sample from metropolitan Auckland, New Zealand (MacMahon et al, 1995). The aim was to invite all Fletcher Challenge employees to participate, and to "top up" the participation to a target of 10,000

people using the electoral role. In total, 10,525 individuals were recruited (8011 from Fletcher Challenge) representing a response rate of 74% from invitations.

Self administered questionnaires regarding medical histories were given to participants and physical examinations were carried out by qualified health professionals (MacMahon et al, 1995). Non fasting blood samples were taken at baseline, and serum and plasma samples (EDTA and citrate) were collected and stored at $-70^{o}C$. These samples were used in a nested case-control study that derived from prospective follow-up of the Fletcher Challenge subjects, using national record linkage systems based on personal health identification numbers. Cases were all members of the study who had CHD, defined as a fatal coronary event or non-fatal hospital-diagnosed myocardial infarction, during the follow-up period (median 5.5 years). In total, 256 cases with available stored plasma samples were identified. Matched controls were randomly sampled as described for nested –case control studies (Woodward, 2005). Using this method, any case may be selected as a control for other cases for whom the CHD event predates their own, and cases may have common controls. This serves to give a more accurate random picture of the "control" population at the point when an incident case occurs, and hence minimises selection bias (i.e. it is unknown who from the general population of potentially eligible controls will become a case at the time of sampling – which should theoretically be when an incident occurs). Besides matching by time of the index case's event, matching variables were age, sex and study population (occupational or general population). Age was matched, to the index case, within one year for all but 6 controls, these being matched within five years. In total, 703 controls were matched to cases; the minimum and maximum number of controls matched to any one case were 2 and 7, and the average control:case ratio was 2.75. Amongst the 703 matched controls were 615 separate individuals who were still free of CHD at the end of follow-up ("unique controls").

IL-18, TNFα and IL-6 were all measured as described in Chapter 2. Aliquots used in this study were previously unthawed.

7.2.2 Statistical analysis

Cases and unique controls were compared, for continuous variables, using t-tests after power transformations to better approximate normal distributions, where necessary and possible. Binary variables were compared through chi-square or Fisher's exact tests.

Distributions of TNFα and IL-18 would not normalise, and so non-parametric Wilcoxon tests were used for these. Spearman rank correlations between the inflammatory variables were calculated for unique controls. Odds ratios for CHD were estimated from conditional logistic regression models (Woodward, 2005) using the matched sets as units of observation. In addition to unadjusted (except for matching variables) analyses, adjustments were made for classical cardiovascular risk factors, with and without CRP.

7.3 RESULTS

7.3.1 Sample Availability

Due to sample attrition (these were residual unthawed samples), IL-18 and TNFα were assayed in 229 cases and 442 unique controls (77% of the total case-control study). IL-6 was measured in 225 cases and 427 controls. Those included here were no different from those without available samples in terms of age, sex, smoking status, systolic blood pressure, total cholesterol or body mass index (all $p>0.10$).

7.3.2 Baseline Characteristics of the Population

The baseline characteristics of the study population are shown in Table 8.1. Lipids (total cholesterol, HDL cholesterol, and triglycerides) and acute phase reaction markers (fibrinogen, CRP and IL-6) were all significantly different in the expected directions ($P<0.001$ for all). Plasma IL-18 and TNFα were also significantly higher in cases compared to controls (10.9%; $p=0.01$ and 10.0%; $p=0.024$ respectively). Although there was a trend for both BMI and SBP to be higher in the cases than in the controls, the observation did not reach significance. After removing subjects with a previous doctor diagnosis of heart disease, the mean SBP (standard deviation) amongst cases increased to 138.1 (21.2) mm Hg and BMI to 27.3 (4.0) kg/m^2 ($P<0.05$ for both).

7.3.3 Cytokine Correlations with Conventional Risk Factors

Table 8.2 shows that, among controls, IL-6 had significant age and sex-adjusted associations with BMI, HDL cholesterol (inversely), fibrinogen, D-dimer, t-PA, and CRP. IL-18 showed significant associations with BMI, HDL cholesterol (inversely), triglycerides, t-PA, and CRP. TNFα showed no association with BMI, but was associated

with triglycerides and HDL cholesterol (inversely). Neither IL-18 nor TNFα showed significant associations with other haemostatic variables (fibrinogen, D-dimer, or vWF). IL-18 and TNFα were both associated with other inflammatory markers (CRP and IL-6) and with each other (r = 0.28).

Both IL-18 (r=0.22) and TNFα (r=0.31) were significantly ($p<0.0001$) associated with age, after controlling for sex. Neither was significantly associated with current smoking ($p\geq0.26$).

7.3.4 Cytokine Associations with Absolute and Adjusted Risk of CHD

29.0% of people in the bottom tertile of IL-18 expression were cases, whereas 40.6% were cases in the top tertile. There was a consequent stepwise increase in the odds ratio (OR) across thirds of IL-18 (p for trend 0.02) after controlling only for the matching variables (OR 1.63; 95% confidence interval [CI] 1.08 - 2.46) (Table 8.3). Adjustment for classical CHD risk factors marginally attenuated this result to below the threshold for statistical significance (OR 1.50; 95% CI 0.91 - 2.48). Further adjustment for CRP did not attenuate the result.

32.8% of people were cases in the bottom tertile of TNFα expression, and only 40.7% were cases in the top tertile. There was no significant stepwise increase in the ORs across thirds of TNFα after controlling for matching variables (OR 1.33; 95% CI 0.87 – 2.02), with, again, marginal attenuation on adjustment for classical CHD risk factors and no further attenuation after adjustment for CRP.

When the 50 prevalent cases of CHD at baseline were deleted from the analysis, odds ratios comparing the extreme thirds all increased slightly, without altering the conclusions. For IL-18 the ORs (95% CIs) for CHD, top third v bottom third, were 1.79 (1.12-2.84) controlling for matching variables only, 1.76 (0.99-3.14) adjusted for classical risk factors and 1.82 (0.94-3.52) after further adjustment for CRP. The corresponding results for TNFα were 1.35 (0.84-2.15), 1.33 (0.76-2.34) and 1.32 (0.69-2.52).

In comparison, IL-6 was a stronger marker of CHD risk than either IL-18 or TNFα. As seen in Table 8.3, 40.9% of people in the bottom tertile of IL-6 expression were cases,

whereas the figure was 68.5% in the top tertile. There was a stepwise increase in the odds ratio (OR) across thirds of IL-6 (p for trend 0.0003) and the risk in the top tertile after controlling only for the matching variables was 2.84 (1.73 - 4.68) (Table 8.3). Adjustment for classical CHD risk factors marginally attenuated this result to 3.01 (CI 1.53 – 5.93), but largely only added noise to the signal. Further adjustment for CRP did not materially affect the result either, and although the confidence intervals of the associations widened, the trend across thirds was still significant (p=0.03).

Table 7.1 Baseline characteristics: means (standard deviations) or percentages and p-value for coronary heart disease cases and controls who were CHD free at end of follow-up

	Cases (n range: 215 - 246)	**Controls (n range: 410 - 472)**	**p-value**
Questionnaire			
Age (yrs)	60.6 (16.5)	59.8 (16.2)	matched
Sex (% Male)	76.8	76.5	matched
Baseline CHD (%)†	20.4	0	<0.0001
Smoking (% Current)	25.2	17.4	0.01
Physical measurements			
BMI (kg/m^2)	26.9 (4.1)	26.4 (3.9)	0.16
SBP (mmHg)	137.4 (21.4)	135.7 (20.3)	0.28
Blood Sample			
Lipids:			
Total cholesterol (mmol/L)	5.96 (1.19)	5.59 (1.10)	<0.0001
HDL cholesterol (mmol/L) *	0.90 (0.30)	1.10 (0.31)	<0.0001
Triglyceride (mmol/L) *	2.11 (1.80)	1.69 (0.99)	<0.0001
Haemostatic variables:			
Fibrinogen (g/L)*	3.52 (0.81)	3.28 (0.62)	0.0003
vWF (IU/dL) *	140.0 (60.8)	127.3 (51.9)	0.01
Fibrin D-dimer (ng/ml) *	333.9 (260.1)	252.7 (176.6)	<0.0001
t-PA (ng/ml) *	5.29 (2.26)	4.68 (2.15)	0.002
Inflammatory variables:			
C-reactive protein (mg/L)	3.82 (9.92)	2.02 (2.98)	<0.0001
Interleukin-6 (pg/ml) *	2.82 (3.54)	1.93 (2.28)	<0.0001
Interleukin-18 (pg/ml) ††	331.5 (167.8)	299.0 (175.2)	0.001
TNFα (pg/ml) ††	2.74 (3.96)	2.49 (3.94)	0.024

P values compare cases and controls: (1) for categorical variables, from chi-square tests, except † when Fisher's exact test was used; (2) for continuous variables, from t-tests, * after power transformations, except †† when Wilcoxon tests were used

Table 7.2 Age- and sex-adjusted Spearman correlations (r) of IL-6, IL-18 and TNFα with each other and established coronary risk factors in controls. p denotes the p-value for the test of a zero correlation.

	Cytokines					
	IL-6		**IL-18**		**TNFα**	
Variable	r	p	r	p	r	p
Body mass index	0.213	<0.0001	0.139	0.003	0.002	0.96
Systolic blood pressure	0.097	0.045	-0.017	0.716	0.029	0.54
Total cholesterol	0.006	0.90	-0.013	0.78	-0.073	0.13
HDL cholesterol	-0.174	0.0006	-0.312	<0.0001	-0.191	<0.0001
Triglyceride	0.079	0.11	0.175	0.0003	0.114	0.018
Fibrinogen	0.287	<0.0001	0.021	0.66	-0.032	0.502
Von Willebrand factor	0.058	0.23	0.100	0.047	0.070	0.17
Fibrin D-dimer	0.157	0.0014	0.071	0.17	0.088	0.09
t-PA	0.182	0.0002	0.146	0.004	0.097	0.05
C-reactive protein	0.461	<0.0001	0.206	<0.0001	0.124	0.015
IL-6	n/a	n/a	0.132	0.009	0.251	<0.0001
IL-18	0.132	0.009	n/a	n/a	0.282	<0.0001

Table 7.3 Odds ratios of coronary heart disease in men based on thirds of IL-18, TNFα and IL-6. Matching variables are age, sex, source of sample and time to event

	Cases	**Controls**	**Odds ratio (95% CI) adjusted for:**		
	n	n	Matched variables only	Classical risk factors*	Classical risk factors and CRP
IL-18 (pg/ml)					
>331	89	130	1.63 (1.08, 2.46)	1.50 (0.91, 2.48)	1.69 (0.94, 3.03)
236 - 330	73	148	1.15 (0.77, 1.73)	0.97 (0.60, 1.58)	1.04 (0.60, 1.82)
< 235	67	164	1	1	1
Total	229	442			
TNFα (pg/ml)					
>2.16	88	128	1.33 (0.87, 2.02)	1.23(0.75, 2.04)	1.25 (0.70, 2.25)
1.57 – 2.15	64	156	0.81 (0.54, 1.23)	0.68 (0.41, 1.12)	0.64 (0.36, 1.15)
< 1.56	77	158	1	1	1
Total	229	442			
IL-6 (pg/ml)					
> 2.15	89	130	2.84 (1.73 - 4.68)	3.01 (1.53 - 5.93)	2.79 (1.11 - 6.99)
1.16-2.15	73	148	2.10 (1.33 - 3.30)	2.62 (1.45 - 4.76)	2.13 (1.01 - 4.48)
< 1.16 pg/ml	67	164	1	1	1
Total	229	442			

* Systolic blood pressure, total cholesterol, HDL-cholesterol, triglycerides, body mass index and smoking status (never/ex/current cigarette smoker)

7.4 DISCUSSION

7.4.1 Population Distribution of IL-18 and TNFα

Comparing the present study to the MONICA-4 study, median IL-18 levels in the present study are very similar to medians in the same age range in the North Glasgow Survey (271 pg/ml among ~60 year old controls vs 251 pg/ml in the 55-64 year old MONICA-4 males) and are also highly comparable to those seen in the BRH study (Chapter 6).

TNFα levels between the two studies were also comparable, although the median circulating level appears to be slightly higher in the present study (1.74 pg/ml among controls vs ~1.59 pg/ml in the 55-64 year old males). Due to the marginal magnitude of this difference, it is likely to be due to drift of the calibrants between TNFα kit lots over time, or simply due to improved sample quality. Since levels of TNFα noted in this study are slightly higher than those seen in MONICA-4, they are very much higher than those seen in the BRH study (Chapter 6).

7.4.2 Associations of IL-18 and TNFα with Conventional Risk Markers

Despite MONICA-4 (and other studies; Woodward et al, 1997, [2]Wannamethee et al, 2005) confirming that circulating levels of cytokines, including IL-18 and TNFα, are increased in smokers, levels of both cytokines increased with age, but were not associated with current smoking in the present study. The BRH study found that while TNFα was not associated with current smoking (although there was an increasing trend across thirds of the population), whereas IL-18 was ($p<0.0001$). The discrepant results in the FC study may be reflective of reduced power of smoking observations in the present study since only ~17% of controls smoked, whereas the figure in GLAMIS was 45.6%. This means there were only 77 control smokers in the current population; probably not a statistically sufficient number to study smoking as a dichotomised (current vs ex/never) variable.

Previous prospective publications generally report that IL-18 shows relatively weak associations with HDL cholesterol (inversely) and triglycerides (but not total cholesterol), as well as weak but statistically significant associations with CRP and IL-6, but not with

age, BMI, or fibrinogen levels (Blankenberg et al, 2002, 2003; Koenig et al, 2006), associations which have also been noted in retrospective studies (Hulthe et al, 2006). Our findings in this prospective study are broadly similar to these, and we also report no association with systolic blood pressure (which has previously been suggested to show very weak associations with IL-18: Koenig et al, 2006 & BRH, Chapter 6) or with some haemostatic variables (D-dimer and vWF).

Likewise for TNFα, this study generally confirms previous prospective reports ([3]Ridker et al, 2000; [2]Cesari et al, 2003; Tuomisto et al, 2006& Chapter 6) that TNFα shows weak (but in this study statistically significant) associations with HDL cholesterol (inversely), and with other inflammatory markers (IL-6, IL-18 and CRP, although not fibrinogen). Also consistent with these reports, TNFα levels showed no strong association with BMI or systolic blood pressure, and associations with fibrinolytic variables (D-dimer and t-PA) were weak. These observations are also broadly similar to those noted in the control populations of retrospective studies (Jovinge et al, 1998; Skoog et al, 2002).

IL-6 demonstrated expected associations with HDL cholesterol and inflammatory risk markers (including very strongly with CRP and fibrinogen) but, in contrast to IL-18 and TNFα, also had strong associations with BMI and fibrinolysis markers (t-PA and D-dimer). In terms of BMI associations, this result at least demonstrates that the control group had sufficient BMI diversity for strong association with some cytokines to be possible.

Weak associations of IL-18 and TNFα with BMI and measurements of adiposity will be discussed further in Chapter 11.

7.4.3 Associations of Cytokines with Risk of CHD

In this nested case – control study, plasma levels of IL-18 showed a significant association with CHD risk. This association was only marginally attenuated after adjustment for classical CHD risk factors and CRP to borderline significance. This is in agreement with the PRIME study of middle-aged men, where although IL-18 correlated with HDL cholesterol and inflammatory markers, adjustment did not result in an

attenuation of the association ([1]Blankenberg et al, 2003). However, this study found that univariable risk (OR 1.63), like in the BRH study (OR 1.55), was lower than in the PRIME study (OR 1.80). Despite this seeming "regression to the mean" discrepancies in the data still exist. The current FC data suggest that adjustment for confounders has little effect on risk associations. This is in agreement with PRIME, but not with the BRH study, where IL-18 was heavily attenuated on adjustment. Why this discrepancy? It is interesting to note that the confounding adjustment models for the three studies are very similar, however, the BRH study additionally adjusts for both social class and physical activity. Possibly it is the residual association of IL-18 with social class which allows it to remain associated with CHD in the FC and PRIME studies (as was seen in the MONICA-4 chapter). Indeed, social class may be associated with risk of CHD in adjusted models to a similar extent to IL-18 (OR 1.28-1.36 in the top two fifths of a population in a recent study [Tunstall Pedoe et al, 2006]). Despite this evidence of a moderate association between circulating levels of IL-18 and incident CHD, one recent case - control study in the MONICA/KORA cohort of healthy men and women (with a similar number of cases to PRIME) reported no multivariable association of IL-18 with risk (Koenig et al, 2006). It is of note that Koenig et al adjusted for alcohol and physical activity in their model of IL-18 risk associations (Koenig et al, 2006) and alcohol consumption is a major correlate of social class.

Hence overall, any association between IL-18 and CHD risk in general population studies is likely to be moderate overall and confounded by social class and alcohol consumption. In addition, the association of IL-18 with atherosclerotic burden has recently been debated. Espinola-Klein et al, 2007 found in 720 patients that IL-18 levels were not different in patients with no significant CAD, those with CAD, or those with clinically relevant multi-vessel disease. Studies in general argue for (Yamashita et al, 2003; Yamaoko et al, 2003; Suchanek et al, 2005) or against (Chapman et al, 2006, Espinola-Klein et al, 2007) significant associations of IL-18 with atherosclerotic burden. Data in this study cannot really add to this debate, however Espinola-Klein et al argue that IL-18 may be a better risk marker in those with prevalent disease. In the current study, exclusion of those with baseline CHD did not significantly alter risk associations.

Plasma levels of TNFα showed a weak, nonsignificant association with CHD risk in the present study (OR 1.33 [0.87, 2.02]), which was attenuated after adjustment for CHD risk factors and CRP. One prospective study with 188 CHD events in people aged 70 -79 years at entry did find a significant, linear, independent association between circulating TNFα levels and CHD ([2]Cesari et al, 2003). Despite this, overall results are conflicting (Table 1.4). A recent population study reported a significant (but non-linear) association of TNF with risk, with the top 3 quartiles (combined) of the population at increased risk (Tuomisto et al, 2006). In contrast, the BRH Study demonstrated a borderline inverse association. Hence overall, the results remain inconsistent.

In the present study, although the top tertile were not at significantly increased risk, it remains possible that people with very high circulating levels of TNFα are at increased risk of CHD events, and this would be consistent with one previous study of people with angina pectoris ([3]Ridker et al, 2000) and with data in those with active inflammatory diseases such as RA who are at increased risk of CHD (Sattar & McInnes, 2005). No study to date has been sufficiently powered to test this hypothesis by accurately examining risk in the top 10-20% of the population for TNFα expression. It also possible that low circulating levels of TNFα may have some atheroprotective effects, since some data from mouse models suggests this possibility (Schreyer et al, 1996, 2002). This may help explain some non-linear associations with risk found in the literature. Other conclusions may be that the ELISA method used is insufficiently sensitive to detect levels in normal populations accurately. If this is the case then one would have to conclude that epidemiological observations linking TNFα to CHD risk (in various ways) were predominantly chance findings, which become more likely when making a large number of statistical inferences from one data set (Woodward et al, 2005). However, the likelihood of this happening repeatedly over a number of studies for one marker is small. Clearly if there is a relationship between TNFα and CHD, it is a complex one that requires further studies, and a meta-analysis of these in due course.

IL-6, in contrast to IL-18 and TNFα, demonstrated both strong unadjusted (2.84 [1.73 - 4.68]) and fully adjusted (2.79 [1.11 - 6.99]) associations with risk of CHD, Cocsistent with the Mann et al meta-analysis (1.2.8).

7.4.4 General Conclusions

Of the two prospective studies of CHD in this thesis the Fletcher Challenge Cohort is the more general population, with relatively young plasma samples (~13 years) that have been stored most rigorously (-80^{o}C) and are previously unthawed. Hence despite a relatively small size, the study is a good candidate to detect any strong risk associations. Indeed, this is re-enforced by the result that IL-6 was a (relatively) strong independent predictor of CHD, which is consistent with the literature. Despite this, any associations of IL-18 or TNFα with risk of CHD have been shown to be moderate and confounded, such that residual associations are probably not clinically significant. This does not preclude a causal role of IL-18 or TNFα in the process of atherogenesis, nor as potential therapeutic targets.

As a final step in the examination of IL-18 and TNFα with risk of CHD, the next chapter will estimate risk associations for cardiovascular disease in the MONICA-4 cross-sectional population (Chapter 4).

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CHAPTER 8

RISK ASSOCIATIONS OF IL-18, TNFα, IL-6, FIBRINOGEN AND CRP WITH CARDIOVASCULAR DISEASE IN THE MONICA-4 SURVEY: A PROSPECTIVE COHORT STUDY

8.1 INTRODUCTION

8.1.1 Risk Associations Within a Cohort

Prospective nested case-control studies are a powerful means of elucidating risk associations of prospective risk markers within a cohort, and the method is relatively cheap since the entire cohort is not measured. Although this method is less bias-prone than retrospective case-control studies, residual bias is introduced in the process of selecting controls from the cohort. Although there are methods available to limit this bias (Woodward, 2005) it is difficult to exclude as a confounder entirely.

Estimating the associations of putative risk markers in a cohort is the least bias-prone method of establishing a risk association, since the entire "non-case" cohort are controls and there is no selection process. Causality still cannot be implied in the absence of placebo-controlled trials to specifically alter biological function of the marker (Lowe et al, 2005), and there are other trade-offs for limitation of bias confounding. Due to controls being unselected there is increased confounding from conventional risk factors which could be potentially matched in nested case-control studies (age, sex, geographical location, baseline CHD etc), which results in wider confidence intervals after adjustment. In addition funding constraints generally limit the cohort size, the rates of CHD events are lower than in more selected studies (e.g. BRH study of middle-aged men), and hence statistical analysis is powered by fewer cases.

Despite these limitations, cohort studies are the "best" method to estimate risk associations, since the control cohort is a more realistic representation than in nested case-control studies (because they are unselected).

After performing the analysis of the demographic associations of IL-18 and TNFα in the MONICA-4 study, data for a 10 year follow-up study of incident CHD became available. Due to the cytokines already having been measured, this data was used to estimate associations of IL-18 and TNFα (as well as IL-6, fibrinogen and CRP) with CVD risk in a general population survey of North Glasgow, as described in Chapter 4.

8.1.2 Previous Cohort Studies of IL-18 and TNFα

There have been no previous reports of full cohort studies of IL-18 and TNFα which have examined risk associations. Previously published data in case-control studies is as discussed in Chapters 6&7.

All inflammatory markers were measured as described in Chapter 2. As described in Chapter 4, MONICA-4 aliquots had not been previously thawed before the measurement of IL-18 or TNFα, and were thawed on 1 further occasion to measure IL-6.

8.1.3 Aim

The aim of this study was to elucidate CHD risk associations of IL-18, and TNFα, as well as IL-6, fibrinogen and CRP for comparison in the MONICA-4 cohort study, the cohort previously described in Chapter 4. This was with the aim of comparing a range of novel and established inflammatory risk markers and their relative associations with CVD risk, as well as generating additional data on CVD risk associations in IL-18 and TNFα to perform a meta-analysis in the concluding stages of this thesis.

8.2 METHODS

8.2.1 Subjects and Methods

The characteristics of the cohort have been described in detail in Chapter 4. In brief, 1836 men and women aged 25-64 years randomly sampled from GP registers gave blood samples and undertook a standard MONICA questionnaire at a health screening clinic in 1995 (full details see chapter 4).

The MONICA-4 cohort was followed for 10 years from baseline for incidence of MI, stroke, development of unstable angina or sudden coronary death, which were the qualifing events for entry into the case-control study as a case subject. Diagnosis of fatal and non-fatal CVD events was based on medical records of general practioner reports and hospitalizations according to WHO criteria (Walker et al, 2004) and from death certificates using ICD-9 codes 410-414. During follow-up there were 228 qualifying CVD events in the cohort (12.4% event rate over 10 years).

8.2.2 Statistical Analysis

Distributions of IL-18, TNFα and IL-6 were log-transformed to approximate normality, as were other inflammatory markers where appropriate. Inflammatory markers were analysed by pre-specified equal thirds of the control population distribution, as well as analysing markers as continuous variables (expressed per 1 quartile increase in the distribution for the marker). Odds ratios for CHD were estimated from conditional logistic regression models (Woodward, 2005). These were computed both with partial adjustment (age and sex) and with full adjustment for potential confounding factors (age, sex, serum cholesterol, HDL cholesterol, smoking, diabetes, SBP, family history, and socioeconomic status i.e. the ASSIGN risk factors).

8.3 RESULTS

8.3.1 Cardiovascular Disease in the Population

Of the 1836 men and women sampled, 228 experienced a CVD event during the 10 year follow-up. The overall event rate for MONICA-4 was therefore 12.4%. Excluding those with baseline CVD, there were 131 CVD cases from 1596 participants.

8.3.2 Risk Associations of IL-6, Fibrinogen, and CRP.

Table 8.1 summarises all inflammatory markers for risk associations with risk of all-CVD both by tertiles and as continuous variables.

IL-6 was significantly associated with risk of CVD as a continuous variable (OR 1.11 [95% CI 1.03-1.20]). Exclusion of those with prevalent disease did not attenuate these associations, and neither did adjustment for conventional risk markers attenuate the association (1.12 [1.03-1.22]). The top tertile of IL-6 expression were at a ~2-fold increase in risk compared to the bottom tertile, which attenuated to ~1.5-fold after full adjustment. A quartile increase in levels of fibrinogen was associated with an increase in risk (1.37 [1.19-1.57]), which was marginally attenuated but remained significant after full adjustment (1.24 [1.07-1.43]). Excluding those with prevalent disease did not markedly improve associations (1.24 [1.03-1.55]). CRP was also associated with risk, a 1 quartile increase resulting demonstrating an OR of risk of 1.22 (1.15-1.29), which was again only marginally attenuated on full adjustment (1.19 [1.12-1.26]) or exclusion of those with prevalent disease.

Comparing middle with bottom thirds, no marker was significantly associated with risk of CVD in any model. Additional adjustment for alcohol consumption did not further attenuate any observed associations (data not shown).

8.3.3 Risk Associations of IL-18 and TNFα

IL-18 showed only borderline significant risk associations with CVD as a continuous variable after age and sex adjustment (1.09 [0.97-1.23]). After full adjustment the association was abrogated to unity. Excluding those with prevalent disease did not materially impact these observations. Comparing extreme thirds of the population in the all-CVD group, IL-18 was a significant risk marker on age and sex-adjustment (OR 1.61), although the result was again attenuated to non-significance (although not abrogated) after adjusting (OR 1.25).

In contrast, TNFα was significantly associated with risk of CVD as a continuous marker after age and sex adjustment (1.11 [1.03-1.19]) and after full adjustment (1.12 [1.03-1.21]). Exclusion of those with prevalent disease did not materially impact these results. The OR for TNF-α after adjustment for fibrinogen, IL-6 and CRP, as well as all conventional risk factors and SES, was 1.12 (1.02-1.24) (data not shown in Table 8.1),

indicating that TNFα risk associations were independent of other inflammatory risk markers in this study. The only other marker for which this was true was CRP (1.17 [1.07-1.27] adjusted for conventional risk markers, fibrinogen, IL-6 and TNF-α). In tertile analysis, TNFα was a significant risk marker comparing extreme thirds after only age and sex adjustment (OR 1.70), but the result was attenuated to borderline significance after full adjustment (OR 1.35).

Like the other inflammatory markers, comparing middle with bottom thirds, neither IL-18 not TNFα was significantly associated with risk of CVD in any model, and adjustment for alcohol consumption did not further attenuate any observed associations (data not shown)

Table 8.1 MONICA-4 follow-up risk associations (ORs) for inflammatory risk markers, assessed as continuous variables (per 1 quartile increase of marker in the population) or by tertile analysis. Min adjustment model is age and sex-adjusted. Full adjustment model adjusts for ASSIGN risk factors (methods). Bold value indicates significance ($p<0.05$). Incident CVD excludes those with baseline CVD (total 131 events from 1596 people after exclusions)

	Continuous analysis				Tertile analysis			
	All CVD		Incident CVD		All CVD			
Comparison					2 vs 1	3 vs 1	2 vs 1	3 vs 1
Adjustment	Min	Full	Min	Full	Min	Min	Full	Full
IL-6	**1.11**	**1.12**	**1.11**	**1.12**	1.18	**2.00**	0.94	1.55
Fib	**1.37**	**1.24**	**1.43**	**1.27**	1.13	**1.81**	1.01	**1.49**
CRP	**1.22**	**1.19**	**1.23**	**1.20**	1.63	**3.54**	1.42	**2.82**
IL-18	1.09	1.02	1.08	1.00	1.14	**1.61**	0.98	1.25
TNFα	**1.11**	**1.12**	**1.10**	**1.12**	1.29	**1.70**	1.12	1.35

8.4 DISCUSSION

8.4.1 MONICA-4 Population Event Rate:

An overall event rate of 12.4% in a general population is quite high, especially since the eldest subjects in the survey were 64 years. However, the latest estimates from the British Heart Foundation (www.heartstats.org; 2007 CVD Report) suggest prevalence rates of CHD (which would account for most observed CVD events) in Scotland are 8% in men and 6% in women. Given this, and the fact that Glasgow has a poorer public health record even than the rest of Scotland (sub-optimal health of the cohort illustrated in Table 4.1), a 12.4% rate of CVD over ten years is perhaps a proportionate outcome.

8.4.2 CVD Risk Associations in Established Inflammatory Risk Markers

As broadly expected IL-6, fibrinogen, and CRP showed significant and independent CVD risk associations in the MONICA-4 study. This is in line with the published literature in meta-analyses (Mann et al, submitted; Fibrinogen Studies Collaboration, 2005; Danesh et al, 2004). The absence of a significant association of IL-6 with risk in the top tertile of the fully adjusted model is suggestive that the study is a little underpowered, since the marker is significantly associated with risk on meta-analysis in the literature (Mann et al, submitted). Commensurate with a suggestion of lack of power, no marker was associated with risk comparing middle with bottom tertiles. Lack of power is generally an issue in full cohort studies, which need to be very large to attain large numbers of cases over reasonable follow-up periods. 12.4% may be a high event rate for a cohort study over 10 years, but at this rate the population would require >5000 participants to attain the same number of cases, and hence power, as the BRH study. Studies of such size are impractical in terms of expense for examining potential novel risk markers.

8.4.3 IL-18 and TNFα Associations with Risk of CVD

To my knowledge, this is the first cohort study examining CVD risk associations of IL-18 or TNFα. Despite the lack of power discussed above, the design of the study makes it an important resource in examining risk associations.

Interestingly, the OR of 1.6 comparing extreme thirds of IL-18 in the age and sex adjusted model is highly comparable to that seen in the Fletcher challenge study in the matched variables model (OR 1.63). Contrasting the studies however, adjustment in the full model in this study heavily attenuated the association to a borderline significant trend, whereas the association remained uninfluenced (but borderline significant due to widening confidence intervals) in the fully adjusted Fletcher Challenge model (Table 7.3). The main difference in the adjustment models is the additional adjustment for socio-economic status (by postcode) in the present study. As discussed in Chapter 7, in 7.4.3, the attenuation of moderate IL-18 associations with risk after adjusting for socio-economic status may be commensurate with the published literature ([1]Blankenberg et al, 2003; Koenig et al, 2006) and with the data in this body of work (BRH study: Chapter 6). This observation is further discussed in Chapter 12.

In this study TNFα demonstrates a positive association with risk of CVD, which is independent of conventional risk markers (and other inflammatory markers) in the continuous model, but not in the tertiles model, where the association was reduced to borderline significance. This positive association is in direct contrast to the BRH study (Chapter 6) where the association was inverse, and became stronger on adjustment. The association noted here was also stronger than that noted in the Fletcher Challenge study, where the OR in the top tertile was non-significant (1.33) and only slightly attenuated on adjustment (1.27). Overall, one would conclude that the risk association patterns shown by TNFα in this body of data, and in the literature in general (Table 1.4) are inconsistent. Reasons for this inconsistency are explored, along with appropriate meta-analysis of both IL-18 and TNFα in Chapter 12.

8.4.4 General Conclusions

Despite moderate independent associations of TNFα with risk of CVD in this study, it seems unlikely, overall, that either IL-18 or TNFα have strong associations with risk of CHD or CVD (Chapters 6-8), although this will be formally tested by meta-analysis in Chapter 12. Having extensively examined risk associations with of these cytokines CHD and CVD, I moved on to explore the risk associations with stroke.

CHAPTER 9

ASSOCIATIONS OF IL-18, TNFα, AND IL-6 WITH PROGRESSING STROKE AND WITH OUTCOME FROM ACUTE STROKE: THE STROKE IN PROGRESS (SIP) STUDY

9.1 INTRODUCTION

9.1.1 Acute and Progressing Stroke

Acute ischaemic stroke remains clinically problematic to treat, as suggested by the above quote. Decades of research have investigated the issues of how to ensure patients admitted with such strokes are quickly diagnosed, treated effectively to minimise further ischaemia, ensure there is not recurrent thrombosis (without causing haemorrhage), prevent subsequent myocardial damage, and prevent further inflammation-driven neurological degeneration (Khaja et al, 2007). Despite continued work in these areas and many promising therapies being evaluated in animal trials, few pharmacological interventions available for acute stroke have not been developed. Indeed, recombinant tissue plasminogen activator therapy (rt-PA) is still the only drug recommended for thrombolytic therapy in acute stroke, and at that with many contraindictions, such as a three hour symptom onset to administration window, and a requirement for systolic blood pressure < 2185mmHg and diastolic blood pressure <110mmHg to prevent complications.

Progressing stroke is a clinical complication of acute stroke, generally defined as a neurological degeneration in the days following admittance. Although it has been problematic to agree an international standard of definition there have been recent advances in the European Progressive Stroke Study (Birschel et al, 2004). A current working definition is a ≥2 Scandinavian Stroke Score (SSS)-point worsening in eithe conscious level, arm, leg or eye movement scores, and/or a ≥3 SSS-point worsening in speech score comparing the day 3 assessment with the baseline assessment, or death occurring within 72 h of onset. Progressing stroke occurs in around 20-25% of patients and is prognostic of poor outcome (Hashiniski et al, 1980; Birschel et al, 2004). The

pathophysiological mechanism for progressing stroke symptoms is not known, although extension of the brain oedema, absence of recanalization and thrombus propagation may each play a part (Asplund et al, 1992).

No current anticoagulants, such as heparin are of any benefit to acute stroke patients including those with atrial fibrillation or progressing stroke (O'Donnell et al, 2006; Rodden-Jullig et al, 2003). There have however been studies showing that individual inflammatory or haemostatic biomarkers predict risk of poor outcome or are associated with progressing stroke.

9.1.2 Previously Published Data on Acute Stroke and Inflammatory Markers

There is a body of evidence in the literature that elevated levels of fibrinogen and CRP predict risk of poor outcome in patients upon admission with acute ischaemic stroke ([1&2]Di Napoli et al, 2001, 2005; Anuk et al, 2002; Turaj et al, 2006). This has led to speculation that CRP and/or fibrinogen may be of clinical benefit as prognostic markers (Di Napoli et al, 2005, 2006). However, the evidence for the predictive value of fibrinogen in the acute stages is weak ([1]Di Napoli et al, 2001); studies so far have shown fibrinogen to be predictive of outcome at 1 year follow-up and levels may not be elevated in acute stroke until 1 week after the event (Anuk et al, 2002; Turaj et al, 2006). It is likely in these subacute phases that fibrinogen is predicting the risk of recurrent CVD events in outcome, and the aetiology of this may be very distinct from outcome in acute stroke. Illustrating this, 12% of stroke patients have recurrence within the first year, but only 4% in the first month (Hankey et al, 2003).

IL-6 has been shown to be a good predictor of acute ischaemic stroke outcome. IL-6 predicts neurological deterioration in the first 48 hours independently of clinical risk markers (Vila et al, 2000), and peak levels in the first week predict modified Rankin score at 3 months and computed tomography (CT) brain infarct volume as well as 12 month outcome (Smith et al, 2004; Emsley et al, 2003).

One study of 23 ischaemic stroke patients and 15 controls has examined both IL-18 and TNFα levels (Zaremba & Losy, 2001; Zaremba and Losy, 2004). This study found that both IL-18 and TNFα were elevated in serum and CSF of the patients, and correlated

significantly with SSS and Barthel index (BI) scores at baseline, as well as week 1 and 2. There was some evidence that IL-18 levels were associated more strongly with outcomes in non-lacunar stroke subtypes (Zaremba and Losy, 2004) although numbers are obviously very small. In contrast with these findings, a more recent study of TNFα found that in 41 patients it did not correlate with lesion size or neurological impairment (Intiso et al, 2004). Animal models of IL-18 suggest that IL-18 does not contribute to acute ischaemic brain damage (Wheeler et al, 2003) (although this obviously does not preclude involvement in pre-thrombotic inflammatory processes). These results in small studies require re-examination in larger studies with hard end-points.

The Stroke in Progress (SIP) study in Glasgow Royal Infirmary (Barber et al, 2004) showed that D-dimer, but not CRP or other haemostatic variable was independently associated with risk of progression in patients with acute ischaemic stroke. The availability of stored plasma for this study was a good opportunity to measure pro-inflammatory cytokines (IL-6, IL-18, and TNFα). Due to a lack of serum samples, MMP-9 and sCD40L were not measured in the SIP study.

9.1.4 Aims

The aim of this study was to assess whether IL-18, TNFα or IL-6 were associated with stroke progression or 30 day outcome in acute ischaemic stroke. This was done concurrently with a panel of inflammatory and haemostatic markers since this area is relatively unexplored in the literature. Furthermore, the study aimed to ascertain which inflammatory or haemostatic markers added greatest prognostic information to current clinical markers.

9.2 METHODS

9.2.1 Subject Recruitment, Assessment, and Methods

The aim of the SIP study (Barber et al, 2004) was to identify the biomarkers most strongly associated with poor outcome and poor prognosis (progressing stroke) independently of clinical risk markers. It was hoped that these markers may be able to

identify subsets of patients at greatest likelihood to experience clinical benefit from interventions such as, anticoagulants, or rt-PA therapy outwith current recommendations, or for future drug therapies. Approval to measure novel biomarkers in acute stroke was obtained from the local research ethics committee The aim was to assess which from a large range of inflammatory and haemostatic biomarkers were the best independent prognostic indicators of acute ischaemic stroke outcome (death/dependency/progressing stroke; with an initial emphasis on progressing stroke) in a reasonably size cohort.

Patients were recruited from consecutive ischaemic stroke admissions to Glasgow Royal Infirmary hospital between April 2002 and October 2003. Patients were excluded on the basis of any of the following: > 24 hours from symptom recognition to admission, age < 18 years, coma (only responding to pain on admission) or suspected epileptic seizures. Patients anticoagulated prior to admission were also excluded. No recruited patients were prescribed thrombolysis or heparin in the first 72 hours after stroke onset. All patients were treated on a standardized protocol for the management of dehydration (2.5 litres of 0.9% sodium chloride solution intravenously, over the first 24 hours, unless contraindicated), hyperglycemia, hypoxia and pyrexia.

A single experienced examiner saw all patients. Assessment included clinical classification (Oxfordshire Community Stroke Project (OCSP) classification) and measurement of stroke severity using the Scandinavian Stroke Scale (SSS). Follow up was at 30 days using the Rankin scale and Barthel Index, which were also administered by the same examiner.

Of 474 consecutive admissions assessed, 280 were deemed potentially suitable for recruitment. 61 patients were subsequently excluded (blind to blood sample results). Of the remaining 219 patients, 54 (25%) met the criteria of progressing stroke. For the outcome of death/ dependency (Rankin >2) a further 39 cases were excluded from analysis because of a premorbid Rankin of 3-5. This left a group of 180 subjects of whom 17 (9.4%) died before day 30. In total 94 of the 180 cases were dead or dependent at day 30.

Progressing stroke was defined using a modification of the European Progressing Stroke Study (EPSS) criteria (see 8.1.1; Birschel et al, 2004). Progression is excluded if the

conscious level improves significantly between the 2 assessments, even if there has been worsening in other criteria. In this study eye movement changes were excluded, because there is concern about reliability of this facet of the SSS (Barber et al, 2004) and removing the gaze palsy component of the EPSS definition does little to change the validity of conclusions made (Birschel et al, 2004).

Investigating an initial emphasis on progressing stroke, the primary results from SIP examined the association of haemostatic and inflammatory markers with progressing stroke (Barber et al, 2004). These included coagulation factors VII, VIII, XI, prothrombin F1+2, thrombin-antithrombin complexes, fibrin D-dimer, APC ratio, t-PA, vWF, PV, fibrinogen, CRP and blood leucocyte count, as well as clinical risk markers. CRP, F1+2, TAT, VWF and D-dimer levels were univariately associated with progressing stroke (but not fibrinogen; p=0.08). In logistic regression analysis, with all significant clinical and laboratory variables included, only D-dimer and mean arterial blood pressure remained independent predictors of progressing stroke. Associations of D-dimer with progressing stroke have subsequently been validated in diagnostic as well as epidemiological measurement methodologies (Barber et al, 2006).

IL-6, IL-18, and TNFα were assayed as described in chapter 2 and assays were performed on previously unthawed aliquots.

9.2.3 Statistical analysis

Results are expressed as medians throughout the results unless otherwise stated. For non-normally distributed variables log transformations to normality were made where possible. In univariate comparison of categorical variables the chi-squared test was used. Univariate analysis of normally distributed and transformed variables was by the unpaired t-test, and for remaining non-normally distributed continuous variables the Mann Whitney U test was used. Multivariate analysis was by forward stepwise binary logistic regression. The probability for stepwise entry was 0.05 and for removal was 0.10. Oxfordshire Community Stroke Project (OCSP) classification was included in the model as a categorical variable. All analyses were carried out using SPSS for Windows version 13.0.

9.2.4 Power Calculations

In this study, which was specifically designed to assess haemostatic markers in progressing stroke, prospective power calculations were performed in aiding study design by the principal investigator, Dr Mark Barber. Power calculations assumed a 25% rate of progressing stroke among acute stroke patients. For 90% power ($p<0.05$), to determine similar abnormalities in TAT and D-dimer levels (25%), it was estimated that a total group size of 189 patients would be required. The 280 patients recruited fulfills this criteria even allowing for exclusions.

9.3 RESULTS

9.3.1 Sample Availability

The majority of the 280 stored plasma samples were available for analysis, with a minimum of 94% being assayed for TNFα, which required the largest sample volume. All blood samples had been stored at -80^{o}C for up to five years, and the aliquots used for analysis of IL-18, TNFα and IL-6 were previously unthawed. The sample quality was hence excellent.

9.3.2 Clinical and Demographic Differences Between Patients with Alternative Clinical Outcomes

Table 9.1 summarises data comparing patients dead or dependent (Modified Rankin Score 3-5) at 30 days compared to surviving independent patients. Age was the only significant measured demographic predictor of stroke outcome at 30 days ($p<0.001$). Clinically, both OCSP ($p=0.007$) classification and SSS ($p<0.001$) were highly significant predictors of stroke outcome, as was atrial fibrillation on admission electrocardiogram ($p=0.02$). Comparing only those who died within 30 days to survivors, predictors of outcome were similar to death/dependency composite (data not shown), with the exceptions that premorbid Rankin score 3-5 increased risk of death (35% versus 16% $p=0.02$) as well as presence of a left hemisphere lesion (31% versus 57% $p=0.01$; inverse association) were also associated with outcome.

Comparing the progressing stroke patients to the non-progressing group, the clinical and demographic differences were again broadly similar to those described in Table 9.1 (data not shown; table given in Barber et al, 2004). The only significant differences were that progressing stroke was more likely to occur in females (67% vs 51% p=0.04) and in those with pyrexia in the first 72 hours (44% vs 28% p=0.02), and hemisphere lesion had no bearing on progression.

Known cases of diabetes did not associate with any outcome, but this could be a chance finding due to the small numbers of diagnosed cases, or the presence of undiagnosed latent disease within the group.

9.3.3 Inflammatory Marker Associations with Progressing Stroke

Univariate comparisons of inflammatory markers in the progressing versus the non-progressing stroke subgroups are given in Table 9.2. CRP was significantly associated with progression (as described previously; Barber et al, 2004), but the association is much stronger for IL-6; median levels of circulating IL-6 levels were ~69% higher at admission in those who go on to experience stroke progression. In contrast with these findings, neither IL-18 nor TNFα were associated with stroke progression.

9.3.4 Inflammatory and Haemostatic Marker Associations with Poor 30 Day Outcome

As seen in Table 9.3, CRP, IL-6, TNFα, fibrinogen, leukocyte count, D-dimer, prothrombin fragment F_{1+2}, TAT, vWF, factors VIII and XI, and viscosity all had significant associations with 30 day adverse (death or dependency) outcome on univariable analysis. The strongest univariate predictors were CRP, IL-6, D-dimer, and factor VIIIc ($p<0.0001$), although the association was also considerable for TNFα ($p=0.007$). Factor VIIc levels had a borderline inverse association with outcome ($p=0.07$). IL-18 and APC ratio were the only variables measured not to have univariate associations with 30 day death/dependency outcome.

Comparing only those who died versus survivors, all markers remained significantly associated with outcome (data not shown) with the exceptions of TNFα (2.6 versus 2.1pg/ml; p=0.17) and fibrinogen (3.81 versus 3.92 g/L; p=0.53) which became non-significant, while PV (p=0.07) retained only borderline significance.

In Table 9.4 a logistic regression analysis in 2 separate models is shown for the risk of death or dependency in 30 days. Model 1 includes all the variables in Table 9.1 with significant univariate associations with death/dependency ($p<0.1$), as well as selected pro-inflammatory markers (CRP, IL-6, IL-18, TNFα). In this analysis after stepwise addition/removal of individual markers, age (OR 1.03; 95% CI 1.00-1.07), stroke subtype according to OCSP (OR 2.83; 95% CI 1.00-8.05) and SSS (OR 0.94; 95% CI 0.90-0.98) were significantly associated with outcome. Of the inflammatory risk markers included in the model, only IL-6 retained a significant association with death/dependency (OR 1.07; 95% CI 1.01-1.13).

Model 2 in Table 9.4 includes all the variables in Table 9.1, as well as *all* markers in Table 9.3 with significant univariate associations with death/dependency ($p<0.1$). Here, of the demographic and clinical markers, only SSS is significantly associated with outcome (OR 0.92; 95% CI 0.88-0.96). The haemostatic marker D-dimer (OR 1.72; 95% CI 1.24-2.39) was the only variable required to add prognostic information to SSS in this model.

No multivariate analysis of a death only outcome is presented here due to the fairly small number of cases, and consequently large confidence intervals.

Table 9.1 Demographic and clinical differences between patients dead or dependent (Rankin 3-5) at 30 days compared to surviving independent patients. For the purpose of this analysis those with a premorbid Rankin of 3-5 were excluded. For continuous variables results are expressed as median (interquartile range [IQR]) except for blood pressure where results are expressed as mean (standard deviation).

Variable	Dead /Dependent at 30 days (n=94)	Alive / independent at 30 days (n=86)	p value
Age (yrs)	72 (65 - 80)	66 (56 - 73)	***<0.001***
Female gender	53 (56%)	43 (50%)	*0.39*
Previous hypertension	49 (52%)	45 (52%)	*0.98*
Known diabetic	13 (14%)	11 (13%)	*0.84*
Previous stroke	17 (18%)	16 (19%)	*0.93*
Antiplatelet therapy	44 (47%)	34 (40%)	*0.58*
Current smoker	38 (43%)	32 (39%)	*0.63*
Clinical Differences:			
Left hemisphere lesion	52 (55%)	50 (58%)	*0.70*
OCSP classification			
TACI	40 (43%)	7 (8%)	
PACI	39 (41%)	44 (51%)	***<0.001***
LACI	12 (13%)	26 (30%)	
POCI	3 (3%)	9 (11%)	
Admission SSS score (minus gait)(points)	30 (15 - 40)	41 (36 - 43)	***<0.001***
Admission MABP (mmHg)	106 (20)	108 (20)	*0.64*
Atrial fibrillation on ECG	16 (17%)	5 (6%)	***0.02***

OCSP; Oxfordshire Community Stroke Project score, TACI; total anterior circulation infarct, PACI; partial anterior circulation infarct, LACI; lacunar infarct, POCI; posterior circulation infarct, SSS; Scandinavian Stroke Scale, MABP; mean arterial blood pressure, ECG; electrocardiogram.

Table 9.2 Circulating inflammatory markers in those who experience either progressing or non-progressing acute stroke. For TNFα and IL-6 statistical analysis is by the Mann Whitney U test. Values are medians (IQR)

Variable	Progressing Stroke (n=52-54)	Stable/ improving stroke (n=154-163)	p value
IL-18 (pg/ml)	278 (216 – 406)	277 (207 - 375)	*0.30*
TNFα (pg/ml)	2.36 (1.42 – 3.64)	2.10 (1.48 – 2.86)	*0.53*
IL-6 (pg/ml)	11.69 (6.16 – 20.52)	6.90 (3.29 – 13.82)	***0.001***
CRP (mg/l)	8.66 (3.69 – 30.45)	5.26 (1.64 – 18.4)	***0.05***

Table 9.3 Circulating inflammatory, haemostatic and haemorrheological variables in patients who died or were dependent at 30 days compared to those who were not. Results are expressed as median (interquartile range). For TNFα and IL-6 statistical analysis is by the Mann Whitney U test.

Variable	Dead /Dependent at 30 days (n=94)	Alive / independent at 30 days (n=86)	p value
CRP (mg/l)	8.0 (3.5 - 26)	3.3 (1.1 – 8.2)	***<0.001***
IL-6 (pg/ml)	10.4 (6.0 – 16.7)	4.4 (2.4 – 9.2)	***<0.001***
TNFα (pg/ml)	2.3 (1.5 - 3.4)	1.8 (1.4 – 2.6)	***0.007***
IL-18 (pg/ml)	248 (202 - 358)	284 (196 - 378)	*0.26*
Leucocyte count ($x10^9$/l)	10.2 (7.9 – 12.6)	9.1 (7.4 – 10.9)	***0.002***
Fibrinogen (g/l)	3.98 (3.34 – 4.90)	3.58 (2.95 – 4.30)	***0.001***
Fibrin D-dimer (ng/ml)	273 (148 - 788)	128 (59 - 306)	***<0.001***
Prothrombin F1+2 (nmol/l)	1.1 (0.9 – 1.6)	1.0 (0.7 – 1.4)	***0.003***
TAT (μg/l)	4.5 (3.5 – 7.8)	3.7 (2.9 - 5.6)	***0.003***
APC ratio (n=133)	2.8 (2.5 – 3.1)	2.8 (2.6 – 3.2)	*0.40*
t-PA antigen (ng/ml)	12.4 (8.8 – 17.4)	10.0 (7.8 – 12.8)	***0.01***
vWF antigen (iu/dl)	208 (171 - 264)	186 (140 - 216)	***0.001***
Factor VIIc (iu/dl)	142 (120 - 165)	149 (129 - 173)	***0.07***
Factor VIIIc (iu/dl)	206 (174 - 253)	176 (144 - 218)	***<0.001***
Factor IXc (iu/dl)	165 (148 - 188)	152 (138 - 171)	***0.003***
Plasma viscosity (mPa.s)	1.29 (1.21 – 1.37)	1.23 (1.19 – 1.32)	***0.03***

APC; activated protein C, TAT; thrombin-antithrombin complex, t-PA; tissue plasminogen activator, vWF, von Willebrand factor

Table 9.4 Forward stepwise logistic regression models of predictors of death or dependency at 30 days.

	Coefficient B (SE)	Odds Ratio	95% CI	p value
Model 1				
Constant	-0.76 (1.37)			
Age	0.03 (0.02)	1.03	1.00 - 1.07	0.03
OCSP (TACI or other)	1.04 (0.53)	2.83	1.00 - 8.05	0.05
SSS score	-0.07 (0.02)	0.94	0.90 – 0.98	0.002
IL6	0.06 (0.03)	1.07	1.01 – 1.13	0.02
Model 2				
Constant	3.62 (2.19)			
SSS score	-0.09 (0.02)	0.92	0.89-0.96	0.00
D-Dimer	0.48 (0.17)	1.72	1.24-2.39	0.01

Model 1: All demographic and clinical variables with p value <0.10 in univariate analysis, as well CRP, IL-6, IL-18, and TNFα.

Model 2: All demographic and clinical variables as well as all variables in Table 9.3 with p value <0.10 in univariate analysis.

9.4 DISCUSSION

9.4.1 Patient Population Distribution of Inflammatory Markers

Median circulating levels of TNFα (2.1 pg/ml in all patients) and IL-18 (277pg/ml) were not substantially higher than in those in the older age group of MONICA-4 (Chapter 4). There was a ~15% increase in median levels for each cytokine; which is minimal considering the patients were experiencing acute stroke. In contrast, median IL-6 levels were 7.69pg/ml, which is roughly a 3-fold increase from levels seen in a similar MONICA-4 age-group. CRP levels were similarly elevated in this population.

9.4.2 IL-18 and TNFα and Associations with Acute and Progressing Stroke and 30 Day Outcomes

That IL-18 and TNFα levels were not elevated during acute stroke (compared with MONICA-4) suggests that these cytokines are not substantially elevated within the first 24 hours of stroke onset, and are therefore unlikely to play immediate roles in acute stroke pathology, such as early neurological deterioration.

Lack of an association between admission levels of these cytokines and 72 hour stroke progression is suggestive of lack of a causal role in the condition, since the pathophysiological background required for persistent thrombi-mediated occlusion would probably be set in the acute stages of stroke. Consistent with this, one study has shown in 41 patients that TNFα levels at admission do not associate with lesion size or neurological impairment (Intiso et al, 2004). It should be noted that these results are inconsistent with a very small study (Zaremba & Losy, 2001; Zaremba and Losy, 2004), showing associations of the IL-18 and TNFα with clinical markers of stroke severity and neurological deterioration, but there are too few patients in that study to draw rigorous conclusions.

Despite univariable associations with death/dependency composite outcome, levels of TNFα no longer predicted outcome in the multivariable model including other

inflammatory markers. The possibility that baseline TNFα levels were not maximal within the 24 hour inclusion time limit, which might reduce the study's power to detect associations with outcome, cannot be discounted (see 9.4.3). It is interesting to note that TNFα was one of the only markers with strong associations with 30 day outcome that did not also predict early progression, although there were trends towards increased levels in the latter condition. This may be indicative of a differential underlying aetiological role for TNFα in acute versus more chronic pathological processes, or alternatively it may be a chance finding.

IL-18 was apparently not elevated during the acute stages of stroke, and was not a prognostic indictor of progression, 30 day outcome, or death at 30 days. IL-18 has no role in knock-out models of stroke induced brain injury in mice (Wheeler et al, 2003), and likewise is not a stimulator of the acute phase response (Stuyt et al, 2005). Therefore it seems lack of association with both the degree of stroke injury and with the acute phase response means IL-18 is not even univariately associated with outcome.

9.4.2 Other Inflammatory and Haemostatic Markers and Associations with 30 Day Outcomes

In this study of a cohort of acute ischemic stroke patients, a wide range of inflammatory and haemostatic biomarkers predicted poor prognosis as measured by 30 day death or dependency in univariate analysis. Such widespread general associations with outcome probably reflect the association between these markers and underlying biological processes in acute stroke, and hence likelihood of poor outcome. For instance, generalised inflammation (such as that measured by CRP and IL-6) probably reflects the extent of systemic atherosclerosis, the extent of neurological damage in acute stroke, the risk of further atherothrombotic rupture, and the age of the patient (Lowe et al, 2005; Hansson et al, 2005) while levels of haemostatic markers may reflect current thrombotic potential in the formation of thromboemboli and ongoing fibrinolysis as well as patient age (Franchini et al, 2006; Matijevic et al, 2006). Indeed, one study has shown the extent of brain injury may largely determine the extent of the acute phase response following stroke (Smith et al, 2006). Clinically relevant biological mechanisms that increase the risk of poor outcome as well as some confounding associations hence probably explain

much of the association between inflammatory and haemostatic risk markers and prognosis in acute stroke.

In contrast to TNFα and IL-18, both IL-6 and CRP were elevated on admission, and in particular IL-6 was associated strongly with progression. These results for IL-6 were in agreement with previous studies showing that it predicts neurological deterioration in the first 48 hours independently of clinical risk markers (Vila et al, 2000), and peak levels in the first week predict modified Rankin score at 3 months and computed tomography (CT) brain infarct volume as well as 12 month outcome (Smith et al, 2004; Emsley et al, 2003). IL-6 association with 30 day outcome over and above clinical risk markers and other inflammatory markers is in line with observations that IL-6 is the pro-inflammatory cytokine largely responsible for promotion of the acute phase response, as well as perhaps playing roles in endothelial dysfunction, atherogenesis, and the promotion of a hypercoagulable state (Yudkin et al, 2000).

Interestingly, fibrinogen shows only borderline association with progressing stroke (Barber et al, 2004) and was not associated with 30 day outcome. This suggests that although fibrinogen has been considered a good candidate marker of prognosis in stroke (Di Napoli et al, 2006), not all the acute phase response is equal in prediction of stroke progression. In other studies, fibrinogen appears predictive of longer term outcome and risk of recurrent CVD events (Anuk et al, 2002; Di Napoli et al, 2005; Turaj et al, 2006). This may be indicative that fibrinogen levels are not elevated to maximal levels within 24 hours of stroke onset, which is in agreement with one study which has reported fibrinogen to be significantly elevated only a week after acute stroke (Lip et al, 2002) while another has demonstrated that CRP and IL-6 follow distinct patterns of expression following acute stroke, with inter-individual differences also present within these patterns (Smith et al, 2004). Hence there may be other markers (such as TNFα and perhaps IL-18) which do not show prognostic ability in the acute phase.

When all clinical risk markers significantly associated with prognosis in this study were included with all haemostatic and inflammatory markers in the same multivariate model, only SSS and D-dimer were required to give the greatest prognostic information. D-dimer is perhaps a good prognostic indicator of short-term outcome in ischaemic stroke because

it is both an acute phase reactant and a marker of turnover of cross-linked fibrin, generated by activation of coagulation and fibrinolysis, and it hence may represent the biological activation of both the inflammatory and haemostatic systems. A previous study has suggested that D-dimer levels may be associated specifically with ischaemic stroke subtypes (Ageno et al, 2002) and another study found that acute stroke D-dimer levels were not associated with long term stroke prognosis after adjustment for stroke subtype (Squizzato et al, 2006). Although we have not examined this hypothesis it is possible this is a confounding factor for its association with outcome here, but this is also equally possible for all markers measured considering the potential differences in aetiology (Suwanwela et al, 2006).

9.4.3 Study Strengths and Limitations

The strengths and weaknesses of the present study have been considered previously (Barber et al, 2004). A single researcher assessed consecutive admissions to the hospital using relatively few exclusion criteria, although those included should be sufficient to remove extraneous confounding of acute ischaemic stroke phenotypes seen on hospital admittance. Samples were taken within 24 hours of symptom onset and not at a regular interval after symptom onset, and this criteria may allow inter-individual confounding and, as discussed, may not temporally allow aspects of the inflammatory response to peak. Despite this the study time is representative of conditions upon general medical ward assessment, and is perhaps more clinically relevant than strict temporally controlled exclusion criteria. Detailed functional imaging was not performed as part of the study protocol, and hence such data cannot be included. This particular weakness may detract from the strength of the multivariable models used, but as such does not influence conclusions drawn about IL-18 and TNFα in particular.

9.4.4 General Conclusions

Neither IL-18 nor TNFα were significantly elevated within 24 hours of onset of acute stroke, nor were they associated with stroke progression. Therefore hospital admittance levels of IL-18 and TNFα are unlikely to be clinically useful in determining stroke severity or likihood of progression. Despite a univariate association of admission TNFα

with 30 day outcome, the association was not as strong as it was using IL-6 or D-dimer as a prognostic biomarker. Negative findings here and elsewhere (Wheeler et al, 2003) collectively suggest that IL-18 has minimal involvement in the acute stages of stroke, even as a passive bystander.

These results do not preclude a role for either cytokine in predicting recurrent stroke. Chapter 11 therefore explores the associations of IL-18 and TNFα with recurrent stroke, preceded by a study of incident stroke in Chapter 10.

CHAPTER 10

ASSOCIATIONS OF IL-18 AND TNFα WITH INCIDENT STROKE IN AN ELDERLY POPULATION WITH PREVALENT VASCULAR DISEASE: PROSPECTIVE STUDY OF PRAVASTATIN IN THE ELDERLY AT RISK (PROSPER)

INTRODUCTION

10.1.1 Stroke Incidence in the Elderly

In the elderly, chronic vascular disease, and particularly stroke, is by far the major cause of morbidity and mortality and is also a major contributor to cognitive decline and dementia (Wenger, 1992; Martyn, 1996; Elkind, 2003). Stroke incidence rates double in populations for every 10 years after 55 years (Elkind, 2003). The aetiology of stroke, particularly in the elderly, therefore requires specific epidemiological consideration.

10.1.2 Previously Published Data on Inflammatory Markers and Incident Stroke

The literature examining the epidemiological relationships of inflammatory markers and stroke risk is examined in section 1.7.4/1.7.5. In brief, although they have different underlying aetiologies, the association of CRP and fibrinogen with both CHD and stroke appears remarkably similar in general populations (Di Napoli et al, 2005; Danesh et al, 2004; Fibrinogen Studies Collaboration, 2005). The Rotterdam Study recently showed prospectively in a study with 498 primary strokes that CRP was very modestly associated with risk of all strokes (age- and sex-adjusted OR per SD, 1.14; 95% CI 1.04 - 1.24) and risk of ischemic stroke (age- and sex-adjusted OR per SD, 1.17; 95% CI 1.04 - 1.32) (Bos et al, 2006). Significant univariable associations of IL-6, CRP and fibrinogen with stroke were also recently reported in the Edinburgh Artery Study (Tzoulaki et al, 2007). In the whole PROSPER cohort CRP predicts risk of stroke independently of conventional risk factors: 1.32 (1.03 - 1.79) comparing extreme tertiles and this, again, is similar to risk association seen for CHD in the study 1.33 (1.09 -1.62) ([1]Sattar et al, 2007) and risk of CHD on meta-analysis for CHD (Danesh et al, 2004).

The Fibrinogen Studies Collaboration (2005) have published data on the association between fibrinogen and incident stroke, and again found broadly similar associations to those seen in CHD: for all types of stroke (n=2775) the age and sex-adjusted OR per 1g/L increase in fibrinogen was 2.06 (95% CI, 1.83-2.33), for ischemic stroke 2.08 (95% CI, 1.74-2.48), for hemorrhagic stroke 1.44 (95% CI, 1.05-1.96), and for stroke attributed to unclassified causes 2.36 (95% CI, 1.94-2.86). These results are comparable to data for the combined OR for all vascular deaths and events: 2.35 (95% CI, 2.21-2.49) and was also similar to that for all nonvascular mortality 2.03 (95% CI, 1.90 – 2.08).

For both IL-18 and TNFα there are data that report associations with the composite endpoints "MI plus stroke risk" or "all CVD risk," (e.g. [2]Cesari et al, 2003, Wise et al, 2005, Blankenberg et al, 2006) and although this is reasonable methodology in terms of clinical risk assessment, it is not helpful in delineating associations with stroke risk specifically. The only published data examining stroke risk specifically and TNFα is from the ABC study ([2]Cesari et al, 2003) and contained only 60 incident strokes in which TNFα was not associated with outcome (see Table 1.4). There is no such published data for IL-18. Similarly, despite recent interest in positive associations of IL-6 gene polymorphisms with stroke (Chamorro et al, 2005; Lalouschek et al, 2006; Yamada et al, 2006) IL-6 itself has been strongly implicated in stroke risk. For instance, the ABC study (which found TNFa to be not associated with risk of stroke in 60 cases [Table 1.4]) found that IL-6 predicted stroke risk in healthy elderly people even after adjustment for classical risk factors: OR 2.86 (1.71–4.80) ([2]Cesari et al, 2003). This was significantly higher than its association with risk of CHD: OR 1.71 (1.27–2.29).

During this thesis, a PROSPER stroke case/control study was formed from the principal cohort, and a full complement of PROSPER plasma case/control samples were available in our laboratory. It was a good opportunity to complete the initial assessment of IL-6, IL-18 and TNFα in stroke, also examining cytokine associations with incident stroke in a population (in addition to acute and recurrent stroke; Chapters 9 and 11). Consent to perform the work on new potential cardiovascular risk markers had been obtained at the time of the trial recruitment, which was approved by the local research ethics committees. Due to the availability of only plasma samples, sCD40L and MMP-9 were not examined in this study.

10.1.3 Aims

The aim of this study was to assess the associations of IL-18, IL-6 and TNFα with incident all-cause and ischaemic stroke in an elderly population with prevalent vascular disease and to assess the level of independence of this association from conventional risk markers.

10.2 MATERIALS AND METHODS

10.2.1 Establishment of the PROSPER Trial

It is well documented that treatment with statins reduces the burden of CHD in middle-aged populations both with and without prevalent vascular disease (e.g. Scandinavian Simvastatin Survival Study Group (4S), 1994; West of Scotland Coronary Prevention Study [WOSCOPS]; Shepherd et al, 2005), and these observations have previously been extrapolated to the elderly. However, this assumption may be inaccurate, and the elderly may be very different in terms of CHD aetiology and the consequent benefits gained from statin use. For instance it is known that the BMI is a poorer index of CHD risk in the elderly than in middle-aged populations; this may reflect underlying changes in the aetiology of disease as a population ages, for instance loss of skeletal muscle mass as well as gain of fat mass being a risk factor, confounding associations of BMI with risk in the elderly (Wannamethee et al, 2005).

The PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) (Shepherd et al, 1999) was set up to investigate the benefits of statin use to reduce cardiovascular risk in the elderly with prevalent vascular disease. The study reported a 34% reduction in serum cholesterol after 3 years, and a reduction in risk of CHD events (OR 0.81, 95%CI 0.69-0.94) in active treatment group versus placebo (Shepherd et al, 2002). Stroke risk was unaffected in this study, but this may be due to the 3 year follow-up; some studies indicate that it may take in excess of 3 years to see benefit from statin use in terms of stroke risk (Byington et al, 2001; Pedersen et al, 1998) and pravastatin is a less potent drug for lowering cholesterol than some other statins. In subsequent meta-analysis of

randomised controlled tirals of statins (primarily in men), including the prosper trial, the Cholesterol Trialists Collaboration reported that statins reduced the risk of all stroke type 19% (99% CI 11-26%) and ischaemic stroke 22% (99% CI 12–30%) (CTC, Lancet, 2005). Subsequent clinical issues of whether lipoprotein profiles in the elderly can be used to assess those who are best the best targets for statin use have also been examined in PROSPER (Packard et al, 2005). Also published from the PROSPER study are data (previously discussed in section 1.7.4) demonstrating a weak but significant and independent relationship of CRP to CVD event risk in the whole cohort, which was shown to add little to the C-statistic of the Framingham risk factors in the elderly, or to indicate predictive statin benefit ([1]Sattar et al, 2007).

10.2.2 Subjects and Methods

The PROSPER study was a randomised placebo-controlled trial of Pravastatin in 5,804 subjects (2,520 in Scotland, 2,184 Ireland, and 1,100 Netherlands) aged 70-82 years, with vascular risk factors or known vascular disease (Shepherd et al, 1999). Half had evidence of chronic vascular disease (stable angina or intermittent claudication) and the other half having at least one vascular risk factor (hypertension, smoking or diabetes mellitus). Blood samples were taken at baseline, prior to study entry. After 3.2 years follow-up a nested case-control study was extracted comprising 266 patients with incident stroke who were compared with 532 controls from the PROSPER cohort in a 2:1 ratio. Matching variables included age (within 5 years), gender and treatment allocated (pravastatin or placebo).

Stroke was defined as: (a) rapid onset of focal neurological deficit lasting ≥ 24 hours, (b) focal neurological deficit mode of uncertain onset but with no plausible evidence of alternative (non-stroke) cause, or (c) rapid onset of global neurological deficit (e.g., coma) lasting ≥ 24 hours with no other plausible non-stroke cause. Transient ischaemic attacks were defined as rapid onset of focal neurological deficit lasting < 24 hours. Strokes were categorised as ischaemic or haemorrhagic on the basis of neuroimaging (computed tomography or magnetic resonance imaging) or post mortem. Where no neuroimaging or post-mortem was available strokes were categorised as ‘unclassified’. Of the 266 strokes in PROSPER 179 (67%) were infarcts, 18 (7%) primary intracerebral haemorrhages and 69 (26%) undefined.

IL-18 and TNFα were measured on previously unthawed aliquots as described in Chapter 2.

10.2.2 Statistical analysis

Cases and unique controls were compared, for continuous variables, using t-tests after log transformations to better approximate normal distributions where necessary. Binary variables were compared through chi-square or Fisher's exact tests. Odds ratios for stroke were estimated from univariable logistic regression models (Woodward, 2005) using the matched sets as units of observation in both total and ischaemic stroke subgroups. Reported odds ratios for continuous variables are those associated with 1 SD change in the control population. In addition to unadjusted (except for matching variables) analyses for IL-18 and TNFα, multivariable adjustments were made for history of vascular disease and for APR-associated inflammation (IL-6 and CRP).

10.3 RESULTS

10.3.1 Baseline Characteristics

Baseline clinical characteristics of the 266 patients with all incident strokes, compared with 532 age and-sex matched controls, are presented in Table 10.1. Prior history of stroke (<0.0001) and to a lesser extent history of MI or any vascular disease (p=0.08, 0.01 respectively) were associated with increased risk of stroke, as was diabetes mellitus (p=0.03). Lower cognitive function (Mini-Mental State Examination, p=0.02), reduced speed of information processing (STROOP test, p=0.05), lower Instrumental Activities of Daily Living score (p=0.02) and reduced Barthel Index (p=0.001) at baseline all were associated with increased likelihood of stroke. Alcohol consumption (p=0.006) was more likely to be prevalent among the case group, and there was a non-significant trend for cases to be current smokers as well. No common cardiovascular medications were associated with risk of stroke. SBP showed non-significant trends to be higher among cases than in controls, but BMI and DBP did not. Likewise, HDL and LDL cholesterol

measurements showed no differences in cases and controls, but non fasting glucose measurements were higher in the case group (p=0.009).

Among the inflammatory markers neither fibrinogen, CRP, IL-6, IL-18 nor TNFα showed any significant differences between cases and controls, although with the exception of TNFα all showed non significant trends to be slightly higher in the case than in the control group (overall difference between 1 and 4%).

Similar patterns were seen in the subset of 179 ischaemic stroke patients when compared with the control group, and none of the overall results from Table 10.1 differed in this analysis (data not shown).

10.3.2 Risk Ratios of Stroke

As expected from the results in Table 10.1, Table 10.2 demonstrates that neither IL-6, IL-18, nor TNFα are associated with risk of stroke on univariable analysis. IL-6 demonstrated only non-significant borderline associations with risk in univariable analysis; OR 1.10 (95% CI 0.95-1.28) per SD increase. IL-18 and TNFα show no associative trends; 1.06 (0.92-1.22) and 0.91 (0.76-1.10) respectively. Multivariable analysis was only performed on IL-18 and TNFα in the ischaemic stroke subgroup compared to the control group. Unsurprisingly, after further adjustment for IL-6, CRP and history of vascular disease, there was no change in the null-association of the cytokines with risk of incident ischaemic stroke (Table 10.2).

10.3.3 Correlations

Correlations of cytokines with conventional/clinical risk markers were not explored in this study due to the age and prevalence of vascular disease. The correlations are therefore unlikely to elicit meaningful results in this population.

Table 10.1 Baseline characteristics of all stroke case and control populations: back-transformed (from log-normalised analysis) means (standard deviations) or percentages. Corresponding p-values obtained by two tailed t-test after log-transformations of continuous variables where appropriate. Categorical variables compared using chi-squared test.

	Cases (n=266)	**Controls (n=532)**	**p-value**
Questionnaire/Clinical exam			
History of vascular disease	143 (53.8%)	236 (44.4%)	0.01
History of stroke/TIA	53 (19.9%)	49 (9.2%)	<0.0001
History of MI	78 (29.3%)	126 (23.7%)	0.08
History of diabetes	46 (17.3%)	62 (11.7%)	0.03
Alcohol intake (any)	161 (60.5%)	271 (50.9%)	0.006
Current smokers	78 (29.3%)	126 (23.7%)	0.08
Anti-hypertensive medication	188 (70.7%)	399 (75.0%)	0.19
Anti-platelet medication	95 (35.7%)	171 (32.1%)	0.31
Barthel Index	19.6 (1.07)	19.8 (0.52)	0.001
MMSE	27.8 (1.75)	28.1 (1.52)	0.02
TIDAL score	13.5 (1.33)	13.7 (0.91)	0.02
STROOP	70.9 (29.6)	66.9 (25.6)	0.05
Physical measurements			
Body mass index (kg/m^2)	26.5 (4.1)	26.8 (4.0)	0.36
Systolic blood pressure (mmHg)	157.2 (21.29)	154.9 (23.0)	0.16
Diastolic blood pressure (mmHg)	84.0 (11.8)	84.3 (11.2)	0.70
Blood Sample			
LDL cholesterol (mmol/L)	3.76 (0.83)	3.75 (0.82)	0.88
HDL cholesterol (mmol/L)	1.26 (0.33)	1.27 (0.34)	0.46
Glucose (mmol/L)	5.81 (2.12)	5.46 (1.44)	0.009
Fibrinogen (g/L)	3.60 (0.76)	3.56 (0.77)	0.56
C-reactive protein (mg/L)	1.25 (1.13)	1.13 (1.17)	0.21
Interleukin-6 (pg/ml)	1.03 (0.70)	0.99 (0.65)	0.45
Interleukin-18 (pg/ml)	375 (175)	365 (157)	0.39
TNFα (pg/ml)	1.75 (2.22)	1.96 (2.89)	0.33

MMSE, Mini mental-state exam; TIDAL, Instrumental Activities of Daily Living score; STROOP, speed of information processing

Table 10.2 Associations of IL-6, IL-18 and TNFα with risk of incident stroke as continuous variables (ORs represent risk of 1 standard deviation increase in circulating cytokine levels among control population).

	Univariable analysis (age- and sex-matched samples)		**Multivariable analysis***	
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
All strokes				
IL-6	1.10 (0.95-1.28)	0.21		
TNFα	0.91 (0.76-1.10)	0.33		
IL-18	1.06 (0.92-1.22)	0.39		
Ischaemic Strokes				
TNFα	0.96 (0.79-1.16)	0.65	0.96 (0.79-1.16)	0.66
IL-18	1.08 (0.92-1.28)	0.34	1.07 (0.90-1.26)	0.45

* Adjusted for IL-6, CRP and history of vascular disease

10.4 DISCUSSION

10.4.1 Population Distributions

The median levels of circulating IL-18 in this population were 330 pg/ml (IQR 255-434) for the controls and 336 pg/ml (276-438) for the cases. This is ~30% higher than in the eldest age-group (55-64 years) in the MONICA-4 survey (Chapter 4), a result entirely consistent with the age (70-82 years) and prevalence of vascular disease within the current case-control study. In contrast, circulating levels of TNFα were 1.24 pg/ml (0.89-2.03) and 1.30 pg/ml (0.93-1.78) for controls and cases respectively. This is ~30% *lower* than the eldest age group in the MONICA-4 study. This unexpected result is unlikely to be explicable through sample quality issue. Samples were only ~8 years old, and were previously unthawed aliquots. Since TNFα generally increases with age (Chapter 4) the simplest logical explanation is a drift in the standards provided by the manufacturer between kit lots.

10.4.2 General Null Association of Inflammatory Biomarkers with Risk of All Stroke or Ischaemic Stroke

Data from this PROSPER case-control study indicate that not only are IL-18 and TNFα not associated with risk of stroke in the elderly, but also that IL-6, fibrinogen and CRP demonstrate a similar lack of association. All of the inflammatory markers, with the exception of TNFα, showed a non significant trend to be higher in the case than in the control group, although on no occasion did the trend reach borderline significance ($p<0.1$).

This result is surprising considering that both the Leiden 85-P (Gussekloo et al, 2000) and the Cardiovascular Health (Cao et al, 2003) studies of the elderly found CRP to be a significant predictor of risk of stroke in the elderly (median 5.7 vs 2.7 mg/L, $P<0.005$ for Leiden 85-P, and 2.07 vs 1.87 mg/L, $p<0.006$ respectively) and the current study certainly has sufficient power for incident stroke compared with the Leiden 85-P study (80 cases). Similarly, the Fibrinogen Studies Collaboration (2005) has shown that a 1g/L increase in fibrinogen in the elderly (>70 years) is associated with a RR of 1.90 (95% CI

1.54-2.34), which is a less strong association than that seen in the middle aged (OR 2.77 per 1g/L increase).

A recent cross-sectional study has suggested that IL-6 (but not other inflammatory markers) is weakly associated with MMSE ([2]Wright et al, 2007) so the lack of a univariate association of IL-6 with risk was also surprising considering MMSE was associated with stroke risk in this cohort. However, this may be explained by the weak association of IL-6 with MMSE, and the weak association of MMSE with stroke risk.

10.4.3 Limitations of the Study

Lack of an association of CRP or fibrinogen with risk in this cohort requires careful consideration, since IL-18 and TNFα may demonstrate lack of association for potentially the same underlying reasons.

This particular cohort were carefully selected elderly patients considered at risk of stroke to due the presence of vascular risk factors, or the confirmed presence of vascular disease. As in GLAMIS, selection of those with prevalent disease will result in selection biases in the data. Indeed the current study found smoking not to be associated with risk of stroke, but smoking is a major risk factor for stroke. That the increased smoking/risk trend did not reach significance is probably a similar phenomenon to that reported in the GLAMIS study, where those with recent health problems may have recently given up smoking. However, no "ex-smoker" data is available in this cohort. BMI also was not associated with risk in this cohort, and this probably because the population was elderly (low muscle as well as fat mass, hence BMI has little to do with vascular risk in this population; [3]Wannamethee et al, 2005)

As seen for fibrinogen (Fibrinogen Studies Collaboration, 2005) it is likely that associations between circulating levels of fibrinogen (and potentially other inflammatory markers) are less strongly associated with risk of stroke in the elderly. It is also likely that the differences between cases and controls in terms of conventional risk factors is likely to be smaller than in an unselected general cohort or a cohort comprising people without verified vascular disease. It is possible therefore that associations of inflammatory

markers with conventional stroke risk factors seen in other studies are largely lost due to the suboptimal health of the case and control populations in this study; a facet of the study design. This is reflected by the fact that ~ ¾ of both cases and controls were on medication for hypertension and that mean SBP among cases and controls was >150mmHg. Even among controls, 44% of the population had a history of vascular disease. These disease and risk factor levels were not seen in control populations in previous studies of stroke (Gussekloo et al, 2000; Cao et al, 2003; Fibrinogen Studies Collaboration, 2005)

A previous report from the PROSPER cohort has shown that within the entire cohort, CRP is associated with global vascular risk ([1]Sattar et al, 2007), and is also associated with risk of stroke, although not strongly: OR 1.38 (95% CI 1.08-1.85) in top versus bottom thirds of the population after only adjustment for pravastatin. Therefore the null association of CRP with risk of stroke in the present study must only be a facet of the control population (because the cases are the same ones as in the cohort). This observation supports the preceding hypothesis and is further alluded to in the overall cohort study, which shows that CRP was more strongly associated with risk of CVD events in those with no history of vascular disease (51% higher risk in the fully adjusted model) compared with those with a history of vascular disease (30% higher risk) ([1]Sattar et al, 2007).

Hence the present study has some limitations in terms of comparison to more general populations. It would be useful to measure TNFα and IL-18 in the whole PROSPER cohort.

10.4.3 IL-18 and TNFα and Risk of Stroke in the Elderly

Overall this study suggests that both IL-18 and TNFα are of limited use as biomarkers of risk of stroke in elderly patient populations with prevalent vascular disease or vascular disease risk. This finding suggests that in other "unselected" studies, potential associations of IL-18 and TNFα with risk of stroke may be confounded by both age and other vascular risk markers. Previously the PROSPER cohort has demonstrated that CRP adds nothing to the Framingham risk score in terms of the C-statistic, and does not add

information to indication of who would benefit from pravastatin prescription in the cohort ([1]Sattar et al, 2007). Combined with results from the present study, the conclusion must be that inflammatory markers in general may be of little use as risk predicotrs in elderly populations with prevalent vascular disease, either as prognostic markers, or as indicators of those who benefit from statin administration. More studies in more general elderly populations are required to confirm and expand on these findings, as well as further studies in middle-aged populations; although attaining large numbers of incident first strokes to sufficiently power observations in the latter population is problematic.

As a final step in the examination of associations of IL-18 and TNFα with stroke, I examined associations with risk in a case-control study of recurrent stroke.

CHAPTER 11

ASSOCIATIONS OF IL-18, TNFα AND THE APR WITH RECURRENT STROKE: THE PERINDOPRIL PROTECTION AGAINST RECURRENT STROKE (PROGRESS) TRIAL

11.1 INTRODUCTION

11.1.3 Inflammatory Markers and Recurrent Stroke

CRP and fibrinogen have been shown to be associated with recurrent stroke (Woodward et al, 2005) and with mortality in follow-up after major stroke (Elkind et al, 2006; Turaj et al, 2006) although these studies failed to classify initial strokes by major subtypes (Elkind et al, 2006) or to report the number of incident recurrent strokes in the 900 patients during follow-up (Turaj et al, 2006). There were no studies found which assessed either IL-18 or TNFα as risk markers for recurrent stroke in the literature. Also, despite recent interest in positive associations of IL-6 gene polymorphisms with stroke (Yamada et al, 2006; Lalouschek et al, 2006; Chamorro et al, 2005), the biomarker itself is has not been studied in either primary or recurrent stroke.

11.1.4 Aims

The aim of this study was to assess IL-6, IL-18 and TNFα as risk markers for recurrent stroke (according to major subtype) and their relative independence in risk prediction from conventional risk markers, acute phase reactants, and each other, in multivariate models. In addition to univariate associations we thus aimed to elucidate the contribution of "generalised" inflammation to risk of recurrent stroke.

11.2 MATERIALS AND METHODS

11.2.1 Study Design and Subject Recruitment

Of the estimated 15 million people who experience stroke worldwide each year, 10 million survive, although over a third of those survivors are classified as disabled (WHO,

Global Burden of Stroke, 2004). The survivors of stroke or TIA are at highly increased risk of recurrent strokes; around 4% in the first month, 12% in the first year, and 5% for every year thereafter (Hankey, 2003). Therefore effective treatment for prevention of secondary stroke is a medical priority.

Although it had been known for some time that blood pressure is a key risk factor in stroke (Eastern Stroke and Coronary Heart Disease Collaborative Research Group, 1998; Prospective Studies Collaboration, 1995), fewer data were available linking it as a risk factor to recurrent stroke. One meta-analysis of 2742 patients with history of cerebrovascular disease suggested that a SBP 6-8 mm Hg reduction was associated with one fifth reduction in stroke, although confidence intervals were wide (Rodgers et al, 1997). The diuretic inadapamide was reported to be effective in prevention of recurrent stroke (PATS Collaborating Group, 1995), but final results of that study were not published. The Perindopril Protection Against Recurrent Stroke (PROGRESS) study (PROGRESS Management Committee, 1996) was set up as a double blinded placebo controlled study of 6105 patients with previous stroke or TIA, who were given the ACE inhibitor perindopril, indapamide, combination therapy, or placebo. The aim of the study was to estimate the effects of blood pressure lowering on risk of recurrent stroke.

Stroke occurred in 307 patients (10%) in the active treatment group and 420 (14%) in the placebo group (relative risk reduction, 28%; 95% CI, 17 to 38%; $P<0.0001$) (PROGRESS Collaborative Group, 2001). Further studies showed active treatment reduced the risk of major coronary events by 26% (95% CI: 6-42%; p=0.02) and the risk of congestive heart failure by 26% (5-42%; p=0.02). Interestingly, there was no evidence of differences between the treatment effects in participants classified as hypertensive or non-hypertensive, and those with or without a history of coronary heart disease (PROGRESS Collaborative Group, 2003).

Since cessation of follow-up (average 3.9 years), the study has provided a valuable case – control study of recurrent stroke, with 591 cases matched with 1182 controls.

The PROGRESS case-control study was recently used to assess the hereto relatively unknown associations between stroke recurrence and CRP, fibrinogen, plasma viscosity, t-PA, and D-dimer (Woodward et al, 2005). Only CRP and fibrinogen were risk

predictors of ischaemic stroke (top thirds OR 1.34 [95% CI 1.01 – 1.78], and 1.39 [1.05 – 1.85] respectively) but not for haemorrhagic stroke. These haemostatic markers showed trends to be inversely associated with haemorrhagic stroke, but the association was not significant.

Stored plasma samples were available fro this case-control study, affording the oppertunity to measure both IL-18 and TNFα in the study and incorporate them into multivariate regression models with CRP and fibrinogen, to see if upstream inflammatory markers are responsible for association between acute phase reactants and recurrent stroke. The cytokine IL-6 (the major stimulator of the APR) was also measured. As there were no serum samples available in the PROGRESS study, MMP-9 and sCD40L were not measured here. All markers were measured as described in Chapter 2. Aliquots used for the measurement of IL-18 and TNFα had had two previous thaws.

The design of the PROGRESS study has been described in detail elsewhere (PROGRESS Management Committee, 1996). Briefly, 6105 patients with a history of stroke or transient ischaemic attack (TIA) were recruited and each patient randomly assigned active treatment (n=3051) or placebo (n=3054). Entry criteria was based on evidence of an acute disturbance of focal neurological or monocular function (with symptoms lasting less than 24 h consistent with vascular disease or thrombosis) within the previous 5 years. Patients had to have no history of intolerance to angiotensin-converting enzyme (ACE) inhibitors, and were required to show tolerance to open label perindopril for two weeks before enrolment into the study. For those deemed suitable by the responsible physician, mono or - dual therapy was allocated: perindopril and/or indapamide or single/double placebo. In the principal study 6105 patients with a history of stroke or transient ischaemic attack (TIA) were recruited and each patient randomly assigned active treatment (n=3051) or placebo (n=3054). At baseline, venous blood samples collected from 97% of patients, were anticoagulated with K_2 EDTA, and centrifuged at 2000*g* for 10 min at 4°C. 1.5ml aliquots of plasma were stored at –80°C. Patients were followed for a mean of 3.9 years.

The base population for the case-control study was all those who had plasma frozen and stored within 48 hours of venipuncture and whose most recent qualifying event (stroke or TIA) at baseline in the clinical trial occurred >1 month ago. These restrictions give

protection against deterioration of the blood samples and confounding attributable to acute-phase reactant increases in blood variables as a result of the incident stroke or its acute consequences.

The final case-control study comprised 591 incident cases matched with 1182 controls, which were selected as outlined below.

11.2.2 Statistical Analysis

During IL-18 measurements, two kits of IL-18 lot 39A were used, whereas the rest of the study was measured using lot 42A. There were significant differences between these lots (~50% see 12.3.1) and the assays using 39A were adjusted for to combine the different lots. This represents ~160 samples proportionately adjusted.

Stroke cases comprised anyone with an ischaemic or haemorrhagic stroke or transient ichaemic attack (TIA) recorded during follow-up. Using standard methodology for nested case-control studies (as described in chapter 7, Woodward et al, 2005), each case was randomly matched to between 1 and 3 controls selected from all those free of recurrent stroke at the time of recurrent stroke for that case. The overall sample size was restricted so that the total number of people selected who had no stroke during follow-up outnumbered the cases in the ratio of 1 to 2. Matching variables for controls were sex, age (within 5 years), principal study treatment arm, region (Australia or New Zealand / China / Japan / France or Belgium/Italy/Sweden/UK or Ireland) and most recent qualifying event (ischaemic stroke /TIA/hemorrhagic stroke/stroke of unknown type). Prespecified ischaemic and hemorrhagic stroke substudies isolated those cases and their matched controls, who had ischaemic or hemorrhagic strokes as their first recorded stroke event.

Odds ratios were calculated according to equal thirds of the distributions of each of the inflammatory markers, and trends across these thirds were tested, using conditional logistic regression models. These were computed both with and without adjustment for potential confounding factors (systolic blood pressure, smoking, prevalence of peripheral artery disease, statin use and antiplatelet use) not controlled for in the matching variables

11.3 RESULTS

11.3.1 Sample Availability

The nested case-control study includes 591 cases (83 haemorrhagic strokes, 472 ischaemic strokes, and 36 of unknown type). Controls satisfying all matching criteria were found for 572 of these cases and 19 were incompletely matched (~3%). After the addition of cases who acted as matched controls for other cases and taking account of controls who acted for >1 case, there were 33 cases with 1, 213 with 2, and 345 with 3 matched controls. Altogether, 89 cases served as controls for ≥1 other case. In the presentation of this data, "unique" cases and controls are shown, that is, after removing duplicates consequent to the unbiased nested design. 1182 "unique" controls who did not subsequently become cases (exact ratio 2:1) are hence included in the study.

After performance of assays on all available samples, there were few missing values for IL-6, IL-18, fibrinogen or CRP (>95%), but several for TNFα, due to its being the last of the assays performed on the limited residual blood supplies, and it requiring 200ul of plasma. Sample sizes for TNFα were 433 cases and 859 controls (73% of the total), representing equal numbers of missing cases and controls with similar matched demographic distributions for missing and assayed samples (p>0.1 for all?).

11.3.2 Population Characteristics

Table 11.1 summarises the baseline demographic data, and shows risk factor data according to whether the subject became a case or not during PROGRESS. In the ischaemic stroke group, several conventional risk factors for stroke were higher among cases than in controls including systolic BP, smoking, presence of diabetes at study entry, and presence of PAD at study entry, although not evidence of CHD. Among the inflammatory markers IL-6 was significantly (mean level 2.44 vs 2.20 pg/ml; *p*=0.003) higher in cases than in controls, as were fibrinogen and CRP (3.87.vs 3.73 g/L; p=0.005 and 2.20 mg/L vs 1.91 mg/L; p=0.03 respectively from previously published data; Woodward et al 2005). Neither IL-18 nor TNFα was significantly higher in the ischaemic

stroke case group, although there was a trend for TNFα to be higher among cases (2.56 versus 2.45 pg/ml; p=0.15).

For the haemorrhagic stroke case group, blood pressure showed non-significant trends to be higher among cases (SBP 2.5% increase p=0.11, DBP 2.8% increase p=0.08), and fibrinogen levels showed trends to be lower among cases (5% lower p=0.11). The study may be underpowered to detect these potential associations. No other conventional or inflammatory markers were significantly different in the haemorrhagic case group.

11.3.3 Risk Associations of Inflammatory Markers: Haemorrhagic Stroke

On analysis by thirds of their distributions, none of the cytokines showed significant associations with hemorrhagic stroke (Table 11.2). The highest third of TNFα had a moderately elevated risk compared to the lowest third, the adjusted OR being 1.23, although the 95% CI were wide (0.55-2.75) due to small numbers. Fibrinogen also showed trends towards and association, the highest third of expression having a borderline lower risk of haemorrhagic stroke; OR 0.56 (0.29-1.10) in models adjusted only for matched variables.

11.3.4 Risk Associations of Inflammatory Markers: Ischaemic Stroke

For ischaemic stroke, IL-6 showed a stepwise increase in OR across thirds, and although the trend was reduced to borderline significance after adjustment for conventional risk factors, the highest third still had an increased risk compared to the lowest: OR 1.33 (95%CI 1.00 – 1.78). TNFα showed somewhat similar associations, although the significance of its trend was entirely due to the excess risk comparing the highest to lowest thirds; OR 1.46 (95%CI 1.02 – 2.10) after adjustment. IL-18 was completely un-associated with risk of recurrent ischameic stroke even in model adjusted only for matched variables where the risk association was unity; OR 0.99 (0.77-1.27). As previously discussed, both CRP and fibrinogen were associated with risk of stroke in fully adjusted models comparing extreme thirds; OR 1.39 (1.05-1.85) and 1.34 (1.01-1.78) respectively.

11.3.5 Risk Associations of Inflammatory Markers: Total Stroke

For total stroke, both IL-6 and TNFα had a greater significance in trend of risk across thirds compared to the major subtypes of stroke. This is because there are slight trends for both cytokines to be associated with risk of haemorrhagic stroke in extreme thirds, and consequently higher case numbers and smaller confidence intervals when the categories are combined. Despite this, the actual estimates of associations were relatively unaffected by the addition of hemorrhagic and unknown strokes to ischaemic stroke, probably largely due to the predominance of ischaemic stroke as a case in the cohort. The significant associated risks were OR 1.47 (1.07-2.03; p=0.01) for TNFα and OR 1.31 (1.01-1.69; p=0.04) for IL-6 in fully adjusted models comparing extreme thirds (Table 11.2). Again, both CRP and fibrinogen were borderline risk predictors in the total stroke group; OR 1.26 (0.98 –1.61) and 1.24 (0.97-1.59) respectively.

11.3.6 Combinations of Inflammatory Markers as Risk Predictors of Ischaemic Recurrent Stroke

Summaries of multivariable models, including either three markers at a time (adjusted for one "missing" marker) or all four inflammatory markers at once, in addition to adjusting for potential confounders, are shown in Table 11.3. Adjusting TNFα for either IL-6, fibrinogen or CRP results in borderline residual associations of TNFα with ischaemic stroke. Adjusting either IL-6, CRP or fibrinogen for each other, or for TNFα, resulted in non-significant associations of the markers with ischaemic stroke when comparing extreme thirds. Inclusion of all four markers in the same model (adjusting each marker for the other three) reduced odds ratios for any of the markers to near unity, with the exception of the borderline significant residual OR comparing the extreme thirds of TNFα, which was 1.43 (95% CI: 0.98-2.10).

Table 11.1 Baseline summary data for all subjects in the nested case-control study classified by whether or not they developed either of the major subtypes of stroke during PROGRESS.

Variable	Haemorrhagic stroke		p-value	Ischaemic stroke		p-value
	Cases (n=83)	Controls (n=199)		Cases (n=472)	Controls (n=1011)	
Mean (95% CI)						
Age * (years)	62.5 (60.6–64.5)	62.4 (61.2–63.6)	0.92	66.5 (65.7–67.3)	66.2 (65.7–66.7)	0.48
SBP (mm Hg)	149.7 (145.4–154.0)	145.9 (143.4–148.4)	0.11	149.9 (148.1–151.6)	147.4 (146.3–148.6)	0.02
DBP (mm Hg)	88.4 (85.9–91.0)	85.9 (84.5–87.2)	0.08	86.0 (85.1–87.0)	85.0 (84.4–85.7)	0.10
Fib ‡ (g/L)	3.58 (3.40–3.76)	3.76 (3.63–3.89)	0.11	3.87 (3.79–3.96)	3.73 (3.70–3.78)	0.005
CRP † ‡ (mg/L)	1.47 (1.10–1.96)	1.62 (1.35–1.94)	0.57	2.20 (1.98–2.45)	1.91 (1.77–2.05)	0.03
IL-6 † (pg/ml)	2.00 (1.70-2.37)	2.00 (1.83-2.18)	0.99	2.44 (2.31-2.59)	2.20 (2.11-2.29)	0.003
IL-18 † (pg/ml)	455 (399-521)	474 (436-517)	0.59	484 (459-511)	481 (463-500)	0.84
TNFα † (pg/ml)	2.61 (2.24-3.07)	2.48 (2.35-2.62)	0.54	2.56 (2.44-2.69)	2.45 (2.38-2.53)	0.15
No (%)						
Males *	63 (76)	154 (77)	0.88	348 (74)	750 (74)	0.85
Smokers	15 (18)	43 (22)	0.63	110 (23)	173 (17)	0.006
Diabetes	6 (7)	19 (10)	0.65	85 (18)	113 (11)	0.0004
Previous CHD	11 (13)	39 (20)	0.23	91 (19)	172 (17)	0.31
PAD	1 (1)	7 (4)	0.94	32 (7)	39 (4)	0.02
Statins	5 (6)	13 (7)	1.00	36 (8)	77 (8)	1.00
Anti-PT	39 (47)	104 (52)	0.44	362 (77)	780 (77)	0.84

CI; confidence intervals, SBP; systolic blood pressure, PAD; peripheral arterial disease, Anti-PT; antiplatelet therapy.
* Matching variable
†Analysed on logarithmic scale and back-transformed to show estimates
‡ Previously published: Woodward et al, 2005

Table 11.2 Odds Ratios (95% confidence intervals) for haemorrhagic, ischaemic and total stroke by thirds of each variable

	Haemorrhagic Stroke		Ischaemic stroke		Total stroke	
Variable/third	Matched	Adjusted*	Matched	Adjusted*	Matched	Adjusted*
IL-6 (pg/ml)						
1 (<1.70)	1	1	1	1	1	1
2 (1.70-2.94)	1.10 (0.58-2.06)	1.15 (0.60-2.21)	1.36 (1.03-1.79)	1.33 (1.01-1.77)	1.29 (1.01-1.65)	1.28 (1.00-1.64)
3 (>2.94)	1.17 (0.61-2.24)	1.18 (0.61-2.27)	1.43 (1.08-1.90)	1.33 (1.00-1.78)	1.37 (1.06-1.76)	1.31 (1.01-1.69)
p (trend)	0.62	0.60	0.02	0.06	0.02	0.04
IL-18 (pg/ml)						
1 (<366)	1	1	1	1	1	1
2 (366-582)	0.70 (0.37-1.33)	0.78 (0.40-1.50)	1.09 (0.83-1.44)	1.07 (0.81-1.41)	1.02 (0.79-1.30)	1.02 (0.79-1.30)
3 (>582)	0.74 (0.38-1.45)	0.77 (0.39-1.53)	1.04 (0.78-1.39)	0.97 (0.72-1.29)	0.99 (0.77-1.27)	0.94 (0.72-1.21)
p (trend)	0.36	0.44	0.78	0.84	0.92	0.63
TNFα (pg/ml)						
1 (<2.17)	1	1	1	1	1	1
2 (2.17-2.89)	0.88 (0.41-1.88)	0.81 (0.37-1.80)	0.98 (0.68-1.40)	0.96 (0.67-1.39)	1.01 (0.74-1.39)	1.00 (0.73-1.38)
3 (>2.89)	1.43 (0.66-3.12)	1.23 (0.55-2.75)	1.47 (1.03-2.11)	1.46 (1.02-2.10)	1.49 (1.08-2.05)	1.47 (1.07-2.03)
p (trend)	0.35	0.58	0.02	0.03	0.01	0.01
Fib (g/L)†						
1 (<3.32)	1	1	1	1	1	1
2 (3.32–4.04)	1.20 (0.65-2.23)	1.17 (0.61–2.22)	1.22 (0.94–1.60)	1.20 (0.92-1.58)	1.19 (0.93-1.51)	1.17 (0.92-1.49)
3 (>4.04)	0.56 (0.29-1.10)	0.57 (0.29–1.14)	1.43 (1.08–1.89)	1.34 (1.01-1.78)	1.28 (1.00-1.64)	1.24 (0.97-1.59)
p (trend)	0.13	0.13	0.009	0.03	0.03	0.06
CRP (mg/L)†						
1 (<1.14)	1	1	1	1	1	1
2 (1.14–3.34)	0.57 (0.30-1.09)	0.59 (0.31–1.16)	1.37 (1.04–1.80)	1.31 (0.99-1.73)	1.20 (0.94-1.52)	1.16 (0.91-1.49)
3 (>3.34)	0.77 (0.41-1.46)	0.81 (0.42–1.57)	1.52 (1.15–2.00)	1.39 (1.05-1.85)	1.34 (1.05-1.70)	1.26 (0.98-1.61)
p (trend)	0.13	0.22	0.03	0.12	0.14	0.32

*Adjusted for systolic blood pressure, smoking, prevalence of peripheral artery disease, statin use and antiplatelet use

† Previously published; Woodward et al, 2005

Table 11.3 Odds Ratios (95% confidence intervals) for ischemic Stroke for combinations of inflammatory variables in multivariable models, by thirds, adjusted for the "missing" variable (1st third not shown; by definition, odds ratio = 1), and then for every other marker (final section of rows). All models are adjusted for systolic blood pressure, smoking, peripheral artery disease prevalence, statin use and antiplatelet use.

Third of the population	**TNFα**	**IL-6**	**Fibrinogen**	**CRP**
2nd	0.95 (0.65-1.37)		1.22 (0.92-1.62)	1.09 (0.81-1.48)
3rd	1.44 (0.99-2.10)		1.33 (0.97-1.80)	1.16 (0.84-1.61)
p (trend)	p=0.05		p=0.08	p=0.31
2nd	0.97 (0.67-1.39)	1.27 (0.95-1.69)		1.14 (0.85-1.53)
3rd	1.44 (1.00-2.08)	1.21 (0.89-1.64)		1.18 (0.85-1.62)
p (trend)	p=0.04	p=0.27		p=0.34
2nd	0.96 (0.67-1.39)	1.27 (0.94-1.71)	1.20 (0.90-1.59)	
3rd	1.43 (0.99-2.08)	1.24 (0.90-1.72)	1.29 (0.94-1.78)	
p (trend)	p=0.05	p=0.23	p=0.12	
2nd		1.17 (0.83-1.65)	1.07 (0.76-1.50)	0.99 (0.70-1.40)
3rd		1.08 (0.74-1.57)	1.09 (0.76-1.56)	1.13 (0.79-1.63)
p (trend)		p=0.63	p=0.60	p=0.46
2nd third	0.93 (0.64-1.34)	1.11 (0.76-1.61)	1.07 (0.76-1.53)	0.95 (0.65-1.38)
3rd third	1.43 (0.98-2.10)	0.99 (0.65-1.50)	1.02 (0.68-1.53)	1.13 (0.73-1.76)
p (trend)	0.04	0.92	0.89	0.50

11.4 DISCUSSION

11.4.1 Comparison of the Population Distributions with MONICA-4

Comparing this population's distribution of inflammatory markers to that of the MONICA-4 study, it is of note that the median IL-18 expression in this cohort (~450 pg/ml in cases and controls) is much higher than that even of the eldest age groups in the MONICA-4 cohort (median male and female in 55-64 years ~250 pg/ml). This could be in part be attributed to assay QC drift, due to the lack of an international standard, but use of the in-house QC should prevent drift of anything like this magnitude of difference. In a similar fashion, TNFα levels were also higher here than medians in the eldest age group of MONICA-4 (~2.47 pg/ml in controls and 2.61 pg/ml in cases versus ~1.90 pg/ml in MONICA-4). This may suggest that TNFα and particularly IL-18 are elevated in people with prevalent cerebrovascular disease after (>1 month in this study) the index event. The effect is probably reverse causal-discussion.

11.4.2 Association of Only APR-Associated Inflammatory Markers with Risk or Recurrent Stroke

To my knowledge, this is the first reported study of the association between the pro-inflammatory cytokines IL-6, IL-18 and TNFα and risk of recurrent stroke. We found that TNFα and IL-6, but not IL-18, were significant risk predictors of recurrent ischemic stroke. In TNFα only the top third of the population was at statistically increased risk. This finding is consistent with a previous study of recurrent myocardial infarction, which found that only those with very high TNFα expression were at increased risk (Ridker et al, 2000). IL-6 appeared to show stepwise increases in risk through thirds of this population, although the trend was less notable after adjusting for potential confounders.

A major biological property of IL-6 and TNFα is that these cytokines may actively participate in the hepatic stimulation of the APR (Hansson et al, 2005). There is little evidence to suggest IL-18, which was not associated with any major stroke subtype in this study, directly contributes to the APR (Stuyt et al, 2005). As previously noted in the original PROGRESS case-control study (Woodward et al, 2005) and in this thesis, the

acute-phase reactant behaviour of fibrinogen, CRP, or any inflammatory marker may be viewed as a potential confounder of associations with recurrent stroke. This is because the underlying association may be due to "generalised inflammation" (as proximally measured by the APR) rather than the causal behaviour of any individual inflammatory marker *per se* (Lowe et al, 2005). However, adjusting acute phase reactants only for each other in multivariable models to remove potential acute phase response confounding assumes that the APR is an absolute event, with roughly equal effects on circulating CRP and fibrinogen levels. This may not be an accurate assumption, particularly when making only one baseline observation. A recent publication has shown in acute stroke patients that the pattern of expression of CRP is stable in days 1-90 following stroke, while levels of fibrinogen increase following the acute phase, and levels of leucocyte counts fall (Marquardt et al, 2005). These observations are broadly confirmed by the SIP study. This suggests that a "textbook" APR is not seen following an acute stroke, and markers of the acute phase response can behave temporally differently. The patients in the current study had incident ischaemic stroke up to 5 years ago, and the dynamics of the response may be different. Indeed, IL-18 levels (which were not elevated in acute stroke), are substantially elevated in the chronic phase as shown in this study.

By adjusting for combinations of CRP, fibrinogen, IL-6 and TNFα, this study shows that markers of the APR and stimulators of the APR almost completely explain each other's association with recurrent stroke. This suggests that despite evidence that IL-6, IL-18, and TNFα are involved in experimental atherogenesis and atherothrombosis (section 1.2.8, 1.3.5, and 1.4.5), potential associations with recurrent stroke are almost fully explained by conventional risk markers, and proximity to the APR. Equally this suggests that CRP and fibrinogen themselves are associated with recurrent stroke due to being acute phase reactants rather than necessarily making additional inflammatory contributions to atherothrombosis. Clearly an individual inflammatory marker may be a "better" risk predictor than others, but this would depend on the time point the observation is made in moving from the acute to the chronic response to stroke and underlying prevalent CVD.

Just as caution must be employed in drawing conclusions of causality in epidemiological studies (Lowe et al, 2005) it is equally important not to exclude causality on the same

basis. It is quite possible (perhaps probable) that some inflammatory markers actively participate in atherogenesis and atherothrombosis, at least partially through other conventional risk factors (e.g. the association of inflammation with smoking or cholesterol levels). However, associations of inflammatory markers of the acute phase response with recurrent stroke were fully explained by conventional risk markers and APR confounding in this model, and this may be consistent with observations that CRP adds little to Framingham assessments of primary stroke risk (Bos et al, 2006). This suggests that from the inflammatory markers measured in this study, it is only "general inflammation" rather than a particular marker that is associated with secondary ischemic stroke.

11.4.2 Limitations and Strengths

This was a large nested case-control study from a well-defined cohort of patients, and as such was ideal for the measurement of inflammatory cytokines among a novel patient group. Despite this there are limitations to this study to consider. Entry into the study was only on the basis of a qualifying event in the last 1 month – 5 years. It is possible that recurrent events that occurred during the 3.9 year median follow-up would have occurred at a greater rate among those with more recent stroke. It is hence possible that those who experienced a recurrent event had elevated levels of certain inflammatory markers due to the more proximal length of time since their index event, rather than it reflecting risk profile *per se*. Associations between inflammatory markers and risk could hence reflect the greater risk those who have had stroke in the last year experience compared with subsequent years (12% risk in the first year, and 5% for every year thereafter [Hankey, 2003]). However, a minimum 1 month since index event should be sufficient to allow any residual reverse-caused inflammatory reaction to settle in most patients. More specifically, for IL-18 and TNFα, the results from the SIP study argue against profound acute phase reaction complications for these cytokines in acute stroke, suggesting consequent elevation of these cytokines after the acute event may reflect *bona fide* physiological processes potentially related to recurrent stroke risk.

Entry to the study was based on physician diagnosis of stroke or TIA thought to be thrombotic or vascular in origin of ischaemia but was not based on clinical imaging,

giving rise to the possibility of inaccurate diagnosis. Despite this the accuracy of a clinical examination by an emergency physician is good, with a sensitivity of about 85% and a specificity of 99% for ischaemic stroke (von Arbin et al, 1981).

11.4.3 Conclusions

This study finds that TNFα, but not IL-18, is moderately associated with risk of recurrent cerebrovascular disease (particularly ischaemic stroke) in those with a history of ischaemic stroke or TIA. However, inflammatory markers generally associated with the acute phase response (promoters; IL-6 and TNFα, acute phase reactants; CRP and fibrinogen) are all associated with risk of recurrent stroke (but not IL-18). These markers are dependant on each other in multi-variable models, and once all are included, none are independently significant. This suggests that the generalised inflammation of the acute phase response predicts recurrent stroke, rather than any particular marker.

CHAPTER 12

GENERAL DISCUSSION

12.1 INFLAMMATION AND CARDIOVASCULAR RISK

12.1.1 General Overview

In the mid-1970s, Russell Ross put forth the theory that an injury to the blood vessel wall begins the insidious process of inflammation, ulcerated plaque formation and activated platelet aggregation at the endothelial layer (Ross, 1976). 30 years on, it is remarkable to observe that the implications of this ground breaking theory are still being debated and refined. The injury to the blood vessels that Ross proposed is thought to occur as a result of anatomical or physiological stress (Lowe, 2003) such as non-laminar flow or DNA methylation bringing about genetic aging (Stenvinkle et al, 2007), by exposure to oxidised lipids (Cominacini et al, 2001), or perhaps by latent or active infection (Vita and Loscalzo, 2002; Liuba and Pesonen, 2005). It seems likely that the combined effects of these types of processes are greater than the sum of their parts, and they all converge at one central process: the stimulation of inflammation.

As reviewed in Chapter 1, there is increasing awareness of the role of inflammation in the promotion of atherosclerosis in the scientific, clinical and lay communities. No longer considered a post-script to lipid accumulation and arterial occlusion, inflammation is a central component of atherogenesis and its acute complications. Animal models, epidemiological evidence, and the non-lipid effects of statins all seem to suggest that chronic low-grade systemic inflammation is associated with risk of CVD events, and it is easy to illustrate biological mechanisms for these processes (Figs 1.1-1.5). Indeed, accumulating evidence suggests that high grade inflammation (such as that seen in RA and type 1 diabetes) is also associated with increased cardiovascular risk in a potentially causal manner (Solomon et al, 2003; Sattar and McInnes, 2005; Sherer et al, 2006). The problem remains however that although the evidence for a role for inflammation in general is strong, there are real problems in resolving which are the causally important factors, and which are bystanders associated with the inflammatory process. This is an

important issue in terms of what markers may be therapeutic targets, and which may add clinically useful information to risk prediction algorithms.

Fibrinogen and CRP have both consistently demonstrated moderate associations with risk of CHD events in both healthy populations and in populations with prevalent vascular disease (Fibrinogen Studies Collaboration, 2005; Danesh et al, 2004), although CRP in particular demonstrates evidence of early publication bias (Danesh et al, 2004). Furthermore, the causal role of these molecules in atherogenesis have been called into question. They are both products of the APR, and are potentially bystanders of low grade systemic inflammation. Genetic manipulation of promoters of either molecule in animal models suggests that modest overexpression has no significant effect on atherosclerosis (Gulledge et al, 2003; Rezaee et al, 2002; Trion et al, 2005). While Pepys et al (Pepys et al, 2006) have recently reported a potential inhibitor of CRP in a mouse model, no specific therapeutic inhibitors of either molecule exist for field trials in humans to resolve this issue. Furthermore there is debate as to the potential clinical utility of these markers, since the C-statistic indicates that little may be gained from adding CRP to Framingham risk assessments (Folsom et al, 2005; [1]Wilson et al, 2005; Tzoulaki et al, 2007). Proponents of the clinical measurement of CRP for CVD risk prediction, most notably Ridker's Boston group, have sparked widespread debate as to the clinical utility of the C-Statistic (Cook et al, 2006; Cook 2007; Ridker, 2007). The C-statistic may be a blunt tool for estimating clinical utility of a marker, but it is currently the best statistical means available to discriminate additional predictive utility of a marker.

In this thesis I have examined the utility of pro-inflammatory markers which are upstream of the APR as potential predictors of CVD events. These markers are 1) directly pro-inflammatory in most physiological settings (as opposed to CRP and fibrinogen), 2) convincingly pro-atherogenic in animal and tissue models, and 3) upstream of potential associations of APR markers. I identified four inflammatory markers (TNFα, IL-18, MMP-9, and sCD40L) which have strong putative mechanistic roles in atherogenesis and atherothrombosis in experimental studies, which are detectable in the circulation of healthy and diseased persons, and for which there was limited literature to suggest that they may be associated with CVD risk.

12.2 PRE-ANALYTICAL AND PRACTICAL CONSIDERATIONS OF USE OF INFLAMMTORY BIOMARKERS

12.2.1 General Considerations

If there is a case for any biomarker (inflammatory or otherwise) to be added to clinical risk prediction algorithms, a primary concern is the practical ability to measure the markers both in scientific (particularly epidemiological) and clinical settings. Many proponents of CRP measurement in risk prediction cite this as one of CRP's key distinguishing properties from other inflammatory markers; a relative biochemical stability, indifference to sample source type (plasma/serum), and a well standardised, cheap assay (Pearson et al, 2003). Fibrinogen is also a stable, standardised, cost-effective biomarker, with an abundance of data linking it to CVD risk (Fibrinogen Studies Collaboration, 2005). Other inflammatory markers may also become standardised and cheap to measure if demand for the marker from the scientific community (on the basis of consistently strong risk associations) grows.

The points considered in this thesis for the four markers are summarised in Table 12.1.

Table 12.1 Overview of pre-analytical variables for IL-18, TNFα, MMP-9 and sCD40L, comparing these markers to more established inflammatory risk markers of CHD (fibrinogen, CRP and IL-6). + and – ratings are given as a relative comparison.

	Assay characteristics				Marker stability				Variability	
	Sample type		Accuracy		Storage		Freeze-thaw		Short-term Bio variation	4-Year regression dilution
	Use in serum?	Use in plasma?	Intra-assay CV	Inter-assay CV	Absolute values stable?	Correlations stable?	Absolute values stable?	Correlations stable?	Reliability coefficient (95% CI)	Regression dilution coefficient
IL-18	+	+	~5%	~10%	++	+/-	+/-	+/-	0.66 (0.52-0.93)	0.71
TNFα	+	+	~8%	~12%	+ (?)	+ (?)	+	++	0.58 (0.43-0.89)	0.25
MMP-9	+	-	~4%	~10%	--	++	--	+/-	?	0.46
sCD40L	+	-	~4%	~15%	+	++	---	++	?	0.47
Fibrinogen†	-	+*	~3%	~4%	++	++	++	++	0.73 (0.60-0.94)	0.61‡
CRP†	+	+	~5%	~8%	++	++	++	++	0.58 (0.43-0.89)	0.52‡
IL-6†	+	+	~8%	~9%	++	+/-	++	+/-	0.28 (0.17 – 0.83)	?

*Citrate only for von Clauss detection method
† Data from Poorhang MSc thesis, 2005, unless otherwise stated
‡ Data from Emberson et al, 200

12.2.2 IL-18 as a Biomarker

Chapter 3 demonstrated that the IL-18 ELISA employed shows IL-18 to be a relatively flexible biomarker, suitable for use in serum or in plasma. It has assay CVs generally comparable to other inflammatory markers (including CRP and fibrinogen which are measured by automated means), which suggests that the assay is sensitive and the data reproducible. IL-18 was a relatively biochemically stable marker, despite an initial decrease in detectable levels after a second thaw. One would therefore expect there may also be a decrease in detectable levels after a thaw for the first time from fresh plasma (although this was not investigated here due to the primarily epidemiological rather than clinical focus of this thesis). This finding requires further investigation.

IL-18 had a level of short-term biological variation somewhat lower than other inflammatory markers, including CRP, although more data is required to narrow confidence intervals in order to confirm this result. IL-18 also demonstrated a relatively small degree of variation of over 4 years in regression dilution studies. This is in agreement with observations that IL-18 levels increase only marginally as middle-aged men approach old age (Chapter 4). The conclusion that IL-18 is relatively stable in terms of biological expression in the long- and short-term, would make it an attractive clinical biomarker.

Based on the evidence presented here, I concluded that IL-18 was suitable for use as an epidemiological marker in prospective studies and in cross-sectional studies.

12.2.3 TNFα as a Biomarker

Like IL-18, TNFα is a versatile biomarker and serum or plasma samples are suitable for its measurement, although it was noted in Table 3.2 that EDTA levels of TNFα in our plasma validation study sample were lower than those reported by the kit manufacturer. This difference is further explored in section 12.3.1.

The assay error in measurement of TNFα is slightly higher than for the other markers, despite the assay being "high-sensitivity." This is likely to be a reflection of the very low circulating levels of the marker. In so far as the available data available allowed investigation (due to a kit manufacturing alteration), TNFα was the most robust of the four novel inflammatory markers, relatively resistant to both storage and freeze-thaw cycles.

Short-term biological variability of TNFα was comparable to most of the other inflammatory markers, including CRP. In spite of this, TNFα demonstrated a regression dilution coefficient which makes it the most variable marker over 4 years measured to date (Emberson et al, 2004). This may mean that associations of TNFα with risk of CHD are greatly underestimated in prospective studies.

Based on the evidence presented here, I concluded that TNFα was suitable for use as an epidemiological marker in prospective studies and in cross-sectional studies.

12.2.4 MMP-9 as a Biomarker

According to the manufacturers (RandD Systems), MMP-9 can only be measured in serum (or heparinised plasma) by ELISA due to chelation by citrate and EDTA. We accepted this declaration without further investigation (although some authors recently report plasma should be used, but do not investigate the chelation issue [1.5.5]). In this work, serum levels were certainly detectable. The assay sensitivity and reproducibility was comparable to most other inflammatory markers. MMP-9 levels were biochemically labile, and decreased with both time in storage and number of freeze-thaws. Despite this, significant rank correlations of the data sets were maintained within the investigative limits of the current work.

Short-term biological variation of MMP-9 was not investigated due to non0availability of serum. The degree of regression dilution was similar to that observed of CRP, suggesting that despite biochemical labiality, variation in circulating concentrations of MMP-9 over time is fairly comparable to CRP. Hence the degree of underestimation in prospective studies is also likely to be similar.

Based on these findings, I concluded that MMP-9 was suitable for use in epidemiological studies (in serum) presuming all samples had the same storage and freeze-thaw history within the study.

12.2.5 sCD40L as a Biomarker

sCD40L could only be detected with any sensitivity in serum samples. Despite some reports that sCD40L should be measured in plasma (1.6.5), we have found plasma levels to not be accurately detectable in normal people, and other studies have suggested that, in practice, blood sampling procedures will artificially elevate sCD40L levels compared to the *in vivo* setting (1.6.5). The ELISA kit we used showed similar sensitivity and reproducibility to other inflammatory markers, although the inter-assay variation may be somewhat higher, but this result may be a chance finding and is not in agreement with the manufacturer's report. sCD40L levels were labile when subjected to biochemical stress, and decreased with both time in storage and number of freeze-thaws, particularly the latter, detectable levels falling almost by half after 4 thaws. Despite this, significant rank correlations of the data sets were maintained within the investigative limits of the current work.

Short-term biological variation of sCD40L was not investigated due to non-availability of serum. The degree of regression dilution was similar to that observed in CRP, suggesting that despite biochemical labiality, variation in circulating concentrations of MMP-9 are fairly comparable to CRP over time. Hence the degree of underestimation in prospective studies is also likely to be similar.

Based on these findings, I concluded that sCD40L was suitable for use in prospective case-control studies (in serum) presuming all samples within the study had the same storage and freeze-thaw history.

12.2.6 Suggestions for Further Research on the Practical Limitations of the Biomarkers

Fresh blood samples were not used in the present thesis. Similar studies to those reported here in fresh samples are required to verify the suitability of these markers for clinical

use. Some studies have already investigated this for sCD40L (Ahn et al, 2004; Mason et al, 2005; Varo et al, 2006; Weber et al, 2006), although they report on the presumption that plasma levels of sCD40L are more suitable for use. I do not believe this to be the case (3.2.4). Overall, it is unlikely that sCD40L or MMP-9 could be useful clinical markers of risk due to their labile nature and inflexibility in blood letting procedure. IL-18 and TNFα are more versatile in terms of sample type, and more robust in terms of stability, and in that sense are comparable to CRP and fibrinogen. More data subjecting both markers to more rigorous tests of stability in the healthy and those with prevalent disease are required.

The short-term biological variation data reported is revelant, both from an epidemiological and from a clinical aspect. Due to the small number of volunteers studied, the confidence intervals from the estimates in the study are wide. More data in sick as well as healthy people are required to expand findings and to narrow these confidence intervals, although it is problematic practically (in terms of both labour intensiveness and finding suitable willing volunteers and patients) to achieve this.

In particular, it may be interesting to study variability over a longer period than 20 weeks. This would allow further investigation into the issue of whether some individuals have "permanently" elevated variability. If so, are these individuals at elevated CVD risk beyond that predicted by their "habitual" inflammatory marker levels? One could speculate that they may be; increased variability (aside from transient infection) may be indicative of living a "high-risk" lifestyle (e.g. binge drinking). Indeed, one could speculate that it may be (at least partially) that those with higher levels of cytokines in prospective studies with one baseline measurement are more likely to be "variable" (since the variation away from habitual levels is positively skewed). Prospective studies of these markers in general cohorts, taking 3-4 baseline blood samples (one week apart) from each participant would be interesting, although a formidable undertaking. This would allow ~90% reliably estimated habitual plasma levels for all the inflammatory markers (perhaps with the exception of IL-6) in all participants. This would not only improve "true" risk association estimates, but would allow investigation into the hypothesis that variability itself may be associated with CVD risk.

As discussed in Chapter 4, further study of regression dilution ratios is warranted, both in terms of improved estimates (greater n) and investigating regression dilution over other timeframes. This is possible to do using the BRH study (Emberson et al, 2004). In particular, more data is required to confirm and expand on the relative temporal stability of biological expression of IL-18, and the relative instability of TNFα. As noted in Chapter 3, the similarity in short-term biological CV of the two cytokines is expected. That they should become so divergent in expression stability over time is interesting, and may reflect TNFα associations with underlying biological ageing processes (such as developing atheroma) while IL-18 is relatively unaffected.

The interaction of short-term variation and long-term regression dilution also requires further work. Although regression dilution increases over the years, for most inflammatory markers, a large degree of regression dilution is seen within the first week (Emberson et al, 2004). It would be an interesting further project to try to develop an algorithm to estimate the total overall degree of associative underestimate (both short- and long-term) of inflammatory markers

12.3 POPULATION DISTRUBUTIONS OF IL-18 AND TNFα

12.3.1 Comparing Levels of IL-18 and TNFα Across all Thesis Study Populations

Table 12.2 compares median and IQRs of IL-18 and TNFα in all populations in the thesis.

IL-18 levels are generally comparable throughout the study. The middle-aged men in MONICA-4 have similar levels of IL-18 to those in the prospective CHD studies, BRH and Fletcher Challenge. Those with a history of stroke (but not acute stroke) have much higher levels of IL-18 (in PROGRESS) which is direct contrast to those with a history of CHD (GLAMIS). In agreement with findings of an IL-18 and age correlation in MONICA-4 (typical of most inflammatory markers), the elderly (with a history of vascular disease; PROSPER) have elevated levels of IL-18.

The generally tight agreement of IL-18 levels between studies makes it unlikely that there are problems regarding its measurement using the current assay. Despite this, I have noted that both the levels of the quality control (QC) sample, and levels in the mean of the assay within a population (a secondary QC measurement in large studies) may vary between kit lots of IL-18. For example, using IL-18 lot 38A and 39A our QC (777712) had a mean value of 197 ± 22 pg/ml (n=32), but using IL-18 lot 42A the mean was 294 ± 24 pg/ml (n=21). This represent a difference of 49%; hardly negligible, and potentially very problematic in terms of directly comparing studies. These differences have not affected the majority of this thesis, since it was almost all performed using lot 37, 38A, and 39A (highly comparable). Adjustment was used on the few assays where there was an issue with lot 42A in the PROGRESS study. Despite this, the issue of cross-lot differences and drift is clearly a problem with this particular assay (which is the most widespread in use in the literature). IL-18 therefore requires an international standard before it can be considered a clinically credible biomarker. Assay standardisation is of paramount importance in epidemiological studies. Due to such problems previously with other markers, our laboratory has adopted a policy of ordering one kit lot of a marker in large epidemiological studies. This at least ensures samples within the same study are comparable.

This problem in standardisation of assay lots can also be seen for TNFα. The first TNFα studies performed in this thesis were for the pre-analytical study of plasma validation (Chapter 3), GLAMIS, and the BRH study. TNFα levels in EDTA plasma in the plasma validation study were noted to be lower than the manufacturer's levels (Table 3.2). Table 12.2 also shows TNFα levels were also far lower in the GLAMIS and BRH studies than elsewhere. These three studies were performed using kit lots 226764 and 227166. Analysing the QC data, QC 777710 had mean levels of 0.42 ± 0.18 pg/ml using these earlier kit lots (n=42). Subsequently, using kit lot 228849, QC 777710 had a mean of 1.14 ± 0.21 pg/ml. This is an increase by a factor of 2.71. Adjusting the detected levels of TNFα in Table 12.2 for GLAMIS and the BRH would clearly help rectify problems in cross-comparison with MONICA-4. Other apparent discrepancies to do with other kit lots would remain however. For instance, the elderly in PROSPER do not appear to have elevated levels of TNFα compared with MONICA-4, which contradicts an age-related increase in circulating levels of TNFα suggested by MONICA-4 (Chapter 4). Those with

acute (SIP) and with history of stroke (PROGRESS) appear have elevated circulating levels of TNFα, again perhaps suggesting one would expect to see elevated levels in persons with vascular disease.

In conclusion, although I have shown that these markers are stable enough to be examined in cross-study comparisons and using reference ranges, such studies do require the assays to be reproducible for the potential of such studies to be realised.

Table 12.2 Population distributions of IL-18 and TNFα, in each of the case-control and cohort studies. Values are medians and IQRs.

Study	Population	IL-18 (pg/ml)	TNF (pg/ml)
MONICA-4	Cohort	222 (169-293)	1.59 (1.28-2.03)
	Men (55-64 yrs)	251 (191-340)	1.90 (1.50-2.35)
	Control	219 (166-287)	1.56 (1.26-1.97)
	Case	249 (188-332)	1.87 (1.49-2.39)
GLAMIS	Control	271 (200-373)	0.77 (0.52-1.22)
	Case	287 (212-404)	0.99 (0.65-1.64)
BRH Study	Control	283 (204-381)	0.92 (0.69-1.36)
	Case	308 (224-406)	0.89 (0.60-1.24)
Fletcher Challenge	Control	271 (203-346)	1.74 (1.39-2.25)
	Case	295 (228-409)	1.89 (228-409)
SIP	Cohort	277 (202-370)	2.10 (1.47-2.84)
Prosper	Control	330 (255-434)	1.24 (0.89-2.03)
	Case	336 (276-438)	1.30 (0.93-1.78)
PROGRESS	Control	450 (335-687)	2.47 (1.94-3.08)
	Case	451 (326-685)	2.61 (2.04-3.28)

12.3.2 Future Work on Population Distributions

It is a poor commentary on commercial suppliers when such obvious QC issues are spotted by customers, especially when a premium is paid assuming the kits are fully QC-compliant, and where international standards are available to minimise the risk of these mistakes (as for TNFα). It may be the case that development of in house-assays may rectify these problems by allowing the researcher to maintain adequate QC themselves. As a marker becomes used in a more widespread fashion however (as will happen if the marker is epidemiologically interesting) manufacturers will be required to tighten QC regulations to ensure that studies in the literature between groups are comparable.

It remains to be seen in future studies if recent changes in the TNFα kit (3.4.1) rectify these problems, and the manufacturers have not reported the reason these changes were necessary other than "to improve sensitivity". Eventual development of an IL-18 standard should also help this marker. There is nothing inherently wrong with the currently reported studies in delineating population distributions (although more studies, larger than MONICA-4 are required to confirm and expand on findings), or the presupposed reasoning that the populations should be directly comparable. Observations have been hampered only by poor manufacturer QC.

12.4 IL-18 AND TNFα ASSOCIATIONS WITH CONVENTIONAL CARDIOVASCULAR RISK MARKERS AND MARKERS OF THE METABOLIC SYNDROME

12.4.1 Study Heterogeneity in Risk MarkerAssociations

Several studies in the current thesis have delineated associations of IL-18 and TNFα with conventional risk markers. The results from these are summarised generally in Table 12.3. Strengths of associations are summarised in categories (rather than using absolute r-values) due to differences between studies in term of data analysis methodology. As can be seen, there are many similarities in correlations in different studies, but also areas of heterogeneity. For instance, IL-18 correlated strongly with age in the Fletcher challenge study, not at all in the BRH study or GLAMIS, and moderately in MONICA-4. This

highlights the caution that should be employed when examining associations of risk markers in highly selected cohorts, as discussed in section 6.4.3. For instance the BRH is a highly age-selected, male only study. Although these are actually fairly broad qualifying criteria they are likely to impact on observed associations by making age, and perhaps other characteristics of the study population (such as BP) more narrow than in a general cohort. This limits the ability of the data to powerfully assess associations of markers with wide ranges of characteristics (Woodward, 2005). For these reasons, more weight must be placed on associations observed in the MONICA-4 cohort.

12.4.2 Associations of IL-18 and TNFα

Both IL-18 and TNFα are generally positively associated with age (Table 12.3), which is in agreement with observations in most inflammatory markers (Lowe et al, 1997, Woodward et al, 1999, 2003; Fibrinogen Studies Collaboration, 2005; Gimeno et al 2007; Miles et al, 2006). It has been known for some time that TNFα increases with age (Paolisso et al, 1998), and blood vessels from older mice manufacture increased TNFα, which is a potentially contributing factor to circulating concentrations (Belmin et al, 1995). The specific observation that IL-18 is relatively weakly associated with age has also been noted in other recent population studies (Hung et al, 2005; Thorland et al, 2005). Both cytokines are also higher in men than in women (MONICA-4), which is in agreement with other studies (Chapmen et al, 2006; Yamagami et al, 2005). This is in contrast to fibrinogen, which is higher in women than in men (Fibrinogen Studies Collaboration, 2005).

IL-18 is strongly associated with smoking in both sexes, which is in contrast to TNFα, which only shows weak overall associations. Nicotine may inhibit mononuclear cell production of TNFα (Madrestma et al, 1996), and clearly this may be a very specific inhibitory pathway. General inflammatory marker associations with smoking (CRP, IL-6, fibrinogen etc; Woodward et al, 1997 and 2003; [2]Wannamethee et al, 2005) are suggestive that inflammation may be a key pathophysiological mediator between the habit of smoking and the increased risk of CVD, although associations cannot be taken to imply causality (Lowe et al, 2005).

Blood pressure is a key conventional risk factor, and like smoking, inflammation may partially be a cause or consequence of high blood pressure (e.g. due to increased endothelial dysfunction in hypertension and increased inflammatory mediator production as well as extravasation of monocytes into the tunica intima) (Bautista et al, 2003). For instance, CRP may be produced by mouse SMCs in response to angiotensin II (Peng et al, 2007) and inhibition of the renin-angiotensin system is thought to suppress general inflammation (Sironi et al, 2005) including TNFα production (Schindler et al, 1995). Indeed, angiotensin II has also been implicated in promoting IL-18-mediated cross-talk between SMCs (Sahar et al, 2005). It may therefore be somewhat surprising that neither cytokine was strongly associated with blood pressure in any population in this thesis. A recent review discusses the positive relationship between CRP and blood pressure in epidemiological studies (Virdis et al, 2007) and points to the particular association between CRP and arterial stiffness (and perhaps consequently more with systolic than diastolic blood pressure). Whether CRP is produced by stressed vascular endothelium is unclear. It seems likely however that a lack of an association of IL-18 and TNFα with arterial blood pressure means that hypertension will not directly result in increases of the cytokines in circulation. Whether the cytokines are involved in local inflammatory vascular cross-talk (such as between SMCs [Sahar et al, 2005]) remains to be verified.

As expected, IL-18 and TNFα were in general both fairly strongly inversely associated with the atheroprotective HDL cholesterol. HDL cholesterol, apart from reverse cholesterol efflux, is thought to have a wide range of anti-atherogenic properties, including potential modulation of the plaque inflammatory response (Barter et al, 2004; Vaisar et al, 2007), and is known to show inverse associations with most inflammatory markers. The inverse association of HDL with IL-18 and TNFα is hence likely to be a consequence of general anti-inflammatory pathways elicited by HDL, which are common to most inflammatory markers. Overall, IL-18 and TNFα show little association with total cholesterol, suggesting overall positive associations with LDL cholesterol. IL-18 and TNFα also have moderate associations with triglycerides, which itself has a moderate association with CHD risk (Sarwar et al, 2006).

As expected in this thesis, all inflammatory markers generally showed reasonably strong inter-correlations. IL-18 and TNFα have strong associations with CRP, but interestingly

not fibrinogen. This may underscore potential extra-hepatic sources of CRP production (independent of the APR) during chronic low-grade inflammation (such as the vascular endothelium). Cytokine associations with BMI are discussed in the next sections

Table 12.3 Qualitative overview of associations of IL-18 and TNFα with conventional cardiovascular risk markers (as well as CRP and fibrinogen) reported in this thesis. In case-control studies only the results from the control population are shown. IL-18 and TNFα always correlated highly significantly ($p<0.001$) with each other and with IL-6 (where measured). All associations are age- and sex-adjusted Spearman or Pearson models.

	Age		Smoking		TC		HDL		Trig		BMI		BP		CRP		Fib	
	IL-18	TNF	IL-18	TNF	IL-18	TNF	IL-18	TNF	IL-18	TNF	IL-18	TNF	IL-18	TNF	IL-18	TNF	IL-18	TNF
MONICA-4	✓	✓	✓	✓	✓	✓	✓	✓	-	-	✓	✓	✓		✓	✓	✓	
GLAMIS		✓	✓				✓	✓	✓	✓	✓				✓		✓	
BRH Study			✓			✓	✓		✓		✓		✓		✓	✓	-	-
Fletcher Challenge	✓	✓					✓	✓	✓	✓	✓				✓	✓		

Tc, total cholesterol; HDL, high density lipoprotein cholesterol; Trig, triglycerides; BMI, body mass index; BP, blood pressure; Fib, fibrinogen

Blank = not significant
- = not reported
✓ = $r<0.1$
✓ = $0.2>r>0.1$
✓ = $r>0.2$

12.4.2 Metabolic Syndrome and Cardiovascular Associations

Due to recent advances in the field it is necessary to comment on insights gained from this thesis in terms of novel inflammatory markers and associations with aspects of the metabolic syndrome, although it is important to conceding that metabolic syndrome was not a primary consideration of the thesis in terms of methodology, and any inferences drawn must be with great caution..

Accumulating data in the field indicates that cardiovascular disease and the metabolic syndrome may have common underlying causes ([1]Grundy et al, 2004), to the extent that some researchers have taken to employing the phrase "cardiometabolic syndrome" to indicate a clinical state indicative of relatively high likelihood to experience type 2 diabetes or cardiovascular events. Intra-individual clustering of risk factors for cardiovascular disease and diabetes have been noted for many years (obesity, hyperlipidemia, diabetes, and hypertension; Avogaro, et al, 1967). Indeed, the figure is often quoted that those with metabolic syndrome have a 50% increased risk of cardiovascular events (although the risk is actually cumulative with increasing numbers of "cardiometabolic" risk factors present, and the syndrome is not greater than the sum of its parts; Wannamethee et al, 2005). This is logical when considering that metabolic syndrome (comprising measurements of adiposity, dyslipidaemia, hypertension, and hyperglycemia depending on the definition used) has strikingly overlapping features with Framingham/ASSIGN-type cardiovascular risk assessments.

The Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) presents a simple clinical definition of the metabolic syndrome. The simplicity of this definition has led to the suggestion (particularly in the USA) that the NCEP definition could replace more complex Framingham assessments (Meigs et al, 2004). This proposition has been called into question by data indicating collectively that, as is logically expected, Framingham assessments are stronger predictors of CHD, and NECP metabolic syndrome scores are better predictors of diabetes (Stern et al, 2004; [2]Grundy et al, 2004; McNeill et al, 2005; Wannamethee et al, 2005) suggesting that segregation of the scores remains preferable in terms of optimising risk management.

This clinical issue is separate however to academic and research interest into the common causal pathways in metabolic syndrome and cardiovascular disease.

12.4.3 Metabolic Syndrome and Inflammatory Marker Associations

A common factor that subclinical cardiovascular disease and the pre-diabetic metabolic syndrome may have is the presence of low-grade systemic inflammation. Over the last few years there has been a shift of interest in the literature reflecting this. As considered in a recent review (Hotamisligil, 2006) a common inflammatory root for the conditions is perhaps not unrealistic or surprising. Evolutionary data indicate that it is possible that the haematopoetic, immune, adipose and hepatic cells compartmentalised into distinct organs in mammals may have arisen from related precursors, as seen in the interweaving mesh of the homologues of these cells in the fat body of the *Drosophila melanogaster* (Hotamisligil, 2006).

The possibility that low grade inflammation is the key mechanism linking CVD risk to metabolic syndrome and risk of type II diabetes is widely considered a realistic proposition (Lyon et al, 2003; Rajala and Scherer et al, 2003; Wise, 2004) (Fig 12.1). At the heart of this is the theory, for many investigators, is the proposition that excess visceral adipose tissue (or resident macrophages therein) acting as an active endocrine organ, releases inflammatory markers such as IL-1, IL-18, IL-6 and TNF-α (Hotamiglisi et al, 1993 and 2006; Esposito et al, 2002). These inflammatory factors may have the ability to mediate, not only features of metabolic syndrome like insulin resistance (Borst et al, 2004; Paolisso et al, 1998), but also atherogenesis, hence providing a potential common causal pathway for the conditions. However, whether this production of inflammatory mediators has true systemic consequences, or is to a greater extent a localised autocrine and paracrine cross-talking mechanism for infiltrating macrophages within the adipose tissue is not clear. So how important is degree of body fatness in terms of defining circulating concentrations of inflammatory markers?

To help answer this question, I aimed to use the limited data in this thesis to assess the association of inflammatory markers with a key feature of the metabolic syndrome; visceral fat deposits.

12.4.4 Association of a Key Component of the Metabolic Syndrome (Visceral Fat) with Inflammatory Markers in This Thesis

In MONICA-4, CRP, and to a lesser extent IL-6, were strongly associated with BMI and WHR (Table 4.2, section 4.4.4). Both IL-18 and TNFα were weakly associated with BMI, but the association improved (particularly for IL-18) when WHR was considered as opposed to BMI as a measure of fatness. Unfortunately this "extra" data (waist size or WHR) was not available in other studies, although these do show generally poor associations of IL-18 and TNFα with BMI. The observation that the relationship of the two cytokines with fatness improves when considering WHR (a better marker of metabolically active visceral fat and a better marker of cardiometabolic risk than BMI) suggests there may be a relationship of IL-18 and TNFα with visceral fat in particular. The observation of improved associations of IL-18 when WHR is considered instead of BMI is in line with other recent observations for IL-18 (Hung et al, 2005; Thorland et al, 2005; Villarrasa et al, 2006). Despite this, the improved relationship is marginal, and visceral adipose tissue is unlikely to be a major contributor to circulating levels of IL-18, a conclusion supported by the MONICA/KORA study (Herder et al, 2006). IL-18 in the MONICA-4 study had an association of $r = 0.1$ for BMI and $r = 0.2$ for WHR. This means variations in BMI explain 1% of circulating IL-18 concentrations while WHR explains 4% in a general population - so while visceral fat is responsible for some circulating IL-18, it is unlikely to be very biologically significant. Indeed the MONICA/KORA study failed to find any association of IL-18 with absolute or percentage fat mass. This is in line with observations that levels of IL-18 secreted by adipocytes are low (Skurk et al, 2005). Similarly, another study in a generally healthy population failed to find strong relationships of TNFα with BMI or WHR (Tuomisto et al, 2006).

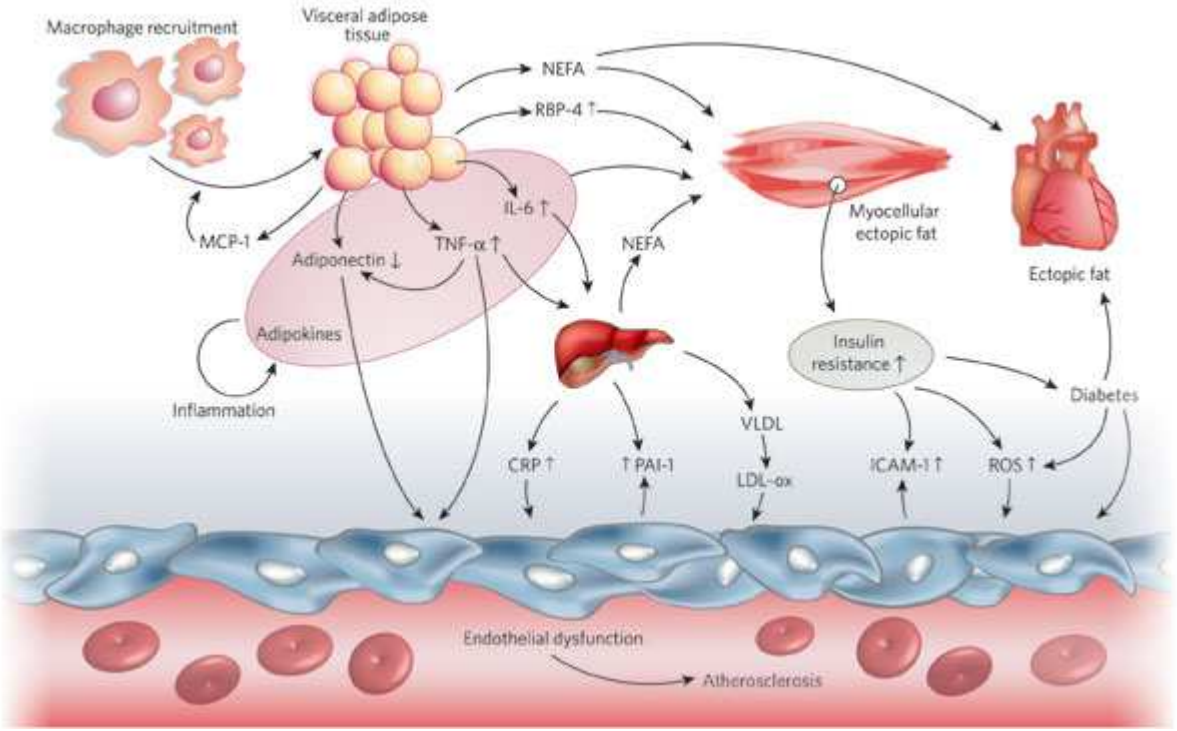
12.4.5 Conclusions

Taken together these results suggests that the "central" role that adipose-tissue related inflammatory markers (such as IL-18 and TNFα) are purported to play in mediating insulin resistance and risk of diabetes as well as cardiovascular disease through inflammatory cytokines are debatable. While some inflammatory markers, such as CRP

and IL-6, are strongly associated with BMI, their relationship with visceral fat is not particularly specific (in that associations with WHR are not particularly better than with BMI). Likewise, TNFα may be produced by adipose tissue, but is also produced by many other cell types such as muscle cells – the metabolic antithesis of fat (Saghizadeh et al, 1996). Associations of excess visceral adipose tissue to circulating levels of IL-18 and TNFα are far from being clearly biologically significant, thus it is hard to envisage how adipose tissue may mediate the systemic development of diabetes through these cytokines as a major causal mechanism.

Interestingly, these cytokines themselves may remain associated with risk of diabetes (perhaps more strongly than with risk of CHD; Thorland et al, 2005). It remains possible that metabolic syndrome and diabetes increase risk of CHD (with or without the presence of obesity) through influences on the arterial wall and endothelial dysfunction, which in turn may be associated with systemic inflammatory processes. This mechanism does not rely on adipose tissue "causing" systemic chronic low grade inflammation, but may associate with it as another risk factor. For instance, fatty acids may be causal in bringing about diabetes via ectopic-fat induced insulin resistance in muscle cells and key organs (Fig12.1) (Seppala et al, 2002; Sinha et al, 2002). Despite this it remains possible that these cytokines are related to adipose tissue in individuals who are outside the normal range and may be defined as clinically obese (Vilarrasa et al, 2007), although this thesis has not examined these associations. Clearly data from this thesis is limited, and more work is required both epidemiologically and biologically to help delineate the metabolic and inflammatory consequences of large reserves of visceral fat.

Fig 12.1 An integrated view of obesity-mediated inflammation acting in the promotion of ectopic fat deposition, insulin resistance (type II diabetes), dyslipidaemia, as well as endothelial dysfunction and reduced arterial elasticity via increased atherosclerosis. (adapted from Van Gaal et al, 2006)



NEFA, non-esterified fatty acids; ROS, Reactive oxygen species; RBP-4, rentional binding protein-4

12.4.4 Further research into IL-18, TNFα and Metabolic Syndrome

Clearly this thesis was not designed to contribute significant information to the metabolic syndrome debate. More research is required into possible correlates of IL-18 and TNFα with other risk factors associated with metabolic syndrome, since body fat appears largely unrelated. For instance, insulin resistance is often considered a main mediator of risk of type II diabetes, and inflammation may promote this metabolic disorder (Steinberg et al, 2007). Alternatively, age is the often forgotten simple risk factor that is also a major risk factor for insulin resistance (Kahn, 2006), and the cytokines clearly are age-associated. Data in large studies are required to elucidate possible associations, and to delineate the (possibly strong) associations of IL-18, and TNFα, with risk of diabetes in eventual meta-analysis. This should allow further debate as to the inflammatory aetiological similarities or differences between CVD and metabolic syndrome.

12.5 INFLAMMATORY MARKER ASSOCIATIONS WITH CVD

12.5.1 MMP-9 and sCD40L and Associations with Risk of CHD

Most of the conclusions possible to draw from this thesis regarding these two marker's risk associations are included in Chapter 6, as this is the only opportunity I had to measure risk associations for them in serum.

Notwithstanding the aforementioned limitations of MMP-9 as a risk biomarker, the results from the BRH study of middle aged men indicate MMP-9 is moderately associated with risk of CHD in middle-aged men. MMP-9 was associated with risk of incident CHD (OR 1.37, 95% CI 1.04-1.82), although this association was almost completely dependant on smoking and on generalised inflammation, since association with risk was abrogated after adjustment for smoking and CRP and IL-6. There may have been a slight residual association following these adjustments, OR 1.13 (0.82, 1.56), but the study was underpowered to detect this, and the association would probably be weak. MMP-9 confounding by smoking is in agreement with recent observations that smoking increases vascular expression of MMP-9 in a mouse model ([2]Wright et al, 2007). This upregulation was via a TNFR1 dependant mechanism, and hence also alludes to a potential inflammatory link for MMP-9, which is consistent with much of the literature (1.5.3).

As seen in Table 1.8, much of the data showing the association of MMP-9 with risk is inconsistent due to small studies, most of which comprised patients with documented CAD. The largest study ([2]Blankenberg et al, 2003) found a linear increase in risk across quartiles, and although risk was attenuated after adjustment for classical and inflammatory risk markers, this study still found the top two quartiles for MMP-9 to be at significantly increased risk. Despite this, lack of association between MMP-9 and CVD risk in some small studies (Wu et al, 2005; Cavusoglu et al, 2006) may be indicative of regression to the mean overall when considered concurrently with this BRH study data. As discussed previously (6.4.4), it is still possible and interesting to speculate that MMP-9 may be a "better" risk marker in those with established arterial disease due to its putative role in plaque rupture, although the BRH study was underpowered to make a robust inference regarding this. Certainly the reduction is risk observed after exclusion of

those with baseline disease was not nearly as strong as one would suspect considering the associations seen in the Atherogene data ([2]Blankenberg et al, 2003).

The possibility that serum levels of sCD40L would be inversely related to risk of CHD had been previously considered as part of a platelet exhaustion hypothesis (1.6.5). This hypothesis has been partially corroborated in other work using serum, where those with prevalent disease have lower serum levels of sCD40L (Aukrust et al, 1999; Tanne et al, 2006) and at least non-significant trends have been observed in other work (Weber et al, 2007; Kiani et al, 2007). The only other available published prospective work examining serum sCD40L associations with CHD showed that in those where serum sCD40L tended to be higher (at 90th percentile) there was a non-significant trend for lower risk of CHD: OR 0.70 (0.34–1.41), although the study only had 233 cases (Tanne et al, 2006). The BRH study, with twice as many cases and twice as long a follow-up, found that univariable inverse associations with risk were significant although moderate (OR 0.75 [0.57, 0.99]), and relatively unconfounded by conventional or inflammatory risk factors, although significance was lost due to the loss of power on adjustment.

12.5.2 Further Work to Assess CHD Risk Associations of Serum MMP-9 and sCD40L

More data are obviously needed to confirm and expand on these findings with meta-analysis in due course.

Further confirmation that MMP-9 was a moderate predictor of CHD, confounded by smoking and other inflammatory markers, would essentially preclude it as a useful clinical marker (from an epidemiological perspective). Interestingly it may still represent a potentially strong therapeutic candidate. It remains to be seen if small therapeutic inhibitors can be made specifically enough to inhibit individual MMPs while not interacting with others .

It would be particularly interesting to see if the inverse associations of serum sCD40L with risk of CHD are reproducible in other large studies. Delineating whether inverse associations of sCD40L with risk of CHD were continuous and linear throughout the population is also of interest. Assessment would be required of those with very low relative levels of serum sCD40L to see if such levels are artefactual or truly reflect

exhausted platelets and greater risk. It may also be interesting (if the platelet exhaustion hypothesis is verified) to see if those who develop venous thrombosis in prospective studies also have exhausted platelets, platelet interactions being a major factor in these events (Lowe, *submitted review*, 2007).

12.5.3 IL-18 and TNFα Power Calculations

Due to the large number of studies conducted on IL-18 and TNFα, the retrospective power calculations offer potential for comparison. One must of course bear in mind the limitations of these calculations (2.6.2).

Table 12.4 illustrates power calculations across all relevant studies for these two cytokines, including IL-6 where appropriate. In the study of prevalent CHD, GLAMIS had 70% power to detect risk associations of 1.5 for both IL-18 and TNFα. Among prospective CHD studies, the BRH study and MONICA-4 had greater power than GLAMIS, demonstrating similar power to each other to detect risk associations of 1.5 (92% and 83% respectively for IL-18, and 88% and 83% for TNFα respectively). The Fletcher Challenge study was somewhat less powerful than this, demonstrating 53% power in both markers. Not shown in the figure, sCD40L and MMP-9 had similar power in the BRH as did IL-18 and TNFα.

Among the stroke studies, SIP had limited power to detect associations with risk due to the small number of "cases" of poor outcome. It is hence possible that the study was underpowered to find associations of cytokines with outcome – further studies are therefore required. Prospective power calculations however showed the study had 90% power to detect 25% differences in D-dimer levels between groups on outcome (9.2.4). PROGRESS was the most powerful of the stroke studies, demonstrating 90% power to detect associations of 1.5 for IL-18 and 1.6 for TNFα. The null association of IL-18 with recurrent stroke is therefore unlikely to be a chance finding. PROSPER showed 59% power to detect risk ratios of 1.5 for IL-18 and for TNFα, and therefore there is a possibility the study was underpowered.

Table 12.4 Power calculations for all relevant studies in the thesis showing both the minimum OR association required at 90% power (column "90%") and the power of the study to detect an OR of 1.5 (column "1.5").

Study	**Outcome**	**IL-18**	**90%**	**1.5**	**TNF**	**90%**	**1.5**	**IL-6**	**90%**	**1.5**
GLAMIS	MI	446 cases 477 controls	1.69	70	445 cases 477 control	1.69	70	n/a		
BRH	MI	595 cases 1238 controls	1.47	92	535 cases 1155 controls	1.52	88	n/a		
FC	MI	229 cases 442 controls	1.93	53	229 cases 442 controls	1.93	53	229 cases 442 controls	1.93	53
MONICA-4	MI	1484	1.56	83	1484	1.56	83	1437	1.57	82
SIP	Death/dep (acute stroke)	94 dead/dep 86 indep	3.39	19	94 dead/dep 86 indep	3.39	19	94 dead/dep 86 indep	3.39	19
PROGRESS	Recurrent Stroke	591 cases 1182 controls	1.50	90	433 cases 859 controls	1.60	80	591 cases 1182 controls	1.50	90
PROSPER	Incident stroke	266 cases 532 controls	1.83	59	266 cases 532 controls	1.83	59	n/a		

Dep, dependant; indep, independant

12.5.4 IL-18 Risk Associations with CHD and Stroke and Meta-Analysis

IL-18 demonstrated consistently significant, but moderate, univariable associations with CHD in all studies in this thesis. In GLAMIS, IL-18 levels were elevated in those who were retrospective CHD cases relative to controls, although the difference was not large (9.6%). In the BRH prospective study, the difference between cases and controls was again moderate (9.8%), as it was in Fletcher challenge (10.8%), and MONICA-4 (13.7%). The difference of ~10% between cases and controls is hence a consistent observation, both in populations with prevalent disease and in normal populations. This data is broadly similar to other publications comparing cases and controls for levels of IL-18 retrospectively (4.2%: Hulthe et al, 2006) and prospectively both in patients with prevalent disease (16%: Blankenberg et al, 2002; 10.6%: Blankenberg et al, 2006) and in healthy patients (10.4%: Blankenberg et al, 2003; 6.9%: Koenig et al, 2006). This suggests that IL-18 may have minimal confounding associations with prevalent disease, since associations with hard end points are similar regardless of the patient population studied.

In all studies of CHD (BRH study, FC study and MONICA-4) IL-18 was associated with risk after adjustment for matching variables (age and sex) (ORs 1.55, 1.63, and 1.61 respectively comparing extreme tertiles, all $p<0.05$). After adjustment for only short-term biological variability ($R=0.66$; Chapter 3; not adjusted for regression dilution due to a lack of data), an OR of ~1.60 would become ~2.04.

Commensurate with the observation that IL-18 may be relatively free of confounding by some conventional vascular risk factors, I observed that IL-18 risk associations on multivariable analysis were not markedly attenuated by adjustment for classical risk markers in the FC study, but were attenuated in the BRH study and MONICA-4 studies where social class was adjusted for. This is again consistent with other studies (Blankenberg et al, 2001; [1]Blankenberg et al, 2003; Tiret et al, 2005; Koenig et al, 2006; Table 1.6). Despite this, the univariable association of IL-18 was moderate enough that in full adjustment models (with or without social class) observations were generally

rendered borderline significant by increasing the confidence intervals. Therefore more power is needed to confirm these adjusted observations. This can be achieved by meta-analysis of the data.

For stroke, IL-18 levels were not substantially elevated in the SIP acute stroke cohort compared to the MONICA-4 population or other case-control studies, although the possibility that this is due to drifts in kit calibration or differences in sample quality is hard to exclude. The latter is unlikely since reduced sample quality would be expected to lead to drops in detectable IL-18 levels, and the SIP samples are relatively recent (~4 years), and the aliquots used were previously unthawed. IL-18 also demonstrated no associations with 72 hour stroke progression or with 30 day death dependency outcome. Absence of an association with stroke in the acute stages is commensurate with animal data since elevated IL-18 is not required for or associated with acute ischaemic brain injury in mice (Wheeler et al, 2003). All inflammatory markers measured apart from IL-18 were univariably associated with the 30 day death/dependency outcome. Hence the inflammatory marker prediction of 30 death/dependency outcome seems likely to be a general APR-associated phenomenon (Stuyt et al, 2005). Indeed, in the multivariable SIP model, D-dimer was one of the only independent indictors of outcome; and as well as being a marker of fibrinlysis it is also APR-associated. This speculation requires some degree of caution because the study was small and poorly powered (Table 12.4). IL-18 was not associated with recurrent stroke (haemorrhagic, ischaemic, or total recurrent stroke [PROGRESS]), despite IL-18 levels being elevated in the cohort. The latter observation may be directly due to changes in kit lot however (lot 42A; 12.3.1) and requires verification. The PROSPER study also failed to implicate IL-18 as a risk predictor of incident stroke in a population of elderly people with vascular disease. This may have been due to limitations of the study, since the case-control PROSPER study found CRP and fibrinogen to not be associated with risk of stroke, although these inflammatory markers are established risk predictors of stroke (1.7.4). IL-18 associations with incident stroke require further study in general populations. Overall however, this thesis fails to find any evidence of an association of IL-18 with stroke.

As previously discussed, pooling of the data on risk associations is required to better estimate the associations of IL-18 with risk of CVD. Firstly, this requires studies appropriate for this meta-analysis to be identified. GLAMIS and SIP were excluded on the basis of their retrospective and acute investigative natures (consistent with methodologies in other published meta-analyses e.g. Fibrinogen Studies Collaboration, 2004). PROSPER was excluded on the basis of the limitations of its study design (10.4.3).

Meta-analysis of the remaining studies was performed by pooling and weighting of ORs (comparing adjusted extreme population thirds) in a fixed model (Woodward, 2005). Analysis of heterogeneity was performed using the I^2 statistic (as opposed to Cochrane's Q) due to the small number of studies (Higgins and Thompson, 2002).

Analysis of heterogeneity between the studies suggests that there were no significant differences between the studies of IL-18 ($p=0.36$), and hence pooling of the data is a valid methodology, since pooling of the data will theoretically only improve power and not bias the data by inclusion of widely differing studies.

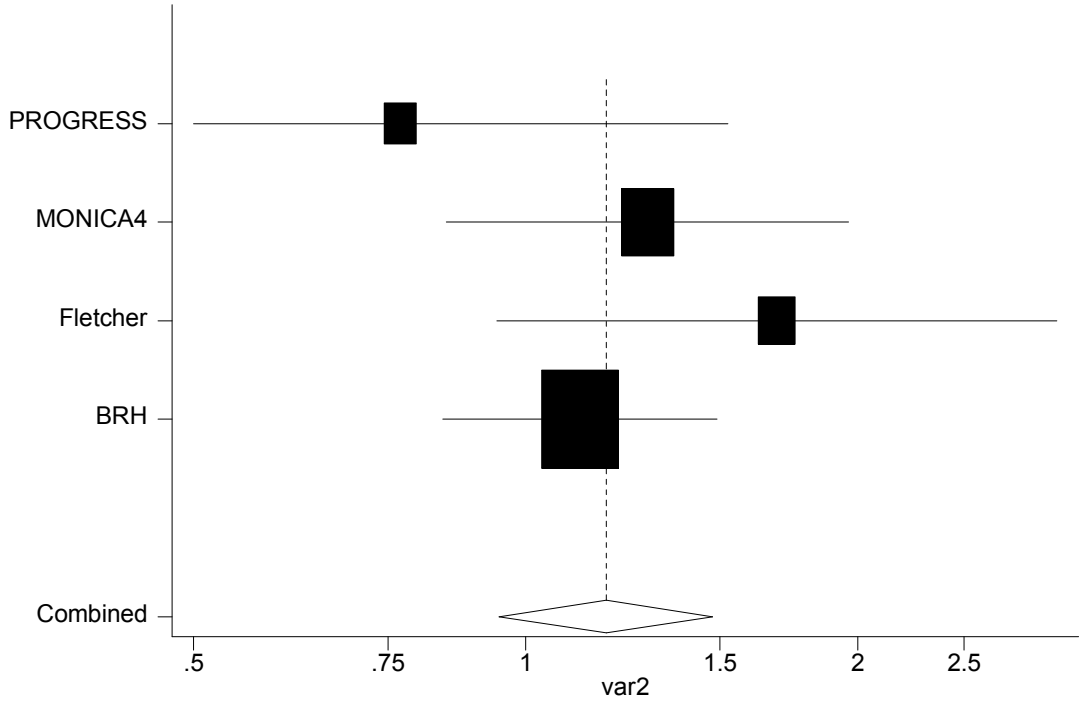
Data from the IL-18 meta-analysis from fully adjusted models in each of the studies is shown in Fig 12.2. In the fully adjusted models IL-18 shows a weak, borderline significant association with risk of CVD, such that comparing extreme thirds the additional risk is OR 1.18 (95% CI 0.95-1.48). Looking at the data given in the figure, it is obvious that the PROGRESS study of recurrent stroke is an outlier compared to the studies of CHD. Hence, further sub-analysis of CHD-only associations should be performed. There are plans for such analysis to be performed in the near future, increasing the amount of data by including data from the published literature (in collaboration with Prof P Whincup). Therefore this sub-analysis is not included here. Adjustment for short-term or biological variability in the present meta-analysis is not possible due to absence of such data for conventional risk markers (which would also be required to be adjusted for in a further model).

The possibility that IL-18 may be (albeit moderately) associated with CHD but not stroke is intriguing. Markers of the acute phase response (e.g. fibrinogen and CRP [Fibrinogen Studies Collaboration, 2005, Danesh et al, 2004]) predict CHD and stroke to a similar degree. If IL-18 associates with only CHD, it may reflect the different underlying causes of stroke and CHD. While nearly all CHD is linked to coronary atheroma, only half of ischaemic stroke cases are due to large vessel atheroma such as in the middle cerebral artery (Di Napoli et al, 2002) cardiac disorders (e.g atrial fibrillation, heart valve disease) or small vessel disease causing lacunar strokes. These different aetiologies may have potentially different inflammatory associations.

Overall, with or without stroke data, IL-18 appears a moderate predictor of CHD, and risk associations generally weaken to borderline significance on adjustment for conventional risk markers.

There are speculations current in the literature that IL-18 may act in both a pro-inflammatory manner (as in Chapter 1) as well as an anti-inflammatory manner (due to stronger associations with TGF-β than most other inflammatory risk markers), as considered in the WISE study (Kip et al, 2005). This study also considers TNFα a potential candidate for these opposing mechanistic effects on risk (Kip et al, 2005). Whether opposing pro- and anti-inflammatory actions of these cytokines have any impact on risk associations requires more study. It is possible however, that these cytokines are important in atherogenesis at a local level, but that circulating concentrations of the cytokines may not reflect this, and hence display limited risk associations.

Fig 12.2 Meta-analysis of IL-18 in the four pooled studies (full adjustment models from this thesis) comparing risk in the top versus bottom tertiles of the population distribution. Filled box size indicates relative weighting (by study power), and lines indicate 95% CI. Unfilled box indicates overall combined OR 1.18 (95% CI 0.95-1.48).



12.5.5 TNFα and Risk Associations with CHD and Stroke and Meta-Analysis

TNFα demonstrated inconsistent associations with CHD in this thesis. Levels of TNFα were more elevated than IL-18 in the GLAMIS study of patients with prevalent MI compared to matched controls (28.6%). This is in agreement with published literature (Jovinge et al, 1998; Bennett et al, 2006, Hulthe et al, 2006), although the two cytokines had not been directly compared in the same study previously. In prospective studies TNFα was *lower* among cases in the BRH study (8.8%), and higher in the FC study as well as the MONICA-4 study (10.0% and 19.9% respectively). Hence TNFα appears, in comparison to IL-18, to show stronger association with prevalent CHD (previous MI) than with incident CHD in general populations. More data is required to confirm these observations.

Although some reports have shown that elevated levels of TNFα are associated with prospective risk of CHD risk in people with recent MI ([3]Ridker et al, 2000), in a general elderly population ([2]Cesari et al, 2003), and in a general population (Tuomisto et al, 2006), the data are not entirely clear. A small recent study suggests that TNFα does not predict events in people with prevalent MI (Sukhija et al, 2007), although the study is small (n cases = 55). The FINRISK study, which is the most general population in the published literature, shows that the association of TNFα with risk of CHD is strong (OR>2.0 above first quartile) but non-linear, with particularly notable non-significant inverse association with risk in the second quartile of women for cardiovascular (OR 0.74 [0.18-30.5]) and coronary (OR 0.25 [0.01-5.27]) risk (Tuomisto et al, 2006). Clearly this observation may be a chance finding, since confidence intervals are very wide and numbers per quartile small. The BRH study result may be a symptom of "regression to the mean" (i.e. a more or less null association overall, including the published literature),

or alternatively it may be a reflection of poor sample quality (although the results of Chapter 3 and the positive associations of IL-18 suggest otherwise).

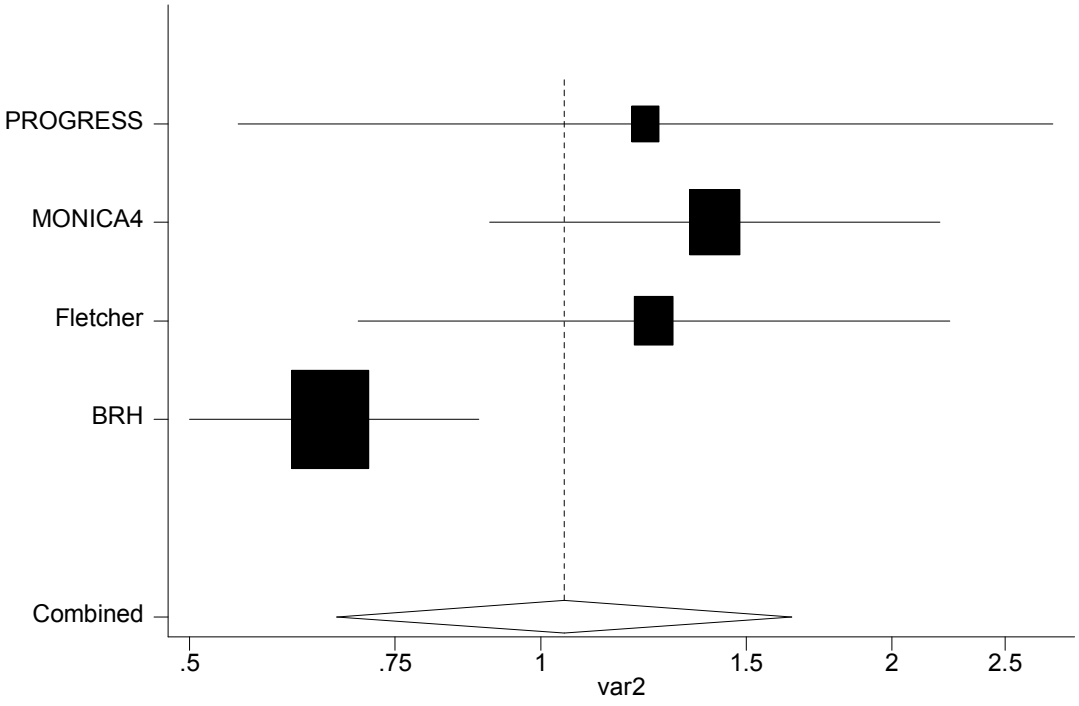
In other studies in this thesis, TNFα results were no more conclusive. In the FC study, TNFα showed a non-significant positive trend to be associated with CHD risk in the top third of the population; OR 1.33 (0.87, 2.02), and the result was only marginally attenuated by adjustment for classical risk markers (OR 1.23). Interestingly however, there was an inverse trend in the middle third, which became stronger after adjustment for classical risk factors; OR 0.68 (0.41-1.12). In MONICA-4 the top third of the population were at increased risk of CHD: OR 1.70 (1.11-2.59), a result marginally attenuated by adjustment (OR 1.40). Also, in MONICA-4, in contrast with FC, risk associations were linear through the middle third of the population. Due to these inconsistencies no attempt was made to adjust univariable associations for short-term biological variation, as was done for IL-18.

Overall then, these results are inconclusive in terms of delineating an association of TNFα with risk of CHD. It is possible there is an association of high levels of TNFα with risk in general populations, although this is likely to be weak. It is also possible that the association is non-linear, such that those with moderate expression levels have some protection. This may be in line with observations from previous authors suggesting that TNFα and IL-18 have both pro- and anti-inflammatory properties in the WISE study (Kip et al, 2005).

Performing meta-analysis of the TNFα data may help to observe overall trends from data in this thesis. Meta-analysis was therefore performed on TNFα in the same manner as IL-18. Data from this analysis is shown in Fig 12.3. Analysis from pooling of the data showed significant heterogeneity of the data ($p<0.02$), hence meta-analysis should be performed with caution. Clearly the BRH study is a major outlier in this data set. Yet it should not be excluded from analysis on the basis of its difference for risk of obtaining a biased false positive result, and cross-study heterogeneity in associations is perhaps consistent with the published data (Table 1.4). Differences between studies may due to differing study populations such as their age ranges, genders and the prevalence of

disease. This thesis has shown TNFα increases with age, suggests that levels are higher in males than females, and perhaps that it is associated more strongly with prevalent disease (compared to IL-18). How these differences impact on prospective risk associations is not clear, and requires more research, but perhaps generally explains study heterogeneity. As seen in Fig 12.3, fully adjusted models show that TNFα has an association with risk very close to unity comparing extreme tertiles: OR 1.05 (0.67-1.64). It is hence possible that all the speculation of differing risk associations in different populations is misplaced, and overall regression to the mean in the data show that TNFα does not predict risk at all beyond associations with conventional risk markers. More data would be required to establish this one way or the other.

Fig 12.3 Meta-analysis of TNFα in the four pooled studies (full adjustment models from this thesis) comparing risk in the top versus bottom tertiles of the population distribution. Filled box size indicates relative weighting (by study power), and lines indicate 95% CI. Unfilled box indicates overall combined OR 1.05 (0.67-1.64). Studies are heterogeneous ($p<0.02$).



12.5.6 High grade Inflammatory Risk and IL-18 and TNFα in the Literature

IL-18 and TNFα may be poorly associated with CVD in these populations, which are typical of research into low-grade inflammation-associated CVD. Data is currently accumulating which suggests that those with high-grade inflammatory disease (such as rheumatoid arthritis [RA] and type-1 diabetes) are at increased risk of CHD. This increased risk is not fully explained by cardiovascular risk factors in studies of rheumatoid patients: the excess risk after adjustment for these factors in RA populations is thought to be ~3-fold (del Rincon et al, 2001). There is hence the possibility that high-grade inflammation may contribute to systemic cardiovascular risk in a causal manner. IL-18 (Gracie et al, 2003) as well as TNFα (Sattar and McInnes et al, 2005) are likely to be major contributors to such systemic Th1 type inflammation. There is good evidence that treatment with statins reduces levels of inflammatory markers, including in those with RA (McCarey et al, 2004). Recent studies have however indicated that simavastatin offers little additional anti-inflammatory or endothelial function/aortic stiffness improvement above ezetimebe (the latter being a non-statin cholesterol lowering agent restricted to the small intestine) (Maki-Petaja et al, 2007). This would suggest that the systemic anti-inflammatory effects of statins are largely linked to cholesterol-altering mechanisms. The potentially causal role of inflammation in high grade inflammatory CVD requires clarification.

In this respect, TNFα is in a unique research position, in that it is one of the only cytokines that have specific monoclonal antibodies liscensed for treatment of human disease. As previously discussed, the current generation of TNFα-inhibitors are unsuitable for use in those with subclinical disease, and may also be pathological in those with advanced CHF (1.3.8). Despite this, the drugs are fairly commonly used in those with RA unresponsive to methotrexate treatment. A recent double-blinded placebo controlled trial in 127 patients with psoriatic arthritis used onercept (a mAb TNFα inhibitor) to show that short-term treatment resulted in lowered Lp(a) and homocysteine levels and elevated Apo A-I, changes suggestive of cardiovascular protection ([2]Sattar et al, 2007). In addition however, levels of triglycerides and Apo B were raised (adverse phenotype), and the authors recommend that further studies using measures of extent of

atherosclerotic disease (such as IMT) or hard end-points are required. It is possible that general dampening of an inflammatory state would show greater efficacy than targeting an individual pro-inflammatory molecule. This remains to be investigated.

12.5.7 Further Work for IL-18 and TNFα

In general, more data are required to delineate associations of both IL-18 and TNFα with risk of CVD in a range of populations. This is particularly true of TNFα; in particular, research into those with very high circulating levels of TNFα is required ([3]Ridker et al, 2000) as well as data in general populations in order to evaluate risk associations in large populations with valid sub-analysis by gender and by age, and prevalence of disease. This data may help to elucidate possible mechanistic risk associations.

Overall, one would conclude from all current data that any independent risk associations of either cytokine in general populations are likely to be very small, and unlikely to be clinically significant. Even if associations are stronger in sub-populations, these are unlikely to be of clinical benefit in terms of risk prediction. The molecules may nonetheless be therapeutic targets. Research into TNFα blockade in RA patients and assessment of risk reduction in hard end-points is required. This may be problematic for a number of reasons relating to recruitment of sufficient numbers and study design. Primarily, RA patients receiving TNFα blockade medication are generally highly selected, having non-methotrexate responsive and clinically severe disease, and hence may be at increased risk of CVD. Ethical recruitment of controls (of similar disease severity to not bias the data) is therefore problematic.

Furthermore, research is needed into the experimental and epidemiological implications of the potential “anti-inflammatory” properties of IL-18 and TNFα, and the implications of this for atherogenesis. It seems likely that these properties are likely to act in the promotion of the Th2 response (and hence suppression of Th1) rather than Th1 suppression directly. Population-wide patterns of low grade Th1 versus Th2 circulating inflammatory expression may be required to compare the elevated Th1 responses seen in those with prevalent vascular disease (Szodoray et al, 2006). The role of the systemic

Th1/Th2 balance in atherogenesis is speculative however, and it is important to remain conservative as to its importance in atherogenesis. Mast cells have recently been shown to be involved in atherogenesis (Bot et al, 2007), and mast cells are conventionally activated by Th2 stimuli such as IgE. It is hence possible that localised inflammation within the intima and adventitia of the vascular lesion will advance atherosclerosis in the presence of endothelial dysfunction (irrespective of the type of inflammation).

12.6 CONCLUDING REMARKS

This thesis has shown that circulating levels of IL-18, TNFα, and MMP-9 generally have limited positive associations with risk of CVD in population studies, which are likely to be both moderate and confounded. Univariable associations with prospective risk of CVD suggest the markers may have some role in arterial disease progression, although this requires more study. After adjustment for conventional risk markers the associations with risk are likely to be small, although further studies are required to delineate risk associations accurately in sub-populations. Serum sCD40L was shown to be inversely associated with risk, perhaps due to platelet exhaustion in those at increased risk. More studies are required to confirm this observation, although the marker is unlikely to be of any clinical utility due to pre-analytical issues.

IL-18 and TNFα were the most extensively studied markers. At the outset of this thesis there was published data estimating risk associations from 450 CVD cases for IL-18. Since then there have been an extra 1236 cases added in published literature, and the current thesis adds 2239 cases to that. Similarly for TNFα there were 542 cases in prospective data, 260 cases have been added in the last 3 years, and the current body of work adds a further 2179 cases. This work therefore adds significant data to currently published work, potentially making risk observations much more accurate.

In this thesis, both cytokines were associated with aspects of the metabolic syndrome (in line with cardiometabolic risk factor clustering), although their association with population markers of adiposity was limited, despite literature linking them to obesity and visceral adipose tissue. The demographic associations of IL-18 and TNFα were

similar to those seen for other risk associated inflammatory markers (CRP, fibrinogen and IL-6), both increasing with age and showing some gender associations, as well as associations with smoking, alcohol intake and social class. Animal and tissue models indicate IL-18 and TNFα are important in the development of atheromatous lesions. The limited association of these cytokines with CVD risk, while markers associated with acute phase response (CRP, fibrinogen and IL-6) are more strongly associated with risk (although arguably still moderate) suggests that the APR may be a "better" indicator of CVD risk than circulating levels of inflammatory markers associated with Th1 inflammatory responses.

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