Inflammatory Markers and Cardiovascular Disease

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ABSTRACT

Background

Inflammation is now recognised to play a central part in the initiation, progression and clinical manifestation of atherosclerotic cardiovascular disease. Correspondingly, on a population level, circulating levels of a wide range of inflammatory markers have been shown to be predictive of future cardiovascular events, regardless of whether they are measured in asymptomatic people, patients with stable angina, or patients with acute coronary syndromes. These include both systemic markers of inflammation such as the white blood cell count (WBC), fibrinogen, and C-reactive protein (CRP), and locally produced mediators of inflammation such as the cellular adhesion molecule soluble intercellular adhesion molecule 1 (sICAM-1), the cell-surface protein soluble CD40 ligand (sCD40L), and the metalloproteinase pregnancy associated plasma protein-A (PAPP-A). Investigation of these inflammatory markers has given many useful insights into the mechanisms that underlie the development of atherosclerosis and atherosclerotic clinical events. However, although the association (on a population level) of raised inflammatory markers with increased atherosclerotic events is widely accepted, the clinical utility of these markers (their ability to provide meaningful additional information that will help individualise treatment strategies and lead to better clinical outcomes) remains a subject of vigorous debate. Consequently, the research presented in this thesis has two broad purposes: to determine the value of inflammatory markers in a particular clinical situation (the prediction of restenosis following percutaneous coronary intervention), and to examine whether vascular inflammation is a modifiable risk factor (whether marker levels can be lowered by health interventions such as drug therapy, exercise, or smoking cessation).
Methods and results

a. Inflammatory markers and restenosis

To investigate whether inflammatory markers are predictive of restenosis following PCI, inflammatory markers (CRP, sICAM-1, sCD40L and PAPP-A) were measured prior to and 48 hours, 1 week and 1 month after elective PCI, and angiographic follow-up was performed at 6 months, in 133 stable angina patients. PCI led to a significant rise in CRP, sCD40L and PAPP-A levels 48 hours post-procedure, but neither pre-PCI nor post-PCI inflammatory marker levels were predictive of restenosis. This lack of association could not be attributed to concurrent use of medications such as statins, thienopyridines or glycoprotein IIb/IIIa inhibitors, since 50% of patients were not on statins and no patients received thienopyridines or glycoprotein IIb/IIIa inhibitors during the study.

b. The effects of lipid lowering agents on inflammatory marker levels

The effects of lipid-modifying agents on inflammatory marker levels were tested in 215 participants with stable angina randomised to simvastatin or placebo, and a further 100 participants randomised to simvastatin or bezafibrate, over a treatment period of at least 2 years. In addition, the effect of statins on the inflammatory response to PCI was assessed in a subset of 92 patients by comparing inflammatory marker levels before and 48 hours, 1 week, and 1 month after PCI in those randomised to simvastatin versus those randomised to placebo. Although simvastatin led to a reduction in CRP levels with long-term therapy, the effect was modest and variable compared to the predictable effect on cholesterol levels. Average CRP levels fell ~5%, compared to a 40% reduction in LDL cholesterol, and CRP levels increased in nearly a quarter of patients on simvastatin. In addition, simvastatin did not lower levels of any other inflammatory marker, and had no appreciable effect on the inflammatory response to PCI. Similarly, bezafibrate therapy did not lower levels of any inflammatory marker.
c. The effect of exercise training on inflammatory marker levels.

The effects of exercise training on inflammatory markers were assessed in two separate randomised controlled trials. The first trial involved CRP measurement in 63 healthy elderly participants randomised to either 6 months’ exercise training or to a control group. The second trial involved measurement of several inflammatory markers (WBC, fibrinogen, CRP, sCD40L, sICAM-1) in 152 healthy female smokers randomised to either 12 weeks’ exercise training or to a health education (control) group as part of a smoking cessation program. In both trials, exercise led to a significant improvement in fitness but had no effect on inflammatory marker levels.

d. The effect of smoking cessation on inflammatory marker levels

The smoking cessation trial also investigated the effect of abstinence from smoking on inflammatory marker levels. Forty-eight individuals (35%) achieved 6 weeks verified abstinence from smoking. Abstinence caused a significant decrease in WBC and fibrinogen levels but had no effect on other inflammatory markers (CRP, sICAM-1, and sCD40L).

Conclusions

There are several important findings from this research. Firstly, inflammatory markers are not useful in the prediction of restenosis following PCI in stable angina. Secondly, neither simvastatin nor bezafibrate have major antiinflammatory effects in vivo. This brings into question the mechanism(s) by which statins lower CRP, and has implications for recent proposals in the literature advocating the clinical use of CRP to titrate statin therapy. Thirdly, smoking cessation leads to a reduction in WBC and fibrinogen levels (which may reflect changes in pulmonary inflammation), but neither exercise nor smoking cessation are associated with a broad reduction in inflammatory markers linked
to cardiovascular risk. It is therefore unlikely the appreciable cardiovascular benefits of these interventions are due in any substantial part to antiinflammatory effects. It remains to be demonstrated whether there are interventions which can reliably lower inflammatory marker levels, whether this decreases cardiovascular risk, and whether measurement of inflammatory markers improves upon current management of cardiovascular disease and leads to actual clinical benefit.
ACKNOWLEDGEMENTS

The studies presented in this thesis were undertaken during a research fellowship in the Department of Cardiovascular Research, Green Lane Hospital, Auckland, New Zealand from December 2000 to December 2002.

I am indebted to Professor John French and Associate Professor Ralph Stewart, whose mentorship during this period provided me with continuing support and encouragement. I am also grateful to Professor Harvey White for his support and advice throughout my research, and to Mrs Mary Denton and her team of experienced cardiovascular research nurses who ensured that my research was always performed to the highest ethical standards and was in accordance with the current guidelines of good clinical practice.

This research would not have been possible without the collaboration of several key investigators who performed the inflammatory marker assays. To this end I would like to thank Dr Rohan Ameratunga and Mr Roy The of LabPlus, Auckland City Hospital, who performed the CRP analyses, Drs Uwe Schönbeck and Nerea Varo of Brigham and Women’s Hospital, Harvard Medical School, Boston MA, USA who performed the sCD40L and sICAM-1 analyses, and Professor Michael Christiansen of Statens Serum Institut, Copenhagen, Denmark who performed the PAPP-A analyses. I would also like to thank Ms Teena West and Mrs Wanzhen Gao, biostatisticians in the Department of Cardiovascular Research, Green Lane Hospital, who provided statistical assistance for this thesis.

The research presented in chapters 3 and 4 uses data from the Fragmin and Simvastatin Trial (FAST), which was designed and conducted by Professor Harvey White (Principal Investigator) at Green Lane Hospital throughout the 1990’s. The inflammatory marker sub-studies presented in this thesis were performed after completion of the main trial; I was responsible for the conception, design, organisation,
data handling and analysis of these sub-studies. This involved identification of case-report form (CRF) data from the FAST trial, incorporation of this information into a database suitable for analysis, identification of stored blood samples, co-ordination of inflammatory marker assays, statistical analyses (in conjunction with the statisticians), and interpretation of the results. I am indebted to the staff of the Cardiovascular Research Unit, in particular the research nurses Mary Denton and Lynette Pearce, for their invaluable assistance in collating and clarifying the data from the FAST trial and in identifying the blood samples for analysis (a painstaking and time-consuming task, as outlined in chapter 2). I am also grateful to Mr Bruce Webber who performed the quantitative angiographic analysis to determine restenosis rates for this study.

Chapter 5 uses data from the Exercise in the Elderly Trial. Drs Helen Oxenham and Rob Doughty were the principal investigators for this trial, and they oversaw all aspects of trial design, patient recruitment, co-ordination, and follow-up. Inflammatory marker analysis was performed as a separate sub-study, and I was responsible for design, co-ordination, data handling, statistical analysis and interpretation of results for this sub-study.

The Smoking Cessation Trial presented in chapters 5 and 6 was designed and conducted by Drs Harry Prapavessis (Principal Investigator) and Chris Baldi of the Department of Sports and Exercise Science, Auckland University, with outstanding assistance from Kendra McFarlane and Dean Fourie who were responsible for the day to day running of the trial. I was principal investigator for the inflammatory markers sub-study, which was conducted alongside the Smoking Cessation Trial with separate ethics approval, patient recruitment, and patient follow-up. I was responsible for design of the sub-study, patient recruitment, blood collection, additional data collection (including smoking data and activity score questionnaires), organisation of inflammatory marker measurement, statistical analysis, and interpretation of results. I am particularly grateful
to research nurses Pauline O’Brien and Loretta Bush who gave up many evenings and weekends to assist me with blood collection and patient follow-up for this sub-study.

I was financially supported during my research by a Fellowship from the Cardiac Society of New Zealand and Merck Sharpe and Dohme, and by the Green Lane Cardiovascular Research Unit. I am also grateful for the assistance of Dade Behring Diagnostics (New Zealand), which arranged loan of an autoanalyzer for measurement of high sensitivity CRP, and the financial support of the Auckland Medical Research Foundation and the National Heart Foundation of New Zealand, which awarded me grants to perform the research, and to travel to conferences to present the results.

Finally I would like to thank Ms Charlene Nell, who provided outstanding secretarial and editing services, in particular with submission of manuscripts to peer reviewed journals, preparation of abstract presentations, and editing of this thesis.
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- **Cardiac Society of Australia and New Zealand / MSD Research Fellow 2001**
  Provided salary throughout 2001 to allow commencement of this doctoral research

- **Auckland Medical Research Foundation Grant (No. 81410)**
  Provided full funding for the inflammatory markers sub-study of the Smoking Cessation trial, including collection of blood samples, inflammatory marker measurement, and analysis of results

- **2001 National Heart Foundation small projects grant (No. 989)**
  Funded measurement of CRP levels in samples from the FAST trial

- **2001 National Heart Foundation small projects grant (No. 990)**
  Funded measurement of CRP levels in samples from the Exercise in the Elderly trial

- **2002 National Heart Foundation of New Zealand Travel Grant (No. 1039)**
  Funded attendance at the 75th Annual Scientific Session of the American Heart Association, Chicago I.I., USA to present results of the CRP analysis in the Exercise in the Elderly trial

- **2004 CSANZ Travelling Scholarship**
  Funded my attendance at the 52nd Annual Scientific Meeting of the Cardiac Society of Australia and New Zealand, Brisbane, Qld, Australia to present five abstracts arising from this thesis
PUBLICATIONS

The following papers have been published from studies in this thesis:


The following abstracts have been presented at international meetings:

**75th American Heart Association Scientific Sessions, Chicago, IL, USA November 2002**


**52nd American College of Cardiology Scientific Sessions, Chicago, IL, USA March 2003**


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE-I</td>
<td>Angiotensin converting enzyme inhibitor</td>
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<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
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<tr>
<td>AFCAPS/TexCAPS</td>
<td>Air Force / Texas Coronary Atherosclerosis Prevention Study</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ARIC</td>
<td>Atherosclerosis Risk in Communities trial</td>
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<tr>
<td>ASAP</td>
<td>Atorvastatin versus Simvastatin on Atherosclerosis Progression study</td>
</tr>
<tr>
<td>BIP</td>
<td>Bezafibrate Infarction Prevention study</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BPM</td>
<td>Beats per minute (heart rate)</td>
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<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>CAPTURE</td>
<td>The Chimeric c7E3 AntiPlatelet Therapy in Unstable angina Refractory to standard treatment trial</td>
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<tr>
<td>CEASE</td>
<td>Collaborative European Anti-Smoking Evaluation trial</td>
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<td>CARE</td>
<td>Cholesterol and Recurrent Events Trial</td>
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<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CO</td>
<td>Carbon monoxide</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>CRF</td>
<td>Case report form</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>EPIC</td>
<td>Evaluation of 7E3 for the Prevention of Ischaemic Complications trial</td>
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<tr>
<td>FAST</td>
<td>Fragmin and Simvastatin Trial</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<td>IQR</td>
<td>Interquartile range</td>
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<tr>
<td>JUPITER</td>
<td>Justification for the Use of Statins in Prevention: an Interventional Trial Evaluating Rosuvastatin trial</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LIPID</td>
<td>Long-term Intervention with Pravastatin in Ischaemic Disease trial</td>
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<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
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<tr>
<td>Ln-CRP</td>
<td>Natural-log-transformed CRP</td>
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<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
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<tr>
<td>MET</td>
<td>Metabolic-equivalent</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MIRACL</td>
<td>Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering study</td>
</tr>
<tr>
<td>MLD</td>
<td>Minimum lumen diameter (of a coronary stenosis)</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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</tbody>
</table>
MONICA  Multinational monitoring of trends and determinants in cardiovascular disease (a World Health Organisation project)

MRFIT  Multiple risk factor intervention trial

NSAIDs  Non-steroidal antiinflammatory agents

NRT  Nicotine replacement therapy

PAPP-A  Pregnancy associated plasma protein-A

PAPP-A/proMBP ratio  The ratio of pregnancy associated plasma protein-A and its endogenous inhibitor, the proform of eosinophilic major basic protein

PCI  Percutaneous coronary intervention

PRINCE  Pravastatin Inflammation CRP Evaluation trial

proMBP  Proform of eosinophilic major basic protein (the endogenous inhibitor of Pregnancy associated plasma protein-A)

PROVE IT TIMI 22  Pravastatin or Atorvastatin Evaluation and Infection Therapy – Thrombolysis in Myocardial Infarction 22 trial

REVERSAL  Reversing Atherosclerosis with Aggressive Lipid Lowering trial

RR  Relative risk

sCD40L  Soluble CD40 ligand

SD  Standard deviation

sICAM-1  Soluble intercellular adhesion molecule 1

TIMP  Tissue inhibitors of matrix metalloproteinases

TNF-α  Tumour necrosis factor-α

VO_{2}\text{max}  Maximum aerobic capacity

WBC  White blood cell count

WOSCOPS  West of Scotland Coronary Prevention study
Chapter 1

Introduction, and Overview of Inflammatory Markers

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1.1 Background

Coronary artery disease is the leading cause of death in New Zealand and the rest of the Western world.\textsuperscript{1,2} In most cases the underlying cause is build-up of cholesterol-laden atherosclerotic plaque at focal points within the coronary artery wall. Sudden rupture of this plaque with superimposed thrombus formation leads to occlusion of the blood flow and causes the acute coronary events (unstable angina, myocardial infarction, sudden cardiac death) responsible for most cardiovascular mortality.\textsuperscript{3} In the last decade the key role of inflammation in coronary artery disease has become generally accepted; inflammation plays an important part both in the development and progression of atherosclerotic plaque, and in the plaque rupture and thrombosis that underlie coronary events.\textsuperscript{4-6} Of potentially great clinical significance, it appears possible to quantify this inflammatory activity within the vessel wall through measurement of circulating markers in the blood stream.\textsuperscript{7} This is a rapidly evolving field of research, and an increasing number of these “inflammatory markers” have in recent years been shown to be predictive of future cardiovascular risk, whether measured in asymptomatic individuals, patients with stable angina, or patients with acute coronary syndromes.\textsuperscript{7} However, epidemiological associations do not necessarily imply clinical utility.\textsuperscript{8} It remains to be seen whether these epidemiological associations, seen on a population level, translate to clinically relevant improvements in management for the individual patient.\textsuperscript{8-10} In particular, it has yet to be shown that measurement of inflammatory markers improves upon current risk prediction models,\textsuperscript{11} or has clinical utility for guiding therapy,\textsuperscript{12-14} and that treatments exist that can predictably lower inflammatory marker levels, with corresponding reduction in clinical events.\textsuperscript{9,13,15}

This thesis addresses two current areas of uncertainty regarding inflammatory markers and coronary artery disease. Firstly, are inflammatory markers useful in the prediction of adverse events following percutaneous coronary intervention (PCI)? Despite ongoing dramatic growth in the use of PCI for the treatment of coronary artery disease, the ability to predict which patients will experience complications (clinical events, restenosis) following such procedures is limited. Inflammation is thought to contribute to these complications, but it is not clear whether measurement of inflammatory markers will help predict their occurrence.\textsuperscript{16} Secondly, can
vascular inflammation be reduced? Despite the central role of inflammation in coronary disease, it is not clear what interventions (if any) are reliably effective in reducing inflammatory marker levels. Potential strategies include use of medications such as lipid-modifying agents, and lifestyle modifications such as exercise and smoking cessation. However, it remains to be proven whether inflammatory marker levels can be predictably lowered using these interventions.\textsuperscript{17,18} These questions are addressed in this thesis. Original research is presented on the use of inflammatory markers for prediction of clinical events and restenosis following PCI (chapter 3), and on the effect of lipid-modifying therapy (chapter 4), exercise training (chapter 5) and smoking cessation (chapter 6) on inflammatory marker levels. The literature related to each of these questions is reviewed in the relevant chapters of the thesis. The purpose of this introductory chapter is to provide an overview of the role of inflammation in the development and complications of atherosclerosis, and to review current knowledge on each of the inflammatory markers (white blood cell count [WBC], fibrinogen, C-reactive protein [CRP], soluble intercellular adhesion molecule 1 [sICAM-1], soluble CD40 ligand [sCD40L], pregnancy associated plasma protein-A [PAPP-A]) studied in this thesis.
1.2 Inflammation and atherosclerosis

1.2.1 Inflammation in the initiation and early development of atherosclerotic plaque

The prominence of inflammatory cells within atherosclerotic lesions has been noted for many years, and consequently early theories of atherosclerosis development accorded inflammation an important role.\textsuperscript{19-21} However, with the dominance of the ‘lipid hypothesis’ in the latter part of the 20\textsuperscript{th} century, inflammation was largely overlooked and atherosclerosis came to be widely regarded as a passive build-up of excess cholesterol within an inert vessel wall.\textsuperscript{3} In the last ten to twenty years this theory has been challenged by numerous studies that have again shown atherosclerosis to be an active process in which inflammation plays a key role.\textsuperscript{3,4,6} Consequently, the ‘lipid hypothesis’ has been superseded in current theories of atherosclerosis development by the ‘response to injury’, ‘response to retention’ and ‘inflammation’ hypotheses, in which an initial injury to the vessel wall (such as build-up of oxidized low density lipoproteins within the interstitial proteoglycan matrix) triggers an ongoing inflammatory response that is responsible for atherosclerosis progression and its clinical manifestations.\textsuperscript{5,22}

The normal, healthy endothelium is highly active in maintaining vessel homeostasis and resisting inflammatory cell entry to the vessel wall. Many of these processes are mediated by nitric oxide, which has powerful antioxidant, antiinflammatory and anti-thrombotic properties.\textsuperscript{23} Nitric oxide production is promoted by normal laminar blood flow along the vessel wall, and in areas where laminar blood flow is interrupted, such as vessel branch points, atherosclerotic lesions are prone to develop. In the early stages, the endothelium at these sites becomes dysfunctional and develops increased permeability to lipids such as low density lipoprotein (LDL). LDL particles accumulate in the intimal layer of the vessel wall, where they become trapped by irreversible binding to proteoglycans.\textsuperscript{3} Subsequent oxidation of these bound particles renders them antigenic and pro-inflammatory and triggers an inflammatory response (figure 1.1).\textsuperscript{6} Leucocyte adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) are then expressed on the vessel wall, allowing macrophages and T lymphocytes to attach and migrate through the endothelium and accumulate within the intimal layer of the vessel wall (figure 1.2).
The normal protective functions of the endothelium may become impaired at focal points along the vessel wall, especially at branch points where normal laminar flow is interrupted. Risk factors such as hypertension, smoking and diabetes accentuate this process by further impairing endothelial function. The vessel wall then becomes permeable to lipids such as low density lipoprotein (LDL) which accumulate within the intimal layer of the vessel wall. The LDL particles are retained within the intima by irreversible binding to proteoglycans, and subsequent enzymatic modification renders them antigenic and proinflammatory. This leads to activation of leucocyte adhesion molecules on the endothelial surface and recruitment of circulating macrophages and T lymphocytes into the vessel wall. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells. Adapted from Hansson (2005).
The uptake of LDL particles by macrophages triggers an inflammatory reaction which further impairs endothelial function. Endothelial permeability and leucocyte adhesion increase, leading to accumulation of more LDL particles and macrophages within the vessel wall. This leads to release of more inflammatory mediators and the development of a persistent inflammatory lesion within the vessel wall. Adapted from Ross (1999).  

These macrophages actively ingest large quantities of oxidized LDL and can become engorged with lipids (so called ‘foam cells’). As they do so the macrophages present antigenic particles of LDL to the T lymphocytes, leading to elaboration of numerous cytokines that cause further inflammatory cell activation, accumulation and proliferation. The earliest visible atherosclerotic lesion, the so-called ‘fatty streak’, consists entirely of these lipid-engorged inflammatory cells. These lesions develop early in the lifespan, with fatty streaks present in around 65% of children between 12 and 14 years of age (figure 1.3).²⁵

Many cardiovascular risk factors contribute to this endothelial dysfunction and inflammation and thereby promote atheroma growth. In hypertension, raised angiotensin II levels can increase intimal inflammation. In diabetes, advanced glycation end products (AGE) augment the production of proinflammatory cytokines. In obesity, adipose tissue is an important source of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6)²⁶,²⁷ Chronic infections such as gingivitis, and inflammatory diseases such as rheumatoid arthritis, are also associated with increased levels of inflammatory cytokines that may exacerbate atherosclerotic inflammation.⁶,²⁸ Therefore it is possible (but remains to be proven) that inflammation represents a ‘final common pathway’ through which the effects of many different cardiovascular risk factors may be explained.
Figure 1.3  Fatty streak formation in atherosclerosis.

The persistent inflammatory state leads to the accumulation of lipid-laden macrophages (foam cells) and T-lymphocytes within the vessel wall. They are subsequently joined by smooth muscle cells, which migrate from the medial layer of the vessel wall in response to chemottractant proteins such as monocyte chemotactic protein 1. Together, these cells cause a macroscopically visible lesion within the vessel wall (the so-called ‘fatty streak’). Adapted from Ross (1999).
1.2.2 Progression to the complex plaque

With ongoing inflammation the fatty streak progresses to the more complex lesion of established atheroma. This is characterised by the presence of smooth muscle cells within the lesion, and by development of a ‘necrotic core’ of tissue covered by a thin fibrous cap. Smooth muscle cells are attracted from the medial layer of the vessel wall into the intima by chemotactants, with matrix metalloproteinases (a group of enzymes secreted by macrophages) playing a key role in their migration by performing local digestion of the extracellular matrix. Once incorporated into the atherosclerotic plaque, replication and programmed cell death (apoptosis) of smooth muscle cells progress hand-in-hand, driven by inflammatory cytokines and growth factors. Lipid-laden macrophages also undergo apoptosis and the lesion evolves a central core of lipid and necrotic tissue surrounded by cells and extracellular matrix (the ‘complex plaque’). This necrotic core is sealed off from the bloodstream by a fibrous cap of smooth muscle cells and collagen, the integrity of which is crucial to plaque stability (figure 1.4).4,5

Inflammation remains active within complex atherosclerotic plaque, and intercellular interactions between the cell surface molecule CD40-ligand (CD40L) and its receptor (CD40) are thought to be central to this ongoing inflammatory activity.29 Both CD40L and CD40 are co-expressed on a multitude of cells (T lymphocytes, macrophages, endothelial cells and smooth muscle cells) within atherosclerotic plaque and their interaction promotes numerous proatherogenic inflammatory processes that accelerate growth of the plaque. These include enhanced secretion of proinflammatory cytokines by T-lymphocytes and macrophages, increased expression of adhesion molecules such as ICAM-1 on the endothelial surface, and recruitment of further monocytes and T-lymphocytes into the lesion. In addition CD40/CD40L interaction causes thinning of the fibrous cap and tissue factor secretion into the plaque. These changes promote plaque rupture and thrombosis (see 1.2.3 below).23,30
Figure 1.4  Formation of the advanced, complex lesion of established atheroma.

With ongoing inflammation the fatty streak progresses to the more complex lesion of established atheroma. Smooth muscle cells and macrophages undergo programmed cell death (apoptosis), leading to a central core of lipid and necrotic tissue, walled off from the lumen by a fibrous cap that represents a type of healing or fibrous response to the injury. Adapted from Ross (1999).
As a consequence of this persistent inflammatory state within the vessel wall, the cytokines IL-6 and TNF-α spill over into the blood stream and induce a low grade continuous release of acute-phase reactants such as C-reactive protein (CRP) and fibrinogen from the liver. Circulating levels of CRP and fibrinogen provide a convenient measure of inflammatory activity and therefore vascular risk and they have been used for this purpose both in research and in clinical settings.

1.2.3 The vulnerable plaque and plaque rupture

Plaque disruption (erosion or rupture) with superimposed thrombus is the proximate cause of most acute coronary events (unstable angina, myocardial infarction, sudden cardiac death). A number of factors increase a plaque’s vulnerability to erosion or rupture. Characteristic features of vulnerable plaques include active inflammation with a large inflammatory cell infiltrate, a relative scarcity of smooth muscle cells, and a thin cap overlying a large necrotic lipid core (figure 1.5). This scarcity of smooth muscle cells and thinning of the fibrous cap appear to be consequences of the inflammatory activity within the plaque. Inflammatory mediators provoke smooth muscle cell death, while matrix metalloproteinases secreted by activated macrophages break down collagen and elastin fibres to cause thinning of the fibrous cap. Pregnancy associated plasma protein-A (PAPP-A) may be particularly important in this regard, as this matrix metalloproteinase is minimally expressed in stable plaque but is abundantly expressed in eroded and ruptured plaque, particularly at the site of disruption.

Plaque rupture exposes both oxidized lipid and tissue factor to the blood stream, triggering platelet activation, adhesion, and thrombosis. As activated platelets accumulate they release from their storage granules large quantities of inflammatory mediators including soluble CD40L. This exacerbates local inflammation and produces a measurable systemic inflammatory response, the severity of which appears predictive of outcome.
With ongoing inflammation, the plaque develops thinning of the fibrous cap, leaving it vulnerable to rupture. Plaque rupture exposes oxidised lipid and tissue factor within the necrotic core to the blood stream, triggering platelet activation, adhesion and thrombosis. The resultant blood clot can obstruct blood flow within the vessel lumen, causing pain and ischaemia (an ‘acute coronary syndrome’). Adapted from Ross (1999).
1.2.4 Inflammatory markers and cardiovascular risk

As can be seen from the preceding overview, inflammation appears central to the initiation, progression and eventual rupture of atherosclerotic plaques. A number of inflammatory mediators involved in these processes can be measured and quantified in the peripheral blood. Such markers have proved useful in study of the mechanisms that underlie coronary events. They may also improve clinical risk prediction and offer new therapeutic targets.\(^7,22,23,29\)

The inflammatory markers that have been used for risk prediction in cardiovascular disease fall into two broad categories:

1. Systemic markers, such as the white blood cell count (WBC), fibrinogen and C-reactive protein (CRP). Inflammation in local tissues stimulates production of these markers from the bone marrow (WBC) or liver (fibrinogen, CRP) and levels reflect the overall inflammatory activity within the body. An advantage of these markers is their presence at relatively high concentrations in the systemic circulation, making them easy to measure and quantify. One potential disadvantage is their lack of specificity. Levels can be markedly elevated in response to any systemic illness, and may not directly reflect vascular inflammation. Nonetheless, ‘basal levels’ (taken in the absence of an acute illness) have consistently been shown to be predictive of cardiovascular risk\(^31,40,41\) and CRP in particular is now being considered for routine clinical use, in risk prediction\(^7\) and guidance of therapy.\(^42\)

2. Local mediators of inflammation, such as soluble CD40 ligand (sCD40L), soluble intercellular adhesion molecule 1 (sICAM-1), and pregnancy associated plasma protein-A (PAPP-A). As discussed in section 1.2, these proteins are produced locally within atherosclerotic plaque and contribute directly to disease progression, but they are also detectable at low levels in the systemic circulation and these circulating levels have been shown to be predictive of cardiovascular risk. However, they are only present at very low concentrations (close to the limit of detection and measurement) and therefore it may not be possible to quantify levels as accurately as for the systemic inflammatory markers.\(^7\)
Several key issues related to these markers have yet to be resolved, including standardisation of assays, the definition of the 'normal range', and the definition of elevated levels for purposes of risk prediction. Nonetheless because these proteins are directly involved in the atheromatous process, they offer two potential advantages over systemic markers; firstly, they may be more specific for cardiovascular inflammation, and secondly, they may serve as therapeutic targets.\textsuperscript{29,33,43}

In this thesis three systemic inflammatory markers (white blood cell count, fibrinogen and C-reactive protein) and three local mediators of inflammation (soluble CD40 ligand, intercellular adhesion molecule 1, and pregnancy associated plasma protein-A) are studied. The literature on each of these inflammatory markers is reviewed in the following sections of this chapter.
1.3 White Blood Cell Count (WBC)

The white blood cell count was the first inflammatory marker to be associated with cardiovascular risk. Over thirty years ago, Friedman et al. (1974) performed a retrospective case control study involving 120,000 participants who had a complete health assessment and were then followed for an average of 4 years. 44 464 cases developed myocardial infarction (MI) during follow-up, and their average WBC was compared with the WBC of an equal number of controls matched for age, sex, race and standard coronary risk factors. WBC was found to be significantly higher in those who went on to develop a myocardial infarction (8.0 x 10^9/l versus 7.5 x 10^9/l, p<0.001). These results have subsequently been confirmed by several prospective epidemiological studies which show a graded relationship between increasing WBC and the risk of future cardiovascular events. 44-47 In a meta-analysis of these trials, the mean WBC was 7.0 ± 1.8 x 10^9/l, and a WBC in the upper tertile (mean level 8.4 x 10^9/l) conferred 1.4x the risk of a cardiovascular event over the next 8 years compared to a WBC in the lower tertile (mean level 5.6 x 10^9/l), after adjustment for other risk factors including age, smoking, obesity and blood pressure (p<0.0001). 48 WBC has also been shown to be predictive of mortality in patients with acute coronary syndromes, 49-52 and in post MI patients with ischaemic left ventricular dysfunction. 53 WBC is elevated, proportional to infarct size, in patients with acute ST-elevation myocardial infarction, although it does not appear to be as good a predictor of long term outcome as other acute-phase reactants such as CRP in this setting. 54

WBC is easily measured using standardized techniques (usually flow cytometry using an automated cell counter) with a co-efficient of variation <3%. 7 However total WBC in the body is distributed between the circulation, the bone marrow and the tissue. Within the circulation white blood cells are further distributed between marginated and freely circulating subpools. White blood cells can move freely between these pools and the measured WBC can therefore differ substantially from day to day or even within a single day in an individual. 41 This greatly limits the utility of WBC as a clinical tool for individual prediction of cardiovascular risk, since it lacks the specificity for individual (as opposed to population) risk prediction. 48 Nonetheless WBC remains useful as a research tool and was included in the assessment of exercise and smoking cessation in this thesis (chapters 5 and 6) as previous reports suggest that both physical inactivity 55,56 and
smoking\textsuperscript{46,57,58} are associated with raised WBC levels, and that WBC may decrease rapidly with smoking cessation.\textsuperscript{59-61} WBC therefore provides a useful benchmark against which to compare the effects of exercise and smoking cessation on other inflammatory markers.
1.4 Fibrinogen

Fibrinogen has a central role in both platelet aggregation and thrombus formation,\textsuperscript{31,62} and initial interest in fibrinogen as a cardiovascular risk factor stemmed from this role as a coagulation factor.\textsuperscript{63,64} However fibrinogen is also an acute-phase reactant that reflects inflammation throughout the body.\textsuperscript{65} Given this link with both thrombosis and inflammation, it is therefore not surprising that fibrinogen is associated with cardiovascular risk. A recent landmark meta-analysis confirmed a consistent, moderately strong association between plasma fibrinogen levels and cardiovascular events.\textsuperscript{66} This meta-analysis involved 154,211 participants without known cardiovascular disease at baseline, from 31 prospective studies, with a total of 20,154 events (non-fatal myocardial infarction, stroke, or death) over a mean follow-up period of 8.9 ± 4.9 years. Mean fibrinogen levels were 8.9 ± 2.3 μmol/l, and across all studied age groups (40-59, 60-69, and ≥ 70 years), there was a log-linear relationship between fibrinogen levels and risk of a cardiovascular event or death, with no evidence of a threshold effect. On average, there was a doubling of risk for every 3 μmol/l increase in fibrinogen levels. The association between fibrinogen levels and cardiovascular events was seen regardless of baseline levels of other established risk factors such as sex, smoking, blood pressure, and serum lipid levels. There is therefore little doubt that raised fibrinogen levels are associated with increased risk of cardiovascular events; whether this association represents a causal link between fibrinogen and cardiovascular disease remains to be determined.\textsuperscript{66}

Individual fibrinogen levels are affected by a number of intrinsic and environmental factors. Fibrinogen shows moderate positive correlation with traditional cardiovascular risk factors including age, smoking, cholesterol, physical inactivity and obesity. In addition, female sex, black ethnicity, lower socioeconomic status, and alcohol abstinence are each associated with modestly higher fibrinogen levels.\textsuperscript{67} On average, fibrinogen levels are 0.5 μmol/l higher in females than in males,\textsuperscript{31,68,69} but it has been argued that this apparent difference may be a laboratory artifact from the use of citrated blood samples for fibrinogen measurement, since there is a greater dilutional effect from the added citrate in men (who tend to have higher haematocrit values, and therefore less serum per ml collected) than in women.\textsuperscript{67}
Not surprisingly, fibrinogen levels show good correlation with levels of other acute-phase reactants such as WBC and particularly CRP, but measurement of fibrinogen in addition to CRP has been shown to improve the accuracy of cardiovascular risk prediction compared to measurement of CRP alone, providing a rationale for a multi-marker strategy in cardiovascular risk assessment.

Of relevance to this thesis, fibrinogen levels are positively correlated with BMI and smoking history (figure 1.6), and are ~ 0.5 μmol/l lower in physically active individuals compared to their less active counterparts. The association between BMI and fibrinogen levels is likely explained by chronic low-level IL-6 production by adipose tissue, which stimulates hepatic production of acute-phase reactants such as fibrinogen and CRP (see figure 1.7). This is an important potential confounder in assessing the effect of physical activity on fibrinogen levels, and therefore any effect exercise training has on inflammatory marker levels will be corrected for changes in body fat in the studies reported in this thesis.

Smoking, which induces low grade inflammation in the lungs and other organs, is not surprisingly associated with higher fibrinogen levels, although the impact is greater in men than in women, even after adjustment for number of cigarettes smoked per day. Compared to non-smokers, fibrinogen levels are ~ 0.4 μmol/l higher for female smokers and ~ 0.9 μmol/l higher for male smokers. Fibrinogen levels decrease rapidly (within weeks) with smoking cessation, but, for several years after cessation, mean levels still remain slightly higher in ex-smokers than in those who have never smoked.
Figure 1.6  The effect of BMI and smoking on fibrinogen levels.

Mean fibrinogen levels, adjusted to age 50, for males (black diamonds) and females (grey squares) for varying levels of BMI and current cigarette smoking. For both males and females there is a clear association between increasing levels of BMI (left) and cigarette smoking (right) and higher fibrinogen levels. The association between BMI and fibrinogen levels is approximately twice as strong in females, possibly related to differences in body composition, whereas the association between smoking and fibrinogen levels is stronger in males. Adapted from The Fibrinogen Studies Collaboration (2007).
A number of factors limit the usefulness of fibrinogen for clinical screening. Firstly, there are several different assay techniques in use, with poor reproducibility between techniques. The more commonly used functional clotting assays (for example the Clauss method) do not have an available World Health Organisation reference standard and therefore results may not be comparable between centres. In addition, fibrinogen levels are reported to vary substantially from one measure to the next. This has important implications for risk prediction given the relatively small difference between fibrinogen levels conferring ‘low’ versus ‘high’ cardiovascular risk. Finally, in comparative studies fibrinogen has only modest predictive value for cardiovascular risk compared to other inflammatory markers such as CRP.

Nonetheless, measurement of fibrinogen levels may be useful in the research setting. Levels have been shown to fall rapidly with both smoking cessation and exercise training, and so as with WBC, fibrinogen measurement was included in this thesis as a reference standard against which to compare changes in other markers with exercise and smoking cessation.
1.5 C-Reactive Protein (CRP)

1.5.1 Overview of CRP and CRP assays

C-reactive protein (CRP) is the most extensively studied serological marker of cardiovascular risk. It was first described in 1930 from the serum of patients with bacterial pneumonia, and was named for its reaction to the C polysaccharide of *Streptococcus pneumoniae*. It consists of 5 identical subunits arranged as a cyclical pentamer with molecular mass 1.15kD, and is predominantly synthesized by the liver. CRP is an ‘acute-phase reactant’, present in serum at low concentrations in health but with levels increasing several hundredfold with severe infection. Like other acute-phase reactants CRP production is triggered by the cytokine interleukin 6, which is produced at sites of inflammation and travels via the bloodstream to the liver where it regulates CRP synthesis and release proportional to the degree of inflammation. CRP levels in the blood therefore reflect the overall inflammatory activity throughout the body, and CRP measurement has been used clinically for a number of years to monitor disease activity in inflammatory conditions such as rheumatoid arthritis. Particularly useful features of CRP include its short circulation half-life of only 5.7 hours, and its lack of circadian variation (which means that levels can be taken at any time of the day). Thus levels rise rapidly following an acute stimulus, reaching a peak within 50 hours, and also fall rapidly with resolution of the inflammation.

The standard nephelometric CRP assays used for monitoring systemic acute inflammatory disorders (e.g. rheumatoid arthritis) lack the sensitivity to accurately detect and quantify low CRP levels in the normal population. In recent years high sensitivity ELISA assays for CRP have been developed, and with use of these assays there has emerged a strong body of evidence that basal CRP levels (<10 mg/l i.e. within what was defined as the ‘normal range’ using nephelometric assays) are predictive of cardiovascular events. This is thought to be because these basal CRP levels reflect inflammatory activity within atherosclerotic plaque in the vasculature (although a plausible alternative explanation is that basal CRP levels provide a summative measure of a number of other cardiovascular risk factors that affect CRP levels, such as abdominal obesity, insulin resistance, and non-LDL atherogenic particles, that are not
adequately controlled for in risk adjustment models). Inflammatory cytokines are generated within atherosclerotic lesions in proportion to the degree of local inflammation, and these cytokines travel via the bloodstream to the liver where they stimulate the production of CRP (figure 1.7). In addition, adipose tissue also continuously releases inflammatory cytokines such as interleukin-6 and thereby contributes to basal levels of CRP and other acute-phase reactants (figure 1.7). This may in part explain the excess cardiovascular risk associated with obesity, since inflammatory cytokines released from adipose tissue could exacerbate inflammation within the atherosclerotic plaque and thereby contribute to plaque instability and plaque rupture.

There is also growing evidence that CRP itself may directly contribute to the atherosclerotic process. CRP co-localises with complement protein and macrophages in atherosclerotic plaque, and in experimental models it augments the innate inflammatory response via opsonisation, attenuates endothelial cell production of nitric oxide, increases expression of cellular adhesion molecules, is chemotactic for monocytes, and increases the susceptibility of endothelial cells to destruction by cell lysis. All of these processes contribute to atheroma development. CRP may therefore be pathogenic in its own right, raising the possibility it could become a therapeutic target in the treatment of cardiovascular disease.

CRP has several features that make it attractive for routine laboratory assay. It can be measured in both serum and plasma yielding similar results, and it remains stable throughout freezing and rethawing cycles which means it can be accurately measured in samples that have been in long term storage (whether kept at -20°C or -80°C). In addition high sensitivity assays are now widely available and have been standardized against World Health Organisation reference samples, providing accurate quantification down to 0.15 mg/l with a coefficient of variation of <10%.
Activated immune cells within atherosclerotic plaque produce inflammatory cytokines (interferon-γ, interleukin-1, and tumour necrosis factor [TNF]), which in turn induce the production of interleukin-6 within the vessel wall. Adipose tissue also continuously releases inflammatory cytokines such as interleukin-6 and thereby contributes to basal levels of CRP and other acute-phase reactants. Interleukin-6 from both sources travels through the bloodstream to the liver where it stimulates the production of acute-phase reactants including C-reactive protein (CRP), serum amyloid A, and fibrinogen. Cytokines at each step of this process have important biological effects and have been shown to be predictive of cardiovascular risk, but amplification at each step of the cascade makes the measurement of downstream mediators such as CRP particularly useful for clinical diagnosis. Adapted from Hansson (2005).
On an individual level, basal levels of CRP show high reproducibility over time, comparable to that of blood pressure and LDL cholesterol.\textsuperscript{91} There do not appear to be important differences in CRP levels according to sex or race.\textsuperscript{92} Median levels in the normal population are $\sim 1.5$ mg/l, with 75\textsuperscript{th}, 90\textsuperscript{th} and 99\textsuperscript{th} percentile levels of $\sim 3.5$, 6.6 and 10 mg/l respectively.\textsuperscript{38,80,93} The distribution of basal CRP levels in the population is rightward skewed, with low levels in most but with some individuals having basal levels several fold higher than the population median. Natural log transformation of CRP levels produces a normal distribution and this is often used for statistical analyses. (figure 1.8).
C-reactive protein values are not normally distributed but are skewed rightwards (left-hand graph). Natural log transformation results in a normal distribution, allowing use of parametric tests for statistical analyses (right-hand graph). Adapted from Rohde, Hennekens and Ridker (1999).
1.5.2 CRP and cardiovascular risk

A number of studies have investigated the association between CRP and conventional cardiovascular risk factors.\textsuperscript{94-97} CRP shows strong correlation with measures of obesity such as body mass index (BMI), and modest association with current smoking status, age and blood pressure.\textsuperscript{94} There is also a weaker association with hypercholesterolaemia.\textsuperscript{98} However, CRP remains predictive of coronary events after adjustment for these risk factors, suggesting inflammation is an important cardiovascular risk factor in its own right.\textsuperscript{80} Indeed, vascular inflammation may be the final common pathway through which the effects of various other cardiovascular risk factors may be explained.\textsuperscript{99}

The use of CRP for cardiovascular risk prediction has been explored in a number of clinical settings. For example, CRP levels are elevated above normal population levels in the majority of patients with acute coronary syndromes (ACS), with >65\% of patients with unstable angina and >90\% of patients with acute myocardial infarction having CRP > 3mg/l at the time of hospital admission.\textsuperscript{36,39,100} These elevated CRP levels appear to reflect widespread coronary inflammation in patients with ACS\textsuperscript{101,102} and are predictive of both short and long term adverse outcomes and recurrent events. However the ideal CRP cutpoints for use in this setting have not yet been established, with cutpoints of 3mg/l, 10mg/l and 15mg/l each being shown to have prognostic value in different trials.\textsuperscript{36,39,103}

In apparently healthy asymptomatic men and women, the CRP levels associated with low, intermediate and high cardiovascular risk are better established. A number of large trials have investigated the association between CRP and cardiovascular risk by dividing CRP levels into tertiles or quartiles and calculating the relative risk of cardiac events for each tertile or quartile. These trials consistently demonstrate that independent of other cardiac risk factors (such as age, body weight, blood pressure, cholesterol, smoking status, and diabetes), increased CRP levels are associated with increased cardiovascular risk. Individual trials have reported as high as a 3-4 fold increased risk of future cardiovascular events such as unstable angina or MI for individuals with CRP levels in the upper tertile or quartile compared to CRP levels in the lowest tertile or quartile.\textsuperscript{78,80,98} However, more recent analyses (including a meta-analysis of available studies)\textsuperscript{104} put the risk at a more modest 1.5-fold increase from lower to upper tertile.\textsuperscript{71,104} To
standardise comparisons, a scientific statement from the American Heart Association has recommended use of set cutpoints of <1 mg/l, 1-3 mg/l, and >3 mg/l for low, medium and high CRP respectively, based on approximate tertiles in the adult population. Clinical application of these CRP levels appears to add to and improve upon conventional risk prediction models such as the Framingham risk score. On the basis of these findings the American Heart Association scientific statement suggested CRP measurement might be considered for clinical use to better define cardiovascular risk in those classified as being ‘intermediate risk’ using conventional risk scores. However, it was acknowledged that the benefits from this strategy remain uncertain, and prospective trials will be required to prove that this approach leads to improved clinical outcomes.

Although measurement of CRP levels for coronary risk prediction is beginning to move from the research setting to clinical application, many questions remain unanswered. These include whether CRP will prove useful for coronary risk prediction in other settings (such as prediction of adverse outcomes following PCI), and whether CRP is a modifiable risk factor (i.e. can it be predictably lowered with medication or lifestyle changes). Research addressing these questions form the basis of this thesis, with the background literature for each research question reviewed in the relevant chapters.
1.6 Soluble CD40 ligand (sCD40L)

1.6.1 The CD40/CD40L signalling dyad

CD40 ligand (CD40L, also known as CD154) and its receptor CD40 comprise a cell surface-based signalling system involved in immune system activation and inflammation.\textsuperscript{30} Both CD40 and CD40L are trimeric transmembrane proteins (being structurally related to the cytokine TNF-\(\alpha\), they are classified as belonging to the tumour necrosis family of proteins),\textsuperscript{105} and upon binding to each other both the ligand and the receptor actively mediate inflammatory processes within their respective cells.\textsuperscript{23} They are important in both humoral and cellular immunity, with CD40/CD40L interaction being a co-factor for T-lymphocyte activation by antigen presenting cells, and for the subsequent T-cell dependent B-cell activation involved in lymphocyte switching.\textsuperscript{23,30} However their role appears to extend beyond being a co-factor in immune system activation. CD40 and CD40L are widely expressed on various cell types found in the vasculature including monocytes, macrophages, endothelial cells, and platelets, and are thought to be directly involved in vascular inflammation.\textsuperscript{106} Within atherosclerotic plaque, CD40/CD40L interaction causes increased synthesis of adhesion molecules, cytokines, and matrix metalloproteinases, which leads to weakening of the fibrous cap and vulnerability to plaque rupture.\textsuperscript{30} Once plaque rupture has occurred CD40L is also potently prothrombotic, playing an important part in clot formation (figure 1.9).\textsuperscript{107} Thus CD40/CD40L signalling directly contributes to the plaque rupture and thrombosis that are the immediate cause of cardiovascular events.\textsuperscript{43,108,109} It is possible that this discovery could lead to new treatments for cardiovascular disease. This has been explored in experimental animal models, where blocking of CD40/CD40L signalling by anti-CD40L antibody leads to a marked reduction in the size of atherosclerotic lesions.\textsuperscript{109}
CD40 and CD40L are widely expressed on a range of cell types in atheromatous plaque including monocytes, macrophages, endothelial cells and platelets, and they contribute to many aspects of the inflammatory process within atherosclerotic lesions:

1. Interaction of CD40 and CD40L leads to increased expression of adhesion molecules including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and of chemokines such as monocyte chemoattractant protein 1 (MCP-1).

2. These adhesion molecules and chemoattractants recruit further leucocytes into the vessel wall through facilitating leucocyte adhesion and transmigration.

3. Activated leucocytes release increased quantities of proinflammatory cytokines such as interleukin 1 (IL-1) and interleukin 6 (IL-6) creating a positive feedback loop which reinforces inflammatory activity within the lesion.

4. CD40/CD40L interaction on leucocytes increases matrix metalloproteinase (MMP) activity and tissue factor (TF) expression, and decreases endothelial cell (EC) migration. Together these changes promote thinning of the fibrous cap and increase vulnerability to plaque rupture.

5. Once plaque rupture occurs, exposure of tissue factor to the blood stream causes platelet activation. These activated platelets release soluble CD40L from storage granules, reinforcing the inflammatory reaction.

Adapted from Szmitko et al. (2003).\textsuperscript{23}
1.6.2 Soluble CD40L; overview and assays

In addition to its membrane bound cellular expression, CD40L can be detected and quantified in human plasma in a soluble form, sCD40L, that maintains its trimeric structure. Although some may arise from activated lymphocytes, it is estimated that more than 95% of circulating sCD40L is derived from platelets. CD40L remains quiescent within platelet granules until platelet activation occurs; it is then rapidly expressed on the platelet surface and is shed over a period of minutes to hours to generate sCD40L.

There is evidence from both animal and human studies that sCD40L is biologically active. CD40L-deficient mice have a thrombosis defect that can be corrected by infusion of recombinant sCD40L. In humans, sCD40L is released within stored platelet concentrates and is responsible for the febrile inflammatory reactions sometimes seen with platelet infusions. As with membrane bound CD40L, platelet derived sCD40L is thought to contribute to local vascular inflammation, endothelial cell shedding, impaired re-endothelialisation, plaque instability, and thrombosis upon plaque rupture (figure 1.10).
Figure 1.10  The pathological processes involving sCD40L in atheroma.

Diagram of a coronary artery in cross-section, demonstrating the pathological processes involving sCD40L in atheroma. sCD40L is released from activated platelets on the endothelial surface, and contributes to various steps in atherosclerotic lesion progression: (1) Inflammation. sCD40L stimulates release of inflammatory cytokines and matrix metalloproteinases from cells within the atheroma. (2) Thrombosis. sCD40L contributes to platelet aggregation and stabilises platelet-rich thrombi. (3) Restenosis. sCD40L inhibits re-endothelialisation of the injured vessel wall following balloon injury from PCI, which may lead to activation and proliferation of smooth muscle cells and consequently restenosis. Adapted from André et al. (2002).
Given its apparent central role in both atherosclerosis progression and acute complications, it is not surprising that sCD40L has been shown to be increased in the presence of known atherosclerotic risk factors such as hypercholesterolaemia and diabetes, is predictive of cardiovascular risk in apparently healthy asymptomatic individuals, is raised in acute coronary syndromes, and is predictive of outcome following an acute event.\textsuperscript{23,43,105} This evidence linking sCD40L to cardiovascular risk is outlined below (sections 1.6.3 - 1.6.5).

sCD40L can be measured in fresh or frozen blood samples using commercially available ELISA assays with high sensitivity and low coefficient of variation. However, many outstanding issues need to be resolved before sCD40L measurement can be used outside the research setting.\textsuperscript{111} As yet, there is no formal standard against which the accuracy of different sCD40L assays can be compared. In addition, the biokinetics of sCD40L, including its circulation half life, are poorly understood. sCD40L levels differ in serum compared to plasma, and it is not clear whether serum or plasma measurement is better for cardiovascular risk prediction. To date, most studies have used plasma samples which are theoretically preferable because the clotting process may affect sCD40L levels in serum. Finally, the normal range and definition of elevated sCD40L levels for risk prediction have yet to be clearly delineated, although median levels in healthy populations are below 2 ng/ml in most studies, and levels >5 ng/ml are clearly elevated.\textsuperscript{111}
1.6.3 Soluble CD40L and other cardiovascular risk factors

There have been no comprehensive studies of the association between sCD40L levels and conventional cardiovascular risk factors. From those studies that have been published, it appears that sCD40L levels are unaffected by age or body fat, but are elevated in the presence of hypercholesterolaemia and diabetes. They may also be elevated by smoking, although only one small study has investigated this. The effect of exercise on sCD40L levels is not known.

At least two studies have shown sCD40L levels to be elevated in hypercholesterolaemic subjects. Cipollone et al. (2002) compared sCD40L levels in 80 hypercholesterolaemic subjects (mean cholesterol 6.7 mmol/l) and 80 healthy age and gender matched controls (mean cholesterol 4.8 mmol/l). Levels of sCD40L were significantly higher in hypercholesterolaemia (8 ± 5 ng/ml vs. 2 ± 1 ng/ml, p<0.0001). Similarly Semb et al. (2003) found mean levels of sCD40L several fold higher (10ng/ml vs. <1ng/ml) in 110 patients with familial hypercholesterolaemia (mean baseline cholesterol ~10.5 mmol/l) compared to 20 age and gender matched healthy controls (mean baseline cholesterol 5.2 mmol/l).

sCD40L levels have also been shown to be elevated in patients with diabetes, and to come down with treatment using thiazolidinediones. In a study of 49 type 1 and 48 type 2 diabetics Varo et al. (2003) found mean sCD40L levels were ~7 ng/ml, significantly higher than levels of ~1 ng/ml in healthy age-matched controls. The difference was independent of cholesterol and other cardiovascular risk factors including CRP. Administration of the thiazolidinedione troglitazone 600mg/d for 12 weeks significantly decreased sCD40L levels in diabetics by 29% whereas placebo had no effect. These findings were confirmed in a randomized controlled trial by Marx et al. (2003) who showed that treatment with rosiglitazone 4mg twice a day for 12 weeks significantly lowered sCD40L by 27.5% in 39 diabetics with angiographically proven coronary artery disease. The thiazolidinediones are thought to have antiinflammatory properties related to their binding to the nuclear receptor peroxisomal proliferation activating receptor- γ (PPAR-γ) and this may account for their effect on sCD40L levels.
1.6.4 Soluble CD40L in asymptomatic individuals

As with CRP, sCD40L has been shown to predict future cardiovascular risk in asymptomatic individuals. From the Women’s Health Study, Schönbeck et al. (2001) performed a nested case-control study of 130 cases and an equal number of controls matched for age and smoking status. Women with sCD40L levels above the 95th percentile (>3.7 ng/ml) had more than 3x relative risk of developing future cardiovascular events compared to women with lower sCD40L levels (p=0.01), and this increased risk remained after adjustment for usual cardiovascular risk factors. In addition there was no significant correlation between sCD40L levels and levels of either CRP or sICAM-1, suggesting sCD40L reflects aspects of risk distinct from those gauged by other inflammatory markers.

1.6.5 Soluble CD40L in acute coronary syndromes

Two large studies of sCD40L in acute coronary syndromes have been reported. The CAPTURE trial (2003) studied 1,265 high risk acute coronary syndrome patients with recurrent chest pain at rest, ECG changes, and a culprit lesion > 70% suitable for PCI. Patients were randomly assigned to the platelet GPIIb/IIIa receptor blocker abciximab or placebo and had PCI 18-24 hours after study treatment begun. A significant proportion (40.6%) had markedly elevated sCD40L levels (>5 ng/ml), and in patients on placebo a sCD40L level > 5 ng/ml was associated with 2.7x risk of death or non fatal MI. Therefore sCD40L helped identify patients at increased risk of recurrent events. Most of the circulating sCD40L appeared to come from activated platelets, since in a subgroup of 161 patients there was a strong correlation between measures of platelet activation and soluble sCD40L levels (r=0.75, p<0.001). Importantly, abciximab attenuated the increased risk in patients with elevated sCD40L levels but provided no benefit to patients with low levels of sCD40L. sCD40L may therefore prove a convenient platelet function test that helps identify high risk ACS patients most likely to benefit from antiplatelet therapy. In the second large study, sCD40L levels were measured in 2,908 ACS patients in the Myocardial Ischaemia Reduction with Aggressive Cholesterol (MIRA CL) study (2004). A raised sCD40L level (>4.4 ng/ml) was predictive of an increased risk of recurrent cardiovascular events, but this increased risk was abolished by randomization to atorvastatin 80mg per day.
It is notable that in both these studies sCD40L remained predictive of increased risk even among troponin-negative patients, suggesting the risk associated with raised sCD40L is independent of myocardial necrosis and that measurement of sCD40L and troponin together may provide incremental value over measurement of troponin alone. Furthermore sCD40L levels did not correlate with other markers of inflammation such as CRP, suggesting they may reflect different aspects of the inflammatory process (CRP being a general marker of inflammation and sCD40L more specifically reflecting platelet activation and thrombotic risk). Measurement of both may therefore be of benefit.

Because of the potential incremental value of CRP and sCD40L for risk prediction, sCD40L levels were measured alongside CRP in the studies in this thesis. The role of sCD40L in prediction of PCI outcomes, and the effects of lipid-modifying therapy, exercise and smoking cessation on sCD40L levels are discussed in chapters 3, 4, 5, and 6 respectively.
1.7 Soluble Intercellular Adhesion Molecule 1 (sICAM-1)

Intercellular adhesion molecule 1 (ICAM-1) is a cell surface protein involved in cell-cell binding. Like other members of the immunoglobulin superfamily, it is a transmembrane glycoprotein with extracellular immunoglobulin-like domains. In response to inflammatory stimuli, ICAM-1 is rapidly expressed on both endothelial cells and leucocytes, facilitating leucocyte binding to the vessel wall (through interaction with integrin adhesion molecules on the opposing cellular surface). These leukocytes subsequently transmigrate across the endothelium and into inflamed tissue, thereby increasing inflammatory cell infiltration within atheroma. Through these mechanisms, ICAM-1 may directly contribute to atherosclerosis progression and coronary events.

Within the body, a proportion of transmembrane ICAM-1 gets cleaved from the cell surface enzymatically, yielding soluble fragments (sICAM-1) that can be quantified in the plasma using ELISA techniques. Levels of soluble ICAM-1 correlate with surface ICAM-1 expression and consequently are thought to reflect inflammatory activity within the vasculature. sICAM-1 may therefore prove useful as an inflammatory marker of cardiovascular risk.

A number of studies have investigated the association between sICAM-1 and future cardiovascular events. In a nested case-control study of 474 cases and an equal number of controls matched for age and smoking status from the Physician’s Health Study, Ridker et al. (1998) demonstrated that raised sICAM-1 levels were associated with increased cardiovascular risk in apparently healthy individuals. Median sICAM-1 was 225 ng/ml, and participants with sICAM-1 levels in the upper quartile (> 260 ng/ml) had a relative risk 1.8 times that of participants with low sICAM-1 (< 193 ng/ml). Similarly in the Atherosclerosis Risk in Communities (ARIC) study (1997), plasma sICAM-1 levels were significantly higher in 170 subjects with coronary heart disease (mean 289 ng/ml) than in healthy controls (mean 244 ng/ml), and the difference was not explained by adjustment for other known coronary risk factors. However, in the largest study to date (626 cases and 1,253 age-matched controls) Malik et al. (2001) found only a weak association between sICAM-1 and cardiovascular risk which was non-significant after adjustment for smoking and other conventional risk factors (RR 1.1 for upper vs. lower tertile of sICAM-1, 95% CI 0.75-1.64).
In all three studies there was a significant association between sICAM-1 levels and smoking, but it should be noted that these studies were predominantly or exclusively of men. It is not clear if a similar association exists between sICAM-1 and smoking in women. There is no strong association between sICAM-1 levels and other cardiovascular risk factors including age, cholesterol, and hypertension, and reports on the association between diabetes or obesity and sICAM-1 are conflicting.

There are several unresolved issues with the use of sICAM-1 for cardiovascular risk assessment. Little is known about the determinants of sICAM-1 levels, including release and breakdown kinetics and plasma half life. Day to day and longer term variability in levels has not been adequately assessed, and there is no standardized assay for sICAM-1 measurement. Nonetheless, because sICAM-1 levels may directly reflect vascular inflammation, and because they have been implicated in experimental models of both restenosis and smoking exposure, measurement of sICAM-1 levels was included in the FAST trial (chapters 3 and 4) and the smoking cessation trial (chapters 5 and 6) in this thesis.
1.8 Pregnancy associated plasma protein-A (PAPP-A)

Matrix metalloproteinases (MMPs) are a family of enzymes (endopeptidases) responsible for the resorption and remodelling of the extracellular matrix in both health and disease. Within atherosclerotic lesions, metalloproteinases are secreted by activated macrophages and smooth muscle cells and are thought to play an important role in facilitating the smooth muscle migration into the intima that is a hallmark of advanced atherosclerotic lesions. They are also implicated in the weakening of the plaque’s fibrous cap that leads to plaque erosion or plaque rupture and acute coronary events. MMP proteolytic activity is inhibited by a family of naturally occurring specific inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). These inhibitors are synthesised by macrophages and connective tissue cells and tightly regulate enzyme activity by forming high affinity, irreversible noncovalent complexes with activated matrix metalloproteinases in vivo. The net level of proteolytic activity is therefore dependent on the relevant concentrations of active matrix metalloproteinases and inhibitors within the connective tissue matrix.

Matrix metalloproteinases can be detected in the circulation, and (given their role in plaque destabilization) could prove useful as a blood test of cardiovascular risk. One matrix metalloproteinase that has shown promise in this regard is pregnancy associated plasma protein-A (PAPP-A). This MMP was initially identified in maternal blood during pregnancy and has been proposed for use in the prenatal diagnosis of Down’s Syndrome and pre-eclampsia. However, PAPP-A is also abundantly expressed by fibroblasts and smooth muscle cells in advanced atherosclerotic lesions, and circulating PAPP-A can be identified and quantified in the serum of healthy men and women. Although there are few published data either on the source and metabolism of circulating PAPP-A, or on the normal ranges in serum and plasma, recent reports suggest that PAPP-A may be an important predictor of cardiovascular risk.

In one of the first reports, Bayes-Genis et al. (2001) demonstrated from autopsy samples that PAPP-A is not present in appreciable quantities in stable coronary plaques or normal vascular tissue, but is abundantly expressed at sites of plaque erosion or plaque rupture associated with acute coronary syndromes. Further, in a comparison of circulating PAPP-A levels in the plasma of 37 patients with acute coronary syndrome, 19
patients with stable angina, and 13 healthy age-matched controls, they showed that median PAPP-A levels were significantly higher in patients with MI or unstable angina (20.6 mIU/l and 14.9 mIU/l respectively) than in patients with stable angina or controls (8.4 and 7.4 mIU/l respectively). Although PAPP-A levels showed strong correlation with CRP (r=0.61, p<0.001), a PAPP-A of >10mIU/l was more accurate at identifying acute coronary syndrome than a raised CRP (sensitivity 89.2%, specificity 81.3%). These results have subsequently been replicated by Iversen et al. (2008), who showed elevation of PAPP-A levels in patients with ST-elevation myocardial infarction is more sensitive than standard biochemical measures (Troponin T, CKMB) for detection of early presenting infarcts (< 2 hours).

In addition to proving useful in the diagnosis of acute coronary syndromes, PAPP-A may also predict subsequent outcomes. At least two studies have looked at this. The first, by Lund et al. (2003), found that PAPP-A was an independent predictor of death, MI or revascularization over the next 6 months in 136 patients presenting with troponin negative acute coronary syndromes. More recently, Heeschen et al. (2005) have reported the predictive value of PAPP-A for subsequent cardiovascular events in 545 patients with confirmed acute coronary syndrome from the CAPTURE trial, and from a further 644 consecutive patients presenting to a hospital emergency room with acute chest pain. In the acute coronary syndrome patients, median PAPP-A levels were 9.3 mIU/l and there was a stepwise increase in cardiovascular risk with increasing PAPP-A. Using a cutpoint of 12.6 mIU/l to define elevated PAPP-A levels (upper two quintiles, 40% of patients), patients with raised PAPP-A had 2.5x the risk of events (death, non-fatal MI) compared to patients with lower PAPP-A levels (event rates of 17.4% vs. 7.9% respectively, p=0.001). As in previous studies, there was correlation between PAPP-A and CRP levels (r=0.21, p<0.001) but PAPP-A remained predictive of risk after adjustment for CRP, and in fact proved a better predictor of risk than CRP in this ACS population. Of note, PAPP-A levels also remained an independent predictor of cardiovascular events when troponin T and sCD40L levels were taken into account. Furthermore, PAPP-A remained predictive of adverse events in patients who received the anti-platelet agent abciximab (contrasting with sCD40L levels, for which increased risk was attenuated by abciximab as discussed in section 1.6.5). Therefore sCD40L and PAPP-A levels reflect separate and complementary aspects of cardiovascular risk, and measurement of both may predict risk better than either alone. In the second part of the
study (the 644 patients presenting with acute chest pain), PAPP-A levels were significantly higher in those with a subsequent diagnosis of ACS (median 4.9 mIU/l) as compared to patients with stable angina (median 1.9 mIU/l) and non-cardiac chest pain (median 1.4 mIU/l). Troponin T, sCD40L and PAPP-A were all independent predictors of cardiovascular events during 30 days of follow-up, and patients who were negative for all three markers were at very low cardiac risk. These findings support a multimarker approach for cardiovascular risk assessment, but will require confirmation in other prospective trials. In addition, further work is needed to define what constitutes a normal or raised PAPP-A level, since median and ‘raised’ levels in different trials have varied markedly. This is partly because, in the absence of an agreed standard, trials have used discordant assay techniques measuring different aspects of circulating PAPP-A levels (total PAPP-A, free PAPP-A, and PAPP-A activity). In the research presented in this thesis, an in-house immunoassay was used to measure total PAPP-A levels (Appendix 1). More recent studies of PAPP-A use one of two commercially available ELISA techniques that were not widely available when our research was done, and PAPP-A levels measured with these techniques may differ from the in-house immunoassay used in our research.

Few studies have looked at the relationship between PAPP-A levels and other cardiovascular risk factors. In a recent report, Cosin-Sales et al. (2005) compared PAPP-A levels against other risk factors and the extent of coronary disease in 643 patients with stable angina undergoing diagnostic angiography. PAPP-A levels were weakly correlated with age and gender (being higher in men [mean 6.2±2.8 mIU/l] than in women [5.2±1.8 mIU/l], p<0.001). PAPP-A levels were also higher in patients with hypertension, but were unaffected by diabetes, smoking status, cholesterol levels, or BMI. In contrast to previous studies, PAPP-A showed no association with CRP levels (p=0.6). Notably, PAPP-A levels were related to coronary disease extent whereas CRP levels were not. PAPP-A levels were significantly higher in those with multivessel disease (6.4±2.6 mIU/l) than in those with single vessel disease (5.5±1.5 mIU, p<0.001) or normal coronary arteries (4.6±1.2 mIU/l, p<0.001), and the differences remained after multivariate analysis adjusting for potential confounding factors. These results suggest PAPP-A levels might be useful in identifying the presence and extent of coronary plaque. It is also possible that PAPP-A levels can predict the vulnerability of plaque to rupture. PAPP-A levels have been
shown to be higher in patients with carotid plaques thought vulnerable to rupture (hyperechoic or isoechoic on ultrasound) compared to patients with more stable plaques (10.3 ± 2.7 versus 8.3 ± 2.9 mIU/l, p<0.05). However once again there is marked variation in PAPP-A levels in these trials and it is unclear whether PAPP-A levels will prove able to discriminate plaque extent or vulnerability in the ‘real world’ of clinical practice.

The proform of eosinophilic major basic protein (proMBP) has been identified as the endogenous inhibitor of PAPP-A. The relative concentrations of PAPP-A and proMBP (the PAPP-A/proMBP ratio) may therefore reflect PAPP-A proteolytic activity better than PAPP-A levels alone. Cosin-Sales et al. (2004) have shown that the PAPP-A/proMBP ratio is significantly higher in patients with complex coronary stenoses on coronary angiography than in those without (3.1 ± 1.2 versus 2.7 ± 0.8 x 10^-3, p<0.001), and in a multivariate analysis the PAPP-A/proMBP ratio was an independent predictor of the number of complex stenoses. However the relative value of the PAPP-A/proMBP ratio compared to measuring PAPP-A levels alone for cardiovascular risk assessment remains to be determined.

In conclusion, the matrix metalloproteinase PAPP-A is expressed in atherosclerotic plaque, and has been implicated in plaque vulnerability and plaque rupture, the heralding features of acute coronary events. PAPP-A can be detected and quantified in the systemic circulation, and levels correlate with the extent of coronary atheroma, are raised in acute coronary syndromes, and may be predictive of long term events following a presentation with chest pain or acute coronary syndrome.

Acute coronary syndromes and percutaneous coronary interventions have many features in common, including vessel wall injury, platelet activation, and a systemic inflammatory response. The apparent predictive value of PAPP-A in ACS suggests it may also be of value in predicting clinical events following PCI. PAPP-A may also play a part in the restenotic process, since migration of smooth muscle cells into the intima (a hallmark of restenosis) is dependent on matrix metalloproteinase activity. For these reasons, the predictive value of PAPP-A and the PAPP-A/proMBP ratio for PCI outcomes is assessed in chapter 3 of this thesis. In addition, the effects of lipid-modifying therapy on PAPP-A levels are assessed in chapter 4.
1.9 Scope and aims of this thesis

Despite the increasing evidence that inflammation plays a central role in the initiation, progression and complications of atherosclerotic cardiovascular disease, and that inflammatory markers may be useful in cardiovascular risk prediction, many unanswered questions remain. To address some of these important questions, the research presented in this thesis has two broad purposes: to determine the value of inflammatory markers in a particular clinical situation (the prediction of restenosis following percutaneous coronary intervention), and to examine whether vascular inflammation is a modifiable risk factor (whether marker levels can be lowered by health interventions such as drug therapy, exercise, or smoking cessation). The background to these questions is outlined briefly below, and will be explored in detail in subsequent chapters of this thesis.

Inflammatory markers and PCI outcomes

Renarrowing ('restenosis') of coronary arteries is an important complication following percutaneous coronary intervention (PCI). Basic science reports have implicated inflammation in the development of this restenosis, but clinical studies that have assessed the association between inflammatory marker levels and the development of restenosis have shown conflicting results. This may have been because unrecognised confounding from inclusion of both stable and unstable angina patients caused a false-positive result in some studies, or because use of medications with antiinflammatory properties masked the association between inflammation and restenosis and caused a false-negative result in other studies. These issues are addressed in this thesis by study of the association between inflammatory markers and restenosis in a cohort of stable angina patients in whom the influence of concurrent medications could be excluded.

The effects of lipid-modifying agents on inflammatory marker levels

Given the association between inflammatory markers and cardiovascular risk, there is considerable interest in finding interventions that will lower inflammatory marker levels. To this end, much has been made of potential antiinflammatory effects of medications such as statins and lifestyle interventions such as exercise and smoking cessation. There is now good evidence that statin use is associated with a reduction in circulating CRP levels. However, this reduction
in CRP is modest compared to the effect that statins have on cholesterol levels and could conceivably be an indirect consequence of lipid lowering. In this thesis, evidence of a direct antiinflammatory effect of statins was sought by examining whether they lower a broad range of inflammatory markers simultaneously, and whether they reduce the inflammatory response to an acute stimulus such as PCI. In addition, the effect of simvastatin on inflammatory marker levels was compared with that of another lipid lowering agent, bezafibrate (which is also postulated to have antiinflammatory properties).

The effects of exercise and smoking cessation on inflammatory marker levels

Both exercise and smoking cessation are lifestyle interventions known to reduce the risk of cardiovascular disease. It has been suggested that this is at least in part due to a reduction in vascular inflammation brought about by these interventions. Indirect support comes from cross sectional studies in which regular exercise and smoking cessation are associated with lower inflammatory marker levels. Prospective trials would provide stronger evidence that these interventions have antiinflammatory effects, but to date such trials have been small, non randomised and inconclusive. Consequently, prospective trials were performed in this thesis to assess the effects of exercise and smoking cessation on inflammatory marker levels.

In summary, the aims of this thesis are:

1. To determine whether inflammatory marker levels are predictive of adverse outcomes (cardiac events, restenosis) following percutaneous coronary intervention.

2. To explore whether inflammation is a modifiable cardiovascular risk factor. This will be done by determining whether inflammatory marker levels can be predictably lowered by:
   a. Medication (lipid-modifying therapy using simvastatin or bezafibrate).
   b. Lifestyle modification (exercise training, smoking cessation).
Chapter 2

Overview of the Fragmin and Simvastatin Trial (FAST)

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2.1 Introduction

The data used in chapters 3 and 4 of this thesis are derived from the Fragmin and Simvastatin Trial (FAST). This chapter gives an overview of aspects of the FAST study relevant to the thesis.

FAST was a 2x2 factorial design randomized controlled trial performed by the Cardiovascular Research Unit at Green Lane Hospital, Auckland between 1990-2000. The aims of FAST were:

1. To test the hypothesis that prolonged subcutaneous low molecular weight heparin (LMWH) reduces restenosis at six months after percutaneous revascularization procedures.
2. To test the hypothesis that lipid-modifying treatment with simvastatin reduces restenosis at six months after percutaneous revascularization procedures.
3. To test the hypothesis that long-term (two years) lipid-modifying therapy with simvastatin reduces the progression of coronary artery disease in patients with stable angina.

As has been shown by other investigators, neither lipid-modifying therapy nor LMWH affected the primary study endpoint of restenosis. Analysis of the effect of long term lipid-modifying therapy (two years) on the progression of coronary artery disease is ongoing.

The aims for this thesis are distinct from the original study aims for FAST. For this thesis, stored blood samples from FAST trial patients were used to determine:

1. The association between inflammatory marker levels and PCI outcomes (clinical events, restenosis).
2. The effect of simvastatin on the inflammatory response to PCI.
3. The effects of long term (2 years) lipid-modifying therapy on inflammatory marker levels.
2.2 Study design and patient population

The FAST study population consisted of patients listed for elective PCI on a de novo lesion. The exclusion criteria included need for urgent PCI, PCI planned within 4 days, PCI planned on a restenotic lesion, previous unsuccessful PCI, previous coronary artery bypass grafting (CABG), insulin-dependent diabetes, or abnormal hepatic function tests (i.e. with transaminase, alkaline phosphatase, or bilirubin levels >20% above the normal range). A total of 315 patients were recruited to the trial. The majority (87%) were recruited between 1990 and 1995 and consequently most had balloon angioplasty alone without stenting.

Upon enrolment two separate randomizations took place. The first randomization, to lipid-modifying therapy, was stratified according to baseline lipids. Patients with an ‘acceptable’ total cholesterol level were randomised in a double blind manner to simvastatin 40 mg/day or identical matching placebo (Group A), whereas patients with an ‘unacceptably high’ total cholesterol were randomised to simvastatin 40 mg/day or to bezafibrate 400 mg/day (Group B). The allocated treatment was continued for two years post-PCI. The trial recruited from 1990 to 1998, and over this time the definition of ‘acceptable’ and ‘unacceptably high’ cholesterol levels progressively changed. This was because of emerging trial data on statin therapy, in which each new trial recruited patients with lower baseline cholesterol levels than the trial before, and demonstrated clinical benefit of statin therapy in these patients. Initially, ‘acceptable’ cholesterol levels were defined as a total cholesterol ≤ 7 mmol/l; only those patients with a total cholesterol > 7 mmol/l were placed in group B, with all others in group A. Subsequently the threshold was reduced to 5.5 mmol/L, and eventually 4 mmol/l. Consequently there is considerable overlap in the baseline cholesterol levels of group A and group B.

At PCI, a median 1.3 months (interquartile range 0.6-2.8) after enrolment and randomization to lipid-modifying therapy, patients were separately randomized to LMWH (dalteparin 5,000 IU twice daily) or to placebo. These subcutaneous injections began within 24 hours of the PCI procedure and were continued for 1 month.
2.3 Collection of blood samples in the FAST trial

Multiple blood samples were stored from FAST trial patients as they progressed through the trial. As part of the original study design, serum and citrate plasma samples were taken at both baseline and two years for future study of novel cardiovascular risk factors. These samples were stored at -80°C. Additional blood samples were also collected for two separate sub-studies. The first involved collection of serum samples at baseline, 1 month, 6 months and 2 years for study of insulin resistance. The second involved collection of citrate plasma samples pre-PCI and 48 hours, 1 week and 1 month post-PCI for comparison of anti-Xa activity with fragmin versus placebo. Any residual samples from these two sub-studies were stored at -20°C for potential future use.
2.4 Identification of stored blood samples

The FAST trial randomized 315 patients over a 10 year period and over 6,000 serum or plasma tubes were stored. During this period multiple different laboratory computer systems and labelling codes were used, which meant there was no single database by which the samples could be identified. Initial work for this thesis involved cataloguing of all the samples stored at -20°C and -80°C. Samples were identified by matching their laboratory code numbers with the laboratory result sheets in the original patient records. This process was very time consuming, taking over 6 months to complete, but it resulted in an up-to-date database of all available samples which allowed the comprehensive analyses in this thesis to take place.

From this database it became apparent that not all patients had samples available at every time point. The most critical period was pre- and post-PCI, where the only samples available came from the anti-Xa activity sub-study. In many instances no samples could be found, and in others only a small amount of plasma (insufficient for measurement of all inflammatory markers) had been stored. In contrast there were often multiple samples at baseline and two years (where several aliquots of serum and plasma had been stored at -80°C, in addition to the -20°C serum samples from the insulin resistance sub-study). The numbers of samples available for each analysis are detailed in the relevant chapters.
2.5 Preliminary analyses of the FAST samples

Following identification of the stored samples, preliminary CRP analyses were performed on selected samples to determine whether they had been affected by storage conditions. Details of the assays used for hsCRP measurement, and for other inflammatory markers in this thesis, are given in appendix 1. All available samples on 40 randomly selected patients (350 samples in total) were analysed to allow comparison of CRP levels in matching serum and plasma samples, and in duplicate samples that had been taken at the same time but stored at different freezer temperatures (-20°C and -80°C).

These analyses confirmed that the samples had remained stable in storage. For duplicate samples (taken at the same time but stored at different temperatures) the co-efficient of variation was only 6% (interquartile range 5-10%). In addition CRP levels in serum and plasma samples showed strong correlation although CRP levels were on average 10% lower in plasma; this has been described by others and is thought to be due to a dilutional effect in plasma samples, both from the anticoagulant itself and from an osmotic effect of the anticoagulant drawing water from erythrocytes.\textsuperscript{18}

Data from 10 patients are presented in table 2.1 by way of illustration. CRP results were highly reproducible regardless of sample type (serum or plasma) and storage conditions (-20°C or -80°C).

These preliminary analyses demonstrated that it was appropriate to use the FAST samples for the inflammatory marker analyses detailed in subsequent chapters of this thesis.
Table 2.1  CRP results from 10 FAST trial patients. Results of four separate stored serum samples and 1-2 stored plasma samples collected at the same timepoint are shown.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Timepoint</th>
<th>Serum samples mg/l</th>
<th>Plasma samples mg/l</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CRP1 80°C</td>
<td>CRP 2 80°C</td>
<td>CRP 3 20°C</td>
</tr>
<tr>
<td>A173</td>
<td>2 years</td>
<td>2.95</td>
<td>2.97</td>
<td>2.66</td>
</tr>
<tr>
<td>A192</td>
<td>Baseline</td>
<td>0.54</td>
<td>0.54</td>
<td>0.50</td>
</tr>
<tr>
<td>A216</td>
<td>Baseline</td>
<td>0.55</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>A239</td>
<td>Baseline</td>
<td>0.70</td>
<td>0.72</td>
<td>0.69</td>
</tr>
<tr>
<td>A259</td>
<td>Baseline</td>
<td>4.46</td>
<td>4.41</td>
<td>4.05</td>
</tr>
<tr>
<td>B080</td>
<td>Baseline</td>
<td>0.47</td>
<td>0.48</td>
<td>0.42</td>
</tr>
<tr>
<td>B085</td>
<td>Baseline</td>
<td>2.99</td>
<td>3.07</td>
<td>2.72</td>
</tr>
<tr>
<td>B091</td>
<td>Baseline</td>
<td>2.32</td>
<td>2.38</td>
<td>1.93</td>
</tr>
<tr>
<td>B096</td>
<td>Baseline</td>
<td>1.05</td>
<td>1.09</td>
<td>1.03</td>
</tr>
<tr>
<td>B101</td>
<td>Baseline</td>
<td>0.76</td>
<td>0.99</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Chapter 3

Inflammatory Markers for the prediction of adverse outcomes (cardiac events, restenosis) following Percutaneous Coronary Intervention

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3.1 Introduction

3.1.1 Percutaneous coronary intervention (PCI)

Over the last two decades, percutaneous coronary intervention (PCI) has revolutionized the treatment of symptomatic coronary artery disease. Over 500,000 PCI procedures are performed each year in the United States alone, and over a million procedures are performed worldwide. Use of PCI continues to increase as both technological advances and adjuvant therapies make treatment of ever more complex lesions possible. For example, 4,668 PCI procedures were performed in New Zealand in 2003, up from 3,656 in 2000 and only 1,673 in 1995 (NZ PCI registry data, unpublished).

However PCI complications remain a problem. The most important complications of PCI are acute thrombotic events and restenosis, restenosis in particular has been described as the ‘Achilles’ heel’ of PCI. Angiographic restenosis (a >50% renarrowing at the intervention site) occurs in 20-40% of balloon angioplasty and 10-20% of coronary stenting procedures, causes significant morbidity, and because of the need for further revascularisation procedures such as repeat PCI or coronary surgery, considerably increases the angioplasty procedural cost. In the United States alone the cost of treating restenosis in the pre drug-eluting stent era was estimated at US$ 3.5 billion/year. Although the advent of drug-eluting stents has significantly reduced the incidence of restenosis, it has not abolished it. Until recently, the high cost of drug-eluting stents in New Zealand limited their use to only a subset of lesions; in 2003, less than 15% of coronary stent implantations in New Zealand involved a drug-eluting stent (NZ PCI registry data, unpublished). Furthermore, drug eluting stents have their own problems, such as an increased incidence of late stent thrombosis, making it desirable to limit their use to those most like to accrue benefit from their anti-restenotic properties. Strategies to predict those at increased risk of restenosis therefore remain of utmost importance.

3.1.2 Inflammation and restenosis

The mechanisms of restenosis are only partly understood, though contributing factors include anatomical features (e.g.: lesion length), procedural variables (e.g.: residual stenosis) and clinical factors (diabetes, unstable angina, smoking, older age). Unfortunately these factors
are of only limited use in predicting those in whom restenosis will occur.\textsuperscript{156} Meanwhile evidence is accumulating that inflammation may play an important role in restenosis,\textsuperscript{157,158} raising the possibility that measurement of inflammation may improve our ability to predict those at increased risk. Balloon injury to the vessel wall caused by PCI leads to a ‘healing’ response characterised by inflammation in both the intimal and adventitial layers of the artery wall.\textsuperscript{159} In addition to being prothrombotic, this inflammation can have two important consequences: adventitial constriction and intimal hyperplasia.\textsuperscript{159} These are the underlying processes that lead to restenosis. Adventitial constriction results in an overall reduction in the arterial diameter at the PCI site and is a major component of restenosis following balloon angioplasty without stenting. This constrictive remodelling is prevented by stent deployment, but conversely intimal hyperplasia is more marked following stenting and accounts for virtually all in-stent restenosis.\textsuperscript{160} In both animal models and human pathology studies the extent of the inflammatory reaction to angioplasty correlates significantly with the degree of restenosis.\textsuperscript{159} Experimental treatments aimed at decreasing inflammation have shown some promise in reducing restenosis,\textsuperscript{163,164} adding further weight to the theory that inflammation is a central component of the restenotic process.\textsuperscript{161,162} If this is the case, a better understanding of the components of the inflammatory response that contribute to restenosis may help in the development of anti-restenosis treatments. In addition, if measurement of circulating markers of inflammation improves our ability to predict those who will develop restenosis, they may be of use in targeting prevention strategies such as drug-eluting stents to those most likely to benefit. For these reasons, the associations between several inflammatory markers and restenosis are assessed in this thesis.

3.1.3 Pre-PCI inflammatory markers and restenosis

A number of previous investigators have studied whether inflammatory marker levels are predictive of restenosis in patients undergoing PCI.\textsuperscript{165-170} However these studies, which focused on CRP, have shown conflicting results (table 3.1).
Table 3.1  Studies of CRP and restenosis. Although some studies have shown an association between CRP and restenosis, most have not.

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>n</th>
<th>Restenosis assessment</th>
<th>CRP measurement</th>
<th>Association between CRP and restenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-PCI CRP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffon et al. (1999)</td>
<td>Mixed stable and unstable angina</td>
<td>121</td>
<td>Clinical</td>
<td>Pre-PCI</td>
<td>Associated</td>
</tr>
<tr>
<td>Walter et al. (2001)</td>
<td>Mixed stable and unstable angina</td>
<td>276</td>
<td>Angiographic</td>
<td>Pre-PCI</td>
<td>Associated</td>
</tr>
<tr>
<td>Zairis et al. (2002)</td>
<td>Mixed stable and unstable angina</td>
<td>483</td>
<td>Angiographic</td>
<td>Pre-PCI *</td>
<td>Not Associated</td>
</tr>
<tr>
<td>Dibra et al. (2003)</td>
<td>Stable angina</td>
<td>1,152</td>
<td>Angiographic</td>
<td>Pre-PCI</td>
<td>Not Associated</td>
</tr>
<tr>
<td>Gomma et al. (2004)</td>
<td>Stable angina</td>
<td>133</td>
<td>Angiographic</td>
<td>Pre-PCI</td>
<td>Not Associated</td>
</tr>
<tr>
<td>Segev et al. (2004)</td>
<td>Stable angina</td>
<td>216</td>
<td>Angiographic</td>
<td>Pre-PCI</td>
<td>Not Associated</td>
</tr>
<tr>
<td>Rittersma et al. (2004)</td>
<td>Predominantly stable angina</td>
<td>345</td>
<td>Angiographic</td>
<td>Pre-PCI</td>
<td>Not Associated</td>
</tr>
<tr>
<td><strong>Post-PCI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gottsauner-Wolf et al. (2000)</td>
<td>Stable angina</td>
<td>40</td>
<td>Angiographic</td>
<td>Up to 96 hours post-PCI</td>
<td>Associated</td>
</tr>
<tr>
<td>Gomma et al. (2004)</td>
<td>Stable angina</td>
<td>58</td>
<td>Angiographic</td>
<td>Up to 6 months post-PCI</td>
<td>Not Associated</td>
</tr>
<tr>
<td>Segev et al. (2004)</td>
<td>Stable angina</td>
<td>43</td>
<td>Angiographic</td>
<td>Up to 6 months post-PCI</td>
<td>Not Associated</td>
</tr>
<tr>
<td>Dibra et al. (2005)</td>
<td>Mixed stable and unstable angina</td>
<td>1,800</td>
<td>Angiographic</td>
<td>Up to 72 hours post-PCI</td>
<td>Associated</td>
</tr>
</tbody>
</table>

* CRP was measured by conventional assay, not hsCRP, limiting conclusions that can be drawn from this study

54
Initial reports included a mix of stable and unstable angina patients and showed an association between raised CRP levels and subsequent angiographic restenosis. For example, in 276 “high-risk” PCI patients, including many with ACS, Walter et al. (2001) found that pre-PCI CRP levels were predictive of angiographic restenosis, with a CRP level ≥5 mg/l associated with significantly higher restenosis rates than CRP <5 mg/l (40% vs. 19% respectively, p=0.002). In contrast, studies limited to a stable angina population have found no association between pre-PCI CRP levels and restenosis. In by far the largest study reported, Dibra et al. (2003) assessed baseline CRP, 6 month angiographic restenosis and 1 year clinical outcome in 1,152 patients undergoing elective PCI for stable angina. Stent deployment was universal, and all patients underwent 6 month angiographic follow-up. In this study, CRP appeared predictive of clinical events (death or non-fatal MI) but not of angiographic restenosis (CRP >5 mg/l, restenosis rate 25%, CRP ≤5 mg/l, restenosis rate 24%, p=0.66).

Why have some studies shown CRP to be predictive of restenosis while others have not? Firstly, the discrepancy may relate to differences in the population studied. The studies in which CRP was not associated with restenosis involved stable angina patients, whereas the studies that did show an association included mixed populations of stable angina and acute coronary syndrome patients. The underlying pathological processes in ACS are quite distinct from those of stable angina. ACS patients have widespread coronary inflammation with evidence of unstable coronary plaques in non-culprit arteries, and this is reflected in significantly higher pre-procedural CRP levels and more marked elevation of CRP levels after PCI than those observed in stable angina patients. This may introduce unrecognised confounding in studies that include a mixed population of stable and unstable angina patients, since unstable angina is associated with both raised CRP levels and increased rates of restenosis. It may also be that CRP is predictive of restenosis in acute coronary syndromes but not in stable angina; only study of a ‘pure’ ACS cohort would answer this question.

A second possible explanation for the discrepant results of previous studies lies in the pre-treatment that nearly all patients with stable angina receive before undergoing PCI. This was raised in an editorial in the American Heart Journal in 2004, in which the author postulated that the lack of association in stable angina patients could have been due to near universal use of
statins and other drugs with potential antiinflammatory effects (such as thienopyridines and glycoprotein IIb/IIIa inhibitors), which might lower circulating inflammatory marker levels and therefore mask the true association between inflammation within the vessel wall and the development of restenosis.\textsuperscript{16}

In this thesis, the potential for confounding was avoided by study of a stable angina population, and the possibility of a masking effect from statin use was explored by separately assessing the association between inflammatory markers and restenosis for those randomised to simvastatin and those randomised to placebo.

Despite the focus on CRP in studies to date, it is possible other markers directly involved in the restenotic process might be more predictive of restenosis. For example, sCD40L may exacerbate inflammation and prevent re-endothelialisation of the injured vessel wall thereby promoting restenosis,\textsuperscript{43} and sICAM-1 has been implicated in the pathogenesis of neointimal proliferation following vascular injury in animal PCI models.\textsuperscript{127} Only one study to date has examined whether either sCD40L or sICAM-1 levels are predictive of restenosis.\textsuperscript{178} This study involved 70 predominantly unstable angina patients who underwent PCI (stent deployment rate 50\%) to a single non-occlusive coronary stenosis. All patients had sCD40L and sICAM-1 measured at the time of PCI and underwent 6 month angiographic follow-up. Pre-procedural levels of both sCD40L and sICAM-1 were significantly higher in patients who subsequently developed angiographic restenosis than in those who did not (sCD40L: 2.13 ± 0.3 versus 0.87 ± 0.12 ng/ml, p<0.0001; sICAM-1: 365 ± 32 versus 115 ± 23 ng/ml, p<0.0001), raising the possibility that sCD40L and sICAM-1 levels may be useful in predicting restenosis in patients with unstable angina. However, further study is required to confirm these results and to determine whether they are applicable to patients with stable angina. Therefore, sCD40L and sICAM-1 levels were measured for this thesis.

It is also possible that levels of circulating metalloproteinases such as PAPP-A may be useful in predicting restenosis. Despite evidence that matrix metalloproteinases are expressed rapidly after PCI and may contribute to subsequent constrictive remodelling and intimal hyperplasia,\textsuperscript{131,179,180} the association between circulating metalloproteinase levels and restenosis has not previously been assessed. The metalloproteinase PAPP-A in particular has
shown promise for risk prediction in other clinical situations such as ACS, and for these reasons was included among the markers studied in this thesis.

3.1.4 Post-PCI inflammatory markers and restenosis

Since it is the inflammatory response to PCI that is thought to contribute to restenosis, measurement of post-PCI inflammatory markers may be more indicative of outcome than baseline inflammatory marker levels. CRP levels rise significantly following PCI, with levels peaking around 48 hours post-procedure. sCD40L levels also rise post-PCI but data on other markers are scarce. Of note, it appears that this inflammatory response is modifiable and can be blunted by administration of antiplatelet agents at the time of PCI. In a substudy of the EPIC trial (2001), the rise in CRP 48 hours post-PCI was 32% less in patients receiving the glycoprotein IIb/IIIa inhibitor abciximab compared to those receiving placebo (CRP rose from 9 to 31 mg/l in the placebo group compared to 12 to 27 mg/l in the abciximab group, p=0.025). Similarly in registry data from the Cleveland Clinic (2004), the periprocedural increase in CRP was attenuated by 65% in patients pre-treated with clopidogrel. These studies raise the possibility that inflammatory marker measurement post-PCI could be used to guide treatment, perhaps with increased dose, prolonged infusion, or addition of further anti-platelet therapy in those with a pronounced inflammatory marker rise. A better understanding of the inflammatory response to angioplasty may also lead to new treatment strategies targeting specific aspects of the inflammatory process.

Only a small number of studies have examined whether the inflammatory response to PCI is predictive of subsequent restenosis. In the largest of these, involving 1,800 patients with both stable and unstable angina, there was an association between the post-PCI inflammatory response and six month angiographic restenosis. CRP was measured at multiple timepoints post-PCI (8 hours, 16 hours, and then daily until hospital discharge) and both pre-PCI CRP levels and the post-PCI CRP rise (pre-PCI to peak post-PCI level) were compared to angiographic restenosis rates. Pre-PCI CRP levels were not predictive of restenosis (p=0.88), whereas the post-PCI CRP rise did correlate with angiographic restenosis (p=0.002). However, this association was strongly influenced by the presence of unstable angina, since when analysis was restricted to stable angina patients (who made up 73% of patients in the study) the
association was of only borderline statistical significance (p=0.05). Three other small studies have examined the association between post-PCI CRP and restenosis in stable angina (table 3.1). Gottsauner-Wolf et al. (2000) studied 40 consecutive patients with stable angina, and found that CRP levels 96 hours post-procedure were significantly higher in those who subsequently went on to develop restenosis. However, Gomma et al. (2004) measured CRP serially for up to 6 months post-PCI in 58 stable angina patients undergoing elective coronary stenting, and found no association between post-PCI CRP levels and restenosis. Similarly, Segev et al. (2004) found no association between post-PCI CRP levels and angiographic restenosis in 43 stable angina patients undergoing coronary stenting. CRP was measured at multiple time-points (6 hours, 24 hours, 3 days, 7 days, 1 month, 3 months and 6 months post stenting). Given these discrepant results, further study is needed to determine whether post-PCI levels of CRP and other inflammatory markers are predictive of restenosis in stable angina patients.

Data on other markers are scarce. There is one study which has shown post-PCI sCD40L and sICAM-1 levels to be predictive of restenosis. This study, by Cipollone et al. (2003), measured levels sCD40L and sICAM-1 before, and 1, 5, 15 and 180 days after, PCI. As previously discussed (section 3.1.3, page 56), patients who went on to develop restenosis had higher baseline levels of these markers than patients who did not. With PCI, there was a significant rise in levels of both markers at 24 hours, and this rise was more pronounced and more prolonged in those who developed restenosis. Although sCD40L and sICAM-1 levels had returned to baseline by day five in those who did not develop restenosis, they remained elevated through to 6 months in those with restenosis. This is an interesting finding which lends support to the hypothesis that these markers are involved in the restenotic process. However, as discussed in section 3.1.3, this study included predominantly unstable angina patients; the applicability of these findings to stable angina patients is uncertain.

In this thesis, levels of several markers (CRP, sCD40L, sICAM-1, PAPP-A) measured 48 hours, 1 week and 1 month post-PCI were compared against 6 month angiographic restenosis rates in stable angina patients undergoing elective PCI.
The mechanisms that underlie coronary events following PCI are distinct from the mechanisms that underlie restenosis. Restenosis is the consequence of proliferative neointimal hyperplasia whereas coronary events are triggered by plaque instability, embolisation and thrombosis. It is now well established that CRP levels are predictive of long-term events in patients with coronary artery disease, whether measured during the stable phase of their disease or at the time of an acute coronary syndrome event. Therefore it is not surprising that, in contrast to restenosis, a number of studies have shown inflammatory markers are predictive of risk of subsequent cardiac events following PCI (table 3.2).

For example, Dibra et al. (2003) reported that although CRP was not predictive of restenosis, elevated pre-PCI CRP levels (>5 mg/l) were associated with almost a two-fold increase in the rate of death or myocardial infarction after coronary stenting. During 1 year of follow-up, 62 (9.5%) of the 651 patients with an elevated CRP and 24 (4.8%) of the 501 patients with normal levels died or had a myocardial infarction (p=0.002). Most of this difference in event rates developed within the first 30 days, consistent with reports that inflammation may play a direct role in the pathogenesis of periprocedural myocardial injury. A study of registry data from 727 consecutive patients from the Cleveland Clinic (2001) also found pre-PCI CRP to be predictive of death or myocardial infarction within the first 30 days after PCI, with a cut-point of 3 mg/l successfully distinguishing those at increased risk of events (4.7% risk for CRP < 3 mg/l, versus 12.1% risk for CRP ≥ 3 mg/l; p=0.001). The same authors have subsequently shown that pretreatment with clopidogrel substantially reduces this risk of events associated with an elevated CRP, raising the possibility that CRP could be used to guide therapy. Routine use of platelet glycoprotein IIb/IIIa receptor inhibitors such as abciximab may also be of therapeutic benefit in those with evidence of heightened inflammation, since glycoprotein IIb/IIIa inhibitors have been shown to decrease the inflammatory response to PCI.
Table 3.2  Studies of CRP and clinical events following PCI.

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>n</th>
<th>CRP measurement</th>
<th>Outcome</th>
<th>Findings *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versaci et al. (2000)</td>
<td>Unstable angina</td>
<td>62</td>
<td>Pre-PCI</td>
<td>1 year death, MI, recurrent angina</td>
<td>CRP &gt; 5mg/l predictive</td>
</tr>
<tr>
<td>Chew et al. (2001)</td>
<td>Mixed stable and unstable angina</td>
<td>727</td>
<td>Pre-PCI</td>
<td>30 day death or MI</td>
<td>CRP &gt; 3mg/l predictive</td>
</tr>
<tr>
<td>de Winter et al. (2002)</td>
<td>Stable angina</td>
<td>501</td>
<td>Pre-PCI</td>
<td>2 year death, MI, unstable angina</td>
<td>CRP &gt; 3mg/l predictive</td>
</tr>
<tr>
<td>Dibra et al. (2003)</td>
<td>Stable angina</td>
<td>1,152</td>
<td>Pre-PCI</td>
<td>1 year death or MI</td>
<td>CRP &gt; 5mg/l predictive</td>
</tr>
<tr>
<td>Palmerini et al. (2005)</td>
<td>Mixed stable and unstable angina undergoing Left Main stenting</td>
<td>83</td>
<td>Pre-PCI</td>
<td>9 month death or MI</td>
<td>CRP &gt; 3mg/l predictive</td>
</tr>
<tr>
<td>Gaspardone et al. (1998)</td>
<td>Stable angina</td>
<td>81</td>
<td>72 h post-PCI</td>
<td>1 year death, MI, recurrent angina</td>
<td>CRP &gt; 5mg/l predictive</td>
</tr>
<tr>
<td>Dibra et al. (2005)</td>
<td>Mixed stable and unstable angina</td>
<td>1,800</td>
<td>Post-PCI rise</td>
<td>30 day death, MI, re-PCI</td>
<td>CRP rise &gt;11.8mg/l predictive</td>
</tr>
<tr>
<td>Gach et al. (2007)</td>
<td>Stable angina</td>
<td>89</td>
<td>Post-PCI rise</td>
<td>6 year death, MI, unstable angina</td>
<td>CRP rise of &gt; 3mg/l predictive</td>
</tr>
<tr>
<td>Fournier et al. (2008)</td>
<td>Mixed stable and unstable angina</td>
<td>68</td>
<td>1 month post-PCI</td>
<td>1 year death, MI, revascularisation</td>
<td>CRP &gt; 2.5mg/l predictive</td>
</tr>
</tbody>
</table>

* ‘Predictive’ = Predictive of increase risk of subsequent cardiovascular events.
It is well recognised that PCI initiates an inflammatory response in the coronary arteries, with a associated rise in circulating CRP levels.\textsuperscript{109,181} It is possible this inflammatory response to PCI (measured by post-PCI CRP levels or CRP rise) more accurately reflects the inflammatory milieu within the coronary arteries than baseline CRP levels, and may therefore be more predictive of adverse outcomes and long term events. A small number of studies have shown interesting results in this regard (table 3.2). For example, Gach et al. (2007) demonstrated the post-PCI CRP rise to be more predictive of late events than pre-PCI CRP.\textsuperscript{195} The largest reported study is again from Dibra et al. (2005), who reported a CRP rise of >11.8 mg/l post-PCI to be associated with an increased risk of adverse clinical events (death, MI, need for urgent target vessel revascularisation) at 30 days, and an increased incidence of MI at 1 year.\textsuperscript{173} However this may have been influenced by the inclusion of both stable and unstable angina patients, and it is not clear whether the findings still hold when analysis restricted to patients with stable angina.

Percutaneous coronary intervention shares many features with acute coronary syndromes including direct injury to the vessel wall, platelet activation, and evidence of a systemic inflammatory response.\textsuperscript{100,181,200} In acute coronary syndromes, inflammatory markers such as sCD40-L and PAPP-A are thought to contribute directly to the disease process and may give prognostic information above and beyond that obtained from non-specific markers such as CRP.\textsuperscript{33,112,139} Therefore it could be supposed that these markers will be of use in predicting PCI outcomes.\textsuperscript{43,131} However few studies of markers other than CRP have been published. In the CAPTURE trial (2003) an sCD40L level >5 ng/ml was associated with 2.7x risk of death or myocardial infarction compared to sCD40L ≤ 5 ng/ml in patients with ACS undergoing PCI,\textsuperscript{112} but it is not clear whether levels of sCD40L are predictive of adverse events following PCI in stable angina.

In this thesis, 30 day and 2 year cardiac event rates were assessed against inflammatory marker levels in a stable angina population undergoing elective PCI.
3.1.6 Summary

Following percutaneous coronary intervention (PCI), restenosis remains an important clinical problem that is not well predicted using conventional clinical and angiographic features. PCI triggers a measurable inflammatory response, and both in animal models and human pathology studies this inflammation is implicated in the restenotic process. Consequently, measurement of inflammatory marker levels may help improved prediction of restenosis. Disappointingly, the literature to date suggests that non-specific markers of inflammation such as CRP are poor predictors of restenosis when measured prior to PCI. Whether this is due to a lack of association, or a masking of association by use of concomitant medications with antiinflammatory effects (statins, thienopyridines, glycoprotein IIb/IIIa inhibitors), has been debated. Reports that have focussed on the post-PCI inflammatory response (post-PCI CRP level or CRP rise) have been more likely to show an association between CRP and restenosis, but as yet there are incomplete data to determine whether the post-PCI inflammatory response is truly predictive of restenosis. In addition, it has been hypothesised that specific local mediators of inflammation such as sICAM-1, sCD40L, and PAPP-A may be more directly involved in the restenotic process, and therefore predictive of restenosis, but very little published data exist.

In contrast to restenosis, there is consistent evidence that CRP measured at the time of PCI is predictive of subsequent clinical events. There are theoretical reasons (and limited data) to suppose more direct mediators of inflammation (such as sICAM-1, sCD40L, and PAPP-A), and particularly the post-PCI rise in inflammatory markers, may provide better risk prediction than baseline CRP, but this remains to be proven.
3.1.7 Study aims and research questions

This research has a number of general aims:

1. To determine the effect of PCI on a range of inflammatory markers.

2. To determine whether inflammatory marker levels, measured before and at multiple times after PCI in a stable angina population, are predictive of:
   a. 30 day cardiac events
   b. 6 month angiographic restenosis
   c. Longer term (2 year) cardiac events.

The following research questions will be addressed:

a. What is the effect of PCI on plasma levels of CRP, sICAM-1, sCD40L, PAPP-A, and the PAPP-A/proMBP ratio 48 hours, 1 week, and 1 month post-procedure?

b. Are plasma levels of CRP, sICAM-1, sCD40L, PAPP-A, and the PAPP-A/proMBP ratio measured pre-PCI or 48 hours post PCI predictive of 30-day events (death, MI, revascularization) in patients with stable angina undergoing elective angioplasty?

c. Are plasma levels of CRP, sICAM-1, sCD40L, PAPP-A, and the PAPP-A/proMBP ratio measured pre-PCI, or 48 hours, 1 week or 1 month post-PCI, predictive of 6 month angiographic restenosis in patients with stable angina undergoing elective angioplasty?

d. Are plasma levels of CRP, sICAM-1, sCD40L, PAPP-A, and the PAPP-A/proMBP ratio measured pre-PCI, or 48 hours post-PCI, predictive of 2 year events (death, MI, revascularization) in patients with stable angina undergoing elective angioplasty?

e. Is any association (if present) between inflammatory marker levels and either restenosis or clinical events affected by the use of statin therapy (which is postulated to have an anti-inflammatory effect and therefore may mask associations between inflammatory markers and clinical outcomes)?
3.2 Methods

3.2.1 Study overview

Inflammatory marker levels, 6-month angiographic restenosis rates, and 30-day and 2-year event rates were determined in participants in the FAST trial (chapter 2). CRP, sCD40L, sICAM-1, and PAPP-A levels, and the PAPP-A/proMBP ratio, were measured in the available pre-PCI and 48 hour, 1 week, and 1 month post-PCI samples (table 3.3). In addition, troponin T levels were measured on the pre-PCI and 48 hour post-PCI samples. Details of the inflammatory marker and troponin assays are given in appendix 1.

3.2.2 PCI procedure and follow-up angiography

The majority of patients (87%) in this study were recruited between 1990 and 1995, and none were recruited after 1997. The PCI technique used was at the operator’s discretion, and most cases involved balloon angioplasty alone without stenting. All patients received bolus intravenous doses of unfractionated heparin in the catheterization laboratory, followed by a 24-hour infusion at the operator’s discretion. Aspirin (150-300 mg daily) was given for the duration of the trial unless there was a specific contraindication. No patients received thienopyridines as these were not in use at our institution when the study was performed. Glycoprotein IIb/IIIa inhibitor use was also infrequent and none of the study patients received these agents.

Coronary angiograms were recorded before and immediately after PCI. Follow-up angiography was performed at 6 months or sooner if clinically indicated. Before PCI, the orthogonal projections that best demonstrated the stenosed segment in end-diastole, (free of superimposed structures and foreshortening) were identified, and these views were repeated at follow-up angiography. Sublingual glyceryl trinitrate was given before each angiogram, and care was taken to reproduce the field size, rotation, angulation, image intensifier height, and table height used in each projection.
Table 3.3  Number of samples available for inflammatory marker analysis at each timepoint *

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pre PCI</th>
<th>48 hours</th>
<th>1 week</th>
<th>1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>133</td>
<td>120</td>
<td>112</td>
<td>114</td>
</tr>
<tr>
<td>sCD40L</td>
<td>127</td>
<td>103</td>
<td>107</td>
<td>91</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>133</td>
<td>108</td>
<td>113</td>
<td>96</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>133</td>
<td>123</td>
<td>113</td>
<td>96</td>
</tr>
<tr>
<td>PAPP-A/proMBP ratio †</td>
<td>133</td>
<td>123</td>
<td>113</td>
<td>96</td>
</tr>
</tbody>
</table>

* As outlined in chapter 2, samples were not available from all participants in the FAST trial, and in some instances there was insufficient sample to allow measurement of all inflammatory markers. Only patients in whom pre-PCI blood samples were available were included in this analysis. This ensured that the same group of patients was studied at each timepoint, and that each post-PCI blood result can be compared to a corresponding pre-PCI result from the same patient. For example pre-PCI CRP results were available for 133 patients, and of these 133 patients, 120 had blood samples available 48 hours post-PCI, 112 had samples available 1 week post-PCI, and 114 had samples available 1 month post-PCI.

† The proform of eosinophilic major basic protein (proMBP) is the endogenous inhibitor of PAPP-A, and therefore the relative concentrations of PAPP-A and proMBP (the PAPP-A/proMBP ratio) may reflect PAPP-A proteolytic activity better than PAPP-A levels alone. See section 1.8 for further information.
3.2.3 *Quantitative angiographic analysis*

Quantitative coronary angiographic measurements were made using the Cardiovascular Measurement System (CMS-Medis Medical Imaging Systems, Nuenen, the Netherlands) by a single, experienced analyst blinded to treatment allocation, inflammatory marker levels, and order of angiograms. The maximum percentage diameter stenosis, lesion minimum lumen diameter (MLD), corresponding reference diameter, and lesion length were determined using a non-tapering portion of the angiographic guide catheter as a calibration reference. The reproducibility of quantitative coronary angiographic findings from our institution have been reported previously; measurements were highly reproducible with an intraobserver accuracy (mean signed difference between repeated measurements) for both minimal lumen diameter and reference diameter of 0.01mm.\(^{201}\) Statistical analyses were performed using the mean MLD and maximum percentage diameter stenosis calculated from the two orthogonal projections of each lesion. Where more than one lesion had been treated by PCI (39% of cases), the maximum percentage diameter stenoses of all treated lesions were averaged to allow analysis on a “per patient” basis. The primary outcome measure was renarrowing at the intervention site, determined by the percentage diameter stenosis on the 6-month angiogram. Because of the possibility that the six month diameter stenosis could be influenced by poor procedural outcome (i.e. residual narrowing at the end of the PCI procedure) as well as subsequent restenosis, a secondary analysis was performed comparing inflammatory marker levels with late lumen loss. Late lumen loss reflects the degree of luminal renarrowing following the PCI procedure and is calculated by the formula

\[
\text{Late lumen loss} = \left( \frac{\text{Post intervention MLD} - \text{6 month MLD}}{\text{Reference Diameter}} \right) \times 100\%.^{202,203}
\]

Finally, to further explore the possibility of an interaction between inflammatory marker levels and restenosis, restenosis rates (defined as a target lesion diameter stenosis > 50% on the follow-up angiogram) were compared by inflammatory marker tertiles at each timepoint.

3.2.4 *Determination of clinical events*

The composite clinical endpoints of death, myocardial infarction (MI), and target vessel revascularization were determined at 30 days and 2 years. MI was defined as the presence of
two of the following three World Health Organization (WHO) criteria: typical symptoms of ischaemia, electrocardiographic changes, or a creatine kinase level exceeding twice the upper limit of the reference range. In addition to the above, the definition of periprocedural MI included troponin T levels above the upper limit of the reference range (>0.03 μg/l) at 48 hours; all patients had normal troponin T levels before PCI. Target vessel revascularization was defined as repeat PCI or coronary artery bypass grafting (CABG) necessitated by symptomatic narrowing (predominantly restenosis) within the same major coronary artery as the original intervention. Where more than one event occurred in the same patient, events were considered in a hierarchical manner as follows: death, MI, target vessel revascularization (CABG, PCI).

3.2.5 Statistical analyses

Statistical analyses were performed using SAS software version 8.1 (SAS Institute Inc., Cary, North Carolina, USA). All probability values were two-tailed. Since inflammatory marker levels are skewed rightwards, levels of each marker were natural-log-transformed to assume a normal distribution prior to analysis. Although statistical tests were performed on these log-transformed data, anti-logged values (‘geometric mean’ and 95% CI) are presented in the results for ease of understanding.

The following analyses were performed:

1. To assess for any baseline differences associated with inflammatory marker levels, pre-PCI levels of each inflammatory marker were divided into tertiles (low, middle and high) and baseline clinical and angiographic characteristics were compared for each tertile using analysis of variance (ANOVA) for means and the χ² test for proportions.

2. To determine the effect of PCI on inflammatory marker levels, levels at each time point post-PCI were compared with the pre-PCI level using a paired t test.

3. To assess whether inflammatory marker levels influenced early adverse outcomes post-PCI, 30 day event rates were compared by tertiles of each inflammatory marker pre-PCI and 48 hours post-PCI using the χ² test for trend.
4. To assess whether inflammatory marker levels influenced restenosis rates, Pearson’s correlation coefficients were used to evaluate any association between inflammatory marker levels at each timepoint and the lesion diameter stenosis on the follow-up angiogram. With 133 patients, the study was adequately powered to detect a modest association between these variables (80% power to detect an association of \( r \geq 0.24 \)).

5. To exclude the possibility of poor procedural outcomes influencing this association, analysis was also performed comparing late lumen loss (as opposed to lumen diameter stenosis) against inflammatory marker levels at each timepoint.

6. To further assess whether inflammatory marker levels influenced restenosis, restenosis rates were compared by low, middle and high tertiles of inflammatory markers at each timepoint using the \( \chi^2 \) test. In this analysis, the standard clinical definition of restenosis as a binary outcome (present or absent) was used. Here, restenosis is defined as a percentage diameter stenosis of >50% on the follow-up angiogram.

7. To ensure that the averaging of treated lesions in the 39% of patients who had more than one PCI performed had not influenced the results, analyses 4-6 were repeated on a per-lesion rather than per-patient basis.

8. To assess whether inflammatory marker levels influenced longer term events post-PCI, 2-year event rates were compared by tertiles of each inflammatory marker pre-PCI and 48 hours post-PCI using the \( \chi^2 \) test for trend.
3.3 Results

3.3.1 Baseline clinical characteristics

The FAST Trial enrolled 315 patients. This sub-analysis focused on 133 patients with stable angina who underwent successful PCI and in whom stored pre-procedural blood samples, 6-month angiograms, and 2-year clinical follow-up data were available. There were no significant differences in the baseline characteristics of these 133 patients and the 315 patients initially randomised in the FAST trial.

Baseline characteristics did not appear to have a significant influence on inflammatory marker levels. Looking at each inflammatory marker in turn, there were no significant differences in baseline characteristics between patients with low, medium or high inflammatory marker levels (tertiles) pre-PCI. By way of illustration table 3.4 shows the baseline characteristics of the patients stratified by pre-PCI CRP tertile. The mean age was 60, and the majority of patients (82%) were male. Few patients were current smokers, and diabetes rates were low since diabetes mellitus requiring insulin therapy was an exclusion criterion of the trial. As determined by the randomised nature of the trial, half the patients were on simvastatin. All patients were on aspirin unless there was a specific contraindication (aspirin use > 90%).
Table 3.4  Baseline clinical characteristics, stratified by pre-PCI CRP tertile.

<table>
<thead>
<tr>
<th></th>
<th>CRP &lt;0.97 mg/l</th>
<th>CRP 0.97-1.96 mg/l</th>
<th>CRP &gt;1.96 mg/l</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=44</td>
<td>n=44</td>
<td>n=45</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>58 (9)</td>
<td>61 (10)</td>
<td>60 (10)</td>
<td>0.24</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>39 (89%)</td>
<td>36 (82%)</td>
<td>34 (76%)</td>
<td>0.28</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26 (3)</td>
<td>27 (3)</td>
<td>28 (6)</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>3 (7%)</td>
<td>1 (2%)</td>
<td>3 (7%)</td>
<td>-</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)*</td>
<td>1 (2%)</td>
<td>2 (5%)</td>
<td>4 (9%)</td>
<td>-</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>13 (30%)</td>
<td>18 (41%)</td>
<td>23 (51%)</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous MI, n (%)</td>
<td>20 (45%)</td>
<td>16 (36%)</td>
<td>20 (44%)</td>
<td>0.58</td>
</tr>
<tr>
<td>LVEF, % (mean ± SD)</td>
<td>75 (9)</td>
<td>73 (9)</td>
<td>71 (10)</td>
<td>0.12</td>
</tr>
<tr>
<td>Statin therapy, n (%)</td>
<td>21 (48%)</td>
<td>24 (55%)</td>
<td>23 (51%)</td>
<td>0.81</td>
</tr>
<tr>
<td>Aspirin therapy, n (%)</td>
<td>40 (91%)</td>
<td>42 (95%)</td>
<td>38 (84%)</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Laboratory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>results</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (mmol/l) *</td>
<td>0.09 (0.02)</td>
<td>0.10 (0.02)</td>
<td>0.09 (0.02)</td>
<td>0.86</td>
</tr>
<tr>
<td>Cholesterol (mmol/l) *</td>
<td>6.0 (0.9)</td>
<td>5.8 (1.0)</td>
<td>5.7 (0.8)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD) unless otherwise indicated.

* See Appendix 2 for conversion factors to mg/dl.
3.3.2 *Baseline angiographic characteristics and PCI procedure*

Most patients (60%) had PCI to a single lesion, although 31% of patients had PCI to two lesions and 9% had PCI to three or more lesions. The mean reference vessel diameter was 2.7 mm, with a mean diameter stenosis of 73% and a lesion length of 14 mm. 14% of lesions treated were total occlusions. With respect to PCI procedure, 81% of patients had balloon angioplasty without stenting, 13% had stents deployed, 4.6% had directional coronary atherectomy, and 1.5% had rotational atherectomy. The number of patients having angioplasty with stent deployment (18 patients, 13%) was too low to allow separate analyses by procedure type. When analysis was restricted to patients having balloon angioplasty alone, the results were unchanged.

There were no significant differences in angiographic characteristics according to pre-PCI inflammatory marker levels. In particular, pre-PCI inflammatory marker levels were not associated with number of lesions, reference vessel diameter, baseline lesion severity, or lesion length. This is illustrated for pre-PCI CRP levels in table 3.5, which compares pre-PCI angiographic characteristics by CRP tertile. Angiographic characteristics were similar across all CRP levels. Although there was a trend towards a lower total occlusion rate with higher CRP levels, this is likely due to chance, since a similar trend was not seen for other inflammatory markers.
### Table 3.5 Baseline angiographic characteristics stratified by pre-PCI CRP tertile.

<table>
<thead>
<tr>
<th>CRP &lt;0.97 mg/l</th>
<th>CRP 0.97–1.96 mg/l</th>
<th>CRP &gt;1.96 mg/l</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=44</td>
<td>n=44</td>
<td>n=45</td>
<td></td>
</tr>
<tr>
<td>Number of treated lesions, n (%)</td>
<td>31 (70%)</td>
<td>26 (59%)</td>
<td>23 (51%)</td>
</tr>
<tr>
<td>1</td>
<td>31 (70%)</td>
<td>26 (59%)</td>
<td>23 (51%)</td>
</tr>
<tr>
<td>2</td>
<td>11 (25%)</td>
<td>14 (32%)</td>
<td>16 (36%)</td>
</tr>
<tr>
<td>≥3</td>
<td>2 (5%)</td>
<td>4 (9%)</td>
<td>6 (13%)</td>
</tr>
<tr>
<td>Reference vessel size, mm</td>
<td>2.72 (0.52)</td>
<td>2.78 (0.62)</td>
<td>2.72 (0.51)</td>
</tr>
<tr>
<td>MLD, mm</td>
<td>0.68 (0.42)</td>
<td>0.70 (0.43)</td>
<td>0.83 (0.35)</td>
</tr>
<tr>
<td>Diameter stenosis, %</td>
<td>75.4 (14.2)</td>
<td>74.7 (14.1)</td>
<td>69.4 (11.2)</td>
</tr>
<tr>
<td>Lesion length, mm</td>
<td>14 (8)</td>
<td>13 (5)</td>
<td>14 (5)</td>
</tr>
<tr>
<td>Total occlusion, n (%)*</td>
<td>13/61 (21%)</td>
<td>10/65 (15%)</td>
<td>5/68 (7%)</td>
</tr>
<tr>
<td>Stent deployment, n (%)*</td>
<td>6/61 (10%)</td>
<td>6/65 (9%)</td>
<td>6/68 (9%)</td>
</tr>
</tbody>
</table>

*Calculated on a per-lesion basis.
3.3.3 Effect of PCI on inflammatory marker levels

Figure 3.1 shows the effect of PCI on inflammatory marker levels. All inflammatory markers showed a significant rise in levels at some point post-PCI, but the magnitude and time course of this inflammatory response differed between markers. The acute-phase reactant CRP showed a marked increase (over 5-fold rise) in levels within 48 hours of the procedure. At 1 week CRP levels were still elevated compared to pre-PCI, but they had returned to pre-PCI levels by 1 month. Although they did not show such a marked increase as CRP, sCD40L and PAPP-A levels were also significantly higher 48 hours post-PCI than they were pre-PCI, and unlike CRP levels of these markers remained elevated for at least one month post-procedure. The PAPP-A/proMBP ratio was also elevated at 1 week and 1 month, although it was not elevated at 48 hours due to a concomitant rise in levels of the PAPP-A inhibitor proMBP immediately following PCI. Of the markers studied, sICAM-1 showed the least response to PCI. sICAM-1 levels did not become elevated above pre-PCI levels until 1 month post-procedure.

In subsequent analyses, cardiac event and restenosis rates are compared by inflammatory marker tertiles at each timepoint. The inflammatory marker levels upon which these tertiles are based are given for reference in table 3.6.
Figure 3.1  Effect of percutaneous coronary intervention on inflammatory marker levels.

Geometric mean and 95% CI; p values = comparison with pre-PCI levels.
Pre-PCI inflammatory marker levels are represented at “0 days”.

A. C-reactive protein (CRP).
B. Soluble intercellular adhesion molecule 1 (sICAM-1).
C. Soluble CD40 ligand (sCD40L).
E. The ratio between plasma associated plasma Protein-A and its endogenous inhibitor, the proform of eosinophil major basic protein (PAPP-A/proMBP ratio).

A. CRP
B. sICAM-1

C. sCD40-L
Table 3.6  Inflammatory marker levels used to define tertiles at each timepoint.

<table>
<thead>
<tr>
<th></th>
<th>Lower tertile</th>
<th>Middle tertile</th>
<th>Upper tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRP levels, mg/l</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>&lt; 0.97</td>
<td>0.97 - 1.96</td>
<td>&gt; 1.96</td>
</tr>
<tr>
<td>48 hour post-PCI</td>
<td>&lt; 4.85</td>
<td>4.85 – 9.23</td>
<td>&gt; 9.23</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>&lt; 1.59</td>
<td>1.59 – 3.89</td>
<td>&gt; 3.89</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>&lt; 0.77</td>
<td>0.77 – 1.99</td>
<td>&gt; 1.99</td>
</tr>
<tr>
<td><strong>sICAM-1 levels, ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>&lt; 214</td>
<td>214 – 286</td>
<td>&gt; 286</td>
</tr>
<tr>
<td>48 hour post-PCI</td>
<td>&lt; 235</td>
<td>235 – 340</td>
<td>&gt; 340</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>&lt; 210</td>
<td>210 – 320</td>
<td>&gt; 320</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>&lt; 278</td>
<td>278 – 422</td>
<td>&gt; 422</td>
</tr>
<tr>
<td><strong>sCD40L levels, ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>&lt;0.20</td>
<td>0.20 – 0.95</td>
<td>&gt; 0.95</td>
</tr>
<tr>
<td>48 hour post-PCI</td>
<td>&lt; 0.62</td>
<td>0.62 – 1.55</td>
<td>&gt; 1.55</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>&lt;0.52</td>
<td>0.52 – 1.57</td>
<td>&gt; 1.57</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>&lt; 0.78</td>
<td>0.78 – 2.4</td>
<td>&gt; 2.4</td>
</tr>
<tr>
<td><strong>PAPP-A levels, miU/l</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>&lt; 3.7</td>
<td>3.7 – 4.8</td>
<td>&gt; 4.8</td>
</tr>
<tr>
<td>48 hour post-PCI</td>
<td>&lt; 4.7</td>
<td>4.7 – 6.8</td>
<td>&gt; 6.8</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>&lt; 5.3</td>
<td>5.3 – 7.7</td>
<td>&gt; 7.7</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>&lt; 6.0</td>
<td>6.0 – 9.0</td>
<td>&gt; 9.0</td>
</tr>
<tr>
<td><strong>PAPP-A/proMBP ratio, x10⁻³</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>&lt; 2.6</td>
<td>2.6 – 3.9</td>
<td>&gt; 3.9</td>
</tr>
<tr>
<td>48 hour post-PCI</td>
<td>&lt; 2.9</td>
<td>2.9 – 4.2</td>
<td>&gt; 4.2</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>&lt; 3.7</td>
<td>3.7 – 5.3</td>
<td>&gt; 5.3</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>&lt; 3.7</td>
<td>3.7 – 6.3</td>
<td>&gt; 6.3</td>
</tr>
</tbody>
</table>
3.3.4  Inflammatory marker levels and 30 day events

There were 13 periprocedural MIs (12 of which were diagnosed on the basis of elevated troponin T levels), but no deaths, target vessel revascularization, or further MIs up to 30 days. The occurrence of a periprocedural MI did not affect inflammatory marker levels, since the 48 hour post-PCI inflammatory marker levels of the 13 patients who suffered a periprocedural MI did not differ from those of the other 110 patients with available samples.

Interactions between pre-PCI inflammatory marker levels and 30 day events are explored in table 3.7 (A), which shows the 30 day event rate according to tertiles for each inflammatory marker (low, middle or high). There was no association between pre-PCI levels of any inflammatory marker and 30-day events.

Interactions between 48 hour post-PCI inflammatory marker levels and 30 day events are explored in table 3.7 (B). The 30 day event rate is compared for low, middle and high tertiles of each inflammatory marker post-PCI. There was no association between 48 hour post-PCI levels of any inflammatory marker and 30-day events.
Table 3.7  Comparison of 30 day event rates by pre-and post-PCI inflammatory marker levels.

A. Comparison of 30 day event rates by pre-PCI inflammatory marker levels (in tertiles).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lower tertile</th>
<th>Middle tertile</th>
<th>Upper tertile</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>7 (16%)</td>
<td>2 (5%)</td>
<td>4 (9%)</td>
<td>0.27</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>4 (9%)</td>
<td>3 (7%)</td>
<td>4 (9%)</td>
<td>0.99</td>
</tr>
<tr>
<td>sCD40L</td>
<td>2 (5%)</td>
<td>2 (5%)</td>
<td>5 (12%)</td>
<td>0.20</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>4 (9%)</td>
<td>5 (11%)</td>
<td>3 (7%)</td>
<td>0.66</td>
</tr>
<tr>
<td>PAPP-A/proMBP ratio</td>
<td>5 (12%)</td>
<td>5 (12%)</td>
<td>2 (5%)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

B. Comparison of 30 day event rates by 48 hour post-PCI inflammatory marker levels (in tertiles).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lower tertile</th>
<th>Middle tertile</th>
<th>Upper tertile</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>5 (12%)</td>
<td>3 (8%)</td>
<td>5 (12%)</td>
<td>0.99</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>2 (5%)</td>
<td>5 (14%)</td>
<td>5 (14%)</td>
<td>0.26</td>
</tr>
<tr>
<td>sCD40L</td>
<td>4 (10%)</td>
<td>3 (9%)</td>
<td>5 (14%)</td>
<td>0.59</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>4 (10%)</td>
<td>3 (8%)</td>
<td>5 (12%)</td>
<td>0.68</td>
</tr>
<tr>
<td>PAPP-A/proMBP ratio</td>
<td>3 (8%)</td>
<td>5 (12%)</td>
<td>4 (10%)</td>
<td>0.68</td>
</tr>
</tbody>
</table>
3.3.5 Inflammatory marker levels and angiographic restenosis

All patients underwent follow-up angiography at 6 months, or sooner if clinically indicated. The median time to follow-up was 6.4 months, with an interquartile range of 6.0 to 6.8 months.

To test for any association between inflammatory marker levels and the restenotic process, correlation coefficients were calculated between inflammatory marker levels at each timepoint and renarrowing of the target lesion on the six month angiogram (table 3.8). Two separate definitions of renarrowing were used to test for an association - percent diameter stenosis, and late lumen loss. Separate analyses were performed for each definition.

Overall there was no evidence of an association between levels of any inflammatory marker (either pre- or post- PCI) and luminal renarrowing on the six month angiogram (whether measured by percent diameter stenosis or late lumen loss), although PAPP-A levels 1 week post PCI did show weak associations with both percent diameter stenosis and late lumen loss. Analyses were repeated on a per-lesion basis and this did not alter the findings.

The lack of association between inflammatory marker levels and percent diameter stenosis on the follow-up angiogram is illustrated for pre-PCI and 48 hour post-PCI CRP levels in figure 3.2. Although PCI led to a significant elevation in CRP levels 48 hours post procedure in most participants, there was no association between the percent diameter stenosis at six months and either pre-PCI CRP levels ($r = -0.04, p=0.62$) or 48 hour post-PCI CRP levels ($r=0.07, p=0.44$).
### Table 3.8

Associations between inflammatory marker levels at each timepoint with percent diameter stenosis and late lumen loss on the six month angiogram.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Percent diameter stenosis</th>
<th>Late lumen loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p value</td>
</tr>
<tr>
<td><strong>CRP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>-0.04</td>
<td>0.62</td>
</tr>
<tr>
<td>48 hours post-PCI</td>
<td>0.07</td>
<td>0.44</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>-0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>-0.11</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>sICAM-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>-0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>48 hours post-PCI</td>
<td>-0.19</td>
<td>0.05</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>0.07</td>
<td>0.48</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>sCD40L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>0.03</td>
<td>0.72</td>
</tr>
<tr>
<td>48 hours post-PCI</td>
<td>-0.07</td>
<td>0.51</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>0.04</td>
<td>0.69</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>0.01</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>PAPP-A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>0.10</td>
<td>0.22</td>
</tr>
<tr>
<td>48 hours post-PCI</td>
<td>0.11</td>
<td>0.23</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>0.12</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>PAPP-A/proMBP ratio</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-PCI</td>
<td>-0.06</td>
<td>0.50</td>
</tr>
<tr>
<td>48 hours post-PCI</td>
<td>0.04</td>
<td>0.67</td>
</tr>
<tr>
<td>1 week post-PCI</td>
<td>0.07</td>
<td>0.50</td>
</tr>
<tr>
<td>1 month post-PCI</td>
<td>0.04</td>
<td>0.80</td>
</tr>
</tbody>
</table>
Figure 3.2 Scatter plots of the percentage diameter stenosis at follow-up according to CRP levels (A) pre-PCI and (B) 48 hours post-PCI.
A subsequent analysis was performed using the binary definition for restenosis (> 50% diameter stenosis on the six month angiogram = restenosis). Using this definition, restenosis occurred in 56 patients (42%). Restenosis rates were compared for low, middle and high levels (tertiles) of each inflammatory marker at each timepoint (figure 3.3). There was a trend towards greater restenosis rates with higher PAPP-A levels at both 1 week and 1 month but this was not reflected in a similar trend for the PAPP-A/proMBP ratio. Overall, there was no evidence of inflammatory marker levels influencing restenosis rates.

Simvastatin, bezafibrate and dalteparin had no significant effect on CRP levels either pre- or post- PCI, and no effect on clinical events or restenosis rates at 6 months. The lack of association between CRP and restenosis was not caused by simvastatin use, since it was equally apparent in those randomised to placebo.
**Figure 3.3** Interaction between inflammatory marker levels and restenosis rates.

The six month restenosis rates are compared by inflammatory marker tertile (low, middle or high) at each timepoint.

A. C-reactive protein (CRP).

B. Soluble intercellular adhesion molecule 1 (sICAM-1).

C. Soluble CD40 ligand (sCD40L).


E. The ratio between plasma associated plasma Protein-A and its endogenous inhibitor, the proform of eosinophil major basic protein (PAPP-A/proMBP ratio).
Figure 3.3 (continued)

B. sICAM-1

<table>
<thead>
<tr>
<th></th>
<th>Lower sICAM-1 tertile</th>
<th>Middle sICAM-1 tertile</th>
<th>Upper sICAM-1 tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>48 hr</td>
<td>1 week</td>
<td>1 month</td>
</tr>
<tr>
<td>Restenosis Rate (%)</td>
<td>p=0.13</td>
<td>p=0.10</td>
<td>p=0.49</td>
</tr>
</tbody>
</table>

C. sCD40L

<table>
<thead>
<tr>
<th></th>
<th>Lower sCD40L tertile</th>
<th>Middle sCD40L tertile</th>
<th>Upper sCD40L tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>48 hr</td>
<td>1 week</td>
</tr>
<tr>
<td>Restenosis Rate (%)</td>
<td>p=0.27</td>
<td>p=0.13</td>
<td>p=0.99</td>
</tr>
</tbody>
</table>
3.3.6 Inflammatory marker levels and 2-year events

There were 31 clinical events in the 2 years post-PCI: two deaths, two MIs and 27 target vessel revascularization procedures (six CABG, 21 re-PCI).

Interactions between pre-PCI inflammatory marker levels and 2 year cardiac events are explored in table 3.9 (A), which shows the 2 year event rate according to tertiles for each inflammatory marker (low, middle or high). Although there was a trend towards an increased 2-year event rate with increasing pre-PCI CRP levels (p=0.09), this did not reach statistical significance and was not reflected in similar trends for other markers.

Interactions between 48 hour post-PCI inflammatory marker levels and 2 year events are explored in table 3.9 (B). The 2 year event rate is compared for low, middle and high tertiles of each inflammatory marker post-PCI. There was no association between 48 hour post-PCI levels of any inflammatory marker and 2 year events.
Table 3.9 Comparison of 2 year event rates by pre- and post-PCI inflammatory marker levels.

A. Comparison of 2 year event rates by pre-PCI inflammatory marker levels (in tertiles).

<table>
<thead>
<tr>
<th></th>
<th>Lower tertile</th>
<th>Middle tertile</th>
<th>Upper tertile</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>7 (16%)</td>
<td>10 (23%)</td>
<td>14 (31%)</td>
<td>0.09</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>9 (20%)</td>
<td>9 (20%)</td>
<td>12 (27%)</td>
<td>0.44</td>
</tr>
<tr>
<td>sCD40L</td>
<td>5 (12%)</td>
<td>11 (26%)</td>
<td>10 (24%)</td>
<td>0.18</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>9 (21%)</td>
<td>9 (19%)</td>
<td>13 (29%)</td>
<td>0.37</td>
</tr>
<tr>
<td>PAPP-A/proMBP ratio</td>
<td>9 (21%)</td>
<td>10 (24%)</td>
<td>8 (19%)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

B. Comparison of 2 year event rates by 48 hour post-PCI inflammatory marker levels (in tertiles).

<table>
<thead>
<tr>
<th></th>
<th>Lower tertile</th>
<th>Middle tertile</th>
<th>Upper tertile</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>8 (20%)</td>
<td>11 (27%)</td>
<td>9 (22%)</td>
<td>0.79</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>11 (30%)</td>
<td>11 (31%)</td>
<td>4 (11%)</td>
<td>0.06</td>
</tr>
<tr>
<td>sCD40L</td>
<td>13 (33%)</td>
<td>4 (12%)</td>
<td>8 (23%)</td>
<td>0.26</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>9 (21%)</td>
<td>10 (25%)</td>
<td>10 (24%)</td>
<td>0.75</td>
</tr>
<tr>
<td>PAPP-A/proMBP ratio</td>
<td>11 (28%)</td>
<td>7 (17%)</td>
<td>10 (26%)</td>
<td>0.84</td>
</tr>
</tbody>
</table>
3.4 Discussion

In this study, inflammatory marker levels did not predict PCI outcomes in stable angina patients, with neither pre-PCI nor post-PCI inflammatory marker levels predictive of clinical events or restenosis. In particular, pre-PCI levels of CRP, sICAM-1, sCD40L, PAPP-A, and the PAPP-A/proMBP ratio were not predictive of 30 day cardiac events, six month angiographic restenosis or 2 year cardiac events. Although PCI caused a systemic inflammatory response, with levels of CRP, sCD40L, and PAPP-A all significantly higher 48 hours post-PCI than pre-PCI, post-PCI levels of CRP, sICAM-1, sCD40L, PAPP-A, and the PAPP-A/proMBP ratio were also not predictive of 30 day cardiac events, six month angiographic restenosis, or 2 year cardiac events.

It has been suggested “the overall and local inflammatory status at the time of PCI plays a significant role in the development of restenosis”. However previous studies of inflammatory marker levels and restenosis have shown conflicting results. There may be several explanations for this, including differences in the population studied (stable angina versus unstable angina), concurrent use of medications that may blunt the inflammatory response (statins, glycoprotein IIb/IIIa inhibitors, thienopyridines), timing of inflammatory marker measurement (pre-PCI versus post-PCI), PCI procedure (balloon angioplasty versus stent deployment), and outcomes studied (angiographic restenosis versus clinical restenosis or events). The relevance of these issues to the current study is discussed below.

3.4.1 Inflammatory marker response to PCI

This study confirmed that in stable angina patients, PCI leads to a systemic inflammatory response. CRP levels were several-fold higher 48 hours post-PCI than pre-PCI, but returned to pre-PCI levels somewhere between one week and one month post-PCI. This is consistent with previous reports of the CRP response to PCI in stable angina. sCD40L and PAPP-A levels also became elevated by 48 hours post-PCI, but unlike CRP they remained elevated for at least one month. Surprisingly sICAM-1 levels did not become elevated immediately following PCI, but at one month sICAM-1 levels were significantly higher than they had been pre-PCI. The reasons for these differing patterns of response to PCI are unexplained. Although the systemic
inflammatory response following PCI (CRP rise) is short-lived, it is possible the ongoing involvement of local mediators of inflammation such as sCD40L and PAPP-A in the remodeling process at the intervention site causes elevation of their levels in the bloodstream through to at least one month post-procedure.

3.4.2 Pre-PCI inflammatory marker levels and restenosis

Nearly all previous studies of inflammatory markers and restenosis have involved CRP. In this study of stable angina patients there was no association between pre-PCI CRP levels and 6-month angiographic restenosis. This is consistent with previous studies of stable angina patients. In contrast, an association between CRP levels and restenosis has been reported in studies that have included patients with acute coronary syndromes (ACS). For example, Walter et al. (2001) found that pre-PCI CRP levels were predictive of angiographic restenosis in 276 “high-risk” patients (including many patients undergoing PCI for ACS). ACS patients have widespread coronary inflammation with evidence of unstable coronary plaques in non-culprit arteries, and this is reflected in significantly higher preprocedural CRP levels and more marked elevation of CRP levels after PCI than those observed in stable angina patients. The pre-PCI CRP levels in the study by Walter et al. (2001) were several times higher (middle tertile 5-14 mg/l) than the levels in the current study (middle tertile 1-2 mg/l) and in previous studies of patients with stable angina. Pre-PCI CRP levels may be predictive of angiographic restenosis in patients with ACS but not those with stable angina, or the inclusion of a heterogeneous group of stable and unstable angina patients in studies such as that by Walter et al. may have resulted in unrecognized confounding. Studies of CRP levels and angiographic restenosis in ‘pure’ ACS cohorts are needed to answer this question.

The lack of association between pre-PCI levels of sCD40L and siCAM-1 and restenosis in the current study are at odds with the study by Cipollone et al. (2003), who reported both sCD40L and siCAM-1 levels to be predictive of restenosis. However their study included predominantly unstable angina patients, providing further evidence that the predictive value of inflammatory marker levels for PCI outcomes may differ for stable angina and unstable angina patients. The current study is the first to report the lack of association between pre-PCI PAPP-A levels and restenosis.
It has been questioned whether the negative results in patients with stable angina could have been a consequence of concurrent use of medications that may blunt the inflammatory response. The almost universal use of statins in patients undergoing elective PCI has received particular attention in this regard.\textsuperscript{16,168} The current study shows that statins do not mask an association between inflammatory markers and restenosis, because patients were randomized either to statins or placebo and results were the same regardless of randomisation. Similarly, the lack of association in this study was not due to thienopyridine\textsuperscript{198} or glycoprotein IIb/IIIa inhibitor\textsuperscript{183} use, because the majority of patients were enrolled before these medications were in common usage and no study patients received these medications.

3.4.3 \textit{Post-PCI Inflammatory marker levels and restenosis}

It has also been postulated that post-PCI inflammatory marker levels may be more predictive of restenosis than pre-PCI levels.\textsuperscript{16} However although our study confirms previous reports of a significant increase in inflammatory marker levels after PCI,\textsuperscript{169,178,181,182} these elevated levels did not correlate with angiographic stenosis at 6 months. This again contrasts with the findings of Cipollone et al. (2003) in unstable angina patients, in whom restenosis was associated with a more marked and more prolonged rise in sCD40L and sICAM-1 levels post-PCI.\textsuperscript{178} It also contrasts with the study by Gottsauner-Wolf et al. (2000), who demonstrated an association between post-PCI CRP levels and angiographic restenosis in stable angina patients.\textsuperscript{172} The apparent discrepancy with the current study may be due to differences in stent utilisation, since all patients in the study by Gottsauner-Wolf et al. underwent stenting, whereas only 13\% of patients in the current study received stents (too low a rate to assess the association between inflammatory markers and restenosis in stented lesions alone). However two otherwise similar small studies of stable angina patients undergoing coronary stenting (with 43 and 58 patients respectively) found no relation between post-PCI CRP levels and angiographic restenosis.\textsuperscript{168,169} In the largest study to date (which included 1,321 stable angina patients), the association between the post-PCI CRP rise and angiographic restenosis was of only borderline significance in patients with stable angina (p=0.05).\textsuperscript{172} It remains possible that the results of the study by Gottsauner-Wolf et al. (2000) are a chance finding, given the small sample size (40 patients) and the use of serial CRP measurements with multiple comparisons to retrospectively
determine the CRP measurement time of greatest statistical significance. Further study using prospectively designed trials with pre-specified CRP measurement times is required to determine in which clinical circumstances (if any) post-PCI CRP levels predict restenosis.

The association between PAPP-A levels and restenosis has not previously been studied. There were weak associations between PAPP-A levels 1 week post-PCI and both percent diameter stenosis and late lumen loss, (table 3.8) and patients with higher PAPP-A levels (in the upper tertile) at either 1 week or 1 month had increased restenosis rates (figure 3.3). These findings are of interest given reports that matrix metalloproteinases directly contribute to the restenotic process, but should be interpreted with caution given the lack of association at other timepoints and the absence of similar findings for the PAPP-A/proMBP ratio. Given the number of comparisons made these may represent chance findings and require confirmation in further prospective studies.

3.4.4 Inflammatory marker levels and cardiac events.

The mechanisms that underlie coronary events following PCI are distinct from the mechanisms that underlie restenosis, so it is possible inflammatory marker levels could be predictive of clinical events following PCI but not of restenosis. Elevated pre-PCI CRP levels have been reported to predict an increased risk of early adverse outcomes (30-day death or MI) following PCI. However, the registry of 727 patients on which this report was based included a mix of both stable and unstable angina patients, and the association between CRP levels and events was more evident in patients with ACS. The strength of the association for patients with stable angina is unclear. Unfortunately the current study was underpowered to detect any association between pre- or post-PCI inflammatory marker levels and 30-day events in stable angina patients, as only 13 events occurred. However the low event rates reported in the current study suggest that even if inflammatory markers are found to have predictive value for cardiac events in stable angina patients, their clinical utility may be limited.

The current study was also relatively underpowered for the composite 2-year endpoint of death, MI, or target vessel revascularization. There was a trend towards an increase in 2-year events
in patients with higher pre-PCI CRP levels, but there was no association between levels of other markers either pre- or post-PCI and 2 year events.

Larger studies by Dibra et al. \(^{167}\) and de Winter et al. \(^{192}\) have shown that pre-PCI CRP levels predict 1-2 year cardiac event rates in stable angina patients undergoing PCI. Therefore, although CRP is not predictive of restenosis \emph{per se} in stable angina patients, it does appear to be predictive of plaque instability and, consequently, of clinical manifestations of ischaemia. This is consistent with our current understanding of atherosclerosis and the pathogenesis of ACS, \(^{6}\) and with observations that CRP levels are also predictive of medium to long term cardiac events in asymptomatic individuals, \(^{80}\) in patients with stable angina, \(^{93}\) and in patients with ACS not undergoing PCI. \(^{39}\) Further research is needed to determine whether other markers such as sCD40L are also predictive of either short or long term cardiac events following PCI in stable angina patients.

\section*{3.4.5 Study limitations}

As noted above (section 3.4.4), the low clinical event rate resulted in the study being underpowered to detect associations between inflammatory marker levels and clinical events. However, it was adequately powered to assess restenosis using quantitative coronary angiography. Although imaging techniques such as intravascular ultrasound may more sensitively measure time-dependent changes in coronary artery lesions, \(^{205,206}\) these were not available at our institution when this study was conceived. In some patients, missing inflammatory marker levels and quantitative coronary angiographic results may have introduced unappreciated bias, although the baseline clinical and angiographic characteristics of these patients were similar to those of the main study cohort.

The low stent deployment rate in the study limits the applicability of our findings to patients undergoing balloon angioplasty without stenting. However, with this caveat, our study included a relatively homogeneous population of patients with stable coronary disease undergoing elective PCI for a \emph{de novo} native coronary lesion, limiting the likelihood of confounding or systematic bias. Other strengths of this study include randomization to statins and absence of glycoprotein IIb/IIIa inhibitor and thienopyridine use (which allowed us to exclude the possibility that use of
these agents masked a positive association between inflammatory markers and restenosis), and the performance of 6-month angiographic follow-up with assessment of angiographic restenosis in all participants (which avoids the potential for observer bias that arises with use of less objective outcome measures such as clinical restenosis).165

3.4.6 Conclusions

In stable angina patients, PCI leads to a systemic inflammatory response with significant increases in circulating levels of the inflammatory markers CRP, sCD40L and PAPP-A within 48 hours of the procedure. Despite this inflammatory response, neither pre-PCI nor post-PCI inflammatory marker levels were predictive of six month angiographic restenosis. This lack of association between inflammatory marker levels and restenosis was not due to prior statin, thienopyridine, or glycoprotein IIb/IIIa inhibitor use. In addition there was no association between inflammatory marker levels and either 30 day or 2 year cardiac events, but event rates were low in this stable angina population limiting any conclusions that can be drawn. Further prospective studies are required to evaluate what role inflammatory markers have (if any) in the risk stratification of patients undergoing PCI.
Chapter 4

Effects of Lipid Modifying Agents on Inflammatory Marker Levels

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4.1 Introduction

4.1.1 Cardiovascular risk reduction with statins

In the last two decades several large randomised trials have established beyond doubt the cardioprotective benefits of the lipid lowering 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitor class of drugs known as statins. The primary mode of action of these agents is a rapid and striking reduction in serum low density lipoprotein cholesterol (LDL), and this is clearly responsible for most of the benefits of statin therapy, as illustrated by the predictable linear relationship between the LDL cholesterol level achieved and the cardiovascular event rate (figure 4.1). Overall, there is a 21% risk reduction for vascular events and a 12% reduction in mortality per 1 mmol/l reduction in the LDL cholesterol over 5 years, regardless of base (absolute) risk. Of note, there is no evidence of a threshold LDL level below which further reductions in LDL do not result in further reductions in event rates. For example, in the Heart Protection Study (2002) simvastatin led to a significant reduction in cardiovascular events and mortality irrespective of initial cholesterol concentrations, including in those with low LDL prior to treatment (<3.0 mmol/l).
Figure 4.1  Event rates according to LDL cholesterol levels achieved with statin therapy in secondary prevention studies.

Event rates plotted against LDL cholesterol levels during statin therapy in secondary prevention studies. There is a linear relationship between the LDL cholesterol achieved and the risk of cardiovascular events. From LaRosa et al. (2005).\textsuperscript{221}

HPS denotes Heart Protection Study,\textsuperscript{211} CARE Cholesterol and Recurrent Events Trial,\textsuperscript{209} LIPID Long-term Intervention with Pravastatin in Ischaemic Disease,\textsuperscript{210} 4S Scandinavian Simvastatin Survival Study,\textsuperscript{207} and TNT Treating to New Targets study.\textsuperscript{221}

Event rates for HPS, CARE and LIPID are for death from CHD and nonfatal myocardial infarction. Event rates for 4S and the TNT Study also include resuscitation after cardiac arrest.
Despite the demonstrated importance of LDL cholesterol levels, it appears that not all of the benefits of statin therapy can be attributed to cholesterol lowering. This was first highlighted in the West of Scotland Coronary Prevention study (WOSCOPS), a primary prevention study which showed a significant reduction in cardiovascular events with pravastatin therapy. In this trial, for any given cholesterol level the risk of cardiovascular events was lower if that cholesterol level was achieved with statin therapy than if it occurred on placebo treatment. This suggested some of the benefits of statin therapy were independent of lipid changes. Further support for this concept comes from an analysis of data from the Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) trial. In this secondary prevention trial, not only did pravastatin therapy reduce the risk of cardiac events, it also reduced the risk of cerebrovascular events despite the fact that LDL cholesterol is not a risk factor for stroke. This suggests that statins lower the risk of stroke by mechanisms independent of their effect on circulating LDL cholesterol.

These observations have focused attention on the non lipid lowering (‘pleiotropic’) effects of statins. In experimental models, statins inhibit smooth muscle proliferation, enhance endothelial function, reduce inflammation, promote plaque stability and counter platelet aggregation and thrombus formation. However doubts remain as to whether these in vitro effects, often produced using high drug concentrations, are relevant in vivo. Clinical studies demonstrating lipid-independent effects of statins in vivo are needed to confirm that ‘pleiotropic’ effects of statins exist.

4.1.2 Effect of statin therapy on CRP levels.

To this end, several studies have investigated whether statin therapy reduces CRP levels. One of the first reports was from Ridker et al. in 1999, which looked at CRP levels at baseline and 5 years in 472 patients from the Cholesterol and Recurrent Events (CARE) trial, a randomized comparison of pravastatin versus placebo for the secondary prevention of coronary disease. CRP levels decreased an average of 17.4% over 5 years in the pravastatin group, independent of change in lipid levels, and did not change in the placebo group. This was followed by the Pravastatin Inflammation CRP Evaluation (PRINCE) study in 2001, the first large prospectively designed trial of the effect of statins on CRP levels. This study included separate primary and
secondary prevention cohorts. In the primary prevention cohort, 1,702 participants with no history of cardiovascular disease were randomized to 40mg pravastatin or to placebo for 24 weeks. CRP did not change in the control group, whereas pravastatin reduced median CRP levels by 16.9% at 24 weeks (a decrease of 0.2 mg/l, p<0.001). There were similar reductions in CRP levels in the secondary prevention cohort, in which 1,182 patients with known coronary disease were treated with open-label pravastatin for 24 weeks. There was little correlation between change in CRP and change in LDL cholesterol (r=0.04) and, in linear regression models, change in LDL cholesterol was not a predictor of change in CRP (p=0.44).

By far the strongest evidence that statin therapy lowers CRP levels comes from the Justification for the Use of Statins in Prevention: an Interventional Trial Evaluating Rosuvastatin (JUPITER) trial. This randomised, placebo-controlled trial included 8,901 apparently healthy men and women with ‘low’ LDL cholesterol levels (< 3.4 mmol/l, median baseline level 2.8 mmol/l), but ‘high’ CRP levels (>2 mg/l, median baseline level 4.2 mmol/l), treated with Rosuvastatin 20mg/day for just under two years. Use of Rosuvastatin led to not just a significant reduction in LDL cholesterol levels (to a median of 1.4 mmol/l, p<0.001), but a similar, significant reduction in CRP levels (to a median of 2.2 mg/l, p<0.001). The reduction in CRP levels showed only modest correlation (r=0.15) correlation with reduction in LDL cholesterol levels. There was a significant decrease in clinical events (cardiovascular death, myocardial infarction, unstable angina, stroke, or arterial revascularisation) with Rosuvastatin use, and the benefit appeared greatest for those in whom low levels of both LDL cholesterol (<1.8 mmol/l) and CRP (< 2 mg/l) were achieved.

The onset of effect of statins on CRP appears to be rapid, with a significant fall in CRP levels within 2-4 weeks of initiating therapy. Until recently it was thought the effect of statins on CRP was a ‘class effect’ with all statins being equally effective, and the CRP response was thought to be independent of the dose of statin used. However several recent trials (published since this thesis was commenced) have now shown that high dose atorvastatin (80mg/day) causes greater reduction in CRP than either low dose atorvastatin or other statins, whether in primary prevention, secondary prevention or acute coronary syndromes. Despite these trial results, most of the primary and secondary prevention data behind the
proven efficacy of statins comes from trials of simvastatin or pravastatin, and it is from analysis of these studies that non lipid lowering or “pleiotropic” benefits of statins were proposed. The effect of simvastatin on CRP levels therefore remains of interest in assessing the pleiotropic benefits of these agents. In addition, our understanding of how statins work may be further helped by study of a range of markers (to determine if they have a broad or selective antiinflammatory effect), investigation of their effect on the acute-phase response, and comparison with other lipid lowering agents such as fibrates. These issues are all examined in this thesis.

4.1.3 **Effect of statin therapy on other inflammatory markers**

There are no published reports of the effect of statins on a broad range of inflammatory markers measured simultaneously. This would be of interest because if statins work selectively against only certain inflammatory markers, we may learn more about how and where they act. However, most reports concern the effect on one or two markers only, and there are scant data on some markers such as sICAM-1, sCD40L and PAPP-A.

One risk factor that has been extensively studied is fibrinogen. In the largest of these reports, from the WOSCOPS study, randomization to pravastatin had no effect on fibrinogen, and a subsequent meta-analysis also concluded that statins do not affect fibrinogen levels. Only small studies have been carried out for sICAM-1, with most but not all showing no effect of statin therapy.

For sCD40L, previous studies also have shown conflicting results. In the Atorvastatin versus Simvastatin on Atherosclerosis Progression (ASAP) study (2003), comprising 110 patients with familial hypercholesterolaemia randomized to atorvastatin 80mg/day or simvastatin 40mg/day for two years, a significant reduction in sCD40L of around 40% was seen in both the atorvastatin and simvastatin groups. However in the Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study (2004), 16 weeks’ atorvastatin had no significant effect on sCD40L levels in 1,160 acute coronary syndrome patients (despite abolishing the excess risk associated with a high sCD40L level). Further research is needed to establish the effect of statins on sCD40L levels in a range of clinical settings.
Only one study has looked at the effect of statins on PAPP-A levels. PAPP-A was unaffected by 10 weeks atorvastatin therapy (20mg/day) in 27 subjects with isolated hypercholesterolaemia, but the numbers in this study were too low to exclude a false negative result.

This thesis addresses some of the uncertainties outlined above, through examination of the effects of simvastatin therapy on a broad range of inflammatory markers (CRP, fibrinogen, sICAM-1, sCD40L and PAPP-A).

4.1.4 Statin therapy and the acute-phase response

Despite the evidence that statins reduce inflammatory marker levels, it is not clear whether this represents a direct antiinflammatory effect, or whether it is secondary to other mechanisms such as reduction in LDL cholesterol in the vessel wall. The effect of statins on the acute-phase response would be informative in this regard, since attenuation of the acute-phase response would provide support for a direct antiinflammatory effect. Acute coronary syndromes and PCI represent two situations in which this can be studied, since they involve vessel injury with an acute phase response.

In acute coronary syndromes, there is immune system activation in response to the vascular injury caused by plaque rupture. Inflammatory marker levels are raised, and return gradually to normal over a period of weeks. Early initiation of statin therapy appears to hasten the return of inflammatory markers to baseline; a number of recent studies have confirmed that high-dose statin therapy leads to a 30-40% greater reduction in CRP over a 3-4 month period compared to placebo alone. For example, in the Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study (2004), the fall in CRP levels four months after an acute coronary syndrome was much greater in patients treated with atorvastatin (80mg/day) than in patients on placebo (-83% versus -74%, p<0.0001). This has led some authors to propose the intriguing hypothesis that "the early benefits of statin therapy are derived largely from the antiinflammatory effects of the drugs, whereas the delayed benefits are lipid-modulated". However, the reductions in CRP may have developed gradually over several weeks and we cannot be sure whether they are ascribable to a direct antiinflammatory effect or
secondary to other changes. The speed with which statins have their effect is relevant to determining whether they have direct antiinflammatory effects, since agents with direct antiinflammatory effects lead to a very rapid reduction in inflammatory marker levels. For example, methylprednisolone therapy in acute coronary syndrome patients leads to a 33% reduction in CRP levels within 48 hours.\textsuperscript{244}

PCI offers a more convenient model with which to examine the vascular antiinflammatory effects of statins, since it causes a discrete vascular injury that is followed by a measurable inflammatory response. As demonstrated in chapter 3, levels of several inflammatory markers rise following PCI, with CRP in particular rising several-fold within 48 hours of the procedure. Drugs that curtail this acute-phase response can be presumed to have an antiinflammatory effect. For example, the anti-platelet agent abciximab has been shown to reduce to post-PCI CRP rise by 32% (presumably through inhibiting platelet release of inflammatory mediators).\textsuperscript{183} Similarly in registry data from the Cleveland Clinic (2004), the periprocedural increase in CRP was attenuated by 65% in patients pre-treated with clopidogrel.\textsuperscript{184} It is not known whether statins have a similar effect on the acute–phase response to PCI, since this has not previously been investigated. A demonstration that statins attenuate the acute-phase response would provide evidence that they have a direct antiinflammatory effect, and thereby provide a useful insight into their mechanisms of action.

### 4.1.5 Effect of fibrates on inflammatory marker levels

Other lipid-modifying agents such as fibric acid derivatives (fibrates) are also frequently used in the treatment of dyslipidaemia either as stand-alone agents or in combination with statins. Such agents are effective and have been shown to reduce cardiovascular events and slow the progression of established atherosclerosis.\textsuperscript{245,246} The predominant mechanism of action is elevation of HDL cholesterol levels. However, fibrates may also have antiinflammatory effects related to their binding of the nuclear receptor peroxisomal proliferation activating receptor-α (PPAR-α), which regulates the expression of a number of genes implicated in atherogenesis and plaque stability.\textsuperscript{118}
Despite this theoretical antiinflammatory action, the effect of fibrates on inflammatory marker levels is uncertain. The few existing reports have shown disparate results. In the Efficacy and Safety of a Combination of Fluvastatin and Bezafibrate in Patients with Mixed Hyperlipidaemia (FACT) study (2000), 333 patients with stable angina and mixed hyperlipidaemia were treated with fluvastatin, bezafibrate or combination for 24 weeks. Fibrinogen levels decreased significantly with bezafibrate (alone or in combination), but not with fluvastatin. Neither fluvastatin nor bezafibrate had any effect on CRP levels.247 Similarly, Gomez-Gerique et al. (2002) reported that bezafibrate 400 mg/day did not significantly lower CRP levels in 103 patients with mixed hyperlipidaemia (14% reduction, p=0.11).248 The most conclusive evidence that bezafibrate does not affect CRP levels comes from an analysis of the Bezafibrate Infarction Prevention (BIP) study, published after the research in this thesis was undertaken.249 Baseline and 2-year CRP levels were compared in over 1,300 patients treated with bezafibrate 400mg daily, with no appreciable change in CRP levels over that time. In contrast, fibrinogen levels were lowered significantly.250

The effect of fibrates on other markers, such as sICAM-1, sCD40L and PAPP-A, has not been reported.

4.1.6 Clinical implications of reductions in inflammatory marker levels

In the PRINCE trial, the median decrease in CRP levels with simvastatin therapy was only 0.2mg/l (16.9%).223 As the accompanying editorial noted, “It is difficult to accept that this small change in the level of an acute-phase reactant is important and explains the ability of pravastatin to improve clinical outcomes”.251 Nonetheless, several retrospective analyses of statin trials have suggested that CRP reduction with statin therapy does account for clinical benefit.252-254 In the Air Force / Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS), patients with “low” LDL (<3.8 mmol/l) but “raised” CRP levels (> 1.6 mg/l) at baseline benefited from statin therapy, whereas those with low LDL and low CRP did not.252 In the Pravastatin or Atorvastatin Evaluation and Infection Therapy – Thrombolysis in Myocardial Infarction 22 (PROVE IT – TIMI 22) trial, patients with low CRP levels after statin therapy had better clinical outcomes than those with higher CRP levels, regardless of the resultant level of LDL cholesterol, and the best outcomes occurred in patients with both a low
LDL cholesterol (<1.8 mmol/l) and a low CRP (<1 mg/l) following therapy. In the Reversing Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial, a reduction in both LDL cholesterol and CRP was important in slowing of the rate of atherosclerosis progression with statin therapy.

The growing evidence from these post-hoc analyses led to the recently published Jupiter trial, a prospective randomised-controlled trial of rosuvastatin versus placebo for the primary prevention of cardiovascular events in 17,802 apparently healthy participants with LDL cholesterol levels below the currently accepted ‘threshold’ for initiating statin therapy (LDL cholesterol <3.4 mmol/l), but with high CRP levels (>2 mg/l). Rosuvastatin use led to a 50% reduction in LDL cholesterol levels (from a median 2.8 mmol/l to 1.4 mmol/l), a 37% reduction in CRP levels (from a median 4.2 mg/l to 1.8 mg/l), and a 50% reduction in cardiovascular events (cardiovascular death, myocardial infarction, unstable angina, stroke, or arterial revascularisation) after an average follow-up of just under two years. Consistent with previous lipid lowering trials (figure 4.1, page 97), the magnitude of reduction in LDL cholesterol was directly related to the magnitude of clinical benefit. However, intriguingly, a prespecified subanalysis revealed that both LDL cholesterol lowering and CRP lowering were clinically important, and that individuals who achieved the combined goal of low LDL cholesterol (<1.8 mmol/l) and low CRP (<2 mg/l) achieved the greatest reduction in cardiovascular events.

What do the results of the JUPITER trial tell us? Firstly, they confirm that even within what are deemed to be clinically ‘acceptable’ cholesterol levels, there remains a linear relationship between LDL cholesterol levels and cardiovascular event rates, with no evidence of a threshold LDL cholesterol level below which no further benefits are achieved with further cholesterol lowering (figure 4.1). Secondly, they support the concept that inflammation plays a central role in atherosclerotic cardiovascular events, since the greatest benefits were seen when there was evidence of a reduction in inflammation. They also raise the possibility that CRP levels could be used to target and monitor statin therapy in much the same way LDL cholesterol is used. However, further evidence will be needed before the concept of CRP-guided statin therapy is considered for clinical use, since the trial did not test the effect of rosuvastatin on a matched group with similar LDL cholesterol levels but normal levels of CRP, and did not assess...
a strategy of adjusting therapy based on the effect on CRP levels. Finally, the findings do not in themselves prove that the effects of statins on CRP are independent of lipid lowering, since the effect could be mediated by changes in non-LDL lipid particles,¹³,¹⁴ or the association between LDL cholesterol lowering and reduction in CRP could be masked by intra-individual variation in the measurement of these indices.²⁵⁷,²⁵⁸

4.1.7 Summary

A number of studies have demonstrated statins have non lipid lowering or pleiotropic effects in vitro, including improved endothelial function and direct antiinflammatory actions, but it remains uncertain whether statin therapy has clinically relevant antiinflammatory effects in vivo. Although a number of studies have shown modest reductions in CRP levels with statin therapy, it is not clear whether this effect is truly independent of their effect on lipid levels. In addition, it is not known whether statins have a broad antiinflammatory effect or whether they selectively lower CRP, since an effect of statins on other inflammatory markers such as fibrinogen, soluble intercellular adhesion molecule 1 (sICAM-1), and soluble CD40 ligand (sCD40L) has not been convincingly demonstrated. Nor is it clear whether other lipid lowering agents have antiinflammatory effects. Despite theoretical antiinflammatory effects of fibrates, few studies have looked at the effect of fibrates on inflammatory marker levels, and there are no direct comparisons of the effect of statins versus fibrates on a broad range of cardiovascular risk factors.
4.1.8 Study aims and research questions

The general aims of this research are to compare the effect of simvastatin versus placebo, and of simvastatin versus bezafibrate, on lipids, homocysteine and five inflammatory markers linked to future cardiovascular risk: CRP, fibrinogen, sICAM-1, sCD40L and PAPP-A.

The following research questions will be addressed:

1. Does long-term (2 year) simvastatin treatment lower serum levels of the inflammatory markers CRP, fibrinogen, sICAM-1, sCD40L and PAPP-A compared to placebo? If so does it have a proportionally equal effect on all inflammatory markers or does it affect some markers more than others?

2. Does long term (2 year) bezafibrate treatment lower serum levels of the inflammatory markers CRP, fibrinogen, sICAM-1, sCD40L and PAPP-A? If so, are the effects of bezafibrate equal to those of simvastatin?

3. Does pre-treatment with simvastatin for an average of 2 months prior to percutaneous coronary intervention reduce the post-PCI rise in plasma levels of the inflammatory markers CRP, sICAM-1, sCD40L and PAPP-A? If so does it have a proportionally equal effect on all inflammatory markers or does it affect some aspects of the inflammatory response but not others?
4.2 Methods

4.2.1 Study design and measurement of cardiovascular risk factors

Inflammatory markers and other serological cardiovascular risk factors were measured on the baseline and two year serum samples from the Fragmin and Simvastatin Trial (FAST, chapter 2). In FAST, randomisation to lipid lowering therapy was stratified according to baseline lipids. Patients with an ‘acceptable’ total cholesterol level were randomised in a double blind manner to simvastatin 40 mg/day or identical matching placebo (Group A), whereas patients with an ‘unacceptably high’ total cholesterol were randomised to simvastatin 40 mg/day or to bezafibrate 400 mg/day (Group B). Because of emerging trial data and changes in clinical practice over the years that the trial was conducted, the threshold for ‘acceptable’ cholesterol levels was progressively lowered during the trial, as outlined in chapter 2.

Elective PCI was performed a median 1.3 months (interquartile range 0.6-2.8) after enrolment and randomisation to lipid-modifying therapy. The PCI technique was at the operator’s discretion and most cases involved balloon angioplasty alone without stenting. At PCI a second randomisation to low molecular weight heparin (LMWH) or placebo took place. Those randomised to LMWH received subcutaneous dalteparin 5,000 u twice a day, continued for 1 month. As reported in chapter 3, randomisation to dalteparin had no effect on inflammatory marker levels. Aspirin 150mg daily was given for the duration of the trial unless there was a specific contraindication. No patients received glycoprotein IIb/IIIa inhibitors or thienopyridines (ticlopidine or clopidogrel).

The blood samples collected at baseline and study end (two years after PCI) were used to evaluate the longer term effects of lipid-modifying agents on inflammatory markers. The samples were collected following a 12 hour overnight fast and serum and plasma samples were stored at -80°C immediately after collection as detailed in Chapter 2. Lipid and fibrinogen levels were determined at the time of collection, while other cardiovascular risk factors (homocysteine, CRP, sICAM-1, sCD40L, PAPP-A) were measured on the stored serum samples (see appendix 1 for details of the assays used).
To investigate the effect of statins on the acute-phase response, inflammatory marker levels (CRP, sICAM-1, sCD40L and PAPP-A) were measured in stored plasma samples taken at the time of PCI. These samples were taken immediately prior to, and 48 hours, 1 week and 1 month after, PCI, and stored at -20°C until the time of analysis.

4.2.2 Statistical analyses

Analyses were performed using SAS statistical software version 8.1 (SAS Institute Inc., Cary, NC, USA). All probability tests are two tailed and a p value of 0.05 or lower was considered statistically significant.

Long term effects - Baseline to two year change

The distributional properties of the study variables were assessed using the Shapiro-Wilk statistic and visually with box and whisker and normal probability plots. Most variables were not normally distributed and the primary outcome (baseline to two-year change in inflammatory markers) did not approximate a normal distribution after log-transformation. Therefore statistical analyses were conducted using non-parametric tests and results are presented as median (interquartile range).

The chi-squared test (Fisher exact test where appropriate) was used to compare categorical variables at baseline and the Mann-Whitney U test was used for continuous variables. The change of each risk factor from baseline to two years was assessed using the Wilcoxon matched pairs test. To compare the median change of each risk factor for those on simvastatin versus those on placebo (group A), the Mann-Whitney U test was used. A similar analysis was performed for those on simvastatin versus those on bezafibrate (group B).

Acute effects – post-PCI rise in inflammatory markers

Two separate analyses were made. First, at each time-point (pre-PCI, and 48 hours, 1 week and 1 month post-PCI) inflammatory marker levels in the simvastatin group and the placebo group were compared. Analysis was performed on natural log-transformed inflammatory marker levels (to give a normal distribution) using an unpaired student’s t-test. Results are presented as geometric mean ± 95% CI. Second, the change in inflammatory marker levels (pre-PCI to 48
hours, pre-PCI to 1 week, pre-PCI to 1 month) was compared for the simvastatin and placebo groups. Because the change in inflammatory marker levels did not approximate a normal distribution after log-transformation, non-parametric tests (Mann-Whitney U test) were used.
4.3 Results

4.3.1 Baseline characteristics

A total of 315 patients were enrolled in the FAST trial, 215 to Group A (simvastatin versus placebo) and 100 to Group B (simvastatin versus bezafibrate). Table 4.1 shows the baseline characteristics of patients in each group. There were no differences in baseline characteristics of those randomised to simvastatin compared to placebo in group A, nor those randomised to simvastatin compared to bezafibrate in group B.

4.3.2 Long term (two year) effects of simvastatin and bezafibrate

The effects of simvastatin versus placebo (group A) on cardiovascular risk factors are shown in table 4.2 and figure 4.2, while the effects of simvastatin versus bezafibrate (group B) are shown in table 4.3 and figure 4.3. Simvastatin had a large, predictable effect on cholesterol levels with a median reduction in LDL cholesterol from baseline to 2 years of 1.8 mmol/l (43%, p<0.0001) in group A, and 1.6 mmol/l (39%, p<0.0001) in group B. Bezafibrate did not have such a marked effect on LDL cholesterol (median reduction of 0.6 mmol/l, 15%, p<0.0001) but it did increase HDL cholesterol by 0.1 mmol/l (12%, p<0.0001).

The effect of either agent on other risk factors was modest. Simvastatin was associated with a 1.8 μmol/l (13%, p<0.01) reduction in homocysteine levels in both groups, whereas bezafibrate elevated homocysteine by 1.7 μmol/l (12%, p=0.02). The change in CRP from baseline to 2 years was highly variable. In group A, there was a median 0.1 mg/l (5%, p=0.09) reduction in CRP with simvastatin and no change with placebo (simvastatin vs. placebo, p=0.06). In group B there was a median reduction of 0.6 mg/l (24%, p=0.02) with simvastatin and 0.3 mg/l (16%, p=0.3) with bezafibrate (simvastatin vs. bezafibrate, p=0.3). There was a clear lack of effect of either simvastatin or bezafibrate on fibrinogen and PAPP-A. There was also no discernable effect of either agent on sICAM-1 or sCD40L levels, although variability in levels of both markers was appreciable and a modest effect of either simvastatin or bezafibrate cannot be excluded.
Table 4.1 Baseline characteristics for Group A (simvastatin versus placebo) and Group B (simvastatin versus bezafibrate).

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Simvastatin (n = 102)</td>
<td>Placebo (n = 113)</td>
</tr>
<tr>
<td>Age, years</td>
<td>58 (51, 66)</td>
<td>60 (53, 68)</td>
</tr>
<tr>
<td>Male</td>
<td>89%</td>
<td>81%</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26 (24, 29)</td>
<td>26 (24, 29)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>3%</td>
<td>2%</td>
</tr>
<tr>
<td>Diabetes mellitus *</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35%</td>
<td>40%</td>
</tr>
<tr>
<td>Previous MI</td>
<td>36%</td>
<td>41%</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>74 (70, 80)</td>
<td>72 (66, 77)</td>
</tr>
<tr>
<td>Aspirin Therapy</td>
<td>94%</td>
<td>95%</td>
</tr>
<tr>
<td>β-Blocker therapy</td>
<td>86%</td>
<td>82%</td>
</tr>
<tr>
<td>ACE-I therapy</td>
<td>12%</td>
<td>18%</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>0.09 (0.08, 0.10)</td>
<td>0.09 (0.08, 0.11)</td>
</tr>
<tr>
<td>Serological Risk Factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol, mmol/l</td>
<td>5.8 (5.2, 6.2)</td>
<td>5.4 (5.0, 6.2)</td>
</tr>
<tr>
<td>LDL Cholesterol, mmol/l</td>
<td>4.1 (3.5, 4.5)</td>
<td>3.8 (3.2, 4.4)</td>
</tr>
<tr>
<td>HDL Cholesterol, mmol/l</td>
<td>1.0 (0.8, 1.1)</td>
<td>1.0 (0.9, 1.3)</td>
</tr>
<tr>
<td>Homocysteine, µmol/l</td>
<td>12.9 (10.8, 14.8)</td>
<td>13.1 (11.0, 16.1)</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>1.9 (1.1, 3.5)</td>
<td>1.8 (1.0, 3.9)</td>
</tr>
<tr>
<td>Fibrinogen, µmol/l</td>
<td>8.5 (7.3, 10.0)</td>
<td>9.1 (8.2, 10.6)</td>
</tr>
<tr>
<td>PAPP-A, mIU/l</td>
<td>5.8 (4.8, 6.7)</td>
<td>5.7 (4.6, 6.8)</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>290 (191, 368)</td>
<td>297 (227, 410)</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>2.4 (1.3, 4.9)</td>
<td>2.4 (1.2, 5.0)</td>
</tr>
</tbody>
</table>

Results are presented as median (interquartile range).

*Diabetes mellitus requiring insulin therapy was an exclusion criterion of the trial.
Table 4.2  Changes in cardiovascular risk factors, baseline to 2 years, for Group A (simvastatin versus placebo).

(A positive value represents an increase in level, a negative value represents a reduction in level).

<table>
<thead>
<tr>
<th>n *</th>
<th>Simvastatin</th>
<th>Placebo</th>
<th>Simvastatin vs. Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median change (IQR)</td>
<td>p value †</td>
<td>Median change (IQR)</td>
</tr>
<tr>
<td>Total Cholesterol, mmol/l</td>
<td>199</td>
<td>-1.7 (-2.2, -1.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL Cholesterol, mmol/l</td>
<td>196</td>
<td>-1.8 (-2.1, -1.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>201</td>
<td>0.1 (0, 0.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Homocysteine, µmol/l</td>
<td>141</td>
<td>-1.8 (-3.1, -0.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>150</td>
<td>-0.1 (-1.6, 0.4)</td>
<td>0.09</td>
</tr>
<tr>
<td>Fibrinogen, µmol/l</td>
<td>197</td>
<td>0.3 (-0.9, 1.5)</td>
<td>0.14</td>
</tr>
<tr>
<td>PAPP-A, mIU/l</td>
<td>150</td>
<td>-0.3 (-1.0, 1.2)</td>
<td>0.99</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>150</td>
<td>23 (-114, 119)</td>
<td>0.62</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>150</td>
<td>-0.6 (-1.8, 1.3)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* Number of patients in whom baseline and 2 year results were available. Paired samples were not available for all markers on all patients.

† P value for the change of each risk factor from baseline to two years (Wilcoxon matched pairs test).

‡ P value comparing the median change of each risk factor in the simvastatin versus the placebo group (Mann-Whitney U test).
Figure 4.2  Change in LDL cholesterol and other variables from baseline to two years in group A (simvastatin versus placebo).

Solid boxes represent median change and bars represent interquartile range for those on simvastatin (grey boxes) and those on placebo (white boxes). Asterisks beside the boxes indicate a significant change from baseline to two year values; *P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001

The p values comparing change in each variable on simvastatin versus placebo are given along the top of the graph.
<table>
<thead>
<tr>
<th></th>
<th>n *</th>
<th>Simvastatin</th>
<th>Bezafibrate</th>
<th>Simvastatin vs. Bezafibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median change (IQR)</td>
<td>p value †</td>
<td>Median change (IQR)</td>
</tr>
<tr>
<td>Total Cholesterol, mmol/l</td>
<td>97</td>
<td>-2.0 (-2.4, -1.3)</td>
<td>&lt;0.0001</td>
<td>-0.8 (-1.7, -0.4)</td>
</tr>
<tr>
<td>LDL Cholesterol, mmol/l</td>
<td>92</td>
<td>-1.6 (-2.1, -1.1)</td>
<td>&lt;0.0001</td>
<td>-0.7 (-1.6, -0.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>96</td>
<td>0 (-0.1, 0.2)</td>
<td>0.19</td>
<td>0.1 (0.0, 0.3)</td>
</tr>
<tr>
<td>Homocysteine, µmol/l</td>
<td>73</td>
<td>-1.8 (-5.1, -0.7)</td>
<td>&lt;0.01</td>
<td>1.7 (-0.4, 3.9)</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>73</td>
<td>-0.6 (-1.2, 0)</td>
<td>0.02</td>
<td>-0.3 (-1.2, 0.6)</td>
</tr>
<tr>
<td>Fibrinogen, µmol/l</td>
<td>92</td>
<td>0.2 (-0.3, 0.75)</td>
<td>0.11</td>
<td>-0.1 (-0.5, 0.6)</td>
</tr>
<tr>
<td>PAPP-A, mIU/l</td>
<td>68</td>
<td>-0.1 (-1.3, 0.7)</td>
<td>0.47</td>
<td>-0.2 (-0.8, 1)</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>72</td>
<td>-42 (-177, 19)</td>
<td>0.10</td>
<td>-46 (-172, 104)</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>72</td>
<td>-0.5 (-3.7, 2.0)</td>
<td>0.61</td>
<td>0 (-3.9, 3.6)</td>
</tr>
</tbody>
</table>

* Number of patients in whom baseline and 2 year results were available. Paired samples were not available for all markers on all patients.

† P value for the change of each risk factor from baseline to two years (Wilcoxon matched pairs test).

‡ P value comparing the median change of each risk factor in the simvastatin versus the bezafibrate group (Mann-Whitney U test).
**Figure 4.3** Change in LDL cholesterol and other variables from baseline to 2 years for patients in group B (simvastatin versus bezafibrate).

Solid boxes represent median change and bars represent interquartile range for those on simvastatin (dark grey boxes) and those on bezafibrate (light grey boxes). Asterisks beside the boxes indicate a significant change from baseline to two year values; *P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001

The p values comparing change in each variable on simvastatin versus bezafibrate are given along the top of the graph.
4.3.3 Stability of CRP and LDL cholesterol levels in those on placebo

Figure 4.4 compares the change in CRP and LDL cholesterol levels for group A patients on placebo, and figure 4.5 compares the change for those on simvastatin. Baseline CRP and LDL cholesterol levels were divided into five groups according to quintiles. Two year levels were also divided into five groups using the same cut-points, and the agreement between the baseline and two year groups was determined. For those on placebo, the stability in CRP levels from baseline to 2 years was at least as good as that of LDL cholesterol (figure 4.4). Baseline and two year CRP groups were the same in 49% of people, and were within one group of each other in 84%. For LDL cholesterol, 33% had baseline and 2 year measurements in the same group and 72% had baseline and 2 year measurements within one group of each other. Homocysteine, fibrinogen and PAPP-A levels showed similar long term stability (proportion of patients with levels in the same group at both baseline and 2 years 49%, 33% and 44% respectively). Variability was higher for sICAM-1 and sCD40L (less than 25% of baseline and 2 year measurements in agreement for either marker).

4.3.4 CRP and LDL cholesterol response to simvastatin

The effects of simvastatin therapy on CRP and LDL cholesterol levels are compared in figure 4.5. As expected, simvastatin had a predictable effect on LDL cholesterol with over 80% of patients at 2 years having a reduction in levels by one or more groups from baseline. LDL cholesterol levels did not increase in any patient on simvastatin. The effect on CRP levels was considerably less, and the response was variable. A reduction in CRP (a decrease of one group or more from baseline to two years) occurred in 42% of those on simvastatin compared to only 27% on placebo. However CRP levels remained the same in 32% of those on simvastatin, and they increased by 1 or more groups in a further 24%. The response of other inflammatory markers to simvastatin was equally variable and did not differ appreciably from their response to placebo.
Figure 4.4  Change in LDL cholesterol and CRP levels for group A patients on placebo.
See section 4.3.3 for explanation.

Figure 4.5  Change in LDL cholesterol and CRP levels for group A patients on simvastatin.
See section 4.3.4 for explanation.
4.3.5 *Simvastatin and the acute-phase response.*

Paired pre-and post PCI samples were available for analysis on 99 patients from group A (53 on simvastatin, 46 on placebo). Baseline characteristics were evenly matched between the simvastatin and placebo groups and did not differ from the baseline characteristics given for Group A as a whole in table 4.1. No patients had evidence of pre-existing inflammatory disease (no patients had pre-PCI CRP > 10 mg/l). The median duration of treatment (simvastatin or placebo) prior to PCI was 1.9 months (IQR 0.9 to 3.6). Randomisation to dalteparin (‘fragmin’) had no effect on inflammatory marker levels (data not shown).

As previously demonstrated in figure 3.1, PCI led to a significant rise in inflammatory markers. This was most marked for CRP, with a six-fold rise between pre-PCI and 48 hours post-PCI, and least apparent for sICAM-1, for which an elevation of levels was only apparent 1 month post-PCI.

In figure 4.6, inflammatory marker levels at each time-point are compared for patients on simvastatin and patients on placebo. Treatment with simvastatin had no effect on the inflammatory response to PCI. For each inflammatory marker, at each time-point, there were no differences in levels for those on simvastatin compared to those on placebo. Exclusion of patients on treatment for less than 4 weeks pre-PCI (n=25) did not affect the results. Reanalysis by change in inflammatory marker levels (pre-PCI to 48 hours, pre-PCI to 1 week, pre-PCI to 1 month) in the simvastatin versus placebo groups did not affect the results.
Figure 4.6  Comparison of inflammatory marker levels pre-PCI and 48 hours, 1 week and 1 month post-PCI for patients on simvastatin versus patients on placebo.

Black bars represent geometric mean for patients on simvastatin, grey bars represent geometric mean for patients on placebo, and error bars show 95% CI.

PCI led to an inflammatory response with a rise in CRP, sCD40L and PAPP-A levels within 48 hours post-PCI (as discussed in section 3.3.3). Treatment with simvastatin had no effect on this inflammatory response; at each timepoint, inflammatory marker levels were similar for those on simvastatin versus those on placebo (P>0.05 for all comparisons).

A. C-reactive protein (CRP).
B. soluble intercellular adhesion molecule 1 (sICAM-1).
C. soluble CD40 ligand (sCD40L).
D. pregnancy associated plasma protein-A (PAPP-A).
E. The ratio between plasma associated plasma Protein-A and its endogenous inhibitor, the proform of eosinophil major basic protein (PAPP-A/proMBP ratio).

A. CRP
B. sICAM-1

C. sCD40L
D. PAPP-A

![Graph showing PAPP-A levels over time with and without Simvastatin or Placebo.]

- **Simvastatin**:
  - Pre-PCI: p=0.79
  - 48 hours: p=0.57
  - 1 week: p=0.85
  - 1 month: p=0.85

- **Placebo**:
  - Pre-PCI
  - 48 hours
  - 1 week
  - 1 month

E. PAPP-A / proMBP ratio

![Graph showing PAPP-A/ProMBP ratio over time with and without Simvastatin or Placebo.]

- **Simvastatin**:
  - Pre-PCI: p=0.62
  - 48 hours: p=0.60
  - 1 week: p=0.81
  - 1 month: p=0.79

- **Placebo**:
  - Pre-PCI
  - 48 hours
  - 1 week
  - 1 month


4.4 Discussion

In this randomised controlled trial, 2 years’ treatment with simvastatin led to significant reductions in LDL cholesterol and homocysteine levels, but had very little effect on inflammatory marker levels. There was a modest reduction in CRP levels (which was significant in group B but not in group A), and no effect on other inflammatory markers (fibrinogen, sICAM-1, sCD40L, PAPP-A). In addition, simvastatin had no effect on the inflammatory response to PCI. In contrast to simvastatin, bezafibrate had less effect on LDL cholesterol, elevated homocysteine levels, and had no effect on inflammatory markers.

4.4.1 Effect of simvastatin on C-reactive protein levels

The degree of CRP lowering with simvastatin in the current study is consistent with previous trials. In group B the median reduction was 0.6mg/l (25%, p=0.02), while in group A, who had lower baseline CRP levels, the median reduction was only 0.1mg/l (5%, p=0.09). In both the PRINCE (pravastatin 40mg daily for 24 weeks) and AFCAPS/TexCAPS (lovastatin 20-40 mg daily for 1 year) studies, statin therapy led to a median reduction in CRP of 0.2 mg/l (~14%).

Although it has been claimed that “all statins are equally effective at lowering C-reactive protein levels”, several studies have challenged this assumption. In the Atorvastatin vs. Simvastatin on Atherosclerosis Progression (ASAP) study (2002), atorvastatin 80mg per day for two years caused a significantly greater reduction in CRP levels than simvastatin 40mg per day. Similarly, both the REVERSAL trial (in stable angina) and PROVE IT TIMI 22 (in acute coronary syndromes) found CRP lowering to be significantly greater with high dose atorvastatin (80mg per day) than with conventional dose pravastatin (40mg per day). We might therefore have seen a greater effect in the current study had high dose atorvastatin been used in place of simvastatin. However most of the primary and secondary prevention data behind the proven efficacy of statins comes from trials of simvastatin or pravastatin, and it is from analysis of these studies that non lipid lowering or “pleiotropic” benefits of statins were proposed. It is therefore important to determine the effect of simvastatin on CRP and other inflammatory markers before the pleiotropic benefits of this agent are ascribed to an
antiinflammatory effect. Previous studies of the effects of simvastatin on CRP have been relatively small but have shown similar results to the current study.\textsuperscript{225,227,259,260}

In the current study, the CRP response to statin therapy was variable (figure 4.5). Despite the trend towards a lower median CRP with simvastatin, CRP increased by one or more groups in 24\% of cases. In contrast, LDL cholesterol did not increase in any patient on simvastatin. This was not due to variability in CRP levels per se, since for those on placebo CRP levels showed the same two year stability as LDL cholesterol (figure 4.4). The unpredictable response of CRP levels to statin therapy has implications for targeted therapy based on a combination of LDL cholesterol and CRP levels,\textsuperscript{252} and titration of statin therapy based on CRP response.\textsuperscript{7} It remains to be seen whether statins provide greater risk-reduction in those with higher baseline CRP, and if so whether this risk-reduction is limited to those in whom CRP falls.\textsuperscript{261}

### 4.4.2 Effects of simvastatin on other inflammatory markers

There was no effect of simvastatin therapy on other inflammatory markers in the current study. The lack of effect on fibrinogen is consistent with a number of previous trials,\textsuperscript{236,262-264} and adds to the “strong evidence that statins do not affect fibrinogen levels”.\textsuperscript{228} There has been only one previous study of the effect of statins on PAPP-A levels.\textsuperscript{241} In this small study, PAPP-A was unaffected by 10 weeks atorvastatin therapy in 27 hypercholesterolaemic subjects. Previous studies of statins on sICAM-1 have been small (less than 50 patients each), with conflicting results. Most\textsuperscript{238,239,265} but not all\textsuperscript{240} have found no effect of statin therapy on sICAM-1 levels. Results for sCD40L are also conflicting. In the ASAP trial (110 patients), simvastatin and atorvastatin both caused an equal 40\% reduction in sCD40L in patients with familial hypercholesterolaemia.\textsuperscript{115} However, the MIRACL trial showed no significant reduction in sCD40L levels in 1,160 acute coronary syndrome patients treated with 16 weeks' atorvastatin.\textsuperscript{120} The differences between the effects of simvastatin in the ASAP study compared to the current study may be due to differences in the population studied (familial hypercholesterolaemia vs. stable angina), since baseline sCD40L levels were several-fold higher in ASAP (10ng/ml) than in the current study (2.4ng/ml). Overall, there is no convincing evidence that simvastatin has a significant effect on inflammatory markers other than CRP.
4.4.3 *Effects of simvastatin on homocysteine levels*

Few studies have reported the effect of statin therapy on homocysteine levels. In the largest study to date, there was a small but significant 0.4 μmol/l (4%) reduction in homocysteine in 2,705 AFCAPS/TexCAPS patients treated with lovastatin for one year.\(^{266}\) In the current study simvastatin was associated with a much larger 1.8 μmol/l (~14%) reduction in homocysteine levels. The more pronounced effect in the current study may be attributable to use of a different statin, a longer duration of treatment, or study of a secondary prevention cohort with higher baseline homocysteine levels.

4.4.4 *Effects of bezafibrate*

Recent interest has focused on theoretical antiinflammatory effects of the fibrate class of drugs related to their peroxisomal proliferation activating receptor-\(\alpha\) (PPAR-\(\alpha\)) agonist action.\(^{118}\) However few studies have examined the effects of fibrates on inflammatory marker levels. In the current study, bezafibrate did not reduce levels of CRP or any other inflammatory marker. This is consistent with previous reports that both bezafibrate\(^{249}\) and fenofibrate\(^{267}\) fail to lower CRP levels. A lack of effect on fibrinogen has also been noted in some\(^{268}\) but not all\(^{247,250}\) previous reports. To my knowledge this is the first report to document the lack of effect of fibrates on PAPP-A, sICAM-1 and sCD40L levels.

The effect of bezafibrate on other risk factors was as expected. Bezafibrate caused a greater elevation of HDL cholesterol than simvastatin, but it also had less effect on LDL cholesterol and significantly raised homocysteine levels. This adverse effect on homocysteine levels has been reported for both bezafibrate\(^{269,270}\) and micronised fenofibrate\(^{268,271,272}\) and may relate to an adverse effect on renal function. Gemfibrozil, which does not impair renal function, does not elevate homocysteine levels.\(^{273}\)

4.4.5 *Simvastatin and the acute-phase response*

PCI offers a convenient model with which to examine the vascular antiinflammatory effects of statins, since it causes a discrete vascular injury that is followed by a measurable inflammatory response.\(^{157}\) It is reflected by increased cytokine production within the coronary circulation.\(^{274}\)
and a systemic inflammatory response including granulocyte activation and an increase in circulating levels of sICAM-1, sCD40L and CRP. This inflammatory response can be attenuated by medications with antiinflammatory activity. Pre-treatment with the glycoprotein IIb/IIIa inhibitor abciximab has been shown to diminish the rise in cytokines and CRP levels following PCI. Similarly in acute coronary syndromes, which are also associated with immune system activation and an acute-phase response, treatment with methylprednisolone leads to a significant reduction of CRP levels within 48 hours. It therefore seems reasonable to suppose that if statins had direct antiinflammatory activity they would suppress the post-PCI rise in inflammatory markers. In the current study, pre-treatment with simvastatin for an average 2 months prior to PCI had no effect on the inflammatory response or inflammatory marker levels (CRP, sICAM-1, sCD40L, PAPP-A) measured 48 hours, 1 week and 1 month post-PCI.

4.4.6 Potential mechanisms for pleiotropic effects of simvastatin

In experimental models of atherosclerosis, statins have been shown to have direct antiinflammatory effects on components of the atherosclerotic plaque. However doubts remain as to whether these effects, often produced using high drug concentrations, are relevant in vivo. It remains possible the reduced inflammation and plaque stabilisation seen with clinical use of statins occur as a consequence of lipid lowering. Many aspects of the CRP response to statin therapy mimic that of LDL cholesterol. This raises questions as to whether the antiinflammatory effects of statins are truly independent of the lipid lowering effects. For example, the onset of CRP lowering with statins, while rapid, lags behind LDL-reduction. This was demonstrated in a crossover study by Plenge et al. (2002) involving 40 patients with elevated LDL cholesterol treated with 14 days' simvastatin therapy. Reduction in LDL cholesterol levels occurred 1 week earlier than reduction in CRP levels (reduction in LDL cholesterol apparent within 7 days of initiating simvastatin, reduction in CRP only apparent after 14 days of simvastatin therapy). It is also notable that upon withdrawal of statin therapy, lipid levels increase rapidly whereas CRP levels remain suppressed for at least three weeks. Again, this may be because the effects of statins on inflammatory markers are indirect. Furthermore, in comparative studies of different statins, atorvastatin (which has a greater effect on LDL cholesterol) also has a greater effect on CRP. For example in the
REVERSAL trial, atorvastatin 80mg per day resulted in a 36.4% reduction in CRP levels over 18 months, compared to only a 5.2% reduction on pravastatin 40 mg per day (p<0.001).206 Similarly CRP shows dose responsiveness to statin therapy across the same dosage range as LDL cholesterol, with atorvastatin 80mg per day having considerably more effect than atorvastatin 10mg per day.229 Of particular note, it has been reported that dual lipid lowering therapy using the combination of ezetimibe and simvastatin has a significantly greater effect on CRP levels than simvastatin therapy alone (-34.8% vs. -18.2%, p<0.01).277 Ezetimibe inhibits cholesterol absorption at the intestinal brush border and consequently produces significant incremental LDL cholesterol lowering when added to statin therapy. Surprisingly, there was little correlation between change in LDL and change in CRP in this study,277 which mirrors several previous statin studies in which the change in CRP was largely unrelated to the change in LDL cholesterol.223,253,254 Nonetheless, unless ezetimibe also has pleiotropic effects, we must assume the incremental CRP reduction with ezetimibe is a consequence of its effects on cholesterol. Further support for this concept comes from animal studies in which lipid lowering by diet alone produces antiinflammatory and plaque stabilising changes including reduced adhesion molecule, CD40L, and metalloproteinase expression, and improved endothelial function.278-280 These changes may be secondary to decreased oxidised LDL within the vessel wall. The lack of a close correlation between change in circulating levels of LDL cholesterol and circulating levels of CRP cannot be taken as evidence that the antiinflammatory effects of statins are independent of lipid lowering effects. It is plausible that lowering LDL cholesterol in the bloodstream leads to a reduction in oxidised LDL in the vessel wall, resulting in reduced local inflammation, reduced release of proinflammatory cytokines into the bloodstream, and ultimately reduced circulating CRP levels, but that variability at each step of this process masks the association between changes in circulating LDL cholesterol and circulating CRP (figure 4.7). This is consistent with two meta-analyses of the effect of statin therapy on LDL cholesterol and CRP levels, which demonstrated that when data are pooled to minimise the effect of intra-individual variation, the antiinflammatory effect of LDL lowering therapies is closely related to the change in LDL levels.257,281
In the current study, two years’ treatment with simvastatin affected CRP levels but not other inflammatory markers (fibrinogen, PAPP-A, sICAM-1, sCD40L). Furthermore, the average reduction in CRP was small and the individual response was variable. Simvastatin had no effect on the acute rise in inflammatory markers following PCI. This contrasts with the broad, rapid and marked reduction in inflammatory markers seen with direct antiinflammatory agents such as methylprednisolone,\textsuperscript{244} and would seem to point away from a direct antiinflammatory action for simvastatin. In addition, other pleiotropic effects of simvastatin such as lowering of homocysteine levels cannot be explained on the basis of an antiinflammatory mechanism. The reduction in both CRP and homocysteine seen with simvastatin may be secondary to other statin effects such as reduction in oxidised LDL within the vessel wall, or improved endothelial function.\textsuperscript{215,282,283} There is a clear need for further research into the mechanisms by which statins exert their ‘pleiotropic’ effects.
In population studies, both LDL cholesterol and CRP levels are reduced with statin therapy, but at an individual level there is a poor correlation between the change in LDL cholesterol and the change in CRP. This has lead to the suggestion that the CRP reduction with statins is independent of lipid lowering (a ‘pleiotropic’ effect). However, as outlined in the text, several lines of evidence suggest that a reduction in LDL cholesterol may indeed be responsible for lower circulating CRP levels. A direct association between the two may be masked by the intervening steps involving changes within the vessel wall, which introduce variability to the response.

Figure 4.7 Possible mechanisms by which lipid-modifying agents such as statins lower circulating CRP levels.

- Lipid-modifying agents
  - Circulating LDL
    - LDL in vessel wall
      - inflammation in vessel wall
        - CRP in the bloodstream
4.4.7 Study limitations

The current study employed a randomised controlled trial design, which is important to avoid bias from cohort effects or regression to the mean. Unrecognised bias may have been introduced by the relatively high proportion of patients without complete inflammatory marker data, however this is unlikely as baseline characteristics and treatment allocation for those with missing data did not differ from the main study population.

Although the stability of most markers was comparable to that of LDL cholesterol, sICAM-1 and sCD40L levels in particular showed considerable test-test variability and this may have limited our ability to reliably detect treatment effects on these markers. In addition, because the response to treatment for CRP and other inflammatory markers was so variable, a much larger study would be needed to definitively exclude a minor treatment effect of either simvastatin or bezafibrate on inflammatory markers. However, the clinical relevance of any such minor effect would be questionable.

4.4.8 Conclusions

In patients with stable angina, two years’ treatment with simvastatin significantly reduced LDL cholesterol and homocysteine levels, but caused only a modest reduction in CRP and did not affect levels of any other inflammatory marker (fibrinogen, PAPP-A, sICAM-1, sCD40L). In addition, pre-treatment with simvastatin had no effect on the inflammatory marker rise following PCI. Long-term (two years’) treatment with bezafibrate elevated homocysteine levels and had no effect on inflammatory markers. Neither simvastatin nor bezafibrate appear to have broad or major antiinflammatory effects in patients with stable angina. The mechanism(s) that underlie the modest, variable reduction in CRP with statin therapy require further study.
Chapter 5

Effects of Exercise Training on Inflammatory Marker Levels

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5.1.2 Associations between activity and inflammatory markers
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5.3 The Smoking Cessation Exercise Trial
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5.4.2 Study limitations
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5.1 Introduction

5.1.1 Inactivity and cardiovascular risk

There is strong evidence that regular physical activity reduces the risk of cardiovascular disease. Many large epidemiological studies have found a step-wise decrease in cardiovascular risk with increasing levels of physical activity or fitness, and progression of coronary artery disease assessed by serial angiography is lower in persons who have higher levels of physical activity. In addition, the risk of cardiovascular disease has been shown to decrease in individuals who increase their usual levels of exercise. The possibility that benefit may occur relatively rapidly is suggested by randomized clinical trials of exercise-based cardiac rehabilitation after myocardial infarction. A systematic overview of clinical trials showed that exercise training for 2 to 6 months reduced cardiovascular mortality by 25 to 30%.

Although exercise has favourable effects on a number of cardiovascular risk factors, the mechanisms by which exercise reduces cardiovascular risk are not well understood. In epidemiological studies only about one third of the risk reduction associated with increased physical activity is explained by adjustment for conventional risk factors. Consequently a recent American Heart Association Scientific Statement (endorsed by the American College of Sports Medicine) on exercise and cardiovascular disease called for “research [to] address the mechanisms by which exercise reduces CAD risk”. One postulated mechanism is a direct antiinflammatory effect of exercise, with the consequent favourable effect on the inflammatory component of atherosclerosis responsible for the reduction in cardiovascular events.

5.1.2 Associations between activity and inflammatory marker levels

The effect of regular exercise on immune function has been the subject of much interest in the sport’s medicine literature. Although it is well known that a single episode of intense physical activity may cause a transient inflammatory response, regular exercise appears to down-regulate immune system activity. Several investigators have shown that regular exercise suppresses in-vitro neutrophil function, and in epidemiological studies, regular physical activity has been consistently associated with lower levels of circulating inflammatory markers. For example, Wannamethee et al. (2002) reported that physical activity showed a significant
inverse relationship with a number of inflammatory risk factors including CRP, fibrinogen, and white blood cell count. This association persisted after adjustment for potential confounders including age, BMI, smoking, alcohol and pre-existing cardiovascular disease. In an analysis of data from the Third National Health and Nutritional Examination Survey (NHANES III) carried out in the United States, Abramson and Vaccarino (2002) also found progressively lower WBC, CRP and fibrinogen levels with increasing physical activity. CRP also shows an inverse association with the more objective measure of cardiorespiratory fitness, assessed by maximum aerobic capacity (VO$_2$max) during treadmill exercise. However, as in all observational studies, the possibility of unrecognized confounding cannot be excluded. Potential confounders include undiagnosed illness (which even if mild may cause inflammation as well as decrease regular activity and physical fitness), and adiposity. Adipose tissue is an important source of proinflammatory cytokines which stimulate a low grade inflammatory response. Consequently levels of inflammatory markers such as WBC, fibrinogen and CRP are strongly affected by body fat.

Some observational studies have attempted to correct for this by adjusting for BMI, but BMI is a relatively imprecise measure of body fat and may not fully correct for differences in body fat with exercise. Because of the potential for confounding, a prospective and preferably randomized trial is the most reliable way to assess the effect of exercise on inflammatory marker levels.

5.1.3 Effects of exercise training on inflammatory marker levels

To date, only a few such prospective trials have been undertaken. Stratton et al. (1991) reported that 6 months’ exercise training caused a reduction in average fibrinogen levels in 13 men over the age of 60 years. However exercise had no effect on fibrinogen levels in younger subjects. Mattusch et al. (2000) reported a significant reduction in CRP levels after nine months’ marathon training in 12 athletes. Smith et al. (1999) reported that six months’ exercise training reduced mononuclear cell production of atherogenic cytokines and a trend towards lower CRP (p=0.12) in 43 volunteers with cardiovascular risk factors. These studies were non-randomised, did not include a control group, and did not adjust for changes in body fat. In addition, in the study by Smith et al. (1999) evidence of immune system activation was an enrolment criterion and it is therefore possible their results are explained by regression to the
A recent non-randomized study by Milani et al. (2004) reported that 12 weeks cardiac rehabilitation (including exercise training) led to a significant reduction in CRP levels independent of statin therapy or changes in weight. However, the effects of different components of the rehabilitation program (dietary change, exercise training) could not be individually assessed.

At the time this research was undertaken, there were only two randomized trials of the effects of exercise training on inflammatory markers in patients with established cardiovascular disease in the literature. Worsomu et al. (1992) reported a significant decrease in fibrinogen levels in 35 patients randomized to aerobic exercise following coronary bypass surgery. The reduction in fibrinogen was apparent by 12 weeks, sustained at 6 months, and independent of changes in body weight. In a more recent randomised crossover study (2002), exercise training significantly reduced the pro-inflammatory cytokines tumour necrosis factor α and interleukin-6 in 24 patients with heart failure but not in 20 healthy controls. These studies did not report serum CRP levels or other inflammatory markers such as sCD40L and sICAM-1.

Although exercise clearly has important cardioprotective effects in apparently healthy individuals, at the time this research was undertaken there were no randomized trials of the effect of exercise training on inflammatory markers in healthy individuals in the literature.

5.1.4 Summary

It is possible that the cardioprotective benefits of exercise derive at least in part from anti-inflammatory effects of regular exercise. General population studies have reported an inverse association between levels of WBC, fibrinogen and CRP and self reported physical activity or physical fitness, and non-randomised prospective trials have suggested that short periods of exercise training may lower markers such as CRP. These studies have been taken as evidence of a direct ‘anti-inflammatory’ action of regular physical exercise. However, since WBC, fibrinogen and CRP are all strongly influenced by body fat it is equally plausible that lower levels are an indirect consequence of the effect of exercise on body fat. The effect of physical activity on inflammatory markers that are not strongly related to body fat such as sICAM-1 and sCD40L has not been examined. For these reasons randomised trials
of exercise training are needed to determine whether increasing physical activity lowers a range of inflammatory markers independent of its effects on body fat.

In this chapter two such trials are reported. The first investigates the effect of six months’ exercise training on CRP and body fat in healthy elderly subjects. The second reports the effects of 6 and 12 weeks’ exercise training on five inflammatory markers (WBC, fibrinogen, CRP, sICAM-1, sCD40L) in female smokers taking part in a smoking cessation trial.

5.1.5 Study aims and research questions

The general aim of this research is to determine the effect of exercise training on inflammatory markers shown to be linked to future cardiovascular risk.

The following research questions will be addressed:

1. Does 6 months exercise training lower blood levels of C-reactive protein in healthy elderly? If so, does it do so independently of the effects of exercise on body fat?

2. Does 6 or 12 weeks of exercise training lower blood levels of the inflammatory markers WBC, fibrinogen, CRP, sICAM-1 and sCD40L in female smokers? If so, does it have a proportionally equal effect on all inflammatory markers or does it affect some markers more than others?
5.2. Exercise in the Elderly Trial

Methods

5.2.1 Participants

Healthy elderly subjects aged 60-85 were recruited from the general population by newspaper advertisements. Exclusion criteria included inability to undertake an exercise training program, current illness known to be associated with a systemic inflammatory response, a history of hypertension, diabetes, valvular or cardiovascular heart disease, or any other condition requiring regular cardiovascular medication, including aspirin. In addition, participants underwent a screening physical examination and investigations including an electrocardiogram (ECG), treadmill exercise stress test and echocardiogram. Abnormalities suggestive of cardiovascular disease on examination or investigation were further exclusion criteria. These included signs of hypertension (systolic blood pressure >160 mmHg, diastolic blood pressure > 90mmHg), atrial fibrillation, left bundle branch block or changes consistent with previous myocardial infarction on ECG, development of symptoms or ECG changes consistent with ischaemia on exercise stress test, and myocardial hypertrophy, regional wall motional abnormalities or valvular disease on echocardiogram. Of 86 people who attended pre-trial screening 63 met the entry criteria and were enrolled in the trial. The study protocol was approved by the Auckland University ethics committee and all participants gave written informed consent.

5.2.2 Study design

Following the baseline assessment eligible subjects were randomly assigned to either an exercise or a control group. The control group was asked to maintain their usual level of physical activity for the six month trial period. The exercise group underwent six months of exercise training consisting of three supervised sessions and one unsupervised session per week. During the first two to four weeks, training sessions lasted 30 minutes at an intensity of approximately 50% VO\textsubscript{2}max. After this period, exercise duration and intensity were gradually increased so that participants were training for 45 minutes at approximately 80% VO\textsubscript{2}max by the fourth month of training. During training sessions participants were free to choose from
treadmill, cycle and rowing ergometers. The use of heart rate monitors ensured that all exercise modalities were carried out at the appropriate aerobic intensity.

5.2.3 Measurements

Assessment of physical activity

Participants were asked at the start and end of the trial to report the average amount of time spent per week over the last six weeks in a variety of physical activities using a standardised validated questionnaire used in both the US Nurse’s Health study285 and the Women’s Health Initiative Observational Study.308 Activities included climbing stairs, walking outdoors, jogging, running, bicycling, swimming laps, playing tennis or squash and participating in aerobics. The responses were used to calculate a weekly metabolic-equivalent (MET) score, in MET hours per week (Appendix 3).308 The MET score is a measure of the absolute intensity of physical activity, represented as multiples of basal metabolic activity (1 MET equals the resting metabolic rate of ~ 3.5 ml O_2/kg). The activity score gave a quantitative measure of baseline activity levels for both groups and was used to check whether activity levels had changed in the control group over the six months of the trial.

Assessment of cardiorespiratory fitness

Cardiorespiratory fitness was assessed in all participants at baseline and study end by measurement of maximum aerobic capacity (VO_2max) during treadmill exercise. After warm up, participants began exercise at an initial workload (calculated in METs) of 60% age-predicted maximum heart rate, and workload was increased by 1.0 to 1.5 METs over successive two minute stages until exhaustion. Expired fractions of O_2 and CO_2 were measured using a Schiller CS100 metabolic analyser. It was assumed that VO_2max had been achieved if two of the following three criteria were met 1) a plateau in oxygen consumption (VO_2) despite increased workload, 2) a respiratory quotient (VO_2/VCO_2) of 1.1 or greater, or 3) attainment of greater than maximum age-predicted heart rate.

Assessment of body fat

The effect of exercise on weight and adiposity is an important potential confounder because plasma levels of CRP are influenced by total body fat and are reduced by weight loss.26,310
Therefore BMI and total body and trunk fat composition were measured on participants at the start and end of the study. Total body fat composition was determined by dual energy x-ray absorptometry (DEXA) scan\textsuperscript{311} using a Lunar DPX-IQ scanner (GE Medical Systems, Waukesha, WI).

**Measurement of serum glucose, cholesterol and CRP**

Venous blood samples were collected at baseline and study end for measurement of serum glucose, cholesterol, and CRP concentrations. Samples were collected at least 24 hours after the last exercise session and were postponed for at least 2 weeks for any illness or injury associated with an acute-phase response. After collection glucose and cholesterol were measured immediately and the remaining serum was stored at \(-80^\circ\) Celsius for subsequent CRP analysis as a single batch blind to collection order and treatment allocation.

**5.2.4 Statistical analyses**

Results are reported as number, mean ± standard deviation or median (interquartile range) unless otherwise indicated. The distribution of variables was checked visually with plots (box and whisker) and the Shapiro-Wilk statistic. Because the distribution of CRP levels was skewed natural-log-transformed (Ln) CRP was used for statistical analysis to satisfy assumptions of normality and equal variance. In the text CRP levels are presented as median and interquartile range for ease of interpretation. The Pearson correlation coefficient was used to assess the association between Ln-CRP and other baseline variables including BMI, percent body fat, physical activity scores and fitness. The baseline to six month change in CRP and other variables were compared between the exercise and control groups using a Mann Whitney U-test. All analyses were performed on SAS release 8.0 and a p-value of 0.05 or less was considered statistically significant.
Results

5.2.5 Study population

Thirty-two patients were randomised to exercise training and 31 to the control group. Two participants (both from the exercise group) were lost to follow-up, giving a final study cohort of 30 exercise and 31 control subjects. Four of the exercise participants failed to complete the training program but attended six-month follow-up. There were no baseline differences between those lost to follow-up, those not completing the training program, and the main study cohort. Primary analysis was performed on an intention to treat basis for all patients with follow-up data; a sensitivity analysis excluding the dropouts was subsequently performed and did not alter the study findings.

5.2.6 Baseline characteristics and associations

The baseline characteristics of participants are given in table 5.1. The mean age was 66 years and just over half the participants were female. Exercise and control groups were evenly matched by adiposity, physical activity levels, cardiorespiratory fitness, lipid profile and CRP levels.

Baseline correlations between CRP, adiposity, and cardiorespiratory fitness are shown in figures 5.1 to 5.3. There was a strong inverse association between fitness and percent body fat, with less body fat on fitter individuals (figure 5.1). Also as expected, CRP was influenced by adiposity with higher CRP levels as percent body fat increased (figure 5.2). In contrast, there was only a weak inverse association between fitness and CRP levels that did not reach statistical significance (figure 5.3).

The associations between various cardiovascular risk factors and both CRP and fitness are explored further in table 5.2. Higher activity scores, younger age, and lower body fat were all associated with increased levels of fitness. However, the strongest association was between fitness and percent body fat. In addition, CRP levels were not significantly associated with activity scores or fitness but were strongly correlated with all measures of adiposity (BMI, percent body fat, and especially percent truncal fat). Therefore of the variables assessed, fitness was the strongest determinate of body fat, and body fat was the strongest determinate of CRP levels.
Table 5.1  Baseline characteristics of participants in the exercise in the elderly study.

<table>
<thead>
<tr>
<th></th>
<th>Intervention group n=30</th>
<th>Control group n=31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>17 (57%)</td>
<td>17 (55%)</td>
</tr>
<tr>
<td>Age, years</td>
<td>67 ± 5.2</td>
<td>66 ± 4.0</td>
</tr>
<tr>
<td>Caucasian</td>
<td>28 (93%)</td>
<td>31 (100%)</td>
</tr>
<tr>
<td>Current smoker, number</td>
<td>1 (3%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Ex-smoker, number</td>
<td>9 (30%)</td>
<td>15 (50%)</td>
</tr>
<tr>
<td>Ex-smoker, years since smoked</td>
<td>25 (20, 30)</td>
<td>20 (15, 29)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.2 ± 2.9</td>
<td>25.8 ± 3.8</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>33 ± 10.4</td>
<td>33 ± 8.9</td>
</tr>
<tr>
<td>Trunk fat (%)</td>
<td>17 ± 4.8</td>
<td>18 ± 4.5</td>
</tr>
<tr>
<td>Activity score, MET.hours/week</td>
<td>24 (7, 42)</td>
<td>23 (15, 39)</td>
</tr>
<tr>
<td>Fitness (VO₂max, ml/kg/min)</td>
<td>26.4 ± 5.6</td>
<td>28.4 ± 7.9</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.2 ± 0.7</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.4 ± 0.7</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>1.6 (0.5, 2.9)</td>
<td>1.5 (0.7, 2.1)</td>
</tr>
</tbody>
</table>
Figure 5.1  Scatter plot demonstrating the strong inverse association between cardiorespiratory fitness and percent body fat for the combined groups at baseline.

$\text{Fitness (VO}_2\text{max, ml/kg/min)}$  

$\text{Percent Body Fat (%) }$

$r = -0.61$

$p < 0.001$
Figure 5.2 Scatter plot demonstrating the moderately strong association between C-reactive protein and percent body fat for the combined groups at baseline.

$r = 0.46$

$p = 0.001$
Figure 5.3 Scatter plot demonstrating the weak trend towards lower C-reactive protein with increasing cardiorespiratory fitness for the combined groups at baseline.
Table 5.2  Association between plasma levels of C-reactive protein, cardiovascular fitness and other predictors of cardiovascular risk measured at the baseline assessment.

<table>
<thead>
<tr>
<th>Correlation with CRP</th>
<th>Correlation with VO$_2$max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
</tr>
<tr>
<td>Age, years</td>
<td>-0.04</td>
</tr>
<tr>
<td>BMI</td>
<td>0.40</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Trunk fat (%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Activity score</td>
<td>-0.01</td>
</tr>
<tr>
<td>VO$_2$max</td>
<td>-0.22</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>0.33</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.24</td>
</tr>
</tbody>
</table>
5.2.7 Effect of exercise training

The median proportion of training sessions attended by the exercise group was 70±14% (range 37-90%). Weekly activity levels remained unchanged in the control group. At six months there was an 18% improvement in cardiorespiratory fitness in the exercise group compared to no change in the control group (median increase in VO_{2max} 3.7 ml/kg/min versus 0.05 ml/kg/min; p=0.004). There were no significant changes in body weight, % body fat, lipid profile or serum glucose in either group (table 5.3).

There was considerable individual variability in the change in CRP from baseline to six months, but on average serum levels of CRP did not change for either the exercise or control groups (Figure 5.4). Overall there was a median change of -0.12 mg/l (-0.8, 0.3) for the exercise group and -0.06 mg/l (-0.4, 0.5) in the control group, a -6% and -4% change respectively, p=0.30.

The possibility of a threshold effect, in which only large improvements in fitness result in antiinflammatory effects and reduction in CRP, was explored further by plotting change in fitness (from baseline to 6 months) against change in CRP (from baseline to 6 months) (figure 5.5). There was no evidence for an association between change in fitness and change in CRP, and in particular there was no change in CRP levels even for those participants with a large improvement in fitness through the six month trial.
Table 5.3  Change in fitness, body mass index and body fat, and plasma levels of C-reactive protein, glucose and lipids between baseline and 6 months for subjects randomised to exercise training and control groups.

(A positive value represents an increase in level, a negative value represents a reduction in level).

<table>
<thead>
<tr>
<th></th>
<th>Exercise Training</th>
<th>Control</th>
<th>Exercise vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median Change</td>
<td>% Change</td>
<td>Median Change</td>
</tr>
<tr>
<td>VO₂max, ml/kg/min</td>
<td>3.7 (1.3, 8.3)</td>
<td>18 (4, 37)</td>
<td>0.05 (-2.2, 3.0)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>-0.14 (-0.6, 0.3)</td>
<td>-1 (-3, 1)</td>
<td>-0.07 (-0.7, 0.2)</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>-0.7 (-2.8, 0.1)</td>
<td>-2 (-10, 0)</td>
<td>0 (-1.7, 1.3)</td>
</tr>
<tr>
<td>Trunk fat (%)</td>
<td>-0.9 (-1.7, -0.5)</td>
<td>-6 (-10, -2)</td>
<td>-0.8 (-0.19, 0.6)</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>-0.12 (-0.8, 0.3)</td>
<td>-6 (-34, 33)</td>
<td>-0.06 (-0.4, 0.5)</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>0.05 (-0.5, 0.3)</td>
<td>1 (-9, 7)</td>
<td>0.1 (-0.6, 0.3)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>-0.2 (-0.4, 0.2)</td>
<td>-6 (-10, 8)</td>
<td>-0.3 (-0.5, 0.0)</td>
</tr>
</tbody>
</table>

Change is calculated as six months – Baseline level. Results presented as median change (interquartile range)
Figure 5.4  Box and Whisker plot of change in serum C-reactive protein (baseline - 6 months) for exercise and control groups.

The solid black boxes represent the interquartile range for each group, with median values represented by a white line. The majority of values are encompassed by the whisker bars (1.5 times the interquartile range), with outliers indicated by dots. There was no difference in the change in CRP between the exercise and control groups (p=0.3).
Figure 5.5  Plot of change in C-reactive protein against change in fitness ($\text{VO}_2\text{max}$), comparing the exercise group (solid circles, ●) with the control group (open circle, ○).

There is no apparent interaction between improvement in fitness and CRP change. In particular, there is no decrease in CRP even with large improvements in fitness.
5.3 The Smoking Cessation Exercise Trial

Methods

5.3.1 Participants and study design

Serial measurement of inflammatory markers and other cardiovascular risk factors was performed on participants in a randomized controlled trial of exercise versus health education for smoking cessation. The effects of exercise on inflammatory marker levels are reported in this chapter. The effects of smoking cessation on inflammatory markers are reported in chapter 6.

Female smokers were chosen for this smoking cessation trial because smoking rates were increasing in young women (but not in young men) at the time the trial was conceived, and the relative risk of cardiovascular disease associated with smoking is higher in women than in men. Female smokers were recruited from the general population by newspaper advertisement. Women were eligible for inclusion if they were aged 18-65, had smoked at least five cigarettes per day for the last two years, were motivated to stop smoking and were in generally good health. Exclusion criteria included inability to undertake an exercise training program, pregnancy, chronic illness known to be associated with a systemic inflammatory response, a history of hypertension (systolic blood pressure > 160 mmHg, diastolic blood pressure > 90 mmHg), diabetes, valvular or cardiovascular heart disease, a history of dependence on alcohol or a non-nicotine substance within the past year, and current use of cardiovascular or psychotropic medication. Of the 166 participants who met the entry criteria and joined the smoking cessation trial, 152 took part in the inflammatory markers sub-study that is the subject of this report (figure 5.6). Twenty five percent of the women were on HRT (9%) or oral contraceptives (16%). Use of these medications remained constant throughout the study. No participants were on non-steroidal antiinflammatory agents (NSAIDs), aspirin, statins or other cardiovascular medications, since use of these agents was an exclusion criterion.

The study protocol was approved by the Auckland ethics committee and all participants gave written informed consent.
At baseline, participants were randomly assigned to either an exercise training group or to a health education group. The exercise group took part in three 45 minute supervised exercise sessions per week, and were free to choose from a range of equipment including treadmill, cycle and rowing ergometers. Heart rate was continuously monitored using Proform AccuRate wireless heart rate monitors (Icon Health and Fitness, Logan, Ut., USA). Exercise was maintained at the desired intensity by participants keeping their heart rate between 60-70% of their estimated maximum heart rate (as determined by baseline fitness testing) throughout the exercise session. The health education group, who also took part in three 45 minute sessions each week (consisting of group support and general lifestyle advice), were asked not to change their usual level of physical activity for the duration of the trial. The trial lasted 12 weeks, divided into two distinct phases. For the first six weeks participants of both groups were asked to continue smoking at their usual levels to allow attainment of cardiorespiratory fitness or group support prior to smoking cessation attempts. For the second six weeks, commencing with a preset “quit day”, smoking cessation was actively attempted (figure 5.6). At the “quit day” a second randomisation stratified by study group determined whether participants received nicotine patches or not. For those assigned to receive patches, the starting dose of 21 mg / day was continued throughout the remaining six weeks of the trial. The nicotine dose was then reduced to 14 mg / day for two weeks, 7 mg / day for two weeks, and then stopped.
Of the initial population of 166, 14 elected not to take part in this inflammatory markers sub-study. There were 152, 104 and 88 participants remaining in the trial at baseline, 6 weeks and 12 weeks respectively.

( = smoking quit day at 6 weeks).
5.3.2 Measurements

Assessment of physical activity and cardiorespiratory fitness

Participants reported their usual weekly physical activity at baseline using the validated questionnaire from the Nurses’ Health study (Appendix 3). Cardiorespiratory fitness was measured at baseline, 6 weeks and 12 weeks using the YMCA submaximal exercise test protocol. Under standardised conditions, participants exercised on a cycle ergometer at increasing workloads set according to their heart rate response. From the heart rates achieved at given workloads, fitness (maximal oxygen uptake, VO$_2$max) was calculated (Appendix 4).

Measurement of inflammatory markers and other cardiovascular risk factors

At each session throughout the 12 week trial, self-reported cigarette consumption over the previous 48 hours, exhaled carbon monoxide levels and weight were recorded. Weight was measured on a single calibrated set of scales, with participants dressed in light gym clothing without shoes.

At baseline, 6 weeks, and 12 weeks, salivary cotinine concentrations were measured, and non-fasting venous blood samples were collected for inflammatory markers and other cardiovascular risk factors. WBC, fibrinogen and cholesterol were measured at the time of collection, then samples were centrifuged at 3,000 rpm for 10 minutes at 4°C with the resultant serum and plasma stored at -80°C Celsius until analysis for inflammatory markers as a single batch blind to group and smoking status. In addition to WBC and fibrinogen, the following inflammatory markers were measured: CRP, sICAM-1, sCD40L. Details of the measurement of these inflammatory markers are given in appendix 1.

5.3.3 Statistical analyses

Baseline associations

Distributional properties of study variables were assessed with box plots and the Shapiro-Wilk statistic. For the baseline comparisons, activity score, CRP and sCD40L levels were not normally distributed and these variables were analysed using non-parametric tests. All other variables approximated normal distribution and were analysed using parametric tests. Baseline differences between the exercise and control groups were compared using an unpaired t-test,
or a Mann-Whitney U test for variables with a non-normal distribution (activity score, CRP, sCD40L). Spearman correlation coefficients were used to assess the association between inflammatory marker levels. Levels of each inflammatory marker, BMI, and smoking exposure were compared by tertiles of fitness using ANOVA (or Kruskal-Wallis rank ANOVA if not normally distributed). The associations between each inflammatory marker, fitness, and BMI were explored further using linear regression, with results reported as β-coefficient and 95% confidence intervals.

Effect of exercise
The difference in levels of inflammatory markers and other variables between the baseline and 6 week assessments in the exercise compared to the control group was the primary endpoint. Smoking exposure remained constant during this time. The difference between baseline and 12 week inflammatory markers in the exercise compared to the control group was a secondary endpoint. Because the change in variables was not normally distributed, the Mann-Whitney U test was used. The effect of exercise on inflammatory markers was also compared in a multivariate analysis using ANCOVA to adjust for baseline physical fitness, BMI, smoking intensity and inflammatory markers, and change in cigarettes per day between assessments.

All analysis was performed using SAS statistical software and a p value less than 0.05 was considered statistically significant.
Results

5.3.4 Study population

Baseline characteristics of the study population are described for subjects in the exercise training and health education groups in table 5.4. The two groups were well matched for age, BMI, physical fitness, smoking history, inflammatory marker levels, and all other variables. Withdrawals from the study during follow-up are summarised in figure 5.6. There was no significant difference in any baseline variable or inflammatory marker between subjects who did and did not withdraw from the study, either at the primary endpoint at 6 weeks (table 5.5) or at the secondary endpoint at 12 weeks (table 5.6).
Table 5.4  Baseline characteristics for women randomised to exercise and control groups in the smoking cessation trial.

<table>
<thead>
<tr>
<th></th>
<th>Exercise n=79</th>
<th>Control (Education) n=73</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>38 ± 12</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>71 ± 16</td>
<td>69 ± 15</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26 ± 6</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Activity, MET.hours/week</td>
<td>6 (1, 17)</td>
<td>8 (3, 15)</td>
</tr>
<tr>
<td>Fitness, VO₂ max, ml/kg/min</td>
<td>28 ± 7</td>
<td>29 ± 7.0</td>
</tr>
<tr>
<td>Years smoking</td>
<td>20 ± 11</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>16 ± 7</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>CO level, ppm *</td>
<td>19 ± 8</td>
<td>24 ± 10</td>
</tr>
<tr>
<td>Cotinine level, ng/ml *</td>
<td>199 ± 116</td>
<td>196 ± 115</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>5.3 ± 1.1</td>
<td>5.2 ± 1.1</td>
</tr>
</tbody>
</table>

**Inflammatory Markers**

<table>
<thead>
<tr>
<th></th>
<th>Exercise n=79</th>
<th>Control (Education) n=73</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, x 10⁹/L</td>
<td>8.9 ± 2.3</td>
<td>8.9 ± 2.5</td>
</tr>
<tr>
<td>Fibrinogen, μmol/l</td>
<td>10.0 ± 1.9</td>
<td>10.3 ± 2.0</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.9 (0.5, 3.8)</td>
<td>1.3 (0.8, 2.2)</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>182 ± 51</td>
<td>191 ± 82</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>1.9 (1.3, 2.8)</td>
<td>1.8 (1.1, 2.9)</td>
</tr>
</tbody>
</table>

Mean ± SD or median (interquartile range).

* See section 6.1.3 for a discussion of carbon monoxide (CO) and cotinine levels as measures of smoking exposure.
Table 5.5  Comparison of baseline characteristics for participants remaining in the study at six weeks (primary endpoint) and dropouts.

<table>
<thead>
<tr>
<th></th>
<th>Remained in study n=104</th>
<th>Dropouts n=48</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>38 ± 12</td>
<td>40 ± 11</td>
<td>0.30</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>69 ± 15</td>
<td>73 ± 17</td>
<td>0.22</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 ± 5</td>
<td>27 ± 6</td>
<td>0.21</td>
</tr>
<tr>
<td>Activity, MET.hours/week</td>
<td>8 (2, 17)</td>
<td>7 (1, 15)</td>
<td>0.25</td>
</tr>
<tr>
<td>Fitness, VO₂ max, ml/kg/min</td>
<td>29 ± 6</td>
<td>27 ± 8</td>
<td>0.06</td>
</tr>
<tr>
<td>Years smoking</td>
<td>21 ± 11</td>
<td>23 ± 11</td>
<td>0.37</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>16 ± 7</td>
<td>14 ± 6</td>
<td>0.09</td>
</tr>
<tr>
<td>CO level, ppm</td>
<td>21 ± 9</td>
<td>19 ± 10</td>
<td>0.55</td>
</tr>
<tr>
<td>Cotinine level, ng/ml</td>
<td>198 ± 118</td>
<td>203 ± 94</td>
<td>0.92</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.69</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>5.2 ± 1.1</td>
<td>5.3 ± 1.0</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Inflammatory markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC, x 10⁹/L</td>
<td>9.0 ± 2.3</td>
<td>8.8 ± 2.6</td>
<td>0.66</td>
</tr>
<tr>
<td>Fibrinogen, µmol/l</td>
<td>10.2 ± 2.1</td>
<td>10.5 ± 2.1</td>
<td>0.67</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.7 (0.7, 3.3)</td>
<td>1.1 (0.6, 2.4)</td>
<td>0.43</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>193 ± 76</td>
<td>172 ± 44</td>
<td>0.08</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>1.9 (1.1, 2.8)</td>
<td>2.2 (1.5, 2.9)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Mean ± SD or median (interquartile range).
Table 5.6  Comparison of baseline characteristics for participants remaining in the study at twelve weeks (secondary endpoint) and dropouts.

<table>
<thead>
<tr>
<th></th>
<th>Remained in Study n=88</th>
<th>Dropouts n=64</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>39 ± 12</td>
<td>39 ± 11</td>
<td>0.99</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>69 ± 15</td>
<td>72 ± 17</td>
<td>0.18</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 ± 5</td>
<td>27 ± 6</td>
<td>0.22</td>
</tr>
<tr>
<td>Activity, MET.hours/week</td>
<td>8 (3, 17)</td>
<td>7 (1, 15)</td>
<td>0.22</td>
</tr>
<tr>
<td>Fitness, VO₂ max, ml/kg/min</td>
<td>29 ± 7</td>
<td>27 ± 7</td>
<td>0.10</td>
</tr>
<tr>
<td>Years smoking</td>
<td>21 ± 11</td>
<td>21 ± 11</td>
<td>0.82</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>16 ± 6</td>
<td>14 ± 7</td>
<td>0.06</td>
</tr>
<tr>
<td>CO level, ppm</td>
<td>21 ± 9</td>
<td>18 ± 9</td>
<td>0.09</td>
</tr>
<tr>
<td>Cotinine level, ng/ml</td>
<td>208 ± 120</td>
<td>150 ± 84</td>
<td>0.08</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.77</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>5.2 ± 1.2</td>
<td>5.3 ± 1.0</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**Inflammatory markers**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, x 10⁹/L</td>
<td>8.9 ± 2.3</td>
<td>8.9 ± 2.5</td>
<td>0.87</td>
</tr>
<tr>
<td>Fibrinogen, µmol/l</td>
<td>10.0 ± 2.1</td>
<td>10.2 ± 1.8</td>
<td>0.78</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.5 (0.6, 3.3)</td>
<td>1.3 (0.7, 2.6)</td>
<td>0.49</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>195 ± 79</td>
<td>174 ± 47</td>
<td>0.07</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>1.9 (1.0, 2.8)</td>
<td>2.0 (1.4, 2.9)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Mean ± SD or median (interquartile range).
5.3.5 **Baseline associations**

There were modest correlations between levels of CRP, WBC count and fibrinogen at baseline (r between 0.32 and 0.45, p<0.01 for all) and weaker correlations between sICAM-1 and CRP, fibrinogen, and sCD40L (r between 0.18 and 0.21, p<0.05 for all). There was no significant association between sCD40L and any marker (r<0.15, p>0.05) other than sICAM-1.

Table 5.7 summarizes BMI, cigarette consumption and blood levels of inflammatory markers by tertile of physical fitness at baseline for all subjects. Greater fitness was associated with a lower BMI, whereas average cigarette consumption was the same for all levels of fitness. CRP and fibrinogen levels decreased as fitness increased, with a similar trend for WBC count. In contrast sICAM-1 and sCD40L levels showed no association with fitness.
Table 5.7  Interactions between physical fitness and body fat, smoking exposure and inflammatory marker’s levels.

Measures of body fat (weight, body mass index), smoking exposure (cigarette consumption, exhaled carbon monoxide levels, salivary cotinine levels) and blood levels of inflammatory markers are compared by tertile of physical fitness at baseline for all 152 subjects.

<table>
<thead>
<tr>
<th>Tertile of fitness (VO\textsubscript{2} max)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25.3</td>
<td>25.3 - 31.5</td>
</tr>
<tr>
<td><strong>BMI, kg/m\textsuperscript{2}</strong></td>
<td></td>
</tr>
<tr>
<td>29 ± 6</td>
<td>25 ± 3</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>79 ± 19</td>
<td>68 ± 10</td>
</tr>
<tr>
<td><strong>Cigarettes/day</strong></td>
<td></td>
</tr>
<tr>
<td>16 ± 7</td>
<td>15 ± 6</td>
</tr>
<tr>
<td><strong>CO, ppm</strong></td>
<td>0.80</td>
</tr>
<tr>
<td>21 ± 9.1</td>
<td>22 ± 9.6</td>
</tr>
<tr>
<td><strong>Cotinine, ng/ml</strong></td>
<td>0.15</td>
</tr>
<tr>
<td>220 ± 146</td>
<td>212 ± 104</td>
</tr>
</tbody>
</table>

**Inflammatory markers**

| WBC, x 10\textsuperscript{9}/l           | 0.06    |       |       |
| 9.3 ± 2.8                                | 8.7 ± 2.1| 8.4 ± 2.0|
| **Fibrinogen, µmol/l**                    |<0.001   |<0.001 |<0.001 |
| 10.9 ± 2.1                               | 10.0 ± 1.8| 9.4 ± 1.8|
| **CRP, mg/l**                            |<0.001   |<0.001 |<0.001 |
| 2.2 (1.0, 5.9)                           | 1.2 (0.7, 2.5)| 0.9 (0.5, 2.2)|
| **sICAM-1, ng/ml**                       | 0.95    |       |       |
| 190 ± 54                                 | 187 ± 89| 184 ± 61|
| **sCD40L, ng/ml**                        | 0.62    |       |       |
| 2.0 (1.4, 3.6)                           | 1.7 (1.1, 2.8)| 2.1 (1.4, 2.9)|

Mean ± SD or median (interquartile range)
Table 5.8 displays the association of each inflammatory marker with BMI and with fitness. CRP, fibrinogen and WBC count were associated with both BMI and physical fitness, but the strength of these associations was weak (figure 5.7). There was no association between either sICAM-1 or sCD40L and physical fitness or BMI. Differences in physical fitness accounted for ~10% of the difference in CRP ($r^2=0.11$) and fibrinogen ($r^2=0.08$), and differences in body mass index accounted for 10 to 20% of the difference in CRP ($r^2=0.18$) and fibrinogen ($r^2=0.12$). The associations between physical fitness and CRP, fibrinogen and WBC count were substantially weaker and no longer statistically significant after adjusting for BMI (table 5.8).
Table 5.8  Associations between body mass index, physical fitness, and blood or plasma level of each inflammatory marker at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Body Mass Index</th>
<th>Fitness</th>
<th>Fitness adjusted for BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β (95% CI)</td>
<td>p value</td>
<td>β (95% CI)</td>
</tr>
<tr>
<td>WBC</td>
<td>0.14 (0.07, 0.21)</td>
<td>&lt;0.001</td>
<td>-0.06 (-0.11, -0.00)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.05 (0.03, 0.07)</td>
<td>&lt;0.001</td>
<td>-0.03 (-0.04, -0.01)</td>
</tr>
<tr>
<td>Ln CRP</td>
<td>0.09 (0.05, 0.12)</td>
<td>&lt;0.001</td>
<td>-0.06 (-0.08, -0.03)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>-0.40 (-2.47, 1.67)</td>
<td>0.23</td>
<td>-0.62 (-2.33, 1.10)</td>
</tr>
<tr>
<td>sCD40L</td>
<td>-0.06 (-0.15, 0.04)</td>
<td>0.70</td>
<td>0.02 (-0.06, 0.09)</td>
</tr>
</tbody>
</table>
Although statistically significant, the association between LnCRP and fitness was modest, with fitness accounting for only ~10% of the difference in LnCRP between subjects. The association was strongly influenced by differences in body weight, and was no longer significant after adjusting for BMI.
5.3.6 Effect of exercise training

Primary endpoint

Changes in inflammatory marker levels and other variables between baseline and 6 weeks are compared for the exercise and control groups in table 5.9. Consistent with the study design, smoking was held steady and there was no change in daily cigarette consumption or cotinine levels during this time. There was a significant improvement in physical fitness in the exercise group and no change in the control group (11% versus 0% increase respectively, p<0.001). There was a small increase in body weight in both groups, more pronounced in the control group (1% versus 2% increase in the exercise and control groups respectively, p=0.001). Despite these changes, exercise had no effect on inflammatory marker levels. There was no difference in 6 week levels or the baseline to 6 week change of any inflammatory marker (WBC, fibrinogen, CRP, sICAM-1, sCD40L) between the exercise and control groups in the univariate analysis (P>0.05 for all). In multivariate analysis adjusted for physical fitness, body mass index, smoking intensity and inflammatory marker levels at baseline, and change in number of cigarettes/day between assessments, there was a small increase (of borderline statistical significance) in sICAM-1 (p=0.04), and a small decrease in sCD40L (p=0.03) for patients randomised to exercise training compared to controls, but no change in other inflammatory markers.
Table 5.9 Changes in fitness, weight, smoking and inflammatory marker levels at 6 and 12 weeks in the exercise and control (education) groups.

<table>
<thead>
<tr>
<th></th>
<th>Primary endpoint</th>
<th></th>
<th>Secondary endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change from baseline to 6 weeks</td>
<td>Change from baseline to 12 weeks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exercise</td>
<td>Control</td>
<td>p *</td>
</tr>
<tr>
<td>Fitness (VO₂max)</td>
<td>3.0 (0.9, 6.4)</td>
<td>0.2 (-1.7, 2.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>0.7 (-0.2, 1.5)</td>
<td>1.5 (1.1, 2.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>0 (-4, 1)</td>
<td>0 (-4, 1)</td>
<td>0.59</td>
</tr>
<tr>
<td>CO, ppm ‡</td>
<td>1 (-3.6)</td>
<td>-3 (-7, 2)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cotinine, ng/ml ‡</td>
<td>-1.4 (-53, 46)</td>
<td>-3 (-45, 24)</td>
<td>0.86</td>
</tr>
<tr>
<td>Inflammatory marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC, x 10⁹/l</td>
<td>0.4 (-0.6, 1.1)</td>
<td>0.4 (-0.5, 1.0)</td>
<td>0.99</td>
</tr>
<tr>
<td>Fibrinogen, µmol/l</td>
<td>0.3 (-0.6, 1.2)</td>
<td>0.3 (-0.9, 0.9)</td>
<td>0.41</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>0.0 (-0.6, 0.3)</td>
<td>0.0 (-0.5, 0.4)</td>
<td>0.50</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>28 (0, 61)</td>
<td>16 (-5, 53)</td>
<td>0.36</td>
</tr>
<tr>
<td>sCD40L, ng/ml</td>
<td>-0.4 (-1.8, 0.2)</td>
<td>-0.1 (-1.1, 0.6)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Results are presented as median change (interquartile range). A positive value represents an increase in level, a negative value represents a reduction in level.

* Univariate analysis, exercise versus control. Mann-Whitney U test.
† Multivariate analysis adjusted for fitness, body mass index, number of cigarettes smoked per day and inflammatory marker level at baseline, as well as change in number of cigarettes smoked per day between assessments. Analysis of Covariate (ANCOVA).
‡ See section 7.1.3 for a discussion of carbon monoxide (CO) and cotinine levels as measures of smoking exposure.
Secondary endpoint

Change in inflammatory markers between baseline and 12 weeks was treated as a secondary endpoint because changes in smoking exposure and nicotine patch use might influence inflammatory marker levels. There was a significant reduction in smoking from 6 to 12 weeks (table 5.9) which was the same for the exercise and control groups (average reduction of 10 cigarettes per day in both groups, \( p=0.84 \)). Abstinence rates for those remaining in the study were also similar (24/48, 50%, for the exercise group; 24/40, 60%, for the control group; \( p=0.7 \)). Therefore change in smoking remained balanced in the exercise and control groups. As will be reported in detail in Chapter 6, smoking cessation led to a small but significant reduction in WBC and fibrinogen but not CRP, sICAM-1 or sCD40L. Randomisation to nicotine patches was equal in both groups and nicotine patch use had no appreciable effect on inflammatory marker levels at 12 weeks.

Physical fitness improved in the exercise group at 12 weeks (17% increase from baseline, compared to no change in the control group, \( p<0.001 \), table 5.9). A small increase in body weight at 12 weeks was greater in the control group (+4%) compared to the exercise group (+2%, \( p<0.001 \)). At 12 weeks there was no difference in levels of any inflammatory marker, and no difference in the baseline to 12 week change in marker levels, between the exercise and control groups. Results were similar for univariate analysis and multivariate analysis which adjusted for baseline characteristics and change in smoking intensity between assessments (table 5.9).
5.4 Discussion

5.4.1 Effect of exercise on inflammatory marker levels

Both studies in this thesis show no effect of exercise training on inflammatory marker levels. In the exercise in the elderly study, six months’ exercise led to an 18% increase in cardiorespiratory fitness but did not affect CRP levels. In the smoking cessation exercise study, fitness also improved significantly (11% and 17% increases at 6 and 12 weeks respectively) but exercise did not lower levels of any of the five inflammatory markers studied either at 6 or 12 weeks. Results were similar following multivariate analysis which adjusted for fitness, BMI, number of cigarettes per day and the plasma level of the inflammatory marker at baseline, as well as change in the number of cigarettes per day between assessments. The borderline significance of change in sICAM-1 and sCD40L at 6 weeks on the multivariate analysis may be due to chance given the number of comparisons made. There were no baseline associations between levels of these markers and fitness in the cross-sectional analyses, and similar changes were not observed at the 12 week assessment.

The results of these two studies suggest that the associations seen in large cross sectional population studies between WBC, fibrinogen and CRP levels and physical fitness are largely dependent on differences in body weight, and that exercise does not have a direct antiinflammatory action in healthy individuals. The following observations support this conclusion.

1. In the smoking cessation exercise study only those markers associated with BMI (WBC, fibrinogen, CRP) showed an association with fitness. Markers unaffected by BMI (sICAM-1 and sCD40L) showed no association with physical fitness.

2. In the exercise in the elderly study body fat was a more important determinant of CRP levels than physical fitness (for which the weak association failed to reach statistical significance).

3. In the smoking cessation exercise study there was a strong inverse association between BMI and fitness, and the association of fitness with WBC, fibrinogen and CRP was
substantially weakened and not significant after adjustment for BMI. Although differences in BMI do not completely account for the association between inflammatory markers and fitness (in large epidemiological studies associations remain after adjustment for BMI)\textsuperscript{55,300,301} this may be because BMI is an imprecise measure of abdominal obesity rather than because exercise has an independent effect on inflammatory markers. Body fat, and visceral fat in particular, directly influence inflammatory marker levels through production of proinflammatory cytokines.\textsuperscript{26,27,82,83,302,303} Consequently small differences in body fat or in body fat distribution could lead to significant differences in inflammatory marker levels which would not be accounted for by adjustment for BMI. This is consistent with the observation in the exercise in the elderly study that CRP showed a stronger correlation with abdominal fat (assessed by DEXA scan) than with BMI.

4. The results reported in this thesis are consistent with other randomised studies of the effect of exercise training on CRP levels, published since this research was undertaken.\textsuperscript{315-318} Each individual trial reported exercise training had no significant effect on CRP levels, and a 2006 meta-analysis of the five randomised trials available (which included the published results from the exercise in the elderly study of this thesis)\textsuperscript{319} concluded that although exercise training improves measures of body composition and physical fitness, it does not lower CRP levels.\textsuperscript{320} Since this meta-analysis was undertaken, a further study of exercise training in nearly 200 sedentary overweight men and women has been reported.\textsuperscript{315} Consistent with the research in this thesis and other published data, 6 months aerobic exercise led to significant improvements in fitness and visceral adiposity but had no effect on CRP levels. Available data therefore suggest exercise does not have a direct effect on inflammatory marker levels.

5. Interventions aimed at reducing body fat do decrease inflammatory marker levels, contrasting with the lack of effect of exercise training in this thesis and other reported trials. At least three recent studies have shown a reduction in CRP following weight loss in overweight or obese women.\textsuperscript{310,321,322} In a prospective study of 83 healthy, obese women placed on a very low-fat, energy-restricted diet for twelve weeks, an average weight loss of 7.9 ± 0.3 kg led to a 26% reduction in CRP (from 5.6 ± 0.4 to 4.1 ± 0.4 mg/l, p<0.001), and
there was correlation between weight loss achieved and the change in CRP ($r=0.31$, $p=0.005$). Similarly, in a randomised trial of intensive lifestyle intervention (including dietary change and increased physical activity) involving 120 obese premenopausal women, there was a significant reduction in interleukin-6 and CRP levels in the intervention group, which was largely attributable to reduced BMI. Weight loss by diet alone has also been shown to reduce serum levels of CRP in postmenopausal women, with an average weight loss of $14.5 \pm 6.2$ kg (-15.6%, $p<0.0001$) over a one year period accompanied by a 32% reduction in median CRP (from 3.1 mg/l to 1.6 mg/l, $p<0.0001$) in 25 obese postmenopausal women studied by Tchernof et al. (2002).

Because of the potential for confounding from differences in body fat and other variables, a prospective randomized trial is the most reliable way to assess the effect of exercise on inflammatory marker levels. The studies in this thesis are among the largest randomised trials of exercise training on inflammatory markers to be reported. The lack of effect of exercise on inflammatory marker levels despite significant improvements in cardiorespiratory fitness was consistent across both the exercise in the elderly and the smoking cessation exercise studies and provides further evidence that exercise does not have an appreciable direct antiinflammatory effect in asymptomatic individuals.

5.4.2 Study limitations

The current studies have several potential limitations.

1. The smoking cessation exercise study was undertaken in a population of smokers. It is possible that the effect of smoking on inflammatory marker levels outweighed the beneficial effects of exercise training. However, the cardioprotective benefits of exercise are equally apparent in smokers and non-smokers, if these are attributable to a direct antiinflammatory effect of exercise then a reduction in inflammatory markers should occur regardless of smoking status. In this study exercise had no consistent effect on inflammatory marker levels either during the first six weeks of the trial when smoking was maintained or the second six weeks during smoking cessation. Results were unchanged in multivariate analysis which included adjustments for baseline smoking intensity and change
in smoking between visits. Therefore it seems unlikely an effect of exercise on CRP levels was masked by smoking status.

2. It is possible that inflammatory marker levels were not sufficiently elevated in these healthy populations to be affected by exercise. However the levels of inflammatory markers seen in these trials have been shown to be predictive of cardiovascular risk in apparently healthy individuals. Since there is a graded increase in cardiovascular risk with increase in levels of each marker it is important to know the effects of exercise on ‘normal levels’ of inflammatory markers in apparently healthy populations.

3. The effects of exercise may be more pronounced in patients with established cardiovascular disease, in whom inflammatory marker levels are elevated. Adamopoulos et al. (2003) reported that twelve weeks’ exercise reduced inflammatory cytokine levels in patients with heart failure. Exercise has also been reported to lower fibrinogen and CRP levels in patients with coronary artery disease. However because these studies were small or non-randomized further research is needed to confirm or disprove these findings.

4. It is also possible the duration or intensity of exercise in the present studies was insufficient to demonstrate changes in inflammatory markers. However, in epidemiology studies there is a continuous and graded association between exercise and inflammatory markers, with even low grade physical activity associated with lower marker levels than no activity. There was a similar improvement in VO\textsubscript{2}max in the current studies (17% at twelve weeks in the smoking cessation exercise study, 18% at six months in the elderly study) as in studies by De Souza et al.(2000) and Kraus et al.(2002) which reported improvements in endothelial function and plasma lipoproteins respectively with exercise training. In addition, the duration of training in both trials should have been sufficient to demonstrate any direct antiinflammatory effect of exercise, because the inflammatory markers studied have a short half-life and can be rapidly lowered with other interventions such as drug therapy. It is therefore likely that in the current studies exercise was of sufficient intensity and duration to influence inflammatory markers if exercise had direct antiinflammatory effects.
5. The difficulty of undertaking randomized clinical trials which include intensive exercise training is reflected in the high drop out rate in the smoking cessation exercise study in particular. Although unrecognized bias is possible, it is unlikely this could result in failure to detect an effect of exercise on inflammatory markers. Baseline characteristics and inflammatory marker levels were similar for subjects who did and did not drop out, and the number of drop outs was similar for the exercise and control group.

6. Finally, although the results of these trials are consistent with a meta-analysis of all available trials on exercise training and CRP levels, variability in levels of some inflammatory markers (sCD40L in particular) was such that larger clinical trials would be needed to identify or exclude a small decrease in inflammatory markers with exercise training.

5.4.3 Conclusions

The effect of exercise training on inflammatory marker levels was assessed in two randomized controlled trials. In the first study, 63 healthy elderly participants were randomized to either six months' exercise training or to a control group. Despite a significant improvement in fitness, exercise did not affect CRP levels. In the second study, 152 healthy female smokers were randomized to either 12 weeks' exercise or to a health education control group as part of a smoking cessation program. Smoking was held steady for the first six weeks, then smoking cessation was actively attempted. There was no difference in levels of any inflammatory marker (WBC, fibrinogen, CRP, sICAM-1, sCD40L) between the exercise and control group with either 6 or 12 weeks' exercise. These observations suggest that exercise training does not have a direct antiinflammatory action in healthy individuals. In addition, baseline associations between physical fitness and the inflammatory markers WBC, fibrinogen and CRP were largely explained by differences in body fat. These findings suggest the associations between greater physical fitness and lower inflammatory marker levels are at least partly explained by long-term regular exercise reducing body fat.
Chapter 6

Effects of Smoking Cessation on Inflammatory Marker Levels

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6.1 Introduction

6.1.1 Smoking and cardiovascular risk

Cigarette smoking is the leading preventable cause of cardiovascular disease and death in the Western world. The risk of myocardial infarction is substantially increased in all smokers, including those conventionally thought of as being at low risk, such as premenopausal women. Nonetheless cardiovascular risk decreases rapidly with smoking cessation and approaches that of non-smokers within 3-5 years, regardless of the intensity or duration of smoking or the age at which smoking cessation occurred. For example Rosenberg et al. (1990) showed that although female smokers aged 25-64 years had a 3 to 4-fold higher cardiovascular risk higher than non-smokers, this excess risk decreased rapidly with smoking cessation and was virtually abolished within 3 years of abstinence. The reasons why cardiovascular risk drops rapidly with smoking cessation are uncertain, but it has been suggested that a reduction in vascular inflammation could be responsible.

Despite these known risks of smoking and the benefits of smoking cessation, smoking rates in young women are not decreasing, and in some parts of the Western World have shown a slow but continual increase. This is of considerable concern given that heart disease is already the leading cause of death for women 35 or older, and success rates with smoking cessation are lower in female smokers than in males. New strategies are needed to combat these problems.
6.1.2 **Effect of smoking on inflammatory marker levels**

A number of inter-related mechanisms are likely to contribute to the excess cardiovascular risk associated with smoking, including vascular inflammation, vasomotor dysfunction, modification of lipid profile, and prothrombotic effects.\textsuperscript{331} Inflammation may be especially important amongst these, since the inflammatory response is a central component in the initiation, progression and complications of atherosclerosis. Consequently there has been recent interest and a growing body of literature on smoking and inflammation. A review of this literature supports the following conclusions:

1. Exposure to cigarette smoke causes inflammation on and within the arterial wall.\textsuperscript{332-335}

2. This triggers a systemic inflammatory response, with smokers having increased levels of a number of inflammatory markers compared to non-smokers.\textsuperscript{46,57,94,123,124,336-338}

3. The inflammation associated with smoking may be reversible upon smoking cessation, since in cross-sectional studies inflammatory marker levels are lower in ex-smokers than in current smokers.\textsuperscript{94,338,339} However cross-sectional studies provide only indirect evidence and are vulnerable to a number of potential confounding factors; prospective studies of smoking cessation are needed.

4. Relatively few prospective studies on the effect of smoking cessation on inflammatory marker levels have been performed. Although WBC and fibrinogen levels have been shown to fall rapidly with smoking cessation,\textsuperscript{59,61,340} there are only limited data and conflicting results for other markers.\textsuperscript{341-343}

The evidence behind these conclusions is reviewed in subsequent paragraphs.
Exposure to cigarette smoke causes inflammation within the arterial wall

A number of avenues of research suggest that smoking causes inflammation within blood vessels and provokes a significant inflammatory response. The pro-inflammatory changes in vascular tissue induced by smoking include increased monocyte-endothelial cell adhesion, increased platelet activation, altered endothelial-derived fibrinolytic and antithrombotic factors, and increased tissue factor expression. In addition, short periods of smoking appear to cause significant increases in levels of circulating inflammatory markers such as WBC and fibrinogen. Together, these studies provide good evidence of direct vascular injury from components of cigarette smoke, with a consequent inflammatory response.

Smoking is associated with a systemic inflammatory response

Several large observational studies have examined the association between smoking status and proatherogenic inflammatory marker levels. These studies, predominantly of men, have shown conclusive evidence of raised levels of inflammatory markers with smoking. WBC, fibrinogen, CRP and sICAM-1 levels are all significantly higher in smokers than in non-smokers. sCD40L may also be elevated with smoking, although current data are inconclusive.

One of the first large studies to demonstrate an association between smoking and raised inflammatory marker levels was the Multiple Risk Factor Intervention Trial (MRFIT, 1985). In this study, average WBC was significantly higher in smokers (n=3,920; average consumption 34 cigarettes/day) than in non-smokers (WBC 7.8x10^9/l vs. 6.1x10^9/l respectively, p<0.01). This report was followed by the Caerphilly and Speedwell Heart disease study (1987), in which inflammatory marker levels were compared by smoking habit in 4,759 men drawn from the general UK population. Both WBC and fibrinogen levels were significantly higher in smokers than in non-smokers, with levels increasing progressively according to cigarette consumption. In the same year, the Framingham study (examination 10, 1987) also demonstrated an association between smoking and raised fibrinogen levels, although the association was more marked in men (8.7 μmol/l versus 8.1 μmol/l, p<0.001) than in women (8.9 μmol/l versus 8.6
μmol/l, p=0.06), despite similar numbers of male and female smokers, with similar smoking habits. More recently attention has focused on CRP, with cross-sectional studies consistently showing an association between smoking and higher CRP levels. For example in 1,172 apparently healthy men from the Physician’s Health Study, CRP levels were higher in current smokers (geometric mean 2.0±2.7 mg/l) than in ex-smokers (1.3±2.5 mg/l) or in those who had never smoked (1.0±2.5 mg/l); p<0.001. In addition, among current smokers there was a significant positive association between CRP levels and the number of cigarettes smoked per day (Spearman rank coefficient 0.29, p<0.01). Similar results were seen for women in the Women’s Health Study. In a nested case-control group of 340 women from this study, there was a significant trend towards increasing levels of CRP across never (geometric mean CRP 2.4 mg/l), ex- (3.2 mg/l) and current smokers (3.6 mg/l), p=0.04. The association between CRP and smoking status remained after adjustment for several potential confounders including age, BMI, cholesterol, history of hypertension, alcohol use and exercise frequency.

The cell adhesion molecule sICAM-1 also appears to be influenced by smoking status, with a number of cross-sectional studies showing higher levels in smokers compared to non-smokers, and positive associations between the number of cigarettes smoked per day and sICAM-1 levels. For example, in the Women’s Health Study (2002), sICAM-1 levels were significantly higher in current smokers (median [IQR] 386 [309-484] ng/ml) than in non-smokers (285 [246-336] ng/ml); p<0.001. In contrast, few studies have looked at the association between sCD40L and smoking and the evidence for this marker is inconclusive. In one small study, Harding et al. (2004) reported a trend (p=0.07) towards higher sCD40L levels in 25 smokers compared to 25 non-smoking controls, but smoking status did not appear to influence sCD40L levels in ACS patients enrolled in the much larger CAPTURE trial (2003).

Few studies have included both men and women and examined several markers concurrently in the same cohort. The recently published MONICA Augsburg Survey 1994/95 (2003) was one such study comparing levels of a number of markers (WBC, fibrinogen, CRP, albumin and plasma viscosity) by smoking habit in 2,305 men and 2,211 women. For male smokers, the results were consistent with previously published cross-sectional studies. Male smokers had
significantly higher WBC (7.9x10^9/l versus 6.5x10^9/l, p<0.001), fibrinogen (8.8 μmol/l versus 7.9 μmol/l, p<0.001) and CRP levels (1.9mg/l versus 1.0 mg/l, p<0.001) than non-smokers. In addition there was clear evidence of a dose-response relationship, with number of cigarettes per day, duration of smoking and pack-year exposure all correlated with inflammatory marker levels. In contrast, the results for female smokers were at odds with previous studies. Although levels of both WBC and fibrinogen were higher in smokers compared to non-smokers, only WBC showed an association with number of cigarettes smoked per day. Surprisingly, CRP levels were similar in female smokers and non-smokers and showed no association with measures of smoking intensity. The reason why these results should differ for men compared to women are unclear, although the authors suggest that differences in smoking habit may play a role. They point to evidence of differing smoking habits for men compared to women and postulate that women may have a more variable exposure to the components of cigarette smoke due to the use of more low-yield cigarettes and to smaller and shorter inhalations when smoking.

In summary, in male smokers there is a strong body of evidence from cross-sectional studies that WBC, fibrinogen, CRP and sICAM-1 levels are all elevated by smoking. There are insufficient data to determine if sCD40L is similarly affected. For female smokers fewer studies have been published and consequently the evidence is less clear cut. Higher levels of WBC, fibrinogen, CRP and sICAM-1 have been reported in female smokers compared to non-smokers but the differences are less marked in women than in men, and the MONICA Augsburg Survey 1994/95 (2003) in particular failed to show a difference in CRP levels in female smokers compared to non-smokers, and failed to find a dose-response relationship between cigarettes smoked per day and levels of any inflammatory marker other than WBC.

**Inflammatory marker levels are lower in ex-smokers compared to current smokers**

Cross sectional studies also provide indirect evidence that the inflammatory response induced by smoking may be reversible with smoking cessation. Levels of WBC, fibrinogen, and CRP are all lower in male ex-smokers compared to current smokers. It is interesting to note however that while WBC and fibrinogen levels rapidly normalise upon smoking cessation, CRP levels may not reach those of non-smokers for many years. For women, study results are less
consistent. WBC and fibrinogen levels are lower in ex-smokers than in current smokers, but there are conflicting reports as to whether CRP levels are lower in female ex-smokers compared to current smokers. In the Women’s Health Study there was a significant trend towards increasing CRP levels across never, former and current smokers (2.4, 3.2 and 3.6 mg/l respectively, p=0.04), but the much larger MONICA Augsburg Survey 1994/95 failed to demonstrate any difference in CRP levels across never, former, and current female smokers (1.4, 1.4 and 1.5 mg/l respectively, p=0.25). The MONICA Augsburg Survey 1994/95 did not examine sICAM-1 levels, but in the Women’s Health Study this was also lower in ex- compared to current smokers (290 ng/ml vs. 386 ng/ml, p<0.001). It is not known whether sCD40L levels are lower in ex-smokers, as this has not been examined in previous trials.

These cross-sectional studies provide only indirect evidence of a link between smoking and raised inflammatory marker levels. Although the raised inflammatory marker levels seen in current smokers and the lower inflammatory marker levels seen in ex-smokers may be due to a direct pro-inflammatory effect of smoking cigarettes, it is also possible they are due to unrecognised confounding. For example, raised inflammatory markers may be due to an increased occurrence of chronic inflammatory diseases such as bronchitis, atherosclerosis or gingivitis caused by long-term cigarette smoking, which may not be clinically evident. Alternately the associations observed in epidemiological studies might be explained by other pro-inflammatory ‘lifestyle’ factors associated with cigarette smoking such as obesity and physical inactivity. Prospective studies demonstrating a rapid reduction in proatherogenic inflammatory marker levels with smoking cessation are needed to provide stronger evidence of a direct link between smoking and vascular inflammation.

Prospective trials of smoking cessation and inflammatory markers

There have been relatively few prospective trials on the effect of smoking cessation on inflammatory marker levels. In addition, most trials have been small and have only included WBC and/or fibrinogen, not other markers. In 1987 Ernst and Matrai reported a significant fall in WBC (7.4±2.0 x10^9/l to 6.2±1.7 x10^9/L, p<0.01) and fibrinogen (11.2±3.5 μmol/l to 7.1±2.9 μmol/l, p<0.01) in 14 healthy male smokers 8 weeks after smoking cessation. There was no change in WBC or fibrinogen levels in 17 controls who continued to smoke. Similar results were
demonstrated by Terres, Becker and Rosenberg (1994) who showed a significant fall in WBC from 7.8 to 6.3 x10^9/l (P<0.001) in 52 participants (56% female) after 12 weeks’ abstinence from smoking.\(^60\)

The largest study to date was published by Jensen et al. in 1998.\(^61\) WBC levels were serially measured in 160 smokers (95% male) through 26 weeks of successful smoking cessation (verified by measurement of exhaled carbon monoxide). WBC fell significantly with smoking cessation (from 7.6 to 6.1 x10^9/L, p<0.01), with most of the decrease occurring within the first six weeks.

For inflammatory markers other than WBC and fibrinogen, few studies have been performed. Only one prospective trial has looked at the effect of smoking cessation on CRP levels.\(^342\) In this study, CRP levels were measured in 30 participants before and after one year of validated smoking cessation, and in a control group of 30 ongoing smokers. Smoking cessation did not affect CRP levels, with no significant change in either group. The authors separately reported the effect of smoking cessation on sICAM-1 levels.\(^341\) In contrast to the lack of effect on CRP, smoking cessation caused a significant fall (-71.1 ng/ml, p<0.001) in sICAM-1 levels at one year. These findings were followed by a retrospective analysis of 68 participants (49% female) who successfully achieved one year’s abstinence in the Collaborative European Anti-Smoking Evaluation (CEASE) trial.\(^343\) sICAM-1 levels showed a rapid decline within four weeks of smoking cessation (from 300 ng/ml to 230 ng/ml, p<0.01), and then plateaued, with no further decrease from 4 to 52 weeks.

The effect of smoking cessation on sCD40L has not been reported, and no study has examined the effect of smoking cessation on a broad range of inflammatory markers simultaneously.

*Nicotine replacement therapy and inflammatory marker levels*

Nicotine replacement therapy (NRT) is common therapy for those attempting to quit smoking, and therefore, to interpret changes in inflammatory marker levels with smoking cessation, it is important to know whether NRT in itself has any effect on inflammatory markers. To this end Jensen et al.\(^61\) performed a sub-analysis of their 1998 study to determine whether NRT (used by 44% of participants) influenced the fall in WBC levels seen in this trial. Overall, WBC fell
regardless of nicotine use, although the authors reported a weak inverse relationship between the cumulative amount of nicotine replacement during the trial and the decrease in WBC ($r=0.26$, $p<0.05$). From this they concluded that nicotine use blunted the fall in WBC associated with smoking cessation. However at least two other trials have found no effect of NRT on WBC or fibrinogen levels. Blann, Steele and McCollum (1997) measured WBC and fibrinogen levels in 18 smokers at three separate time points; at baseline (while still smoking), after successful smoking cessation using NRT, and again at least three weeks after ceasing NRT. From this they concluded that nicotine use blunted the fall in WBC associated with smoking cessation. However at least two other trials have found no effect of NRT on WBC or fibrinogen levels. Blann, Steele and McCollum (1997) measured WBC and fibrinogen levels in 18 smokers at three separate time points; at baseline (while still smoking), after successful smoking cessation using NRT, and again at least three weeks after ceasing NRT. 348 Both WBC and fibrinogen fell significantly with smoking cessation and there was no further decrease upon ceasing nicotine replacement therapy. Similarly, in a randomised controlled trial of NRT for smoking cessation, Ludviksdotter et al. (1999) showed a significant decrease in WBC (from 8.4 to $6.6 \times 10^9$/L, $p<0.001$) in 46 participants who successfully achieved three months’ abstinence; the decrease was identical in patients with and without NRT. 349 Finally, in the Collaborative European Anti-Smoking Evaluation (CEASE) trial (which showed a significant fall in sICAM-1 levels with smoking cessation), 343 half the participants were on NRT and half on placebo. The decrease in sICAM-1 levels was unaffected by the use of NRT. These reports suggest that NRT has minimal if any effect on the fall in inflammatory markers following smoking cessation.

6.1.3 Measures of smoking cessation

Self-reports of smoking status alone are generally considered inadequate for clinical research, both because of the possibility that the reports may be inaccurate or untruthful, and because variations in smoking habit from person to person (number of puffs taken, depth and duration of inhalations) leads to marked differences in nicotine intake per cigarette smoked. 350-352 The unreliability of self-reported smoking status is particularly relevant to smoking cessation trials, where participants may feel pressure to report abstinence even if it has not been achieved. 350,351 Therefore a number of biochemical measures that reflect smoking exposure are commonly employed in smoking cessation trials; these include exhaled carbon monoxide, carboxyhaemoglobin, thiocyanate, nicotine and cotinine. 350-352 Of these tests, exhaled carbon monoxide (CO) is the most convenient and cotinine is the most accurate; 350,351 therefore these measures were used to verify smoking cessation in the current study.
Exhaled carbon monoxide levels are an indirect, non-invasive measure of the blood carboxyhaemoglobin (%COHb) formed in response to cigarette smoke inhalation.\textsuperscript{353} Levels remain elevated for up to 24 hours following smoking exposure,\textsuperscript{354} and a level of 10ppm has been shown to have the highest sensitivity and specificity in distinguishing smokers from non-smokers.\textsuperscript{350,355} Exhaled CO levels of <10 ppm have therefore been used to verify smoking cessation in several recent smoking cessation trials.\textsuperscript{356-358}

Cotinine is the main metabolite of nicotine, and has been widely used as a biomarker of tobacco exposure.\textsuperscript{359-361} It has an elimination half-life of ~17 hours, which means that levels remain elevated for 3-4 days following smoking exposure.\textsuperscript{352} In smokers it is detectable in a variety of body fluids including blood, urine, saliva, amniotic fluid, and cervical mucus.\textsuperscript{360} Although blood cotinine levels are considered the ‘gold-standard’ for detecting smoking exposure, cotinine levels in saliva have been shown to be highly correlated with blood levels and the two can therefore be used interchangeably.\textsuperscript{361} Salivary cotinine levels therefore offer a convenient non-invasive measure of tobacco exposure\textsuperscript{350,351} which has been used in a number of trials to validate smoking cessation; a cut-off of <10ng/ml accurately verifies abstinence for the last 3-4 days.\textsuperscript{352}

Although the presence of a raised cotinine level has been shown to be a reliable indicator of smoking, there is not a close correlation between the number of cigarettes smoked per day and the absolute cotinine level.\textsuperscript{360} This may be due to differences in smoking habit (for example, variations in the amount of tobacco smoke inhaled) or due to differences in nicotine and cotinine metabolism.\textsuperscript{351,352,360} Because of this, use of the absolute cotinine level as a measure of smoking intensity is not recommended by some authors\textsuperscript{360} although others contend that cotinine levels may reflect the biologic effects of tobacco exposure better than the number of cigarettes smoked and are therefore a valid quantitative measure of smoking.\textsuperscript{352} In addition, cotinine cannot be used to verify smoking cessation for patients on nicotine replacement therapy because cotinine is derived from the metabolism of nicotine and levels are therefore elevated with nicotine patch use.\textsuperscript{362} Indeed, a number of trials have used cotinine levels to confirm the adequacy of nicotine replacement therapy, although the ideal cut-points for this purpose are yet to be defined.\textsuperscript{363-365} In the current study, cotinine was used to verify abstinence at the 6 and 12
month follow-up visits, but could not be used for this purpose at the end of the intervention phase at 12 weeks, since half the participants were on nicotine replacement therapy at this point.

6.1.4 Potential clinical applications

Despite widespread knowledge of the harmful effects of smoking, many individuals find it difficult to stop smoking and even more difficult not to start again. 70-80% of those who try to stop relapse within 1 year, and the failure rate is higher in women than in men.312 One of the major hurdles to keeping off smoking is that the negative effects such as symptoms of nicotine withdrawal and weight gain occur rapidly, whereas health benefits seem distant and speculative. The will to keep off smoking could be strongly reinforced if asymptomatic individuals could receive positive feedback from tests showing them that their health is improving as a result of giving up smoking.312 Measurement of inflammatory markers has been proposed for this.7,37 WBC and fibrinogen levels have been shown to be elevated proportional to cigarette exposure and to fall rapidly with smoking cessation. However as both WBC and fibrinogen are labile markers whose levels can vary markedly from test to test, it is unlikely they will prove clinically useful for monitoring smoking cessation on an individual level. The more stable or more specific markers such as CRP, sICAM-1 or sCD40L included in this study may prove more useful in this respect, and may shed light on the specific aspects of the immune response most influenced by smoking and smoking cessation.
6.1.5 Summary

Cigarette smoking is the leading preventable cause of cardiovascular disease and death in the Western world. In experimental models smoking causes pro-inflammatory changes in vascular tissue. There is also limited clinical evidence, derived predominantly from epidemiological studies of male smokers, linking smoking to raised levels of proatherogenic inflammatory markers. Compared to non-smokers, smokers have higher circulating levels of a number of inflammatory markers including white blood cell count,\textsuperscript{46,57,58} fibrinogen,\textsuperscript{57,58,338} CRP\textsuperscript{93-97,339,344} and sICAM-1.\textsuperscript{123,124,128,339,345} However we can not be certain that these raised levels are due to direct proinflammatory vascular effects of cigarette smoking. Prospective studies demonstrating a rapid reduction in proatherogenic inflammatory marker levels with smoking cessation would provide stronger evidence of a direct link between smoking and vascular inflammation, but few such studies have been performed and the data for markers other than WBC and fibrinogen are inconclusive. Therefore a prospective study was performed to determine whether smoking cessation leads to decreased levels of a range of inflammatory markers associated with cardiovascular risk. WBC, fibrinogen, CRP, sICAM-1 and sCD40L were measured before and 6 weeks after attempted smoking cessation in 138 healthy female smokers participating in a clinical trial of smoking cessation strategies.
6.1.6 Study aims and research questions

The general aims of this research are to determine the effect of smoking cessation on a broad range of inflammatory markers linked to future cardiovascular risk, and to identify markers that, by reflecting the health benefits of smoking cessation, may help encourage individuals to make and maintain this lifestyle change.

The following research questions will be addressed:

1. Is there an association between baseline levels of the inflammatory markers WBC, fibrinogen, CRP, sICAM-1 and sCD40L and measures of smoking intensity (number of cigarettes smoked per day, cotinine levels, pack year smoking experience) in female smokers?

2. Does 6 weeks smoking cessation lower blood levels of the inflammatory markers WBC, fibrinogen, CRP, sICAM-1 and sCD40L? If so, does it have a proportionally equal effect on all inflammatory markers or does it affect some markers but not others?

3. If a change in inflammatory marker levels with smoking cessation does occur, is it sufficiently predictable to consider clinical use of inflammatory markers to demonstrate the health benefits of abstinence in smoking cessation programs?
6.2 Methods

6.2.1 Participants and study design

Inflammatory markers were measured in participants of the smoking cessation trial outlined in chapter 5.3. In summary, 166 female smokers aged 15-65 were randomized to either 12 weeks’ exercise training or health education. Participants were asked to continue smoking at their usual level for the first 6 weeks, then from a preset “quit day” after the 6 week assessment, smoking cessation was actively attempted. At the “quit day” participants were also randomized to a nicotine patch (21mg/day) from week 6 to week 12, or to no nicotine. This randomisation was stratified according to baseline group, to ensure that nicotine patch use was balanced in the exercise and education groups. The nicotine dose was then reduced and stopped over one month.

For analysis of the effects of smoking cessation all participants were followed up, including those who had withdrawn from the smoking cessation program. Consequently data were available for 138 participants who provided blood samples and smoking status at baseline, 6 weeks and 12 weeks. This included the 88 participants who completed the 12 week exercise or health education programme, and a further 50 participants (36%) who had withdrawn by 12 weeks.

The 88 participants who completed the programme were contacted again for follow-up visits and repeat blood samples 6 and 12 months after baseline. All except two participants at 6 months, and an additional one at 12 months completed late follow-up.

6.2.2 Assessment of cigarette consumption

At baseline, current smoking habits (number of cigarettes per day) and cumulative smoking exposure (pack years smoking, defined as [average number of cigarettes per day / 20] x number of years smoked) were recorded.

Participants attended three times per week throughout the 12 week trial. At each visit they reported the number of cigarettes smoked each day since the previous visit, and carbon monoxide levels were measured in end expiratory air after a 20 second breath-hold, using a
piCO Smokerlyzer hand-held analyser (Bedfont Scientific, Rochester, UK). In addition, salivary cotinine concentrations were measured at baseline, 6 weeks, 12 weeks, and at the 6 and 12 month follow-up visits. Participants were asked to chew a cotton roll for 1 minute at least half an hour after eating or drinking, and the samples were immediately frozen at -20°C until analysis as a single batch at study end. Each sample was analysed in duplicate and the average of the two measurements was used to define smoking status (Salimetrics, State College, PA, USA).

Smoking abstinence at 12 weeks was defined as self-reported continuous abstinence with carbon monoxide readings <10 ppm at all visits after quit day. Abstinence at six and 12 months was additionally confirmed by measurement of salivary cotinine levels <10 ng/ml.

6.2.3 Statistical analyses

The distributional properties of the study variables were assessed using the Shapiro-Wilk statistic and visually with box and whisker and normal probability plots. Since CRP was positively skewed, statistical tests were conducted using natural log transformed (Ln) values, but for ease of interpretation un-transformed values of CRP are presented in the results.

Spearman rank correlation coefficients were used to consider the association between baseline smoking and inflammatory markers.

Paired t-tests were used to compare inflammatory marker levels before and after smoking cessation for those who achieved complete abstinence at 12 weeks. The number of smokers maintaining complete abstinence at 6 and 12 months were too small to allow assessment of the longer term effects of smoking cessation. In a separate analysis, the association between change in smoking, change in cotinine levels, and change in inflammatory markers was assessed for each participant, including those unsuccessful with smoking cessation. Data from six months and one year were included and analysis of covariance was used to account for the serial observations per subject.

All analyses were performed using SAS release 8.0 statistical software and a p-value of 0.05 was considered statistically significant.
6.3 Results

6.3.1 Success with smoking cessation

48 of the 138 participants (35%) achieved verified complete smoking abstinence between quit day and completion of the intervention phase of the trial 6 weeks later.

The baseline data were analysed at the end of the study to determine whether those participants who successfully stopped smoking had any characteristics, or combination of characteristics, that were different from the group who were unsuccessful (table 6.1). Participants who went on to achieve smoking cessation had lower baseline carbon monoxide levels and a trend towards lower cotinine levels than those who were unsuccessful, despite smoking the same number of cigarettes per day. Otherwise the baseline characteristics of participants who did and did not achieve smoking abstinence were similar. Success with smoking abstinence was the same for subjects randomised to exercise training compared to health education \((p=0.7)\), but there was higher abstinence for subjects randomised to nicotine compared to no nicotine patches \((p = 0.04)\).

Of the 138 participants upon whom this analysis is based, 88 (64%) had completed the smoking cessation programme and 50 (36%) had dropped out. There was no difference in any baseline characteristic (including age, cigarettes per day, years smoked, and inflammatory marker levels) between those who completed the 12 week programme and those who had not, but there were higher abstinence rates in those completing the programme \((45\% \text{ vs. } 16\%, p<0.001)\). The 88 participants who completed the smoking cessation programme were followed up at 6 and 12 months. In this group, abstinence was maintained in 17 (19%) participants at 6 months and in 14 (16%) at 12 months.
Table 6.1  Baseline characteristics for all study participants according to smoking status at 12 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Not smoking</th>
<th>Continued smoking</th>
<th>$P$ *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=138</td>
<td>n = 48</td>
<td>n = 90</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>39 ± 12</td>
<td>38 ± 12</td>
<td>39 ± 12</td>
<td>0.49</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>26 ± 5</td>
<td>27 ±10</td>
<td>26 ± 5</td>
<td>0.94</td>
</tr>
<tr>
<td>Cigarettes / day</td>
<td>15 ± 6</td>
<td>15 ± 6</td>
<td>16 ± 7</td>
<td>0.64</td>
</tr>
<tr>
<td>Years smoked</td>
<td>21 ± 11</td>
<td>21 ± 10</td>
<td>22 ± 11</td>
<td>0.52</td>
</tr>
<tr>
<td>Fitness †</td>
<td>29 ± 7</td>
<td>29 ± 7</td>
<td>29 ± 7</td>
<td>0.90</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.25</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.8 ± 0.9</td>
<td>4.9 ± 1.0</td>
<td>4.8 ± 0.7</td>
<td>0.26</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>5.3 ± 1.1</td>
<td>5.2 ± 1.2</td>
<td>5.3 ± 1.0</td>
<td>0.77</td>
</tr>
<tr>
<td>CO level, ppm</td>
<td>22 ± 9</td>
<td>19 ± 7</td>
<td>23 ± 10</td>
<td>0.03</td>
</tr>
<tr>
<td>Cotinine level, ng/ml</td>
<td>198 ± 115</td>
<td>178 ± 110</td>
<td>214 ± 118</td>
<td>0.16</td>
</tr>
<tr>
<td>Randomised to exercise</td>
<td>74 (54%)</td>
<td>24 (50%)</td>
<td>50 (56%)</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Mean ± SD or n (%)

* For comparison between not smoking and continued smoking groups.

† VO$_2$max, ml/kg/min
6.3.2 Baseline associations

Baseline associations between intensity of smoking (cigarettes per day, salivary cotinine levels, pack year smoking exposure) and blood levels of each inflammatory marker are presented in table 6.2. Measures of current smoking exposure (number of cigarettes per day and salivary cotinine levels) showed good agreement with each other ($r=0.58$, $p<0.001$). sICAM-1 levels showed the strongest association with measures of smoking exposure. sICAM-1 correlated with number of cigarettes smoked each day ($P=0.01$), salivary cotinine levels ($p<0.01$), and pack year exposure ($p=0.04$). WBC count and fibrinogen levels also showed a weak association with numbers of cigarettes each day, with a similar trend for CRP, but were not significantly correlated with salivary cotinine levels. sCD40L levels showed no association with smoking.
**Table 6.2** Associations between inflammatory marker levels and the intensity of smoking at the baseline assessment.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Number of cigarettes/day</th>
<th>Salivary cotinine level</th>
<th>Pack years smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$p$</td>
<td>$r$</td>
</tr>
<tr>
<td>WBC</td>
<td>0.20</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.19</td>
<td>0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>CRP</td>
<td>0.16</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.23</td>
<td>0.01</td>
<td>0.34</td>
</tr>
<tr>
<td>sCD40L</td>
<td>0.12</td>
<td>0.17</td>
<td>0.02</td>
</tr>
<tr>
<td>Cotinine</td>
<td>0.58</td>
<td>&lt;0.001</td>
<td>...</td>
</tr>
</tbody>
</table>
6.3.3 Effect of abstinence from smoking

Smoking abstinence was confirmed by carbon monoxide readings <10 ppm at all visits after quit day. 48 individuals achieved verified smoking cessation. For these individuals, average carbon monoxide levels were 19 ppm prior to quit day, and 3 ppm at 12 weeks. For those not on patches, cotinine levels were 180 ± 77 ng/ml prior to quit day and 2 ± 3 ng/ml at 12 weeks, while for those on patches cotinine levels were 185 ± 121 ng/ml prior to quit day and 117 ± 84 ng/ml at 12 weeks (63% nicotine replacement). \(^{364,365}\)

Inflammatory marker levels were compared before and after smoking cessation for these 48 participants who had not smoked during the six weeks after the quit day. Abstinence from smoking was associated with a significant decrease in WBC count and fibrinogen (table 6.3). In contrast there was no significant difference between smoking and non-smoking levels of CRP, sICAM-1, and sCD40L, although variation in levels of these markers was higher than for WBC count and fibrinogen which could potentially have masked a minor effect of smoking cessation. Even for WBC and fibrinogen, the response at the individual level was highly variable. Although on average there was a 6-7% reduction in WBC and fibrinogen levels with smoking cessation, In 35% of participants the WBC increased with smoking cessation, and the magnitude of change varied from a 93% increase to a 34% fall (figure 6.1). The variability of response was similar for fibrinogen.

In terms of other risk factors, smoking cessation led to a small but statistically significant increase in BMI of 1 kg/m\(^2\) but had no effect on serum glucose levels.
Table 6.3 Comparison of inflammatory marker levels and other baseline variables before and after smoking cessation in the 48 subjects who achieved complete abstinence.

<table>
<thead>
<tr>
<th></th>
<th>Smoking Level</th>
<th>Non smoking level</th>
<th>Absolute change</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, ( \times 10^9/\text{l} )</td>
<td>8.9 ± 2.2</td>
<td>8.2 ± 2.0</td>
<td>-0.7 ± 1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen, µmol/\text{l}</td>
<td>10.0 ± 2.1</td>
<td>9.4 ± 2.1</td>
<td>-0.6 ± 1.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CRP, mg/\text{l}</td>
<td>1.4 (0.7, 3.7)</td>
<td>1.5 (0.7, 4.3)</td>
<td>0.1 (-0.2, 0.9)</td>
<td>0.08</td>
</tr>
<tr>
<td>sICAM-1, ng/\text{ml}</td>
<td>202 ± 61</td>
<td>219 ± 65</td>
<td>17 ± 75</td>
<td>0.12</td>
</tr>
<tr>
<td>sCD40L, ng/\text{ml}</td>
<td>2.3 ± 2.0</td>
<td>2.7 ± 2.2</td>
<td>0.4 ± 2.1</td>
<td>0.21</td>
</tr>
<tr>
<td>CO level, ppm</td>
<td>19 ± 2</td>
<td>3 ± 2</td>
<td>-18 ± 7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cotinine (patches) *</td>
<td>185 ± 121</td>
<td>117 ± 84</td>
<td>-82 ± 93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cotinine (no patches) *</td>
<td>180 ± 77</td>
<td>2 ± 3</td>
<td>-165 ± 69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/\text{m}^2</td>
<td>27 ± 10</td>
<td>28 ± 11</td>
<td>1 ± 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose, mmol/\text{l}</td>
<td>4.9 ± 0.8</td>
<td>5.0 ± 0.8</td>
<td>0.1 ± 0.8</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SD or median (interquartile range). ‘Smoking level’ is the average of baseline and 6 week (pre-quit) levels. ‘Non smoking’ level is the level at 12 weeks.

* Cotinine levels (ng/\text{ml}) are presented separately for participants achieving abstinence who were randomised to nicotine replacement therapy (‘patches’, \( n=26 \)) or no nicotine replacement therapy (‘no patches’, \( n=22 \)).
Figure 6.1 Comparison of the change in WBC ($x10^9/l$) from baseline to study end for the 48 individuals who achieved abstinence and the 90 individuals who did not.

Although the mean reduction in WBC was greater in those who achieved abstinence (-0.7 ± 1.2 versus -0.2 ± 1.8, $p=0.03$), there was considerable variability in the WBC change in both groups. There was a large overlap in the change in WBC for those who achieved abstinence and those who did not, limiting the usefulness of WBC as a clinical marker to encourage smoking abstinence.
6.3.4 Effect of change in smoking intensity

Many individuals reduced smoking but did not achieve complete abstinence at 12 weeks. Therefore the effect of smoking on inflammatory marker levels was further explored by examining whether inflammatory marker levels were affected by changes in smoking and nicotine exposure (cigarettes per day, salivary cotinine levels) for all 138 trial participants, regardless of whether they achieved smoking cessation or not. The correlations between change in the number of cigarettes smoked each day, change in salivary cotinine levels, and change in the level of each inflammatory marker for all participants are presented in table 6.4. Data from 6 and 12 month follow-up were included and analysis of covariance was used to account for the serial observations per subject.  

A decrease in smoking exposure (number of cigarettes per day, salivary cotinine level) was associated with a decrease in WBC count and fibrinogen (p<0.001 for both), but there was no association between change in smoking exposure and change in CRP, sICAM-1 or sCD40L levels.

6.3.5 Effect of exercise and nicotine patches

As detailed in chapter 5, there was no statistically significant change in the blood level of any inflammatory marker by randomisation to exercise training compared to health education, or randomisation to a nicotine patch compared to no nicotine patch. Twenty five percent of the women were on HRT (9%) or oral contraceptives (16%), and use of these medications remained constant throughout the study. Neither HRT nor oral contraceptive use influenced smoking cessation rates. Use of other medications such as aspirin or non-steroidal antiinflammatory agents was an exclusion criterion and no participants used these medications during the study.
Table 6.4  Associations between change in smoking, change in cotinine levels, and change in inflammatory marker levels for all 138 subjects.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Number of cigarettes/day</th>
<th>Salivary cotinine level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$p$</td>
</tr>
<tr>
<td>WBC</td>
<td>0.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>0.02</td>
<td>0.66</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>sCD40L</td>
<td>0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>
6.4 Discussion

In this prospective study of smoking cessation in healthy female smokers, there was a small but statistically significant decrease in WBC count and plasma fibrinogen levels 6 weeks after stopping cigarette smoking. In addition for all participants, whether abstinent or not, there was a positive association between change in smoking (cigarettes per day, salivary cotinine levels) and change in WBC and fibrinogen levels. In contrast, change in smoking or smoking cessation had no consistent effect on CRP, sICAM-1 or sCD40L levels.

6.4.1 Baseline associations

There were weak baseline correlations between the number of cigarettes per day and WBC, fibrinogen, CRP and sICAM-1 levels, but only sICAM-1 showed significant association across all three measures of smoking exposure (cigarettes per day, salivary cotinine levels, pack years). Although a number of large cohort studies have reported dose-dependent relationships between the number of cigarettes smoked each day and WBC, fibrinogen, CRP, and sICAM-1, these findings predominantly relate to male smokers. The relationship between smoking and inflammatory markers in female smokers is less evident. Neither CRP nor fibrinogen were associated with cigarettes per day for women in the MONICA Augsburg Survey 1994/95, and in the Framingham study (examination 10, 1987) the effect of smoking on fibrinogen levels was considerably stronger for men (p<0.001) than for women (p=0.06). In the Women's Health Study there was only a weak association between CRP and smoking (with a stronger correlation for sICAM-1). These observations are consistent with the current study. There is evidence to suggest that smoking habit is different in women, with smaller and shorter inhalations resulting in less exposure to inhaled smoke than in men for a given number of cigarettes per day, which could explain why the association between inflammatory markers and smoking is stronger for men than for women. These differences disappear in heavy smokers (greater than 20 cigarettes per day).

There are few reports on the effect of smoking on sCD40L levels. In the CAPTURE study (2003) there was no association between current smoking status and sCD40L levels. Harding
et al. (2004) have reported increased sCD40L expression in smokers compared to non-smokers, but it is not clear if the association was gender specific because this information was not included in their report.\textsuperscript{116} sCD40L showed no association with smoking in the current study.

6.4.2 Smoking cessation

The rapid reduction in WBC and fibrinogen levels that occurred within 6 weeks of smoking cessation in this study is consistent with previous epidemiological studies and smoking cessation trials.\textsuperscript{31,59} WBC and fibrinogen appear to reflect a direct, rapidly reversible inflammatory response to smoking. These markers may predominantly reflect reversible airways inflammation, given previous reports that lung function and WBC are inversely related in smokers and that smoking cessation leads to a simultaneous improvement in lung function and decrease in WBC.\textsuperscript{61}

In contrast, smoking cessation had no appreciable effect on CRP, sICAM-1 and sCD40L levels. Variability in levels of these inflammatory markers was much higher than for WBC or fibrinogen (table 6.3) and consequently a modest effect of smoking cessation cannot be excluded. However the lack of effect was consistent across all measures of smoking change (smoking cessation, change in number of cigarettes per day, change in salivary cotinine levels).

The findings for CRP are consistent with epidemiological studies in which CRP levels remain elevated for many years after smoking cessation,\textsuperscript{58,347,367} and with a small prospective trial in which CRP was unchanged by 1 year of validated smoking cessation.\textsuperscript{342} Serum levels of CRP may predominantly reflect the consequences of smoking on atherosclerosis or other diseases that are not rapidly reversible with smoking cessation.\textsuperscript{18} In comparison, the observation that sICAM-1 did not decrease with smoking cessation is surprising given the strong baseline associations between sICAM-1 and smoking exposure, and is at odds with the findings of the Collaborative European Anti-Smoking Evaluation (CEASE) trial, in which sICAM-1 levels decreased within 4 weeks of smoking cessation.\textsuperscript{343} There are several possible explanations for our findings. First, sICAM-1 may, like CRP, reflect long term effects of smoking and levels may not be rapidly reversible with smoking cessation. Second, sICAM-1 levels in the current study population may not have been elevated sufficiently by smoking to demonstrate a significant fall
with smoking cessation. The baseline median sICAM-1 level of 202 ng/ml is considerably lower than levels seen in smokers in the Women’s Health Study\textsuperscript{339} and in the CEASE trial.\textsuperscript{343} Finally, variability in plasma levels of sICAM-1 in the current study was large and this may have limited the statistical power of the study to detect modest effects of smoking cessation.

The current study is the first prospective trial to examine the effect of smoking cessation on sCD40L levels. Smoking cessation was not associated with any significant change in sCD40L levels, and there was no evidence of a trend towards lower sCD40L levels with smoking cessation.

6.4.3 Implications for public health

Despite widespread knowledge of the harmful effects of smoking, most smokers find it difficult to stop. In the current study only 35% of women achieved 6 weeks’ abstinence and only 16% remained continuously abstinent at one year, a low success rate but one consistent with abstinence rates using nicotine replacement therapy in other studies.\textsuperscript{312,356,357} One aim of the current study was to determine whether one or more inflammatory markers known to be associated with increased cardiovascular risk could be measured as a simple objective indicator of the benefits of stopping smoking, giving positive feedback to encourage people to stay off smoking. However while WBC and fibrinogen decrease after smoking cessation, the small size of the effect (8-15%) and large individual variability of these and other inflammatory markers suggest that serial measurement of inflammatory markers will not be useful for this purpose (see figure 6.1, page 191).

6.4.4 Study strengths and limitations

We studied female smokers because at the time the study was performed smoking rates were increasing in young women,\textsuperscript{312} the relative risk of cardiovascular disease associated with smoking is higher in women than in men,\textsuperscript{313} and little is known of the effects of smoking and smoking cessation on inflammatory markers in this population. Previous studies suggest the association between smoking and some inflammatory markers may differ by gender,\textsuperscript{58,338,367} and
therefore the results of smoking cessation trials involving male smokers may not be applicable to women.

This is the first prospective study to directly compare the effect of smoking cessation on several inflammatory markers linked to cardiovascular risk. However, significant variability in circulating levels of sICAM-1 and sCD40L over time reduced the statistical power of the study to exclude modest changes in levels of these markers with smoking cessation. Nonetheless the lack of effect of smoking cessation on sICAM-1 and sCD40L (table 6.3, page 190) is supported by a similar lack of effect of change in smoking intensity on these markers (table 6.4, page 193). There were no associations between change in smoking exposure (number of cigarettes per day, salivary cotinine levels) and change in sICAM-1 or sCD40L for all 138 participants.

There are a number of potential confounding factors in this study, such as the use of exercise training, nicotine replacement therapy, and changes in body fat with smoking cessation. Participants were randomized to exercise training or health education for 6 weeks before and 6 weeks after the smoking quit day, and it is possible this exercise training could have affected inflammatory marker levels. However there was no difference in levels of any inflammatory marker, or in success with smoking abstinence, between the exercise and control groups at study end. It is therefore unlikely the exercise training influenced the observed changes in inflammatory markers with smoking cessation. Similarly it is unlikely that nicotine patch use influenced the study findings. Changes in inflammatory marker levels were similar for subjects randomized to a nicotine patch or no patch, both for the whole study cohort and when analysis was repeated for subjects who achieved complete abstinence. This is consistent with most previous studies, which have also found no association between use of nicotine replacement and inflammatory marker levels.\textsuperscript{340,348} In addition, serum levels of inflammatory markers are influenced by obesity, and it is possible that weight gain after smoking cessation could increase blood levels of some inflammatory markers. In the current study the reduction in WBC count and fibrinogen occurred despite a small increase in body weight.

Finally, because the number of participants not smoking at 6 and 12 months was small there was insufficient data for analysis of the effect of longer periods of smoking cessation on
inflammatory marker levels. Further study is required to determine the longer term effects of smoking cessation on inflammatory marker levels in women.

6.4.5 Conclusions

Although a number of inflammatory markers have been linked to cigarette smoking in epidemiological studies, in this prospective trial only WBC count and fibrinogen levels fell significantly with smoking cessation. Smoking cessation did not lead to a reduction in levels of other inflammatory markers linked to cardiovascular risk (CRP, sICAM-1, sCD40L), despite strong baseline associations between smoking intensity and sICAM-1 in particular. The vascular inflammation associated with smoking may not be rapidly reversible with smoking cessation, and changes in WBC and fibrinogen may reflect a decrease in pulmonary inflammation rather than changes in the vasculature. This could be tested by further research directly comparing changes in lung function, lung injury, vascular function, and vascular injury with changes in inflammatory marker levels during smoking cessation.

Finally, it has been proposed that measurement of inflammatory markers could be used to encourage lifestyle changes such as smoking cessation. However, even for WBC count and fibrinogen, individual responses to stopping smoking were variable, limiting their value for monitoring and encouraging the health benefits associated with abstinence.
Chapter 7

Summary and Conclusions

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7.1 Overview

The key role that inflammation plays in all stages of atherosclerotic coronary artery disease has been increasingly recognized throughout the last decade.\(^4\) This has stimulated intense interest in the study of serological markers of inflammation, several of which have been shown to be predictive of future cardiovascular events across the spectrum of atherosclerotic disease progression. These include systemic markers of inflammation such as WBC,\(^{48}\) fibrinogen\(^{48}\) and CRP,\(^{80}\) and locally produced mediators of inflammation detectable at low levels in the systemic circulation such as sICAM-1,\(^{123}\) sCD40L,\(^{119}\) and PAPP-A.\(^{33}\) Beyond being merely associated with risk, the addition of inflammatory markers to traditional risk factors has been reported to improve upon conventional risk prediction models.\(^{373,374}\) However, it is still not clear whether measurement of these inflammatory markers makes a meaningful difference to patient management decisions, leads to better patient outcomes, and ultimately, is cost-effective. Some of the outstanding issues include:

1. **Clarification of the full range of clinical scenarios in which measurement of inflammatory markers are, or are not, predictive of outcomes.**

   Although inflammatory markers appear to be predictive of cardiovascular events in a range of situations including in asymptomatic individuals,\(^{80,119}\) people with stable angina,\(^{93,187}\) and acute coronary syndrome patients,\(^{39,112,120}\) it is not clear whether they are predictive of outcome in other clinical settings such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). The underlying mechanisms that drive adverse outcomes (in which restenosis predominates) following PCI differ from the mechanisms that drive de novo coronary events. However, pathology studies suggest inflammation is a central component in both scenarios.\(^{22,157}\) Therefore the associations between inflammatory marker levels and PCI complications are assessed in this thesis.
2. **Uncertainty regarding the clinical relevance of any increased risk predicted by inflammatory markers.**

The clinical relevance of risk prediction using inflammatory markers has not yet been demonstrated. Independent associations between inflammatory markers and cardiovascular risk at a population level do not automatically translate to clinical benefit at an individual level.\(^8\) To determine clinical relevance, it is also necessary to show that the addition of inflammatory markers to established cardiovascular risk algorithms leads to reclassification of risk for a substantial proportion of individuals, and that this reclassification triggers changes in treatment decisions that result in improved clinical outcomes.\(^{375}\) Until we know that the use of inflammatory markers appropriately targets treatments to those at most risk and consequently leads to a reduction in cardiovascular events, widespread use of inflammatory marker measurement cannot be recommended.\(^7,255\) No inflammatory marker yet fulfils these criteria.\(^{376}\)

3. **Uncertainty regarding means by which increased risk associated with inflammation can be attenuated.**

It is not known whether vascular inflammation (as measured by inflammatory markers) is a modifiable risk factor which can be reliably reduced by interventions or therapies. Although a number of interventions (such as statin therapy,\(^{377}\) fibrates,\(^{118}\) exercise training\(^{378}\) and smoking therapy\(^{378}\)) have been postulated to have antiinflammatory properties, few have been adequately tested in prospective or randomised trials.

4. **Lack of evidence that reduction of inflammatory markers leads to better clinical outcomes.**

It remains to be shown whether lowering inflammatory marker levels, in the absence of concurrent beneficial changes in conventional cardiovascular risk factors such as lipid profile, reduces cardiovascular risk.\(^7,255\) Research is underway to identify novel agents which have direct antiinflammatory actions within the atherosclerotic plaque, but results thus far have been disappointing.\(^{22}\) To date, the most promising agents for lowering inflammatory marker levels have proven to be statins. These are already one of the most widely used medicines worldwide because of their potent lipid lowering actions, with associated
substantial reduction in cardiovascular events and mortality.$^{379}$ At a population level, statins can be shown to lead to modest reductions in levels of at least some inflammatory markers.$^{224}$ However, the magnitude and predictability of this effect, and its independence of lipid lowering, remain in doubt.$^{257}$

5. **Uncertainty over which marker(s) to measure.**

Finally, if inflammatory markers do prove useful in the clinical setting, the question remains as to which inflammatory marker(s) to measure. Several different inflammatory markers have been shown to be predictive of cardiovascular events, yet few trials have directly compared the predictive value of one marker against another. CRP has many advantages over other markers, including low biological variation in the absence of overt inflammation (leading to reproducibility of results over weeks or months), stability in stored serum or plasma, and widespread availability of standardized high-sensitivity assays with low coefficients of variation.$^{62}$ Consequently CRP shows the greatest promise for use in clinical practice.$^{7}$ Nonetheless measurement of other markers such as sCD40L or PAPP-A may improve risk prediction above measuring CRP alone,$^{33,112}$ and in the research setting measurement of a range of markers reflecting different aspects of the inflammatory process may give some insight into the underlying pathophysiological processes. For this reason, a range of inflammatory markers (WBC, fibrinogen, CRP, sICAM-1, sCD40L, PAPP-A) were studied in this thesis.
A substantial amount of ongoing research will be required to finally address all of these issues and determine whether measurement of inflammatory markers should move from the research setting into standard clinical practice. The research in this thesis was performed to add incremental knowledge to our overall understanding of inflammatory markers in cardiovascular disease, by addressing the following specific questions:

- Are inflammatory markers predictive of adverse outcomes (cardiac events, restenosis) following percutaneous coronary intervention?

- Is inflammation a modifiable cardiovascular risk factor? Can inflammatory marker levels be predictably lowered by:
  
  a. Medication (lipid-modifying therapy using simvastatin or bezafibrate)?
  
  b. Lifestyle modification (exercise training, smoking cessation)?

The major findings from this research are summarised in the following sections.
7.2 Summary of the findings in this thesis

7.2.1 Inflammatory markers and percutaneous coronary intervention

Chapter 3 of this thesis examines whether measurement of inflammatory markers at the time of PCI is predictive of subsequent adverse outcomes, including restenosis and cardiovascular events.

PCI is a very effective and widespread treatment for coronary artery disease in both stable angina and acute coronary syndrome patients, with over a million procedures performed worldwide each year. Nonetheless, PCI is not without its complications. Restenosis is the most prevalent and problematic of these, and despite knowledge of a number of anatomical, procedural and clinical features associated with an increased likelihood of its occurrence (including vessel size, lesion length, and presence of diabetes or an acute coronary syndrome), it remains difficult to predict. Basic science, animal and human pathology studies all suggest inflammation is an important component of the restenotic process, and therefore it seems logical to suppose detection of an increased inflammatory state within the atherosclerotic plaque by raised inflammatory markers in the bloodstream at the time of PCI would be predictive of subsequent restenosis. This is not adequately addressed in the literature, where reports are either conflicting (for markers such as CRP), or lacking (for other markers such as sICAM-1, sCD40L, and PAPP-A).

The research presented in Chapter 3 examines the effect of PCI on a range of inflammatory markers (CRP, sICAM-1, sCD40L, and PAPP-A) in 133 stable angina patients undergoing elective PCI, and assesses associations between levels of these inflammatory markers and subsequent adverse outcomes. Inflammatory markers were measured before, and 48 hours, 1 week, and 1 month after, PCI, and levels at these timepoints were compared against 30 day clinical events, six month angiographic restenosis, and two year cardiovascular event rates. A number of conclusions can be drawn from this research:
PCI leads to a significant increase in levels of a broad range of inflammatory markers.

PCI is known to elicit a 3-4-fold rise in CRP levels, peaking 48-72 hours post-procedure.\(^{169,181}\) However, there are few reports of the effect of PCI on other markers. In this study, PCI lead to a significant rise in several different inflammatory markers, with CRP, sCD40L and PAPP-A levels all elevated 48 hours post-procedure (P<0.001 for all). sICAM-1 levels were also elevated by PCI, but this was a less immediate response with elevated levels first detected one month post-procedure. Interestingly, the acute-phase reactant CRP peaked rapidly following PCI, falling by one week and back to baseline levels by one month, indicating a pronounced but short-lived systemic inflammatory response to PCI. In contrast, levels of local mediators of inflammation such as sCD40L and PAPP-A were still progressively rising 1 month post-PCI. These markers are postulated to have a direct role in the post-PCI remodelling process that can lead to restenosis,\(^{43,179}\) and their prolonged elevation through to 1 month may indicate ongoing inflammation at the tissue level for at least 1 month post-procedure. However, this is speculative and its clinical relevance is uncertain.

Pre-PCI CRP levels are not predictive of restenosis in stable angina patients undergoing elective PCI. This lack of association is not a consequence of masking of inflammation through concomitant medication use.

Previous studies of CRP and restenosis have shown conflicting results. Initial reports suggested that CRP levels were predictive of restenosis,\(^{165,166}\) but these studies were flawed by inclusion of a mixed population of stable and unstable angina patients that may have introduced unrecognised confounding. More recent studies limited to stable angina populations have all failed to show an association between pre-PCI CRP levels and restenosis.\(^{167-169}\) However, it has been questioned whether these negative results may have been a consequence of masking of the underlying inflammation through near-universal use of concomitant medications postulated to have antiinflammatory effects, such as statins, thienopyridines and glycoprotein Iib/Illa inhibitors.\(^{16}\) The research in this thesis confirms that there is no association between pre-PCI CRP levels and restenosis in stable angina patients, and importantly it extends knowledge in this area by demonstrating that this lack of
association is not due to statin, thienopyridine or glycoprotein IIb/IIIa inhibitor use. The randomised nature of the trial meant that half the patients received simvastatin 40mg daily and half received placebo for a median two months prior to PCI. There was no association between inflammatory markers and restenosis in either group. In addition, no patients in the study received glycoprotein IIb/IIIa inhibitors or thienopyridines as the study was conducted before these agents were widely available at our institution. This is an important aspect of this research which could not be repeated in the current era of almost universal thienopyridine and statin use in PCI patients.

3. Post-PCI CRP levels are not predictive of restenosis in stable angina patients.

Given that PCI results in an inflammatory response associated with a 3-4 fold rise in CRP levels, and that in pathology studies this inflammatory response appears to be a driving factor for restenosis, it is plausible that post-PCI CRP levels may be more predictive of restenosis than pre-PCI levels. Indeed a large study by Dibra et al. (which included 1,800 patients with both stable and unstable angina) showed an association between the post-PCI rise in CRP and subsequent restenosis. However, this may have been largely driven by the inclusion of unstable angina patients, since when analysis was restricted to patients with stable angina (n= 1,321) the results were of only borderline significance (p=0.05). Other studies which have examined the association between post-PCI CRP and restenosis in stable angina patients have included only small numbers of patients and have shown conflicting results. In this thesis, CRP levels were measured 48 hours, 1 week and 1 month post-PCI and compared against 6 month angiographic restenosis rates. Despite PCI causing an inflammatory response characterised by a significant rise in CRP levels, there was no association between post-PCI CRP levels at any timepoint and restenosis. This demonstrates that in stable angina patients, post-PCI CRP levels are no more predictive of restenosis than pre-PCI levels.

4. Other inflammatory mediators more directly implicated in the restenotic process (sICAM-1, sCD40L and PAPP-A) do not predict restenosis following PCI for stable angina.

CRP is a non-specific systemic marker of inflammation, reflecting inflammation anywhere throughout the body. Other inflammatory mediators are thought to contribute more directly
to the restenotic process, with sCD40L preventing endothelialisation of the injured vessel wall and promoting local inflammation,\textsuperscript{43} and both sICAM-1 and PAPP-A facilitating the development of intimal hyperplasia.\textsuperscript{127,179} Measurement of these markers might therefore predict restenosis better than CRP, but this has not been previously investigated. For these reasons, measurement of sCD40L, sICAM-1 and PAPP-A levels were included in the research reported in chapter 3.

Regardless of whether levels were measured pre-PCI or 48 hours, 1 week or 1 month after PCI, no association was seen between levels of sICAM-1 or sCD40L and restenosis. There was a weak trend towards increased restenosis rates with higher PAPP-A levels at 1 week and 1 month, but this should be interpreted with caution given the number of comparisons made. No association was seen for the PAPP-A/proMBP ratio, or for PAPP-A at other timepoints. Therefore despite the possible involvement of these inflammatory mediators in the restenotic process, neither pre- nor post-PCI circulating levels of sICAM-1, sCD40L and PAPP-A predicted restenosis following PCI for stable angina.

Given the lack of association between any inflammatory marker (regardless of measurement before or after PCI) and restenosis, further study is required to determine whether inflammation truly contributes to the development of restenosis, or is merely an epiphenomenon.

5. The association between inflammatory marker levels at the time of PCI and subsequent clinical events could not be adequately assessed.

Early (30 day) and late (two year) clinical events following PCI are caused by different pathological processes from those that cause restenosis.\textsuperscript{185} Early clinical events are dominated by procedural issues (e.g. vessel dissection, abrupt vessel closure, stent malaposition), and late clinical events are predominantly caused by de novo rupture of unstable atherosclerotic plaque with superimposed thrombus formation. The association between inflammatory markers and clinical events therefore requires separate assessment from restenosis.
For this thesis, the occurrence of periprocedural complications and subsequent 2 year events were compared against pre-PCI and 48 hour post-PCI levels of each marker. Events were separated into those occurring within the first 30 days post-PCI (almost all of which represent PCI complications) and those which occurred over the subsequent 2 years. Any differences in event rates according to inflammatory marker levels were then assessed.

Thirteen events (all periprocedural MI) occurred within the first 30 days, and the occurrence of these events was unaffected by inflammatory marker levels. Thirty one clinical events occurred in the subsequent 2 years (two deaths, two MIs and 27 target vessel revascularization procedures). There was a trend towards increasing 2 year event rates (p=0.09) with increasing levels of CRP pre-PCI, but similar trends were not seen for other markers. There were no associations between post-PCI levels of any inflammatory marker and 2 year event rates.

Because the event rates were low in this stable angina population, the study was underpowered to detect a modest association between inflammatory markers and clinical events. The trend towards increased 2 year events with raised CRP levels is likely to represent a true association rather than be due to chance, given that CRP is predictive of clinical events in a number of other settings, and that two previous studies have reported similar associations between pre-PCI CRP and subsequent cardiac events.\textsuperscript{167,192} Larger trials will be needed to assess the association between other markers such as sCD40L, sICAM-1 and PAPP-A and both 30 day and 2 year events. Nonetheless in light of the low event rates reported in the current study, the clinical utility of such markers for predicting events may be limited even if a modest association is found.
7.2.2 Effects of lipid modifying agents on inflammatory marker levels

Chapter 4 of this thesis examines the effects of lipid lowering agents (simvastatin and bezafibrate) on inflammatory marker levels, to determine whether they have broad and predictable antiinflammatory effects.

The predominant mechanism of action of statins is a rapid and striking reduction in LDL cholesterol levels. Nonetheless, a number of studies have suggested that not all the benefits of statins are accounted for by LDL cholesterol lowering alone.\textsuperscript{212,213} This has focused interest on possible non lipid lowering or ‘pleiotropic’ effects of statins.\textsuperscript{118} There is now strong evidence that statin use is associated with a reduction in circulating CRP levels,\textsuperscript{222-224} which has been taken as evidence that statins have direct antiinflammatory properties.\textsuperscript{377} However, a plausible alternative explanation is that the reduction in CRP with statin therapy is an indirect consequence of decreased inflammation within the vessel wall caused by cholesterol lowering (including reduction in atherogenic lipid particles other than LDL cholesterol). Salient to this is the observation that other LDL cholesterol lowering agents such as ezetimibe, with quite different mechanisms of action from statins, also reduce CRP levels.\textsuperscript{277}

In this thesis, blood samples were analysed on two separate groups of stable angina patients from the FAST trial to determine the effect of lipid lowering therapies on inflammatory marker levels. Levels of CRP, fibrinogen, sICAM-1, sCD40L, and PAPP-A were measured at baseline and after two year’s therapy in 215 participants randomised to simvastatin or placebo, and in 100 participants (with higher baseline cholesterol levels) randomised to simvastatin or bezafibrate. From this, a number of conclusions could be drawn:

1. Statins lower CRP levels, but the effect is modest and, on an individual level, unpredictable.

   Simvastatin led to significant reductions in both LDL cholesterol and homocysteine (by ~40% and 13% respectively) in both groups. Simvastatin also led to an overall reduction in CRP levels, but the effect was modest, with a 0.1 mg/l (5%) reduction with simvastatin vs. placebo. In addition, the CRP response to statin therapy was very variable. Less than half the patients had a decrease in CRP levels on simvastatin therapy, and levels \textit{increased} in
nearly a quarter. This contrasts with the predictable reduction in LDL cholesterol levels seen with simvastatin (figure 4.5, page 117).

2. **Statins do not have appreciable effects on other inflammatory markers.**

Previous studies of the effect of statins on inflammatory marker levels have concentrated on CRP; the effects of statins on locally active inflammatory markers such as sICAM-1, sCD40L and PAPP-A have only been studied in small trials, with conflicting results.\(^{115,120,237,241}\) Evidence of action against a broader range of markers would provide further support for a generalised anti-inflammatory effect of statins. However, the research presented in this thesis does not suggest statins have broad anti-inflammatory effects, since simvastatin had no effect on levels of other inflammatory markers (fibrinogen, sICAM-1, sCD40L and PAPP-A). It should be noted that variability in some markers (sICAM-1 and sCD40L) limited the ability to reliably detect treatment effect on these markers, but the results are consistent with most previous reports.\(^{120,228,239,241}\)

3. **Bezafibrate has no appreciable effect on inflammatory marker levels.**

Fibrates have been postulated to have anti-inflammatory effects related to their binding of the nuclear receptor peroxisomal proliferation activating receptor-α (PPAR-α), which regulates the expression of a number of genes implicated in atherogenesis and plaque stability.\(^{118}\) However, although bezafibrate led to a modest reduction in LDL cholesterol (median reduction of 15%) and had favourable effects on HDL cholesterol levels (median increase 12%), it had no effect on levels of CRP or other inflammatory markers in the research conducted for this thesis. This is consistent with other recent reports and suggests bezafibrate does not have appreciable anti-inflammatory effects *in vivo.*

4. **Statins do not attenuate the inflammatory response triggered by PCI.**

Agents with direct anti-inflammatory effects, such as methylprednisolone, have been shown to significantly attenuate the acute-phase response during acute coronary syndromes, with a rapid and marked reduction in CRP levels within 48 hours.\(^{244}\) Statins have also been postulated to have direct anti-inflammatory effects, but their influence on the acute-phase response to vascular injury has not been previously investigated. PCI offers a convenient
model to examine this, since it causes a discrete vascular injury that is followed by a measurable inflammatory response.\textsuperscript{157} For this thesis, the inflammatory marker response to PCI was compared for 53 patients on simvastatin and 46 patients on placebo. Simvastatin had no effect on post-PCI levels of any inflammatory marker (CRP, sICAM-1, sCD40L, or PAPP-A) whether measured 48 hours, 1 week, or 1 month post-PCI.

These results suggest that neither simvastatin nor bezafibrate have major antiinflammatory effects \textit{in vivo}. Bezafibrate did not affect levels of any inflammatory marker studied, and while treatment with simvastatin had a modest and variable effect on CRP levels, it had no effect on levels of other inflammatory markers. In addition pre-treatment with simvastatin had no effect on the acute inflammatory response to PCI. This contrasts with the broad, rapid and marked reduction in inflammatory markers seen with direct antiinflammatory agents such as methylprednisolone.\textsuperscript{244} Methylprednisolone, however, has no effect on cardiovascular event rates.\textsuperscript{244} It has therefore been suggested CRP reduction may be analogous to tightening your belt to reduce waist circumference; it lowers the measurement but does nothing to the underlying risk.\textsuperscript{380} The mechanisms(s) that underlie the modest, variable reduction in CRP with statin therapy, and the relevance of this reduction to the cardiac benefits seen with statin therapy, require further study.
7.2.3 Effects of exercise training on inflammatory marker levels

Chapter 5 of this thesis examines whether inflammatory marker levels can be lowered through exercise training.

Although there is strong evidence that regular physical activity reduces the risk of cardiovascular disease, the mechanisms by which this occurs are not well understood. Exercise has favourable effects on a number of cardiovascular risk factors, but in epidemiological studies only about one third of the risk reduction associated with increased physical activity is explained by adjustment for conventional risk factors. Given that inflammation is an important component of atherosclerosis, it has been suggested that some of the benefits of exercise may derive from an antiinflammatory effect. This is supported by observational studies in which increasing physical activity or cardiorespiratory fitness has been associated with progressively lower levels of inflammatory markers such as WBC, CRP and fibrinogen. However, as in all observational studies, it is possible these associations are due to unrecognized confounding. Prospective and preferably randomized trials would provide more reliable evidence of the effect of exercise on inflammatory marker levels.

Two randomized controlled trials assessing the effect of exercise training on inflammatory marker levels were undertaken for this thesis. The first study involved 63 healthy elderly participants aged 60-85, randomized to either six months’ exercise training or to a control group. Fitness, CRP levels, and total body fat (measured by DEXA scan) were compared at baseline and at six months. In the second study, 152 healthy female smokers were randomized to either 12 weeks’ exercise training or to a health education control group as part of a smoking cessation program. The primary endpoint was change in inflammatory marker levels (WBC, fibrinogen, CRP, sICAM-1, sCD40L) from baseline to 6 weeks, during which time smoking rates were held steady. Change in inflammatory marker levels from baseline to 12 weeks was a secondary endpoint (over the second half of the 12 week period smoking levels decreased, potentially affecting inflammatory marker levels, but the reduction in smoking proved equal between the exercise and control groups through this time).
From this research, the following conclusions can be drawn:

1. **Baseline levels of inflammatory markers are more strongly associated with body fat than with cardiorespiratory fitness.**

   In the exercise in the elderly study, there was a moderately strong baseline association between CRP levels and percent body fat (assessed by whole body DEXA scan), and a stronger inverse association between percent body fat and physical fitness (p ≤ 0.001 for both). In contrast, there was only a weak trend towards lower CRP levels with increasing cardiorespiratory fitness (p=0.09).

   In the smoking cessation exercise trial, body fat (assessed by BMI) also showed a strong inverse relationship with fitness (p<0.001). Inflammatory markers affected by body fat (WBC, fibrinogen, CRP) were lower with increasing fitness levels, but these associations were not significant after adjustment for BMI. In addition, there were no associations between levels of markers unaffected by body fat (sICAM-1, sCD40L) and baseline cardiorespiratory fitness.

   Although large epidemiological studies have reported that the associations between increasing levels of physical activity or fitness and lower levels of inflammatory markers remain significant after adjusting for BMI, this may be because BMI is a relatively poor measure of body fat. Visceral fat in particular has a major influence on inflammatory marker levels through production of proinflammatory cytokines, and consequently small differences in body fat could lead to significant differences in inflammatory marker levels which would not be accounted for by adjustment for BMI. This is consistent with the observation in the exercise in the elderly study that CRP showed a stronger correlation with abdominal fat (assessed by DEXA scan) than with BMI.

2. **Inflammatory marker levels do not decrease with exercise training.**

   In the exercise in the elderly study, six months’ exercise training led to an 18% increase in cardiorespiratory fitness (p<0.01), but had no effect on CRP levels (median change of -0.12 mg/l for the exercise group and -0.06 mg/l for the control group, p=0.30).
In the smoking cessation exercise trial, there was also a significant improvement in fitness with exercise (11% and 17% at 6 and 12 weeks respectively, p<0.001 for both), but no change in inflammatory marker levels at either 6 or 12 weeks.

The duration of exercise in these trials should have been sufficient to demonstrate that exercise training lowers inflammatory marker levels, if that is a mechanism by which exercise is cardioprotective. Cardiovascular risk has been shown to decrease after only short periods of exercise training, otherwise cardiovascular risk factors improve rapidly with exercise training, and inflammatory markers themselves can be rapidly lowered with other interventions such as drug therapy.

The results of these studies are consistent with other randomised studies of exercise training published recently, all of which reported that exercise training had no significant effect on CRP levels. These findings contrast with the effect of weight loss therapies on inflammatory marker levels, where a number of studies have shown significant decreases in inflammatory marker levels with reduction in body fat.

Taken together, these observations suggest that the associations seen in large cross sectional population studies between WBC, fibrinogen and CRP levels and physical fitness are largely dependent on differences in body weight, and that exercise does not have a direct antiinflammatory action in healthy individuals.
7.2.4 Effects of smoking cessation on inflammatory marker levels

Chapter 6 of this thesis examines whether inflammatory marker levels can be lowered through smoking cessation.

Cigarette smoking remains the leading preventable cause of cardiovascular disease and death in the Western world, with smokers having a two- to three-fold increase in heart attack rates compared to non-smokers.\(^6\) Thankfully, cardiovascular risk decreases rapidly with smoking cessation and approaches that of non-smokers within 3-5 years.\(^327-329\) The increased risk associated with smoking is thought to be attributable least in part to vascular inflammation caused by the components of cigarette smoke,\(^332-335\) which may be reversible upon smoking cessation. Smokers have increased levels of a number of inflammatory markers compared to non-smokers, including WBC, fibrinogen, CRP and sICAM-1.\(^46,57,84,123,124,338,339\) Cross-sectional studies have also shown levels of these markers to be lower in ex-smokers than current smokers,\(^94,338,339\) but such studies are vulnerable to a number of potential confounding factors and provide only indirect evidence that smoking causes an elevation in inflammatory marker levels that is reversible with smoking cessation. More definitive evidence would come from prospective studies which assess the effects of smoking cessation on inflammatory marker levels.

Chapter 6 of this thesis reports the results of one such study. Inflammatory marker levels were measured prospectively in 138 health female smokers participating in the smoking cessation exercise study. Forty eight individuals achieved 6 weeks verified abstinence from smoking, and the effect of this on levels of WBC, fibrinogen, CRP, sICAM-1, and sCD40L were assessed. From this, the following conclusions can be drawn:

1. **Baseline inflammatory marker levels are associated with smoking exposure.**

   Consistent with the previous much larger epidemiological studies mentioned above, baseline levels of WBC, fibrinogen, and sICAM-1 all showed an association with number of cigarettes smoked per day (\(p < 0.05\) for all). There was a similar but weaker trend for CRP (\(p = 0.06\)). The strongest association was seen with sICAM-1. These findings are consistent with those of the Women's Health Study, in which the association between sICAM-1 and...
smoking exposure was much stronger than the association of CRP with smoking exposure.\textsuperscript{339} In contrast to the other markers, sCD40L showed no association with the amount of smoking.

2. \textit{Only WBC and fibrinogen levels are reduced by short term smoking cessation.}

Six weeks' abstinence from smoking resulted in significant decreases in WBC (from 8.9 to 8.2 x10^9/L, p < 0.01) and fibrinogen levels (from 10.0 to 9.4 μmol/l, p < 0.01). In contrast, there was no change in levels of other inflammatory markers (CRP, sICAM-1, sCD40L). Variability in levels of these inflammatory markers was much higher than for WBC or fibrinogen and consequently a modest effect of smoking cessation cannot be excluded. However the lack of effect was consistent across all measures of smoking change (smoking cessation in the 48 participants who achieved abstinence, and both change in number of cigarettes per day and change in salivary cotinine levels for the entire study cohort of 138 participants, including those who reduced smoking but did not achieve abstinence). Therefore it seems unlikely an appreciable effect of smoking cessation on levels of these inflammatory markers was missed.

In conclusion, WBC and fibrinogen levels fall quickly with smoking cessation, but levels of CRP, sICAM-1 and sCD40L do not. If the raised levels of these inflammatory markers seen in smokers are due to vascular inflammation, then this inflammation does not appear to be rapidly reversible with smoking cessation. It may be that the rapid changes in WBC and fibrinogen are due to decreased inflammation elsewhere in the body (for example, in the lungs), rather than changes in the vasculature. Further study will be needed to clarify this.

Finally, it has been proposed that measurement of inflammatory markers could be used to encourage lifestyle changes such as smoking cessation.\textsuperscript{7,372} However, even for WBC count and fibrinogen, individual responses to stopping smoking were variable (with levels \textit{increasing} in 35% of those who gave up smoking), limiting their value for monitoring and encouraging the health benefits associated with abstinence.
7.3 Clinical Relevance of Inflammatory Markers

The association between markers of inflammation and risk of cardiovascular events is now beyond doubt. The best studied of these markers, CRP, has consistent evidence from at least 23 cohort studies showing that raised CRP (>3 mg/l) is associated with higher risk of cardiovascular events than low CRP (< 1 mg/l), and this association persists after adjustment for other known cardiovascular risk factors. Nonetheless, the magnitude of the association is relatively weak; in a recent systematic review and meta-analysis of these 23 studies the summary estimate of relative risk for CRP > 3 mg/l vs. CRP < 1 mg/l was only 1.58 (95% CI, 1.37 to 1.83). This raises doubts about whether inflammatory markers will be of value in clinical practice, because for a risk factor to be of use for prediction in individual patients, it must have a relatively strong association with the disease. If there is only a weak association, the risk factor will have poor discriminatory value and either low detection rates (poor sensitivity) or a high number of false-positive results (poor specificity), depending on where the cutpoint for a ‘positive’ value is set (figure 7.1). Inflammatory markers may therefore add little information to what can already be determined from conventional risk factors. This was illustrated in a recent report from the Framingham Heart Study (2006), in which measurement of 10 novel biomarkers (including CRP) was added to conventional risk assessment in 3,209 participants followed for a median 7.4 years. Higher levels of these markers were associated with an increased risk of death or cardiovascular events, but their measurement had very little effect on the ability to classify risk beyond what can already be achieved using conventional risk scores (figure 7.2).
Figure 7.1 Poor discriminatory value of a risk factor which has only moderate association with event rates.

The graph shows normal distribution curves for a hypothetical risk factor with moderate association with cardiovascular events for people who do not subsequently develop cardiovascular events (leftmost curve), and for those who do subsequently develop cardiovascular events (rightmost curve). The 10th and 90th percentiles of the distribution in the event free group are labelled as $x_1$ and $x_2$, respectively. The cutoff value (diagnostic threshold) for a diagnostic test with 95% specificity is labelled as $T$.

As can be seen, for values of the risk factor higher than the diagnostic threshold there is considerable overlap between the groups, and although the risk factor is associated with an increased risk of events, it has very poor discriminatory value with a sensitivity of 13% and a false positive rate of 5%.

Modified from Ware (2006).
Figure 7.2  Difference between association with, and discriminatory value for, cardiovascular risk of a multimarker panel of novel risk factors.

These diagrams demonstrate the association between a panel of novel biomarkers (C-reactive protein, B-type natriuretic peptide, N-terminal proatrial natriuretic peptide, aldosterone, renin, fibrinogen, D-dimer, plasminogen-activation inhibitor type 1, homocysteine, and the urinary albumin to creatinine ratio) and subsequent cardiovascular risk (panel A), and the impact of this association on the prediction of risk at an individual level (panel B) in 3,209 patients from the Framingham Study.71

Although higher levels of the markers (as calculated by a multimarker score) were associated with increased risk of subsequent cardiovascular events (panel A), they had minimal impact on risk prediction at an individual level compared to conventional cardiovascular risk algorithms, as demonstrated by the overlapping receiver-operating characteristic curves in panel B.

Modified from Wang (2006).71
The American Heart Association has recently issued a scientific statement outlining criteria that should be met during the evaluation of novel markers of cardiovascular risk, before they can be recommended for clinical use. In its initial investigation, a novel marker must be associated with an increased risk of cardiovascular events, and add predictive information to established standard risk markers. CRP, the best studied of the inflammatory markers, has been reported to meet these criteria. However, demonstration of an association at a population level is not in itself sufficient to determine that addition of a novel risk marker will add useful information to guide clinical practice. Therefore the scientific statement also outlines that a novel marker must have clinical utility (by changing predicted risk sufficiently to change recommended management), and measurement of the novel marker should be shown to lead to better clinical outcomes. Finally, incorporation of the novel marker into standard practice must also be cost effective; in other words, use of the marker must improve outcomes sufficiently to justify the additional costs of testing and treatment.

These issues are not yet proven for CRP or other inflammatory markers. Individuals at low (<10%) or high (>20%) ten year risk using the conventional Framingham Risk Score are not likely to benefit from additional measurement of CRP, since it is unlikely to change the risk estimate sufficiently to lead to a change in management. It is therefore those at intermediate risk (10-20% ten year risk), in whom treatment decisions are uncertain, upon whom attention has focussed. Among this group, CRP levels greater than 3 mg/l may define a group at higher risk who may benefit from more intense preventive therapy, but to date this has not been prospectively tested.

The recently published JUPITER study (2008) requires special mention in this regard. JUPITER was a randomised placebo controlled trial of the lipid lowering agent rosuvastatin 20mg/l vs. placebo in 17,802 men and women without evidence of coronary heart disease, and with LDL cholesterol levels below the threshold for treatment (< 3.4 mmol/l) but with raised CRP levels (>2 mg/l). The trial was stopped early (after a median follow-up of 1.9 years) because of a near 50% reduction in cardiovascular events in the rosuvastatin group.
It has been suggested that these findings confirm a clinical role for routine CRP measurement, to guide statin therapy in intermediate risk patients who might not otherwise qualify for treatment. However, this conclusion is premature, for the following reasons:

1. The event rate in the placebo arm of the study was unexpectedly high. This may have been because a substantial proportion of the patients (over 40%) had documented metabolic syndrome. Metabolic syndrome is associated with a distinct pattern of dyslipidaemia consisting of a high concentration of small dense atherogenic lipid particles, not detected by measurement of LDL cholesterol, that are associated with a marked increase in cardiovascular risk. The benefits of statin therapy in JUPITER are consequently likely to be exaggerated compared to what can be expected from most patients with similar LDL levels. This, along with chance differences compounded by the trials early termination, may limit ability to generalise the findings to genuinely low cholesterol populations.

2. Since the trial only included participants with CRP levels > 2 mg/l, it cannot be determined whether raised CRP distinguished patients who benefit from statin therapy from those who do not. It may be that an LDL cholesterol treatment threshold of 3.4 mmol/l is too high, and that, regardless of CRP levels, there is substantial benefit in lowering cholesterol levels further. The trial results are consistent with several previous primary and secondary prevention trials of lipid lowering therapy, which show incremental benefit with lower LDL cholesterol levels, with no threshold below which further reduction in LDL cholesterol levels does not lead to further reduction in cardiovascular events (see figure 4.1, page 97). Both the Heart Protection Study (HPS) and the Treating to New Targets (TNT) trial are relevant in this regard, as participants in these trials who had LDL cholesterol levels < 3 mmol/l at baseline still benefited from further LDL cholesterol reduction from statin therapy, with a significant reduction in cardiovascular risk. As would be expected, the magnitude of clinical benefit in JUPITER was directly related to the reduction in LDL cholesterol achieved. It is therefore plausible that had the JUPITER study included participants with CRP < 2 mg/l, they would also have gained benefit from lipid lowering therapy.
3. The JUPITER study did not assess the incremental value of CRP measurement to conventional risk algorithms such as the Framingham Risk Score. It is therefore difficult to determine the proportion of participants in whom risk would have been reclassified (and treatment altered) by addition of CRP measurement.382

Further research will be needed confirming that addition of CRP measurement leads to a reclassification of risk in a substantial proportion of individuals, and that this reclassification leads to treatment changes that cost-effectively result in significant reductions in cardiovascular risk, before widespread clinical measurement of CRP should be considered. Consequently, guidelines such as those of the American Heart Association and the U.S. Preventive Services Task Force emphasise that there is currently insufficient evidence to recommend the use of inflammatory markers in clinical practice.15,376
7.4 Conclusions

There has been considerable ongoing research into the role of inflammatory markers for cardiovascular risk prediction over the last decade. This research has helped reinforce our understanding of inflammation as a key pathogenic mechanism in atherosclerosis. In addition, on a population level inflammatory markers such as C-reactive protein have been shown to be predictive of increased future risk of cardiovascular events in a variety of settings including in apparently healthy individuals, in patients with stable coronary artery disease, and in patients with acute coronary syndromes. Consequently it has been suggested that inflammatory markers such as CRP “may be of assistance in global risk assessment programs designed to better target intervention efforts, including smoking cessation, weight loss, diet and exercise”. Indeed a number of clinical applications for inflammatory marker measurement in cardiovascular disease have been proposed. Measurement of inflammatory markers might be used to better target the use of statins in primary prevention to those most likely to benefit. It might also be used to monitor the effects of statin therapy and to adjust therapy accordingly. In addition, inflammatory markers may be useful to “motivate persons with moderate to high risk levels to improve their lifestyles (e.g., smoking cessation, dietary modification, exercise, weight loss)”, and to subsequently provide “an inflammatory fitness score to monitor improvement in their cardiovascular health”.

This thesis explored specific aspects of the relationship between inflammatory markers and cardiovascular disease. From this research, the following conclusions can be drawn:

1. In stable angina patients undergoing PCI, measurement of inflammatory markers is not useful for predicting adverse outcomes such as restenosis. This remains true regardless of which inflammatory marker is measured (CRP, sICAM-1, sCD40L, or PAPP-A), or when it is measured (before PCI, or 48 hours, 1 week or 1 month after PCI).
2. Despite purported direct antiinflammatory mechanisms, the effect of lipid lowering agents on inflammatory marker levels is modest and unpredictable.

3. Inflammatory marker levels are not affected by up to six months of regular exercise training. Baseline levels of these markers are more strongly influenced by body fat than by cardiorespiratory fitness. This could account for the inverse associations seen between physical activity and inflammatory marker levels in cross-sectional studies (which have only used incomplete adjustment for body fat).

4. Inflammatory marker levels are associated with measures of smoking exposure, but are not reduced by up to six weeks of smoking cessation.

What are the implications of this research?

- Vascular inflammation does not appear to be an easily modifiable risk factor. The effects of medications such as statins on inflammatory markers are modest and variable, and other interventions such as exercise and smoking cessation (in the short term at least) do not affect inflammatory marker levels.

- The clinical scenarios in which inflammatory marker measurement is useful requires further clarification. Despite strong basic science evidence that inflammatory markers are implicated in restenosis and other adverse outcomes of PCI, in a clinical setting inflammatory marker levels did not appear related to PCI outcomes, and were not useful in predicting their occurrence.
In summary, after a decade of intense interest and research into the role of inflammation in cardiovascular disease, it is still not clear whether measurement of inflammatory markers has any clinical role in the detection or treatment of cardiovascular disease. There are several issues yet to be resolved regarding the measurement of inflammatory markers for clinical risk prediction or for targeting and monitoring intervention strategies. Randomised clinical trials are needed to test whether risk prediction and initiation of preventive treatment on the basis of inflammatory marker levels leads to actual clinical benefit. Randomised trials are also needed to determine whether there are pharmacological or non-pharmacological interventions which will reliably lower inflammatory marker levels, and if so whether this inflammatory marker reduction is associated with a decrease in cardiovascular risk. Until these important questions are answered, inflammatory marker measurement should remain a research tool only, and not be employed in a clinical setting.
Appendices

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Appendix 1

Measurement of Inflammatory Markers and other serological risk factors.

*C Reactive Protein (CRP)*

For all the studies detailed in this thesis CRP levels were measured using the commercially available high-sensitivity Dade Behring assay (Dade Behring, Deerfield, Illinois, USA). This is an automated latex-enhanced immunonephelometric assay which was performed on a BN ProSpec analyzer kindly loaned by Dade Behring New Zealand. All analyses were performed by Mr Roy The at LabPlus, Auckland City Hospital. The assay was standardized against laboratory references and 10% of samples from each study were analysed in duplicate to ensure the reproducibility of results. Our results were in keeping with the reported detection threshold of 0.15 mg/l and coefficient of variation of ~5%.383

*Soluble CD40 Ligand (sCD40L) and intercellular adhesion molecule 1 (sICAM-1)*

sCD40L and sICAM-1 concentrations were measured by Drs Uwe Schoenbeck and Nerea Varo of the Department of Cardiovascular Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA. sCD40L was measured using a commercially available ELISA from BenderMedSystems, Vienna, Austria and sICAM-1 was measured using a commercially available ELISA from R&D Systems, Minneapolis, MN, USA. Samples were measured in triplicate and the intra-assay variation among the triplicates for all samples was less than 10%. Marker levels and reproducibility of results for both the FAST and smoking studies were consistent with previous published reports on the use of these assays at this institution.119,123

*Pregnancy associated plasma protein-A (PAPP-A)*

PAPP-A levels were measured by Dr Michael Christiansen at the Staten Serum Institut, Copenhagen, Denmark using an ‘in-house’ biotin-tyramide-amplified enzyme immunoassay. PAPP-A polyclonal antibodies were used for capture and a combination of monoclonal antibodies was used for detection. The assay was calibrated against the World Health
Organisation's international reference standard 78/610, which is standard for pregnancy-associated proteins. The assay has a limit of detection of 0.03 mIU/l and intra-assay and inter-assay coefficients of variation 10% and 15% respectively as has been previously described. 33,143,146 It should be noted that more recent studies of circulating PAPP-A have used commercially available ELISA techniques that were not widely available when our research was done, and that PAPP-A levels measured with these techniques may differ from the in-house’ immunoassay used in our research.141,142

**Other Serological Cardiovascular Risk Factors**

Other serological cardiovascular risk factors were measured at LabPlus, Auckland City Hospital, an International Accreditation New Zealand (IANZ) accredited medical laboratory. Total WBC count was measured immediately after sample collection using the ADVIA 102 Haematology System (Bayer Corporation, Tarrytown, NY, USA). Fibrinogen levels were determined in citrated plasma using the Multifibren U method (a modification of the Clauss method) on a Behring BCT analyser (Dade Behring Corporation, Deerfield, Ill., USA). Total cholesterol and glucose levels were measured enzymatically using a Hitachi 917 autoanalyser. In addition to these assays, homocysteine and troponin T levels were measured on stored samples from the FAST study. Homocysteine was measured using the Abbott AxSYM homocysteine assay L2002 (Abbott Laboratories, Ill., USA), a newer generation of fluorescence polarization immunoassay modified from the Abbott IMx analyser.384 Troponin T levels were measured using the third-generation Roche assay (Mannheim, Germany) which has a discrimination level for MI of 0.03 µg/L. All assays were standardized against laboratory references and 10% of samples were analysed in duplicate to ensure reproducibility of results.
Appendix 2

Conversion factors

The following conversion factors can be used to change values from SI units to mg/dl:

<table>
<thead>
<tr>
<th>Variable</th>
<th>SI Units</th>
<th>Divide by</th>
<th>To convert to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>mmol/l</td>
<td>0.0555</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mmol/l</td>
<td>0.0884</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mmol/l</td>
<td>0.02586</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>μmol/l</td>
<td>0.0294</td>
<td>mg/dl</td>
</tr>
</tbody>
</table>
Appendix 3

Activity Questionnaire (adapted from the Nurses’ Health Study).  

The activity questionnaire used in the Nurses’ Health Study is a self-reported measure of weekly recreational physical activity estimated for the past year, which collects information on eight moderate and vigorous activities, usual walking pace, and number of flights of stairs climbed daily. The questionnaire has been shown to provide an accurate estimate of physical activity when compared to more time consuming measures such as use of an activity diary (correlation between the two methods $r=0.62$, $P<0.001$). Activity scores calculated from the questionnaire have been shown to be predictive of future cardiovascular events in both the US Nurse’s Health Study (1999) and the Women’s Health Initiative Observational Study (2002). In these studies, both regular walking and vigorous exercise were associated with a substantially lower incidence of cardiovascular events in women, irrespective of race, ethnic group, age, and body mass index. Conversely, prolonged sitting predicted an increased cardiovascular risk.

For this thesis, the activity questionnaire was used to assess activity levels of participants in both the Exercise in the Elderly and Smoking Cessation Trials (chapter 5). The questionnaire was administered both at baseline and at follow-up visits, to determine whether there had been any change in physical activity in the control groups throughout the study. Because of this need for repeat assessment, the questionnaire used in this thesis recorded activity levels over the past six weeks (rather than the past year as in the original Nurses’ Health Study Questionnaire). Otherwise the questionnaire was identical to that used in both the US Nurse’s Health Study (1999) and the Women’s Health Initiative Observational Study (2002).

The questionnaire asked participants to detail time spent per week in a range of activities including climbing stairs, walking outdoors, jogging, running, bicycling, swimming laps, playing tennis or squash and participating in aerobics. From this an activity score was calculated, using units of ‘metabolic equivalent’ (MET) hours per week. The MET score is a measure of the absolute intensity of physical activity, represented as multiples of basal metabolic activity (1 MET equals the resting metabolic rate of $\sim 3.5 \text{ ml O}_2/\text{kg}$). It gives an
objective quantification of the amount of physical activity per week and consequently allows comparisons between subjects and across time.

The activity questionnaire is reproduced on the following pages. The activity score was calculated by multiplying the time spent in each activity (hours per week) by its estimated intensity (in METs) according to the following formula:

**Estimated intensity (in METs) of each activity:**

Walking (Dependent on pace):
- Casual: 2.5 MET
- Average: 3 MET
- Brisk: 4 MET
- Very Brisk: 4.5 MET
- Unable: 1 MET

Jogging: 7 MET
Running: 12 MET
Bicycling: 7 MET
Aerobics: 6 MET
Tennis, squash: 7 MET
Swimming: 7 MET
Other exercise: 4.5 MET

For stair climbing, the number of stairs climbed per day was multiplied by seven (to convert to number of stairs per week), and by the time to climb one flight of stairs (0.002), and by the aerobic intensity of stair climbing (8 MET). For example, if a participant climbed an average 10-14 stairs per day, the associated activity score would be:

12 (average number of stairs per day) * 7 * 0.002 * 8 = 1.3 MET. hours per week.

The overall activity score was calculated by adding up the MET. hours per week of each individual activity (walking + jogging + running + bicycling + aerobics + tennis + swimming + other exercise + stair climbing).
Figure A3.1  Activity Questionnaire.

During the past 6 weeks, what was your average time **PER WEEK** spent at each of the following recreational activities (If you are in the exercise group DO NOT include the exercise done as part of the program).

Please tick one box in each row

<table>
<thead>
<tr>
<th>Activity</th>
<th>Zero</th>
<th>1-4 min</th>
<th>5-19 min</th>
<th>20-59 min</th>
<th>One Hr</th>
<th>1-1.5 Hr</th>
<th>2-3 Hr</th>
<th>4-6 Hr</th>
<th>7-10 Hr</th>
<th>11+ Hr</th>
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</thead>
<tbody>
<tr>
<td>Walking or Hiking outdoors (include walking to work)</td>
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<td>Jogging (slower than 1k in 6 mins)</td>
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<td>Running (faster than 1 k in 6 mins)</td>
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<td>Bicycling (include stationary machine)</td>
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<tr>
<td>Callisthenics/aerobics/aerobic dance/rowing machine</td>
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<tr>
<td>Tennis, squash or racquetball</td>
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<tr>
<td>Lap swimming</td>
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<td>Other aerobic recreation (e.g. lawn mowing)</td>
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</tbody>
</table>
On average, how many hours **PER WEEK** do you spend:

Please tick one box in each row

<table>
<thead>
<tr>
<th>Activity</th>
<th>Zero</th>
<th>1 hr</th>
<th>2-5 hr</th>
<th>6-10 hr</th>
<th>11-20 hr</th>
<th>21-40 hr</th>
<th>41-60 hr</th>
<th>61-90 hr</th>
<th>Over 90 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing or walking around at work</td>
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<tr>
<td>Standing or walking around at home</td>
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<td>Sitting at work or while driving</td>
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<tr>
<td>Sitting at home</td>
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</tbody>
</table>
Figure A3.1  Activity Questionnaire (continued).

**What is your usual walking pace outdoors?**

- [ ] Easy, casual (less than 3km per hr)
- [ ] Normal, average (3-5km per hr or faster)
- [ ] Brisk (5-7km per hr)
- [ ] Very brisk/striding (7km per hr or faster)
- [ ] Unable to walk

**How many flights of stairs (not individual steps) do you climb daily?**

- [ ] 2 flights or less
- [ ] 3-4
- [ ] 5-9
- [ ] 10-14
- [ ] 15 or more flights
Appendix 4

YMCA cycle ergometer submaximal exercise test protocol

In the Smoking Cessation trial (chapters 5 and 6), cardiorespiratory fitness was measured using the YMCA cycle ergometer submaximal exercise test. In this test, subjects exercise on a cycle ergometer at a series of submaximal workloads, with their heart rate response extrapolated to estimate the workload that would be needed to achieve the subject’s age-predicted maximal heart rate. This workload is then used to calculate cardiorespiratory fitness (maximal aerobic capacity, VO$_{2\,\text{max}}$). The use of submaximal exercise tests such as the YMCA protocol has been verified against formal measurement of VO$_{2\,\text{max}}$ using expired gas measurement during treadmill exercise. Submaximal tests are simple to administer and can be conducted at a standard training facility, without the use of specialised equipment. It was for these reasons that the YMCA submaximal test was used to predict cardiorespiratory fitness in the smoking cessation trial, which required serial fitness measurements in a large number of participants recruited concurrently. The details of the test are outlines below.

Participants in the trial exercised for between two to four sequential 3-minute stages of continuous exercise, with the aim of raising their steady-state heart rate to between 110 beats per minute (bpm) and 85% of maximal predicted heart rate for at least two consecutive stages. Heart rates were recorded within the final 15 to 30 seconds of the second and third minutes of each exercise stage. If these two heart rates were within 6 bpm of each other this confirmed achievement of steady-state heart rate and the subjects could progress to the next stage of the protocol. If not, the current workload was maintained and heart rate was remeasured each minute until steady-state was achieved. At the end of each stage, workload was adjusted according to the heart rate response as detailed in figure A4.1. Exercise was performed on a cycle ergometer with a flywheel of 6 m/rev maintaining a speed of 50 rpm. The initial workload was set at 150 kgm / min (0.5 kg weight at 50 rpm). At the end of the first stage, the workload was adjusted according to the heart rate: if less than 80 bpm, workload was set at 750 kg.m/min (2.5 kg of weight at 50 rpm); if between 80-89 bpm, workload was set at 600 kg.m/min (2.0 kg at 50 rpm); if between 90-100 bpm, workload was set at 450 kg.m/min (1.5 kg at 50 rpm); and if greater than 100 bpm, workload was set at 300 kg.m/min.
(1.0 kg at 50 rpm). The workload for the third and fourth stages (if required) was set in relation to the workload required in the second stage as detailed in figure A4.1.

The test was completed once two steady-state heart rates between 110 beats/min and 85% age-predicted maximal heart rate were achieved. These heart rates were then plotted against the corresponding workload as displayed in figure A4.2 (workloads were converted from kg.m/min to Watts using the following conversion factor: 1 kg.m/min = 0.1635 Watts). The resultant line was extrapolated to the age-predicted maximal heart rate (220 – age) and a perpendicular line was dropped to the x-axis to estimate the workload that would have been achieved had the person exercised to maximum capacity.

Using this estimated workload, cardiorespiratory fitness (maximal aerobic capacity, VO$_2$max) was then calculated using the following formula.$^{314}$

$$\text{VO}_2\text{max} \text{ (ml/kg/min)} = [0.6 + (\text{maximum workload} - 25) \times 0.012] \times 1000 / \text{weight}$$

Where maximum workload is in Watts and weight is in kilograms. 1 Watt = 0.012 l/min.
Figure A4.1  YMCA cycle ergometry protocol.

<table>
<thead>
<tr>
<th>Stage</th>
<th>HR &lt; 80</th>
<th>HR 80 – 89</th>
<th>HR 90-100</th>
<th>HR &gt; 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd Stage</td>
<td>750 kg.m/min (2.5 kg)</td>
<td>600 kg.m/min (2.0 kg)</td>
<td>450 kg.m/min (1.5 kg)</td>
<td>300 kg.m/min (1.0 kg)</td>
</tr>
<tr>
<td>3rd Stage</td>
<td>900 kg.m/min (3.0 kg)</td>
<td>750 kg.m/min (2.5 kg)</td>
<td>600 kg.m/min (2.0 kg)</td>
<td>450 kg.m/min (1.5 kg)</td>
</tr>
<tr>
<td>4th Stage</td>
<td>1050 kg.m/min (3.5 kg)</td>
<td>900 kg.m/min (3.0 kg)</td>
<td>750 kg.m/min (2.5 kg)</td>
<td>600 kg.m/min (2.0 kg)</td>
</tr>
</tbody>
</table>
Figure A4.2  Estimation of workload at which maximal aerobic capacity (VO\textsubscript{2max}) is achieved, based on the heart rate response to submaximal exercise on a cycle ergometer using the YMCA protocol.\textsuperscript{314}
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